RNA Virus Vectors

1. A Direct Comparison of Foamy and Lentiviral Vector Genotoxicity in SCID-Repopulating Cells Shows Foamy Vectors Are Less Prone to Clonal Dominance

Elizabeth M. Everson, Miles E. Olzsko, David J. Leap, Jonah D. Hocum, Grant D. Trobridge
Washington State University, Spokane, WA

Hematopoietic stem cell (HSC) gene therapy has immense potential for numerous diseases including primary immunodeficiencies. However, in some SCID-X1 patients treated with gammaretroviral vectors, leukemia occurred as a result of vector mediated genotoxicity. Lentiviral vectors are less genotoxic than gammaretroviral vectors and have become the retroviral vector of choice in HSC clinical trials. Foamy retroviral vectors have a promising integration profile and are less prone to read-through transcription when directly compared to gammaretroviral or lentiviral vectors. Here we directly compared the safety and efficacy of foamy vectors to lentiviral vectors in human cord blood CD34$^+$ repopulating cells in immunodeficient mice (NSG). To increase their genotoxic potential, foamy and lentiviral vectors with identical transgene cassettes that utilized a known genotoxic spleen focus forming virus promoter were used. Our rationale was that in order to compare the relative safety of these two vector systems in normal human CD34$^+$ cells, within the relatively short time span of repopulating cells in mice, a highly genotoxic promoter would be the best way to elicit differences in genotoxicity. Both foamy and lentiviral vectors resulted in efficient marking in vivo with 12.2% of peripheral blood human CD45$^+$ cells expressing EGFP for foamy vectors, and 9.6% for lentiviral vectors at 19 weeks after transplantation. A total of 837 foamy vector and 423 lentiviral vector unique integration sites were recovered in human SCID repopulating cells 19 weeks after transplantation. Foamy vector proviruses were observed less often within genes (40%) than lentiviral vectors (74%), and were also observed less often within proto-oncogenes (6.6%) than lentiviral vectors (9.7%). Analysis of clonality in repopulating cells within individual mice showed that the foamy vector group was more polyclonal with fewer dominant clones (2/6 mice) than in the lentiviral vector group (8/8 mice) (Figure 1). Dominant clones were defined as a single integration site having over 20% of captures. Five of the thirteen dominant clones from the lentiviral group had integrations near known proto-oncogenes (Table 1) but in the foamy vector group, none of the three dominant clones had a known proto-oncogene near the integration site. In summary, in a direct comparison in human CD34$^+$ SCID repopulating cells, foamy vectors resulted in fewer dominant clones and more polyclonal repopulation than lentiviral vectors. Our data further supports the relative safety of foamy vectors for HSC gene therapy.

Table 1. Foamy vector (FV) and lentiviral vector (LV) RIS contributing to more than 20% of all retroviral integration sites (RIS)

Vector	Mouse	RIS	Contribution to all RIS[a]	Closest proto-oncogene (distance)[b]
LV	6	1	99%	within MKL1
LV	8	2	66%	RPS6KA1 (30 kb)
LV	1	3	63%	n/a
		4	28%	CYP4F22 (61 kb)
LV	3	5	63%	n/a
		6	33%	FLT3 (76 kb)
FV	5	7	39%	n/a
		8	27%	n/a
LV	4	9	34%	n/a
		10	22%	n/a
LV	5	11	30%	MST1 (73 kb)
		12	25%	n/a
LV	2	13	23%	n/a
		14	22%	n/a
LV	7	15	22%	n/a
FV	6	16	20%	n/a

Abbreviations: n/a, not applicable
[a] capture frequency per mouse
[b] proto-oncogenes within 100 kb of RIS

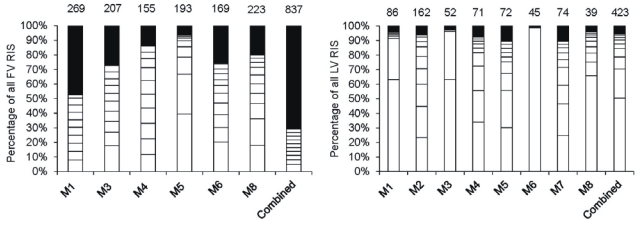


Figure 1. Clonality in the bone marrow of NOD-SCID gamma (NSG) mice transplanted with foamy vector (FV) and lentiviral vector (LV) transduced human CD34$^+$ cells. Each bar represents one mouse (for example M1 is mouse 1) and combined are RIS for all 6 mice for foamy, and all 8 mice for lenti. The numbers above the bars represent the total number of unique RIS found in each mouse, or for all mice (combined).

2. Identification and Ranking of Different Chromatin Insulators to Block Vector-Driven Enhancer-Mediated Insertional Mutagenesis *In Vivo*

Monica Volpin[1], Andrea Calabria[1], Daniela Cesana[1], Erika Tenderini[1], Fabrizio Benedicenti[1], Giulio Spinozzi[1], Jack Lenz[2], George Stamatoyannopoulos[3], Eugenio Montini[1]
[1]HSR-TIGET, Milan, Italy, [2]Albert Einstein College of Medicine, New York, NY, [3]University of Washington, Seattle, WA

Aimed at refining the safety profile of self-inactivating (SIN) lentiviral vectors (LV) for gene therapy applications we investigated the impact of chromatin insulators (CI) on vector-mediated genotoxicity. Specifically, we studied four recently identified CI whose function is mediated by CCCTC-binding factor (CTCF), the best characterized insulator protein in vertebrates, and cloned these CI in the LTRs of a SIN.LV with a strong enhancer/promoter in internal position (CI.SIN.LV).

We took advantage of two sensitive *in vivo* genotoxicity assays based on the systemic injection of LVs in newborn tumor-prone *Cdkn2a*$^{-/-}$ and *Cdkn2a*$^{+/-}$ mice, that allow to measure vector-induced genotoxicity as accelerated tumor onset proportional to the genotoxic potential of the tested LV.

CI.SIN.LVs displayed slightly not statistically significant improvement in the median survival time vs. the uninsulated SIN. LV counterpart (ranging from 193.5 to 214 days vs. 186 days, respectively) in *Cdkn2a*$^{-/-}$ mice.

In *Cdkn2a*$^{+/-}$ mice, two insulated vectors significantly improved the median survival time, which resulted non-statistically different from Mock mice (450 and 511 vs. 505.5 days respectively), while the other two CI.SIN.LVs studied resulted to be still slightly genotoxic (median survival time: 412 and 429.5 days). To gain more insights on the safety profile of these LVs we retrieved and analyzed the vector integration sites (IS) (n>14000 IS) and identified common integration sites (CIS) in the murine tumors generated in our experimental framework and in both murine models.

In *Cdkn2a*$^{-/-}$ mice, uninsulated SIN.LV-induced tumors harbored activating integrations targeting *Map3k8* oncogene, while tumors obtained in mice treated with two out of four different insulated

LVs significantly reduced the frequency of tumors with *Map3k8*-activating insertions. The reduced targeting frequency of *Map3k8* was accompanied by a skewing of integrations inactivating *Pten*, *Rasa1* or other tumor-suppressors, an escape genotoxicity mechanism on which insulators cannot act.

In *Cdkn2a*$^{-/+}$ mice we identified different predominant CIS genes targeted by the different insulated vectors. These data show that heterozygous *Cdkn2a*$^{-/+}$ mice allow discriminating between more subtle shades of genotoxicity of the different vector designs and are therefore instrumental to understand the different molecular mechanisms of insertional mutagenesis and ways to avoid them. Interestingly by comparing the results from both *in vivo* assays we observed that one CI displayed superior safety profile in terms of significant improvement in the median survival time and/or in terms of reduced oncogenic CIS identified.

In summary we validated new human-origin insulator elements able to block SIN.LV genotoxicity *in vivo*. Overall, these data highlight the importance of stringent *in vivo* genotoxicity testing of improved vector versions and support the use of CI for future gene therapy applications.

3. Development of a Cas9 Protein Delivery System with Lentiviral Vectors for RNA-Guided Genome Editing

Naoya Uchida, Anna Shvygin, Luke Skala, Lydia Raines, Josiah Ballantine, John Tisdale
NIH, Bethesda, MD

Gene correction is an ideal gene therapy strategy for hereditary disease, including sickle cell disease. Recently, the CRISPR/Cas9 system was developed to allow site-specific DNA breakage, which can enhance homologous recombination-based gene correction with template DNA. However, efficient delivery remains crucial for successful genome editing. In this work, we sought to develop a new gene/protein delivery system with lentiviral vectors for efficient genome editing. We first used an HIV-1 based lentiviral vector system to deliver the CRISPR/Cas9 system including guide RNAs and the Cas9 endonuclease since efficient delivery in various cells, including hematopoietic stem cells, has previously been established. However, using this system, the guide RNA/Cas9-transduced cells continuously express the Cas9 endonuclease, increasing the likelihood of off target effects. In addition, the large size of the Cas9 DNA (4.3kb) limits efficient lentiviral packaging, likely reducing transduction efficiency. Therefore, we sought to develop a Cas9 protein delivery system with lentiviral particles that did not integrate the Cas9 DNA. Cyclosphilin A (CypA) has a function to bind to lentiviral capsids; thus, we hypothesized that fusion proteins between Cas9 and CypA could be packaged in lentiviral particles to allow delivery. We designed two Cas9/CypA fusion proteins: "CypA to Cas9" and "Cas9 to CypA", and we prepared the lentiviral vectors encoding a GFP-targeting guide RNA with the fusion proteins. We transduced a GFP+ stable cell line with GFP-targeting guide RNA vector containing Cas9 fusion proteins. At 14 days after transduction, GFP-positivity (%GFP) was reduced with both Cas9 protein delivery vectors (48-53%, $p<0.01$), as compared to a guide RNA alone vector control (83%) and no transduction control (83%). The disruption of GFP was comparable to a guide RNA/Cas9 integrating vector (40%). These data suggest that Cas9/CypA fusion proteins can be delivered with lentiviral particles, and the Cas9 fusion proteins have an endonuclease function to efficiently induce a GFP DNA break. To model DNA correction, we then designed a Cas9 protein delivery non-integrating lentiviral vector encoding both GFP-targeting guide RNA and YFP gene template, which contains all essential components for GFP to YFP gene correction in one vector. Conversion of GFP to YFP would thus model gene correction. Silent mutations in the target site in YFP template were required to produce the gene correction vector. We transduced a GFP+ stable cell line with the gene correction vector using the Cas9/CypA fusion protein or Cas9 protein alone control (without CypA fusion). We observed a significant reduction of GFP positivity (20-24% GFP, $p<0.01$) along with high rates of conversion to YFP positivity (29-30%, $p<0.01$) among all Cas9 protein delivery vectors, even with Cas9 protein alone control, as compared to no Cas9 control (YFP 4.9%) and no transduction control (YFP 4.3%). The GFP to YFP gene correction was confirmed by DNA sequencing. These data suggest that Cas9 protein alone can be delivered with lentiviral particles and the Cas9 protein delivery system (with or without CypA fusion) allows for efficient one-time gene correction with non-integrating vectors encoding both guide RNA and template. Additionally, we transferred guide RNA and YFP template again 6 days after Cas9 protein delivery; however, no increase of %YFP was observed, suggesting that Cas9 function was lost over the short term (<6 days). In summary, we developed Cas9 protein delivery system with lentiviral vectors, which resulted in efficient GFP gene breakage. The Css9 protein delivery system allowed for efficient one-time GFP to YFP gene correction with non-integrating lentiviral vector encoding both guide RNA and template DNA. Our findings improve the prospects for efficient delivery and safe genome editing.

4. β-Deliverin: A Small Molecule for Improving Gene Transfer to Hematopoietic Stem Cells and Probing Mechanisms of Lentiviral Vector Restriction

Nina Timberlake[1], Stosh Ozog[1], Saritha D'souza[2], Igor Slukvin[2], Sergio D. Catz[1], Scott Snyder[3], Bruce E. Torbett[1]
[1]The Scripps Research Institute, La Jolla, CA, [2]Wisconsin National Primate Research, Madison, WI, [3]University of Chicago, Chicago, IL

A major obstacle to the success of gene therapy for hematologic disorders is the inefficiency of lentiviral vector (LV) gene transfer to hematopoietic stem cells (HSCs). Achieving clinically relevant gene delivery levels requires prolonged *ex vivo* culture of HSCs with cytokines and large LV doses. Rapamycin, PGE2, and other small molecules, have been reported to increase LV transduction of HSCs, however the mechanism of action of these drugs and the basis for LV restriction in HSCs are poorly understood.

Here, we report a novel small molecule, β-deliverin, which improves LV gene transfer to HSCs up to 3 fold over DMSO control *in vitro*. This effect is most pronounced in human adult peripheral blood-derived HSCs, but also observed in human cord-derived HSCs, and in non-human primate bone marrow-derived HSCs. Importantly, treatment with β-deliverin does not significantly affect the viability or expansion of HSCs in culture. Furthermore, for cord-derived HSCs, there was no reduction in the number or type of colony-forming cells. *In vivo,* β-deliverin treated HSCs engraft in the peripheral blood of NSG mice at levels comparable to control at 16 weeks post-engraftment. Despite an only modest increase in transduction efficiency in β-deliverin-treated cord-derived HSCs transduced *in vitro* (26% versus 20% for control), the marking frequency for control cells dropped to <5% in the peripheral blood of NSG mice 16 weeks post-engraftment, while mice engrafted with β-deliverin treated HSCs retained on average 20% marking. Our data suggest that β-deliverin treatment increases gene marking in long-term repopulating cells without reducing proliferation, differentiation or engraftment potential.

Mechanistically, β-deliverin acts at the stage of viral fusion; the rate of fusion is increased more than 2-fold in the presence of β-deliverin. We show, by confocal microscopy, that the proportion and intensity of virus signal localized to EEA1-labelled early endosomes is unchanged by drug treatment, suggesting that viral entry is not affected. In further

support of this, LDLR surface expression and receptor density is unchanged following β-deliverin treatment. In β-deliverin treated cells, 2 hours post-transduction, we observed reduced accumulation of virus in late endosomes, a 3-fold reduction in the total number of late endosomes, as measured by LAMP1 puncta, and a nearly 2-fold increase in the proportion of early endosomes when compared to control cells. Together, our data imply that β-deliverin facilitates more efficient exit of virus from the endosome, possibly by inhibiting endosomal maturation. Further mechanistic studies are ongoing. Notably, a monomer of β-deliverin has no effect on HSC transduction, suggesting an important structure-function relationship.

β-deliverin is a promising tool not only for the development of novel gene delivery applications, but also to further elucidate LV-HSC interactions. We are investigating whether the mechanisms we observed here hold true for other small molecule transduction enhancers, and using these drugs to probe the biology of LV restriction in HSCs.

5. Development of Enhancing Intraosseous Delivery Efficiency of LV-Factor VIII Variants in Platelets of Hemophilia A Mice

Xuefeng Wang, Richard Y. Fu, Carol H. Miao
Seattle Children's Research Institute, Seattle, WA

Introduction: Our previous studies demonstrated that human factor VIII (FVIII) specifically expressed in megakaryocytes and then stored in platelets of Hemophilia A (HemA) mice can partially correct their phenotype over 5 months in mice with or without pre-existing inhibitors. This was achieved by intraosseous (IO) delivery of lentiviral vectors (LVs) carrying a transgene encoding human FVIII variant (BDDhFVIII/N6; abbreviated as F8) driven by a megakaryocyte-specific promoter (Gp1bα) without preconditioning as required in *ex vivo* gene therapy. **Methods:** In this study, we aimed at enhancing transgene expression by two strategies. One was to enhance LV transduction efficiency by suppressing the innate and adaptive immune responses against LVs and LV-transduced cells using pharmacological agents. The other was to improve FVIII gene expression by incorporating a new human FVIII variant, F8X10K12 (a 10-amino acid change in the A1 domain and a 12-amino acid change in the light chain; a kind gift from Dr. Weidong Xiao). **Results:** First, the immune competent C57BL6 mice were pretreated with both dexamethasone (Dex) (IP, 5 mg/kg at -24h, -4h, 4h and 24h) and anti-CD8α monoclonal antibody (mAb) (IP, 4 mg/kg on day -1, 4, 11, 16 and 21), or Dex only. IO infusion of GFP-LVs (1.1×10^8 i.f.u./mouse) driven by a ubiquitous MND promoter was performed on day 0. On day 7, Dex only and combination drugs + LVs treated mice (n=3) produced higher numbers of GFP^+ total bone marrow cells (17.7±3.5% and 11.8±2.1% vs 6.9±3.1%, P=0.0001 and 0.005) and $GFP^+Lineage^-Sca1^+cKit^+$ HSCs (55.5±3.1% and 48.3±6.1% vs 44.4±17.2%, P=0.1 and 0.31) compared with LV-only treated mice (n=3). Most importantly, in the long term, higher numbers of GFP^+ cells (2.4±0.4% vs 0.5±0.1%, P<0.001) in the total bone marrow and GFP^+HSCs (10.7±3.3% vs 2.6±0.6%, P<0.001) were observed in combination drugs + LVs treated mice (n=3) compared with LV-only treated mice on day 160 after LV infusion (n=3), which was confirmed by higher LV copy number in bone marrow cells of drugs + LVs treated mice. Second, we tested the FVIII expression levels from two human FVIII variants in HemA mice by hydrodynamic injection of plasmids driven by a human elongation factor-1 promoter (pEF1α-F8X10K12 or pEF1α-F8, 50 μg/mouse, n=8), respectively. Compared with F8, F8X10K12 produced a 25-fold increase (147±27% vs 3,734±477%) in the clotting activity determined by an aPTT assay on day 4 post injection. Then two LVs containing F8X10K12 or F8 transgene driven by EF1α promoter (E-F8X10K12-LV or E-F8-LV) were constructed and used to transduce 293T cells, respectively. Flow cytometry data showed that E-F8X10K12-LV produced a significant increase of $hFVIII^+$293T cells (77.8% vs 15%) and MFI (795 vs 541) compared to E-F8-LV at the same doses. These results indicated that F8X10K12 may further enhance FVIII gene expression in platelets for more effective therapy. LVs containing F8X10K12 or F8 transgene driven by Gp1bα promoter (G-F8X10K12-LV or G-F8-LV) were subsequently generated and were intraosseously delivered into HemA mice to test the FVIII efficiency in platelets by ELISA, thrombin generation assay and tail clipping. **Conclusion:** We found that administration of Dex that efficiently inhibited initial innate immune responses to LVs *in vivo* combined with anti-CD8α mAb that depleted subsequent cytotoxic $CD8^+$ T cells improved the transduction efficiency of LVs and persistence of transduced cells, leading to over 10% GFP^+HSCs in treated mice up to 160 days. In addition, a new FVIII variant, F8X10K12, can significantly enhance FVIII expression in mice following hydrodynamic injection of plasmids and in LV-transduced cells. Taken together, IO infusion of G-F8X10K12-LV into HemA mice pretreated with Dex and antiCD8α mAb can be used to further enhance and prolong transgene efficiency in platelets for effective correction of hemophilia A.

6. Retargeted Foamy Virus Vectors Integrate Less Frequently Near Proto-Oncogenes

Jonah D. Hocum, Ian Linde, Dustin T. Rae, Casey P. Collins, Lindsay K. Matern, Grant D. Trobridge
Pharmaceutical Sciences, Washington State University, Spokane, WA

Hematopoietic stem cell gene therapy offers immense potential to treat many genetic diseases and has already shown efficacy in clinical trials. However, retroviral vector mediated gene dysregulation, known as genotoxicity, remains a major challenge and clinically relevant approaches to reduce integration near genes and proto-oncogenes are needed. The frequency at which a retroviral vector integrates near proto-oncogenes is a major factor in determining the safety of the vector. Here we describe a clinically relevant method to retarget foamy retroviral vector (FV) integration away from proto-oncogenes using modified Gag and Pol helper constructs. The chromatin binding site (CBS) of FV Gag was altered using a previously described triple alanine substitution mutation of RTY (Gag-RTY) shown to drastically reduce chromatin binding of the FV pre-integration complex. The modified FV Pol construct expresses chromobox protein homolog 1 fused to the C-terminus of integrase (CBX1-IN). CBX1 interacts with tri-methylated lysine 9 of histone H3 (H3K9me3) and is associated with gene sparse regions in the human genome. We hypothesized a modified FV expressing Gag-RTY and CBX1-IN would have an altered integration site profile and be retargeted to H3K9me3 regions and away from genes and proto-oncogenes. FV integration sites from modified and control FVs were compared in normal human fibroblasts and in $CD34^+$ human cord blood cells. We observed retargeting of FV integration into H3K9me3 regions with the modified FV (Figure 1). Importantly, retargeting FV integration significantly reduces the number of integration sites near proto-oncogene transcription start sites (Table 1). Retargeted FVs can be produced at clinically relevant titers (> 10^7 transducing units / ml), resulting in efficient transgene expression (75 % of control in $CD34^+$ cells) with no evidence of vector silencing. Another published method to retarget lentiviral vector integration required using a modified cell line, which is not practical for clinical use. Our approach is cell line independent and the modified Gag and Pol helper constructs will allow an investigator to simply use these modified helper plasmids during vector production to retarget any therapeutic FV in any target cell. Retargeted FVs integrate less frequently near proto-oncogenes than gammaretroviral vectors and unmodified FVs, and may be the safest current option for hematopoietic stem cell gene therapy.

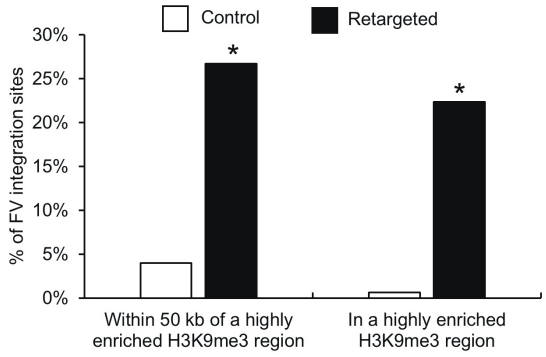


Figure 1. Retargeted FV integrates into H3K9me3 regions, associated with gene sparse regions, much more frequently than control FV in CD34+ cells. *statistically significant at $p < 0.001$ compared to control vector. FV, foamy retroviral vector.

		Percent of FV integration sites in human CD34+ cells		
Vector	Total sites	In RefSeq genes	< 10 kb from TSS	< 50 kb from proto-oncogene TSS
Control FV	4217	40.9	24.5	4.7
Retargeted FV	1534	30.6*	12.5*	2.2*

Table 1. Retargeted FV integrates further away from genes and proto-oncogenes than control FV. *statistically significant at $p < 0.001$ compared to control vector. FV, foamy retroviral vector; TSS, transcription start site.

AAV Vector Biology

7. High-Throughput Sequencing of AAV Proviral Libraries from the Human Population Reveals Novel Variants with Unprecedented Intra- and Inter-Tissue Diversity

Guangchao Xu[1], Li Luo[1], Phillip W. L. Tai[2], Wanru Qin[1], Yuanyuan Xiao[1], Chunmei Wang[1], Qin Su[2], Hong Ma[2], Ran He[2], Yuquan Wei[1], Guangping Gao[2]
[1]State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, China, [2]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA

Recombinant adeno-associated viruses (rAAVs) remain the most promising gene delivery vehicle for human gene therapy. The development of novel rAAVs with high transduction efficiency and specific tropisms primarily relies on PCR cloning of natural variants from primate tissues, or the engineering of capsids via directed evolution and rational design. Faithful clinical translation of leading therapeutic AAV serotypes derived from non-human tissues remains a hot-button issue. Here, we employed high-throughput variant profiling of natural AAV proviral libraries from the human population to identify novel AAV variants with unique tissue tropisms. We screened 844 surgical specimens, encompassing a wide range of tissue types and disease states from 455 patients by robust signature PCR. We found that >80% of human tissues are AAV positive with notable serotype frequencies (AAV2/3 chimera > AAV8 > AAV2 > AAV6) distributed among different tissues. Notably, we also used qPCR primers against the conserved AAV rep sequence to quantify the abundance of pAAV proviral genomes in human tissues.Next, to ensure the highest return on identifying novel variants, we employed single-molecule, real-time (SMRT) sequencing to characterize full-length rep and cap sequences. Our preliminary findings from a single patient tissue sample revealed more than 800 unique DNA sequence variants (originating from single nucleotide polymorphisms, genetic recombination, and de novo mutations) that account for more than 50 unique amino acid sequence variants. We also detected significant inter-patient variation, as well as high inter-tissue variability within individual patients. These findings suggest that AAV exhibits the capacity for high genomic diversity and accelerated intra-host evolution. These characteristics, which are shared among many other viruses, are presumed to promote survival against the host immune response, and fitness in tissue-specific cellular environments.

We also validated a selection of AAV8 variants by assessing their capacity for vector packaging and in vivo gene transfer. We found that a subset of variants has a stronger competency for transcytosis and tropism to liver and muscle than the conventional AAV8 serotype. Remarkably, one AAV8 variant can cross the blood-brain barrier and target the CNS more efficiently than AAV9 after intravenous injection of adult mice. Another noteworthy discovery is the sequence variability found in the assembly-activating protein (AAP) gene, which is encoded by an alternative open reading frame of the cap gene. The diversity within AAP may define the efficacy of AAV packaging among variants, and implicate its role in AAV evolution and fitness.

By exploiting AAV-host tissues as a natural incubator for viral evolution to potentiate new AAV capsid sequences, and employing a strategy to profile intact capsid sequences on a high-throughput scale, this study highlights the unprecedented diversity and evolutionary capacity of AAV. Importantly, these new findings reshape the versatility of rAAV as the ideal biotherapy for targeting a range of normal and/or diseased tissues.

8. Further Characterization of U2 snRNP Mediated Restriction of AAV Vector Transduction

Claire A. Schreiber[1], Yoshihiro Izumiya[2], Aravind Asokan[3], Yasuhiro Ikeda[1]
[1]Molecular Medicine, Mayo Clinic, Rochester, MN, [2]Dermatology, UC Davis School of Medicine, Sacramento, CA, [3]Gene Therapy Center and Department of Genetics, University of North Carolina School of Medicine, Chapel Hill, NC

Improving adeno-associated virus (AAV) vector transduction efficiency is central to the development of its continued, widespread use in gene therapy. More effective AAV transduction would reduce vector doses required for efficient gene delivery, minimizing the risks associated with high dose AAV vectors. Several treatments have been reported to increase AAV vector transduction, including adenoviral co-infection. Through screening of a siRNA library, we recently identified U2 snRNP as a host restriction factor for AAV vector transduction. Disruption of U2 snRNP spliceosome and associated proteins, including PHF5A, SF3B1, SF3B2 and U2AF1, potently enhanced AAV vector transduction. Relevant to gene therapy applications of AAV vectors, meayamycin B, a powerful SF3B1 inhibitor, allowed for substantial increases in AAV vector transduction (up to 400-fold). This post-entry restriction appeared to occur after the second-strand synthesis but before transgene expression or accumulation of transgene transcripts and independently of the cellular splicing machinery. No notable changes were found in the cytoplasmic trafficking, nuclear entry, or genome release of AAV vector infection by U2 snRNP inhibition. Here, we further studied the mechanism(s) underlying the U2 snRNP-mediated block of AAV vectors. We first tested another commercially available U2 snRNP inhibitor, pladienolide B (PladB) and verified that PladB treatment also showed substantial dose dependent increases (up to 700-fold) in AAV vector transduction. We next studied the relationship between adenoviral co-infection and U2 snRNP inhibition. Adenoviral co-infection alone enhanced AAV vector transduction up to 30-fold, while adenoviral co-infection, in addition to genetic or pharmacological U2 snRNP inhibition, showed marginal additive effects, suggesting a common pathway targeted by adenoviral co-infection and U2

snRNP inhibition in enhanced AAV vector infection. Using a series of plasmids providing adenoviral helper functions, we have identified Ad5 E4, but not the E2A and VA RNA genes, as partially responsible for the enhanced AAV transduction by adenoviral co-infection through U2 snRNP inhibition. Since the effects of U2 snRNP inhibition are most likely on the regulation of transgene expression, we also assessed the epigenetic modifications of the AAV2 genome in the presence or absence of PladB. Through chromosome immunoprecipitation (ChIP) assays we mapped histone recruitment patterns at different regions of the AAV vector epigenome. PladB treatment reproducibly enhanced recruitment of histone H2b and H3 proteins to the AAV CMV promoter, luciferase transgene, and polyA regions up to 3-fold. Our preliminary study also suggested that U2 snRNP inhibition altered specific histone modification patterns on the AAV vector genome. We are in the process of verifying the influence of U2 snRNP inhibition, as well as AAV capsid variations, on epigenetic modifications of the AAV vector epigenome. Better understanding the underlying mechanism would provide novel insights into host-virus interactions and could lead to the rational design of next generation AAV vectors with improved transduction efficiencies and safety profiles.

9. rAAV Designs Harboring DNA Secondary Structures with High Thermal Stabilities Produce Heterogenic Viral Genome Populations

Jun Xie, Ran He, Qin Su, Phillip W. L. Tai, Hong Ma, Jia Li, Guangping Gao

Horae Gene Therapy Center, UMASS Medical School, Worcester, MA

It has been known for more than a decade that AAV genomes in ssAAV particles are heterogeneous, and can contain smaller than unit-length molecules to varying degrees. However, the cause underlying this phenomenon remained unknown. Our earlier work demonstrated that short DNA hairpin (shDNA) sequences designed into rAAV vectors, resulted in truncated genomes through a template-switching mechanism during AAV genome replication. Importantly, the knowledge we have gained about shDNA-mediated genomic truncation has helped to improve shRNA-cassette design. Specifically, we have found a correlation between the thermal stability of shDNA structures with the prevalence of truncation events. By introducing point mutations into the passenger strand of shRNA sequences to create DNA bulges within shDNAs in scAAV vectors, we found that lowering the thermal stability of DNA hairpins lowered the proportion of truncated to complete genomes. In addition, we found that scAAV vectors incorporated with pri-miRNA transgenes, which contain natural bulges in their stem-loop structures, also produced fewer truncated genomes, relative to shDNA-rAAV constructs. By embedding the guide strand of small RNAs into pri-miRNA scaffolds, we have defined critical improvements to the genomic integrity of rAAV vectors expressing small RNAs. Furthermore, we developed a method, named AAV-GPSeq (AAV genome populations sequencing), to directly sequence whole vector genome populations from purified rAAVs using the PacBio platform for high-throughput sequencing. We discovered through this methodology that truncation events can originate from palindromic sequences and inverted repeats that reside in the expression cassette elements of widely used ssAAV and scAAV vector designs (i.e. transcriptional regulatory elements, transgene sequences, and post-transcriptional regulatory regions). The resulting diversity of genomic truncations produces populations of packaged virions with variable transgene efficacies, and gives us the first insights into the phenomenon of rAAV heterogeneity. By examining the genomes from rAAV vectors harboring shDNAs, pri-miRNA fragments, and palindromic/inverted repeat sequences, we now highlight the importance of DNA secondary structure on vector design and genomic heterogeneity in packaged viral vectors. Improvements upon quality control standards are thus necessary and critical to efficacious and safe clinical uses for rAAV as a biomedicine. Further implications for our novel findings towards understanding AAV replication, and new considerations for future therapeutic rAAV vector designs will be discussed. To improve the homogeneity of clinical rAAV vectors, we are optimizing the vector production procedure, testing non-palindrome promoters, changing the codon usage in transgenes to lower the thermostability of rAAV genomes, and modifying rAAV packaging cell lines to minimize template-switching events to produce more intact rAAV genomes.

10. High-Efficiency Transduction of Primary Human CD34$^+$ Hematopoietic Stem/Progenitor Cells by AAV6 Serotype Vectors: Strategies for Overcoming Donor Variation and Implications in Genome Editing

Zifei Yin[1], Kanit Bukhai[2], George Aslanidi[3], Chen Ling[3], Mengqun Tan[4], Mervin Yoder[5], Philippe Leboulch[2], Emmanuel Payen[2], Arun Srivastava[3]

[1]Traditional Chinese Medicine, Second Military Medical University, Shanghai, China, [2]Institute of Emerging Diseases and Innovative Therapies, University of Paris-Sud, Paris, France, [3]Pediatrics, University of Florida College of Medicine, Gainesville, FL, [4]Physiology, Central South University, Changsha, China, [5]Pediatrics, Indiana University School of Medicine, Indianapolis, IN

We first reported that of the 10 most commonly used AAV serotype vectors, AAV6 is the most efficient in transducing primary human bone marrow-derived CD34$^+$ hematopoietic stem/progenitor cells (HSPCs), both *in vitro* and in murine xenograft models *in vivo* (*Cytotherapy*, 15: 986-998, 2013; *PLoS One*, 8(3): e58757, 2013). More recently, two independent groups also reported successful transduction of primary human CD34$^+$ cells using the wild-type (WT) AAV6 vectors (*Sci. Transl. Med*., 7: 307ra156, 2015; *Nat. Biotechnol*., 33: 1256-1263, 2015), except that multiplicities of infection (MOIs) ranging from 100,000-200,000 vgs/cell were used to achieve ~40-55% transduction efficiency. Furthermore, the transduction efficiency of the WT AAV6 vector varies greatly in HSPCs from different donors, ranging between ~6-87%. Here we report two distinct strategies to further increase the transduction efficiency in HSPCs from donors that are transduced poorly with the WT AAV6 vectors. The first strategy involved modification of the viral capsid proteins where specific surface-exposed tyrosine (Y) and threonine (T) residues were mutagenized to generate a triple-mutant (Y705F+Y731F+T491V) AAV6 vector. The second strategy involved the use of *ex vivo* transduction at high cell density, which revealed a novel mechanism, which we have termed, 'cross-transduction'. The combined use of these strategies resulted in transduction efficiency exceeding 90% at an MOI of 20,000 vgs/cell in primary human cord blood-derived HSPCs at day 4 (Fig. 1A). scAAV6 vectors were more efficient than ssAAV6 vectors, but at high cell density, there was a modest enhancement in EGFP-positivity even with ssAAV6 vectors. However, 14 days post-transduction, virtually no EGFP-positive cells could be detected (Fig. 1B), suggesting the loss of vector genomes, and hence, the lack of stable integration of vector genomes in HSPCs. Our studies have significant implications in the optimal use of capsid-optimized AAV6 vectors in genome editing in HSPCs. *These authors contributed equally to this work #Co-corresponding authors

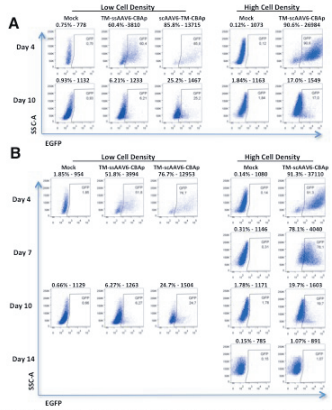


Figure 1. Transduction efficiency of TM ss and scAAV6 vectors in primary human CD34⁺ cells. (A) Primary human cord blood-derived CD34⁺ cells were either mock-transduced, or transduced at day 0 at low ($1x10^6$ cells/ml,) or high ($1x10^7$ cells/ml) cell density with 20,000 vgs/cell of the indicated AAV6 vectors in serum-free medium. Two hrs later, cells were diluted to $4x10^5$ cells/mL and switched to the expansion medium. EGFP expression was determined by FACS at day 4 and day 10 post-transduction. **(B)** Following mock-transduction, or transduction of CD34⁺ cells as described above, cells were switched to the expansion medium for 10 days, and cultured in an erythroid differentiation medium for an additional four days. EGFP expression was determined by FACS at day 14.

11. The Role of Glucocorticoid Receptor Signaling in Adeno-Associated Virus 2 Infection

Chen Ling[1], Jun Li[1], Daniel Zhang[1], George Aslanidi[1], Changquan Ling[2], Arun Srivastava[1]
[1]Pediatrics, University of Florida College of Medicine, Gainesville, FL, [2]Traditional Chinese Medicine, Second Military Medical University, Shanghai, China

We previously observed that purified recombinant glucocorticoid receptor (GR) protein specifically binds to the D-sequence within the inverted terminal repeat of the AAV2 genome because the D-sequence shares partial homology to the consensus half-site of the glucocorticoid receptor-binding element (GRE). The half-site is an essential core element, which has been reported to be sufficient to mediate GR signaling. Substitution of the D-sequence with the authentic GRE further increased AAV2 vector-mediated transgene expression. Based on these observations, we systemically examined whether AAV2 infection involves the GR signaling pathway. Our results showed that following infection by wild-type (WT) AAV2, or transduction with recombinant AAV2 vectors *in vitro*, GR was activated. The serine phosphorylated form of GR in whole cell lysates was significantly increased in a dose-dependent manner 18 hours post viral infection/vector transduction. Translocation and accumulation of GR in the nucleus was also increased correspondingly. To further corroborate our findings in an *in vivo* model, C57BL/6 mice were injected via tail-vein with either WT-AAV2 ($1x10^{12}$ vgs/mouse) or AAV2-EGFP vectors ($5x10^{11}$ vgs/mouse) and sacrificed 16 hours post virus/vector administration. A significant increase in the serine phosphorylated form of GR was also observed in liver tissues. We also observed that in cell cultures, dexamethasone, a well-known activator of GR, significantly enhanced rAAV2 vector-mediated transgene expression in various GR-positive cell lines, but not in a GR-deficient human osteosarcoma cell line, U2OS. Furthermore, stable transfection of U2OS cells with a GR expression plasmid not only facilitated AAV2 vector transduction, but also restored the ability of dexamethasone to further increase AAV2 vector-mediated transgene expression. Together with our previous data, we propose a model for elucidating the role of glucocorticoid receptor signaling pathway in AAV2 infection/AAV2 vector transduction (Fig. 1). Upon viral transduction or stimulation with glucocorticoids, the cytoplasmic GR is phosphorylated and translocated into the nucleus. Following AAV2 entry into the nucleus, the viral genome is released. Upon viral second-strand DNA synthesis or annealing of the complementary DNA strands, the activated GR binds to the double-stranded D-sequence in the viral inverted terminal repeats and consequently, enhances transgene expression. The activated GR also regulates its downstream pathways, such as NF-κB, which we have previously shown to have a substantial effect on AAV2 vector-mediated transgene expression (*Proc Natl Acad Sci USA*, 108: 3743-3738, 2011). A better understanding of the complex interaction between GR and NF-kB signaling pathways notwithstanding, the availability of the fully functional GRE site-substituted novel AAV vectors to achieve high-efficiency transgene expression has implications in the potential use of these vectors in human gene therapy.

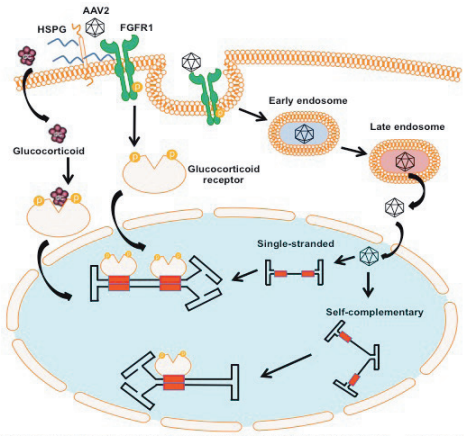


Figure 1: A schematic model for the role of glucocorticoid receptor signaling in AAV2 infection. Following AAV2 infection, or glucocorticoid stimulation, the cellular GR is phosphorylated and translocated into the nucleus, where it binds to the double-stranded D-sequence (red boxes) in the viral inverted terminal repeats and consequently, enhances transgene expression.

12. Assembly-Activating Protein Is Not an Essential Requirement for Capsid Assembly of Adeno-Associated Virus Serotypes 4, 5, and 11

Lauriel F. Earley[1], John Powers[2], Kei Adachi[2], Nancy L. Meyer[3], Qing Xie[3], Michael S. Chapman[3], Hiroyuki Nakai[2]
[1]Molecular Microbiology & Immunology, Oregon Health & Science University, Portland, OR, [2]Molecular & Medical Genetics, Oregon Health & Science University, Portland, OR, [3]Biochemistry & Molecular Biology, Oregon Health & Science University, Portland, OR

The mechanisms of capsid assembly for adeno-associated virus (AAV) is still incompletely understood, but delineating the role of the assembly-activating protein (AAP) in this process may lead to insight on this important step in vector production. AAP is a non-structural protein encoded within the cap gene in an overlapping open reading frame (ORF) that initiates near the N-terminus of the VP2 ORF and continues into the VP3 ORF. This protein has been shown to be essential for assembly of AAV2, 8, and 9 capsids; however, interestingly, we have recently found that AAV5 is capable of forming VP3-only capsids without AAP, raising a new question of what role AAP plays in the AAV5 virus life cycle. To further understand the functional role of AAP of AAV5 and other serotypes, it is important to investigate whether AAV5 would require AAP to form infectious capsids containing all three capsid proteins (VP1, VP2, and VP3) and whether other AAV serotypes exist that could form AAP-independent capsids. Here we show that AAV5 particles assembled in the absence of AAP are infectious and AAP-independent capsid assembly takes place for AAV4 and 11 as well but not for AAV12, a serotype that is phylogenetically closely related to AAV4 and 11. In the study, we created an AAV5 helper plasmid expressing AAV2 Rep and AAV5 Cap in which the AAP5 ORF was codon-modified extensively to prevent AAP expression while leaving the VP ORF intact. Using this AAP-KO helper plasmid and the standard AAV5 helper plasmid with an intact AAP ORF, we produced two types of AAV5 vectors expressing a marker gene, AAV5(AAP-) and AAV5(AAP+) vectors, respectively. These vectors were applied on CHO cells, and the marker

gene expression was quantified 2 days post-infection. This assay revealed that the AAP-independent AAV5(AAP-) vector was able to efficiently transduce CHO cells although the transduction efficiency was about a half of that with the AAV5(AAP+) vector. The yield of AAV5(AAP-) vector production was lower than AAV5(AAP+) vector by ~10 fold but restored by co-expression of AAP in trans, indicating that AAP retains the assembly promoting role even though AAP is not an absolute requirement for AAV5 capsid assembly. We next sought to determine if AAV4 and 11 could also assemble capsids without AAP. To address this question, we transfected HEK293 cells with codon-modified AAV4 or AAV11 VP3-expressing plasmids and purified the potential viral particles by cesium-chloride ultracentrifugation. Electron microscopy revealed viral particles consistent with AAV capsids. Given the close evolutionary relationship between AAV4, 11 and 12, we hypothesized that AAV12 would also be capable of AAP-independent capsid formation, but preliminary evidence has shown this hypothesis to be incorrect. We anticipate that closer examination of the dependence or independence of viral capsid assembly on AAP among various serotypes will reveal differences in VP structure or the assembly process that will illuminate the mechanistic function of AAP.

Adenoviruses

13. Functional Role of Adenovirus Penton in Modulating *In Vivo* Properties of Liver-Targeted and Liver-Detargeted Adenovirus Variants

Svetlana Atasheva[1], Corey Emerson[2], Nelson C. Di Paolo[1], Eric Irons[3], Neetu Gulati[2], Phoebe Stewart[2], Dmitry M. Shayakhmetov[1]
[1]Lowance Center for Human Immunology, Departments of Pediatrics and Medicine, Emory, Atlanta, GA, [2]Department of Pharmacology and Cleveland Center for Membrane and Structural Biology, Case Western Reserve University, Cleveland, OH, [3]Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY

Pharmacokinetic studies of adenovirus (Ad) vectors after intravascular delivery demonstrate that the majority of an administered virus dose is rapidly sequestered from the circulation by the liver. The molecular determinants that target Ad to the liver have been found on each of the virus capsid proteins, including hexon, penton, and fiber. The penton protein in the majority of Ad serotypes comprises a hypervariable loop containing an RGD motif that has been shown to interact with a number of cellular integrins upon virus entry into the cell. The binding of penton to integrins triggers virus internalization and relaxes the virus structure for subsequent endosomal disassembly. However, RGD motif interaction with integrins on tissue macrophages leads to production of inflammatory cytokines and significantly contributes to systemic Ad toxicity. Despite its importance for the virus entry into the cell, the penton is not well employed in the development of therapeutic Ad vectors. Therefore, in this study we evaluated strategies for modulating Ad penton interactions with cellular integrins. Ad vectors were created bearing substitutions of the RGD motif with mimetic non-RGD-containing peptides derived from extracellular proteins laminin 1 and laminin 3. These peptides are interacting with a different subset of integrins compared to those binging penton RGD motif. Unlike wild type (WT) Ad5, these penton-modified vectors were able to utilize β4 integrin for cell entry. The *in vivo* studies demonstrated that penton-modified Ads cannot activate a full inflammatory cascade and their *in vivo* toxicity, therefore, is dramatically reduced. Although the penton-mutated vectors were infecting a set of cancer cell lines *in vitro* and hepatocytes *in vivo* with a similar efficacy as the WT Ad5, interactions of virus with Kupffer cells and virus accumulation in the liver after intravascular vector delivery remain similar for penton-mutated and WT Ad vector. We also developed Ad vectors containing mutations abrogating virus interactions with all types of liver cells. Additionally to penton modifications, we introduced a T425A mutation in the hexon hyper variable loop HVR7 to completely abrogate virus interaction with blood coagulation FX and prevent transduction of hepatocytes. Furthermore, a set of mutations in the hexon HVR1 region were introduced to prevent Ad uptake by Kupffer cells. Due to penton modifications these vectors had very low toxicity and combinations of three mutations diverted the vector away from the liver. These data demonstrate that RGD loop modification represents a useful approach for introducing beneficial properties to the Ad vectors for greatly reducing their systemic toxicity and enable liver de-targeting. CryoEM analyses are currently underway to identify the structural differences in the capsid between the penton mutants and the wild type Ad.

14. Engineering and Characterization of a Cloned Adenoviral-Library to Explore Natural Virus Diversity

Wenli Zhang[1], Jun Fu[2], Jing Liu[1], Sebastian Janz[1], Maren Schiwon[1], Eric Ehrke-Schulz[1], Johannes Dörner[1], Philip Boehme[1], Manish Solanki[1], Thorsten Bergmann[1], Hailong Wang[2], Andreas Petzold[2], Roma Yumul[3], Andre Lieber[3], Francis Stewart[2], Anja Ehrhardt[1]
[1]University Witten/Herdecke, Witten, Germany, [2]Dresden University of Technology, Dresden, Germany, [3]University of Washington, Seattle, WA

Large double-stranded DNA (dsDNA) viruses such as adenovirus (Ad), herpesvirus, poxvirus and their recombinant counterparts were well explored in basic virology and biomedical research. Especially for the development of novel vaccines and gene therapies, Ad gained special attention and represents the most widely explored vector worldwide. Although ~70 types of human Ad and numerous nonhuman Ad (>200) have been identified so far a system for efficient Ad genome cloning and manipulation was lacking and therefore the majority of recombinant adenoviral vectors (AdVs) are based on a small fraction of Ad types. Here we report the generation and characterization of an engineered human adenoviral-library allowing exploration and system studies of the natural Ad diversity. Towards that end we first established that adenoviral genomes can be cloned and tagged in a high-throughput manner utilizing advanced homologous recombination techniques. Wild type Ads from clinical isolates including around half of the currently known adenovirus types that represent all seven human adenovirus species were propagated and direct high-throughput cloning (HTC) applied. The integrity of cloned Ad genomes was confirmed by DNA restriction enzyme pattern and virus reconstitution was conducted using optimized conditions. Most importantly next-generation sequencing (NGS) and phylogenetic analysis was performed. As a further step, half of all cloned adenoviruses representing each species were tagged with a 2A peptide-mediated multicistronic expression cassette providing a Turbo Green fluorescent protein as *in vitro* marker, a NanoLuc luciferase for *in vivo* studies and kanamycin/neomycin as selection marker. For HTC of tagged viruses the reporter cassette was inserted into the adenovirus E3 region using different orientations because the orientation of the transgene was essential for reconstitution efficiencies. After successful reconstitution these double reporters-labeled AdVs were evaluated in cultured cell lines and mouse models. *In vitro* characterization revealed distinct tropisms for tested viruses. Among the currently evaluated cell lines, specie B viruses demonstrated high transduction efficiencies in epithelial (Hela- and A549 cells) and endothelial cells, while Ad5 still displayed highest transduction rates in other human and murine cell types (hepatocytes, lymphocytes, neuroblastoma cells and myoblasts). For the further characterization of unknown receptor usage, we injected recombinant viruses into DSG2 or CD46

transgenic mice for *in vivo* bio-distribution analyses on genome level by quantitative PCR and on protein level by immunohistology analyses. We anticipate that our engineered adenoviral-library will provide a spacious novel view to the adenovirus field. As a broader perspective it will bring AdV and also other large dsDNA viruses from mono- to multi-types and enables broader applications in molecular medicine including gene therapy and vaccination studies, as well as basic virology.

15. A Novel Fast Production Pipeline for High Capacity Adenoviral Vectors to Deliver All Components of CRISPR/Cas9 System for Somatic Gene Editing Using One Single Viral Vector with Multiple Guide RNAs

Eric Ehrke-Schulz, Theo Leitner, Stephan Dávid, Jing Liu, Maren Schiwon, Thorsten Bergmann, Anja Ehrhardt
Human Medicine, Institute for Virology, Witten/Herdecke University, Witten, Germany

The discovery of the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system changed the field of *in vivo* genome editing. Nevertheless viral delivery of all required components including Cas9 and one or multiple guide RNA (gRNA) expression units using one single viral vector has not been fully exploited. Gene deleted high-capacity adenoviral vectors (HCAdVs) have the potential to efficiently deliver all expression units of the complete CRISPR/Cas9 machinery including multiple gRNAs into a broad variety of target cells using a single viral vector. However, the complicated handling of large DNA constructs and the time consuming production procedure hampered the use of HCAdV to deliver the CRISPR/Cas9 machinery for genome editing approaches. This work aimed at adapting a toolbox for HCAdV genome manipulation for the fast and simple introduction of the customized CRISPR/Cas9 machinery to provide new instruments to improve somatic genome editing approaches in mammalian cells. We generated a new CRISPR/Cas9 shuttle plasmid toolbox containing the Cas9 nuclease gene either utilizing a constitutive or an inducible promotor and a gRNA expression unit enabling customizing the CRISPR/Cas9 for a desired target sequence. This allows cloning or recombining all CRISPR/Cas9 components into the HCAdV genome in one step. To use several gRNA expression units for multiplexing the CRISPR/Cas9 system, further gRNA expression units can be easily included. To enable fast assembly of recombinant CRISPR-HCAdV genomes we used DNA recombineering to introduce all CRISPR/Cas9 expression units into the HCAdV genome contained in the bacterial artificial chromosome pBHCA. For insertion of multiple gRNA expression units into the HCAdV genome we utilized the established pAdV-FTC plasmid in concert with homing endonuclease directed cloning. CRISPR-HCAdVs were produced using a shortened amplification and purification procedure. Exploiting our toolbox we produced several CRISPR-HCAdVs carrying single and multiplex gRNA units specific for different targets including hCCR5, hDMD, and HPV16- and HPV18-E6 genes yielding sufficient titers within a short time. T7E1 assays were applied to prove CRISPR/Cas9-mediated cleavage of respective targets and infection of cultured human cells with respective CRISPR-HCAdVs resulted in efficient site specific gene editing. In summary, this new platform enables customization, cloning and production of CRISPR-HCAdV vectors for single or multiplex approaches within a short time. It simplifies the delivery of the CRISPR/Cas9 machinery by only using one single viral vector. Inducible Cas9 expression helps to avoid targeting of the genome of producer cell lines during vector production and may be beneficial for special approaches where constitutive expression is unwanted. We speculate that this may pave the way for broader applications of the CRISPR technology in preclinical and eventually clinical studies.

16. STAT2 Knockout Syrian Hamsters Support Enhanced Replication and Pathogenicity of Human Adenovirus Type 5, Revealing an Important Role of Type I Interferon Response in Viral Control

Karoly Toth[1], Sang R. Lee[2], Baoling Ying[1], Jacqueline F. Spencer[1], Ann E. Tollefson[1], John E. Sagartz[3], Il-Keun Kong[4], Zhongde Wang[5], William Wold[1]
[1]Molecular Microbiology & Immunology, Saint Louis University, St. Louis, MO, [2]Animal, Dairy & Veterinary Sciences, Utah State University, Logan, UT, [3]Comparative Medicine, Saint Louis University, St. Louis, MO, [4]Animal Science, Gyeongsang National University, Jinju, Korea, Republic of, [5]Animal Science, Utah State University, Logan, UT

Oncolytic vectors based on human species C adenovirus type 5 (Ad5) are being developed for cancer gene therapy. Studies on wild-type Ad5 in animal models are likely to provide important insights into the behavior of these vectors in patients. The most frequently used permissive immunocompetent animal model for Ad5 infection is the Syrian hamster. Ad5 (and Ad6) replicates in these animals and causes pathology that is similar to that seen with humans. Here, we report findings with a new Syrian hamster strain in which the STAT2 gene was functionally knocked out (KO) by site-specific CRISPR/Cas9-mediated gene targeting. STAT2 is a critical element of the Type I and Type III interferon signal transduction pathways. STAT2 KO hamsters infected intravenously with Ad5 demonstrated an accentuated pathology compared to the wild-type control animals, and the virus load in the organs (liver, lung, kidney) of STAT2 KO animals was 100- to 1000-fold higher than that in wild-type hamsters. We show that the Type I interferon pathway is disrupted in these hamsters, inasmuch as the interferon response genes PKR, OAS, and Mx2 were induced by Ad5 in the liver of wild-type but not STAT2 KO hamsters, revealing a critical role of interferon-stimulated genes in controlling Ad5 infection. Notably, the adaptive immune response to Ad5 is not adversely affected in STAT2 KO hamsters, and surviving hamsters cleared the infection by 7 to 10 days post challenge. In fact, anti-Ad5 neutralizing antibodies were 10-fold higher at 7 days postinfection in the STAT2 KO hamsters than in wild-type hamsters. T cell infiltration into the liver was similar at 3 and 7 days in the STAT2 KO and wild-type hamsters. Treatment of Ad5-infected STAT2 KO hamsters with high dose cyclophosphamide resulted in markedly increased mortality, pathogenesis, and virus replication in the liver (~10^{11} $TCID_{50}$/g liver) at 10 days postinfection, likely because the adaptive immune response as well as other aspects of the innate response were abolished. This is the first study to report findings with a genetically modified Syrian hamster infected with human adenovirus. Further, this is the first study to show that the Type I interferon pathway plays a role in inhibiting Ad5 replication in a permissive animal model. This conclusion is in accord with a previous study in which we showed using a custom microarray platform that there is a robust up-regulation of genes involved in the innate immune response (e.g. OAS, PKR, IFN-inducible protein 10) in the liver at 18 h post-intravenous infection of wild-type Syrian hamsters with Ad5 (Ying, B. et al., Virology 485, 305, 2015). Besides providing an insight into adenovirus infection in humans, our results underscore the usefulness of Syrian hamsters as a permissive model for the study of species C human adenovirus and adenovirus-based vectors. In future studies, STAT2 KO hamsters bearing tumors could be used to explore the role of Type I and Type III interferon signaling on the efficacy of oncolytic adenovirus vectors.

17. Human Adenovirus D17 Has Tropism for Endothelium Cells and Can Use Both hCAR and CD46 as Receptors

Jing Liu[1], Philip Boehme[1], Jun Fu[2], Wenli Zhang[1], Manish Solanki[1], Roma Yumul[3], Achim Schmidtko[4], Francis Stewart[2], Andre Lieber[3], Anja Ehrhardt[1]
[1]University Witten/Herdecke, Witten, Germany, [2]Dresden University of Technology, Dresden, Germany, [3]University of Washington, Seattle, WA, [4]University Frankfurt, Frankfurt, Germany

Human adenovirus type 5-derived vectors (HAdV5) from species C were broadly explored for gene therapeutic approaches and vaccination. However, disadvantages associated with this vector type are the strong liver tropism in vivo in mice, preexisting immunity and induction of robust immune responses. There is growing interest in exploring other adenovirus types of which >69 were identified. Here we constructed a new first generation adenovirus labelled with green fluorescent protein marker based on human adenovirus D17 which was first isolated from conjunctival scrapings in 1955 and aimed at characterizing the vector in vitro and in vivo. We applied a new homologous recombineering technology to construct GFP labelled early E1 gene deleted HAdV17 and HAdV5, rescued viruses in complementary stable cell lines, and then screened a panel of different cell lines by FACS analyses and quantitative PCR. Competition assays based on soluble recombinant fiber knob blocking reagents (5knob, 17knob, JO4, Augmab) were used to characterize the receptor interaction in vitro. In vivo biodistribution analyses were performed after intravenous injection of recombinant viruses into normal and CD46 transgenic mice. We observed that HAdV17 has tropism for endothelium cells which are normally refractory to HAdV5 infection. This finding was further verified using primary human umbilical vein endothelial cells (HUVEC). Moreover, after performing competition assays we found that HAdV17 can utilize both CD46 (a membrane cofactor protein which is expressed on all nucleated cells) and CAR (coxsackievirus and adenovirus receptor) as cell attachment receptors. The endothelium tropism was CD46-dependent and could be blocked by the CD46 blocking reagent Ad35K++/Augmab. In vivo biodistribution studies showed significantly increased vector genome copies (VCN) in various organs of human CD46 transgenic mice compared to normal mice indicating involvement of CD46 as a receptor. Immunohistological analyses using cell-specific marker are ongoing. Neutralizing antibody assays revealed that there was less seroprevalence with HAdV17 compared to HAdV5. In total, we believe that HAdV17-based vectors, which can use both hCAR and CD46 as receptors, hold great promise for gene therapy in endothelial disease. The understanding of the molecular interaction between virus and host will be beneficial for vaccination and drug development.

18. Delivery of CRISPR/*Cas*9 or TALENs Against Hepatitis B Virus cccDNA by High-Capacity Adenoviral Vectors

Maren Schiwon, Thorsten Bergmann, Eric Ehrke-Schulz, Anja Ehrhardt
Witten/Herdecke University, Witten, Germany

High-capacity adenoviral vectors (HCAdV) are superior tools to deliver large DNA cargos into living cells. These vectors are devoid of all adenoviral coding sequences, leaving only essential adenoviral DNA sequences which are required for virus replication and packaging of HCAdV genomes containing foreign DNA. Here HCAdVs were utilized in combination with designer nucleases to develop a cure for chronic hepatitis B virus (HBV) infection. Although advanced prevention measures are available, chronic HBV infection still presents a serious global health burden for which no cure exists. The hepatitis B virus genome forms a persistent DNA species in infected cells (covalently closed circular DNA, cccDNA) and in that way is able to convert the infection into a chronic state. A chronic carrier state makes the sufferers susceptible for cirrhosis and liver cancer. To date various versions of designer nucleases against the HBV genome were already devised but yet no adequate transfer to HBV-infected cells *in vitro* and especially *in vivo* was presented. In the present study the potential of designer nucleases as a tool to cure chronic HBV infection was investigated and the aim was to cut and therefore destroy the HBV-DNA intracellularly. We employed *transcription factor-like effector nucleases* (TALENs) and the *clustered regularly interspaced short palindromic repeats / Cas*9 (CRISPR/*Cas*9) system and additionally adapted HCAdV as an efficient delivery method. This is the advancement of this study in which either both TALEN subunits of a pair or multiple guide RNA expression cassettes alongside with a *Cas*9 coding sequence including promoter and termination signal were co-delivered in one vector. The expression cassettes including all compounds of both systems were inserted into HCAdVs. Vectors were amplified in large scale, their integrity monitored and then tested on liver cell lines which were infected with HBV. For this purpose we established an HBV infection model which is based on a HBV genome which was also inserted into a HCAdV. The effect of the designer nucleases on the HBV genome and its transcription was assayed by qPCR, a mutation detection assay and HBsAg ELISA. Our data revealed 80% reduction of hepatitis B surface antigen production in designer nuclease treated cells in comparison to untreated or mock treated cells. Furthermore cells treated with nucleases resulted in a decreased HBV genome copy number and the introduction of mutations could be demonstrated by a mutation detection assay using T7 endonuclease I. Note that the CRIRSPR/*Cas*9 system was superior to the TALEN based construct. In conclusion, we demonstrated delivery of a complete TALEN pair as well as a CRISPR/*Cas*9 construct containing three guide RNAs by use of just one HCAdV, respectively, which after application resulted in effective reduction of HBV parameters. Future objectives are to test our vectors in animal models of HBV infection and eventually to optimize the vector for the needs of this application.

Cardiovascular and Pulmonary Diseases

19. Using a Novel Combinatorial Non-Viral Vector and Small Molecules to Treat Familial Hypercholesterolaemia (FH)

Alastair G. Kerr[1], Lawrence Tam[1], Milena Cioroch[1], Ashley Hale[2], Gillian Douglas[2], Joe Mason[1], Carole Bataille[1], Graham Wynne[1], Angela Russell[1], Keith Channon[2], Richard Wade-Martins[1]
[1]Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom, [2]Welcomme Trust Centre for Human Genetics, University of Oxford, Oxford, United Kingdom

Familial hypercholesterolaemia (FH) is a life-threatening genetic disorder characterised by elevated levels of plasma low density lipoprotein cholesterol (LDL-C). Loss-of-function mutations in the gene encoding the low density lipoprotein receptor (LDLR) are responsible for ~85% of all FH cases. We have previously generated mini-gene vectors carrying the human *LDLR* cDNA, driven by 10 kb of genomic DNA from the native human *LDLR* locus, encompassing a promoter region with all essential elements required for physiologically regulated expression (pLDLR-LDLR). We demonstrated that incorporation of the genomic DNA promoter elements resulted in long-term physiologically-regulated *LDLR* transgene expression that complemented Ldlr deficiency. Here we further enhance *LDLR* transgene expression by characterising and cloning in a miRNA, targeting *Hmgcr* (miR82) generating a combinatorial RNAi-LDLR vector (pLDLR-LDLR-miR82). We

show in vivo that the combinatorial vector efficiently suppresses endogenous Hmgcr transcripts, which leads to an increase in *LDLR* transgene expression through induction of the *LDLR* promoter. In a preliminary study the pLDLR-LDLR-miR82 vector was able to significantly reduce total and LDL-C, in *Ldlr*$^{-/-}$ mice fed a 1% cholesterol diet at two and four weeks post vector delivery. We then carried out a longer term study in *Ldlr*$^{-/-}$ mice fed a 0.25% cholesterol diet, LDLR expression could be detected 12-weeks post-delivery with the plasmid able to be rescued as a functioning episome. LDL-C was significantly lowered throughout the study and this resulted in reduced atherosclerosis in the pLDLR-LDLR-miR82 vector treated mice. Here we demonstrate for the first time, that an episomal non-viral vector is able to significantly reduce LDL-C and the progression of atherosclerosis in a mouse model of FH. Based on the success of the miRNA to further enhance the lipid lowering of our non-viral vector, we went on to screen small molecules which can drive the expression of the human LDLR promoter. 216 molecules were screened, where we identified a novel series of small molecules that increased the LDLR in human and mouse cell lines at nano molar potencies. We then went on to elucidate the mechanism of action as squalene synthase inhibitors and demonstrated, that when used in combination with statins, these compounds give a much greater increase in LDLR expression than statins alone. These small molecules could be used in conjunction with low dose statins, alongside our non-viral vector to further enhance expression of the LDLR and provide greater therapeutic outcome for patients of FH.

20. A Novel Approach in the Treatment of Dystrophic Cardiomyopathy

Mona El Refaey, Li Xu, Jing Xu, Lixia Zhao, Yandi Gao, Renzhi Han

Surgey, Wexner Medical Center, Columbus, OH

Duchenne muscular dystrophy (DMD) is the most common muscular dystrophy with a worldwide incidence of one in 3500 live male births. It is caused by the lack of dystrophin, a critical muscle protein that connects the cytoskeleton and the extracellular matrix (ECM). Cardiomyopathy develops in at least 90% of patients and alone can shorten the life expectancy of DMD patients by at least 2 years and up to 40% of DMD patients eventually die from heart failure. Recently, RNA-guided, nuclease-mediated genome editing based on type II CRISPR (clustered regularly interspaced short palindromic repeat)/Cas (CRISPR-associated) system, has been emerged to alter the genome. In this study, we hypothesize that CRISPR-mediated genome editing could offer a novel therapy for DMD-associated cardiomyopathy in live mice. Two gRNA target sites were chosen from intron 20 and 23 of mouse *Dmd*. Co- transfection of the two gRNA with cas9 plasmids into mouse C2C12 cells resulted in the detection of a small PCR product as predicted, indicating successful CRISPR-mediated genome editing. DNA sequencing confirmed that the transcripts from C2C12 cells treated with gRNA/cas9 were formed due to successful deletion of exons 21-23 of mouse *Dmd*. Moreover, we injected the adenoviral vectors carrying GFP-2A-cas9 and gRNAs systemically and locally into the newborn pups. Four weeks after adenovirus transduction, dystrophin expression was restored in the heart muscles positive for GFP. Our PCR and western blotting data demonstrated that in-frame deletion of the genomic DNA covering exon 23 restored functional dystrophin expression in the hearts of mdx mice. Immunofluorescence staining also demonstrated that β-dystroglycan, which is normally located to the sarcolemma in healthy heart muscles via interaction with dystrophin-glycoprotein complex, was also restored at the sarcolemma of GFP-positive heart muscle fibers. These data provide the proof evidence of systemic restoration of dystrophin in the hearts of live mice.

21. Pulmonary Macrophage Transplantation (PMT) Therapy of Hereditary Pulmonary Alveolar Proteinosis (hPAP) Is Effective with Mature Macrophages and Does Not Require Myeloid Precursor/Progenitor Expansion

Takuji Suzuki[1], Paritha Arumugam[1], Anthony Sallese[1], Nico Lachmann[2], Claudia Chalk[1], Diane Black[1], Brenna Carey[1], Thomas Moritz[2], Bruce C. Trapnell[1]

[1]Translational Pulmonary Science Center, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, [2]Institute of Experimental Hematology, Hannover Medical School (MHH), Hannover, Germany

Rationale: Hereditary Pulmonary Alveolar Proteinosis (hPAP) is caused by mutations in the *CSF2RA* or *CSF2RB* genes (encoding GM-CSF receptor α or β, respectively) leading to defective GM-CSF-dependent surfactant clearance by alveolar macrophages, resulting in severe respiratory failure. Recently, using a validated model of hPAP *(Csf2rb*$^{-/-}$ mice), we reported a novel Pulmonary Macrophage Transplantation (PMT) approach as a safe, effective, tissue-specific and durable therapy for hPAP disease (*Nature*. 2014, 514: 450). Our results showed that wild-type bone marrow-derived macrophages (BMDMs) delivered by PMT successfully engraft, proliferate, gradually replace functionally deficient endogenous alveolar macrophages and efficiently clear surfactant in *Csf2rb*$^{-/-}$ recipient mice resulting in durable hPAP disease correction, in the absence of myeloablation. However, these studies did not exclude the possibility of an expanded myeloid progenitor population as the effector cell in PMT therapy of hPAP. Because the transplanted cells were capable of clearing surfactant, we hypothesized that the therapeutic efficacy of PMT is mediated by mature macrophages without obligate expansion of any myeloid progenitor/stem cell. **Methods:** BMDMs or alveolar macrophages were obtained from wild-type mice and intrapulmonary administered into *Csf2rb*$^{-/-}$ mice by PMT ($2.5x10^5$ per mouse). The therapeutic efficacy was evaluated at 2 months after PMT by measuring the optical density (OD λ= 600 nm) of bronchoalveolar lavage (BAL) turbidity - an excellent measure of overall PAP disease severity. **Results:** Characterization prior to transplantation showed that BMDMs were highly purified, mature macrophages: they had the morphologic appearance and surface markers of mature macrophages, clonogenic analysis indicated they contained less than 0.005% CFU-GM and no BFU-E, or CFU-GEMM progenitors, and they were able to clear surfactant in vitro. To further increase macrophage purity, BMDMs were sorted by flow cytometer by applying a conservative gating strategy to isolate $CD11c^{Hi}F4/80^{Hi}$ macrophages. PMT using these highly uniform, mature $CD11c^{Hi}F4/80^{Hi}$ BMDMs into *Csf2rb*$^{-/-}$ mice showed extraordinary therapeutic efficacy as evidenced by marked reduction of BAL turbidity compared to untreated, age-matched *Csf2rb*$^{-/-}$ mice (OD = 0.9±0.1 vs 2.3±0.2; n=4; P<0.01). As a second approach to exclude the possibility of an expanded hematopoietic progenitor as the therapeutic effector cell of PMT therapy, highly purified $CD11c^{Hi}F4/80^{Hi}$ alveolar macrophages were delivered by PMT into *Csf2rb*$^{-/-}$ mice. Mature alveolar macrophages were equally efficacious in hPAP disease correction, as shown by the marked reduction of BAL turbidity compared to untreated, age-matched *Csf2rb*$^{-/-}$ mice (OD = 1.5±0.1 vs 2.3±0.2; n=4; P<0.01). All donor cells analyzed at 2 months after PMT were of $CD11c^{Hi}F4/80^{Hi}$ phenotype. **Conclusions:** Results demonstrate that mature macrophages are the effector cells of PMT therapy, excluding a requirement for progenitor population expansion in hPAP disease correction. These observations have important implications in clinical trial design for translating PMT therapy to human children with hPAP.

22. Post-Exposure Vaccination by Capsid-Modified AdC7 Vector Expressing *Pseudomonas aeruginosa* OprF in Chronic *P. aeruginosa* Lung Infection

Anurag Sharma, Rika Gomi, Wenzhu Wu, Biin Sung, Stefan Worgall
Weill Cornell Medicine, New York, NY

Chronic infections by *Pseudomonas aeruginosa* are a common problem in cystic fibrosis and other chronic lung diseases associated with bronchiectasis. With antimicrobial resistance of *P. aeruginosa* becoming more common, alternate prophylactic and therapeutic approaches are needed. Despite extensive efforts, a vaccine against *P. aeruginosa* is not yet available. A post-exposure vaccine that eliminates already established *P. aeruginosa* from the respiratory tract, could be useful in the management of chronic *P. aeruginosa* colonization. Replication-deficient adenoviral (Ad) vectors are an attractive platform for vaccines against respiratory pathogens. We have previously found that, in addition to circumvent pre-existing anti-human Ad immunity, a non-human primate-based AdC7 vector expressing outer membrane protein F (OprF) of *P. aeruginosa* (AdC7OprF) was more potent in inducing lung mucosal and protective immunity compared to a human Ad5-based vector. In addition, genetic modification of the AdC7 fiber to display an integrin-binding arginine-glycine-aspartic acid (RGD) sequence can further enhance mucosal protective immunogenicity of AdC7OprF. In this study we investigated if post-exposure vaccination by AdC7OprF.RGD can clear the already established *P. aeruginosa* in a mouse model. Intratracheal inoculation of *P. aeruginosa* (clinical strain RP73) encapsulated in agar beads (10^6 cfu/mouse) was used to establish persistent infection. Intranasal immunization of *P. aeruginosa* infected mice with AdC7OprF.RGD (10^10 pu/mouse) induced significantly high serum anti-OprF IgG antibodies as early as 1 week of immunization that further increased to higher levels after 2 week of immunization compared to AdC7Null or PBS inoculated mice ($p<0.05$; all comparisons). In addition to robust humoral response, immunization with AdC7OprF.RGD induced OprF-specific T-cell responses, as indicated by the higher secretion of IFN-γ or IL-4 from the OprF-stimulated cultured splenocytes compared to AdNull or PBS inoculated mice ($p<0.05$; all comparisons). Importantly, the AdC7OprF.RGD immunized mice showed significantly higher clearance of *P. aeruginosa* from the infected lungs after 1 week or 2 weeks of immunization ($p<0.05$; all comparisons). In fact, after 2 weeks of immunization, 50% of AdC7OprF.RGD immunized mice (3 out of 6) completely cleared the *P. aeruginosa* from the infected lungs. These data suggest that immunization with AdC7OprF.RGD induced robust humoral and cellular anti-*P. aeruginosa* immunity that could clear established pulmonary *P. aeruginosa* infections.

23. SiRNA and CRISPR/Cas9 Mediated Knockout of αENAC

Aristides Tagalakis[1], Joost van Haasteren[1], Ahmad Aldossary[1], Luca Rosa[1], Mustafa Munye[1], David McCarthy[2], Stephen Hart[1]
[1]UCL Institute of Child Health, London, United Kingdom, [2]UCL School of Pharmacy, London, United Kingdom

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis conductance regulator (CFTR) gene, which encodes for a chloride channel. Loss of CFTR upregulates the epithelial sodium channel (ENaC) causing hyperabsorption of sodium, reducing the watery lining of the lung, leading to impaired mucociliary clearance and enabling inflammatory lung damage. Therefore, inhibition of ENaC has been proposed as a treatment in CF. In this study we are assessing the use of siRNA and CRISPR/Cas9 system as complementary methods of achieving disruption of ENaC expression in airway epithelial cells using receptor-targeted nanocomplexes. We sought to compare the efficiency and persistence of silencing, repeatability of delivery and its toxicity. We assessed silencing of the αENaC subunit by transfecting primary CFBE cells growing in Air-Liquid Interface cultures with siRNA-bearing nanocomplexes and achieved 30% silencing at the mRNA level. We then assessed their silencing efficiency in lungs of normal mice (C57BL/6) delivered by oropharyngeal instillation. Following a single dose, the siRNA formulations achieved ~30% knockdown of mouse αENaC. Following repeated delivery (3 doses at 48h intervals) we found out that 48h after the last administration ~50% silencing was achieved, with no adverse effects (as judged by body weight and histology), whilst following a single administration there was still ~30% silencing 7 days later. An algorithm was used to design 6 different guide RNA targets for αENaC. These were used to transfect cells along with Cas9 and showed varied levels of indel mutation rates in the αENaC gene by the T7 endonuclease I assay although two, T3 and T4, were optimal. In order to increase the amount of indels created we subjected the cells (CFTE and HBE) to sequential transfections of the same target at 48h intervals. This showed an accumulation of indels in cells targeted with T3 at a rate of 33.3% in HBE cells. This correlated with a 60% decrease in ENaC mRNA compared to controls and with 40% after a single dose to 65% decrease in protein levels after three doses. The siRNA-mediated silencing showed that we can repeatedly deliver the formulations with no adverse effects and that the effect of a single dose, although transient, can last for at least 1 week. CRISPR/Cas9 also mediated efficient gene disruption of αENaC, but with the advantage that this knockdown will be permanent in cells where the life span is quite long. Another encouraging finding was the accumulation of gene disruption with both systems when delivered by a non-immunogenic vector. This is very important as CF therapy would need repeated administrations. It is reasonable to anticipate that these systems offer new prospects to CF gene therapy.

24. Sustained Expression with Partial Correction of Neutrophil Defects 5 Years After Intramuscular rAAV1 Gene Therapy for Alpha-1 Antitrypsin Deficiency

Christian Mueller[1], Jeffrey D. Chulay[2], Noel G. McElvaney[3], Gwladys Gernoux[1], Emer P. Reeves[3], Farshid N. Rouhani[4], Margaret Humphries[1], Alisha M. Gruntman[5], Martha Campbell-Thompson[6], James M. Wilson[7], Terence R. Flotte[8]
[1]Pediatrics and Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA, [2]Vice President and Chief Medical Officer, AGTC, Alachua, FL, [3]Medicine, Royal College of Surgeons in Ireland, Dublin, Ireland, [4]University of Florida, Gainesville, FL, [5]Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA, [6]Pathology, University of Florida, Gainesville, FL, [7]Gene Therapy Program, Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, [8]Pediatrics, Gene Therapy Center and Department of Microbiology and Physiologic Systems, University of Massachusetts Medical School, Worcester, MA

Alpha-1 antitrypsin (AAT) deficiency is a common monogenic disorder resulting in emphysema, which is currently treated with weekly infusions of protein replacement. We previously reported achieving plasma wild-type (M) AAT concentrations at 2.5-3.8% of the therapeutic level at 1 year after intramuscular (IM) administration of 6×10^{12}vg/kg of a recombinant adeno-associated virus serotype 1 (rAAV1)-AAT vector in AAT-deficient patients, with an associated regulatory T cell (Treg) response to AAV1 capsid epitopes in the absence of any exogenous immune suppression. Here, we report sustained expression, at >2% of the therapeutic level, for 5 years

after one-time treatment with rAAV1-AAT in an AAT-deficient patient from that study, with the partial correction of neutrophil defects previously reported in AAT-deficient patients. There was also evidence of an in-situ Treg response and an exhausted $CD8^+$ T cell response to AAV1 capsid. These findings suggest that muscle-based AAT gene replacement is tolerogenic and that very stable levels of M AAT may exert beneficial effects at lower concentrations than previously anticipated.

Delivery Questions for Neural Applications

25. Intranasal Gene Delivery of AAV Iduronidase: An Effective and Non-Invasive Approach for Treatmentof CNS Disease in a Murine Model of MPS Type I

Lalitha Belur[1], Megan Buckvold[1], Kelly M. Podetz-Pedersen[1], Maureen Riedl[2], Lucy Vulchanova[2], Leah Hanson[3], Carolyn Fairbanks[2], Karen Kozarsky[4], William H. Frey II[3], Walter C. Low[5], R. Scott McIvor[1]
[1]Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, [2]Neuroscience, University of Minnesota, Minneapolis, MN, [3]Center for Memory and Aging, Health Partners Research Foundation, Regions Hospital, St. Paul, MN, [4]REGENXBIO Inc., Rockville, MD, [5]Neurosurgery, University of Minnesota, Minneapolis, MN

Mucopolysaccharidosis type I (MPS I) is a progressive, multisystemic, inherited metabolic disease caused by deficiency of alpha-L-iduronidase (IDUA). The most severe form of this disease (Hurler syndrome) results in death by age 10. Current treatments for this disease are ineffective in treating CNS disease due to the inability of lysosomal enzymes to traverse the blood-brain barrier. Our goal is to supplement current therapy, and treat CNS manifestations of the disease, by AAV-mediated gene delivery and expression of IDUA.We have taken a non-invasive and effective approach to the treatment of CNS disease by intranasal administration of an IDUA-encoding AAV9 vector. Adult IDUA-deficient mice were immunotolerized at birth with human iduronidase, to prevent anti-IDUA immune response, and at 3 months of age were infused intranasally with AAV9-IDUA vector. Animals sacrificed 3 months post-infusion exhibited IDUA enzyme activity levels that were 100-fold that of wild type in the olfactory bulb, with wild type levels of enzyme restored in all other parts of the brain. Neurocognitive testing using the Barnes maze demonstrated that treated IDUA-deficient mice were not different from normal control animals, while untreated IDUA-deficient mice exhibited a significant learning deficit. There was strong IDUA immunofluorescence staining observed in tissue sections of the nasal epithelium and olfactory bulb, but no staining was observed in other portions of the brain. This indicates that the widespread distribution of IDUA enzyme most likely was the result of enzyme diffusion from sites of transduction and IDUA expression in the olfactory bulb and the nasal epithelium into deeper areas of the brain. In order to increase access, delivery and vector distribution throughout the brain, IDUA-deficient animals were pretreated with intranasal infusions of an absorption enhancer. At different time points following pretreatment, animals were infused intranasally with AAV9 or AAVrh10 vector encoding IDUA. Animals were sacrificed at 2 months post-infusion, brains microdissected, and assayed for IDUA enzyme activity, clearance of glycosaminoglycans, and immunofluorescence staining for IDUA and GFP. This novel, non-invasive strategy for intranasal AAV9-IDUA administration could potentially be used to treat CNS manifestations of MPS I.

26. Protein Delivery of an Artificial Transcription Factor Restores Widespread Ube3a Expression in an Angelman Syndrome Mouse Brain

Barbara Bailus, Benjamin Pyles, Michelle McAlister, Henriette O'Geen, Sarah Lockwood, Alexa Adams, Jennifer Trang Nguyen, Abigail Yu, Robert Berman, David J. Segal
Genome Center, University of California, Davis, Davis, CA

Angelman Syndrome is a neurological genetic disorder caused by loss of expression of the maternal copy of UBE3A in the brain. Due to brain-specific genetic imprinting at this locus, the paternal UBE3A is silenced by a long antisense transcript. Inhibition of the antisense transcript could lead to unsilencing of paternal UBE3A, thus providing a therapeutic approach for Angelman Syndrome. However, widespread delivery of gene regulators to the brain remains challenging. Here we report an engineered zinc finger-based artificial transcription factor that, when injected intraperitoneally or subcutaneously, crossed the blood-brain barrier, and increased Ube3a expression in the brain of an adult mouse model of Angelman Syndrome. The factor displayed widespread distribution throughout the brain. Immunohistochemistry of both the hippocampus and cerebellum revealed an increase in Ube3a upon treatment. An artificial transcription factor containing an alternative DNA-binding domain did not activate Ube3a. We believe this to be the first report of an injectable engineered zinc finger protein that can cause widespread activation of an endogenous gene in the brain. These observations have important implications for the study and treatment of AS and other neurological disorders.

27. Therapeutic Efficacy of Intracranial and Intrathecal AAV2/9-PPT1 in Infantile Batten Disease

Charles Shyng[1], Hemanth R. Nelvagal[2], Josh T. Dearborn[1], Jonathan D. Cooper[2], Mark S. Sands[1]
[1]Washington University School of Medicine, Saint Louis, MO, [2]King's College London, London, United Kingdom

Background The neuronal ceroid lipofuscinoses (NCLs) are a group of the most common pediatric neurodegenerative lysosomal storage disorders. Infantile NCL (INCL), caused by a deficiency in the lysosomal enzyme palmitoyl-protein thioesterase-1 (PPT1), is characterized clinically by progressive cognitive and motor decline, profound neurodegeneration and neuroinflammation, and accumulation of autofluorescent storage material (AFSM). Infantile NCL murine model recapitulates the human disease. AAV2/5-PPT1 intracranial (IC) delivery delayed the onset of INCL histopathological markers in the forebrain and cerebellum and improved preclinical outcome measures. However, overall disease progression was only partially corrected suggesting peripheral nervous system involvement. In collaboration with Dr. Jon Cooper (King's College, London), we discovered substantial progressive pathology in the spinal cord: neuronal loss and axon density, significant microgliosis and astrocytosis, and AFSM (Nelvagal H *etal*, manuscript in prep). These data suggest that the spinal cord could be an important therapeutic target. We hypothesize that IC and intrathecal (IT) gene therapy in combination will significantly improve the lifespan, preclinical outcome measures, and histopathological markers as compared to either therapy alone. **Methods** We generated five groups (n=10): PPT1-/- , wild type, and PPT1-/- injected with IC, IT, or the combination IC/IT AAV2/9-PPT1. For IC injections, 3-2µl bilateral intracranial injections were performed. For IT injections, one 15µl bolus injection into the lumbar subarachnoid space was performed. The AAV2/9-PPT1 virus was diluted to $1x10^{12}$ viral particles/ml. To date, we have collected 3, 5, and 7-month time points for all groups, and have generated a 9-month time point. Samples will be

analyzed for PPT1 enzyme activity, AFSM, neuroinflammation and neurohistopathology, spinal cord pathology, and a histochemical stain for PPT1. Lifespan, behavior, and brain weight (gross measure of atrophy) will be analyzed. Significance was determined using a 2-way ANOVA test. **Results** PPT1-/- mice have a median lifespan of 35.8 weeks and rapid decline in rotarod performance beginning at 5 months. There is a progressive decline in PPT1-/- brain weight beginning at 3 months. IT AAV2/9-PPT1 mice have a median lifespan of 48.4 weeks and have a steady decline in rotarod performance beginning at 7 months. There is a progressive decline in the IT mice brain weight compared to wild-type, reaching significance at 7 months ($p<.001$); however, it had significantly less atrophy than PPT1-/- brains until 7 months ($p<0.05$). IC AAV2/9-PPT1 mice have a median lifespan of 58.5 weeks and a rapid decline in rotarod performance beginning at 9 months. IC AAV2/9-PPT1 mice brain weight are not significantly different than wild-type. To date, at 66 weeks, all IC/IT AAV2/9-PPT1 mice are alive. There is a significant decline in IC/IT mice rotarod performance at 15 months. The IC/IT mice brain weight is not significantly different than wild-type. Data for the enzyme activity, neuroinflammatory markers, histopathology, and histochemical stain will be complete by April 2016. **Conclusions** To date, these data confirm that targeting the entire CNS will provide a significant step for INCL therapy. The combination therapy significantly increases the lifespan beyond that of an additive benefit. As expected, modifying the gene therapy vector from IC-AAV2/5 to IC-AAV2/9 significantly improved preclinical outcome measures. Lastly, the IT AAV2/9-PPT1 injections suggest that spinal cord disease plays an important role in INCL pathogenesis. These findings could form the basis for an effective therapeutic strategy that incorporates targeting multiple facets of INCL disease.

28. Mouse and Pig Photoreceptor Transduction Mediated by Triple AAV Vectors

Andrea Maddalena, Patrizia Tornabene, Fabio Dell'Aquila, Ivana Trapani, Alberto Auricchio
TIGEM, Pozzuoli, Italy

Purpose: The majority of inherited blinding diseases are caused by mutations in genes expressed specifically in photoreceptors (PRs). However the size of several of these genes exceeds the DNA cargo capacity of AAV vectors, to date the safest and most effective gene therapy vectors in the retina. We have previously demonstrated that dual AAV vectors expand AAV PR transfer capacity to about 9 kb. Our aim is to further expand this to around 13.5 kb using a triple AAV vector system. **Methods:** To assess the percentage of PR transduction mediated by three independent vectors, we initially injected subretinally in 4 week-old C57/BL6 mice single AAV8 vectors encoding either EGFP, dsRed or beta-gal reporter proteins. Then, in order to evaluate the PR transduction efficiency of triple AAV vectors we generated a reporter EGFP-dsRed fusion protein under the control of either the ubiquitous CMV or the PR-specific IRBP(interphotoreceptor retinoid-binding protein) promoters. The corresponding EGFP-dsRed expression cassettes were either included in single or triple AAV8 vectors (Fig1) which were administered by subretinal injection to C57/BL6 mice or large white pigs. Direct fluorescence, Western blot, and ELISA analysis were used to evaluate transgene expression.**Results**: In mice injected with three independent AAV8 vectors encoding for the different EGFP, dsRed and beta-gal reporter proteins, the percentage of PR co-transduced was 12%. In mouse and pig eyes injected with triple and single AAV8 vectors encoding EGFP-dsRed, full length protein expression was confirmed by Western Blot analysis independently of the CMV or IRBP promoters used; importantly, 75% of mouse and 65% of pig eyes injected with triple IRBP vectors showed bands of the expected size. Quantification in mice of EGFP-dsRed protein expression from triple AAV8 vectors mediated by either the CMV or the IRBP promoter shows 30% and 1%, respectively, of the levels observed with a single AAV8. Interestingly, in pigs, the levels of EGFP-dsRed protein expression from triple AAV8-IRBP vectors were 40% ratio of those observed with a single AAV8. **Conclusions:** Our results show that triple AAV vectors, which increase AAV transfer capacity up to 13.5 kb, transduce mouse and pig PR at levels that are 1% and 40% respectively of those obtained with single AAV vectors. This bode well for further testing this platform in animal models of inherited retinal degenerations.

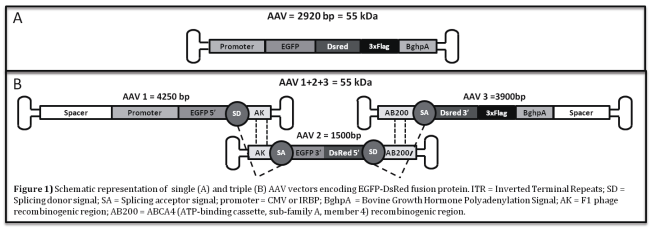


Figure 1) Schematic representation of single (A) and triple (B) AAV vectors encoding EGFP-DsRed fusion protein. ITR = Inverted Terminal Repeats; SD = Splicing donor signal; SA = Splicing acceptor signal; promoter = CMV or IRBP; BghpA = Bovine Growth Hormone Polyadenylation Signal; AK = F1 phage recombinogenic region; AB200 = ABCA4 (ATP-binding cassette, sub-family A, member 4) recombinogenic region.

29. Novel Surgical Method for Intravitreal AAV Administration Overcomes Transduction Barriers in Non-Human Primates

Jason Comander[1,2], Livia Carvalho[1,2,3,4], Sarah Wassmer[1,2,3,4], Ru Xiao[1,2,3,4], Eva Plovie[1,2,3,4], Aliete Langsdorf[1,2,4], Lawrence Lim[2,4], Brian Hafler[1,2], David M. Wu[2], Dean Eliott[2], Leo A. Kim[2,4], Luk H. Vandenberghe[1,2,3,4]
[1]Ocular Genomics Institute, Boston, MA, [2]Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA, [3]Grousbeck Gene Therapy Center, Boston, MA, [4]Schepens Eye Research Institute, Boston, MA

Introduction For gene therapy applications in the primate retina, intravitreal injections (IVT) of adeno-associated virus (AAV) have been shown to target the retina inefficiently, mainly in retinal ganglion cells within a small perifoveal region. The internal limiting membrane (ILM) has been previously hypothesized to be a barrier to broader transduction. We further hypothesized that the local concentration and residence time of AAV at the vitreo-retinal interface was insufficient for efficient retinal transduction. Here, we investigate gene delivery to the retina in rhesus macaque with regular IVT injection as compared to a novel surgical technique "Peel and Puddle" aimed at overcoming these potential barriers. **Methods** Three eyes of rhesus macaques were injected with AAV-CMV-GFP via a traditional IVT at a concentration of 1E+11 particles (2/2 or Anc80 capsid). Another two eyes from different rhesus macaques had the ILM surgically removed followed by the concentrated application of AAV2 onto the peeled area, known as "Peel and Puddle" (PP), also at 1E+11 particles. The PP procedure consisted of a vitrectomy, posterior hyaloid separation, ILM staining and subsequent ILM peel over the majority of the macula. This was followed by fluid-air exchange, and deposition of the virus on the peeled area with 30 minutes of supine positioning generating a "puddle" onto the retina aimed at maximizing the AAV residence time at an undiluted concentration. **Results** GFP expression in the PP condition was dramatically greater than with a regular intravitreal injection, both in intensity and the region of transduction. In the PP eyes, fluorescence was noted in an area that matched the extent of the ILM peel and puddle, as shown by imaging and histology whereas traditional IVT demonstrated the expected parafoveal dim pattern of transduction. All eyes, irrespective of the type of IVT injection, presented clinically significant signs of inflammation, which was managed effectively by intravitreal steroid injections. Histological sections show increase microglia infiltration in both groups compared to un-injected controls. **Conclusions** The vitreous, ILM, and/or physical parameters of the vector delivery significantly limit gene transfer to the retina. Surgical minimization

of these barriers demonstrated highly increased levels and distribution of transduction. Inflammation following intravitreal injection with a non-self transgene is of concern and requires further study.

30. Modulation of Intracellular Calcium Enhances AAV Transduction in the CNS

Garrett Berry, Giridhar Murlidharan, Aravind Asokan
Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC

Recent preclinical studies and clinical trials utilizing recombinant AAV vectors have highlighted vector dose-related toxicity as a potential safety concern. Therefore, strategies to enhance AAV transduction may mitigate this toxicity, as they may allow for administration of lower vector doses. While some strategies rely on modification of the capsid to achieve such, pharmacological modulation of the cellular environment often has the benefit of enhancing AAV transduction independent of capsid serotype. Intracellular calcium is important in the life cycles of several viruses, such as Ebola virus and rotavirus. Several small molecule drugs and biologics exist that can modify intracellular calcium levels in a specific manner. In the current study, we sought to determine if modulation of intracellular calcium levels affects AAV transduction. Increasing intracellular calcium levels using either thapsigargin or ionomycin decreased AAV transduction by an order of magnitude. Conversely, decreasing intracellular calcium levels using the cell permanent calcium chelator BAPTA-AM increased transduction by 10 to 100-fold in a cell independent fashion. In addition, the effects of modification of intracellular calcium are not dependent on AAV serotype. Furthermore, *in vivo* studies performed in mice demonstrate that BAPTA-AM augments transduction by different AAV serotypes, when administered intracranially. In summary, our results support the preclinical evaluation of drugs and biologics that modulate intracellular calcium in the CNS and evaluate their potential for affecting AAV transduction. Results from ongoing studies in mouse models of disease will be presented.

Pre-Clinical Development Activities for Gene Therapy Trials

31. AAV Gene Therapy for Choroideremia: Dose Determination Analyses

Irena Ignatova, Paolo Falabella, Katherine A. High, Linda B. Couto
Spark Therapeutics, Philadelphia, PA

Choroideremia (CHM) is a degenerative, X-linked retinal disease characterized by deletions or mutations in the choroideremia (*CHM*) gene, resulting in defective or absent Rab escort protein-1 (REP-1). In normal retinas, the CHM gene is expressed in multiple cell types, including retinal pigment epithelium, photoreceptors, and choroidal cells, though there is evidence that the RPE cell is the primary disease-causing cell type. Absence or deficiency of REP-1 leads to cellular apoptosis and degeneration of the retinal pigment epithelium (RPE), choroid, and retinal photoreceptors. Choroideremia is an excellent disease candidate for gene transfer as it shares several key features with other inherited retinal degenerative diseases that are also candidates for AAV-mediated vector delivery. These features include: a) the small and localized nature of the target area, namely the retina; b) the tissue boundaries surrounding the area to be injected, which minimizes exposure of other cells to the vector and facilitates treatment with a relatively small vector dose; and c) the favorable immune status of the eye with respect to viral vector- mediated gene transfer. We are conducting a Phase 1 clinical trial to treat CHM subjects using either $5x10^{10}$ or $1x10^{11}$ vg/eye of an AAV2-hRep1 vector, and delivering the vector via a subretinal injection. Due to the lack of suitable animal models that mimic CHM disease in humans, we took a different approach to assess the potential therapeutic dose. A molecular analysis of non-human primate retinas was performed following subretinal injection of AAV2-hCHM to determine the relative amount of human Rep1 compared to endogenous monkey Rep1. Cynomolgus monkey eyes were injected subretinally with either $5x10^{11}$, $7.5x10^{11}$ or $2x10^{12}$ vg of AAV2-hRep1. Fundus images were taken following the injection of 300 ul of vector, and these images were used at the time of sacrifice to dissect the tissue associated with the injection bleb. The remaining retina was also collected and analyzed separately. RNA was isolated and absolute quantitative RT-PCR was performed using both cynomolgus monkey-specific Rep1 primers/probe and human Rep1-specific primers/probe. No human Rep1 mRNA was observed in non-injected eyes, and the amount of human Rep1 mRNA in the bleb area was determined to be at least 50 fold higher than cynomolgus Rep1 mRNA following a dose of $5x10^{11}$ vg/eye. At the two higher doses, similar levels of hRep1 mRNA, ranging from 270-380 times more than cynomolgus Rep1 mRNA, were observed. These data suggest that in the Phase 1 clinical trial, the low dose subjects ($5x10^{10}$ vg/eye) will express hRep1 at a level approximately five fold higher than normal and the high dose subjects will express at least 10 fold higher levels of hRep1 than normal. Ongoing studies are evaluating the level of hRep1 in normal human retinas to confirm these ratios. Thus, the results from this non-conventional pharmacology model provide an estimate of the potential therapeutic dose of AAV2-hCHM for use in clinical studies.

32. Towards a Phase I Clinical Trial for Cystinosis

Tatiana Lobry[1], Jay Sharma[1], Sarah Ur[1], Athena Lau[1], Celine Rocca[1], Donald B. Kohn[2], Denise Carbonaro[2], Laura Hernandez[1], Stephanie Cherqui[1]
[1]UCSD, San Diego, CA, [2]UCLA, Los Angeles, CA

Cystinosis is an autosomal recessive lysosomal storage disorder characterized by the accumulation of cystine in the lysosomes leading to cystine crystals formation. The gene involved, CTNS, encodes a lysosomal cystine transporter, cystinosin. Cystinosis leads to a renal Fanconi's syndrome before the age of one characterized by polyuria and nutrients loss, and to multi-organ degeneration especially the eyes and kidneys. The cysteamine treatment allows the exit of cystine out of the lysosomes but only delays the evolution of the disease. We showed that transplantation of wild-type Hematopoietic Stem and Progenitor Cells (HSPCs) in a lethally irradiated $Ctns^{-/-}$ mouse leads to cystine content decrease in every tissues tested and kidney, eye and thyroid function and structure improvement in treated mice compared to mock-treated mice. An autologous HSPCs gene therapy approach has then been developed with $Ctns^{-/-}$ HSPCs gene-modified *ex vivo* to express a functional CTNS cDNA using the lentiviral vector pCCL-CTNS. Tissue cystine decrease and kidney function rescue were observed with this strategy. The toxicology and pharmacology studies required by the FDA are in progress with a targeted Vector Copy Number (VCN) included between 1 and 3. The *in vitro* studies, Colony Forming Unit assay and In Vitro Immortalization assay, have been completed using peripheral blood $CD34^+$ cells from five healthy donors and four cystinosis patients. The *in vivo* studies are in progress: $Ctns^{-/-}$ HSPCs are isolated and transduced with pCCL-CTNS and transplanted into primary $Ctns^{-/-}$ mice and 6-months later their bone marrow cells are transplanted into secondary $Ctns^{-/-}$ recipients. Primary and secondary mice are carefully monitored and comprehensive histological, biochemical, molecular and clinical analyses are performed at 6 months post-transplant. So far, seven primary and one secondary $Ctns^{-/-}$ mice transplanted with pCCL-CTNS-transduced $Ctns^{-/-}$ HSPCs have reached the 6 month post-transplantation time point. The primary

mice had a mean VCN of 1.713 and the secondary 2.04. Clinical evaluations, histopathology and Vector Integration Site (VIS) analyses revealed no adverse event so far suggesting a good safety profile of our product. Moreover, cystine content was significantly decreased in all tissues tested. Analysis of the remaining primary and secondary recipient mice is in progress and these data will be included in an Investigational New Drug (IND) for a phase 1 clinical trial for autologous transplantation of pCCL-CTNS-modified $CD34^+$ HSPCs in patients with nephropathic cystinosis, For the design and conduct of the future clinical trial, the Cystinosis Stem Cell and Gene Therapy Consortium was recently created and is composed of experts in cystinosis, bone marrow transplant and gene therapy. The clinical grade pCCL-CTNS virus preparation is about to be produced at the Gene Therapy Resources Program (GTRP), Clinical Grade Lentivirus Vector Core directed by Dr. Kenneth Cornetta who prepared the Good Manufactory Practice-comparable (GMPc) virus used for the pharmacology/toxicology studies. We are currently preparing the documents necessary for the IND such as the clinical protocol, the toxicology/pharmacology report, the Chemistry, Manufacturing and Controls (CMC) report, etc. This clinical trial will represent the first stem cell and gene therapy treatment strategy for cystinosis.

33. Advancing a State of the Art Gene Therapy Called OXB-202 That Resists Corneal Rejection in High Risk Patients

Naghmeh Fouladi, Katie Binley, Laura McCloskey, Julie Loader, Michelle Kelleher, Kyriacos Mitrophanous, Scott Ellis
Oxford BioMedica, Oxford, United Kingdom

Due to both the avascularity of the cornea and the relatively immune-privileged status of the eye corneal transplantation is one of the most successful clinical transplant procedures. However in high risk patients, which account for >20% of the 100,000 transplants carried out worldwide each year, the rejection rate is high due to vascularization of the recipient corneal bed. In some of these patients the prognosis is extremely poor, with grafts failing at an accelerating rate to the point where patients are no longer considered suitable for further transplants and are left blind, despite an otherwise normally functioning visual system. The main reason for graft failure is irreversible immunological rejection and it is therefore unsurprising that neovascularization (both pre- and post-grafting) is a significant risk factor for subsequent graft failure. Neovascularization is thus an attractive target to prevent corneal graft rejection. OXB-202 (previously known as EncorStat®) is a human donor cornea modified prior to transplant by *ex vivo* genetic modification with genes encoding secretable forms of the angiostatic human proteins, endostatin and angiostatin. This is achieved using a lentiviral vector derived from the Equine Infectious Anaemia Virus (EIAV) called pONYK1EiA, which subsequently prevents rejection by suppressing neovascularization. Previously we have shown that rabbit corneas treated with pONYK1EiA substantially suppress corneal neovascularization, opacity and subsequent rejection in rabbit models of cornea graft rejection (Parker et al, 2014). We will present data from a 3-month GLP toxicology and biodistribution safety study of pONYK1EiA modified rabbit corneas in a rabbit corneal transplant model. In particular, the GLP study has been designed to include a number of high content in-life assessments that include regular slit-lamp ophthalmic examinations, evaluation of corneal thickness and endothelial cell density using pachymetry and specular microscopy respectively and intraocular pressure measurements. We will present a summary of these data to show that there are no safety issues with pONYK1EiA modified corneas. The GLP safety study data to be presented supports the evaluation of OXB-202 corneas in a First-in-Man trial. The toxicology study, GMP manufactures and clinical development of OXB-202 has been supported by the UK Technology Strategy Board (Innovate UK).

34. Development and Validation of Identity and Homogeneity Assays for AAV Preparations

Simon Pacouret[1,2], Mathieu Mevel[3], Mohammed Bouzelha[3], Anna Maurer[1,2], Rajani Shelke[1,2], Ru Xiao[1,2], Magalie Penaud-Budloo[3], Heikki Turunen[1,2], Frederic Broucque[3], Véronique Blouin[3], Philippe Moullier[3], Eduard Ayuso[3], Luk H. Vandenberghe[1]
[1]Department of Ophthalmology, Ocular Genomics Institute, Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA, [2]Grousbeck Gene Therapy Center, Boston, MA, [3]INSERM UMR 1089/Atlantic Gene Therapies, University of Nantes, Nantes University Hospital, Nantes, France

Adeno-associated virus (AAV) vectors have emerged as key clinical candidates for gene therapy. Yet, the efficiency and safety of these 20-25 nm biological nanoparticles remain difficult to harmonize across pre-clinical studies due to the limitations of current analytical tools. The presence of residual DNA, protein contaminants, empty particles and VP subunits resulting from incomplete capsid assembly are variables that can strongly modulate the reliability of *in vivo* data and that, therefore, need to be closely monitored in AAV research laboratories. In this work, 70 AAV preparations, obtained with various production (baculo/Sf9 and triple transfection system) and purification (iodixanol gradient and double cesium-chloride gradient) techniques were analyzed using a thermal shift assay based on the fluorescent dye Sypro® Orange. The fluorescence fingerprint obtained did not only allow to discriminate various AAV serotypes based on their capsid melting temperatures, but also enabled to probe the homogeneity and purity of AAV vector preparations, investigated in parallel using dynamic light scattering (DLS) and polyacrylamide gel electrophoresis. In particular, a double fluorescence transition indicated the presence of capsid-associated protein contaminants whereas a high initial fluorescence background correlated with the presence of free protein contaminants and capsid subunits, possibly resulting from capsid degradation during vector purification or storage. The variability, sensitivity and precision of this assay were further investigated in two different AAV research laboratories. This simple, fast (analysis of 94 preps in ~6 hrs) and low-cost assay emerges as a relevant tool for characterization of AAV vector preparations and will help to increase the reliability of *in vivo* gene transfer studies.

35. Establishment of the Dose-Response Relationship Needed for Human Translation of Pulmonary Macrophage Transplantation (PMT) Therapy of Hereditary Pulmonary Alveolar Proteinosis (hPAP)

Paritha I. Arumugam[1], Takuji Suzuki[1], Anthony Sallese[1], Nico Lachmann[2], Claudia Chalk[1], Diane Black[1], Brenna Carey[1], Thomas Moritz[2], Bruce C. Trapnell[1]
[1]Pulmonary Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, [2]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany

Hereditary pulmonary alveolar proteinosis (hPAP) is a severe pediatric lung disease caused by mutations in *CSF2RA/B* (encoding GM-CSF receptor α/β, respectively) without pharmacologic therapy. Disease patho-genesis is mediated by loss of GM-CSF-dependent clearance by alveolar macrophages (AMs) resulting in progressive pulmonary alveolar surfactant accumulation and hypoxemic respiratory failure. We recently reported a conceptually

and technically novel, exceptionally efficient gene and cell therapy approach -PMT- as a promising alternative for the existing highly invasive, inefficient whole lung lavage procedure for hPAP. Our preclinical studies revealed that macrophage cell doses from 0.5 to 4 x10^6/mouse were similarly highly efficacious as therapy of hPAP in *Csf2rb*-/- mice, an authentic model of human hPAP (*Nature*, 2014, 514: 450-5). The present study was undertaken to identify a dose-response relationship and to address the hypothesis of an expected 'trade-off' between the minimum effective dose and time to treatment effect. We isolated Lineage-, Sca1+, cKit+ hematopoietic stem/progenitors from the bone marrow of wild-type (WT) CD45.1+ mice, differentiated them *in vitro* into mature macrophages and sorted for highly homogenous $CD11c^{Hi}$, $F4/80^{Hi}$ macrophage population by flow cytometry. We administered cell doses (2.5 x10^3, 2.5 x10^4, 2.5 x10^5, 1 x10^6 cells/mouse; n=3-4/dose) by PMT to CD45.2+ *Csf2rb*-/- recipients. At 8 weeks after PMT, therapeutic efficacy was evaluated by measuring the optical density (OD) of bronchoalveolar lavage (BAL turbidity) - an excellent measure of overall PAP disease severity. The CD45.1+ donor macrophages recovered from the BAL were of $CD11c^{Hi}$, $F4/80^{Hi}$ phenotype. BAL turbidity decreased smoothly with increasing PMT cell dose over the entire range but reached significance only at the two highest doses compared to age-matched, untreated *Csf2rb*-/- controls (OD λ=600 nm = 0.88±0.07 and 0.5±0.08 vs. 2.32±0.18; P<0.001, P<0.0001; respectively). BAL turbidity correlated inversely with the percentage of CD45.1+ donor macrophage engraftment in CD45.2+ Csf2rb-/- recipients (Spearman rank correlation, R^2=0.74). Since we have identified an increased ratio of cholesterol (relative to phospholipid) in surfactant as the primary lipid abnormality in hPAP, we also evaluated the relationship of BAL cholesterol level and PMT cell dose. Like turbidity, BAL cholesterol levels decreased smoothly with PMT cell dose over the entire range but reached significance only at the two highest cell doses compared to age-matched, untreated Csf2rb-/- controls (ng/ml BAL = 25±3 and 10±0.3 versus 54±0.3; P<0.01, P<0.001; respectively, R^2=0.67). Our pre-clinical results establish a direct relationship between the number of macrophages transplanted and efficacy of PMT therapy in *Csf2rb*-/- mice and facilitate the design of a clinical trial to test PMT therapy in children with hPAP. Based on these results and the previously demonstrated strong survival advantage of transplanted over endogenous macrophages, we anticipate a 'trade-off' between the minimum effective dose and the time to equivalent treatment effect.

36. Translation of an Adenovirus-Based Cocaine Vaccine dAd5GNE to a Clinical Trial

David F. Havlicek, Bishnu De, Jonathan Rosenberg, Odelya Pagovich, Dolan Sondhi, Stephen Kaminsky, Ronald Crystal
Weill Cornell Medical College, New York, NY

dAd5GNE, an anti-cocaine vaccine based on a disrupted serotype 5 adenovirus gene transfer vector covalently conjugated to the cocaine analog GNE, evokes high titers of high affinity anti-cocaine antibodies that prevent cocaine from reaching its cognate receptor in the CNS. dAd5GNE has been shown to be effective in preclinical efficacy studies in mice, rats and nonhuman primates. In order to translate dAd5GNE to the clinic, this study focused on optimizing the choice of adjuvant, timing of vaccination regimen and dose. **Adjuvant.** To evaluate vaccine formulation, the adjuvants Adjuplex and Alum were assessed at a fixed dose to identify the formulation that evoked the fastest and highest titer response. BALB/c mice vaccinated with dAd5GNE/Adjuplex demonstrated high serum anti-cocaine antibody titers (>5x10^5) after a single administration while animals vaccinated with dAd5GNE/Alum required multiple injections to achieve high titers, and mice vaccinated with dAd5GNE/PBS plateaued at a lower titer. Therefore, a formulation based on the Adjuplex adjuvant was chosen for the clinical protocol. **Timing.** The measurement of titer half-life in nonhuman primates following individual vaccinations at variable intervals was used to inform the timing between vaccine boosts. The anti-cocaine antibody titer half-life in 8 dAd5GNE/Adjuplex vaccinated nonhuman primates was assayed regularly over the course of 1 yr and the titer half-life was calculated following each administration. The average half-life for all animals was 4.0 ± 0.2 wk. Continued cocaine use did not impact the titer half-life. These results indicate that a 4 wk interval for vaccinations is necessary to maintain the high titer anti-cocaine antibody levels. **Dose.** We measured the dose response in mice to provide the baseline for a phase I/II clinical trial and identified the range of doses that bracket the anticipated minimum effective dose and maximum tolerated dose. Doses from 0.04 - 40 μg of the vaccine were evaluated in BALB/c for the capacity to evoke antibody titers and to minimize access of radiolabelled cocaine to the CNS. Doses at and above 4 μg produced high anti-cocaine antibody titers above the threshold for efficacy (>5x10^5) which substantially reduced cocaine levels in the brain (p<0.0001 *vs* PBS). Using a modified weight adjusted dose from these results, the vaccine doses to be evaluated in the clinical trial were determined to be 100 to 1000 μg. The results of these studies combine to provide the specifications for critical vaccine design parameters required to translate the anti-cocaine vaccine to evaluation in a clinical trial. Based on these specifications the FDA has allowed the Adjuplex-formulated dAd5GNE vaccine to proceed to clinical study with a monthly vaccination regimen in recovering cocaine addicts.

Targeted Genome Editing: Gene Editing in Hematopoietic Cells

37. Towards Clinical Translation of Hematopoietic Stem Cell Gene Editing for the Correction of SCID-X1 Mutations

Pietro Genovese[1], Giulia Schiroli[1], Anthony Conway[2], Samuele Ferrari[1], Dmitry Y. Guschin[2], Tiziano Di Tomaso[1], Luisa Albano[1], Virna Marin[3], Erica Giuliani[3], Philip D. Gregory[2], Michael C. Holmes[2], Bernhard Gentner[1], Chiara Bovolenta[3], Gregory J. Cost[2], Angelo Lombardo[1], Luigi Naldini[1]
[1]TIGET, Milan, Italy, [2]Sangamo BioSciences, Richmond, CA, [3]MolMed, Milan, Italy

The scope of genetic engineering of hematopoietic stem/progenitor cells (HSPC) has broadened from gene replacement to targeted genome editing using a choice of artificial nucleases, which enables precise modifications of endogenous genes. We recently showed that gene editing in the primitive HSPC is constrained by gene transfer efficiency and limited proficiency of homology directed DNA repair. By combining Integrase Defective Lentiviral Vectors (IDLV) for donor template delivery and mRNA transfection for nucleases expression and tailoring culture conditions, we overcame in part these barriers and provided evidence of increased targeted integration (TI) in human HSPC. We achieved TI of a corrective cDNA into a mutational hotspot of IL2RG gene in long-term repopulating HSPC with efficiency and specificity potentially suitable for clinical translation. Here, in order to improve the tolerability of the procedure and establish a transferable-to-the-clinic gene correction protocol we optimized reagents and scaled-up the gene editing procedure. We developed new ZFNs targeting the upstream region of the IL2RG gene to correct the majority of SCID-X1 mutations with only one ZFN/donor set. By targeting a corrective cDNA into intron-1 of IL2RG gene in primary T cells, we found that targeted cells were functionally indistinguishable from wild-type ones, proving the functionality of the edited gene. We compared the performance of IDLV and AAV6 as donor vehicles for HSPC targeting and found that both vectors are similarly proficient in the delivery of donor templates for HDR, allowing up to 20% TI in bulk HSPC. To improve nuclease expression while decreasing cellular innate response to mRNA transfection

we included modified nucleotides during mRNA production and performed HPLC purification after in-vitro transcription. The use of this optimized mRNA and clinical grade purification of IDLV (based on DNA removal followed by anion exchange chromatography) allowed decreasing type-1 interferon activation and electroporation toxicity, respectively. To further optimize ex-vivo HSPC manipulation we tested pyrimidoindole derivatives added to the culture and found a combination promoting HSPC expansion in conditions that preserve their primitive phenotype, increasing the yield of edited cells that are able to repopulate NSG mice. Finally, we demonstrated the therapeutic potential of our strategy by correcting the IL2RG gene in HSPC from a genotyped SCID-X1 patient. Currently, we are processing large scale lots of gene corrected cells using a high volume electroporator and have successfully treated up to 40 million of HSPCs. We envisage early clinical translation of this approach, which may benefit SCID-X1 patients while avoiding the risks of allogeneic transplantation or random transgene integration mediated by HSPC gene therapy with conventional integrating vectors.

38. CCR5 Gene Edited Hematopoietic Stem Cells Engraft in Diverse Anatomical Locales and Undergo SHIV-Dependent Positive Selection in Nonhuman Primates

Christopher W. Peterson[1], Jianbin Wang[2], Patricia Polacino[3], Shiu-Lok Hu[3], Michael C. Holmes[2], Hans-Peter Kiem[1]
[1]Fred Hutchinson Cancer Research Center, Seattle, WA, [2]Sangamo BioSciences, Richmond, CA, [3]University of Washington, Seattle, WA

Background: Gene editing in hematopoietic stem/progenitor cells (HSPCs) has rapidly emerged as a promising therapy for a number of diseases, including human immunodeficiency virus (HIV) infection. We have previously demonstrated the feasibility of this approach in nonhuman primates. Here, we leverage our expertise with gene editing in large animal models to interrogate the clonal persistence, trafficking, and antiviral efficacy of CCR5-edited cells in the pigtailed macaque, *M. nemestrina*. Our objectives were to map the tissue distribution of HSPC-derived, gene edited progeny, understand how individual gene edited HSPCs persist following autologous transplantation, and develop strategies to select for these cells *in vivo.*

Methods: Zinc Finger Nucleases (ZFNs) are used to target the CCR5 locus in macaque HSPCs. Engraftment and persistence of these autologous stem cells, and stem cell-derived lymphoid and myeloid cells, are measured *ex vivo* and *in vivo*. Animals are challenged with simian/human immunodeficiency virus (SHIV). Gene edited HSPCs are transplanted either prior to SHIV infection, or in SHIV-infected animals that are treated with combination antiretroviral therapy (cART) in order to approximate a well-suppressed HIV^+ patient. Edited cells are measured longitudinally in peripheral blood, bone marrow, gastrointestinal (GI) tract, and lymph nodes, and at necropsy in a panel of 25 tissues, using methods including deep sequencing.

Results: We observe up to 14-fold enrichment of CCR5-gene edited memory $CD4^+$ T-cells in SHIV-infected animals, consistent with virus-dependent selection against CCR5 wt memory $CD4^+$ T-cells. Gene edited cells are found in a broad array of anatomical sites, including GI tract and lymph nodes. Spatial and temporal tracking of CCR5 mutations suggests that gene edited cells persist in an analogous fashion to control lentivirus gene-marked cells. Homology directed repair (HDR) pathways can be exploited in macaque $CD34^+$ HSPCs, facilitating knock-in of selectable markers at the disrupted CCR5 locus.

Conclusions: Our results in SHIV-infected animals reinforce that this gene editing strategy results in stable engraftment of CCR5-mutated and SHIV-resistant HSPCs and their progeny. Additionally. gene edited $CD4^+$ T-cells undergo positive selection during active infection, further supporting the validity of this approach in the clinic. Moreover, our preliminary *ex vivo* HDR data suggest that these gene edited cells could be engineered to undergo virus-independent selection.

39. FACS-Based Enrichment of a Highly Purified HBB-Targeted Hematopoietic Stem and Progenitor Cell Population Using rAAV6 and CRISPR/Cas9

Daniel P. Dever[1], Rasmus O. Bak[1], Joab Camarena[1], Nivi Saxena[1], Andreas Reinisch[2], Carmencita E. Nicolas[1], Alec B. Wilkens[1], Ayal Hendel[1], Nobuko Uchida[1], Ravindra Majeti[2], Kenneth I. Weinberg[1], Matthew H. Porteus[1]
[1]Pediatrics, Stanford University, Palo Alto, CA, [2]Medicine, Division of Hematology, and Cancer Institute, Stanford University, Palo Alto, CA

Precise engineered nuclease-mediated gene correction via homologous recombination (HR) in hematopoietic stem and progenitor cells (HSPCs) has the power to transform curative therapies for monogenic diseases of the immune system. Sickle cell disease (SCD) is one of the most common monogenic diseases, affecting millions of people worldwide. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only curative treatment for patients with SCD; however, immunocompatibility issues, graft-versus-host disease, and graft rejection are major roadblocks for efficacious therapy. In theory, the ideal curative strategy for SCD and most monogenic immune diseases is ex vivo gene correction of patient-derived HSPCs followed by autologous HSCT of a highly purified targeted population to avoid possible complications of competition between unedited and gene-targeted HSPCs in vivo. By supplying a homologous GFP-expressing HBB donor via recombinant adeno-associated virus serotype 6 (rAAV6) in combination with a double strand break created by the CRISPR/Cas9 system, we achieved HR frequencies of 15-49% of HSPCs, and more importantly, identified a population of HBB-targeted HSPCs (HSPCHBB) through a log-fold MFI increase in chromosomal transgene expression. Fluorescence activated cell sorting (FACS) of this population revealed consistent GFP expression in >95% of cells over two weeks in vitro in multiple CD34+ donors isolated from either bone marrow, cord blood or peripheral blood. Single-cell FACS of HSPCHBB into methylcellulose led to both myeloid and lymphoid colony formation, and on-target PCR analysis revealed >95% of these HSPCHBB clones had either a mono or biallelic-targeting event. Notably, a fraction of HSPCHBB displayed the CD34+/CD38-/CD90+/CD45RA- immunophenotype, suggesting successful targeting of long-term repopulating hematopoietic stem cells (LT-HSCs). Furthermore, HSPCHBB displayed long-term engraftment in the bone marrow of immunodeficient NSG mice at 12 weeks post-transplant, where we identified that ~70% of human cells were GFP+ and also produced both myeloid (CD33+) and lymphoid (CD19+) cell types, implying that the HSPCHBB population contains true LT-HSCs that can repopulate a functional immune system. Altogether, these proof-of-concept studies showed that by combining a homologous rAAV6 donor, the CRISPR/Cas9 system, and FACS, it is feasible to generate and enrich a highly purified population of gene-corrected HSPCs that include LT-HSC potentials.

40. Nuclease-Targeted Gene-Editing of *FOXP3* in Primary T Cells Creates a Stable and Functional T_{reg} Phenotype

Nicholas W. Hubbard, David Hagin, Karen M. Sommer, Yumei Song, Iram Khan, Andrew M. Scharenberg, David J. Rawlings, Troy R. Torgerson
Seattle Children's Research Institute and Departments of Pediatrics and Immunology, University of Washington School of Medicine, Seattle, WA

Reprogramming T cells to adopt regulatory T cell (T_{reg}) functions represents a promising strategy for treating autoimmune disorders and graft-versus-host disease. The development and maintenance of T_{reg} cells is dependent on FOXP3 expression, which in turn is tightly controlled through epigenetic modification of intronic conserved non-coding sequences (CNS) surrounding the first coding exon. Two major challenges associated with FOXP3 gene-transfer approaches for T_{reg} reprogramming are: achieving adequately high FOXP3 expression, and avoiding eventual gene silencing. We devised two gene editing strategies with the goal of driving stable expression of FOXP3 in primary human T cells. Using a combination of TALEN-mediated gene disruption and adeno-associated-virus (AAV) delivered donor repair templates, we introduced either an MND promoter upstream of the first coding exon, or we deleted intronic CNS implicated in transcriptional silencing. The donor templates were also designed to co-express FOXP3 with either GFP or EGFRt (truncated Epidermal Growth Factor receptor), to allow tracking and purification of edited cells. Both strategies resulted in T cells stably expressing FOXP3 at high levels (~60 %). Introduction of the MND promoter resulted in the greatest levels of cellular FOXP3 expression (MFI), and these cells showed phenotypic and functional changes consistent with Treg cells, including: surface marker expression ($CD25^{high}$, $CD127^{low}$, $CTLA4^{high}$, $LAG3^{high}$), suppression of cytokine production (IL-2, IL-17 and IFN-γ), and resistance to Rapamycin. Edited cells also suppressed the proliferation of stimulated T cells *in vitro*, demonstrating their effective 'reprogramming' towards a T_{reg} lineage. Thus, our gene modification strategy allowed us to over-ride (using the MND promoter) or modulate (by deleting CNS elements) endogenous *FOXP3* regulatory mechanisms to enforce stable, long-term FOXP3 expression in T cells that were not previously committed to the Treg lineage. This approach, used alone or in combination with selection for disease-relevant TCR specificity or with delivery of a chimeric antigen receptor, is likely to be broadly applicable for producing stable, functionally active Tregs for a range of future clinical applications.

41. Therapeutic Level CRISPR-Oligomer-Mediated Correction of X-CGD Patient Hematopoietic Stem Cells Using Non-Viral, cGMP Compliant, Scalable, and Closed System

Linhong Li[1], Suk see De Ravin[2], Cornell Allen[1], Uimook Choi[2], Sherry Koontz[2], Narda Theobald[2], Janet Lee[2], Angelia Viley[1], Pachai Natarajan[1], Hannah NewCombe[2], Ashley McMichael[2], Xiaolin Wu[2], Harry L. Malech[2], Madhusudan V. Peshwa[1]
[1]MaxCyte, Gaithersburg, MD, [2]Lab of Host Defenses, NIAID, NIH, Bethesda, MD

Gene therapy using integrating viral vectors in hematopoietic stem cells (HSC) has shown clinical benefit in genetic diseases. However, there remain safety concerns associated with random integration and the lack of regulation of gene expression. Efficient and site-specific correction of mutation(s) in HSC using non-viral methods may improve safety and regulation of gene expression. Chronic granulomatous disease (CGD) due to defective phagocyte NADPH oxidase complex and lack of bactericidal superoxide and other reactive oxidative species (ROS) is characterized by severe infections and hyperinflammation. Although the X-linked form of CGD with gp91phox deficiency results from mutations that span the CYBB gene, we identified a 'hotspot' mutation at Exon 7 c.676C>T, causing a premature stop codon in 17 out of 285 patients with X-linked CGD at the NIH. Here we report the result of the efficient correction of the hotspot CYBB mutation using highly efficient CRISPR (Cas9 and sgRNA) system with an oligomer as donor repair template, using MaxCyte's commercially/clinically validated cGMP/regulatory compliant and closed platform technology. Plasmids encoding Cas9 and gRNA were purchased from the Genomic Engineering Center at Washington University (St. Louis, MO). The mRNA encoding Cas9 and gRNA were in vitro transcribed at MaxCyte using mMESSAGE mMACHINE® T7 Ultra kit, (Ambion, Austin, TX). We screened and selected best gRNA from four gRNA candidates for correction and then optimized transfection conditions with EBV-transformed B cell line (B-LCL) derived from an adult patient (P1) with the hotspot CYBB mutation. Transfected B-LCL exhibit 80±6% viability, minimal detectable toxicity as determined by cell proliferation rate referenced to control cells, and efficient site-specific gene correction with 20-50% WT gp91 expression. These developed protocols were used to treat G-CSF and pleraxifor mobilized CD34+ HSC from P1. Following optimization, *in vitro* treated HSC from P1 achieved 20-30% WT gp91 expression, with >50% viability, and minimal loss of cell proliferation capacity compared with control cells. CD34+ HSCs undergo myeloid differentiation in DMEM supplemented with G-CSF prior to functional evaluation using flow cytometric dihydrorhodamine (DHR) assay, demonstrating ~20% ROS+ cells in treated samples compared to ~80% in normal controls. P1's HSC treated the same way were transplanted into immunodeficient mice, and analyzed 8 weeks later. Bone marrow from mice transplanted with P1 treated cells showed CD45+ human cell engraftment rates at 50-80%, and of the forward/side scatter-gated granulocytes, 11-26% express gp91phox relative to 68% in normal control. Peripheral blood from mice demonstrated 11-23% human CD45+ cells, of which 9-21% expressed gp91phox, compared to 79% in normal controls. Deep sequencing of human CD45+ cells sorted from mouse bone marrow confirmed high rates (up to 21%) of genetic correction from the 'T' mutation to the wildtype 'C'. Since female carriers of X-CGD with ~10-15% normal functioning neutrophils appear to have normal resistance to infections, this level of correction at 10-20% in human CD45+ cells from transplanted mice suggest CRISPR/oligo approach a feasible therapeutic option for treatment of CGD patients with the Ex7, c.676C>T mutation.

42. Correction of SCID-X1 by Targeted Genome Editing of Hematopoietic Stem/Progenitor Cells (HSPC) in the Mouse Model

Giulia Schiroli[1,2], Pietro Genovese[1], Valentina Capo[1], Maria C. Castiello[1], Luisa Albano[1], Michael C. Holmes[3], Anna Villa[1], Giovanni Sitia[4], Angelo Lombardo[1,2], Luigi Naldini[1,2]
[1]TIGET, Milan, Italy, [2]Vita Salute San Raffaele University, Milan, Italy, [3]Sangamo BioSciences, Richmond, CA, [4]San Raffaele Scientific Institute, Milan, Italy

Targeted genome editing by engineered nucleases has brought the goal of gene correction within the reach of gene therapy. A candidate disease for HSPC gene correction is SCID-X1, because gene therapy trials with integrating vectors showed robust clinical efficacy even from few corrected cells but also the occurrence of leukemias due to insertional mutagenesis and unregulated transgene expression. To model SCID-X1 gene correction in preclinical studies, we developed a mouse model carrying the *IL2RG* human gene harboring a common disease-causing mutation in place of the murine *Il2rg*, allowing to use of the same reagents developed for gene correction of human cells. These mice have impaired lymphoid development which phenocopies

that reported for *Il2rg-/-* mice. To assess the minimal level of corrected HSPC required to achieve immune reconstitution we performed competitive transplants with wild-type (WT) and *Il2rg-/-* HSPC and found that 1% of WT cells are sufficient to reconstitute in part the T and B cell compartments. We then tested gene correction of the murine Lin- HSPC by the delivery of donor DNA template by IDLVs followed by transfection of ZFN mRNAs. This protocol yielded high on-target nuclease activity (40%) and a mean of 6% transgene integration by HDR but also high cytotoxicity (65% cell loss) under the conditions we used. The surviving cells remained capable of expansion in culture and maintained their clonogenic potential. Importantly, upon transplant into lethally irradiated mice, only the gene corrected cells were able to generate lymphoid lineages (B and T cells), showing a clear selective advantage over the un-corrected SCID cells. These data indicate functional correction of the defective *IL2RG* gene by targeted editing. Furthermore, upon challenging the mice with a murine pathogen we observed viral-specific γIFN production by CD8+ gene corrected cells, proving their *in vivo* functionality. Yet, measuring the percentage of edited cells (either by NHEJ or HDR) within the HSC compartment long-term, we found that it was nearly undetectable. Despite the lack of HSC marking, gene corrected lymphoid cells persisted in the mice up to 7 months post transplantation within all the hematopoietic organs, indicating successful editing of at least 1% progenitors able to sustain long-term lymphopoiesis and partially correct the disease phenotype. We then developed a new protocol exploiting CRISPR/Cas9 technology that enabled to achieve substantial levels of targeted DNA repair by NHEJ (up to 70%) and HDR (up to 25%) with minimal cytotoxicity and provided stable engraftment of the edited cells in transplanted mice. By this strategy we are now assessing the impact of HSC vs. progenitor targeted editing and conditioning regimen for the extent and stability of disease correction. These studies will help establish the key factors underlying safe and effective rescue of the disease by HSPC gene editing and assist in the design of the protocol for its first clinical testing.

43. CRISPR/Cas9 and rAAV6-Mediated Targeted Integration at the CCR5 Locus in Hematopoietic Stem and Progenitor Cells

Rasmus O. Bak[1], Daniel P. Dever[1], Nivedita Saxena[1], Joab Camarena[1], Andreas Reinisch[2], Alec B. Wilkens[1], Carmencita E. Nicolas[1], Ayal Hendel[1], Ravindra Majeti[2], Kenneth I. Weinberg[1], Matthew H. Porteus[1]

[1]Department of Pediatrics, Stanford University, Stanford, CA, [2]Department of Medicine, Stanford University, Stanford, CA

CRISPR/Cas9-mediated genome editing relies on guide RNAs to direct site-specific DNA cleavage mediated by the Cas9 endonuclease. In this study, we identified a highly potent single guide RNA (sgRNA) targeting exon 3 of *CCR5*. This sgRNA was chemically synthesized with three modified nucleotides at each terminus with 2´O-Methyl and phosphorothioate modifications, and electroporated into cells either with Cas9 mRNA or complexed with Cas9 protein (RNP). Using Tracking of Indels by DEcomposition (TIDE) to quantify insertions and deletions (INDELs), we observe up to 80% INDELs in CD34+ hematopoietic stem and progenitor cells (HSPCs).

To achieve targeted integration by homology-directed repair (HDR), we produced rAAV6 vectors carrying a GFP expression cassette flanked by *CCR5* homology arms. Electroporation with Cas9 RNP followed by rAAV6 transduction led to targeted integration in up to 30% of the cells. Interestingly, we observed that cells with targeted integration expressed GFP at fluorescence intensities more than 10-fold higher than from episomal AAV vectors. This allowed us to sort targeted cells as early as four days after nucleofection and transduction. Upon fluorescence-activated cell sorting and culture of this population, >99% of cells remained GFP^+ 20 days post sort. In a methylcellulose-based colony-forming unit (CFU) assay, we identified multipotent and lineage-committed progenitor cells in this population, and PCR of gDNA extracted from colonies confirmed targeted integration at *CCR5* in at least 98% of cells. Phenotypic characterization of this targeted population confirmed the presence of $CD34^+$ $CD38^-$ $CD90^+$ $CD45RA^-$ cells, indicating genome editing of hematopoietic stem cells. We transplanted edited cells into immunodeficient NSG mice and analyzed the bone marrow 8 weeks post-transplant. In mice transplanted with cells that were not enriched for targeted integration, we found 0.1-1.9% GFP^+ cells among the engrafted human cells. This was a significant decline compared to the 12-13% GFP^+ cells in the input cell population following culture, which is a phenomenon consistent with findings reported by other groups using different nuclease platforms. In contrast, when transplanting cells enriched for targeted integration, we found that 75% of the engrafted human cells were GFP^+, confirming the presence of cells with long-term engraftment potential in the enriched population.

In conclusion, we have found that the combination of CRISPR/Cas9 and rAAV6 is an effective platform for HDR-mediated targeted integration of a transgene into the *CCR5* locus. Furthermore, the GFP MFI shift observed when episomal rAAV6 vectors are integrated into the chromosome following HDR enables early isolation of a population highly enriched for targeted integration at this locus. Since *CCR5* is considered a 'safe harbor' for targeted insertion of a gene, this approach might find general use in therapeutic genome editing. Additionally, since CCR5 is an important co-receptor during HIV infection, our findings may be used to generate immune cells resistant to HIV infection.

44. Novel Combination of megaTAL Nuclease-Driven Genome Engineering with a Drug Selection Cassette Increases Efficiency of HIV Gene Therapy

Biswajit Paul[1,2], Guillermo Romano[3], Kevin Haworth[1], Patrick Younan[1], Nicholas Hubbard[3], Donald Gisch[1], Iram Khan[3], Alexander Astrakhan[4], David J. Rawlings[3], Hans-Peter Kiem[1]

[1]Fred Hutch, Seattle, WA, [2]University of Washington, Seattle, WA, [3]Seattle Children's Research Institute, Seattle, WA, [4]Bluebird Bio, Seattle, WA

Human Immunodeficiency Virus (HIV) infection remains a substantial health problem worldwide. The C-C chemokine receptor 5 (CCR5) serves as a co-receptor for HIV entry into CD4+ T cells and represent an alternative therapeutic target. Early clinical trials using CCR5-targeting zinc finger nucleases demonstrated transient control of HIV infection in the course of antiretroviral treatment interruption (Tebas, NEJM, 2014). Our current work improves on these advances by combining high level of CCR5 gene disruption with preferential selection of gene modified cells. The CCR5-targeting megaTAL combines a LAGLIDADG homing endonuclease scaffold with an eleven repeat transcription activator-like (TAL) effector array to achieve site specific DNA cleavage. This nuclease produces highly efficient CCR5 targeting in primary human CD4+ T cells *in vitro* (70-90% disruption). To test the protective effects of megaTAL treatment, primary human CD4+ T cells treated with CCR5-megaTAL were transplanted into NOD/SCID/γc-null (NSG) 'humanized' mice and challenged with HIV-1. We observed a 100-fold increase of megaTAL-treated cells compared to untreated controls during an active *in vivo* infection demonstrating the functionality of this approach. Based on the decline of CCR5 modified cells in the clinical trials to date, we hypothesized that we could improve maintenance of HIV resistant cells by expanding them either *ex vivo* or *in vivo*. We propose that coupling megaTAL nuclease treatment with drug selection will help us achieve therapeutically relevant levels of HIV-

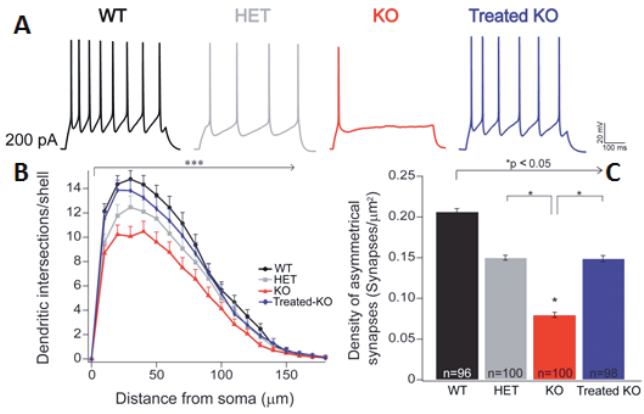
protected cells by enabling efficient selection only of CCR5-modified T-cells. The mutant human dihydrofolate reductase (mDHFR) chemoselection system has been used to render cells resistant to lymphotoxic concentrations of the drug methotrexate (MTX). We tested our experimental approach by transducing cells with lentiviral vector encoding a mDHFR cassette followed by chemoselection in MTX at 0.02uM. This approach resulted in a six fold enrichment of gene modified primary CD4+ T cells *ex vivo*. Previously we have shown that combining megaTAL treatment with adeno-associated virus (AAV) transduction produces very high rates of homology-driven repair (HDR) in primary human T cells. Hence, we combined megaTAL/AAV treatment to integrate the mutant DHFR into the CCR5 locus, producing a population of MTX-resistant CD4+ cells that also lack CCR5. Primary human CD4+ T cells were transfected with CCR5-megaTAL mRNA and transduced with AAV6 containing a mutant DHFR donor template flanked by 0.6kb CCR5 homology arms. They demonstrated a greater than five-fold enrichment in MTX compared to untreated controls *ex vivo*. Next, we have transplanted NSG mice with $1x10^6$ gene modified cells/ mouse to assess the therapeutic potential of our approach. Mice that engraft effectively will be treated with daily injections of 0, 0.5 and 2 mg/kg of MTX to monitor preferential selection and enrichment of our target cell population. Subsequent studies will assess the long term control of viremia in these mice following HIV challenge. In conclusion, the CCR5-megaTAL nuclease platform produces very high levels of gene-modified CD4+ T-cells and protects these cells from subsequent HIV infection *in vivo*. Furthermore, combining targeted integration and chemical selection results in the specific selection of gene modified primary human T cells. To our knowledge we are the first group to report MTX-mediated chemoselection and expansion of CD4+ T cells following targeted integration at the CCR5 locus.

Diabetes, Metabolic and Genetic Diseases

45. Rescue of the Functional Alterations of Motor Cortical Circuits in Arginase 1 Deficiency with AAV-Based Gene Therapy

Gloria Cantero[1], Xiao-Bo Liu[2], Steven D. Cederbaum[1], Peyman Golshani[1], Gerald S. Lipshutz[1]
[1]UCLA, Los Angeles, CA, [2]UC Davis, Davis, CA

The urea cycle is the main mechanism for terrestrial mammals to detoxify excess nitrogen. Disorders of the proximal urea cycle characteristically have periodic episodes of hyperammonemia leading to often severe and permanent neurological deterioration & disability. Ammonia has been implicated by compromising potassium buffering of astrocytic membranes and causing clinical neurological abnormalities by impairing cortical inhibition. Complete arginase 1 (Arg1) deficiency, a distal urea cycle disorder, is the least severe of these abnormalities, demonstrating neurological impairment including spasticity, loss of ambulation and seizures; while characterized by the presence of hyperargininemia, *hyperammonemia is not a frequent clinical finding*. While mortality is unfortunately common due to acute episodes of hyperammonemia in proximal urea cycle disorders, patients with hyperargininemia often are long-lived, however, suffering from progressive intellectual disability and spastic diplegia, and the mechanisms underlying the neurological dysfunction are not understood. To gain better insight on how the loss of arginase expression causes dysfunction in the developing brain, *and if gene therapy could prevent these abnormalities*, we studied how the excitability and functional and anatomical connectivity of motor cortical neurons are altered in the disorder using the murine knockout model. In addition, we examined if AAV expressing *Arg1*, administered IV on postnatal day 2, could rescue these findings. Results: Single- and double-copy loss of *Arg1* caused dose-dependent decreases in intrinsic excitability, dendritic arborization complexity, and synapses in motor cortex layer V neurons. These findings show that 1) the intrinsic excitability of neurons of homozygous *Arg1* knockout mice is abnormal and that, unexpectedly, heterozygous neurons (single copy loss) exhibit an intermediate phenotype compared to wild type and homozygous knockouts (double copy loss) (Fig . A); corresponding loss of *Arg1* decreased the frequency of miniature excitatory postsynaptic currents and the amplitude of miniature inhibitory postsynaptic currents; 2) neuronal branching and spine phenotypes differ between genotypes with, unexpectedly, an intermediate phenotype for heterozygotes (Fig. B); and 3) with electron microscopic analysis and comparison of layer V synapses from arginase 1 knockout, heterozygous, and wild type mice, there is a very low density of excitatory (i.e. asymmetrical) synapses (Fig. C) in the knockout and decreased number of inhibitory (perisomatic) synapses (i.e. symmetrical) on somata of pyramidal cells, both dramatic findings. Finally, changes in synaptic morphology and abnormal ultrastructural features were found in knockout mice, also suggesting neuronal degeneration and inflammation. Neonatal intravenous administration on the second postnatal day with AAV expressing arginase 1 by a hepatocyte-specific promoter led to a near-resolution of these abnormalities when administered to homozygous *Arg1* knockout animals. Summary: Our studies suggest that arginase 1 deficiency leads to severe and specific changes to intrinsic excitability and synaptic connectivity of motor cortical circuits. Importantly, we find that neonatal AAV-based *Arg1* gene expression is effective in reversing both the physiological and anatomical hallmarks of the disorder.



46. The First Viable Mouse Model of *cblC* Type Combined Methylmalonic Acidemia and Homocysteinemia: AAV Gene Therapy Rescues Neonatal Lethality and Provides Insight into Disease-Associated Retinal Degeneration

Madeline Arnold[1], Jennifer L. Sloan[1], Nathan P. Achilly[1], Gene Elliot[1], Ighovie F. Onojafe[2], Brian P. Brooks[1,2], Charles P. Venditti[1]
[1]National Human Genome Research Institute, Bethesda, MD, [2]National Eye Institute, Bethesda, MD

Combined methylmalonic acidemia and homocysteinemia, *cblC* type (*cblC*), is the most common inborn error of cobalamin metabolism and is caused by mutations in the *MMACHC* gene. MMACHC transports and processes intracellular cobalamin (vitamin B12) into its two active cofactors, 5'-adenosylcobalamin and methylcobalamin, necessary for the enzymatic reactions of methylmalonyl-CoA mutase and methionine synthase, respectively. Mutations in *MMACHC* result in methylmalonic acidemia, hyperhomocysteinemia and hypomethioninemia. Disease manifestations include growth failure, anemia, heart defects, developmental delay and a progressive

maculopathy and pigmentary retinopathy that causes blindness, usually by the end of the first decade. Despite the use of conventional therapies including cobalamin injections and other cofactors, the manifestions of *cblC*, in particular the eye disease, remain unresolved with treatment. In order to explore disease pathophysiology and develop AAV gene therapy for *cblC* deficiency, we first created a viable mouse model using TALENs to edit the murine *Mmachc* gene, near the location of the most common mutation in humans, c.271dupA. Two alleles were investigated: c.165_166delAC p.P56CfsX4 (Δ2) and c.162_164delCAC p.S54_T55delinsR (Δ3). $Mmachc^{\Delta 2/\Delta 2}$ and $Mmachc^{\Delta 3/\Delta 3}$ homozygous mutant mice displayed reduced survival, severe growth retardation, and massive metabolic perturbations. The median survival was less than 7 days with 90% of the mutant mice perishing before 3 weeks (Δ2 n=13; Δ3 n=42). The weights of $Mmachc^{\Delta 3/\Delta 3}$ mice were reduced relative to heterozygote and wild type littermates (n=15, p<0.0001). $Mmachc^{\Delta 2/\Delta 2}$ and $Mmachc^{\Delta 3/\Delta 3}$ mice (n=4, n=6) recapitulated the biochemical features of *cblC*, with significantly elevated plasma methylmalonic acid, homocysteine, cystathionine and decreased methionine compared to littermate controls (n=7) (p<0.05 for all metabolites). To assess the potential for gene therapy as a treatment for *cblC*, we generated two AAV vectors: rAAVrh10-CBA-m*Mmachc* and rAAV9-CBA-h*MMACHC* and compared AAV with biweekly injections of OH-cobalamin, the standard therapy. $Mmachc^{\Delta 3/\Delta 3}$ mice were then treated with a single vector dose (1 x 10^{11} GC) delivered via intrahepatic injection in the neonatal period. $Mmachc^{\Delta 3/\Delta 3}$ mice treated with AAV vectors (AAVrh10 n=11, AAV9 n=5) displayed dramatically improved clinical appearance with improved growth (p= 0.0568), and increased survival (p<0.0001 for both vectors), with the oldest treated mutants currently living beyond 9 months. Successful gene therapy in the $Mmachc^{\Delta 3/\Delta 3}$ mice also enabled us to model, for the first time, *cblC* associated ocular pathology: surviving $Mmachc^{\Delta 3/\Delta 3}$ mice displayed thinning of the outer nuclear layer and shortening of photoreceptor outer segments, consistent with the pathology described in patients. Our results demonstrate that AAV gene delivery of *MMACHC* represents a new therapy for *cblC* which can treat the systemic, and possibly ocular, manifestations of this relatively common and devastating inborn error of metabolism.

47. Gene Transfer-Mediated Diversion Towards Non-Toxic Metabolites for Therapy of Primary Hyperoxaluria Type 1

Nicola Brunetti-Pierri, Raffaele Castello, Patrizia Annunziata, Roberta Borzone
Telethon Institute of Genetics and Medicine, Pozzuoli, Italy

Primary hyperoxaluria type 1 (PH1) is an inborn error of liver metabolism due to mutations of the *AGXT* gene encoding the peroxisomal enzyme alanine:glyoxylate-aminotransferase (AGT) which catalyzes the conversion of glyoxylate to glycine. In PH1 patients, glyoxylate cannot be efficiently converted into glycine and is instead oxidized to oxalate resulting in systemic oxalosis with deposition of insoluble calcium oxalate in kidneys and in other tissues, leading to nephrolithiasis, nephrocalcinosis, kidney failure, and systemic tissue damage. Combined liver/kidney transplantation is the only therapeutic strategy available to prevent disease progression. We hypothesize that overexpression of specific genes encoding enzymes involved in glyoxalate metabolism will steer glyoxylate towards alternative pathways to diminish oxalate production. To test this hypothesis, we overexpressed murine glyoxylate reductase/hydroxypyruvate reductase (GRHPR), that converts glyoxylate into glycolate, by a helper-dependent adenoviral vector (HDAd-GRHPR) in livers of $Agxt^{-/-}$ mice. The intravenous injection of HDAd-GRHPR resulted in significant reduction of hyperoxaluria and concomitant increase of serum glycolate that was not associated with signs of toxicity. Glutamate-pyruvate transaminase (GPT) in the cytosol transaminate glyoxylate using glutamate and alanine as amino-group donors. We hypothesize that GPT overexpression will steer glyoxylate towards transamination to diminish oxalate production. The intravenous injection of a helper-dependent adenoviral vector expressing murine GPT (HDAd-GPT) in $Agxt^{-/-}$ mice also resulted in significant and sustained reduction of hyperoxaluria. Interestingly, co-administration of both HDAd-GRHPR and HDAd-GPT resulted in further reduction and normalization of hyperoxaluria. Glycolate is one of the substrates leading to glyoxylate production, via peroxisomal glycolate oxidase (GO). We also show that an HDAd expressing a short hairpin RNA against GO resulted in reduction of hyperoxaluria in $Agxt^{-/-}$ mice. In summary, the results of this study show that metabolic diversion towards non-toxic metabolites has potential for treatment of PH1 and potentially other forms of hyperoxalurias, both primary and secondary. The metabolic diversion could be also obtained by RNA-based molecules expressing GRHPR and/or GPT or inhibiting GO activity. In addition, this study shows that HDAd vectors can be used to functionally validate therapeutic enzyme targets in inherited metabolic diseases.

48. Treatment of Methylmalonic Acidemia by Promoterless Gene-Targeting Using Adeno-Associated Viral (AAV) Mediated Homologous Recombination

Randy J. Chandler[1], Adi Barzel[2], Mark A. Kay[2], Charles P. Venditti[1]
[1]*MGMMGB, National Institutes of Health, Bethesda, MD,* [2]*Departments of Pediatrics and Genetics, Stanford University, Stanford, CA*

Methylmalonic acidemia (MMA) is an autosomal recessive inborn error of metabolism most typically caused by mutations in methylmalonyl-CoA mutase (*MUT*). While the hallmark of this disease is elevated levels of methylmalonic acid in the plasma, other disease related metabolites such as methylcitrate are elevated in the plasma as well. Patients with MMA suffer from frequent and potential lethal bouts of metabolic instability that can be eliminated by liver transplantation. Adeno-associated viral (AAV) gene therapy has shown great promise as the treatment for MMA in a murine model of the disease. However, a majority of the AAV-treated mice developed hepatocellular cancer, which was determined to arise from AAV-mediated insertional mutagenesis. In an attempt to create a safer gene therapy platform for the treatment of MMA, we created a novel vector for site-specific gene addition of human *MUT* into the mouse albumin (*Alb*) locus. This promoterless AAV vector contains a 2A-peptide coding sequence proximal to a codon-optimized human *MUT* gene. The 2A-MUT sequence is flanked by arms of homology immediately upstream of mouse *Alb* stop codon. Since albumin is expressed exclusively in hepatocytes, we prepared an AAV serotype 8 vector to take advantage of the murine liver tropism conferred by this capsid. This newly created vector was named AAV8-Alb-A2-MUT. After site-specific integration of the vector into the *Alb* locus in the liver, ribosomal skipping generates both Alb and MUT as separate proteins derived from the same transcript. We delivered a dose of 2.5e12 GC of AAV8-Alb-2A-MUT to five mice with MMA by intraperitoneal injection at birth. At one month post-injection, we observed increased hepatic expression of the MUT by western blot, improved growth and a significant reduction of disease related metabolites, in the treated MMA mice (Table 1). This gene delivery approach is anticipated to provide permanent hepatic transgene expression while reducing the risk of off-target integration and vector-mediated insertional mutagenesis.

Table 1			
Markers of Correction in MMA Murine Model	Untreated (n=3-8)	AAV8-Alb-A2-MUT (n=5)	P Value
Percent of Wild-type Mut Expression	ND	11.3+/-9.1	NA
Plasma Methylmalonic Acid (uM)	969+/-396	563+/-98	<0.05
Plasma Methylcitrate (nM)	5,020+/-873	2,904+/-638	<0.0001
Weight at 1 Month	6.9+/-1.6	10.7+/-0.6	<0.003

49. Minimum Effective Dose of Liver-Targeted Gene Therapy for Pompe Disease Reduced 10-Fold from Prior Estimates

Sang-oh Han[1], Benjamin Arnson[1], Korie Busch[1], Giuseppe Ronzitti[2], Songtao Li[1], Federico Mingozzi[2], Dwight D. Koeberl[1]
[1]Duke University Medical Center, Durham, NC, [2]Genethon, Evry, France

Pompe disease (glycogen storage disease type II; acid maltase deficiency) is a devastating myopathy resulting from acid alpha-glucosidase (GAA) deficiency in striated and smooth muscle. Despite the availability of enzyme replacement therapy (ERT) with recombinant human GAA, many patients have poor outcomes including mortality. ERT has increased survival rates, improved hypotonia, and resolved heart involvement in patients with Pompe disease. However ERT is limited by the short half-life of GAA and the formation of antibody responses that reduced its clinical benefits. The present study compared the efficacy of ERT against an adeno-associated viral (AAV) vector containing a liver-specific promoter, AAV2/8-LSPhGAApA. We hypothesized that liver-specific expression of GAA with AAV2/8-LSPhGAA would suppress the antibody response, continually express GAA, and improve efficacy in comparison with ERT. Preclinical experiments with Pompe disease therapies demonstrated that a very low amount of AAV2/8-LSPhGAApA, $2x10^{10}$ vector particles (vp), equivalent to $8x10^{11}$ vp/kg body weight, was as effective as long-term ERT. Therefore, we evaluated the biochemical efficacy of 3 lower dosages of AAV2/8-LSPhGAA in GAA knockout (KO) mice, reduced to as low as $2x10^{10}$ vp/kg, either alone or in combination with ERT. Biochemical correction in GAA-KO mice was evaluated 8 weeks following vector administration. The minimum effective dose (MED) was at least 10-fold lower than previously estimated, because $8x10^{10}$ vp/kg significantly reduced glycogen content in the striated muscle of GAA-KO mice. This MED of AAV2/8-LSPhGAA significantly increased GAA activity in liver ($p<0.01$), in comparison with untreated GAA-KO mice. Importantly, the MED significantly reduced the glycogen content of heart ($p<0.01$; Fig. A), and diaphragm ($p<0.01$; Fig. B), which demonstrated that the glycogen storage in muscle associated with Pompe disease was substantially cross-corrected by GAA secretion from liver accompanied by receptor-mediated uptake in striated muscle. Glycogen clearance, or reduction in comparison with no treatment, was 36% in heart, and 35% in diaphragm. Furthermore, administering ERT by itself had no significant effect on the glycogen content of quadriceps, but ERT following administration of the MED of AAV2/8-LSPhGAA significantly reduced glycogen content of quadriceps ($p<0.05$; Fig. C) by 38%, indicating that gene therapy with AAV2/8-LSPhGAA made ERT more effective. Vector dose correlated inversely with anti-GAA antibody formation, confirming that immune tolerance to GAA was critical to the efficacy from AAV2/8-LSPhGAApA. In conclusion, the MED for AAV2/8-LSPhGAA has been estimated at least 10-fold lower than previous data suggested, and this dosage would be acceptable as a starting dose for a Phase I clinical trial of gene therapy in Pompe disease.

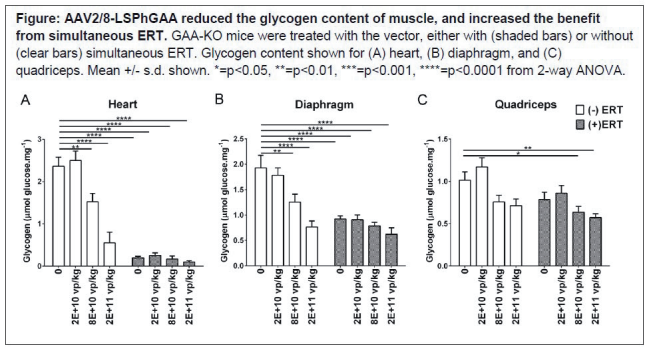


Figure: AAV2/8-LSPhGAA reduced the glycogen content of muscle, and increased the benefit from simultaneous ERT. GAA-KO mice were treated with the vector, either with (shaded bars) or without (clear bars) simultaneous ERT. Glycogen content shown for (A) heart, (B) diaphragm, and (C) quadriceps. Mean +/- s.d. shown. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001 from 2-way ANOVA.

50. Long-Term Follow-Up of Diabetic Dogs Treated with Adeno-Associated Viral Vectors Encoding for Insulin and Glucokinase

Veronica Jimenez[1,2,3], Maria Luisa Jaen[1,2,3], Laia Vila[1,2,3], Jordi Rodo[1,2,3], Miquel Garcia[1,2,3], Ivet Elias[1,2,3], Luca Maggioni[1,2,3], Xavier Leon[1,2,3], Sergio Munoz[1,2,3], David Callejas[1,2,3], Iris Grifoll[1,2,3], Eduard Ayuso[1,2,3], Tura Ferre[1,2,3], Virginia Haurigot[1,2,3], Fatima Bosch[1,2,3]
[1]Center of Animal Biotechnology and Gene Therapy (CBATEG), Universitat Autonoma de Barcelona, Bellaterra, Spain, [2]Department of Biochemistry and Molecular Biology, School of Veterinary Medicine, Universitat Autonoma de Barcelona, Bellaterra, Spain, [3]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Madrid, Spain

Diabetes is a chronic disease for which there is no cure and is associated with severe secondary complications, caused largely by poor glycaemic control. Treatment with exogenous insulin fails to prevent these complications completely, leading to significant morbidity and mortality.

We previously demonstrated that it is possible to generate a "glucose sensor" in skeletal muscle through co-expression of glucokinase (Gck) and insulin (Ins) by adeno-associated viral vectors of serotype 1 (AAV1), increasing glucose uptake and correcting hyperglycaemia in diabetic mice and dogs. Here we show the results of a 8-year follow-up study of diabetic dogs previously treated with a single administration of AAV1 vectors encoding for Ins and Gck. Dogs showed normalization of fasting glycaemia, recovery of body weight, reduced fructosamine levels and increased insulin and C-peptide circulating levels throughout the observation period. Vector genome biodistribution analysis confirmed that most of the detectable vector was present in injected muscles, which showed normal muscle morphology. Accordingly, Ins and Gck transgene expression was only detected in skeletal muscle of treated dogs. This was associated with long-term survival without occurrence of secondary diabetic complications or adverse events for 8 years after AAV-mediated gene transfer.

Our data represent the first demonstration of long-term correction of diabetes in a large animal model using gene transfer. This study also confirms the long-term safety of the AAV-mediated overexpression of Ins and Gck for the treatment of diabetes, laying the foundation for the clinical translation of this approach to veterinary medicine and to human patients in the future.

51. LV.InsB9-23/Anti-CD3 mAb Inhibits Recurrence of Autoimmunity in NOD Mice After Islet Transplants

Fabio Russo[1], Maria Grazia Roncarolo[2], Andrea Annoni[1]
[1]SR-Tiget, Milano, Italy, [2]Division of Stem Cell Transplantation and Regenerative Medicine, Department of Pediatrics, Stanford School of Medicine, Stanford, CA

Type 1 diabetes (T1D) is an autoimmune disease resulting in complete destruction of insulin-producing pancreatic beta-cells, which is mediated by auto-reactive T cells. In T1D in human and in the non-obese diabetic (NOD) mouse, the spontaneous murine model of T1D, induction of tolerance to beta-cell associated antigens represents a potential curative option. We previously showed that systemic administration of a single dose of integrase-competent (IC) or integrase-defective (ID) LV.ET.InsB9-23.142T (LV.InsB), enabling stable or transient expression of InsB9-23 in hepatocytes, arrests beta-cell destruction in NOD mice at advanced pre-diabetic stage. In these mice stable normoglycemia is maintained long term and InsB9-23-specific FoxP3$^+$ T regulatory cells (Tregs) are generated. Moreover, LV.InsB in combination with a suboptimal dose (1X 5µg) of anti-CD3 mAb reverts overt diabetes and preserves residual beta-cell mass. In the present study we tested the efficacy of the LV.InsB/anti-CD3 combination therapy to inhibit recurrence of autoimmunity and maintain insulin independence after pancreatic islets transplants. Pancreatic islets isolated from NOD-*scid* donor mice were transplanted under the kidney capsule of diabetic NOD mice with blood glucose levels >350mg/dL. Successfully transplanted mice (normoglycemic: blood glucose levels ~100 mg/dL) were treated with LV.InsB/anti-CD3; anti-CD3 mAb (1X 5µg) alone or left untreated as controls. LV.InsB/anti-CD3 combination therapy allowed stable normoglycemia up to 250 days whereas in mice receiving anti-CD3 mAb alone or untreated controls recurrence of diabetogenic responses cleared transplanted islets in 2 weeks. Autoreactive T cells were still present in the spleen of transplanted mice treated with LV.InsB/anti-CD3, but the frequency of FoxP3$^+$ Tregs within the CD4$^+$ T cells in renal (RLN) and pancreatic (PLN) lymph nodes significantly increased, as shown by phenotypic analysis. These data show that LV.InsB/anti-CD3 treatment induces active suppression of autoimmune responses in long-term normoglycemic transplanted NOD mice. Ag-specific FoxP3$^+$ Tregs accumulating in the PLN and RLN suppress effector T cells present in the target tissue. This mode of action is similar to what we previously described after LV.InsB treatment in a model of autoimmunity (Akbarpour M. et al. Science TM. 2015). Further studies are currently ongoing to evaluate the relative contribution of endogenous and exogenous beta-cell mass to insulin independence and to investigate the efficacy of the LV.InsB/anti-CD3 treatment after transplant of allogeneic pancreatic islets. The definition of novel gene therapy strategies to induce Ag-specific tolerance that at the same time control autoimmunity and transplant rejection would represent a major step toward the cure of type 1 diabetes.

52. Therapeutic Factor VIII Expression After AAV Delivery in Non-Human Primates

Xavier M. Anguela[1], Liron Elkouby[1], Raffaella Toso[1], Marti DiPietro[1], Robert J. Davidson[2], Katherine A. High[1], Denise E. Sabatino[2]
[1]Spark Therapeutics, Inc., Philadelphia, PA, [2]The Children's Hospital of Philadelphia, Philadelphia, PA

Adeno-associated viral (AAV) vector delivery of factor VIII (FVIII) has been challenging due to its intrinsic properties that result in inefficient expression compared to similarly sized proteins. Early studies of AAV delivery in hemophilia A mice and dogs suggested that the therapeutic vector dose for FVIII will be higher than for factor IX. However, higher vector loads may induce stronger immune responses against capsid antigens, as evidenced in the clinical studies of AAV delivery for hemophilia B. The use of codon-optimization and novel FVIII variants with enhanced biological properties may provide strategies to increase FVIII expression or secretion to support clinical studies for hemophilia A. One published study has reported clinically relevant levels of hFVIII following AAV-hFVIII delivery in non-human primates (NHPs). This study utilized a hFVIII variant that included a 17 amino acid synthetic sequence within the 14 amino acid B-domain region that increased hFVIII expression compared to the parental B-domain deleted FVIII-SQ transgene (McIntosh, 2013). While this and other variants may increase expression after AAV delivery, the use of non-native FVIII sequences may also increase the risk of development of neutralizing antibodies to potential neo-antigens. In order to generate an AAV-hFVIII vector capable of expressing therapeutic levels of FVIII at a clinically relevant vector dose without introduction of any neoantigens, 28 hFVIII-SQ sequences were generated and introduced into our optimized expression cassette containing a modified transthyretin (TTRm) promoter. The constructs were initially screened by hydrodynamic delivery of plasmid DNA which identified 11 candidates that expressed FVIII 2-7 fold higher than our first generation codon optimized construct, CO3. AAV vectors (n=9) were generated using a novel AAV capsid, Spark100, with the best performing FVIII constructs. Hem A/CD4 KO mice were administered the vectors alongside CO3 (4x10e12vg/kg). At 8 weeks post vector administration, 2/9 expressed hFVIII similar to CO3, 5/9 were 4-8 fold higher than CO3 while 2/9 (SPK-8003 and SPK-8005) were >10 fold more potent than CO3. SPK-8005 was then evaluated in a dose escalation study in cynomologus macaques (n=3/group) treated with 3 doses: 2x10e12, 5x10e12 and 1x10e13 vg/kg and compared to vehicle controls (n=2). At 2 weeks post AAV administration, average hFVIII levels in the low, mid and high dose cohorts were 12.7 ± 2.1, 22.6 ± 0.8 and 54.1 ± 15.6 percent of normal, respectively. By 3-4 weeks, hFVIII expression started to decline in most of the animals concomitant with generation of antibodies against human FVIII. Of note, this is an expected and well-described observation that occurs in immune competent animal models due to differences between human and endogenous FVIII protein sequences. The 2 macaques that did not develop anti-hFVIII antibodies had sustained FVIII expression through the last time point evaluated. Finally, no vector-related toxicity events were observed. In summary, extensive codon-optimization identified novel AAV-hFVIII constructs capable of achieving therapeutic FVIII levels in macaques at clinically relevant doses. To our knowledge, the hFVIII levels observed in this study are the highest reported in a large animal model after treatment with an AAV vector expressing an unmodified FVIII-SQ protein. These safety and efficacy results in NHPs support the use of SPK-8005 hepatic gene transfer for the potential treatment of hemophilia A.

Gene Therapy for CNS Diseases

53. A Neuro-Specific Gene Therapy Approach to Treat Cognitive Impairment in Down Syndrome by RNA Interference

Andrea Contestabile[1], Martina Parrini[1], Shovan Naskar[1], Micol Alberti[1], Anna Rocchi[1], Marina Nanni[1], Giuseppe Ronzitti[2], Federico Mingozzi[2], Laura Cancedda[1]
[1]Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia (IIT), Genova, Italy, [2]Genethon, Evry, France

Down syndrome (DS) is a genetic disorder caused by the presence of a third copy of chromosome 21. DS affects multiple organs, resulting in characteristic facial features, muscular hypotonia, heart defects, brain development impairment, and varying degrees of

intellectual disability. Trisomic mouse models of DS reproduce the main cognitive disabilities of the human syndrome. In particular, DS mice show structural and functional synaptic impairment as well as learning and memory deficits, largely determined by altered GABAergic transmission through chloride-permeable $GABA_A$ receptors ($GABA_AR$). In particular, we have recently found that intracellular chloride accumulation shifts $GABA_AR$-mediated signaling from inhibitory to excitatory in the adult brain of the Ts65Dn mouse model of DS. Accordingly, intracellular chloride accumulation was paralleled by increased expression of the chloride importer NKCC1 (Na-K-Cl cotransporter) in the brains of both trisomic mice and DS patients.

Our findings on NKCC1 as a pivotal molecular target for the rescue of cognitive deficits in DS opens the possibility of a gene therapy approach to treat the disease. Here, to normalize NKCC1 expression and rescue synaptic dysfunctions as well as cognitive deficits in Ts65Dn mice we have developed and characterized a knock-down approach to normalize NKCC1 activity. Reducing the expression of the chloride importer NKCC1 by RNA interference restored $GABA_AR$-mediated inhibition and also rescued the structural dendritic deficits found in trisomic neurons *in vitro*. Most importantly, focal administration of an AAV expressing a silencing RNA under the transcriptional control of a neuron-specific promoter in the hippocampus of Ts65Dn animals mediated NKCC1 knockdown *in vivo* and rescued behavioral performance on different learning and memory tests at levels undistinguishable from those of WT mice.

Our findings demonstrate that NKCC1 overexpression drives excitatory $GABA_AR$ signaling in trisomic cells, leading to structural neuronal abnormalities and behavioral impairments in DS mice. Moreover, our study identifies a new gene therapy target for treatments aimed at rescuing cognitive disabilities in individuals with DS.

54. Prevention of Sensory Ataxia in a Novel Mouse Model of Friedreich Ataxia Using Gene Therapy Approach

Françoise Piguet, Charline de Montigny, Nadège Vaucamps, Laurence Reutenauer, Hélène Puccio
IGBMC, Illkirch, France

Friedreich's ataxia (FRDA), the most common autosomal recessive ataxia, is characterized by a sensory and spinocerebellar ataxia, hypertrophic cardiomyopathy and increase incidence of diabetes. FRDA is caused by reduced levels of frataxin (FXN), an essential mitochondrial protein involved in the biosynthesis of iron-sulfur (Fe-S) clusters. Impaired mitochondrial oxidative phosphorylation, bioenergetics imbalance, deficit of Fe-S cluster enzymes and mitochondrial iron overload occur in individuals with FRDA. Proprioceptive neurons within the dorsal root ganglia (DRG) and cardiomyocytes are the most affected tissues in FRDA patients. To date there are not effective treatment for FRDA. We have previously established the primary proof-of-concept for developing gene therapy of FRDA cardiomyopathy and showed that adeno-associated virus (AAV) rh.10 vector expressing human FXN injected intravenously not only prevented the onset of the cardiac disease in a faithful FRDA cardiac mouse model, but also, when administered at the time of heart failure, rapidly and completely reversed the cardiac disease. To date, there were unfortunately no adequate neuronal mouse model to address the possibility of gene therapy for the neuronal aspects of FRDA. We therefore recently generated a novel mouse model that recapitulates faithfully the sensory ataxia associated to FRDA using the conditional approach to delete frataxin specifically in the proprioceptive neurons of the DRG. By behavioural analysis, the mice exhibit an ataxic phenotype beginning at 3 weeks of age, which is rapidly progressive. Electrophysiological studies reveal a significant decrease of sensory wave already at 4.5 weeks and almost a complete loss at 8 weeks of age. A significant loss of sensory neurons within dorsal root ganglia is observed at 17.5 weeks of age compare to age matched controls. Ultrastructural analysis of sciatic and saphenous nerves showed abnormalities at early time points. Using this mouse model, we have developed an AAV gene therapy approach based on an intravenous delivery of AAV9-CAG-hFXN-HA vector at an early symptomatic stage of the disease. Mice displayed a complete prevention of the ataxic phenotype and electrophysiological analysis showed maintenance of the sensory wave in the treated animals. Histological studies revealed a complete prevention of neuronal loss in the DRG as well as a normal ultrastructure of saphenous and sciatic nerve. As results are encouraging, we plan to evaluate the potential therapeutical effect at a more advanced stage of the disease.

55. Aquaporins and CSF Flux Are Critical Determinants of AAV Mediated CNS Gene Transfer

Giridhar Murlidharan, Aravind Asokan
Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC

The central nervous system (CNS) lacks conventional lymphatics for solute clearance. Convective currents of interstitial fluid (ISF) shunt macromolecules across the brain parenchyma into cerebrospinal fluid (CSF) compartments. Here the cargo is either reabsorbed into CNS via subarachnoid ducts or disseminated into the blood or peripheral lymph vessels. Recent studies have identified that flow of water via aquaporin (AQP) channels at the astroglial endfeet enables exchange of biomaterials between ISF and CSF. Conversely, loss of AQP expression due to aging or disease is correlated with ineffective clearance of neurodegenerative accumulations such as Amyloid β and tau. AAV mediated gene therapy of CNS disorders requires a deeper understanding of such factors and their effects on transduction efficiency and biodistribution/clearance. Here, we demonstrate that AQP mediated water transport dictates various aspects of AAV gene transfer in the CNS. We compared the CNS spread, transduction profile and systemic leakage of clinically relevant AAV vectors in wildtype (WT) or AQP knockout (AQP-/-) mice. Within minutes following intracerebroventricular (ICV) administration, AQP-/- mice exhibited highly restricted spread of fluorophore labeled AAVs when compared to wild type mice. Transgene expression was markedly increased from AAV administrations in AQP-/- mice. Further, systemic leakage and off-target transgene expression in peripheral tissues were markedly reduced for some AAV serotypes in AQP-/- mice when compared to WT mice. These results suggest that AQP-mediated water transport plays a critical role in determining the spread, transduction efficiency and systemic leakage of AAV vectors following CNS administration. We hypothesize that altered CSF flux under conditions pertinent to aging and CNS disease, can impact the residence time of AAV vectors and consequently gene transfer efficiency. Further results evaluating the CNS transport properties of AAV in mouse models of aging and disease will be presented.

56. Intracerebroventricular and Intravenous AAV Gene Therapy in Canine Globoid Cell Leukodystrophy

Allison M. Bradbury[1], Mohammad Rafi[2], Gary P. Swain[1], Jessica H. Bagel[1], Maria Prociuk[1], Patricia O'Donnell[1], David Peterson[3], Daniel S. Ory[1], James M. Provenzale[3], David A. Wenger[2], Charles H. Vite[1]
[1]Clinical Studies, University of Pennsylvania, Philadelphia, PA, [2]Thomas Jefferson University, Philadelphia, PA, [3]Duke University School of Medicine, Durham, NC

Globoid cell leukodystrophy (GLD), or Krabbe disease, results from a deficiency in the hydrolytic enzyme galactosylceramidase

(GALC), which is responsible for degrading the central and peripheral nervous system (CNS, PNS) myelin lipids galactosylceramide and galactosylsphingosine (psychosine). Symptoms in infants include irritability and stiffness with disease progressing rapidly to a vegetative state and death typically resulting by 2 years of age. GLD is naturally occurring in dogs and signs include ataxia, tremors, pelvic limb paralysis, loss of hearing, and blindness, with disease progression warranting euthanasia at 15.9 ± 4.6 weeks. In GLD dogs, GALC concentrations are decreased in leukocytes, CSF, and tissues and psychosine concentrations are elevated in serum and CSF. Conduction velocities in peripheral nerves of GLD dogs are < 50% of normal and G-ratios of the sciatic nerve are significantly higher than normal. Brain stem auditory evoked response testing exhibits a loss of waveform integrity and increased central conduction time consistent with auditory system demyelination. MRI of the brain shows T2-weighted hyperintensity of the white matter, widened sulci, and enlarged ventricles and MRS identified decreases in N-acetylaspartate and increases in choline. Diffusion tensor imaging established decreased fractional anisotropy and increased radial diffusivity in multiple white matter tracts including the corpus callosum and posterior internal capsule. Histologically, GLD dogs revealed severe loss of myelin, a decrease in oligodendrocytes, astrogliosis, and microgliosis. Combination gene therapy has been evaluated in GLD affected dogs using AAVrh10 encoding canine *GALC* (AAVrh10-cGALC). Two GLD dogs received a low dose of AAVrh10-cGALC, 1.2E12, by combination intravenous (IV) and intracerebroventricular (ICV) injection routes and displayed a modest increase in survival to 17.9 and 22.1 weeks of age. Two additional GLD dogs were treated with an increased dose of AAVrh10-cGALC, 1.9E13, and survival was further increased to 30.3 and 43.1 weeks of age. Both low and high dose combination IV and ICV therapy delayed the onset of neurological signs and prevented the onset of tremors, one of the debilitating neurologic signs in untreated GLD dogs. High dose AAVrh10-cGALC, but not low dose, resulted in complete normalization of pelvic and thoracic limb NCV and near normal sensory NCV. Combination therapy of either dose had negligible effect on the auditory system, as treated animals showed little to no improvement in distance between waveform peaks or hearing threshold over untreated animals. After high dose treatment with AAVrh10-cGALC, GALC activity reached near normal levels in the cerebellum and sciatic nerve, with levels decreasing in more distal brain regions. GALC levels in the liver and heart were intermediate between affected untreated GLD and normal control dogs. Interestingly, the highest GALC activity was found in the quadriceps muscle. GLD dogs treated with high dose AAV had CSF psychosine concentrations lower than untreated and low dose AAV-treated GLD dogs, despite being substantially older. Efficacy of hematopoietic stem cell transplant (HSCT) alone and in combination with IV AAVrh10-cGALC is currently being evaluated in GLD dogs. Ongoing studies suggest that addition of IV infusion of AAVrh10-cGALC substantially increases survival as compared to HSCT alone.

57. Intrathecal Administration of AAV/GALC Vectors in Juvenile Twitcher Mice Improves Survival and Is Enhanced by Bone Marrow Transplant

Subha Karumuthil-Melethil[1], Mike Marshall[2], Ernesto R. Bongarzone[2], Steven J. Gray[3]
[1]Gene Therapy Center, University of North Carolina, Chapel Hill, NC, [2]Dept of Anatomy & Cell Biology, University of Illinois at Chicago, Chicago, IL, [3]Gene Therapy Center & Dept of Ophthalmology, University of North Carolina, Chapel Hill, NC

Globoid cell Leukodystrophy (GLD) or Krabbe disease is a rapidly progressing, neurodegenerative disease caused by the deficiency of the lysosomal enzyme Galactocerebrosidase (GALC). The pathological characteristics include presence of globoid cells and decreased myelin. In its severe infantile form, the symptoms appear within the first 6 months of life and complete loss of GALC function is fatal by 2-3 years old. The murine model of infantile GLD, the twitcher mouse, has been used to evaluate potential therapeutic approaches for GLD. Hematopoietic stem cell transplantation (HSCT) provides modest benefit in presymptomatic patients and mice indicating a slowdown in the progression of the disease, but no complete cure. Neonatal gene transfer experiments using viral vectors also has shown some limited success in extending the survival of the twitcher mice. The translatability of neonatal therapy in mice to human has met with difficulties as the stage of the disease in human and mice differ due to the difference in their gestation periods. So there is a need for testing out later stage interventions for GLD treatment. In the present study, we compare multiple vector designs along with a combination treatment of AAV plus bone marrow transplant (BMT) in juvenile twitcher mice. Initially, three single stranded (ss) AAV serotypes, two natural and one engineered (with oligodendrocyte tropism), were packaged with a codon-optimized murine *GALC* gene driven by the beta actin promoter. The vectors were delivered via a lumbar intrathecal route for global CNS distribution on post-natal day (PND) 10-11, at a dose of $2x10^{11}$ vg per mouse. The results show a significant extension of life span of the twitcher mice for all three serotypes (AAV9, AAVrh10, and AAV-Olig001) when compared to control cohorts. The treatment produced similar survival benefit regardless of which capsid was used. The rAAV gene transfer facilitates GALC biodistribution and detectable enzymatic activity throughout the CNS as well as in sciatic nerve and liver. When combined with BMT from syngeneic wild type mice, there was significant improvement in survival and enzymatic activity over either treatment alone. Immunohistochemical analysis of the brain and spinal cord showed reduced inflammation and pathology. Additionally, we have also tested a novel self-complementary (sc) AAV vector with a minimal synthetic promoter, which would mediate a weaker overall level of GALC expression but express in more cells. Preliminary results indicate that this vector design provides a survival advantage over the ssAAV vector designs. In summary, we demonstrate that lumbar intrathecal delivery of rAAV/mGALCopt can significantly enhance the life span of twitcher mice treated at juvenile stage (PND10-11) and BMT synergizes with this treatment to further improve the survival. This effect is mediated by increased GALC activity in various parts of the nervous system as well as by the reduction in neuro-inflammation. Together, these studies detail a therapeutic approach for GLD in mice which is feasible and relevant for human translation.

58. Pushing the Limits for Canavan Gene Therapy into Adulthood: Is There an Age Limit for Gene Therapy in CNS Disorders?

Dominic J. Gessler[1], Danning Li[1], Hongxia Xu[1], Qin Su[1], Reuben Matalon[2], Guangping Gao[1]
[1]Gene Therapy Center, UMass, Worcester, MA, [2]University of Texas Medical Branch, Galveston, TX

Canavan Disease (CD) is a rare and lethal inherited leukodystrophy caused by autosomal recessive mutations in the aspartoacylase (Aspa) gene. To date, no effective treatment is available. Thus, gene therapy is an attractive approach to treat this devastating disease. Commonly associated with early death in childhood, an increasing group of patients reach teen age or even adulthood and in the process pose new challenges for families and the scientific community. The availability of a new mouse model (Nur7) with barely reduced life expectancy but full blown Canavan-like clinical manifestation prompted us to determine if there is a point-of-no-return in the gene therapeutic treatment of Canavan disease and to identify potentially limiting

factors in the development of the central nervous system (CNS) and severity of the neuropathology. We previously reported that early postnatal systemic delivery of the human aspartoacylase (hASPA) gene by recombinant adeno-associated virus (rAAV) to the CNS of a CD mouse model with neonatal death rescued lethality and partially restored motor function. Now in its 3rd generation, our Canavan gene therapy completely reverses the disease phenotype in the CD KO mouse. For clinical translation to treat juvenile and adult patients, we sought to study and understand the age-limitations for Canavan disease gene therapy. We hypothesized that there is a point-of-no-return, when gene replacement therapy alone is no longer sufficient to successfully alter the disease outcome. First, Nur7 mice were treated at post-natal (p) day 1 as the gold standard. In the next step, experimental groups were dosed at 6, 12, and 24 weeks of age with a dose 10-fold higher than that for neonates. Motor function was tested for all mice 4 weeks after treatment and subsequent intervals up to one year of age for direct comparison. As expected, the earlier mice were treated, the better the therapeutic outcome. To our surprise, juvenile mice at 6 weeks of age recovered completely within 4 weeks post-injection. Although mice treated at 3 months of age and older did not respond immediately within the first 4 weeks post-treatment, they eventually showed significant improvements over Nur7 mutant control mice. Of note, cognitive function testing revealed that treated mice recover cognitively before motor function improves; this was even true for late treatment time points. Furthermore, response to rAAVhASPA gene therapy was confirmed via MRS for N-acetyaspartate, MRI, and neuropathology. Finally, our preliminary data characterizing oligodendrocytes and myelin in the Nur7 mouse model shows rapid reconstitution of myelin in mice treated at 6 weeks, underlining the potential of our therapy for later treatment. Currently, we are working on identifying molecular mechanisms limiting complete disease rescue in adult Nur7 mice to further explore options in order to successfully treat older patients. Overall, our data demonstrate that rAAV mediated hASPA expression of our 3rd generation gene therapy vector not only prevents but also rescues the clinical manifestation and pathology of the juvenile and adult model of Canavan disease at an unprecedented level, which might also have implications for other CNS disorders that require treatment in later stages of life.

59. APPsα Gene Therapy for Alzheimer's Disease

Romain Fol[1,2,3], Susann Ludewig[4], Tobias Abel[5], Sascha W. Weyer[6], Jan-Peter Roederer[6], Florian Brod[5], Mickael Audrain[1,2,3], Alexis-Pierre Bemelmans[7], Christian J. Buccholz[5], Martin Korte[4,8], Ulrike C. Müller[6], Nathalie Cartier-Lacave[1,2]
[1]INSERM U1169/MIRCen CEA, Fontenay aux Roses, France, [2]Université Paris Sud, Orsay, France, [3]Université Paris Descartes, Paris, France, [4]Zoological Institute, Division of Cellular Neurobiology, TU Braunschweig, Braunschweig, Germany, [5]Molecular Biotechnology and Gene Therapy, Paul-Ehrlich-Institut, Langen, Germany, [6]Dept. of Bioinformatics and Functional Genomics, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Heidelberg, Germany, [7]CNRS UMR9199 MIRCen CEA, Fontenay aux Roses, France, [8]HZI, AG NIND, Braunschweig, Germany

Alzheimer's disease (AD) is characterized by synaptic failure, dendritic and axonal atrophy, neuronal death and progressive loss of cognitive functions asociéted with β-amyloid accumulation and neurofibrillary tangles of phosphorylated Tau protein. Increasing evidence indicates that loss of physiological APP functions mediated predominantly by neurotrophic APPsα produced in the non-amyloidogenic α-secretase pathway may contribute to AD pathogenesis. We used an AAV vector to directly overexpress APPsa in the brain and explored its potential to rescue structural, electrophysiological and behavioral deficits in APP/PS1ΔE9 AD mouse model. Sustained APPsα overexpression in aged mice with already preexisting pathology and amyloidosis restored synaptic plasticity and rescued spine density deficits. Importantly, AAV-APPsα treatment also resulted in a functional rescue of spatial memory. A significant reduction of both toxic soluble β42 and plaque load was evidenced. APPsα induced the recruitment of microglia with ramified morphology towards plaques and upregulated IDE and TREM2 expression suggesting enhanced plaque clearance. APPsα overexpression in the brain using an AAV vector improves synaptic and cognitive deficits, despite established pathology and may be of therapeutic relevance for AD.

60. Engineering AAV Vector for the Delivery of Human BuChE to Protect Against Exposure to Organophosphates

Angelica Medina-Jaszek, Omua Ahokhai, Maryam Taheri, Muhammad Arif, Anna Tretiakova, James M. Wilson
Gene Therapy Program, Department of Medicine, University of Pennsylvania, Philadelphia, PA

Human butyrylcholinesterase (hBuChE) is a widely-distributed enzyme that may play a role in nerve conduction, can hydrolyze various toxic esters (e.g., cocaine), and acts as a potent scavenger of organophosphates (OP) including pesticides and chemical warfare agents. Major limitations prohibiting the development of purified hBuChE as a mainstream biologic drug include: limited availability of human plasma, limited scalability of manufacturing, high cost of purified human-derived product, and the need for intravenous administration. In addition, the preventive uses of hBuChE are limited by its relatively short half-life. In an attempt to address some of these limitations, several methods for the production of recombinant hBuChE *in vitro* (cell lines) and *in vivo* (transgenic animals, plants) were developed; however, the resulting recombinant proteins presented with unfavorable pharmacokinetic profiles making them unsuitable for preventive uses. Alternatively, adenovirus-mediated delivery of hBuChE is very effective, but is not a candidate for development due to the inherent immunogenicity of the vector. Expression of hBuChE peaks at around 4 days post IV administration of the adenoviral vector, and the peak is followed by a rapid decline, with minimal expression being detectable 10 days post-administration.

AAV vectors present a radically different, non-immunogenic hBuChE delivery platform, which is not reliant on protein purification and has the flexibility to deliver the product into both liver and muscle. AAV technology engineers the recipient's own cells to produce sustainable pharmacologic amounts of hBuChE on demand and/or replenish the exhausted scavenger via a single dose of hBuChE-expressing vector under the control of a constitutive promoter. Through the systematic optimization of AAV-hBuChE vectors, we achieved not only rapid onset of hBuChE expression in mice, but also sustainable, long-term expression for at least 2 months with no decline. AAV-hBuChE expressed at 100 units/ml of serum at 24 hours post vector administration into mouse muscle, with steady-state expression levels of up to 30,000 units/ml of serum at 1 month. The optimization parameters included selection of the protein isoform, tetramerization partner, coding sequence, as well as promoters and other regulatory elements. Overall, approximately 40 vectors were evaluated in comparative studies. Based on previous findings, the levels of hBuChE expressed by our platform are predicted to be protective in OP challenges, which we plan to perform in future studies. In the future, the use of our platform can be expanded to include populations that are occupationally exposed to pesticides. This approach may also be applicable for the delivery of other protective human enzymes, such as the use of rhodanese to protect against cyanides or glutathione against 2-chloro-acetophenone.

Cancer-Oncolytic RNA Viruses

61. Ascending Dose Trials of a Retroviral Replicating Vector (Toca 511) in Patients with Recurrent High-Grade Glioma: Clinical Update, Molecular Analyses, and Proposed Mechanism of Action

Douglas J. Jolly[1], Joan M. Robbins[1], Derek Ostertag[1], Carlos Ibañez[1], Noriyuki Kasahara[2], Harry Gruber[1], Steven N. Kalkanis[3], Michael Vogelbaum[4], Manish K. Aghi[5], Timothy Cloughesy[6], Alice Chu[1], Asha Das[1], Jamey Skillings[1]
[1]Tocagen Inc, San Diego, CA, [2]University of Miami, Miami, FL, [3]Henry Ford Hospital, Detroit, MI, [4]Cleveland Clinic, Cleveland, OH, [5]UCSF, San Fransisco, CA, [6]UCLA, Los Angeles, CA

We have conducted Phase 1 studies in patients with high grade glioma of a retroviral replicating vector (RRV), Toca 511 (vocimagene amiretrorepvec), based on an amphotropic murine gamma retrovirus encoding an optimized cytosine deaminase. The vector appears highly selective for tumor cells. Infected cells convert the antifungal drug 5-fluorocytosine (5-FC) delivered as an orally available extended-release formulation (Toca FC), into the antineoplastic drug 5-fluorouracil (5-FU). Toca 511 has been delivered by intratumoral injection (NCT01156584), injection into the tumor bed post-resection (NCT01470794), or by IV administration (NCT01985256). In all cases the treatment is well-tolerated. In animal models extensive infection of tumors (close to 100%) leads to control of tumor growth both in xenograft and syngeneic models. Limited access to tumor tissue post Toca 511 treatment in trial subjects, and lack of good markers of human glioblastoma cells in these heterogeneous primary tumors make it difficult to determine the extent of cancer cell infection but there is good evidence of selective tumor infection by PCR, RTPCR and IHC against the CD protein. Clinical data in both the first two trials (intratumoral and resection bed administration with 39 and 43 evaluable subjects respectively) show a favorable safety profile and extended overall survival (OS) compared to historical controls. In the resection study, for example, median OS was 13.6 months compared to 7-8 months in matched historical controls, and the OS at 24 months was 32%. In addition an RNA expression signature in untreated resected tumors that predicts long term survival in trial subjects has been identified from subjects that subsequently underwent tumor bed administration of Toca 511 and Toca FC. This signature does not normally correlate with survival in available public data sets. In immune competent orthotopic animal models, Toca 511 and 5-FC treatment leads to apparent tumor elimination and induction of strong antitumor immune responses by several mechanisms, including local elimination of myeloid derived suppressor cells. Subcutaneous re-implantation of the same tumors did not lead to tumor growth in animals treated up to a year before, whereas tumors did develop in control naïve animals. Available data in the human trials are consistent with the immune response playing a significant role in the apparent clinical efficacy. Thus, clinically, treatment with Toca 511 and extended-release 5-FC (Toca FC) appears to selectively destroy tumor cells within the body, while leaving healthy cells unharmed. Toca 511 and Toca FC have been administered to more than 120 high grade glioma subjects in the three studies and, based on results from these trials, a phase 2/3 trial (Toca 5 has recently started recruitment (NCT02414165). The combination of clinical and preclinical data supporting this decision will be reviewed.

62. MiRNA Regulation of Oncolytic Measles Virus Infectivity

Hirosha Geekiyanage, Evanthia Galanis
Mayo Clinic, Rochester, MN

Oncolytic measles virus (MV) strains represent a promising novel oncolytic platform that is currently evaluated in several clinical trials including glioblastoma. The recently identified MV receptor, poliovirus receptor-related 4 (PVRL4), has accumulated great interest due to its strong biochemical binding affinity to MV and tumor specificity. However the regulation of PVRL4 is not understood. Immunohistochemistry and western blot analysis studies demonstrated increased PVRL4 protein levels in glioblastoma with no significant change in *PVRL4* mRNA levels detected by qRT-PCR, suggesting that PVRL4 is likely post-transcriptionally regulated. Therefore, we investigated the potential role of miRNA in PVRL4 regulation and thereby MV infectivity. From miRNAs predicted, by the algorithms microRNA.org and RegRNA, to bind the 3'UTR of *PVRL4*, the miRNAs miR-31 and miR-128 which are reportedly down-regulated in glioblastoma, were selected as potential regulators of PVRL4. Luciferase functional binding studies showed that miR-31 and miR-128 binds to the 3'UTR of *PVRL4*. Similarly, over-expression of miR-31/-128 down-regulated PVRL4 protein levels while anti-miRs-31/-128 increased PVRL4 protein levels suggesting that *PVRL4* is a miRNA targeted gene. Furthermore, miR-31 and miR-128 expression levels were down-regulated in glioblastoma patient samples (n= 18) compared with control gliosis (n=15) samples and showed statistically significant negative correlations with their respective PVRL4 protein levels signifying the post-transcriptional regulation of PVRL4 by miR-31 and miR-128. Gain-and-loss of function studies using a wild-type MV strain that exclusively utilizes PVRL4 as its receptor showed that over-expression of miR-31/128 decreases MV infectivity while inhibition of the respective miRNAs via anti-miRs increase MV infectivity in cell culture. In order to assess the miRNA regulation on MV infectivity *in vivo*, athymic nude mice were treated with 3-4 consecutive MV injections intratumorally at 1 x 10^6 TCID50 following establishment of subcutaneous glioblastoma (U87) tumor xenografts stably transduced with lentiviral-vectors encoding miRs alone or in combination with *PVRL4* and anti-miRs. Tumor sizes were significantly decreased in tumors expressing anti-miRs-31/-128, while tumors expressing miR-31/ -128 showed increase in size after MV administration. Additionally, statistically significant correlations were observed between miR-128 levels, MV infectivity measured by qRT-PCR and tumor sizes validating the impact of miRNAs on MV infectivity and thus viral oncolysis. In addition to this regulation of MV infectivity by host miRNAs, we observed that MV infection up-regulated host miR-31expression levels in glioblastoma cells. This up-regulation of miR-31 expression resulted in down-regulation of PVRL4 suggesting that MV infection regulate host miRNA and in turn host PVRL4 levels. In conclusion, this study suggests that PVRL4 is post-transcriptionally regulated by miR-31/-128 and represent possible miRNA targets that could modulate MV infectivity to enhance MV based oncolytic therapeutic strategies, while the latter signifies the simultaneous regulation of host miRNA by MV infection.

63. Immunogenicity of Self Tumor Associated Antigens Is Enhanced Through Protein Truncation

Tim Kottke[1], Kevin G. Shim[1], Laura Evgin[1], Vanesa Alonso-Camino[1], Shane Zaidi[1], Rosa Diaz[1], Jose Pulido[1], Jill Thompson[1], Karishma Rajani[1], Amanda Huff[1], Elizabeth Ilett[2], Hardev Pandha[3], Kevin Harrington[4], Peter Selby[2], Alan Melcher[2], Richard Vile[1]
[1]Mayo Clinic, Rochester, MN, [2]University of Leeds, Leeds, United Kingdom, [3]University of Surrey, Guildford, United Kingdom, [4]The Institute of Cancer Research, London, United Kingdom

We showed previously that expressing tumor-associated antigens (TAA) from Vesicular Stomatitis Virus (VSV) eradicates established tumors. We show here that truncation of TAA expressed from VSV can occur to preserve the ability of the virus to replicate efficiently. We observed that truncation of VSV-expressed TAA affects the processing of the antigen, causing a bias towards an IL-17 anti-tumor response which was raised by cumulative signaling from different types of APC, each presenting specific, truncated antigens. Whereas processing of full length, self-TAA invoked an IFN-γ based, CD8+ dependent response, truncated versions of the same self-TAA (likely to be poorly and incompletely folded) were processed through a class II-dependent pathway, and invoked an IL-17 based response. Significantly, the IL-17-mediated anti tumor response was both more therapeutic, and durable, than the response against full length self-TAA. These data show that the type/potency of anti-tumor immune responses against self-TAA can be manipulated *in vivo* through the nature of the self protein (full length or truncated), inclusion of multiple TAA to recruit the optimal combination of APC, and the resultant skewing of the T cell response to either an IFN-γ or IL-17 producing phenotype. Therefore, in addition to generation of neoantigens through sequence mutation, immunological tolerance against self-TAA can be broken through manipulation of protein integrity, allowing for rational design of better self immunogens for cancer immunotherapy.

64. Generation of Tumor Cells Resistant to Oncolysis Is Mediated Through Virus Induced APOBEC Expression

Karishma Rajani[1], Kevin G. Shim[1], Nazanin Yeganeh Kazemi[1], William Gendron[1], Tim Kottke[1], Amy Molan[1], Christopher Driscoll[1], Reuben Harris[2], Richard Vile[1]
[1]Mayo Clinic, Rochester, MN, [2]University of Alberta, Edmonton, ON, Canada

In both pre-clinical, and clinical models, we have observed that treatment of primary tumors with oncolytic viruses can lead to very significant tumor regressions, often with apparent disappearance of the tumor. However, in many cases, tumor subsequently recurs, often extremely aggressively. We have been able to mimic this effect *in vitro* by growing several different types of tumor cell lines in the presence of different oncolytic viruses at low m.o.i. over several weeks. Under such conditions, we consistently observe the emergence of cells which survive over long periods of time in culture, despite the demonstrable presence of ongoing viral replication. We identified APOBEC3 from a screen of genes which are induced in tumor cells undergoing continual exposure to either reovirus or Vesicular Stomatitis Virus (VSV). In this respect, overexpression of APOBEC3B, a cytidine deaminase, has been identified in human tumors associated with mutations that may drive tumorigenesis. Therefore, we tested the hypothesis that, upon infection with oncolytic viruses, APOBEC3 may help drive tumor cell mutation leading to protection from viral cytotoxicity. Consistent with this, both mRNA and protein levels of APOBEC3 were rapidly induced within 24-72 hours of low M.O.I infection by reovirus, or VSV, of B16 melanoma, GL261 glioma and TC2 prostate tumor murine cell lines. Similar low level infection of human tumor cell lines was associated with rapid induction of the human APOBEC3B mRNA. Interestingly, engineered over-expression of APOBEC3B in tumor lines significantly enhanced the ability of these cells to resist killing by either VSV or reovirus. This effect was inhibited by blockade of PKC signaling upon viral infection but was enhanced by the presence of type I interferons. Correspondingly, inhibition of APOBEC3 using shRNA decreased the frequency of emergence of VSV-resistant tumor populations. These data suggest that infection of tumor cells by oncolytic viruses at low M.O.I (as is likely to be the case during clinical treatments) leads to the induction of cellular proteins, which enhance the ability of resistance to oncolysis to develop. Deep sequencing studies are currently underway to determine the genetic changes in both virus, and target tumor cells, which are associated with APOBEC3 over-expression during chronic exposure to oncolytic virus infection. Finally, data will be presented on how these findings allow the construction of improved viruses for cancer therapy by targeting APOBEC3 induction to improve primary killing and prevent emergence of treatment resistant populations.

65. Oncolytic Measles Virus Differentially Affects Mitochondrial Biogenesis in Transformed versus Non-Transformed BM-Derived MSCs

Sarah Aref[1], Aditi Dey[1], Katharine Bailey[1], Benjamin Rosen[2], Anna Castleton[1], Gyorgy Szabadkai[3], Adele K. Fielding[1]
[1]UCL Cancer Institute, Paul O'Gorman Building, University College London, London, United Kingdom, [2]University College London, London, United Kingdom, [3]Division of Biosciences, University College of London, London, United Kingdom

Vaccine strain measles virus (MV) is oncolytic in numerous models of malignancy. The mechanism behind the selectivity of MV for transformed cells is poorly understood. To investigate further, an established step-wise model of cellular transformation was used; in which progressive oncogenic hits were stably and additively expressed in human bone marrow derived mesenchymal stromal cells following retroviral transfer of human telomerase reverse transcriptase (hTERT), human papilloma virus16 E6 and E7 (3H), SV40 small T antigen (4+V), finally, H-RAS (5H) (Funes at al, 2007). The most highly transformed cells (5H) were more permissive to oncolytic MV infection than any of the less transformed counterparts, with significantly greater viral titres. MV-induced cell-death increased progressively with progressive transformation. This was not explained by any differences in MV receptors CD46, SLAM or Nectin-4 expression. Investigation of anti-viral type 1 IFN response in this model 24 and 48 hours post MV infection (hpi) by ELISA demonstrated a robust induction of IFNβ (to a lesser extent IFNα) in hTERT cells, which was significantly and progressively reduced in 3H, 4+V and 5H according to level of transformation, suggesting that defective IFN pathway is a potential mechanism for the enhanced MV permissiveness observed in transformed cells. Examination of integrity of the RLR signalling pathway, which triggers IFNα/β production, revealed that expression levels of RIG-I, MDA5 and MAVS, determined by RQ-PCR at 6, 12, 24 and 48 hpi, were lowest in 5H and highest in hTERT cells, proposing a role for the RLR pathway in MV-mediated oncolysis. To gain insight into the differential effects of MV infection in transformed versus non-transformed cells, metabolic effects post MV infection were investigated. A significant increase in oxygen consumption rate (OCR) was observed 24 hpi, proportional to the stage of transformation, followed by a steep decline at 48 hpi. This increase was abrogated in the presence of fusion inhibitory peptide (FIP), implicating the role of syncytia formation, the cytopathic effect of MV infection, in MV-mediated oncolysis *in vitro*. A similar pattern was observed in ATP levels post MV infection; highest at 24hpi in 4+V and 5H and dropping off dramatically at 48hpi when cell viability is compromised. To further characterize the consistent increase in OCR and ATP levels

upon MV infection of the more transformed cell lines, we sought to assess mitochondrial biogenesis using the fluorescent probes TMRM, Calcein AM and Hoechst. Preliminary results indicated significantly increasing numbers of mitochondria per cell at 24 hpi principally in 4+V (16%) and 5H (~29%) cells compared to their uninfected counterparts potentially explaining the observed high levels of OCR and ATP. Altogether, our data suggests that altered mitochondrial numbers and functions correlate with the degree of MV permissiveness and thus MV-induced cell death. Mitochondrial mass was also seen to be increased with MV infection. However, this effect was not specific to transformed cells. Metabolomics and further evaluations will be crucial to comprehensively assess differences in metabolism between cells in this model and to understand the role of bioenergetics in the differential levels of MV-mediated oncolysis.

66. Recombinant Newcastle Disease Virus as an Oncolytic Therapy for Ovarian and Prostate Cancers

Lisa A. Santry, Jacob P. van Vloten, Kathy Matuszewska, Byram W. Bridle, James J. Petrik, Sarah K. Wootton
Pathobiology- Ontario Veterinary College, University of Guelph, Guelph, ON, Canada

Newcastle disease virus (NDV) is a member of the *Avulavirus* genus in the *Paramyxoviridae* family. NDV selectively replicates in tumor cells due to defects in antiviral and apoptotic signalling. It is the oncolytic virus with the longest history of use in clinical trials with a proven safety record as a monotherapy due to its strong induction of antiviral responses in non-transformed mammalian cells. In addition to its direct oncolytic effect, NDV also activates both innate and adaptive immune responses and therefore has strong immunotherapeutic potential. To enhance the fusogenicity of the virus, the fusion protein of recombinant LaSota NDV (rNDV) was engineered to express a multibasic cleavage and activation site (rNDV/F3aa). This virus is highly fusogenic leading to the formation of large syncytia that allows for efficient intratumoral spread and increased lytic potential. While NDV has demonstrated efficacy in a number of different cancer models, including melanoma and hepatocellular carcinoma, little information about its efficacy in ovarian and prostate cancer is available. To this end, we evaluated the oncolytic potential of rNDV/F3aa expressing enhanced green fluorescent protein (GFP) in a panel of prostate and ovarian cancer cell lines using a resazurin dye-based cell viability assay. Infection with rNDV/F3aa-GFP at a range of MOIs significantly reduced the viability of these cancer cells *in vitro,* but had little effect on normal untransformed cells. To evaluate the efficacy of NDV *in vivo*, we used a well-established orthotopic mouse model of epithelial ovarian cancer and a syngeneic murine RM9 prostate carcinoma model. Intravenous delivery of 1x10^8 PFU of rNDV/F3aa-GFP to ovarian tumor-bearing mice resulted in a significant reduction in the primary tumor size and number of metastatic abdominal tumours. Furthermore, 50% of the mice had a complete loss of ascites, which is an accumulation of fluid in the abdomen, as a result of advanced stage ovarian cancer. The apparent decrease in secondary disease after treatment with rNDV/F3aa-GFP is particularly promising as it is the metastatic lesions that ultimately cause high morbidity and mortality in patients with advanced ovarian cancers. A pilot study in the RM9 prostate model revealed that rNDV/F3aa-GFP was able to replicate and spread within the tumor and neovasculature after intratumoral or intravenous delivery of 1x10^8 PFU. Studies to confirm these results, as well as to evaluate the ability of rNDV/F3aa-GFP to induce tumor regression and increase survival are currently underway. Taken together, these data suggest that NDV/F3aa is a promising oncolytic for further development as an anti-cancer agent for the treatment of prostate and ovarian cancers.

67. Rational Design of MicroRNA-Retargeted Coxsackievirus A21 Infectious RNA for Cancer Therapy

Noura Elsedawy[1], Autumn Ruiz[2], Stephen Russell[2]
[1]Sanford School of Medicine of University of South Dakota, Sioux Falls, SD, [2]Molecular Medicine Department, Mayo Clinic, Rochester, MN

Coxsackievirus A21 (CVA21) is a positive-sense single stranded RNA virus. Intratumoral (IT) administration of CVA21 viral particles or *in vitro* transcribed RNA encoding the CVA21 genome to SCID mice bearing subcutaneous KAS6/1 human multiple myeloma xenografts results in tumor regression and viremia, but causes lethal myositis. We previously showed that inserting target sequences complementary to muscle-specific microRNAs (miRT), specifically miR-206 and miR-133, into the 3' UTR of the viral genome inhibits viral replication in cells expressing the cognate miRNAs. IT administration of miRT-virus to KAS6/1 tumor bearing mice regressed tumors and ameliorated muscle toxicity. However, IT injection of RNA encoding the miRT-CVA21 genomes failed to induce tumor regression. *In vitro* transfected miRT-CVA21 RNA had delayed rescue kinetics and blind serial passaging was required to produce titratable virus. Therefore, the goal of this study was to optimize miRT positioning within the genomic construct to achieve retargeting without compromising virus rescue efficiency. We inserted two copies of each miRT in the variable domain (VD) of the 5' UTR to make CVA21-T2-VD. We also truncated the VD ad replaced it with either one or two copies of each miRT generating CVA21-T1-ΔVD and CVA21-T2-ΔVD. Finally, we hypothesized that the original 3' UTR insertion position deforms a stabilizing secondary RNA structure that incorporates part of the ORF and the 3'UTR thus hindering viral replication. To preserve the stability of this structure we duplicated the terminal sequences of the ORF and inserted either one or two copies of each miRT in between, forming CVA21-T1-TR and CVA21-T2-TR. Transfection of RNA encoding each of the recombinant genomes or the wildtype genome into H1-HeLa cells resulted in visible cytopathic effects at 48 hours. Viral titers produced at 12 hours post-transfection with CVA21, CVA21-T1-ΔVD, CVA21-T2-ΔVD, CVA21-T2-VD, CVA21-T1-TR and CVA21-T2-TR RNA were $1.6x10^5$, $1.4x10^4$, $4.0x10^4$, $3.1x10^5$, $9.2x10^4$ and $3.7x10^4$ $TCID_{50}$/ml, respectively. The integrity of the miRT sequences and the absence of additional mutations were confirmed by sequencing. All five miRT-CVA21 viruses displayed replication kinetics similar to that of unmodified CVA21 during one-step growth curve analysis. miRNA targeting was analyzed by measuring the viability of H1-HeLa cells transfected with synthetic miR-206, miR-133 or both followed by viral infection. Cells transfected with muscle specific miRNAs resisted oncolysis by CVA21-T1-ΔVD, CVA21-T2-ΔVD, CVA21-T2-VD and CVA21-T1-TR to variable degrees but not by wildtype CVA21 or CVA21-T2-TR. Maximal protection was achieved in cells transfected with both miRNAs. Correspondingly, viral titers measured in supernatants of cells resisting oncolysis were lower than viral titers in supernatants of cells succumbing to it. Conclusion: In this study we have generated microRNA-retargeted CVA21 infectious RNA that results in the rescue of viral particles at a similar rate to unmodified CVA21 RNA. In conjunction with our previous studies, this data supports the use of microRNA-retargeted infectious RNA as a potential formulation for oncolytic virotherapy. Animal studies to test in vivo efficacy and selectivity of intratumorally administered RNA corresponding to the aforementioned CVA21 recombinants are currently underway.

68. Immunovirotherapy: Enhancing Personalized T Cell Therapy with Oncolytic Measles Virus Vaccination

Elena S. Czink, Tobias Speck, Johannes Heidbuechel, Jessica Albert, Birgit Hoyler, Dirk Jaeger, Christof von Kalle, Guy Ungerechts, Christine E. Engeland
National Center for Tumor Diseases, Heidelberg, Germany

Oncolytic viruses directly reduce tumor burden by selective lytic replication in malignant cells, which designates these vectors as a potent platform for targeted cancer therapy. Lately the focus of oncolytic research has shifted towards the immunostimulatory potential of viral infection within the tumor microenvironment. Oncolytic viruses can be considered to act as an autologous tumor vaccine with the capacity to induce a tumor-specific T cell response. We hypothesize that oncolytic measles virus (MV) expressing a tumor-associated antigen (TAA) can specifically support T cell activation and persistence after adoptive transfer of TAA-specific T cells. We modified the Schwarz vaccine-derived measles virus to encode either OVA as a model antigen or one of the endogenous melanoma antigens trp-2 or gp100. TAA insertion did not impede viral replication and oncolysis compared to the parental virus. For *in vivo* studies, we have generated a new immunocompetent mouse model for MV oncolysis, B16-hCD46. Transduction of hCD46-positive B16 tumor cells with the novel viruses induces a strong virus-mediated expression of the tumor antigens, serving as a specific and lasting stimulus for adoptively transferred T cells. With the increasing availability of tumor sequencing data, this study has direct implications for future clinical trials of personalized immunovirotherapy.

Cancer-Targeted Gene and Cell Therapy

69. TNFR Costimulatory Domains Impair Expansion of CD5 CAR T Cells Due to Enhanced Fas-Mediated Apoptosis

Maksim Mamonkin[1,2], Sandhya Sharma[1,2], Malini Mukherjee[3,4], Madhuwanti Srinivasan[1,2], Diogo Silva[1,2], Jordan S. Orange[3,4], Malcolm K. Brenner[1,2]
[1]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, [2]Texas Children's Hospital, Houston, TX, [3]Department of Pediatrics, Baylor College of Medicine, Houston, TX, [4]Center for Human Immunobiology, Texas Children's Hospital, Houston, TX

CD5 is expressed at high levels on most normal and malignant T cells. We previously demonstrated that T cells transduced with a CD5 CAR harboring signaling domains from CD28 and TCR zeta chain (CD28.zeta) genes produce only limited fratricide against normal CD5+ T cells and expand >10,000-fold in vitro. Although these CAR T cells also have significant anti-tumor activity against CD5+ cell lines, tonic signaling from the CAR enhances terminal differentiation during in vitro expansion. The accumulation of CCR7- effector T cells and reduced frequency of CD4+ CAR T cells limits the in vivo persistence of CAR T cells ultimately allowing resurgence of CD5+ tumor cells. Since strong PI(3)K activation from the CD28 domain likely contributes to the terminal differentiation of CAR T cells, we aimed to improve CD5 CAR T cell persistence by replacing the CD28 domain with a TNFR superfamily domain 4-1BB, which elicits weaker PI(3)K activation and preserves the central memory population.

Here, we show that replacing the CD28 signaling domain with one of the TNFR-superfamily genes (4-1BB, OX40, CD27, CD30 or HVEM) allowed CD5 CAR T cells to retain the CCR7+ central memory population. Unexpectedly, however, TNFR signaling domains dramatically increased CAR T cell death and reduced proliferation, resulting in 5- to 15-fold reduction in cell expansion after 2 weeks of culture. These results correlated with a 3-fold upregulation of Fas on the T cell surface and the formation of stable immunologic synapses between CAR T cells. Disrupting a TRAF2 binding site in the 4-1BB domain prevented Fas upregulation and restored the expansion of CD5 CAR T cells, but also compromised costimulation and undermined long-term cytotoxicity.

In contrast, CD28.zeta CD5 CAR T cells displayed normal Fas levels and their expansion was comparable to that of ICOS.zeta and zeta-only (first generation) CD5 CAR T cells. Limited fratricide of CD28.zeta CD5 CAR T cells was dependent on PI(3)K activity, as chemical blockade of PI(3)K resulted in dose-dependent enhancement of apoptosis (56% vs 31%) and a 3-fold reduction in CD5 CAR T cell numbers after 72h. Combining CD28 and 4-1BB domains in a third generation CD5 CAR still did not rescue T cell expansion. Therefore, we developed a regulated retroviral expression system that uses a small molecule to reversibly inhibit CAR expression, thereby preventing tonic signaling and the resulting fratricide and differentiation. Withdrawing the inhibitor restores CAR expression and the anti-tumor function of T cells, and preserves the central memory population by the time the CAR is fully expressed. Overall, these studies provide a mechanism regulating T cell fratricide and offer a means of reducing the negative effects of tonic CD5 CAR signaling.

70. Knocking Down the Circular RNA ciRS-E2 Blocks Cancer Cell Proliferation Demonstrating Circular RNAs as a New Therapeutic Target

Alessia B. Baccarini[1], Ravi Sachidanandam[2], Brian Brown[1]
[1]Genetics and Genomic Sciences, Mt. Sinai School of Medicine, New York, NY, [2]Department of Oncological Sciences, Mt. Sinai School of Medicine, New York, NY

Circular RNAs (circRNA) are a newly discovered class of abundant and well conserved non-coding RNAs (Memczak et al. and Hansen et al. Nature 2013). They are comprised of RNA sequences in which the 3' end of a downstream exon has been backspliced to the 5' end of an upstream exon, causing the formation of a continuous RNA loop. Thousand of different circRNAs have been identified so far in different cell types, including cancer cells, and their expression levels often reaches or exceeds many coding RNAs in the cell. A small number of circRNAs have been shown to control the activity of specific microRNAs by acting as molecular sponges, however the functions of the vast majority of circRNAs are still not known. Because the sequence of the 3'-5' exon junction of a circRNA is unique in the transcriptome, we hypothesized that targeting this region may be used to generate RNAi vectors that can knockdown circRNAs without impacting their host gene.

Using a deep-sequencing approach that we call Capture-seq and a novel mapping algorithm, we detected an abundant circRNA expressed in highly proliferative cells, which we dub circular E2 (ciRS-E2). ciRS-E2 is comprised of exon 2 of a coding gene which has circularized as a result of the 3' end of exon 2 backsplicing to the 5' end of the exon. Using a PCR that was specific for the backspliced junction, we found ciRS-E2 to be highly expressed in a number of cancers, including leukemia, melanoma, and ovarian.

We generated two different shRNAs to target the unique 3'-5' exon junction of ciRS-E2 and designed them to have different seed regions so that potential off-target effects could be accounted for. The shRNAs were cloned into lentiviral vectors encoding GFP (LV.GFP), and introduced into acute myelocytic leukemia or ovarian adenocarcinoma cells. More than 95% of cells were transduced. For comparison, the cells were transduced with two different control LV.GFP vectors encoding scrambled sequences. Impressively, both ciRS-E2 shRNAs knocked down ciRS-E2 by more than 80%, and

there was no change in the expression of ciRS-E2's host gene's mRNA or protein. Strikingly, within 7 days of transduction there was a major loss in cells transduced with either ciRS-E2 shRNA, which did not happen with control cells. By 10 days, there were less than 20% GFP+ cells, indicating that ciRS-E2 is important for cancer cell fitness. Further analysis revealed that this was due to impaired cell proliferation as a result of the cells arresting in G_0/G_1. Transcriptomics analysis by RNA-seq indicated that loss of ciRS-E2 resulted in a significant decrease ($P<0.05$, >2-fold) in the expression of a number of key oncogenes including Myc and Max.

This study is one of the first to assign an important cellular function to a circRNA, and the first to identify a circRNA that controls cancer cell fitness. Importantly, we show that RNAi vectors can be generated to efficiently and specifically knock down a circRNA without impacting their host gene. circRNAs represent a potential new therapeutic target, and our work suggests that knockdown of ciRS-E2 may be used to control cancer cell proliferation.

71. Validation of Manufacturing NKG2D Chimeric Antigen Receptor T Cells for a First-In-Human Clinical Trial in AML/MDS and Multiple Myeloma

Heather Daley[1], Sarah Nikiforow[1,2], Joana Murad[3], Helene Negre[1], Jake Reider[3], Charles Sentman[4], Sarah Snykers[5], Rachel Allen[5], Ilene Galinsky[1], Nikhil Munshi[1,2], Richard Stone[1,2], Robert Soiffer[1,2], Jerome Ritz[1,2], Suzanne Baumeister[1,2,6]
[1]Dana-Farber Cancer Institute, Boston, MA, [2]Harvard Medical School, Boston, MA, [3]Celdara Medical, LLC, Lebanon, NH, [4]Geisel School of Medicine at Dartmouth, Hanover, NH, [5]Celyad, SA, Mont-Saint-Guibert, Belgium, [6]Boston Children's Hospital, Boston, MA

Canonical CAR-T cells are engineered using constructs encoding a single chain variable fragment derived from a murine or humanized antibody, a costimulatory domain connected by a hinge region, and the signaling domain of CD3 zeta. This restricts T cells transduced with these constructs to recognizing one tumor antigen, such as CD19, and a limited set of cancers. Prior work in murine models demonstrated preclinical efficacy of T cells transduced with a novel activating CAR construct that exploits the ability of NK cells to recognize a variety of tumor antigens. Specifically, a CAR was generated by fusion of native full-length human Natural Killer Group 2D (NKG2D) gene with the human CD3 zeta cytoplasmic signaling domain. The NKG2D receptor in association with natural costimulatory molecule DAP10 creates a unique 6 protein receptor complex enabling NK and CD8+ T cells to kill many cells types via recognition of ligands including MIC-A, MIC-B, and UL-16 binding proteins 1-6. NKG2D-ligands are upregulated in malignancy but protein expression is absent or minor in healthy tissues. Complete remissions and durable CD4+ and CD8+ T-cell memory have been demonstrated in murine models of lymphoma, myeloma and ovarian cancer following adoptive therapy with NKG2D CAR T cells. At the same time, NKG2D CAR T cells significantly alter the tumor microenvironment through cytokine secretion to further promote anti-tumor immunity. This technology is being evaluated clinically in a phase I dose-escalation study (ClinicalTrials.gov NCT02203825) arising through collaboration between academic investigators and commercial sponsors. GMP manufacturing procedures for NKG2D CAR T cells were developed to support this first-in-human trial of NKG2D CAR T cells to assess safety and feasibility in acute myeloid leukemia/myelodysplastic syndrome and multiple myeloma. Following isolation of mononuclear cells, T cells were activated with anti-CD3 mAb and IL-2, subjected to 2 rounds of transduction with SFG retroviral vector containing the NKG2D CAR construct (CM-CS1), and expanded in media containing IL-2. GMP manufacturing procedures were validated using T cells from healthy donors as well as patients with AML and myeloma. Validation studies and initial clinical manufacturing demonstrated consistent viability, robust cell expansion, vector-mediated surface expression of NKG2D on a median of 53.6% CD8+ and 90.3% of CD4+ T cells, consistent viral copy number/cell less than 5, and the absence of replication-competent retrovirus in CM-CS1 T cells. Functionally, CM-CS1 T cells exhibit potent IFN-γ production following exposure to NKG2D ligand-expressing tumor cells. We also demonstrated minimal carry-through of malignant myeloid or plasma cells after deriving autologous CM-CS1 T cells from patients with malignancies. The Phase I trial using CM-CS1 T cells is ongoing, with dose-escalation proceeding as planned. Future goals include application of this novel cell therapy to additional malignant diseases.

72. A Phase I Study of RB94 in Genitourinary Cancers Using a Tumor-Targeted Systemic Nanodelivery System

Arlene Siefker-Radtke[1], Xin-qiao Zhang[1], Charles C. Guo[2], Kathleen F. Pirollo[3], Chris P. Leung[4], Esther H. Chang[3], Randall E. Millikan[1], William F. Benedict[1]
[1]Genitourinary Medical Oncology, MD Anderson Cancer Center, Houston, TX, [2]Pathology, MD Anderson Cancer Center, Houston, TX, [3]Georgetown University Medical Center, Washington, DC, [4]SynerGene Therapeutics., Inc., Potomac, MD

RB-94 is a truncated form of the RB tumor suppressor gene which has shown marked cytotoxicity in every human tumor type tested to date without affecting normal cells. A systemically administered, tumor-targeted nanocomplex for systemic gene delivery (termed SGT) has been developed. In the SGT nanocomplex, the payload is encapsulated within a cationic liposome. The surface of the liposome is decorated with an anti-transferrin receptor (TfR) single chain antibody fragment (scFv) designed to target cancer cells by binding to the TfR which is highly expressed on tumor cells. Here we describe the results of a DNA dose escalating first-in-man Phase l study of SGT encapsulated RB-94 (SGT-94) for the treatment of metastatic genitourinary tumors. The majority of the cancers treated in this study were bladder cancers. Treatment with SGT-94 was well tolerated with minimal side effects observed. Moreover, among the 11 evaluable patients of 13 treated, a complete remission (CR) in a lung metastasis, two partial remissions (PRs) and three incidences of stable disease (SD) were observed. The CR and PRs occurred at the highest dose (2.4 mg DNA) tested. In addition, there is strong evidence for tumor-specific targeting including data from two resected lung metastases. In these metastatic lesions, the presence of RB94 was documented by both PCR and Western blotting in the tumors. However, RB94 was absent in contiguous normal lung tissue. Therefore, this Phase I study demonstrates not only the safety, but also the anti-cancer potential of the SGT-94 nanocomplex and suggests that additional studies using SGT-94 alone or in combination with chemotherapy, radiation or other modalities are warranted.

73. Enhanced Engineering of Chimeric Antigen Receptor (CAR)-Modified T Cells Using Non-Viral Sleeping Beauty Transposition from Minicircle Vectors

Razieh Monjezi[1], Csaba Miskey[2], Tea Gogishvili[1], Martin Schleef[3], Marco Schmeer[3], Julia Wegner[1], Hermann Einsele[1], Zoltan Ivics[2], Michael Hudecek[1]
[1]Department of Medicine II, University Hospital Würzburg, Würzburg, Germany, [2]Medical Biotechnology, Paul-Ehrlich-Institute, Langen, Germany, [3]PlasmidFactory, Bielefeld, Germany

Adoptive therapy with T cells that were modified with gamma-retroviral and lentiviral (LV) vectors to express a CD19-specific

chimeric antigen receptor (CAR) has shown remarkable efficacy in pilot clinical trials. However, there are persistent concerns with the use of viral vectors with regard to safety, as well as the cost and scale of vector production required for making CAR T-cell therapy available to patients on a global level. In this study, we have refined non-viral *Sleeping Beauty* (SB) transposition to provide an effective and broadly applicable gene-transfer strategy with superior safety profile. We demonstrate that SB transposition of CAR transgenes from minicircle (MC) DNA vectors enables dramatically improved transposition rates compared to conventional plasmids. MC-derived CD19-CAR transposons display a highly favorable integration profile and confer stable CAR expression that permits potent anti-tumor functions and rapid manufacture of CAR T-cell products.

We prepared MC transposon donor vectors encoding a CD19-CAR or eGFP from corresponding parental pT2 plasmids through site specific recombination, and a MC encoding SB100X transposase. Each MC contained only the promotor and gene of interest, and was devoid of antibiotic resistance gene and bacterial origin of replication. We then performed transfections into CD8+ and CD4+ T cells of healthy donors (n=7) and analyzed transposition rate and stability that could be accomplished when CD19-CAR transposon and SB100X transposase were encoded by MCs vs. equimolar amounts of corresponding conventional plasmids. In each donor, we achieved a significantly higher transposition rate with MCs (mean 49.8% on day 14 post transfection) compared to plasmids (mean 12.8%; 4.4-fold difference; p<0,001). Expression of the CD19-CAR was stable over multiple rounds of expansion and several weeks in culture. Importantly, MC transfection was not only more effective, but also substantially less toxic compared to plasmids and on average, a 6-fold higher yield of CAR T cells could be obtained within 14 days of culture without the need for feeder cell expansion. In functional experiments, CD19-CAR T cells modified with our enhanced MC-based SB transposition strategy were equally effective as LV transduced CD19-CAR T cells, and exerted high levels of specific cytolytic activity, cytokine production including IFN-g and IL-2, and productive proliferation after stimulation with CD19+ lymphoma. Moreover, a single administration of SB-modified CD19-CAR T cells leads to complete eradication of systemic lymphoma in a murine xenograft model (NSG/Raji-ffLuc), and was at least equivalently potent as LV transduced CD19-CAR T cells prepared from the same donor. To address safety, we determined the gene copy number (n=5, range 3-8 in CD8+ T cells) and performed a comprehensive genomic insertion site analysis. The data show a close-to-random integration profile of MC-derived CD19-CAR transposons, without preference for highly expressed or cancer related genes. Intriguingly, a significantly higher proportion of SB integrations had occurred in genomic safe harbors compared to LV integrations (7-fold difference; p<0,001), close to the perfect score expected for random integration. In conclusion, we demonstrate the potential to manufacture CAR T cells using virus-free SB-mediated transposition from MC DNA vectors. The superior safety profile, high level stable transposition rate and ease-of-handling MC vectors position our novel approach to become a preferred gene-transfer strategy in advanced cellular and gene-therapy.

74. The Impact of Different Hinge and Transmembrane Components on the Function of a Novel Fully-Human Anti-CD19 Chimeric Antigen Receptor

Leah M. Alabanza, Melissa A. Pegues, Claudia Geldres, Victoria Shi, James N. Kochenderfer

Experimental Transplantation and Immunology Branch, National Cancer Institute, Bethesda, MD

T cells expressing anti-CD19 chimeric antigen receptors (CARs) have significant activity against B-cell malignancies in humans, but increased efficacy and decreased toxicity of anti-CD19 CAR T cells are needed. Anti-CD19 CARs that have been clinically tested have single-chain variable fragments (scFvs) derived from murine antibodies or in 1 case a humanized murine antibody. Evidence of human anti-mouse immune responses against CARs with murine scFvs has been reported (Jensen et al. Biology of Blood and Marrow Transplantation 2010; Lee et al. Lancet, 2015), providing a rationale for development of less immunogenic CARs. A CAR with a scfv derived from a fully-human antibody would eliminate these human anti-mouse immune responses and perhaps increase the persistence of anti-CD19 CAR T cells. A non-immunogenic CAR might be particularly important if multiple, temporally-separated doses of CAR T cells are administered to the same patient. We have designed and constructed fully-human anti-CD19 CARs encoded by a lentiviral vector. These CARs specifically recognize CD19, and T cells expressing the CARs exhibit a full range of functions including degranulation, cytotoxicity, proliferation, and release of a variety of cytokines. The extracellular scFv component of a CAR is connected to intracellular domains by an extracellular hinge region and a transmembrane (TM) region. In comparing anti-CD19 CARs, we noticed a difference in cytokine production by CARs with hinge and TM regions from the human CD8α molecule compared to CARs with hinge and TM regions from the human CD28 molecule. We designated a fully-human CAR with CD8α hinge and TM domains hu19-CD828Z, and we designated a CAR that was identical to hu19-CD828Z except that it had CD28 hinge and TM domains hu19-28Z. Compared to T cells expressing hu19-CD828Z, T cells expressing hu19-28Z produced much higher levels of interferon gamma (IFNγ), tumor necrosis factor (TNF), and interleukin-2. For example, after an overnight culture with CD19+ NALM6 cells, T cells expressing hu19-CD828Z yielded a mean of 5688 pg/mL IFNγ in the culture supernatant while T cells expressing hu19-28Z yielded 19396 pg/mL IFNγ (P=0.03). Similarly, after an overnight culture with CD19+ NALM6 cells, T cells expressing hu19-CD828Z yielded a mean of 2033 pg/mL TNF in the culture supernatant while T cells expressing hu19-28Z yielded 5008 pg/mL TNF (P=0.007). Cytokine levels were normalized for the percentage of T cells expressing each CAR in each experiment. This same pattern of hinge and TM domains affecting cytokine production was observed for CARs incorporating a different anti-CD19 scFv. In contrast to cytokine production, significant differences in other T-cell functions including degranulation, cytotoxicity, and proliferation were not found when hu19-CD828Z and hu19-28Z were compared. When T cells expressing hu19-CD828Z and hu19-28Z were assessed in a murine lymphoma model, there was not a statistically-significant difference in tumor eradication or survival. We are currently assessing other measures of T-cell activation to obtain a more mechanistic understanding of the differences in T-cell activation with different hinge and TM domains. Most of the clinical toxicity of anti-CD19 CAR T cells is caused by cytokine release. Compared to anti-CD19 CARs with CD28 hinge and TM domains, anti-CD19 CARs with hinge and TM regions from CD8α cause lower levels of cytokine release from T cells in vitro. This decreased cytokine release might reduce cytokine-mediated toxicity in patients. We have initiated a

clinical trial of T cells expressing hu19-CD828Z. We will assess cytokine release, immunogenicity, peak blood levels, and long-term persistence of hu19-CD828Z-expressing T cells in humans.

75. Development of an LMP-Specific T Cell Bank for Third Party Use as a Curative Strategy Post-Transplant Lymphoproliferative Disease After Solid Organ Transplant

Lauren McLaughlin[1], Lauren Roesch[1], E. Kathy Mintz[1], Sarah McCormick[1], Cecilia Barese[1], Elizabeth Williams[1], Neha Joshi[1], Fahmida Hoq[1], Michael Keller[1], C. Russell Cruz[1], Mitchell S. Cairo[2], Jessica Hochberg[2], Patrick J. Hanley[1], Catherine M. Bollard[1]

[1]Program for Cell Enhancement and Technologies for Immunotherapy, Children's National Medical Center, Washington, DC, [2]Division of Pediatric Hematology, Oncology and Stem Cell Transplantation, New York Medical Center, Hawthorne, NY

EBV-associated tumors in the immune deficient host express type II and III latency antigens including latent membrane protein 1 (LMP1) and LMP2, which can serve as potential targets for immunotherapy. Several studies have documented the safety and efficacy of LMP-specific T cells for patients with EBV-associated malignancies, but clinical applications may be limited by the time to generate LMP-specific T cell products as well as the availability of an appropriate donor source. We hypothesize that the administration of "off the shelf" third party LMP-specific cytotoxic lymphocytes (LMP-CTLs) will rapidly restore EBV-specific T-cell immunity and prevent relapse in patients with post-transplant lymphoproliferative disease PTLD post solid organ transplant (SOT). To develop the third party T cell bank, we manufactured healthy donor-derived LMP-specific T cells from eligible donors with a wide range of HLA types in our good manufacturing practices (GMP) facility. This T-cell bank is for several clinical trials including a proposed multicenter Children's Oncology Group (COG) trial (ANHL1522) for patients with PTLD after SOT. Currently, 15 LMP-specific T-cell products have been manufactured from healthy donors and released for third-party use using autologous monocytes and lymphoblastoid cell lines (LCL), transduced with an adenoviral vector expressing ΔLMP1 and LMP2. T-cell products were active against LMP2 (mean: 172 SFU/1x10^5 cells; range: 13-655), LMP1 (33; 1-322), and LCL (87; 0-424) as determined by IFN-γ ELISPOT assay. Epitope mapping of LMP-specific T cells using IFN-γ ELISPOT assay demonstrates that these products recognize a broad epitope repertoire within LMP1 and LMP2. At the time of cryopreservation, the T-cell products comprised a mean of 45% CD8+ T-cells, 35% CD4+ T-cells, and 9% NK cells. No B cells or monocytes were detected in the final products. Thus far, one patient with NK/T cell non-Hodgkin Lymphoma received third party LMP-specific T cells achieving a very good partial response. No infusion-related toxicities were observed, and LMP-specific T cells were detectable post- infusion. Thus, third party LMP-specific T cells appear to be a safe and promising therapeutic modality for patients with EBV-associated lymphomas, and a third party bank will make this therapeutic more readily available to patients with PTLD post-SOT.

76. Transgenic Expression of IL15 Improves Antiglioma Activity of IL13Rα2-CAR T Cells

Giedre Krenciute[1], Irina Balyasnikova[2], Gianpietro Dotti[3], Stephen Gottschalk[1]

[1]Baylor College of Medicine, Houston, TX, [2]Northwestern University, Chicago, IL, [3]University of North Carolina, Chapel Hill, NC

BACKGROUND: Glioblastoma (GBM) is the most aggressive primary brain tumor in humans, and is virtually incurable with conventional therapies. Immunotherapy with T cells expressing chimeric antigen receptors (CARs) specific for the GBM antigen IL13Rα2 is an attractive approach to improve outcomes. We recently generated the first scFv-based CAR that is specific for IL13Rα2 and demonstrated that IL13Rα2-CARs with a CD28.ζ endodomain had potent anti-GBM activity in preclinical models. However, CAR T-cell persistence was limited, resulting in recurrence of IL13Rα2-positive GBMs. Since IL13Rα2-CARs with other endodomains (41BB, 41BB and OX40) did not improve outcomes, the goal of this project was to evaluate if transgenic expression of IL-15, a cytokine that is critical for T-cell proliferation and survival, enhances persistence and anti-tumor activity of IL13Rα2-CAR.CD28.ζ T cells.

METHODS: We generated IL13Rα2-CAR.CD28.ζ T cells expressing IL-15 (IL13Rα2-CAR.IL15 T cells) by double transducing T cells with retroviruses containing expression cassettes encoding i) IL13Rα2-CAR.CD28.ζ or ii) IL-15, ΔNGFR, and iC9 separated by 2A sequences. We determined the effector function of IL13Rα2-CAR.IL15 T cells *in vitro* using standard assays, and in the U373 GBM xenograft model.

RESULTS: Double transduction of CD3/CD28-activated T cells resulted in T-cell lines that expressed both transgenes in 45-50% of T cells. At base line IL13Rα2-CAR.IL15 T cells produced on average 69.5 pg/ml of IL15. Production was significantly increased after CD3 or antigen-specific T-cell stimulation (176.7 pg/ml; n=6; $p<0.001$). IL13Rα2-CAR.IL15 T cells were as efficient as IL13Rα2-CAR T cells in killing IL13Rα2-positive GBMs *in vitro*. After intratumoral injection into U373 glioma-bearing mice IL13Rα2-CAR.IL15 T cells persisted significantly longer than IL13Rα2-CAR T cells ($p<0.05$). This resulted in a significant increase in progression free (98 vs 49 days; $p=0.004$) and overall survival ($p=0.006$) of treated mice. Up to date, 4/10 IL13Rα2-CAR.IL15 T-cell treated mice remain glioma free with a follow up of at least 80 days. Recurring U373 gliomas post IL13Rα2-CAR.IL15 T-cell therapy had down regulated IL13Rα2 expression, indicating immune escape. This was specific for the targeted antigen since IL13Rα2-CAR.IL15 T-cell-treated U373 gliomas continued to express other tumor antigens such as EphA2 and HER2 at unchanged levels in comparison to controls.

CONCLUSION: Here we demonstrate that transgenic expression of IL15 enhances the *in vivo* persistence of IL13Rα2-CAR T cells resulting in improved anti-glioma activity. However, enhanced *in vivo* persistence of T cells also resulted in the development of antigen-loss variants highlighting the need to target multiple antigens in tumors with heterogeneous antigen expression such as GBM.

Immunological Aspects of Gene Therapy I: AAV Vectors

77. Antigen-Specific Modulation of Capsid Immunogenicity with Tolerogenic Nanoparticles Results in Successful AAV Vector Readministration

Amine Meliani[1], Florence Boisgerault[1], Giuseppe Ronzitti[1], Fanny Collaud[1], Christian Leborgne[1], Takashi Kei Kishimoto[2], Federico Mingozzi[1]
[1]Genethon, Evry, France, [2]Selecta Biosciences, Boston, MA

Gene transfer approaches based on the adeno-associated virus (AAV) vector platform have shown great therapeutic potential in both preclinical studies and clinical trials. Neutralizing immune responses to AAV, however, are an important limitation to the use of AAV vectors as therapeutic tools, as even low-titer anti-capsid neutralizing antibodies (NAb) can lead to vector clearance and lack of efficacy. In particular, anti-AAV NAbs develop at high titers following vector administration and persist for several years after AAV vector administration, making vector re-administration hard if not impossible. Here we tested a novel strategy to modulate immune responses directed against AAV vectors based on the co-administration of biodegradable tolerogenic poly(lactic acid co-glycolytic acid) (PLGA) nanoparticles (tNP) containing rapamycin at the time of vector administration. C57BL/6 mice (n=5/group) received an AAV8 vector encoding for luciferase (AAV8-Luc) at a dose of $4x10^{12}$ vg/kg injected intravenously alone, or formulated with empty PLGA nanoparticles (NP), or formulated with tNP containing 100 μg of rapamycin. Three weeks after treatment, anti-AAV8 antibodies were measured and animals received a second intravenous infusion with an AAV8 vector encoding for human coagulation factor IX (AAV8-hFIX) at a dose of $4x10^{12}$ vg/kg formulated with NP or tNP. An anti-AAV8 antibody ELISA and an in vitro neutralization assay was used to follow humoral immune responses to the vector. While no development of anti-AAV8 antibodies was observed in the tNP-treated animals after the first and second vector administration, control groups developed robust humoral immune responses to the AAV8 capsid, which prevented vector readministration. Consequently, efficient hFIX transgene expression deriving from the second AAV8 vector administration was observed only when tNP were used, at levels identical to animals that received only a single administration of AAV8-hFIX. Lack of antibody formation against the AAV8 capsid in animals treated with tNP was also accompanied by a downregulation of both CD4+ and CD8+ T cell responses in the liver. A mild decrease in the frequency of CD4+ T cells in spleen, with no change in frequency of regulatory T cells, were also noted. In a separate set of experiments, we tested the antigen-specificity of the treatment of tNP with AAV8 administration. Mice (n=5/group) received $4x10^{12}$ vg/kg of an AAV8-luc vector with tNP followed by either challenge with hFIX protein in complete Freund's adjuvant, AAV5-hFIX vector intravenous injection, or AAV8-hFIX vector intravenous injection. All animals pretreated with AAV8-luc and tNP developed antibodies against the hFIX and the AAV5 antigens, while they anti-AAV8 antibody titers were significantly decreased. In conclusion, tNP administration together with AAV vector prevents anti-capsid immune responses in an antigen-specific manner and allows for AAV vector readministration, addressing one of the most important challenges of the in vivo gene transfer field.

78. Distinct Roles of Dendritic Cell Subsets in Innate Immune Sensing and Cross-Presentation of AAV Capsid *In Vivo*

Jamie L. Shirley, Geoffrey L. Rogers, Monaro Biswas, Roland W. Herzog
Pediatrics, University of Florida, Gainesville, FL

Immune responses to AAV vectors currently represent one of the greatest hurdles facing successful gene therapy. Adeno-associated virus (AAV) vectors have been investigated in clinical trials for treatment of hemophilia B. However, in these trials, it was shown that transduced hepatocytes are eliminated by capsid specific $CD8^+$ T cells, resulting in a loss of transgene expression and therapeutic efficacy. AAV capsid proteins are not endogenously expressed and therefore must be loaded on MHC class I via cross-presentation, a poorly understood mechanism relatively unique to conventional dendritic cells (cDCs). Previous work in our lab has shown that selective depletion of cDCs using CD11c-DTR mice ablates the anti-capsid $CD8^+$ T cell response, and that innate sensing of the AAV genome is mediated through TLR9-sensing of the DNA genome and downstream signaling through its adaptor protein, MyD88. A second subset of dendritic cells, called plasmacytoid DCs (pDCs), highly express TLR9 and secrete large amounts of type I interferon following activation from a viral stimulus. Herein, we investigated the respective roles of cDCs and pDCs in the cross-presentation of capsid antigen. To measure capsid-specific T cell responses, we have created a modified AAV2 capsid that contains the peptide sequence SIINFEKL (AAV2-SIINFEKL), which is the $CD8^+$ immunodominant epitope of the model antigen ovalbumin in C57BL/6 mice. With this vector, capsid-specific $CD8^+$ T cells can be identified using H2-K^b-SIINFEKL tetramer. We first confirmed the requirement of cDCs in capsid cross-presentation by measuring the proliferation of adoptively transferred T cells from TCR transgenic OT-1 mice, which produce $CD8^+$ restricted, OVA-specific T cells, into cDC-depleted CD11c-DTR mice (n=4) that received AAV2-SIINFEKL. After 7 days, OT-I T cell proliferation was ~3% in the absence of cDCs compared to ~27% in the presence of cDCs. Strikingly, we found that the specific depletion of pDCs in BDCA2-DTR mice (n=4) that received AAV2-SIINFEKL also resulted in suppressed capsid $CD8^+$ T cell formation, suggesting discrete yet essential roles for both pDCs and cDCs in the initiation of a cytotoxic T lymphocyte (CTL) response directed against AAV capsid antigen. To dissect the specific roles of cDCs and pDCs, we first investigated the requirement for MyD88 in cDCs by generating a transgenic mouse expressing Cre-recombinase under the control of the CD11c promoter with the MyD88 gene flanked by LoxP sites, specifically ablating MyD88 expression in cDCs (DC-$MyD88^{-/-}$ mice). We observed no significant difference in tetramer positive $CD8^+$ T cells between DC-$MyD88^{-/-}$ mice (n=4) and WT mice, indicating that cDC-intrinsic MyD88 signaling is not required for the formation of anti-capsid CTL responses. Importantly, pDCs retained MyD88 expression in this model. To precisely define the role of TLR9 in these DC subsets, we adoptively transferred either WT pDCs (n=9) or WT cDCs (n=9) into $TLR9^{-/-}$ mice followed by injection of AAV2-SIINFEKL. Adoptive transfer of WT pDCs but not of WT cDCs restored induction of tetramer positive $CD8^+$ T cells in the $TLR9^{-/-}$ mice. Therefore, we propose a 2-step model for anti-capsid $CD8^+$ T cell responses to AAV vectors in which innate recognition of the genome occurs in pDCs via the TLR9-MyD88 pathway, promoting the cross-presentation of capsid antigen by cDCs. These results establish both DC subsets as critically important in the initiation of anti-capsid $CD8^+$ T cell responses to AAV vectors and unveil potential targets to mitigate the deleterious effects of the immune response to AAV gene therapy.

79. Vector Dose Delineates Between Chronic, Non-Functional CD8+ T Cell Response and Tolerance to the Transgene Product Upon Liver Gene Transfer

Sandeep Kumar, Brad E. Hoffman, Roland W. Herzog
Pediatrics, College of Medicine, University of Florida, Gainesville, FL

The liver is characterized by a microenvironment that promotes immune tolerance, which can be exploited by gene therapy. In the case of hepatitis viral infections such as HBV or HCV however, ineffective or non-functional responses can result in chronic disease. To understand the parameters and mechanisms that govern the $CD8^+$ T cell response to a virally encoded antigen expressed in the liver, we administered an adeno-associated virus expressing ovalbumin (AAV8-EF1α-ovalbumin) to immune competent C57BL/6J mice at various doses (low: $1x10^8$ vg, medium: $1x10^9$ vg, and high: $1x10^{10}$ vg). Interestingly, all doses resulted in sustained vector-dose dependent systemic ova expression, albeit with distinct immune profiles. At the low dose and the high doses, no response to ova was observed. At the medium dose, circulating ova-specific $CD8^+$ T cells were detected in 40-50% of mice at high frequency (5-35%) by tetramer stain. These emerged within 1 month and, while declining in frequency over time, can persist for up to one year. Despite their inefficiency in eliminating vector-derived ova expression, functional assays demonstrated that induced $CD8^+$ T cells retained cytolytic activity to SIINFEKL (the dominant ovalbumin MHC class I epitope in C57BL/6J mice) peptide-loaded target cells *in vivo* or *in vitro*, and that they produced Th1 type cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) in response to *ex vivo* stimulation with SIINFEKL epitope. Further phenotypic characterization revealed that most of these cells expressed PD-1 and 2B4. Other markers of exhaustion such as CD160, LAG3 and TIM3 were expressed to a lesser extent. Cells acquired memory phenotype over time as judged by CD44, CD62L, and CCR7 markers. Since higher vector doses failed to induce the CD8+ T cell response entirely, a potent antigen-dependent "off switch" has to exist in the liver. Previously, we and others identified $FoxP3^+$ Treg, activation induced apoptotic cell death, and expression of the immune suppressive cytokine IL-10 as contributors to immune tolerance induction by hepatic gene transfer. Therefore, we used *Foxp3*-DTR mice (for transient depletion of Treg), $FasL^{-/-}$, and $IL\text{-}10^{-/-}$ mice to determine if these pathways were required in preventing ova-specific $CD8^+$ T cell activation in the high-dose group. Transient depletion of $Foxp3^+$ Treg in Foxp3-DTR mice led to a low but detectable $CD8^+$ T cell response in all animals. In contrast, only 1/5 (20%) of $FasL^{-/-}$ or $IL\text{-}10^{-/-}$ mice developed a response. Therefore, it is likely that multiple (and perhaps redundant) mechanisms work in concert to achieve high-dose tolerance to proteins expressed in the liver. These results have implications for viral infections of the liver and for gene and cell therapies, illustrating the potential for chronic albeit only partially functional T cell responses at low doses, which can be entirely prevented at sufficient antigen doses.

80. Delayed Inflammatory Response to Intravitreal AAV Gene Transfer in Non-Human Primates

Sarah Wassmer[1,2,3,4], Jason Comander[2,3], Livia Carvalho[1,2,3,4], Ru Xiao[1,2,3,4], Eva Plovie[1,2,3,4], Aliete Langsdorf[2,3], Lawrence Lim[2,4], Brian Hafler[2,3], David M. Wu[2], Dean Eliott[2], Leo A. Kim[2,4], Luk H. Vandenberghe[1,2,3,4]
[1]Grousbeck Gene Therapy Center, Boston, MA, [2]Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA, [3]Ocular Genomics Institute, Boston, MA, [4]Schepens Eye Research Institute, Boston, MA

Introduction Adeno-associated virus (AAV) gene therapy in the eye via intravitreal injection has recently advanced into clinical trials. Here, we wanted to investigate the immunological response to AAV capsids within the vitreous of non-human primates. **Methods** Bilateral injections of AAV gene delivery were evaluated in five rhesus macaques. An intravitreal injection (IVT) was performed in one eye of the non-human primate, while a subretinal injection was performed in the contralateral eye. For the IVT eye, AAV2 or synthetic ancestral Anc80 capsid with GFP transgene was used at a concentration of 1E+11 particles. The surgical procedure for two animals was a normal IVT injection as performed in the clinic and the other two animals received an inner limiting membrane peel before viral injection. In the subretinal injection (SR) eye, animals were treated with AAV8 or 9, carrying the GFP or LacZ transgene at a concentration of 1E+10 or 1E+11 particles. Animals were monitored for 5/6 weeks before being sacrificed for histological analysis. **Results** In all eyes, inflammation in the first 2 weeks was mild or moderate and self-resolving. At weeks 3-5, all IVT treated eyes developed vitritis. None of the eyes injected subretinally developed vitritis. The most severe eye belonged to the IVT group, which developed hyphema and most of the IVT eyes developed anterior cataracts. Inflammation was well controlled with 4mg to 12mg of Triescence (triamcinolone acetonide) injected intravitrally. One animal was not given Triescence at the onset of inflammation (week 3), which became unmanageable within 48 hours, leading to the ultimate termination of the animal. Within the IVT group, similar amounts of late inflammation were seen between the intravitreal and peel/puddle eyes. **Conclusion** Our studies demonstrate that, in stark contrast to subretinal injection, an intravitreal injection of AAV expressing a non-self transgene has the potential to develop clinically significant inflammation at doses normally tolerated in the subretina. This inflammation can be treated with early administration of Triescence. Left untreated, the animal will develop severe immunological infiltration. These results warrant further study to determine the antigenic nature and inflammatory mechanism leading to the low inflammatory threshold to intravitreal AAV gene transfer.

81. Downregulation of Immune Responsive Chemokines Are Associated AAV8-Mediated Transgene Immune Tolerance

Yuanyuan Xiao[1], Shaoyong Li[2], Qin Su[2], Li Luo[1], Gaungchao Xu[1], Wanru Qin[1], Chunmei Wang[1], Yi Lu[2], Li Yang[1], Yuquan Wei[1], Guangping Gao[2]
[1]State Key Lab of Biotherapy, Sichuan University, Sichuan, China, [2]Horae Gene Therapy Center, UMASS Medical School, Worcester, MA

Recombinant adeno-associated virus (rAAV)-mediated liver transduction can induce transgene product-specific immune tolerance, which can be further extended to the systemic tolerance. Because of such tolerogenic properties and its physiological functions, the liver has been a preferred target organ for AAV- gene therapy. The hepatic regulatory T cells (Tregs) are regarded as the major factor

that contributes the immune tolerance in the liver. Here, we first tested if AAV capsid plays a role in inducing systemic immune tolerance. To this end, we used highly immunogenic Ovalbumin (OVA) as a model antigen expressed under the direction of a strong ubiquitous chicken β-actin (CB) promoter and packaged the vector with both AAV1 and AAV8 capsids for *in vivo* delivery to C57BL/6 mice. Compared to AAV1, AAV8 achieved significantly higher OVA expression without IgG production, regardless of intravenous (IV) or intramuscular (IM) injection. In addition, IV and IM delivered AAV1.OVA both generated OVA-specific CD8+ T cells in spleen, while AAV8-mediated deliveries blunted CD8+ T cell response and, instead, provoked B- and T-Reg responses. In an attempt to explore other possible cellular mechanism(s) led to hepatic tolerogenic properties, we profiled expression of innate and adaptive immune responsive genes that are possibly associated with liver immune tolerance in the context of rAAV transduction. We revealed that rAAV1.CB.OVA injections significantly elevated *IL1a* and *CXCL1* gene expression as compared to the PBS and rAAV8 groups. As rAAV8 is more liver tropic than rAAV1, we hypothesized that hepatic OVA expression is essential to inducing immune tolerance. We tested this hypothesis by creating rAAV8.CB.OVA vectors (+/-) the binding sites for the liver enriched miR-122 and IV dosing C57BL/6 mice with the vectors at 4 doses ($1x10^9$, 10^{10}, 10^{11} and 10^{12} gcs/mouse). We confirmed that miR-122 indeed effectively inactivated OVA expression from rAAV8.CB.OVA(+)MiR-122BS transduced liver; however, rAAV8.CB.OVA(-) miR122BS but not rAAV1CBOVA achieved sustained OVA expression in a dose dependent manner without eliciting OVA specific IgG. Finally, considering that the route of rAAV administration may affect transgene expression and immunity, we IM injected C57BL/6 mice with AAV8.CB.OVA (+/-) miR122BS vectors and found that AAV8.CB.OVA (-)miR-122BS expressed OVA efficiently without OVA-specific IgG production. In contrast, just as rAAV1.CB.OVA did, rAAV8.CB.OVA(+) miR-122BS produced significant OVA-specific IgG without OVA expression. Interestingly, IM delivered rAAV8.CB.OVA(+) miR-122 group expressed significantly higher levels of *CXCL1*, Myeloperoxidase(*Mpo*) and *CCL5* in liver, as compared the PBS and rAAV8OVA(-)miR-122 groups as detected by qRT-PCR. Our data suggest that the downregulation of CXCL1, Mpo, CCL5 and IL1α in the liver are involved in the immune tolerance to rAAV8 delivered transgene. Those cytokines may represent therapeutic targets against autoimmune diseases.

82. Mapping the Humoral Immune Response to AAV by Molecular Docking and Cryo-Electron Microscopy for the Design of Next-Generation AAV Vectors

April R. Giles, Lakshmanan Govindasamy, Roberto Calcedo, Anna Tretiakova, Surina Boyd, Qiuyue Qin, Mark Schneider, James M. Wilson

Gene Therapy Program, Department of Medicine, University of Pennsylvania, Philadelphia, PA

Despite constant improvements in gene therapy with AAV vectors, including use of new serotypes, routes of administration, and transgene cassette engineering, immune responses continue to be a major obstacle to therapeutic translation. Neutralizing antibodies (NAb) against the capsid generated by prior viral infection or AAV vector administration significantly reduce not only the effective patient population but also the overall efficacy of an AAV-based gene therapy. Here, by cloning out and evaluating anti-AAV antibodies from singly-sorted memory B cells from seropositive individuals, we have designed an approach that allows us to look at the humoral immune response globally, to hone in on the immunogenic regions of the capsid itself, and to compare responses between individuals in an unbiased and therapeutically-relevant setting. In this study, we screened a panel of 30 normal human donors, selected one with high pre-existing NAb titers (1:320 for AAV2, 1:40 for AAV3B), then sorted out switched memory B cells by negative selection, seeding on irradiated ms3T3-CD40L feeder cells and culturing for 2 weeks in the presence of IL-2 and IL-21. Following supernatant screening for anti-AAV antibody production, over 100 AAV-reactive clones were identified. After isolation and cloning using nested PCR, antibodies were evaluated for AAV capsid binding as well as neutralizing capacity. To date, all antibodies demonstrated binding to AAV2 and AAV3B as well as a panel of additional AAV serotypes (8, 9, rh10, rh32.33), suggesting that AAV-binding, yet non-neutralizing antibodies may possess broad serotype specificity.

To identify the epitopes for these anti-AAV antibodies, we first took a high-throughout, predictive approach. Antibody variable region sequences were placed into a generic antibody scaffold and their three-dimensional structure modeled using Kotai Antibody Builder and RosettaAntibody followed by validation using COOT. The resulting structures were then iteratively docked onto the published structure of AAV3B using PIPER to identify the most energetically-favorable binding conformation. Thus far, the vast majority of footprint residues lie in variable regions of the capsid comprising and surrounding the 3-fold spikes. For a number of antibodies, initial prediction-directed capsid alanine scanning experiments have shown decreased antibody binding at the predicted residues, supporting the use of this approach. More comprehensive mutagenesis experiments are underway to further validate the approach and more completely map immunogenic epitopes of the AAV capsid proteins. In addition, cryo-EM analysis is currently underway for a number of these Fab-AAV complexes for additional, direct observation of the repertoire of binding footprints. These studies will provide information critical to understanding the antibody response to AAV and guide future attempts to rationally design next-generation capsids that are able to evade the anti-AAV antibody response.

83. Relationship Between Immune Responses and Ocular Inflammation: What Is Learnt from Intravitreal Injection of rAAV2-2-*ND4* (GS010) in Non-Human Primates and Leber Hereditary Optic Neuropathy (LHON) Patients

Céline Bouquet[1], Anne Galy[1], Serge Fitoussi[1], Sandrine Meunier[1], Roxane Noel[1], Scott Uretsky[1], Catherine Vignal[2], José A. sahel[2], Jean-Philippe Combal[1], Nitza Thomasson[1]

[1]Gensight-Biologics, Paris, France, [2]Centre Hospitalier National d'Ophtalmologie des Quinze-Vingts, Paris, France

GenSight Biologics develops a rAAV2/2-*ND4* vector (GS010) administered via intravitreal route (IVI) against vision loss in LHON patients bearing *ND4* mutation. Gene therapy Adeno Associated Viral vectors are known to trigger host immune response which may interfere with safety and efficacy. Therefore, relationship between anti-AAV2 antibody systemic levels and eye inflammatory reactions following GS010 administration in non-human primates and LHON patients has been investigated.36 non-human primates (NHP) and 15 LHON-*ND4* patients (Phase I/IIa; ClinicalTrials.gov NCT02064569) underwent IVI of GS010 with two (4.3^E10; 3.1^E11 vg/eye) and four dose levels (9^E9; 3^E10; 9^E10; 1.8^E11 vg/eye) respectively. Ocular examinations were performed up to 6 months post-injection in NHP and up to 48 weeks in patients. NHP eye histopathology was performed and sera collected for neutralizing antibodies (Nabs) analysis (using a seroneutralization luciferase cell-based assay). Serum was collected from all patients and aqueous humor samples prior to injection in the last 8 patients, for quantification of anti-AAV2 IgG by ELISA and NAbs using the same method principle than for NHP. At baseline, NAbs were undetectable in 55% of NHPs and

ranged between 1:5 and 1:400 titers for 45% of them. All animals showed titer increase up to 1:12800 starting week 2 post-injection. Between 2 and 6 months titers remained stable. Ocular inflammation (corneal punctuate focal opacity, vitreal haze, and superficial retinal mononuclear infiltration in histopathology) was observed in up to 80% of NHPs without deleterious effects on retinas. Preliminary Phase I/IIa data showed that, at baseline, 7 out of the 15 patients had undetectable serum NAb levels and 2 patients had titers above 1:1000. Prior treatment, none of the 8 tested patients had NAbs in aqueous humor, even the one showing a titer >2000 in serum. Two weeks after injection, IgG titers increased up to 19 times their baseline level in 7 patients and NAbs up to 39 times in 10 patients. This humoral response increase was not correlated to the dose level. At week 8, NAb titers remained above 1:1000 titer for 6 out of 15 patients; then titers tended to decrease progressively overtime. 13 out of 15 patients experienced mild to moderate ocular inflammation (anterior chamber inflammation and/or vitritis), responsive to standard medication, without apparent correlation with the humoral serum response. In conclusion, a humoral serum response against AAV2 was observed from 2 weeks post-injection of GS010 in both NHP and LHON patients. Ocular inflammation was reported, but currently no consistent correlation with anti-AAV2 antibody levels (at baseline or at the time-points when inflammation was recorded) were noted. Additional follow-up of humoral and cellular immunogenicity in upcoming nonclinical study and Phase III trials will help to confirm these observations and delineate the potential predictable impact for contralateral eye injection.

84. Alpha 1 Antitrypsin Protein & Gene Therapies for the Treatment of Lupus in Animal Models

Ahmed Elshikha[1], Yuanqing Lu[1], Mong-Jen Chen[1], Mohammad Akbar[1], Leilani Zeumer[2], Ye Yuan[1], Laurence Morel[2], Sihong Song[1]
[1]Pharmaceutics, University of Florida, Gainesville, FL, [2]Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville, FL

Systemic lupus erythematosus (SLE) is an autoimmune rheumatic disorder. Although the pathogenesis of SLE remains elusive, over-reactive dendritic cells (DCs), which promote B cell activation and autoantibodies secretion, play critical roles in the disease development. Alpha-1 antitrypsin (AAT) is a multifunctional protein with anti-inflammatory, cytoprotective and immunoregulatory properties. We has shown that AAT has protective effect in autoimmune disease models including type 1 diabetes and rheumatoid arthritis. In this study, we tested therapeutic potentials of AAT in controlling DC and B cells function and the development of SLE in a spontaneous lupus mouse model. Using bone marrow cells from normal (C57BL/6) and lupus prone (B6.TC, MRL/lpr) mice, we showed that AAT treatment significantly inhibited lipopolysaccharide (LPS) (TLR4 agonist) or CpG (TLR9 agonist) induced DC (cDC and pDC) maturation. In this *in vitro* system, AAT significantly inhibited the production of IFN-I, IL-1β, IL-6 and TNF-α from DCs. AAT treated DC also have significantly lower potential in stimulating B cell proliferation and functions. Based on these results, performed following experiments using spontaneous lupus mouse models. In order to test this therapeutic potential, we treated female MRL/lpr mice (at 7-weeks of age, n=10) with clinical grade of AAT (2 mg/mouse, every 3 days) or PBS for 11 weeks. Consistent with our *in vitro* observations, results from this experiment showed that AAT treatment significantly inhibited DC maturation and reduced DC susceptibility to LPS stimulation. Importantly, AAT treatment significantly lowered serum antibody (anti-dsDNA IgG and anti-nuclear antibodies, ANAs) levels and urine albumin levels. Detailed pathological examinations showed that AAT treatment prevented kidney disease development. To confirm this observations and test the long-term effect of AAT treatment, we performed second experiment using both AAT protein and gene therapies. In this experiment, we treated adult female NZM2410 (another commonly used spontaneous lupus model) mice with AAT protein and rAAV8-CB-AAT vector ($1x10^{11}$ vg/mouse, single IP injection). While 100% mice in control group develop lupus and died at 46 week of age, 50% mice in AAT protein treatment group remain lupus free at 55 weeks of age. Intriguingly, AAT gene therapy (rAAV8-CB-AAT) significantly prevent lupus development (70% mice remain lupus free at 63 weeks of age, P=0.0059). These results clearly demonstrated the therapeutic effect of AAT protein and gene therapy in lupus mouse model. Our results also indicated the advantage of rAAV8 mediated gene therapy for the chronic autoimmune diseases. In summary, we showed AAT inhibited the activation and function of DCs and B cells *in vitro* and *in vivo*. We also showed AAT treatment (protein therapy and gene therapy) prevent lupus development in spontaneous lupus mouse models. Considering the safety profile of AAT and rAAV, our results may be translated into clinical application and lead to a novel (safe and effective) therapy for SLE in humans. This work was supported by grants from University of Florida and Grifols, Inc.

AAV Vectors I

85. AAV9-Utrophin Prevents Myonecrosis in Dystrophic Mice and Dogs without Immunosuppression

Yafeng Song[1], Alock Malik[1], Andrew Mead[1], Marilyn Mitchell[1], Mihail Petrov[1], Xiangping Lu[1], Daniel VanBelzen[1], Maxwell Joffe[1], Margaret Choi[1], Leonard Su[1], Joe Kornegay[2], Tejvir Tejvir Khurana[1], Hansell Stedman[1]
[1]Perelman School of Medicine & Pennsylvania Muscle Institute, University of Pennsylvania, Philadelphia, PA, [2]Departments of Pathology and Laboratory Medicine and Neurology and the Gene Therapy Center, Chapel Hill, NC

The majority of mutations causing Duchenne muscular dystrophy (DMD) are multi-exon, frameshifting deletions, complicating therapy with recombinant dystrophin because of the potential for chronic immune recognition of the "non-self" protein. The paralogous protein utrophin is ubiquitously expressed at levels insufficient to prevent myonecrosis in animal models for DMD, but may confer central immunological tolerance through early developmental expression in the thymus. Here we show for a first time histological evidence for the complete prevention of myonecrosis in dystrophin-deficient striated muscles following systemic administration of an AAV9 vector carrying a 3.5 kb synthetic utrophin transgene (AAVμU). The cDNA was miniaturized by removal of domains least conserved in a comprehensive evolutionary comparison, and further optimized for maximal expression in striated muscle by using the codon bias of mammalian genes encoding contractile proteins. Administration of 10^{15} AAVμU vector genomes (vg) per kg to neonatal mice prevented centronucleation and saturated global recovery of the sarcoglycan complex, despite a subsequent tenfold increase in striated muscle mass with growth. In neonatal dystrophic dogs, intravenous injection of $10^{13.5}$ AAVμU vg/kg without immunosuppression restored sarcoglycan levels and normalized the myofiber size-distribution following a fourfold increase in muscle mass. Interferon-gamma ELISpot assays using utrophin-derived peptides revealed no reactivity in injected dogs, consistent with central immunological tolerance. These findings provide a rationale for high dose, neonatal gene therapy using utrophin as a "self" protein to forestall disability and mortality in DMD, while minimizing the risk of chronic immunotoxicity.

86. AAV9 Mediated Correction of Iduronate-2-Sulfatase Deficiency in the Central Nervous System to Prevent the Onset of Neurologic Deficits in a Murine Model of Mucopolysaccharidosis Type II.

Kanut Laoharawee[1], Kelly M. Podetz-Petersen[1], Kelley Kitto[2], Lucy Vulchanova[2], Carolyn Fairbanks[3], Karen Kozarsky[4], Walter C. Low[5], R. Scott McIvor[1]
[1]Genetics Cell Biology and Development, University of Minnesota, Minneapolis, MN, [2]Neuroscience, University of Minnesota, Minneapolis, MN, [3]Pharmaceutics, University of Minnesota, Minneapolis, MN, [4]REGENXBIO Inc., Washington D.C., DC, [5]Neurosurgery, University of Minnesota, Minneapolis, MN

Mucopolysaccharidosis type II (MPS II; Hunter Syndrome) is an X-linked recessive inherited lysosomal storage disease caused by deficiency of iduronate-2-sulfatase (IDS) and subsequent accumulation of glycosaminoglycans (GAGs) dermatan and heparan sulphate. Affected individuals exhibit a range in severity of manifestations such as organomegaly, skeletal dysplasias, cardiopulmonary obstruction, neurocognitive deficit, and shortened life expectancy. There is no cure for MPS II at the moment. Current standard of care is enzyme replacement therapy (ELAPSRASE; idursulfase), which is used to manage disease progression. However, enzyme replacement therapy (ERT) does not show neurologic improvement. As hematopoetic stem cell transplantation (HSCT) has not shown neurologic benefit for MPS II, there is currently no clinical recourse for patients exhibiting neurologic manifestations of this disease, and new therapies are desperately needed. We have been developing the use of AAV9 vectors for delivering the human IDS coding sequence (AAV9.hIDS) into the central nervous system of MPS II mice to restore IDS levels in the brain and prevent the emergence of neurocognitive deficits in the treated animals. A series of CMV-enhancer, beta actin-regulated vectors were generated that encode human IDS with or without the human sulfatase modifying factor-1 (SUMF-1), required for activation of the sulfatase active site. Three routes of administration; Intrathecal (IT), Intracerebroventricular (ICV) and Intravenous (IV) were used in these experiments. We found no significant difference in the enzyme level between mice that were treated with AAV9 vector transducing hIDS alone and mice that were treated with AAV9 vector encoding human IDS and SUMF-1, regardless of the route of administration. IT-administrated NOD.SCID (IDS Y+) and C57BL/6 (IDS Y+) did not show elevated IDS activity in the brain and spinal cord when compared to untreated animals, while plasma showed ten-fold higher (NOD.SCID) and 150-fold higher (C57BL/6) levels than untreated animals. IDS-deficient mice intravenously administered AAV9-hIDS exhibited IDS activities in all organs that were comparable to wild type. Moreover, the plasma of IV injected animals showed enzyme activity that was 100-fold higher than wild type. IDS-deficient mice administered AAV9-hIDUA intracerebroventricularly showed IDS activities comparable to wild type in most areas of the brain and peripheral tissues, while some portions of the brain showed two- to four-fold higher activity than wild type. Furthermore, IDS activity in plasma was 200-fold higher than wild type. Surprisingly, IDS enzyme activity in the plasma of all administrated animals showed persistence for at least 12 weeks post injection; therefore, IDS enzyme was not immunogenic at least on the C57BL/6 murine background. We also conducted additional neurobehavioral testing using the Barnes maze to differentiate neurocognitive deficits of untreated MPS II animals from that of wild type littermates. We found that the learning capability of affected animals is distinctively slower than that observed in littermates. Thus, Barnes maze will be used to address the benefit of these therapies in the MPS II murine model in future experiments. These results indicate potential of therapeutic benefit of AAV9 mediated human IDS gene transfer to the CNS to prevent neurologic deficiency in the MPS II.

87. Rationally Designed Inverted Terminal Repeats Enhance Transduction of Embryonic Stem Cells

Nolan Brown, Matthew Hirsch
University of North Carolina, Chapel Hill, NC

Despite the prevalence of AAV vectors in gene therapy, treatments combining AAV vectors and cell therapy have not progressed into the clinic despite previous reports of successful transduction of stem cells by AAV vectors. One potential explanation is AAV vector toxicity in stem and stem-like cells, as demonstrated in previous studies. In particular, the AAV inverted terminal repeat (ITR) sequence was shown to induce apoptosis in human embryonic stem cells (hESCs) in a p53 dependent manner. A bioinformatic analysis of putative transcription factor binding sites in the AAV2 ITR sequence revealed six putative p53 binding sites. A panel of rationally designed AAV2 ITRs was generated which abrogated putative p53 binding sites and demonstrated reduced p53 binding in an in vitro binding assay. The synthetic ITRs were capable of all of the vector production requirements and were verified by sequencing directly from vector preparations. AAV vector transduction and the hESC DNA damage response was altered by the synthetic ITRs. Importantly, our ITRs demonstrated reduced toxicity in hESCs compared to an AAV vector having the AAV2 ITR sequence. The collective results allude to modulation of the host's DNA damage response using rationally designed ITRs, which may enhance the relevancy of AAV vectors in stem cell therapies.

88. Safety and Biodistribution Study of rAAV2tYF-PR1.7-hCNGB3 in Nonhuman Primates

Guo-jie Ye[1], Ewa Budzynski[2], Peter Sonnentag[2], T. Michael Nork[3], Paul E. Miller[3], Alok K. Sharma[2], James N. Ver Hoeve[3], Leia M. Smith[4], Tara Arndt[2], Roberto Calcedo[5], Chantelle Gaskin[1], Paulette M. Robinson[1], David R. Knop[1], William W. Hauswirth[6], Jeffrey D. Chulay[1]
[1]Applied Genetic Technologies Corporation, Alachua, FL, [2]Covance Laboratories Inc, Madison, WI, [3]OSOD, Madison, WI, [4]LabCorp, Seattle, WA, [5]University of Pennsylvania, Philadelphia, PA, [6]University of Florida, Gainesville, FL

Background: AGTC is developing a recombinant AAV vector expressing the human *CNGB3* gene for treatment of achromatopsia, an inherited retinal disorder characterized by markedly reduced visual acuity, extreme light sensitivity and absence of color discrimination. Here we report results of a toxicology and biodistribution study of this vector administered by subretinal injection in cynomolgus macaques. **Methods:** Three groups of animals (n=2 males and 2 females per group) received a subretinal injection in one eye of 300 µL containing either vehicle or rAAV2tYF-PR1.7-hCNGB3 at one of two concentrations (4×10^{11} or 4×10^{12} vg/mL) and were evaluated for safety and biodistribution over a 3-month period prior to euthanasia. Toxicity assessment was based on mortality, clinical observations, body weights, ophthalmic examinations, intraocular pressure (IOP) measurements, electroretinography (ERG), visual evoked potentials (VEP), and clinical and anatomic pathology. Vector shedding and biodistribution was assessed by qPCR analyses. Immune responses to AAV and hCNGB3 were measured by ELISA, Elispot, or neutralization antibody assay for AAV2tYF. **Results:** There was no evidence of local or systemic toxicity and no changes in IOP, VEP responses, or hematology, coagulation or clinical chemistry parameters and no clinically important changes in ERG responses.

Aqueous cells, sometimes with aqueous flare, were observed at the Day 3 evaluation in all groups and generally resolved or were at the mild (1+) levels by Week 4 and absent on Week 8 and thereafter except in one high dose animal. Posterior segment findings consisted of varying degrees of dose-related white vitreous cell, vitreous haze, white retinal perivascular sheathing, and white to grey-white subretinal infiltrates within and outside of the injection site. Vitreous haze resolved by Day 8 in eyes given vehicle control, by Week 4 in the low dose group and by Week 13 in the high dose group. Vitreous cells were observed at the mild (trace or 1+) level in the vehicle control group and resolved by Study Weeks 9 or 13 but persisted through Week 13 in a dose-related fashion in the low and high dose groups. Serum neutralizing antibodies against AAV2tYF were detected in all animals given vector. There were no T cell responses to AAV capsid peptides and no antibody or T cell responses to hCNGB3. Mononuclear cell infiltrates in the vitreous body/optic disc, of minimal intensity, in the vector-injected eye of all animals at both dose levels. All other tissues collected for histopathological examination showed no abnormalities. Results of biodistribution studies demonstrated that the vector did not spread widely or consistently outside the injected eye. High levels of vector DNA were found in vector-injected eyes but minimal or no vector DNA was found in any other tissue. **Conclusions:** Subretinal injection of rAAV2tYF-PR1.7-hCNGB3 at concentrations of 4×10^{11} or 4×10^{12} vg/mL was associated with a dose-related anterior and posterior segment inflammatory response that improved over time. There was no evidence of systemic toxicity and no changes in IOP, VEP responses, or hematology, coagulation or clinical chemistry parameters and no clinically important changes in ERG responses. These results support the use of rAAV2tYF-PR1.7-hCNGB3 in clinical studies in patients with achromatopsia. A Phase 1/2 clinical trial evaluating rAAV2tYF-PR1.7-hCNGB3 in patients with achromatopsia is scheduled to begin in 2016.

89. Expanding the Toolkit of Protease-Activatable Viruses to Optimize Their Versatility and Efficiency

Caitlin Guenther[1], Michelle Ho[1], Annicka Evans[1], Paul Chariou[2], Momona Yamagami[1], Michael Lam[1], Junghae Suh[1]
[1]Rice University, Houston, TX, [2]Case Western Reserve University, Cleveland, OH

Adeno-associated virus (AAV) has emerged as a promising gene delivery vector because of its non-pathogenicity, simple structure and genome, and low immunogenicity compared to other viral vectors. However, its wide adoption as a safe and effective gene therapy treatment for disease may rely on targeting the vector to deliver transgenes to desired cell populations. To this end, our group has developed a protease-activatable virus (PAV) based on AAV that responds to elevated protease activity commonly found in many diseased tissue microenvironments. Small peptide "locks" were inserted into the viral capsid near its primary receptor binding domain, interfering with the virus-cell receptor interaction and preventing binding and transduction. The peptide locks are flanked by two proteolytically cleavable peptides, which are cleaved off in the presence of matrix metalloproteinases (MMPs), restoring virus-receptor interaction and allowing transduction to resume. Cleavage sequences were chosen from literature based on well-characterized linear peptide substrates. Initial testing demonstrated the desired MMP-activated cellular binding and transduction behavior, but it was discovered that cleavage efficiency and transduction activity of the PAV variants were uncorrelated with the reported specificity constants of the cleavage sequences. We therefore devised several ways to tune the sensitivity and specificity of our PAV. For example, by controlling the ratio of wild type to MMP-activatable protein subunits in the self-assembling 60-mer capsid, we can modulate the level of transduction achieved by the PAVs to attain targeted gene delivery to a range of human pathologies. A new lock format was also designed to allow for more predictable kinetics. This new "linearizable" lock format is modified by replacing one of the two flanking MMP cleavage motifs with the enterokinase cleavage motif, DDDDK, on the C-terminal side of the lock. When the enterokinase-linearized PAVs (ePAVs) are pretreated with enterokinase, the peptide locks are cleaved on one side, allowing the peptides to assume a more flexible conformation protruding from the capsid. The newly linearized peptide locks are cleaved with greater efficiency and kinetics that more closely correlate with expected activity. The PAV platform was also expanded into several AAV serotypes to allow for more diverse clinical applications. Our PAVs have demonstrated targeted delivery to several cancer models, including ovarian and pancreatic cancers, as well as ischemic tissue resulting from myocardial infarction.

90. Vector Biodistribution After Recombinant AAVrh10 Intrathecal Delivery in Non-Human Primates

Gwladys Gernoux[1], Florie Borel[1], Guangping Gao[1], Robert H. Brown[2], Christian Mueller[1]
[1]Gene Therapy Center, UMass Medical School, Worcester, MA, [2]Department of Neurology, UMass Medical School, Worcester, MA

Intrathecal (IT) delivery of adeno-associated viral (AAV) vector is currently used for the treatment of neurodegenerative disorders. To date, there is no standardized method to deliver the vector into the cerebrospinal fluid (CSF). Several parameters could impact the transduction efficacy among them the dose, the AAV serotype or the volume of injection. In this study, we analyzed the impact of large vector volume administered in the lumbar CSF on the vector biodistribution. Cynomolgus monkeys were injected with a serotype 10 AAV vector (AAVrh10) a serotype known to transduce efficiently the CNS via a pre-implanted catheter. The catheter was inserted after a hemilaminectomy at approximately the L_5 vertebra, and advanced intrathecally with the tip located near the thoracolumbar junction. The rAAVrh10 (5.0 ml) was administered as two 2.5 ml infusions (the second infusion was approximately six hours after initiation of the first infusion). The infusion rate was 7.5 ml/hr over approximately 20 minutes. For the duration of both infusion, the NHPs were restrained in a prone position with the restraint table tilted approximately 30 degrees head-down. The vector biodistribution was analyzed by digital PCR and the results demonstrate that viral genomes are detected along the entire length of the spinal cord but also in substantial levels in peripheral organs. Due to viral genomes detected in lymphoid organs, we are currently analyzing the immune response to AAV capsid. These results are important for assessing the safety of IT delivery in large animal models before translation to human clinical trials.

91. Development of Optimized ["Opt"] AAV Vectors by Combining Capsid-Modified NextGen and Genome-Modified GenX AAV Vectors for High-Efficiency Transduction at Further Reduced Doses

Chen Ling, Baozheng Li, Wenqin Ma, George Aslanidi, Arun Srivastava
Pediatrics, University of Florida College of Medicine, Gainesville, FL

There is little doubt that AAV did not evolve for the purposes of delivery of therapeutic genes. The use of first generation AAV vectors, albeit successful, is unlikely to reach its full potential. We have described the development of capsid-modified next generation [NextGen] AAV vectors for both AAV2 (*Proc Natl Acad Sci USA*, 105: 7827-7832, 2008; *Mol Ther.*, 18: 2048-2056, 2010; *Vaccine*,

30: 3908-3917, 2012; *PLoS One*, 8: e59142, 2013) and AAV3 (*Gene Ther.*, 19: 375-384, 2012; *Hum Gene Ther.*, 25: 1023-1034, 2014) serotypes, in which specific surface-exposed tyrosine (Y), serine (S), threonine (T), and lysine (K) residues on viral capsids were modified to achieve high-efficiency transduction at lower doses Fig. 1A). We have also described the development of genome-modified generation X [GenX] AAV vectors (*J Virol.*, 89: 952-961, 2015), in which the transcriptionally-inactive, single-stranded AAV genome was modified to achieve improved transgene expression (Fig. 1B). Thus, we reasoned that encapsidation of GenX AAV genomes into NextGen AAV capsid might lead to further increased transduction at further reduced vector doses. To this end, the following sets of ssAAV2 as well as ssAAV3 serotype vectors containing the firefly luciferase (Fluc) reporter gene were generated: (i) wild-type (WT) genome and Y444+500+730F+T492V AAV2 quadruple-mutant (QM) capsid (WT-Fluc-AAV2/QM); (ii) two GenX genomes and AAV2 QM capsid (LC1-Fluc-AAV2/QM and LC2-Fluc-AAV2/QM); (iii) WT genome and S663V+T492V AAV3 double-mutant (DM) capsid (WT-Fluc-AAV3/DM); and (iv) two GenX genomes and AAV3 DM capsid (LC1-Fluc-AAV3/DM and LC2-Fluc-AAV3/DM). The combination of the modified-genomes with the capsid-mutants led to ~5-6-fold increase with both AAV2 and AAV3 serotype vectors following transduction of a human hepatocellular carcinoma (HCC) cell line, Huh7, at an MOI of 1,000 vgs/cell under identical conditions *in vitro*. When male C57BL/6 mice were injected via tail-vein with WT-Fluc-AAV2/QM, LC1-Fluc-AAV2/QM, and LC2-Fluc-AAV2/QM vectors at a relatively low dose of $5x10^9$ vgs/mouse, the AAV2 QM capsid-mutant vectors led to ~6-10-fold increase in transgene expression in the liver. Similarly, when WT-Fluc-AAV3/DM, LC1-Fluc-AAV3/DM, and LC2-Fluc-AAV3/DM vectors were injected via tail-vein in NSG mice xenografted with human liver tumors at relatively low dose of $3x10^9$ vgs/mouse, led to ~5-8-fold increase with the combination of modified-genomes with the AAV3 DM capsid-mutant vectors, which was restricted to human liver tumors. Taken together, these data document that the combination of NextGen capsids and GenX genomes leads to the generation of optimized [Opt] AAV serotype vectors (Fig. 1C), which transduce cells and tissues more efficiently, both *in vitro* and *in vivo*, at significantly reduced doses. These studies have significant implications in the potential use of the Opt AAV serotype vectors in human gene therapy.

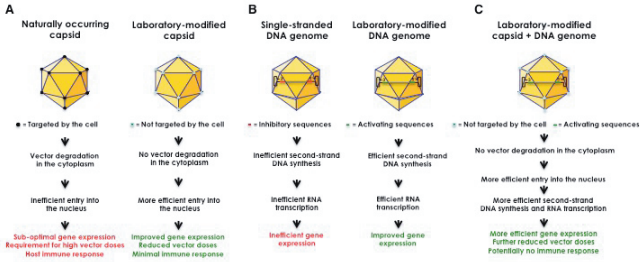


Figure 1: (A) NextGen AAV vectors. Surface-exposed, specific tyrosine, serine, and threonine residues on AAV capsids can be phosphorylated, which is a signal for ubiquitination. Similarly, surface-exposed, specific lysine residues on AAV capsids can be ubiquitinated, and subsequently degraded by the host cell proteasome machinery. Site-directed mutagenesis of these residues circumvents these problems. **(B) GenX AAV vectors.** The D-sequence at the 3'-end in the viral inverted terminal repeat in the transcriptionally-inactive ssAAV genome contains the binding site for a cellular protein, FKBP52, phosphorylated forms of which, strongly inhibit the viral second-strand DNA synthesis. The D-sequence at the 5'-end in the ITR contains the binding site for NF-κB–repressing factor, a negative regulator of transcription. Removal of these sequences leads to the generation of GenX AAV vectors that circumvent these problems. **(C) Opt AAV vectors.** Encapsidation of GenX viral genomes into NextGen capsids circumvents the problems associated with the first generation AAV vectors. The OptAAV vectors are more efficient at further reduced vector doses, thus potentially further minimizing host immune responses.

92. Evaluation of Producer Cell Line Platform for Production of Oversized AAV-FVIII Vectors

Bindu Nambiar, Cathleen Sookdeo, Patricia Berthelette, Robert Jackson, Brenda Burnham, Shelley Nass, David Souza, Catherine R. O'Riordan, Karen Vincent, Seng H. Cheng, Donna Armentano, Sirkka Kyostio-Moore
Gene Therapy, Sanofi Genzyme, Framingham, MA

Recombinant adeno-associated virus (rAAV) vectors are being evaluated as gene delivery vehicles in several clinical trials. The 4.7 kb wild-type (WT) size genome of AAV presents a challenge for incorporating larger transgenes with incomplete vector genome (vg) packaging being a frequent outcome. To test the feasibility of producing oversized rAAV production using the producer cell line (PCL) method, we generated slightly oversized rAAV vectors (harboring 5.1 or 5.4 kb sized vgs) containing a liver-restricted promoter (mTTR) and a codon-optimized cDNA encoding human B-domain deleted FVIII (FVIIIco). Genomes were packaged into the AAVrh8R serotype vector using the PCL process and compared to matched vectors generated via the triple transfection (TXN) method. Vectors were then characterized for production yields, integrity of packaged genomes and homogeneity. The data showed that the PCL platform was able to produce oversized AAV vectors at levels that were 10- to 100-fold higher than the TXN process with yields greater than 100,000 vg/cell. The PCLs were stable with consistent production maintained up to 60 passages. Southern and dot blot analyses of the packaged genomes demonstrated encapsidation of genomes larger than 4.7 kb in the PCL generated vector while the majority of genomes packaged via the TXN method were 4.7 kb in size. Furthermore, the PCL process generated more vector DNA-containing particles and less packaging of non-vector DNA. Testing the PCL generated vectors in the hemophilia A knock-out (KO) mouse model showed a 2-fold higher plasma FVIII activity (Coatest) and vg copies in the livers than obtained with the vectors made by the TXN process. In summary, the PCL production process generated higher yields of oversized rAAV/FVIIIco vectors as well as higher quality vectors than the TXN method. Hence, the PCL platform may be used for producing greater quality oversized rAAV vectors at levels that can meet the needs for clinical studies.

93. Development of AAVidua Vector for the Treatment of Cornea Associated Vision Loss in Bone Marrow Treated-Hurler Patients

Melisa Vance[1], Llanga Llanga[2], Joanne Kurtzberg[3], Richard Jude Samulski[4], Matthew Hirsch[5]
[1]Gene Therapy Center, Hematology and Oncology Division, University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, [3]Department of Pediatrics, Duke University Medical Center, Durham, NC, [4]Gene Therapy Center, Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, [5]Gene Therapy Center, Department of Ophthalmology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Mucopolysaccharidosis type 1 (MPS1), also known as Hurler syndrome, is a genetic lysosomal storage disease that results from the loss-of-function mutations present on the L-iduronidase (IDUA) gene. As a consequence, glycosaminoglycans accumulate aberrantly in lysosomes in multiple organs, leading to hepatosplenomegaly, dwarfism, mental and psychomotor retardation, life-threatening cardiac and pulmonary complications, several skeletal and ocular manifestations and nervous system problems. Untreated pediatric patients often die at 5-10 years of age from progressive heart and lung involvement. Since the early 1980s, successful treatment of MPS1 patients with allogeneic hematopoietic stem cell transplantation after myeloablative chemotherapy has been established. The benefits of this therapy rely on cross-correction of IDUA deficient cells with functional IDUA protein produced by a donor cell circulating through the blood and also from engraftment of donor-derived glial cells in the MPS1 patient brain. As a result, if the stem cell transplantation is performed before two years old, the individual manifest cardiac, liver, pulmonary and neurological improvement as well as his lifetime is significantly prolonged. However, still >30% of the patients have progressive corneal clouding, which leads to blindness. Cornea

transplantation is not an option for patients with severe hurler disease and the ones that receive a cornea transplant are at the risk of developing future cloudiness. Recently, we have developed an adeno-associated virus (AAV) capable of delivering idua cDNA to MPS1 patient fibroblasts and restoring IDUA protein function. Furthermore, immunohistochemistry and functional analysis of intrastromal IDUA protein in a normal human cornea showed low levels of the protein. Even though the levels of IDUA that are required to correct the loss of sight in MPS1 patients are relatively low, administration of a >50-fold increase in IDUA activity (over wild type levels) with gene therapy does not result in any detectable cellular toxicity. We then investigated AAV serotype tropism by incubating human cornea with different AAV viral capsids carrying AAV-CMV-eGFP. From this experiment, we have identified certain capsids with higher levels of transduction and GFP expression on the human cornea. Moreover, we determined the optimal injection volume required for complete coverage of the corneal center area, which is sufficient for allowing regain of the vision. Considering that: 1) the eye is an easily accessible, immune-privileged organ; 2) intrastromal injections are commonly performed at the clinic to treat fungal keratitis; 3) gene therapy for eye diseases has been performed for nearly two decades, and 4) there are 127 registered clinical trials currently taken place for gene therapy in the eye; we foresee that injection of AAV delivering idua cDNA directly to the cornea will likely reverse the MPS1-associated vision loss and provide a better quality of life for the patient.

94. A Scalable Recombinant HSV-Based Manufacturing System for Production of Highly Potent Adeno-Associated Type 9 Vectors

Nathalie Clement
Pediatrics, University of Florida, Gainesville, FL

Introduction: Adeno-associated virus vectors (AAV) have been utilized as a gene delivery tool to treat a multitude of diseases. As the potential uses for these vectors expand, the need for improved manufacturing methods becomes essential for large-scale implementation. Recombinant Herpes Simplex Virus (rHSV)-based method delivering AAV capsid and transgenes for recombinant AAV (rAAV) production has been shown to be a robust and scalable method for producing multiple rAAV serotypes for pre-clinical and clinical studies. Here we will present the first scalable production and purification method for rAAV serotype 9 utilizing the rHSV system. ***Methods:*** Recombinant rAAV9-GFP stocks were produced by transfection or rHSV co-infection on HEK 293. Vector genome titers were assessed by quantitative PCR, digital droplet PCR and dot blot. Infectivity was assessed by an infectious center assay, flow cytometry, and green cell transduction assay (GCA). Coomassie SDS-PAGE, or western blot analyses were performed to evaluate identity and purity of rAAV samples and electron microscopy to assess full versus empty ratio for each method. ***Results:*** *rAAV* vectors produced by the HSV method showed a 7 and 10 fold increase in total vector genomes and transducing units per CellStack®, respectively. Increased infectivity was observed in rHSV-made vectors as demonstrated by an increasing in transducing unit titers by GCA and infectious center assay. Flow cytometry of C12 cells infected with equal vector genome amounts of both transfection and rHSV made rAAV showed a 30% increase in GFP expressing cells. Coomassie staining and western blot analysis showed a 20-32% reduction in total capsid proteins in rAAV made by rHSV co-infection. Electron microscopy confirmed this reduction in total capsid protein was due to a decrease in empty particles in HSV-made rAAV samples. Overall, this method produced greater than $1x10^{14}$ rAAV9 vector genomes per liter equivalent of 293 cells of a final, purified product. ***Conclusions:*** Our data demonstrated the significant improvement in rAAV9 manufacturing by utilization of the rHSV based manufacturing methods. An improvement in both vector titer and infectivity was observed in rHSV made rAAV vectors compared to transfection-based production. Importantly, this scalable purification method is readily suitable for pre-clinical GLP studies and GMP manufacturing in our laboratory. Further improvements will be presented specifically evaluating rHSV production in a suspension-adapted HEK 293 cell system in view of a large scale manufacturing protocol of rAAV9 vectors for clinical studies.

95. Incorporation of AAV Serotype 2 Viral Protein 2 into Other AAV Serotype Capsids

Daniel Zhang[1], Jun Li[2], Yinglu Feng[3], Adam Snowden[1], Georgy Aslanidi[1], Nicholas Muzyczka[4], Arun Srivastava[5], Changquan Ling[2], Chen Ling[1]
[1]Division of Cellular and Molecular Therapy, Department of Pediatrics, University of Florida, Gainesville, FL, [2]Department of Traditional Chinese Medicine, Second Military Medical University, Shanghai, China, [3]Department of Traditional Chinese Medicine, 401 Hospital of the Chinese People's Liberation Army, Qingdao, China, [4]Department of Molecular Genetics & Microbiology, University of Florida, Gainesville, FL, [5]Powell Gene Therapy Center, University of Florida, Gainesville, FL

It was previously documented that large ligands inserted after residue 138 in the adeno-associated virus serotype 2 (AAV2) viral protein 2 (VP2) is tolerant to produce nearly wild-type levels of recombinant AAV2 (rAAV2) vectors (*J Virol*. 78(12):6595-609, 2004). Furthermore, insertion of enhanced green fluorescent protein (EGFP) into VP2 resulted in a rAAV2 particle, whose trafficking could be temporally monitored using confocal microscopy. Here, we examined whether AAV2 VP2 (2VP2) could be incorporated into other AAV serotype capsids during viral production.

A pcDNA3.1-based plasmid was generated to over-express only the 5'-EGFP-tagged 2VP2 (EGFP-2VP2), but not other viral proteins, such as AAV2 Reps, VP1, or VP3. Then, rAAV1~10 vectors were produced in HEK293 cells using PEI-mediated, quadruple-plasmid transfection. The four plasmids for each rAAV vector included a packaging plasmid that contains AAV2 *rep* genes and a corresponding *cap* gene, a helper plasmid that provides essential genes from adenovirus, an ITR-containing plasmid that carries a reporter gene encoding firefly luciferase (Fluc), and the pcDNA3.1-EGFP-2VP2 plasmid. The expression of corresponding VP2 in each serotype capsid was not blocked. Purified viral stocks were separated by SDS-12% acrylamide gel electrophoresis and analyzed by Western blot assays with the B1 antibody or EGFP antibody. The results indicated that the EGFP-2VP2 protein was successfully inserted into all tested rAAV vectors, except for rAAV5. Approximately one-tenth to one-half of the total VP2 was substituted by EGFP-2VP2, depending on the serotype. Next, we characterized the novel rAAV2 and rAAV3 particles that had EGFP-2VP2 insertions. Four vectors (WT-rAAV2, rAAV2-EGFP-2VP2, WT-rAAV3, rAAV3-EGFP-2VP2) carrying the *fluc* gene were used to transduce a human hepatocellular carcinoma cell line, Huh7, under identical conditions *in vitro*. There was no difference in the transgene expression between the WT and EGFP-2VP2-inserted vectors. Furthermore, when heparin was added in the cell culture, the transduction efficiency of both rAAV2 vectors was inhibited in a dose-dependent manner. On the other hand, the transduction efficiency of rAAV3-2VP2-EGFP vectors showed a similar pattern to that was previously reported for WT-rAAV3 vectors, in which low doses of heparin significantly enhanced rAAV3 transduction, but high doses inhibited it. The tropism of rAAV2-EGFP-2VP2 vectors was further characterized *in vivo* following tail-vein injection of Balb/c mice and showed no difference compared with the WT-rAAV2 vectors. Finally, the trafficking of both rAAV2-EGFP-2VP2 and rAAV3-EGFp-2VP2 was temporally monitored using confocal microscopy.

In summary, we have demonstrated an easy method that can be used to insert large peptides into most AAV serotype particles. This system may provide significant information to study rAAV transduction.

96. An Approach to Compare Multiple Adeno-Associated Virus (AAV) Capsids Side-by-Side in a Single Eye Following Intravitreal Injection

John M. Powers[1], Kei Adachi[1], Benjamin Sivyer[2], William Taylor[2], Mark Pennesi[3], Hiroyuki Nakai[1]
[1]Medical & Molecular Genetics, Oregon Health & Science University, Portland, OR, [2]Department of Visual Sciences, Casey Eye Institute - OHSU, Portland, OR, [3]Ophthalmology and Visual Sciences, Casey Eye Institute - OHSU, Portland, OR

Current gene therapy approaches for ocular diseases rely largely on the use of subretinal injection for treatment of outer nuclear layer cells of interest. While subretinal injection allows for efficient transduction of retinal cells close to the site of the injection, there is a significant risk that the injection can further damage retinas with compromised integrity. Several studies have identified new adeno-associated virus (AAV) capsid mutants that are potentially able to transduce outer nuclear layer cells following intravitreal injections. To further explore AAV vector-mediated intravitreal injection approaches for clinical translation, it is imperative to directly compare retinal transduction efficiencies between newly identified and previously published AAV mutants. Using clinically relevant large animal models, these mutant capsids, as well as other commonly used AAV serotypes, can be tested in a comprehensive manner. To this end, we propose to utilize a viral DNA/RNA barcoding approach established by our lab. In this approach, AAV capsids derived from different serotypes and mutants can be identified by DNA and RNA barcodes unique to each AAV capsid. These barcodes are contained in viral genomes and also expressed as RNA barcodes following cellular transduction. As each AAV capsid strain has a unique viral genome, a mass transduction of a number of different AAV strains can be conducted in order to directly compare transduction levels side-by-side in a single eye. To test feasibility of the barcode approach and establish proof-of-principle in the ocular gene therapy research, we produced a DNA/RNA-barcoded AAV library that contained 20 serotypes including AAV serotypes 1-11 and AAV mutants including DJ, LK03, 2i8 and 2G9. Each AAV strain expresses a pair of 12 nucleotide-long RNA barcodes unique to each strain under the control of the human U6 snRNA promoter. Seven C57BL/6J mice have been injected into vitreous humor bilaterally with the DNA/RNA-barcoded AAV library vector at doses ranging from 2 to 9 x109 vg. To date, eyes were harvested from one mouse 3 weeks post injection. Total DNA and RNA were extracted from the retina as a whole from each eye, and were subjected to DNA-PCR or reverse transcriptase (RT)-PCR to amplify viral DNA and RNA barcodes, followed by Illumina barcode sequencing. A preliminary data analysis revealed that AAV2, AAV-DJ and AAV2G9 transduced the murine retina better than other AAV strains including AAV9, the reference control AAV strain contained in the library. Although further optimization of the approach may be required to move this project forward, we plan to apply this method to assess transduction efficiencies of various AAV strains side by side in specific subsets of sorted retinal cells and in various animal species. The safer approach of intravitreal injections with the most optimal AAV vectors would significantly increase the ability to treat over 200 genetic retinal diseases and therefore the data that will be obtained from these studies will considerably benefit the field of retinal gene therapy.

97. Large Scale Purification of AAV with Continuous Flow Ultracentrifugation

Haifeng Chen[1], Sandra Marino[2], Ching Yi Ho[1]
[1]Virovek Inc., Hayward, CA, [2]Alfa Wassermann Inc, West Caldwell, NJ

Since its first approval in Europe as gene therapy drug in human use in 2012, adeno-associated viral (AAV) vectors have gained more and more attentions in the field for gene therapy research. So far AAV vectors have usually been purified through either density gradient ultracentrifugation in small volume centrifuge tubes or column chromatography. Though these purification methods have their unique benefits, there is still a need for technology that can process large volume of lysate with high AAV recovery rate. We reasoned that continuous flow ultracentrifugation could meet these requirements. We tested the Alfa Wasserman's AW Promatix 1000™, a research scale continuous flow ultracentrifuge, as a prove of concept for AAV vector purification. In the initial experiments, we tested cesium chloride (CsCl) solution as density gradient media for AAV vector purification but found out that CsCl solution was not stable enough to form a linear gradient even when sucrose was added to increase its viscosity for AAV purification. We then tested iodixanol solution as density gradient media and got satisfactory purification of AAV vectors. Our results indicate that we can obtain near-purified AAV vectors in a single-step of centrifugation with AAV recovery rate exceeding 50%. Further experiments indicate that minor impurities associated with the purified AAV vectors could be removed by adding salts to the iodixanol solution such as CsCl to increase the ionic strength of the density gradient. The data presented here indicate that continuous flow ultracentrifugation can be used for large scale purification of AAV vectors and it should provide an additional tool to facilitate the translation from research to the clinic.

98. Evaluation of Proteasome Inhibitors on AAV-Mediated Transduction Efficiency in Retinal Bipolar Cells

Shengjie Cui[1], Qi Lu[1], Tushar Ganjawala[1], Gary Abrams[2], Zhuo-Hua Pan[2]
[1]Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, MI, [2]Department of Ophthalmology, Kresge Eye Institute, Detroit, MI

Purpose: Recombinant adeno-associated virus (rAAV) vectors have been a powerful gene delivery vehicle to the retina for basic research and gene therapy. For many of these applications, achieving cell-type specific targeting and high transduction efficiency is desired. Recently, there has been increasing interest in targeted gene expression in retinal bipolar cells, especially for optogenetic gene therapy for vision restoration. However, rAAV-mediated transduction efficiency in retinal bipolar cells is relatively low. The transduction efficiency could be affected by a number of factors, one of which is the proteasome-dependent virus degradation. In this study, we are examining the effect of proteasome inhibitors on the transduction efficiency of rAAV vectors in retinal bipolar cells.Methods: The expression of the transgene, mCherry, was used to evaluate the AAV transduction efficiency. Targeted expression of mCherry in retinal bipolar cells was achieved by rAAV2 vectors carrying an mGluR6 promoter. rAAV vectors at the concentration of 5 x 1012 vg/ml with or without containing proteasome inhibitors were intravitreally injected into the eyes of C57BL/6J mice at about one month of age. Animals were euthanized about one month after virus injection for assessing the expression of mCherry. Results: We tested the effects of three proteasome inhibitors, MG132, doxorubicin, and aclarubicin, on rAAV-mediated transduction efficiency in retinal bipolar cells. Retinas treated with doxorubicin from 200 μM to 800 μM exhibited a concentration-dependent increase in the transduction efficiency.

Doxorubicin at the concentration of 2000 μM produced cytotoxicity as evidenced by the thinning of the retinas and decreased the number of mCherry-expressing bipolar cells. The optimal concentration of doxorubicin to enhance the AAV transduction efficiency was 500 μM. MG132 (100 μM, 200 μM, 500 μM) and aclarubicin (50 μM, 100 μM) were not found to enhance the transduction efficiency. Conclusions: Doxorubicin, a proteasome inhibitor, is effective in enhancing rAAV transduction in retinal bipolar cells in mice. Investigation of the mechanism of doxorubicin action may help to further improve the rAAV transduction efficiency in retinal bipolar cells in particular and rAAV-mediated gene delivery in general.

99. Construction and Evaluation of Recombinant AAV Vectors for Central Nervous System Gene Delivery

Diego Sucunza[1,2,3], Jose Diego Pignataro[1,3], Esperenza Lopez-Franco[1], Alberto Jose Rico[1,2,3], Lucia Vanrell[4], Iria Gonzalez[1,2,3], Elvira Roda[1,2,3], Africa Vales[1,3], Jose Luis Lanciego[1,2,3], Gloria Gonzalez-Aseguinolaza[1,2,3]

[1]CIMA, Pamplona, Spain, [2]CIBERNED, Madrid, Spain, [3]IdiSNA, Pamplona, Spain, [4]Universidad de la República, Montevideo, Uruguay

The Adeno-associated virus (AAV) have become a highly promising tool for research and clinical applications in the Central Nervous System (CNS). Here we used the reporter gene EGFP under the transcriptional control of five minimal promoters (MinP) that show efficient and specific expression in neurons or astrocyte *in vitro*. We also describe the extent of viral spread, transduction efficiency and cell type specificity of each promoter into the mouse striatum using the AAV serotype 8 (AAV8). Robust and specific neuronal EGFP expression was observed with the BM88 (88pb) and B2RN (170bp) MinPs, both *in vivo* and *in vitro*. Cell typing with immunofluorescence confirmed the efficient AAV8 gene expression into the striatal neurons. Furthermore, we detected axonal transport of the EGFP protein when using these promoters. Additionally, two variants of the minimal human GFAP promoter (<600bp) were evaluated to analyze the role of a 75bp segment (D region) spanning bp -132 to -57 with respect to the RNA start site, in the control of the transgene expression. A reduction on the transcriptional activity was observed *in vivo* when the region was eliminated. In addition, a minimal murine GFAP promoter (581bp) was generated that exhibited mostly glial expression; however, we also observed EGFP expression in other type of cells like microglia. In summary, we have developed a set of AAV vectors designed for SNC specific cell type expression using minimal promoters to drive gene expression when the size of the inserts matters.

100. Evaluation of Ion-Exchange Membranes for the Purification of AAV8 from Culture Media

Bryan A. Piras[1], David J. McNally[1], Timothy D. Lockey[1], Amit C. Nathwani[2], Michael M. Meagher[1]

[1]Therapeutics Production & Quality, St. Jude Children's Research Hospital, Memphis, TN, [2]UCL Katharine Dormandy Haemophilia and Thrombosis Centre, Royal Free Hospital, London, United Kingdom

Introduction: AAV is the vector of choice for over 6% of all gene therapy clinical trials, with 111 clinical trials open worldwide as of July 2015. However, production of clinical material often relies on inefficient (e.g., size exclusion chromatography, cesium chloride centrifugation) or expensive and highly serotype-specific (e.g., affinity) downstream processes. Therefore, we investigated scalable and potentially serotype-agnostic alternative primary capture and purification methods. Here, we evaluate three ion-exchange membranes with different ligands for their ability to capture and purify AAV8 from culture media, and show highly efficient capture and purification using a Sartobind salt-tolerant interaction chromatography (STIC) with a primary amine (PA) ligand membrane.

Methods: AAV8 particles were produced in adherent 293 cells using either serum-free DMEM or DMEM supplemented with 1% FBS. AAV was harvested at day 6 post-transfection, at which point approximately 90% of AAV8 particles were in the culture media. Media was sterile filtered and, in equal volumes using an AKTA Explorer, run over one of three ion-exchange membranes: Sartobind Q or STIC PA anion-exchange membranes, and a Sartobind S cation-exchange membrane. Load, flow-through, wash, and elution fractions were analyzed by qPCR or AAV8 capsid ELISA to determine purification efficiency and BCA was used to quantify total protein in each fraction.

Results: Recovery with Sartobind Q and S membranes was very poor, with 12% and 15% recovery, respectively, in the elution fractions and the vast majority of AAV present in the flow-through fractions. The STIC PA membrane performed considerably better, with 99.9% of particles captured by the membrane and approximately 67% of particles in the eluate when the media was adjusted to pH 9 prior to loading and the elution was performed with a gradient of 2M NaCl. However, adjusting the pH to 7 and using a gradient of 3M NaCl led to recoveries of greater than 90% as measured by AAV8 particle ELISA. In addition, the eluate showed an 80% decrease in total protein compared to the load. Results were comparable using both serum-free DMEM and DMEM supplemented with 1% FBS, which showed a dynamic binding capacity of approximately $1x10^{14}$ capsids per mL of membrane at 6% breakthrough.

Conclusions: While Sartobind Q and S membranes inefficiently captured AAV8, the Sartobind STIC PA membrane captured AAV8 highly efficiently and eluted AAV in a pH and salt-dependent manner with concomitant reduction in total protein. While it remains to be determined whether this modality is suitable for serotypes other than AAV8, the membrane format would allow for processing of an equivalent batch of AAV in less than 1% of the time of a size exclusion column, without the requirement for column packing, and with more efficient recovery.

101. Rational Design and Cloning of a Stable RPGR ORF15 cDNA Encoding the Full-Length Native RPGR Protein

Jilin Liu, Andrew Espejo, Weihong Pan, Guojie Ye

AGTC, Alachua, FL

Introduction: The retinitis pigmentosa GTPase regulator (RPGR) cDNA contains a long purine-rich repetitive sequence in ORF15 exon that is unstable during recombinant DNA manipulation. This complicates efforts to develop AAV-based vectors for gene therapy of X-linked retinitis pigmentosa (XLRP) caused by mutations in the RPGR gene. We reasoned that the stability of RPGR-ORF15 cDNA could be significantly improved by rational design of the cDNA sequence through codon modification without changing the amino acid sequence. **Methods:** A human RPGR cDNA was designed based on Genbank reference mRNA sequence NM_001034853 that encodes hRPGR isoform C. The 3459 bp coding sequence was codon-optimized based on human codon usage and further modified to reduce tandem repeats, and adjust G/C content, where possible. The codon-modified cDNA (hRPGRco), which encodes a full-length hRPGR protein of 1152 amino acids that is 100% identical to the published sequence, was synthesized and cloned into conventional cloning vector pUC57 and sub-cloned into various vectors, including an AAV plasmid (used for AAV production by transfection) and an HSV shuttle plasmid (used to make an HSV helper for AAV production using AGTC's proprietary HSV-based AAV manufacturing system).

Stability of the hRPGRco cDNA sequence was verified by DNA sequencing at multiple steps: after sub-cloning, large scale plasmid production, production of recombinant HSV helper virus clones that carry the hRPGRco expression cassette and production of AAV vector. Stability of plasmids carrying the hRPGRco expression cassette was also analyzed after serial passage of bacterial clones cultured at various temperatures and during multiple passages. **Results:** Plasmids carrying the redesigned hRPGRco cDNA were grown stably in bacteria at 37ºC. The hRPGRco cDNA was 100% correct in AAVplasmids, in shuttle vectors used to produce HSV helpers, and in the rHSV helper virus passed multiple rounds in mammalian cells. The sequence was also confirmed 100% correct in AAV vectors produced by plasmid transfection for animal efficacy studies. **Conclusions:** A stable RPGR cDNA that encodes full-length, native RPGR protein was obtained and will allow large scale production of an AAV vector for treatment of patients with XLRP.

102. Method Validation for Quantification of Recombinant Adeno-Associated Virus 5 in Mouse Genomic DNA Using Real-Time PCR

Hyun-Jung Kim, Sang-Jin Park, Sunyeong Lee, Ok-sun Kim, Su-Yeon Han, Kyoung-Sik Moon
Division of Non-Clinical Studies, Korea Institute of Toxicology, Daejeon, Korea, Republic of

The recombinant adeno-associated viral (rAAV) vectors are one of the most promising tools in human gene therapy because of their excellent safety profiles. Recently, one of these, rAAV serotype 5 (rAAV5) has attracted attention as a therapeutic vector by many researchers. To develop rAAV5 vector for treatment in clinical trials, it is necessary to perform GLP compliant preclinical toxicity and biodistribution studies in animals. Quantitative real-time PCR (qPCR) technique is gold standard for quantification of gene therapy products and also has been used as the most sensitive method for bioanalysis of other rAAV vectors. In present study, an analytical method by qPCR was validated for determination of rAAV5 encoding human transgene in mouse genomic DNA. The validation was conducted to evaluate specificity, linearity, accuracy, and precision. There were no interfering reacts with the targeted sequence between true positive and negative control (no template control) samples. The correlation coefficient of rAAV5 in mouse genomic DNA was 0.9995 ($1/x^2$ weighted) over a concentration range of 100 to 10,000,000 copies/ug. This method was accurate and precise verified by intra- and inter-batch analysis. The LLOQ for rAAV5 in mouse genomic DNA was 100 copies/ug, measured with acceptable accuracy and precision. Thus, the analytical method was quantifiable, linear, accurate, and precise. In conclusion, the validation method for rAAV5 in mouse genomic DNA has been successfully performed and will be also useful for further preclinical toxicity and biodistribution studies.

Adenovirus Vectors and Other DNA Virus Vectors

103. High-Capacity Adenoviral Vectors (HCAdV) Armed with a High Risk Human Papillomavirus (HPV) Oncogene Specific CRISPR/Cas9 Machinery Specifically Kill Cervical Cancer Cells

Eric Ehrke-Schulz, Anja Ehrhardt
Human Medicine, Institute for Virology, Witten/Herdecke University, Witten, Germany

Persistent high risk human Papillomavirus (HPV) infections are the main cause of cervical cancer and partially also head and neck cancer. Recent studies showed that plasmid transfection of HPV oncogene specific designer nucleases effectively induced in/del mutations in the early promotors or oncogenes of HPV genomes integrated into the genome of cervical cancer cells by double-strand DNA break mediated non homologous end joining (NHEJ). Destruction or suppression of HPV oncogenes lead to decreased suppression of HPV oncogenes mediated by tumor suppressor protein p53 and retinoblastoma protein pRB inducing recovery from HPV oncogene mediated inhibition of apoptosis induction and cell cycle control. However, only few attempts have been made to improve delivery of respective designer nucleases by means of viral delivery allowing for translating these promising findings towards *in vivo* applications. Here we aimed at arming gene deleted high-capacity adenoviral vectors (HCAdVs) with HPV-specific CRISPR/cas9 machineries. By using a new toolbox that facilitates customization, cloning and production of CRISPR-HCAdVs we assembled HCAdV genomes containing the *Streptococcus pyogenes* Cas9 (spCas9) gene including either one gRNA expression unit specific for HPV18-E6 or two gRNA expression units specific for HPV18 and HPV16-E6 that have been shown efficiency to destroy respective genes. HPV specific CRISPR-HCAdVs were amplified in medium scale using a shortened protocol yielding high virus titers. Hela and Caski cervical cancer cells containing HPV18 or HPV16 genomes integrated into their cellular genome, as well as HPV negative A549 cells were infected with HPV specific CRISPR-HCAdVs. Adenoviral delivery of HPV specific CRISPR/Cas9 resulted in strong cell death in HPV positive cervical cancer cell lines whereas HPV negative A549 cells were unaffected. Moreover, HPV-specific CRISPR-HCAdVs infected Hela and Caski cells showed decreased proliferation compared to untreated cells and HPV negative control cells and cytotoxicity assays revealed strongly decreased cell viability of cervical cancer cells. In sharp contrast to control groups, increased apoptosis in HPV specific CRISPR-HCAdV treated cervical cancer cells was measured after performing apoptosis detection assays. Our results suggest that also HCAdVs can serve as oncolytic agents when armed with target specific designer nucleases such as CRISPR/Cas9. We believe that our approach will pave the way towards *in vivo* applications of CRISPR/Cas9 mediated oncolysis of HPV induced cervical cancer. As our CRISPR/Cas9-HCAdV production pipeline is adaptable to incorporate other or even more gRNA expression units specific for further HPV types, it provides a valuable platform to develop personalized antiviral or oncolytic vectors for any specific HPV target.

104. A Gene Deleted High Capacity Adenoviral Vector for Efficient Delivery of a Multiplex DMD Specific CRISPR/Cas9 Machinery

Eric Ehrke-Schulz[1], Rudolf A. Kley[2], Anja Ehrhardt[1]
[1]Human Medicine, Institute for Virology, Witten/Herdecke University, Witten, Germany, [2]Department of Neurology, Neuromuscular Centre Ruhrgebiet, University Hospital Bergmannsheil, Ruhr-University Bochum, Bochum, Germany

Recent advances in the field of designer nuclease directed genome editing hold great promise to correct underlying mutations leading to Duchenne muscular dystrophy. Especially the CRISPR/Cas9 system provides an easy way to design and to assemble RNA guided nucleases offering the potential to develop personalized treatments to correct the multiple different mutations leading to this fatal disease. Recent studies showed efficient genome editing in a myoblast cell line derived from DMD patients and mdx mice. Nevertheless viral delivery of all required CRISPR/Cas9 components including Cas9 and one or multiple guide RNA (gRNA) expression units has not been fully exploited. Gene deleted high-capacity adenoviral vectors (HCAdVs) offer the packaging capacity to deliver the complete CRISPR/Cas9 machinery including several gRNA expression units using a single viral vector. By using a new toolbox that facilitates customization, cloning and production of CRISPR-HCAdVs, we

assembled a HCAdV genome containing a Streptococcus pyogenes Cas9 (spCas9) gene including two guide RNA (gRNA) expression units specific for DMD that have shown efficiency to delete exon 51 in dystrophic human myoblasts. CRISPR-HCAdV was amplified in medium scale using a shortened protocol yielding high titers. Infection of cultured HEK293 cells and primary human myoblasts with purified DMD specific CRISPR-HCAdV at different MOIs resulted in strong locus specific deletion efficiency for DMD exon 51 as shown with locus specific PCR. As a comparison we also designed and produced DMD-specific TALEN encoding HCAdVs which allow delivery of a complete TALEN pair using a single vector. We found that it was more complicated to produce double TALEN-HCAdVs compared to multiplex CRSIPR/Cas9-HCAdV as they require controlled expression of TALEN genes by inducible promotors. Furthermore the TALEN system is not suitable for multiplexing and showed less efficiency in T7E1 assays. Our platform enables cloning and production of gene deleted adenoviral vectors for the delivery of a DMD specific CRISPR/Cas9 system within a short time providing a valuable tool for viral delivery of customized CRISPR/Cas9 for DMD treatment. Additional gRNAs or gRNAs with other specificities can be easily included in the vector allowing personalized molecular design of the gene transfer vector. We expect that this may pave the way towards broader applications of the CRISPR technology for DMD treatment including preclinical and eventually clinical studies.

105. Enhanced Oncolytic Activity Mediated by a Novel Human Adenovirus Type 6 Based Vector

Johannes Doerner, Wenli Zhang, Philip Boehme, Anja Ehrhardt
Institute of Virology and Microbiology, Witten, Germany

Most existing oncolytic adenoviruses (AdV) are based on human AdV type 5 (hAdV-5). Clinical efficacy of hAdV-5 based oncolytic viruses is limited by variable expression levels of coxsackie- and adenovirus receptor (CAR) in different tumor cells and insufficient replication rates. Additionally, high prevalence of neutralizing antibodies against hAdV-5 resulting in lower efficiency makes hAdV-5 a less suitable candidate for systemic application. Recent studies have highlighted human adenovirus type 6 (hAdV-6) as a promising candidate for oncolytic and vaccine vectors. Thus, development of novel oncolytic AdV based on hAdV-6 may help overcoming these limitations. We further hypothesised that oncolytic efficacy of the candidate virus can be augmented by expression of RNAi suppressor protein P19 (Lecellier et al., Nature 2005) as has been shown previously for hAdV-5 (Rauschhuber et al, Sci. Rep. 2013). Here we aim at evaluating a novel hAdV-6-based, p19-containing oncolytic AdV as novel candidate for oncolytic applications in different tumour cell lines. We cloned a P19-containing hAdV-6 based virus (hAdV-6FP19) by a novel seamless recombineering technique (Zhang et al, unpublished). In order to allow P19 expression from the adenoviral vector genome, the *P19* cDNA was fused via an internal ribosome entry site (IRES) to the late fiber gene. After release of the respective recombinant adenoviral genomes from plasmids containing the complete DNA molecule, linearized viral DNA was transfected into HEK293 cells for virus reconstitution. After initial amplification steps which were monitored by virus specific PCRs upscaling and virus purification using cesium-chloride density-gradient ultracentrifugation was performed. Rescue and amplification efficiencies were comparable to commonly used hAdV-5 based vectors. Various cancer cell lines from different origin were used to perform oncolysis assays. This included A549 (lung carcinoma), HCT 116 (colon carcinoma), Hela (cervical carcinoma) and Huh7 (hepatocellular carcinoma) cells which were infected with hAdV-6FP19, hAdV-6 and hAdV-5 at various multiplicities of infection (MOI). 2-3 days after infection cells were fixed and stained with methylene blue. We observed significantly higher cell lysis (up to 100-fold) for hAdV-6FP19 infected cells as compared to hAdV-5 and 6 at identical MOIs. Higher cell lysis rates for hAdV-6FP19 compared to wildtype virus were present in all evaluated cell lines, suggesting significantly enhanced oncolytic potential for hAdV-6FP19. In total we believe that hAdV6-based vectors hold great promise for oncolytic applications and that their oncolytic effectiveness can be further improved by RNAi-suppression.

106. A *Sleeping Beauty* Transposase System in the Context of HD-Ad5/35++ Vectors Achieves Stable In Vivo Transduction of Hematopoietic Stem Cells in Mouse Models

Maximilian Richter[1], Jing Liu[2], Zsuzsanna Izsvák[3], Roberto Cattaneo[4], Wolfgang Uckert[3], Philip Ng[5], Anja Ehrhardt[2], Thalia Papayannopoulou[1], André Lieber[1]
[1]University of Washington, Seattle, WA, [2]Universität Witten/ Herdecke, Witten, Germany, [3]Max-Delbrück-Center for Molecular Medicine, Berlin, Germany, [4]Mayo Clinic, Rochester, MN, [5]Baylor College of Medicine, Houston, TX

Hematopoietic stem cells (HSCs) are important targets for the gene therapy of infectious diseases, genetic disorders, and cancer. Our aim is to transduce HSCs in vivo after mobilization from the bone marrow and intravenous injection of a gene transfer vector. We employed helper-dependent adenovirus vector containing Ad35 fiber mutants with enhanced affinity to CD46 (HD-Ad5/35++) for gene transfer into HSCs. HD-Ad5/35++ vectors efficiently transduced human HSCs and human CD46-transgenic mouse HSCs in vitro. To trigger chromosomal transgene integration we utilized the hyperactive *Sleeping Beauty* transposase system (SB100x). The system consists of two vectors; the transposon vector carrying a GFP gene and the SB100x vector that mediates integration of the GFP cassette into a TA dinucleotide of the genomic DNA. As a limitation, this system requires the co-infection of the same target cell with two vectors to achieve stable transduction. This is exemplified in a study with two Ad5/35++ reporter vectors, expressing either GFP or mCherry. Human CD46tg mice were mobilized with GSCF/AMD3100 and intravenously injected with either vector alone or the vector combination. Reporter gene expression in bone marrow localized HSCs was analyzed 3 days later. While 2% of lineage depleted cells expressed either GFP or mCherry only 0.25% expressed both. In spite of these limitations, we tested whether the co-injection of a HD-Ad5/35++ -GFP transposon vector and a HD-Ad5/35++ SB100x vector, supplying a Sleeping Beauty transposase in trans into mobilized mice would lead to stable HSC transduction. Twelve weeks post-injection, we detected stable GFP expression in bone marrow HSCs in the range of 1 to 2%. GFP integration through SB100X was confirmed by genome-wide sequencing of integration sites. Our data indicate that HD-Ad5/35++ vectors preferentially target primitive HSCs and that this increases the chance of co-infection with both vectors. This is supported by a comparison of GFP marking in HSCs and more differentiated marrow and blood cells four weeks after in vivo transduction. While in the HSC-containing LSK fraction of the bone marrow the marking rate was on average 7.7%, marking rates for total mononuclear cells of the bone marrow and peripheral blood were only 2.5% and 0.3%, respectively. The preferential HSC targeting in hCD46tg and humanized mice can be explained by higher CD46 levels on primitive HSCs compared to more differentiated cells in the bone marrow and the dependence of Ad5/35 infection efficiency (at low MOIs) on CD46 density on the cell surface.

107. Tracking Target Cell Fate After Oncolytic Herpes Simplex Virus Infection

Anna Grosskopf, Samuel D. Rabkin, Robert L. Martuza, Hiroaki Wakimoto
Neurosurgery, Massachusetts General Hospital, Boston, MA

Oncolytic virus (OV) cancer therapy relies on virus-mediated selective killing of cancer cells and subsequent secondary effects that involve induction of anti-tumor immunity. Although cell death induction is a prerequisite in this therapeutic process, experimental evidence is lacking as to whether OV infection of cancer cells results in their inevitable death. To address this issue, we developed an experimental platform that allows us to track the fate of a cell following OV infection. This system is composed of: 1) Recombinant oncolytic herpes simplex viruses derived from G47delta (ICP6-, gamma34.5-, alpha47-) and MG18L (ICP6-, Us3-) that express a Cre recombinase-EGFP fusion protein (G47delta-CreGFP and MG18L-CreGFP), and 2) tumor cell lines that have been engineered to stably carry a reporter construct, in which Cre-mediated excision of a polyA STOP sequence converts the cell into an expresser of the mCherry fluorescence protein. Using patient-derived and established T98 glioblastoma lines, we show that upon G47delta-CreGFP or MG18L-CreGFP infection, the reporter cells become mCherry positive, indicating virus-induced Cre recombination in the host genome. Monitoring of these cells in culture revealed that although an overwhelming majority of the cells die, a small fraction of mCherry+ cells survive and eventually proliferate. Our results demonstrate the existence of "resistance" after OV infection of cancer cells. Our experimental platform should have versatile utilities for tracking the fate of both neoplastic and non-neoplastic cells during OV therapy in in vitro and in vivo settings.

108. Pseudotyping Baculovirus Based Vectors for Enhanced *In Vitro* and *In Vivo* Delivery

Jondavid de Jong, Laura van Lieshout, Jacob P. van Vloten, Sarah K. Wootton
Pathobiology, University of Guelph, Guelph, ON, Canada

Autographa californica multinucleopolyhedrovirus (AcMNPV) is the best-characterized baculovirus and the platform for well established recombinant protein and vaccine production technologies. In addition to countless examples of recombinant protein production, two commercially available vaccines (Cervarix, and Provenge) are produced via AcMNPV based expression technologies. More recently, Baculovirus based vectors have also garnered attention as gene delivery vectors, including for use in human gene therapy, because of several key characteristics. Baculoviruses can transduce cells of human origin, albeit at MOIs of 100-200, can accommodate large gene insertions (>38 kb), allowing for the inclusion of multiple genes, large promoters, and regulatory elements, are non-replicative in mammalian cells, do not integrate into mammalian chromosomes, and humans lack pre-existing immunity to baculoviruses. In vivo, Baculoviruses have been used to successfully transduce a wide variety of organs from mammalian species, such as mice, rats and rabbit. Despite these reports there is little information on the overall tissue distribution of in vivo delivered baculovirus. Here we test the transduction efficiency of wildtype and pseudotyped baculovirus vectors in various cell lines, and define the biodistribution of these vectors in C57BL/6 mice via intravenous, intrahepatic, and intranasal installation. In vitro, wildtype virus demonstrated good transduction of HEK293 cells and moderate to low transduction of cells originating from the lung and liver. Amongst the pseudotypes tested, the greatest transduction achieved across all cell types (in vitro) was a recombinant displaying a cell-penetrating-peptide-GP64 fusion protein (CPP-GP64), which demonstrated high levels of transduction across all cell lines tested. In vivo, overall transduction by wildtype virus was restricted to the kidneys and liver, and only at moderate to low levels. Similar to the in vitro results, inclusion of a CPP-GP64 greatly enhanced the transduction levels as well as expanded the tissue distribution of in vivo delivered vector.

109. Therapeutic Efficacy and Safety Profile of EGFR-Targeted Oncolytic Ad Nanocomplex in Orthotopic Lung Tumor Model

A-Rum Yoon, Dayananda Kasala, Yan Li, Jinwoo Hong, Wonsig Lee, Soo-Jung Jung, Chae-Ok Yun
Department of Bioengineering College of Engineering Hanyang University, Seoul, Korea, Republic of

Adenovirus (Ad)-mediated cancer gene therapy has been proposed as a promising alternative to conventional therapy for cancer. However, success of systemically administered naked Ad has been limited due to the immunogenicity of Ad and the induction of hepatotoxicity caused by Ad's native tropism. In this study, we synthesized an epidermal growth factor receptor (EGFR)-specific therapeutic antibody (ErbB)-conjugated and PEGylated PAMAM dendrimer (PPE) for complexation with Ad. Transduction of Ad was inhibited by complexation with PEGylated PAMAM (PP) dendrimer due to steric hindrance. However, PPE-complexed Ad selectively internalized into EGFR-positive cells with greater efficacy than either naked Ad or Ad complexed with PP. Systemically administered PPE-complexed oncolytic Ad elicited significantly reduced immunogenicity, nonspecific liver sequestration, and hepatotoxicity than naked Ad. Furthermore, PPE-complexed oncolytic Ad demonstrated prolonged blood retention time, enhanced intratumoral accumulation of Ad, and potent therapeutic efficacy in EGFR-positive orthotopic lung tumors in comparison with naked Ad. We conclude that ErbB-conjugated and PEGylated PAMAM dendrimer can efficiently mask Ad's capsid and retarget oncolytic Ad to be efficiently internalized into EGFR-positive tumor while attenuating toxicity induced by systemic administration of naked oncolytic Ad.

110. Enhanced Intratumoral Accumulation of Nucleic Acid and Oncolytic Adenovirus by Complexation with Biocompatible and Bioreducible Polymer

Jung-Woo Choi[1], Jaesung Kim[2], Yan Li[1], Chae-Ok Yun[1], Sung Wan Kim[2]
[1]Department of Bioengineering College of Engineering Hanyang University, Seoul, Korea, Republic of, [2]Center for Controlled Chemical Delivery (CCCD), Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake, UT

As an effective and safe strategy to overcome the limits of therapeutic nucleic acid or adenovirus (Ad) vectors for in vivo application, various technologies to modify the surface of vectors with nonimmunogenic/biocompatible polymers have been emerging in the field of gene therapy. However, the transfection efficacy of the polymer to transfer genetic matrials is still relatively weak. To develop more advanced and effective polymer to deliver not only Ad vectors, but also nucleic acids, 6 biocompatible polymers were newly designed and synthesized to different sizes (2k, 3,4k, or 5k) of poly(ethylene) glycol (PEG) and different numbers of amine groups (2 or 5) based on methoxy poly(ethylene glycol)-b-poly{N-[N-(2-aminoethyl)-2-aminoethyl]-L-glutamate (PNLG). We characterized size distribution and surface charge of 6 PNLGs after complexation with either nucleic acid or Ad. Among all 6 PNLGs, the 5amine group PNLG showed the strongest efficacy in delivering nucleic acid as well as Ad vectors. Interestingly, cellular uptake results showed higher uptake ability in Ad complexed with 2 amine group PNLGs is more essential than the surface charge for cellular uptake in polymers

with charges greater than 30 mV. Moreover, the endosome escape ability of Ad/PNLGs increased depending on the number of amine groups, but decreased by PEG size. Cancer cell killing efficacy and immune response studies of oncolytic Ad/PNLGs showed 5 amine group PNLG to be a more effective and safe carrier for delivering Ad. Overall, these studies provide new insights into the functional mechanism of polymer-based approaches to either nucleic acid or Ad/nanocomplex. Furthermore, the identified ideal biocompatible PNLG polymer formulation (5 amine/2k PEG for nucleic acid, 5 amine/5k PEG for Ad) demonstrated high transduction efficiency as well as therapeutic value (efficacy and safety) and thus has strong potential for in vivo therapeutic use in the future.

111. HSV Vectors Retargeted for Infection of TrkA Receptor-Bearing, Pain-Sensing C-Fibers

Marco Marzulli, Justus B. Cohen, Bonnie Reinhart, William F. Goins, Joseph C. Glorioso
Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA

Chronic pain represents a major cause of morbidity and effective pain therapy remains a significant unmet medical need. The standard of care relies primarily on systemic drug therapies that do not target the site of pain sensation. These therapies often have limited effectiveness, deleterious side effects, and ,induce tolerance. Herpes simplex viruses (HSV)-based gene therapy vectors offer an attractive alternative to drug therapy since therapeutic genes can be delivered to sensory nerve afferents where pain is arising. Our goal is to develop a transductionally retargeted HSV vector to selectively deliver therapeutic genes to those neurons activated during chronic pain states. NGF/TRKA signaling mediates the pain response associated with inflammatory hyperalgesia and neuropathic pain conditions, making TrkA-expressing cells an important target for chronic pain gene therapy. To obtain a fully retargeted HSV, the virus attachment/entry component glycoprotein D (gD) can be modified to eliminate recognition of its cognate receptors (HVEM and nectin1) and introduce a new ligand into the N-terminus of gD to allow entry through its corresponding cellular receptor. Therefore, we replaced the signal peptide and HVEM binding domain of gD with pre-pro-(pp) NGF to create a TrkA targeting protein, gD:ppNGF(Y38), that can still bind nectin1. Virus expressing gD:ppNGF(Y38) was propagated on cells expressing nectin1 and purified virus was shown to enter J1.1-2, nectin1-deficient cells, only when transduced with TrkA receptor (J/TrkA cells). To enhance the propagation of these vectors on complementing cells, we developed genetic selection methods to isolate retargeted virus variants that display enhanced entry and spread on J/TrkA cells. We found that a selected variant (J4H) had acquired mutations in other HSV envelope glycoproteins, including one glycoprotein involved in envelope-cell fusion events (gH) and two that were previously shown to contribute to virus spread (gE and gI). Moreover, we show that the gH mutation alone, when introduced into the parental virus backbone, enhances entry and minimally improves virus spread. We are currently investigating the specific phenotypes of the individual gE and gI mutations. A fully TrkA-retargeted J4H-based virus (J4HΔ38), further modified to completely eliminate nectin1-binding, also displays enhanced entry and spread on J/TrkA cells. We are currently testing the J4HΔ38 virus in primary sensory neurons in culture and in infections of animals in vivo. We suggest that the TrkA-vector retargeting will provide a means for transduction of pain sensing C-fibers to more precisely introduce pain-relieving gene products.

112. Potent Antifibrotic Effect on Human Dermal Fibroblasts and Keloid Spheroids by Adenovirus-Mediated Inhibition of Wnt and TGF-β Signaling

Hyo Min Ahn[1], Won Jai Lee[2], Youjin Na[1], Jinwoo Hong[1], Chae-Ok Yun[1]
[1]Department of Bioengineering, College of Engineering, Hanyang University, Seoul, Korea, Republic of, [2]Institute for Human Tissue Restoration, Department of Plastic & Reconstructive Surgery, Yonsei University College of Medicine, Seoul, Korea, Republic of

Aberrant activation of the canonical Wingless type (Wnt) signaling pathway plays a key role in the development of hypertrophic scars and keloids. This aberrant activation of the canonical Wnt pathway and its potent profibrotic effects suggest that Wnt pathway can be a potential target for the development of novel anti-fibrotic agents. In this study, we evaluated the anti-fibrotic potential of a soluble Wnt decoy receptor (sLRP6E1E2)-expressing non-replicating adenovirus (Ad; dE1-k35/sLRP6E1E2) on human dermal fibroblasts (HDFs) and keloid spheroid. Wnt family member 3a and its effector β-catenin expression in keloid tissues were analyzed by hematoxylin and eosin and immunohistochemical staining. HDFs were transduced with dE1-k35/sLRP6E1E2 or control Ad vector (dEl-k35/LacZ), and then luciferase reporter assay for β-catenin activity was performed. Furthermore, change in type-I and -III collagen mRNA levels in HDFs were measured by quantitative real time PCR. Wnt intracellular signaling and secreted transforming growth factor-β1 (TGF-β1) protein expression were examined by western blot and the enzyme-linked immunosorbent assay, respectively. The effect of sLRP6E1E2 on the nuclear localization of β-catenin and Smad 2/3 complex was evaluated by immunofluorescence staining. Lastly, the expression levels of major extracellular matrix components were investigated by immunohistochemistry in keloid spheroids after transduction with dEl-k35/LacZ or dE1-k35/sLRP6E1E2. Higher Wnt3a and β-catenin expression was observed in the keloid region compared to the adjacent normal tissues. The activity of β-catenin and mRNA expression of type-I and -III collagen were significantly decreased following treatment with dE1-k35/sLRP6E1E2. The expression of LRP6, β-catenin, phosphorylated glycogen synthase kinase *3 beta*, Smad 2/3 complex, and TGF-β1 were decreased in Wnt3a- or TGF-β1-activated HDFs, following administration of dE1-k35/sLRP6E1E2. Moreover, dE1-k35/sLRP6E1E2 markedly inhibited nuclear translocation of both β-catenin and Smad 2/3 complex. The expression levels of type-I and -III collagen, fibronectin, and elastin were also significantly reduced in keloid spheroids after treatment with dE1-k35/sLRP6E1E2. These results indicate that Wnt decoy receptor-expressing Ad can degrade extracellular matrix in HDFs and primary keloid spheroids, and thus it could be highly beneficial for treatment of keloids.

113. Potent Therapeutic Efficacy of Neurotensin Receptor-Targeting and Extracellular Matrix-Degrading Oncolytic Adenovirus in an Orthotopic Pancreatic Tumor Model

Youjin Na[1], Joung-Woo Choi[1], Dayananda Kasala[1], Jinwoo Hong[1], Eonju Oh[1], Yan Li[1], Soo-Jung Jung[1], Sung Wan Kim[2], Chae-Ok Yun[1]
[1]Department of Bioengineering College of Engineering Hanyang University, Seoul, Korea, Republic of, [2]Center for Controlled Chemical Delivery (CCCD), Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, Seoul, UT

Pancreatic cancer is highly aggressive, malignant, and notoriously difficult to cure using conventional cancer therapies. These conventional therapies have significant limitations due to excessive

extracellular matrix (ECM) of pancreatic cancer and poor cancer specificity. The excess ECM prevents infiltration of drugs into the inner layer of the solid tumor. Therefore, novel treatment modalities that can specifically target the tumor and degrade the ECM are required for effective therapy. In the present study, we used ECM-degrading and Wnt signal-disrupting oncolytic adenovirus (oAd/DCN/LRP) to achieve a desirable therapeutic outcome against pancreatic cancer. In addition, to overcome the limitations in systemic delivery of oncolytic Ad (oAd) and to specifically target pancreatic cancer, neurotensin peptide (NT)-conjugated polyethylene glycol (PEG) was chemically crosslinked to the surface of Ad, generating a systemically injectable hybrid system, oAd/DCN/LRP-PEG-NT. We tested the targeting and therapeutic efficacy of oAd/DCN/LRP-PEG-NT toward neurotensin receptor 1 (NTR)-overexpressing pancreatic cancer cells, both *in vitro* and *in vivo*. The oAd/DCN/LRP-PEG-NT elicited increased NTR-selective cancer cell killing and increased transduction efficiency when compared with a cognate control lacking NT (oAd/DCN/LRP-PEG). Furthermore, systemic administration of oAd/DCN/LRP-PEG-NT significantly decreased induction of innate and adaptive immune responses against Ad, and blood retention time was markedly prolonged by PEGylation. Moreover, NTR-targeting oAd elicited greater *in vivo* tumor growth suppression when compared with naked oAd, and 9.5×10^6-fold increased tumor-to-liver ratio. This significantly enhanced antitumor effect of oAd/DCN/LRP-PEG-NT was mediated by active viral replication and viral spreading, which was facilitated by ECM degradation and inhibition of Wnt signaling-related factors (Wnt, β-catenin, and/or vimentin) in tumor tissues. Taken together, these results demonstrate that oAd/DCN/LRP-PEG-NT has strong therapeutic potential for systemic treatment of NTR-overexpressing pancreatic cancer, due to its NTR-targeting ability, enhanced therapeutic efficacy, and safety.

114. Replication-Defective HSV Vector Development for Targeted Gene Delivery

Bonnie Reinhart, Daniela Leronni, Justus B. Cohen, Joseph C. Glorioso
Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA

Gene therapy treatment strategies for Huntington's disease (HD) depend on a vector that (i) allows targeted delivery of large therapeutic transgenes to specific cell populations and (ii) is replication-defective and non-cytotoxic. Thus, our goal is to develop a replication-defective HSV-based gene therapy vector to deliver therapeutic transgenes to the cells most affected by expression of the mutant Huntington gene (Htt). Strategies for full retargeting of HSV require virus detargeting from its cognate receptors (HVEM and nectin1), recognized by the virus attachment/entry component glycoprotein D (gD), and introduction of a new ligand into gD that allows entry through recognition of the corresponding cellular receptors. To target an HSV vector for entry exclusively into cells expressing the receptor GFRα1 we employed the ligand GDNF. We replaced the signal peptide and HVEM binding domain of gD with pre-pro-(pp)GDNF to create a GFRα1 targeting protein, gD(Y38)_GDNF, that can still bind nectin1. Virus expressing gD(Y38)_GDNF was propagated on cells expressing nectin1 and purified virus was shown to enter nectin1-deficient J1.1-2 and B78H1 cells in a GFRα1-dependent manner. U2OS cells engineered to express GFRα1 demonstrated the most robust virus entry and spread, allowing us to create and propagate a fully retargeted virus that can no longer bind nectin1, gD(Δ38)_GDNF. Once the functionality of gD(Δ38)_GDNF was confirmed, we transferred this mutation to a replication-defective backbone lacking essential immediate early gene expression. Complementing cells were screened and those cells that best supported growth of the replication-defective backbone were selected. Virus stocks were propagated and tested for receptor specificity *in vitro* and experiments are currently underway to assess GFRα1-specific entry *in vivo*. We anticipate that the use of neuronal-specific targeting ligands, such as GFRα1, in replication-defective backbones will promote homing of non-toxic HSV gene therapy vectors to cells affected in HD.

115. Tumor Microenvironment-Targeting Hybrid Vector System Utilizing Oncolytic Adenovirus Complexed with pH-Sensitive and Bioreducible Polymer

Jung-Woo Choi[1], Dayananda Kasala[1], Chang Yoon Moon[1], Soo-Jung Jung[1], Sung Wan Kim[2], Chae-Ok Yun[1]
[1]Department of Bioengineering College of Engineering Hanyang University, Seoul, Korea, Republic of, [2]Center for Controlled Chemical Delivery (CCCD), Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake, UT

Oncolytic adenoviruses (Ads) have shown great promise in cancer gene therapy but their efficacy has been compromised by potent immunological, biochemical, and specific tumor-targeting limitations. To take full advantage of the innate cancer-specific killing potency of oncolytic Ads but also exploit the subtleties of the tumor microenvironment, we have generated a pH-sensitive and bio-reducible polymer (PPCBA)-coated oncolytic Ad. Ad-PPCBA complexes showed higher cellular uptake at pH 6.0 than pH 7.4 in both high and low coxsackie and adenovirus receptor-(CAR)-expressing cells, thereby demonstrating Ad-PPCBA's ability to target the low pH hypoxic tumor microenvironment and overcome CAR dependence for target cell uptake. Endocytic mechanism studies indicated that Ad-PPCBA internalization is mediated by macropinocytosis instead of the CAR-dependent endocytic pathway that internalizes naked Ad. VEGF-specific shRNA-expressing oncolytic Ad complexed with PPCBA (RdB/shVEGF-PPCBA) elicited much more potent suppression of U87 human brain cancer cell VEGF gene expression in vitro, and human breast cancer MCF7 cell/Matrigel plug vascularization in a mouse model, when cancer cells had been previously infected at pH 6.0 versus pH 7.4. Moreover, intratumorally and intravenously injected RdB/shVEGF-PPCBA nanocomplexes elicited significantly higher therapeutic efficacy than naked virus in U87-tumor mouse xenograft models, reducing IL-6, ALT, and AST serum levels. These data demonstrated PPCBA's biocompatibility and capability to shield the Ad surface to prevent innate immune response against Ad after both intratumoral and systemic administration. Taken together, these results demonstrate that smart, tumor-specific, oncolytic Ad-PPCBA complexes can be exploited to treat both primary and metastatic tumors.

116. Overcoming CAR-Dependency of Oncolytic Adenovirus via Adenoviral Fiber Modification with Vesicular Stomatitis Virus Glycoprotein Epitope for Enhanced Antitumor Efficacy

A-Rum Yoon, Jinwoo Hong, Chae-Ok Yun
Department of Bioengineering College of Engineering Hanyang University, Seoul, Korea, Republic of

Utility of traditional oncolytic Ad has been limited due to low expression of coxackies and adenovirus receptor (CAR) in cancer cells which results in poor infectivity of Ads. Here with an aim of improving the efficiency of Ad's entry to the cell, we generated a novel tropism-expanded oncolytic Ad which contains the epitope of vesicular stomatitis virus glycoprotein (VSVG) at the HI-loop of Ad fiber. We generated 9 variants of oncolytic Ads with varying linkers and partial deletion to the fiber. Only one VSVG epitope-incorporated variant, RdB-1L-VSVG, which contains 1 linker and no deletion to fiber, was produced efficiently. Production of 3-dimensionaly stable fiber in RdB-1L-VSVG was confirmed by immunoblot analysis.

RdB-1L-VSVG shows a remarkable improvement in cytotoxicity and total viral yield in cancer cells. RdB-1L-VSVG demonstrates enhanced cytotoxicity in cancer cells with subdued CAR-expression as it can be internalized by an alternate pathway. Competition assays with a CAR-specific antibody (Ab) or VSVG receptor, phosphatidyl serine (PS), reveals that cell internalization of RdB-1L-VSVG is mediated by both CAR and PS. Furthermore, treatment with RdB-1L-VSVG significantly enhanced anti-tumor effect in vivo. These studies demonstrate that the strategy to expand oncolytic Ad tropism may significantly improve therapeutic profile for cancer treatment.

117. Development of Novel Cancer Therapy Combination of Ad-SOCS Gene Therapy and LAK Cell Immunotherapy for the Treatment of Prostate Cancer

Hiroki Saito[1], Koichi Kitagawa[1], Tetsuji Naka[2], Satoshi Serada[2], Toshiro Shirakawa[1]
[1]Division of Translational Research for Biologics, Kobe University, Kobe, Japan, [2]Laboratory of Immune Signal, National Institute of Biomedical Innovation, Health and Nutrition, Osaka, Japan

Background Suppressor of cytokine signaling 3 (SOCS3) is a promising molecule for cancer gene therapy. SOCS3 suppresses tumor growth through inhibition of mutiple signaling pathways including Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK), and focal adhesion kinase (FAK). The constitutive activation of JAK/STAT3 pathway is known to be involved in oncogenesis of various types of cancers including prostate cancer. We investigated the anti-tumor activity of Ad-SOCS, which is the recombinant adenovirus vectors carrying the SOCS3 gene, against prostate cancer cell line *in vitro*. Furthermore, to improve the antitumor effect against prostate cancer, we combined LAK (Lymphokine activated killer) cell immunotherapy with Ad-SOCS gene therapy. **Materials and Methods** LNCaP, human prostate cancer cell line was used in this study. The cytotoxicity of Ad-SOCS against LNCaP was measured by XTT assay. LAK cells were generated from PBMCs isolated from fresh blood by Ficoll-paque (GE Healthcare, Munich, Germany) density gradient centrifugation. PBMC were incubated in medium with interleukin (IL)-2 and anti CD3 antibody (OKT3) to generate LAK cells. The cytotoxicity of LAK cells against LNCaP cells infected with Ad-SOCS was investigated by the *in vitro* cytotoxicity assay. Expression level of SOCS3, STAT3 and ULBPs were measured by real-time PCR. **Results** Ad-SOCS showed the significantly higher cell growth inhibitory effect compared to Ad-LacZ and PBS controls in LNCaP cells. In real-time PCR, Ad-SOCS significantly increased the level of SOCS3 mRNA expression. Sequentially, the expression of STAT3 mRNA was decreased by Ad-SOCS infection. Upregulation of SOCS3 mRNA and downregulation of STAT3 mRNA were not observed in both Ad-LacZ and PBS infected LNCaP cells. In cytotoxicity assay, LAK cells exhibited the significantly higher cytotoxicity against LNCaP cells infected with Ad-SOCS at all effector : target cell ratios, 1:1,5:1, 10:1, compared with that of non-infected or Ad-LacZ infected cells. The mRNA expression levels of ULBP 1-5, which are NKG2D ligands to stimulate LAK cell activities, were measured and the mRNA expressions of ULBP 1, 3 and 4, in LNCaP infected with Ad-SOCS were significantly increased compared to the treatments. **Conclusion** In the present study, we demonstrated that Ad-SOCS could inhibit the cell growth of LNCaP cells and increased the sensitivity of LAK cell *in vitro*. These findings suggested that the Ad-SOCS and LAK cell combination therapy warranted the further development of novel therapeutic approach for advanced prostate cancer.

Targeted Genome Editing I

118. Characterization of Chromosomal Alterations Using a Zinc-Finger Nuclease Targeting the Beta-Globin Gene Locus in Hematopoietic Stem/Progenitor Cells

Joseph Long[1], Megan D. Hoban[1], Caroline Y. Kuo[2], Beatriz Campo Fernandez[1], Aaron R. Cooper[3], Dianne Lumaquin[1], Roger P. Hollis[1], Donald B. Kohn[1], Zulema Romero[1]
[1]Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, Los Angeles, CA, [2]Pediatrics, Division of Allergy and Immunology, University of California, Los Angeles, Los Angeles, CA, [3]Molecular Biology Interdepartmental Ph.D. Program, University of California, Los Angeles, Los Angeles, CA

The use of engineered nucleases for targeted gene correction of the sickle cell disease (SCD) mutation in hematopoietic stem and progenitor cells (HSPCs) combined with a homologous DNA donor template can result in successful targeted gene correction. However, due to a high sequence homology existing between the beta- and delta-globin genes, off-target endonuclease cleavage events can occur when using endonucleases targeted to beta-globin. Moreover, the introduction of multiple double stranded breaks by site-specific endonucleases has the potential to induce chromosomal alterations such as translocations, deletions, and inversions. This work focused on the use of a novel Droplet Digital PCR (ddPCR) assay to characterize the frequency of deletions, inversions and translocations between the beta- and delta-globin paralogs when delivering nucleases that can cleave at beta- and show off-target activity at the delta-globin locus in HSPCs. The impact of co-delivering these nucleases with or without a homology-directed repair (HDR) donor template (as an integrase defective lentiviral vector [IDLV] or as a single stranded oligonucleotide) was also assessed. Bone marrow (BM) $CD34^+$ cells from a SCD patient were treated with ZFNs with high levels of site-specific cleavage activity at both the beta- and delta-globin loci (32-44% indels at beta-globin and 2-12% indels at delta-globin by CEL1 assay). Samples treated with nuclease-only, nuclease+Oligo and nuclease+IDLV respectively, showed that the different events were present at the following frequencies: 41.3%, 45.3% and 50.2% for the deletion; 9.7%, 6.5% and 8.8% for the inversion; and 0.6%, 0.5% and 0.2% for the translocations involving beta- and delta-globin. The same analysis performed in colony forming units (CFUs), derived from the SCD BM $CD34^+$ cells treated with nuclease or nuclease with HDR Donor, showed that the deletion was the most frequent event, followed by the inversion, with the translocation least frequent, mirroring what was observed in the $CD34^+$ pooled populations. Thus, for each event, the frequency was independent of the presence of oligo or IDLV donor template, in both the pooled and the clonal populations. Additionally, the ddPCR analysis of CFUs gave insight into the mono-allelic *vs*. bi-allelic nature of each rearrangement event, and thus the mono-allelic *vs*. bi-allelic modification frequency of the nuclease-driven editing. Analysis of the nuclease only treated CFUs showed that 30% of deletion events were bi-allelic; while the majority of inversions and translocations were mono-allelic. These results confirmed that due to the high sequence homology existing between the target locus in beta-globin and the off-target locus in delta-globin, there was a significant frequency of chromosomal alterations induced by cleaving both paralogs in the same human HSPCs. This may partly reflect the unique chromosomal conformation of the globin genes due to their co-regulation in hematopoiesis, and looping to the same cis-regulatory element. More generally, these findings demonstrate the need to develop site-specific endonucleases with high specificity to avoid unwanted chromosomal alterations.

119. Engineering a Self-Inactivating CRISPR System for AAV Vectors

Benjamin E. Epstein, David V. Schaffer
Bioengineering, University of California, Berkeley, Berkeley, CA

Significant strides in nuclease engineering have enabled a broad range of biomedical applications. However, numerous challenges remain, and in particular the long term expression mediated by AAV delivery of nuclease-encoding constructs to post-mitotic cells raises concerns with specificity and immunogenicity. That is, off-target nuclease activity can induce genotoxicity, and expression of an exogenous nuclease has the potential to elicit an immune response against transduced cells. Thus, it would be advantageous to limit the duration of nuclease expression following delivery.

We have engineered self-inactivating nuclease constructs using the CRISPR/Cas9 system, which consist of a Cas9 nuclease such as that from *Streptococcus pyogenes* (SpCas9), a chimeric single guide RNA (sgRNA) molecule for targeting, and flanking sites targeted by that sgRNA. For example, we modified an SpCas9/sgRNA construct targeted at the VEGFA locus by introducing copies of the target site flanking the nuclease construct. The result is a negative feedback loop where Cas9 cuts both the target genomic locus and its own coding construct and thereby self-limits its expression. We demonstrate that this construct can eliminate >90% of its expression within 72 hours, and tuning of different parameters enables retention of up to 65% on-target efficiency and reduction of off target-cutting by up to 80%. We further show that by engineering the flanking target sites to contain mismatches to the sgRNA, the Cas9 expression duration can be modulated and the on-target/off-target cutting ratios improved.

By retaining strong activity while minimizing off-target effects and by eliminating potentially immunogenic, long-term expression of foreign protein, these self-inactivating constructs have the potential to address two substantial concerns with therapeutic application of engineered nucleases.

120. Efficient *In Vivo* Liver-Directed Gene Editing Using CRISPR/Cas9

Kshitiz Singh, Hanneke Evens, Melvin Rincón, Nisha Nair, Shilpita Sarcar, Ermira Samara-Kuko, Marinee K. Chuah, Thierry VandenDriessche
Department of Gene Therapy & Regenerative Medicine, Free University of Brussels, Brussels, Belgium

Clustered, regularly interspersed, short palindromic repeat RNA-guided nucleases have emerged as highly efficient genome editing tools. However, *in vivo* tissue-specific genome editing at the desired loci is still a challenge. Here, we report that truncated guide RNAs (gRNAs) and Cas9 under the control of a computationally designed hepatocyte-specific promoter lead to liver-specific and target site-specific indel formation in the mouse *factor IX (FIX)* gene. The truncated gRNAs targeting unique sites in exon 1 and exon 6 of the mouse *FIX gene* were designed using a computational CRISPR design tool and the target sites overlap with mutations known to cause hemophilia B in patients. The gRNA and Cas9-expressing constructs were delivered *in vivo* using AAV9 vectors. The efficiency of *in vivo* targeting was assessed by T7E1 assays, site-specific Sanger sequencing and deep sequencing of on-target and putative off-target sites. Though AAV9 transduction was apparent in multiple tissues and organs, Cas9 expression was restricted mainly to the liver, with only minimal or no expression in other non-hepatic tissues. Consequently, the indel frequency was robust in the liver (up to 50%) in the desired target locus of the FIX gene, with no evidence of targeting in other organs. This resulted in a substantial loss of FIX activity and the emergence of a bleeding phenotype, consistent with hemophilia B. Deep sequencing of putative off-target sites revealed no off-target editing. Our findings have potentially broad implications for somatic gene editing in the liver using the CRISPR/Cas9 platform.

121. T Cell Receptor Modification by Highly Specific TALEN and CRISPR/Cas9

Friederike Knipping[1], Karl Petri[1], Eliana Ruggiero[1], Mark Osborn[2], Jakub Tolar[2], Manfred Schmidt[1], Christof von Kalle[1], Richard Gabriel[1]
[1]Department of Translational Oncology, German Cancer Research Center and National Center for Tumor Diseases, Heidelberg, Germany, [2]Department of Pediatrics, University of Minnesota, Minneapolis, MN

Adoptive transfer of T cells with transgenic high avidity T cell receptors (TCR) is a promising therapeutic approach, however it comprises certain challenges. Endogenous and transferred TCR chains compete for surface expression and may pair inappropriately, potentially leading to autoimmunity. This can be prevented by designer nucleases such as transcription activator-like effector nucleases (TALEN) and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) system. Their ability to introduce specific DNA double strand breaks (DSB) at their target sites can be harnessed for targeted genome editing. DSB repair through non-homologous end joining (NHEJ) can result in permanent gene knockout due to frame shift mutations. Alternatively, if a homology-containing donor template is provided, transgenes can be integrated into the specific target locus by homology directed repair (HDR). In order to disrupt endogenous TCR expression, we assembled nine TALEN and two CRISPR/Cas9 guide RNA (gRNA) targeting the constant region of the TCR α-chain (*TRAC*) and four TALEN and three CRISPR/Cas9 gRNA targeting both constant regions of the TCR β-chain (*TRBC1*/*TRBC2*). Here we show specific DSB induction by TALEN and CRISPR/Cas9 in K562 and primary T cells using T7 endonuclease I targeting efficiency assay and deep sequencing. Electroporation of primary T cells with *TRAC*-TALEN or *TRBC*-TALEN mRNA led to successful elimination of surface TCR expression in about 76% of the cells. To analyze nuclease specificity, K562 cells were transduced with an integrase-defective lentiviral vector (IDLV) prior to nucleofection with TALEN- or CRISPR/Cas9-expressing plasmids. IDLV can be integrated into DSB during DNA repair, thereby serving as stable markers for transient DSB. IDLV-marked DSB were subsequently localized using LAM-PCR and deep sequencing. Clustered integration sites (CLIS) were detected around all nuclease target sites, confirming on-target activity. Although up to 3268 IDLV integration sites were analyzed for each TALEN and CRISPR/Cas9 gRNA, only one CLIS for one gRNA was mapped at an off-target position, indicating a very high level of specificity. To establish HDR-mediated targeted integration, we assembled donor templates containing a GFP expression cassette flanked by 800bp *TRAC*- or *TRBC1*-homologous sequences. We verified targeted gene addition in 10% of K562 cells treated with TALEN and the respective donor template. Delivery of the *TRAC*-donor packaged into IDLV resulted in targeted integration of the GFP expression cassette in 5% of primary T cells. To sum up, here we present highly efficient and specific TALEN and CRISPR/Cas9 and their utility for T cell engineering.

122. Seamless Correction by Donor DNA of a Class I CFTR Mutation Facilitated by a Double Nicking CRISPR/Cas9 in CF-iPSCs

Shingo Suzuki[1], Keisuke Chosa[1,2], Albert Lee[1], Michael Yezzi[1], Hirofumi Kai[2], Dieter Gruenert[1]
[1]*University of California, San Francisco, San Francisco, CA,* [2]*Kumamto University, Kumamoto, Japan*

Development of effective gene modification via Homology Directed Repair (HDR) as well as induced pluripotent stem cells (iPSCs) have been significant advances in development of gene- and cell-based therapies for inherited diseases. Patient-specific iPSCs with a corrected disease causing mutation have the potential to functionally repair damaged tissues and organs. Cystic fibrosis (CF) is the most common inherited disease in the Caucasian population with significant multiorgan damage, caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene. We and others have shown seamless gene correction of the *F508delCFTR mutation*, the most common mutation found in CF patients and classified as Class II *CFTR* mutation, in CF-iPSCs. Correction of the *F508del* mutation by small/short DNA fragments (SDFs) was enhanced by sequence-specific transcription activator-like effector endonucleases (TALENs). Given that there are > 2,000 mutations associated with CF, the approach used above makes it possible to develop personalized, mutation specific gene and cell therapeutic strategies for CF patients. The studies here describe the generation of iPSCs from a CF patient homozygous for the *W1282X*, Class I, *CFTR* mutation and its correction via HDR. As an alternative to using an SDF/TALEN based system described above, the studies described here use a drug selection-based *wtCFTR* PiggyBac donor DNA system with clustered randomly interspersed short palindromic repeat (CRISPR)/Cas9 nickases targeting *CFTR* exon23 (the site of the W1282X mutation). CF W1282X/W1282X patient-derived fibroblasts were retrovirally reprogramed with Yamanaka factors (Oct4/Sox2/Klf4/c-Myc) into CF-iPSCs. To correct the W1282X mutation in the CF-iPSCs, a donor DNA comprising the *wtCFTR* genomic DNA region including exon23, and a CAG-puroΔTK cassette flanked by PiggyBac repeats ending in the TTAA sequence also found in intron 23 of genomic *CFTR*. Several pairs of CRISPR/Cas9 nickase were also designed to cleave at specific sites adjacent to the W1282X locus. These nickases were assayed for optimal targeting efficiency. The pair that most effectively introduced nicks in double strand DNA were co-transfected with the donor DNA in CF-iPSC. Candidate corrected clones were selected with puromycin, and then assayed by PCR with one primer inside of PiggyBac cassette and the other outside the homology arms in donor DNA 5' and 3' of the PiggyBac Cassette. The donor DNA alone did not catalyze site-directed HDR in clones exhibiting puromycin resistance, however, co-transfection of the donor DNA with the optimized CRISPR/Cas9n pairs gave a targeting efficiency of 4.76% (1/21) and 36.6% (15/41) (colonies with HDR/ puromycin resistant colonies) in independent experiments. The drug-resistance cassette in the clones was excised by overexpressing PiggyBac transposase resulting in seamless correction of the CF-iPSCs. This study was supported by grants from Pennsylvania Cystic Fibrosis, Inc, and Cystic Fibrosis Research, Inc, and PPG DK 088760.

123. Gene Editing as a Therapeutic Approach to Treat IPEX Syndrome

Marianne Goodwin[1], Francesca Santoni de Sio[2], Daniel Dever[1], Matthew Porteus[1], Maria Grazia Roncarolo[1], Rosa Bacchetta[1]
[1]*Division of Stem Cell Transplantation and Regenerative Medicine, Department of Pediatrics, Stanford University, Stanford, CA,* [2]*San Raffaele Telethon Institute for Gene Therapy, Milan, Italy*

Site-specific gene editing combined with autologous stem cell transplantation holds tremendous potential to treat both inherited and acquired diseases. However, the translational applications of gene editing are limited by the challenge of achieving sufficient levels of gene targeting in human primary cells. Recent advances in gene editing, including use of the CRISPR-Cas9 system, nuclease-resistant chemically modified small guide RNAs (sgRNAs), and serotype-optimized AAV delivery of donor repair DNA templates, are enabling clinically relevant levels of gene editing in human primary cells. Here, we build upon these advances to develop a gene editing strategy to treat the immune-dysregulation polyendocrinopathy-enteropathy-X-linked (IPEX) syndrome, a severe primary immune deficiency manifesting with life-threatening multi-organ autoimmunity in children. IPEX is caused by mutations in the forkhead box protein 3 gene (*FOXP3*), resulting in dysfunction of T regulatory cells (Tregs) and T effector cell lymphoproliferation. Due to the presence of disease causative mutations throughout the entire *FOXP3* gene and the complex nature of *FOXP3* regulatory elements, we designed a strategy to functionally correct the gene in IPEX patient cells using on-target, homology directed repair (HDR)-mediated insertion of the *FOXP3* coding sequence into the gene locus. By targeting the *FOXP3* gene using a CRISPR system comprised of Cas9 mRNA and chemically modified sgRNAs, we were able to attain high targeting frequencies, reaching 70-80% in human primary CD4+ T cells. We use this CRISPR system in combination with a repair donor DNA template delivered with AAV to demonstrate *FOXP3* gene correction in human CD34+ hematopoietic stem and progenitor cells (HSPCs). In addition to gene correction of *FOXP3*, we use a similar strategy in parallel to knockout *FOXP3* in wild-type human CD34+ HSPCs. We use the knockout HSPCs to generate a humanized mouse model of *FOXP3* dysfunction, which enables us to study the pathophysiology of IPEX and to ultimately test the efficacy of gene-corrected Treg cell-based therapies. These results will help demonstrate the feasibility of *FOXP3* gene editing, which we propose for the translational application of autologous transplant of Tregs and HSPCs as a therapy for IPEX syndrome.

124. Therapeutic Correction of an LCA-Causing Splice Defect in the CEP290 Gene by CRISPR/Cas-Mediated Gene Editing

Morgan L. Maeder, Rina Mepani, Sebastian Gloskowski, Maxwell Skor, McKensie Collins, Shen Shen, Gregory Gotta, Eugenio Marco, Luis Barrera, Hari Jayaram, David Bumcrot
Editas Medicine, Cambridge, MA

Leber congenital amaurosis (LCA) comprises a genetically heterogeneous group of early-onset retinal disorders characterized by severe loss of vision in the first years of life. Approximately 20% of LCA patients harbor mutations in the *CEP290* gene, which exceeds the packaging limit of AAV and is therefore not amenable to traditional gene therapy. Here, we demonstrate a gene editing approach in which the CRISPR/Cas9 system is used to modify the endogenous *CEP290* locus and restore normal function of the gene.

The most common mutation in *CEP290* is the IVS26 c.2991+1655 AtoG mutation. This point mutation in intron 26 of the gene generates a novel splice donor, resulting in aberrant splicing and the presence of a premature stop codon. Using the *S. aureus* CRISPR/Cas9 system, we employed a dual-cut approach in which two gRNAs are used to excise the mutation-containing region in primary fibroblasts derived LCA10 patients. Upon gene editing, these cells show correction of the splicing defect as evidenced by a two-fold increase in expression of wildtype *CEP290* transcript and a concomitant two-fold decrease in levels of the mutant RNA species. Additionally, Western blotting shows increased expression levels of full-length CEP290 protein.

Using targeted amplicon sequencing of computationally predicted sites, as well as the minimally biased GUIDE-Seq method, we have performed broad specificity profiling, allowing us to narrow down

the list of top gRNA candidates. The use of the S. aureus CRISPR/Cas9 system enables efficient packaging of the Cas9 gene, as well as two gRNA genes, into a single AAV vector and provides a method for therapeutic delivery of this system into patient photoreceptors.

125. Purging Latent HIV Infection by CRISPR-Mediated Viral Reactivation

Prajit Limsirichai[1], Thomas Gaj[2], David V. Schaffer[3]
[1]Department of Plant and Microbial Biology, University of California, Berkeley, Berkeley, CA, [2]Department of Chemical and Biomolecular Engineering, University of California, Berkeley, Berkeley, CA, [3]Departments of Chemical and Biomolecular Engineering, Bioengineering, Molecular & Cell Biology, University of California, Berkeley, Berkeley, CA

Complete eradication of HIV-1 infection is impeded by the existence of cells that harbor chromosomally integrated but transcriptionally inactive provirus. These latently-infected cells can persist for years without producing viral progeny, rendering them refractory to immune surveillance and antiretroviral therapy and providing a permanent reservoir for the stochastic reactivation and reseeding of HIV-1. Strategies for purging this latent reservoir are thus needed to eradicate infection. Here we show that engineered transcriptional activation systems based on CRISPR/Cas9 can be harnessed to activate HIV-1 expression in cell line models of latency. We utilized two distinct CRISPR transcriptional activation systems, dCas9-VP64 and the synergistic activation mediator (SAM) complex, to target numerous sites across the HIV-1 long terminal repeat (LTR) promoter and observed robust expression from the full-length HIV-1 promoter in multiple cell line models of HIV-1 latency. We further demonstrated that complementing Cas9 activators with latency-reversing compounds can enhance latent HIV-1 transcription and that epigenome modulation using CRISPR-based acetyltransferases could also promote viral gene activation. Finally, we showed that latent HIV-1 expression could also be stimulated by CRISPR-mediated activation of endogenous factors not previously implicated in HIV-1 pathogenesis but whose expression could nonetheless reactivate viral gene expression. Collectively, these results demonstrate that CRISPR systems are potentially effective tools for inducing latent HIV-1 expression and that their use, in combination with antiretroviral therapy, could lead to improved therapies for HIV-1 infection.

126. Identification of Human Papillomavirus Entry Receptors with CRISPR-Cas9 Library

Xinping Fu, Lihua Tao, Aiwu Jin, Xiaoliu Zhang
Center for Cell Receptors and Cell Signaling, Department of Biology & Biochemistry, University of Houston, Houston, TX

Human papillomaviruses (HPVs) are a family of small nonenveloped viruses that induce mostly benign papillomas. However, infection of several high-risk HPV types, most prominently HPV 16 and 18, can cause cervical cancer and other epithelial tumors. The cell biology of HPV entry is still a subject of scientific debate, and in particular, the cellular receptors for HPV infection have not been firmly determined. The present study was designed to use CRISPR-Cas9 gene knock-out library to identify cellular proteins that may be used by HPV for entry. HEK293 cells were initially transduced with CRISPR-Cas9 knockout screening libraries, and then selected with puromycin for two weeks. The cells were then transduced with HPV-16 pseudoviruses containing the GFP marker gene, and GFP-negative cells were subsequently selected by flow cytometry. This process was repeated multiple rounds to enrich the GFP^- cell population, and the percentage of GFP^+ cells went down from over 90% to less than 10% during this enrichment process. A combination of PCR and DNA sequencing was then used to determine the genes that had been knocked out from this cell population. Our data show that solute carrier family 35 (adenosine 3'-phospho 5'-phosphosulfate transporter) member B2 (SLC35B2), malectin (MLEC), xylosylprotein beta 1,4-galactosyltransferase and transmembrane protein 8C (TMEM8C) were the most frequently knocked out genes in these cells. We then validated the involvement of one of these genes, SLC35B2 in HPV entry, by either deleting genes from fresh HEK293 cells or by reintroducing the gene back to the cells that had it knocked out. Interestingly, SLC35B2 knockout was found to affect entry of some other viruses such as herpes simplex viruses. In conclusions, CRISPR-Cas9 knockout screening system is a useful tool for identifying cellular receptors for viruses such as HPV, in which the entry mechanism has not been fully elucidated.

127. Lentiviral Protein Transduction for Tailored Genome Editing and Site-Directed Gene Insertion

Yujia Cai, Rasmus O. Bak, Anders Laustsen, Yan Zhou, Chenglong Sun, Yonglun Luo, Martin R. Jakobsen, Jacob G. Mikkelsen
Department of Biomedicine, Aarhus University, Aarhus, Denmark

Therapeutic use of site-directed endonucleases relies on safe and effective cellular delivery, preferentially resulting in short-term enzymatic activity. Based on the packaging of Gag/GagPol-fused heterologous proteins into VSV-G-pseudotyped lentivirus-derived particles, we have established lentiviral protein transduction for delivery of DNA transposases and custom-made endonucleases. Up to 24% of targeted *CCR5* and *AAVS1* alleles were disrupted in primary cells, including normal human dermal fibroblasts and primary keratinocytes, exposed to lentiviral particles loaded with zinc-finger nucleases (ZFNs). By exposing human 293 cells to 'all-in-one' integrase-defective lentiviral vectors (IDLVs) containing a complete gene repair kit consisting of ZFNs and viral RNA carrying the donor sequence for homology-directed repair, correction of genomic mutations was obtained in more than 8% of treated cells. As shown by confocal microscopy, ZFN proteins were abundant within transduced cells one hour after initial virus exposure, but were short-lived and gone after 24 hours. In accordance, under conditions supporting comparable *CCR5* indel rates, disruption of the nearby *CCR2* off-target site was reduced by lentiviral delivery of ZFNs targeting *CCR5* relative to a conventional transfection-based approach. As biased and uncontrolled integration into genes remains a key challenge for gene therapies based on lentiviral vector technologies, we engineered ZFN-loaded IDLVs with the capacity to insert transgenes into the human *CCR5* and *AAVS1* loci by a homology-driven mechanism. Targeted gene integration into safe genomic loci was observed in human cell lines (85% of analyzed clones) and in human stem cells, including CD34+ hematopoietic progenitors and induced pluripotent stem cells (iPSCs). Notably, targeted transgene insertion into safe harbors was identified in all of 23 analyzed iPSC clones. Altogether, our findings generate a new platform for targeted genome engineering based on lentiviral delivery of complete gene repair or gene insertion kits.

128. Targeting a High-Expression FVIII Transgene to Exogenous Locations in the Genome without Disrupting Endogenous Gene Expression

Jennifer M. Johnston, Matthew Porteus
Pediatrics, Stanford University, Stanford, CA

The technology that is now available for targeted genome editing consists of artificial nucleases that create double stranded breaks at specific genomic locations that can then be corrected by intrinsic cellular repair processes. Genome editing can be achieved by exploiting the repair mechanism of homologous recombination by providing a donor vector containing the gene(s) of interest flanked by sequences homologous to the site of the double strand break. To date, gene addition in this manner is targeted to defined inert

safe harbor loci. We developed an innovative targeting strategy in which homologous recombination could be utilized to target a transcriptionally active genomic locus without disrupting expression of the endogenous gene. In this manner, essentially any location in the genome becomes a potential safe harbor for homologous recombination-mediated genome editing. One advantage to this strategy is that it allows for transgene expression to be under the precise control of endogenous regulatory elements, rather than using heterologous promoters. Therefore, the optimal locus with the most advantageous regulatory elements to drive transgene expression can be selected for gene addition. Pilot experiments confirmed the expression of exogenous reporter genes (such as the green fluorescent protein or the more therapeutically relevant biologically inert surface selectable marker delta NGFR) from either an exogenous ubiquitous promoter or from the exogenous promoter due to the addition of a T2A element incorporated downstream of the endogenous gene. Notably, expression of the endogenous gene was confirmed to be unaltered. These experiments were performed at three separate genomic locations. To demonstrate the feasibility of this strategy therapeutically however, a high-expression FVIII chimeric transgene (HPFVIII) was incorporated into two distinctly active genomic loci: the IL2R gamma chain locus and the adenosine deaminase locus. The donor vectors utilized contained the cDNA for the endogenous gene locus (either the IL2R gamma chain gene or the adenosine deaminase gene) followed by a ubiquitous promoter intended to drive the expression of HPFVIII, all of which was flanked by one kilobase arms of homology. Both the myeloid erythroid-leukemic K562 cell line and the acute T cell leukemic Jurkat cell line were modified upon nucleofection and confirmed to be targeted by sequencing. Single cell clones were produced and assessed for HPFVIII expression by a one-stage APTT based coagulation assay. Clones were found to express therapeutic levels of FVIII from both loci in both cell types (1 Unit / 2 million cells / 24 hrs). These experiments set the stage for targeting of HPFVIII into hematopoietic stem and progenitor CD34+ cells for the purpose of treating Hemophilia A.

129. Does Transcription Influence AAV-Mediated Homologous Recombination?

Laura P. Spector[1], Mark A. Kay[2]
[1]Department of Genetics, Stanford University, Stanford, CA, [2]Departments of Pediatrics & Genetics, Stanford University, Stanford, CA

Recombinant adeno-associated viral (AAV) vectors constitute one of the most promising tools for gene transfer. While the majority of AAV transduction events are episomal, our laboratory recently exploited the vector's ability to induce homologous recombination (HR) to design promoterless vectors that utilize chromosomal homology arms flanking a ribosomal skipping P2A-therapeutic coding sequence construct to integrate sequences just upstream of the stop codon of an endogenous gene. When targeting the albumin gene in the liver, a chimeric mRNA transcript capable of producing both albumin and a second therapeutic protein is consequently created. Not only do these vectors offer the permanence of gene transfer associated with integration, but a vector lacking a promoter reduces the chance for oncogene activation from off-target vector integration. AAV-mediated HR appears to be more efficient when targeting transcriptionally active loci, yet it is unclear if transcription itself or other factors that secondarily influence transcription, such as chromatin state, are directly linked to AAV-mediated HR. We therefore set out to establish if the transcriptional rate in particular is a major determinant for this type of HR.

To do this, we developed a strategy to quantify precision AAV-mediated transgene integration by exploiting an engineered locus whose transcriptional rates could be controlled by drug administration. To this end, we used lentiviral vectors to generate a series of clonal HeLa cell populations each harboring a single-copy, doxycycline-inducible genomic site expressing the human alpha 1-antitrypsin (hAAT) cDNA. Upon induction with doxycycline, transgene expression from this site increases by greater than 1,000-fold. Each clonal cell population was infected with an AAV serotype DJ vector containing homology arms to the integrated hAAT cDNA and a P2A- codon-optimized human coagulation factor IX (hFIX) cDNA or P2A-eGFP construct that would allow for multicistronic genomic expression of both peptides from a single mRNA only if HR occurred. Transgene hFIX or eGFP expression as a measure of HR is established by quantitative RT-PCR and by transgene protein measurements. By modulating the rate of transcription just prior to rAAV vector administration, we can establish if transcription from a given genomic locus influences the frequency of AAV-mediated HR. Our preliminary results suggest that transcription per se might not be the predominant factor influencing the rate of HR. Further studies will provide more insight into the mechanism of gene targeting by AAV, optimal target site selection, and potentially expand the use of AAV-mediated gene targeting for treating various genetic and acquired diseases.

130. Purification of Large Scale mRNA Encoding ZFN Nucleases by dHPLC Technology

Erica Giuliani[1], Claudia Piovan[1], Sergio Bossi[1], Stefano Corna[1], Cinzia Scavullo[1], Monika Pema[1], Tiziano Di Tomaso[2], Pietro Genovese[2], Angelo Lombardo[2], Luigi Naldini[2], Claudio Bordignon[1], Gian Paolo Rizzardi[1], Chiara Bovolenta[1]
[1]MolMed S.p.A., Milan, Italy, [2]Telethon Institute for Gene Therapy (TIGET), San Raffaele Scientific Institute, IRCCS Ospedale San Raffaele, Milan, Italy

A novel strategy of targeted gene correction of the interleukin-2 receptor common gamma chain (IL2RG) gene for the treatment of X-linked Severe Combined Immunodeficiency (SCID-X1) is achieved by the combination of a pair of IL2RG-specific Zinc Finger Nucleases (ZFN) and the correct-gene template DNA delivered by integration-defective lentiviral vector (IDLV).The transient expression of the ZFN pair targeting the disease-causing gene is obtained by the electroporation of the two corresponding mRNAs, produced by *in vitro* transcription starting from plasmid DNA template.A major limitation of the mRNA transcribed *in vitro* is the presence of residual contaminants such as short RNAs and double stranded (ds)RNAs that may affect the function and spectrophotometric quantification of the product hampering therefore the delivery of high quality and precise amount of mRNA to target cells. Moreover, dsRNA contaminants represent a possible risk in terms of immunogenicity of the product, leading to activation of unwanted innate immune response with consequent reduction/abrogation of mRNA translation as well as potential alteration of the properties of the transfected cells. To improve nuclease expression while decreasing cellular innate response to mRNA transfection we combined different strategies: (i) inclusion of UTRs and polyA tails in the DNA template used for mRNA production; (ii) use of modified nucleotides during mRNA production and (iii) purification of the mRNAs by dHPLC with a reverse phase column made of non-porous matrix consisting of polystyrene-divinylbenzene copolymer beads alkylated with C-18 chains (Transgenomic, LTD.). In particular, the purification of *in vitro* transcribed mRNAs by means of dHPLC has been shown to strongly improve the translation of mRNA and significantly reduce the contaminant presence thus preventing innate immunity and eventually increasing modified cells persistence *in vivo*.We have developed feasible and reproducible, small and large scale mRNA production and downstream purification processes of the ZFN pairs obtaining accurate RNA quantification and reduced risk of immunogenicity. The full process achieved a 60% yield, loading with a 500µg RNA for each run with a single clean chromatographic peak. Furthermore, the level of residual organic solvent (i.e. Acetonitrile) used in the

purification process is compatible with that applicable into clinic. The highly translatable non-immunogenic dHPLC-purified mRNA can be delivered without toxicity and represents a powerful and safe tool for the application of gene therapy protocols.

131. Chromatin-Dependent Loci Accessibility Affects CRISPR-Cas9 Targeting Efficiency

Ciaran M. Lee, Timothy H. Davis, Harshavardhan Deshmukh, Gang Bao
Bioengineering, Rice University, Houston, TX

The CRISPR-Cas9 system from *Streptococcus pyogenes* has been successfully modified to target specific genomic loci in a wide range of organisms, making it a powerful tool for biotechnology and personalized medicine. However, there are several challenges to be addressed before successful application of CRISPR-Cas9 systems for clinical use. Specifically, significant concerns have been raised over off-target effects that may lead to aberrant cellular function, and the efficiency of gRNAs varies widely between target loci. The factors influencing the targeting efficiency of a particular gRNA design are not fully understood. Although several groups have developed algorithms that attempt to identify highly active gRNAs based on the sequence composition of the gRNA, the predicted scores for gRNA designs do not correlate with observed activity in cells, suggesting that factors other than gRNA sequence are at play. Here, we demonstrate that the underlying genomic context, especially the chromatin state of the target locus influences the cleavage efficiency of CRIPSR-Cas9 in a sequence independent manner. Furthermore, we demonstrate that knowledge of the chromatin state may inform the selection of highly active gRNAs, and reveal the specific chromatin marks associated with CRISPR-Cas9 target accessibility. Finally, we postulate that *in silico* tools that consider both gRNA sequence and chromatin state of the target locus will have greater predictive power and facilitate the selection of highly active gRNAs for therapeutic applications.

132. Targeting the *BTK* Locus in Primary Human Hematopoietic Cells with TALENs and AAV Donor Template

Courtnee Clough, Yupeng Wang, Iram F. Khan, Swati Singh, King Hung, David J. Rawlings
Center for Immunity and Immunotherapies and Program for Cell and Gene Therapies, Seattle Children's Research Institute, Seattle, WA

X-linked agammaglobulinemia (XLA), a rare immunodeficiency, is caused by mutations in the Bruton's Tyrosine Kinase (*BTK*) gene. BTK is required for B-cell maturation, activation and BCR-mediated signaling. Patients lacking functional BTK have predominantly immature B-cells with near absence of mature B cells, minimal antibody production and are prone to recurrent and life-threatening bacterial infections. Available treatments are limited to intravenous immunoglobulin therapy, which lessens the severity of these infections and judicious use of antibiotic therapy. *BTK* is an ideal target for gene editing as BTK-expressing B cells exhibit a marked selective advantage in vivo, and even a small number of corrected HSC or B cell progenitors rescues B cell development and function in murine models and is expected to rescue the B cell compartment in XLA patients. Here we outline a strategy to edit the endogenous *BTK* locus in primary human cells, targeting a codon-optimized BTK cDNA into the first coding exon of *BTK* using *BTK* specific DNA nucleases and donor template. Four pairs of transcription activator-like effector nucleases (TALENs) cleaving within introns 1 or 2 of *BTK* were constructed and their cleavage efficiency in primary human T cells evaluated. Using the T7 Endonuclease assay, we determined that disruption (indel) rates of ~75% were obtained using the TALENs. Next, utilizing co-delivery of TALEN mRNA and an AAV donor template (consisting of an MND promoter-GFP cDNA expression cassette flanked by 1.0 kb *BTK* homology arms), we achieved up to 25% homology directed repair (HDR) in primary T cells. The translation of this gene editing strategy to human mobilized peripheral $CD34^+$ cells is currently being optimized. If successful, this targeting approach will be used to test expression levels of the targeted cDNA as regulated by the endogenous *BTK* promoter.

133. Highly Efficient Homology-Driven Genome Editing in Human T Cells with Combined Zinc-Finger Nuclease mRNA and AAV6 Donor Delivery and Improved Efficiency Under Serum-Free Conditions

Jianbin Wang, Joshua J. DeClercq, Samuel B. Hayward, Patrick Wai-Lun Li, David A. Shivak, Gary Lee, Michael C. Holmes
Sangamo BioSciences Inc., Richmond, CA

Immunotherapy using gene-modified T cells for adoptive cell transfer is a rapidly expanding field that is currently being tested in early- and late-stage clinical studies, with recent successes seen in the treatment of hematologic malignancies using T-cell receptor or chimeric antigen receptor (CAR)-retargeted T cells. Ex vivo T cell modification can potentially further enhance the activity and safety of these cells via the insertion of the desired transgenes at a specific pre-selected site such as a safe harbor locus. We describe the development of a highly efficient method to genome edit both primary human CD8 and CD4 T cells by homology-directed repair at a pre-defined site of the genome. We have identified AAV serotype 6 as a capsid variant with high tropism for both human $CD8^+$ and $CD4^+$ T cells. Two different homology donor templates were evaluated, representing both minor gene editing events (restriction site insertion) to mimic gene correction, or the more significant insertion of a larger gene cassette. By combining this AAV6 donor delivery method with the delivery of ZFNs as mRNA, we could 'gene correct' >40% of CCR5 or 55% of *PPP1R12C* (AAVS1) alleles in CD8+ T cells. We were also able to achieve targeted insertion of a GFP transgene cassette in >40% of $CD8^+$ and $CD4^+$ T cells at both the *CCR5* and *AAVS1* safe harbor loci, potentially providing a robust genome editing tool for T cell-based immunotherapy. In addition, we found that transducing T cells with AAV6 donor and ZFN encoding mRNA under serum-free conditions resulted in a marked improvement in HDR-mediated genome editing at significantly lower vector doses (2-log lower doses of AAV6 donor), representing an essential stride in the production of clinical-scale genome edited T cells for use in adoptive T cell therapy.

134. Gene Editing Approaches for Investigating Therapy-Resistance in Soft-Tissue Sarcoma

Tim Rath, Stefan Fröhling, Claudia Scholl, Priya Chudasama, Hanno Glimm, Christof von Kalle, Ali Nowrouzi, Manfred Schmidt
National Center for Tumor Diseases and German Cancer Research Center, Heidelberg, Germany

Soft-tissue sarcoma (STS) is a rare type of tumor that accounts for approximately 1 % of adult cancers. STS tumors frequently recur or result in distant metastasis after surgical resection. Cytotoxic chemotherapy, the current "gold standard", is often confronted with development of resistance, leading to patient survival periods below 15 months. Therefore, further insight into new therapies and mechanisms leading to drug resistance in sarcoma is required to improve therapeutic success. In the present study, we analyzed drug resistance *in vitro* using (i) a genome-wide assay for identification of drug resistance-associated genetic alterations and (ii) generation of isogenic cell lines using gene editing approaches to functionally

characterize the genetic alterations. To address drug response and resistance in sarcoma, we have established a genome-wide insertional mutagenesis screening approach. To this end, a panel of STS cell lines is screened for the sensitivity towards drugs currently used for standard therapy of STS. Sensitive cell lines will be treated with transforming lentiviral vectors to identify genes involved in drug resistance. The genetic alterations potentially involved in acquired resistance mechanisms will be functionally characterized using isogenic cell lines created by gene editing approaches. Towards this end, we have successfully employed transcription activator-like effector nucleases and CRISPR/Cas9 system RNA guided endonucleases targeting the *BRAF* locus to introduce the previously described V600E mutation in 293T cells, as a "proof of concept". Analysis of the target site showed that 31 % of the sequences were positive for the mutation. The mutation was also confirmed in single cell clones by tetra-primer ARMS-PCR and western blot using an antibody specific for V600E mutated B-Raf. To test the method in a model relevant to STS, we applied gene editing to introduce a previously undescribed *KRT8* mutation found in STS patients into a sarcoma cell line. For this mutation, wild type and D10A-mutated Cas9 expressing constructs were generated to allow use of single nucleases and paired nickases. We applied single stranded oligodeoxynucleotides as donor template for *KRT8*. Using high-throughput sequencing, we detected successful recombination of the mutated *KRT8* gene locus in 0.6 % of the treated STS cells. We are using improved guide RNA and donor design as well as compounds affecting non-homologous end-joining and homologous recombination to improve efficiency of successful recombination. To summarize, we successfully edited wild-type *BRAF* to *BRAF* V600E in 293T cells and showed that gene editing of *KRT8* is possible in sarcoma cells. The methodology will be further optimized to characterize genes involved in resistance to therapy identified in the genome-wide screens. In addition, our gene editing approaches will also be used to model genetic alterations identified in STS patients by next generation sequencing. Our findings will provide interesting insights in nominating drugs for combinatorial therapy to circumvent drug resistance in STS.

135. Optimization of Dual-gRNA Lentiviral Vectors for Targeted Genomic Deletions

Giulia Pavani[1], Annalisa Lattanzi[1], Fatima Amor[1], Chiara Antoniani[2], Fulvio Mavilio[1], Annarita Miccio[2], Mario Amendola[1]
[1]Genome Editing, Genethon, Evry, France, [2]Laboratory of Chromatin and Gene Regulation During Development, Imagine Institute, Paris, France

CRISPR-Cas9 technology is a powerful tool for genome editing based therapeutic approaches. Despite its potential, the delivery of Cas9 components into primary cells, such as hematopoietic stem progenitor cells (HSPC), is still a major challenge. In addition, several applications - e.g. the use of Cas9-nickase and the generation of genomic deletions/inversions - require the delivery and expression of two different gRNA. Lentiviral vectors (LV) can efficiently transduce HSPC, but the presence and the orientation of direct repeated elements in the gRNA expression cassettes can potentially trigger recombination events that affect vector stability. To address this issue, we designed different LV encoding for two gRNA pairs, which generate deletions of different size (3 and 13 kb) in the beta-globin gene cluster. gRNA expression was driven by murine and human U6 promoters, having little sequence similarity, to avoid potential recombination events of the LV genome. To further reduce LV rearrangements and optimize expression levels, gRNA cassettes were positioned in different orientations with respect to each other (LV Inward, Tandem and Outward). We compared LV from different vector batches in HCT116 cells and we observed that Tandem configuration resulted in higher viral titers and infectivity, although this difference was not statistically significant. Analysis of proviral integrity on genomic DNA of transduced cells showed the intact dual-gRNA cassette and no sign of recombination for all the LV configurations. To understand if different orientations could affect gRNA expression (measured by RT-qPCR) and consequently deletion/inversion efficiency, we co-transduced erythroleukemic K562 cells with LV-Cas9-Blast and the three different LV. Surprisingly, LV Inward showed poor gRNA expression, negligible deletion and inversion frequency and low efficiency of InDel formation at each gRNA target site (2.4 % and 6.8%). Conversely, LV Tandem and Outward configurations allowed efficient cell transduction and significant and comparable levels of gRNA expression. As a results, we observed a good deletion frequency (11.0% ±1.8 of total alleles for LV Tandem and 12.3 %± 2.5 for LV Outward), a lower proportion of inversions events (5.4% ±1.3 for LV Tandem and 3.8 %± 0.4 for LV Outward) and up to 58% of InDels events at each gRNA target sites. Comparable results were also obtained in adult HSPC-derived erythroid cell line (HUDEP-2). Overall, our study indicates LV Tandem and LV Outward as promising tools for genome editing of primary cells; both vectors are now being evaluated in primary HSPC.

136. Nuclease-Assisted Vector Integration (NAVI): A Robust Platform for Multiplexed Targeted Genome Engineering

Alexander Brown, Wendy S. Woods, Pablo Perez-Pinera
Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL

The expansion of genome engineering spurred by CRISPR/Cas9 continues to accelerate. While mutagenesis generated via NHEJ remains a highly efficient and effective strategy for select applications, the insertion of large or complex sequences and the ability to easily select for modified cells often necessitates the use of homology directed repair (HDR) based strategies. The time-consuming construction of donor vectors for HDR gene editing is often technically challenging, costly, and leads to poor modification rates. By using customized single-stranded oligonucleotides (ssODN) the efficiency of gene editing increases, but the scale of possible genetic changes is greatly diminished. Additionally, as both donor vectors and ssODN require two discontiguous regions of homology, neither is well suited to multiplexing. Here, we present Nuclease-Assisted Vector Integration (NAVI) as a unique strategy to bypass HDR and the need for customized donor vectors required for traditional genome editing technologies. We demonstrate, through multiplexed insertion of a single plasmid into multiple loci, that NAVI eliminates the need for homologous sequence within donor vectors. Furthermore, by employing a single and universal guide-RNA, multiple vectors harboring different selection markers were integrated into distinct loci simultaneously, greatly facilitating production of isogenic mammalian cell lines. We were able to integrate sequences ranging from 3-50 kbp, but no upper limit was identified. Through the elimination of the HDR bottleneck and increased efficiency of editing, generation of double knockouts within two loci using NAVI takes approximately 3 weeks from design to completion, at minimal cost. Additionally, as NAVI is independent of homology, vector integration at double strand breaks occurs in either the plus or minus orientation. Finally, the resulting sequence of the juncture of genomic DNA with the vector is variable. We conclude that NAVI, despite sacrificing single base pair resolution, can be readily adapted for use in a variety of research and therapeutic platforms, due to its greatly enhanced versatility, ease of use, efficiency, and robust multiplexing capabilities for targeted integration of large sequences within mammalian genomes.

137. Analysis of Cas9-FokI and TALE-MutH Endonuclease Activity and Toxicity as Key Elements in the Development of a Gene Therapeutic Approach to Treat XLRP

Knut Stieger, Lennart Trimborn, Fei Song, Birgit Lorenz, Mert Yanik
Department of Ophthalmology, Justus-Liebig-University Giessen, Giessen, Germany

Mutations in the retinitis pigmentosa GTPase regulator (RPGR) gene cause severe X-linked retinitis pigmentosa (XLRP). More than 80% of the mutations are located in the terminal exon ORF15 of the *RPGR* gene. Genome editing, which represents a novel approach to treat monogenic disorders, is based on highly specific nucleases that cleave or nick at a chosen position within the complex genome followed by the repair of the double or single strand break (DSB or SSB) by endogenous repair mechanisms. The major pathways include error prone non-homologous end joining (NHEJ), microhomology-mediated end-joining (MMEJ), and homologous recombination (HR), the latter two with the help of a donor template. Currently, endonucleases for inducing the DSB are based on the CRISPR-Cas9 system or TALE proteins fused to the non-specific FokI nuclease (TALEN). However, specificity and toxicity of both endonuclease types raise concerns about their use for therapeutic *in vivo* applications. In order to study in vivo genome editing for the treatment of XLRP, our lab has generated a mouse model containing a point mutation in the ORF15 exon. In the present study, we characterize advanced variants of both endonuclease types (Cas9-FokI and TALE-MutH) for their activity and toxicity at the murine *Rpgr*-ORF15 locus for later usage in the mouse model. In total, ten sequences within or near the ORF15 exon have been targeted for the induction of DSB or SSB. Nine target sites for CRISPR/Cas9-FokI were chosen: three before, within, and behind the exon, respectively, and one target site for TALE-MutH within the exon. These sequences have been cloned into the traffic light reporter (TLR) gene expression system at the homing endonuclease I-SceI site. The TLR system has been modified to express either GFP in case of successful HR or BFP in case of NHEJ. Plasmids containing substrate, nucleases and template DNA were transfected into HEK293T cells. Efficiency of DNA modification was measured by FACS analysis and T7 surveyor assay, and toxicity was assessed by cell survival assay. In addition to the episomal TLR system within a human cell line (HEK293T), the genome of murine C2C12 cells was targeted by all endonuclease variants and toxicity was analysed via the T7 surveyor assay. Toxicity of Cas9-FokI and TALE-MutH are comparable to the golden standard ISceI while standard Cas9 nucleases showed slightly increased toxicity in HEK293T cells. Two different concentrations of the nucleases were used in a toxicity assay and were equally tolerated. Cas9-FokI showed preferences in its activity at the nine target sites with activities well above ISce-I level, while the one target site of TALE-MutH was as efficient as ISceI. Activity results were confirmed in the murine cell line C2C12. Off target toxicity in C2C12 cells was non-detectable. The characterization of the activity and toxicity of the tested endonucleases helped us to identify the most promising tailored nuclease and its target sequence in our gene targeting approach to treat XLRP. With the help of mouse retinal explants and subsequently in vivo experiments, we will confirm the efficacy of endonuclease mediated genome editing in photoreceptors.

138. Potential Therapeutic Treatment of Friedrich's Ataxia Using Highly Specific Engineered Meganucleases

Roshni Davey, Armin Hekele, Jo Ann Hux, Caitlin Turner, Wendy Sharer, Victor Bartsevich, Ginger Tomberlin, Gene Liau, Michael G. Nicholson, Clayton W. Beard, Jeff Smith, Derek Jantz
Precision BioSciences, Durham, NC

Friedreich's ataxia (FRDA) is a recessive genetic disorder that results in progressive neuromuscular deterioration. The most common causative genetic alteration of this disorder is an expansion of a trinucleotide (GAA/TTC) repeat within the first intron of the Frataxin (FXN) gene. This triplet expansion causes transcriptional defects, resulting in low FXN mRNA and protein levels. In patients, the correlation between number of repeats and severity of the disease, age of onset and cardiomyopathy indicates that the repeat expansion is the primary cause of FRDA, thus making it a potential therapeutic target. We are investigating the potential use of our engineered meganuclease technology to precisely remove these trinucleotide repeats as a therapeutic treatment for FRDA. We have designed a pair of meganucleases that introduce double-strand breaks on either side of the FXN trinucleotide repeat region, generating compatible 3' overhangs that can be repaired by direct ligation, resulting in precise excision of the intervening region. Introduction of this pair of meganucleases into FRDA patient fibroblasts in cell culture results in the successful deletion of the causative repeat region in greater than 15% of the transfected cells. This removal of the FRDA repeat region has been confirmed using both digital PCR-based analytics and deep sequencing. Additional analysis of Frataxin mRNA production, Frataxin protein levels, and cellular metabolism will further demonstrate the utility of the repeat excision approach for FRDA gene therapy.

139. Development of a Therapy for Duchenne Muscular Dystrophy Using Either TALEN of Cas9 Proteins

Daniel Agudelo, Joel Rousseau, Jacques P. Tremblay
Faculté de Médecine, Université Laval, Québec, QC, Canada

Duchenne muscular dystrophy (DMD) is a hereditary disease due to a mutation of the *DMD* gene located in the X chromosome. It is mainly caused by the deletion of one or more exons of the DMD gene, which changes the reading frame and results in a premature stop codon and a truncated and inactive dystrophin protein. Nowadays, genome editing by programmable endonucleases like TALENs or the CRISPR/Cas9 systems is a powerful method for developing a treatment for this type of disease. However, the use of DNA encoding these systems leads to a prolonged expression, which may increase the off-target activity of these nucleases. Despite the risk of integration into the genome hence increased probability of side effects, viral vectors remain the most effective delivery system for the nucleases and the sgRNA. The objective of this work was to develop a novel approach using purified TALEN proteins or Cas9 complex (Cas9 protein with crRNA and tracrRNA) for genome editing as potential treatment for DMD. TALEN proteins or Cas9 complex were transduced to generate double strand breaks (DSBs) in the dystrophin gene. Repair of this DSB by non-homologous end joining (NHEJ) could restore the normal reading frame of the DMD gene producing a functional dystrophin protein as already described for Becker patients (Koenig et al. 1989). To develop this approach, DNA coding for a pair of TALEN proteins targeting exon 54 of the DMD gene were engineered and cloned in the pet16b expression vector. The resulting TALEN proteins contained a His-Tag for purification purposes. The proteins were produced in bacteria and the TALEN-His-Tag proteins were purified using a Nickel column. The purity was analyzed by SDS-PAGE electrophoresis.

The Cas9 protein, the crRNA and the tracrRNA were obtained commercially. To verify the cleavage activity of the programmable nuclease-complexes, the target genomic region including exon 54 was PCR amplified and the resulting amplicon was incubated with either the TALEN proteins or the Cas9 complex. Cleavage products were analysed by gel electrophoresis. Both types of proteins were able to cut 100% of the amplicons *in vitro*. The TALEN proteins or the Cas9 complex were tested in Hela IR8 and in human myoblast cell lines. The TALEN proteins were transduced with Cys-(Npys)-$(d\text{-}Arg)_9$, Bioporter or the iTop delivery system (propanebetaine). The Cas9 complex was transduced with RNAiMax. The presence of DSBs was evaluated using the Surveyor assay. The Cas9 complex generated the expected cleavage products. These results are similar to those obtained when the plasmids coding for both systems were transfected. This indicates that the Cas9 complex could be used effectively to target a specific gene. In contrary, the TALEN proteins did not induce INDELs detectable by the surveyor assay. This could be due to an insufficient protein transduction. In conclusion, protein therapy using the CRISPR/Cas9 complex could be a promising approach to develop alternative treatments for genetic diseases. We are currently validating this approach *in vivo using* the hDMD mouse model containing the complete human *DMD* gene. ('t Hoen et al. 2008).

140. Point Mutation Correction for ADA SCID

Miguel Calero-Garcia, Marlene Carmo, Adrian Thrasher, H Bobby Gaspar
Molecular and Cellular Immunology, UCL Institute of Child Health, London, United Kingdom

Adenosine deaminase (ADA) deficiency is a rare genetic disease characterised by the absence of the ADA enzyme (EC 3.5.4.4) involved in purine salvage and degradation. Patients lacking ADA build up adenosine and deoxyadenosine metabolites in the organism, something that in turn leads to an accumulation of other deoxynucleotides and gives rise to functional defects in all lymphocyte lineages and severe combined immunodeficiency (ADA SCID). This SCID phenotype can be corrected by hematopoietic stem cell transplant, enzyme replacement therapy, and integrative gene therapy protocols employing autologous CD34+ cells. ADA deficiency confers a positive selective advantage to corrected lymphocytes over deficient cells, thus offering a great system for the study of novel molecular therapies such as gene editing. The advent of the CRISPR/Cas9 system has allowed for the swift tailoring of novel gene editing strategies in laboratories without access to advanced cloning platforms. We believe this democratisation of gene editing has also made more practicable the therapeutic reversal of genetic mutations into wild type sequences, the ultimate form of genetic correction. We have designed a CRISPR/Cas9 strategy that can target the nonsense Q3X (ADA c7C>T) point mutation endemic to ADA SCID patients of Somali origin residing in the UK, as well as its equivalent site in wild type sequences. In the present work we show evidence of the Q3X site non-homologous end joining (NHEJ) gene ablation in Jurkat cells by means of DNA sequencing and ADA protein readouts (approximately 70% efficiency). Moreover, we have designed a donor for the Q3X repair step that contains an adjacent traceable silent mutation in order to track any homology directed repair (HDR) when working with wild type cells. Here we show preliminary evidence of Q3X site HDR in wild type Jurkat cells in DNA sequencing results by detection of the aforementioned traceable mutation, albeit with low efficiency (less than 3% efficiency). We are currently attempting the translation of this approach into human CD34+ cells, the ultimate target cell population for the correction of the SCID phenotype in ADA deficient patients.

141. A Screening Platform for Generating Cas9 Mutants with Improved Function

Jeffrey M. Spencer, Xiaoliu Zhang
University of Houston, Houston, TX

CRISPR-Cas systems, RNA-guided endonucleases (RGENs), have been utilized for gene editing in a multitude of organisms. The robust editing ability of these nucleases promises to deliver novel therapeutics. CRISPR-derived therapeutics will benefit from performance enhancing modifications made to the system such as improved nuclease activity. We developed an *in vivo* plasmid-cleavage-based screening platform with the ability to screen libraries of Cas9 mutants for improved function. Expression of Cas9 is controlled by a titratable promoter which provides the ability to modulate the stringency of selection. The selection system has the ability to select for both the cleavage of a specific substrate or the inability to cleave a similar, inaccurate target. Together these parameters provide a flexible platform for screening and discovery of Cas9 or alternate RGEN variants with improved endonuclease characteristics. Optimization of Cas9, other CRISPR-associated proteins, and their system components will facilitate adaptation of these technologies into powerful precision research tools with the potential to transform clinical therapeutics.

142. Functionalized Selenium Nanoparticles for mRNA Delivery

Fiona C. Maiyo, Moganavelli Singh
Biochemistry, University of KwaZulu-Natal, Durban, South Africa

The use of inorganic nanoparticles as gene and drug delivery agents has raised expectations in cancer treatment and diagnostics, and is considered as a promising research orientation for oncotherapy. Selenium, an essential trace element plays a fundamental role in cell metabolism, being responsible for the functioning of many enzymes and other proteins, and is also known for its chemopreventative and chemotherapeutic activities. However, its use as a gene delivery vehicle remains largely unexplored. In this study, chitosan, a positively charged natural polysaccharide was grafted onto selenium nanoparticles. Functionalized and non-functionalized SeNPs were synthesized and characterized using Transmission and Scanning Electron Microscopy, UV spectroscopy, IR spectroscopy and Nanoparticle Tracking Analysis. This study demonstrates that the coating of SeNPs with chitosan influences nanoparticle size, distribution and charge. Gel retardation and enzyme protection studies confirmed that these chitosan functionalized SeNPs were capable of binding, condensing and protecting the mRNA *luc* from degradation. Evaluation of the effects of functionalized and non-functionalized SeNPs on the cell viability of the human colon adenocarcinoma (HT-29) cancer cell line and Human Embryonic Kidney (HEK293) non-cancer cell line was studied using the MTT cell viability assay. SeNPs at concentrations of up to 200 μg mL^{-1}, demonstrated low toxicity to both cell lines with percentage cell survival over 70 %. Complexes of NP: mRNA at different weight ratios ($^{w}/_{w}$) similarly exhibited low toxicity levels to both cell lines. Transfection studies were accomplished using the luciferase reporter gene assay. Results showed that the functionalized SeNPs (FSeNPs) produced greater transgene activity than the cationic polymer/mRNA complexes on their own. The results of this study suggest that FSeNP's low cytotoxicity coupled with their small particle size and surface properties, make them suitable non-viral gene delivery vectors. However further engineering and modifications of the FSeNPs may be required to enable *in vivo* gene delivery.The use of SeNPs has opened a new direction for synergistic treatment of cancer with higher efficiency and reduced side effects.

Oligonucleotide Therapeutics I

143. Durable Expression of TT-034 in Cynomolgus Monkey Hepatic and Cardiac Tissues without Long-Term Adverse Effects on Endogenous MicroRNA Levels

Tin Mao, Shih-Chu Kao, David Suhy
Benitec Biopharma, Hayward, CA

Background: The hepatitis C virus (HCV) is an attractive target for RNA interference (RNAi) based therapeutics because its genome consists of a single, positive stranded RNA and its replication occurs strictly within the cytoplasm. TT-034 is a DNA-directed RNAi (ddRNAi) based gene therapy product for the treatment of chronic HCV infection and is currently in a phase I/IIa clinical trial. TT-034 is comprised of a self-complementary recombinant adeno-associated virus (AAV) serotype 8 vector for transduction of hepatic tissues and is systemically administered as a single intravenous dose. Inside the hepatocyte, TT-034 uses the cell's own transcriptional machinery to continuously express three independent short hairpin RNAs (shRNAs) that simultaneously target the 5' UTR and NS5B regions of the HCV genome.

Results: Following a single administration of up to 3.75E12 vg/kg of TT-034, biodistribution analyses demonstrated that greater than 90% of the quantified vector is found in the Cynomolgus monkey liver tissues. *In situ* hybridization analyses demonstrated almost 100% transduction of the hepatocytes when dosed at 1.25E12 vg/kg. Furthermore, qPCR data revealed that shRNA levels persisted for the duration of the 180-day experiment, demonstrating the durability of expression following a single injection. We examined the impact of continuous TT-034 expression on endogenous miRNAs in liver and cardiac tissues because previous reports have suggested that high expression of shRNA may cause global dysregulation of endogenous microRNA (miRNA) levels within cells. Non-human primates were dosed with saline control or either 1.00E11, 1.00E12, 1.00E13 vg/kg of TT-034. Expression profiles of mature miRNAs were generated from liver biopsies taken 15 days after TT-034 administration. Additionally, samples from liver and heart tissues were collected 60 and 180 days after administration. Approximately 260 different miRNAs were reliably detected in each sample type and used for the analyses. The day 15 liver samples showed significant differences in the expression levels of 27 miRNAs. When analyzing all 260 miRNAs, the day 60 and day 180 liver samples showed no significant statistical differences from the control group at any dose. In the heart tissues, no significant statistical differences between the TT-034 treated group and the control group were noted at any timepoint.

Conclusions: These data suggest that a single, intravenous infusion of TT-034 results in almost complete transduction of Cynomolgus monkey hepatocytes. Furthermore, TT-034 results in durable expression of three anti-HCV shRNAs. These doses of TT-034 do not cause long-term perturbation of endogenous miRNA levels.

144. Development of Novel Dicer- and Ago2-Independent Small Hairpin RNAs

Shin-ichiro Ohno[1], Karen Itano[1], Yuichirou Harada[1], Koutaro Asada[1], Keiki Oikawa[1], Mike Kashiwazako[1], Sudo Katsuko[2], Masakatsu Takanashi[1], Norihiko Ikeda[3], Masahiko Kuroda[1]
[1]Molecular Pathology, Tokyo Medical University, Tokyo, Japan, [2]Animal Research Center, Tokyo Medical University, Tokyo, Japan, [3]Respiratory Surgery, Tokyo Medical University, Tokyo, Japan

The innate cytokine response to nucleic acid is the most challenging problem confronting the practical use of nucleic acid medicine. The degree of stimulation of the innate cytokine response strongly depends on the length of the nucleic acid. In this study, we developed a 30-nucleotide single-strand RNA, termed "guide hairpin RNA (ghRNA, ghR)", that has a physiological function similar to that of miRNA and siRNA. The ghRNA caused no innate cytokine response either *in vitro* or *in vivo*. In addition, its structure does not contain a passenger strand seed sequence, reducing the unwanted gene repression relative to existing short RNA reagents. Systemic and local injection of ghRNA-form miR-34a (ghR-34a) suppressed tumor growth in a mouse model of RAS-induced lung cancer. Furthermore, ghR-34a functioned in a Dicer- and Ago2-independent manner. This novel RNA interference (RNAi) technology may provide a novel, safe, and effective nucleic acid drug platform that will increase the clinical usefulness of nucleic acid therapy.

145. A Comparison of scAAV8-TT-034 Mediated Transduction and shRNA Expression in Human Liver Biopsy Samples versus a Chimeric Mouse Model with Humanized Liver

Shih-Chu Kao, Tin Mao, Terrie-Anne Cock, Peter Roelvink, David Suhy
Benitec Biopharma, Hayward, CA

Background: TT-034 is a DNA-directed RNA interference (ddRNAi) agent designed for the treatment of chronic HCV infection and is currently being tested in a phase I/IIa clinical study. TT-034 is comprised of a vector that expresses three independent short hairpin RNAs (shRNAs) simultaneously targeting three well-conserved regions of the HCV genome. The recombinant genome is packaged in a self-complementary aden-associated virus serotype 8 (AAV8) capsid with tropism for hepatic tissues and delivered as a single dose intravenous (IV) infusion.

Methods: Chimeric mouse models in which human hepatocytes replace the majority of mouse hepatocytes are used to study human hepatic function. In order to assess validity of dose/transduction relationships in this murine model to those observed in a human clinical study, chimeric mice were infected with identical doses of TT-034 used in a phase I/IIa study using the same clinical lot of TT-034. In the clinical study, eight subjects have received a single IV infusion of TT-034 at 4.00E10, 1.25E11, 4.00E11 or 1.25E12 vg/kg. At 21 days post dosing, a liver biopsy was collected to assess TT-034 DNA levels and shRNA expression by qPCR. PXB chimeric mice (Phoenix Bio) repopulated with a minimum of 80% hepatocytes were dosed identically with the TT-034 drug product (5 groups, n=4). After 21-28 days, the livers of two mice in each group were removed and hand curated to purify human hepatic tissues (>93% purity). The liver tissues of the other two mice were dissociated and human hepatocytes were enriched to >99% using mouse hepatocyte-capturing Dynabeads. TT-034 DNA and shRNA expression were assessed by qPCR.

Results: In the human study, modest levels of TT-034 DNA copies were detected in the 3 subjects dosed at 1.25E11 vg/kg, yielding 0.48, 3.65 and 10.44 copies per cell respectively. Variability in transduction was noted at the higher dose of 4.00E11 vg/kg, with the two subjects yielding 17.74 and 1.01 copies per cell. qPCR analysis of the three anti-HCV shRNAs confirms concomitant, dose dependent expression. In the hand curated samples from the chimeric mouse model (>93% purity), a dose of 1.25E11 vg/kg yielded 1.1 or 1.3 DNA copies per cell while the 4.00E11 vg/kg dose resulted in 1.9 and 5.5 copies DNA per cell. Dynabead-enriched human hepatocytes (>99%) resulted in a considerable drop in DNA copy levels: the 1.25E11 vg/kg dose averaged 0.35 copies per cell while the 4.00E11 vg/kg resulted in 0.85 copies per cell. The lowered DNA levels in the chimeric mouse model led to a concomitant reduction in shRNA expressed from the hand curated tissues. Likewise, shRNA expression was reduced even further in enriched human hepatocytes.

Conclusion: Our data suggests that residual mouse hepatocytes present in the chimeric livers are transduced with the scAAV8 vector

more efficiently than human hepatocytes and results in lower overall transduction as compared to human clinical samples. Thus, while these models can serve as a surrogate to assess the activity of gene therapy constructs against functions of normal human liver, the doses required for optimal activity may be modestly higher than required in the human clinical setting.

146. Regulation of Inflammation in Cystic Fibrosis Lung Epithelial Cells by miR-155

Motohiro Tsuchiya[1], Parameet Kumar[1], Sharmistha Bhattacharyya[1], Swathi Kalurupalle[1], Shobha Vasudevan[2], Jennifer Martindale[3], Myriam Gorospe[3], Roopa Biswas[1]
[1]Anatomy, Physiology and Genetics, Uniformed Services University of the Health Sciences, Bethesda, MD, [2]Genetics, Massachusetts General Hospital, Boston, MA, [3]National Institute on Aging, NIH, Baltimore, MD

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in cystic fibrosis transmembrane conductance regulator (CFTR). The most common mutation, F508del, is associated with failure of the mutant CFTR to traffic to the plasma membrane, with concomitant loss of cAMP-activated chloride conductance, and hypersecretion of pro-inflammatory IL-8. Our data indicate that the mis-expression of microRNAs (miRNAs, miRs) in CF cells, both in culture and in *ex vivo* bronchial biopsies, contribute to the inflammatory disease phenotype of CF. Our studies have demonstrated that the aberrant up-regulation of miR-155, in CF cells compared to CFTR-repaired control cells, induces hyper-expression of IL-8 through reduction in AKT1 activation. Our data indicate that, in addition to regulation of IL-8 mRNA stability by targeting SHIP1 gene, miR-155 also promotes increased translation of IL-8 mRNA. Therefore, we have analyzed the role of pro-inflammatory RNA-binding proteins (RBPs) and RNAs in miR-155-mediated regulation of IL-8 expression in CF lung epithelial cells. We have employed an *in vivo* cross-linking and affinity purification strategy to isolate and identify proteins and RNA in the miR-155-induced IL-8-mRNP complexes that regulate IL-8 expression in CF cells. We have thus identified the antagonistic role of HuR and miR-16 in the regulation of IL-8 expression in CF. Additionally, we have also identified CF disease-specific inflammatory target genes of miR-155, viz. RPTOR, SIRT1 and NR2F2, in CF lung epithelial cells that regulate the CF disease phenotype. Understanding these mechanisms will ultimately lead to the development of novel miR-based anti-inflammatory therapeutics for CF and other pulmonary disorders.

147. Abstract Withdrawn

148. AptaMetrics: A Web-Based Aptamer Bioinformatics Platform

Thomas Bair[1], Julie S. Wertz[1], Robert J. Miller[2], James Schappet[2], Paloma H. Giangrande[3], William H. Thiel[3]
[1]Iowa Institute of Human Genetics, University of Iowa, Iowa City, IA, [2]Institute for Clinical and Translational Science, University of Iowa, Iowa City, IA, [3]Department of Internal Medicine, University of Iowa, Iowa City, IA

RNA and DNA aptamer development as a therapeutic moiety and as a diagnostic tool is a rapidly growing field. The discovery process for aptamers involves iterative rounds of selection in a process termed SELEX, Systematic Evolution of Ligands by EXponentional enrichment. The modern SELEX process was revolutionized with the adoption of high-throughput sequencing (HTS), which is able to identify millions of aptamer sequences across multiple rounds of aptamer selection. The scale of aptamer HTS datasets has necessitated bioinformatics algorithms, such as clustering algorithms, to identify candidate aptamers for experimental validation. Herein we describe a web-based aptamer bioinformatics platform, AptaMetrics, which utilizes a collection of programs and algorithms to facilitate the analysis of aptamer HTS datasets.

The AptaMetrics platform builds upon our previously described aptamer bioinformatics work (Thiel *et. al.* PLoSOne, 2012). Central to AptaMetrics is our clustering algorithm, ProcessSeqs, which we engineered to include several novel and distinct features: 1) clustering may be performed using either an "all-versus-all" or a "seed" sequence strategy; 2) clustering may be accomplished through either sequence similarity (edit distance) or structural similarity (tree distance); 3) the output of ProcessSeqs provides a visual and interactive representation of related aptamer networks; and 4) these networks of related aptamers may be analyzed using conventional network analysis tools. We also developed an aptamer structure prediction program, ProcessFold, that provides the predicted structure of millions of aptamer sequences utilizing a variety of proven oligonucleotide structure prediction algorithms (e.g., RNAfold, Mfold, RNAstructure). Integration of these two programs thereby allows users to create networks of structurally related aptamers based on the different structure prediction algorithms. We also created and incorporated an aptamer truncation algorithm, AptmR, into the AptaMetrics platform. The purpose of AptmR is to determine a predicted truncated aptamer sequence based on aptamer sequence and structure information provided by ProcessFold. AptmR can be applied to either a single aptamer sequence or to millions of aptamer sequences. The predicted truncations may then be clustered into networks of related aptamers using the ProcessSeqs clustering algorithm. Importantly, to allow the greatest versatility, each program and algorithm within AptaMetrics may be used independently or as a pipeline. The collection of programs and algorithms within AptaMetrics provide a significant degree of flexibility in analyzing aptamer HTS data while maintaining the ease of accessibility of a web-based environment.

149. *In Vitro*-Synthesized Sendai Virus RNA Fragments Induced Antitumor Immunity and Cancer Cell-Selective Apoptosis

Tomoyuki Nishikawa, Li-Wen Liu, Yasufumi Kaneda
Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan

Three different clinical trials to treat malignant melanoma, castration-resistant prostate cancer and malignant mesothelioma using inactivated Sendai virus (hemagglutinating virus of Japan; HVJ) particles called HVJ-envelope (HVJ-E) are on-going because of the multiple-anti-cancer activities of HVJ-E. One the activities is the activation of anticancer immunity through inactivation of Treg (regulatory T cell) and promotion of NK cells activation and generation of CTL against cancers by producing interferon (IFN)-β, CXCL10, RANTES and IL-6. Another is the cancer selective apoptosis by induction of proapoptotic genes such as TRAIL and Noxa in various human cancer cells but not in normal cells. Most of these anti-cancer activities are conducted by RNA genome fragments of HVJ-E through RIG-I/MAVS (retinoic-acid inducible gene-I, mitochondrial antiviral signaling protein) signal pathway. Among various Sendai virus strains, Sendai virus Cantell strain showed the highest production of IFN-β in dendritic cells (DCs) and the strongest DC maturaion and we found that DI (defective interfering) particles of Cantell strain resulted in those immunostimulatory activities. Then, we investigated RNA molecules in Cantell HVJ DI particles which have no replication activities. The Cantell stain HVJ DI RNA has complementary termini (approximately 100nt) and exhibits the highest binding affinity to RIG-I. DI RNA genome (544 base) was isolated and transferred to human prostate cancer cell PC3 by lipofection. The DI RNA induced higher expression level of those apoptosis-related proteins such as

Noxa and TRAIL and more cancer cell death than whole-genome RNA (approximately 15 kb) of complete Cantell strain without DI particles. Furthermore, we examined whether a specific structure of the DI RNA genome stimulates the RIG-I/MAVS downstream-related cancer suppressive pathways using HVJ-derived *in vitro* transcribed (IVT) RNAs. IVT-B2 which is derived from Cantell HVJ DI genome has a special secondary structure with a double-stranded RNA terminus and a single-stranded RNA loop. This IVT-B2 strongly stimulated RIG-I dependent proapoptotic proteins induction in prostate cancer cells. Modified IVT-B2 RNAs which had shorter dsRNA stem lost cancer cell killing activity and proapoptotic gene expressions. On the other hand, other modified IVT-RNA which had deleted ssRNA region in loop structure did not induce cancer cell-selective killing. We also found that calf intestinal alkaline-phosphatase-treated IVT-B2 RNA lost capability of inducing RIG-I/MAVS-related downstream Noxa and TRAIL expression. Finally, *in vivo* electroporation of IVT-B2 RNA induced intra-tumoral apoptosis and tumor suppression in human prostate cancer cell-xenograft mouse model by both direct tumor-cell killing and NK cell activation. These findings provide a novel nucleic acid medicine for the cancer treatment.

150. Further Development of an Allele-Specific Gene Silencing Strategy to Correct a Dominant-Negative Mutation Causing Collagen VI-Related Muscular Dystrophy

Véronique Bolduc, Katherine Sizov, Apurva Sarathy, Yaqun Zou, Carsten G. Bönnemann
NINDS/NIH, Porter Neuroscience Research Center, Bethesda, MD

Collagen VI-related congenital muscular dystrophies (COL6-RD), caused by mutations in any of the three genes coding for collagen type VI (*COL6A1*, *COL6A2*, *COL6A3*), underlie a spectrum of disorders ranging from severe life-threatening early onset Ullrich muscular dystrophy via intermediate phenotypes to the milder Bethlem type, typically presenting with generalized muscle weakness, proximal joint contractures, and respiratory failure. At present, there are no treatment options available for individuals affected with these diseases. The fact that the majority of COL6-RD cases are carriers of inherited or *de novo* dominant mutations, acting as dominant-negative, poses a challenge for the development of targeted therapies. In contrast to gene replacement, allele-specific silencing has the potential to treat these disorders, as it would convert the dominant-negative state into a clinically asymptomatic haploinsufficient state. Therefore, in the laboratory we aim at exploring targeted RNA interference (RNAi) approaches as a potential therapeutic approach for dominant COL6-RD. We have previously demonstrated the allele-specificity and efficacy of siRNA oligos to downregulate the expression of a mutant *COL6A3* transcript *in vitro* in patient-derived fibroblasts. In preparation of in vivo testing we have now extended our study to cells isolated from a mouse model of the disorder, which carries the most frequent dominant-negative mutation, a deletion of exon 16 on *Col6a3*. We transfected a series of small interfering RNA (siRNA) oligonucleotides into Col6a3del16+/- fibroblast cells, isolated from skin and muscle. To assay efficacy we used outcome measures such as unsaturated PCR, quantitative RT-PCR, and immunofluorescence. In addition, we performed in vivo electroporation of siRNA oligonucleotides in FDB muscles of Col6a3del16+/- mice. We found that siRNA oligonucleotides were effective in Col6a3del16+/- cells, to specifically knockdown the expression from the mutant allele and to restore the production of a collagen VI matrix in culture. This study provides further proof-of-concept of the use of RNAi as a potential treatment for COL6-RD.

151. Abstract Withdrawn

152. Tumor Inhibition by Using Chitosan:siRNA PDGFR-ß in Breast Cancer Model of Rat

Jülide Akbuğa[1], Suna Ozbas Turan[1], Emine Salva[2], Naziye Ozkan[3], Levent Kabasakal[4], Saadet Alan[5]
[1]Pharmaceutical Biotechnology, Marmara University, Istanbul, Turkey, [2]Pharmaceutical Biotechnology, İnönü University, Malatya, Turkey, [3]Pathology, Marmara University, Istanbul, Turkey, [4]Pharmacology, Marmara University, Istanbul, Turkey, [5]Medical Pathology, Malatya State Hospital, Malatya, Turkey

Platelet-derived-growth factors (PDGFs) may represent a novel target in human cancer because they regulate many cellular processes, including cell proliferation, transformation, apoptosis and angiogenesis. PDGF-D's exert their cellular effects by two receptors PDGFR-α and PDGFR-β. Moreover PDGF ligands and receptors are proto-oncogenes that can be activated by various types of alternatives in cancer cells. Breast cancer is the most common and fatal type of cancer. The PDGF pathway is essential in tumor angiogenesis. It is known that the expression of PDGF receptors is altered and upregulated in breast cancer. Delivery is very important for success in gene therapy. Among the different non-viral gene delivery system, chitosan has very useful properties as a gene carrier. In this study; we investigated the effect of chitosan:siRNA complexes on tumor angiogenesis in breast tumor models of rats. Firstly chitosan:siRNA complexes (1/5-1/20 rate) targeting to PDGFR-β were prepared and characterized *in vitro*. Transfection efficiency of these nanoplexes in breast cancer cells such MCF-7, MDA-MB-231 and MDA-MB-435 was assayed. In *in vitro* transfection studies, 54%-65% of PDGFR-β inhibition was measured. Then these chitosan:siRNA complexes were injected intratumorally to tumor bearing Sprague Dawley rats. After tumor reached to constant size, complexes (20/1) were injected (40µg siRNA) and tumor volumes were measured periodically. After sacrification of animals, tissue and blood samples were taken and investigated histologically and immuno-histochemically. Expression levels of PDGFR-β determined by ELISA, mRNA levels were measured by RT-PCR also. Nanoplexes having 207 ± 3.5 nm size and 14.2 mV surface charge were used in this study. Tumor volume of nanoplex treated rats decreased at 92.49% at the end of the experiment (28th day). After free siRNA treatment, however, tumor volume increased very slowly until 21 days (68.1 % of decreasing value) then went up fastly. RT-PCR, Western-blot studies and immuno-histochemical data showed similarity with tumor volume measurements. Similar IFN response with tumor (untreated) was obtained after nanoplex administration. In conclusion, chitosan can be complexed with siRNA targeting PDGFR-β and chitosan:siRNA PDGFR-β nanocomplexes may be useful alternative in the treatment of breast cancer. The receptors of PDGF may be also suitable target in breast cancer.

153. Third Generation Antisense Targeted to Double Homeobox Protein 4 (DUX4) Reduced DUX4 Expression and Improved Differentiation of FSHD Myoblasts

Sachchida Nand Pandey[1], Kelly Murphy[2], Hunain Khawaja[1], Mallikarjuna Putta[3], Lakshmi Bhagat[3], Sudhir Agrawal[3], Yi-Wen Chen[1,2]
[1]Children's National Health System, Washington, DC, [2]George Washington University, Washington, DC, [3]Idera Pharmaceuticals, Inc., Cambridge, MA

Facioscapulohumeral muscular dystrophy (FSHD) is caused by aberrant expression of double homeobox protein 4 (DUX4) gene at chromosome 4q35. To date there is no effective treatment for the disease. In the current study, we have evaluated selected Third Generation Antisense compounds (3GAs) targeted to DUX4 for

their effectiveness in knocking-down DUX4 in FSHD myoblasts. In addition, we examined the effects of the treatments on myogenic differentiation of FSHD myoblasts. Multiple 3GA compounds (3GA1-5) showed significant knock-down of DUX4 in the FSHD myoblasts, as examined by quantitative RT-PCR. We evaluated two 3GA candidates (3GA3 and 3GA5) at 4 different concentrations, 2.5 nM, 5nM, 25nM, and 50nM, and observed dose-response effects. One of the 3GA compounds completely knocked-down DUX4 expression at 50nM. The effects of 3GAs at25nM on cell differentiation were determined by fusion index, myotube size and number of atrophic myotubes after 7 days of muscle differentiation in culture. Our results showed that the treatments increased fusion index and reduced the number of atrophic myotubes. The studies showed that 3GAs successfully knocked-down DUX4, which improved the myogenic capacity of FSHD myoblasts. The findings demonstrated that the 3GAs are promising therapeutic molecules to be further developed for FSHD treatment.

154. Applying Insights from Pri-miRNA Processing to shRNA Design

Xavier Bofill-De Ros, Lisheng Dai, Shuo Gu
Gene Regulation and Chromosome Biology Laboratory, Center For Cancer Research, National Cancer Institute, Frederick, MD

Short hairpin RNA (shRNA) technology permits efficient and stable gene regulation, providing a useful tool for gene therapy. shRNAs can be embedded in miRNA scaffolds that allow their expression from Pol II promoters, which is more amendable to control transcription. Under this platform, shRNAs are processed through the canonical miRNA maturation pathway, which includes stepwise cleavages by Drosha-DGCR8 complex (Microprocessor) and Dicer. Despite many successes, design of Pol II driven shRNA is far from optimal. Most Pol II driven shRNAs are based on primary transcript sequence of hsa-miR-30a. Recent studies have shown that different pri-miRNAs have distinct Drosha cleavage efficiencies leading to various levels of its mature form. Here, we seek to systematically explore how different pri-miRNA scaffolds can be adapted and optimized to provide the best shRNA expression and function. By taking advantage of DGCR8 KO cells and luciferase-based reporters, we were able to directly monitor Microprocessor cleavage efficiency of primary transcripts *in vivo*. We have analyzed the Microprocessor cleavage of 20 different primary miRNA transcripts that are the most abundant and widely expressed. Moreover, we have explored how the preservation of stem structure and endogenous surrounding sequences and motifs contribute to shRNA maturation. This study aims to pave the way for a universal miRNA-based shRNA expression platform.

155. Cancer-Specific Theranosis Using Quantum Dots and siRNA Co-Loaded in Micellar Nanoparticles

Seong Jae Kang, Hwa Yeon Jeong, Min Woo Kim, In Ho Jeong, Moon Jung Choi, Young Myoung You, Chan Su Im, Yong Serk Park
Yonsei University, Wonju, Korea, Republic of

Theranostics is a proposed process of diagnostic therapy for an individual patient. It has been considered a powerful tool of personalized medicine. In this study, we have prepared lipid micellar nanoparticles encapsulating quantum dots (QDs) as a diagnostic agent and incorporating cholesterol-siRNA (Cho-siRNA) as a therapeutic agent. Furthermore, for targeted delivery of QDs and Cho-siRNA, anti-EGFR antibodies or aptamers were conjugated to the surface of the nanoparticles. First of all, for stable nanostructure of lipid micelles, we determined an optimal molar ratio of lipids, QDs, and Cho-siRNA as 350:1:0.35. The size of QD micelles was approximately 50 nm that is suitable for efficient localization in tumors via enhanced permeability and retention (EPR) effect. Their serum stability and pH stability suggest that the QD micelle structure is stable enough to be applied under biological conditions. The RNase protection assay showed that Cho-siRNA molecules in the QD micelles were effectively protected by steric shielding of hydrophilic polyethylene glycol molecules. The analyses with a flow cytometry and a confocal microscope showed that the nanoparticles can effectively deliver Cho-siRNA to the target cells, followed by effective residing of siRNA molecules in the cytoplasm. A further systematic evaluation of the micellar nanoparticles established in this study would verify their value as a theranostic vehicle.

156. A Plasmid Manager, a Comprehensive Database for Archiving Plasmid DNA Sequence

Emily J. Heathward[1], Giun Sun[2], Ron U. Domingo[2], Calvin A. Johnson[2], Christopher T. Richie[1], Brandon K. Harvey[1]
[1]National Institute on Drug Abuse, Baltimore, MD, [2]Center of Information Technology, National Institutes of Health, Bethesda, MD

A freely available plasmid database that is interoperable with popular freeware (such as *a Plasmid Editor*, or *ApE* [1]) has been developed by the Center of Information Technology (CIT) for the NIDA Optogenetics and Transgenic Technology Core (OTTC). As a core facility at the National Institute on Drug Abuse, the NIDA OTTC group has accrued a wealth of plasmid DNA data that can no longer be efficiently analyzed without a comprehensive cataloging system. The OTTC Plasmid Manager provides an intuitive plasmid database with analytical and search capabilities, offering a versatile platform for the storage and organization of plasmid sequences and their associated metadata. The database has been designed with informatics and visualization support to enhance the efficiency and analytic capabilities of the user, with a user-friendly interface. Annotations performed in *ApE* can be imported directly into the database, and unlike most commercially available and free DNA editing software, A Plasmid Manager (APM) also includes a mechanism to standardize the annotations of uploaded sequences, facilitating the direct comparison of multiple plasmids at the feature level. The visualization component generates a graphic representation of the plasmid map together with its features and annotations, allowing the visual comparison of multiple plasmids side-by-side. The contents of the database can be browsed and searched using a variety of criteria, including plasmid name, database accession number and associated features. This innovative plasmid database presents a new platform for molecular biologists to catalog plasmids relevant to their laboratory, saving time and enhancing the analytical capabilities of researchers.

Diabetes, Metabolic and Genetic Diseases I

157. High-Field In Vivo Neuroimaging to Determine CNS Gene Therapy Outcome and Probe Disease Pathomechanism

Dominic J. Gessler[1], Wei Huang[2], Danning Li[1], Hongxia Xu[1], Qin Su[1], Jean King[2], Constance Moore[3], Guangping Gao[1]
[1]Gene Therapy Center, UMass Medical School, Worcester, MA, [2]Department of Psychiatry, UMass Medical School, Worcester, MA, [3]Deparment of Psychiatry, UMass Medical School, Worcester, MA

Gene therapy targeting the central nervous system (CNS) is one of the most challenging gene therapies due to the blood-brain barrier (BBB). However, recombinant adeno-associated virus (rAAV) has proved to be an excellent tool to target the CNS. Another obstacle in the setting of CNS gene therapy is the non-invasive evaluation of therapeutic outcome. While biopsies and sections of the CNS are the

gold-standard to assess brain pathology and response to CNS gene therapy, the invasiveness and potentially associated complications limit its frequent use in pre-clinical as well as clinical studies. Thus, it is of no surprise that non-invasive monitoring of CNS gene therapy *in vivo* holds great promise for longitudinal and functional assessment of treatment response. We used high-field *in vivo* neuroimaging to monitor intravenously (i.v.) and intracerebroventricularly (i.c.v.) administered rAAV based CNS directed gene therapy in a mouse model of Canavan disease (CD). Characteristically, Canavan disease presents with a very high NAA peak detected by magnet resonance spectrometry (MRS) and hyper intensity on T2-weighted anatomic images using magnet resonance imaging (MRI). Consequently, we first determined the efficacy of our i.v. and i.c.v. gene therapy by those two means. In congruence with motor function and pathology data, both MRI and MRS alterations have been entirely normalized by gene therapy. Another characteristic neuropathological change on Canavan brain sections is the loss of white matter tracts, which is thought to explain neurological symptoms seen in Canavan disease patients. Thus, we hypothesized that diffusion tensor imaging (DTI) enables the assessment of white matter tract degeneration and recovery upon gene therapy without brain biopsies. Selecting thalamus and corpus callosum as regions of interest (ROI), DTI indeed shows a recovery of brain white matter integrity when utilizing 3rd generation Canavan gene therapy. Furthermore, our 3rd generation gene therapy converts this CD mouse model with the severest phenotype into "supermouse", outperforming wild-type mouse on motor function testing. We hypothesized that functional connectivity identifies brain regions that not only show response to treatment but also indicates possible explanations for this enhanced phenotype. Using resting-state functional MRI (rs-fMRI), we show that treated CD mice have a functional connectivity pattern that is more similar to or even enhanced beyond what is seen in WT brain. This suggests facilitated inter-brain-region functional connectivity, which might provide a neural mechanism that sub-serves the observed enhanced motor function. Currently, we are investigating how the identified brain regions can promote increased motor function, and how high-field brain imaging can provide biomarkers to track the disorder and treatment response in a manner that would help facilitate the prediction of CNS directed gene therapy outcome. In summary, our data show that high-field in vivo neuroimaging is a valuable tool to monitor pre-clinical CNS gene therapy and pathology in detail, that it can provide insights into pathophysiology and that it has potential implications for the use in clinical trial outcome prediction and assessment.

158. Cryptic ATG Removal from Synthetic Introns Increase the Therapeutic Efficacy of AAV Vector Mediated Gene Transfer

Giuseppe Ronzitti, Fanny Collaud, Giulia Bortolussi, Severine Charles, Patrice Vidal, Marcelo Simon Sola, Andres Muro, Federico Mingozzi
Genethon, Evry, France

Inclusion of synthetic introns in adeno-associated virus (AAV) vector expression cassettes is a commonly used maneuver to drive increased transgene expression, possibly via an increased stability and/or increased nuclear translocation of mRNA. Although the positive effect of intronic sequences on transgene expression has been demonstrated in several models, less clear is whether splicing in these synthetic expression cassettes occurs with 100% efficiency and if the presence of the unspliced intron can influence the efficiency of translation of the transgene. In an effort to optimize a liver gene transfer approach to treat Crigler-Najjar (CN) syndrome, an autosomal recessive disorder caused by mutations in the UDP-glucuronosyltransferase 1 isotype A1 (UGT1A1) gene, we analyzed the sequence of a synthetic intron derived from human beta-globin intron 2 (hBB2) for motifs that may affect the transgene expression levels. This analysis revealed the presence of out of frame and in frame ATG codons before the start site of the UGT1A1 transgene. Unexpectedly, the removal of these cryptic ATGs from the intronic sequence enhanced transgene expression, which resulted in stable, long-term correction of serum bilirubin levels in animal models of CN syndrome. These results demonstrate that intron optimization increases mRNA and protein levels in vitro and enhance the efficacy of the vector in vivo. Next, we conducted a similar analysis on other intronic sequences commonly used in AAV-mediated gene transfer, showing that the removal of cryptic ATGs in the intron at the 5' of a transgene generally increases its expression. The scanning ribosome model may provide a solid explanation for our findings given that the intronic sequence is not completely removed by splicing. We thus analyzed the structure of the region at the 5' of the transgene by RT-PCR and primer elongation assay and we observed the accumulation of unspliced forms of the mRNA. We also confirmed that the presence of cryptic ATGs has a critical impact on the translation efficiency of the transgene. In conclusion, our results confirm the positive effect of an intron fused to the 5' of a transgene on its expression levels. On the other hand, our results provide evidence that the synthetic introns used in gene therapy are not spliced with 100% efficiency, thus the optimization of the sequence of the intron by removal of cryptic ATGs is important to increase the transgene expression in AAV vector-mediated gene transfer.

159. Sustained Weight Loss and Altered Lipid Taste Sensitivity Induced by Modulation of Salivary PYY and Excendin-4 in Mice

Sean M. Crosson[1], Seth Currlin[1], Taimoor Khan[1], Cedric D. Dotson[2], Sergei Zolotukhin[1]
[1]Pediatrics, University of Florida, Gainesville, FL, [2]Neuroscience and Psychiatry, University of Florida, Gainesville, FL

Peptide tyrosine-tyrosine (PYY), known to induce satiation when present in plasma, has recently been described to modulate body weight (BW) and food intake (FI) of diet induced obese (DIO) mice when included in saliva. Similarly, glucagon-like peptide 1 (GLP-1), a GI peptide present in both plasma and saliva, also modulates BW and FI. The purpose of this project was to study the anorexigenic effect of sustained elevation of PYY and Excendin-4 (Ex-4, a GLP-1R agonist) in saliva of mice. Using recombinant Adeno-associated virus serotype 8 (rAAV8) we performed gene transfer of GFP (control), PYY, Ex-4, and PYY-Ex-4 dual vectors to the submandibular salivary glands of C57BL/6 mice fed a high fat diet. We observed a significant ($p < 0.05$) decrease in BW of mice treated with either Ex-4, or PYY-Ex-4 dual vectors when compared to controls. Notably, mice treated with PYY-Ex-4 dual vector displayed a significant decrease in BW as early as 1-week post vector administration while Ex-4 mice alone did not demonstrate a significant loss until 8 weeks post-injection. PYY mice, while demonstrating a decreasing trend in BW gain with respect to GFP mice, did not show a significant difference in BW during the 12-week experiment. To determine whether the anorexigenic effect of salivary PYY and Ex-4 is modulated through taste perception, we utilized a Davis Rig gustometer to generate brief access taste response curves for a panel of tastants for all groups of treated mice. Notably, the Ex-4 as well as the PYY-Ex-4 dual group displayed a significant increase in sensitivity to intralipid stimulus, suggesting that this taste modality plays a role in BW modulation. This observed weight loss and altered taste perception in PYY and Ex4 treated mice while significant, is complicated by the unintended transduction of hepatocytes. Unintended transduction of the liver may allow for the elevated levels of PYY and Ex4 in the bloodstream in addition to saliva. In order to observe the saliva-specific effect of dual PYY Ex4

vector administration, we have developed viral constructs containing miR122 target sequences, a liver specific micro RNA (miRNA), and miR206, a skeletal muscle specific miRNA, target sequences in the 3' UTR of each construct. These micro RNA target sequences will suppress vector expression in off target tissues such as the skeletal muscle and liver. Additionally, all viral constructs were packaged into AAV5 instead of AAV8 which has the same transduction efficiency of salivary glands but decreased transduction of the murine liver. Using these miRNA constructs, we have observed no detectable expression of GFP in the murine liver.

160. Successful Treatment of Neonatal Metachromatic Leukodystrophy Model Mice by Low Dose of Self-Complementary AAV Type9 Vector Expressing ASA

Noriko Miyake, Koichi Miyake, Motoko Yamamoto, Takashi Shimada, Takashi Okada
Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan

Metachromatic leukodystrophy (MLD) is a lysosomal storage disease (LSD) caused by a functional deficiency of the lysosomal enzyme arylsulfatase A (ASA) and characterized by severe neurological symptoms due to widespread and progressive demyelination in the both central and peripheral nervous systems. Enzyme replacement therapy (ERT) has been applied to treat certain types of LSDs, but correction of neurological abnormalities is usually hampered by the blood brain barrier (BBB). We have previously shown that MLD mice can be treated by intravenous (IV) injection of classical single-stranded AAV type 9 vector expressing ASA (ssAAV9/ASA) for neonate and self-complementary AAV9/ASA (scAAV9/ASA) for adult. To develop the clinical MLD gene therapy, it is important to decrease the amount of viral vectors because of safety. In this study, we evaluated the therapeutic potential of scAAV9/ASA-mediated systemic transduction of neonatal MLD mice at 10 times lower dose (2×10^{11} v.g./body) compared to the treatable dose of the ssAAV9/ASA (2×10^{12} v.g./body). We generated both ssAAV9/ASA and scAAV9/ASA to intravenously inject then into the neonatal MLD mice (n=6-9). ELISA analysis showed that sustained high-level expression of ASA was detected in the brain and spinal cord of the scAAV9/ASA-treated mice (cerebral cortex, 2.1 times; cerebellum, 2 times; spinal cord, 5.5 times) compared to the ssAAV9/ASA for more than one year. Furthermore, in the behavior test, both scAAV9/ASA and ssAAV9/ASA-treated mice showed a significant improvement in their ability to traverse narrow balance beams, as compared to the non-treated MLD mice (latency; 7.2±0.9, 9.3±1.1 *vs.* 16.6±0.8 sec, $P<0.05$: slips; 3.4±0.8, 3.6±0.6 *vs.* 7.3±1.8 times, $P<0.05$). These data clearly demonstrate that MLD model mice can be treated by systemic neonatal transduction with low dose of scAAV9/ASA. Therefore, neonatal gene therapy would be an important option for parents faced with the prenatal diagnosis of a genetically affected child. Since ten times lower dose of scAAV9/ASA is enough to treat the neonatal MLD mice compared to ssAAV9/ASA, transduction strategy to use scAAV9/ASA should be safe and more practical for the clinical MLD gene therapy.

161. Novel Gene Transfer Strategy for Hemophilia A by Omental Implantaion of Autologous Endothelial Cell Sheet

Hideto Matsui[1], Masahi Noda[1], Midori Shima[2], Rie Utoh[3], Teruo Okano[3], Mitsuhiko Sugimoto[1]
[1]Regulatory Medicine for Thrombosis, Nara Medical University, Kashihara, Japan, [2]Pediatrics, Nara Medical University, Kashihara, Japan, [3]Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan

Preclinical and clinical studies using adeno-associated viral (AAV) vectors for hemophilia B showed that the safety profile is vector dose-dependent and that immune responses to AAV-capsid proteins with subsequent hepatocyte toxicity required transient immunosuppression for sustained transgene expression. However, there still remain concerns over the safety of systemic vector injection. Potential side effects include adverse immunological reactions, vector-mediated cytotoxicity, germ-line transmission, and insertional oncogenesis. Moreover, especially in hemophilia A, an alternative transgene delivery approach may be necessary due to the large size of the factor VIII (FVIII) cDNA. Blood outgrowth endothelial cells (BOECs) are considered to be an attractive candidate to treat hemophilia A, because BOECs express von Willebrand factor**,** which is known to act as a carrier protein for FVIII and prevents its proteolytic degradation. We previously demonstrated that therapeutic levels of plasma FVIII were documented from hemophilia A mice over 300 days, in which lentivirally-engineered blood outgrowth endothelial cells (BOECs) sheet were implanted subcutaneously (Tatsumi K et.al. PLoS One 2013 8(12):e83280). While this new technology is effective and safe in small animal such as mouse (20-25g body weight), the major challenge is in applying this functioning procedure in the patients with hemophilia A. For this purpose, the current study focus on an assessment of the safety of cell sheet implantation in canine models as the first step toward gene therapy in clinic. GFP-transduced BOECs were cultured on temperature-responsive poly (N-isopropylacrylamide) (PIPAAm)-grafted dish to engineer BOECs sheet. This dish allows for simple detachment of the cultured cells without the use of proteolytic enzymes such as trypsin and enables us to harvest the cell sheet as a contiguous monolayer that retains its native intercellular communications and intracellular microstructure, which are indispensable for normal cellular function. When the cultured BOECs reached confluency, the cultured cells were detached from the PIPPAm dish as a uniformly connected tissue sheet by lowering the culture temperature to 20°C for 30 minutes. Under general anesthesia using isoflurane, beagle dog (10-12Kg body weight) was placed on the operation table. To exteriorize the greater omentum, an abdominal ventral midline incision was made and 20 BOECs sheets in total were implanted. Upon completion, the omentum was placed back into the abdomen and the incision was closed. For the assessment of GFP expression, the liver samples were collected by open liver biopsy both 30 and 120 days after sheet implantation. We confirmed that implanted BOECs were differentiated into mature endothelial cells and contribute to new blood vessel formation by histological examination. In conclusion, tissue engineering approach by omental endothelial cell sheet implantation are viable for persistent tissue survival and providing therapeutic values in canine model. This novel ex vivo gene transfer strategy can provide the safe and efficacious delivery of FVIII in hemophilia A. Now, we are conducting FVIII gene transfer by cell sheet implantation in canine hemophilia A model.

162. Functional Correction of Mucopolysaccharidosis I in Adult Mice by a Systemic rAAV9-IDUA Gene Delivery

Aaron Meadows, Ricardo Pineda, Marybeth Camboni, Kathryn Waligura, Darren Murrey, Douglas M. McCarty, Haiyan Fu
Center for Gene Therapy, Research Institute at Nationwide Children's Hospital, Columbus, OH

Mucopolysaccharidosis (MPS) I is a lysosomal storage disease caused by autosomal recessive defect in iduronidase (IDUA). The lack of IDUA activity results in the accumulation of GAGs in cells in virtually all organs, leading to profound somatic and neurological disorders. No treatment is currently available for the neurological disorders of MPS I. In this study, we have developed a self-complementary (sc) AAV9 vector expressing human IDUA, targeting the root cause. A single intravenous injection of scAAV9-hIDUA vector at $5x10^{12}$vg/kg led to the rapid and persistent restoration of IDUA activity and the clearance of lysosomal storage pathology throughout the CNS, peripheral nervous system (PNS) and broad peripheral tissues, as well as the correction of astrocytosis in the CNS and PNS. Furthermore, we demonstrate that a single systemic scAAV9-IDUA gene delivery provides long-term neurological benefits in MPS I mice, resulting in significant improvement in cognitive and motor function, and extension of survival (ongoing). More importantly, functional benefits were also achieved in MPS I mice that were treated at advanced disease stages. These data demonstrate the promising clinical potential of systemic scAAV9-hIDUA gene delivery for treating MPS I and other neurogenetic diseases.

163. Hematopoietic Stem Cells Transplantation Can Normalize Thyroid Function in a Cystinosis Mouse Model

Heloise P. Gaide Chevronnay[1], Virginie Janssens[1], Patrick Van Der Smissen[1], Celine J. Rocca[2], Xiao-Hui Liao[3], Samuel Refetoff[3], Christophe E. Pierreux[1], Stephanie Cherqui[2], Pierre J. Courtoy[1]
[1]Cell Biology Unit, de Duve Institute & Université catholique de Louvain, Brussels, Belgium, [2]Pediatrics, UCSD, La Jolla, CA, [3]Medicine, The University of Chicago, Chicago, IL

Cystinosis is a multi-systemic lysosomal storage disease caused by defective transmembrane cystine transporter, cystinosin (*CTNS* gene). In the mouse model of cystinosis, $Ctns^{-/-}$ mice, it has already been shown that hematopoietic stem and progenitor cell (HSC) transplantation provides long-term protection of kidneys and eyes, which are affected early on in cystinosis. Tissue repair involves transfer of cystinosin-bearing lysosomes from HSPCs differentiated as macrophages into deficient adjacent cells, via tunnelling nanotubes (TNTs). Hypothyroidism is the most frequent and earliest endocrine complication in cystinotic patients and was recently shown as a complication in the $Ctns^{-/-}$ mice too. Here, we are evaluating the benefit of HSPC transplantation in the thyroid of $Ctns^{-/-}$ mice. Abundant engraftment of bone marrow-derived cells in $Ctns^{-/-}$ thyroid correlated with drastic decreased of cystine content, normalization of TSH level and correction of the structure of a large fraction of thyrocytes. In the thyroid microenvironment, HSPCs differentiated into a distinct, mixed macrophage/dendritic cell lineage expressing CD45 and MHCII, but not-detectably CD11b and F4/80. Like in cystinotic kidneys, HSPC-derived cells produced TNT-like extensions capable of crossing the follicular basement laminae. Interestingly, HSPCs themselves further squeezed into follicles, allowing extensive contact with thyrocytes, but did not transdifferentiate into Nkx2.1-expressing cells, the hallmark of thyrocytes. This is the first report demonstrating the potential of HSPC transplantation to correct thyroid disease, and supports a major multisystemic benefit of stem cells therapy for cystinosis.

164. Improvement of Gene Therapy for Wilson Disease

Oihana Murillo[1], Daniel Moreno[1], Cristina Gazquez[1], Iñigo Navarro-Blasco[2], Jesus Prieto[1], Ruben Hernandez-Alcoceba[1], Gloria Gonzalez-Aseguinolaza[1]
[1]Gene therapy, FIMA, Pamplona, Spain, [2]Biochemestry, University of Navarra, Pamplona, Spain

Wilson's disease (WD) is an autosomal recessively inherited copper storage disorder due to mutations in ATP7B gene that causes hepatic and neurologic symptoms. Current treatments are based on lifelong copper chelating drugs, which may cause side effects and do not restore normal copper metabolism. We have recently demonstrated that the administration of an AAV vector expressing ATP7B under the control of a liver specific promoter induces full restoration of copper homeostasis in a mouse model of Wilson's disease. However, the size of the vector genome surpasses the optimal size for AAV packaging, limiting the use of large promoters or additional regulatory sequences such as introns or bigger poly A sequences. In the present work we have designed two truncated versions of ATP7B protein (T1 and T2), and have analyzed their functionality and therapeutic efficacy. ATP7B contains 6 metal binding sites (MBS) in the N-terminal region. T1 presents a deletion of 1 MBS and a fragment of the adjacent one, whereas T2 lacks the first 4 MBS. Both proteins were active since they were able eliminate intracellular excess of copper in an in vitro system. The administration of recombinant AAV vectors carrying the truncated proteins under the control of a liver specific promoter was able to reduce liver damage and urinary copper excretion in the murine model, but T1 was less efficient than the WT ATP7B gene. In contrast, T2 showed full capacity to normalize these parameters. Furthermore, T2 was more efficient than WT in restoring ceruloplasmin oxidase activity in serum. In conclusion, we have identified a fully functional truncated version of ATP7B protein that will reduce the size of the therapeutic AAV vector genome and allow the introduction of additional regulatory elements.

165. Liver-Directed Gene Therapy for Murine Glycogen Storage Disease Type IB

Joonhyun Kwon[1], Jun-Ho Cho[1], Young Mok Lee[2], Goo-Young Kim[1], Javier Anduaga[1], Janice Chou[1]
[1]NICHD/NIH, Bethesda, MD, [2]University of Florida, Gainesville, FL

Glycogen storage disease type Ib (GSD-Ib) deficient in the glucose-6-phosphate transporter (G6PT or SLC37A4) is characterized impaired glucose homeostasis, myeloid dysfunction, and long-term complication of hepatocellular adenoma (HCA). We have shown that gene therapy mediated by a recombinant (r) AAV8 vector expressing G6PT directed by the chicken β-actin promoter/CMV enhancer enabled the *G6pt*-/- mice lived to over 51 weeks but all 5 transduced *G6pt*-/- mice expressed only low levels of hepatic G6PT activity and two developed multiple HCAs with one undergoing malignant transformation. We now examined the safety and efficacy of rAAV8-GPE-G6PT, a rAAV8 vector expressing G6PT directed by the gluconeogenic tissue-specific human *G6PC* promoter/enhancer (GPE). Of the fifteen rAAV8-GPE-G6PT-treated *G6pt*-/- mice that lived over age 60 weeks expressed 2-62% of wild-type hepatic G6PT activity with only one developed HCA. The treated mice, including the HCA-bearing mouse exhibit a leaner phenotype along with normal blood metabolite, display normal glucose tolerance profiles, maintain normoglycemia over a 24-hour fast, and retain insulin sensitivity.

We further show that activation of hepatic ChREBP signaling that improves glucose tolerance and insulin sensitivity is one mechanism that protects the rAAV-GPE-G6PT-treated *G6pt*-/- mice against age-related obesity and insulin resistance.

166. Lentiviral Hematopoietic Stem Cell Gene Therapy for Sjögren-Larsson Syndrome

Yoon-Sang Kim[1], Irudayam Maria Johnson[1], Dana S'Aulis[2], William B. Rizzo[2], Arthur W. Nienhuis[1]
[1]Hematology, St. Jude Children's Research Hospital, Memphis, TN, [2]Pediatrics, University of Nebraska Medical Center, Omaha, NE

Sjögren-Larsson syndrome (SLS) is a rare autosomal recessive disorder characterized by scaling skin (ichthyosis), mental retardation, and spasticity. SLS is caused by mutations in the *ALDH3A2* gene, which encodes fatty aldehyde dehydrogenase (FALDH), an enzyme that is involved in the oxidation of fatty aldehyde and fatty alcohol. In SLS, FALDH deficiency and impaired fatty aldehyde oxidation results in lipid accumulation, which is responsible for the symptoms. Spurred by previous hematopoietic stem cell gene therapy trials for multi-systemic diseases, such as Adrenoleukodystrophy (ALD), Metachromatic leukodystrophy (MLD), and Cystinosis, we investigated the plausibility of hematopoietic stem cell gene transfer to correct FALDH deficiency and alleviate the phenotype in SLS. We designed the hematopoietic stem cell gene therapy using a lentiviral vector containing human *ALDH3A2* cDNA based on our previous MND-WASP (Wiskott-Aldrich syndrome protein) vector design. The lentiviral vector contains the normal human *ALDH3A2* coding sequence which was derived from PCR products using human mobilized peripheral blood CD34+ cDNA. The vector was tested for FALDH protein expression in transfected human HEK293T cells by western blot and the enzyme activity was confirmed using HPLC-MS/UV. A transplantation experiment was performed using the *Aldh3a2*-/- KO mouse model, where the lineage negative BM cells from CD45.1 *Aldh3a2*-/- KO mice (C57BL/6 background) were sorted and transduced with a lentiviral vector followed by transplantation into lethally irradiated CD45.2 *Aldh3a2*-/- KO mice (C57BL/6 background). These mice are being monitored for cell engraftment, vector copy numbers, and any phenotypic changes. An initial analysis for the grafts showed 56% transduction in the MND(a synthetic promoter that contains the U3 region of a modified MoMuLV LTR with myeloproliferative sarcoma virus enhancer)-GFP group and 31% transduction in the MND-ALDH3A2 group based on the CFU-C data. Flow cytometric analysis of peripheral blood on these mice at 6 and 12 post- transplant week showed 32% transduction in MND-GFP group. Further analysis including vector copy numbers and end point analysis for various tissues including liver, spleen, and brain are underway. This work provides the foundation for moving forward with potential lentiviral hematopoietic stem cell gene therapy for non-hematological disorders.

167. Genome Editing to Generate the First Mouse Model of Alpha-One Antitrypsin Deficiency, the Leading Cause of Genetic COPD

Florie Borel[1], Brynn Cardozo[1], Andrew Cox[1], Weiying Li[1], Andrew Hoffman[2], Mai ElMallah[1], Christian Mueller[1]
[1]Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA, [2]Tufts University, Grafton, MA

Alpha-one antitrypsin (AAT) deficiency is a common autosomal co-dominant genetic disorder. This condition affects 1:2500 individuals of European ancestry, leading to the development of lung and liver disease. Within North American and Northern European populations, an estimated 4% of individuals are carriers of mutant alleles. AAT deficiency presents with an emphysema phenotype in the lungs of older subjects. AAT deficient subjects can also suffer from liver disease of varying severity; however, lung disease is the principle cause of death. AAT is a protease inhibitor predominantly synthesized in the liver that belongs to the serine protease inhibitor (serpin) family. Upon secretion into the blood stream, AAT enters the lungs where it inactivates excess neutrophil elastase, thereby preventing damage to the alveoli. Mutations of the *Serpina1* gene can lead to reduced serum levels of AAT and decreased protein functionality, allowing for unrestricted elastin breakdown, pulmonary inflammation and eventual emphysema. Currently, an animal model simulating the lung condition does not exist, which severely limits the development of therapeutics. This is due to the higher genomic complexity of mice compared to humans. Indeed due to amplification events, C57BL/6 mice have five genes that are homologous to human *SERPINA1*. To address this we generated a quintuple gene knockout using CRISPR/Cas9 system via zygote microinjection. We generated three founding lines in which all 5 copies of the gene were disrupted. Mice from all three lines demonstrate absence of hepatic and circulatory AAT protein as well as a reduced capability to inactivate neutrophil elastase. We also characterized the lung phenotype in response to a lipopolysaccharide challenge, where the model recapitulated many characteristics of the human lung disease including decreased elastance and increased compliance, and lung morphometry was also affected. Genomic and transcriptomic characterization will be presented. Future work will include challenges with cigarette smoke, a well-known disease accelerator in patients. Further, the ongoing generation of a new transgenic model carrying both the quintuple disruption of the murine *Serpina1* genes and a single copy of the Z variant of human *SERPINA1* will bring to the field the ultimate disease model that will finally allow researchers to evaluate the effects of liver-directed gene augmentation in the presence of Z-AAT polymers.

168. Assaying Hepatic Correction Mediated by Varied AAV Vectors in a Knock-Out Transgenic Mouse Model of Methylmalonic Acidemia (MMA)

Brandon T. Hubbard, Randy J. Chandler, Charles P. Venditti
National Human Genome Research Institute, National Institutes of Health, Bethesda, MD

Methylmalonic acidemia (MMA) is an inborn error of metabolism most commonly caused by deficient methylmalonyl-CoA mutase (MUT) activity. The disorder can have multiple clinical manifestations, including metabolic instability, stroke of the basal ganglia, pancreatitis, end-stage renal failure, growth impairment, osteoporosis and developmental delay. Unfortunately, current non-invasive therapies fail to chronically manage the disease, and patients still suffer from increased morbidity and early mortality. Solid organ transplantation, including elective liver, combined liver-kidney and isolated kidney transplantation, has been used to provide sustained benefit to patients, but the procedures come with substantial risks as well as the post-operative requirement for life-long immunosuppression. To address the large and unmet need for new therapies for patients with MMA, we have developed an effective adeno-associated viral (AAV) gene therapy that has been previously validated in a neonatal lethal mouse model of *Mut* deficiency (*Mut*$^{-/-}$). The current project compares how two distinct AAV8 vectors that express the human *MUT* gene under the control of either the liver specific, human alpha 1-antitrypsin (hAAT) promoter, or the ubiquitous CMV-enhanced chicken β-actin (CBA) promoter differentially affect metabolite levels following systemic delivery to adult mice. The animals used in this study (*Mut*$^{-/-}$;Tg$^{\text{INS-MCK-}Mut}$) express wild-type *Mut* in a muscle-specific fashion via a stable germline transgene and completely lack transgene expression in the liver. *Mut*$^{-/-}$;Tg$^{\text{INS-MCK-}Mut}$ mice accurately model the hepatorenal manifestations of MMA, but afford an opportunity to assess gene therapy vectors at or after weaning because mice are

rescued from neonatal lethality, yet experience massive elevations of the characteristic metabolites (methylmalonic and 2-methylcitric acid) because of the lack of hepatic Mut activity. Disease related metabolites were measured in plasma samples derived from *Mut*$^{-/-}$;Tg$^{INS\text{-}MCK\text{-}Mut}$ mice (n=7) prior to AAV gene delivery. The mice were then injected via the retro-orbital route with 1.5X10^{11} GC of either AAV8-hAAT-MUT or AAV8-CBA-MUT. At 10 days and 30 days post-treatment, mice treated with either vector showed a significant reduction in methylcitrate and methymalonic acid levels, with AAV8-CBA-MUT (n=2) treated mice manifesting methylcitrate and methylmalonic acid levels that trended lower than those measured in mice injected with AAV8-hAAT-MUT (n=4) at both time points. Further studies will be needed to precisely compare the differences between the hAAT and CBA promoters in MMA mouse models, but our preliminary results demonstrate that the *Mut*$^{-/-}$;Tg$^{INS\text{-}MCK\text{-}Mut}$ mice can be used to easily ascertain hepatic correction of Mut deficiency, which should help inform the selection of regulatory elements that will provide maximal therapeutic efficacy to treat patients with MMA.

169. Expression Studies of Ornithine Transcarbamylase in Liver Using Minicircle Vectors for Gene Therapy: Flag-Tagging of a Mitochondrial Enzyme and Stable Expression Under Control of an Endogenous *Otc* Promoter-Enhancer

Hiu Man Viecelli[1], Sereina Deplazes[1], Andrea Schlegel[2], Sharon Cunningham[3], Ian Alexander[3], Johannes Häberle[1], Beat Thöny[1]
[1]Division of Metabolism and Children's Research Center, University Children's Hospital Zurich, Zurich, Switzerland, [2]Department of Sugery, Swiss HPB and Transplant Center, Zurich, Switzerland, [3]Children's Medical Research Institute, Westmead, Australia

Ornithine transcarbamylase deficiency (OTCD) is the most common inherited defect of the urea cycle resulting in severe hyperammonemia and death if left untreated. We are aiming at correcting OTCD using naked-DNA minicircle (MC) vector mediated gene therapy in the *spf*^{ash} mouse model with low residual OTC activity. A critical parameter is delivery to periportal hepatocytes where the urea cycle is located because of metabolic zonation in the liver. To distinguish between MC-born and endogenous OTC enzyme, we generated an expression cassette with an internally Flag-tagged OTC enzyme. Internal epitope tagging is required because of the N-terminal mitochondrial import sequence. A corresponding Flag-tag sequence was introduced C-terminally of the mitochondrial import signal. The tagged protein performed similar to its non-tagged OTC enzyme upon hydrodynamic tail vein injection for liver targeting in *spf*^{ash} mice, indicating that the tag neither interferes with the mitochondrial import nor the formation of a functional OTC trimer. Furthermore we generated an endogenous *Otc* promoter-enhancer construct, termed PmO1, for potential specific or "natural" OTC transgene expression. According to others, the promoter (672 bp) sequence derived from mouse *Otc* was not sufficient for liver specificity (Veres et al, J Biol Chem 261: 7588-7591, 1986) and requires a corresponding enhancer sequence (Nishiyori et al, J Biol Chem 269: 1323-1331, 1994). An enhancer sequence (232 bp) originating from rat *Otc* was used that contains several binding sites for liver-selective transcription factors (Murakami et al, Mol Cell Biol 10: 1180-1191, 1990). The resulting MC-vector expressing OTC from this PmO1 promoter-enhancer construct was delivered to *spf*^{ash} mice via hydrodynamic tail vein injection, resulting in similar enzymatic activity in whole liver extracts compared to wild-type mice. Vectors designed to express Flag-tagged OTC driven from a natural *Otc* promoter might be useful to study biodistribution and/or long-term stable expression after MC-based gene therapy for OTCD.

170. PhaseRx mRNA Technology Platform Uses SMARTT Polymer Technology® to Target and Deliver mRNA to the Liver and Treat a Urea Cycle Disorder in a Mouse Disease Model

Mary Prieve, Allen Li, Alex Baturevych, Eric Bell, Teri Blevins, Anna Galperin, Pierrot Harvie, Jean-Rene Ella Menye, Sean Monahan, Amber Paschal, Maher Qabar, Debashish Roy, Matt Waldheim, Mike Houston
PhaseRx, Seattle, WA

Messenger RNA (mRNA) is a promising alternative in both the viral and non-viral DNA-based gene delivery fields. Current viral vectors for gene therapy are associated with serious safety concerns and nonviral vectors are limited by low gene transfer efficiency. mRNA gene expression in the liver can be used for treatment of genetic diseases involving disorders of metabolism. The majority are due to defects of single genes that code for enzymes expressed solely or predominantly in the liver. SMARTT Polymer Technology® has been developed into a robust platform for RNA therapeutics. Optimization of the mRNA technology platform has led to stepwise improvements in mRNA delivery to the liver using GalNAc targeted polymers. We demonstrated a 5,000-fold improvement in activity over our first generation delivery system.

Urea cycle disorders result from single gene mutations that lead to deficiency in one of the six enzymes in the urea cycle pathway. This deficiency can trigger elevated blood ammonia levels, also known as hyperammonemia, a life-threatening illness that leads to brain damage, coma or even death in humans. The deficient protein is intracellular and IV protein therapeutics are ineffective. Liver transplantation is the only cure for urea cycle disorders but is limited by the shortage of donors and complications associated with rejection and infection of the transplant. There is a dire need for new treatment options.

Using the mRNA technology platform, we have demonstrated preclinical proof of concept in a urea cycle disorder mouse disease model. Treatment with therapeutic mRNA shows normalization of blood ammonia levels in hyperammonemic mice. Therapeutic mRNA expression is detected in the liver after a single mRNA dose with good duration of expression. The treatment was well tolerated, with no toxicities associated with both single and multiple dosing regimens.

This mRNA technology platform provides a significant opportunity for the treatment of urea cycle disorders and other orphan liver diseases.

171. Quantification of Human FVIII and Estimation of Its Molecular Weight: Potential Applications in Gene Therapy for Hemophilia A

Rajeev Mahimkar, Barrie Carter, Gordon Vehar
Biomarin Pharmaceutical Inc, Novato, CA

Current protocols for measurement of FVIII rely on chromogenic or coagulation assays, and are not designed for quantification of FVIII antigen. Development of assays for measuring FVIII antigen may be necessary and beneficial for determining the efficiency of newer therapeutic approaches for Hemophilia A such as gene therapy. Herein, we describe the development of proprietary assays for quantification of human FVIII in plasma matrices from heterologous species, and estimation of the human FVIII molecular weight.

A variety of monoclonal antibodies against the FVIII heavy and light chain were screened for their ability to recognize human FVIII from normal human plasma pool, or CHO cell derived recombinant human FVIII-B domain deleted (FVIII-BDD) spiked in mouse and non-human primate (NHP) plasma. Based on the initial screen, a monoclonal each against the human FVIII heavy or the light chain was selected for enrichment, followed by detection using a sheep anti-

FVIII polyclonal antibody. Using this sandwich ELISA procedure, a standard curve was generated for human FVIII-BDD antigen in the range of 100 ng/ml - 0.1 ng/ml, and minimal cross reactivity was observed when tested with mouse and NHP plasma pools containing endogenous FVIII antigen. Furthermore, individual mouse strain plasma pools, and individual NHP samples representing different genetic origins were also screened for cross-reactivity to endogenous FVIII antigen and found to be negative. The sandwich ELISA was used to quantify FVIII antigen in defined plasma samples from normal or Hemophilia A human subjects, and antigen levels correlated with the reported FVIII activity.

The ELISA procedure was modified and adapted for estimation of the human FVIII antigen molecular weight. FVIII heavy and light chains, from normal human plasma pool or recombinant human FVIII-B domain deleted (FVIII-BDD), were enriched followed by resolution using a denaturing reducing PAGE and western blotting. The apparent migration of heavy and light chain was at the appropriate molecular weights; 90-185 kDa for heavy chain and 70 kDa for light chain. In case of heavy chain from normal human plasma pool, a ladder of differing molecular weights was seen and may be indicative of the differential furin and thrombin dependent processing of the B-domain.

Thus, these newly developed assays for quantification of human FVIII and estimation of its molecular weights will be useful for further characterization of gene therapy for Hemophilia A.

Cardiovascular and Pulmonary Diseases

172. B-Type Natriuretic Peptide Gene Therapy as a Novel Early Treatment for Familial Hypertrophic Cardiomyopathy

Cassandra A. Bongers[1], Sara J. Holditch[1], Claire A. Schreiber[1], John P. Konhilas[2], John C. Burnett[3], Yasuhiro Ikeda[1]
[1]Department of Molecular Medicine, Mayo Clinic, Rochester, MN, [2]Department of Physiology, University of Arizona, Tucson, AZ, [3]Department of Cardiovascular Diseases, Mayo Clinic, Rochester, MN

Hypertrophic cardiomyopathy (HCM) is the most common familial cardiomyopathy, with the prevalence between 0.2 to 0.5 %. HCM presents as an increase in left ventricular mass with thickening of the interventricular septum (IVS), myocellular disarray, interstitial fibrosis, heart rhythm abnormalities, and sudden death, necessitating therapeutic intervention. HCM is commonly caused by mutations in genes *MYH7*, *MYBPC3*, *TNNT2*, and *TNNI3*.

The cardiac hormone, B-type natriuretic peptide (BNP) is a guanylyl cyclase A (GC-A) agonist. Physiological effects of BNP include natriuresis, vasodilation and blood pressure-lowering action. Additionally, BNP shows direct cardioprotective properties including anti-hypertrophic and anti-fibrotic effects, independently of its anti-hypertensive effects. We have recently found that genetic BNP ablation results in progressive cardiac hypertrophy with upregulation in HCM-associated genes in rats. In the current study, we tested the hypothesis that sustained BNP treatment with novel gene delivery of BNP may preserve cardiac function and structure in HCM in a mouse model of HCM.

We used transgenic HCM mice expressing a mutated α-myosin heavy chain. Adeno-associated virus serotype 9 (AAV9) vectors were utilized to exploit their natural myocardial tropism. Three groups of mice were treated via tail-vein injections at four weeks of age (week one of study); Group 1, No HCM control (C57bl/6, no transgenic α-myosin heavy chain, n=6); Group 2, Untreated HCM mice with transgenic α-myosin heavy chain (PBS treated, n=10); Group 3, AAV9-BNP vector-treated HCM mice (n=6). To observe therapeutic benefit we assayed non-invasive blood pressure, myocardial architecture and remodeling (echocardiography), exercise capacity (treadmills), and any cardiorenal interplay through renal parameters. At 6, 9, 12, 15 and 25 weeks after AAV vector administration, left ventricular mass by echo was significantly higher in the untreated HCM group than the AAV-BNP-treated HCM and wildtype C57bl/6 mice. Significant increases in IVS and posterior wall thickness were also observed in control HCM group. At 11, 19, and 25 weeks after AAV vector administration, exercise capacity, monitored by treadmill tolerance, was significantly lower in the control HCM group than the AAV-BNP-treated HCM mice. No significant changes were seen in urine outputs. HCM AAV9-BNP treated mice showed a trend towards a lower blood pressure than both C57bl/6 and HCM mice.

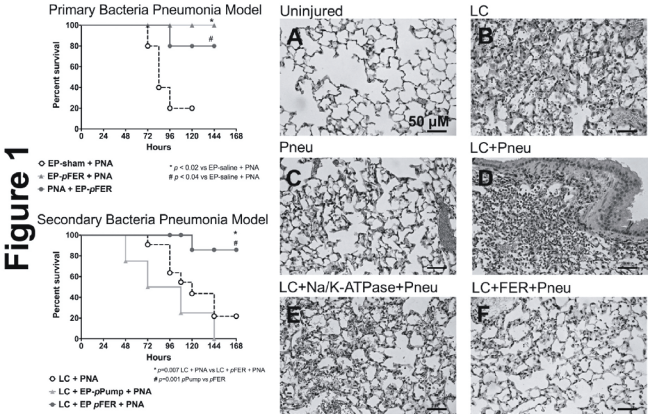
Our data demonstrate that AAV9-mediated BNP over-expression blocks progressive left ventricular enlargement and improves exercise capacity in a mouse model of HCM. The present study supports the BNP/GC-A/cGMP axis as a novel therapeutic target for the treatment of HCM.

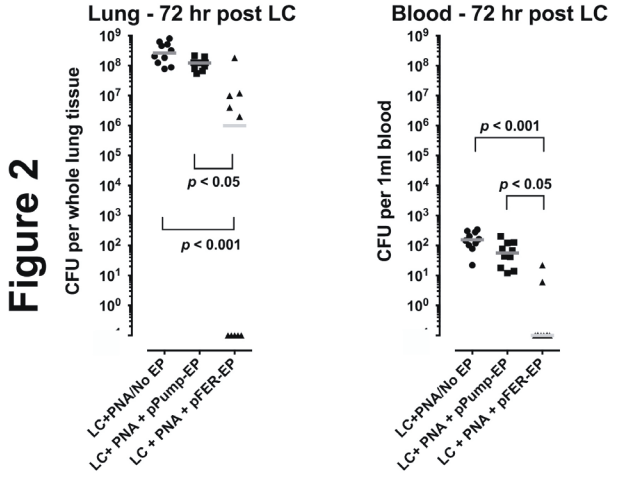
173. Electroporation-Mediated FER Gene Delivery in the Resolution of Severe Gram Negative Pneumonia

Vladislav Dolgachev, Rebecca Goldberg, Suresh Madathilparambil, Krishnan Raghavendran, Mark R. Hemmila, Mark Johnson, David Machado-Aranda
Surgery, Division of Acute Care Surgery, University of Michigan, Ann Arbor, MI

Introduction: The emergence of multi drug resistant bacterial pneumonia is a major concern among Intensive Care Units across the nation. Severely injured patients including those with lung contusion (LC) and requiring mechanical ventilation are particularly vulnerable in developing above forms of virulent pneumonia (PNA). The continued deterioration of these patients evolves into highly mortal Acute Respiratory Distress Syndrome. A recent GWAS study showed that FER expression, a non-receptor cytosolic tyrosine-kinase, was associated with survival from pneumonia and sepsis. We elected to study the role of FER gene in a murine model of bacterial PNA. We hypothesized that electroporation-mediated (EP) delivery of the FER gene would decrease mortality in severe PNA complicated by trauma. **Methods**: Two separate PNA models were created in C57Bl/6 mice by airway inoculation of 500 CFU of *Klebsiella sp.*, in naive (Primary PNA) or after blunt trauma-induced lung contusion (Secondary PNA). Treatment group(s) consisted on delivering naked plasmid DNA encoding FER to the lung (*p*FER) followed by square wave EP at 200 V/cm, eight 10 ms pulses, 1 s apart using external forelimb flat electrodes, either before or after insult. These plasmids were delivered with appropriate controls (saline EP {EP-sham} and plasmids encoding the Na^+/K^+-ATPase- which has shown favorable response in sterile inflammatory conditions {*p*Pump}). 7-day survival curves were recorded. Specific readouts included parameters of lung inflammation (Histology, Bronchial Alveolar Lavage [BAL], Taq-man PCR) and infection (blood-lung bacterial quantification) at 24 and 72 hrs. **Results**: Survival was markedly improved by *p*FER treatment, both in primary and secondary bacterial PNA as compared to EP-sham or *p*Pump controls. (**Figure 1 left**). Assessed parameters of inflammation showed also favorable changes being more prominent in secondary bacterial PNA, as visualized by H-E staining (**Figure 1 right**) .With *p*FER treatment there was a robust recruitment of monocytes in BAL (~20 fold increase over LC+PNA control, $p<0.0001$), with cells showing activation of bactericidal Fizz (~110 fold change over LC + PNA control, $p<0.0001$) and NOS (~160 fold change over LC + PNA, $p<0.0001$) genes. Blood and lung, bacterial counts were significantly decreased in the *p*FER group with some animals able to completely clear the lung of infection (**Figure 2**). **Conclusion**: EP-mediated delivery of the FER gene significantly

improved mouse survival in severe primary and secondary models of bacterial PNA. Mechanisms include recruitment of monocytes, activation of lung immune cells and improved bacterial clearance.





174. Post-Deployment Modifications of Stent with Endothelial Cells

Ilia Fishbein, Ivan S. Alferiev, Richard F. Adamo, David T. Guerrero, Yanqing Tang, Michael Chorny, Robert J. Levy
Pediatrics, The Children's Hospital of Philadelphia, Philadelphia, PA

Accelerated restoration of a functional endothelial layer in the injured arteries following stent angioplasty has been pursued over the past 20 years as an approach for preventing uncontrolled expansion of neointimal tissue and subsequent in-stent restenosis. To achieve enhanced re-growth of endothelium over the denuded arterial segments, strategies based on the pre-procedure seeding of stents with syngeneic/autologous endothelial cells (EC) and protocols involving stent surface modification to expedite capture and proliferation of circulating endothelial progenitor cells (EPC) have been explored. The main hurdle for the clinical translation of these "pro-healing" stents is the impediment in establishing an integral EC/EPC monolayer arrangement immediately after stent deployment, which is paramount for aborting platelet attachment to the exposed sub-endothelium and preventing inflammatory cell recruitment. The current work investigates a novel practical way to attain coverage of stent struts with syngeneic/autologous EC simultaneously with stent implantation. Stainless steel surfaces of tubing and stents were consecutively modified with 1% polyallylamine bisphosphonate, 4 mM sulfo NHS-LC-LC-biotin and 100 μg/ml avidin. Control samples were prepared omitting avidin attachment step. Rat aortic endothelial cells (RAEC) were surface modified in suspension with 1 mM sulfo NHS-LC-LC-biotin for 10 min, followed by pelleting and resuspension in PBS. Stainless steel tubing samples (avidin-coated or control; n=3 for both) were placed in succession inside 2 separate loops of polypropylene tubing that were mounted on a Chandler loop apparatus that models continuous blood flow. RAEC were resuspended in 20 ml of DMEM ($2.5x10^5$ EC/ml) and 10 ml of EC suspension was used to fill each loop. After 45 min of biotinylated RAEC recirculation through the avidin-modified and control steel samples (25 dynes/cm^2, 37°C), the samples were removed, fixed in 4% PFA, inverted and stained with Hoechst 33258. The density of fluorescent nuclei associated with avidin-modified and control stainless steel surface was determined by fluorescence microscopy, demonstrating a 50-fold higher attachment density of biotinylated EC on avidin-modified surfaces. Firefly luciferase expressing RAEC (via lentiviral transduction) were surface modified with 1 mM sulfo NHS-LC-LC-biotin and intraluminally delivered ($2x10^5$ cells) into temporally isolated segments of rat carotid arteries immediately after deployment of PAB/ sulfo NHS-LC-LC-biotin/avidin modified (N=4) or PAB only modified control stents (N=4). The animals underwent bioluminescent imaging 2 days post-stent deployment showing a 14.5-fold higher luminescent signal in animals treated with avidin-modified, than with control stents, demonstrating preferential attachment of biotinylated EC to the avidin-modified steel substrate and attesting to the viability of the delivered cells. Post-deployment binding of biotinylated EC to the surface of the avidin-modified stent can present a viable method of stent re-endothelialization and may have a therapeutic value in the prevention of in-stent restenosis and thrombosis.

175. Abstract Withdrawn

176. Novel Molecules That Enhance Adenovirus Transduction of the Airway Epithelium: Reinvigorating Adenovirus-Mediated Gene Therapy

Katherine J. D. A. Excoffon, Priyanka Sharma, James M. Readler, Mahmoud S. Alghamri
Biological Sciences, Wright State University, Dayton, OH

Adenovirus remains the most common vector system used in gene therapy clinical trials worldwide and several adenoviral vectors show promise in phase III clinical trials. One major limitation to the efficacy of adenovirus-mediated gene therapy is low transduction due to the limited primary receptor availability in most tissues. Interventions able to boost adenovirus transduction would have significant implications for both improving transduction and lowering the viral dose below the immunogenic threshold. Most human and several key animal adenoviruses share a common receptor with group B coxsackieviruses named the coxsackievirus and adenovirus receptor (CAR). CAR has several alternatively spliced isoforms. We have recently discovered that the eight-exon isoform (CAR^{Ex8}) can specifically localize to the apical membrane of polarized primary human airway epithelia where it can mediate apical adenovirus infection. Moreover, we have discovered that CAR^{Ex8} is both positively and negatively regulated by the cellular scaffolding protein MAGI-1. We hypothesized that cell permeable decoy peptides targeting the interaction between MAGI-1 and CAR^{Ex8} would increase CAR^{Ex8} protein levels and increase AdV transduction. Tat-conjugated peptide entry into polarized epithelia was confirmed by mass spectrometry and fluorescence microscopy. Relative to control, peptides increased the levels of CAR^{Ex8} at the apical surface of polarized epithelia and significantly increased AdV entry and transduction, as measured by apical surface biotinylation, qPCR, and AdV5-β-Gal transduction. Moreover, AdV5 transduction

was increased by 300-500% after intranasal peptide administration in mice demonstrating *in vivo* activity. To determine the mechanism of action, we followed the synthesis and co-localization of CAR^{Ex8} with key molecules in the cellular ER-Golgi-vesicular trafficking pathways. We found that CAR^{Ex8} was in compartments distinct from MAGI-1 and spread throughout the apical trafficking pathway and at the apical surface of the epithelium. Removal of the peptides reversed both the amount and localization of CAR^{Ex8} and returned the susceptibility of the epithelium to baseline. Taken together, these data indicate that decoy peptides able to block the MAGI-1-CAR^{Ex8} interaction transiently increase adenovirus-mediated gene transfer and offer the potential to increase the efficacy of adenovirus-mediated gene therapy.

177. Cross-Talk Between Human Relaxin Gene Therapy and Infarct-Related Coronary Artery

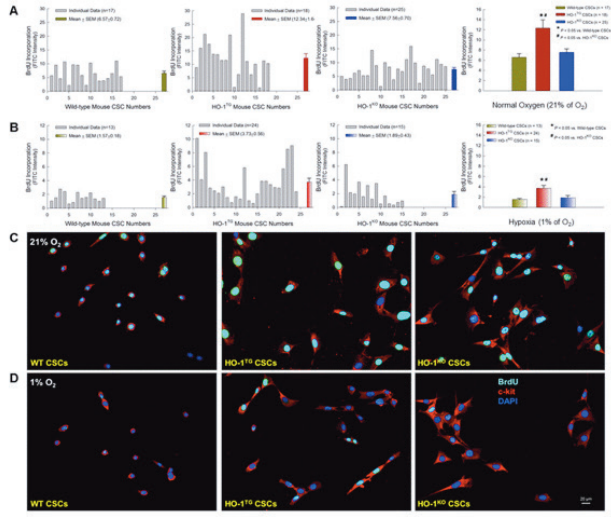
Young Sook Lee[1], Joung-Woo Choi[1], Jung-Eun Oh[2], Sung Wan Kim[1], Chae-Ok Yun[2]
[1]Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT, [2]Department of Bioengineering, Hanyang University, Seoul, Korea, Republic of

Background & Aim : The occlusion of coronary artery causes myocardial infarction (MI), leading cause of morbidity and mortality in the world. In the coronary angiography (CAG), the revascularization by reperfusion of infarct-related coronary artery is the key therapeutic modality. The systolic function evaluated by ejection fraction is a well-known traditional prognostic parameter. The non-invasive transthoracic Doppler echocardiography has been feasible to evaluate coronary artery in clinic (*J Am Soc Echocardiogr* 17(2):178-185, 2004). The purpose of this study is to valuate the efficacy of human Relaxin gene therapy in an infarct-related coronary artery, correlate the assessment of human Relaxin gene therapy with a typical cardiac function-structure relationship, and expand the significance of human Relaxin gene therapy on infarct-related artery to be a promising candidate for cardioprotective therapeutics after MI. **Method:** MI was induced by 30 min surgical occlusion of proximal left anterior descending (pLAD) at 7-8 wks-old male Sprague-Dawley rats (ischemia-reperfusion (I/R) injury group). Compared with I/R group and intramyocardial injection of *human Relaxin plasmid DNA* alone, the effects of intramyocardial injection of *human Relaxin* gene delivered by a bioreducible polymer were evaluated by 2D speckle-tracking Doppler echocardiography. **Results:** The diameter and velocity of the pLAD, infarct-related coronary artery, was assessed both during diastole and systole on 1 and 4 wks after I/R injury of MI. The diameter of pLAD in *human Relaxin* gene delivery group was more preserved than I/R group, up to the level of thoracotomy sham group on 1 and 4 wks after MI. In addition, the time velocity integral (TVI), stroke volume, and output in pLAD of *human Relaxin* gene delivery group were significantly higher than I/R group on 1 and 4 wks after MI. This diagnostic approach of infarct-related coronary artery of *human Relaxin* gene delivery was well correlated to the systolic function (EF), hemodynamics, and structural parameters in rat hearts after MI. **Conclusion:** This study suggests that the *human Relaxin* gene delivery system exerts reverse remodeling after MI on infarct-related coronary artery as well as systolic function, hemodynamics, and cardiac chamber quantification.

178. Effect of Heme Oxygenase-1 Overexpression on DNA Synthesis of Adult Cardiac Progenitor Cells Under Hypoxia

Ding Li, Landys Z. Guo, Qinghui Ou, Wei Xie, Roberto Bolli, Qianhong Li
University of Louisville, Louisville, KY

Although the studies in animals and humans have shown that the beneficial effects of adult c-kit^+ cardiac progenitor cells (CPCs) on left ventricle function and remodeling post-myocardial infarction persist for several weeks and up to 1 year after CPC administration, the vast majority (>95%) of transplanted CPCs in animal studies die or vanish shortly after cell administration. Additionally, the less blood flow in the scar tissue after heart attack results in the oxygen reduced to 1 - 2% (hypoxia) which makes the cardiac microenvironment inadequate for cell DNA synthesis, thereby implying that gene manipulations on the transplanted CPCs that limit cell death under hypoxia may enhance the efficacy (and thus clinical applicability) of cell therapy. One of the most powerful cytoprotective proteins known is heme oxygenase-1 (HO-1). To test the hypothesis that *HO-1* gene may improve newly synthesized DNA level in CPCs under hypoxia, adult CPCs were isolated and sorted from wild-type (WT), HO-1 transgenic (TG) or knockout (KO) mouse hearts. The c-kit positivity in all live CPCs was 60 - 65% detected by FACS with specific monoclonal anti-c-kit antibody in which Mast cells were used as positive control and CHO cells as negative control. HO-1 protein overexpression in $HO\text{-}1^{TG}$ CPCs was confirmed by FACS and immunoblotting (2.8-fold greater than that in WT CPCs); in contrast, $HO\text{-}1^{KO}$ CPCs had no HO-1. A hypoxia chamber system containing 1% of O_2, 5% of CO_2, and 94% of nitrogen was employed to mimic the damaged environment in the cardiac scar tissue. Furthermore, Bromodeoxyuridine (BrdU), an analog of thymidine that can be incorporated into the newly synthesized DNA of replicating cells in S-phase, was used to determine DNA synthesis levels in CPCs. Cells were cultured for 16 h and then labeled with BrdU for 30 min before BrdU immunofluorescent staining. Under normal oxygen tension (21% of O_2), $HO\text{-}1^{TG}$ CPCs exhibited an significant increase in nuclear BrdU intensity relative to either WT or $HO\text{-}1^{KO}$ CPCs (+88% vs WT group, and +63% vs $HO\text{-}1^{KO}$ group), indicating that the new DNA synthesis in $HO\text{-}1^{TG}$ CPCs are very active during cell mitosis as compared to WT or $HO\text{-}1^{KO}$ CPCs. Under hypoxic condition (1% of O_2), although new DNA synthesis in S-phase in all of the groups was much less active, $HO\text{-}1^{TG}$ CPCs showed even greater differences (+2.4-fold vs WT, +2.0-fold vs $HO\text{-}1^{KO}$) in the extent of BrdU nuclear incorporation as compared with WT or $HO\text{-}1^{KO}$ CPC group (Fig), providing a strong evidence that *HO-1* gene plays an important role in protecting CPCs under hypoxia via promotion of new DNA synthesis in BrdU-labeled cell mitosis. These results demonstrate that *HO-1* gene promotes new DNA synthesis during mitoses in adult CPCs not only under normal oxygen condition but also in the hypoxia environment, therefore $HO\text{-}1^{TG}$ CPCs may provide higher survival rate than WT and $HO\text{-}1^{KO}$ CPCs. The data support the hypothesis that *HO-1* gene improves the survival and reparative ability of adult CPCs via enhancing newly synthesized DNA in replicating cells in S-phase under hypoxia. Thus, the adult CPCs carried *HO-1* gene as a new approach may have potentially clinical application to increase the efficacy of cell therapy for patients with heart attack.



179. Helper-Dependent Adenovirus-Mediated Gene Transfer of an LDL Receptor/Transferrin Chimeric Protein Reduces Aortic Atherosclerosis in LDL Receptor-Deficient Mice

Eleonora Leggiero[1], Giuseppe Labruna[2], Laura Iaffaldano[1], Martina Esposito[1], Dalila Savoia[1], Lucia Sacchetti[1], Lucio Pastore[1]
[1]CEINGE-Biotecnologie Avanzate, Napoli, Italy, [2]IRCCS SDN, Istituto di Ricerca Diagnostica e Nucleare, Napoli, Italy

Familial hypercholesterolemia (FH) is a well-characterized genetic hyperlipidemia due in most of the cases to mutations in the LDL receptor (LDLR) gene; FH is characterized by elevated concentration of plasma LDL cholesterol (LDL-C) with consequent deposition of LDL-C in tendons, skin and arteries. Statins can lower cholesterol levels but are not effective in all patients whose prognosis is still quite poor. In the past, we have developed safe and effective gene-therapy strategies for hepatocytes transduction and consequent expression of anti-atherogenic proteins using PEGylated helper-dependent adenoviral (HD-Ad) vectors. We have recently devised a therapeutic strategy for reducing LDL using a secreted protein that can potentially be expressed in non-hepatic tissues used as bioreactors. At this aim, we developed an HD-Ad vector for the expression of the soluble form of the extracellular portion of the human LDLR fused with transferrin (LDLR/Tf). We evaluated the efficacy of LDLR/Tf in cellular models such as CHOldla7 in which we restored the cell ability to uptake of labeled LDL; subsequently, we administered intravenously 1X10E11 vp/kg of the HD-Ad vector expressing LDLR/Tf in LDLR-deficient mice and demonstrated the efficacy of the above-mentioned vector in reducing total and LDL cholesterol levels; in addition, expression of LDLR/Tf significantly reduced aortic atherosclerotic lesions in treated LDLR-deficient mice compared to controls 1.78±0.48 vs. 5.38±0.54 sq.mm.). We therefore demonstrated the efficacy of serum secretion of LDLR/Tf in reducing aortic atherosclerosis in FH mice. These results will allow the evaluation of HD-Ad vector-mediated expression of LDLR/Tf in non-hepatic tissues using alternative routes of administration in order to develop safer gene transfer protocol more compatible with clinical applications.

180. The Role of the Pyruvate Dehydrogenase Kinase 4 (PDK4) Gene Mutation in the Development of Dilated Cardiomyopathy in Dobermanpinschers and Potential Application of Cardiac Gene Therapy

Luiz Bolfer[1], Amara H. Estrada[1], Brandy Winter[1], Kathryn Taggart[1], Francisco Lourenço[1], Naohiro Terada[2], Barry J. Byrne[3], Thomas J. Conlon[4], Christina A. Pacak[5]
[1]Small Animal Clinical Sciences, University of Florida, Gainesville, FL, [2]Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville, FL, [3]Pediatrics and Molecular Genetics & Microbiology, University of Florida, Gainesville, FL, [4]Pediatrics and PGTC Toxicology Center, University of Florida, Gainesville, FL, [5]Pediatrics, University of Florida, Gainesville, FL

Dilated cardiomyopathy (DCM) is estimated to be the third most common inherited type of heart disease in humans reported to affect 35.6 and 0.57 in 100,000 adults and children, respectively. The Doberman Pinscher (DP) is a canine breed affected with idiopathic, non-ischemic DCM that closely resembles the human counterpart acting as a clinically relevant human model. Recently, a 16 base pair deletion in the 5' donor splice site of intron 10 of the pyruvate dehydrogenase kinase 4 (PDK4) gene, encoding for a mitochondrial protein involved in energy metabolism, has been identified but the relationship between the PDK4 mutation, mitochondrial function and phenotypic development of DCM is largely unknown. Our research has focused on understanding the relationship between the consequences of the PDK4 mutation on mitochondrial function and phenotypic development of DCM as well as the potential of cardiac gene therapy to correct the phenotype. A total of 64 DP were tested for the PDK4 mutation and screened for DCM. Healthy dogs and dogs diagnosed with DCM were further divided into 3 groups based on the presence of the genetic mutation: Wild-type (PDK4wt/wt), Heterozygous (PDK4wt/del), and Homozygous (PDK4del/del). Preliminary analyses using isolated mitochondria from skin fibroblasts of these dogs demonstrated a lack of detectable PDK4 protein expression in PDK4del/del, and a significant decrease in PDK4wt/del (48% of normal). Phase contrast microscopy and immunofluorescence studies revealed significantly different morpho-phenotypic characteristics and reduced capacity to adapt to unfavorable metabolic conditions as compared to healthy dogs. Assessment of mitochondrial metabolic potential and function (oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)) were performed using an extracellular flux analyzer. Cells were stressed using serum glucose starvation. Compared to healthy dogs, PDK4wt/del and PDK4del/del revealed a much lower basal respiration rate (OCR reduction of 46% and 68% of normal, respectively) and decreased metabolic potential to meet energy demands (85% and 72% of normal, respectively). Maximal mitochondrial respiration and spare respiratory capacity were all significantly lower in the affected dogs (PDK4wt/del 42% and 14%, and PDK4del/del 40% and 1.4% of normal OCR, respectively). Our results indicate that the mitochondria of affected dogs have a lower metabolic capacity and thus exhaust the two major energy-producing pathways, aerobic and glycolytic metabolism, at a much faster rate when under stress compared to healthy control dogs. Although we detected a significant decrease in PDK4 protein levels, PDK4 transcription levels have yet to be determined in these samples. Our immediate future goals include 1) quantitative PCR to compare PDK4 transcript levels, 2) PCR and sequencing to identify abnormalities in PDK4 mRNA splicing in affected dogs, 3) assessments of mitochondria ATP production and PDK4 protein kinase function. Our long-term goal is to perform in vitro experiments to assess if mitochondrial abnormalities can be improved with gene

therapy. To this end, we have cloned canine healthy PDK4 into an adeno-associated virus construct. Ultimately, our efforts will lead us to a cardiac gene therapy clinical trial for Doberman PDK4-DCM.

181. Optimization of *In Vivo* Delivery of Baculovirus Gene Therapy Vectors for the Treatment of Alpha-1 Antitrypsin Deficiency

Laura van Lieshout, Jondavid de Jong, Jacob van Vloten, Sarah K. Wootton
University of Guelph, Guelph, ON, Canada

Alpha-1 antitrypsin deficiency (AATD) is a single-gene disorder commonly associated with adult onset lung diseases, including emphysema, chronic obstructive pulmonary disease and airway inflammation. Alpha-1 antitrypsin (AAT) is produced primarily in hepatocytes and is subsequently secreted into the bloodstream and travels to the lung where it inhibits neutrophil elastase. Unregulated neutrophil elastase activity in individuals with AATD causes degradation of various components of the extracellular matrix producing damage to alveolar and airway epithelial cells. Furthermore, mutated AAT folds incorrectly causing intracellular aggregation within hepatocytes, which can result in liver damage in addition to pulmonary symptoms. The development of gene therapy strategies has been a popular approach for the treatment of AATD due to monogenic nature of this genetic disease. Recently, baculovirus based vectors have garnered attention for possible application in gene therapy due to multiple factors. Baculoviruses can tolerate large gene insertions (>38kb), are capable of transducing mammalian cells but not replicating or integrating into host chromosomes and finally, humans lack pre-existing immunity to these insect viruses. Although there are many characteristics that make baculovirus vectors attractive for use in gene therapy, there is little *in vivo* data on bio-distribution and tissue tropism in mammalian models. As AATD is a disease affecting both the liver and the lung, we examined several delivery routes of a baculovirus vector expressing the human placental alkaline phosphatase reporter gene in C57BL/6 mice. To target the lung we investigated intranasal and intratracheal administration and for liver delivery intravenous, intrahepatic and intraportal vein injections were utilized. Lung delivery of wild type baculovirus yielded little transduction, however intrahepatic injection resulted in moderate liver transduction as well as unexpected spread to other tissues. Different formulations of the baculovirus vector were examined for pulmonary delivery including a variety of viscoeleastic gels. Additionally, pharmacological agents such as clodronate liposomes to deplete macrophages and cobra venom factor to ablate complement activity were examined in an effort to bolster transduction.

182. Effects of Inflammation-Mediated Autophagy of Human Placental Mesenchymal Stem Cells of Fetal Origin on Their Immunomodulatory Properties and Therapeutic Potential in Bleomycin-Induced Pulmonary Fibrosis Models

Xiaoming Liu, Yongzhao Zhu, Xueyun Liang, Fei Han, Jun Wei
Stem Cell Research, Ningxia Medical University, Yinchuan, China

Pulmonary fibrosis (PF) is an interstitial pulmonary disease caused by lung injury and inflammatory response, which is currently incurable in clinical settings. Fibroblast proliferation and extracellular matrix accumulation are the main clinical manifestation and pathogenic features of PF. An compelling number of evidence has demonstrated that mesenchymal stem cells (MSCs) pose therapeutic potentials in PF disease, owing to their strongly immunomodulatory roles in injury repair of tissues, including the lung. In this regards, the autophagy of MSCs induced by inflammatory microenvironment of pulmonary injury plays a critical role in their immunomodulatory properties. Our works have recently revealed that human placental MSCs of fetal origin (fPMSCs) have an ability to alleviate and reverse the pathological progress in a bleomycin-induced PF model. In order to better understand mechanisms underpinning the therapeutic effect of fPMSCs in pulmonary fibrosis, the inflammation-mediated MSC autophagy in murine pulmonary fibrosis model was interrogated. Our results demonstrated that the inflammatory microenvironment of PF was able to induce MSC autophagy *in vitro* and *in vivo*, implying that the inflammatory microenvironment of PF may trigger autophagy of fPMSCs, which in turn affects their therapeutic potentials to PF. These results also suggest that an interaction between autophagy of fPMSCs and PF inflammatory microenvironment may have a fundamental impact on immunomodulatory functions and therapeutic effects of fPMSCs on pulmonary. **Key words:** placenta; mesenchymal stem cells; cell therapy; pulmonary fibrosis; autophagy.

183. Elucidation of Alpha-1 Antitrypsin Levels in Human Infant Blood Samples

Heather S. Loring[1], Alisha M. Gruntman[1], Qiushi Tang[1], Christian Mueller[1], Terence R. Flotte[2]
[1]Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA, [2]Gene Therapy Center; Department of Pediatrics; Microbiology & Physiologic Systems, University of Massachusetts Medical School, Worcester, MA

Alpha-1 antitrypsin (AAT) is a serum anti-protease that is produced in the liver and secreted into the circulation to function in the lung, protecting the alveolar interstitium from degradation by neutrophil elastase. AAT deficiency is a monogenic disease that results from mutations to the AAT gene. In the case of the PiZ mutation, the mutant Z-AAT produced in the liver undergoes loop-sheet polymerization inhibiting the secretion of AAT from the hepatocytes into the blood stream. Since AAT is sequestered in the liver, the protein is incapable of inhibiting neutrophil elastase's destruction of the alveolar epithelium, resulting in progressive lung disease. Accumulation of Z-AAT within the hepatocyte endoplasmic reticulum leads to hepatocellular stress, which can result in acute or chronic liver disease. About 5% of children afflicted with AAT deficiency develop acute liver failure within the first year of life. The cause for this acute liver failure in young patients has not yet been elucidated. Previous analysis of AAT levels in a mouse model expressing human Z-AAT from the endogenous AAT promoter has shown a considerable increase in hAAT concentration within the first few weeks of life, peaking at about $8.0*10^6$ ng/mL at eight weeks, followed by a decrease in AAT concentration leveling out at $4.0*10^6$ ng/mL at eighteen weeks of age. If the AAT promoter is regulated in a similar fashion in humans, one might predict that higher AAT expression in the early neonatal period may lead to increased accumulation of mutant AAT within the hepatocytes and precipitate fulminant liver disease early in life. In order to investigate this possibility, AAT levels were measured in healthy human infants over the course of the first two years of life. AAT levels were determined using a modified sandwich enzyme-linked immunosorbent assay (ELISA). Infant AAT levels demonstrate a biphasic trend with an initial increase to a maximum mean level of $2.8*10^6$ ng/mL at five months, followed by a gradual decreasing trend. Mean levels remain higher than mean adult levels ($1.3*10^6$ ng/mL) up to twenty-four months of age and higher than previously reported infant levels ($0.93*10^6$ - $2.51*10^6$ ng/mL) up to thirteen months of age.

Gene Therapy for Neurosensory Diseases

184. PGN-503, a Herpes Simplex Virus Based Vector Expressing Neurotrophin-3, Prevents and Reverses Neuropathy in a Mouse Model of Paclitaxel-Induced Peripheral Neuropathy

James R. Goss, David Krisky, Karen Bouch, Mark O'Malley, Sarah Coghlan, James Wechuck
Periphagen, Inc, Pittsburgh, PA

Paclitaxel-induced peripheral neuropathy (PIPN) is devastating constellation of symptoms that arise as a complication in patients receiving paclitaxel chemotherapy for cancer, most notably breast cancer. It presents as a glove and stocking neuropathy characterized by numbness, tingling, and occasionally burning pain in the feet and hands. Approximately 230,000 women will be diagnosed with breast cancer this year and over half, or about 120,000 women, will develop peripheral neuropathy as a consequence of paclitaxel therapy; this neuropathy will last for 5 years or longer in a quarter of these women. The development of PIPN may result in dose reduction of the paclitaxel, a switch to less efficacious agent, or even cessation of all chemotherapy treatment. Currently there are no approved therapies for the treatment of PIPN.

PGN-503 is a new investigation drug designed to prevent and/or treat PIPN. It is a herpes simplex virus type 1 based vector expressing human neurotrophin-3. Following a skin injection, PGN-503 is taken up by sensory nerves, transported back to the cell body, and directs the expression of neurotrophin-3 (NT3). NT3 is a well-studied neurotrophic factor necessary for the growth and maintenance of sensory neurons, and thus represents an ideal candidate for the treatment of primary sensory neuropathies. In a series of preclinical studies, we examined the efficacy of PGN-503 in a mouse model of PIPN.

Paclitaxel, dosed at 30 mg/kg/day for 6 days over two weeks, causes a peripheral neuropathy in BALB/c mice characterized by a long-lasting decrease in evoked sensory nerve action potentials (SNAPs) and conduction velocities (SNCVs). Mice, pretreated with a single injection of PGN-503 into the plantar surface of the hind paws 3 days prior to paclitaxel dosing, were completely protected against this decrease in SNAPs and SNCVs for the length of the study (approximately 4 months). This protection was dose-dependent on the total plaque forming units of PGN-503 applied to the paws. In a separate set of animals, we determined that a single application of PGN-503 was completely protective against the development of neuropathy following a second round of paclitaxel dosing beginning at 47 days post vector injection and partially protective against a third round beginning at 99 days post vector injection. Importantly, we have also demonstrated that PGN-503, applied one week after paclitaxel dosing, is capable of reversing the peripheral neuropathy. A Phase I/II trial of PGN-503 in preventing the development of PIPN in adjuvant breast cancer treatment is currently planned to begin next year.

185. Safety Study by Validated Immunoassays in a Phase III Study of Subjects with Inherited Retinal Dystrophy Due to Mutations in the Gene Encoding Human Retinal Pigment Epithelium-Specific Protein 65 (RPE65) Injected with Adeno-Associated Viral Vectors

Daniel J. Hui[1], Yifeng Chen[1], Thomas Antrilli[1], Yun Liu[1], Romuald Corbau[1], Stephen R. Russell[2], Albert M. Maguire[3], Jean Bennett[3], Katherine A. High[1]
[1]Spark Therapeutics, Philadelphia, PA, [2]Wynn Institute for Vision Research, University of Iowa, Iowa City, IA, [3]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

Mutations in the gene encoding Retinal Pigment Epithelium-Specific Protein 65 (RPE65) cause impaired vision from an early age and eventually total vision loss later in life. Recent work by our group and others demonstrated the use of *SPK-RPE65*, an adeno-associated viral (AAV) vector, to deliver the gene stably in small and large animal models, while Phase 1 and Phase 3 clinical trials with *SPK-RPE65* showed promising results (Maguire et al., 2008; Maguire et al., 2009; Simonelli et al., 2010; Maguire, AAO 2015). The Phase 3 trial was an open-label, randomized study using a subretinally-delivered AAV2 vector to augment the RPE65 gene. Thirty one subjects were enrolled from centers at The Children's Hospital of Philadelphia or University of Iowa. Twenty of the 21 intervention group subjects received 1.5E11 vg non-simultaneous injections to each eye. In addition to a separate efficacy assessment that measured the primary and secondary endpoints for the trial, an independent safety study was performed that included previously established immunological assays designed to monitor cellular immune responses. Two assays were subjected to further validation in-house and used for immune analysis of clinical samples obtained from all intervention subjects. An Enzyme-Linked Immunosorbent Assay (ELISA), capable of detecting a titer of at least 1.55 ug/mL anti-AAV2-capsid human IgG, was performed on 21 subjects covering up to 4 timepoints (baseline prior to injection, day 30, day 90 and 1 year). To better evaluate the results, the subjects were placed into 6 categories based on their antibody titer profile (Table 1).

Table 1: Anti-AAV2 Profile of Intervention Group (n=21)

Number of Subjects	Anti AAV Titer Range (ug/mL)	Anti-AAV2 Profile
7	<1.55	Below quantification limit
3	1.72 - 2.91	Low pre-existing antibody titer
2	16.37 - 19.61	Moderate pre-existing antibody titer
4	54.39 - 248.55	High pre-existing antibody titer
4	1.73 - 87.02	Antibody titer developed after vector administration
1	N/A	Withdrew

The other immunoassay performed on the intervention group was an interferon-γ Enzyme-Linked Immunospot Assay (ELISPOT) to assess T cell responses against AAV2 capsid or RPE65 transgene product. The same set of intervention subjects/timepoints was analyzed with positive T cell responses defined as ≥50 spot forming units (SFU) and 3-fold the background (media) control for AAV2 and greater than the statistically determined cutoff of 161.3 SFU for RPE65. Eighteen of the 21 intervention subjects tested negative for T cell responses against AAV2 and RPE65 across all timepoints. One subject was positive against AAV2 capsid at baseline (55.0 SFU) and positive against RPE65 at the 1 year timepoint (171.7 SFU). Another subject was positive at 1 year for RPE65 only (170.0 SFU). These positive responses were considered very weak with respect to threshold cutoff values. One subject displayed a moderate response

(518.3 SFU) against RPE65 at baseline only, while all subsequent timepoints were negative. The positive T cell responses observed at baseline prior to vector administration are unlikely to be related to gene transfer. Considering the cumulative immune response data collected from the two validated immunoassays performed here, the Phase 3 trial provided results supporting the immunologic tolerability of the delivery of AAV2 vector to the subretinal space in the eye to treat inherited retinal dystrophy due to mutations in RPE65. Efforts are ongoing to correlate these findings with available efficacy data.

186. Translating an Optogenetic Gene Therapy Approach for Treatment of Neuropathic Pain in Humans

Chris Towne, Jesus Aguado, Annie Arguello, Claire Discenza, Tim Galfin, Stephan Gehrke, Sabeena Khan, Michael Kaplitt
Circuit Therapeutics Inc, Menlo Park, CA

Optogenetics has been established as a powerful tool to study the central and peripheral nervous systems with its potential for directly treating human disease tantalizingly on the horizon. Optogenetic inhibition of pain in mice has been shown to be effective and provides an attractive initial application. We aim to translate this approach to treat neuropathic pain in humans. Firstly, we demonstrated that the inhibitory opsin, NpHR, delivered by intraneural injections of AAV6 could reduce pain (mechanical allodynia) in a clinically meaningful paradigm i.e. vector delivery after establishment of neuropathic pain in the chronic constriction injury (CCI) model. The inhibitory opsin, iC1C2 (a chloride channel requiring less light than NpHR to pass currents) could also reduce pain in this system. We next tested a surgical approach more amenable to patients than nerve injection. Lumbar puncture is a routine clinical practice and AAV8 delivery by this method results in efficient gene delivery to sensory neurons in rodents, dogs and pigs. We found that intrathecal administration of AAV8 expressing iC1C2 could decrease mechanical allodynia in the CCI mouse model. The magnitude of pain inhibition correlated with transduction levels and only 10% transduction of sensory neurons was required for pain relief. To further validate this approach we utilized a second rodent pain model with translational relevance. The mouse tibia fracture/cast immobilization model of Complex Regional Pain Syndrome (CRPS) presents extreme allodynia and recapitulates human disease effectively. We found that intrathecal delivery of AAV8 encoding iC1C2 following development of the CRPS model resulted in light-mediated pain inhibition. Taken together, our results confirm optogenetic therapy for neuropathic pain as an attractive clinical application of this technology.

187. TFEB-Mediated Clearance of the Lipofuscin Fluorophore A2E

Ivana Trapani, Elisabetta Toriello, Renato Minopoli, Alberto Auricchio
Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli, Italy

Abnormal accumulation of various by-products of the visual cycle, including the diretinoid-pyridinium-ethanolamine (A2E), in retinal pigment epithelium (RPE) is an hallmark of both Stargardt disease (STGD) and age-related macular degeneration (AMD). This is responsible for RPE and, consequently, photoreceptor (PR) cell death. A2E storage in RPE lysosomes has been shown to reduce both the capacity of RPE to degrade phagocytized PR outer segments and autophagosome biogenesis, trafficking and autophagic flux. The transcription factor EB (TFEB) is a master regulator of cellular clearance. Here we tested if TFEB overexpression induces A2E clearance from the RPE cells. We have generated a plasmid encoding for a double-serine mutant (S142A, S211A) form of TFEB, which is constitutively active. We have transfected this plasmid in the human RPE-derived ARPE19 cells to evaluate TFEB activation of its transcriptional targets and ability to clear A2E after loading. We found that TFEB overexpression in ARPE19 cells results in the induction of TFEB transcriptional targets involved in cellular clearance. Importantly, TFEB overexpression in ARPE19 cells was associated with reduction of intracellular A2E accumulation. Taken together these results suggest that TFEB overexpression is an effective strategy to promote A2E clearance in vitro. Further studies will clarify if this holds true in vivo. These results may have implications for the therapy of both STGD and the more common AMD.

188. AAV Mediated Gene Therapy to Modulate Neurotropic Factors in the Retina and in Neuronal Cells in Culture

Nitin Chitranshi, Roshana Vander Wall, Yogita Dheer, Stuart L. Graham, Vivek Gupta
Clinical Medicine, Macquarie University, Sydney, Australia

Neurotrophic factors, particularly Brain Derived Neurotrophic Factor (BDNF) and its high affinity receptor TrkB, play an important role in protecting the retinal ganglion cells (RGCs) in glaucoma. Translation of the immense neuro-protective potential of BDNF/TrkB activation has been largely unsuccessful and gene therapy to modulate BDNF and TrkB have also shown only short-lived protective effects on the RGCs. *PTPN11* phosphatase plays a key role in the regulation of BDNF/TrkB signaling in the retina. Here, we investigated the effects of *PTPN11* modulation involving both over-expression and knock-down paradigms on the TrkB activation. *PTPN11* and PTPN11-shRNA sequences were separately incorporated into the AAV2 vector under cytomegalovirus chicken β actin hybrid promoter linked with green fluorescent protein (GFP). SH-SY5Y cells were transfected with each of the *PTPN11* modulating and control GFP constructs. PTPN11 over-expression resulted in significant reduced TrkB phosphorylation and accordingly reduced viability of the SH-SY5Y cells was observed while *PTPN11* knockdown did not exhibit any significant effects on either TrkB phosphorylation or cellular survival. *PTPN11* over-expression in neuronal cells was also accompanied by elevation of GADD, PERK and XBP-1 endoplasmic reticulum (ER) stress marker proteins as detected using western blotting. The roles of *PTPN11* and TrkB in mediating ER stress were evaluated by treating cells with PTPN11 inhibitor (PHPS1) or TrkB agonist (7,8-dihydroxyflavone) independently, both of which ablated the up-regulation of ER stress proteins upon *PTPN11* over-expression. The effects of PTPN11 over-expression on the rat retina were evaluated by administering *PTPN11* AAV2 construct along with corresponding GFP controls individually into the rat eyes. Briefly, 5µl of AAV2 constructs were injected intravitreally into SD rats ($2x10^{10}$ vg). Anti-GFP staining in retinal sections demonstrated AAV2 transduction in the RGC layer. Anti-NeuN/ anti-PTPN11 staining confirmed the ganglion cell specific over-expression of *PTPN11*. Rat retinas were assessed for inner retinal functional changes using scotopic threshold response (STR) measurements. Retinal structural changes were assessed using histological analysis. Results indicate that pSTR amplitude which primarily reflects the function of the RGCs was significantly diminished in the *PTPN11* over-expression model. Hematoxylin and eosin staining also revealed degenerative changes primarily associated with the inner retina. Assessment of optic nerve sections using Bielschowsky's staining further identified reduced axonal density. Concluding, our findings strongly indicate that *PTPN11* genetic modulation holds the promise to regulate neuronal cell survival through its effects on TrkB activation. *PTPN11* over-expression exerts detrimental impact on the inner retinal health which has significant implications in glaucoma and other retinal disorders. Further studies involving rescue of the disease phenotype

using *PTPN11* knockdown in animal models will substantiate these observations and provide mechanistic insights into the roles of neurotrophic factor regulation in retina.

189. Therapeutic Gene Transfer as a Treatment Option for Age-Related Macular Degeneration

Erik Wielechowski[1], Angelica Medina-Jaszek[1], Omua Ahonkhai[1], Muhammad Arif[1], Jean Bennett[2], Albert Maguire[2], Anna Tretiakova[1], James M. Wilson[1]
[1]Gene Therapy Program, Department of Medicine, University of Pennsylvania, Philadelphia, PA, [2]Department of Ophthalmology, University of Pennsylvania, Philadelphia, PA

Age-related macular degeneration, otherwise known as AMD, is a progressive, irreversible eye condition in which the retina is permanently damaged by either atrophy of its pigment epithelial layer (dry AMD) or abnormal blood vessel growth in the retina's membrane (wet AMD). Wet AMD, the more advanced, severe version of the disease, is induced by overexposure to vascular endothelial growth factor (VEGF) in the eye. This VEGF-induced blood vessel growth leads to rupture and leaking of the retina's membrane, endangering the integrity of the macula, which is vital for acute central vision. Once the macula is scarred and central vision is lost, it cannot be recovered. Current treatments for wet AMD include several drugs (i.e. lucentis) that inhibit VEGF, yielding a reduction in retinal thickness and moderate increases in visual acuity. While these treatments succeed in prohibiting disease progression, they require frequent intravitreal injections. In the future, the goal is to prevent disease progression while avoiding repeated injections with the use of therapeutic gene transfer.

To develop this new generation of AMD therapeutic, we sought to create an efficacious AAV vector expressing anti-VEGF antibody via systematic optimization of all vector elements. Six promoters, 12 coding sequence variants, an F2A linker, and 3 internal ribosome entry sites (IRES) were evaluated for tissue-specific expression in mouse eye. Mean expression from the 2 best promoters, UbC and CB7, was 400 and 450 ng/mg of eye lysate, respectively, compared with the CMV promoter at 10 ng/mg. In the context of a CMV promoter, mean expression for best-performing IRES (EMCV) was 2 ng/mg of tissue lysate, which was 5-fold lower than that from the construct containing an F2A linker (10 ng/mg). The best-expressing coding sequence variants, named AMD11 and AMD42, yielded 12 and 13 ng/mg of tissue lysate, respectively.

In order to identify a clinical candidate vector, the best-performing elements were combined to generate 4 additional vectors, which were evaluated in both eyes of 8 rhesus macaques for each vector. Following subretinal injection with $1x10^{11}$ GC/eye, anterior chamber fluid was sampled on days 15 and 29. Among the 16 injected eyes, average expression of AAV-UbC-AMD11 was 473.6 ng/ml (ranging from 123.5 to 933 ng/ml) of anterior chamber fluid on day 15 after vector administration and 546.2 ng/ml (262 to 1263 ng/ml) on day 29. Average expression of AAV-UbC-AMD42 was 360.6 ng/ml (144.6 to 646.8 ng/ml) and 536.8 ng/ml (238.3 to 920.7 ng/ml) on days 15 and 29, respectively. For AAV-CB7-AMD11, average expression on days 15 and 29 was 972.4 ng/ml (495.9 to 1684.7 ng/ml) and 1100.3 ng/ml (422.3 to 2187.8 ng/ml). The final vector, AAV-CB7-AMD42, expressed at 1922.2 ng/ml (707.9 to 2892.7 ng/ml) on day 15 and 1396 ng/ml (431.2 to 2346.4 ng/ml) on day 29. After final selection, the clinical candidate vector will be evaluated in formal nonclinical studies prior to a clinical trial. Selection criteria will include expression levels and vector yields.

Cancer-Immunotherapy, Cancer Vaccines I

190. Adaptive CAR T Cell Design

Norihiro Watanabe, Sujita Sukumaran, Pradip Bajgain, Salma Ansari, Helen E. Heslop, Cliona M. Rooney, Malcolm K. Brenner, Ann M. Leen, Juan F. Vera
Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

Chimeric antigen receptor (CAR) T cell therapy has recently emerged as an attractive approach for the treatment of CD19-expressing hematological malignancies. However extending the success of this strategy to other targets has proven to be more complicated that simply replacing the scFv. To address this issue we have implemented a form of adaptive CAR design whereby a series of sequential modifications are made to a single domain and subsequently tested in vitro and in vivo to assess activity. We illustrate the utility of such a strategy using our published CAR-PSCA (Anurathapan U. et al, Mol Ther. 2014) as a template (CAR-PSCA v1.0) with subsequent iterations coded as versions 2.0, 3.0, 4.0 and 5.0. We now demonstrate how modifications made to a single CAR structural domain can result in enhanced (i) T cell migration, (ii) antigen recognition, and (iii) cell phenotype, ultimately producing superior anti-tumor effects. First, with CAR v2.0 and v3.0 we were able to improve T cell migration, which was evident in NSG mice engrafted s.c. with Capan-1 and treated i.v. with FFluc+ T cells. Ten days post CAR administration we saw a 2 log increase in the T cell signal at the tumor site ($4.5\pm2.3x10^5$ p/s vs $4.8\pm0.5x10^7$ p/s vs $4.0\pm1.1x10^7$ p/s, CAR v1.0, v2.0 and v3.0 respectively). Subsequently to enhance in vivo T cell persistence, we next generated CAR v4.0, which resulted in a less differentiated T cell phenotype (T_{naive}: 1.8±0.6% to 19.2±4.0%, T_{CM}: 10.4±1.4% to 14.1±3.0%, T_{EM}: 83.5±1.2% to 53.7±6.9% and T_{EMRA}: 4.3±0.9% to 12.9±1.7% - CAR v3.0 and CAR v4.0, respectively). When administered to Capan-1-engrafted NSG mice CAR v4.0 T cells exhibited enhanced in vivo longevity as measured using bioluminescence imaging ($7.3\pm4.6x10^7$ p/s CAR v3.0 vs $2.8\pm1.7x10^8$ p/s CAR v4.0 T cells - day 35 post-administration). Finally, antigen recognition of CAR-PSCA was further improved in v5.0 where a final modification to the same domain produced superior anti-tumor effects against a PSCA-dim target tumor cell line (DU145) in a 6hr ^{51}Cr-release assay (20.7±5.8% vs 48.4±5.2%, CAR v4.0 vs CAR v5.0, 40:1 E:T). Overall, therefore, implementation of this adaptive design produced a CAR T cell product with enhanced in vivo anti-tumor activity. This was clearly illustrated when we compared the tumor volume of NSG mice treated with CAR v1.0 or CAR v5.0 T cells (1309 ± 143 mm^3 vs 510 ± 53 mm^3 on Day 66). Specific details of the modifications conducted in this adaptive CAR design will be presented.

191. Development of an Effective Cancer Vaccine Platform Using Attenuated Salmonella to Deliver Recombinant Tumor-Associated Antigens

Xin Xu[1], Michael S. Wood[1], Suhrab Kurbanov[1], Linjie Guo[1], Xiuhua Gao[1], James E. Galen[2], Leonid S. Metelitsa[1]
[1]Pediatrics, Texas Children's Cancer Center, Baylor college of medicine, Houston, TX, [2]Center for Vaccine Development, Division of Geographic Medicine, University of Maryland School of Medicine, Baltimore, MD

Inadequate antigen delivery is one of the major limitations of modern cancer vaccine vectors. To overcome this challenge, we exploited Salmonella Pathogenicity Island 2 (SPI2) and its type III secretion system (T3SS) to deliver a tumor-associated antigen (TAA) of choice into the cytosol of antigen-presenting cells (APC) in situ. The goal of this study was to explore and exploit the potential of

SPI2-encoded T3SS of clinically validated S. typhi strain CVD908 for construction of an effective cancer vaccine.We engineered the clinically validated S. typhi strain CVD908 to express SPI2-regulated dominant-negative oncoproteins survivin and MYCN. To adapt CVD908 to stably express recombinant antigens without antibiotic-dependent selection, we used a recently reported plasmid stabilization system that encodes the single-stranded binding protein (SSB), an essential protein in DNA metabolism, which was deleted from the bacterial chromosome. The SPI2-regulated expression cassette was then cloned into the SSB plasmid, so that the resultant construct maintained bacterial vector stability. We found that CVD908Δssb vector could effectively infects dendritic cells and induce antigen-specific CD8 T cell responses in vitro and in vivo. Furthermore, therapeutic vaccination with CVD908Δssb vector expressing survivin or MYCN produced potent antitumor activity in murine models of neuroblastoma and lymphoma.Thus, oral antigen delivery via SPI2-encoded T3SS of Salmonella typhi may provide the foundation of an effective cancer vaccine platform.

Supported by grants from CPRIT (RP121035) and The Leukemia and Lymphoma Society.

192. Deep Phenotyping of Manufactured Enhanced-Affinity NY-ESO-1-Specific T Cells Reveals a Pattern of Effector and Memory Programming That Correlates with Clinical Outcome in Observed Cancer Indications

Luca Melchiori[1], Daniel E. Lowther[1], Gwendolyn K. Binder-Scholl[1], Daniel D. Williams[1], Yoav Peretz[2], Marylene Fortin[2], Willian D. Tap[3], Aaron P. Rapoport[4], Crystal L. Mackall[5], Bent K. Jakobsen[1]

[1]Adaptimmune Therapeutics, Abingdon, United Kingdom, [2]ImmuneCarta, Montreal, QC, Canada, [3]Memorial Sloan-Kettering Cancer Center, New York, NY, [4]University of Maryland, Baltimore, MD, [5]National Cancer Institute, Bethesda, MD

It is well established that human tumors express unique antigens; however, immunosuppressive mechanisms prevent natural immune responses. We hypothesized that infusion of T cells that have been genetically modified to express affinity-optimized tumor antigen-specific TCRs may overcome these barriers. We initiated several ongoing clinical studies to evaluate T cells engineered with an affinity-enhanced TCR specific for the NY-ESO-1 and LAGE-1 cancer testis antigens (NY-ESO^{c259}-T), in synovial sarcoma (SS) and multiple myeloma (MM) patients with antigen-positive tumors. Here we report updated phenotyping results from two MM and SS cohorts of patients. Manufactured product (MP) and baseline samples were analyzed by flow cytometry evaluating memory and activation markers (e.g. CD45RA, CCR7, ICOS, OX40, etc) and polyfunctionality markers (e.g. IFN-ɣ and IL-2). NY-ESO^{c259}-T was detected by pentamer staining and its phenotype was correlated with clinical response. Upon culture NY-ESO^{c259}-T generated different memory phenotypes. The phenotype at baseline (day -50) did not predict the phenotype acquired at the end of culture. Interestingly, in MM a positive trend with clinical response was observed in MPs bearing a higher percentage of Central Memory (CM) cells secreting IL-2 and IFN-ɣ, while a negative trend was found in Effector Memory (EM) and Effectors (EMRA)-dominated MPs secreting high levels of TNF-α. This trend was confirmed by analysing the absolute number of CM cells infused in a small number of patients, hinting at the existence of an "effective CM cells dose" of around $3x10^8$ CM cells necessary to achieve clinical response. Of note, this trend was observed also in the SS cohort. Upon stimulation T cells proliferate and differentiate *in vitro*, but at the same time cells need time in culture to reach an effective dose. To understand these dynamics we retrospectively analysed the evolution of CM cells in relation to the length of culture. We observed a negative correlation between percentage of CM cells and culture time, while EM and EMRA cells increased with the duration of culture. This suggests the importance of reconciling time of culture with phenotype and number of cells in order to achieve that "sweet spot" critical for clinical response. Activation/costimulation markers in the SS cohort were also analysed in relation to culture conditions. MPs consistently displayed up-regulation of ICOS, CD40L and OX40, suggesting that an activated phenotype is achieved even after several days of culture. Of note, markers of immunological memory like CD27 and CD28 were also maintained up to the end of culture highlighting the fitness of the product despite the duration of the culture. These data suggest that NY-ESO^{c259}-T may acquire a CM phenotype that positively correlates with clinical response in the cancer indications observed. Additionally, we show how the manufacturing process successfully produces a population of activated cells that express important activation markers without losing markers of fitness and memory - properties that are key to driving the anti-tumour response observed in cancer patients.

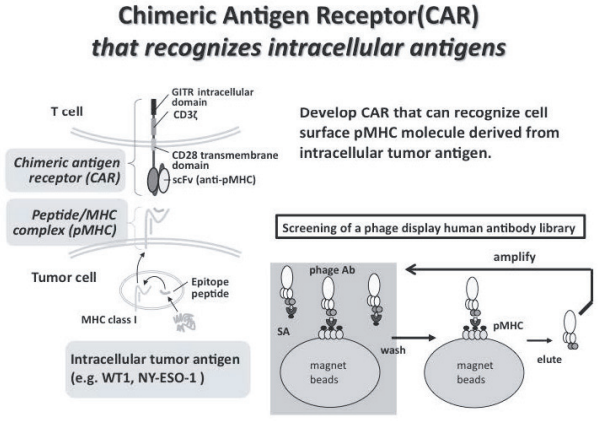
193. Efficacy and Safety of Immunotherapy with Chimeric Antigen Receptor Targeting WT1 and HLA-A24:02 pMHC Complex

Hiroaki Ikeda[1], Yasushi Akahori[1], Yuki Orito[1], Yoshihiro Miyahara[1], Yasunori Amaishi[2], Sachiko Okamoto[2], Junichi Mineno[2], Kazutoh Takesako[2], Hiroshi Fujiwara[3], Masaki Yasukawa[3], Hiroshi Shiku[1]

[1]Department of Immuno-Gene Therapy, Mie University Graduate School of Medicine, Tsu, Japan, [2]Takara Bio Inc., Kusatsu, Japan, [3]Department of Hematology, Clinical Immunology and Infectious Disease, Ehime University Graduate School of Medicine, Toon, Japan

Recent success in the treatment of patients with B cell malignancy by CD19-CAR encourages the development of successive CAR therapy targeting broad range of tumor-associated antigens. However, the search for the appropriate target molecule for CAR, other than B cell markers, has not met the needs. The molecules recognized by CAR is generally limited to the cellular surface molecules, making difficult the search for the tumor-specific targets. Inspired by the physiological recognition of epitope peptide and MHC molecule (pMHC) by T cells, we have generated a series of antibodies that recognize the pMHC complexes with peptides derived from tumor antigens expressed intracellularly. Screening a phage display library of human antibody scFv, we isolated an scFv antibody clone "WT#213" that can specifically recognize WT1 p235-243 peptide (CMTWNQMNL) complexed with HLA-A24:02 molecule. We have constructed a retroviral vector that encodes the CAR consists of WT#213 and intracellular signal transduction domains of CD3ζ and GITR (WT#213 CAR). We confirmed the specific recognition of endogenous WT1-expressing cells by the CAR-T cells with the intracellular cytokine staining and the ^{51}Cr release cytotoxic assay. Moreover, we demonstrated the effectiveness of adoptive cell therapy with WT#213 CAR against the WT1 expressing HLA-A24:02 positive human leukemia cells, utilizing NOG immunodeficient mice. To evaluate the safety of the WT#213 CAR, we predicted the potential property of WT#213 CAR to cross-react to normal tissues in humans. We conducted alanine scan analysis of WT1p235-243 peptide that was recognized by WT#213 CAR as well as the TCR derived from CTL clone TAK-1 which recognizes same epitope peptide in association with HLA-A24:02 to define the amino acids that were critically important in the recognition by the WT#213 CAR or TAK-1-derived TCR. After BLAST search, we synthesized the normal protein-derived peptides with potential risk of cross-reactivity, and tested the recognition of these peptides by WT#213 CAR or TAK-1-derived TCR. Although the critical peptides, and therefore the peptides with

potential risk, were quite different between the WT#213 CAR and TAK-1-derived TCR, none of these peptides showed the stimulation of WT#213 CAR or TAK-1-derived TCR. The results here suggest that the immunotherapy with WT#213 CAR will be effective for the treatment of the leukemia patients without the predicted risk in the evaluation we performed.



194. Adenoviruses Armed with TNFα and IL-2 Successfully Enable Adoptive T-Cell Therapy

Aino Kalervo[1], Mikko Siurala[1], Riikka Havunen[2], Suvi Parviainen[1], Simona Bramante[2], Dipongor Saha[2], Siri Tähtinen[2], Markus Vähä-Koskela[2], Michael Behr[2], Dirk Nettelbeck[3], Anja Ehrhardt[4], Akseli Hemminki[1]
[1]TILT Biotherapeutics Ltd, Helsinki, Finland, [2]Cancer Gene Therapy Group, Medicum, Haartman Institute, University of Helsinki, Helsinki, Finland, [3]German Cancer Research Center (DKFZ), Heidelberg, Germany, [4]Witten/Herdecke University, Witten, Germany

The field of T-cell therapy of cancer holds great promise, as demonstrated by the results obtained in Chimeric Antigen Receptor (CAR) modified T-cell therapy of CD19+ leukemia. However, the approach has not yielded similar results in the treatment of solid tumors with the exception of tumor infiltrating lymphocyte (TIL) therapy of melanoma. Recent work has identified key obstacles which has limited the efficacy of the approach: i) lack of recruitment of transferred T-cells to the tumor due to lack of trafficking signals ii) anergy of transferred cells at the tumor due to tumor immunosuppression iii) lack of propagation of transferred T-cells at the tumor. Previously, we have identified interleukin 2 (IL-2) and Tumor Necrosis Factor alpha (TNFα) as the most promising factors to stimulate the graft used in adoptive T-cell therapy. Both cytokines are important activators of immune cells and also known for their direct anti-tumor properties. Notably, these cytokines can cause severe side effects when administered systemically. Previously, we and others have achieved long lasting, high level cytokine expression locally but low level systemically when using armed oncolytic viruses *in vivo*. Oncolytic viruses armed with immunostimulatory molecules constitute a potent form of immunotherapy. In essence, the danger signal caused by virus replication, coupled with the actions of the transgene, and effective presentation of tumor epitopes by lysis of the cells, results in a personalized cancer vaccine. One attractive aspect of this approach is the ease of combinations with standard therapies including chemotherapy, radiation and immune checkpoint inhibiting antibodies for example. Therefore, we developed oncolytic adenoviruses expressing one or both above mentioned human cytokines. For more detailed immunological studies in immune competent mouse models, we constructed non-replicative adenoviruses with murine cytokines (Ad5-CMV-mIL2 and Ad5-CMV-mTNFα). These viruses were studied in combination with adoptive transfer of OT-1 TCR transgenic T-cells to treat C57BL/6 mice bearing B16-OVA melanoma tumors. The animals were administered intraperitoneally with CD8+-enriched OT-1 cells with or without intratumoral injections of cytokine-coding viruses. Combination treatment with Ad5-CMV-mIL2 and OT-1 resulted in statistically significant antitumor efficacy when compared with either monotherapy or untreated control. In further experiments a triple combination of Ad5-CMV-mIL2 + Ad5-CMV-mTNFa and OT-1 T-cells improved antitumor efficacy was observed over dual agent therapies. Furthermore, in Syrian hamster model, oncolytic virus successfully improved the efficacy of TIL therapy. Additionally, splenocytes derived from animals treated with the combination of Ad5-D24 and TIL killed autologous tumor cells more efficiently than monotherapy-derived splenocytes.

195. Allogeneic CD19-CART Therapy to the Rescue of a Pediatric Patient with Relapsed and Refractory Acute B Lymphoblastic Leukemia

Lung-Ji Chang[1], Jian-Ping Zhang[2], Yu-Chen Liu[1], Lujia Dong[3], Tong Wu[2], Dao-Pei Lu[2]
[1]Genoimmune Medical Institute, Shenzhen, China, [2]Beijing Lu Dao-Pei Hospital, Beijing, China, [3]America Yuva Biomed, Beijing, China

Acute B lymphoblastic leukemia (B-ALL) is a common hematological malignancy in children. Using a fourth generation, safety-switch inserted, chimeric antigen receptor (4SCAR) technology, we have treated more than 200 patients with B-ALL in China, with an overall complete remission rate of more than 80% (ASH 2015). Here we report the case of a 7-year-old girl with a high load of refractory B-ALL, who was heavily pretreated with chemotherapy but relapsed with >80 leukemic blasts in her bone marrow. The patient received CD19-4SCAR T cells generated from her mother's peripheral blood T cells (allo-4SCAR19) for three times at total of 0.76 x10^6 CART/kg on day 0, 4, and 7, followed by an infusion of autologous 4SCAR19 (auto-4SCAR19) at total of 0.16 x10^6 CART/kg on day 56, which was derived from the patient's own T cells after her immune recovery. Complete remission was achieved as revealed by bone marrow examination on day 77. Graft versus host disease signs were not observed throughout the days following the infusions, and the syndrome of suspected cytokine storm was generally mild. The only adverse responses were intermittent fever and skin rashes following allo-4SCAR19 infusions which lasted for about 20 days. The outcome of this case suggests that allo-4SCAR19 may be a safe approach in treating B-ALL patients who could not provide their own T cells for CART engineering, and allo-4SCAR19 combined with auto-4SCAR19 is a novel strategy that may rescue chemo-refractory, high-risk and highly immune suppressed leukemia patients.

196. Nordihydroguaiaretic Acid (NDGA) Improves Function of Antigen-Specific T-Cells Stimulated by Capsid-Optimized AAV6 Vectors

Daniel Zhang, Chen Ling, George Aslanidi
University of Florida, Gainesville, FL

Cancer, a leading cause of death in the human population today, is generally treated with rigorous surgery followed by intensive chemotherapeutic methods, both of which have adverse toxic side effects. Immunotherapy represents an attractive alternative since it is based on activation of the host immune system. We recently showed that capsid-optimized AAV6-S663V+T492V vectors expressing a tumor-associated antigen are able to initiate specific peripheral T-cell response and suppress tumor progression as well as extend survival in

a prostate cancer animal model (*J Immunother*., 38: 292-298, 2015). However, vaccination based on AAV6 vectors alone was not able to completely overcome the adverse effect of tumor microenvironment and tumor-induced immune dysfunction. Tumor-associated macrophages (TAMs) are prominent components of solid tumors. TAMs exhibit distinct phenotypes and function, and negatively contribute to immune system evasion. Previously published data indicate that inhibition of lipoxygenase (15-LOX2), a key enzyme contributing to TAM function, is critical to overcome tumor resistance and facilitate immunotherapy treatment. In our studies presented here, we first showed that 15-LOX2 is expressed in mouse prostate cancer cells, RM1, growing subcutaneously in C57BL/6 mice, but not in cells growing in culture. We also showed that LOX inhibitors, such as a plant-derived compound, nordihydroguaiaretic acid (NDGA), could restore the function of specific cytotoxic T-cells co-cultured with TAMs in a concentration-dependent manner. Splenocytes from C57BL/6 mice injected intra-muscularly with capsid-optimized AAV6 vectors expressing prostatic aid phosphatase (PAP) were isolated 14 days after vector injection. T-cells were incubated in the presence or absence of RM1 tumor cell extracts and NDGA for 2 hours and then used in killing assays against cultured RM1 cells. Two-color fluorescence assays of cell-mediated cytotoxicity was used to estimate the percentage of dead/alive target cells and a killing curve was generated with different effectors to target cell ratios. Our data indicated that the killing ability of T-cells initiated by capsid-optimized AAV6 vector injection and incubated with RM1 tumor-derived TAMs was approximately 3-fold lower than original activity T-cell. NDGA at a concentration of 10 uM had limited effect on the T-cell activity, but 25 μM NDGA restored the function of the T-cells to approximately 90% of the original activity. In our currently ongoing studies, we are using a combination of capsid-optimized AAV6 vector-mediated immune activation with the NDGA-treatment to corroborate whether a synergetic therapeutic effect can be achieved in an animal model. If successful, this approach could prove useful in the potential clinical application.

197. Balancing Anti-Tumor Efficacy with Local Inflammatory Toxicity for the Treatment of Diffuse Intrinsic Pontine Glioma and Other Brain Tumors

Matthew Schuelke[1], Laura Evgin[1], Tim Kottke[1], Jill Thompson[1], Christopher Driscoll[1], Elizabeth Ilett[2], Julia Cockle[2], Amulya NageswaraRao[1], Richard Bram[1], Alan Melcher[2], Richard Vile[1]
[1]Mayo Clinic, Rochester, MN, [2]University of Leeds, Leeds, United Kingdom

We have shown previously that multiple rounds of a systemic treatment of GM-CSF, followed by intravenous reovirus, leads to effective treatment of subcutaneous melanomas (Ilett et al., Mol. Ther. 2015). We show here that a similar regimen is also effective at treating both melanoma (B16) and glioma (GL261) tumors growing intra-cranially. As a result of these pre-clinical studies, we initiated a Phase I clinical trial in paediatric patients with gliomas of GM-CSF and reovirus therapy. To date, three patients have been treated. In two of three of these patients there were possible indications of pseudoprogression and intra-cranial inflammation, both of which were clinically resolved upon treatment with dexamethasone. Whilst this trial continues to recruit, we have investigated additional treatments for paediatric brain tumors, especially those in which direct virus injection may allow greater local access to tumor. In this respect, there is a constant tension between the pro-inflammatory, anti-tumor nature of oncolytic viroimmunotherapy and the need to reduce potentially toxic local inflammatory reactions within the brain. Our overall hypothesis is that it will be possible to balance the anti tumor effects of viral oncolysis, in part caused by inflammatory reactions to the virus, with the associated toxicity. Nowhere is this more relevant than for the treatment of Diffuse Pontine Gliomas (DIPG), which grow in the brain stem. Our experiments show that it is indeed possible to treat tumors growing in the pons/and or medulla, with inflammatory, immune based therapies (both T cell mediated and locally cytotoxic) without increasing toxicity over that caused by tumor growth alone. Therefore, we have screened multiple oncolytic virus types for their ability to kill DIPG cell lines both *in vitro* and *in vivo*. In particular, we have compared viruses with different speeds of oncolysis and different inflammatory properties to investigate how the efficacy can be balanced with local toxicity, using standard of care anti inflammatory treatments (with dexamethasone) as well as with novel agents and viruses designed to suppress local inflammatory reactions.

198. CD4+ IL13Rα2-Specific CAR T Cells Exhibit Potent Effector Function Against Glioblastoma

Dongrui Wang, Renate Starr, Brenda Aguilar, Alfonso Brito, Brenda Chang, Aniee Sarkissian, Behnam Badie, Michael E. Barish, Stephen J. Forman, Christine E. Brown
City of Hope National Medical Center, Duarte, CA

Adoptive T cell transfer, including chimeric antigen receptor (CAR) T cell therapy, has been successfully applied to the treatment of multiple types of cancer, but the variation in response rates among patients remains an important issue. Recent studies have demonstrated that CAR T cells vary in function and persistence, depending on the specific T cell subpopulation that is engineered and the phenotype of the final T cell product. Specifically, a starting population of central memory T cells (Tcm) has been shown to improve persistence and efficacy of CAR T cell therapy. Tcm consist of both CD4+ and CD8+ pools, but little is known about the contribution of each subset for CAR T cell function. Notably, although CD8+ T cells have long been considered the primary cytotoxic effector population, CD4+ T cells have also been shown to mediate anti-tumor response against some solid tumors which is CD8-independent. It is thus critical to identify the optimal composition of CD4+ and CD8+ CAR T cells for mediating the maximal anti-tumor effect. Here, using both *in vitro* and *in vivo* glioblastoma (GBM) models, we examined the anti-tumor activity of IL13Rα2-specific CAR T cells engineered from purified CD4+ or CD8+ Tcm pools. Surprisingly, we discovered a superior antitumor response from CD4+ IL13Rα2-CAR T cells as compared to CD8+ CAR T cells. After co-culture with primary patient-derived GBM cells *in vitro*, CD4+ CAR T cells displayed more potent tumor killing as compared to CD8+ CAR T cells, especially when effector cell numbers were more limited. Consistently, CD4+ CAR T cells maintained better effector function *in vitro* when re-challenged with tumor. These CD4+ CAR T cells produced higher levels of cytokine as compared to CD8+ T cells, a difference that was further maintained after tumor re-challenge. We also assessed whether the CD4 versus CD8 composition of the IL13Rα2-CAR T cell product would impact therapeutic efficacy *in vivo* using primary patient-derived GBM lines orthotopically implanted in NSG mice. When injected intracranial into GBM-bearing mice, the CD4+ CAR T cells resulted in durable antitumor efficacy, mediating tumor-free survival for over 200 days in 7of 7 experimental animals, which was unaffected by mixing with 10% of CD8+ CAR T cells. While CD8+ CAR T cells, as well as 50%-50% or 10%-90% CD4-CD8 mixed populations, also mediated significant antitumor activity, the majority of mice recurred and showed a less durable therapeutic response. These results demonstrate that CD4+ CAR T cells can have potent effector function and suggest that, for at least IL13Rα2-CARs, CD4+ T cells mediated superior anti-tumor immune responses as compared to CD8+ T cells. These studies provide important insight to further elucidate the optimal composition of the CAR T cell product for the treatment of cancer.

199. Genetic Engineering of Hematopoietic Stem Cells to Generate Invariant Natural Killer T Cells

Drake J. Smith
Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, Los Angeles, CA

Invariant natural killer T (iNKT) cells comprise a small population of αβ T lymphocytes. They bridge the innate and adaptive immune systems and mediate strong and rapid responses to many diseases, including cancer, infections, allergies, and autoimmunity. However, the study of iNKT cell biology and the therapeutic applications of these cells are greatly limited by their small numbers in vivo (0.01-1% in mouse and human blood). Here, we report a new method to generate large numbers of iNKT cells in mice through T-cell receptor (TCR) gene engineering of hematopoietic stem cells (HSCs). We showed that iNKT TCR-engineered HSCs could generate a clonal population of iNKT cells. These HSC-engineered iNKT cells displayed the typical iNKT cell phenotype and functionality. They followed a two-stage developmental path, first in thymus and then in the periphery, resembling that of endogenous iNKT cells. When tested in a mouse melanoma lung metastasis model, the HSC-engineered iNKT cells effectively protected mice from tumor metastasis. This method provides a powerful and high-throughput tool to investigate the in vivo development and functionality of clonal iNKT cells in mice. More importantly, this method takes advantage of the self-renewal and longevity of HSCs to generate a long-term supply of engineered iNKT cells, thus opening up a new avenue for iNKT cell-based immunotherapy.

200. Generation of CAR-T Cells Lacking T Cell Receptor and Human Leukocyte Antigen Using Engineered Meganucleases

Christina Pham, Aaron Martin, Jeyaraj Antony, Daniel MacLeod, Audrey Brown, Michael Nicholson, Jo Ann Hux, Caitlin Turner, Wendy Sharer, Bruce McCreedy, Victor Bartsevich, Ginger Tomberlin, Janel Lape, Jeff Smith, Derek Jantz
Precision BioSciences, Inc., Durham, NC

The manufacture of CAR-T cells depends on peripheral blood donations that contain T cells of sufficient quality and quantity. Currently, many CAR-T programs rely on autologous T cells, but several technical and commercial challenges hinder development. The majority of CAR-T trials have enrolled leukemia or lymphoma patients, many of which are unsuitable donors for CAR-T production due to their disease state or to previous treatments with lymphodepleting agents. In addition, a custom CAR-T production run for each patient is time consuming, lacks standardization and may present regulatory challenges. An alternative strategy is to source T cells from healthy donors and produce large batches of allogeneic CAR-T cells. Allogeneic T cells, however, will display mismatched human leukocyte antigens (HLA) that will be recognized by the recipients' immune systems, contributing to immune rejection of engrafted CAR-T cells. Additionally, donor T cells will recognize the mismatched HLAs present in the recipient, contributing to graft-versus-host immune pathology. Both undesired immune responses are predicated on interactions between HLA and T cell receptors (TCR), and while the therapeutic effectiveness of CAR-T cells with targeted deletions in TCR genes has been reported by several groups, studies featuring both TCR and HLA deletion are limited. Here, we describe the use of meganucleases engineered to target regions of the TCR α chain constant region and β-2 microglobulin genes to generate TCR and HLA class I knockout primary human T cells. Both nucleases generate knockouts with approximately 75% efficiency and are well-tolerated by primary T cells from at least four separate donors. Purified double knockout cells do not demonstrate functional disadvantages in terms of proliferation or cytokine production, but do exhibit reduced allostimulatory potential toward HLA-mismatched T cells. Together, these findings demonstrate the feasibility of generating therapeutic quantities of CAR-T cells with reduced allo-reactive potential and collateral toxicity to normal tissues in recipients.

201. Modulation of the Antitumor Immune Response Using Semireplication-Competent VSV Armed with Immunostimulatory Transgenes

Johanna Reul, Christian J. Buchholz, Alexander Muik
Molecular Biotechnology and Gene Therapy, Paul-Ehrlich-Institut, Langen, Germany

Immunotherapy has been shown to be an effective treatment modality for various cancer types. A branch of immunotherapy is oncolytic immunovirotherapy, which relies on arming oncolytic viruses with immunomodulatory cargo. A potent oncolytic virus candidate is the vesicular stomatitis virus (VSV), showing impressive antitumor activity in a variety of animal models. However, to overcome the neurotoxicity of VSV a semireplication-competent VSV vector system (srVSV) has been generated, which is based on two trans-complementing, propagation deficient VSV vectors. As a consequence, infectious progeny can only be produced in double infected cells. We aim at establishing a system that couples the oncolytic function of srVSV with its ability to accommodate multiple transgenes. Immunostimulatory transgene expression in combination with the oncolysis-induced inflammatory milieu at the tumor site might suffice to interfere with tumoral immunotolerance mechanisms and to induce a long-term antitumor immune response eliminating residual and metastatic cancer. The immunostimulatory transgenes GM-CSF, Flt3L, B7-1-Ig and the tumor-associated antigens ErbB2, CTLA4-ErbB2 were cloned into the genome of the srVSV vector system. All VSV vectors were successfully generated *de novo* and functional analyses revealed that the vectors were able to transcomplement, induce oncolysis and express the respective transgene *in vitro.* To investigate antitumor efficacy *in vivo,* a suitable VSV-permissive syngeneic tumor mouse model was identified. As VSV is exquisitely sensitive to type I interferon induced antiviral responses, a total of five different murine tumor cell lines were analyzed concerning their interferon sensitivity. *In vitro* and *in vivo* analyses revealed that the murine colon cancer cell line MC38 is an appropriate tumor model, as MC38 cells were productively infectable even when pretreated with high doses of IFN-α and established s.c. MC38 tumors allowed for sustained transgene-expression over an extended period of time. Initial *in vivo* data confirm the potential of transgene-armed as well as control srVSV to induce an antitumor immune response eventually leading to cure in some animals. The immune response was analyzed by evaluating tumor-specific T cells using ELISpot and MHC class I tetramer staining. Furthermore, immunological tumor rejection was shown after tumor rechallenge of cured mice. Taken together, the data indicate that srVSV in combination with different transgenes can induce a long-term antitumor immune response. Further research is warranted to identify the most promising transgene combinations.

202. Development of T-Cells Carrying Two Complementary Chimeric Antigen Receptors Against GPC3 and ASGR1 for the Treatment of Hepatocellular Carcinoma

Zonghai Li[1], Cheng Chen[1], Kesang Li[1], Hua Jiang[1], Huiping Gao[1], Fei Song[1], Xiaorong Pan[1], Bizhi Shi[1], Yanyu Bi[1], Bo Song[2]
[1]Shanghai Cancer Institute, Shanghai, China, [2]CARsgen Therapeutics, Shanghai, China

Recently, we reported that GPC3-targeted CAR-T cells could eradicate hepatocellular carcinoma xenografts in mice. However, we do not know whether on-target off-tumor toxicity, a major toxicity associated with CAR-T cells will happen when using these GPC3-CAR T cells in human being. We propose that dual-targeted CAR-T cells co-expressing a GPC3- and a ASGR1 (a liver tissue-specific protein)-targeted CAR that signal using CD3ζ and 28BB (containing both CD28 and 4-1BB signaling domain) respectively might be used to reduce on-target off-tumor toxicity. We first demonstrated that GPC3 and ASGR1 were co-expressed in 54.7% of the tested HCC samples. We further demonstrated that dual-targeted CAR-T cells could exhibit no cytotoxic activities on $ASGR1^{+}GPC3^{-}$ tumor cells. However, the cytotoxic activities of GPC3-targeted CAR-T cells and dual-targeted CAR-T cells have similar anti-tumor activities in vitro on $ASGR1^{-}GPC3^{+}$ or $GPC3^{+}ASGR1^{+}$ HCC cells. Interestingly, dual-targeted CAR-T cells showed significantly higher cytokine secretion, proliferation and anti-apoptosis ability against tumor cells bearing both antigens *in vitro* than single-targeted CAR-T cells. Furthermore the dual-targeted CAR-T cells displayed potent growth suppression activity on $GPC3^{+}ASGR1^{+}$ HCC tumor xenograft while no obvious growth suppression on single or double antigen-negative tumor xenografts. Additionally, the dual targeting T cells exerted superior anti-cancer activity and persistence than single targeting T cells in two $GPC3^{+}ASGR1^{+}$ HCC xenograft models. Together, T-cells carrying two complementary CARs against GPC3 and ASGR1 might reduce the risk of on-target off-tumor toxicity while remain relative potent anti-tumor activities on $GPC3^{+}ASGR1^{+}$ HCC

203. Shortened T Cell Culture with IL-7 and IL-15 Provides the Most Potent Chimeric Antigen Receptor (CAR)-Modified T Cells for Adoptive Immunotherapy

Saba Ghassemi, Felipe Bedoya, Selene Nunez-Cruz, Carl June, Jos Melenhorst, Michael Milone
Pathology and Lab Medicine and Center for Cellular Immunotherapy, Perelman School of Medicine of the University of Pennsylvania, Philadelphia, PA

Adoptive T cell immunotherapy involves the isolation, ex vivo expansion and reinfusion of patient T cells. The efficacy of adoptive immunotherapy is dependent on the ability of T cells to engraft, expand and persist upon adoptive transfer. In this therapy, T cells are cultured ex vivo using natural or artificial antigen presenting cells that deliver signal 1 (TCR/CD3) and signal 2 (e.g. CD28 co-stimulation) along with exogenously added cytokines. IL-2 is the most commonly used cytokine for ex vivo T cell culture; however, there is renewed interest in IL-7 and IL-15 due to their ability to enhance the survival and proliferation of stem cell memory (T_{scm}) and central memory (T_{cm}) T cells. We show that primary human T cells freshly isolated from peripheral blood are heterogeneous with substantial numbers of T_{scm} and T_{cm} cells in addition to effector differentiated T cells. During ex vivo culture, these cells progressively differentiate into a population of T cells with a predominantly CD45RO+, CD27-, CCR7- effector differentiated phenotype. Exogenous IL-7 and IL-15 delay this transition in T cell phenotype and preserve a greater proportion of T_{scm} and T_{cm} cells in the final ex vivo culture product. We hypothesize that limited ex vivo culture of T cells in the presence of IL-7 and IL-15 rather than IL-2 will enhance engraftment and persistence of T cells in vivo contributing to enhanced efficacy in adoptive transfer. We show that T cells can be harvested and viably frozen from ex vivo cultures as early as day 3 following activation. Early activated T cells expressing a chimeric antigen receptor targeting CD19 (CART-19) show potent yet specific cytotoxicity and cytokine production in vitro. We investigated the therapeutic potential of cells harvested at day 3 versus later time points using a Nalm-6 leukemic cell xenograft mouse model. We demonstrate that day 3 CART-19 cells show potent anti-leukemic activity compared to day 5 or day 9 cells. Comparing CART19 cells cultured in either IL-2 or IL-7/15, we show that mice treated at a 10-fold lower dose with day 3 cells cultured in IL-7/15 exhibit the greatest anti-leukemic efficacy compared with day 9 cells where the latter fail to control leukemia. In summary, we show that limiting T cell culture ex vivo to the minimum required for lentiviral transduction in the presence of IL-7 and IL-15 provides the most efficacious T cells for adoptive T cell immunotherapy.

204. HER2-Specific Chimeric Antigen Receptor T Cells for the Treatment of Breast-to-Brain Metastasis

John P. Murad, Dileshni Tilakawardane, Brenda Aguilar, Anthony Park, Ethan Gerdts, Aniee Sarkissian, Alfonso Brito, Josh Neman, Renate Starr, Brenda Chang, Wen-Chung Chang, Sarah Wright, Behnam Badie, Stephen J. Forman, Christine E. Brown, Saul J. Priceman
Beckman Research Institute, City of Hope, Duarte, CA

Adoptive transfer of chimeric antigen receptor (CAR)-engineered T cells has demonstrated robust and durable clinical efficacy in patients with CD19+ B-cell malignancies. Broader application of this approach to brain and other advanced solid tumors is an immediate goal for the field and is presently under intense investigation. In 2014, 40,000 individuals in the U.S. alone succumbed to breast cancer, primarily as a result of metastatic disease. Approximately 30 percent of breast cancer patients carry an amplification of the HER2 gene and/or HER2 over-expression, which confers a particularly poor prognosis. Among the most common sites of HER2-positive breast cancer metastases is the brain. For patients with breast cancer that has metastasized to the brain, the 1-year survival rate is a dismal 20 percent. Despite clinical successes in both preventing relapse and treating systemic disease with HER2-targeted therapies, the currently available agents are only modestly effective in managing brain metastasis. Our group has demonstrated safety and transient anti-tumor responses in two U.S. FDA-authorized phase I clinical trials evaluating local intracranial adoptive transfer of CAR T cells in glioma patients.

Our current project builds on this clinical experience with locally administered CAR T cells that specifically target HER2 for the treatment of breast-to-brain metastasis. Our lab has initiated design and testing of CAR T cell therapy targeting HER2 based on an scFv derived from trastuzumab. We anticipate that local intracranial delivery will enhance therapeutic response, while reducing the likelihood of off-tumor systemic toxicities as previously observed. Importantly, HER2 expression in normal brain tissue is limited, supporting HER2 as a therapeutic target in brain metastasis. Our innovative CAR T cell platform focuses on engineering central memory T cells (Tcm) for therapeutic application, with the intent of improving persistence of T cells after infusion, a critical parameter correlated with ultimate therapeutic success. Herein, we have evaluated several HER2-CAR constructs incorporating either the CD28 or 4-1BB co-stimulatory domain using both *in vitro* T cell functional assays as well as orthotopic patient-derived xenograft models of breast-to-brain metastasis. While HER2-28ζ and HER2-BBζ CAR T cells similarly kill HER2+ breast cancer cells *in vitro*,

BBζ CARs demonstrate lower induction of the exhaustion marker PD-1 and proliferate better compare with 28ζ CARs. Both HER2-CARs similarly lead to tumor eradication and prolonged survival. Based on these data, we have successfully developed HER2-specific CAR T cells, and plan to clinically develop these CARs for the treatment of HER2+ metastatic disease.

205. Cytotoxic and Immunotherapeutic Effects of Toca 511 and 5-Fluorocytosine in an Intraperitoneal Model of Metastatic Colorectal Cancer

Kader Yagiz, Daniel Mendoza, Maria Rodriguez-Aguirre, Fernando Lopez Espinoza, Carlos E. Ibañez, Harry E. Gruber, Douglas J. Jolly, Joan M. Robbins
Tocagen Inc., San Diego, CA

Toca 511 (vocimagene amiretrorepvec), a gamma retroviral replicating vector encoding an optimized yeast cytosine deaminase (CD) gene, selectively replicates and spreads in tumors. In infected cells, CD enzyme is expressed and converts 5-FC (5-fluorocytosine, an orally-available anti-fungal drug) to 5-FU (5-fluorouracil), leading to both direct tumor cytotoxicity and extended immunotherapeutic effects in preclinical models. Toca 511 in combination with Toca FC (extended-release 5-FC) recently entered a Phase 2/3 trial (NCT02414165) in patients with recurrent HGG (high grade glioma). Toca 511 is also under investigation delivered via intravenous (IV) infusion followed by injection into the wall of the resection cavity (NCT01985256), followed by Toca FC, in patients with recurrent HGG. IV administration of vectors is minimally invasive, can easily be repeated if desired, and may be applicable to other tumor types including metastatic colorectal cancer (mCRC). Previously we have shown that IV delivery of Toca 511 in a mouse syngeneic mCRC liver model resulted in expression of CD in tumor foci, but not in adjacent normal liver, and followed by courses of 5-FC resulted in direct tumor response, improved survival, and a systemic anti-tumor immune response. Elevated circulating myeloid-derived suppressor cells (MDSC) are associated with advanced disease stages in CRC patients and failure of immunotherapeutic strategies. We further investigated the effect of intralesional administration of Toca 511 with 5-FC treatment on tumor recurrence and immune infiltrates in a model of CRC brain metastases. A significant decrease in MDSC in spleens and tumors was observed with Toca 511 and 5-FC compared to controls, via in situ production of 5-FU ($p=0.03$, $p<0.0001$; respectively). Currently, we are investigating the efficacy of Toca 511 and 5-FC in a mouse syngeneic intraperitoneal (IP) model. We identified the optimal delivery method for treatment of IP metastases, and evaluated survival after IV and IP vector delivery followed by courses of 5-FC. Treatment with Toca 511 and 5-FC led to an improved median survival compared to control. Systemic 5-FU has hematological toxicity even at low doses (20 mg/kg) in both naïve and CRC tumor-bearing mice which could have an adverse effect on anti-tumor immune responses. We further evaluated Toca 511 and 5-FC treatment compared to systemic 5-FU in terms of efficacy, hematologic toxicity, and induction of anti-tumor immune responses in the IP mCRC model. The effect of Toca 511 and 5-FC compared to systemic 5-FU on MDSC and other immune cell populations will be presented. Our data provides support for the development of Toca 511 and 5-FC as a unique approach targeting both the tumor and the immune system for the treatment of metastatic cancers such as mCRC. A phase 1 study of IV Toca 511 and Toca FC in solid tumors, including mCRC, is planned (NCT02576665).

206. Determining the Effect of Endogenous PD-1 Expression on the Co-Stimulatory Potential of the PD1:CD28 Chimera

Megan E. Keys, Jonathan Arroyo, Bridgette Kielhack, Megan Prosser
Department of Biology and Chemistry, Azusa Pacific University, Azusa, CA

Adoptive immunotherapy is an emerging field of study involving genetic alteration of the immune system to enhance its effectiveness in eliminating cancer. A previous study created a novel chimera (PD1:CD28) containing the extracellular portion of an inhibitory molecule, Programmed Death 1 (PD-1), and the intracellular portion of a co-stimulatory molecule, CD28, resulting in tumor induced co-stimulation of T cells. Given that endogenous PD-1 expression is likely to up-regulate on T cells in the tumor microenvironment, the current study aims to determine whether the presence of PD-1 on the cell surface negatively impacts T cell stimulation by the PD1:CD28 chimera. Endogenous PD1 was tagged with a red fluorescent protein, mCherry, and PD1:CD28 with a green fluorescent protein, GFP. Fusion proteins were transfected into H9 cells and positive expression was detected via flow cytometric analysis and fluorescence microscopy. Stable cell lines expressing both PD1:mCherry and PD1:CD28:GFP fusion proteins are being examined for downstream signaling markers, proliferation, and cytokine expression following exposure to Programmed Death Ligand 1 (PD-L1), as compared to cells engineered to express only PD1:mCherry or PD1:CD28:GFP. This study will provide further insight into the ability of the PD1:CD28 chimera to overcome PD-1 mediated tumor immune evasion.

Cancer-Targeted Gene and Cell Therapy I

207. Variable Lymphocyte Receptors Enable Development of Chimeric Antigen Receptors for the Treatment of T-Cell Malignancies

Sunil S. Raikar[1], Daniel Tylawsky[1], Robert Moot[2], Lauren Fleischer[2], David McCarty[1], Christopher Doering[1], H. Trent Spencer[1]
[1]Aflac Cancer and Blood Disorders Center, Department of Pediatrics, Emory University, Atlanta, GA, [2]Graduate Program in Molecular and Systems Pharmacology, Emory University, Atlanta, GA

Relapsed T-cell leukemia has a poor prognosis. Chimeric antigen receptor (CAR) therapy could be an effective treatment modality, although targeting T-cell disease without a T-lymphoblast specific antigen is difficult. The use of natural killer (NK) cells as the effector cell and CD5 as the target antigen provides a potential solution. While traditionally CARs use an immunoglobulin (Ig) based single chain variable fragment (scFV) to target tumor cells, we designed a novel CAR structure using a variable lymphocyte receptor (VLR) as our antigen recognition sequence. VLRs represent the functional unit of the adaptive immune system in jawless vertebrates (lamprey and hagfish) and are analogous but not homologous to immunoglobulins. VLRs have a fundamentally different structure and geometry compared to Ig-based antibodies while still demonstrating high degrees of specificity and avidity. Additionally, VLRs exist naturally as single chain structures that allows for rapid creation of CAR cassettes. In the current study our objective was to develop a VLR-CAR-NK cell against T-cell leukemia using an anti-CD5 VLR sequence. We constructed a second generation CAR using a VLR sequence targeting CD5 as our antigen recognition sequence. The CAR structure consisted of the anti-CD5 VLR sequence, the CD28 co-stimulatory transmembrane domain, and the intracellular CD3ζ signaling domain. Using this construct, high titer CD5 VLR

CAR self-inactivating lentivirus was produced at titers >10^8 TU/ml. To test the functionality of the CAR construct, CD5 expressing Jurkat cells were transduced with various doses of lentivirus, and activation was measured by expression of CD69. For cytotoxicity studies, NK-92 cells were used as the effector cell with CCRF-CEM cells being the target cell. Cytotoxicity was measured at different effector: target ratios using a flow cytometry based assay. Activation positively correlated with lentiviral copy numbers and CAR expression in Jurkat cells. Both copy number and activation decreased over time, which was anticipated as we show that highly activated cells had a growth disadvantage. NK-92 cells expressing the CD5 VLR CAR showed approximately two fold increase in cytotoxicity towards CCRF-CEM cells when compared with naïve NK-92 cells; however, low transduction efficiency and copy numbers (<0.5) were problematic, similar to previous data showing that NK-92 cells are resistant to lentiviral transduction. In order to improve upon these results, we created a bicistrionic vector co-expressing GFP and the CD5-VLR-CAR. Jurkat cells genetically modified to express the new construct showed a direct correlation between GFP expression and activation, thus confirming dual expression and function of both proteins. Transduced NK-92 cells were sorted and expanded. These cells expand robustly, demonstrate CAR expression and have the functional characteristics required for targeting T-cell disease. Our studies show the utility of VLR-derived CARs and provide the foundation to progress these studies into preclinical testing as a treatment for relapsed T-cell leukemia.

208. Neural Stem Cell Mediated Oncolytic Virotherapy for Ovarian Cancer

Asma Abdul Majid[1], Rachael Mooney[1], Megan Gilchrist[1], Maciej Lesniak[2], Karen Aboody[1]
[1]Developmental and Stem Cell Biology, City of Hope, Duarte, CA, [2]Neuro-Oncology, Northwestern University, Evanston, IL

Intro. Oncolytic virotherapy is a promising novel cancer treatment that uses replication-competent viruses to induce cancer cell death. While clinical trials are underway for a variety of solid tumors; success has been hampered by rapid immune-mediated clearance/neutralization of the viral vectors, and poor viral distribution to tumor satellites dispersed throughout normal tissue. Neural stem cells (NSCs) are ideal cell carriers that could overcome viral delivery hurdles due to their intrinsic tumor-tropism and penetration capabilities. Our lab has established a well-characterized, non-immunogenic human NSC line that can selectively distribute to many different solid tumors. Most recently, we observed impressive selectivity and penetration of peritoneal ovarian cancer metastases after intraperitoneal NSC administration. We have engineered our NSCs to produce a conditionally replication-competent adenovirus, CRad-Survivin-pk7 (NSC.CRad-S-pk7). This virus has two notable genetic modifications: (1) a polylysine fiber addition that enables high affinity binding to cell-surface proteoglycans, thus promoting viral entry into the target cell; and (2) a E1A transcriptional modification which prevents viral replication in the the absence of the *surviving* promoter, a gene over-expressed in many cancers. Clinical grade equivalent research banks of the NSC.CRad-S-pk7 cells have demonstrated safety and efficacy in orthotopic glioma models, but have not yet been tested in a metastatic ovarian cancer model. We hypothesize that NSCs are able to selectively distribute this virus to ovarian metastases, and provide protection from immune-mediated clearance and neutralization. Our long-term goal is to demonstrate efficacy and safety of NSC.CRad-S-pk7 for targeted selective tumor killing in patients suffering from stage III ovarian cancer. ***Methods.*** Two million NSC and NSC.CRad-S-pk7 cells were injected IP into nude mice containing established human peritoneal ovarian (OVCAR8 and SKOV3) metastases. After 7 days, tissue was assessed for viral distribution and activity using standard immunological and tumor-lysate viral titration assays, respectively. ***Results.*** The results suggest that the viral payload does not interfere with the NSC tumor-tropism or penetration (Fig 1). NSC-mediated viral distribution was confirmed via immunohistochemistry, and the presence of active virus within lysed peritoneal tumors was assessed. Significant tumor killing was observed in both OVCAR8 and SKOV3 ovarian cancer cells after 5 days of culture with diluted tumor lysates (1:10 and 1:100) derived from mice that received NSC.CRad-S-pk7 injections (Fig 2). No killing was observed when cancer cells were cultured with tumor lysates derived from mice that received parental NSCs. These results demonstrate that NSC.CRad-S-pk7 can selectively penetrate peritoneal ovarian metastases and produce CRad-S-pk7 adenovirus following IP administration. Studies underway include free virus distribution and pharmacokinetic comparisons.

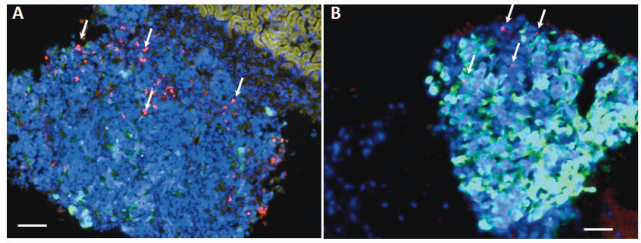


Figure 1. NSCs demonstrate tumor tropism. Neural stem cells demonstrate tumor tropism when injected intraperitoneal (IP) into metastatic ovarian cancer mouse model (2 million SKOV3.eGFP.ffluc IP). NSCs (A) or NSC.CRad-S-pk7 (B) labeled with CellTracker CM-DiI demonstrate good distribution in tumor but not in adjacent normal kidney (2 million cells in 200uL PBS injected IP after 3 weeks: harvested 1 week days post-NSC injection). Scale bar = 100 microns.

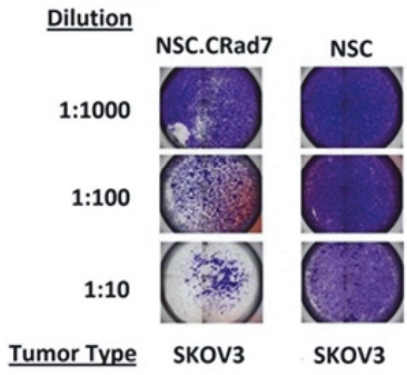


Figure 2. NSC.Crad-S-pk7 Viral Titration. To test if IP administration of NSC.CRad-S-pk7 can selectively penetrate peritoneal metastases and produce CRad-S-pk7 adenovirus, the supernatants of the tumors were collected for viral titration experiments. Parallel viral infections of SKOV3 (results shown above) and OVCAR8 were performed and significant effects were observed for the NSC.CRad-S-pk7 supernatants in comparison to the NSC controls.

209. Stem Cell/Nanoparticle Constructs for Targeted Ovarian Cancer Therapy

Pengpeng Cao[1], Revathiswari Tirughana[2], Ugochi Nwokafor[2], Soraya Aramburo[3], Linda Flores[3], Jacob Berlin[1], Karen S. Aboody[3]
[1]Molecular Medicine, City of Hope, Duarte, CA, [2]Develpmental and Stem Cell Biology, City of Hope, Duarte, CA, [3]Developmental and Stem Cell Biology, City of Hope, Duarte, CA

Intro. Targeted drug delivery is a critical goal for effective cancer therapy. Nanoparticles (NPs) have shown promise as platforms for

targeting drugs to tumors, but major challenges remain for controlling the distribution and retention of NPs within tumors. In general, NPs predominantly accumulate in the liver and spleen and have difficulty penetrating poorly vascularized and hypoxic tumor regions. Neural Stem Cells (NSCs) are ideal candidates for use as carriers for NPs in order to overcome these biodistribution challenges. NSCs have demonstrated inherent tumor tropic properties in preclinical invasive and metastatic tumor models, migrating selectively to invasive tumor foci, penetrating hypoxic tumor regions, and even traversing through the blood-brain barrier to access intracranial tumor foci following intravenous administration. We have been pioneers in genetically modifying NSCs to express prodrug activating enzymes for gene therapy, completing a first-in-human safety clinical trial for recurrent glioma in 2013, and demonstrating proof of concept for localized drug delivery (Phase I ongoing).

As NSC-based therapies move into the clinic, there is an opportunity to develop complementary NSC-mediated treatment strategies for tumor elimination. We have shown that these clinically relevant NSCs maintain their tumor tropism when transporting either surface-bound or internalized NPs. NSC-NPs selectively penetrate tumor foci (Figure 1 shows NSC-NPs, orange, at tumor foci, green, near liver). The combination of NSCs and NPs offers the potential to realize a modular and general drug targeting system. We now demonstrate that the cisplatin-loaded NPs carried by NSCs is a viable approach for the treatment of Stage III ovarian cancer, where tumors have metastasized to the abdominal cavity. We show that encapsulating cisplatin within silica NPs allows for delayed drug release for 24 hours with minimal leakage, allowing sufficient time for the NSCs to effectively penetrate and distribute through tumor foci.

Methods. Ten million NSCs carrying cisplatin-loaded silica NPs (Figure 2) were injected intraperitoneally (IP) into nude mice containing established human ovarian (OVCAR8) peritoneal metastases. Control groups included equivalent levels of free cisplatin or cisplatin-loaded NPs.

Results. IP delivery these NSC-NP constructs showed significantly increased concentrations of cisplatin at multiple tumor sites, regardless of size, throughout the peritoneum, as compared to free drug alone or drug-loaded NPs alone. At all timepoints tested, only tumors treated with the NSC-NP constructs had therapeutically significant levels of drug present (Figure 3 shows 6h and 24h timepoints). This allows for concentration of drug at tumor sites, while minimizing toxicities. Long-term efficacy studies are in progress. Our present goal is to generate the efficacy and safety data needed to move toward clinical trials.

Figure 1:

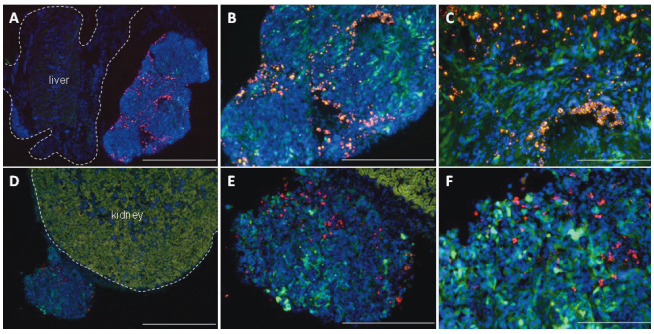


Figure 2:

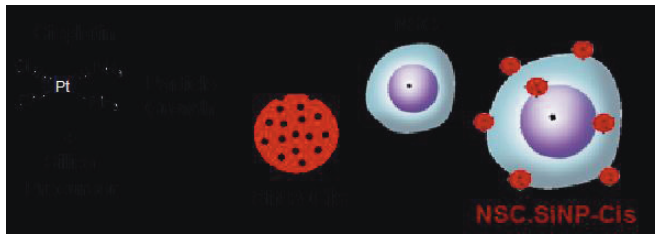
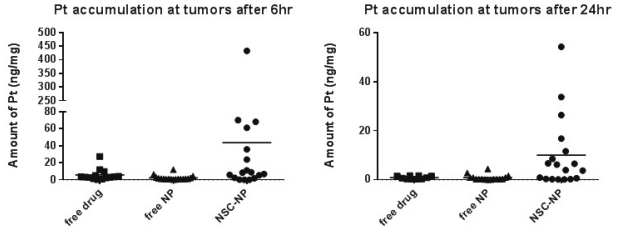


Figure 3:



210. High Expression of Second Generation CD19 CAR with a 4-1BB Costimulatory Domain from a Retroviral Vector Impairs CAR T Cell Expansion by Enhancing Fas-Mediated Apoptosis

Diogo Silva[1,2,3], Malini Mukherjee[4,5], Madhuwanti Srinivasan[1,2], Jordan S. Orange[4,5], Malcolm K. Brenner[1,2], Maksim Mamonkin[1,2]

[1]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, [2]Texas Children's Hospital, Houston, TX, [3]Department of Bioengineering and iBB-Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa, Lisboa, Portugal, [4]Department of Pediatrics, Baylor College of Medicine, Houston, TX, [5]Texas Children's Hospital Center for Human Immunobiology, Houston, TX

Recent clinical trials in patients with B cell malignancies demonstrated that CD19 CAR T cells could expand in vivo and eliminate target cells. Two main variants of the CD19 CAR have been successful - a retroviral construct with CD28 and CD3 zeta signaling domains (28.zeta) and a lentivirus with 4-1BB.zeta. While infused CD28.zeta CAR T cells rapidly expanded and produced immediate anti-tumor activity, 4-1BB.zeta CAR T cells persisted longer and had sustained anti-tumor activity. Although these differences may represent differences in the costimulation provided by each CAR, the choice of viral vector may also contribute to differences in performance. We compared the in vitro expansion and anti-tumor activity of T cells transduced with either 28.zeta or 4-1BB.zeta CD19 CAR using a standard retroviral vector SFG. We demonstrate that retroviral transduction of T cells with a 4-1BB.zeta CD19 CAR reduced T cell expansion 18 fold after 2 weeks of culture and impaired anti-tumor activity compared to cells transduced with a similar vector encoding CD28.zeta CD19 CAR. High expression of the 4-1BB.zeta CD19 CAR led to upregulation of Fas and relocation of intracellular FasL to the cell surface, resulting in co-localization of both proteins on the plasma membrane. Consistent with this finding, we observed a 5-fold increase in apoptosis of 4-1BB.zeta CAR T cells which was completely reversed by chemical blockade of caspase-8, a key initiator caspase activated by Fas signaling. We also observed a gradual downregulation of 4-1BB.zeta CD19 CAR levels on surviving T cells suggesting that high CAR expression is toxic for T cells. In fact, reducing the level of 4-1BB.zeta CD19 CAR expression by inserting an IRES sequence upstream of the CAR normalized Fas levels on T cells and restored T cell expansion and anti-tumor activity. Similarly,

transducing T cells with a self-inactivating lentiviral vector resulted in reduced levels of 4-1BB.zeta CD19 CAR expression allowed excellent expansion, similar to that of T cells with 28.zeta CD19 CAR. We also observed improved cytotoxicity of T cells with lower expression of 4-1BB.zeta CAR in co-culture assays with CD19+ cell lines Daudi (5-fold reduction in remaining tumor cells with lenti-CAR and 22-fold for IRES-CAR) and Raji (18-fold reduction for both lenti- and IRES-CAR constructs) and sustained performance in sequential killing assays.

These studies reveal a mechanism that limits expansion and function of T cells expressing high levels of 4-1BB.zeta CD19 CAR and provide a basis for rational choice of expression systems and design of chimeric molecules that incorporate the 4-1BB signaling domain.

211. Combination of Forced Transduction of P53 and an Agent That Blocks MDM2-p53 Interactions Produces Synergistic Cytotoxicity on Mesothelioma Defective of the INK4A/ARF Region

Masatoshi Tagawa[1], Takao Morinaga[1], Zhihan Li[1], Thao T. T. Nguyen[1], Boya Zhong[1], Shuji Kubo[2], Ikuo Sekine[3], Yuji Tada[4], Koichiro Tatsumi[4], Hideaki Shimada[5], Kenzo Hiroshima[6]
[1]Division of Pathology and Cell Therapy, Chiba Cancer Center Res Inst, Chiba, Japan, [2]Department of Genetics, Hyogo College of Medicine, Nishinomiya, Japan, [3]Department of Medical Oncology, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan, [4]Department of Respirology, Graduate School of Medicine, Chiba University, Chiba, Japan, [5]Department of Surgery, School of Medicine, Toho University, Tokyo, Japan, [6]Department of Pathology, Tokyo Women's Medical University Yachiyo Medical Center, Yachiyo, Japan

A majority of malignant mesothelioma specimens possesses the wild-type p53 gene with a homologous deletion in the INK4A/ARF locus which encodes the p14ARF and the p16INK4A genes. Consequently, the p53 downstream pathways are functionally inactivated since the p14 defect augments activities of MDM2 which mediates p53 degradation through the ubiquitination processes. Furthermore, a loss of p16 induces pRb phosphorylation and uninhibited cell cycle progression through up-regulated CKD activities. We then examined possible anti-tumor effects of MDM2 inhibitors, Nutlin-3a and RITA, on human mesothelioma cells in combination of adenoviruses expressing the p53 gene (Ad-p53). Nutlin-3a, inhibiting the MDM2-p53 interactions, increased p53 phosphorylation levels and suppressed viability of mesothelioma cells in a p53-dependent manner. RITA, although blocked the MDM2-p53 interactions and phosphorylated p53, inhibited viability of mesothelioma cells independently of the p53 geneotype. Transduction of mesothelioma cells with Ad-p53 induced apoptotic cell death irrespective of the genotype. Moreover p21 induced by Ad-p53 dephosphorylated pRb and subsequently inhibited cell cycle progression. A combinatory use of Ad-p53 and Nutlin-3a or RITA, produced synergistic cytotoxicity of mesothelioma irrespective of the p53 geneotype. In addition, we demonstrated that the combination produced anti-tumor effects in an orthotopic animal model, mesothelioma developed in the pleural cavity in nude mice. In contrast, heat shock protein 90 inhibitors, blocking functions of MDM4, a homologous gene product of MDM2 without ubiquitination activities, did not produce the synergistic cytotoxicity with Ad-p53. These data collectively showed that MDM2 inhibitors enhanced expression levels of endogenous and exogenously transduced p53 through increased stability of the gene produce, and consequently activated the p53-mediated apoptosis. The p53-directed therapy is thus a possible therapeutic strategy for mesothelioma with the INK4A/ARF deletion.

212. Lentiviral Insertional Mutagenesis Helps to Uncover the Mechanisms of Resistance to AZD9291 and CO-1686 in EGFR-Mutant Lung Adenocarcinoma

Valentina Pirazzoli, Giulio Spinozzi, Erika Tenderini, Fabrizio Benedicenti, Andrea Calabria, Eugenio Montini
HSR-TIGET, Milano, Italy

The discovery of cancer-driver mutations, accounting for the growth and spreading of cancer cells, led to the development of anti-cancer targeted therapies, which hit in a specific manner cell pathways directly involved in tumor progression. This new class of therapeutic agents has been shown to be more effective and less toxic than conventional chemotherapy in advanced forms of cancer. However, the inevitable development of acquired resistance has limited their success. An example of this concept is the case of Epidermal Growth Factor Receptor (EGFR)-mutant lung cancer. The discovery of EGFR mutations that confer sensitivity to the Tyrosine Kinase Inhibitors (TKI) erlotinib and gefitinib have underlined the importance of defining molecular subgroups to design more efficacious targeted therapies. Unfortunately, on average ~1 year after starting treatment, resistance to these agents, caused by EGFR secondary mutation T790M, occurs at high frequency. This observation suggests that strategies to delay or prevent the emergence of this resistance mechanism would prolong the lives of many lung cancer patients with EGFR mutations. AZD9291 and CO-1686 are two novel third-generation EGFR TKIs designed to irreversibly and specifically target both the initial activating EGFR mutations and the resistance T790M. Phase I/II studies show compelling clinical activity of these compounds. Nevertheless the observed progression-free survival is about 12 months and the reasons of the relapse are under evaluation. We took advantage of a lentiviral vector (LV) -based insertional mutagenesis platform, developed by our lab, to screen genes that confer resistance to CO-1686. To this aim T790M+ (PC9BRc1) and T790M- (PC9) human lung adenocarcinoma cells were transduced with a genotoxic LV, harboring the Spleen Focus Forming Virus enhancer/promoter in the Long Terminal Repeats (LV-SF-LTR) or a non-genotoxic LV with self-inactivating LTR. After 2 weeks, transduced cells were divided in three groups receiving Erlotinib, CO-1686 or DMSO as control. In our rationale, the integration of the genotoxic LV in the cellular genome can potentially deregulate the expression of neighboring genes that contribute to confer resistance to these TKIs. Therefore, exposure to the treatment would result in the selection and expansion of the cell clones harboring those specific traceable mutations. Drug-resistant colonies were obtained after 4 weeks of erlotinib and 6 weeks of CO-1686 treatment. While results on PC9BRc1 are still pending, sequencing analysis performed on 100,000 LV integration sites retrieved by PCR-based technologies on PC9 drug-resistant colonies identified SOS1 as the principal LV-induced gene deregulation responsible for the resistance to both Erlotinib and CO-1686. A similar experiment aimed at identifying the AZD9291-induced resistance in lung cancer cells is on-going. The identification of biomarkers of resistance will allow the development of new therapies to overcome resistance, improving the life expectancy of lung adenocarcinoma patients.

213. Detection of Integration Sites of CD19 Chimeric Antigen Receptor Gene in a Non-Viral Gene Transfer System

Motoharu Hamada
Pediatrics, Nagoya University, Nagoya, Japan

BACKGROUND: Chimeric antigen receptor (CAR)-modified T cells targeting CD19 have exhibited marked activity in hematological malignancies, such as acute lymphoblastic leukemia, chronic

lymphocytic leukemia, and B-cell lymphomas. Many of these results have been obtained using CD19-CAR T cells established by retro- or lentiviral vectors. We developed a non-viral gene-transfer method using the *piggyBac* transposon system wherein the CD19-CAR gene was integrated into the genome by cut paste mechanism of the transposase. In this system, the expression of CAR was permanent, and the *in vitro* and *in vivo* cytotoxic activity on several cancer cell lines was confirmed. In the process of such gene transfer, however, insertional mutagenesis and subsequent activation of proto-oncogenes are a concern. In this report, we aimed to detect the integration sites of CAR genes to evaluate the safety of our CD19-CAR T cells. METHOD: CD19-CAR T cells were produced by transfecting two plasmids containing *piggyBac* and a CD19-CAR gene by electroporation. We detected the integration sites of CAR genes by inverse polymerase chain reaction and subsequent next-generation sequencing using MiSeq. An analytic pipeline was developed to identify and to classify the integration sites. Proto-oncogenes were defined according to the Cancer Gene Census of the Catalogue of Somatic Mutations in Cancer database. RESULTS: We detected 181 independent integration sites of which five were in exons (*CASP10*, *F2R*, *CYP51A1-AS1*, *ULBP2*, *and SLC24A2*) and 113 in introns. The integration sites demonstrated no preference for specific sites. Five integration sites (2.8%) were in the introns of known proto-oncogenes (*CDK6*, *MAML2*, *RAD51B*, *RUNX1*, *and EP300*). This percentage of integrations into proto-oncogenes is comparable with that of random integration (2.4%) and lower than that of retro- and lentiviral vectors (6.3-10.4%) according to previous report using marker genes (Galvan et al. J Immunother 2009). CONCLUSION: We confirmed that our *piggyBac*-mediated transduction system randomly transduced the CAR gene into the genomic DNA. Therefore, this system is considered safer than viral vector system in terms of genotoxicity for CAR transduction into human T cells; however, the possibility of leukemic transformation caused by insertional mutagenesis should be carefully monitored by *in vivo* clonal analysis.

214. Specific Targeting of IL-13 Receptor alpha 2 Expressing Breast Cancer Cells by Paramyxovirus-Pseudotyped Lentiviral Vectors Displaying IL-13

Anna R. Kwilas, Wu Ou, Michael P. Marino, Takele Argaw, Akiko Suzuki, Bharat Joshi, Syed R. Husain, Raj K. Puri, Jakob Reiser
Division of Cellular and Gene Therapies, CBER, FDA, Silver Spring, MD

The ability to selectively target specific cell types is highly desirable for the *in vivo* application of viral vectors for the treatment of multiple diseases including cancer. Lentiviral vectors (LV) have the advantage of being easily pseudotyped allowing their tropism to be altered. LV can also be engineered to deliver therapeutic or cytotoxic genes directly to cancer cells. IL-13 receptor alpha 2 (IL-13Rα2) is overexpressed in many different tumor types, including glioma, sarcoma, kidney, breast and ovarian cancer, making it an attractive target for tumor therapy. We have previously shown that firefly luciferase-expressing LV pseudotyped with a truncated fusion (F) protein derived from measles virus (MV) and a tail-truncated, receptor-blind MV hemagglutinin (H) protein bearing IL-13 at the C-terminus were capable of transducing IL-13Rα2-positive U251 glioma cells when administered intratumorally. In addition, we have shown the ability of LV to be pseudotyped using the F and H proteins from the Tupaia paramyxovirus (TPMV). TPMV does not infect human cells, potentially making it a safer choice for pseudotyping LV for *in vivo* delivery. Similar to the MV H protein, the TPMV H protein can also display IL-13 and bind specifically to IL-13Rα2-positive tumor cells. We plan to confirm and extend these findings by examining the targeting of LV-MV-IL-13 and LV-TPMV-IL-13 in metastatic orthotopic models of breast cancer by intratumoral and intravenous administration. In addition, LV expressing HSV1 thymidine kinase (TK) were constructed and are being evaluated for antitumor efficacy.

215. The Use of miR-122 and Its Target Sequence in Adeno-Associated Virus-Mediated Cytotoxic Gene Therapy

Daniel Zhang[1], Jun Li[2], Zifei Yin[2], Georgy Aslanidi[1], Arun Srivastava[3], Changquan Ling[2], Chen Ling[1]
[1]Division of Cellular and Molecular Therapy, Department of Pediatrics, University of Florida, Gainesville, FL, [2]Department of Traditional Chinese Medicine, Second Military Medical University, Shanghai, China, [3]Powell Gene Therapy Center, University of Florida, Gainesville, FL

There are two challenges in adeno-associated virus (AAV)-mediated cytotoxic gene therapy: i) overexpression of the cytotoxic transgene in the producer cell lines, such as HEK293, hampers efficient AAV vector production; and ii) most, if not all, AAV serotype vectors possess hepatic tropism since liver is the major tissue for cellular metabolism. These two challenges can be partially resolved by tissue specific promoters. However, in the cases that a specific promoter is not available, constitutively active promoters, such as the chicken β-actin (CBA) promoter, have to be used. For example, there is no well-studied specific promoter for hepatic satellite cells, which are the major cell type involved in liver fibrosis. In addition, most tissue-specific are weak promoters and are often too large to be packaged into AAV vectors. To address the above problems simultaneously, we explored the use of miR122 and its target sequence for the conditional regulation of transgene expression not only in the producer cells during AAV production, but also in the liver after systemic delivery. We first established a HEK293 cell line that overexpresses miR122. The functional miR122 expression was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) as well as Western blot assays against cellular proteins that are known to be regulated by miR122, such as c-Met. The expression of miR122 showed little effect on HEK293 cell growth and AAV protein expression. Next, we generated rAAV plasmids harboring suicide genes under the control of CBA promoter and a miR122 target (miR122T) sequence in the 3'-UTR. Two strong cytotoxic genes were selected, which encodes trichosanthin (a type 1 ribosome-inactivating protein) and diphtheria toxin (an exotoxin causing diphtheria). The presence of miR122T sequence had little effect on the protein functions in miR122-free cells, but significantly reduced the protein expression in the cells overexpressing miR122. Consequently, the AAV8 vectors carrying suicide genes and miR122T sequence yielded significantly increased production, up to 75-fold, in the HEK293-miR122 cells, when compared to that in the parental HEK293 cells (Table 1). Finally, we showed that AAV8 vectors that were produced from the HEK293-miR122 cells preserved the same tropisms and full bioactivity *in vivo*. Most importantly, the AAV8 vectors carrying a miR122T sequence mediated little transgene expression in normal liver. Taken together, we conclude that the use of HEK293-miR122 cells and a miR122T sequence should be applied to attenuate the transgene cytotoxicity during AAV vector production and infection of normal liver tissues.

HEK293-miR122 and miR122T sequence together increase yields of AAV vectors carrying suicide genes.					
HEK293	Batch 1	**Batch 2**	**Batch 3**	Ave	SD
ssAAV8-FLuc	2.68E + 12	9.96E + 11	2.78E + 12	2.15E + 12	1.00E + 12
ssAAV8-FLuc-122T	8.72E + 11	2.28E + 12	1.34E + 12	1.50E + 12	7.17E + 11
ssAAV8-TCS	6.23E + 08	1.34E + 09	3.75E + 08	7.79E + 08	5.01E + 08
ssAAV8-TCS-122T	2.34E + 09	1.68E + 09	4.27E + 08	1.48E + 09	9.72E + 08
HEK293-miR122	**Batch 1**	**Batch 2**	**Batch 3**	Ave	SD
ssAAV8-FLuc	4.04E + 12	6.60E + 12	1.05E + 12	3.90E + 12	2.78E + 12
ssAAV8-FLuc-122T	5.61E + 12	2.74E + 12	7.80E + 11	7.80E + 11	2.43E + 12
ssAAV8-TCS	3.45E + 09	1.39E + 09	8.50E + 08	1.90E + 09	1.37E + 09
ssAAV8-TCS-122T	1.66E + 11	8.50E + 10	9.30E + 10	1.15E + 11	4.46E + 10

216. Exploiting Redox-Based Vulnerabilities in Field Cancerization by Targeting Cancer Associated Fibroblasts

Jeremy Soon Kiat Chan, Ming Jie Tan, Nguan Soon Tan
School of Biological Sciences, Nanyang Technological University, Singapore, Singapore

Tumor development and progression to malignancy is increasingly viewed as a consequence of disrupted homeostatic interactions between epithelial cells and the underlying stromal elements. In squamous cell carcinoma of the skin, genomic and histological abnormalities have been observed in keratinocytes and stroma bordering multifocal neoplastic lesions, suggesting a regional carcinogenic signal that promotes aberrant cellular behavior within the tumor microenvironment. This has been termed field cancerization. Cancer associated fibroblasts (CAFs) are the major cell type in the tumor stroma and are known producers of extracellular pro-tumor signaling molecules. While growth factors and cytokines are candidate molecules for targeted therapy, reactive oxygen species (ROS) are gaining attention for their roles in heterotypic paracrine signaling in cancer. The importance of ROS in field cancerization remains poorly understood. Histological analysis of on sections of patient tumor biopsies revealed that ROS was elevated in the tumor stroma. CAFs explanted from patient tumors also released high levels of hydrogen peroxide into the extracellular space. Interestingly, normal fibroblasts exposed to CAF-conditioned medium displayed increased ROS production, an observation that could be reversed by the antioxidant N-acetyl cysteine. Exposure to hydrogen peroxide also resulted in elevated ROS production in normal fibroblasts. Taken together, these findings suggest that CAFs are a potent source of ROS in the tumor microenvironment and can induce a field effect that transforms adjacent normal fibroblasts into a CAF-like state. Transformed fibroblasts in turn produce ROS to spread oxidative stress in the microenvironment, potentially exacerbating field cancerization. Therefore, antioxidant-based therapies targeting the tumor microenvironment may be important adjuvant treatments to current anticancer chemotherapy or targeted therapy.

217. Combinatorial Anti-Angiogenic Gene Therapy in a Human Malignant Mesothelioma Model

Shuji Kubo[1], Yuki Mawatari-Furukawa[1], Noriyuki Kasahara[2]
[1]Genetics, Hyogo College of Medicine, Nishinomiya, Japan, [2]Cell Biology and Pathology, University of Miami, Miami, FL

Anti-angiogenic gene therapy represents a promising strategy for cancer; however, it has rarely been tested in malignant mesothelioma, a highly aggressive tumor associated with asbestos that has poor prognosis. In this study, we investigated whether anti-angiogenic factors such as angiostatin, endostatin, and the soluble form of vascular endothelial growth factor receptor 2 (sFlk1) were able to inhibit endothelial cell proliferation via lentivirus-mediated gene transfer into malignant mesothelioma cells in culture. We also assessed whether a dual-agent strategy had greater therapeutic benefit. Human malignant pleural mesothelioma MSTO-211H cells were transduced using lentiviral vectors that individually expressed angiostatin, endostatin, and sFlk1 and linked to enhanced green fluorescent protein (EGFP) marker gene expression via an internal ribosome entry site. The lentivirus expressing EGFP alone was used as a control. The resultant cells designated as MSTO-A, MSTO-E, MSTO-F, and MSTO-C were confirmed by Western blot and fluorescence microscopy to stably express the corresponding proteins. No differences were observed in the *in vitro* growth rates between any of these cells. However, coculture of MSTO-A, MSTO-E, and MSTO-F showed significant suppression of human umbilical endothelial cell growth *in vitro* compared with that of MSTO-C. Furthermore, a combination of any two among MSTO-A, MSTO-E, and MSTO-F significantly enhanced efficacy. These results suggest that combinational anti-angiogenic gene therapy targeting different pathways of endothelial growth factor signaling has the potential for greater therapeutic efficacy than that of a single-agent regimen.

218. Monitoring ER Stress Activation of the ATF6 Pathway Using Nanoluciferase

Emily S. Wires, Doug Howard, Mark J. Henderson, Xiaokang Yan, Kathleen A. Trychta, Emily J. Heathward, Yajun Zhang, Molly Lutrey, Christopher Richie, Brandon K. Harvey
OTTC, NIDA/NIH, Baltimore, MD

The endoplasmic reticulum (ER) is an invaluable component of the secretory pathway; serving as the main site for protein synthesis and modification, lipid metabolism, lipoprotein secretion, and calcium homeostasis. In response to "stress", the ER maintains homeostasis through the unfolded protein response (UPR). The UPR is an adaptive response employed by the ER to alleviate stress caused by misfolded proteins. UPR activation is mediated by three transmembrane proteins; IRE1 (inositolrequiring protein-1), ATF6 (activating transcription factor- 6), and PERK (protein kinase RNA-like ER kinase). Here we describe the construction and characterization of a Nanoluciferase-based assay to look at the activation of the ATF6 prong of the UPR. We demonstrate the ability to simultaneously monitor ER calcium homeostasis using a novel Gaussia luciferase-based sensor. Using in vitro and in vivo assays, we demonstrate the ability to monitor ATF6 activation in human neuroblastoma cells, rat hepatoma cell line, and rat liver. With this approach we hope to gain better understanding of the contributions of ATF6 activation and ER calcium depletion in disease pathogenesis. Ongoing studies will examinegenetically or pharmacologically augmenting or inhibiting these pathways as a therapeutic approach.

219. Investigation of the Role of Decorin Gene (DCN) in Glioblastoma and Cancer Stem Cells (CSCs)

Jun-Ming Han, Hsin-I Ma
Neurological Surgery, National Defense Medical Center, Taipei, Taiwan

Glioblastoma multiforme (GBM) is the most malignant cancer in the central nervous system with poor clinical prognosis. In this study, we investigated the therapeutic effect of an anti-cancer protein, decorin, by delivering it into a xenograft U87MG glioma tumor in the brain of nude mice through an adeno-associated viral (*AAV2*) gene delivery system. Decorin expression from the AAV vector *in vitro* inhibited cultured U87MG cell growth by induction of cell differentiation. Intracranial injection of AAV-decorin vector to the glioma-bearing nude mice *in vivo* significantly suppressed brain tumor growth and prolonged survival when compared to control non-treated mice bearing the same U87MG tumors. Proteomics analysis on protein expression profiles in the U87MG glioma cells after AAV-mediated decorin gene transfer revealed up- and down-regulation of important proteins. Differentially expressed proteins between control and AAV-decorin-transduced cells were identified through MALDI-TOF MS and database mining. We found that a number of important proteins that are involved in apoptosis, transcription, chemotherapy resistance, mitosis, and fatty acid metabolism have been altered as a result of decorin overexpression. These findings offer valuable insight into the mechanisms of the anti-glioblastoma effects of decorin. In addition, AAV-mediated decorin gene delivery warrants further investigation as a potential therapeutic approach for brain tumors.

Hematologic & Immunologic Diseases I

220. AAV-Mediated miR122 Overexpression Rescues Hepatic Inflammatory Response Induced by Acute Iron-Overload

Min Li[1], Yuxiao Tang[1], Lusha Wu[1], Fengfeng Mo[1], Xin Wang[1], Hongxia Li[1], Ruirui Qi[1], Arun Srivastava[2], Chen Ling[3]
[1]Military Hygiene Department, Faculty of Naval Medicine, Second Military Medical University, Shanghai, China, [2]Powell Gene Therapy Center, University of Florida, Gainesville, FL, [3]Division of Cellular and Molecular Therapy, Department of Pediatrics, University of Florida, Gainesville, FL

Frequent blood transfusions among patients with thalassaemia and sickle cell disease can lead to acute iron overload (IO), also known as transfusional haemosiderosis. Excessive iron is initially distributed to the liver, the primary organ for iron recycling and storage, and then gradually leads to damage of other major organs such as the heart and endocrine organs. IO frequently increases hepatic inflammatory infiltration and expression of pro-inflammatory cytokines. Recently, hepcidin, a well-known iron-regulatory hormone, and iron-catalyzed reactive oxygen species were proven to be independent of the IO-mediated inflammation. These findings triggered us to further pursue the potential underlying molecular mechanisms.

In our preliminary studies, we observed that IO significantly reduced the expression of liver-specific microRNA, miR122, through the inhibition of a nuclear receptor, hepatocyte nuclear factor 4 alpha (HNF4α), but not other regulators of miR122. Furthermore, we demonstrated that the chemokine (C-C motif) ligand 2 (CCL2), which recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation, is negatively regulated by miR122. Thus, we hypothesized that overexpression of hepatic miR-122 may relieve the inflammatory responses to the acute IO. To this end, a recombinant adeno-associated virus serotype 8 (rAAV8) vector was generated, in which a miR-122 expression cassette was embedded in the intron region between a Guassia Luciferase (GLuc) transgene and a chicken β-actin promoter. Another rAAV8 vector, which has the exact GLuc transgene cassette but without miR-122, was used as a negative control. C57BL/6 mice were intra-peritoneal challenged with iron-dextran at 200mg/kg mouse weight or PBS as a control, at Day 0 and Day 8. The IO group was sub-divided into three sub-groups, in which PBS, rAAV8-GLuc, or rAAV8-miR122-GLuc vectors were tail-vein injected at 10^11 viral genome/mouse, respectively, at Day 6. All mice were sacrificed at Week 4. It was evident that following systemic administration, rAAV8-miR122-GLuc vectors led to a 12-fold increase in the expression of miR122. On the other hand, only a modest increase (<2-fold) in the HNF4α expression was observed. Interestingly, although *in vivo* overexpression of miR-122 had no effect on serum iron indices or hepatic expression of iron-related molecules, it significantly reduced the expression of several inflammatory genes, such as IL-6 and IL-1β, in the IO mice. Western blot analysis also revealed that overexpression of miR-122 resulted in a significant reduction of CCL2 and NF-κB proteins in the IO mice. Consequently, both the hepatic inflammation scores and serum activities of AST and ALT in the IO mice were significantly reduced upon miR-122 overexpression.

In sum, our model (Figure 1) and studies should be important and informative for elucidating the mechanism of IO-induced hepatic inflammation and for developing clinical strategies to prevent them in the patients with major haemoglobinopathy.

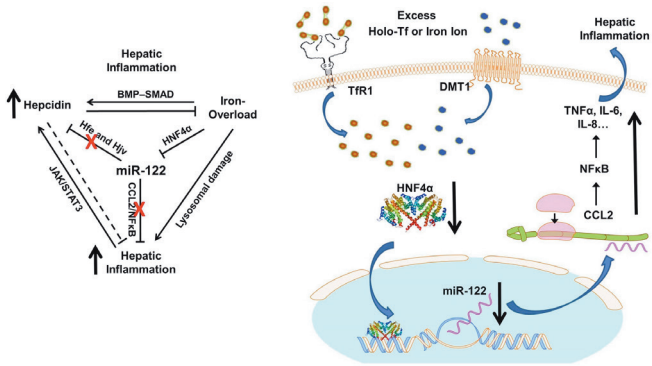


Fig. 1 The role of miR122 in iron-overload-mediated hepatic inflammation

221. Staurosporine Increases Lentiviral Transduction of Human CD34+ Cells

Melissa Bonner[1], Gretchen Lewis[1], Lauryn Christiansen[1], Dakota Campbell[1], Christopher Tipper[2], Amanda Hamel[1], Seema Shah[1], Holly Horton[1], Olivier Negre[1], Gabor Veres[1], Philip Gregory[1]
[1]Bluebird Bio, Inc., Cambridge, MA, [2]Bluebird Bio, Inc. (Current address: Dimension Therapeutics), Cambridge, MA

Lentiviral vector (LVV) mediated transduction of CD34+ hematopoietic stem and progenitor cells holds tremendous promise for the treatment of monogenic diseases of the blood. Critical to the success of this *ex vivo* gene therapy approach is the generation of a sufficient proportion of gene-modified cells. Here we investigated the potential of staurosporine, a protein kinase inhibitor, to enhance the transduction of LVVs in mobilized peripheral blood CD34+ cells both *in vitro* and *in vivo*. Staurosporine treatment has been previously demonstrated to increase HIV-1 integration in metaphase-arrested cells and shown to cause chromatin relaxation in metaphase cells (Manioui et al., Virology 2004). Additionally, in a separate study staurosporine treatment led to a 150% increase in transduction of CAp24+ CD4+ T cells (Permanyer et al., PLoS One 2013). Staurosporine increases vector copy number (VCN) approximately 2-fold when added to human mobilized peripheral blood (mPB) CD34+ enriched cells prior to transduction. The mechanism of this

VCN improvement was investigated using the BLAM assay and a 1.5-fold improvement in viral entry was demonstrated. This effect was observed in at least six different mPB CD34+ cell lots that had various levels of transducibility. Interestingly, the effect of increased LVV transduction was most striking with low transducing cell lots and the effect diminished in higher transducing cell lots. Staurosporine treatment did not affect viability of cells post-transduction and there was no difference in *in vitro* colony formation in staurosporine-treated cells compared to vehicle-treated cells. NSG mice transplanted with cells transduced in the presence of 400 nM or 800 nM staurosporine demonstrated a statistically significant 3-fold and 4-fold increase in VCN in human CD45+ cells in bone marrow at 4 months post-transplantation compared to vehicle-treated cells. Importantly, there was no significant difference in engraftment nor any observed lineage-bias between groups. Based on these observations, the use of staurosporine to enhance LVV transduction of human CD34+ cells is a promising method to improve the potential therapeutic benefit of gene therapy drug products.

222. The LCR-Free γ-Globin Lentiviral Vector Combining Two HPFH Activating Elements Corrects Murine Thalassemic Phenotype *In Vivo*

Panayotis Antoniou[1,2], Eleni P. Papanikolaou[1,2], Maria Georgomanoli[3], Ekati Drakopoulou[3], Nicholas P. Anagnou[4]
[1]University of Athens School of Medicine, Athens, Greece, [2]Biomedical Research Foundation of the Academy of Athens, Athens, Greece, [3]Laboratory of Cell and Gene Therapy, Biomedical Research Foundation of the Academy of Athens, Athens, Greece, [4]Laboratory of Biology, University of Athens School of Medicine, Athens, Greece

The β-thalassemias result from reduced or absent expression of the β-chain of hemoglobin (α2β2;HbA) causing precipitation of excess α-chains and eventually apoptosis. Thus, factors that reduce the degree of chain imbalance such as an innate ability to increase fetal hemoglobin (HbF;α2γ2), as in the HPFH phenotype, have an ameliorating effect on the disease. Hence, gene therapy of β-thalassemia based on γ-globin addition via viral vectors, displays a considerable advantage. In this study, we assessed the efficiency of our previously generated LCR-free γ-globin self inactivating vector GGHI (Papanikolaou et al. Hum Gene Ther 2012) to correct the thalassemic phenotype *in vivo* in the $Hb^{th3/+}$ C57BL/6J mouse model (thal3 model). Recipient mice aged about 12 weeks, were treated with the myelosuppresant factor busulfan, while donor mice, of the same age and of the opposite gender, were treated with 5-fluorouracil, prior to transplantation. Total bone marrow was isolated from 5-fluorouracil-treated donors and was transduced with GGHI in X-VIVO™ medium containing cytokines (mIL-1a, mIL-3, mIL-6 and mSCF) at an MOI=30, employing the spinoculation method. The transduced cells were then transplanted via tail vein intravenous injection to the recipients. To evaluate the therapeutic effect of GGHI, blood was collected from recipient mice prior and post transplantation for 4 months and, hemoglobin levels (g/dl), hematocrit and total red blood cell count were assessed by a hematological analyzer. The expression of human γ-globin in peripheral blood was assessed by flow cytometry using an anti-HbF monoclonal antibody. Our results documented that transplanted thalassemic mice (n=4) with GGHI-corrected hemopoietic stem cells, exhibited an increase in hematocrit values by 22.3% (ranging from 24.5% to 35.7%, p=0.02) with a concomitant increase in hemoglobin levels, reaching an average of 11.1 g/dl in transplanted mice vs 8.8 g/dl to those prior to transplantation, which corresponds to a 25.5% increase (p=0.008). Human γ-globin was detected in the peripheral blood of all transplanted animals by flow cytometry and ranged from 20 to 45%. Transduction efficiency in these experiments was estimated to be 35-50% as assessed *in vitro* in CFUs by PCR for vector-specific sequences. In summary, these results demonstrate for the first time that viral-mediated globin gene transfer via an LCR-free γ-globin lentiviral vector in hemopoietic stem cells effectively corrects a severe hemoglobin disorder.

223. Duration of Factor IX Expression in Macaques Following AAV8-Mediated Liver Transduction

Hiroaki Mizukami[1], Tsukasa Ohmori[2], Ryosuke Uchibori[1], Yasushi Saga[1], Masashi Urabe[1], Akihiro Kume[1], Yoichi Sakata[3], Keiya Ozawa[4]
[1]Div. of Genetic Therapeutics, Jichi Medical University, Shimotsuke, Japan, [2]Dept. of Biochemistry, Jichi Medical University, Shimotsuke, Japan, [3]Div. of Cell and Molecular Medicine, Jichi Medical University, Shimotsuke, Japan, [4]Director, IMSUT Hospital, The University of Tokyo, Minato-ku, Japan

We have been evaluating the efficacy and safety of hemophilia gene therapy using macaques, and demonstrated that liver-targeted approach with AAV8-based vector has resulted in therapeutic level of factor IX expression at the practical vector doses. Another important finding is the inhibitory effect of neutralizing antibody (NAb) against AAV capsid upon AAV vector-based gene transfer approach; none of the subjects with positive NAb showed recognizable transgene expression following IV injection of the vector. At this stage, one of the most crucial questions is the duration of this therapeutic modality. As this approach utilizes hepatocyte transduction by a non-integrating vector, the length of this therapy should depend on the lifetime of the transduced cells. During the series of experiment, we observed 5 animals for long-term outcome (longer than 3 years). All of these animals showed therapeutic levels of factor IX expression following vector injection, and the expression levels lasted throughout the observation period in all of the animals. In one animal, the expression level was kept constant within therapeutic level for more than 7 years. No adverse effect was observed in any of the animals. These findings suggest long-term efficacy and safety of this therapeutic approach, supporting application to human treatment. This study was performed in collaboration with Tsukuba Primate Research Center, National Institute for Biomedical Innovation, and The Corporation for Production and Research of Laboratory Primates, Japan.

224. A Gene Therapy Clinical Study of a Patient with X-Linked Chronic Granulomatous Disease

Toshinao Kawai[1], Kohji Okamura[2], Mari Yagita[1], Fumihiro Goto[1], Yumiko Nakazawa[1], Toru Uchiyama[1], Kazuhiko Nakabayashi[3], Hiroyuki Nunoi[4], Harry Malech[5], Masafumi Onodera[1]
[1]Department of Human Genetics, National Center for Child Health and Development, Tokyo, Japan, [2]Department of Systems BioMedicine, National Center for Child Health and Development, Tokyo, Japan, [3]Division of Developmental Genomics, National Center for Child Health and Development, Tokyo, Japan, [4]Department of Pediatrics, University of Miyazaki, Miyazaki, Japan, [5]National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

BACKGROUND: X-linked Chronic granulomatous disease (X-CGD) is a primary immunodeficiency disease characterized by failure of the nicotinamide adenine dinucleotide phosphate enzyme system in phagocytes to produce reactive oxygen species (ROS). This disorder derives from defects in the CYBB gene encoding gp91phox and leads to recurrent infection and hyper-inflammation, occasionally represented by CGD-associated colitis (CGD colitis). Hematopoietic stem cell transplantation (HSCT) is the only curative therapy for X-CGD, and in Europe and America, gene therapy clinical trials have been performed to patients who have no HLA-matched

donor. To assess the safety and efficacy of hematopoietic stem cell gene therapy, we commenced a retroviral gene therapy clinical study using a busulfan-based preconditioning treatment regimen for CGD patients. **METHODS AND PATIENT:** The patient was a 27-year-old man with X-CGD. His condition was complicated by refractory subcutaneous abscess, fungal lung disease, lymphadenitis and CGD colitis, and he did not have an appropriate HLA-matched donor for HSCT. Granulocyte colony-stimulating factor -mobilized $CD34^+$ stem cells were transduced with an MFGS-based retrovirus vector encoding the therapeutic gene of CYBB *ex vivo*, and infused back to the patient after busulfan-based preconditioning. Clinical and biochemical assessments were performed to evaluate the safety and efficacy of retrovirus gene therapy. **RESULTS:** After engraftment of $6.5x10^6$/kg of $CD34^+$ cells including 76% of $gp91^{phox}$ expressing cells, the gene-modified phagocytes appeared in peripheral blood after day +14. Dihydrorhodamine (DHR) flow cytometry assay demonstrated that the 2.3% of neutrophils in peripheral blood produced ROS, and a vector insert copy number was 2.63 per DHR^+ neutrophil at day +95. His clinical symptoms of subcutaneous abscess and CGD colitis improved at almost the same time. The retrovirus insertion site of gene-modified peripheral blood cells revealed that there was no evidence of abnormal growth of clones with *MDS-EVI1*, *SETBP1* or *PRDM16* integrants at day +63. At day +182, gene marking of peripheral blood decreased to about 0.1% of neutrophils. PCR quantification of vector gene marking showed a vector insert copy number of 0.17 and 0.04 per neutrophil at day +95 and day +182, respectively. A vector insertion copy of bone marrow cells was 0.08 per marrow cell at day +183, suggesting that a reduction of gene-modified $CD34^+$ stem cells would cause a decrease of $gp91^{phox}$ expressing phagocytes in peripheral blood. **CONCLUSION:** The therapeutic gene itself does not confer a growth advantage on the transduced cells in CGD, which would impact on the long-term gene marking and the withdrawal of clonal expansion. As other studies have reported that residual ROS production could contribute to the survival of CGD patients, the presence of gene-modified ROS-producing phagocytes led to the improvement of severe infection in our patient.

225. Preserving CD4+ T-Cells Phenotype and Function Upon *Ex Vivo* Lentiviral Transduction

Isabelle Hmitou[1], Amani Ouedrani[1], Lounes Djerroudi[1], Cécile Roudaut[1], Laure Caccavelli[1], Tayebeh S. Soheili[2], Isabelle André-Schmutz[2], Marina Cavazzana[3], Fabien Touzot[1]
[1]Biotherapy Department, Hôpital Universitaire Necker-Enfants Malades, Paris, France, [2]IMAGINE Institute, Université Paris Descartes, Sorbonne Paris Cité, Paris, France, [3]INSERM-APHP, CIC Biothérapie GHU Ouest, Paris, France

Ex vivo gene modification of T-cell is an appealing strategy for the treatment of both immunodeficiencies and neoplasia. Cell activation remains an essential prerequisite to genetic modification by lentiviral transduction. However, most current protocols for T-cell activation alter significantly the phenotype of transduced cells. This phenotypic change can constitute a drawback when the gene modification aims to correct a gene defect while preserving the T cell-subsets diversity. As we plan to conduct an open label phase I/II gene therapy trial for HIV-1 infected patients presenting with lymphoma where the patients will receive autologous hematopoietic stem cells transplantation with gene modified CD34+ cells and CD4+ T-cells, we investigate the use of the TransAct™ reagent (a CD3/CD28 nanoscale activator reagent from Miltenyi Biotec, Bergisch Gladbach, Germany) for the clinical grade transduction of CD4+ T-cells with the LVsh5/C46 lentiviral vector (Cal-1, Calimmune, Inc. Tucson, USA). LVsh5/C46 is a SIN lentiviral vector that inhibits two crucial steps of CD4+ T cell infection by the HIV virus: (i) attachment of the virus to its target by downregulation of CCR5 via a short hairpin RNA, (ii) fusion of the virus to the target cell through expression of the C46 inhibitor. Compared to previously published T-cells transduction protocols, the use of Miltenyi TransAct™ permits an efficient transduction - evaluated by measurement of vector copy number through quantitative PCR - without major phenotypic modification. Indeed, after activation with the TransAct™ reagent, *ex vivo* transduced CD4+ T-cells display very few changes in their surface markers with conservation of naïve (CCR7+CD62L+CD45RA+), central memory (CCR7+CD62L+CD45RA-) and effector memory (CCR7-CD62L-CD45RA-) subsets in superimposable proportions as initially. Moreover, expression of CD25 remains below 50% of cells suggesting a more "gentle " activation of the transduced CD4+ T-cells. Phenotypic analysis of transduced cells show no overt modification of cell subsets containing TH1, TH2 and TH17 memory T-cells. Furthermore, transduced T-cells retained the ability to be primed towards the TH1, TH2 and TH17 pathways. Cell sorting of *ex vivo* modified cells demonstrate a preferential transduction of naïve CD31+CCR7+CD62L+CD45RA+ T-cells as compared to other subsets. Besides, we did not find any significant impact of the transduction process on the TCRβ repertoire diversity as evaluated by high-throughput sequencing and analysis of diversity through the Gini-Simpson index or the Shannon index. This optimized protocol could be of valuable use for clinical trials aiming to provide the patient with diversified gene-corrected T-cells.

226. Lentiviral Vector Gene Therapy Protects XCGD Mice from Acute *Staphylococcus aureus* Pneumonia and Inflammatory Response

Giada Farinelli[1], Raisa Jofra Hernandez[1], Alice Rossi[2], Serena Ranucci[2], Francesca Sanvito[3], Maddalena Migliavacca[1], Chiara Brombin[4], Aleksandar Pramov[4], Clelia Di Serio[4], Bernhard Gentner[1], Alessandra Bragonzi[2], Alessandro Aiuti[5]
[1]HSR TIGET, Milan, Italy, [2]Infection and Cystic Fibrosis Unit, San Raffaele Scientific Institute, Milan, Italy, [3]Pathology Unit, San Raffaele Scientific Institute, Milan, Italy, [4]CUSSB-University Center for Statistics in the Biomedical Sciences, Vita-Salute San Raffaele University, Milan, Italy, [5]HSR TIGET, Vita-Salute San Raffaele University, Milan, Italy

Chronic Granulomatous Disease (CGD) is a primary immunodeficiency due to the mutation of one of the subunits of the NADPH oxidase complex. CGD patients are characterized by an increased susceptibility to bacterial and fungal infections as well as granuloma formation due to the excessive inflammatory responses. Several gene therapy (GT) approaches with lentiviral vectors have been proposed but in vivo preclinical data on the ability to control infections and inflammation are lacking. We set up a model of acute infection closely mimicking CGD patients airway infection by an intratracheal injection in X-CGD mice of a methicillin-sensitive reference strain (MSSA) of *S.aureus*. GT with hematopoietic stem cells transduced with regulated lentiviral vectors encoding CYBB gene restored the functional activity of NADPH oxidase complex (30-99% of dihydrorhodamine-DHR positive granulocytes and 16-73% of DHR positive monocytes) and rescued all mice from mortality due to *S.aureus* as compared to X-CGD or mock-transduced X-CGD mice (40% survival). Neutrophil infiltrate, bacterial burden and residual lung damage were similar to WT mice. When challenged with bacteria, X-CGD untreated mice showed an exaggerated production of pro-inflammatory cytokines and chemokine, while in GT treated mice inflammation was controlled to levels that were comparable to WT mice. In conclusion, we developed a useful and manageable infection model that can be used for the evaluation of the efficacy of GT treatment for CGD. Taken together, our results further support the clinical development of a gene therapy protocol for X-CGD using lentiviral vectors for the protection from infections and inflammation.

227. A Dose-Escalating Preclinical Study to Determine the Efficacy, MED, and Safety of a Clinical Candidate Vector in a Mouse Model of Hemophilia B

Lili Wang[1], John White[1], Zhenning He[1], Tamara Goode[1], Deirdre McMenamin[1], Christine Draper[1], Cassandra Brown[1], Edward A. Chroscinski[1], Lisa Kattenhorn[2], Gwen Wilmes[2], Reed Clark[2], Samuel Wadsworth[2], James M. Wilson[1]
[1]Gene Therapy Program, Department of Medicine, University of Pennsylvania, Philadelphia, PA, [2]Dimension Therapeutics, Cambridge, MA

Despite the availability of safe and effective recombinant FIX therapeutics, patients with Hemophilia B could obtain additional clinical benefit by having longer-acting therapeutic options. Although longer-acting recombinant protein FIX therapies are becoming available, the ultimate long-acting FIX therapy could be provided by a gene therapy approach, for example with rAAV vectors encoding FIX. Promising clinical data has been generated over the last few years in an academic setting, but these results must be replicated and extended in a commercial setting for these new therapies to become broadly available to patients.

Dose-dependent inflammatory responses that impact the transgene expression have been reported in clinical trials with rAAV FIX vectors, highlighting the need for clinical rAAV vectors to have the highest possible potency such that the overall vector dose can be reduced. We have developed a potent AAV serotype rh10 vector containing a wild-type FIX gene under the control of a highly active liver specific promoter with the goal of achieving approximately 10% of normal FIX levels in patients.

In preparation for a clinical trial, we tested this rAAVrh10FIX vector for aspects of efficacy and safety in the factor IX knockout (FIX-KO) mouse model of Hemophilia B. A dose-dependent increase in FIX protein and activity was observed following intravenous administration at vector doses between $1.6x10^{10}$ and $5.0x10^{13}$ GC/kg. FIX-KO mice that received AAVrh10-hFIXco at 1.6×10^{10} GC/kg achieved between 5% and 8% of normal hFIX levels at 2 weeks post-dose and maintained this level for the 90-day study period. FIX-KO mice that received rAAVrh10FIX vector at 5.0×10^{10} GC/kg achieved between 30% and 42% of normal hFIX levels at 14 days post-dose and maintained this level for the 90-day study period. From these results we can estimate that the vector dose required to achieve the stated goal of 10% of normal FIX levels of 10% would be between 1.6×10^{10} GC/kg and 5.0×10^{10} GC/kg. Expression of hFIX antigen correlated well with measured activity of hFIX antigen, with doses between 1.6×10^{10} GC/kg and 5.0×10^{10} GC/kg providing between 8% and 35% activity of normal hFIX levels.

FIX-KO mice provide an excellent model to examine the effects of AAVrh10-hFIXco on hemophilia B-related complications. FIX KO mice are prone to spontaneous bleeds and often suffer from fatal hemorrhages after handling or trauma. In this study, 4 out of 7 animals in the Day 90 control group succumbed to hemophilia-related complications. Single dose intravenous administration of AAVrh10-hFIXco at doses of 1.6×10^{10}, 5.0×10^{10}, 1.6×10^{11}, 5.0×10^{11}, 5.0×10^{12}, and 5.0×10^{13} GC/kg to FIX-KO mice did not result in test article-related mortality, clinical pathology, gross pathology, or histopathologic findings.

228. Characterization of Hematopoietic Progenitors from Pyruvate Kinase Deficient (PKD) Patients and Transduction of PKD CD34$^+$ Cells with a Therapeutic Lentiviral Vector

Susana Navarro[1,2], Maria Garcia-Gomez[1], Sergio Lopez-Manzaneda[1,2], Rebeca Sanchez-Dominguez[1,2], Maria Garcia-Bravo[1,2], Omaira Alberquilla[1,2], Maria M. Mañu[3], Manuela Muriel-Ramos[4], Joan Ll. Vives-Corrons[3], Julian Sevilla[5], Juan A. Bueren[2,6], Jose C. Segovia[1,2]
[1]Cell Differentiation and Cytometry Unit. Division of Hematopoietic Innovative Therapies, Centro de Investigaciones Energéticas Medioambientales y Tecnológicas/Centro de Investigación Biomédica en Red, Madrid, Spain, [2]Advanced Cell Therapies Mixed Unit, Instituto de Investigación Sanitaria Fundación Jiménez Díaz (IIS-FJD, UAM), Madrid, Spain, [3]IDIBAPS, Hospital Clinic de Barcelona, Barcelona, Spain, [4]Salamanca University Hospital, Salamanca, Spain, [5]Hospital Infantil Universitario Niño Jesús, Madrid, Spain, [6]Division of Hematopoietic Innovative Therapies, Centro de Investigaciones Energéticas Medioambientales y Tecnológicas/Centro de Investigación Biomédica en Red, Madrid, Spain

Pyruvate kinase deficiency (PKD) is an autosomal recessive disorder caused by mutations in the PKLR gene. PKD is the most common erythroid inherited enzymatic defect causing chronic non-spherocytic hemolytic anemia, high reticulocytosis, acute splenomegaly and intense iron overload in the liver, being life-threatening in severe patients. Up to date allogeneic bone marrow transplant represents the only curative treatment of patients affected by the severe form of the disease (5-10% of PKD patients aprox.). Preclinical gene therapy studies conducted in pyruvate kinase deficient mice have shown the safety and the efficacy of a new PGK-coRPK-Wpre therapeutic lentiviral vector that provided the designation by the European Medicinal Agency (EMA) of this PGK-coRPK-Wpre vector as a new orphan drug (EU/3/14/1130). To continue with the preclinical studies required to develop a clinical trial for PKD we have characterized the hematopoietic progenitor's content and the erythroid progenitors' profile in peripheral blood (PB) from PKD patients, showing an increase in both types of cells. For transduction studies, CD34$^+$ sorted cells from PKD PB were transduced with PGK-coRPK-Wpre viral vector supernatants. Hematopoietic progenitors' content and final yield of cells and CFCs was evaluated to analyze the toxicity of the vector. Percentage of transduction ranged from 31% to 100%, showing the efficacy of the transduction protocol and of the PGK-coRPK therapeutic vector. Vector copy number was quantified in both, individual CFUs and CD34$^+$ cells maintained in liquid culture for 14 days, ranging from 0.1 to 3.0 VCN/cells. Additional studies are being conducted to demonstrate PGK-coRPK functionality in human PKD cells.

229. PGE2 Increases Lentiviral Vector Transduction Efficiency of Human HSC

Garrett Heffner, Melissa Bonner, Dakota Campbell, Lauryn Christiansen, F. John Pierciey, Wen Zhang, Gretchen Lewis, Yegor Smurnyy, Amanda Hamel, Seema Shah, Holly Horton, Byoung Ryu, Kendrick Goss, Olivier Negre, Gabor Veres, Christopher Horvath, Mitchell Finer, Philip Gregory
Bluebird Bio, Cambridge, MA

Gene therapy for congenital hematopoietic disorders frequently relies on ex vivo lentiviral transduction of isolated CD34+ hematopoietic progenitor cells. Through a high-throughput small molecule screen, we identified PGE2 as a positive mediator of lentiviral transduction of hematopoietic stem and progenitor cells enriched from mobilized peripheral blood (PB CD34+ cells). CD34+

cells transduced with a VSVG-pseudotyped lentiviral vector in the presence of cytokines and 10 uM PGE2 yielded a vector copy number per cell (VCN) approximately 2-fold higher than CD34+ cells transduced in the absence of PGE2. This effect was seen consistently in 16 of 16 tested normal human donors in vitro, as well as primary CD34+ cells from both thalassemia and sickle cell disease patients. Importantly, PGE2 was observed to improve transduction of prospectively-isolated CD34+CD38- hematopoietic stem cells - a sub-population thought to be enriched for the long term repopulating stem cell. Transduction improvements were not associated with increased viral entry, but were associated with elevated expression of cAMP genes, supporting a post-entry mechanism of action that involves cAMP signaling downstream of prostaglandin receptors. Lastly, in a mouse xenotransplantation model of hematopoietic stem cell transplant, transduction of PB CD34+ cells in the presence of PGE2 improved VCN levels in engrafted human CD45+ cells 4-5 months post-transplant by ~2-fold without adversely affecting overall human cell engraftment. These data suggest that PGE2-mediated improvements in lentiviral transduction of human CD34+ cells could result in higher transduction efficiency and provide potential benefit in clinical gene therapy applications.

230. Designing High-Titer Lentiviral Vectors for Gene Therapy of Sickle-Cell Disease

Fabrizia Urbinati[1], Valentina Poletti[2], Beatriz Campo Fernandez[1], Roger P. Hollis[1], Colin Koziol[1], Michael L. Kaufman[1], Devin Brown[1], Annarita Miccio[3], Michael Antoniou[4], Donald B. Kohn[1], Fulvio Mavilio[2]

[1]UCLA, Los Angeles, CA, [2]Genethon, Evry, France, [3]Imagine Institute, Paris, France, [4]King's College London, London, United Kingdom

Sickle cell disease (SCD, OMIM 603903) is one the most common inherited blood disorders worldwide, caused by a single amino acid substitution (E6V) in the β-globin chain. SCD is associated with abnormal red cell morphology, hemolytic anemia, vascular occlusion, severe pain, progressive organ damage and reduced life expectancy. Gene therapy by transplantation of autologous, genetically corrected hematopoietic stem cells could be a therapeutic alternative, particularly in patients lacking an allogeneic bone marrow donor. Recent attempts to treat β-thalassemia and SCD with gene therapy showed encouraging results as well as potential limits of a technology based on complex lentiviral vectors for stem cell transduction due to the vector size and complexity, and limited globin synthesis. Designing an efficient vector that combines high-level globin expression with high titer and infectivity remains a formidable challenge. The GLOBE vector carries a β-globin gene under the control of its promoter and a reduced version of the β-globin locus control region (LCR); it was designed to reduce the size and complexity of the LCR while maintaining its enhancer and chromatin opening activity (Miccio et al., PNAS 2008). Based on the GLOBE design, we developed novel LVs for SCD gene therapy, carrying a modified transgene encoding an anti-sickling β-globin gene with 3 amino acid substitutions (AS3), alternative β-globin promoters and a short sequence (FB) with insulating and enhancer-blocking activity in the LTR. The vectors are produced at high titer and reproducibly transduce 60-70% of human BM CD34$^+$ cell-derived clonogenic progenitors at an average VCN of 3. The GLOBE vectors were compared to the Lenti/β^{AS3}-FB vector (Romero, Urbinati et al. JCI 2013), previously developed for a gene therapy clinical trial of SCD. We will present data comparing the different vectors for transduction efficiency in BM CD34$^+$ cells from healthy donors and SCD patients, β^{AS3}-globin transgene expression and anti-sickling activity, *in vivo* repopulation activity in NSG mice and vector integration profiles in repopulating cells before and after transplantation. This study provides a comprehensive analysis of different LV vectors, useful to determine the optimal candidate for gene therapy of SCD that can provide high transduction of Hematopoietic Stem and Progenitor Cells (HSPCs) as well as maintaining an adequate expression of the anti-sickling globin gene.

231. Mixed Chimerism After Allogeneic Hematopoietic Stem Cell Transplantation in Sickle Cell Disease: Preliminary Results on Peripheral Blood Sorted Subpopulations and Erythroid Progenitors

Alessandra Magnani[1], Laure Caccavelli[1], Mathieu Fusaro[1], Jeremy Magalon[1], Elisa Magrin[1], Emmanuelle Six[2], Fabien Touzot[1], Chloé Couzin[1], Jean Antoine Ribeil[1], Cécile Arnaud[3], Corinne Pondarré[3], Annie Kamdem[3], Françoise Bernaudin[3], Jean Paul Vernant[4], Eliane Gluckman[5], Dominique Bories[6], Marina Cavazzana[1]

[1]Biotherapy Department, Necker-Enfants Malades Hospital, Paris, France, [2]INSERM Unité Mixte de Recherche 1163, Laboratory of Human Lymphohematopoiesis, Institut Imagine, Paris, France, [3]Centre de Référence de Drépanocytose, CHIC Centre Hospitalier Intercommunal de Créteil, Créteil, France, [4]Hematology Department, Pitié-Salpêtrière Hospital, Paris, France, [5]Hôpital Saint Louis, Paris, France, [6]Hématologie Moléculaire, Hôpital Henri Mondor, UPEC, Créteil, France

Introduction: Patients with sickle cell disease (SCD) may develop a persistent mixed chimerism (MC) after hematopoietic stem cell transplantation (HSCT) associated with clinical control of the disease. In order to further investigate this condition we analyzed the chimerism in sorted myeloid and lymphoid subpopulations, and erythroid progenitors.

Methods: Between 1990 and 2013, 112 sickle cell disease (SCD) patients underwent allogeneic HSCT after myeloablative conditioning. Among the 107 patients with available chimerism at 2 years, 55.1% had a MC, i.e. <95% of cells from donor origin on white blood cells (WBCs). Myeloid (CD14+, CD15+) and lymphoid (CD3+, CD19+) subpopulations, erythroid burst-forming units (BFU-E) and granulocyte monocyte colony forming units (CFU-GM) were analyzed in patients presenting with MC, follow-up ≥1 year and the hemoglobin (Hb) profile comparable to their donors'. High performance liquid chromatography (HPLC) analysis was performed on whole blood.

Results: Thirty patients were included. Preliminary results from 13 patients with clinical control of the disease reveal heterogeneous patterns of subpopulation chimerism (table). A significant discrepancy in chimerism measured in WBCs compared to the other populations is observed. Patients were divided into two groups according to the difference in the donor chimerism between the CD3+ and in the myeloid compartment. This difference in donor chimerism in CD3+ cells might be explained by the expansion of recipient's memory T cells after transplantation and/or a specific T-cell progenitor engraftment in these patients. Interestingly, a trend to a lower percentage of donor chimerism in the BFU-E than in the peripheral erythroid compartment is observed.

Conclusions: These data show that the chimerism analysis on total WBCs is a poor indicator of the myeloid and erythroid engraftment in SCD patients. Chimerism study on sorted subsets and erythroid progenitors allows a more exhaustive investigation. Further analyses on T cells compartment are necessary to complete this information. Results in BFU-E as compared to the peripheral erythroid compartment suggest a selective genetically modified CD34+ cells.

Pt ID	Donor phenotype	Months post HSCT	Hb (g/dl)	HPLC				Donor chimerism (%)						
				HbA (%)	HbS (%)	HbA2 (%)	HbF (%)	Whole blood	CD3+	CD14+	CD15+	CD19+	BFU-E	CFU-GM
1	AA	21	14.2	84.5	0	na	na	66	40.5	98.5	>95	90	na	na
6	AA	91	11.8	93.8	3.1	3.1	0	19	12	23	31	13	37.5	57
7	AS	155	13.5	56.5	41	2.6	0	58	34	71	na	49	75	60
4	AS	132	10.6	54	33.3	na	na	53	44	44	46	58	32	37
17	AS	13	11.5	47.8	41.2	3.2	1.4	85	75	na	92	73	83	76
18	$A/D^{ins/ab}$	80	12.6	47.9	0	na	na	88	77	95	95	94	94.5	99
12	A/b0Thal	57	9.6	95	0	3	2	91	68	96	98	99	99	100
11	AA	54	13.4	97	0	3	0	88	78	100	100	99	98	98
5	AA	34	10.9	78.3	8	na	na	44	46	41	na	50	58	43
2	AS	46	8.4	24	68.1	na	na	21	70	9.4	5.4	31	12.5	na
8	AS	133	9.7	34.7	63	2,4	0	16	31	12	10	13	0	8
9	AS	153	9.5	52.1	45.9	2	0	18	30	26	16	11	17	15
3	AA	33	10.1	89.5	5	3.2	2.3	30	63	17	18	23	13	17

232. Generalized Entropy Based Clonal Diversity Estimation of TCR Repertoire

Saira Afzal, Shahzad Ahmad, Richard Gabriel, Eliana Ruggiero, Christof von Kalle, Manfred Schmidt, Raffaele Fronza
DKFZ (German Cancer Research Center), Heidelberg, Germany

In depth study of T-cell receptor (TCR) repertoire, specifically clonal expansion of T cells and diversity of repertoire, can provide invaluable information in various health and diseases states including infection, cancer and genetic disorders etc. Currently, Shannon and Simpson diversity indices used to estimate TCR clonality have certain limitations. These indices are biased either towards total number of different TCR receptor types (richness) or distribution of each clonal type (evenness). We introduced here a new framework for accurate and precise quantification of clonal diversity of TCR cell population that overcomes shortcomings of already available indices. Our concept is based on the generalized form of entropy known as Rényi numbers and two components of diversity, richness and evenness. Based on the Rényi equation, we can prove that at a specific richness value the highest possible evenness obtained cannot go beyond the stated richness value. Plotting richness versus evenness helps to define maximal theoretical polyclonality and monoclonality regions and diversity of sample can be measured by estimating distances of sample from these theoretical bounds. We have validated our method on in-silico data sets and TCR repertoire study. We conclude that our idea of clonal plane and diversity index measurement provides more reliable and robust estimation of immune population diversity. It can prove to be a useful tool for quantitative characterization of TCR repertoire in immunological studies.

233. The Efficacy of Megakaryocytic Progenitor Products Generated from Cord Blood in Acute Myeloid Leukemia Patients: A Before-After Phase 2 Trial

Xuetao Pei
Beijing Institute of Transfusion Medicine, Academy of Military Medical Sciences, Beijing, China

Infusion of cord blood-derived megakaryocytic progenitors (MPs) products shows safe and feasible for treatment of thrombocytopenia in hematological malignancies in our phase 1 study. This phase 2, before-after study assigned 54 patients with acute myeloid leukemia (AML) to receiving MPs products or not after consolidation chemotherapy with high-dose ara-C (HDAC). One patient from MPs group and one from control group withdraw the study. Compared to control group, the MPs group achieved a decreased platelet infusion rate (7.5% [4/53] *vs.*22.6% [12/53], p=0.03), platelet infusion units (1.0 *vs.* 2.0 Units, p=0.001), time of platelet count recovery normal (>100×10^9/L, p=0.01). Only two patients had reversible fever (below 38℃) and one patient had reversible local skin rash. No patients withdraw due to adverse event. MPs products were active and well tolerated, with decreased platelet infusion rate and platelet count recovery after chemotherapy. This study was registered at http://www.chictr.org as # ChiCTR-TRC-11001691.

Cell Therapies I

234. Efficient Generation of Stable Genetically Modified Human iPSC-Derived Macrophages for Innovative Gene and Cell Therapeutic Strategies

Mania Ackermann[1], Uta Müller-Kuller[2], Sylvia Merkert[3], Ulrich Martin[3], Manuel Grez[2], Axel Schambach[4], Thomas Moritz[5], Nico Lachmann[1]
[1]JRG Translational Hematology of Congenital Diseases, Institute of Experimental Hematology, REBIRTH, Hannover Medical School, Hannover, Germany, [2]Institute for Tumor Biology and Experimental Therapy, Georg-Speyer-Haus, Frankfurt, Germany, [3]LEBAO, Department of Cardiac, Thoracic, Transplant and Vascular Surgery, REBIRTH, Hannover Medical School, Hannover, Germany, [4]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany, [5]RG Reprogramming and Gene Therapy, Institute of Experimental Hematology, REBIRTH, Hannover Medical School, Hannover, Germany

Hematopoietic differentiation of human induced pluripotent stem cells (iPSC) holds great promise for disease modeling, drug testing, and in particular for the development of novel gene and cell therapeutic strategies. In the past, interest has been directed primarily at the generation of reconstituting hematopoietic stem cells, a cell type as of yet problematic to obtain from hPSCs. Recently however, also long-lived mature myeloid cells such as tissue resident macrophages have been described and further characterized. Therefore, transplantation of macrophages may serve as an innovative treatment approach in several diseases such as pulmonary alveolar proteinosis (PAP) and adenosine deaminase deficiency (ADA). In this line, we have established an efficient, embryoid body-based human iPSC-differentiation protocol employing IL-3 in combination with M-SCF to continuously (up to 6 months) generate large numbers (5-10x10^6/week/6 well plate) of >95% pure monocyte/macrophages (iPSC-MΦ) via an intermediate "myeloid cell forming complex". The iPSC-MΦ revealed typical morphology, surface phenotype and functionality comparable to their *in vivo* derived counterparts. In order to evaluate the suitability of this protocol also for gene therapy, we transduced human iPSC with 3rd generation SIN lentiviral vectors employing a minimal and safety optimized ubiquitous chromatin opening element (CBX3-UCOE) to prevent differentiation-associated epigenetic silencing. Here, GFP reporter gene expression driven by the elongation factor 1α short (EFS) promoter resulted in >90% GFP^+ iPSC-MΦ after myeloid differentiation. In contrast, the EFS promoter without the CBX3-UCOE was subject to massive epigenetic silencing. Moreover, also a myeloid-biased myeloid-related protein 8 (Mrp8) promoter was efficiently protected from silencing, whilst tissue specificity was not affected by the CBX3-UCOE. Of note, the protective activity of the CBX3-UCOE was associated with decreased levels of repressive histone marks (H3K27me3, H3K9me3) and increased levels of active marks (phosphorylated polymerase). Alternatively, we also targeted an expression cassette to the AAVS1 locus employing zinc finger nuclease mediated homologous recombination. Selected clones showed targeted and robust CMV early enhancer/chicken β actin (CAG) promoter-driven fluorochrome expression in the pluripotent status as well as in terminally differentiated iPSC-MΦ. In summary, we here describe silencing resistance lentiviral vectors and genome editing strategies as well as a differentiation protocol allowing for the efficient and long-term generation of stable genetically modified iPSC-MΦ, thus paving the way for novel gene and cell based therapies.

235. Improved Ex Vivo Gene Therapy Using Highly Purified Hematopoietic Stem and Progenitor Cells

Erika Zonari, Oriana Meo, Samantha Scaramuzza, Eugenio Montini, Giuliana Ferrari, Luigi Naldini, Bernhard Gentner
San Raffaele Telethon Institute for Gene Therapy, Milan, Italy

Ex vivo gene addition into CD34+ hematopoietic stem and progenitor cells (HSPC) followed by autologous transplantation has proven a safe and efficacious therapy for immunodeficiencies, storage diseases and hemoglobinopathies. According to xenograft models and population size estimates of vector-marked cells in gene therapy-treated patients, less than 0.01% of infused CD34+ cells drive long-term (LT) repopulation. Advances in clinical-grade cell sorting technology may make HSC-enriched CD34+ subpopulations accessible for gene therapy, with advantages in terms of lentiviral vector (LV) cost, safety (lowering of integration load) and, potentially, efficacy. By differentially marking mobilized peripheral blood (mPB) CD34 subpopulations distinguished by increasing levels of CD38 expression in order to quantitatively assess their hematopoietic output in an NSG xenograft model over 6 months, we previously mapped most (>90%) LT repopulating capacity to CD34+CD38- cells (lowest 10% CD38 staining), while CD34+CD38int cells drove short-term (ST) reconstitution during the first 2 months after transplantation. We now characterize these subpopulations in terms of CD90 expression, a marker, which has been used in conjunction with CD34 for HSC purification in past clinical trials. CD34+ mPB cells were sorted into CD38-90+ (5% of CD34+), CD38-90- (5%), CD38+90+ (30%) and CD38+90- (60%) fractions and exhaustively transduced with GFP-, OFP-, BFP- and mCherry-expressing LVs, respectively. Differentially marked subfractions were pooled maintaining their original proportions and transplanted into NSG mice. ST engraftment mainly came from CD38+ cells, with equal contribution from the CD90+ and CD90- compartment. LT engraftment was almost exclusively derived from CD34+CD38- cells, of which 70% came from CD90+ and 30% from CD90- cells. Hence, CD34+CD38- is a more sensitive and specific marker combination than CD34+CD90+ to purify LT-HSC. CD34+CD38- cells can be purified by a sequential bead-based selection (CD34 selection of CD38-depleted cells) potentially applicable to clinical practice. We show that CD34+CD38- cells can be efficiently transduced with clinical grade LVs using shortened ex vivo manipulation protocols, reaching similar gene marking levels as with the standard protocol currently used in clinical trials that comprises a double dose of LV. Transduction was stable for at least 5 months when serially measured in xenotransplanted mice, and mice showed multi-lineage hematopoiesis indistinguishable from CD34+ grafts. Based on these results, we are aiming towards clinical development of a new gene therapy protocol based on CD34+CD38- HSPC efficiently transduced with minimum ex vivo culture time (<36h). Our platform will substantially improve the efficacy, safety and feasibility of future ex vivo gene therapy studies.

236. Role of P53 on T Lymphopoiesis from Human Embryonic Stem Cells

Hiroshi Kohara[1], Jiyuan Liao[2], Shohei Miyamoto[1], Yoko Nagai[2], Tomotoshi Marumoto[2], Kenzaburo Tani[1]
[1]Project Division of ALA Advanced Medical Research, Institute of Medical Science, the University of Tokyo, Tokyo, Japan,
[2]Division of Molecular and Clinical Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan

Although pluripotent stem cells are well recognized as a potential source of cell therapy, it is still needed to improve efficiency to differentiate into target cell lineages. Tumor suppressor P53 regulates multiple signaling pathways triggered by diverse cellular stresses including DNA damages, oncogenic stimulations, and hypoxic stress, resulting in cell-cycle arrest, apoptosis, and senescence. P53 signaling is also important for double-stranded DNA breaks (DSBs) induced during physiologic events, i.e., rearrangement of antigen-specific receptors. It has been reported that P53-mediated DSB checkpoint contribute to normal murine T lymphopoiesis, especially at the double-negative (DN) stage which is defined as CD4-CD8- fraction in thymus and requires rearrangements of the T cell receptor (TCR) β locus and successful pre-TCR signaling. Here we defined the role of P53 on lymphopoiesis from human embryonic stem cells (ESCs).

Firstly we modified P53 gene of human ESC H1 by utilizing of zinc finger nuclease targeting the P53 gene, kindly provided by Sangamo BioSciences. Sequencing analysis of the P53 knockout (KO) ES cells showed the successful deletion which induced the frame shift of the downstream sequence in both of its alleles. Western blot analysis of P53 phosphorylation status in P53 KO ESCs showed undetectable levels of phosphorylated or non-phosphorylated P53 proteins when cultured in the presence or absence of apoptotic signal triggered by mitomycin C (MMC). In consistent with this, P53 KO ESCs showed significant resistance to MMC-induced cell death. In addition, P53 KO ESCs lacked apoptotic stimulation-induced upregulation of P53 downstream target genes including P53 up-regulated modulator of apoptosis (PUMA).

We then induced hematopoietic differentiation of P53 KO ESCs through embryoid body formation. Erythroid lineage cells developed from human ESCs were significantly suppressed in the absence of P53 signaling during embryoid body maturation. Pharmacological inhibition of P53 had the same effect as genetic disruption of P53 gene. CD34+ hematopoietic precursors were isolated from embryoid bodies originated from H1 and P53 KO ECSs, plated on OP9-DL1 stromal cells, and cultured in the presence of stem cell factor (SCF), FLT3 ligand, and interleukin (IL)-7. After 3-4 weeks of culture, CD45+CD3+ T lineage cells were induced from both H1 and P53 KO ECSs-derived CD34+ cells. Among these cells, most of the cells were in CD4+CD8+ double-positive (DP) stage, with increase in the yield of DP cells in the absence of P53 signaling (H1: 343 cells/1 x 10^6 input CD34+ cells; P53 KO: 2476 cells /1 x 10^6 input CD34+ cells; Figure). Whether pharmacological inhibition of P53 had the similar effect on T lymphopoiesis as genetic disruption of P53 gene needs to be investigated furthermore.

Our data indicate that P53 mediated signaling regulate in vitro early T lymphopoiesis from human pluripotent stem cells, especially at the transition from double negative into DP stage. These observations promoted us to perform high throughput transcriptome analysis including cDNA microarray analysis between early T lineage cells derived from H1 and P53 KO ESCs. Genes associated with the early T lymphopoiesis from human ESCs were identified and currently under further characterization.

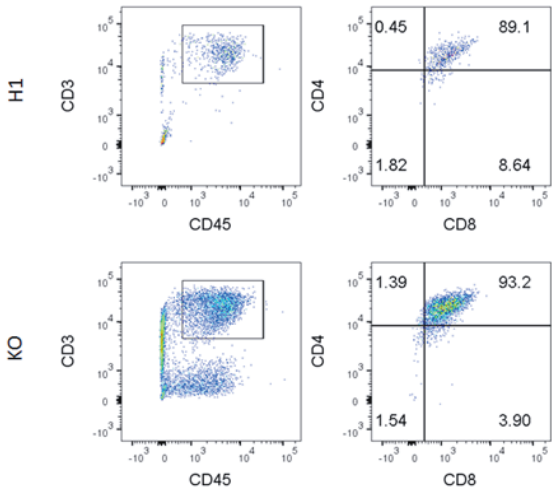


Figure 1: Flow cytometric analysis after induction of T lymphopoiesis

237. Abstract Withdrawn

238. Cell Therapeutic Approach Using Dental Pulp Stromal Cells for Duchenne Muscular Dystrophy

Yuko N. Kasahara[1,2], Mutsuki Kuraoka[2], Hiromi H. Kinoh[1,2], Chiaki Masuda[1], Kiwamu Imagawa[3], Katsuhiko Tachibana[3], Shin'ichi Takeda[2], Takashi Okada[1,2]
[1]Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan, [2]Molecular Therapy, National Institute of Neuroscience, NCNP, Tokyo, Japan, [3]JCR Pharmaceuticals Co., Ltd., Hyogo, Japan

Background: Duchenne muscular dystrophy (DMD) is an incurable genetic disease with early mortality that exhibits skeletal muscle weakness with chronic inflammation. Dental pulp stromal cells (DPCs) could be potential therapeutics because of their immunosuppressive properties and multipotency. In the present study, we examined the strategies for effective cell transplantation to develop a novel approach for functional recovery of the skeletal as well as cardiac muscles using a dog model of Duchenne muscular dystrophy. **Methods:** DPCs were intravenously injected into two littermates of canine X-linked muscular dystrophy in Japan ($CXMD_J$) at weekly interval for 8 weeks without immunosuppression. Clinical phenotypes in the transplanted dogs were analyzed by using blood exams, physical capacity, magnetic resonance imaging (MRI) analysis compared with non-injected littermates as controls. **Results:** The downregulation of inflammation in the lower legs of DPCs-treated $CXMD_J$ was confirmed by MRI analysis. Serum levels of pro-inflammatory cytokine IL-6 were well regulated in the treated $CXMD_J$ during experiments, unlike non-injected dogs. Although $CXMD_J$ showed progressive muscle atrophy in the all four limbs, exercise intolerance and abnormal locomotion, we observed improved phenotypes in the DPCs-treated $CXMD_J$ along with the improved pace of walking and running. **Conclusion:** We suggested that the systemic injection of DPCs ameliorated the progressive phenotype in $CXMD_J$. The therapeutic effects might be associated with the production of paracrine or endocrine factors that regulate inflammation, and might also stimulate the proliferation of endogenous stem cells at the injured muscle tissue. This strategy of DPCs treatment would be promising for the future DMD cell therapy.

239. Induction of Allograft Immune Tolerance with Bioengineered Thymus Organoids

Asako Tajima[1], Isha Pradhan[1], Ipsita Banerjee[2], William A. Rudert[1], Massimo Trucco[1], Yong Fan[1]
[1]Institute of Cellular Therapeutics, Allegheny Health Network, Pittsburgh, PA, [2]Chemical Engineering, University of Pittsburgh School of Engineering, Pittsburgh, PA

One of the major obstacles in solid organ transplantation is to establish immune tolerance of allografts. While immunosuppressive drugs can prevent graft rejection to a certain degree, their efficacies are limited, impermanent, and associated with severe side effects. As a primary immune organ essential to adaptive immunity, the thymus continuously generates a diverse population of naïve T-cells, which can effectively react to invading pathogens, but remains unresponsive to self-antigens. Many factors (e.g. infection, irradiation, drug treatments, or aging) can irreversibly compromise thymic function, resulting in immune deficiency, cancer, autoimmunity and other immunodysregulatory diseases. While numerous efforts have been made to modulate/rejuvenate thymic function, manipulating the thymus, either in vitro or in vivo, proves to be difficult. The major challenge is to reproduce its unique extracellular matrix microenvironment that is critical for the survival and function of thymic epithelial cells (TECs). TECs are the predominant population of thymic stromal cells that are essential for T-cell lineage determination and maturation. TECs cultured in the traditional 2-D culture rapidly lose their molecular properties and fail to grow, which prohibits them from undergoing genetic modification.We have recently developed a thymus decellularization technique, which allows us to reconstruct a functional thymus organoid de novo with isolated TECs. The 3-D thymic scaffolds can support the survival of TECs in vitro, and maintain their unique molecular properties. When transplanted into athymic nude mice, the bioengineered thymus organoids could effectively promote the homing of lymphocyte progenitors and support the development of a diverse, self-tolerant T-cell repertoire. The thymus-reconstructed nude mice could promptly reject skin allografts, and were able to mount antigen-specific humoral responses against ovalbumin upon immunization. Notably, tolerance to allogeneic skin grafts could be achieved by transplanting thymus organoids constructed with either TECs co-expressing both syngeneic and allogenic MHCs, or mixtures of donor and recipient TECs. Our results demonstrate the technical feasibility of inducing donor-specific allogeneic tolerance with bioengineered thymus organoids and highlight the clinical implications of this thymus reconstruction technique in solid organ transplantation and regenerative medicine.

240. Lenti-BMP2/GFP Gene Therapy Enhance the Chondrogenic and Osteogenic Differentiation of Human Muscle Derived Stem Cells In Vitro

Xueqin Gao[1], Ying Tang[2], Aiping Lu[1], Bing Wang[2], Xiaohong Bi[3], Guijin Lv[3], Johnny Huard[1]
[1]Orthopaedic Surgery, University of Texas Health Science Center at Houston, Houston, TX, [2]Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA, [3]University of Texas Health Science Center at Houston, Houston, TX

Human muscle derived stem cells (hMDSCs) have been shown to be multipotent in vitro and can repair critical size bone defects as efficiently as human bone marrow stem cells, but require BMPs. In this study, we constructed a new lenti-BMP2/GFP vector and tested its ability to promote in vitro chondrogenesis and osteogenesis of the hMDSCs. **Material and methods:** 1. Lenti-BMP2/GFP was made by inserting human BMP2 gene up-stream of an eGFP tag gene in a Lenti-viral vector with a CMV promoter. 2. hMDSCs were isolated from skeletal muscle via the preplate technique. All 6 populations were transduced with the Lenti-BMP2/GFP virus,

expanded and sorted via FACS based on GFP fluorescence. BMP2 secretion was measured using an R&D ELISA kit. 3. Osteogenesis: 3D pellet cultures were used for osteogenic differentiation of non-transduced and Lenti-BMP2/GFP transduced hMDSCs. After 4 weeks, the cell pellets were fixed and scanned using VivaCT40 to detect mineralization. The pellets were embedded in NEG medium and snap frozen in liquid nitrogen, sectioned and von Kossa stained. Osteogenic differentiation was detected by staining for osteocalcin. 4. Chondrogenesis was performed on non-transduced and Lenti-BMP2/GFP transduced hMDSCs using a 3D pellet culture method. Cell pellets were cultured for 24 days in chondrogenic medium, embedded in freezing medium and cryosectioned. Alcian blue staining and Col2A1 immunohistochemistry was also performed. Raman Spectroscopy was used to quantify chondroitin sulphate and collagen. **Results**: 1. Lenti-BMP2/GFP transduction enhanced osteogenesis of the hMDSCs. BMP2 secretion levels of the sorted lenti-BMP2/GFP transduced cells are showed in Fig.1A. MicroCT results of the lenti-BMP2/GFP transduced cells showed they had an increase in mineralized pellet volume (Fig.1B,C). Von Kossa staining indicated that the non-transduced hMDSCs had variable brown-black mineralization while the lenti-BMP2/GFP transduced cells all showed stronger mineralization (Fig.1D). Osteocalcin staining showed lenti-BMP2/GFP transduction enhanced osteogenic differentiation of the hMDSCs (Fig.1E). 2. Lenti-BMP2/GFP transduction enhanced chondrogenesis of the hMDSCs. Alcian blue staining showed a significantly bluer chondrogenic matrix in the Lenti-BMP2/GFP transduced cell pellets compared to non-transduced hMDSCs (Fig2A and 2B). Raman Spectroscopy indicated the Lenti-BMP2/GFP transduced hMDSC exhibited higher amounts of chondroitin sulphate and collagen (Fig.2C, D). Col2A1 staining showed more Col2A1 in the lenti-BMP2/GFP transduced hMDSCs compared to non-transduced hMDSCs (Fig.2E).**Conclusion:** We found that all the Lenti-BMP2/GFP transduced hMDSCs showed enhanced osteogenesis and chonrogenesis compared to non-transduced cells. This in vitro study lays the foundation for using Lenti-BMP2/GFP gene transfer to repair bone and cartilage defects.

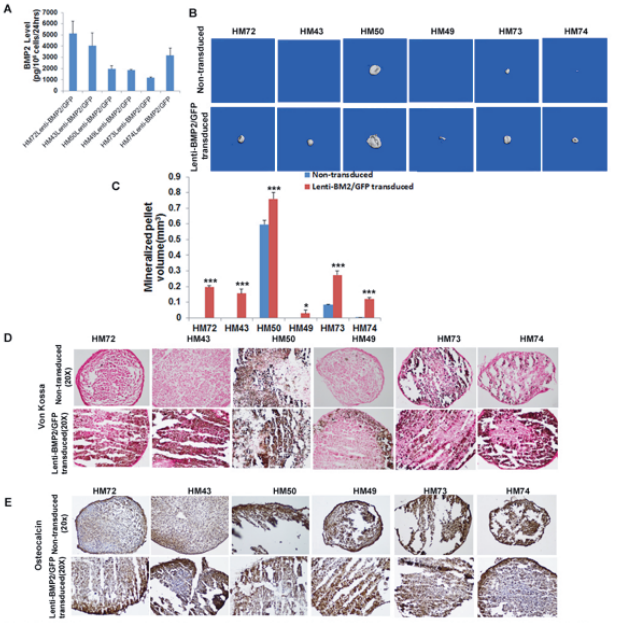


Fig.1: A. BMP2 secretion level of 6 populations of Lenti-BMP2/GFP transduced hMDSCs. B. MicroCT 3D images of pellets. Lenti-BMP2/GFP transduced hMDSCs showed larger minaralized pellets.C. Quantification of mineralized pellet volume showed significant bigger pellet volume of Lenti-BMP2/GFP transduced hMDSCs compared to their non-transdiced hMDSCs counterpart. *p<0.05, *** P<0.001. D. Von Kossa staining showed more brown-black mineralized cells in the Lenti-BMP2/GFP transduced hMDSCs than in the non-transduced hMDSCs.E.Osteocalcinimmunohistochemistry demonstrated more osteocalcin positive cells in the Lenti-BMP2/GFP transduced hMDSCs compared to the non-transduced hMDSCs.

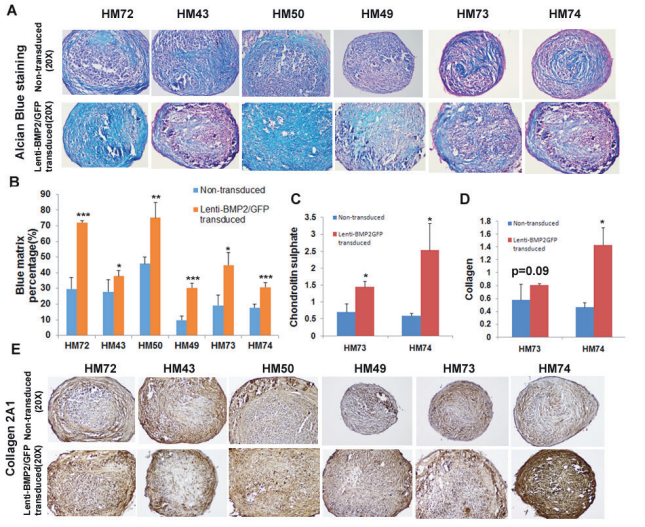


Fig.2. A. Alcian blue staining showed more blue matrix in the 6 populations of Lenti-BMP2/GFP transduced hMDSCs compared to non-transduced hMDSCs. B. Quantification of blue matrix showed significant higher percentage of blue matrix in the Lenti-BMP2/GFP transduced cells compared to non-transduced hMDSCs.C. Raman spectroscopy quantification indicated higher chondroitin sulphate amount in lenti-BMP2/GFP transduced hMDSCs than non-transduced hMDSCs. D. Raman spectroscopy indicated higher collagen content in the Lenti-BMP2/GFP transduced hMDSCs.E. Collagen 2A1(Col2A1) immunohistochemistry indicated stronger collagen 2A1 in the Lenti-BMP2/GFP transduced hMDSCs compared to its respective non-transduced hMDCs. *p<0.05, **p<0.01,*,p<0.0001.**

241. An Efficient Construction of Lentiviral Vectors That Identify and Eliminate Tumorigenic Cells in Pluripotent Stem Cells

Kanako Ide, Kaoru Mitsui, Ken-ichiro Kosai
Department of Gene Therapy and Regenerative Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan

Human pluripotent stem cells (hPSCs) are promising sources for cell transplantation therapy. However, incomplete abolition of tumorigenicity, including teratomas and cancers, causes potential safety concerns in their clinical trials. Importantly, most previous approaches focused on "INDIRECT" inhibition of tumorigenicity by reducing the reprogramming-associated oncogenic potential of hiPSCs. Because they cannot completely eliminate tumorigenic potentials due to the intrinsic characteristics of hPSCs, innovative safety approaches should be developed. In this regard, we first developed "adenoviral conditional targeting", which securely isolated target cells (*Mol Ther.* 14: 673-683. 2006), can increase the efficacy and safety by decreasing tumorigenicity. Second, we have recently developed a novel "oncolytic virus" strategy that specifically and efficiently eliminates undifferentiated cells, thereby inhibiting *in vivo* teratoma formation after hPSC transplantation (*Mol Ther Methods Clin Dev.* 2, 15026, 2015).

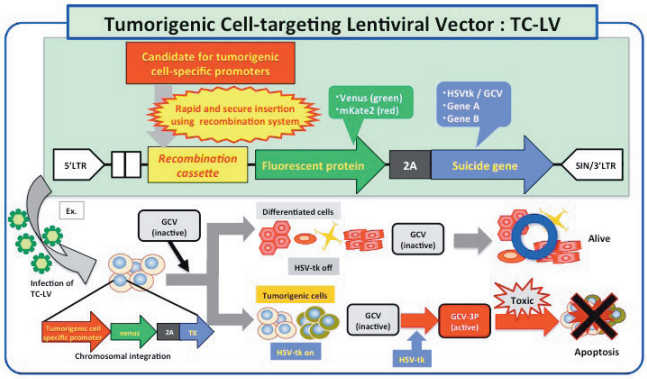
Here we present the third antitumorigenic strategy by a novel methodology that can efficiently generate diverse "Tumorigenic Cell-targeting Lentiviral Vectors (TC-LVs). Tumorigenic cells in the transduced hPSCs can be specifically identified and efficiently killed by fluorescent genes and suicide genes, respectively, under the candidate promoters, which should be specifically and strongly activated in hPCSs in the undifferentiated and/or the transformed status, but not in the differentiated one.

This system consists of two plasmids. One is the "LV-acceptor plasmid" (pLVA) that has a recombination cassette, upstream to the unit consisting of one of two fluorescence genes, 2A sequence and one of candidate suicide genes. The other is the "promoter-subcloning plasmid" (pPS) that has multicloning sites flanked by recombination

Molecular Therapy Volume 24, Supplement 1, May 2016

sequences. We tested the feasibility and the utility of this construction system using the five candidate promoters. First, the promoters cloned into the pPS were surely transferred to pLVA having one of three candidate suicide genes and one of two fluorescent genes by using recombinase, resulting in the feasible and rapid generation of the different types of TC-LVs. To test the efficacy of this system, human and mouse PSCs were transduced with TC-LVs composing Venus (EGFP) and Herpes Simplex Virus Thymidine Kinase (HSV-tk) genes driven by either of the ubiquitously active CA or the cancer-specific *survivin* promoter, and the visualized PSCs were subsequently purified by a cell sorter. All of the PSCs in the undifferentiated status were almost perfectly killed by an addition of ganciclovir (GCV) in the GCV dose-dependent and the promoter activity-dependent manners, whereas they were viable in the differentiated status. The results importantly suggest the necessity of the best combination of the promoter (activity and specificity properties) and the suicide and the fluorescent genes.

In conclusion, we have developed the novel method for a rapid generation of TC-LVs that can systematically identify the best promoter and suicide gene to surely eliminate tumorigenic cells, without harmful effect to the targeting differentiated cell. This methodology may facilitate the safe clinical application of PSCs-based cell therapy.



242. Genetically Engineered Mesenchymal Stem Cell Spheroids for Brain Tumor Therapy

Smruthi Suryaprakash[1], Hon Fai Chan[1], Juli Rodriguez[2], Shawn Hingtgen[2], Kam W. Leong[1]

[1]Biomedical Engineering, Columbia University, New York, NY, [2]Pharmacy, University of North Carolina, Chapel Hill, NC

Gliobastoma is one of the most aggressive forms of brain cancer. Each year 18,000 people are diagnosed with glioblastomas with an average survival of 10 to 12 months. MSC are an attractive candidate for cell based therapy since they can be easily isolated, manipulated and have no immune rejection. MSC transduced with TRAIL are capable of migrating towards the tumor cells and kill the tumor cells due to bystander effect[1]. However, for cell based therapy migration, survival and retention are the major factors. MSC in spheoridal form has shown to have increased expression of CXCR4 which is critical for migration towards tumor cells[2]. The spheroidal form also might help the cells to survive for longer and prevent it from getting washed out due to the extracellular matrix surrounding the cells and larger size respectively. Hence MSC in spheroidal created using a microfluidic platform was used for cell based therapy to treat glioma. The MSC self assembled to form spheroids within the double emulsion in 150 mins. Live/dead staining demonstrated high viability of the cells in the spheroid. Semi-quantitative RT-PCR demonstrated enhanced expression of CXCR4 in the MSC spheroids. This translated to higher migration of the MSC spheroids compared to MSC as seen by increased directionality, velocity, euclidian distance and displacement of the center of mass. MSC was shown to produce TRAIL constantly for 6 days. Condition media collected from MSC-sTRAIL single and spheroids were collected and showed higher caspase 3/7 activation and effectively killed the cancer cells. Hence MSCs in spheroidal form are an excellent candidate for treating glioblastomas. In the future the spheroidal retention and survival will be tested in vivo. This will be followed by survival analysis to ascertain the therapeutic efficacy of MSC-TRAIL.

Reference

1. Sasportas, L. S. *et al.* Assessment of therapeutic efficacy and fate of engineered human mesenchymal stem cells for cancer therapy. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 4822-4827 (2009). 2. Bartosh, T. J. *et al.* Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 13724-13729 (2010).

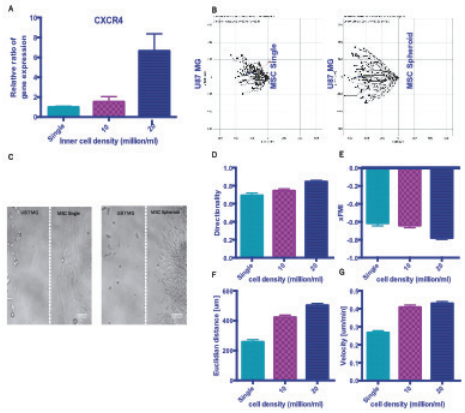


Figure 2. (A) CXCR4 expression of MSC Single and Spheroid cells of different size measured using q-PCR. (B) Path taken by cells tracked using manual tracking and analyzed using chemotaxis tool . (C) Time lapse imaging showing the migration of MSC towards U87 using ibidi's cell culture insert. (D) Value for the directionality of motion 1-> straight motion; 0-> non straight motion. (E) xFMI represent the efficiency of the forward migration of cells. (n>=50) (Data = mean ± SEM) (F) Euclidean distance between points p and q is the length of the line segment connecting them. (n>=50) (Data = mean ± SEM) (G) Velocity of the cell. (n>=50) (Data = mean ± SEM)

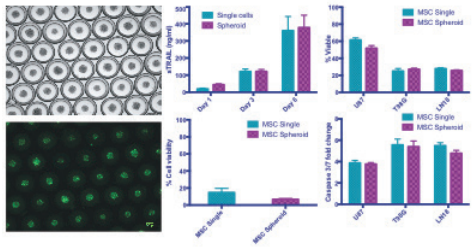


Figure 3. (A) Phase contrast image of MSC-TRAIL spheroids in double emulsion. (B) Fluorescent image of MSC TRAIL spheroid. (C) sTRAIL production of 5000 cells measured using ELISA. (D) Viability of cancer cells incubated with condition media from MSC-TRAIL cells and spheroid measured using CellTiter-GLO 3D. (E) Cell viability of U87 co cultured with MSC Single and MSC Spheroids for 24 hrs measured using CellTiter-GLO 3D. (F) Caspase 3/7 activation of cancer cells incubated with MSC-TRAIL after 24 hrs measured using Caspase-GLO 3/7 assay.

243. The Use of Thymidine Kinase Mutants as a Safeguard Switch for IPS Cells

Masashi Urabe[1], Ryosuke Uchibori[2,3], Akihiro Kume[1], Hiroaki Mizukami[1], Keiya Ozawa[2,4]

[1]Division of Genetic Therapeutics, Jichi Medical University, Shimotsuke, Japan, [2]Division of Immuno-Gene & Cell Therapy (Takara Bio), Jichi Medical University, Shimotsuke, Japan, [3]Divison of Genetic Therapeutics, Jichi Medical University, Shimotsuke, Japan, [4]Division of Genetic Therapeutics, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Induced pluripotent stem (iPS) cells have great potential for a number of applications in gene therapy and regenerative medicine. However, transplanted iPS cells spontaneously form teratoma *in vivo*. Residual undifferentiated iPS cells in terminally differentiated population hampers accelerated translation to human applications. In addition, forced reprogramming of somatic cells to generate iPS cells and their ability to proliferate indefinitely also raises the concern about their tumorigenicity. Introducing a suicide gene to iPS cells in advance is one of strategies to prevent tumor development. Thymidine kinase of herpes simplex virus (HSV-TK) has been applied to humans in many studies, particularly for cancer treatment. The

HSV-TK phosphorylates the prodrug ganciclovir (GCV), an analog of guanosine nucleoside. The phosphorylated GCV is incorporated into host DNA and terminates the elongation of DNA strands, resulting in cell death. The therapeutic range of GCV is narrow and the administration of GCV often induces adverse reactions. Aiming at a more potent and thus less toxic TK, mutations in the HSV-TK had been screened in previous studies. However, no studies have been conducted on the direct comparison of the efficacy of mutant TKs for iPS cells. In the current study, we examined some TK mutants with substitution at the nucleoside binding site that had been reported before (Black, et al., 1996; Black, et al., 2001; Mercer, et al., 2002; Balzarini, et al., 2006). We made iPS cell clones that constitutively express individual mutant TK. Treatment of the TK-iPS clones with GCV revealed that cells with mutant SR11 (IF160 LL+A168Y), SR39 (LIF159IFL+AL168FM), Q7530 (IF160LL+AL168YF), and A168H were 10-fold more sensitive to GCV than wild type TK. Some other mutants that had been shown to have an enhanced killing activity in their original reports did not show an advantage over the wild type. These results suggested that TK mutants were more potent than the wild type and have to be screened and selected for a specific purpose.

244. Development and Qualification of Assays for Human Embryonic Stem Cell (hESC)-Derived Cardiomyocytes Intended for Clinical Use

Jennil Patel, Wei Dang, Aparna Krishnan, Derek Kong, David Hsu, Larry Couture
Center for Biomedicine and Genetics, Beckman Research Institute of City of Hope, Duarte, CA

Stem cell therapy holds the promise for numerous degenerative diseases and injuries. Human embryonic stem cells (hESCs) are the starting cell population for many of these potential therapies since they are capable of seemingly indefinite proliferation in the pluripotent state and have the ability to differentiate into all cell types found in the adult. It is vital that the cellular therapeutics derived from these highly proliferative cells are well characterized prior to clinical use and the assays used to characterize these cellular products are well developed. At the Center of Biomedicine and Genetics (CBG), we have manufactured under cGMP numerous hESC-differentiated products including neuron stem cells (NSC), neuronal progenitor cells (NPC), retinal pigment epithelium (RPE) cells, dopaminergic neurons and cardiomyocytes intended for pre-clinical and early phase clinical studies. We have developed a systematic approach to characterize hESC-derived cell products using well developed technologies such as real-time quantitative polymerase chain reaction (RT-qPCR) and flow cytometry. We have standardized the development and qualification of each assay which involves the selection and banking of positive and negative controls, selection and banking of critical reagents, determination of assay conditions, qualification of assay, generation of assay qualification report and standard operating procedure (SOP). In addition, we have established a general guideline to assist the selection of appropriate markers for different products. Here we describe the application of this systematic approach to develop and qualify identity and purity assays for hESC-derived cardiomyocytes produced through hESC differentiation procedure in suspension culture. Selection of markers for the characterization of cardiomyocyte products will be discussed, and development and qualification of the assays will be reported.

245. MicroRNA Expression in Bone Marrow-Derived Human MSCs

Ian H. Bellayr[1], Jennifer G. Catalano[2], Raj K. Puri[1]
[1]Division of Cellular and Gene Therapies, US Food and Drug Administration, Silver Spring, MD, [2]Office of Industries, US International Trade Commission, Washington, DC

Multipotent stromal cells (MSCs) are being studied in the field of regenerative medicine for their capacity for multi-differentiation. These cells can be isolated from multiple tissue types. The current literature indicates that MSCs have an immunoregulatory capacity, which can suppress the immune system. MicroRNAs (miRNAs) are short non-coding RNAs that are responsible for regulating gene expression. Through targeting binding to gene transcripts, miRNAs have been observed to impact MSC function such as proliferation, differentiation, migration and apoptosis. Studies have shown that various miRNAs are expressed in MSCs; however, the impact of cellular expansion and donor variability on the miRNA expression is not well understood. Six commercially available MSC lines were expanded from passage 3 to 7 and their miRNA expression was evaluated using microarray technology. Statistical analyses of our data revealed that 71 miRNAs out of 939 examined were expressed by this set of MSC lines at all passages and the expression of 13 miRNAs were significantly different between passage 3 and 7. The expression of six miRNAs with the largest fold changes was further evaluated using RT-qPCR for both the original MSC lines and a second set of seven MSC lines expanded from passage 4 to 8. By RT-qPCR only 2 miRNAs, miR-638 and miR-572 were upregulated at passage 7 compared to passage 3 for the original MSC lines by 1.71 and 1.54 fold, respectively; and upregulated at passage 8 compared to passage 4 for the second set of MSC lines, 1.34 and 1.59 fold, respectively. These 2 miRNAs distinguish aging MSCs expanded in culture. These novel results may be useful in establishing critical quality attributes for limiting clinical applications of MSCs beyond specific cellular expansion protocols.

246. Characterization of Peripheral Cardiac Progenitors

José R. Navarro-Betancourt[1], Salomón Hernández[1], Felipe Masso[2], Luis A. Lara-Martínez[1], Alejandra Contreras-Ramos[3], Andrés R. Martinez-Calderón[1], Victor M. Arenas[1], José L. Aceves-Chimal[4]
[1]Molecular Biology, Universidad Panamericana, Mexico City, Mexico, [2]Cellular Biology, National Institute of Cardiology "Ignacio Chávez", Mexico City, Mexico, [3]Cellular Biology, Mexico Children's Hospital "Federico Gómez", Mexico City, Mexico, [4]Cardiothoracic Surgery, "20 de Noviembre" National Medical Centre, Mexico City, Mexico

Introduction We propose that cells expressing cardiomyocyte-specific structural proteins have a specific cardiac differentiation commitment and that an immunophenotype distinguished by the presence of cardiac stem cell (CSC) related surface markers, namely CD117, CD34 and Sca-1, identifies peripheral cardiac progenitors, therefore we explored the presence of these attributes in bone marrow-derived cells, aiming to establish an association between cardiac differentiation potential and a particular cellular profile.

Methods Whole bone marrow was extracted from Wistar rat femur, cells were isolated through enzymatic desegregation and plated for incubation. Twenty-four hours later non-adherent supernatant cells were recovered and re-seeded, seventy-two hours later adherent cells were harvested and underwent dilution cloning. Flow cytometry was used to characterize each clone searching particularly for the surface markers CD117, CD34 and Sca-1. The subpopulation expressing the highest levels of these markers was isolated and labeled bone marrow supernatant (BMSN). Subsequently, a complete immunophenotypic profile of BMSN was performed, their capacity to differentiate into

typical mesenchymal tissues was assessed using a commercial kit. BMSN were evaluated through rt-PCR and q-PCR for the expression of cardiac-related genes. The presence of proteins coded by these genes was evaluated through western blot and immunofluorescence. Co-cultures of green fluorescent protein (GFP) labeled BMSN with mature cardiomyocytes were performed, the expression of prototypal cardiac proteins was verified through immunofluorescence. In a rat model of myocardial infarction, $5x10^6$ GFP-labeled BMSN were injected directly into the infarcted tissue. Cardiac function was tracked with left-ventricle ejection fraction (LVEF), measured though transthoracic echocardiography, and myocardial perfusion measured through single-photon emission computed tomography (SPE-CT). Myocardium histological analysis was carried out to evaluate engraftment.

Results In addition to CD117, CD34 and Sca-1, BMSN express CD29, CD44, CD90, CD105 and CD106. BMSN are able to differentiate into osteocytes, but not into adipocytes or chondrocytes. BMSN express the following genes: GATA4, MEF2, Nkx2.5, a-MHC, connexin-43 and troponin-T, connexin-43 reached a 60% higher expression than mature cardiomyocytes. Through western-blot analysis the presence of GATA4, MEF2, Nkx2.5, a-MHC, connexin-43 and troponin-T in CMSC-like is unequivocal. By means of immunofluorescence protein detection only a meager proportion was positive for GATA4, a-MHC and troponin-T, compared to the quantity presumed by western-blot. BMSN phenotypically differentiate into cardiomyocytes when co-cultured with mature cardiomyocytes, cells that expressed Troponin-T before co-culture acquired spontaneous rhythmic contractile movement at the seventh day of follow-up. Sixty days after BMSN injection LVEF increased from an average value of 29% to a mean of 56% and myocardial perfusion increased significantly. BMSN co-expressing GFP and Troponin T were detected inside and around the infarcted area of treated rats.

Conclusions The available data allows to infer an association between CSC-related surface marker expression, cardiomyocyte-specific gene transcription, prototypal cardiac protein presence and a differentiation potential restricted to cardiac tissue.

Clinical Trials Spotlight Symposium

247. Phase I Study of Second Generation Chimeric Antigen Receptor-Engineered T Cells Targeting IL13Rα2 for the Treatment of Glioblastoma

Christine E. Brown[1], Renate Starr[1], Lihong Weng[1], Darya Alizadeh[1], Jamie R. Wagner[1], Julie Kilpatrick[1], Todd L. Harshbarger[1], Massimo D'Apuzzo[1], Julie A. Ressler[1], Michael C. Jensen[2], Jana Portnow[1], Michael E. Barish[1], Stephen J. Forman[1], Behnam Badie[1]

[1]City of Hope, Duarte, CA, [2]Seattle Children's Research Institute, Seattle, WA

T cell immunotherapy is emerging as a powerful strategy to treat cancer, and may offer new opportunities to improve outcomes for patients with glioblastoma (GBM), the most aggressive of primary brain tumors and among the most lethal of human cancers. We have optimized a chimeric antigen receptor (CAR) T cell therapy targeting the tumor associated antigen IL13Rα2 for the treatment of GBM. This T cell product utilizes CD62L-enriched central memory T cells (Tcm), engineered by lentiviral transduction to express a second-generation 4-1BB-containing CAR (IL13BBζ). We report here initial findings from our first-in-human clinical trial [NCT02208362], evaluating the safety, feasibility and bioactivity of weekly intracranial infusions of autologous IL13BBζ Tcm in patients with recurrent IL13Rα2+ GBM. Enrolled patients undergo leukapheresis to collect autologous PBMC and, concurrent with IL13BBζ Tcm manufacturing, tumor biopsy or resection is performed, with placement of a reservoir/catheter device. Following baseline MR and PET imaging and recovery from surgery, patients are treated on a 4-week therapeutic regimen consisting of 3-weekly intracranial infusions of IL13BBζ Tcm followed by one rest week for toxicity and disease assessment. To date, we have completed treatment of the first low dose cohort of three resection patients, and find that local delivery of IL13BBζ Tcm post surgical resection is safe and well-tolerated with no grade 3 or higher toxicities attributed to the therapy observed. Importantly, we have also obtained early evidence for antitumor activity following CAR T cell administration. For all patients in which sample was available, CAR T cells were detected in the tumor cyst fluid or cerebral spinal fluid (CSF) by flow cytometry for a minimum of 7 days post treatment. One patient of particular interest presented with a recurrent multifocal GBM, including one metastatic site in the spine and extensive leptomeningeal disease. This patient was initially treated per protocol with six local infusions of IL13BBζ Tcm into the resection cavity of the largest recurrent tumor focus in the posterior temporal-occipital region. Encouragingly, this CAR T cell injection site remained stable without evidence of disease recurrence for over 7-weeks, while other disease foci distant from the CAR T cell injection site continued to progress. Based on our preclinical laboratory experiments showing the efficacy of intraventricular delivery of CAR T cells, this patient was then treated on a compassionate use protocol with five weekly intraventricular infusions of IL13BBζ Tcm without any other therapeutic interventions. One week following the final intraventricular CAR T cell infusion, all intracranial and spinal tumors had regressed with most decreasing more than 75% by volume, and this patient remains clinically stable four months following the start of CAR T cell treatment. These early clinical findings suggest that intracranial delivery of second-generation IL13Rα2-targeted CAR T cells is safe and well-tolerated, and that after adoptive transfer, CAR T cells survive and maintain activity, capable of eliciting potent antitumor responses against recurrent multifocal glioblastoma.

248. Results of a Phase 1 and 2a Trial on rAAV.sFlt-1 in Treatment of Wet Age-Related Macular Degeneration

Chooi-May Lai[1], Aaron L. Magno[2], Martyn A. French[3], Samuel B. Barone[4], Steven D. Schwartz[5], Mark S. Blumenkranz[6], Mariapia A. Degli-Esposti[1], Ian J. Constable[1], Elizabeth P. Rakoczy[1]

[1]Centre for Ophthalmology and Visual Science, The University of Western Australia, Nedlands, Australia, [2]Molecular Ophthalmology, Lions Eye Institute, Nedlands, Australia, [3]School of Pathology and Laboratory Medicine, The University of Western Australia, Nedlands, Australia, [4]Avalanche Biotechnologies, Menlo Park, CA, [5]Jules Stein Eye Institute, University of California, Los Angeles, Los Angeles, CA, [6]The Byers Eye Institute, University of Stanford, Palo Alto, CA

Purpose: To assess safety following subretinal rAAV.sFlt-1 injection at one year. Secondary endpoints were assessed for evidence of biologic activity. Methods: Under the protocol, Phase 1 followed a dose escalation design where eligible subjects received subretinal injection of low dose (LD, N=3, 10E10 vg) and high dose (HD, N=3, 10E11 vg) rAAV.sFlt-1, or control regimen (N=2). In Phase 2a eligible subjects were randomized to receive HD (N=21) rAAV.sFlt-1 or control regimen (N=11). All 40 subjects were assessed using ophthalmic exam and laboratory testing, and received intravitreal ranibizumab retreatment according to protocol-driven criteria for worsening wet Age-related Macular Degeneration (AMD). Results: During the 1-year follow-up to the primary endpoint, no serious adverse events related to rAAV.sFlt-1 were observed. However, transient adverse events such as subretinal hemorrhage, inflammation,

and increased intraocular pressure were reported. One LD subject and 11 HD subjects were phakic at baseline; following subretinal injection with vitrectomy all of these subjects experienced cataract progression which was operated by month 10. In the control group, 13 subjects received a mean 3.5 (median 4, range 0-5) ranibizumab retreatments, and had a change in mean Best Corrected Visual Acuity (BCVA) of -8.4 Early Treatment Diabetic Retinopathy Study (ETDRS) letters and a decrease in mean retinal Center Point Thickness (CPT) from 450 μm at baseline (BL) to 355 μm. Subjects in the LD group received a mean 0.33 (median 0, range 0-1) ranibizumab retreatments, and had a mean vision gain of 8.7 ETDRS letters and decreased mean CPT from 449 μm to 315 μm. Retreatment outcomes in the HD group suggested a bimodal distribution: 14 subjects received ≤ 2 ranibizumab retreatments (HD.1 subgroup) with a mean vision gain of 6.6 ETDRS letters and a mean CPT decrease from 406 μm to 360 μm, and 10 HD subjects received ≥ 3 ranibizumab retreatments (HD.2 subgroup) with a mean vision change of -2.7 ETDRS letters and increase mean CPT from 407 μm to 456 μm. At BL, serum neutralizing antibodies to AAV were detected in 5/13 (38%) controls, 1/3 LD group (33%), 11/14 HD.1 subgroup (77%) and 2/10 (20%) HD.2 subgroup. Five HD group subjects who were seronegative at BL seroconverted after rAAV.sFlt-1 therapy. Conclusions: Subretinal rAAV.sFlt-1 was well-tolerated with a favorable safety profile in subjects with wet AMD. Encouraging signs of a response with vision gain and fewer ranibizumab retreatment were observed in the majority of the subjects. Serum neutralizing antibodies to AAV prior to treatment did not adversely affect clinical outcomes.

249. Preliminary Results of a Phase 1, Open-Label, Safety and Tolerability Study of a Single Intravitreal Injection of AAV2-sFLT01 in Patients with Neovascular Age-Related Macular Degeneration

Abraham Scaria[1], Annaig LeHalpere[2], Annie Purvis[3], Cheryl Delacono[3], Seng Cheng[1], Samuel Wadsworth[1], Peter Campochiaro[4], Jeff Heier[5], Ronald Buggage[2]

[1]Gene Therapy, Sanofi-Genzyme, Framingham, MA, [2]Sanofi, Paris, France, [3]Sanofi-Genzyme, Cambridge, MA, [4]Ophthalmology, Johns Hopkins School of Medicine, Baltimore, MD, [5]Opthalmology, Ophthalmic Consultants of Boston, Boston, MA

Clinical Trial: www.clinicaltrials.gov NCT01024998

Introduction: Vascular endothelial growth factor (VEGF) plays a critical role in the development of the neovascular AMD (wet AMD), the most common cause of new onset blindness in elderly adults. While intravitreal (IVT) administration of anti-VEGF agents are effective for improving vision in wet AMD patients, the need for repeated monthly IVT injections imposes a burden on patients and ophthalmologists which may limit the realization of the full benefits of VEGF inhibition. AAV2-sFLT01 is a replication defective, recombinant adeno-associated virus (serotype 2) vector (AAV2) that encodes sFLT01, a novel soluble chimeric protein composed of domain 2 of VEGF receptor 1 and the Fc region of human Immunoglobulin G type 1 (IgG1). We investigated the safety and tolerability of a single IVT administration of AAV2-sFLT01 in wet AMD patients. **Methods:** The study was a Phase 1, open-label,, dose-escalating, safety and tolerability study conducted in 4 sites in the USA. 5 cohorts of patients with BCVA of ≤20/100 in the study eye received a single IVT injection of one of four AAV2-sFLT01 doses ($2x10^8$, $2x10^9$, $6x10^9$, or $2x10^{10}$ vg). Patients in cohorts 1-4 had subfoveal disciform scarring, while cohort 5 patients had responsiveness to an anti-VEGF therapy within 12 months prior to enrollment. All patients were monitored for 52 weeks following the injection in the core study, and encouraged to participate in the extended follow-up program for up to 4 years. The primary safety endpoint was the occurrence of adverse events. Biological activity was evaluated by changes from baseline in sub/intraretinal fluid by optical coherence tomography (OCT), best corrected visual acuity (BCVA) and sFLT01 protein level detected in the aqueous fluid. **Results:** 19 patients with end-stage/advanced wet AMD (53% males, median age 77.0 years, median time since diagnosis of AMD of 5.4 years and median basal BCVA of 22.0 letters) were treated. All patients completed the 52-week study with 17 patients consenting to the 4 year extended follow-up study. Overall, the treatment was considered safe and tolerable with no dose-limiting toxicity observed in any dose level. 11/19 patients were expected to show an anti-VEGF response clinically. Of them, transient (2 patients) and sustained (4 patients) reduction in retinal thickness suggested a biological effect of the treatment. Aqueous sFLT01 protein expression was identified in 5 patients receiving $2x10^{10}$ vg. Post-treatment changes in BCVA did not correlate with other parameters of biological activity. **Conclusion:** Despite advanced disease state, a sustained reduction in retinal fluid in four patients provides evidence of biological activity supporting the concept that a sustained therapeutic anti-VEGF level can be achieved following a single intravitreal administration of an ocular gene therapy.

250. A Phase 2/3 Study of the Efficacy and Safety of Ex Vivo Gene Therapy with Lenti-D™ Lentiviral Vector for the Treatment of Cerebral Adrenoleukodystrophy

Florian S. Eichler[1], Christine Duncan[2], Hernan Amartino[3], Razina Aziz-Bose[1], André Baruchel[4], Colleen Dansereau[5], Jean-Hugues Dalle[4], H. Bobby Gaspar[6], Paul Gissen[6], Donald B. Kohn[7], Jerome Larghero[8], Troy C. Lund[9], Weston P. Miller[9], Patricia L. Musolino[1], Tara O'Meara[10], Paul J. Orchard[9], Asif M. Paker[10], Ami J. Shah[7], Esther Shamir[10], Gerald V. Raymond[9], Raman Sankar[7], Nicholas J. C. Smith[11], Adrian J. Thrasher[6], Patrick Aubourg[12], David A. Williams[2]

[1]Massachusetts General Hospital, Boston, MA, [2]Boston Children's Hospital, Boston, MA, [3]Fundagen, Buenos Aires, Argentina, [4]Hôpital Universitaire Robert-Debre, Paris, France, [5]Boston Children's Hospital, Boston, MA, [6]University College London, London, United Kingdom, [7]University of California, Los Angeles, CA, [8]Hôpital Saint-Louis, Paris, France, [9]University of Minnesota, Minneapolis, MN, [10]Bluebird Bio, Cambridge, MA, [11]Women and Children's Hospital, South Adelaide, Australia, [12]INSERM, Paris, France

Cerebral X-linked adrenoleukodystrophy (CALD) is a severe brain demyelinating disease caused by a deficiency in ALD protein (ALDP), an adenosine triphosphate-binding cassette transporter encoded by the ABCD1 gene. Brain lesion progression can be halted by allogeneic hematopoietic cell transplantation (HCT). We used gene transfer with a third-generation SIN-lentivirus vector with MND as an internal promoter expressing the ABCD1 gene (Lenti-D vector) to transduce autologous hematopoietic stem cells (HSCs) in 17 patients with CALD and gadolinium enhancement on brain MRI lacking HLA antigen-matched sibling donors (ages 4-13y; mean, 5.8y). At enrollment, Neurological Function Score (NFS) was 0 in all subjects and median Loes Score was 2 (1-8). Cells were transduced under GMP conditions at an academic site or a central contract manufacturing site and the drug product frozen, certified for release and transported to study sites for infusion. 6.0-20.4X10e6 CD34+ cells/kg of drug product were infused after myeloablative conditioning with busulfan and cyclophosphamide. Vector copy number (VCN) in infused cells was 0.5 to 2.5. As of 10/1/15, 6 subjects had 12-23m follow up, 8 had 6-12m, and 3 had <6m. All patients engrafted with gene marked cells. Time to absolute neutrophil count (ANC) >500/μl was 19-38d (without G-CSF; N=4) and 10-19d (with G-CSF started

day +5; N=13). Time to platelet counts >20,000/µl without platelet transfusions was 14-54 days. Median VCN in DP and peripheral blood leukocytes (PBL) and proportions of PBLs and CD14+ myeloid cells expressing ALDP as detected by flow cytometric analysis are below. Integration site analysis shows polyclonal reconstitution in all subjects, with no indication of vector mediated clonal skewing to date. Neurologic outcomes (NFS and major functional disabilities [MFD]) were assessed at each study visit. MRI results (Loes Score and gadolinium enhancement) were periodically reviewed centrally. MRI data were available on 8 subjects with ≥6m follow up. All 8 had resolution of gadolinium enhancement on brain MRI. Median change in Loes Score was 1 (0-6), and no patient demonstrated progression of NFS. Two SAEs were assessed possibly related to Lenti-D drug product (DP), BK virus cystitis and tachycardia. To date, the safety profile of Lenti-D DP appears consistent with myeloablative conditioning. These early results demonstrate feasibility of centralized manufacturing for a HSC gene therapy trial and short-term safety and efficacy of gene therapy in CALD. Assessment of long-term outcomes and safety continues.

Median (IQR) VCN and ALDP expression over time			
	VCN	% PBLs expressing ALDP	% CD14+ expresing ALDP
Pre-treatment	DP: 1.0 (0.6, 1.6), N=17	0.9 (0.5, 1.5), N=17	1.0 (0.7, 1.6), N=11
6 months	PBL: 0.5 (0.3, 1.0), N=13	13.5 (9.3, 20.1), N=12	26.4 (14.0, 36.0), N=10
12 months	PBL: 0.7 (0.4, 0.8), N=9	22.9 (18.8, 29.5), N=5	32.5 (22.3, 45.2), N=6

Evolving Better Parvoviral Vectors for Gene Therapy

251. Defining Conserved Structural Components of the AAV Capsid That Enable Tissue-Specific Transduction Following Systemic Administration

Jayme K. Warischalk[1,2], Samuel C. Eaton[2], R. Jude Samulski[1,2]
[1]Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]Dept. of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC

We have previously correlated the destabilization of hydrogen bond networks within recombinant adeno-associated viral (rAAV) capsid surface loop variable region 1 (VR1) to transduction efficiency following intramuscular injection. To investigate the utility of this finding for systemic applications, the capsid structures of rAAV serotypes 1 through 9 were computationally analyzed and VR1 residues participating in hydrogen bonding were individually deleted. Mutant capsids were assessed for tissue tropism and transduction efficiency following intravenous administration into mice. *Ex vivo* luciferase assays revealed increased muscle transduction and targeting in five of the nine serotypes (rAAV1, rAAV6, rAAV7, rAAV8, rAAV9); cardiac and skeletal muscle transduction were enhanced by up to 170-fold and 9027-fold over parental serotypes, while hepatic transduction was decreased up to 427-fold. VR1 stability also appeared critical to the remaining serotypes, as VR1 deletion mutations rendered rAAV2 and rAAV3b defective in transduction, and rAAV4 and rAAV5 defective in virion production. Intriguingly, amino acid insertions into VR1 produced an opposing phenotype: rAAV1, rAAV2, rAAV3b, and rAAV6 capsids could instead be targeted to transduce the liver with high efficiency, increasing hepatic transduction up to 132-fold. Finally, VR1 mutation synergized with established tyrosine-to-phenylalanine mutations to further increase transduction efficiency while maintaining preferential cardiac/skeletal muscle targeting.

252. Using Cre-Dependent In Vivo Selection to Identify AAV Variants That Enable Efficient and Widespread Gene Transfer to the Adult Central Nervous System

Benjamin E. Deverman[1], Piers L. Pravdo[1], Bryan P. Simpson[1], Sripriya Ravindra Kumar[1], Yicheng Luo[1], Ken Y. Chan[1], Abhik Banerjee[1], Wei-Li Wu[1], Bin Yang[1], Nina Huber[2], Sergiu P. Pasca[2], Viviana Gradinaru[1]
[1]Biology and Biological Engineering, California Institute of Technology, Pasadena, CA, [2]Department of Psychiatry and Behavioral Sciences, Stanford, Stanford, CA

Recombinant adeno-associated viruses (rAAVs) are commonly used vehicles for *in vivo* gene transfer. However, the tropism repertoire of naturally occurring AAVs is limited, prompting the development of novel AAV capsids with more desirable transduction characteristics. We have developed a capsid selection method, called Cre-recombination-based AAV targeted evolution (CREATE), that enables the identification of AAV capsids that more efficiently transduce defined cell populations *in vivo* (Deverman et al. in press, *Nature Biotechnology*). We generated AAV capsid libraries and used CREATE to identify variants that cross the blood brain barrier and efficiently and widely transduce astrocytes in the mouse central nervous system (CNS) after intravenous injection. One variant, AAV-PHP.B, transfers genes throughout the adult CNS with an efficiency that is 40- to 92-fold greater (depending on the CNS region) than that of the current standard, AAV9. It transduces the majority of astrocytes and neurons across multiple CNS regions, and *in vitro*, it transduces human neurons and astrocytes more efficiently than does AAV9. We are now evolving AAV-PHP.B for even greater transduction of specific CNS cell types as a means to both develop more effective vectors and to gain insight into the mechanism of enhanced transduction. Our identification of AAV-PHP.B and several other enhanced vectors after only two rounds of selection establishes CREATE as a powerful method to customize AAV vectors for biomedical applications.

253. Expanded Packaging Capacity of AAV by Lumenal Charge Alteration

Matthew Tiffany, Mark A. Kay
Stanford University, Stanford, CA

Adeno-associated virus (AAV) is widely believed to be the safest viral vector for gene therapy. Moreover, the array of AAV serotypes available allows transduction of different tissues *in vivo*. However, one major limitation of AAV is the relatively small DNA packaging size (4,700 nt). Existing serotypes have been over-packaged with limited success and varying reproducibility. The consensus appears to be that AAV can be over packaged by ~10%, but with a concomitant reduction in both viral titers and *in vivo* transduction.

We have taken a novel approach to increase the packaging limitations of AAV by focusing on the lumen of AAV where vector DNA interacts with the capsid. We have created a series of AAV capsid variants that alter the capsid lumenal charge using AAV-DJ as the scaffold. The variants add positively charged lysine and arginine residues at lumenally exposed sites within the capsid that, we believe, may allow for interaction and condensation of vector DNA increasing the packaging capacity and/or stabilizing the capsid in an over-packaged state. We present *in vitro* characterization of the library of capsid variants and demonstrate that variants with intermediate charge (+4 to +7 over wildtype) can be produced at high titer when over-packaged up to 6.2 kb. In addition, these variants can transduce

HEK293 cells with a 5.6 kb length vector better than wildtype. Finally, these variants can completely encapsidate at least a 5.4 kb vector by alkaline Southern blot. To validate that these new vectors are functional, we establish that a subset of these vectors are still able to transduce mouse liver *in vivo*. We are currently establishing the relative transduction efficiencies using standard length and expanded genome sizes in order to establish the maximal DNA length that can be functionally packaged by these new vectors. We believe our capsid alteration strategy can be extrapolated to other serotypes with distinct tissue tropism and applied *in vivo* to treat hemophilia A and other monogenic disorders suitable to replacement therapy.

254. New Chimeric Gene Therapy Vectors Based on Four Different Mammalian Bocaviruses

Julia Fakhiri[1], Marc Schneider[2], Shweta Kailasan[3], Michael Meister[2], Mavis A. McKenna[3], Ziying Yan[4], Jianming Qiu[5], Dirk Grimm[1]
[1]Dept. of Infectious Diseases/Virology, University of Heidelberg, Heidelberg, Germany, [2]Thoraxklinik at Heidelberg University Hospital, Heidelberg, Germany, [3]Center for Structural Biology, University of Florida, Gainesville, FL, [4]Dept. of Anatomy & Cell Biology, University of Iowa, Iowa, IA, [5]Dept. of Microbiology, Mol. Genetics & Immunology, University of Kansas, Lawrence, KS

Parvoviruses have long been developed as safe, efficient and versatile DNA delivery vectors for gene therapy applications. In particular adeno-associated viral (AAV) vectors have emerged as lead candidates owing to their apathogenicity and amenability to genetic modifications. Yet, a drawback is their limited cargo capacity of 4.9 kb, which is insufficient to accommodate larger genes. Intriguingly, it was shown recently that oversized single-stranded AAV genomes can be packaged into capsids of human bocavirus 1 (HboV1, also a parvovirus), yielding HboV1/AAV chimeras that specifically and efficiently transduce human airway epithelia (HAE). Motivated by this pioneering work, we aimed to expand the repertoire of HboV/AAV chimeras by vectorizing four additional primate bocaviruses known to infect the gastrointestinal (GI) tract. We thus assembled and cloned the *VP1*/*VP2* ORFs from three human variants (HboV2-4) and gorilla bocavirus into a helper plasmid derived from HboV1, carrying the genes required for HboV1 replication and packaging. To assess viral particle assembly, HEK293T cells were transfected with three plasmids: (i) one of our new HboV helpers; (ii) a self-complementary AAV-YFP vector; and (iii) pDG, a plasmid encoding all genes for AAV packaging and replication. In all cases, correct expression of VP1/VP2 proteins was confirmed by Western blot analyses of cell lysates. Also, following large-scale production and iodixanol gradient purification, qPCR analyses of the 40% phase showed the presence of DNase-resistant particles for all five bocaviral serotypes. Most importantly, titration of these particles revealed comparable quantities, demonstrating that expression of NS and NP1 proteins from HboV1 supports assembly of the four other bocaviruses. Analysis in primary HAE showed YFP transgene expression for all chimeric vectors except for HboV2/AAV, congruent with prior detection of the cognate wild-type viruses in nasopharyngeal aspirates. Further in line with epidemiological data, we noted a marked difference in infectivity, from 15% for HboV1, to below 1% for the others. In looming experiments, the new vectors will be studied in primary epithelial cells from the GI tract, which are the putative natural target cells for HboV2-4 and gorilla bocavirus. Interestingly, infectivity of all chimeras could be boosted by adding proteasome inhibitors, reminiscent of data with AAV. Hence, to enhance escape from the proteasome degradation pathway and improve transduction, we mutated surface tyrosines in the VP2 protein of HboV1. Functional assessment of the resulting mutants is currently ongoing. Collectively, the large capacity, unique cell specificities and ability to cross-package AAV DNA make this novel vector set highly attractive for human gene therapy applications. In the future, it should be moreover rewarding to attempt molecular evolution of bocavirus capsids, taking advantage of the profound experience with AAV vectors.

255. A Method for Developing More Potent AAV Capsids by Prolonging the Vector Half-Lives in the Blood Circulation

Miyuki Kimura[1,2], Kei Adachi[1], Hiroyuki Nakai[1]
[1]Oregon Health & Science University, Portland, OR, [2]Okayama University, Okayama, Japan

Adeno-associated virus (AAV) has been used successfully as a vector for in vivo gene therapy. Many different serotypes are under investigation, but AAV9 vectors especially show robust in vivo transduction by systemic administration via the periphery. Although the mechanism of this robustness is not yet completely understood, the superior ability to cross the endothelial cell barriers including the blood-brain barrier has been presumed to play a key role in efficient systemic gene delivery by AAV9. In this regard, our laboratory has accumulated provocative evidence that AAV permeability across the endothelium is not substantially different among many AAV strains (serotypes and mutants) per se, but that AAV9's in vivo robustness following intravascular administration is due to its distinctively delayed blood clearance. Given that many AAV strains transduce cells better than AAV9 when directly injected into the parenchyma, it should be reasonable to hypothesize that prolonging the half-life of an AAV could substantially increase in vivo transduction efficiency following systemic administration. Here, we show that substitution of a small subset of amino acids in the C-terminal half of the viral capsid can substantially prolong the half-life of AAV1 in the blood and confer a superior in vivo transduction efficiency that is better than that of AAV9 following systemic vector administration. Based on the results from our preliminary AAV1 and AAV9 capsid domain swapping study and a capsid amino acid sequence alignment study, we identified 7 capsid segments comprising 5 to 23 amino acids that might contribute to the persistent circulation of AAV9 in the blood. Each of these 7 segments can be replicated in the AAV1 capsid, which shows a rapid blood clearance rate, by substituting 3 to 11 amino acids of the AAV1 capsid with those of the AAV9 capsid. In the study, we created a panel of 2^7=128 AAV1-derived strains that carry these segment(s) in any possible combinations (note: one of the 128 is the same as the wild-type AAV1 capsid). We then determined blood clearance rates of all the 128 AAV1-derived strains and AAV9 following intravenous injection into mice using the AAV Barcode-Seq approach. The results show that 42 mutants that showed a >30-fold increase in persistence in the blood at 24 hours post-injection compared to the parental wild-type AAV1 all shared either of the following segment combinations, xxx9xx9 or xxxxx99 (note: each letter represents a segment and x can be either AAV1 or AAV9). This indicates that the 7th segment plays an indispensable role but requires either of the 4th and 6th segments for substantially prolonging the half-life. A preliminary tissue transduction analysis in mice revealed that these AAV1-derived mutants with a long half-life showed enhanced in vivo gene delivery compared to AAV9. In a separate study, we also identified a 16 amino acid-long segment in the N-terminal half of the AAV9 capsids that is essential for maintaining high levels of circulating viral particles in the blood at 72 hours post-injection. We are currently investigating whether this amino acid motif, when combined with the above-described xxx9xx9 or xxxxx99 motifs, could further enhance persistence of AAV1-derived mutants and augment in vivo transduction, using another set of 24 AAV1-derived composite mutant capsids in mice. In summary, the study establishes proof-of-principle of a new approach to create more portent AAV vectors by modulating the vector half-life. This approach

will be particularly useful for developing novel AAV capsids that can transduce the brain tissues more efficiently by systemic vector administration.

256. A Novel Rationally Designed AAV Capsid Yields a Potent Neurotropic Gene Therapy Vector

Els Henckaerts[1], Julie Tordo[2], Nuria Palomar[1], Jahedur Rahman[1], Patrick Aldrin-Kirk[3], Mark Basche[4], Antonette Bennett[5], Claire O'Leary[6], Robin Ali[4], Mavis Agbandje-McKenna[5], Brian Bigger[6], Ahad A. Rahim[7], Tomas Björklund[3], Simon Waddington[8], R. Michael Linden[2]

[1]Infectious Diseases, King's College London, London, United Kingdom, [2]Genetics Medicine Institute, Pfizer Inc., London, United Kingdom, [3]Department of Experimental Medical Science, Lund University, Lund, Sweden, [4]Institute of Ophtalmology-Genetics, University College London, London, United Kingdom, [5]Biochemistry and Molecular Biology, University of Florida, Gainesville, FL, [6]Institute of Human Development, University of Manchester, Manchester, United Kingdom, [7]School of Pharmacy, University College London, London, United Kingdom, [8]Institute for Women's Health, University College London, London, United Kingdom

Recent clinical trials have demonstrated safety and efficacy of adeno-associated virus (AAV)-mediated gene therapy, and precipitated the approval of the first gene therapy product for commercial use. Although AAV is the most commonly used vector, there are limitations due to its restricted biological activity. This can be partially overcome by aligning capsid choice and target tissue. Tailoring of AAV capsids has traditionally been accomplished by simply selecting the most suitable available serotype or by further optimization of available serotypes by peptide insertion or capsid reshuffling and *in vivo* selection in mouse or humanised mouse models. A novel approach based on *in silico* reconstruction of ancestral viruses recently yielded a promising vector for gene therapy of diseases that affect liver, muscle or retina. Here we report an alternative evolutionary approach to AAV capsid design based on the introduction of amino acids that have been found at the other end of the evolutionary spectrum. Specifically, we have designed a novel AAV2 capsid by including amino acids that are conserved in natural AAV isolates found in human pediatric tissues. We have determined the structural and immunological characteristics of this novel AAV2-based capsid and performed biodistribution studies. When the bioactivity of the vector was evaluated in various *in vivo* contexts the data revealed extraordinary transduction characteristics in eye and brain tissues. Intracranial injections in mice and rats revealed a significantly increased spread and enhanced transduction of brain tissues as compared to AAV2, with evidence for retrograde transport. Additionally, intra-ocular injections in adult mice revealed a marked enhancement of transduction of photoreceptor cells by the novel vector when compared to AAV2. Peripheral injections also demonstrated strong affinity for the brain and showed little evidence for transduction of non-neuronal tissues. Importantly, we were able to demonstrate superior performance in a disease model that affects the central nervous system when compared to other serotypes that have a strong affinity for neuronal tissues. In summary, we have engineered a novel capsid to include key residues found in natural variants of AAV2, which yields a unique gene therapy vector for the treatment of diseases that affect the central nervous system.

257. Selection of Next Generation AAV Gene Therapy Vectors for Specific and Precise Gene Delivery

Robert Muench, Mark Kay

Departments of Pediatrics and Genetics, Stanford University, Stanford, CA

The ultimate goal in human gene therapy is the specific and exclusive modification of the desired target cells upon systemic vector administration. Especially vectors derived from adeno-associated virus (AAV) are among the most promising gene transfer systems for *in vivo* application and have received broad attention due to substantial clinical benefit. However, AAV specificity for a particular target cell or tissue has been hampered by the broad tropism of different AAV serotypes. Over the last several years, new approaches have been initiated to create and select for more effective and selective recombinant AAV vectors by genetically modifying the capsid protein. These methods include random and/or rationale amino acid substitutions, creation of chimeric capsid variant libraries and various selection screens, and/or peptide insertion. A different approach involves the incorporation of highly specific binding molecules (DARPins) into the AAV capsid. DARPins are derived from ankyrin-repeat proteins that have been developed as alternative to antibody-based scaffolds, which are selected by high-throughput screens from DARPin libraries. At present, the bottleneck remains the cumbersome selection of DARPin molecules and the low number of functional DARPin capsid chimeras, which are able to assemble into a functional vector and still bind their intended extracellular target. Thus after months of screening and analysis the majority of the pre-selected DARPins are found not to be suitable for AAV targeting approaches. Here we describe a novel reliable and faster selection approach. First we eliminate the cumbersome and often misleading prokaryotic selection steps. To do this, we incorporate an entire DARPin library into the AAV capsid as VP2-fusion protein, generating either replication deficient or competent AAV library systems allowing for direct screening on therapeutically relevant cell types *in vitro* or *in vivo*. Our studies show that replicating deficient and replicating AAV libraries can be generated with high diversity up to $5x10^7$ and high functional titers retaining their infectivity. We have generated a DARPin-capsid library in AAV-DJ (serotype with diverse cellular tropism) and AAV-LK03 (human selective serotype) and after only two selection rounds, found up to 10% of selected DARPins sharing identical repeat motifs responsible for target receptor binding in the pancreas and the liver. We are currently making vectors from these chimeric capsids and will determine their target specificity both *in vivo* and *in vitro*. This new approach takes only six days per selection cycle, allowing discovery of novel DARPin molecules and selection for targeted vectors in substantially reduced time. This approach expands the potential diversity in creating and defining novel rAAV vectors with medically relevant transduction properties. Such viral vectors will not only open the door for an array of new approaches to treat acquired human diseases but also push the development of new AAV-based gene therapy vectors to the next level.

Oligonucleotide Therapeutics I

258. Phase I/IIa Study of TT-034, a DNA-Directed RNA Interference (ddRNAi) Agent Delivered as a Single Administration for the Treatment of Subjects with Chronic Hepatitis C Virus (HCV)

Keyur Patel[1], Georgina Kilfoil[2], David L. Wyles[3], Susanna Naggie[1], Eric Lawitz[4], Stephen Bradley[5], Per Lindell[2], David Suhy[2]
[1]Duke University School of Medicine, Durham, NC, [2]Benitec Biopharma, Hayward, CA, [3]University of California San Diego, San Diego, CA, [4]Texas Liver Institute, University of Texas Health Science Center, San Antonio, TX, [5]Synteract HCR, Carlsbad, CA

Background: TT-034, a RNAi-based gene therapy product for the treatment of HCV infection, is comprised of a self-complementary recombinant DNA packaged in an adeno-associated virus capsid (AAV8) for transduction of hepatocytes. TT-034 directs the continuous expression of three independent short hairpin RNAs (shRNAs) that simultaneously target three well-conserved sequences located in the 5' UTR and NS5B regions of the HCV genome. At clinically relevant doses in non-human primates (NHP), a single intravenous infusion of TT-034 resulted in almost complete transduction of hepatocytes and long-term shRNA expression for 180 days (the duration of the experiment). Intended as a one-time treatment, the dosing with TT-034 is the first time that a non-withdrawable DNA-directed RNAi-based gene therapy has been used in man.

Methods: The ongoing phase I/IIa open label dose-escalating trial is enrolling chronic HCV genotype 1 patients without cirrhosis. Patients receive a single intravenous infusion of TT-034 at one of five dose levels. 21 days after dosing, a liver biopsy is collected to assess TT-034 DNA levels and shRNA expression by Quantitative PCR (QPCR).

Results: Eight subjects have received a single dose of TT-034 at 4.00E10, 1.25E11, 4.00E11 or 1.25E12 vg/kg. Additional subjects may be enrolled in dose cohorts of 1.25E12 or at doses as high as 4.00E12 vg/kg. To date there have been no treatment-related serious adverse events in the study. Once administered, TT-034 clears from serum within the first week post dosing. No long term TT-034 shedding has been detected in the urine, stool, semen, or sputum. QPCR measurement of TT-034 DNA levels from the liver biopsies are similar to those reported in NHP models. Specifically, two patients administered a dose of 4.00E10 vg/kg resulted in either 0.01 or 0.02 copies of TT-034 DNA per cell, the equivalent of 1-2% hepatocyte transduction. At a dose of 1.25E11 vg/kg, substantially higher levels of TT-034 were detected in the three subjects, yielding 0.48, 3.65 and 10.44 copies per cell respectively. Some variability in transduction was noted at a dose of 4.00E11 vg/kg, with the two subjects yielding 17.74 and 1.01 copies per cell. We continue to explore the factors influencing this variability, however these data suggest that a significant portion of the hepatocytes may have been transduced in one cohort 3 subject. QPCR analysis of the three anti-HCV shRNAs performed on RNA isolated from the biopsies confirms concomitant, dose dependent expression. From the subject with the highest TT-034 DNA copies per cell quantities of shRNA6, shRNA19 and shRNA22 were measured at 66, 2032, and 99 copies per cell respectively.

Conclusion: These data suggest that TT-034 is well tolerated and safe in human subjects infected with HCV. Furthermore, the vector can effectively transduce hepatocytes and concurrently express three anti-HCV shRNAs.

259. Targeted Gene Silencing by U1 Adaptor Oligonucleotides in Preclinical Models of Parkinson's Disease and Huntington's Disease

Eric Richfield[1], Kavita Prasad[2], Mark Brenneman[3], Rafal Goraczniak[3], Samuel Gunderson[4]
[1]Department of Neurology, Robert Wood Johnson Medical School, Rutgers University, New Brunswick, NJ, [2]Department of Pathology and Laboratory Medicine, Robert Wood Johnson Medical School, Rutgers University, New Brunswick, NJ, [3]Silagene, Inc., Hillsborough, , NJ, [4]Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, , NJ

Many candidate genes are implicated in neurodegenerative disease, but to study potential therapeutic effects of modifying their expression in the central nervous system of animal models has been difficult, often requiring slow, expensive transgenic methods. Transient gene silencing with synthetic oligonucleotides can be a fast, inexpensive alternative to making new transgenic animal models, and a complimentary technique to extend the utility of existing ones. For genes with products that have been validated as therapeutic targets, but are not amenable to small molecule drugs, gene silencing may also be the therapeutic modality of choice.

U1 Adaptors are a third generation of oligonucleotide-mediated gene silencing technology, mechanistically distinct from antisense or siRNA. U1 Adaptors act by selectively interfering with a key step in mRNA maturation: the addition of a 3` polyadenosine (polyA) tail. Nearly all protein-coding mRNAs require a polyA tail, and failure to add one results in rapid degradation of the nascent mRNA inside the nucleus, preventing expression of a protein product. U1 Adaptor oligonucleotides are well suited to *in vivo* applications because they can accept extensive chemical modifications to improve nuclease resistance and the attachment of bulky groups, such as tags for imaging or ligands for receptor-mediated uptake by target cells, without loss of silencing activity.

To explore the feasibility of U1 Adaptor technology for CNS targets, we designed panels of candidate U1 Adaptor oligos for mouse *Scna* (alpha-synuclein) and human *HTT* (Huntingtin), and screened them in cell culture. We identified U1 Adaptors that robustly suppress mouse *Scna* mRNA and reduce alpha-synuclein protein levels in mouse cells. Similarly, we identified U1 Adaptors that suppress the predominant, full length human *HTT* mRNA and reduce HTT protein levels in human cells. We also identified U1 Adaptors that suppress the *HTT* exon-1 truncation isoform recently implicated in HD pathogenesis. For *in-vivo* PK/PD studies, U1 Adaptors were delivered into the CNS of mice, by intracerebroventricular (ICV) injection or by direct stereotaxic injections into the striatum. To examine distribution, cellular uptake and persistence over time, fluorescently tagged U1 Adaptors were administered, then visualized by confocal microscopy in brain sections. ICV injection achieved broad distribution of fluorescent U1 Adaptors throughout the brain, with uptake visible in most cells. Subcellular distribution 24 hours after injection was diffuse in both cytoplasmic and nuclear compartments, but became more punctate and perinuclear by 48 hours. U1 Adaptor oligonucleotides were detected on northern blots of small RNA recovered from brain tissue specimens. Their levels in tissue were estimated by comparison to a standard loading curve, and correlated well with ICV-injected dose. After direct stereotaxic injection to the striatum, U1 Adaptors diffused rapidly and widely, and were taken up by all striatial cells, though preferentially by neurons. Adaptors persisted in tissue for at least five days (the last time point assayed) and reached the nuclei of striatial cells. We then did a series of studies with U1 Adaptors specific for mouse *Scna* mRNA. RNAScope was used to visualize relative levels of mRNA *in situ*. Injection of U1 Adaptors directly into the striatum resulted in clearly reduced expression of *Scna* mRNA and also of mRNA for synaptophysin, known to be down-regulated when α-synuclein expression is reduced.

260. BB-HB-331, a DNA-Directed RNA Interference (ddRNAi) Agent Targeting Hepatitis B Virus (HBV), Can Effectively Suppress HBV *In Vitro* and *In Vivo*

Tin Mao, Shih-Chu Kao, Terrie-Anne Cock, Kermit Zhang, Michael Graham, Peter Roelvink, David Suhy
Benitec Biopharma, Hayward, CA

Background: BB-HB-331 is a recombinant adeno-associated virus serotype 8 (AAV8) vector designed to treat chronic HBV infection using RNA interference. This self-complementary vector expresses three short hairpin RNAs (shRNAs) that simultaneously target three well-conserved sequences on the viral RNAs that correspond to regions that encode the Core, S-antigen and X-proteins. Using an identical capsid for delivery and an identical set of regulatory elements, BB-HB-331 was engineered as a mimic of TT-034, a ddRNAi therapeutic currently in phase I/IIa clinical studies for the treatment for hepatitis C virus (HCV) infection. The only significant difference between TT-034 and BB-HB-331 is that the anti-HCV shRNAs of TT-034 have been replaced with anti-HBV shRNAs in BB-HB-331.

Methods: We first tested the efficacy of BB-HB-331 in an *in vitro* setting using primary hepatocytes (PHs) isolated from the PXB (Phoenix Bio) mice that are largely comprised of human hepatocytes. PHs were subjected to *in vitro* infection with HBV genotype C for 12 days prior to the treatment of BB-HB-331. Since AAV does not efficiently transduce PHs *in vitro*, we utilized adenovirus to deliver the recombinant BB-HB-331 DNA (Ad-BB-HB-331). Increasing doses of Ad-BB-HB-331 were applied to HBV infected primary hepatocytes for 16 days. Hepatocyte cultures treated with adenovirus Ad-TT-034 served as a control. For the *in vivo* study, PXB mice were first infected with HBV genotype C for 28 days to establish HBV baseline infection. This was followed by a single IV infusion of AAV8-BB-HB-331 at a dose of either 2.00E12 or 2.00E13 vg/kg. Untreated HBV infected PXB mice served as the negative control. Serum samples were collected on a weekly basis to assess HBeAg, HBsAg, and extracellular HBV DNA levels.

Results: *In vitro t*reatment of PXB primary hepatocytes with Ad-HB-BB-331 led to significant decreases in HBV parameters. PHs harvested at the conclusion of the experiment demonstrated dose dependent expression of the anti-HBV shRNAs and corresponding inhibition of the HBV viral RNAs. At an MOI of 3, the extracellular levels of HBsAg, HBeAg, and HBcrAg were reduced by 87% or more when compared to the control after 16 days. Although the total cellular DNA did not change, Ad-BB-HB-331 treated cells also demonstrated a 89% reduction of intracellular and extracellular HBV DNA quantities. The levels of cccDNA were correspondingly reduced by 70% in the same time frame. Similarly, effective suppression of HBV infection was observed following *in vivo t*reatment of PXB mice with AAV8-BB-HB-331. Through the first 28 days of an ongoing 56-day experiment, treatment with the high dose resulted in decreases in extracellular levels of HBsAg and HBeAg by 90% and 84%, respectively, when compared to the untreated control. In addition, treatment with the same dose resulted in nearly a log reduction of extracellular HBV DNA at 28 days.

Conclusion: Collectively, these data demonstrate suppression of HBV infection by HB-BB-331 in both a primary hepatocyte model and chimeric humanized mouse model.

261. Gain-of-Function Effect Augments Therapeutic Efficacy of CpG-STAT3 Anti-Sense Oligonucleotide Against Castration-Resistant Prostate Cancers

Dayson Moreira[1], Xingli Zhao[1], Haejung Won[1], Priyanka Duttagupta[1], Summanta Pal[2], Marcin Kortylewski[1]
[1]Immuno-oncology, City of Hope, Duarte, CA, [2]Medical Oncology and Experimental Therapeutics, City of Hope, Duarte, CA

The treatment for castration-resistant prostate cancers (CRPC) remains a clinical challenge. STAT3 expression level correlates with poor prognosis in prostate cancer patients and resistance to hormone therapy. In addition to oncogenic role of STAT3 in cancer cells, it is also commonly activated in the prostate tumor microenvironment. Therefore, STAT3 is a promising target targeting STAT3 is expected to generate dual effects; anti-tumor activity and anti-immunosuppression in CRPC. Targeting transcriptions factors for cancer therapy has not been successful due to the lack of enzymatic activity. Recent advances in oligonucleotide-based therapy using anti-sense oligonucleotide (ASO) targeting STAT3 had shown promising results in phase I clinical trials in various human cancers, including liver metastasis. However, the limitation fo these reagents remained targeting We previously demonstrated that Toll-like Receptor 9 (TLR9) is up-regulated in prostate cancer stem-like cells in the late-stage prostate cancer. STAT3 signaling is activated in TLR9^{+} prostate cancer cells and tumor associated immune cells. Here, we describe a new molecule that enables specific delivery of STAT3 ASO to TLR9^{+} tumor and immune by linking STAT3 ASO to CpG ODN, TLR9 ligand. CpG-STAT3 ASO was quickly and efficiently internalized by human and mouse prostate cancer cell lines showing cytoplasmic localization after 15 minutes after incubation *in vitro*. Human and mouse TLR9^{+} immune cells; DCs, Macrophages and B cells were able to efficiently uptake CpG-STAT3 ASO in vitro. In a biodistribution experiment, CpG-STAT3 ASO^{Cy3} was effectively internalized by TLR9+ myeloid cells in various organs including lymph node and bone marrow. Treatment with CpG-STAT3 ASO conjugates *in vitro* decreased the expression of STAT3 in TLR9^{+} human (DU145 and LNCaP-TLR9) and mouse (RM1 and RM9) prostate cancer cells more efficiently than unconjugated STAT3 ASO. Notably, CpG-STAT3 ASO showed significantly augmented cytotoxicity in prostate cancer cells compared to STAT3 ASO. In a preliminary anti-tumor efficacy study using syngeneic mouse model, repeated intra-tumoral injections of CpG-STAT3 ASO reduced the tumor growth of RM9. In contrast, the inhibitory effect of STAT3 ASO was transient leading to tumor relapse after the initial treatments. Noteworthy, CpG-STAT3 ASO reduced tumor growth and STAT3 expression in the untreated tumors in the contra-lateral site. Moreover, CpG-STAT3 ASO, but not the unconjugated STAT3 ASO, reduced activation of STAT3 in infiltrated myeloid-derived suppressor cells (MDSC) (CD11b^{+}Gr1^{+}) in both tumors and the percentage of T reg (CD4^{+}FoxP3^{+}) cells in the tumor draining lymph nodes. These results implicate that only when combined with concomitant TLR9 stimulation STAT3 inhibition can systemically overcome tumor immune tolerance. Overall, these results suggest that CpG-STAT3 ASO conjugate holds a great promise for an anti-cancer therapy of CRPC by simultaneously targeting STAT3 signaling in TLR9^{+} prostate cancer cells and tumor-associated immune cells.

262. Therapeutic Suppression of the *KRAS-MYC* Oncogenic Axis in Human Pancreatic Cancer Xenografts with U1 Adaptor Oligonucleotide / RGD Peptide Conjugates

Darren Carpizo[1], Ashley Tsang[1], Xin Yu[1], Lan Yi[1], Crissy Dudgeon[1], Kristen Donohue[1], Mark Brenneman[2], Rafal Goraczniak[2], Samuel Gunderson[3]
[1]The Cancer Institute of New Jersey, Robert Wood Johnson Medical School, Rutgers University, New Brunswick, NJ, [2]Silagene, Inc., Hillsborough, NJ, [3]Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ

U1 Adaptors are synthetic oligonucleotides that enable the U1 small nuclear ribonucleoprotein (U1 snRNP) complex to stably bind within the terminal exon of a specific pre-mRNA. This interferes with the obligatory polyadenylation step in mRNA maturation, causing selective destruction of the targeted mRNA inside the nucleus. In contrast to siRNA or antisense oligos, U1 Adaptors can accept extensive covalent modifications for nuclease resistance, targeted delivery or *in-vivo* imaging without loss of silencing activity, offering important advantages as therapeutic agents. A panel of candidate U1 Adaptors targeting human *KRAS* (*KRAS* Adaptors) was screened *in vitro* using the human pancreatic cancer cell line MIA-PaCa2. The best candidates reduced *KRAS* mRNA expression by up to 76% - as effectively as siRNA controls. Reduced KRAS protein expression was confirmed by western blot. Inhibition of cell growth *in vitro* and increased apoptosis were seen for both the MIA-PaCa2 ($KRAS^{G12C}$) and Panc1($KRAS^{G12D}$) cell lines, but not in BxPC3, a $KRAS^{wildtype}$ pancreatic cancer cell line. In a parallel project, U1 Adaptors targeting human *MYC* mRNA were designed and screened in B-cell lymphoma lines, where the best candidates reduced *MYC* mRNA levels by over 95%. Because of the observed relationship between activating *KRAS* mutation and *MYC* overexpression in pancreatic cancers, *MYC* Adaptors were tested in MIA-PaCa2 cells. MYC Adaptors also inhibited cell growth and increased apoptosis *in vitro*. U1 Adaptors were tested for efficacy in mice bearing subcutaneous MIA-PaCa2 xenograft tumors. For *in-vivo* delivery, Adaptor oligos were directly conjugated to a cyclic RGD-motif peptide (cRGD), which is a targeting ligand for specific integrin-family receptors overexpressed on parenchyma and endothelial cells of many solid tumors. Alternately, U1 Adaptor oligos were linked to "internalizing" RGD (iRGD), a variant RGD peptide that also triggers permeabilization of tumor endothelium and internalization by cells through secondary binding to neuropilin-1. *KRAS* Adaptors linked to cRGD or iRGD were administered by tail vein injections twice weekly for three to four weeks. Over a series of experiments, tumor growth was inhibited by averages of 68% to 93%. Tumor stasis or regression occurred in some treated mice. In a pilot study, *MYC* Adaptors conjugated to iRGD peptide were also highly effective in suppressing tumor growth and inducing tumor regression. Excised tumors were analyzed by qPCR and western blot, which confirmed reductions of the targeted mRNAs and proteins. We have shown that U1 Adaptors conjugated to tumor-targeting / tumor-penetrating peptides can effectively target human *KRAS* and *MYC* oncogenes *in vivo*. These results support the continued development of U1 Adaptor technology as a strategy for therapeutic suppression of *KRAS, MYC* and possibly other oncogene targets in pancreatic cancer.

263. Nuclease-Activated Oligonucleotide Probes for the Rapid and Robust Detection of Breast Cancer Circulating Tumor Cells (CTCs)

David D. Dickey[1], Sven Kruspe[1], Kevin T. Urak[1,2], William H. Thiel[1], Karen C. Clark[1], Elliot Burghardt[1], Justin P. Dassie[1], Alexandra Thomas[1], James O. McNamara[1,2], Paloma H. Giangrande[1,2,3]
[1]Internal Medicine, University of Iowa, Iowa City, IA, [2]Molecular & Cellular Biology Program, University of Iowa, Iowa City, IA, [3]Radiation Oncology, University of Iowa, Iowa City, IA

Metastatic breast cancer is the second leading cause of female cancer deaths in the United States. Despite substantial progress in its treatment, metastatic breast cancer remains incurable. Early identification of breast cancer patients at greatest risk of developing metastatic disease is thus an important goal that would enable oncologists to aggressively treat these patients while the cancer is still vulnerable. In addition, this would spare patients who do not need or would not benefit from further treatments from having to endure the harmful side-effects of chemotherapeutic drug regimens. Circulating tumor cells (CTCs) are rare cancer cells found in the blood circulation of cancer patients that provide a non-invasively accessible cancer cell specimen (liquid biopsy) from patients. The number of circulating tumor cells (CTCs) in cancer patients has recently been shown to be a valuable diagnostic indicator of the state of metastatic breast cancer. In particular, patients with few or no CTCs were found to have a better overall prognosis compared to patients with high numbers of CTCs. Despite the implications of CTCs as diagnostics for advanced breast cancer treatment, a critical challenge for adopting CTC-based diagnostic tests has been the development of methods with sufficient sensitivity to reliably detect the small number of CTCs that are present in the circulation. Furthermore, current tests for CTC detection are expensive, have high false positives and negatives, have high background noise, are time consuming and require a significant level of expertise to conduct. To overcome the limitations of current CTC detection assays and develop more sensitive, rapid and cost effective CTC detection methods, we explored the potential of detecting CTCs by measuring their nuclease activity with *nuclease-activated probes*[1]. We present data towards the development of a rapid and highly-sensitive CTC detection assay based on nuclease-activated oligonucleotide probes that are selectively digested (activated) by target nucleases expressed in breast cancer cells. We confirm that these probes are not activated by serum nucleases or nucleases from a lymphoblastic cell line (e.g. K-562). Furthermore, we present extensive data towards the optimization of activity and sensitivity of these probes in cell lysates from various breast cancer cell lines. Data is also presented confirming the detection of CTCs in blood from patients with stage IV breast cancer as well as healthy controls with spiked cancer cells. Future studies will focus on developing similar probes for detection of CTCs in patients with pancreatic and ovarian cancer where early detection would greatly improve survival outcomes. In conclusion, this work describes a robust assay for detection of breast cancer CTCs that will be straightforward to implement in most clinical diagnostic labs.

1. Hernandez FJ, Huang L, Olson ME, Powers KM, Hernandez LI, Meyerholz DK, Thedens DR, Behlke MA, Horswill AR, McNamara JO, 2nd. Noninvasive imaging of staphylococcus aureus infections with a nuclease-activated probe. *Nat Med*. 2014;20:301-306

264. *In Vivo* DNA-Monoclonal Antibody (DMAb) Gene Delivery Protects Against Lethal Bacterial and Viral Challenges in Mice

Ami Patel[1], Seleeke Flingai[1], Sarah T. C. Elliott[1], Trevor Smith[2], Megan C. Wise[1], Stephanie Ramos[2], Jian Yan[2], Katherine Schultheis[2], Amelia A. Keaton[1], Daniel H. Park[1], Kate E. Broderick[2], Niranjan Sardesai[2], Muthumani Karuppiah[1], David B. Weiner[1]
[1]The Wistar Institute, Philadelphia, PA, [2]Inovio Pharmaceuticals, Plymouth Meeting, PA

Therapeutic monoclonal antibodies (mAb) are approved for treatment of several diseases including primary immunodeficiencies, cancer, asthma, and graft transplantation. Yet, only 2 mAbs are approved for administration against infectious diseases: palivizumab (respiratory syncytial virus) and raxibacumab (inhalational anthrax). Numerous protective mAbs targeting recurring and emerging bacterial and viral pathogens have been isolated, however, the high dosage (mg/kg) and associated cost of mAb manufacturing are significant hurdles for routine therapeutic delivery. Recently we have described the development of DNA vector-encoded monoclonal antibodies (DMAbs), as a possible alternative technology. This delivery targets skeletal muscle for invivo transfection to transiently produce and secrete mAb. By optimizing gene design protective levels of antibody are produced *in vivo* by this technology. We show that expression can last a period of weeks. These designed DMAb formulations encoding the mAb heavy and light chain genes are delivered *in vivo* by intramuscular injection followed by electroporation (IM-EP). Several DMAbs were developed targeting antimicrobial resistant bacteria, a serious global health concern. Additionally, we also designed DMAbs against a range of viral infections including frequent, emerging, and neglected tropical diseases. Through a series of sequence and formulation optimizations, we are able to achieve serum levels >5ug/mL and as high as 100ug/mL, depending on the DMAb. DMAb serum levels match the protective trough level range of their purified mAb counterparts and perform on par in functional assays (e.g. killing assays, neutralization). DMAb candidates are protective against bacterial and viral challenges in mice, illustrating functionality *in vivo*. Data from the challenge studies will be presented. Ongoing studies are investigating DMAb gene delivery in larger animal models including rabbits, guinea pigs, and non-human primates. DNA-delivered mAbs is a flexible platform that transforms mAb delivery, allowing for repeat administration, significantly lower production costs, and expands the utility of DNA vector technology for therapeutic gene therapy. This approach may have benefit for routine DMAb gene delivery to prevent nosocomial and community-acquired infections and can be rapidly deployed during an infectious disease outbreak.

Gene Therapy for Neurosensory Diseases

265. Brain Pathways Enabling Vision in LCA Patients Before and After Gene Therapy

Manzar Ashtari[1], Philip Cook[1], Hui Zhang[2], Laura Cyckowski[3], Elena Nikonova[4], Gloria Young[1], Kathleen Marshall[3], James Gee[1], David Leopold[5], Chris Baker[5], Albert Maguire[1], Jean Bennett[1]
[1]CAROT, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, [2]University College London, London, United Kingdom, [3]Children's Hospital of Philadelphia, Philadelphia, PA, [4]University of Pittsburgh, Pittsburgh, PA, [5]National Institute of Health, Bethesda, MD

Leber's Congenital Amaurosis (LCA) is an incurable eye disease. Due to slow retinal degeneration, LCA patients with RPE65 gene mutations (LCA2) are good candidates for gene therapy (GT). LCA2 patients in a Phase 1 clinical trial showed improvement in multiple measures of retinal and visual function of their treated eye after delivery of a normal copy of RPE65 to viable retinal cells with AAV2. We used functional (fMRI) and diffusion (dMRI) MR imaging to study specific brain pathways mediating vision in LCA2 patients before and after GT and assessed processes of brain plasticity that enable vision before and after GT.

10 LCA2 patients and 11 matched controls enrolled in the neuroimaging study. Based on clinical evaluation 9/10 patients received GT to the right eye and one to the left eye. On average, two years after receiving GT only in one eye, patients underwent MR imaging. MRI scans were performed on a research 3T system using a 32-channel head coil. fMRI data was acquired using the checkerboard stimuli. The dMRI sequence was used with a total of 30 non-parallel diffusion gradient directions.

In response to a contrast reversing checkerboard paradigm, control subjects showed to be using the expected geniculostriate (GS) pathway by activating the bilateral lateral geniculate nucleus (LGN) and the primary visual cortices (V1). Using the same stimuli for the untreated eye in LCA2 patients, they failed to activate the GS pathway. Instead, fMRI results from the untreated eye (before GT) showed that the majority of LCA2 subjects used an alternate pathway known as retinotectal (RT) activating superior colliculus (SC), pulvinar and extrastriatal cortices. Stimulation of the treated eye (after GT) in LCA2 patients showed a similar result as the one observed for control subjects (GS pathway). To verify the fMRI results we performed tractography for both GS and RT visual fibers to examine if retinal GT affects brain structures. Tractography results showed higher RT tract density for LCA2 patients in the hemisphere ipsilateral to their untreated eye and a higher GS tract density ipsilateral to their treated eyes. Control subjects showed symmetrical tracts for both RT and GS pathways. fMRI and dMRI results for the one subject treated in the left eye was the reverse of those treated in the right eye.

Conclusion: Preliminary neuroimaging results offer compelling evidence that before GT LCA2 patients do not use the same visual pathway as sighted controls. Instead, our results suggest the use of RT pathway through which newly discovered melanopsin based intrinsically photosensitive ganglion cells are thought to send information to the brain. After GT the mechanism by which visual signals are transmitted to the brain change to the traditional GS pathway used by sighted controls. Our preliminary neuroimaging results also confirm that the long term stimulation of visual pathways prompted by gene therapy are necessary to shape brain structure and how these activities are critical for the proper wiring of the visual pathways.

266. *In Vivo* Rod Photoreceptor Reprogramming Using AAV-Delivered CRISPR/Cas9 Rescues Retinal Degeneration

Wenhan Yu, Suddhasil Mookherjee, Jung-Woong Kim, Suja Hiriyanna, Yasaman Ataeijannati, Xun Sun, Lijin Dong, Tiansen Li, Anand Swaroop, Zhijian Wu
NIH/NEI, Bethesda, MD

Retinitis pigmentosa (RP) is the most common form of inherited retinal dystrophy and the leading cause of inherited blindness, due to mutations in any of the over 60 genes/loci identified so far. The disease is characterized by an initial loss of rod photoreceptors and secondary cone cell death. Since cone photoreceptors are responsible for day time vision and visual acuity, preserving cone functions in RP patients is a priority when developing treatment strategies. NRL is a transcription factor that determines the rod photoreceptor cell fate during retinal development. Acute gene knockout of *Nrl* in mice was shown to reprogram adult rods into cone-like cells, rendering them resistant to effects of mutations in rod-specific genes and consequently preventing secondary cone loss (Montana CL, *et al*.

PNAS, 2013; 110: 1732-7). With a goal to develop this approach for treatment of RP, we used adeno-associated virus (AAV)-delivered CRISPR/Cas9 for *Nrl*-knockdown in rod photoreceptors. AAV vectors were constructed to carry a photoreceptor-specific Cas9 nuclease expression cassette or a single-guided RNA (sgRNA) targeting *Nrl* or eGFP gene. The Cas9 and the sgRNA vectors were co-delivered into mice by subretinal administration. Potency of the AAV-CRISPR/Cas9 system was validated by EGFP knockdown in a mouse line with eGFP-labeled rods. *Nrl* knockdown was conducted in wild-type C57/Bl6 or Crxp-*Nrl*, a mouse line with rod-only photoreceptors. Molecular, histological and functional alterations were examined by next generation sequencing, immunoblot analysis, immunofluorescence, electron microscopy, and electroretinography (ERG). Our results showed that eGFP and *Nrl* were efficiently knocked down following AAV-CRISPR/Cas9 treatment. For *Nrl* knockdown, almost all insertions and deletions were detected in the targeted *Nrl* locus, and very few mutations were identified in ten potential off-target loci. A majority of the transduced rods acquired characteristics of cone photoreceptors following *Nrl*-CRISPR/Cas9 vector treatment, as demonstrated by reduced expression of rod-specific genes and enhanced expression of cone-specific genes, loss of the unique rod chromatin pattern, and diminished rod ERG response. Rescue of retinal degeneration was assessed in three mouse models harboring either recessive or dominant rod-specific mutations. In all three models, the *Nrl*-CRISPR/Cas9 vector treated eyes maintained significantly better photoreceptor viability and cone function than control eyes, as revealed by remarkably thicker photoreceptor layer, higher cone cell number, greater cone ERG amplitude and better optomotor behavior. In conclusion, AAV-CRISPR-mediated *Nrl* gene knockdown can efficiently reprogram rods into cone-like photoreceptors and prevent secondary cone death in retinal degeneration, which could be developed into a viable treatment for RP in humans.

267. Phase I Gene Therapy Preliminary Clinical Results for Treatment of *ND4* Leber Hereditary Optic Neuropathy with rAAV2-2-*ND4*

Scott Uretsky[1], Catherine Vignal[2], Samuel Bidot[2], Serge Fitoussi[1], Anne Galy[1], Sandrine Meunier[1], Roxane Noel[1], Céline Bouquet[1], Nitza Thomasson[1], Jean-Philippe Combal[1], José A. Sahel[2]
[1]Gensight-Biologics, Paris, France, [2]Centre Hospitalier National d'Ophtalmologie des Quinze-Vingts, Paris, France

Introduction Leber Hereditary Optic Neuropathy (LHON) is a rare mitochondrial genetic disorder predominantly affecting young males. Affected patients experience bilateral severe central vision loss. Currently no therapy is approved in the United States to prevent, halt or reverse vision loss due to LHON. Preliminary safety and pharmacodynamic results of a first-in-man trial of GS010, a gene therapy candidate for patients with LHON carrying the ND4 mutation will be presented. Methods GS010 is a recombinant adeno-associated viral vector, serotype 2, carrying the wild-type *ND4* gene (rAAV2/2-*ND4*) and is an experimental gene therapy for the treatment of LHON due to the G11778A *ND4* mitochondrial mutation. GS010 has received orphan drug designation in EU & USA. GS010 contains a Mitochondrial Targeting Sequence (MTS) that allows localization of the wild-type protein to the mitochondrion, enabling restoration of mitochondrial function. An open-label Phase I/IIa safety study (NCT02064569) included patients with vision loss due to *ND4* LHON and has completed recruitment. Four dose escalation cohorts and an extension cohort were comprised of 3 patients each. Patients received a single intra-vitreal injection of rAAV2/2-*ND4* in their worse seeing eye. Primary outcome was the occurrence of adverse events (AE). Secondary outcomes included immune response to AAV2 and evaluation of visual function. Results Systemic safety was excellent as no unexpected adverse events occurred. Ocular tolerability was good with mostly mild inflammation that were responsive to and resolve with standard therapies. Of the first 9 patients with 48 week follow up, preliminary results indicate that symptom duration could impact magnitude of treatment effect . Additionally, baseline vision status at time of treatment also indicate a relation with potential greater magnitude of effect which was noted with relatively shorter disease duration (< 2 years). These data confirm the importance of treating early from onset of vision loss. The RESCUE (NCT02652767) and REVERSE (NCT02652780) Phase III studies of GS010 have been initiated in the United States and some European Union countries. Both studies are randomized, double-masked, sham-controlled trials and will specifically include patients up to 6 months and one year after the onset of vision loss.

268. Optogenetic Engineering of Retinal Ganglion Cells with AAV2.7m8-ChrimsonR-tdTomato (GS030-DP) Is Well Tolerated and Induces Functional Responses to Light in Non-Human Primates

Anne M. Douar[1], Celine Bouquet[1], Didier Pruneau[1], Joel Chavas[1], Deniz Dalkara[2], Jens Duebel[2], Ryad Benosman[2], Guillaume Chenegros[2], Serge Picaud[2], José Sahel[2], Nitza Thomasson[1]
[1]GenSight Biologics, Paris, France, [2]Vision Institute, Paris, France

Introduction: Expression of a light-sensitive opsin in retinal ganglion cells (RGCs) is an attractive strategy to restore vision. We evaluated the ability of ChrimsonR-tdTomato (ChrR-tdT), derived from the algal light-gated cation channel ChrimsonR (Ed Boyden, MIT), to convert light insensitive RGCs into photoactivatable cells in normal macaques. A photostimulation device (GS030-MD) is developed in parallel to complement the biologics. This GS030 combination treatment is intended to treat blindness caused by retinal degenerative diseases such as retinitis pigmentosa. **Methods:** Cynomolgus macaques were injected intravitreally with the AAV2.7m8 vector encoding ChrR-tdT under the control of the CAG promoter (GS030-DP; 5×10^{11} vg/eye). Electrophysiological measurements by microelectrode array (MEA) and patch clamp as well as expression of the ChrR-tdT protein by immunofluorescence were assessed on explanted retinas 2 months after injection. Local tolerance was evaluated by ophthalmic examination and histology at 2 and 6 months post administration. **Results:** ChR-tdT was essentially expressed in RGCs and its expression restricted to the perifoveal area. MEA recordings showed light responses in all treated retinas, with 3 out of 4 retinas displaying high amplitudes of electrical responses to light stimulation (up to 360 Hz). One retina was less responsive (50 Hz). In patch clamp experiments, conducted by targeting tdT-expressing RGCs, large photocurrents were recorded in 3 out of 4 retinas in response to illumination, and according to the expected action spectrum for ChR-tdT. An exploratory study was conducted in parallel in monkeys, which received a single bilateral intravitreal administration of GS030-DP (5×10^{11} vg/eye) to assess local tolerance, systemic toxicity and immunogenicity at 2 and 6 months. No clinical signs indicative of systemic toxicity or local intolerance were observed. No adverse effects were seen by ophthalmic or histological examinations, especially no retinal structural modifications, inflammation or necrosis. Anti-AAV2 neutralizing antibodies (NAbs) measured in serum at 2, 3 and 6 months slightly increased at month 2 ($\leq$ 1:100) and then returned to baseline levels at month 6. No NAbs were detected in aqueous humor at necropsy (at 2 or 6 months). In parallel, in preparation of a first-in-man clinical trial, a complete prototype of the photostimulation device ("goggles") was developed with a full functional optical chain, an electronic subsystem, and firmware and software architecture. These goggles (GS030-MD) capture external scenes through an event-based camera and deliver

visual stimuli onto the transduced retina at irradiances shown to activate ChrR-tdT expressing RGCs in monkey retinas. **Conclusion:** GS030 vector (GS030-DP) transduced efficiently and safely RGCs *in vivo* after intravitreal administration and induced light responses in normal monkey retinas under pharmacological block of endogenous phototransducion. The GS030 treatment combining the AAV2.7m8-ChrR-tdT vector and the photostimulation goggles represents a valuable treatment for vision restoration in retinitis pigmentosa.

269. Novel Synthetic AAV Efficiently Transduces Neurosensory Hair Cells in the Cochlea

Lukas Landegger[1], Bifeng Pang[2], Sarah Wassmer[3], Sarah Gluck[2], Alice Galvin[2], Konstantina M. Stankovic[1], Jeff R. Holt[2], Luk H. Vandenberghe[3]
[1]Department of Otolaryngology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA, [2]Department of Otolaryngology, Boston Children's Hospital, Harvard Medical School, Boston, MA, [3]Grousbeck Gene Therapy Center, Schepens Eye Research Institute/Massachusetts Eye and Ear Infirmary/ Harvard Medical School, Boston, MA

Hearing loss is the most common sensory disorder worldwide. Approximately 15% of American adults report trouble hearing. Prevalence of hearing loss increases from 2% (age 45-54) to 50% (over age 75). Between 2 and 3 per 1000 live births present with hearing loss in one or both ears; 50% of hearing loss at birth is genetic in etiology. While the first cochlear gene therapy for hearing loss is underway, early results, as well as data from preclinical models, indicate a need for improved gene delivery vehicles for safe and efficient targeting of relevant cell types in the cochlea. In particular, outer hair cells (OHC) have been a challenging target for AAV. OHCs are important for auditory sensitivity and frequency selectivity, key parameters for complex and sophisticated hearing function in humans.

Here, we evaluated several common AAV serotypes as well as Anc80L65, an in silico designed AAV based on evolutionary modeling, for its ability to target the neurosensory cells in the inner ear, particularly inner (IHC) and outer hair cells. Cochlear explants of both CBA/CaJ and C57Bl/6 mice were exposed to equal doses of iodixonal purified preparations of AAV1, 2, 6, 8, 9, and Anc80L65 that encoded eGFP. After 48h, explants were evaluated either immediately or after an additional 5 days of culture. In both animal model explants, Anc80L65 demonstrated highly efficient IHC and OHC transduction (60-100% at 48h, and 100% at 48h+5d). AAV2 in this setting was also efficient, although to a lesser extent. Most serotypes targeted supporting cells and the spiral limbus, to a moderate extent, with AAV8 being least efficient. GFP expression was present from apex to base, with moderately increased targeting for basal hair cells.

Next, we tested Anc80L65 in vivo using a clinically relevant round window injection approach. C57Bl/6 mice received 1µl injection of vector at p0-p1 and cochlea were dissected at p6. Results demonstrate transduction from base to apex of IHC, OHC, and supporting cells at rates of close to 100% for IHC, and ranging from approximately 50-100% of OHCs, although at lesser fluorescent intensity. Animals injected at p1 retained similar levels of GFP hair cell positivity at p30 day of sacrifice. Animals in our study were monitored for function of single cells electrophysiogically, hearing function by Auditory Brainstem Response and Distortion Product Otoacoustic Emission measurements, ectopic expression, and immunological parameters. Ongoing studies are evaluating the transduction properties at later times of injection and disease models of hearing loss.

In conclusion, Anc80L65's unique ability to target IHC as well as OHC following RW injection is highly desirable to restore higher level hearing function in genetic forms of deafness through gene therapy.

270. Optogenetic Activation of Remnant Cone Cells Can Restore Ganglion Cell Light Responses in a Canine Model of Retinitis Pigmentosa

Pavitra S. Ramachandran[1], Sergei Nikonov[1], Keirnan Willett[2], Rachel Huckfeldt[2], Arkady Lyubarsky[1], Jessica Morgan[1], Tomas Aleman[1], Albert Maguire[1], Jean Bennett[1]
[1]Center for Advanced Retinal and Ophthalmic Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, [2]University of Pennsylvania, Philadelphia, PA

In retinitis pigmentosa, the rod photoreceptor cells are generally lost first followed by a slow loss of cone cells. Remnant cone cell bodies have been observed even after many years of vision loss in retinitis pigmentosa patients. Proof-of-principle studies in retinal degeneration mice have demonstrated vision restoration upon expression of the optogene halorhodopsin in remnant cone cells. However, whether this will work in a large animal model of retinal degeneration is unclear. Here we look at light responses and histology post-treatment with halorhodopsin in a dog model of autosomal recessive retinitis pigmentosa. Halorhodopsin was expressed in the cone cells unilaterally in rcd1 dogs (PDE6β mutation) with mid- to late stage retinal degeneration using recombinant adeno-associated viral vectors. The retinas were imaged using OCT and function was evaluated using ERG and pupillometry. The spiking activity of retinal ganglion cells (RGCs) in retinal patches adjacent to and distant from the point of injection was tested using multi electrode array recordings. Retinal patches were stimulated with 589 nm light of different durations/intensities.OCT revealed significant thinning of the photoreceptor layer in the retinas of the dogs. While the ERG and pupillometry data did not show a significant difference between the treated and untreated eyes, multi-electrode array recordings revealed a restoration of ganglion cell light responses (ON, OFF and ON/OFF-responses to discrete flashes and responses to 4Hz flicker, 589nm light) in the treated retinal patch compared to an untreated control patch of retina. The lowest intensities where responses become clearly detectable for two animals were 0.1 mW/cm2 and 2.8 mW/cm2 probably reflecting different levels of NpHR expression. We observed halorhodopsin expression by histology in the remnant cone cell bodies of the treated retinas.Restoration of ganglion cell responses in the treated retinal patches suggests that the retina can be sensitized to light even after major photoreceptor cell loss. Ongoing work will determine if this restoration of ganglion cell light responses in the dogs translate to being able to navigate in the light.

271. Strategies Based on NexGen AAV2 to Improve AKT3-Induced CNS Axon Regeneration In Vivo

Yang Hu[1], Linqing Miao[1], Liu Yang[1], Haoliang Huang[1], Feisi Liang[1], Chen Ling[2]
[1]Shriners Hospitals Pediatric Research Center, Temple University School of Medicine, Philadelphia, PA, [2]Division of Cellular and Molecular Therapy, Department of Pediatrics, University of Florida, Gainesville, FL

Injuries of mature central nervous system (CNS) axons result in loss of vital functions due to the failure of CNS axons to regenerate. AKT, a family of serine/threonine-specific protein kinases, induces CNS axon regeneration through activation of the mTOR signaling. However, mTOR participates in two distinct complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2). How these two complexes interact with regard to axon regeneration is unknown.

To exploit the significant advantages of specific tropism of adeno-associated virus serotype 2 (AAV2) for retinal ganglion cells (RGC) after intravitreal injection, we generated a series of triple tyrosine-optimized AAV2 (NexGen AAV2) vectors carrying

different AKT variants and mutants. We first confirmed that the NexGen viral vectors showed significant more infection efficiency compared to vectors based on wild-type AAV2 capsids. More than 90% RGC cells in whole-mount retina was transduced, based on triple immunofluorescent staining of transgene, RGC marker, and active form of AKT. Then, the optic nerve was crushed as an *in vivo* axon regeneration model in mice 2 weeks post-intravitreal injection of AAV vectors. It was observed that the predominant AKT isoform in brain and retina, AKT3, induces much better axon regeneration than AKT1 and AKT2. Next, we evaluated the roles of mTORC1 and mTORC2 in AKT3-induced CNS axon regeneration. NexGen AAV2 vectors carrying AKT3 and Cre were injection into eyes of either mTOR floxed mice, RAPTOR floxed mice, or RICTOR floxed mice. RAPTOR is unique to mTORC1, whose deletion totally blocks mTORC1 activity; whilst RICTOR is a rapamycin-insensitive component essential for mTORC2 activity. As expected, deletion of mTOR or RAPTOR significantly inhibited AKT3-induced axon regeneration. Interestingly, AKT3 and RICTOR KO produced even more extensive and fine axon regeneration than AKT3 alone, suggesting that mTORC2 is inhibitory for AKT3-induced CNS axon regeneration. Furthermore, NexGen AAV2-mediated delivery of dominant negative mutant of ribosomal protein S6 kinase (S6K1) or constitutively active mutant of factor 4E-binding protein (4E-BP1), two best-characterized substrates of mTORC1, also inhibited AKT3-induced axon regeneration, further corroborating our conclusions. In addition, since S472 of AKT3 is phosphorylated by mTORC2, we generated NexGen AAV2 vectors carrying an AKT3-S472A mutant gene. Other mutant genes, such as AKT3 kinase dead mutant (K177M) and T305A mutant, were used as appropriate controls. Our results indicated that neither of the control mutants induced axon regeneration. However, the AKT3-S472A mutant produced a significant increase in axon regeneration compared to wild-type AKT3. Taken together, our study revealed an AKT-based neuron-intrinsic balancing mechanism involving mTORC1 and mTORC2, which coordinates positive and negative cues to regulate adult CNS axon regeneration, respectively. Our results should provide promising therapeutic targets for CNS injuries.

Taken together, our study revealed an AKT-based neuron-intrinsic balancing mechanism involving mTORC1 and mTORC2, which coordinates positive and negative cues to regulate adult CNS axon regeneration, respectively. Our results should provide promising therapeutic targets for CNS injuries.

Cancer-Immunotherapy, Cancer Vaccines I

272. Rapid Identification of Tumor- and Neoantigen-Reactive T-Cell Receptors for Personalized Immunotherapy

Anna Pasetto[1], Alena Gros[1], Paul F. Robbins[1], Drew C. Deniger[1], Todd D. Prickett[1], Maria R. Parkhurst[1], Rodrigo Matus-Nicodemos[2], Daniel C. Douek[2], Jared Gartner[1], Katarzyna Trebska-McGowan[1], Jessica Crystal[1], Steven A. Rosenberg[1]
[1]National Institutes of Health (CRC-Surgery Branch), Bethesda, MD, [2]National Institutes of Health (VRC-NIAID), Bethesda, MD

Adoptive transfer of T-cell receptor (TCR) gene engineered T-cells targeting tumor-associated antigens can mediate cancer regression. Accumulating evidence suggests that the clinical success of many immunotherapies is mediated by T-cells targeting mutated neoantigens unique to the patient. We hypothesized that the most frequent TCR clonotypes infiltrating the tumor were reactive against tumor antigens. We thus developed a multi-step strategy that involved CDR3-TCRB deep sequencing of tumor infiltrating lymphocytes and matching of TCRA-TCRB pairs by pairSEQ and single cell RT-PCR. CDR3 TCRA-TCRB pairs identified with this approach, were then reconstructed to full length based on IMGT database [http://www.imgt.org], synthetized and cloned into non-viral and retroviral expression vectors. T-cells were gene-engineered with these TCRs and tested against tumor cell lines and antigen presenting cells expressing tandem minigenes encoding mutated tumor neoantigens and/or pulsed with corresponding mutated peptides. Analysis of 12 fresh metastatic melanomas revealed that in 11 samples, up to 5 tumor-reactive TCRs were present in the 5 most frequently occurring clonotypes including reactivity against neoantigens. These data demonstrate the feasibility of developing a rapid approach for the identification of tumor-reactive TCRs that can be used for personalized TCR-gene therapy.

273. Genome Editing Using CRISPR-Cas9 to Increase the Therapeutic Index of Antigen-Specific Immunotherapy in Acute Myeloid Leukemia

Miriam Y. Kim, Saad S. Kenderian, Daniel Schreeder, Michael Klichinsky, Miroslaw Kozlowski, Olga Schestova, Marco Ruella, Saar Gill
University of Pennsylvania, Philadelphia, PA

Antigen-specific immunotherapy using chimeric antigen receptor (CAR) T cells or bispecific T cell engaging (BITE) antibodies directed against CD19 leads to elimination of malignant and normal B cells. These approaches have a broad therapeutic index in acute lymphoid leukemia (ALL) due to a combination of high anti-tumor activity and tolerability of prolonged B cell depletion. In contrast, in acute myeloid leukemia (AML) the absence of surface antigens specific to leukemia implies that potent myeloid-directed immunotherapy will eradicate normal as well as malignant cells, leading to protracted myeloablation and bone marrow failure, as has been shown in several preclinical studies of CD33 or CD123 directed CART cell therapy. We developed a novel approach to circumvent this problem by genetically modifying normal hematopoietic stem cells (HSCs) so that they do not express the antigen recognized by CART cells, thus generating normal myeloid progeny that is resistant to CART cell therapy.

CD33 is expressed on the majority of AML as well on normal myeloid progenitors and its function is poorly characterized. We hypothesized that CD33 knockout HSCs and their myeloid progeny would develop normally yet be resistant to treatment with anti-CD33 CART cells (CART33). Electroporation of human CD34+ cells with Cas9 protein and in-vitro transcribed sgRNA generated deleterious mutations in the CD33 gene with 70-80% efficacy as measured by flow cytometry and DNA sequencing. Addition of a single-stranded oligonucleotide template with a short insertional mutation at the sgRNA cut site increased the knockout efficacy up to 90%. In vitro cytotoxicity assays showed CD33 KO HSC progeny are resistant to killing by CART33 compared to wild-type HSC controls (% live cells after CART33 co-culture: 60% vs. 18%, $p=0.005$). CD33 KO HSC progeny showed a similar growth and differentiation profile in Methocult semi-solid media compared to control cells that were electroporated with an unrelated sgRNA. The differentiated cells had normal neutrophil and macrophage morphology and immunophenotype. We also found that phagocytosis and cytokine secretion capabilities of the CD33 KO progeny were identical to WT control. NOD-SCID-g-/- (NSG) mice injected with CD33 KO HSCs showed normal engraftment with differentiation of both myeloid and lymphoid lineages. As expected, the percentage of CD33+ myeloid cells was lower in CD33 KO recipients than WT control (24% vs 63%, $p<0.0001$), yet the percentage of CD14+CD11b+ monocytes (45% vs 47%, $p=0.77$) and CD14-CD11b+ neutrophils (34% vs. 33%, $p=0.87$) were unaltered.

This approach yields a potential strategy to treat AML with potent CD33-specific immunotherapy, followed by infusion of gene-modified CD33 KO HSCs, which will allow persistent antigen-specific immune attack along with reconstitution of effective hematopoiesis.

274. One-Step Generation of Universal CAR T Cells

Justin Eyquem, Jorge Mansilla-Soto, Ashlesha Odak, Michel Sadelain
Center for Cell Engineering, Memorial Sloan-Kettering Cancer Center, New York, NY

Adoptive immunotherapy using chimeric antigen receptors (CARs) has shown remarkable clinical results in the treatment of leukemia and is one of the most promising new strategies to treat cancer. Current clinical protocols utilize autologous T cells that are collected by apheresis and engineered with retroviral vectors to stably express the CAR. This approach therefore requires patient-specific cell manufacturing, which unavoidably results in patient-to-patient variability in the final cell product. Widespread implementation of this approach will further require progress in automation and miniaturization of cell manufacturing to meet the demand for CAR T cells. Furthermore, current approaches utilize randomly integrating vectors, including gamma-retroviral, lentiviral and transposons, which all result in semi-random integration and variable expression of the CAR owing to transgene variegation. Position effects may result in heterogeneous T cell function, transgene silencing and, potentially, insertional oncogenesis. Thus, the conjunction of autologous cell sourcing and random vector integration is prone to generating cell products with variable potency. Here we utilize gene editing to generate histocompatible T cell products with consistent and homogeneous CAR expression. Different tailored nucleases, including CRISPR/Cas9 system, Zinc Finger Nucleases or TAL effector nucleases (TALENs), have been previously used for gene disruption in a wide range of human cells including primary T cells. In some instances, these nucleases have been used to generate so-called "universal T cells" for allogeneic administration, by disrupting T cell receptor (TCR) or HLA class I expression, but viral vectors or the sleeping beauty transposon were used to deliver the CAR cDNA, all of which result in semi-random transgene integration and its downstream consequences. We present here a novel strategy for one-step generation of universal CAR T cells. We first compared the efficiency of TALEN and CRISPR/Cas9 to promote homologous recombination using AAV6 donor template in T cells and established conditions yielding more than 50% of universal CAR T cells combining target gene disruption and CAR insertion in a single single step. We molecularly confirmed the targeted integration of the CAR transgene, which results in highly homogeneous and stable CAR expression in human peripheral blood T cells. These T cells exhibited the same *in vitro* tumor lysis activity and proliferation than retrovirally transduced CAR T cells, which augur favorably for their in vivo anti-tumor activity. Deep sequencing analyses to evaluate off-target effects of the nucleases and random AAV integration are in progress, as are in vivo experiments comparing the anti-tumor activity and graft-versus-host disease potential of edited T cells vs conventional CAR T cells. The process we describe here, which combines the scalability of universal T cell manufacturing with the uniformity and safety of targeted CAR gene integration, should be useful for the development of off-the-shelf CAR therapy.

275. Optimization of IL13Rα2-Specific CAR T Cells for Clinical Development Using Orthotopic Human Glioblastoma Models in NSG Mice

Brenda Aguilar[1], Aniee Sarkissian[1], Alfonso Brito[1], Renate Starr[1], Brenda Chang[1], Yubo Zhai[1], Lihong Weng[1], Michael Barish[2], Behnam Badie[3], Stephen Forman[1], Christine Brown[1]
[1]Hematology & Hematopoietic Cell Transplantation, City of Hope, Duarte, CA, [2]Neurosciences, City of Hope, Duarte, CA, [3]Neurosurgery, City of Hope, Duarte, CA

Standard-of-care therapies for glioblastoma (GBM), the most aggressive and common type of glioma, are inadequate, resulting in an unacceptably poor median overall survival of approximately 15 months following primary diagnosis. Immunotherapeutic targeting of brain tumors offers the opportunity to redirect the potency and specificity of the immune system to improve therapeutic outcomes. Our research program has developed a CAR T cell immunotherapy for GBM targeting IL-13 receptor α2 (IL13Rα2), a cell surface receptor over-expressed by the majority of high-grade gliomas. We have optimized second-generation IL13Rα2-specific CAR T cells, incorporating enhancements in CAR design and T cell engineering to improve T cell persistence and antitumor potency. These include a second-generation CAR containing the 41BB (CD137) costimulatory signaling domain (IL13BBζ-CAR), and a manufacturing strategy using an enriched central memory T cell (T_{CM}) population for genetic engineering. In order to optimize clinical parameters for translating this therapy to patients, we have used orthotopic human GBM models in NSG mice. For these studies we have utilized the IL13Rα2-expressing primary low-passage GBM tumor sphere line PBT030-2, which has been engineered to express both EGFP and firefly luciferase (ffLuc) reporter genes (PBT030-2 EGFP:ffLuc). We first compared the route of delivery, intravenous (i.v.) or intracranial (i.c.), for mediating optimal antitumor activity against invasive TS-initiated PBT030-2. We found that i.c. delivery of the therapeutic CAR T cells elicited superior antitumor efficacy as compared to i.v. administration, which provided no therapeutic benefit. In a multifocal disease model, we also establish that CAR T cells that were injected i.c. at one tumor site were able to traffic to a second tumor site in the contralateral hemisphere. Performing CAR T cell titration studies, we estimate that approximately one CAR T cell is required for every two IL13Rα2 tumor cells based on volumetric analysis of day 8 PBT030-2 tumors. Further, we evaluated whether CAR positivity impacted therapeutic efficacy, as our product release criteria for the clinical trial requires the therapeutic product to be at least 10% CAR positive. Indeed, we found equivalent efficacy for i.c. delivered T cells of either 10% versus 100% CAR positivity when cell doses were standardized by CAR, suggesting that unengineered T cells do not negatively impact the therapeutic potency of the CAR T cells. Finally, we evaluated the impact of corticosteroid, given its frequent use in clinical management of GBM, and demonstrate that low dose dexamethasone does not diminish T cell antitumor activity in vivo. These findings provide the rational for initiating a first-in-human clinical trial with intracranial administration of IL13Rα2-specific CD4+ CD8+ IL13BBζ-CAR T cells for the treatment of GBM.

276. Long-Term Relapse-Free Survival of Patients with Acute Myeloid Leukemia (AML) Receiving a Telomerase-Engineered Dendritic Cell Immunotherapy

Jane S. Lebkowski[1], Hanna J. Khoury[2], Robert H. Collins[3], William Blum[4], Patrick Stiff[5], Edward Wirth[6], Kevin Nishimoto[6], John DiPersio[7]
[1]Asterias Biotherapeutics, Portola Valley, CA, [2]Emory University, Atlanta, GA, [3]UT Southwestern, Dallas, TX, [4]Ohio State University, Columbus, OH, [5]Loyola University, Maywood, IL, [6]Asterias Biotherapeutics, Fremont, CA, [7]Washington University, St Louis, MO

There are few treatment options for patients with intermediate and high risk AML, and remission and relapse rates are dismal especially in patients ≥60 years old. A Phase 2 clinical trial was conducted in subjects with AML to assess a dendritic cell immunotherapy (AST-VAC1) engineered to express a modified form of telomerase that is processed through both the MHC Class I and II antigen presentation pathways. AST-VAC1 was prepared from leukapheresis collections from 33 subjects and were transfected with an mRNA encoding near full length telomerase (hTERT) modified with a lysosomal sorting signal, LAMP-1, which enhances presentation to both CD4+ helper and CD8+ cytotoxic T cells. hTERT is essential for maintaining the extended proliferative lifespan of tumor cells. AML patients were enrolled if they were in complete remission (CR1 or CR2) with intermediate or high risk cytogenetics. AST-VAC1 was prepared after induction therapy and before or after completion of consolidation cycles. AST-VAC1 containing 1 x 10^7 cells was administered as 6 weekly followed by 6 biweekly intradermal injections. If AST-VAC1 doses were available, patients were eligible to receive additional monthly boosts. Twenty one patients (median age: 55) in complete remission (16 CR1 and 3 CR2) or early relapse (2) received at least 3 injections of AST-VAC1. Only one Grade 3 or 4 adverse event, (idiopathic thrombocytopenia), possibly related to the immunotherapy was observed. The majority of adverse events were transient including headache, fatigue, erythema and rash. The two patients who were vaccinated during early relapse progressed rapidly and did not receive the full dosing regimen of AST-VAC1. Of the 19 patients that were in CR, 13 received all 12 doses of AST-VAC1. Fifty-eight percent (11/19) developed T cell immune responses to hTERT as assessed by ELISpot analysis. Eleven of 19 patients (median follow-up 52 mos.) were in remission as of last follow-up; seven developed detectable cellular immune responses to hTERT. Of the 19 CR patients, 7 were ≥ 60 years old at the time of AST-VAC1 immunotherapy. Four of 7 patients ≥ 60 years old remained relapse free 52-59 months post AST-VAC1 immunotherapy with all four developing immune responses to hTERT. The three patients that received AST-VAC1 while in CR2 were in remission as of their last follow-up of 24, 50 and 59 months with two having hTERT immune responses. The median duration of complete remission was greater than that observed in historical controls especially for patients ≥ 60 years old where relapse-free survival at 4 years is typically 5-20%. The results suggest that immunotherapy with AST-VAC1 is safe, can stimulate immune responses to telomerase, and may extend relapse-free survival even in patients with high risk AML.

277. Small Molecule-Regulated Antigen Recognition System for Inducible T Cell Targeting of Cancer Cells

Wai-Hang Leung[1], Michael Certo[2], Holly Horton[2], Iulia Diaconu[2], Tracy VandenBerg[2], Jordan Jarjour[1], Alexander Astrakhan[1]
[1]Bluebird Bio, Seattle, WA, [2]Bluebird Bio, Cambridge, MA

Redirecting T cells against tumors by introducing chimeric antigen receptors (CAR) has demonstrated promising clinical results in certain cancers. However, the constitutive activity of these receptors limits which antigens can be targeted with this approach and may result in persistent elimination of healthy antigen-expressing cells. Successful CAR-T targeting of CD19+ tumors, for example, produces chronic B cell aplasia and necessitates life-long intravenous immunoglobulin (IVIG) treatment. Daric is an alternative antigen targeting approach that aims to: i) minimize the long-term toxicity of CAR-T treatment; ii) allows targeting of previously inaccessible antigens; and iii) is amenable to multiplex antigen targeting and other advanced targeting designs and strategies. The Daric system separates the antigen recognition and signaling functions of a CAR into two distinct polypeptides that contain the FKPB12 and FRB dimerization domains but lack signaling activity in the absence of a dimerization agent. Addition of the FKBP12-FRB bridging drug rapamycin or a non-immunosuppressive rapamycin analogue AP21967 heterodimerizes the signaling and antigen recognition components and restores signaling competency for antigen-dependent T cell activation. Importantly, the FKB12 and FRB dimerization partners are located extracellularly, minimizing interference with endogenously expressed signaling components and eliminating the requirement for rapamycin/AP21967 cell penetrance. A range of extracellular linkers and transmembrane domains were used to design a variety of CD19-targeting Daric constructs that exhibit minimal basal activity and robust drug-dependent antigen-specific cytolytic activity and cytokine production. The CD19-specific Daric T cells exhibited comparable levels of cytokine release, proliferation and cytolytic activity compared to a CD19-targeting CAR T cells. *In vitro*, Daric T cells were activated at sub-nanomolar to nanomolar concentrations of rapamycin or AP21967. No differences in cellular phenotype, expansion or functional responsiveness of Daric T cells compared to CAR T cells were observed. As expected, rapamycin was immunosuppressive to CAR T cell functionality, however Daric T cells continued to produce high level of cytokines even in the presence of rapamycin. These results highlight the potential of the Daric system to target highly expressed antigens and minimize the off-tumor on-target toxicity associated with traditional CAR designs.

278. Next-Generation Non-Viral Gene Transfer to Redirect T-Cell Specificity

Harjeet Singh[1], Mary Helen Huls[2], Matthew J. Figliola[1], Ling Zhang[1], Tiejuan Mi[1], Sourindra Maiti[1], Gabrielle Romain[3], Simon Olivares[1], Shihuang Su[1], Lenka V. Hurton[1], Navin Varadarajan[3], Laurence J. N. Cooper[4], Partow Kebriaei[5]
[1]Pediatrics, UT MD Anderson Cancer Center, Houston, TX, [2]Intrexon, Germantown, MD, [3]Chemical and Biomolecular Engineering, University of Houston, Houston, TX, [4]Ziopharm Oncology, Boston, MA, [5]Stem Cell Transplantation and Cellular Therapy, UT MD Anderson Cancer Center, Houston, TX

Non-viral gene transfer using the *Sleeping Beauty* (SB) transposon/transposase system has been successfully tested in humans to express a chimeric antigen receptor (CAR) to redirect T-cell specificity to CD19. This system has been modified to (i) improve the design of the CD19-specific CAR and (ii) reduce the time in culture to 14 days. Our previous clinical trials infused T cells expressing a 2nd generation CAR (designated CD19RCD28) with an IgG_4-Fc stalk that activated via chimeric CD28 and CD3ζ. To evaluate the length of extracellular

domain on function, we tested four CD19-specific CARs with two long [IgG_4-Fc (CD19RCD28) and EQ (L235E and N297Q) mutant IgG_4-Fc (CD19R*CD28)], medium (CD8α hinge, CD19RCD8CD28) and short (12aa IgG_1 hinge, CD19R12aaCD28) stalks which all signaled through chimeric CD28 and CD3ζ endodomains. Generation of our T cells is based on electro-transfer of CARs coded by the SB system and antigen-specific stimulation through activating and K562-derived propagating cells (AaPC) in the presence of exogenous cytokines. After electro-transfer of SB-derived DNA plasmids, T cells were selectively propagated with either a new two-weekly (2x) or standard four-weekly (4x) additions of AaPC. All genetically modified T cells were capable of specific lysis of $CD19^+$ tumor targets and producing IFN-γ in response to $CD19^+$ stimulator cells. Serial killing was tested using massively parallel microscopy to observe single T cells and we observed that $CD19RCD8CD28^+$ T cells exhibited superior ability to partake in multiple killing events. CAR^+ T cells were further tested *in vivo* for their ability to control $CD19^+$ leukemia in a mouse model of minimal residual disease as well as established disease (**Figure A and B**). We found that T cells expressing modified CARs (CD19R*CD28, CD19RCD8CD28, CD19R12aaCD28) with reduced ability to bind to Fc gamma receptors (FcγR) were able to control leukemia more efficiently in mice compared to T cells expressing CD19RCD28. The CD19RCD8CD28 CAR was superior in controlling disease in the model of minimal residual disease compared with the CAR design evaluated in our prior clinical trials. T cells expressing CD19R*CD28 and CD19RCD8CD28 were then evaluated in 2x stimulation cycle. Both the 4x CAR^+ T cells had similar CAR expression (>70%) whereas the 2x CAR^+ T cells exhibited reduced CAR expression (~40%). The 2x CAR^+ T cells expressed markers associated with less differentiated state of naïve-like and memory T cells when compared to 4x CAR^+ T cells, which was supported by measurement of mRNA species using bar-coded probes. The efficacy of the CAR^+ T cells was tested in mice bearing established $CD19^+$ leukemia and we observed superior survival in mice receiving the 2x CAR^+ T cells compared with the 4x CAR^+ T cells (**Figure C**). These data depict that length of extracellular domain and its associated binding to FcγR improves T-cell effector functions and that decreasing the time in culture can improve control of leukemia *in vivo*. These data support the use of CD19RCD8CD28 testing in a next-generation clinical trial (IND# 16474).

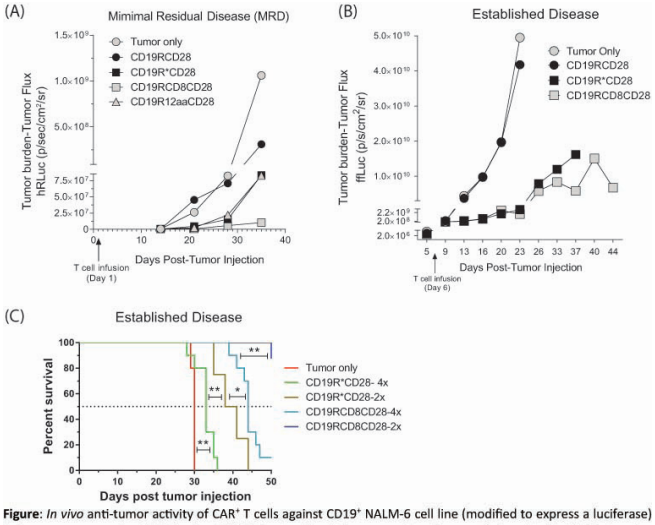


Figure: *In vivo* anti-tumor activity of CAR^+ T cells against $CD19^+$ NALM-6 cell line (modified to express a luciferase) in immunocompromised (NSG) mice. **(A)** Minimal residual disease model, mice were injected with NALM-6 on day 0, followed by injection of T-cells (4x) on day 1. **(B)** Established tumor model in which NSG mice were injected with NALM-6 on day 0 and T cells (4x) were injected on day 6. **(C)** *In vivo* efficacy of 2x and 4x CAR^+ T cells against established $CD19^+$ NALM-6 disease. Tumor burden for individual mice in each group was calculated using serial bioluminescent imaging from NALM-6. Overall survival of mice is shown, *p<0.05, **p<0.01.

Hematologic & Immunologic Diseases I

279. Clinical Outcomes of Gene Therapy with BB305 Lentiviral Vector for Sickle Cell Disease and β-Thalassemia

Marina Cavazzana[1], Jean-Antoine Ribeil[1], Emmanuel Payen[2], Fabien Touzot[1], Bénédicte Neven[1], Francois Lefrere[1], Felipe Suarez[3], Elisa Magrin[1], Yves Beuzard[4], Stany Chretien[2], Philippe Bourget[1], Fabrice Monpoux[5], Corinne Pondarré[6], Pablo Bartolucci[6], Manfred Schmidt[7], Christof von Kalle[7], Laura Sandler[8], Sandeep Soni[8], Michaela Semeraro[1], Wassim El Nemer[9], Olivier Hermine[1], Mariane de Montalembert[1], Stéphane Blanche[1], Salima Hacein-Bey-Abina[1], Philippe Leboulch[2]

[1]Hôpital Universitaire Necker-Enfants Malades, Paris, France, [2]CEA, Institute of Emerging Disease and Innovative Therapies (iMETI), Fontenay aux Roses, France, [3]Paris Descartes Sorbonne Paris Cite University, Paris, France, [4]CEA (iMETI) and University of Paris-Sud, Fontenay-aux-Roses, France, [5]Centre Hospitalier de Nice Sophia-Antipolis, Nice, France, [6]Université Paris Est Créteil, Créteil, France, [7]National Center for Tumor Diseases, Heidelberg, Germany, [8]bluebird bio, Cambridge, MA, [9]INSERM, Paris, France

In patients with hemoglobinopathies, transduction of hematopoietic stem cell (HSC) with a β-globin gene may induce production of functional β-globin, potentially reducing or eliminating disease symptoms. LentiGlobin™ BB305 Drug Product (DP), an investigational gene therapy product for the treatment of sickle cell disease (SCD) and β-thalassemia (βT), consists of autologous CD34+ cells transduced with the BB305 lentiviral vector. BB305 is a replication defective, self-inactivating vector using erythroid-specific globin gene regulatory elements. The vector carries an HBB gene with an anti-sickling amino acid substitution found in γ globin (T87Q) that also allows for HPLC monitoring of transgene globin levels in the subjects' blood. HGB-205 is a phase 1/2 clinical study of safety and efficacy of LentiGlobin BB305 DP in severe SCD and transfusion-dependent βT. Subjects undergo HSC collection via bone marrow harvest (SCD) or mobilization and apheresis (βT). CD34+ cells are selected and transduced with BB305 vector to produce the DP. Subjects undergo myeloablation with IV busulfan followed by DP infusion. As of 11/10/15, 5 subjects had been treated: 1 SCD and 4 βT. No replication competent lentivirus has been detected and integration site analysis shows polyclonal reconstitution without clonal dominance at all time points to date (follow-up between 2 and 23 months). No subjects have experienced drug product-related adverse events; safety observations to date are consistent with myeloablative conditioning. The subject with SCD received 5.6 x 10^6 CD34+ cells/per kg from two DP lots with VCN of 1.0 and 1.2. He achieved neutrophil engraftment on Day +37 and platelet engraftment on Day +91. Prior to study treatment, he had been on prophylactic transfusions to manage severe SCD symptoms including multiple vaso-occlusive crises. He stopped transfusions at Day +88 and at 12 months post DP infusion had a total Hb of 11.7 g/dL, of which approximately 49% is anti-sickling hemoglobin (47%HbA^{T87Q}, 2% HbF). This subject has had no post-infusion pain crises or SCD-related hospitalizations. For the βT subjects the cell dose infused was 8.8 to 13.6 x 10^6 CD34+ cells/per kg, with VCN between 0.8 and 2.1, with neutrophil engraftment at Day +13 to +28 and platelet engraftment at Day +17 to +24. The first two βT subjects treated had genotype β^0/β^E. They have been transfusion independent since ~2 weeks after DP infusion, with consistent levels of total Hb (>10g/dL) and HBA^{T87Q} (7-8 g/dL and 9-10 g/dL). The third βT subject is homozygous for the IVS1 nt 110 G>A mutation. This subject at 4.5 months of follow-up had total Hb 8.0 g/dL and had gone ~2 months without transfusion. The fourth βT subject (β^0/β^E) has less than 2

months follow-up. These interim data suggest that gene therapy with LentiGlobin DP is a promising potential treatment for severe SCD and transfusion-dependent βT.

280. Lentiviral-Mediated Gene Therapy Restores B Cell Homeostasis and Tolerance in Wiskott-Aldrich Syndrome Patients

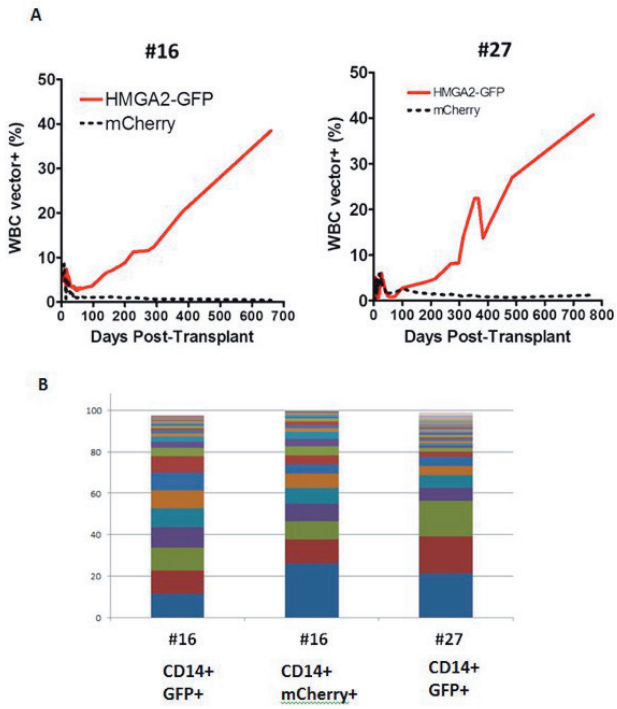
Maria Carmina Castiello[1], Francesca Pala[1], Henner Morbach[2], Jean-Nicolas Schickel[2], Samantha Scaramuzza[1], Nicolas Chamberlain[2], Neil Romberg[3], Francesca Ferrua[1,4,5], Fabio Candotti[6], Mirjam van der Burg[7], Luigi Naldini[1,5], Alessandro Aiuti[1,4,5], Eric Meffre[2], Marita Bosticardo[1], Anna Villa[1,8]
[1]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milan, Italy, [2]Department of Immunobiology, Yale University School of Medicine, New Haven, CT, [3]Department of Pediatrics, Division of Allergy and Clinical Immunology, Yale University School of Medicine, New Haven, CT, [4]Pediatric Immunohematology and Bone Marrow Transplantation Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy, [5]Vita-Salute San Raffaele University, Milan, Italy, [6]Genetics and Molecular Biology Branch, National Human Genome Research Institute, Bethesda, MD, [7]Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, Netherlands, [8]IRGB CNR, Milan Unit, Milan, Italy

Wiskott-Aldrich Syndrome (WAS) is a severe X-linked primary immunodeficiency characterized by micro-thrombocytopenia, eczema and increased risk of infections, autoimmunity and tumors. Allogeneic hematopoietic stem cell (HSC) transplantation is a recognized curative treatment for WAS, but when a matched donor is not available, administration of *WAS* gene-corrected autologous HSCs represents a valid alternative therapeutic approach. Since alterations of WAS protein (WASp)-deficient B lymphocytes contribute to immunodeficiency and autoimmunity in WAS, we followed the B cell reconstitution in 4 WAS patients treated by lentiviral vector-gene therapy (GT) after a reduced-intensity conditioning regimen combined with anti-CD20 administration. We analyzed the B cell subset distribution in the bone marrow and peripheral blood by flow cytometry and the autoantibody profile by a high-throughput autoantigen microarray platform before and after GT. Lentiviral vector-transduced progenitor cells were able to repopulate the B cell compartment with a normal distribution of transitional, naïve and memory B cells. The reduction in the proportion of autoimmune-associated $CD21^{low}$ B cells and in the plasma levels of B cell-activating factor was associated with the decreased autoantibody production in WAS patients after GT. Then, we evaluated the functionality of B cell tolerance checkpoints by testing the reactivity of recombinant antibodies isolated from single B cells. Before GT, we found a decreased frequency of autoreactive new emigrant/transitional B cells in WAS patients, suggesting a hyperfunctional central B cell checkpoint in the absence of WASp. In contrast, high frequency of polyreactive and Hep2 reactive clones were found in mature naïve B cells of WAS patients, indicating a defective peripheral B cell checkpoint. Both central and peripheral B cell tolerance checkpoints were restored after GT, further supporting the qualitative efficacy of this treatment. In conclusion, WASp plays an important role in the regulation of B cell homeostasis and in the establishment of B cell tolerance in humans and lentiviral-mediated GT is able to ameliorate the functionality of B cell compartment contributing to the clinical and immunological improvement in WAS patients.

281. Enforced Expression of a Mutant *HMGA2* Gene Leads to Competitive Expansion and High Level Marking of Long-Term Hematopoietic Stem Cells in Transplanted Nemestrina Macaques

Melissa A. Bonner, Sheng Zhou, Yong-Dong Wang, Zhijun Ma, Lance Palmer, Brian P. Sorrentino
St Jude Children's Research Hospital, Memphis, TN

An important goal in gene therapy and stem cell transplant is to expand hematopoietic stem cells (HSCs) without causing hematopoietic abnormalities. This is particularly challenging in humans and other primates, where expansion protocols that work in mouse models are often less effective in primate HSCs. One approach is to test genes that have led to clonal expansions by vector insertion site effects in human gene therapy trials. In this study, we recreated a mutant HMGA2 cDNA that was associated with a benign clonal expansion in a patient treated on a β-thalassemia gene therapy trial. A lentiviral vector was generated that expressed a cDNA for human *HMGA2* with a 3' UTR deletion that eliminates all seven let-7 microRNA binding sites and thereby avoids the known repressive effects of let-7 miRNAs on HMGA2 expression. A gamma-retroviral MSCV promoter was used to express this mutant HMGA2 cDNA along with an Ires-GFP cassette. Bone marrow CD34+ cells were collected for Nemestrina Macaques and half the cells were transduced with the lentiviral vector containing the HMGA2-GFP construct while the other half was transduced with a mCherry control vector. The transduction efficiencies were approximately the same prior to transplant. At three months post transplantation, the marking in the peripheral blood mononuclear cells was 2.9% GFP+ and 1.1% mCherry+ for animal #16, and was 2.7% GFP+ and 3.2% mCherry+ for animal #27, suggesting relatively equivalent and low levels of transduction of repopulating cells. The HMGA2-GFP marking progressively increased over 21 and 26 months in the peripheral blood leukocytes to 39% for #16 and 41% for #27 while the mCherry marked cells have decreased (Fig 1A). Equivalent levels of marking were seen in various mature peripheral blood lineages, including circulating erythyrocytes, suggesting that expansion had occurred in pluripotent HSCs. This HSC expansion was further demonstrated by a marking analysis in bone marrow cells that showed 44.6% and 75.2% GFP marking in the CD34+CD45RA- HSC compartment for animal #16 and #27 respectively, at the latest time point. Clonality analyses using vector integration sites (VIS) showed overall oligoclonal marking in both the GFP+ and mCherry+ cells in both animals (Fig 1B). The top most frequent VISs were present in all PB lineages indicating that expansion had occurred in multiple HSC clones. The WBC counts, lineage distribution in the peripheral blood, the percentage of CD34+ cells in the bone marrow and all the mature lineages in the peripheral blood are all within the normal range, demonstrating lack of any detectable hematopoietic abnormality. Gene expression microarray analysis of RNA from sorted BM CD34+GFP+ and CD34+mCherry+ cells showed sharp upregulation of several genes in the HMGA2-expressing cells, particularly the IGF2BP gene, a known downstream target of HMGA2. In summary, our data show that long-term HSCs from non-human primates can be progressively expanded in vivo over several years by overexpressing HMGA2. This could be useful for gene therapy, particularly for expanding and obtaining high numbers of transduced erythrocytes and granulocytes for diseases in which a naturally occurring selection advantage is not present.



282. Circumventing Hematopoietic Toxicities and Reversing Sickle Phenotype by Lineage-Specific BCL11A Knock Down and Γ-Globin Induction

Christian Brendel[1], Swaroopa Guda[1], Raffaele Renella[2], Daniel E. Bauer[2], Matthew C. Canver[3], Young-Jo Kim[4], Matthew M. Heeney[5], Denise Klatt[6], Jonathan Fogel[4], Michael D. Milsom[7], Stuart H. Orkin[8], Richard I. Gregory[9], David A. Williams[10]

[1]Boston Children's Hospital, Harvard Medical School, Boston, MA, [2]Boston Children's Hospital, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, [3]Harvard Medical School, Boston, MA, [4]Boston Children's Hospital, Boston, MA, [5]Boston Children's Hospital, Dana-Farber Cancer Institute, Boston, MA, [6]Hannover Medical School, Hannover, Germany, [7]German Cancer Research Center, Heidelberg, Germany, [8]Boston Children's Hospital, Dana-Farber Cancer Institute, Harvard Medical School, Howard Hughes Medical Institute, Harvard Stem Cell Institute, Boston, MA, [9]Boston Children's Hospital, Harvard Stem Cell Institute, Boston, MA, [10]Boston Children's Hospital, Dana-Farber Cancer Institute, Harvard Medical School, Harvard Stem Cell Institute, Boston, MA

The fetal hemoglobin repressor BCL11A represents a therapeutic target for β-hemoglobinopathies as reduced expression of BCL11A leads to simultaneous increased γ-globin and reduced β-globin expression. Reversing this hemoglobin switch is particularly relevant in sickle cell disease to reduce the β^S concentration and increase expression of the protective fetal hemoglobin (HbF, $\alpha_2\gamma_2$). Here we show that despite use of optimized shRNAs embedded into a miRNA (shRNA^{miR}) architecture to reduce non-specific cellular toxicities (Guda et al. MT, 2015), the knockdown of BCL11A profoundly and specifically impaired long-term engraftment of both human and mouse hematopoietic stem cells (HSCs). In competitive transplantation assays cells transduced with shRNA^{miR}s targeting the BCL11A mRNA were consistently underrepresented after hematopoietic reconstitution with gene modified HSCs. Although this effect was particularly pronounced in the B-cell compartment, it was also the case for all other assessed hematopoietic lineages. Mechanistically, while knock-down of BCL11A did not lead to a detectable phenotype in terms of apoptosis, growth or differentiation in human or mouse HSCs *in vitro*, a significant increase in S/G2-phase human HSCs after engraftment into NSG mice was found, a phenotype associated with stem cell exhaustion. To avoid this BCL11A-specific HSC toxicity, we suppressed BCL11A in erythroid cells in a lineage-specific fashion by using transcriptional regulatory elements derived from the β-globin locus. Utilizing this approach for the expression of the optimized shRNA^{miR}s led to stable long-term engraftment of mouse and human gene modified cells in congenic or NSG-mice, respectively. Transduced primary normal or sickle cell disease (SCD) human HSCs gave rise to erythroid cells with up to 90% reduction of BCL11A protein. These erythrocytes demonstrated 60-70% γ-chain expression and a corresponding increase in HbF at low vector copy numbers per cell (VCN<1.5). Similar results were obtained after *in vitro* differentiation of CD34+ cells harvested 16 weeks following engraftment in NSG mice. Transplantation of gene modified murine HSCs from BERK sickle cell mice led to a substantial improvement of sickle-associated hemolytic anemia and reticulocytosis, key phenotypes of SCD. In summary, we have shown an unexpected and severe toxicity of BCL11A knockdown in repopulating HSCs that has direct and important consequences for translation into human gene therapy trials. By utilizing erythroid lineage-specific and micro-RNA embedded expression of targeting shRNAs we demonstrate the capacity of lentivirus vectors to effect significant γ-globin induction leading to clinically relevant increases in HbF while obviating HSC toxicity.

283. In Vivo Selection of MGMT(P140K) Gene Modified Hematopoietic Cells in the Nonhuman Primate Unmasks a Dormant Pool of Repopulating Clones with Progenitor Cell Ontology

Jennifer E. Adair[1,2], Lauren Schefter[1], Daniel Humphries[1], Kevin G. Haworth[1], Hans-Peter Kiem[1,3]

[1]Clinical Research Division, Fred Hutch, Seattle, WA, [2]Department of Medical Oncology, University of Washington, Seattle, WA, [3]Department of Medicine, University of Washington, Seattle, WA

Previously published gene modified cell transplantation studies in nonhuman primate models have described several key features of hematopoietic reconstitution (Kim, S. *et al.*, 2014 and Wu, C. *et al.*, 2014). These studies applied either retrovirus integration site analysis (ISA) or DNA barcode sequencing (DBS) to identify hematopoietic clones in bone marrow or peripheral blood cells. However, these distinct methods detected long-term clones at different time points (1 year after transplant by ISA; ~3 months after transplant by DBS), and neither evaluated reconstitution in the context of selective advantage, which is often the case in gene therapy. Here, we tracked tens of thousands of unique clones in 8 pigtail macaques for up to 10 years following myeloablative transplantation with autologous, lentivirus (LV) gene-modified CD34+ cells. Seven animals received cells gene modified with the P140K mutant methylguanine methyltransferase transgene, conferring resistance to O6- benzylguanine (O6BG) and bis-chloroethylnitrosourea (BCNU) chemotherapy. In two animals, MGMT(P140K)-expressing LVs were DNA barcoded, permitting simultaneous ISA and DBS tracking. Gene marking in peripheral blood cells ranged from 2.3% to 66%. Direct comparison of ISA and DBS demonstrated that abundant clones (&gt 1% of sequence reads) are readily detected by both methods, but DBS captures up to 2-fold more lower abundance clones. Before *in vivo* selection with O6BG/BCNU, we observed a cell dose-dependent, successive pattern of hematopoietic reconstitution by ISA analysis, with short-term clones declining within 100 days after transplantation. Long-term clones

were observed as early as 1 month after transplant. In the first year after transplant, persistent clones ranged from 8% to 54% of clones detected at a &gt 1% frequency, and remained stable in the absence of selective pressure. Importantly, when O6BG/BCNU was administered we observed novel clonal patterns, which directly correlated with transplanted cell dose and time of chemotherapy administration after transplant. In all animals, chemotherapy induced emergence of previously undetected clones. In animals receiving cell doses exceeding $35x10^6$ CD34+ cells/kg (n = 2), chemotherapy more than 1 year after transplant induced a completely novel clonal repertoire. Gene ontology analysis of integration loci among early, long-term and dormant clonal populations identified the greatest functional overlap between early and dormant pools. These data suggest that some short-term repopulating clones revert to a dormant phase within the first year after transplant. Additionally, these data indicate that transplant of excess CD34+ cell numbers results in early dormancy of a large proportion of early repopulating clones. Together, these findings suggest that previous estimates of short- and long-term clonal frequency are an underestimate of true graft repopulation potential.

284. Long-Term Therapeutic Immune Reconstitution in XSCID Canine Model via In Vivo Foamy Virus Delivery of Common Gamma Chain

Frieda Chan[1], Olivier Humbert[1], Christopher Burtner[1], Daniel Humphrys[1], Jennifer Adair[1], Grant Trobridge[2], Patricia O'Donnell[3], Nicholas Hubbard[4], Troy Torgerson[4], Andrew Scharenberg[4], David Rawlings[4], Peter J. Felsburg[3], Hans-Peter Kiem[1]

[1]Fred Hutchinson Cancer Research Center, Seattle, WA, [2]Washington State University, Spokane, WA, [3]University of Pennsylvania, Philadelphia, PA, [4]Seattle Children's Research Institute, Seattle, WA

X-linked combined immunodeficiency disease (XSCID) is caused by mutation in the common gamma chain, γC (interleukin-2 receptor subunit gamma, IL2RG) in both humans and canines. It is characterized by the inability of T-cell development leading to absence of T-cells in peripheral blood, lack of T-cell mediated immune response, low IgA and IgG levels, and early infant mortality. In the 1990s, human XSCID clinical trials utilizing gamma-retroviral vectors to deliver the IL2RG gene caused leukemia in 5 out of 20 patients due to vector integration in or near proto-oncogenes. Recent studies showed Foamy virus based vectors as an excellent alternative for *in vivo* gene-therapy because it is non-pathogenic in humans while exhibiting increased serum stability and favorable integration pattern. Previously, we have demonstrated CD3+ T-cell reconstitution in the canine model via intravenous injection of foamy virus expressing human elongation factor-1 alpha promoter (Ef1α)-γC. Unfortunately, the treated animals contained a low number of gene corrected progenitors at a sub-therapeutic level. Here, we achieved long-term therapeutic immune-reconstitution by intravenous delivery of a human phosphoglycerate kinase promoter (Pgk)-mediated γC foamy viral vector into XSCID neonatal canines. Long-term (2 years) post-injection follow-up demonstrated therapeutic levels of CD3+ T-cell expansion. Within the T-cell population, gene correction with Pgk-γC stabilized at ~80%. We validated T-cell functionality by using spectratyping analysis, which exhibited a diverse repertoire of receptor gene rearrangement. Retroviral integration site analysis (RIS) indicated polyclonal contribution to the reconstituted T-cells. Immunoglobulin ELISA assays showed that IgA and IgG levels in peripheral blood are comparable to normal healthy controls. We immunized the gene-corrected canine recipients with bacteriophage ϕx174 and confirmed production of specific IgG antibodies, showing the ability for isotype switching in B-lymphocytes. Currently, the gene-corrected canines exhibit comparable health and physical attributes to normal controls. Furthermore, semen from the gene-corrected male canine was used via artificial insemination to produce a litter of viable offsprings. In summary, our data demonstrate that Pgk-γC foamy viral vector delivered long-term therapeutic gene correction in a large-animal model for XSCID gene therapy. Most importantly, these results indicate that *in vivo* Pgk-γC foamy vector administration is a viable option for long-term immune reconstitution in future XSCID human clinical trials.

285. Foamy Viral Vector Expressing Human CD18 Results in High Levels of Transduction and Multilineage Engraftment with CD18+ LAD-1 Cells in NSG Mice

Richard H. Smith[1], Md Nasimuzzaman[2], Roop Dutta[1], Gulbu Uzel[3], Thomas R. Bauer[4], Steven M. Holland[3], David W. Russell[5], Dennis D. Hickstein[4], Punam Malik[2], Johannes C. M. van der Loo[6], Andre Larochelle[1]

[1]Hematology Branch, National Institutes of Health, Bethesda, MD, [2]Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, [3]National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, [4]National Cancer Institute, National Institutes of Health, Bethesda, MD, [5]Division of Hematology, University of Washington, Seattle, WA, [6]Center for Cellular and Molecular Therapeutics, Children's Hospital of Philadelphia, Philadelphia, PA

Compared to other classes of retroviral vectors used for HSPC gene therapy, foamy viral vectors (FVV) have distinct advantages, including their non-pathogenicity, a safer integration profile, and a high-efficiency transduction of quiescent cells. Proof-of principle of its therapeutic safety and efficacy in HSPCs was provided with the correction of canine Leukocyte Adhesion Deficiency (CLAD). Dogs with CLAD and individuals with LAD type 1 (LAD-1) suffer from recurrent and life-threatening infections caused by mutations in the β2 integrin CD18 subunit. In this study, G-CSF-mobilized $CD34^+$ HSPCs were isolated from a 19 YO male with severe LAD-1 due to homozygous deletions in CD18. CD34+ cells were transduced for 16 hours at MOI of 0, 5, 10, and 20 with a FVV expressing a human codon optimized CD18 transgene. Flow cytometry of CD34+ cells cultured for 3 days after transduction demonstrated CD18+ cell surface expression in 40-45% of cells. Thirty NSG mice were transplanted with CD18-FVV-transduced human LAD-1 HSPCs (~1.2×10^5 cells/mouse), and human cell engraftment was measured in murine BM 5 months after transplantation using flow cytometry. Human CD45+ cells were detected in all mice (average ~1%). Mice transplanted with mock-transduced (MOI=0) LAD-1 CD34+ cells showed a 3.4-fold, 2-fold and 1.4-fold lower engraftment compared to mice injected with CD34+ cells transduced with FVV at MOI 5 ($p<0.01$), 10 ($p<0.05$) and 20 ($p>0.05$), respectively, suggesting a selective homing/engraftment or survival/proliferative advantage of CD18+ cells. The inverse relationship between engraftment levels and MOI correlated with a gradual decrease in cell survival with increasing MOI, most likely due to toxicity from DMSO (required for FVV cryopreservation) during transduction; cell viabilities of 91%, 84%, 82% and 69% were obtained at MOI 0, 5, 10 and 20, respectively, indicating that further increase in MOI would lead to increasing toxicity. High-level, clinically relevant gene marking levels were obtained; the percentages of human cells expressing CD18 in the murine BM 5 months post-transplantation were 36.0 ± 3.9%, 33.9 ± 5.1%, and 44.5 ± 1.6% at MOI 5, 10 and 20, respectively. Quantitative PCR analysis of vector integrants within engrafted human cells indicated a single integration event occurred in the majority of long-term repopulating HSPCs at all MOI tested. Flow cytometry-based lineage analysis of bone marrow from mice transplanted with FVV-transduced LAD-1 CD34+ cells revealed human CD18+ cells in all lineages, with a predominance

in the CD13+ myeloid compartment (88.3 ± 4.5%) compared to other lineages, including CD20+ lymphoid (9.1 ± 3.9%), CD235a+ erythroid (0.2 ± 0.1%) and CD41+ megakaryocytic (0.6 ± 0.2%) lineages. Interestingly, human myeloid engraftment was superior in recipient mice engrafted with human CD18+ cells (81.5 ± 4.3%) compared to animals transplanted with non-transduced (CD18-) LAD-1 cells (65.3 ± 11.3%). Integration site analysis of engrafted human cells is ongoing. Thus, FVV-mediated transduction of human LAD-1 CD34$^+$ cells leads to clinically significant levels of CD18 expression, supporting the use of this CD18-expressing FVV in a human clinical trial.

Vector and Cell Engineering/Manufacturing

286. Genome Editing of Inducible Cell Lines for Scalable Production of Improved Lentiviral Vectors for Human Gene Therapy

Alessio Cantore[1], Michela Milani[1,2], Andrea Annoni[1], Johannes Lengler[3], Sara Bartolaccini[1], Tiziano Di Tomaso[1], Michael C. Holmes[4], Friedrich Scheiflinger[3], Angelo Lombardo[1,2], Luigi Naldini[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy, [2]Vita-Salute San Raffaele University, Milan, Italy, [3]Baxter Innovation GmbH, Vienna, Austria, [4]Sangamo BioSciences, Inc, Richmond, CA

Lentiviral vectors (LVs) represent efficient and versatile vehicles for gene therapy. Current manufacturing of clinical-grade LVs mostly relies on transient transfection of plasmids expressing the multiple vector components. This method is labor and cost intensive and becomes challenging when facing the need of scale-up and standardization. The development of stable LV producer cell lines will greatly facilitate overcoming these hurdles. We have generated an inducible LV packaging cell line, carrying the genes encoding for third-generation vector components stably integrated in the genome under the control of tetracycline-regulated promoters. These LV packaging cells are stable in culture even after single-cell cloning and can be scaled up to large volumes. In order to minimize the immunogenicity of LVs for *in vivo* administration, we set out to remove the highly polymorphic class-I major histocompatibility complexes (MHC-I) expressed on LV packaging cells and incorporated in the LV envelope. We performed genetic disruption of the β-2 microglobulin (B2M) gene, a required component for the assembly and trafficking of all MHC-I to the plasma membrane in LV producer cells, exploiting the RNA-guided Cas9 nuclease. The resulting B2M-negative cells were devoid of surface-exposed MHC-I and produced MHC-free LVs. These LVs retain their infectivity on all tested cells *in vitro* and efficiently transduced the mouse liver upon intravenous administration. Strikingly, the MHC-free LVs showed significantly reduced immunogenicity in a T-cell activation assay performed on human primary T cells co-cultured with syngeneic monocytes exposed to LV, from several (n=7) healthy donors. To reproducibly generate LV-producer cell lines from these cells, we insert the LV genome of interest in the *AAVS1* locus, chosen for robust expression, exploiting engineered nucleases and homology-directed repair. By this strategy, we have obtained several independent producer cell lines for LVs that express marker or therapeutic genes and are devoid of plasmid DNA contamination. LVs produced by these cells reproducibly show titer and infectivity within the lower bound range of standard optimized transient transfection, and effectively transduce relevant target cells, such as hematopoietic stem/progenitor cells and T cells *ex vivo* and the mouse liver *in vivo*. Overall, we provide evidence that rationally designed targeted genome engineering can be used to improve the yield, quality, safety and sustainability of LV production for clinical use.

287. Production of KTE-C19 (Anti-CD19 CAR T Cells) for ZUMA-1: A Phase 1/2 Multi-Center Study Evaluating Safety and Efficacy in Subjects with Refractory Aggressive Non-Hodgkin Lymphoma (NHL)

Marc Better, Vijay Chiruvolu, James Oliver, Emily Lowe, John M. Rossi, Arianne Perez, Lynn Navale, Adrian Bot

Kite Pharma, Santa Monica, CA

Introduction: An ongoing anti-CD19 CAR T cell study with KTE-C19 in subjects with refractory, aggressive NHL achieved objective responses in 5/7 subjects, including 4 complete remissions (Locke, ASH 2015). To support this trial (ZUMA-1, NCT02348216) and other multicenter trials in lymphoma, we developed a robust and efficient strategy to generate autologous anti-CD19 CAR-engineered T cell products. The newly developed process aims to minimize the time between subject leukapheresis and product administration, and generate a KTE-C19 lot for every enrolled subject. **Methods**: Upon confirmation of eligibility, leukapheresis was performed to process 12-15 L of blood targeting collection of 5-10 x 10^9 peripheral blood mononuclear cells (PBMC). After collection at the investigational site, subject apheresis material was shipped to the central manufacturing site, where it was processed to enrich for the T cell-containing PBMC fraction on a closed system Ficoll™ gradient. In a closed bag system, T cells in the PBMC fraction were then activated using an anti-CD3 monoclonal antibody and cultured in serum-free medium containing 300 IU/ml of IL-2. Magnetic beads were not used for either cell selection or activation. Activated T cells were transduced with a gamma retroviral vector that contains the anti-CD19 CAR gene and further expanded for 4 to 6 days to achieve the target cell dose of 2 x 10^6 CAR-positive T cells/kg (minimum of 1 x 10^6). Final KTE-C19 product was washed, cryopreserved and tested for identity, potency, and adventitious agents. In-process samples were collected for analysis by flow cytometry for CAR expression and other characteristics. After meeting acceptance criteria, the KTE-C19 product was shipped back to the clinical sites using a validated cryo-shipper. **Results**: 7 subjects were dosed in the phase 1 portion of ZUMA-1. KTE-C19 was successfully manufactured in all cases despite a broad range of baseline leukocyte counts (median 5.4x10^3 cells/µl, 2.1-11.1) and low numbers of baseline lymphocytes (median 0.9x10^3 lymphocytes/µl, 0.1-1.4) prior to apheresis. Apheresed cell populations used to generate clinical lots had a broad range of phenotypic characteristics including lymphocyte / monocyte ratio (median 1.1, 0.04-3.5), CD8/CD4 ratio (median 4.2, 0.3-7.7), and naïve plus central memory (Tcm) / more differentiated T cells (median 0.3, 0.04-1.3). Fold-expansion of T cells was consistent among the 7 product lots (average 6-fold) from transduction to harvest. All KTE-C19 lots contained predominantly CD3$^+$ T cells (median 96%; 90-99%), with CD8$^+$ T cells (median 57%, 27-82%) and CD4$^+$ T cells (median 43%, 18-73%). Both CD4$^+$ and CD8$^+$ T cells expressed CAR at similar levels. The product lots contained predominantly effector memory (median 42%, range 30-56%) and Tcm cells (median 34%, 15-58%), with the remainder being naïve (median 14%, 6-19%) and effector T cells (median 5%, 1-22%). All KTE-C19 lots met release specifications and were available for clinical administration within ~2 weeks of apheresis. **Conclusions**: We developed a robust KTE-C19 manufacturing process that successfully generated biologically and clinically active product irrespective of the characteristics of the starting cell populations processed to date. Product lots met release specifications and were available for subject treatment within the target timeframe. This centralized manufacturing process is well suited to support multicenter clinical trials.

288. Development of Cocal Glycoprotein Envelope Producer Cell Lines for Robust Lentiviral Gene Transfer into Hematopoietic Stem Cells and T Cells

Olivier Humbert[1], Donald Gisch[1], Martin Wohlfahrt[1], Phil Greenberg[1], Tom Schmitt[1], Grant Trobridge[2], Hans-Peter Kiem[1]
[1]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, [2]Department of Pharmaceutical Sciences, Washington State University, Spokane, WA

Lentiviral vectors (LVs) are routinely used for stable gene transfer and have demonstrated great promise in hematopoietic stem cell gene therapy and also immunotherapy using genetically modified T cells. LVs are commonly pseudotyped with vesicular stomatitis virus envelope glycoprotein (VSV-G), which confers broad tropism to the vector and allows for vector concentration by centrifugation. However, the use of VSV-G has several limitations, such as susceptibility to inactivation by human serum complement making it unsuitable for in vivo delivery. Furthermore, VSV-G is toxic when constitutively expressed, which has impeded efforts to generate stable producer cell lines. In this study, we first validate the use of cocal vesiculovirus envelope to pseudotype LVs by demonstrating that cocal LVs transduce hematopoietic stem cells and CD4+ T cells more efficiently than VSV-G LVs. We also provide evidence that cocal and VSV-G envelopes use the same receptor for cell entry. We then describe the development of two high-titer, cocal-pseudotyped, LV producer cell lines for a GFP reporter and for a WT1 tumor-specific T cell receptor (TCR). The different 3rd generation lentiviral helper genes were sequentially introduced in HEK293T cells by co-transfection with plasmids encoding antibiotic resistance genes followed by selection to allow for stable protein expression. Cells expressing the cocal envelope produced over 10-times more infectious LV particles as compared to VSV-G expressing cells. High-titer cocal producer cells were isolated by screening for best single clones, which were capable of generating concentrated titers above 10^8 infectious units per mL. We found that these producer cells were stable after serial passages for over 3 months, with no drop in titer detected over time. The resulting GFP and WT1-TCR vectors performed at least as well as identical vectors made with our standard transient transfection protocol for the transduction of CD34+ and CD4+ T cells, respectively. Cocal LV producer cells were also adapted for growth in suspension, serum-free culture, which will facilitate efforts for the scaling up of vector production. In summary, we have successfully developed two independent LV producer cells lines with clinically usable titers. The broad applicability of our cocal packaging cell line offers a promising tool toward the generation of large-scale, clinical grade LV.

289. Sequence Modified Antibiotic Resistance Genes Provide Sustained Plasmid Mediated Transgene Expression in Mammals

Jiamiao Lu[1], Feijie Zhang[1], Andrew Fire[2], Mark Kay[1]
[1]Departments of Pediatrics and Genetics, Stanford University, Stanford, CA, [2]Departments of Pathology and Genetics, Stanford University, Stanford, CA

Conventional plasmid vectors are incapable of achieving sustained levels of transgene expression *in vivo*. Even in quiescent tissues where plasmid DNA levels remain static, transgene expression is transcriptionally extinguished or silenced over a period of several weeks. Extensive work has led to the finding that the length (~1kb or more) rather than the sequence of DNA contained outside of the eukaryotic expression cassette (between 5'end of the promoter and 3'end of the poly A site- such as a canonical bacterial plasmid backbone) is the critical factor that dictates transcriptional silencing *in vivo* (Lu, J *et al. 2012*). Nucleosomal patterns are considered a key element in the establishment and maintenance of silenced chromatin. An/Tn sequence motifs are known to substantially enhance nucleosome mobility and enhance RNA polymerase II accessibility. These sequences sometimes referred to as nucleosome exclusion, remain a mystery in terms of their mode of action. Therefore, we explored whether the addition of a higher number of An/Tn sequences would influence plasmid-mediated transcriptional silencing. First, we found that the placement of a stretch of 20 'T' nucleotides into every 60bp of a random 2.2kb fragment of DNA used in place of the bacterial plasmid backbone abrogated transcriptional silencing. Next, we optimized the An/Tn sequence content of the plasmid containing *Kan* and *Amp* bacterial antibiotic resistance markers without altering their protein coding sequence. These modified antibiotic resistance markers did not affect their propagation in bacterial culture. Most importantly, when transfected into mouse liver they were capable of producing sustained transgene expression at levels 10 times higher than a conventional plasmid. These plasmids maintain expression at levels we previously obtained with minicircle vectors (DNA vectors lacking a bacterial plasmid backbone) without the requirement for special cell lines or plasmid modifications. Thus, we have created a generic plasmid vector that can be produced using standard production schemes that provide sustained expression in quiescent cells and tissues that will be useful in future gene transfer applications.

290. A High-Throughput, Sensitive, Accurate and Reproducible Method for Quantifying Vector Genomes of Recombinant Adeno-Associated Viruses in Crude Lysate

Jianzhong Ai[1], Raed Al-Abbasee[2], Guangping Gao[2]
[1]Department of Urology, West China Hospital, Sichuan University, Chengdu, China, [2]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA

Recombinant adeno-associated viruses (rAAVs) have become an attractive tool for the delivery of therapeutic gene products. Increasing interest and application of rAAV vectors in basic and clinical research has urged efforts to improve AAV production quality and yield. Currently, standard procedures call for genome titration of purified vectors to assess yield at the endpoint of vector production. Mid-production (in-process) methods to gauge AAV yield is severely lacking. In addition, the need to multiplex the production of small-scale AAV batches (e.g. novel serotypes/variants screening) may become cost-prohibitive if vector purification steps were required for hundreds of candidate viral genomes. The high cost of downstream processing and purification during large-scale AAV production can be significantly reduced if low-yield vector batches were identified with earlier monitoring steps during vector production. To meet this need, we have established an enhanced qPCR method for rAAV genome titration in crude lysate. In brief, critical parameters call for the elimination of inhibitory factors inherent to cellular lysates that may reduce qPCR efficiency. These factors may include contaminating nucleic acids originating from packaging vector and helper plasmids and cellular genomes and proteins that are residual from the host cell. Lysate samples are thus directly treated with DNase I and proteinase K in 96-well plates to remove cellular/plasmid DNAs and abundant proteins, followed by TaqMan quantitative PCR analysis on DNase I-resistant vector genomes. To determine treatment efficiencies, we also quantified the copy numbers of vector plasmids diluted in non-transfected crude lysate before and after treatment. We demonstrate that plasmid templates are completely removed by DNase I treatment and qPCR efficiency is markedly enhanced by proteinase K. This enhanced qPCR method is proven to be highly sensitive and reliable, as it can detect as low as 10 copies of plasmid in crude lysate diluent. Furthermore, we demonstrate that this method is scalable

for simultaneous vector genome titration by testing the yields of 13 different serotypes of ssAAV and scAAV. Thus, this procedure can be applied to screen large panels of novel serotypes/variants for vector production. Importantly, we show across independent experiments that titer quantification has high inter-experimental reproducibility among multiple batches of samples. In summary, our described method fulfills unmet needs for quantifying vector genomes in crude lysates from both large- and small-scale AAV preparations in a high-throughput, sensitive, accurate, and reproducible manner. This will significantly improve in-process quality control, batch/lot monitoring in large-scale preparations, and good manufacturing practices (GMP) for AAV production - all key for vector manufacturing, as AAV continues to garner use and impact in both basic research and clinical applications.

291. CD26 Inhibition Enhances Chimerism of Lentivirally Transduced Hematopoietic Stem Cells in a Non-Myeloablative Setting

Garyfalia Karponi, Afroditi Georgakopoulou, Eleni Sgouramalli, Eirini Rantoglou, Loudovikos Soufleros, Achilles Anagnostopoulos, Evangelia Yannaki
Gene and Cell Therapy Center, Hematology-BMT Unit, G. Papanicolaou Hospital, Thessaloniki, Greece

Despite the progress of gene therapy for monogenic diseases over the last years, the engrafting capacity of gene-modified cells, as well as constant and high-level gene expression *in vivo,* require further optimization, especially when a reduced intensity conditioning is mostly preferred. Culture conditions applied for effective gene transfer lead to significant loss of long-term repopulating cells and ultimately, impaired engraftment. The *ex vivo* inhibition of CD26 peptidase activity with Diprotin A was shown to improve homing and engraftment of unmanipulated hematopoietic stem cells (HSCs). We here, sought to assess the homing and engrafting capacity of lentivirally GFP-transduced murine bone marrow (BM) cells under competitive niche settings generated by a non-myeloablative conditioning. Following CD26 inhibition with 5mM Diprotin A of GFP-transduced murine HSCs, we assessed migration towards SDF-1 in transwell systems and the engraftment capacity after partial myeloablation (Busulfan 100mg/kg, equivalent to 8mg/kg in humans) in a syngeneic mouse model, allowing for donor chimera detection (C57Bl6-CD45.2$^+$ donors/PepBoy-CD45.1$^+$ recipients). We also investigated the possible effects of Diprotin A on gene transfer efficiency and transgene expression in bulk and clonogenic cultures of HSCs. *In vitro*, Diprotin A significantly increased the expression of the CXCR4 receptor (25.6±0.75 vs 16.4±1.02, P=0.02), as well as the %migration of gene-modified HSCs towards SDF-1 over untreated transduced cells (71.78±3.79% vs 55.71±5.34%, P=0.03), implying a potentially enhanced engrafting dynamic in the BM. Indeed, *in vivo,* the Diprotin A-treated transduced HSCs exhibited faster hematologic reconstitution by, at least, one week (P=0.03) and both superior long-term engraftment and GFP expression in all hematopoietic tissues (peripheral blood, BM, spleen) of the recipients, over non-Diprotin A-treated transduced cells (blood 4th month post-transplant, %CD45.2$^+$: 77.26±5.26 vs 13.11±11.69, P=0.0002; %GFP$^+$: 30.21±6.86 vs 3.9±2.33, P=0.03). The increased GFP expression observed *in vivo* in the Diprotin A cohort reflected the enhanced engraftment rates, since Diprotin A *per se* did not affect gene transfer efficiency or transgene expression prior to transplantation (%transduction with Diprotin A: 93±3.05 vs without Diprotin A: 89.9±7.47, P=0.73; %GFP in pools of colonies with Diprotin A: 74.8±7.14 vs without Diprotin A: 72.1±8.45, P=0.81). Upon sacrifice, the Diprotin A animals displayed sustained gene marking with ~1 vector copy in all hematopoietic tissues, as opposed to almost undetectable vector copy number (VCN) in the non-Diprotin A cohort. Likewise, individual colonies from the chimeric BM, demonstrated significantly higher VCN in the Diprotin A mice (2.8±0.48 vs 0.37±0.37, P=0.01). Overall, the *ex vivo* treatment with Diprotin A seems to abrogate the negative impact of culture conditions, allowing for enhanced donor chimerism under partial myeloablation and consequently, increased gene marking *in vivo*. This *ex vivo*, easily applicable approach may serve to overcome major constraints for the clinical implementation of gene therapy should the data be confirmed with human CD34$^+$ cells.

292. Towards Large-Scale Manufacturing of Adeno-Associated Virus by Transient Transfection of HEK293 Suspension Cells in a Stirred Tank Bioreactor Using Serum-Free Medium

Parminder Chahal[1], Erica Alessandra Schulze[1,2], Alice Bernier[1], Stephane Lanthier[1], Nathalie Coulombe[1], Amine Kamen[1,3], Renald Gilbert[1]
[1]Human Health Therapeutics Portfolio, National Research Council Canada-Montreal, Montreal, QC, Canada, [2]Technische Universität München, Munich, Germany, [3]Department of Bioengineering, McGill University, Montreal, QC, Canada

Adeno-Associated Virus (AAV) vectors showing safety profile in phase I clinical trials and its ability to transduce gene expression in various tissues have made it a vector of choice for gene delivery. There are different modes of AAV vector production and each has advantages and disadvantages. Here we demonstrated that the production of AAV by transient transfection in a serum-free medium using NRC's patented cGMP compliant human embryonic kidney HEK293 cell line (clone HEK293SF-3F6) adapted for growth in suspension can be readily scaled-up in stirred tank bioreactors. We employed triple-plasmid / polyethylenimine (PEI) based transient transfection technique. As a proof of concept, we demonstrated that nine serotypes of AAV (AAV-1 to AAV-9) encoding GFP can be produced by our cell line HEK293SF with yields of about 1E+13 genome-containing particles per liter (Vg/L). Depending on the serotypes 4-30% of AAV is present in the supernatant of the cell culture at 48hpt. The presence of plasmids and plasmid polyplexes that were not taken up by the cells or were not brought into the cell nucleus were removed by Iodixanol-ultracentrifugation method and Benzonase treatment before analyzing by real-time PCR. About 25% loss in genome containing viral particle counts were observed by Iodixanol purification method based on infectivity assay. Productions of AAV2 and AAV6 encoding GFP were demonstrated in 3L stirred tank bioreactors. Purification scheme was based on column chromatography - a scalable process. Different chromatography media, such as cation exchanger, anion exchanger and hydrophobic interaction chromatography, were tested with each AAV serotypes for their ability to adsorb and elute efficiently. The purification scheme was then adopted by integrating best chromatography medium and sequence dependent upon the AAV serotype in use. We demonstrated the purification scheme for AAV2 based on ion-exchange and hydrophobic interaction chromatography steps. The SDS-PAGE showed the purity of the final product and the presence of three capsid proteins VP1, VP2 and VP3 on Western blot corresponding to the only three bands present in the final product on SDS-PAGE. To extend the storage life of AAV we explored lyophilization technique to study the stability of AAV2 and AAV6 under lyophilized conditions. The AAV2 and AAV6 were stable for over 40 weeks based on infectivity assay. We demonstrated the scalability of the process up to 45L. Productions tested in 20 and 500 mL cultures in shake flasks were scaled up in 2 and 45L cultures (in 3- and 60-L stirred tank bioreactors, respectively). The volumetric yields and purification recoveries were comparable

at all of these production scale levels demonstrating scalability of transient transfection at even larger scale is possible to generate material necessary for dosages required for gene therapy application.

AAV Vectors II

293. Discovery of an Essential Receptor for Adeno-Associated Virus Infection

Sirika Pillay[1], Nancy L. Meyer[2], Andreas S. Puschnik[1], Omar Davulco[2], Jonathan Diep[1], Yoshi Ishikawa[3], Lucas T. Jae[4], Jonathan E. Wosen[1], Claude M. Nagamine[5], Michael S. Chapman[2], Jan E. Carette[1]

[1]Microbiology and Immunology, Stanford University, Stanford, CA, [2]Department of Biochemistry & Molecular Biology, Oregon Health & Science University, Portland, OR, [3]Shriners Hospital for Children, Portland, OR, [4]Netherlands Cancer Institute, Amsterdam, Netherlands, [5]Department of Comparative Medicine, Stanford University, Stanford, CA

Cellular entry of adeno-associated viruses (AAV) is poorly understood, despite the prominent use of AAV vectors in gene therapy for several monogenic diseases. Using an unbiased, haploid genetic screen, we identified critical players in AAV serotype 2 (AAV2) entry including members of distinct protein complexes involved in retrograde trafficking as well as genes involved in the biosynthesis of the AAV2 attachment factor, heparan sulphate. We focused on the single most significantly enriched gene of the screen, an uncharacterized type-I transmembrane protein. Based on the evidence below we renamed this gene AAV receptor (AAVR). We discovered AAVR as capable of rapidly endocytosing from the plasma membrane and trafficking to the trans-Golgi network, taking a similar path as AAV particles utilize. Genetic ablation of *AAVR* using CRISPR/Cas9 technology demonstrated a robust resistance to AAV2 infection in a wide range of mammalian cell types, which could be reversed upon *AAVR* complementation. This confirmed the essentiality of AAVR in AAV2 infection. Further investigation revealed that AAVR was also required for the infections of all tested human and simian-derived AAV serotypes including AAV1, 3B, 5, 6, 8 and 9. Deeper characterization of AAVR showed it to contain Ig-like domains, which are commonly found in many virus receptors including those for poliovirus and measles. We observed that these domains are capable of binding to AAV2 particles, and anti-AAVR antibodies efficiently block AAV2 infection. Moreover, the importance of AAVR for AAV infection *in vivo* was demonstrated by the strong resistance of AAVR$^{-/-}$ mice to AAV9 infection, recapitulating what we showed *in vitro*. Collectively, the data indicates that AAVR is a universal receptor involved in AAV infection. This has significant implications for the improvement of future AAV vector design.

294. Characterizing AAV CaptureSelect Affinity Ligand Interactions

James K. S. Smith, Vibhu Banala, Mario Mietzsch, Paul Chipman, Mavis Agbandje-McKenna

Biochemistry and Molecular Biology, University of Florida, Gainesville, FL

Adeno-associated virus (AAV) is one of the most widely studied viral vector systems for therapeutic gene delivery. This application has already experienced success in several human clinical trials, including the treatment of hemophilia B with an rAAV8 vector expressing therapeutic levels of Factor IX protein and for the treatment of Pompe disease, utilizing AAV1 vectors. Recently, an rAAV1 vector packaging a gene for the treatment of lipoprotein lipase deficiency was approved as the first gene therapy drug, validating the utility of this system. However, the effective production and purification of enough viral vector for use in research, preclinical studies, and human treatment is essential for success. Towards this goal, camelid single domain antibody fragments directed against AAV were used to make affinity resin products suitable for fast, one-step purification of AAV vectors of several serotypes. This affinity based purification method can replace current standards of density purification, which are time consuming and require large volumes of reagents, such as sucrose or iodixanol. At present, the collection of affinity resins suitable for AAV purification, i.e. AVB-Sepharose™, POROS™ CaptureSelect™ AAV8 and -AAV9, show varied affinity for different AAV serotypes, resulting in varied efficacies of virus purification. To better understand these differences and improve broad range utility, it is important to understand how these affinity ligands bind to the surface of the AAV capsids. We have thus mapped their binding footprints on several AAV serotypes, including AAV1, AAV5, and AAV9, using cryo-electron microscopy and image reconstruction combined with pseudo-atomic modeling. The sites for different affinity ligands are clustered on regions of the capsid that are common among most serotypes, but display minor variations which likely account for the varied affinities. This information will assist in the synergistic development of affinity ligands with broader serotype coverage and AAV vectors with improved purification outcomes.

295. Intrathecal Delivery of rAAV9-ABCD1 by Osmotic Pump in a Mouse Model of Adrenomyeloneuropathy

Yi Gong[1], Rene Kok[1], Dakai Mu[1], Guangping Gao[2], Florian Eichler[1]

[1]Neurology, Massachusetts General Hospital, Boston, MA, [2]Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA

Adrenomyeloneuropathy (AMN) is a debilitating neurological disorder caused by mutations in the ABCD1 gene, which encodes a peroxisomal ATP-binding cassette transporter (ABCD1) responsible for transport of CoA-activated very long-chain fatty acids (VLCFA) into the peroxisome for degradation. The Abcd1-/- mouse develops a phenotype similar to AMN, manifesting spinal cord axon degeneration as well as peripheral neuropathy due to affected dorsal root ganglion neurons (DRGs). We previously reported successful transduction of central nervous system cells in vitro and in vivo using recombinant adeno-associated virus serotype 9 (rAAV9) vector for delivery of the human ABCD1 gene. Unfortunately, intravenous delivery in young mice was associated with cardiac toxicity due to transgene overexpression. We therefore set out to optimize delivery to the spinal cord while minimizing systemic leakage using an intrathecal osmotic pump. Self complementary AAV9 GFP(scAAV9GFP) and rAAV9 encoding ABCD1 (rAAV9-ABCD1) were delivered to Abcd1-/- mice intrathecally (IT) either by bolus over a 2min duration or by osmotic pump over 24h duration with PBS injection as sham control. Two weeks after injection, mice were sacrificed and perfused with 4% PFA. Tissues were then collected, sectioned and stained for immunofluorescence analysis. scAAV9-GFP delivered IT by osmotic pump led to widespread expression across CNS-relevant cell types and DRGs in a dose-dependent manner. Spinal cord and DRG had higher expression compared with brain, but GFP expression was also detected in peripheral organs (liver, heart and adrenal gland), with highest expression seen at $3X10^{11}$GC. A similar distribution pattern of ABCD1 protein was detected after rAAV9-ABCD1 intrathecal pump delivery. In general, higher doses ($2X10^{11}$GC and $1X10^{11}$GC) led to more expression in CNS and peripheral organs compared with a lower dose ($0.5X10^{11}$GC). However, widespread expression of ABCD1 across CNS was even detected after direct intrathecal bolus injection of $0.5X10^{11}$GC. Importantly, the same dose delivered by pump led to higher expression in brain and spinal cord far from the injection

site and comparatively less leakage to peripheral organs compared with bolus injection. Preliminary experiments delivering rAAV9-ABCD1 at $0.5X10^{11}$GC by intracerebroventricular approach suggest behavioral improvement in the Abcd1-/- mouse despite localized expression in brain. We therefore anticipate even better performance at this dose using the outlined intrathecal pump delivery. We conclude that rAAV9-mediated ABCD1 gene transfer via intrathecal osmotic pump leads to more uniform and widespread gene delivery to CNS with reduced leakage into the systemic circulation compared with intrathecal bolus injection.

296. Longitudinal Surveillance of Non Human Primate Recipients of Intrauterine Gene Transfer for Coagulation Factor Deficiencies

Citra Mattar[1], Arijit Biswas[1], Nuryanti Johana[1], Yvonne Y. W. Tan[1], Cecilia Rosales[2], Jenny McKintosh[2], Lay-Geok Tan[1], Irene Gil-Farina[3], Mahesh Choolani[1], Manfred Schmidt[3], Amit Nathwani[2], Jerry K. Y. Chan[4]

[1]National University of Singapore, Singapore, Singapore, [2]University College London, London, United Kingdom, [3]NCT Nationales Centrum für Tumorerkrankungen, Heidelberg, Germany, [4]Duke-NUS, Singapore, Singapore

A concerted effort is being made to determine a safe way to treat the fetus with severe monogenic diseases. While the majority of affected pregnancies are currently terminated, intrauterine gene therapy (IUGT) may become a viable future option in well-selected cases. Likely candidates may be perinatally-lethal or chronic-relapsing conditions for which existing postnatal therapy is sub-optimal. We have now amassed robust evidence in a relevant non-human primate model addressing the efficacy and safety of IUGT targeting coagulopathies, with a focus on Factor IX and X deficiencies. Minimally-invasive administration of AAVhFIX or AAVhFX in early and late gestation results in expression of the transgenic protein at therapeutic levels, with minimal immune- and hepatotoxicity. Immune tolerance to gene therapy vectors administered during gestation has been demonstrated with postnatal administration. Further analyses demonstrate the random nature of vector integration without the propensity to cluster at oncogenic sites, while a low level of germline transmission was also demonstrated in post-pubertal subjects. The effects of androgen on cellular transducibility and transgene expression are also studied. Minimal maternal bystander effects have been observed. Though there are ethical concerns to be addressed, there may be enough convincing evidence to take the next step towards clinical fetal therapy.

297. The Influence of ELABELA AAV Gene Therapy on Cardiorenal Function in Rat Models of Salt-Induced Hypertension and Myocardial Infarction

Claire A. Schreiber, Sara Holditch, Alex Generous, Yasuhiro Ikeda

Molecular Medicine, Mayo Clinic, Rochester, MN

Elabela (ELA) is a recently identified apelin receptor agonist essential for cardiac development, but its biology and therapeutic potential are unclear. The apelin receptor when bound to its original ligand, apelin, exerts peripheral vasodilatory and positive inotropic effects. We initially assessed endogenous ELA expression in normal and diseased rats. ELA was predominantly expressed in kidneys, especially in renal collecting duct cells and was not affected by disease. We then characterized the effects of long-term, adeno-associated virus (AAV)-ELA gene delivery on cardiorenal function in Dahl salt-sensitive rats (DS) on a high-salt diet. We overexpressed codon optimized rat ELA in the heart via AAV9 vector by a single intravenous injection. This led to a delayed onset of blood pressure (BP) elevation, and ELA-treated rats had lower BP than controls starting at 5 weeks post-injection (p.i.). As well, ELA significantly reduced fractional sodium and chloride excretion in treated (4 days p.i.), normotensive DS rats on low-salt diet, suggesting ELA's role in sodium and chloride readsorption. Glomerular architecture was better preserved in rats treated with the AAV9-ELA vector. A decrease in renal fibrosis and the significantly suppressed expression of fibrosis associated genes, including transforming growth factor beta (TGF-β), collagen, type 1, alpha 1 (COL1A1) and fibronectin 1 (FN1), was also seen in the treated group. Since ELA is essential for cardiac development, we additionally assessed the impact of the AAV9-ELA vector on cardiac repair in a rat myocardial infarction (MI) model. When compared with control MI rats, ELA treatment did not significantly improve cardiac function or structure. Thus, long-term ELA gene delivery may offer a potential therapy for hypertension and renal remodeling, but its application for MI may be limited.

298. Characterization of a Novel Anti-Sense Promoter Element in Adeno-Associated Virus: Implications for Hepatocellular Carcinoma

Grant Logan[1], Christine M. Smyth[1], Claus V. Hallwirth[1], Neeta Khandekar[1], Samantha L. Ginn[1], Sophia Laio[1], Allison P. Dane[2], Ian E. Alexander[1,3]

[1]Gene Therapy Research Unit, Children's Medical Research Institute, Westmead, Australia, [2]Department of Hematology, University College London Cancer Institute, London, United Kingdom, [3]Discipline of Paediatrics and Child Health, University of Sydney, Sydney, Australia

We have discovered a novel anti-sense promoter element in nucleotides proximal to the right ITR of AAV serotype 2. Presence of the element was initially detected using vector derived from p*sub*201, a widely-used plasmid for AAV vectorization which carries an infectious wtAAV2 clone engineered such that the left ITR is replaced with the right ITR and 46 nucleotides (nt) of proximal AAV2 sequence. While preliminary studies using the p*sub*201-derived vector showed promoter activity originated from a sequence containing 46-nt of wtAAV2 and 59-nt of poly-linker sequence, further investigation demonstrated 105 nucleotides proximal to the right wtAAV2 ITR to be optimal for activity, which was further enhanced by presence of ITR sequence. Demonstration of similar promoter activity in a phylogenetically-related AAV serotype (AAV7) but not a more distantly related serotype (AAV5) indicates potential conservation of the activity amongst related AAV, which may be important for the virus cycle. Activity of the element is restricted to cells of hepatic origin but also operates in a lung sarcoma cell line (A549). Our findings take on new significance in light of a recent study by Nault and colleagues (Nault *et al*. 2015 Nature Genetics) who have associated genomic integration of AAV with HCC in 11/193 patients. Cloncal expansion of AAV integration events occurred in known drivers of carcinogenesis leading to gene dysregulation at the site of integration. A striking feature of the study is that only a fragment of the AAV genome was integrated in the tumor genome with one patient showing a 1442 bp fragment spanning the *rep-cap* junction while the remaining 10/11 tumors contained fragments (201-1975 bp) from the 3' genome of which all contained the 105-nt element described in the current report. Conclusions drawn from the Nault study that AAV is involved in insertional mutagenesis have caused some contention in the field prompting two editorials with alternative interpretation of the data (Buning and Manfred 2015 *Mol.Ther.*; Berns *et al.* 2015 *Human Gene Therapy*). While the Nault report requires further scrutiny and confirmation, the nature of the findings may have implications for AAV gene therapy, especially in the liver over the longer term. Our findings delineating cell-restricted transcriptional activity from the 3' promoter element in AAV is of general interest to the fields of

AAV biology and vectorology but, more importantly, are of specific significance to the HCC controversy and may provide insight and resolution over the matter of AAV-associated HCC.

299. Safety and Biodistribution Study of rAAV2tYF-PR1.7-hCNGB3 in CNGB3-Deficient Mice

Guo-jie Ye[1], Ewa Budzynski[2], Peter Sonnentag[2], T. Michael Nork[3], Paul E. Miller[3], Leslie McPherson[2], James N. Ver Hoeve[3], Leia M. Smith[4], Tara Arndt Arndt[2], Savitri Mandapati[1], Paulette M. Robinson[1], Roberto Calcedo[5], David R. Knop[1], William W. Hauswirth[6], Jeffrey D. Chulay[1]
[1]Applied Genetic Technologies Corporation, Alachua, FL, [2]Covance Laboratories Inc, Madison, WI, [3]OSOD, Madison, WI, [4]Laboratory Corporation of America® Holdings, Seattle, WA, [5]University of Pennsylvania, Philadelphia, PA, [6]University of Florida, Gainesville, FL

Background: AGTC is developing a recombinant adeno-associated virus (rAAV) vector expressing the human *CNGB3* gene, for treatment of achromatopsia, an inherited retinal disorder characterized by markedly reduced visual acuity, extreme light sensitivity and absence of color discrimination. Here we report results of a toxicology and biodistribution study of this vector administered by subretinal injection in CNGB3-deficient mice. **Methods:** Three groups of CNGB3-deficient mice (n= 35 per sex per group) received a subretinal injection in one eye of 1 µL of vehicle (balanced salt solution with 0.014% Tween 20) or rAAV2tYF-PR1.7-hCNGB3 vector at a concentration of 1×10^{12} vg/mL (1×10^{9} vg/eye) or 4×10^{12} vg/mL (4×10^{9} vg/eye). The other eye was untreated. Ten animals/sex/group were used for toxicology evaluation with ophthalmic examinations and pathological evaluations, 10 animals/sex/group were used for biodistribution evaluation, and 15 animals/sex/group were used for efficacy evaluation. Half the animals in the biodistribution and toxicology groups were euthanized 4 weeks after vector administration and the remaining animals were euthanized 12 weeks after vector administration. For animals in the biodistribution groups, blood for qPCR analysis was obtained on Study Days 3, 8 and at euthanasia. At necropsy, samples of eyes, brain, heart, liver, gall bladder, kidneys, spleen, thymus, lungs, adrenals, ovaries, epididymides and testes were obtained for histopathology (for animals in the toxicology groups) or DNA PCR (for animals in the biodistribution groups). For animals scheduled for efficacy evaluations, electroretinography (ERG) testing included scotopic and photopic tests performed at Week 4, 8, and 12 on each eye and serum was collected at euthanasia for measurement of antibodies to AAV and hCNGB3. **Results:** There were no test article-related changes in clinical observations, body weights, food consumption, ocular examinations, clinical pathology parameters, organ weights, or macroscopic observations at necropsy. Cone-mediated ERG responses were detected after vector administration in the treated eyes in 90% of animals in the higher dose group, 31% of animals in the lower dose group, and none of the untreated or vehicle-treated eyes. Microscopic pathology results demonstrated minimal mononuclear cell infiltrates in the retina and vitreous of some animals at the interim euthanasia, and in the vitreous of some animals at the terminal euthanasia. Serum anti-AAV antibodies developed in most vector-injected animals. No animals developed antibodies to hCNGB3. Biodistribution studies demonstrated high levels of vector DNA in vector-injected eyes but little or no vector DNA in non-ocular tissue. **Conclusions:** Subretinal injection of rAAV2tYF-PR1.7-hCNGB3 in CNGB3-deficient mice was associated with no clinically important toxicology findings, rescue of cone-mediated ERG responses in vector-treated eyes, and vector DNA detection limited primarily to vector-injected eyes. These results support the use of rAAV2tYF-PR1.7-hCNGB3 in clinical studies in patients with achromatopsia caused by CNGB3 mutations. A Phase 1/2 clinical trial evaluating rAAV2tYF-PR1.7-hCNGB3 is scheduled to begin in 2016.

300. Changing the Route of Administration to Improve Liver Transduction by Recombinant AAV-Based Vectors

Nerea Zabaleta[1], David Salas[1], Anna Majowicz[2], Rubén Hernandez-Alcoceba[1], Valerie Sier-Ferreira[2], Jesús Prieto[1], Ignacio Bilbao[1], Gloria González-Aseguinolaza[1]
[1]FIMA, Pamplona, Spain, [2]uniQure, Amsterdam, Netherlands

The liver is a central organ in metabolism and there are numerous inherited metabolic disorders that have their origin in this organ. Gene therapy represents a promising therapeutic approach for this type of diseases. Although in preclinical studies using AAV-based vectors high and long-term hepatic expression has been achieved, there are still some concerns about the expression levels and the percentage of hepatocytes that can be transduced. This is of particular importance for diseases in which the deficiency of an enzymatic activity is associated with the generation of toxic products that require the transduction of a high percentage of hepatocytes. Therefore, the main goal of our study was to improve liver transduction efficacy by an AAV serotype. For this purpose, the vector was administered directly to the liver via suprahepatic veins (SHV) using a catheter and balloon occlusion or via the hepatic artery (HA) with balloon occlusion of the suprahepatic vein. The experiment was performed in *Macaca fascicularis*, with 8 animals being infected with a dose of $3x10^{13}$ gc/kg of an AAV5 expressing the reporter gene hSEAP (human secreted embryonic alkaline phosphatase) under the control of a liver specific promoter (AAV5-AAT-hSEAP). The first group (2 animal) received the virus systemically in the saphenous vein, the second group (3 animals) received the virus via the SHV with 10 minutes of balloon occlusion and the third group (3 animals) via the HA with 10 minutes of balloon occlusion of the SHV. The procedure could only be performed on the right branch of SHV and HA due to the small size of the left SHV and HA branches, reaching only 40% of the liver. hSEAP-specific activity in serum samples of the groups that received the virus directly in the hepatic blood flow was higher compared to the animals receiving the vector by peripheral intravenous injection. This was correlated with the presence of a higher number of viral genomes in the liver of the animals. Moreover, when their distribution was analyzed we found that the animals that had received the virus through the SHV or HA displayed higher infection rates in the right side of the liver, where the administration had been performed. In conclusion, direct administration of the vector AAV5-AAT-hSEAP through the hepatic blood flow with balloon occlusion was associated with higher transgene expression and an increase in vector genomes in the injected area. Studies in bigger animals in order to reach both sides of the liver should be performed in order to explore the potential use of alternative routes of administration in the clinic.

301. AAV Capsid Engineering to Improve Transduction in Retina and Brain

Jennifer Sullivan, Lisa Stanek, Michael Lukason, Elizabeth Barry, Shayla Russell, James Morris, Bryan Mastis, Anna Alves, Jie Bu, Lamya S. Shihabuddin, Seng Cheng, Abraham Scaria
Sanofi Genzyme, Framingham, MA

Gene therapy vectors based on adeno-associated virus (AAV) are currently in clinical studies for numerous disease indications including Leber's congenital amaurosis, age-related macular degeneration, hemophilia, muscular dystrophy and Parkinson's disease. AAV vectors hold considerable promise as therapeutic agents; however there is potential to further improve the efficiency of AAV gene

delivery and efficacy by making modifications to the AAV capsid. The AAV capsid can be engineered to incorporate mutations that alter its transduction activity, tropism, biodistribution and immunogenicity. We have constructed variant AAV vectors harboring a variety of capsid modifications including those that negate receptor binding and have tested these vectors in several tissues including the eye and brain. One variant, AAV2HBKO, is an AAV2 based vector containing mutations of critical amino acids known to be required for binding to its receptor, heparin sulfate proteoglycan. Interestingly, an AAV2HBKO vector delivering a secreted transgene, sFLT02, unexpectedly resulted in a 2-log increase in transduction compared to parental AAV2 when delivered subretinally to the mouse eye. Subretinal delivery of an AAV2HBKO vector expressing EGFP demonstrated that these capsid modifications resulted in an increase in photoreceptor transduction compared to the unmodified AAV2 vector. In contrast, the AAV2HBKO vector demonstrated a lack of transduction activity following intravitreal delivery to the mouse eye. In addition, we evaluated the transduction and tropism of AAV2HBKO in the mouse brain. In a head to head comparison with AAV2, the AAV2HBKO vector facilitated widespread striatal and cortical expression following an intrastriatal injection while AAV2-mediated expression was restricted to the site of injection. Similar to AAV2, the tropism of AAV2HBKO was primarily neuronal with little to no transduction of astrocytes or microglia. Biodistribution data suggests that this vector, when delivered systemically in the mouse, has significantly reduced liver transduction but a higher propensity to be delivered to skeletal muscle and heart compared to the wild-type AAV2 vector. We will present data evaluating the transduction activity, tropism and biodistribution of the AAV2HBKO variant. These studies illustrate the potential for improving the efficiency of AAV gene transfer via targeted capsid engineering.

302. Molecular AAV Capsid Evolution Is Not Primarily Restricted by Inadvertent Shuffling of the Assembly-Activating Protein AAP

Stefanie Grosse[1], Magalie Penaud-Budloo[2], Eduard Ayuso[2], Dirk Grimm[1]

[1]Dept. of Infectious Diseases/Virology, Heidelberg University, Heidelberg, Germany, [2]Atlantic Gene Therapies, INSERM UMR1089, Universite de Nantes, Nantes, France

A 2010 report that Adeno-associated virus 2 (AAV2) encodes an additional protein in the second open reading frame of the cap(sid) gene significantly changed our perception of the biology of this virus. This so-called assembly-activating protein or AAP appears to interact with the major viral capsid protein 3 (VP3) and to chaperone its assembly into virus-like particles, via mechanisms that remain unknown. Moreover, it was shown that deliberate mutation of the AAP gene diminishes AAV particle production.

As the AAP open reading frame fully overlaps with that of the AAV capsid proteins VP1/VP2 (and partially VP3), it is likely that the AAP gene is inadvertently disrupted or mutated during molecular AAV evolution protocols that involve capsid gene shuffling (homology-directed recombination of cap gene fragments). To study this possibility comprehensively and to provide solutions, if needed, we first investigated the role of AAP for 9 further AAV serotypes other than AAV2. Therefore, we knocked out AAP expression in serotypes 1, 3-9 and rh10 while leaving the rep and cap genes intact. Indeed, all mutants were markedly impaired in particle production, confirming that functional AAP is critically required for at least 10 different AAV serotypes.

Notably, all vectors were fully restored upon co-delivery of the corresponding AAP via an extra plasmid during particle production. Furthermore, when testing the cross-reactivity of all AAPs with the AAV2-AAP mutant (AAV2mut), we observed a broad interchangeability except for AAP4 and 5 which predominantly rescued their cognate serotype.

These results were confirmed independently in a baculovirus AAV2 vector production system where we also observed a dramatic reduction in particle yields upon AAP mutation. Interestingly, in both, mammalian and insect cells, we consistently noted that absence of AAP reduced the steady-state levels of VP proteins, suggesting a similar biological function of AAP in the two heterologous cellular systems. As we could rule out an effect on the transcriptional level, we postulate that AAP-mediated chaperoning of AAV virion assembly indirectly stabilizes VP proteins in cells of different species.

To explicitly dissect the role of AAP in the context of molecular AAV evolution, we produced five different AAV capsid libraries with increasing complexities, subcloned 46 randomly selected shuffled AAPs from these and tested them with the AAV-AAP mutants. Out of these 46 shuffled AAPs, 37 could rescue AAV2mut, and two more efficiently complemented an AAV5-AAP mutant. In total, 84.8 % of all tested AAPs were functional.

Of note, the highest proportion of defect AAPs was found in libraries that contained the diverse capsids of AAV4 and 5. We thus shuffled serotypes 2, 4, 5, 8 and 9 and produced viral libraries with or without a cocktail of excess AAP from all five serotypes. Strikingly, albeit up to 40% of all AAP genes were predicted to be non-functional as a result of inadvertent shuffling, AAP trans-complementation had no significant effect on particle yields.

Collectively, our data suggest that albeit AAP is disrupted to some extent during AAV capsid gene shuffling, this is likely not a major restriction for the efficiency and success of this technology.

303. Deletion of Pdx1 in Mouse Pancreatic Ducts by CRISPR-Cas9 Mediated Gene Editing

Aiping Lu[1], George K. Gittes[2], Johnny Huard[1], Ping Guo[1]

[1]Orthopedic Surgery, UTHealth at Houston, Houston, TX, [2]Surgery, Childrens' Hospital of Pittsburgh, University of Pittsburgh, Pittsburgh, PA

Introduction: The homeodomain transcription factor Pdx1 is crucial for pancreas formation. Pdx1-expressing cells are first observed at embryonic day 8.5 (E8.5), prior even to the earliest indication of morphogenesis, in endodermal cells designated to give rise to the pancreas. In adult mouse, Pdx1 protein is transiently expressed when pancreas is injured, such as Langerhans islets damage in alloxan induced diabetic mice, implying Pdx1 may be necessary for the neogenetic formation of β-cells from mature ducts. In order to study the role of pdx1 in the pancreatic duct for pancreatic regeneration, most investigators use the Cre-lox system to generate duct-specific Pdx1 deletions in mice. This method is able to delete Pdx1specifically from tissue or cells with or without a tamoxifen-inducible (cre-ERT2) system; however, several caveats and limitations exist with tamoxifen-induced depletion. In addition, the cre-ERT2 system may be leaky, resulting in constitutive rather than inducible activation in some cells. To overcome these flaws from the Cre-ERT2/lox system, we decided to use a novel CRISPR-Cas9 gene editing technique to delete pdx1 in pancreatic ducts. **Methods:** We previously used a mouse short Sox9 promoter (468 bp) to generate a recombinant adeno-associated virus (AAV) carrying a reporter GFP (AAV6-Sox9-GFP), and this recombinant virus construct was infused into the pancreatic ducts, through the common bile/pancreatic duct, to specifically tag the duct cells. In order to address pancreatic duct specific deletion of Pdx1 after alloxan ablation of beta-cells, we first to applied the CRISPR-Cas9 gene editing technique on the HEK293-CMV-GFP cell line in vitro (Figure 2) and ROSA26 LSL tomato reporter mice ex vivo (Figure 3), and then employed AAV6 mediated CRISPR-SaCas9 under the control of the mouse sox9 short promoter, coupled with paired guide RNAs (gRNAs) flanking

the Pdx1 exon2 (Figure 4) through pancreatic duct infusion into the pancreas.**RESULTS AND CONCLUSIONS:** These CRISPR-SaCas9 and gRNAs AAV viruses were co-infused into pancreases of C57BL/6J mice 2 days after alloxan treatment. One week after AAV6-SaCas9+gRNAs infusion, it appeared that only the Pdx1 in pancreatic ductal cells were deleted. Comparatively, PBS or AAV6-CMV-GFP viral infused C57BL/6J mice and ROSA26 LSL tomato reporter mice expressed fluorescence in the entire pancreas as a result of the deleted loxP sites that removed the stop cassette, the Pdx1 is not deleted. Thus, we first report here a highly efficient and specific ablation of Pdx1 in adult mouse pancreatic ducts using the CRISPR-Cas9 technique.Figure 1.Construction of AAV CRISPR-SaCas9 and gRNA vectors. Figure 2. Deletion of ZsGeen in HEK293-CMV-GFP cells using SacCas9 and gRNA in vitro. Figure 3. Valiation of SaCas9 and gRNA in vivo deletion of loxP sites in ROSA26-LSL tomato mice pancreas. Figure 4. Deletion of Pdx1 exon2 in alloxan treated C57 pancreatic ducts.

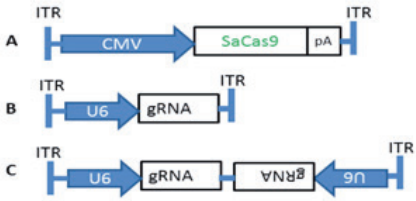


Figure1

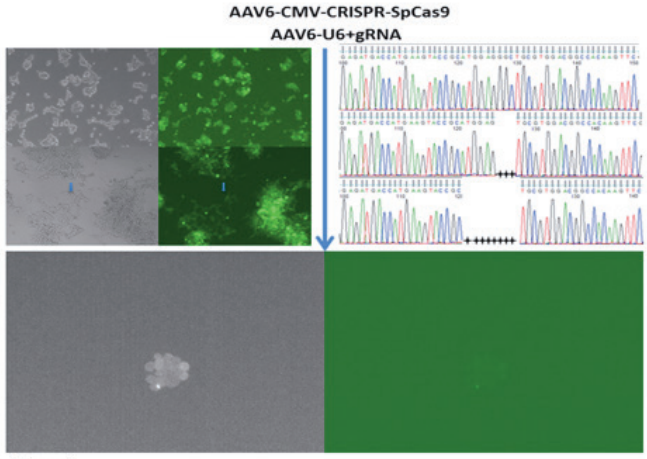


Figure2

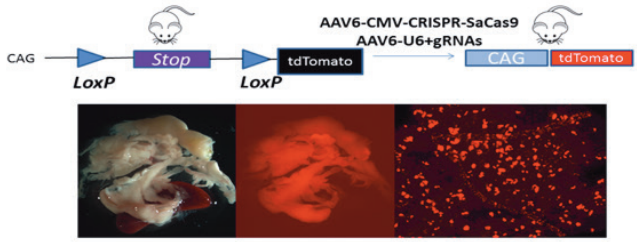


Figure3

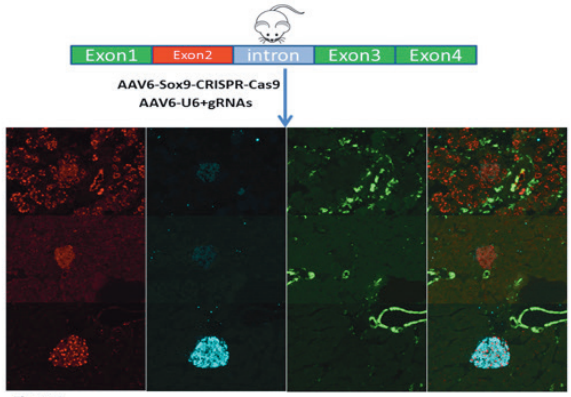


Figure4

304. Particle Titer Determination and Characterization of rAAV Molecules Using Nanoparticle Tracking Analysis

Samantha F. Povlich[1], Jonathan G. Mehtala[2], Gwendolyn M. Wilmes[1], Christopher J. Morrison[1], Samuel C. Wadsworth[1], K. Reed Clark[1]

[1]Dimension Therapeutics, Cambridge, MA, [2]Malvern Instruments, Westborough, MA

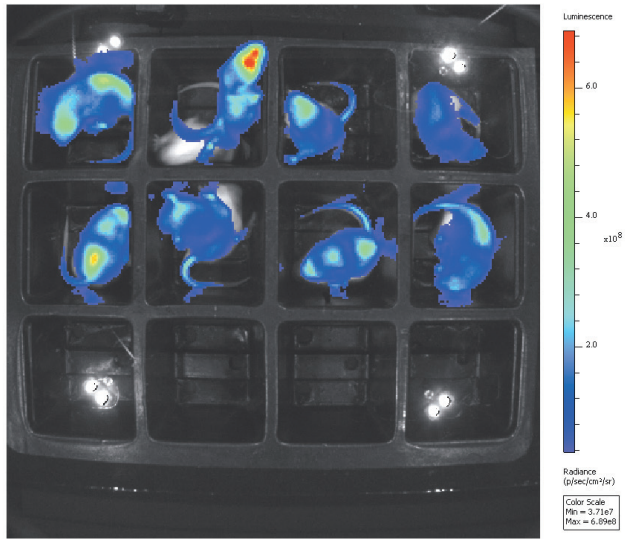
The development and commercialization of a biological pharmaceutical, such as recombinant adeno associated virus (rAAV), requires rigorous quantitative and qualitative testing. An accurate particle titer measurement is important for the development of purification processes. Current methods for titering rAAV include qPCR, for measuring genome copy titer, and ELISA, for measuring particle titer. Additionally, methods such as dynamic light scattering (DLS) have been considered for assessment of particle size and aggregation but with limited success for rAAV. Here, a novel method is described called Nanoparticle Tracking Analysis (NTA), to provide rAAV titer (concentration) within minutes, as opposed to hours or days required by traditional techniques. NTA characterization was enabled by a gold labeling technique to increase the light scattered from rAAVs. With a purified gold labelled rAAV sample, NTA results indicated a monodisperse size distribution and a measured rAAV particle titer equivalent to the concentration based on genome copies by qPCR. Particle titer determination can also be relevant for assessment of product stability. NTA was used to assess the stability of rAAV over a 3 month thermal stress test. The NTA method enabled the detection of a reduction of the primary rAAV particle peak within 1 month, as well as further reductions over time. These initial results demonstrate the potential uses of NTA for product characterization.

305. Generation of Light-Producing, Somatic-Transgenic Mice Using Lentivirus and Adeno-Associated Virus Vectors

Rajvinder Karda[1], Dany P. Perocheau[1], Suzanne M. K. Buckley[1], Juliette M. K. M. Delhove[2], Michael Hughes[3], Ahad A. Rahim[3], Mark R. Johnson[4], Joanne Ng[1], Natalie Suff[1], Tristan R. Mckay[5], Simon N. Waddington[1]

[1]Gene Transfer and Technology, Institute for Women's Health, UCL, London, United Kingdom, [2]Molecular & Cell Sciences Centre, St. George's, London, United Kingdom, [3]Department of Pharmacology, UCL School of Pharmacy, London, United Kingdom, [4]Institute of Reproductive and Developmental Biology, Imperial College London, London, United Kingdom, [5]Manchester Metropolitan University, Manchester, United Kingdom

Germ line light producing transgenic mice, where luciferase expression is controlled by a surrogate promoter or by a minimal promoter downstream of tandem, synthetic, transcription factor binding elements, are used to provide an *in vivo* readout of disease processes. However, as every cell within the organism contains the luciferase reporter gene, it is therefore not specific to individual organs. We have developed a novel technology for the generation of light emitting somatic transgenic animals using lentiviral vectors. This allows signalling pathways in diseased organs to be monitored specifically, continually and in a non-invasive manner [1]. In this study, we aimed to deliver NFκB driving a luciferase reporter constructs to the nervous system of neonatal mice to generate somatic-transgenic mice using both lentivirus and adeno-associated viral (AAV) vectors. Lentivirus vector pseudotyped with VSV-G viral envelope glycoproteins or AAV8 serotyped vector carrying an NFκB response element was injected intracranially or intravenously to outbred CD1 neonatal (P1) mice and luciferase expression was monitored continually by whole body bioluminescence imaging of conscious mice. The ability to image conscious mice holds advantages when studying neuropathology. After weaning, pathological activation of NFκB was induced by intraperitoneal injection of lipopolysaccharide (LPS). Following intracranial injection, the VSV-G lentivirus NFκB biosensor showed transduction of the brain and spinal cord. Luciferase expression was upregulated 24 hours after administration of LPS. Immunohistochemistry revealed only modest spread throughout the brain. Conversely, intracranial injection of AAV8 NFκB biosensor showed a much wider spread and increased luciferase expression. Finally we administered AAV8 NFκB biosensor intravenously at P1. Whereas previous studies show AAV8 serotype transduces many systemic tissues [2] through this route; we observed luciferase expression predominantly in the brain and spine (see figure). Using a standard Gateway® cloning system we have established a library of more than 25 lentivirus biosensors where some have been tested *in vitro* and *in vivo*. We plan on incorporating these response elements into the AAV backbone described above. Therefore, enabling the generation of somatic-transgenic mice which have a wider spread of AAV biosensor. This will complement existing germ line transgenic, light producing technology by maximising the use, and reducing the numbers, of animals used in biomedical research.



1. Buckley, SMK et al. 2015. In vivo bioimaging with tissue-specific transcription factor activated luciferase reporters. Scientific Reports. 2. Inagaki, K et al. 2008. Frequency and spectrum of genomic integration of recombinant AAV serotype 8 vector in neonatal mouse liver. Journal of virology.

306. Production of Safe and Efficient AAV Vectors Using *Minicircle* DNA

Marco Schmeer[1], Tatjana Buchholz[1], Martin Schleef[1], Maria Schnödt[2], Anja Grünert[3], Ram Shankar[1], Thomas Fuchsluger[3], Hildegard Büning[4]

[1]PlasmidFactory GmbH & Co. KG, Bielefeld, Germany, [2]Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany, [3]University of Erlangen, Erlangen, Germany, [4]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany

Especially in gene therapy applications, certain sequence motifs contained in plasmid DNA have to be avoided wherever possible. Such sequences are e.g. the bacterial *ori* or selection markers, only used for controlling the bacterial replication of the plasmid or to select for the plasmid during cloning or during production. Such sequence motifs are redundant in the intended therapeutic application and are completely removed in *minicircles*, i.e. circular and ccc-supercoiled expression cassettes.

Since Adeno-Associated viral (AAV) vectors are produced by co-transfection of HEK293 producer cells, such bacterial sequence motifs may be an issue of an AAV vector-mediated gene transfer as well. Here, as a result of so-called reverse packaging events, an AAV vector-mediated transfer of not only the therapeutic gene but also of the antibiotic resistance gene into the target cells has been reported. Hence, this appears to be a potential risk of plasmid derived AAV vectors which can be overcome by using *minicircle* DNA for AAV production.

Here we present first results showing that both constructs, the Helper & Packaging plasmid as well as the Transfer plasmid can be produced as *minicircles* although certain sequence motifs such as the ITRs have been identified to be an issue in *minicircle* production which has been overcome. These *minicircles* have been used for efficient AAV vector productions. However, only by replacing both, the Helper & Packaging plasmid as well as the transfer plasmid, encapsidation of the antibiotics resistance gene can be avoided.

307. Hitting the Kidney from All Angles: Using Direct Intrarenal Pelvis Injection Combined with Serotype and Promoter Targeting to Achieve Renal AAV Gene Delivery

Claire A. Schreiber, Sara Holditch, Yasuhiro Ikeda
Molecular Medicine, Mayo Clinic, Rochester, MN

Using adeno-associated virus (AAV)-based gene therapy to treat renal diseases is an underdeveloped area. Renal anatomy and physiology make gene delivery to this organ especially difficult. Multiple AAV serotypes and routes of entry in rodents have been tried, with the majority of combinations targeting the renal tubules. To improve upon the current methods of AAV renal transduction for future use in the clinical setting, we employed direct intrarenal pelvis injections of AAV2, 6, 8 and 9 with the luciferase (Luc) or GFP transgene under the control of the cytomegalovirus (CMV) or putative kidney-specific promoters from myo-inositol oxygenase(MIOX), acyl-CoA synthetase medium-chain family member 2 (Acsm2), uromodulin (UMOD), kidney androgen-regulated protein (Kap) and solute carrier family 34 (sodium phosphate), member 1 (Slc34a1) mouse genes in C57Bl/6J mice. Renal luciferase transgene expression was strongest with AAV6, 8 and 9-CMV-Luc. AAV6 and 8-CMV-Luc resulted in stronger expression in the injected kidney, whereas AAV9-CMV-Luc showed equal, bilateral renal expression. AAV9 with the UMOD promoter gave the strongest and most highly renal-specific luciferase expression among all serotype and kidney promoter combinations. Using the GFP transgene we further established AAV transduction of multiple cell types in the kidney. AAV8-CMV-GFP transduced both glomerular and tubular renal cells, while AAV9-CMV-GFP most strikingly transduced periglomerular tubule cells. Both AAV8 and AAV9-UMOD-GFP solely transduced renal tubule cells. We are currently testing AAV variants isolated from clinical urine samples to determine their ability to transduce renal cells *in vivo*. Our results demonstrate efficient renal transduction by direct intrarenal pelvis injection of AAV vectors. Coupling specific renal cell tropisms of AAV serotypes and renal cell-type-specific promoters, along with the intrarenal pelvis injection strategy would pave the way for a renal-targeted AV gene therapy.

308. Ultracentrifugation-Free Chromatography-Mediated Large-Scale Purification of Recombinant Adeno-Associated Virus Serotype 1 (rAAV1) and rAAV9 from the Serum-Free Culture Supernatant

Taro Tomono[1], Yukihiko Hirai[2], Hironori Okada[2], Kumi Adachi[2], Akiko Ishii[3], Takashi Shimada[2], Masafumi Onodera[4], Akira Tamaoka[3], Takashi Okada[2]
[1]Nippon Medical School, Tokyo, National Center for Child Health and Development, Tokyo, University of Tsukuba, Ibaraki, Japan, [2]Nippon Medical School, Tokyo, Japan, [3]University of Tsukuba, Ibaraki, Japan, [4]National Center for Child Health and Development, Tokyo, Japan

[Background]

The current production of rAAV from the transfected cell lysate and purification based on CsCl or iodixanol density ultracentrifugation are not suitable for large-scale processing. Although rAAV1 and rAAV9 are promising therapeutic vectors for genetic neuromuscular disorders, the large-scale purification method for those vectors has not yet been established. In this study, we elaborate the novel chromatography-mediated methods for purification of rAAV1 and rAAV9 from the serum-free culture supernatant with ultracentrifugation-free technique towards large-scale and GMP production.

[Methods]

rAAV1 (scAAV1-CBA-EGFP) and rAAV9 (scAAV9-CBA-EGFP) were produced by the triple-transfection to HEK293 or HEK293EB cells in serum-free medium with polyethyleneimine (PEI). Five days later, the culture supernatant was tangential flow-filtrated (TFF) and concentrated by the Hollow Fiber system. After reducing protein debris by 1/3-saturated ammonium sulfate (1/3 AS) precipitation, rAAV1 or rAAV9 was precipitated in 1/2 AS solution.

Subsequently, the precipitated rAAV1 was dissolved in 10 ml of PBS containing 3 mM $MgCl_2$. After the sample was dialyzed against a buffer containing 5 mM NaCl, the dialysate was diluted with dH_2O and loaded to tandem quintet cation-exchange membranes (Mustang SXT) and quintet anion-exchange membranes (Mustang QXT) with a column volume of 0.86 ml. After rAAV1 was eluted from Mustang QXT, it was finally purified by gel-filtrated chromatography (Superdex 200 10/300 GL).

The precipitated rAAV9 was dissolved in 22 ml of 3.3 mM MES, 3.3 mM HEPES, 3.3 mM sodium acetate (MHN) buffer (pH8.0) containing 50 mM NaCl and 0.01% Pluronic F-68. After the resultant sample was diluted with MHN buffer, it was loaded to tertiary amine charged anion-exchange column. The passed through fraction containing rAAV9 was finally purified by gel-filtrated chromatography.

The physiological and biological properties of the purified rAAV1 and rAAV9 were characterized by qPCR, electron micrograph, FACS, western blot and SDS-PAGE.

[Results]

The purified rAAV1 and rAAV9 displayed three major bands (VP1, VP2, and VP3) on SDS-PAGE and more than 90% of rAAV1 particles or 95% of rAAV9 particles was contained fully packaged viral genomes according to electron micrographic analysis. We confirmed increasing infectivity of rAAV1 products depending on chromatographic purification step and that of rAAV9 is under evaluation. The resultant genomic titer of the purified rAAV1 was 4.17×10^{13} v.g. (3.63×10^{13} v.g./ml) from the 4×10^9 HEK293 cells and the purified rAAV9 was 1.49×10^{15} v.g. (1.14×10^{14} v.g./ml) from the 5.1×10^9 HEK293EB cells.

[Conclusion]

Chromatography-mediated purification from the culture supernatant is a major breakthrough, especially for the production of rAAV9. We obtained rAAV1 and rAAV9 by this protocol with high titer, high purity and minimum contamination of empty particles. This novel chromatography-based method would be consistent with GMP production and facilitate clinical studies in the future.

309. Optimization of Production of AAVrh.10 Viral Vectors

Hyunmi Lee, Lucy Quach, Fan Fan, Sara Cram, Dolan Sondhi, Ronald Crystal, Stephen Kaminsky
Weill Cornell Medical College, New York, NY

With the increasing interest in the use of adeno-associated virus (AAV) vectors to treat human disorders, there is increasing focus on optimizing AAV production to improve yield, purity and potency. The production of AAV vectors can be conceptualized in two stages: upstream cell culture and downstream purification and formulation. As an example of how AAV production can be optimized, we assessed upstream (cell density at transfection, plasmid and transfection reagent concentration, cell culture nutrients and harvest timing) and downstream (virus recovery, purification methods, potency) steps in the production of AAVrh.10, a nonhuman primate vector, using adherent 293T cells and a 2 plasmid transfection system using polyethylenimine (PEI). Upstream, vector yield, as measured by quantitative PCR in the cell harvest from transfections at each of several different cell densities peaked at 8×10^4 cell/cm^2. Total vector yield as a function of time after transfection peaked at day 3 post-transfection with diminishing returns and greater partitioning into the cell supernatant at longer times. Higher vector yield correlated with a greater total quantity of plasmid and PEI, but changing the

PEI:plasmid ratio from 1:1 diminished productivity. Fetal bovine serum concentration had minimal effect. Downstream, freeze-thaw methods were not reproducible. Physically lysing the cells was not as effective for AAV recovery as the detergents, triton or tween, which were equally effective, with the lowest effective detergent concentration important to minimize the residual detergent in the final product. While iodixanol gradients are the traditional AAV purification systems, depth filtration followed by tangential flow filtration worked well for small scale production (>90%), but for large scale was less efficient (>70%). Affinity chromatography using AVB (GE Healthcare Life Sciences), provided an effective purification with maximum vector loads of up to 3 x 10^{12} vector genomes per ml of packed column. Finally, concentration of the final bulk AAV product was critically dependent on a formulation that stabilizes the solubility of the AAV monomer, with 0.01% pluronic acid providing AAV recovery of nearly 80%. Together, the optimized methods for the best yield, purity and potency relied on transfection with equal amounts of plasmid and PEI and a cell density of 8 x 10^4 cells/cm^2 combined with the use of detergent to disrupt and recover AAV and sequential purification by depth filtration, tangential flow filtration and affinity chromatography. This process improved the yield from harvest to final product from 9% to 40% and achieved final purified AAV at 7 x 10^9 vector genomes/cm^2 of adherent cell culture. These systematic refinements to the individual steps of AAV production demonstrate improved yield and facilitates transfer of a method for GMP production for clinical use.

310. Analysis of AAV5 Biodistribution and Viral Shedding in the Presence or Absence of Neutralizing Antibodies

David Salas[1], Anna Majowicz[2], Nerea Zabaleta[1], Estefania Rodríguez-Garcia[1], Harald Petry[2], Valerie Ferreira[2], Gloria Gonzalez Aseguinolaza[1]
[1]Gene Therapy and Regulation of Gene Expression Program, FIMA, Pamplona, Spain, [2]Research Department, uniQure B.V., Amsterdam, Netherlands

Neutralizing antibodies (NAbs) are the main obstacle that must be overcome to achieve a successful delivery of a viral vector to the target organ. NAbs acquisition can be induced naturally after infection with wild type AAV or after the first administration of AAV vector in the course of gene therapy treatment. Therefore, the objective of this project was to evaluate the efficacy and presence of the vector in the organism, as well as the response and vector elimination in the presence and absence of NAbs. We analyzed the presence of the reporter transgene (hFIX), humoral and cellular immune responses against the vector, viral shedding and biodistribution. The administration of AAV2/5 was successful in absence of NAbs. In contrast, vector administration in presence of specific NAbs was unsuccessful, probably due to the vector being completely depleted by anti-AAV2/5 NAbs. Our data demonstrate that while in the absence of antibodies the vector can be detected in serum and in other body fluids for 70 days after vector administration, the presence of antibodies immediately clears the vector from the organism. 24 hours after the AAV-hFIX administration 1X10^{08} gc/mL are detected in the serum in the absence of NAbs but in the presence of NAbs the concentration is reduced 1000-fold. In both groups the presence of NAbs increased upon infection without a cellular immune response against the vector. Finally, the biodistribution studies showed remarkable differences between the two groups: in the absence of NAbs vector genomes are detected mainly in liver, heart, spleen and inguinal LN, while in the presence of NAbs vector genomes are almost undetectable in all tissues analyzed.

Targeted Genome Editing II

311. *In Vivo* Zinc Finger Nuclease-Mediated Targeted Integration of a Glucose-6-Phosphatase Transgene Enhances Biochemical Correction in Mice with Glycogen Storage Disease Type IA

Dustin J. Landau, Elizabeth D. Brooks, Pablo Pablo Perez-Pinera, Hiruni Amarasekara, Adam Mefferd, Songtao Li, Andrew Bird, Charles A. Gersbach, Dwight D. Koeberl
Duke University, Durham, NC

Glycogen storage disease type Ia (GSD Ia) is caused by glucose-6-phosphatase (G6Pase) deficiency in association with severe, life-threatening hypoglycemia that necessitates lifelong dietary therapy. Here we show that use of a zinc-finger nuclease (ZFN) targeted to the ROSA26 safe harbor locus and a ROSA26-targeting vector containing a G6PC donor transgene, both delivered with adeno-associated virus (AAV) vectors, markedly improved survival of G6Pase knockout (G6Pase-KO) mice compared with mice receiving the donor vector alone ($p<0.04$) out to 8 months of age. Transgene integration has been confirmed by sequencing in the majority of the mice treated with both vectors. Surviving G6Pase-KO mice at 8 months of age had decreased glycogen content compared with young untreated G6Pase-KO controls, which correlated with the long-term survival of these mice that otherwise perish before weaning. A short-term experiment resolved difficulty observing biochemical differences between the dual-vector and single-vector groups, caused by a selective advantage for strongly-responding mice: 3-month-old mice receiving the ZFN had significantly reduced hepatic glycogen accumulation and improved G6Pase activity, compared with mice that received the donor vector alone. These data demonstrate that the use of ZFNs to drive integration of G6Pase at a safe harbor locus might improve transgene persistence and efficacy, and lower mortality in GSD Ia.

312. Gene Correction of LGMD2A Patient-Specific iPS Cells for Targeted Autologous Cell Therapy

Sridhar Selvaraj[1], Antonio Filareto[1], James Kiley[1], Daniel Voytas[2], Michael Kyba[3], Rita Perlingeiro[1]
[1]Lillehei Heart Institute, Department of Medicine, University of Minnesota, Minneapolis, MN, [2]Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, [3]Lillehei Heart Institute, Department of Pediatrics, University of Minnesota, Minneapolis, MN

Limb girdle muscular dystrophy type 2A (LGMD2A), an incurable autosomal recessive disorder and the most common form of LGMD, is characterized by symmetric and progressive weakness of limb-girdle muscles leading to loss of ambulation. LGMD2A is caused by mutations in the Calpain 3 (CAPN3) gene which encodes a muscle-specific intracellular calcium-sensitive cysteine protease, hypothesized to act as a gate keeper for proper assembly and maintenance of the sarcomere. A cell therapy based on induced pluripotent stem (iPS) cells has the potential for treating muscular dystrophy. We have demonstrated that transplantation of skeletal muscle progenitors obtained from disease-free human iPS cells can restore muscle contractility in a mouse model for Duchenne Muscular Dystrophy. The goal of this study is to apply gene editing tools to correct the CAPN3 mutation in LGMD2A iPS cells, and to determine the feasibility of an iPS-cell based autologous cell therapy for LGMD2A. Here we investigated two approaches to genetically correct a LGMD2A patient-specific iPS cell line with homozygous deletion of exon 17 to 24 in the CAPN3 gene. The first approach consisted of knocking in the coding region of CAPN3 cDNA with SV40 polyadenylation signal sequence downstream in exon 1 of

the endogenous CAPN3 gene by CRISPR-CAS9 induced double stranded break (DSB) mediated homology directed DNA repair from exogenous donor template, allowing for universal correction of all types of CAPN3 mutations. PCR, sequencing and Southern blot analysis on iPS colonies post- genome editing confirmed the integration of the cDNA in exon 1 of the CAPN3 gene. The second approach involved inserting the missing exons 17-24 with SV40 polyadenylation signal sequence downstream contiguous with the exon 16 of the endogenous CAPN3 gene. PCR and sequencing analysis on iPS colonies post-genome editing confirmed proper integration in this approach as well. LGMD2A iPS cell clones, genetically corrected using these two gene editing methods will be differentiated into skeletal muscle progenitors using conditional expression of Pax7, as previously described by our group, and evaluated *in vitro* and *in vivo* for restoration of full length CAPN3. For the latter, we have generated an immunodeficient LGMD2A mouse model by combining the CAPN3-KO mouse model with NOD/SCID and IL2Rg mutations. This mouse is ideal for studies on the transplantation of human cells. In summary, we have corrected the CAPN3 mutation in LGMD2A iPS cells for the first time, and ongoing transplantation studies with corrected iPS cells will provide proof-of-principal for a potential new cell therapy that can permanently restore CAPN3 function in all LGMD2A patients.

313. Gesicle Mediated Delivery of a Cas9-sgRNA Protein Complex

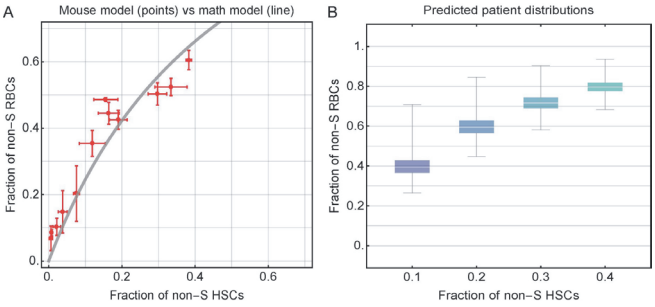
Thomas Quinn, Montse Morell, Tatiana Garachtchenko, Lily Lee, Mei Fong, Michael Haugwitz, Andrew Farmer
Clontech Laboratories, Inc, Mountain View, CA

CRISPR/Cas9-based gene editing has revolutionized the field of cell biology and is quickly being incorporated into the toolboxes of many laboratories. While CRISPR/Cas9 is a powerful technique for genome manipulation, two significant challenges remain: obtaining efficient delivery of Cas9 to all cell types and achieving fewer off-target effects. Recently, it has been demonstrated that genome editing via direct delivery of Cas9 is as effective as plasmid-based delivery, but with the added benefit of fewer off-target effects due to the short duration of the Cas9 protein in the cell (1). Here we report on the delivery of Cas9 using cell-derived nanovesicles termed gesicles. Gesicles are produced via co-overexpression of three components in a mammalian packaging cell: a nanovesicle-inducing glycoprotein, Cas9 endonuclease, and the sgRNA specific to the target gene. Additionally, we have developed a method for actively packaging sgRNA-loaded Cas9 into gesicles via ligand-dependent dimerization (iDimerize™ technology). The active packaging mechanism results in an approximate 4-fold increase in the loading of Cas9 into the gesicles. Moreover, the ligand-dependent dimerization approach also allowed us to efficiently package active Cas9 containing a nuclear localization signal (NLS) into these nanovesicles. We also have developed an optimized, lyophilized, one-step transfection formulation to promote high transfection efficiencies during the production of the Cas9-sgRNA gesicles. Gesicle-based protein delivery does not rely on recombinant protein production from a bacterial source or on the use of a transfection reagent for delivery. In this work, we were able to demonstrate that gesicles carrying a Cas9-sgRNA protein complex can mediate specific target-gene knockout in a wide variety of cell types, including human iPS cells. The observed knockout efficiency is often considerably higher than that observed with plasmid-based delivery of sgRNA and Cas9. This nanovesicle-based method allows for tight control of the dose and duration of the Cas9-sgRNA complex in the cell which has been shown to correlate with the amount of off-target cleavage (1). Through mismatch detection and Sanger sequencing of edited target sequences, we were able to demonstrate a lack of off-target cleavage when compared to plasmid-based delivery. Close analysis of the gesicles shows that they are stable over several freeze/thaw cycles and are consistent in size (~150-170 nm) as determined by nanoparticle tracking analysis. Overall, gesicles can be considered a novel and effective tool for simultaneous Cas9 and sgRNA delivery to any target cells. (1) Zuris, J.A. et al. (2014) Nat. Biotechnol. 33(1): 73-80.

314. Mathematical Modeling of Erythrocyte Chimerism Informs Clinical Strategies for Sickle Cell Disease

Philipp M. Altrock[1,2,3], Christian Brendel[4], Raffaele Renella[5,6,7], Stuart H. Orkin[4,6,8,9], David A. Williams[4,6], Franziska Michor[1,2]
[1]Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA, [2]Department of Biostatistics, Harvard T.H. Chan School of Public Health, Boston, MA, [3]Program for Evolutionary Dynamics, Harvard University, Cambridge, MA, [4]Division of Hematology/Oncology, Boston Children's Hospital and Harvard Medical School, Boston, MA, [5]Boston Children's Hospital and Harvard Medical School, Boston, MA, [6]Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, [7]Département Médico-Chirurgical de Pédiatrie, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, [8]Howard Hughes Medical Institute, Cambridge, MA, [9]Harvard Stem Cell Institute, Cambridge, MA

Recent advances in gene therapy and genome-engineering technologies offer the opportunity to correct sickle cell disease (SCD), which originates from a point mutation in the gamma-globin gene. The developmental switch from fetal gamma-globin to adult beta-globin is governed in part by the transcription factor (TF) BCL11A. This TF can be a therapeutic target for reactivation of corrected gamma-globin and concomitant reduction of sickling beta-globin. Genetic alteration of a portion of the hematopoietic stem cell (HSC) compartment would lead to a mixture of sickling and corrected peripheral red blood cell (RBC) populations. The degree of HSC alteration required to achieve a desired stable fraction of corrected RBCs with high gamma-gobin remained unclear. We developed a mathematical model that describes aging and survival of normal and sickling RBCs: a survival advantage of non-sickled RBCs over sickled RBCs leads to their overrepresentation in periphery. The math model considers HSC and age-structured RBC populations, and we aimed to validate it in an experimental mouse model by transplanting mixtures of Berkeley SCD mouse model and normal murine bone marrow cells to establish chimeric grafts in murine hosts. The math model identified the level of bone marrow chimerism required for successful stem cell-based gene therapies in SCD. We found an equation for the stable fraction of normal RBCs in periphery as a function of the fraction of genetically altered HSCs, predicting that gene therapy leading to 40% altered HSCs results in 55% non-sickling RBCs in periphery (Figure 1A, line). These findings were confirmed in the experimental mouse setting, where we achieved altered HSC mixtures between 0.5 and 38%, leading to RBC fractions between 7 and 62% (Figure 1A, data points). We found that a mathematical model incorporating a constant RBC selection approach was sufficient to predict pheripheral chimerism. This approach was then used to make SCD patient-predictions: 10% HSC alteration leads to 40% normal RBCs in periphery, 30% leads to over 70% normal RBCs (medians, assuming variable S cell life span, Figure 1B), which depend on the mean life spans of sickling (median 20 days) and normal (110 days) RBCs. Our integrative approach informs the target frequency of HSC genetic alterations likely required for effective treatment of sickling syndromes in humans.



315. Highly Efficient Targeted Addition of Large *XIST* Transgenes in a Population of Somatic Cells

Jun Jiang, Jeanne Lawrence
University of Massachusetts Medical School, Worcester, MA

Recent advances in genome editing technologies enable precise and corrective genome modification to treat genetic diseases in a targeted way. High efficiency of genome editing can be achieved through error-prone NHEJ pathway, however, the absolute rate of precise genome editing through homology-directed repair (HDR) pathway remains relatively low, especially for targeted addition of large DNA sequences. Although it is sufficient for the generation of cell lines derived from single targeted clones when coupled with drug selection, poor rates of homologous recombination greatly limit the practical application of targeted gene integration in somatic tissue. *XIST* encodes a large long non-coding RNA (19 kb) that plays an essential role in X chromosome inactivation in mammalian females. Recently, we have shown that *XIST* can be inserted to chromosome 21 and silence the extra copy of entire chromosome in Down syndrome stem cells derived from a single-targeted clones. Successful trisomy silencing *in vitro* holds promise for potential development of 'chromosome therapy" in patients with Down syndrome. Here, using Zinc finger nuclease (ZFN) technology, we tested the feasibility of targeted addition of large *XIST* transgenes into different loci of human genome in a population of transformed cells, as well as primary fibroblasts. Our results demonstrate ZFNs enable highly efficient targeted addition of several large *XIST* transgenes (up to 21 kb) into multiple selected loci of human genome, which are the largest sequences for targeted addition to date. Surprisingly, the frequency of targeted integration is independent of the size of transgenes but possibly depends on ZFN cleavage activity. Highly efficient targeted addition of large *XIST* transgenes allows to directly compare silencing of *XIST* transgenes that contain different functional domains in a pool of many independent integrants. This study provides a powerful tool to test functional mini-*XIST* transgenes that can be packaged in a viral vector, and has particularly important applications in potential development of *in vivo* delivery of *XIST* transgenes into an organ of patients with trisomic chromosomal abnormalities.

316. Successful Editing of the CD40LG Locus in Human Hematopoietic Stem Cells

Gabrielle Curinga[1], Sarah Leach[1], Swati Singh[1], Nick Hubbard[1], David Hagin[1], Karen Sommer[1], Iram Khan[1], Yumei Song[1], Troy Torgerson[2], Andy Scharenberg[2], David J. Rawlings[2]
[1]Seattle Children's Research Institute, Seattle, WA, [2]Seattle Children's Research Institute and Departments of Pediatrics and Immunology, University of Washington School of Medicine, Seattle, WA

X-Linked Hyper-IgM Syndrome (X-HIGM) is a genetic disorder caused by mutations in CD40LG that result in the loss of functional CD40L protein on the T cell surface. CD40L is required for T cells to provide "help" to B cells during an immune challenge; thus X-HIGM patients have impaired immunoglobulin class-switching and somatic hypermutation, and suffer recurrent infections. Murine gene transfer and X-HIGM patient studies suggest that safe and effective gene therapies for this disorder need to replicate cell surface expression patterns of WT endogenous CD40L, as well as knock-out expression of the mutant CD40L; requirements that are unlikely to be achievable using viral gene replacement. We recently reported a gene editing approach combining mRNA-delivered TALEN (targeting just upstream of the coding sequence of CD40LG) with an rAAV donor template for homology directed repair, targeting a promoter-less CD40L cDNA to the ATG start codon of the endogenous allele. This approach restored regulated cell surface expression of CD40L to X-HIGM T cells, disrupted expression of the mutant protein, and resulted in T cells that induced B cell class-switching in vitro, thus demonstrating it's potential as a T cell therapy for X-HIGM.

Although the pathologies of X-HIGM are attributed mostly to the T cell defects, CD40L is expressed in most hematopoietic lineages. Besides providing a stable X-HIGM treatment, editing of autologous hematopoietic stem cells (HSC) would likely rescue regulated CD40L expression in all lineages. A selective advantage for gene corrected cells is not anticipated; however, patients with mixed donor chimerism post-transplantation have substantial improvements when as few as 10% of HSC have the WT allele. Here we report gene editing of CD40LG in adult human CD34+ PBSC at rates that are anticipated to provide such clinical benefit. Initial experiments using mRNA delivery of TALEN pairs targeting exon 1 of the CD40LG locus in human CD34+ cells demonstrated indel frequencies of >50%. To investigate the potential for HDR at the CD40LG locus in adult CD34+ cells, we combined delivery of the TALEN with an AAV6 donor template containing an MND promoter-GFP expression cassette flanked by 1 kb CD40LG homology arms. This donor template allowed us to track editing rates within the CD40LG locus (normally silent in HSCs) by flow cytometry. Using this co-delivery strategy, we consistently achieved editing rates of ~30% across multiple control human stem cell donors. Edited cells demonstrated minimal loss of viability, and expansion rates in culture equivalent to controls. Edited cells have been transplanted into immune deficient, NSG mice in order to track engraftment and differentiation potential. In parallel, we are currently evaluating editing rates using a more clinically relevant, promoter-less donor template encoding the CD40L cDNA. In summary, we have achieved clinically relevant rates of gene editing within the endogenous CD40LG locus in human HSC, setting the stage for additional work required to translate this approach into clinical application.

317. Screening *S. Aureus* CRISPR-Cas9 Paired-Guide RNAs for Efficient Targeted Deletion in Duchenne Muscular Dystrophy

Josh Tycko[1], Nick Huston[1], Jacqueline N. Robinson-Hamm[2], Chris Wilson[1], Charles A. Gersbach[2], Patrick D. Hsu[1], David Bumcrot[1]
[1]Editas Medicine, Inc., Cambridge, MA, [2]Dept. of Biomedical Engineering, Duke University, Durham, NC

Duchenne muscular dystrophy (DMD) is a recessive X-linked neuromuscular disorder that results in progressive muscle degeneration and premature death. Most patients have exonic deletions in the dystrophin gene that result in a frameshift and nonfunctional protein. In contrast, Becker muscular dystrophy (BMD) patients carry a range of exonic deletions in dystrophin that do not disrupt the reading frame, leading to a much milder disease phenotype. Thus, multiplex CRISPR/Cas9 targeted deletions that restore the reading frame could convert DMD genotypes into BMD-like genotypes and potentially treat this disease. Previously, this strategy has been demonstrated *in vitro* with zinc finger nucleases, TALENs, and *S. pyogenes* Cas9

leading to restoration of dystrophin expression in DMD patient myoblasts. However, these genome-editing enzymes are limited by the difficulty of delivering large transgenes with viral vectors *in vivo*. Alternatively, the smaller Cas9 ortholog from *S. aureus* (SaCas9) can be packaged with paired sgRNAs in an all-in-one AAV vector for *in vivo* gene therapy. Recently, three groups have demonstrated that AAV-SaCas9 can mediate targeted deletions in *mdx* mice and restore dystrophin expression. As a first step towards developing a genome editing therapeutic for DMD, we conducted an efficiency screen to identify highly active paired sgRNAs for targeted deletion of exon 51 of the dystrophin gene. First, a sensitive, digital droplet PCR assay to quantify exon 51 deletion was validated with DMD patient samples. An initial pilot study of 15 sgRNAs identified a pair (01+09) that mediated 18% Exon 51 deletion in HEK293T cells three days post-transfection. Next, from the set of 10,553 21-nt SaCas9 guides targeting human DMD introns 50 and 51, we selected 53 sgRNAs (675 pairs) that met the following filtering requirements: an endogenous 5' G, a 3' T in the NNGRR(T) PAM, cross-reactivity with the non-human primate (NHP) genome, and no off-by-1 or off-by-2 mismatch sites in the human genome. These filters were selected to improve U6 promoter expression, improve SaCas9 cleavage efficiency, enable pre-clinical animal model studies, and minimize off-target editing concerns, respectively. Notably, the NHP cross-reactivity requirement skewed the targeted deletion lengths towards larger sizes, all greater than 12.4kb. In order to test smaller deletions, an additional 174 pairs of human-only sgRNAs were designed for deletion lengths of 0.8-14kb. From the 850 guide pairs screened, 78 pairs with a deletion efficiency Z-score >1.5 were selected for follow-up validation. Interestingly, a few individual sgRNAs consistently appeared among the top performing pairs, regardless of whether they were paired with a generally less effective partner sgRNA. The top hit (guides 68+84), a human-only guide pair with a deletion size of 2.3kb, demonstrated a reproducible deletion efficiency of 32%. The 6 best human-only guide pairs and the 6 best NHP cross-reactive guide pairs have been cloned together with SaCas9 in all-in-one AAV vectors for testing in *ex vivo* and *in vivo* models of DMD.

318. Targeted Genome Editing of Human Induced Pluripotent Stem Cells Using CRISPR/CAS9 to Generate a Knock-in Type II Collagen Reporter for the Purification of Chondrogenic Cells

Shaunak S. Adkar[1], Vincent P. Willard[1], Jonathan M. Brunger[1], Kenneth T. Shiao[1], Charles A. Gersbach[1], Farshid Guilak[1,2]
[1]Duke University, Durham, NC, [2]Washington University and Shriners Hospital for Children, St. Louis, MO

INTRODUCTION: Differentiation of human induced pluripotent stem cells (hiPSCs) to articular chondrocytes may provide a strategy for engineering cartilage tissue for therapy and functional characterization of patient-specific cartilage. Development of robust protocols will be particularly important for therapeutic applications. However, the difficulty of generating a pure population of chondrogenic precursors has hindered development of such protocols. We sought to isolate a chondrogenic cell population by engineering a novel hiPSC line to express GFP downstream of the COL2A1 gene using CRISPR/Cas9. **METHODS**: Reporter hiPSCs lines were generated via transfection of guide RNA/Cas9-T2A-GFP expression vector and COL2A1-P2A-GFP targeting vector (Fig 1A). Targeted clones were identified by junction PCR. Reporter validation was performed with lentiviral SOX9 overexpression and recombinant TGF-β3 treatment to activate COL2A1 expression. Cells were imaged with fluorescence microscopy at Days 4, 7, and 10. GFP^+ and GFP^- cells were sorted at Day 14 using fluorescence activated cell sorting (FACS). Gene expression from cell populations was analyzed with qRT-PCR. Reporter lines were differentiated to chondrogenic precursors via ectomesenchymal specification and TGF-β3 treatment. **RESULTS**: COL2A1-P2A-GFP reporter hiPSC lines were generated with CRISPR/Cas9 (Fig 1A/B). Imaging after COL2A1 activation demonstrated the presence of GFP signal only in treated cells (Fig 1C). Clonally derived cell lines displayed varied expression of GFP as measured by FACS (Fig 1D). GFP signal was highly correlated with COL2A1 gene expression (r^2=0.95) (Fig 1E). GFP^+ cells were enriched for chondrogenic markers such as COL2A1 and ACAN ($p<0.05$) (Fig 2A). After differentiation to chondrogenic precursors, 4.5% of cells were GFP^+ (Fig 2B). This GFP^+ population was enriched for COL2A1 transcript (Fig 2C), suggesting isolation of a chondrogenic population. **DISCUSSION**: We show the characterization of a knock-in COL2A1 hiPSC reporter cell line using CRISPR/Cas9. The reporter functions specifically during COL2A1 upregulation and may be useful as a surrogate metric with wide dynamic range and high specificity for determining COL2A1 expression. The GFP^+ population is enriched for chondrocyte-specific markers, namely COL2A1 and ACAN. The reporter enriches for COL2A1 expression after multi-staged differentiation, suggesting its utility for tissue engineering applications.

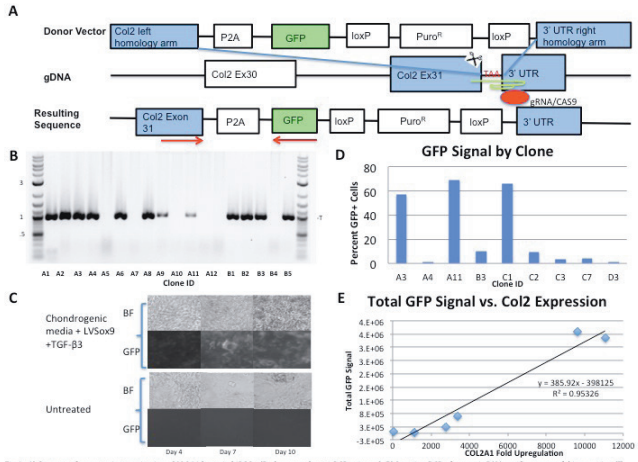


Fig 1. A) Strategy of reporter integration into COL2A1 locus in hiPSCs. (Red arrows denote PCR primers) B) Junction PCR of genomic DNA verifies successful integration (T – expected product of targeted integration) C) GFP fluorescence in culture over time. D) Percent GFP+ cells by clone. E) Correlation of total GFP intensity vs. COL2A1 expression

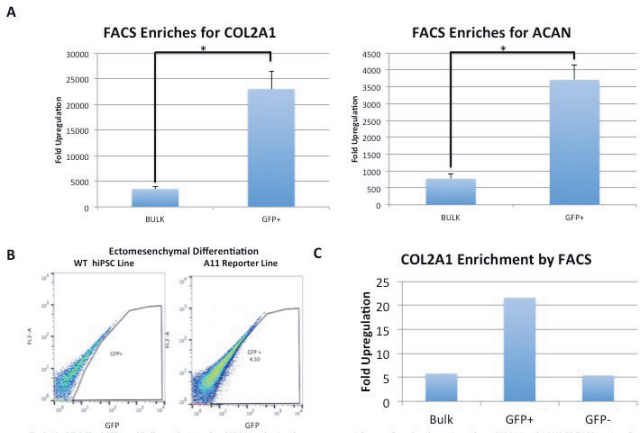


Fig 2. A) qRT-PCR of GFP+ and Bulk populations after COL2A1 induction demonstrates enrichment of prochondrogenic markers, COL2A1 and ACAN. B) FACS sorting of hiPSC derived ectomesenchymal cells C) qRT-PCR of GFP+ cells demonstrates enrichment of COL2A1 expression. qRT-PCR normalized to expression in hiPSCs

Molecular Therapy Volume 24, Supplement 1, May 2016

319. Rationally Designed AAV Inverted Terminal Repeats Enhance Gene Targeting

Matthew Hirsch, Jerry Wu
University of North Carolina, Chapel Hill, NC

Basic and clinical applications of mammalian genetic engineering rely on selective pressure and/or, ironically, DNA damage at or near the modification site to obtain relevant efficiencies. In regards to the later situation, continuously evolving endonuclease technologies remain focused on the generation of a site-specific DNA double strand break to stimulate homology directed repair (HDR). The stimulation of HDR is thus dictated by the ability of the endonuclease platform to specifically recognize its cognate site, with safety concerns dictated by its promiscuity influenced by its persistence. To eliminate the serious safety concerns associated with designer endonucleases while maintaining efficient HDR, it was hypothesized that rational modification of a viral DNA repair substrate would enhance gene editing without the requirement for induced chromosomal damage. Specifically, modifications of the adeno-associated virus (AAV) inverted terminal repeat sequence (ITR), in a viral vector context, were evaluated for stimulation of HDR in human cells. The results demonstrate an approximate 30-fold enhancement of AAV gene targeting using a particular rationally designed ITR sequence. Characterization of this event suggests that differences in the inherent ITR-initiated transcriptional activity and altered interactions with host DNA repair proteins contribute to the enhancement in HDR. The collective results demonstrate for the first time that alterations in the ITR sequence can enhance AAV gene targeting. Importantly, the refinement of such vectors may offer a safer alternative to site-specific endonuclease technologies as on- and off-target DNA cleavage concerns are eliminated.

320. Transcriptional Silencing via Synthetic DNA Binding Protein Lacking Canonical Repressor Domains as a Potent Tool to Generate Therapeutics

Salvatore Botta[1], Elena Marrocco[1], Nicola de Prisco[1], Fabiola Curion[1], Mario Renda[1], Martina Sofia[1], Mariangela Lupo[1], Maria Laura Bacci[2], Carlo Gesualdo[3], Settimio Rossi[3], Francesca Simonelli[3], Enrico Maria Surace[1]
[1]Tigem, Telethon Institute of Genetics and Medicine, Naples, Italy, [2]Department of Veterinary Medical Sciences (DIMEVET), University of Bologna, Bologna, Italy, [3]Multidisciplinary Department of Medical, Surgical and Dental Sciences, Eye Clinic, Second University of Naples, Naples, Italy

Transcription factors (TFs) function by the combined activity of their DNA-binding domains (DBDs) and effector domains (EDs). Here we show that in vivo delivery of an engineered DNA-binding protein uncoupled from the repressor domain entails complete and gene-specific transcriptional silencing. To silence RHODOPSIN (RHO) gain-of-function mutations, we engineered a synthetic DNA-binding protein lacking canonical repressor domains and targeted to the regulatory region of the RHO gene. AAV-mediate retinal delivery at a low dose (AAV2/8-CMV-ZF6-DBD, 1x10e10 vector genomes, vg) in the porcine retina resulted in selective transcriptional silencing of RHO expression. The rod photoreceptors (the RHO expressing cells) transduced cells when isolated by FACS-sorting showed the remarkable 90% RHO transcriptional repression. To evaluate genome-wide transcriptional specificity, we analyzed the porcine retina transcriptome by RNA sequencing (RNA-Seq). The differentially expressed genes (DEGs) analysis showed that only 19 genes were perturbed. In this study, we describe a system based on a synthetic DNA binding protein enabling targeted transcriptional silencing of the RHO gene by in vivo gene transfer. The high rate of transcriptional silencing occurring in transduced cells supports applications of this regulatory genomic interference with a synthetic trans-acting factor for diseases requiring gene silencing in a large number of affected cells, including for instance a number of neurodegeneration disorders. The result support a novel mode of gene targeted silencing with a DNA-binding protein lacking intrinsic activity.

321. Deletion of Mutated GAA Repeats from the Intron 1 of the Frataxin Gene Using the CRISPR System Restores the Protein Expression in a Friedreich Ataxia Model

Dominique L. Ouellet[1], Joel Rousseau[1], Khadija Cherif[1], Catherine Gérard[2], Renald Gilbert[3], Alain Garnier[2], Bruno Gaillet[2], Christian Beauséjour[4], Jacques P. Tremblay[1]
[1]Neurosciences, Laval University, Quebec City, QC, Canada, [2]Laval University, Quebec City, QC, Canada, [3]National Research Council Canada, Montreal, QC, Canada, [4]Université de Montréal, Montreal, QC, Canada

The CRISPR system is now widely used as a molecular tool to edit the genome. We used this technique in Friedreich Ataxia (FRDA), an inherited autosomal disease known to cause a decrease of the mitochondrial frataxin protein. Genetic analysis revealed a GAA repeat expansion within the intron 1 of the frataxin (FXN) gene. We used cells derived from the YG8sR mouse model where the mouse frataxin gene is knockout but contain a human FXN mutated transgene on one allele. We then deleted the GAA trinucleotide repeat using 2 specific guide RNAs (gRNAs) co-expressed with either *S. pyogenes* (Sp) or *S. aureus* (Sa) Cas9. We were able to monitored an increase up to 2-fold of frataxin mRNA and protein levels in clone cells. We also confirmed these results *in vivo* using DNA electroporation in the *Tibialis anterior* muscle of the YG8R mice. Ongoing *in vivo* investigation of a systemically injected AAV-DJ vector expressing the SaCas9 and 2 successful selected gRNAs in the mouse model YG8sR will hopefully provide more details answers on the efficacy of the approach and give us preliminary data to go forward for clinical trial. The deletion of the GAA repeats expansion then might be a highly valuable gene therapy approach for FRDA patients.

322. Genome Editing for Nucleotide Repeat Disorders: Towards a New Therapeutic Approach for Myotonic Dystrophy Type 1

Mirella Lo Scrudato[1], Samia Martin[1], Geneviève Gourdon[2], Denis Furling[3], Ana Buj-Bello[1]
[1]Genethon, INSERM U951, Evry, Paris, France, [2]INSERM UMR 1163, Institut Imagine, Université Paris Descartes-Sorbonne Paris Cité, Paris, France, [3]Sorbonne Universités UPMC Paris 06, Inserm, CNRS, Centre de Recherche en Myologie, Institut de Myologie, Paris, France

Myotonic dystrophy type 1 (DM1) belongs to the group of nucleotide repeat disorders. More specifically this autosomal form of muscular dystrophy is caused by the expansion of the CTG trinucleotide repeat located at the 3' untranslated region (3′-UTR) of the *DMPK* gene. Elongated CUG repeats of the mutated DMPK mRNAs become sequestration sites for splicing factors, and induce the formation of stable ribonucleoprotein complexes visualized as foci. As a consequence, the alternative splicing of numerous transcripts is dysregulated, which leads to the DM1 pathological alterations affecting various tissues. We have developed a strategy to delete the CTG repeat expansion in the human *DMPK* locus by using the CRISPR/Cas9 system. For that purpose, we constructed different expression platforms for small size Cas9 nucleases under either a ubiquitous or a muscle-specific promoter and guide RNAs (sgRNAs) targeting the 3'-UTR of the *DMPK* gene. Co-transfection of these

constructs in DM1 primary myoblasts resulted in the deletion of the CTG repeat. We are currently optimizing the delivery of these Cas9 and sgRNAs constructs in cells by their vectorization in lentiviral and adeno-associated vectors and will present data on *in vitro* (DM1 patient-derived cell lines) and *in vivo* (wild-type mice) assays.

323. Efficient Generation of CART Cells by Homology Directed Transgene Integration into the TCR-Alpha Locus

Baeckseung Lee, Hang Leung, Jordan Jarjour, Alexander Astrakhan
Bluebirdbio.com, Seattle, WA

Genetically engineered cancer targeting CAR-T cells have generated promising results in a series of B cell derived cancers. Development of these therapies for solid tumor applications has been much more difficult and will likely require extensive T cell engineering to improve both efficacy and safety. Advanced gene editing approaches were developed to enable simultaneous disruption of a target gene combined with introduction of exogenous transgenes at the disrupted locus. A gene specific megaTAL nuclease was used to generate double stranded DNA breaks followed by transduction with adeno-associated virus (AAV) encoding new genetic information flanked by regions of homology proximal to the nuclease breakpoint. Highly efficient introduction of a CD19-specific CAR transgene into the T cell receptor-alpha constant (TRAC) locus was demonstrated using this approach. T cells treated with the TRAC megaTAL and corresponding AAV encoding a CD19 CAR and TRAC homology arms generated greater than 50% of CD19-CAR positive T cells that no longer expressed the T cell receptor complex. In vitro assays confirmed that TRAC-targeted CD19-CAR T cells were comparable to CD19 CAR-T cells generated by lentiviral transduction in their cytotoxicity and cytokine responses against CD19+ Nalm-6 cells. These findings demonstrate the potential of megaTAL driven homology directed T cell genome engineering to obviate the need for traditional integrating viral vectors and generate a defined and potentially more potent T cell product by combining gene disruption with targeted transgene integration.

324. Successful Repeated Delivery of Helper-Dependent Adenoviral Vector Toachieve Efficient Long-Term Human CFTR Expression in Mouse Airwaythrough Transient Immunosuppression

Huibi Cao, Jim Hu
The Hospital for Sick Children, Toronto, ON, Canada

Cystic Fibrosis (CF) is a common life-threatening disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which encodes an epithelial chloride channel. As a chronic, lifelong disease, CF should be best treated with a continue level of CFTR expression. Pulmonary gene therapy may ultimately cure the CF lung disease. Thus, efficient long-term expression of the delivered CFTR gene in targeted cell types is essential to achieve this goal either by repeated application or with a long-duration expression system. Even though novel approaches to target airway progenitor/stem cells with gene editing may be more attractive, the efficiency for targeting specific progenitor cells will still be a challenge in airway delivery. Repeated administration of viral vectors may be required to increase the percentage of cells to be targeted and to boost long-term therapeutic effects. However, immune responses to viral proteins are the greatest barrier to repeated vector administration. Here, we demonstrated that repeated transduction of human CFTR gene in mouse airway can be successfully achieved with helper-dependent adenoviral (HD-Ad) vector through transient immunosuppression.

We showed previously that cyclophosphamide significantly enhanced human *CFTR* expression in mice received HD-Ad vector readministration and sustained expression through inhibition of host immune reactions. In this study, we examined cyclophosphamide effects on expression of the human *CFTR* in multiple rounds of HD-Ad vector delivery to mouse airways. Four group mice were nasally delivered with an HD-Ad-K18-CFTR vector at $1.5x10^{10}$ vector particles per mouse at day 0, day 70 and day 120. Two groups were treated with cyclophosphamide 6 hours before and 4 day and 8 days after vector delivery each time (except the last round of vector delivery for mice sacrificed at day 123). The other two groups without treatment were used as controls. One treated group and one control group were sacrificed at day 123, and the others at day 153 for detecting transgene expression and immune reaction. We found that cyclophosphamide has significantly improved the expression of *CFTR* (4.1 times higher) compared to the control group as determined by real-time qPCR 3 days after last delivery. And the *CFTR* expression level was 3.1 times higher 30 days after last vector transduction. Cyclophosphamide also significantly reduced the levels of anti-adenoviral and neutralizing antibodies in both BALF and serum as well as prevented leukocyte infiltration in lung tissues. This data suggested that efficient repeated transduction of human CFTR gene with HD-Ad vector can be achieved by transient immunopuression in mouse lung.

325. Targeted Gene Addition Strategies for the Treatment of X-Linked Lymphoproliferative Disease

Benjamin C. Houghton[1], Claudio Mussolino[2], Toni Cathomen[3], H Bobby Gaspar[1], Claire Booth[1]
[1]Molecular and Cellular Immunology, University College London, London, United Kingdom, [2]Institute for Cell and Gene Therapy & Center for Chronic Immunodeficiency, Medical Center - University of Freiburg, Freiburg, Germany, [3]Institute for Cell and Gene Therapy & Center for Chronic Immunodeficiency, Medical Center - University of Freiburg, Freiburg, Germany

X-linked lymphoproliferative disease is an immunodeficiency arising from mutations in the *SH2D1A* gene encoding SAP, a key regulator of immune function expressed in T cells, natural killer, and natural killer T cells. Haemopoietic stem-cell transplantation is curative, and we have shown proof of concept of haemopoietic stem-cell gene therapy in a mouse model. Preliminary data suggests that adoptive transfer of T cells corrected using lentiviral gene therapy can correct immune defects. However, targeted gene editing of T cells may allow for physiologically regulated SAP expression. We constructed SAP-specific TALE-effector nucleases (TALENs) capable of generating a site-specific double-stranded break close to the translation start codon, allowing correction of most described *SH2D1A* mutations. TALEN activity was validated with a surveyor assay in HEK293T cells. To facilitate optimisation of delivery methods, we designed a fluorescent reporter assay that, when stably integrated into a cellular DNA, provides a fluorescence signal when repair of DNA double-stranded breaks (DSB) created by TALENs occurs. Using the reporter assay in Jurkat cells we observed reduced toxicity and highly efficient delivery using an mRNA nucleofection platform, with 23% of treated cells showing repair mediated fluorescence. We therefore proceeded to deliver TALEN mRNA to human primary T cells, and demonstrated the generation of double-stranded breaks in 25% of the bulk population. To harness the repair of DSB, we constructed a homologous recombination donor in a non-integrating lentiviral vector format to mediate delivery of cDNA with low toxicity. Combined with TALEN mRNA nucleofection, homologous recombination-driven incorporation of donor in T cells was possible. This methodology could be applied to an autologous

T-cell gene therapy strategy, although the low level of homologous recombination remains a limiting factor. We plan to test our strategy in patient cell lines to restore SAP expression and cytotoxic function of cytotoxic T lymphocytes.

326. Improving CRISPR-Cas9 Precision Through Directly or Indirectly Tethered DNA-Binding Domains

Scot Wolfe, Mehmet F. Bolukbasi, Ankit Gupta, Lihua J. Zhu
Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, Worcester, MA

Type II CRISPR-Cas9 systems provide a robust and versatile platform to study gene function in complex genomes, but the standard CRISPR-Cas9 system is sub-optimal for many clinical-level genome editing applications due to insufficient precision. To establish a Cas9-based system that will cut at only a single site within a genome, we have developed an attenuated CRISPR-Cas9 platform fused to a programmable DNA-binding domain. This combination adds an extra specificity determinant to the engagement of a DNA sequence prior to cleavage. The appended DNA-binding domain increases the design density of Cas9 target sites and provides dramatically improved on- vs off-target selectivity as assessed by targeted deep sequencing based on GUIDE-seq analysis. In addition, the DNA-binding domain provides a tunable system that can be used to eliminate residual off-target activity to increase the precision of our nuclease system. We have extended the functionality of this platform through the incorporation of drug-dependent dimerization domains that facilitate the temporal control of gene editing via the presence of a small molecule. This novel platform has the potential to generate regulatable site-specific nucleases with single-site precision for targeted-genome editing, bringing programmable nuclease technology one step closer toward the ultimate goal of facilitating gene therapy-based therapeutics for a multitude of diseases.

327. Genetic Editing for Huntington's Disease

Nicole Deglon[1], Cécile Meunier[2], Virginie Zimmer[1], Guillaume Perriard[1], Mathieu Canales[1], Gabriel Vachey[1], Lucas Hergott[1], Tim Beltraminelli[1], Thomas Dequesne[1], Catherine Pythoud[1], Maria Rey[1], Renaud Du Pasquier[1], Luc Pellerin[2], Anselme Perrier[3]
[1]Clinical Neuroscience, Lausanne University Hospital (CHUV), Lausanne, Switzerland, [2]Department of Physiology, Lausanne University, Lausanne, Switzerland, [3]U861, Institut National de la Santé et de la Recherche Médicale (INSERM), Evry, France

Huntington's disease (HD) is a neurodegenerative disorder caused by a pathological CAG expansion at the 3' end of the first exon of the huntingtin gene (*HTT*). Currently, there is no efficient treatment for HD. Editing of the mutant *HTT* gene with the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system represents a new and promising approach. Recognition of the HTT target sequence by a single-guide RNA sequences (sgRNA) and the Cas9 protein is inducing DNA double-strand breaks (DSB), which activate endogenous cellular repair pathways. Non-homologous end joining (NHEJ) will introduce small insertion/deletion (*indel*) that alter the reading frame of *HTT* gene while homologous directed repair (HDR) is activated in the presence of a DNA template. To validate the approach and optimize the delivery of the CRISPR system with viral vectors, we first targeted artificial sequences containing fluorescent reporter genes in HEK 293T cells. An efficient gene disruption was measured and associated with a loss of fluorescence in neurons, astrocytes, in vitro and in vivo. Furthermore, we developed multiple strategies to disrupt the mutant *HTT* gene. Quantification demonstrated a high rate of indels, leading to a strong reduction of HTT protein in HEK 293T cells, mouse cortical neurons and human iPS-derived neurons. Blocking *HTT* expression in vitro HD models is improving several physiopathological parameters. We are currently evaluating the impact of allele or non-allele specific mutant *HTT* editing in human neurons from HD patients. Altogether, these data demonstrate the potential of the CRISPR technology as therapeutic strategy for HD.

328. Proof-of-Principle Study Shows Efficient Skipping of Exon 2 Using Antisense Morpholino Oligomers

Tabatha R. Simmons[1,2], Nicolas Wein[1], Felecia Gumienny[1], Hannah Huang[1], Kevin M. Flanigan[1,3,4,5]
[1]Center for Gene Therapy, The Research Institute at Nationwide Childrens, Columbus, OH, [2]Molecular, Cellular and Developmental Biology, The Ohio State University, Columbus, OH, [3]Molecular, Cellular, and Developmental Biology, The Ohio State University, Columbus, OH, [4]Pediatrics, The Ohio State University, Columbus, OH, [5]Neurology, The Ohio State University, Columbus, OH

Currently, exon skipping therapies for Duchenne muscular dystrophy (DMD) have been developed for patients with out-of-frame deletions where treatment will lead translation of an internally truncated but partially functional dystrophin protein. In contrast, we are focusing on treating duplications mutations, accounting for around 6% of all mutations, resulting in wild-type transcript and a full-length protein. Modeling the most common single exon duplication we have developed the first duplication mouse containing a duplicated exon 2. We have performed a proof-of-principle study using antisense oligomer (AO)-induced exon 2 skipping using this Dup2 mouse. Intramuscular (TA) injections of exon 2-directed different antisense peptide-morpholino conjugates (PPMO) were performed at either 10 or 20 ug total PPMO (N=6 muscles each) doses. Mice were injected at 8 weeks and sacrificed 1 month later for muscle analysis of DMD mRNA and dystrophin protein expression. Additionally, systemic (tail vein) injections are being conducted of at a dose of 30 ug/kg, at 1 week, 2 week and 1 month timepoints. For the IM studies, a gradient of exon 2 exclusion was seen by RT-PCR at both doses with corresponding high levels of properly localized dystrophin protein by IF and western blot.

Analyses of RT-PCR and protein expression are underway for the systemic delivery PPMOs. These data suggest that skipping of a duplicated exon 2 may be a feasible therapeutic approach, particularly because skipping of exon 2 may be associated with an apparently unlimited therapeutic window. Over-skipping - to the exclusion of exon 2 entirely - results in activation of an internal ribosome entry site (IRES) located in exon 5 of dystrophin that allows for cap-independent translation from an alternative initiation site within exon 6. This alternate dystrophin isoform is highly functional despite being N-truncated, consistent with the observation that patients expressing it have minimally symptomatic (or even asymptomatic) Becker muscular dystrophy (BMD), and suggesting a potential route to therapy for any of the approximately 5% of patients with mutations in the 5' end of the gene.

329. Using CRISPR/Cas9 as a Therapeutic Approach for Leber Congenital Amaurosis 10 (LCA10)

Guoxiang Ruan, Elizabeth Barry, Michael Lukason, Seng Cheng, Abraham Scaria
Rare Diseases Science, Sanofi R&D, Sanofi Genzyme, Framingham, MA

Introduction: Leber congenital amaurosis (LCA) is the most severe form of inherited retinal diseases with early onset of symptoms

in the first year of life. The most frequent genetic cause of LCA, accounting for approximately 15% of all LCA cases in western countries, is a deep-intronic mutation c.2991+1655A>G located in the intron 26 of human CEP290 gene. LCA caused by CEP290 mutation is known as LCA10. The intronic mutation of CEP290 generates a cryptic splice donor site, resulting in the inclusion of a pseudoexon that leads to a premature stop codon and a truncated protein. The size of human CEP290 cDNA (~7.4 kb) exceeds the cargo size (~4.8 kb) of recombinant adeno-associated viral vectors (rAAVs), which makes this gene challenging for gene replacement therapy. In this regard, we tested if we can use the new genome editing technology CRISPR/Cas9 as an alternative strategy for LCA10 by removing the intronic mutation of CEP290 and preventing the cryptic splicing.

Methods: CRISPR/SpCas9 was employed to introduce the intronic splice mutation c.2991+1655 A>G into HEK 293FT cells to create a cellular model for LCA10/CEP290. In this cellular model, we used guide RNA pairs coupled with SpCas9 to delete the intronic region flanking the intronic mutation.

Results: The deep-intronic mutation c.2991+1655 A>G was successfully introduced into 293FT cells through the homology directed repair (HDR) pathway of CRISPR/spCas9. CEP290 expression levels were markedly reduced in the mutant cell line. Using this cellular model, we identified three sgRNA pairs that could efficiently (>50%) delete the intronic mutation, significantly rescue wild-type CEP290 expression levels and reduce mutant CEP290 expression.

Conclusions: Our results demonstrate that the paired sgRNAs coupled with SpCas9 are capable of removing the c.2991+1655A>G mutation and are highly efficient in preventing the splicing of the mutant cryptic exon and restoring wild-type CEP290 expression.

330. Using CRISPR/Cas9 to Edit Hematopoietic Stem and Progenitor Cells

Eduardo Seclen, Hsu-Yu Chen, George N. Llewellyn, Su J. Yang, Morgan Chateau, Cathy X. Wang, Paula M. Cannon
Keck School of Medicine, University of Southern California, Los Angeles, CA

Engineered nucleases such as zinc finger nucleases (ZFNs) and CRISPR/Cas9 have the potential to improve the precision of gene therapies based on hematopoietic stem and progenitor cells (HSPC). ZFNs were the first of this class of reagents to be approved for the clinic, through the disruption of the CCR5 gene as an anti-HIV therapy. Since ZFNs only need to be expressed transiently to permanently disrupt a gene, ZFN mRNA electroporation has proved to be an effective method to deliver CCR5 ZFNs to HSCPs. Optimizing the delivery of CRISPR/Cas9 to HSPC requires, in addition, consideration of how to deliver multiple components - the Cas9 nuclease and one or more single guide RNAs (sgRNAs) - in a transient but coincidental window of expression. This is further complicated if a DNA homology template is also to be delivered, in order to direct site-specific gene editing or insertion. Some of the difficulties of working with HSPC include their sensitivity to DNA or viral vectors that can result in unacceptable levels of cytotoxicity, the possibility for only limited *in vitro* culturing, and the complexity of the studies needed to evaluate any impact of the treatment on HSPC biology. In addition, the relatively large size (4.1kb) of the standard *S.pyogenes* Cas9 (spCas9) makes it a challenge for certain delivery systems, although a smaller variant from *S.aureus* (saCas9, 3.2 kb) is also available. In this work, we evaluated several approaches for the delivery of both Cas9 and sgRNAs to HSPC, including AAV vectors, *in vitro* transcribed Cas9 mRNA, and synthesized sgRNAs. The use of the smaller saCas9 allowed us to evaluate AAV vectors for Cas9 delivery, as well as in combination with sgRNA, using AAV serotype 6 that we identified as having good tropism for human HSPC. Various combinations of the different platforms, and time between deliveries, were optimized to maximize nuclease activity without overt toxicity. Using *in vitro* synthesized Cas9 mRNA and sgRNAs, we found that a single co-electroporation step was preferable to reduce the toxicity of sequential electroporations, and that chemical modification of the sgRNA was necessary to stabilize it and allow its function when co-delivered in this way. For AAV vectors, although the smaller size of saCas9 allowed it to be co-packaged together with a sgRNA in a single AAV6 vector that worked in cell lines, this combination did not effectively function in HSC. However, the sgRNA component alone was effectively delivered by AAV6 vectors, and could be combined with a later delivery of saCas9 mRNA by electroporation. With this latter approach, we were able to disrupt the CCR5 locus at similar levels to those we achieve using the clinically optimized protocol for CCR5 ZFNs.

331. CRISPR-Cas9 Can Inhibit HIV-1 Replication but Also Facilitates Virus Escape

Ben Berkhout, Gang Wang, Na Zhao, Atze T. Das
University of Amsterdam, Amsterdam, Netherlands

Several recent studies demonstrated that the CRISPR-associated endonuclease Cas9 can be used for guide RNA (gRNA)-directed, sequence-specific cleavage of HIV proviral DNA in infected cells. We here demonstrate profound inhibition of HIV-1 replication by harnessing T cells with Cas9 and antiviral gRNAs. However, the virus rapidly and consistently escaped from this inhibition. Sequencing of the HIV-1 escape variants revealed nucleotide insertions, deletions and substitutions around the Cas9/gRNA cleavage site that are typical for DNA repair by the NHEJ pathway. We thus demonstrate the potency of CRISPR-Cas9 as antiviral approach, but any therapeutic strategy should consider the viral escape options.

332. Correction of the Sickle Cell Anemia Mutation in Human Induced Pluripotent Stem Cells

R. Geoffrey Sargent[1], Shingo Suzuki[1], Luke Gruenert[1], Fei Xie[2], Lin Ye[2], Yuet W. Kan[2,3], Dieter C. Gruenert[1,3]
[1]Otolaryngology, University of California, San Francisco, San Francisco, CA, [2]Medicine, University of California, San Francisco, San Francisco, CA, [3]Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, Helen Diller Family Com, University of California, San Francisco, San Francisco, CA

The prevalence of sickle cell anemia (SCA) in the US is approximately 70,000 to 100,000 individuals, affecting mostly the African American and Hispanic communities. While improvements in the treatment of SCA has increased both the lifespan and the quality of life of SCA patients, only hematopoietic stem cell transplants offer a lasting cure. Induced pluripotent stem cells (iPSCs) have significantly advanced the potential of developing a personalized, cell and genetic therapy for diseases like SCA by generating patient-specific stem cells in which the underlying disease-causing mutations could be corrected. Differentiation of the corrected cells into engraftable progenitors underlies their utility for autologous replacement or repair of diseased tissues. In these studies, homology directed repair (HDR) was achieved using sequence-specific TALENs in conjunction with HBB targeting vectors to generate seamless, footprint-free gene correction of the HBs exon 1 SCA A-to-T transversion mutation in sickle cell iPSCs (SC-iPSCs). These studies were supported by NIH Program Project Grant (PPG) DK088760.

333. Intracellular Ankyrin Specific to p24 and zfp Specific to 2-LTR Are Alternatives to CCR5 Knockdown for Stem Cell Gene Therapy

Chatchai Tayapiwatsna
Div of Clinical Immunology, Chiang Mai University, Chiang Mai, Thailand

HIV-1 genome integration and capsid assembly are some of the most important steps to viral life cycle. Designed scaffold proteins, zinc finger and ankyrin repeat protein, were characterized as intracellular antiviral agents that can interfere in these steps. Zinc finger protein, 2LTRZFP, plays a vital role in blocking HIV integration. This protein targets the 2-LTR-circle junction of the HIV-1 DNA but it would have no effect on the HIV-infected cells. Another protein, AnkGAG1D4, will then specifically bind to the viral capsid resulting in inhibition of HIV-1 assembly. Since only one particular scaffold protein cannot confer full protection against the virus. Combination of these two proteins became the key to enhance inhibitory function to block HIV-1 replication at early and late infection stage and also help eliminate latent viral reservoirs. The human T cell line modified by using a lentiviral vector bearing 2LTRZFP alone or combination with N-myristoylatedMyr(+)AnkGAG1D4 were protected from viral infection in HIV-1 challenge. Interestingly, the Myr(+)AnkGAG1D4 showed significantly antiviral effect in HIV-1-preinfected SupT1 cells and also possessed a broad antiviral activity against SIV, and SHIV. These results demonstrate that 2LTRZFP and Myr(+)AnkGAG1D4 proteins can be the novel anti-HIV-1 therapeutic agents for human applications.

334. Double Expression Cassettes Engineering at the Two Alleles with Same Genome Site of Human Cell Using CRISPR/Cas9 Vector System

Zhimin Wang
Cardiovascular Institute, Allegheny Health Network, Pittsburgh, PA

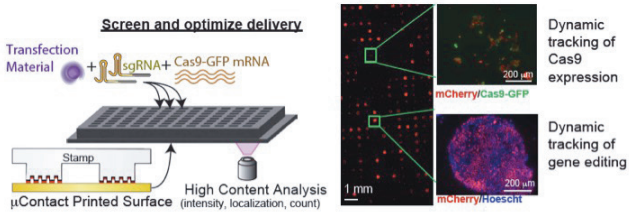
For the past two years, CRISPR/Cas9 mediated genome editing has been widely used for the next-generation strategy for genetic modification. Previous work has showed that both single and multiple genes targeted mutagenesis can be achieved using the CRISPR/Cas9 system. In this study, we demonstrated that two different expression cassettes can be homologous recompensed in human genome in the same gene editing site, but different alleles in HEK293 cells. Our results showed that CRISPR/Cas9 prefer to homologous recombination (HR) of expression cassette in one allele at one time (seven showing heterozygous from eight picked stable cell clones). Targeting the same guided RNA sequence of EMX1gene with CRISPR/Cas9 system, we can generate stable cell lines with homologous recombination of both EGFP and mCherry expression cassettes at the two alleles of EMX1 gene. To further analyze the off target effects of CRISPR/Cas9 derived RNA guided endonucleases, the total cell proteins were exploited to identify with Mass Spectrometry and results showed no novel proteins were identified compared with wild type HEK293 cells. But our results indicated that the expression levels of some large molecular weight proteins are changed. We also analyze the expression pattern of the proteins exits within both upstream and downstream of the insertion sites to study the possible effect of the CMV promoter of the expression cassettes. Results didn't show significant change of the expression of the 21 related genes. Our study indicated that CRIPSR/Cas9 system can mediate double insertion of two expression cassette at the two alleles in the same gene and which can potentially provides more choices for expressing exogenous cNDA in human cells. Also our work confirmed that Cas9 protein expression in culture cells can change expression pattern of some proteins which should be considered when CRISPR/Cas9 system were used in the gene editing.

Non-Viral Gene Transfer and Therapy I

335. High Content Analysis Platform for Optimization of Lipid Mediated CRISPR-Cas9 Delivery Strategies in Human Cells

Benjamin Steyer[1], Jared Carlson-Stevermer[1,2], Nicolas Angenent-Mari[2], Andrew Khalil[2], Ty Harkness[1,2], Krishanu Saha[1,2]
[1]*Wisconsin Institute for Discovery, UW Madison, Madison, WI,* [2]*Department of Biomedical Engineering, UW Madison, Madison, WI*

Background: Non-viral gene-editing of human cells using the CRISPR-Cas9 system requires optimized delivery of multiple components. Both the Cas9 endonuclease and a single guide RNA, that defines the genomic target, need to be present and co-localized within the nucleus for efficient gene-editing to occur. This work describes a new high-throughput screening platform for the optimization of CRISPR-Cas9 delivery strategies. **Methods:** By exploiting high content image analysis and microcontact printed plates, multi-parametric gene-editing outcome data from hundreds to thousands of isolated cell populations can be screened simultaneously. Employing this platform, we systematically screened four commercially available cationic lipid transfection materials with a range of RNAs encoding the CRISPR-Cas9 system. Analysis of Cas9 expression and editing of an endogenous and fluorescent mCherry reporter transgene within human embryonic cells was monitored over several days after transfection. Design of experiments analysis enabled rigorous evaluation of delivery materials and RNA concentration conditions. **Results:** The results of this analysis indicated that the concentration and identity of transfection material have significantly greater effect on gene-editing than ratio or total amount of RNA. Cell subpopulation analysis on microcontact printed plates, further revealed that low cell number and high Cas9 expression 24 hours after CRISPR-Cas9 delivery, were strong predictors of gene-editing outcomes. After purifying the population for high Cas9 expression, 24 hours after CRISPR-Cas9 delivery, greater than 92% of cells were properly edited. **Significance:** These results suggest design principles for the development of materials and transfection strategies with lipid-based materials. This platform could be applied to rapidly optimize materials for gene-editing in a variety of cell/tissue types in order to advance genomic medicine, regenerative biology and drug discovery.



336. Delivery Highways: Tunneling Nanotubes Facilitate Transfer of Therapeutic Molecules for Gene Therapy Treatment of Cystinosis

Spencer Goodman[1], Swati Naphade[1], Jay Sharma[1], Héloïse P. Gaide Chevronnay[2], Pierre J. Courtoy[2], Stephanie Cherqui[1]
[1]Pediatrics, University of California, San Diego, La Jolla, CA, [2]Cell Biology Unit, de Duve Institute, Université Catholique de Louvain, Brussels, Belgium

Cystinosis is a lysosomal storage disorder caused by mutations in the *CTNS* gene, encoding the lysosomal transmembrane transporter cystinosin. As a consequence, cystine builds up in all tissues and eventually causes multi-organ degeneration, especially affecting the kidney and eye.

We previously showed in *Ctns*$^{-/-}$ mice that transplantation of *ex vivo* lentiviral-transduced hematopoietic stem and progenitor cells (HSPCs) resulted in abundant integration of bone marrow-derived cells into all tissues and long-term kidney and eye preservation. We observed that HSPCs differentiated into macrophages that extended intercellular bridges called tunneling nanotubes (TNTs). TNTs are thin long actin-rich structures that provide a dynamic cytosol-cytosol connection enabling cellular communication as well as transfer of protein and organelles. *In vitro* co-culture of DsRed *Ctns*$^{-/-}$ fibroblasts with macrophages expressing cystinosin-GFP allowed visualization of bidirectional lysosome transfer through these TNTs. *In vivo*, we visualized TNTs in multiple tissues in lentiviral-modified HSPC-transplanted *Ctns*$^{-/-}$ mice. We observed the transfer of cystinosin-bearing lysosomes from macrophages into diseased host cells such as proximal tubular cells in the kidney and keratocytes in the eye. These findings represent the first observed instance of TNTs delivering a functional protein to facilitate tissue regeneration following gene therapy.

Utilizing both genomic and proteomic approaches, putative novel constituents of TNTs were discovered and subsequently validated via RNAi. We also designed an application using ImagePro to automatically quantify and measure TNTs *in vitro* via morphological filters. With our ImagePro workflow and FACS-assisted immunoblotting, contributions of candidate proteins to TNT frequency, morphology and transfer efficiency were assessed. We further investigate the molecular nature of the TNTs and established new important protein candidates.

In conclusion, HSPC transplantation can preserve near-normal kidney and eye architecture in cystinosis via a TNT-based transfer of functional protein. Understanding the mechanisms underlying TNT repair could spur the development of novel stem cell-based gene therapies for numerous genetic disorders.

337. Transient Ultrasound-Mediated Microbubble-Assisted Modulation of Blood-Brain Interface in Adult Common Marmoset to Improve rAAV-Mediated Brain Transduction

Hironori Okada[1], Hidetoshi Ishibashi[2], Chiaki Masuda[1], Hiromi Hayashita-Kinoh[1], Tomoko Chiyo[3], Yuko Nitahara-Kasahara[1], Yoko Endo-Takahashi[4], Koichi Kato[5], Yoichi Negishi[4], Shin'ichi Takeda[3], Takashi Okada[1]
[1]Department of Biochemistry and Molecular Biology, Nippon Medical School, Bunkyo-ku, Tokyo, Japan, [2]Animal Research Center, University Hospital, Tokyo Medical University, Shinjuku-ku, Tokyo, Japan, [3]Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan, [4]Department of Drug Delivery and Molecular Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan, [5]Integrative Brain Imaging Center, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan

Background: Non-human primates (NHPs) could provide an appropriate model for neuromuscular diseases because of its cognitive function and physiological resemblance to human. Production of transgenic and knockout NHPs from preimplantation embryos by using genomic modification were reported. However, several decades would be required for establishing homozygous and/or phenotypically stable progenies and for developing the symptoms in case of late onset diseases. In contrast, induction of pathology with recombinant adeno-associated virus (rAAV) has potential to break through this situation, because it is possible to realize with the existing aged animals. In this context, fully maturated blood-brain interface (BBI) significantly limits passive rAAV transport from circulation to the brain. To overcome this issue, we investigated BBI opening as a promising technology to make cerebral capillary open transiently by resonance of i.v. injected microbubble (MB) locally excited with ultrasound irradiation (UI).

Methods: Evans blue (EB) and aminoisobutyric acid (AIB) were used for a tracer that has high affinity for serum albumin (ALB). MB and EB were i.v. injected as bolus into the femoral vein of the anesthetized adult marmosets. Transcranial (TC) UI to the brain was performed for 5 minutes, and the brain was examined one day after the UI. To verify the leakage, cryosections were immunostained against ALB and CD31. For live imaging analysis, MB, carbon 11-labeled AIB, and rAAV1 were i.v. injected into the tail vein. Subsequently, TCUI and PET scan were performed. At two days after TCUI, rAAV1 with distinguishable another vector genome was i.v. injected again. At one week after TCUI, the brain was sampled, and pieced into various parts. Then relative rAAV genome copies were measured by qPCR.

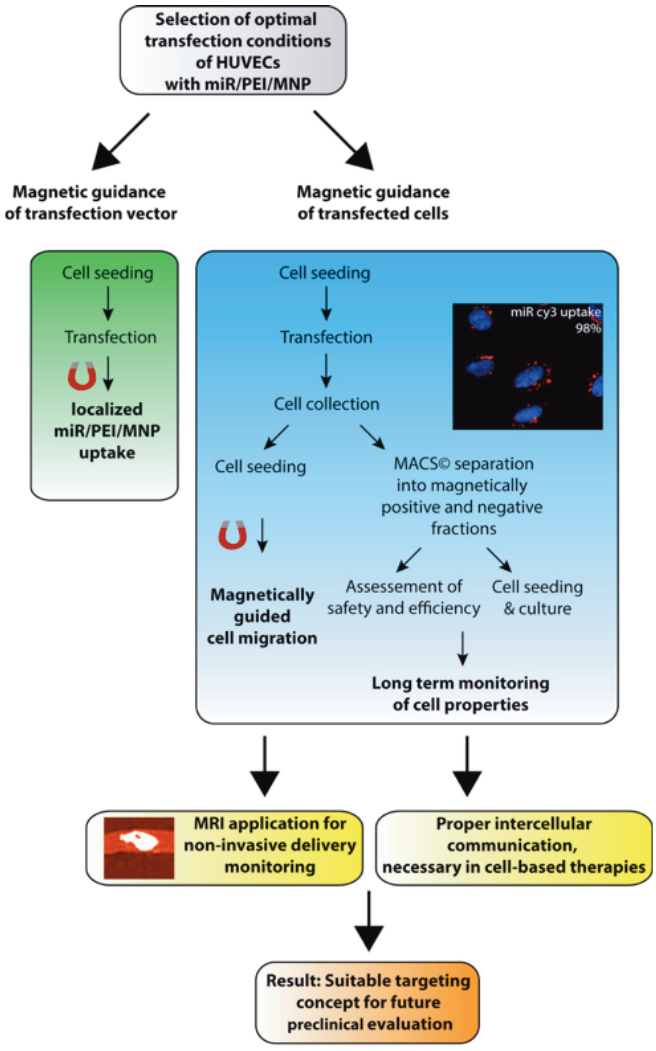
Results: Macroscopically, ALB leakage was recognized in temporal cortex and hippocampus when TCUI was performed towards the temporal region. When TCUI was performed towards all direction, it was found in whole brain except for white matter. Microscopically, the blood vessels of 10-50 μm in diameter especially in the hippocampus were sensitive to BBI opening. From live image, leakage in the hippocampus, anterior cingulate cortex, basal ganglia, optic tract, amygdala, and superior temporal gyrus started just after TCUI. Lower genome copies of the secondary injected rAAV1 at two days after TCUI revealed that the BBI opening was transient.

Conclusion: UI-mediated MB-assisted TC-BBI opening is a promising approach to generate the disease model based on adult common marmoset.

338. Improving Virus-Free Modification of Endothelial Cells - Magnetically Targeted Manipulation

Natalia Voronina[1], Heiko Lemcke[1], Frank Wiekhorst[2], Jens Kühn[3], Robert David[1], Gustav Steinhoff[1]
[1]RTC Rostock, Rostock, Germany, [2]Physikalisch-Technische Bundesanstalt, Berlin, Germany, [3]Radiology and Neuroradiology, Ernst-Moritz-Arndt-University Greifswald, Greifswald, Germany

Regulation of angiogenesis is recognized as a crucial component in the treatment of multiple diseases, e. g. cardiovascular disorders, cancers, diabetes. At the same time, gene therapy is a promising tool for angiogenesis manipulation. Its wide clinical application is, however, limited by the lack of suitable theranostic gene delivery systems.To address this issue, in this study we intended to develop an efficient method for magnetically guided manipulation of endothelial cells. First, we defined optimal conditions for HUVEC transfection with microRNA (miR) using polyethyleneimine-magnetic nanoparticle based vector (PEI/MNP), pre-designed in our group. We found that miRNA can be efficiently delivered with PEI/MNP vector without affecting cell survival and functionality. Furthermore, the presence of magnetic component offers certain targeting and non-invasive MRI monitoring opportunities, which we investigated *in vitro*. Two different approaches were assessed: direct vector guidance and magnetic targeting of transfected cells. As a result, production of miR/PEI/MNP-modified magnetically responsive cells has been selected for further investigation as it was more efficient. We have demonstrated *in vitro* that up to 80% of transfected cells can be driven to the site of interest by magnetic field. In addition, properties of engineered cells were monitored up to 96 hours and proposed modification strategy has been proven safe. Further, transfected cells were tested *in vitro* in terms of MRI detection potential. We observed that 10^4 cells can be detected, when loaded with at least 0.2 pg iron/cell. This and higher intracellular iron concentrations were achieved at optimal transfection conditions defined previously. Moreover, we showed that miR/PEI/MNP-modified endothelial cells do maintain intercellular gap-junctional communication and potentially can exchange miR, serving thereby as its carrier. Taken together, obtained results are indicating that miR/PEI/MNP-modified cells are particularly promising as a multifunctional tool for magnetically guided angiogenesis regulation.



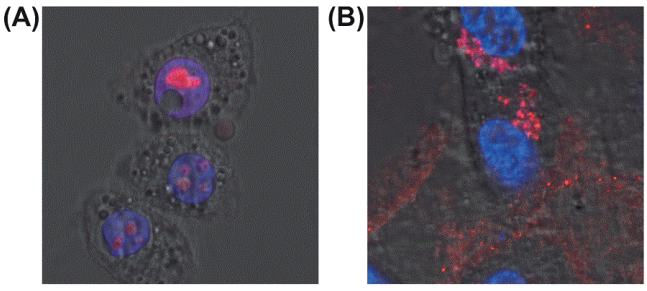
339. Development of Multifunctional Nanoparticles for Targeted Gene Delivery

Rajendra N. Mitra, Min Zheng, Zongchao Han
Ophthalmology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Purpose: Progress in gene therapy is determined by the highly efficient delivery strategies of therapeutic genes to the targeted tissues. The purpose of this study was to develop a nano-delivery system that has the capability of being functionalized to carry multiple imaging and targeting moieties, with the goal of developing rationally decorated multifunctional nanoparticles (NPs) for the delivery of therapeutic genes to the targeted site of ocular tissues.**Methods:** Primarily polymeric CK30PEG and the antioxidant nanoceria, which have a proven history of safe use for the intended application, were fabricated using standard lab protocols. The compounds were further conjugated with folic acid (FA)-NHS and amine reactive DyLight (DL) 550 NHS ester derivative by standard bioconjugation technique (FA-CK30PEG-DyLight550 and FA-nanoceria-DyLight550). In addition, the FA-CK30PEG-DyLight550 was taken as acetate salt and used to compact with plasmid DNA into NP formulation as described previously. The integrity of NP formulations were further characterized by TEM, DLS, FT-IR, and UV-vis. To determine the targeted efficiency and specificity, the NPs were tested in tumor cell

lines (KB cells, neuroblastoma, and Y79-retinoblastoma) known to overexpress FA receptors, and cell lines (NIH 3T3, HTB and ARPE19) that have a low level of FA receptor expression. Western blot and confocal microscopy were performed to detect the FA protein level and to visualize the intracellular trafficking of NPs. **Results:** In line with previous reports, the UV absorbance spectra determined the formation of the conjugations, which were further confirmed by FT-IR analysis compared with the control folic acid molecules. In terms of CK30 NPs, the intact plasmid DNA came out from the digested NP formulation by trypsinsation and reflected the integrity of plasmid DNA inside formulation state. We observed a significant number of labelled NPs (red) into the nucleus (labelled with DAPI). Earlier it was observed that CK30PEG compacted NPs can traffic to the nucleus via nucleolin receptors. Therefore, in our current findings, we can consider that FA conjugation helped the NPs to enter inside the cell via folate receptor mediated endocytosis followed by nucleolin mediated uptake inside the nucleus. When we compared this result with FA-nanoceria-DyLight550 compacted DNA under the same condition, we found a promising result that nanoceria-DyLight550 compacted NPs (labelled red) only showed up inside the cytosol but not in the nucleus (**Fig. 1**). Therefore, our initial finding demonstrates that FA conjugation could boost up the CK30PEG-FA and nanoceria conjugated NPs to cross the cell membrane barriers for targeted delivery and express gene of interest. **Conclusions:** We explored a smart NP gene delivery system with multifunctionalities. The design of multifunctional NP complexes in this study can be custom-built and functionalized to target different diseases by binding the surface of NPs with a cell-specific targeting molecule and therapeutic specificity. We are now conducting these approaches by using different bio-markers in mouse models in vivo for targeted delivery. Project success will provide attractive tools and templates with broad potential for precision medicine.

Fig.1: Cell transfections in KB cells with (A) FA-CK30PEG-DyLight550, and (B) nanoceria compacted plasmid DNA NPs respectively. Images were captured at 63x in Zeiss CLSM 710 sprectral confocal laser scanning microscope.



340. Cell Penetrating Peptide Can Improve the Efficacy of Mitochondrial Transfer

Daisuke Kami[1], Tomoya Kitani[2], Yuki Takaoka[1], Yu Yoshimoto[3], Yasuhiko Tabata[3], Satoshi Gojo[1]
[1]Department of Regenerative Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan, [2]Department of Cardiovascular Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan, [3]Department of Biomaterials, Kyoto University, Kyoto, Japan

Cell-based therapies for organ regeneration are currently emerging as a new promising approach to treat various diseases, including cardiovascular and neurodegenerative diseases. However, the mechanism supporting these therapeutic effects remains poorly understood. Several recent studies suggest that the intercellular transfer of organelles, including mitochondria, might contribute to these regenerative responses.

Mitochondria are considered cellular "power plants" because they largely synthesize the universal energy "currency" of the cells, i.e., adenosine triphosphate (ATP). Mitochondrial dysfunction is associated with many diseases, including metabolic and neurodegenerative disorders. In vitro experiments revealed that mitochondrial DNA (mtDNA)-depleted mammalian cells recovered aerobic respiration after intercellular mitochondrial transfer from intact cells. The therapeutic potential of mitochondrial transfer was supported by an in vivo study conducted on rabbit model of myocardial infarction. Direct injection of autologous mitochondria into the ischemic heart considerably improved post-infarct cardiac functions. Mitochondria dysfunction also plays central role in the pathogenesis of ischemia reperfusion injury and subsequent cardiomyocytes death. The beneficial effect of mitochondria replenishment for damaged cells and tissues has been reported recently.

Our previous studies demonstrated that the mitochondrial transfer could occur by simple co-culture of mammalian cells with isolated mitochondria. We assessed the impact of the transfer on the mitochondrial function in the recipient cells. The mechanism of mitochondrial internalization was investigated using endocytosis inhibitors. We showed that mammalian cells engulfed isolated mitochondria by macropinocytosis, which survived in heteroplasmy state and functioned to generate ATP in recipient cells. However, the internalization for isolated mitochondria remains inefficient to apply as a clinical therapeutic strategy.

Here, we report the use of TAT, which is cell penetrating peptide derived of human immunodeficiency virus, dextran complexes (TAT-DEX) to enhance the cellular uptake of exogenous mitochondria and improve the protective effect of mitochondria replenishment on rat neonatal cardiomyocytes (NRCM) from IR injury in vitro. TAT-DEX modification significantly neutralized surface negative charge in mitochondria isolated from the human endometrium-derived mesenchymal cells and H9c2 rat cardiomyoblast. TAT-DEX modified mitochondria isolated from H9c2 showed a significantly higher level of cellular uptake. Compared with control mitochondria, co-incubation of TAT-DEX modified mitochondria with NRCM during IR significantly reduced IR-induced reactive oxygen species production and apoptosis in NRCM. These results indicate that surface modification of isolated mitochondria at the molecular level can improve the efficiency of cellular uptake of exogenous mitochondria, and enhanced mitochondrial internalization into cardiomyocytes may improve the potential effect of mitochondria replenishment therapy in myocardial IR injury.

341. Nucleic Acid Delivery Using Vesicular Stomatitis Virus-Based Vesicles

Mathias Mangion[1,2,3], Alexandre Audy[1,2,3], Igor Slivac[1,2,3], Rénald Gilbert[3,4], Bruno Gaillet[1,2,3]
[1]Chemical Engineering Department, Laval University, Quebec, QC, Canada, [2]PROTÉO: The Quebec Network for Research on Protein Function, Structure, and Engineering, Quebec, QC, Canada, [3]ThéCell: FRQS Cell and Tissue Therapy Network, Quebec, QC, Canada, [4]Human Health Therapeutics Portfolio, National Research Council Canada, Montréal, QC, Canada

Gene delivery methods are essential to understand fundamental cellular mechanisms and to develop new therapies. Recombinant viruses are efficient to transfer nucleic acids but their safety is a concern. In addition, some commercial transfection reagents (including lipids and cationic polymers) and electroporation methods are cytotoxic. Interestingly, the sole expression of G envelope protein of the vesicular stomatitis virus (VSV) in mammalian cells can lead to the formation of VSV-G pseudotyped vesicles (V-VSV-G). In

presence of polybrene, these vesicles are able to transfer plasmids in a large panel of animal cells. Unfortunately, the production of V-VSV-G and their use for nucleic acid delivery is poorly documented. Here we propose to improve this promising method of transfection. At first we developed a V-VSV-G production process by transient transfection of HEK-293 cells using polyethylenimine (PEI). Three modes of production were compared: cells cultivated in adherence, in suspension and on micro-carriers. Also we demonstrated that the quantity of vesicles produced depends on the VSV-G sequence used. The harvest of V-VSV-G from cell culture media was also optimized. Then, several parameters potentially involved in the formation and the transfer efficiency of V-VSV-G/DNA complex were studied: polybrene concentration, order of addition of mix transfection components, incubation time of the complexes, medium of transfection, etc. Stability studies also demonstrated that V-VSV-G are robust particles: DNA transfer capacity of V-VSV-G is efficient after 10 freezing and thawing cycles and V-VSV-G can be stored for long term at +4 °C, -20 °C and -80 °C. Finally, V-VSV-G/DNA ratio was optimized for three different cell types. Transfection efficiency of 70 % and 55 % were obtained for HEK-293 and HeLa cells respectively, with 1 µg of V-VSV-G and 0.4 µg of DNA. Transfection of refractory cells such as human myoblasts, reached 25 % with 5 µg of V-VSV-G and 0.8 µg of DNA. V-VSV-G can also deliver large plasmids (18 kb). Furthermore, no cytotoxicity was observed in cells transfected with these complexes. Presently, the potential of V-VSV-G to transfer siRNA is investigated. In conclusion, V-VSV-G is a powerful tool for nucleic acid delivery which could be useful for several applications oriented toward cell and gene therapies.

342. Water Soluble and Bio-Friendly Nanoceria Reduces Laser-Induced CNV Mouse Model of Wet AMD

Rajendra N. Mitra, Min Zheng, Zongchao Han
Ophthalmology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Purpose: Recently Nanoceria has been widely explored in various disciplines including biomedical science. Nanoceria has intrinsic antioxidant property, which is restricted by its poor aqueous solubility, and therefore possesses less residual time in the blood stream. For biological applications, nanoceria requires sufficient surface shield to prevent aggregation. Use of toxic solvents during its synthesis are also creating additional detrimental effects in its clinical applications. To avoid these issues and to make more biocompatible nanoceria, we have synthesized a water soluble and stable nanoceria formulation using chitosan derivative in plain water. Our purpose is to utilize this water soluble nanoformulation to scavenge free radicals generated in the Age Related Macular Degeneration (AMD) mice model, a retinal degenerative dystrophy which leads to irreversible central vision loss and finally causes legal blindness in people over 50 years of age. **Methods**: We have used cerium (III) chloride heptahydrate and chitosan derivative to synthesize the ceria nanoparticle (NPs). These NPs are then purified and solubilized in plain water. These NPs were characterized by dynamic light scattering, FTIR, UV-Vis, and TEM. The characterized NPs were then incubated with human retinal pigmented epithelium cells (ARPE19) in presence and absence of H_2O_2 that mimic the *in vitro* AMD model. The MTT assay was carried out to determine the cellular toxicity of the NPs at different concentrations. H_2O_2 induces reactive oxygen species (ROS) inside the APRE19 cells, which were determined by using cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The safe dose of NPs were then injected into adult wild type (C57Bl/6) mice after laser induced choroidal neovascularization to validate the therapeutic effect of this NP formulation in AMD mice model. **Results**: The TEM studies showed that the NPs are conjugated with the organic matrix and shows a diameter of ~4 nm and demonstrated uniform structure throughout the TEM-grid. The NPs showed considerable amount of cell viability with varying concentrations (n=3-5). The NPs (5 different concentrations) were then incubated over night with ARPE19 cells and DCF assay was carried out with 10 µM DCFH-DA and the fluorescence of DCF was determined with excitation 485 nm and emission 530 nm. The results revealed that the NPs could scavenge ROS with a significant amount (n=5, $p<0.0001$) compared to the uninjected and mock injected controls. Furthermore, a single intravitreal injection of the NPs significantly reduced the size of laser-induced AMD mouse model as measured by fundus fluorescence angiography. **Conclusion**: Our result are reflecting consistency with our idea that water soluble ceria NPs are more stable in water and can reduce the injury of the retina in laser induced CNV model. We are working to define the morphological and functional changes of the retina in this animal model. If successful, this will be a major breakthrough in AMD treatment.

343. Non-Viral Transfection of Primary Human Fibroblasts in Suspension Microcarrier Culture

Charlie Y. Hsu[1], Derrick E. Rancourt[1,2]
[1]Biochemistry and Molecular Biology, University of Calgary, Calgary, AB, Canada, [2]McCaig Institute for Bone & Joint Health, Calgary, AB, Canada

Suspension bioreactors have been employed for the large-scale production of recombinant biomacromolecules and expansion of primary tissue-derived cells for clinical applications. However, the bioprocess requires establishing a stably-expressing cell line in static culture first, which is a lengthy process that can add significant upfront cost and limit economies of scale. A transient-expression system is a simpler cost-effective platform for rapid production in a small-medium format, which could make it feasible for generating patient-specific cell-based products. However, current methods for the efficient transfection of primary cells involve either physical methods of delivery that are not adaptable for suspension culture, or viral vectors that are potentially mutagenic, which can present an even greater problem in suspension culture that tend to select for high proliferative tumorigenic clones. Thus, a method to efficiently transfect clinically-relevant primary cells directly in suspension culture by non-viral means is needed to streamline the derivation, expansion and production of cell-based product in one integrated bioprocess. In this study, we explored the feasibility of transfecting primary tissue-derived fibroblasts directly in microcarrier suspension culture using non-viral cationic reagents. We first developed an optimized non-viral transfection system by adopting the gWiz High Expression plasmid, which is capable of 5x higher expression than CAG-based episomal plasmids. We further evaluated several commercial cationic reagents for transfection of primary human fibroblast in static culture and found that XtremeGENE HP and TransIT-3D were among the most efficient transfection reagents (up to 60%). Next, in order to transfect anchorage-dependent cells in suspension, we evaluated a number of microcarriers for their suitability in culturing fibroblast in suspension. We focused on examining cell attachment efficiencies and growth rates on the microcarriers since these variables have the most significant impact on the overall transfection efficiencies. While all of the microcarriers surveyed in this study (i.e. polyGEMs with FACTIII, Collagen, Pronectin F, Glass, Plastic, or Plastic+, and CultiSphere S, Cytodex 3, Hillex II) were capable of supporting cell attachment, Cultisphere S and Hillex II had the most conducive surface for attachment (40% and 25% higher compared to tissue culture dishes). These differences in attachment efficiencies translated into different lag phase and exponential phase among the carriers when we subsequently assayed for growth rate on the carriers over a 12-day period using a MTT-based assay. Due to the differences in coating, charged surfaces, and growth rate on these microcarriers,

in order to properly assess for their compatibility with cationic transfection reagent in facilitating transfection, we transfected cells at multiple time points (day 2, 3, 5 and 7 days post cell seeding) and found that transfection efficiencies correlated with the time frame in which cells were growing the fastest (Days 2-3); highest efficiencies were seen in cells cultured on Glass, Cytodex 3, CultiSphere S and Hillex II (~12-16%). In summary, we demonstrated here a first step towards the efficient transfection of primary tissue-derived human fibroblasts directly in a suspension microcarrier culture using cationic reagent; additional optimization is expected to bring the efficiencies comparable to those in static culture.

344. En Route to Non-Viral Genetic Engineering: Kinetics of DNA Uptake and Transgene Expression Following Repeated Transfection with Multiple Episomal Plasmids in Human Primary Fibroblast

Charlie Y. Hsu[1], Derrick E. Rancourt[1,2]
[1]Biochemistry and Molecular Biology, University of Calgary, Calgary, AB, Canada, [2]McCaig Institute for Bone & Joint Health, Calgary, AB

Non-viral approach to cellular reprogramming or genome editing of mammalian cells often involve co-delivery of multiple types of nucleic acid molecules. Whether the method involves co-transfection with multiple episomal plasmid DNAs, a mixture of mRNA/gRNA oligomers or a combination of both DNA and RNA molecules, the efficacy of these modular approaches hinges upon the efficiency at which all the molecular factors are co-delivered and co-expressed at their optimal stoichiometric ratios. A significant rate-limiting step thus lies in the lack of an efficient co-transfection, in which a subset of the transfected population may be devoid of one, two, or more of the factors required, but the proportion at which these event occur is not clear. Further, because non-viral transfection is a transient process, repeated transfection is often employed to sustain transgene expression, as is often the case in non-viral episomal based cellular reprogramming. However, the effectiveness of these subsequent rounds of transfection in maintaining transgene expression among the transfected cells is presently unclear. In this study, we examined the kinetics of DNA uptake and transgene expression following cationic reagent-mediated non-viral transfection of primary human neonatal foreskin fibroblast with multiple episomal plasmid DNAs. To measure the level of DNA uptake and transgene expression following co-transfection, we employed two fluorescent reporter gene plasmids (eGFP and mtagBFP2) and covalently labeled them with either FITC or Cy5. Cells were transfected using XtremeGENE HP with either one or both of the labeled plasmids. More than 90% of the cells transfected were positive for either FITC or Cy5 plasmid DNA. When the labeled plasmids were mixed at 1:1 ratio or diluted with unlabeled DNA, there was a proportional decrease in the level of fluorescence in the respective fluorescent channel consistent with the relative input ratios between the two labeled DNAs. We also saw a strong correlation in the co-expression of both reporter genes following co-transfection with the majority of the transfected cells dually expressing both GFP and BFP (64%), however, there were subsets of singly transfected cells that express only GFP (8%) or BFP (27%). We next looked at the effectiveness of repeated transfection in enhancing/sustaining transgene expression in transfected cells. In order to distinguish the population of repeatedly transfected ones from new transfection events in subsequent rounds of transfection, we employed the same two reporter gene set-up (eGFP/mTagBFP2) in which cells were transfected with GFP first, followed by a second transfection with BFP a few days later; cells that were repeatedly transfected would then express BFP in addition to GFP. Our result showed that, while the overall transfection efficiency was higher with repeated transfection, to our surprise, the majority of the transfected cells remained GFP+; only a subset of the 40% of the transfected cells were positive for both GFP and BFP (~9% total), with the remaining attributed to newly transfected cells expressing only BFP. Taken together, these data suggest that while cationic reagent can efficiently co-deliver and co-transfect multiple episomal factors, the effectiveness of this modular approach in non-viral genetic engineering may be limited in cases where sustained expression is required due to the majority of the transfected cells being refractory to subsequent rounds of transfection. Addressing these rate-limiting steps should help increase the utility and efficiency of the system.

345. Transient Removal of CD46 Sensitizes Breast Tumor Cells to Complement Attack Combination with Curcumin

Pei-Rong Wang
Department of Pediatrics, Second Hospital of Shandong University, Jinan, China

Wang et al has developed a technology, which is based on a small recombinant protein, Ad35K++, that induces the internalization and subsequent degradation of complement regulatory protein (CRP) CD46. Curcumin, a polyphenol compound derived from the rhizome of the plant *Curcuma longa* L. has been verified as an anticancer compound against several types of cancer. In preliminary studies, we have demonstrated the utility of Ad35K++ in transient removal of CD46 from the cell surface. Then we found that treatment of Ad35K++ increased the sensitivity of the breast cancer cells induced by curcumin to CDC. Apoptosis triggered by curcumin and Ad35K++ was visualized using Annexin V-FITC/7-AAD staining. Further studies demonstrated that the down-regulation of Bcl-2 and up-regulation of Bax that led to the cleavage of caspase-3 and increased cleaved PARP resulted in the apoptosis of breast cancer cells treated with curcumin and Ad35K++. Enzyme-linked immunosorbent assay (ELISA) showed that curcumin and Ad35K++ treatment resulted in the increased expression of C3b, which is mediated by downregulated CD46. These results show that the combination of Ad35K++ and curcumin can augment the anti-tumor effect of CDC on human breast cancer cells and suggest their promising applicability as a new candidate for the therapy against human breast cancer in the future.

Diabetes, Metabolic and Genetic Diseases II

346. AAV9 Delivery into Cerebrospinal Fluid Corrects CNS Disease in a Murine Model of Mucopolysaccharidosis Type II

Christian Hinderer[1], Nathan Katz[1], Jean-Pierre Louboutin[2], Peter Bell[1], Hongwei Yu[1], Mohamad Naval[1], Karen Kozarsky[3], Timothy O'Brien[4], Tamara Goode[1], James M. Wilson[1]
[1]Gene Therapy Program, Department of Medicine, University of Pennsylvania, Philadelphia, PA, [2]Section of Anatomy, Department of Basic Medical Sciences, University of West Indies, Kingston, Jamaica, [3]REGENXBIO, Rockville, MD, [4]Department of Neuroscience, University of Pennsylvania, Philadelphia, PA

Mucopolysaccharidosis type II (MPS II) is an X-linked lysosomal storage disorder typically manifesting in early childhood with bone and joint deformities, cardiac and respiratory disease, and developmental delay. Systemic delivery of the deficient enzyme, iduronate-2-sulfatase (IDS), improves many symptoms of MPS II, but because the enzyme does not cross the blood-brain barrier, there is currently no effective method to prevent the progression of central nervous system (CNS) disease. Using a mouse model of MPS II, we evaluated AAV serotype 9 vector-mediated delivery of the *IDS* gene as a means of achieving continuous IDS expression in the CNS. *IDS* knockout mice received a single injection into the lateral ventricle of

one of three vector doses (3×10^8, 3×10^9 or 3×10^{10} genome copies) and were sacrificed either three weeks after vector administration for assessment of vector biodistribution and IDS expression (n = 7-8 mice per group), or 3 months after vector administration to evaluate the impact of gene transfer on disease progression (n = 7-8 mice per group). IDS activity was detectable in cerebrospinal fluid, reaching 15% of wild-type levels in the low-dose cohort and 268% of normal at the highest dose. Brain enzyme activity ranged from 2.7% of normal in the low-dose cohort to 32% in the high-dose cohort. Quantification of brain storage lesions by staining for the ganglioside GM3 indicated dose-dependent correction, with 35%, 46%, and 86% reductions in the low-, mid-, and high-dose cohorts, respectively. Treated mice also demonstrated improved cognitive function in a novel object recognition test. These findings indicate that intrathecal AAV-mediated gene transfer could serve as a platform for sustained enzyme delivery to the CNS, potentially addressing this critical unmet need for patients with MPS II.

347. CRISPR/Cas9-Based Gene Correction of Arginase-Deficient Human Induced Pluripotent Stem Cells to Recover Enzyme Function

Brian Truong[1], Patrick C. Lee[1], Agustin Vega-Crespo[1], William B. Gilmore[2], Kip Hermann[2], Stephanie Kingman[2], Jonathan K. Tang[1], Katherine M. Chang[1], James A. Byrne[1], Gerald S. Lipshutz[2]
[1]Molecular and Medical Pharmacology, University of California, Los Angeles, Los Angeles, CA, [2]Surgery, University of California, Los Angeles, Los Angeles, CA

Urea cycle disorders (UCDs) are incurable genetic diseases that affect the body's ability to produce urea, leading to hyperammonemia due to a deficiency in any one of six enzymes in the cycle. For arginase deficiency, a mutation in the ARG1 gene, the final step of the cycle, results in hyperargininemia, developmental delays and disabilities, seizures, psychomotor function loss, and in serious cases, death. There is currently no completely effective treatment available. Advances in human induced pluripotent stem cell (hiPSC) research and genome-editing technologies have enabled the genetic modification of stem cells for potential cellular replacement therapies. In this study, we applied such technology to develop a stem cell-based approach for treating arginase deficiency applicable to all arginase deficient patients regardless of their mutation. Methods: Fibroblasts from three patients with arginase deficiency were obtained, defined for their mutation, and reprogrammed into hiPSCs. Selectable, full-length codon optimized human arginase cDNA (coARG) expression cassettes were then developed for site-specific integration into either the HPRT or albumin (ALB) locus. After confirming specificity by Sanger sequencing, genetically corrected hiPSCs were differentiated to hepatocyte-like cells. Results: Fibroblasts were reprogrammed into hiPSCs by applying a STEMCCA lentivirus-based method and were characterized for pluripotency by immunophenotyping for common stemness markers via ICC, alkaline phosphatase staining, and *in vivo* teratoma formation. Using a site-specific CRISPR/Cas9 nickase-mediated gene transferring system, we inserted coARG by two approaches. First, coARG was inserted into the HPRT locus under the control of constitutive hEF1α promoter (LEAPR); targeting HPRT allowed for positive clonal selection of successful on-target integration by 6-thioguanine treatment. Second, we inserted coARG into the ALB locus for expression under the endogenous ALB promoter (ALB-coARG) as ALB is highly expressed in human liver. After LEAPR and ALB-coARG modification and sequence confirmation, hiPSCs were differentiated to hepatocyte-like cells and characterized by immunophenotyping via ICC and RNA expression via RT-PCR for common hepatic markers; cells demonstrated more fetal-like characteristics. Moreover, LEAPR- and ALB-coARG-modified hepatocyte-like cells demonstrated 41% and 1% functional arginase activity recovery compared to human fetal liver, respectively. Discussion: In this study, we demonstrated the ability to genetically correct mutated ARG1 gene expression in hiPSCs derived from patients with hyperargininemia and restored arginase function in hiPSCs and hepatocyte derivatives by CRISPR/Cas9-based gene addition. As the LEAPR construct demonstrated marked coARG expression, recovery of arginase activity of ALB-ARG-modified hepatocyte-like cells was low; we expect significant arginase recovery after *in vivo* maturation of transplanted cells as ALB expression increases with hepatocyte maturation. Also, to demonstrate potential *in vivo* recovery of arginase deficiency pathogenesis, ongoing studies aim to transplant both cohorts of gene-corrected hepatocyte-like cells into an established arginase deficient immunosuppressed mouse model. Successful restoration of enzyme function in patient-specific hiPSCs and relevant cellular derivatives will highlight hiPSCs as a valuable tool in cell replacement therapies and advance applications of genetically modified hiPSCs to treat UCDs and other single enzyme liver deficiencies.

348. Correction of CNS and Somatic Pathology by Intra-Cerebrospinal Fluid Gene Therapy for Mucopolysaccharidosis Type II

Virginia A. Haurigot[1,2], Sandra Motas[1,2], Sara Marcó[1,2], Albert Ribera[1,2], Carles Roca[1,2], Xavier Sánchez[1,2], Miquel García[1,2], Maria Molas[1,2], Victor Sánchez[1,2], Xavier León[1,2], Jesús Ruberte[1,3], Fatima Bosch[1,2]
[1]Center for Animal Biotechnology and Gene Therapy, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain, [2]Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain, [3]Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

Mucopolysaccharidosis type II (MPSII), or Hunter Syndrome, is a X-linked recessive Lysosomal Storage Disease (LSD) caused by deficiency in Iduronate-2-sulfatase (IDS), an enzyme involved in the stepwise degradation of the glycosaminoglycans (GAGs) heparan sulfate (HS) and dermatan sulfate (DS). GAG storage builds up in the CNS and peripheral tissues causing severe neurologic and multisystemic somatic disease. Patients usually die during the second decade of life. Periodic intravenous enzyme replacement therapy (ERT) currently constitutes the only approved therapeutic option for MPSII. However, the inability of recombinant IDS to efficiently cross the blood-brain barrier limits the efficacy of the approach in treating neurological symptoms. Alternatively, the periodic infusion of ERT to the cerebrospinal fluid is under clinical testing, but serious adverse events associated with the use of intrathecal drug delivery devices have been reported. Here we report a gene therapy approach directly addressing the CNS pathology of MPSII. Through a minimally invasive procedure we delivered adeno-associated virus vectors of serotype 9 encoding IDS (AAV9-Ids) to the CSF of MPSII mice with already established disease. Four months after vector administration, treated mice showed a significant increase in IDS activity throughout the encephalon, with full resolution of lysosomal storage lesions, reversal of lysosomal dysfunction, normalization of the brain transcriptomic signature and disappearance of neuroinflammation. Moreover, our approach not only resulted in widespread distribution of vector in the CNS but also in liver transduction, providing a peripheral source of therapeutic protein that corrected the storage disease in visceral organs, with evidence of cross-correction of non-transduced organs by circulating enzyme. Importantly, MPSII mice treated with AAV9-Ids also showed normalization of behavioural deficits. These results provide a strong proof of concept for the clinical translation of our approach for the treatment of Hunter patients with cognitive impairment.

349. The Cure of Canavan Disease: Is It a Scientific Fiction or Clinical Reality?

Dominic J. Gessler, Danning Li, Hongxia Xu, Qin Su, Guangping Gao
Gene Therapy Center, UMass, Worcester, MA

Canavan Disease (CD) is a rare and lethal inherited pediatric CNS disorder with recessive mutations in the aspartoacylase (ASPA) gene. Traditionally, its variable disease phenotypes were descripted with the congenital sub-form showing neonatal onset and the severest phenotype with early death. The two other sub-forms, infantile, and juvenile, manifest with delayed onset and milder symptoms. Some CD patients are now 20 years of age and older. To date, there is no effective treatment available. Thus, gene replacement therapy is an attractive approach for treating this devastating disease. Previously, we have shown that a single intravenous (i.v.) injection of recombinant adeno-associated virus (rAAV) expressing human ASPA (hASPA) rescues early lethality and partially restores motor function (1st generation gene therapy) in the CD knock-out (CD KO) mouse, which resembles the congenital sub-form of CD and displays the severest phenotype of all available CD mouse models, with early death at around post-natal day (p) 28. After this remarkable improvement of symptoms with our 1st generation treatment, we further optimized our gene therapy. Now, in its 2nd and 3rd generation, our gene therapy cures the disease in the CD KO mouse. Interestingly, our 3rd generation gene therapy turns CD KO mice into "supermice", outperforming WT mice on rotarod motor function test. This rescue is persistent and currently mice at 1.5 years of age still show no signs of disease reoccurrence. CNS pathology and magnet resonance imaging (MRI) at p25 and p365 show complete normalization. To further support the efficacy of our 3rd generation gene therapy, we performed neurometabolome profiling with over 400 characterized metabolites that showed reversal of the Canavan disease related metabolic changes including myelin associated lipids. To further evaluate the potency of our 3rd generation gene therapy, we tested different doses and routes of administration. Of note, 200-fold lower doses intraventricularly (ICV) administered still rescues lethality, while mice treated ICV with 20-fold reduced dose draw even with WT mice on motor function testing. Next, we moved to the Nur7 mouse model (resembles infantile and juvenile sub-form) that displays a similar disease pattern as the CD KO mouse with respect to growth curve and neurologic symptoms but eventually re-gains weight and shows survival similar to wild-type mice. Again, we treated mice i.v. with a single dose of rAAVhASPA at p1 as our gold standard and subsequent groups at 6 and 12 weeks of age to determine the therapeutic window. Of note, mice treated at 6 weeks of age recovered within 4 weeks post-treatment. Mice treated later than 6 weeks require more time to recover but still showed significant improvement over Nur7 mutants. This recovery was also correlated by CNS pathology and MRI. Currently, we are evaluating mice that were treated at 24 weeks of age to determine if there is a time point of no return. Overall, our data show clear evidence for the cure of the disease at early and late stages of the disease in two different mouse models. In addition, this is confirmed on different levels of cellular complexity by MRI, fMRI, CNS pathology, and neurometabolic profiling.

350. Kidney-Directed Gene Therapy for Murine Glycogen Storage Disease Type IA

Youngmok Lee[1], Seongho Bae[2], Celine J. Rocca[3], Stephanie Cherqui[3], David A. Weinstein[1], Janice Y. Chou[2]
[1]Glycogen Storage Disease Program, Division of Pediatric Endocrinology, Department of Pediatrics, University of Florida, Gainesville, FL, [2]Section on Cellular Differentiation, Division on Translational Medidine, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, [3]Department of Pediatrics, University of California, San Diego, La Jolla, CA

Glycogen storage disease type Ia (GSD-Ia, MIM232200) is an autosomal recessive disorder caused by deficiencies in glucose-6-phosphatase-α (G6Pase-α or G6PC) that is expressed primarily in the liver and kidney. GSD-Ia patients manifest impaired glucose homeostasis and a long-term complication of renal disease and there is no existing therapy to address this complication. We have previously shown that systemic administration of rAAV8-G6PC, a rAAV8 vector expressing human G6Pase-α directed by the human *G6PC* promoter/enhancer, delivers the G6Pase-α transgene to the liver of G6Pase-α-deficient (*G6pc*-/-) mice and corrects hepatic G6Pase-α deficiency. However, the rAAV8-G6PC vector transduces the kidney poorly and the treated *G6pc*-/- mice continued manifest renal dysfunction. In this study, we used the rAAV9-G6PC vector in a kidney-targeted gene delivery to improve renal function. The *G6pc*-/- die early even with glucose therapy. To overcome this, we performed a two-step kidney-directed gene delivery, first neonatally via the temporal vein with rAAV8-G6PC to sustain the survival of the mice, then at 12 weeks of age via retrograde renal vein injection with rAAV9-G6PC. Metabolic profiles and renal function were examined over a 52-week study. The rAAV-treated *G6pc*-/- mice exhibited normal fasting glucose profiles and could sustain a 24 hour of fast. Moreover, the treated *G6pc*-/- mice displayed normalized blood urea nitrogen concentrations, indicative of improved renal function. Our results strongly suggest that kidney-directed gene delivery with the rAAV9-G6PC vectors offers a promising and efficacious treatment for renal disease in GSD-Ia.

351. Efficacious Non-Oligodendrocyte Gene Therapy Suggests a New Dogma About CNS Compartmentalization of NAA Metabolism and Supports a Metabolic Sink Theory

Dominic J. Gessler[1], Danning Li[1], Hongxia Xu[1], Qin Su[1], Reuben Matalon[2], Guangping Gao[1]
[1]Gene Therapy Center, UMass, Worcester, MA, [2]University of Texas Medical Branch, Galveston, TX

N-acetylaspartate (NAA) is the second most abundant amino acid derivative in the mammalian central nervous system (CNS). Although its physiologic function remains elusive, many CNS disorders have been associated with changes in NAA levels, e.g. Alzheimer's disease, bipolar disorder. The disease that is known to have direct connection to altered NAA metabolism is Canavan disease (CD); a leukodystrophy caused by mutations in the aspartoacylase (ASPA) gene. The current understanding is that, physiologically, the ASPA enzyme hydrolyzes NAA into L-aspartate and acetate in oligodendrocytes. Consequently, it was postulated that Canavan gene therapy has to restore ASPA expression in oligodendrocytes. However, we hypothesized that NAA can move freely and its cell-type independent break-down ameliorates Canavan disease. We constructed several tissue/cell-specific expression cassettes limiting hASPA expression to either astrocytes, neurons, oligodendrocytes, liver, heart, or muscle. We opted for the Canavan disease knock-out (CD KO) mouse model because it shows early lethality at around post-natal day 28 and the

severest disease phenotype of all Canavan mouse models available. To our surprise, mice expressing hASPA restricted to peripheral organs showed extended survival and normalization of the growth curve at later time points, suggesting a contribution of peripheral organs to the disease pathomechanism. Moreover, astrocyte restricted hASPA expression produced the strongest disease recovery matching the performance of wild-type (WT) mice. Thus, our data seem to support our oligodendrocytes independent NAA metabolic sink theory. In an attempt to verify this theory, we used lower dose rAAVhASPA for localized brain injections to demonstrate that localized T2 hyper-intensity signal clearance on MRI was well correlated with reduction of NAA levels by MRS. In other words, the further away from the injection site, the higher the NAA levels, which supports the idea of drainage and hydrolytic activity of NAA towards the injection side. Currently, we are investigating this metabolic sink theory in more detail by creating a functional map of therapeutic gene transfer in the brain by mass spectrometry quantification of NAA, and vector genome and ASPA transcripts analyses in different anatomic regions. Overall, our data present evidence that ASPA expression does not have to be restored in oligodendrocytes in order to rescue lethality and Canavan disease phenotype, which is in congruence with the metabolic sink theory. Furthermore, this calls into question a common hypothesis that NAA breakdown in oligodendrocytes is the mainstay for myelination and myelin defects in Canavan disease and might even support the idea that other metabolic disorders could benefit from treatment under the premise of the metabolic sink theory.

352. Functional Benefits of Systemic rAAV9-hIDS Gene Delivery in MPS II Mouse Model

Douglas M. McCarty[1], Kimberly Zaraspe[1], Aaron S. Meadows[1], Naoka Murakami[1], Ricardo Pineda[1], Marybeth Camboni[1], Joseph Muenzer[2], Haiyan Fu[1]

[1]Center for Gene Therapy, Research Institute at Nationwide Children's Hospital, Columbus, OH, [2]Department of Pediatric, University of North Carolina at Chapel Hill, Chapel Hill, NC

Mucopolysaccharidosis (MPS) II is an X-linked recessive lysosomal storage disease caused by defect in iduronate-2-sulfatase (IDS), leading to in the accumulation of glycosaminoglycans (GAGs) in cells in the CNS and peripheral organs, and profound multisystem disorders in the majority of patients. No treatment is available for the neurological disorders of MPS II. In this study, we developed 2 self-complementary (sc) AAV9 vectors expressing human IDS and tested them in MPS II mice via systemic delivery. At a dose 5e12vg/kg, an IV injection of either scAAV9-U1a-hIDS or scAAV9-mCMV-IDS, led to rapid and persistent IDS expression and the correction of lysosomal GAG storage throughout the CNS, peripheral nervous system (PNS) and periphery tissues, as well as diminishing of astrocytosis in the CNS and PNS, in MPS II mice treated at 1mo, 3mo or 6mo of age. Importantly, we demonstrate significant improvement in cognitive and motor function, and extension of survival (ongoing) in the rAAV9-treated MPS II mice. These data demonstrate substantial functional benefits of a systemic scAAV9-hIDS gene delivery for treating MPS II at early and advanced disease stages, supporting the clinical potential of the approach.

353. 1-^{13}C-Propionate Oxidation as a Measure of Methylmalonyl-CoA Mutase (MUT) Activity in Methylmalonic Acidemia

Jack Gagné[1], Irini Manoli[1], Elizabeth Harrington[1], Sarah Smyth[2], Jacob D. Hattenbach[2], Jennifer L. Sloan[1], Kong Y. Chen[2], Charles P. Venditti[1]

[1]Medical Genomics and Metabolic Genetics Branch, National Human Genome Research Institute, Bethesda, MD, [2]Clinical Endocrinology Branch, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD

Isolated methylmalonic acidemia (MMA) is a grave autosomal recessive inborn error of metabolism, caused by a defect in methylmalonyl-CoA mutase (MUT). In some severe patients, liver (LT), kidney (KT), or combined liver and kidney (LKT) transplantation has been used as a surgical treatment for MMA. Previous studies have shown that 1-^{13}C-propionate oxidation can be used to measure whole body MUT activity in gene therapy treated and transgenic MMA mice and have encouraged translation to patients, with the aim of using this approach as an outcome measure in future gene therapy clinical trials.

We therefore investigated 1-^{13}C-propionate oxidation in 29 patients with isolated MMA (*mut^0* N=17, *mut$^-$* N=6, *cblA* N=5, *cblB* N=1, age range: 4-41 years), including 3 KT, 1 LT, and 4 LKT recipients, as well as one patient who received a heterotopic liver allograft post KT (hLKT). In addition, 7 healthy volunteers (3 males, 4 females, age range: 21-44 years) and 8 heterozygote controls (age range: 35-62 years) were tested to establish test reproducibility. Baseline CO_2 production of the participants was measured using an indirect calorimetry cart before sodium 1-^{13}C-propionate was administered as an oral or G-tube bolus, and breath samples were collected serially over 2 hours via disposable breath collection kits to measure $^{13}CO_2$ enrichment and subsequently propionate oxidation.

Propionate oxidation was decreased in all non-transplanted MMA patients compared with controls (p<0.0001). Both LT and LKT recipients demonstrated complete restoration of oxidation rates to control levels (p=NS), while only minimal oxidation was observed in the single hLKT recipient. KT recipients responsive to vitamin B-12 supplementation (N=2) with *cblA* MMA showed oxidation rates similar to controls. However, the remaining KT recipient, a severely affected mut^0 patient carrying two stop mutations, showed almost no metabolism of label.

Repeat testing conducted both on healthy volunteers in triplicate and 7 MMA patients (6 *mut*, 1 *cblA*) in duplicate supported excellent reproducibility. Notably, similar oxidation rates were observed in 2 patients despite markedly different plasma methylmalonic acid concentrations between one assessment and the next (1741 vs. 2246 and 2260 vs. 719 μmol/L). This suggests that measured 1-^{13}C-propionate oxidation may be a more consistent and reproducible clinical biomarker than plasma metabolites that depend, in part, on protein intake and kidney function.

Our results demonstrate that 1-^{13}C-propionate oxidation, measured using breath testing, has great potential as an outcome measure for therapeutic interventions aimed at increasing hepatic MUT activity, such as gene or cell therapy. It is safe, non-radioactive, minimally invasive, well tolerated by very young patients, and can be frequently repeated over the course of treatment to evaluate the magnitude and sustainability of whole body *in vivo* MUT activity, with every patient's baseline values serving as an individual pre-treatment control. Furthermore, the method is readily applicable to the study of other inborn errors of metabolism affecting propionate oxidation, such as propionic acidemia.

354. Genetically Modified Human Mesenchymal Stromal Cell (MSC) Delivery Improved Glucose Tolerance in Diet Induced Obese (DIO) Mouse Models

Cleyton Domingues, Nabanita Kundu, Neeki Ahmadi, Sabyasachi Sen
George Washington University, Washington, DC

MSCs are undifferentiated, multipotent cells. We have previously showed that High Glucose (HG, 25mM) exposure promotes adipogenic differentiation, increased formation of ROS and decreased cellular oxygen consumption rate (OCR,using Seahorse). HG exposure also upregulated mRNA expression of adipogenic (PPARG, FABP-4, CEBP alpha and beta) and inflammatory (IL-6 and TNF alpha) genes. In this study, in order to reduce intracellular superoxide presence, we used GFP-containing Adenovirus constructs to upregulate both mitochondrial and cytosolic antioxidants (SOD2 and SOD1, respectively) and used GFP gene as a control. We showed both SOD1 and SOD2 upregulation reduced intracellular superoxide presence and improved OCR in presence of HG environment. In addition, the upregulation of IL-6 and TNFa was prevented. Next, we delivered the eGFP, SOD1 and SOD2 upregulated MSCs intra-peritoneally to DIO (60% and 45% high-fat diet fed) C57BL/6J mice. The GFP presence in constructs allowed us to track live, MSCs in-vivo. Prior to MSC delivery, all mice presented fasting blood glucose levels of 200±20 mg/dl . 60% fat fed DIO mice showed higher blood glucose levels than 45% fat fed mice. We fed 60% fat to WT mice for 6 weeks and 45% for 12 weeks. We measured blood glucose from the tail vein using a glucometer and did GTT every 2 weeks and measured body weight and imaged whole body every 3-7 days. Fat depots were harvested at 28 days. Our results indicate that MSCs did reach ("homed-in") distal fat pockets such as omental, epididymal and pericardial. Epididymal imaging was prominent with SOD1 upregulated MSCs and pericardial imaging was very prominent mice receiving SOD2 upregulated within 7 days of delivery. SOD2 upregulated MSCs showed significantly improved glucose tolerance (GTT) at 4 weeks (that is lowest area under the curve) compared to SOD1 and GFP (SOD2>SOD1>GFP) with progressive reduction in fat mass in both 60% and 45% fat fed DIO mice. We are currently processing fat depots and serum samples from MSC delivered mice, for qPCR, Western Blot and ELISA to demonstrate reduction of local and systemic inflammation. In summary, HG evokes superoxide generation, OCR reduction and adipogenic differentiation. Upregulation of superoxide dismutase, particularly mitochondrial, quenches excess of intracellular superoxide and improves MSCs respiration. Delivery of superoxide dismutase using MSCs as a gene delivery vehicle in DIO mouse model improved glucose tolerance. This is particularly important as the 45% fat DIO mouse model is particularly close to human type 2 diabetes patho-physiology. We conclude that delivery of dismutases using MSCs to the inflamed adipocyte depots may be the key to suppression of adipocyte mediated inflammation and may be a novel yet safe therapeutic tool to combat obesity associated diabetes and impaired glucose tolerance.

355. Minicircles Show Improved Hepatic Expression of Their Transgene from a Natural Endogenous Promoter and Are Lost Upon Partial Hepatectomy Due to the Episomal Nature of the Vector

Hiu-Man Viecelli[1], Andrea Schlegel[2], Tanja Scherer[1], Gabirella Allegri[1], Martin Schleef[3], Cary O Harding[4], Johannes Häberle[1], Beat Thöny[1]
[1]Division of Metabolism and Children's Reserach Center, University Children's Hospital Zurich, Zurich, Switzerland, [2]Department of Sugery, Swiss HPB and Transplant Center, Zurich, Switzerland, [3]PlasmidFactory GmbH & Co. KG, Bielefeld, Germany, [4]Departments of Molecular and Medical Genetics and Pediatrics, Oregon Health & Science University, Portland, OR

We have previously showed the successful treatment of phenylketonuria (PKU) in the PKU mouse model (C57Bl/6-*Pahenu2*) by using non-viral naked DNA minicircle (MC) vectors, which are devoid of any viral or bacterial sequences, upon liver-directed phenylalanine hydroxylase (*Pah*) gene transfer via hydrodynamic vein injection (Viecelli et al., Hepatology 2014). Here we improved the efficacy of transgene expression and investigated the fate of MC vectors in mouse liver. We found that the therapeutic doses of MCs could be significantly lowered by using a codon-optimized murine *Pah* cDNA in combination with a truncated 5'-intron. Moreover, when using the natural or endogenous 3.6 kb murine *Pah*-promoter to drive the *Pah* transgene, vector doses could again be lowered compared to two other "minimal" liver-specific promoters, a synthetic hybrid enhancer/promoter (P3) or the classical CBA (modified cytomegalovirus enhancer/chicken β-actin) promoter. Following 70% partial hepatectomy, MC-vector-treated PKU mice showed normal liver regeneration and blood L-Phe concentration increased to pretreatment levels, indicating that treated PKU mice had lost therapeutic MC vectors during liver regeneration. These results corroborate previous observations that MC-DNA do not integrate - or only at a very low frequency that cannot be detected by our assays - and thus express their transgene as episomal vectors. In conclusion, MC-vectors which do not have a defined size-limitation, offer a favorable safety profile due to their non-integrating behavior in combination with a (large) natural or endogenous promoter, and at the same time have the potential for long-term gene-therapy of liver defects.

356. High-Resolution X-Ray Fluorescence Microscopy (XFM) Indicates Enhanced Brain Copper Delivery in AAV9-Treated Menkes Disease Mice

Marie Reine Haddad[1], Martina Ralle[2], David J. Vine[3], Patricia M. Zerfas[4], Stephen G. Kaler[1]
[1]Section on Translational Neuroscience, NIH, NICHD, Bethesda, MD, [2]Department of Molecular and Medical Genetics, OHSU, Portland, OR, [3]X-Ray Science Division, Advanced Photon Source, Argonne National Laboratory, Argonne, IL, [4]Division of Veterinary Resources, Office of Research Services, NIH, Bethesda, MD

Menkes disease is a lethal infantile neurodegenerative disorder caused by mutations in a copper transporter, ATP7A. The mottled-brindled (mo-br) mouse recapitulates the symptoms and early demise (14 days) of untreated human Menkes disease (3 years). We previously demonstrated that viral gene therapy using cerebrospinal fluid (CSF)-directed AAV9-ATP7A in combination with subcutaneous (sc) clinical grade copper histidinate enhanced survival, normalized growth, and improved biochemical and neurobehavioral outcomes in this model. In the current study, we examined copper biodistribution and concentration in specific brain

regions for treated and untreated mutants compared to wild type mice, using X-ray fluorescence microscopy (XFM). XFM enables sensitive, quantitative measurement of the spatial distribution of biometals at image resolutions approaching the subcellular level. Ten days after rAAV9-ATP7A administration, copper levels in cerebral cortex, caudate and choroid plexus in the lateral ventricle in combination treated mice were all normal or slightly higher than normal compared to wild type mice and untreated mutants. Immunohistochemistry demonstrated robust ATP7A expression in choroid plexus epithelia as well as in neurons throughout the brain in the treated animals. These preliminary data support the hypothesis that choroid plexus is the major mediator of brain copper delivery by pumping copper into the CSF via ATP7A. Our findings provide further support for CSF-directed viral gene therapy in human subjects with Menkes disease.

357. A Mouse Model of Cobalamin A (cblA) Class Isolated Methymalonic Academy (MMA) Provides Unique Platform for Testing Gene and Cell Therapies

Madeline Epping[1], Irini Manoli[1], Cindy Wang[1], Pat Zerfas[1], Victoria Hoffmann[1], Gene Elliott[1], Lingli Li[2], Chuck Venditti[1]
[1]National Human Genome Research Institute, Bethesda, MD, [2]Georgetown University Medical Center, Washington, DC

Isolated Methylmalonic Acidemia (MMA) comprises a relatively common and heterogeneous group of inborn errors of metabolism. Most affected individuals display severe multisystemic disease with episodes of metabolic instability, chronic renal disease, and neurological complications. The most severe clinical presentations are typically associated with complete loss of methylmalonyl-CoA mutase (MUT) enzymatic activity. MUT converts methylmalonyl-CoA to succinyl-CoA within mitochondria but defects in the transport or synthesis of its cobalamin co-factor also variably impair MUT activity. Previous mouse models of *Mut* MMA generated by our group have been critical to developing gene therapy for MMA but have either displayed neonatal lethality or required concomitant transgenesis for viability. Thus, the need for ameliorated models to further investigate disease-associated pathophysiology and facilitate gene therapy studies exists. We have therefore constructed a mouse model of an attenuated form of isolated MMA, the *cblA* subtype, by using homologous recombination to create a deletion allele of *Mmaa*, the enzyme that performs the gated transfer of adenosylcobalamin to Mut and protects Mut from oxidative inactivation. Patients with the *cblA* subtype of MMA can have variable presentations, spanning the full spectrum of MMA associated symptoms and pathology, yet always harbor an element of clinical and biochemical responsiveness to injectable B12.

Mmaa-/- mice were born in mendelian proportions, exhibited decreased survival after weaning ($P<0.0001$), weighed 50% less than littermates at 6 months ($P<0.0001$), lacked immunoreactive Mmaa and Mmaa mRNA in multiple tissues, including the liver, and manifested severe metabolic perturbations. Plasma methylmalonic acid concentrations ranged between 63.88-1641.91 μM, representing a 62-fold or more elevation over age matched littermates. *Mmaa-/-* mice also display diminished 1-C-13 propionate oxidative capacity ($P<0.0001$) and hepatic complex IV activity ($P=0.002$). Electron microscopy confirmed the presence of hepatic megamitochondria and abnormal proximal renal tubular mitochondria. The glomerular filtration rate, measured using FITC-sinistrin decay, showed that *Mmaa-/-* mice have diminished kidney function compared to heterozygous littermates. Following hydroxycobalamin administration, *Mmaa-/-* mice had improved survival and increased weight ($P=0.0003$), which was associated with improved 1-13C-propionate oxidative capacity.

This new mouse model fully recapitulates the clinical and biochemical features seen in *cblA* patients, and provides a robust platform for testing gene and cell therapies for MMA because affected mice are relatively severe but survive until weaning. These animals also offer the opportunity to longitudinally examine disease biomarkers and more easily assay vectors across a wide age range.

358. AAV-MitoTimer to Asses Mitochondrial Turnover

Bennett Gosiker, Skylar Rizzo, Amy Donate, Christina Pacak
University of Florida, Gainesville, FL

Mitochondrial turnover is a key marker in the process of cellular senescence. In order to track mitochondrial turnover rates in various cell lines, an indicator is necessary to identify the formation of new mitochondria along with the degradation of old mitochondria. This measure is critical for the study of disease processes in which there is an up or down regulation of mitochondrial turnover rates. One dynamic tool to measure this balance is the pTRE-Tight-MitoTimer plasmid. The MitoTimer transgene combines the red fluorescent protein, dsRed, and a human cytochrome c oxidase subunit VIII (Cox 8) mitochondrial localization sequence to traffic the protein to the mitochondria where the color shifts from green to red over time (approximately 24 hours). Our goal is to use this as a tool to assess the increase or decrease in mitochondrial turnover as a result of mutations that cause mitochondrial dysfunction. We successfully developed two plasmids through the ligation of the MitoTimer transgene into a double stranded AAV-ITR containing plasmid with a 1) Desmin promoter pds-AAV-Des-MitoTimer to restrict expression to striated muscle and 2) CMV promoter pds-AAV-CMV-MitoTimer to enable ubiquitous expression among many cell types. Once cloned, these novel plasmids were then transfected into an immortalized C2C12 mouse muscle cell line to confirm successful generation of functional plasmids. An immediate application for these novel plasmids in our laboratory is the characterization of mitochondrial turnover rates in Barth Syndrome (BTHS) patient derived cells. BTHS is an X-linked mitochondrial disease that results from mutations of the gene TAZ, which encodes tafazzin. This gene serves to orchestrate the production of mature cardiolipin (CL) within the mitochondrial membrane. Mutations in the TAZ gene result in altered CL maturation, with implications on mitochondrial function. Thus far, mitochondrial turnover rates have not been carefully assessed in this disorder. As a preliminary experiment using human patient derived BTHS fibroblasts (harboring an exon 10: 748G &gt T nonsense mutation), pds-AAV-CMV-MitoTimer was successfully transfected and displayed variation in green and red fluorescence ratios as compared to a healthy control cell line. This demonstration of the impact of BTHS on mitochondrial turnover dynamics supports future use of these plasmids in BTHS induced pluripotent stem cells (iPSCs) that have been differentiated into cardiomyocytes or skeletal myotubes - the two cell-types most affected in BTHS. This will enable future comparisons of mitochondrial turnover in clinically relevant BTHS cells harboring a variety of different mutations and provide another metric by which to measure the success of pre-clinical therapeutics for BTHS. We are preparing an AAV Quad-mutant serotype for *in vitro* testing, and other serotypes will be used for *in vivo* models of mitochondrial dysfunction. AAV-MitoTimer will continue to be used as a measure of mitochondrial turnover, and provide an effective characterization method for metabolic diseases.

359. Targeted Genome Editing Using TALENs to Correct a Mouse Model of Methylmalonic Acidemia (MMA)

Jessica L. Schneller, Randy J. Chandler, Charles P. Venditti
National Institutes of Health, Bethesda, MD

Methylmalonic acidemia (MMA) is an autosomal recessive metabolic disorder, in which the body is unable to oxidize valine and isoleucine as well as odd chain fatty acids. Approximately 60 percent of MMA cases are caused by mutations in methylmalonyl-CoA mutase (*MUT*), the enzyme that catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA. Current treatments for *MUT* class MMA include dietary protein restriction and cofactor supplementation; however patients continue to exhibit severe morbidity and high rates of mortality. A murine model of MMA was created by knocking out exon 3 of the *Mut* gene and exhibits the severest clinical phenotype of MMA, neonatal lethality. Gene therapy treatments using recombinant adeno-associated viral vectors to treat mice with MMA have proven effective, however long-term follow-up of mice treated as neonates revealed genotoxicity caused by AAV integrations, leading the mice to develop hepatocellular carcinoma. Programmable nucleases such as TALENs should make it possible to correct at the *MUT* locus, ameliorate disease symptoms and reduce the effects of off-target integration by precise genome modification. Furthermore, AAV-mediated delivery of TALENs with an HR cassette carrying a rescue cDNA for the *Mut* gene in a disease model of MMA should enable *in vivo* correction in the liver, and ameliorate the MMA phenotype. We have designed TALENs that target intron 2 of the *Mut* gene and a partial rescue cassette that contains codon-optimized exons 3 to 13 of the *Mut* gene to restore *Mut* expression in the exon 3 knock out model of MMA after successful homologous recombination. The TALENs are expressed under the control of the liver-specific thyroxine binding globulin promoter to ensure cleavage in hepatocytes, and delivered using a hepatotropic AAV serotype 8 capsid. To restore expression and achieve targeted integration, the rescue cassette is preceded by a splice acceptor site and flanked by homology arms around the TALEN cleavage site. Integration of the rescue cassette will restore *Mut* expression at the locus, using the endogenous promoter and exons 1 and 2 of the *Mut* gene to reconstitute a functional *Mut* gene. AAV packaging limitations necessitate two AAV vectors for delivery of the TALENs, which are 4kb per pair, and required regulatory elements, as well as a third vector for delivery of the *Mut* homologous recombination rescue cassette. All three vectors will be delivered systemically in the neonatal period. We hypothesize that *in vivo* editing at the *Mut* locus with subsequent HR will provide sufficient Mut enzyme activity to improve the clinical and biochemical phenotypes of the MMA mice, with minimal off-target integration, thereby decreasing the risk of genotoxicity associated with AAV gene therapy.

360. Genotype/Phenotype Correlation of Cellular Function and AAV-Mediated Gene Delivery to Treat Xeroderma Pigmentosum - Cockayne Syndrome (XP - CS)

Skylar Rizzo[1], Amy L. Donate[1], Michael Jones[1], Kelsey Manko[1], Katherine E. Santostefano[2], Nao Terada[2], Fowzan S. Alkuraya[3], Peter B. Kang[1], Christina A. Pacak[1]
[1]Pediatrics, University of Florida, Gainesville, FL, [2]Pathology, University of Florida, Gainesville, FL, [3]Genetics, Alfaisal University, Riyadh, Saudi Arabia

Cockayne Syndrome (CS) is a rare, autosomal recessive, neurodegenerative disorder characterized by deficiencies which all contribute to an overall phenotype of premature aging. The underlying cause is a defect in genes involving DNA repair mechanisms. This includes CSA and CSB as well as several genes associated with Xeroderma Pigmentosum (XP). Specifically, Xeroderma Pigmentosum Group G (XPG) is a disorder with two distinct clinical presentations: photosensitivity alone (XP) and photosensitivity with neurodegeneration (XP-CS).

XPG's role as an endonuclease in nucleotide excision repair following UV exposure is well-described and explains the photosensitivity phenotype, yet a separate function for XPG that explains the neurological/early aging deficit that occurs in some patients remains obscure. Other CS proteins known to be involved in nuclear DNA repair have been shown to also function as free radical scavengers in mitochondria. As growing bodies of data illustrate the importance of mitochondria in aging, neuronal cell development and maintenance, and tissue repair, we hypothesize that XPG is trafficked to mitochondria where it could play an important role in mitochondrial function via free radical regulation. Such a deficiency could explain the neurodegeneration and multi-system early aging phenotype observed in CS patients. Our preliminary data (expansion rates, oxygen consumption, ATP generation, free radical sensitivity, Metronidazole [a drug that causes liver failure in CS patients] sensitivity) support this hypothesis through demonstration of decreased viability and mitochondrial function in fibroblasts derived from XPG patients displaying the XP-CS phenotype but not in those from patients displaying the XP phenotype alone. Mitochondrial isolations are being performed to confirm the presence of XPG in these organelles, as well as to better evaluate mitochondrial function.

These characterizations will be used as outcome measures to determine the efficacy of AAV mediated gene therapy for XP-CS. A functional CMV-XPG plasmid has been cloned, and is being packaged into Adeno-associated virus (AAV). Further analyses in XP and XP-CS patient induced pluripotent stem cells differentiated into neurons and an XPG mouse model which closely replicates the human phenotype will yield useful information regarding the dual roles of this protein and provide data to support translation of gene therapy for CS. If successful, this will be the first therapeutic measure to demonstrate correction of the debilitating effects of XP-CS.

Gene Therapy for CNS Diseases

361. IV Gene Therapy Corrects Feline GM1 Gangliosidosis Long Term

Heather Edwards[1], Ashley Randle[1], Miguel Sena-Esteves[2], Douglas Martin[1]
[1]Auburn University, Auburn, AL, [2]University of Massachusetts, Worcester, MA

GM1 gangliosidosis is a fatal neurodegenerative disease that affects all ages. Intracranial injection of adeno-associated viral (AAV) gene therapy has resulted in a >6 fold increase in lifespan. While untreated GM1 cats live to 8.0±0.6 months, many treated cats are still alive at 5-6 years of age (*Sci Transl Med* 2014. **6**, 231). To bypass the invasiveness of brain injection, AAV9 at 1.5e13 vg/kg was injected into the cephalic vein in two GM1 cats at one month of age. Currently over 2 years of age (Fig. 1A), IV treated cats have been followed with biomarkers derived from blood, urine, cerebrospinal fluid (CSF), electrodiagnostics, abdominal ultrasound, 7T magnetic resonance imaging (MRI), and MR spectroscopy. Neurologic abnormalities are limited to mild hindlimb muscle atrophy and fine ear tremors, with remarkable preservation of brain architecture (Fig. 1B). Evaluation of CSF showed complete normalization ($p<0.05$) of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) compared to untreated GM1 cats (Fig 1.C). The defective lysosomal enzyme, β-galactosidase, was increased to ~15 fold normal levels in CSF (Fig. 1D). MR spectroscopy revealed correction of brain metabolites associated with microgliosis (myoinositol; Ins), neuroaxonal loss

(N-acetylaspartate; NAA) and demyelination (glycerophosphocholine and phosphocholine; GPC+PCh) (Fig. 1E), and metabolite levels correlated with clinical signs ($R^2 = 0.66$, 0.50 and 0.63, respectively). No evidence of tumorigenesis or peripheral toxicity has been noted. Intravenous administration of AAV gene therapy shows promise as an alternative for intracranial administration, although further experiments are required to determine maximum survival.

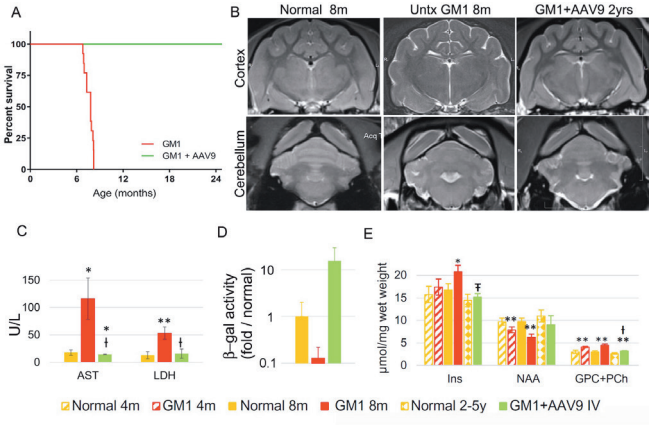


Figure 1. *Effect of IV gene therapy in the GM1 cat*. A) Survival curve of GM1+AAV cats. B) 7T T2 weighted MRI of normal, GM1 and GM1+AAV treated cats. C) CSF levels of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). D) CSF levels of the therepeutic enzyme β-galactosidease (β-gal). D) MR spectroscopy of the feline brain * $p<0.05$ and ** $p<0.01$ from normal; ł $p<0.05$ and Ŧ $p<0.01$ from GM1 cats.

362. Translatable Gene Therapy for Infantile Neuronal Lipofuscinosis

Alejandra J. Rozenberg[1], Sahana Nagabhushan Kalburgi[1], Steven J. Gray[2]
[1]Gene Therapy, University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]Department of Ophthalmology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Infantile Neuronal Ceroid Lipofuscinosis (INCL) is a rare lysosomal storage disease caused by mutations in the *CLN1* gene, which encodes the protein palmitoyl-protein thioesterase-1 (PPT1). In the absence of the PPT1 enzyme, osmiophilic granules accumulate in cells, eventually leading to cell dysfunction and neurodegeneration. In the classic form in human patients, the damage from the storage material and resulting cell death causes visual failure, speech and motor deterioration, and seizures to appear between the ages of 6 and 24 months, with premature death, usually between 3 to 5 years of age. INCL mice (CLN1 knock-out) accurately model the disease symptoms and pathogenesis, leading to premature death at approximately 8 months of age. Previous results from Mark Sands' laboratory have shown that intracranial administration of AAV5 vectors in newborn INCL mice results in a significant extension of lifespan of approximately 50% along with improvements in motor function. While encouraging, the translation of this approach in humans is complicated by the reduced relative biodistribution of the vector after intracranial administration when moving from a rodent to human brain, and because a newborn mouse corresponds to a prenatal time period in humans. With the ability of AAV9 to cross the blood-brain barrier, intravenous (IV) administration of the vector can achieve widespread distribution of the transgene in the Central Nervous System (CNS), while intrathecal (IT) administration of AAV9 vectors into the cerebrospinal fluid (CSF) can achieve a similar CNS distribution at a considerably lower dose more amenable to human translation. Currently, self-complementary AAV9 vectors are being used in ongoing Phase I human clinical trials for Spinal Muscular Atrophy, administered IV (NCT02122952), and for Giant Axonal Neuropathy, administered IT (NCT02122952). We have pursued two global and translationally-relevant gene transfer approaches for INCL: IT delivery of scAAV9/CLN1 into the CSF of the lumbar cistern at a dose of $7x10^{10}$ vg per mouse, and IV delivery of scAAV9.47/CLN1, a liver-detargeted vector, at a dose of $1x10^{12}$ vg per mouse. Both approaches were evaluated at different ages: 1 week and 4 weeks (pre-symptomatic), 20 weeks (early-symptomatic) and 26 weeks (post-symptomatic). While we failed to rescue mice injected at 20 weeks or later, mice treated at 1 and 4 weeks of age increased their survival to approximately twice their expected lifespan (ongoing), concurrent with improvement of their quality of life. These results will be compared to a cohort of mice injected as neonates, which are alive at 1 year and being monitored for long-term benefits. Our results indicate that, at the current doses, either treatment approach is effective early in the disease course, providing a dramatic survival benefit and improved quality of life, but it is wholly ineffective once significant symptoms have emerged. The IT route offers a comparable rescue at < 1/10 the dose, making this approach potentially more favorable for human translation. We propose this as a realistic and readily translatable approach to treat INCL.

363. Improvement of Sandhoff Phenotype Following Intravenous Injection of Adeno-Associated Viral Vector Expressing a Hexosaminidase Isoenzyme in Adult Sandhoff Mice: Preclinical Safety and Efficacy Study

Karlaina J. L. Osmon[1], Evan Woodley[2], Patrick Thompson[1], Subha Karumuthil-Melethil[3], Steven Gray[4], Jagdeep Walia[5]
[1]Centre for Neuroscience Studies, Queen's University, Kingston, ON, Canada, [2]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada, [3]Gene Therapy Center, University of North Carolina, Chapel Hill, NC, [4]Gene Therapy Center and Department of Opthamology, University of North Carolina, Chapel Hill, NC, [5]Centre for Neuroscience Studies, Department of Biomedical and Molecular Science, Medical Genetics, Queen's University, Kingston, ON, Canada

GM2 gangliosidosis disorders stem from a Hexosaminidase A (HexA) isoenzyme deficiency. In humans, HexA is the sole enzyme able to catabolize GM2 ganglioside (GM2). The inability to effectively catabolize GM2 leads to neurodegeneration of the central nervous system. HexA is comprised of 2 subunits (α, β) and works with the GM2 activator protein (GM2AP). In the recent work by Tropak et al. (Mol Ther Met Clin Dev, *in press*), a hybrid subunit, named µ-subunit, was created (patent pending) by combining the stabilization and GM2AP binding sites of the β-subunit while conserving the catalytic properties of the α-subunit. The 'µ'-subunit, coded by *HEXM*, can homodimerize and form a stable, functional enzyme, named HexM, which can interact with GM2AP to hydrolyse GM2. Previous work for successful correction of Sandhoff mice using AAV was only shown in neonatal mice (with immature blood-brain barrier (BBB)) and may not directly help in designing a human clinical trial. In the current study, we examined the efficacy/safety of IV injections of the scAAV9/*HEXM* vector at two doses in adult SD mice (with mature BBB). In addition, we also tested if an adjunct IV injection of mannitol provides any enhancement in efficacy. At 6 weeks old, the vector was injected via tail vein in cohorts of n=17 and n=15 SD mice at 2.5E+12 or 1.0E+13 vg/mouse, respectively. Another cohort of 16 mice received IV mannitol (3g/kg) prior to an IV injection of 2.5E+12 vg scAAV9/*HEXM*. Some mice from low dose group were euthanized at 16 weeks for direct analysis with untreated SD control mice, while the remainder were left until for terminal survival. Analysis of survival benefit, locomotor behaviour, biochemical and

molecular parameters were performed. While untreated SD mice had a 16 week humane endpoint, 4 of 7 mice in higher dose group are now surviving past 56 weeks, 1 of 12 mice in the low dose cohort, and 4 of 9 mice in the mannitol cohort are surviving past 52 weeks. These increases in survival are all highly significant compared to the ~16 week humane endpoint of untreated SD mice. Behaviourally, there are no major significant differences in locomotion between the groups until after 15 weeks, when the adjunct mannitol group significantly outperforms the PBS group. Survival and behaviour monitoring, and the biochemical analyses for this study are ongoing. The preliminary results from this study show delayed onset of the SD phenotype with a single AAV9/*HEXM* injection and a significant benefit of a pre-injection of IV mannitol. This study is the first to show that an IV gene transfer using a scAAV/*HEXM* vector can provide survival and behavioural benefit in adult SD mice especially with adjunct use of mannitol. We propose that these results can advise the design of a human gene therapy trial for SD and the related Tay-Sachs disease.

364. Neurological Correction of Mucopolysaccharidosis IIIB Mice by Haematopoietic Stem Cell Gene Therapy

Stuart M. Ellison[1], Rebecca Holley[1], Daniel Fil[1], John Mc Dermott[1], Nisha Senthivel[1], Alex Langford-Smith[1], Fiona Wilkinson[1], Simon Jones[2], Rob Wynn[3], Shih-hsin Kan[4], Patti Dickson[4], Brian Bigger[1]
[1]Stem Cell and Neurotherapies, University of Manchester, Manchester, United Kingdom, [2]Centre for Genomic Medicine, Royal Manchester Children's Hospital, Manchester, United Kingdom, [3]Paediatric Haematology, Royal Manchester Children's Hospital, Manchester, United Kingdom, [4]Los Angeles Biomedical Research Institute, Harbor-UCLA Medical Center, Torrance, CA

Mucopolysaccharidosis Type IIIB (MPSIIIB) is a paediatric, autosomal recessive Lysosomal Storage Disease (LSD) caused by deficiency of α-N-acetylglucosaminidase (NAGLU), an enzyme in the heparan sulfate (HS) degradation pathway. Absence of NAGLU leads to the accumulation of partially degraded HS glycosaminoglycan in cell lysosomes, giving rise to cellular dysfunction with devastating clinical consequences. Individuals affected by this fatal disease exhibit severe central nervous system degeneration with progressive cognitive impairment and behavioural problems, alongside more attenuated somatic symptoms. There are currently no effective treatments available. Enzyme replacement therapy with recombinant NAGLU enzyme is ineffective for MPSIIIB since enzyme cannot cross the blood brain barrier (BBB) to where it is needed. Modified recombinant NAGLU enzymes that utilise the insulin growth factor II (IGFII) peptide to facilitate improved uptake across the BBB are currently in development. Haematopoietic stem cell gene therapy (HSCGT) is a promising therapeutic strategy that can circumvent the BBB via monocyte trafficking and engraftment in the brain, allowing delivery of enzyme by cross correction. We have developed a novel stem cell gene therapy approach to investigate the therapeutic potential of HSCGT for MPSIIIB. We designed two lentiviral vectors expressing therapeutic enzyme; the first vector expressing codon optimised NAGLU, and the second expressing a NAGLU.IGFII fusion to aid cellular uptake, both driven by the myeloid specific promoter CD11b and compared these in autologous MPSIIIB transplants against a normal WT bone marrow transplant. Here we present for the first time neurological correction of MPSIIIB mice by HSCGT. We observed correction of the MPSIIIB behavioural phenotype in treated mice to wild-type levels with normalisation of path length, average speed, frequency entering the centre and duration of speed >100mm/s in open field tests. In addition, we observed a significant correction of astrogliosis and lysosomal compartment size in the brains of CD11b. NAGLU LV treated mice with an accompanied normalisation of inflammatory cytokines TNFα, IL1B and IL1RN. Furthermore, NAGLU enzyme activity was substantially increased in the brain. Interestingly, WT transplant alone was able to mediate a partial brain correction, although levels of inflammation and lysosomal storage remain high.

365. Long-Term Toxicology Evaluation of AAVrh.10hARSA Administration to the CNS of Nonhuman Primates to Treat Metachromatic Leukodystrophy

Dolan Sondhi[1], Jonathan B. Rosenberg[1], Bishnu P. De[1], Sebastien Monette[2], Alvin Chen[1], Thomas Flagiello[1], Elisa Caffrey[1], Stephen M. Kaminsky[1], Patrick Aubourg[3], Ronald G. Crystal[1]
[1]Weill Cornell Medical College, New York, NY, [2]Memorial Sloan Kettering Cancer Center, New York, NY, [3]INSERM U1169 and University Paris-Sud, Le Kremlin-Bicêtre, France

Metachromatic leukodystrophy (MLD), a lysosomal storage disorder caused by the deficiency of the enzyme arylsulfatase A (ARSA), is a recessive, fatal neurodegenerative lysosomal storage disease associated with intracellular accumulation of sulfatides in the CNS. The disease is fatal, with no approved therapy. The focus of this study is to test the hypothesis that direct CNS administration of AAVrh.10hARSA (AAVrh.10 serotype vector coding for the human ARSA cDNA) to the CNS of nonhuman primates at doses scalable to humans has an acceptable long term safety profile. Safety of intraparenchymal administration of AAVrh.10hARSA was evaluated following its administration to the CNS of non-human primates (NHPs, African Green monkeys, n=24) at 12 locations in the white matter centrum ovale at two different doses (total dose $2.85x10^{10}$ genome copies (gc), equivalent to human clinical dose of $2.85x10^{11}$ gc previously used via a similar route of administration in humans, and $1.5x10^{12}$ gc, a 1.7-log higher dose, equivalent to a human dose of $1.5x10^{13}$ gc). NHPs administered in a similar fashion with AAVNull vector (a vector with an expression cassette without a translatable sequence) and sham (PBS) were used as controls. Target locations were determined using CAT and MRI imaging. The groups (n=6/group) were sacrificed at 1, 13, 26, and 52 wk following vector administration to determine short and long-term effects of treatment. General safety, hematologic, serum chemistry and CNS histopathology parameters were assessed at several time points up to 1 yr after vector administration. Additional in-life safety assessments included behavioral videotaping and CNS monitoring by magnetic resonance imaging (MRI) at 13, 26, and 52 wk post-administration. The vector-administered groups did not differ from the controls in any parameter of general assessment or comprehensive blood profile (complete blood count, chemistry panel). Blinded videotape analysis of NHP behavior pre-surgery and post-administration showed no discernible neurological differences. No significant adverse effects were observed in animals treated with low dose AAVrh.10hARSA; the only abnormal observation was presence of limited and reversible, minimal to mild T and B cell focal infiltrates at the CNS administration sites, findings that were corroborated by MRI. Animals treated with the higher dose of both the ARSA and Null vectors ($1.5x10^{12}$ gc) demonstrated significant infiltrates of T cells, B cells and activated microglial cells and/or macrophages in the brain at the sites of administration. Similar observations were made from MRIs images, i.e., the higher dose may be associated with some local adverse effects, although these findings did not have clinical consequences on in-life data, complete blood count, serum chemistry or behavior in these NHPs. Together these findings demonstrate the safety of AAVrh.10hARSA administration at $2.85x10^{10}$ gc, equivalent to a human dose of $2.85x10^{11}$ gc and support the use of this vector as a vehicle for therapy of MLD.

366. Hitting Two Birds with One Stone: How Efficacious Pre-Clinical Gene Therapy Cures Canavan Disease and Sheds Light onto the Pathomechanism

Dominic J. Gessler[1], Danning Li[1], Hongxia Xu[1], Qin Su[1], Reuben Matalon[2], Guangping Gao[1]
[1]Gene Therapy Center, UMass, Worcester, MA, [2]University of Texas Medical Branch, Galveston, TX

Almost 90 years ago, Canavan Disease (CD) was described for the first time. Since then, the scientific community has unsuccessfully sought to cure this devastating leukodystrophy and understand its pathomechanism. Early hope for gene therapy was fueled with the cloning of the disease causing gene, Aspartoacylase, in 1993. Unfortunately, the only clinical trial for CD gene therapy failed to show significant clinical improvements in Canavan patients. At that time, animal models for CD were just engineered and comprehensive pre-clinical evaluation of CD gene therapy was missing. Earlier, we reported that our 1st generation IV delivered pre-clinical gene therapy was able to rescue early lethality and partially restored motor function in a mouse model of CD. Now in its 3rd generation, our gene therapy cures the disease in Canavan mice by a single intravenous injection, shown by behavioral, cognitive, and neuropathology tests. Taking advantage of this complete reversal of the disease, we used whole brain neurometabolome profiling to closely monitor the molecular efficacy and mechanism(s) in curing Canavan disease in mouse. Hierarchical cluster analysis (HCA) shows complete restoration of the disease associated metabolic derangements, including the array of detected myelin lipids. In the next step, we hypothesized that the metabolic nature of Canavan Disease mandates the origin of its pathomechanism in the metabolic regulation. We identified a specific dysregulation in the energy metabolism *in vitro* and *in vivo* that suggests the self-digestion of myelin for energetic purposes, calling current hypotheses about the Canavan disease pathomechanism into question. Currently, we are intensifying our insight into this mechanism by mircoRNAome, transcriptome analyses, a series of in vitro cell culture models, as well as supplementary and alternative strategies for the treatment of Canavan Disease. In summary, our data demonstrates strong evidence that rAAV mediated pre-clinical gene therapy not only cures the Canavan phenotype but also corrects the extensive neurometabolome, which provides meticulous evidence for gene therapy's high efficacy. Furthermore, we revealed a new pathomechanism that supports a paradigm shift in our perception of the function of ASPA and NAA in Canavan disease and their potential implications in other CNS and metabolic disorders in general.

367. A Dopamine Gene Therapy for Advanced PD: 4 Years Phase I/II Clinical Update

Stéphane Palfi[1], Jean Marc Gurruchaga[1], Scott Gs Ralph[2], Colin Watts[3], Philip Buttery[3], Helene Lepetit[1], James Miskin[2], Gaetane Gouello[1], Sonia Lavisse[4], Gilles Fenelon[1], Claire Thiriez[1], Pierre Brugières[1], Roger Barker[3], Philippe Hantraye[4], Kyriacos Mitrophanous[2]
[1]Paris University UPEC, Creteil, France, [2]Oxford Biomedica, Oxford, United Kingdom, [3]Cambridge University, Cambridge, United Kingdom, [4]CEA/I2BM/MIRCEN, Fontenay aux Roses, France

Parkinson's disease (PD) is a neurodegenerative condition that results in a progressive degeneration of dopaminergic neurons. The dopamine (DA) precursor L-Dopa and dopamine agonists provide the primary standard of care and demonstrate good therapeutic benefit in the early stages of disease. However, their long term use is associated with severe motor side effects that are at least partially caused by the fluctuating nature of dopaminergic stimulation that arises from oral drug administration. As such, a therapy that provides a more continuous and local supply of dopamine to the site of pathology provides a potential approach for the development of new therapeutic strategies. ProSavin® is a gene therapy product that utilises a lentiviral vector to transfer three genes that are critical for de novo dopamine biosynthesis in the striatum, that is depleted of dopamine in PD. Fifteen advanced PD patients have received ProSavin® in three dose cohorts. ProSavin® has been demonstrated to be safe and well tolerated at all doses evaluated to date. No serious adverse events related to the study drug or surgical procedures were observed. All patients demonstrated improvement over baseline at both 6 and 12 months, which were sustained in some patients up to four years. Patients in the highest dose cohort demonstrated the greatest improvement in motor scores, a reduction in requirement for oral dopaminergic medication, and evidences of DA production by PET imaging. In summary, ProSavin® was safe and well tolerated in advanced PD patients. To increase the dose of ProSavin® by administering more vectors is not desirable due to physical constraints. Therefore we have generated OXB-102, an improved version of ProSavin®, that expresses the same enzymes but with an increased DA production per genetically modified cell. An update on OXB-102 will be presented.

368. Advances in Fragile X Gene Therapy Using Adeno-Associated Viral Vectors Coding for the Fragile X Mental Retardation Protein

Jason Arsenault, Yosuke Niibori, David R. Hampson, Shervin Gholizadeh
Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, ON, Canada

Fragile X syndrome (FXS) is a severe debilitating neurodevelopmental disorder characterized by a loss of proper translational control at the synapse. In FXS, an aberrant CGG trinucleotide expansion upstream of the Fragile X Mental Retardation Protein (FMRP) gene causes drastic downregulation of this translational modulator. The absence of FMRP impairs synaptic plasticity and leads to mental retardation and autistic spectrum-related phenotypes. To correct this neurological disorder we recently devised an adeno-associated viral (AAV) vector encoding FMRP where its cellular tropism was controlled by the neuron-specific synapsin promoter. Following intracerebroventricular (i.c.v.) injection in neonatal Fmr1 KO mice, transgene expression remained stable for over 7 months in terminally differentiated neurons. The FMRP transgene corrected PSD-95 protein hypo-expression in the cortex of Fmr1 KO animals, as well as lowered MeCP2 protein over-expression. Behavioral endophenotypes including hyperactivity, non-social anxiety, pre-pulse inhibition, repetitive stereotypies, and social dominance were fully or partially corrected using this viral construct. We combined i.c.v. injection with additional injections into the parenchyma of refractory brain regions to achieve wider transduction, while avoiding potentially pathological transgene over expression effects. This ongoing translational study enables us to determine the proper cellular tropism and the range of FMRP expression required for rescue, as well as the brain region dependent correlation of autistic behaviors implicated in FXS neuropathology. These findings are relevant to gene therapy strategies for treating human FXS, as well as other neurodevelopmental disorders.

369. Therapeutic Effectiveness of AAV-Mediated Iduronidase Delivery to the CNS Following Intravenous Administration in a Murine Model of Mucopolysaccharidosis Type I

Lalitha Belur[1], Thuy An Tran[1], Kelly M. Pedersen[1], Maureen Riedl[2], Lucy Vulchanova[2], Karen Kozarsky[3], Walter C. Low[4], R. Scott McIvor[1]

[1]Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, [2]Neuroscience, University of Minnesota, Minneapolis, MN, [3]REGENXBIO Inc., Rockville, MD, [4]Neurosurgery, University of Minnesota, Minneapolis, MN

Mucopolysaccharidosis type I (MPS I) is an inherited metabolic disease caused by deficiency of alpha-L-iduronidase (IDUA), resulting in accumulation of heparin and dermatan sulfate glycosaminoglycans (GAGs). Individuals with the most severe form of the disease (Hurler syndrome) suffer from neurodegeneration, mental retardation, and death by age 10. Current treatments for this disease include allogeneic hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT). However, these treatments are insufficiently effective in addressing CNS manifestations of the disease. Our goal is to improve therapy for severe MPS I by supplementing current ERT and HSCT with IDUA gene transfer to the CNS, thereby preventing neurological manifestations of the disease. In this study we tested the ability of intravenously administered AAV serotypes 9 and rh10 (AAV9 and AAVrh10) to cross the blood brain barrier for delivery and expression of the IDUA gene in the CNS. 4-5 month old adult MPS I animals were infused intravenously via the tail vein with either an AAV9 or AAVrh10 vector encoding the human IDUA gene. Blood and urine samples were collected on a weekly basis until the animals were sacrificed at 10 weeks post-injection. Plasma IDUA activities in treated animals were close to 1000-fold higher than that of heterozygote controls at 3 weeks post-injection. Brains, spinal cords, and peripheral organs were analyzed for IDUA activity, clearance of GAG accumulation, and IDUA immunofluorescence of tissue sections. Treated animals demonstrated widespread restoration of IDUA enzyme activity in all organs including the CNS. High levels of IDUA enzyme activity were observed in the brain and spinal cord, which ranged from 40 to 300-fold higher than heterozygote controls, while levels in peripheral organs were also high, ranging from 100 to 1000-fold higher than control animals. Levels of urinary and tissue GAGs were significantly reduced starting at 3 weeks after vector infusion, with restoration of normal GAG levels by the end of the study. These data demonstrate the non-invasive effectiveness of systemic intravenous AAV9 and AAVrh10 vector infusion in counteracting CNS manifestations of MPS I.

370. Reconstruction of the Nigrostriatal Pathway in Parkinsonian Macaques

Diego Pignataro[1,2,3], Diego Sucunza[1,2,3], Alberto J. Rico[1,2,3], Iria G. Dopeso-Reyes[1,2,3], Elvira Roda[1,2,3], Africa Vales[1,3], Gloria Gonzalez-Aseguinolaza[1,2,3], Nick Kholodilov[4], Robert E. Burke[4], José L. Lanciego[1,2,3]

[1]CIMA, Pamplona, Spain, [2]Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas, Madrid, Spain, [3]Instituto de Investigación Sanitaria de Navarra (IdiSNA), Pamplona, Spain, [4]Udall Parkinson´s Disease Research Center, Columbia University Medical Center, NY

The field of Gene Therapy in the CNS has recently witnessed a number of major conceptual changes. At present, ongoing strategies are focused on using vectors carrying genes to further modify brain circuits of interest. It is expected that these approaches will result in a great therapeutic potential being sustained by the induced changes in brain circuitry. Indeed, for the first time these advances will allow the implementation of "disease-modifying" therapies, e.g., trying to arrest or even revert the natural course of Parkinson's disease. Here we are using hRheb(S16H)-carrying Adeno-associated virus (AAV) vectors in parkinsonian macaques, in an attempt to reconstruct the damaged nigrostriatal pathway. Preliminary results reported here stand on the intracerebral delivery of h-Rheb-carrying AAV serotype 5 in the substantia nigra of two MPTP-treated macaques showing a severe parkinsonian syndrome. After a follow-up of six months, both macaques showed a lack of motor improvement, together with no changes on the conducted microPET neuroimage scans. However, the histopathological analysis revealed a moderate degree of axonal reinnervation in the putamen nucleus following a viral infection limited to 10-12 dopaminergic neurons per animal. These results, so far insufficient to elicit any motor/neuroimage improvements, are very appealing and indeed represent the first evidence that a damaged dopaminergic circuit can be reconstructed in adult parkinsonian macaques. A number of ongoing strategies are currently under development in an attempt to improve the amount of neurons being infected with the hRheb gene, therefore leading to a more complete reconstruction of the nigrostriatal pathway.

371. AAV9-Mediated Over-Expression of Hippocampal Neuritin Prevents Extremely Low-Frequency Electromagnetic Field Exposure-Induced Impairment of Recognition Memory

Yanai Mei[1], Qianru Zhao[1], JunMei Lu[1], Jinjing Yao[1], Zhengyu Zhang[1], Chen Ling[2]

[1]Institutes of Brain Science, School of Life Sciences and State Key Laboratory of Medical Neurobiolog, Fudan University, Shanghai, China, [2]Division of Cellular and Molecular Therapy, Department of Pediatrics, University of Florida, Gainesville, FL

Extremely low-frequency (50Hz) electromagnetic fields (ELF MFs) are ubiquitously present in various environments during daily life. The major sources of ELF MFs pertaining to the general public are in-house installations, household appliances and power lines. Animal studies have shown that ELF MFs exposure may interfere with the activity of brain cells, thereby generating behavioral and cognitive disturbances. However, the underlying mechanisms and possible preventions are still unknown. In our preliminary studies, we found that ICR mice that were exposed to ELF MFs had deficits in hippocampus-dependent recognition memory but no concurrent changes in their locomotor activity. Further experiments revealed that ELF MFs exposure reduced dendritic spine densities of hippocampal CA1 pyramidal cells. Meanwhile, there was no significant effect on dendritic spine diameter or length. Since previous work has shown that neuritin, an important neurotrophin, has the ability to increase the spine density of hippocampal pyramidal neurons, we decided to explore whether neuritin is capable of reversing ELF MFs exposure-induced decreases in dendritic spine density. To this end, adeno-associated virus serotype 9 (AAV9) vectors carrying human neuritin cDNA were produced and stereotaxically injected into the CA1 region of the mouse hippocampus (0.2μl per mouse) 7 days before exposure to ELF MFs. Two control groups were injected in hippocampus with the same volume of AAV-GFP or saline. Western blot analysis indicated that the expression of neuritin was increased by 60.2 ± 5.8% in the hippocampus. We then quantified the effects of AAV9-mediated neuritin over-expression on hippocampal dendritic spine number and subsequent recognition memory in mice of both non-ELF MFs and ELF MFs exposure groups. After a 2.5-week infection period, a significant increase in spine density was observed in the AAV-neuritin group. On the other hand, there was no significant difference in dendritic diameter or length among all three groups of mice. Additionally, after ELF MFs exposure (1mT) for 12 h/day for 10 days, the spine density of pyramidal neurons

obtained from neuritin-overexpression mice was no longer reduced. Consequently, the recognition memory test of AAV-neuritin mice showed a significant increase in recognition index when compared to AAV-control mice. Collectively, our study provided evidence for the association between ELF MFs exposure, impairment of recognition memory, and resulting changes in hippocampal dendritic spine density. Furthermore, overexpression of neuritin has the ability to prevent the ELF MFs-exposure-induced effect by increasing the hippocampal spine density.

372. U1 snRNA-Mediated Correction of a Splicing Error of the Dopa Decarboxylase Gene

Ni-Chung Lee[1], Yu-May Lee[1], Yin-Hsiu Chien[1], Barry Byrne[2], Wuh-Liang Hwu[1]
[1]National Taiwan University Hospital, Taipei, Taiwan, [2]Powell Gene Therapy Center, University of Florida, Gainesville, FL

Aromatic l-amino acid decarboxylase (AADC) deficiency is an inborn error of monoamine neurotransmitter synthesis that resulted in dopamine and serotonin deficiency. The *DDC* gene founder mutation IVS6+4A>T is highly prevalent in Chinese patients with AADC deficiency. In this study, we employed U1 snRNA to correct this splicing error. We first used a modified U1 snRNA sequence (IVS-AAA) that matched both the mutated nucleotide and exonic U1 binding sequences corrected the splicing error of both mutated human *DDC* minigene and mouse artificial splicing construct *in vitro*. We further injected an adeno-associated viral (AAV) vector to express IVS-AAA in the brain of a knock-in mouse model with either $1x10^{10}$ vg or $2x10^{10}$ vg per mouse. This treatment was well-tolerated and slightly improved the survival and brain dopamine levels of mice with AADC deficiency. Therefore, U1 snRNA-mediated gene therapy can be a promising method to treat genetic diseases caused by splicing errors, but the efficiency of such a treatment needs improvements.

373. Optimization of AAV-Gene Therapy for GM1-Gangliosidosis

Lorelei Stoica[1], Sophia Todeasa[1], Jaclyn Lata[1], Douglas Martin[2], Miguel Sena-Esteves[1]
[1]UMMS, Worcester, MA, [2]Auburn University, Auburn, AL

GM1-gangliosidosis is an autosomal recessive disease caused by mutations in the GLB1 gene encoding for the lysosomal acid beta-galactosidase (βgal) enzyme. The resulting enzymatic deficiency leads to accumulation of GM1-ganglioside in neurons and eventually massive neurodegeneration. The incidence of GM1-gangliosidosis is estimated at 1:100,000-200,000 live births, and there is currently no available treatment. Intracranial delivery of recombinant adeno-associated virus (AAV) vectors has been shown to be highly effective in animal models of this disease. The choice of injection sites and promoters driving transgene expression are important parameters to translate into clinical trials a safe and effective AAV-based therapy for GM1 gangliosidosis. We performed a study using AAVrh10 vectors to determine the safest and most effective promoter and delivery route combination for AAVrh10-βgal gene therapy in a GM1-mouse model. We used a combination of two direct intracranial injection site - bilateral thalamic and deep cerebral nuclei infusions (Th+DCN), or bilateral thalamic and CSF infusion into one cerebral lateral ventricle (Th+ICV). We tested three different promoters of varying strengths. The CBA promoter, composed of the chicken beta actin promoter with an enhancer element and an artificial intron; the CBi, identical to the previous promoter but lacking the enhancer element; the CB, the basic chicken beta-actin promoter without an enhancer or intron. All vectors were delivered at a total dose of 4E9 (Th+DCN) or 1E10 (Th+ICV) vector genomes. At four weeks post injection, we analyzed the brain histologically, for beta-galactosidase expression and decrease in lysosomal storage. Additionally, we analyzed the enzyme activity of beta-galactosidase, as well as the total GM1-gangliosidosis storage by enzymatic assays and LC-MS/MS mass spectrometry, respectively. The AAVrh10-CBA-βgal vector restored GM1 ganglioside levels to normal, and generated the highest βgal activity of all three vectors. Additionally, the distribution was more extensive for the AAVrh10-CBA vector, showing distribution to the frontal cortex, while for the other vectors the enzyme was mostly restricted to the injection site and the immediate area. However, at this vector dose we did not detect any apparent toxicity or cell death. Thus, we determined that the AAVrh10-CBA-βgal vector is safe and effective, and thus appropriate to carry out long-term therapeutic studies in GM1 mice toward a clinical trial.

374. Developing Bone Marrow Transplant and Lentiviral Vectors to Treat Friedreich Ataxia

Sze Hwee Ong[1], Dong C. Zhang[2], Chou H. Sim[2], Matthew Burton[2], Dean Phelan[2], Sarah E. M. Stephenson[2], Gabrielle R. Wilson[2], Donald F. Newgreen[2], Anthony J. Hannan[3], Paul J. Lockhart[2], Martin B. Delatycki[2], Marguerite V. Evans-Galea[2]
[1]Department of Paediatrics, The University of Melbourne, Murdoch Childrens Research Institute, Melbourne, Australia, [2]Murdoch Childrens Research Institute, Melbourne, Australia, [3]Neural Plasticity, The Florey Institute of Neuroscience and Mental Health, Melbourne, Australia

Friedreich ataxia (FRDA) is a debilitating neurodegenerative disorder with disease onset at around 10-15 years of age. It affects 1 in 29 000 individuals of Caucasian descent and is characterised by progressive gait and limb ataxia. This leads to an ongoing loss of motor coordination which results in affected individuals becoming wheelchair-dependent within 15 years after disease onset - decreasing their quality of life. FRDA patients also experience other disease symptoms such as cardiomyopathy, with heart complications being the leading cause of death with life expectancy decrease to 30-40 years. There is currently no treatment which can cure or slow the neurodegeneration inherent to FRDA and patients undergo various symptomatic treatments to manage disease symptoms. It is thus essential to develop new treatments effective at slowing disease progression to improve the quality of life of FRDA patients. FRDA is caused in most cases by a homozygous GAA trinucleotide repeat expansion within intron 1 of *FXN* which encodes the frataxin, a mitochondrial protein. As the expansion only reduces the level and does not alter the frataxin protein structure, it is predicted that increasing frataxin will be therapeutically beneficial to FRDA patients. This research aims to introduce frataxin into cells via cell and gene therapy as a potential treatment for FRDA. This introduction of frataxin will not illicit an immune response as patients do produce frataxin at very low levels. We are investigating if transplanting wild-type bone marrow into an irradiated FRDA mouse model increases frataxin and alleviates the neurological phenotype of slight coordination impairment and locomotor defects which develop around six months of age. Reconstitution of the haematopoietic system with GFP-positive donor bone marrow cells in corrected recipient mice indicated successful engraftment following transplant. GFP-positive cells also successfully engrafted into the dorsal root ganglia (DRG) and spinal cord, both major sites of neuropathology in FRDA, of corrected mice - demonstrating low-level chimerism. Immunofluorescence studies showed increased neuronal marking in the DRG of corrected mice, particularly proprioceptive neurons which are highly affected in FRDA patients. Increased frataxin protein is observed in some tissues of corrected mice. Corrected mice also exhibited significant improvement in motor coordination post-transplant. For autologous gene therapy via bone marrow transplant, we are currently developing a lentiviral vector that over-expresses

frataxin. These data together illustrate the potential of bone marrow transplant in correcting FRDA *in vivo* and provide an avenue for the delivery of therapeutic viral vectors for gene therapy.

375. TDP-43 Interacts with Cannabinoid Type 1 Receptor Modulating Its Signaling Properties

Catalin Filipeanu[1], Matthew R. Deshotels[2], Peter Weed[2], Anastasia R. Robinson[1], Blessing Ongulade[1], Peter J. Winsauer[2], Jessie J. Guidry[2]
[1]Pharmacology, Howard University, College of Medicine, Washington, DC, [2]Pharmacology, LSU HSC, New Orleans, LA

To identify new molecular chaperones of cannabinoid type-1 receptors (CB1R), we used a proteomic approach in HEK293T cells, transfected with two spicing variants of this receptor, CB1R and CB1AR. These two isoforms differ only in the composition of the N-terminus domain. Surprisingly, the only protein found to interact differently with CB1R and CB1AR was transactive response DNA-Binding Protein 43 (TDP-43), which is known to be involved in the pathology of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). The specific interactions between CB1R and TDP-43 were also confirmed by co- immunoprecipitation not only in transfected HEK293 cells, as well at the endogenous level in rat crude spinal cord. TDP-43 overexpression decreased CB1R plasma membrane levels by stimulating receptor internalization, but it did not change the total receptor levels. Further, TDP-43 enhanced CB1R ubiquitination to similar levels, as induced by the full CB1R agonist, CP 55,940. Also, overexpression of TDP-43 resulted in reduced effects of CB1R stimulation on cAMP, P-ERK1/2, and P-CREB responses. Present results revealed TDP-43 as an unexpected molecular chaperone of CB1R and these findings may have significance in the regulation of CB1R intracellular trafficking and possibly in the treatment of ALS and FTLD.

376. Overexpression of Pitx3 in the SNpc and Protection from MPTP Toxicity

Joanne Clark-Matott[1], Allison K. Graebner[1], Caroline E. Bass[2], David K. Simon[1]
[1]Neurology, BIDMC, Boston, MA, [2]Department of Pharmacology and Toxicology, University at Buffalo, Buffalo, NY

In the brain, expression of the transcription factor Pituitary Homeobox 3 or Paired-Like Homeodomain Transcription Factor 3 (PITX3) is crucial to the development and maintenance of dopaminergic neurons. During brain development, PITX3 functions in concert with the transcription factors NURR1 and LMX1b to facilitate dopaminergic neuron maturation. In the mature brain, PITX3 promotes the dopaminergic phenotype by positively regulating tyrosine hydroxylase (TH), dopamine transporter (DAT), and vesicular monoamine transporter 2 (VMAT2), either singly or in concert with NURR1. PITX3 also positively regulates expression of the pro-survival factors brain-derived neurotrophic factor (BDNF) and glial-derived neurotropic factor (GDNF). PITX3 is endogenously expressed in the dopaminergic neurons of the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA). However, in Pitx3 null mouse models, perturbations of cell number are observed only in the SNpc, a situation that resembles the relative sparing of the VTA in early-stage Parkinson's disease (PD). Systemic administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) results in the degeneration of SN dopaminergic neurons via a mechanism thought to involve the inhibition of mitochondrial respiration and the generation of reactive oxygen species, both of which are implicated in the pathogenesis of PD. Recent work in muscle demonstrates that PITX3 regulates expression of antioxidant enzymes and genes involved in mitochondrial biogenesis via the transcription factor NRF1. We have overexpressed murine Pitx3 (or mCherry control) from the CMV promoter in the SN of C57BL/6J mice using an AAV2/10 viral vector, and tested for neuroprotection in a sub-chronic MPTP model of PD (5 x 20 mg/kg MPTP over 9 days) by assaying for striatal dopamine, stratial TH and DAT immunoreactivity, and SN neuronal number. We will present results from these assays and changes in expression of Pitx3 target genes (TH, DAT, VMAT2, BDNF and GDNF) after Pitx3 overexpression in the SN.

Musculo-Skeletal Diseases I

377. AAV-Mediated Transfer of FKRP Shows Therapeutic Efficacy in a Murine Model of Limb-Girdle Muscular Dystrophy Type 2i, but Requires Tight Control of Gene Expression

Evelyne Gicquel[1], Natacha Maizonnier[1], Steven J. Foltz[2], William Martin[2], Nathalie Bourg[1], Fedor Svinartchouk[1], Karine Charton[1], Aaron Beedle[2], Isabelle Richard[1]
[1]Genethon, Evry, France, [2]University of Georgia, Athens, GA

Limb Girdle Muscular Dystrophies (LGMD) type 2I, a recessive autosomal muscular dystrophy, is caused by mutations in the Fukutin Related Protein (FKRP) gene. It has been proposed that FKRP, whose function remains unclear, is a participant in α-dystroglycan (αDG) glycosylation, which is important to ensure the cell/matrix anchor of muscle fibers. A knock-in mouse model of LGMD2I was generated to express the most frequent mutation (L276I) encountered in patients. The introduction of the mutation did not alter the expression of FKRP, neither at transcriptional nor at translational levels, but did alter its function since abnormal glycosylation of αDG was observed. In this model, skeletal muscles were functionally impaired from 2 months of age and a moderate dystrophic pattern was evident by histology starting from 6 months of age. Gene transfer with a rAAV2/9 vector expressing *Fkrp* restored the biochemical defects, corrected the histological abnormalities and improved the resistance to eccentric stress in the mouse model was obtained. However, injection of high doses of the vector induced a decrease of αDG glycosylation and laminin binding. Finally, we showed that intravenous injection of the rAAV-*Fkrp* vector into a dystrophic mouse model suffering of dystroglycanopathy due to skeletal muscle-specific Fukutin (*Fktn*) knock-out caused toxicity. The dose-dependent worsening of the dystrophic phenotype suggests requirement for a precise control of its expression**.**

378. Improved Transduction of Canine X-Linked Muscular Dystrophy with rAAV9-Microdystrophin by Introducing Immune Tolerance

Hiromi Hayashita-Kinoh[1,2], Hironori Okada[1,2], Yuko N. Kasahara[1,2], Tomoko Chiyo[2], Kiwamu Imagawa[3], Katsuhiko Tachibana[3], Shin'ichi Takeda[2], Takashi Okada[1]
[1]Dept. of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan, [2]Dept. of Molecular Therapy, National Institute of Neuroscience, NCNP, Tokyo, Japan, [3]JCR Pharmaceuticals Co., Ltd., Hyogo, Japan

Background: Duchenne muscular dystrophy (DMD) is a congenital disease causing progressive deterioration of skeletal and cardiac muscles because of mutations in the dystrophin gene. Supplementation of dystrophin using rAAV is effective to improve pathogenesis of animal models of DMD. However, we have previously reported that local injection of rAAV2 or rAAV8 to canine skeletal muscles without immunosuppression resulted in insufficient transgene expression with potent immune responses. Here we used

DMD dog ($CXMD_J$) to investigate three strategies of inducing immune tolerance to the rAAV vector and transgene expression with rAAV9-microdystrophin (rAAV-μDys).

Methods: For fetal transduction, we tried two methods to induce immune tolerance against rAAV and microdystrophin. First, direct injection of rAAV into amniotic fluid at embryonic day 35 (oral ingestion of rAAV). Second, pregnant $CXMD_J$ heterozygote with embryonic day 30 fetuses was injected with rAAV by intravenous injection (trans-placental rAAV transduction). Furthermore, for postnatal transduction, we tried mesenchymal stem cells (MSCs) pretreatment with rAAV transduction. Bone-marrow derived MSCs and rAAV9-Luciferase or rAAV9-μDys were intramuscularly or intravenously injected into the normal or $CXMD_J$ dog at 8 weeks old. Seven days after injection, MSCs were systemically injected again. At 8 days after 1st injection, rAAV9-Luciferase or rAAV9-μDys was intramuscularly or intravenously injected into the same dog. To examine the immune response against rAAV, purified canine peripheral leukocytes were exposed to rAAV9 for 4 hours, and then IFN-γ expression was analyzed using qRT-PCR. Skeletal muscles of the rAAV-Luc or rAAV-μDys injected animals were sampled by biopsy for expression analysis at 4 weeks after rAAV injection.

Results: Following the fetal transduction, expression of IFN-γ in the purified peripheral blood leukocytes after the rAAV exposure were not induced in both of the rAAV oral ingestion and trans-placental transduced dogs, suggesting the successful induction of immune tolerance against rAAV. rAAV-derived microdystrophin expression were confirmed by immunohistochemistry in the transduced affected dogs from additional rAAV injection in both methods. In normal or $CXMD_J$ puppy, administration of rAAV-Luc or rAAV-μDys following MSCs treatment resulted in higher expression of transgene, compared to the rAAV transduction alone. Expression of IFNγ in the purified peripheral blood leukocytes after the rAAV exposure were not enhanced in the rAAV with MSCs, suggesting the immune suppressive effects of the MSCs.

Conclusion: Our results demonstrate that induction of immune tolerance against rAAV and/or transgene can be achieved both by fetal or postnatal rAAV injections. These strategies would be effective approach to analyze the expression and function of transgene *in vivo*. These findings also support the future feasibilities of rAAV-mediated protein supplementation strategies.

379. MicroRNA-29 and Micro-Dystrophin Combinatorial Therapy Suppresses Fibrosis and Restores Function to *mdx*/utrn$^{+/-}$ Mice

Kristin Heller[1], Eric Meadows[2], Sarah Lewis[1], Josh Mendell[3], Jerry Mendell[1], Louise Rodino-Klapac[1]
[1]Nationwide Childrens Hospital, Columbus, OH, [2]Baylor College of Medicine, Houston, TX, [3]University of Texas Southwestern Medical Center, Dallas, TX

Duchenne muscular dystrophy (DMD) is caused by dystrophin deficiency resulting in muscle loss and progressive muscle weakness and fibrotic scarring. Muscle fibrosis impairs blood flow and excludes endomysial derived constituents hampering muscle repair and regeneration. Irrespective of the success of gene restoration (molecular or pharmacologic) functional improvement is limited without reduction of muscle fibrosis. miR-29c regulates collagen levels making it an ideal candidate for decreasing muscle fibrosis. miR-29c levels are reduced in DMD and our goal is to develop an anti-fibrotic therapy by overexpressing miR-29c with adeno-associated virus (AAV) mediated delivery in combination with micro-dystrophin to improve membrane stability. We injected scAAVrh.74.CMV.miR-29c alone, co-delivered with rAAVrh.74.MCK.micro-dystrophin, and rAAVrh.74.MCK.micro-dystrophin alone by intramuscular injection (IM) into the left gastrocnemius (GAS) muscle of 3 month old mdx/utrn+/- mice, a DMD mouse model. GAS muscle was analyzed 3 months post-injection to assess collagen accumulation by Sirius Red staining and subsequent quantification with ImageJ. Additional outcomes included miR-29c and collagen transcript levels, force measurements in the GAS muscle, fiber diameter measurements and western blot analysis for proteins involved in muscle regeneration (MyoD, Myogenin). Analogous to DMD tissue, we demonstrated a significant reduction in miR-29c levels in mdx/utrn$^{+/-}$ muscle correlated with increased fibrosis measured by Sirius red staining. Following 3 months of treatment with scAAV.miR-29c alone, there was a significant reduction in fibrosis (treated-23.5%±1.3 vs. untreated-27.8% ±0.6, $p<0.01$) in the GAS muscle. When co-delivered with micro-dystrophin we see further reduction in collagen (41%) by Sirius red staining along with significantly reduced mRNA levels of Col1A,Col3A, fibronectin and TGF-β levels. We observed an increase in specific and absolute force in the muscle treated with miR-29c alone compared to the untreated limb, which when combined with micro-dystrophin led to absolute and specific force that were not significantly different than wild-type (miR-29c treated-204.7±11.7 vs. untreated-151.6±14.5 vs. combined-244.2±6.6 vs. wild type-313.1±40.69 $p<0.01$). We also observed a significant increase in gastroc weight in those muscles that were co-treated. Demonstration of increased fibrosis and decreased miR-29c expression in the mdx/utrn$^{+/-}$ mice and dystrophin-deficient patients validates the mouse model as representative of the human disease. Initial results using AAV.miR-29c as an anti-fibrotic therapy suggest that there is beneficial effect with reduction in collagen levels, a key contributor in fibrosis. Moreover, when combined with micro-dystrophin to improve membrane stability, miR-29 upregulation normalized muscle force. These data provide rationale for overexpression of miR-29c to reduce fibrosis along with dystrophin replacement as a potential treatment for DMD.

380. Therapeutic Capacity of AAV-Delivered Muscle-Specific-Expressed Micro-Utrophin (ΔR4-R21/ΔCT) in *mdx^{4cv}* Skeletal Muscles

Glen B. Banks, Quynh Nguyen, Stephen D. Hauschka, Jeffrey S. Chamberlain, Guy L. Odom
University of Washington, Seattle, WA

Duchenne muscular dystrophy (DMD) is a severe muscle wasting disorder caused by dystrophin mutations. Utrophin is a dystrophin paralogue that can prevent necrosis in the *mdx* mouse DMD model. We designed an expression plasmid containing a miniaturized M-creatine kinase regulatory cassette (CK8e) and a rationally designed micro-utrophin$^{\Delta R4\text{-}21/\Delta CT}$ (μUtrn) to accommodate the limited capacity of recombinant adeno-associated virus (rAAV). Both intramuscular (IM) and intravascular (IV) delivery of rAAV6-CK8-μUtrn at 2 weeks of age profoundly mitigated the dystrophic phenotype in skeletal muscles when examined at 3-6 months of age. These improvements were observed in many but not all skeletal muscle histological and functional parameters. At a pathological level μUtrn production was associated with significant reductions in centrally nucleated muscle fibers and with a greatly reduced prevalence of muscle regions containing inflammatory cells. μUtrn production also restored dystrophin-glycoprotein-complex (DGC) components β-Dystroglycan, δ-Sarcoglycan, α-Dystrobrevin-2, and α1-Syntrophin to the sarcolemma. As expected, nNOS was not restored, presumably because CK8-μUtrn lacks direct nNOS binding sites. Analysis of μUtrn immunostaining intensities 3-months post-treatment suggested lower μUtrn levels in many of the 1, 2a and 2d/x fibers than in the 2b fibers. These differences would be consistent with the relative transcriptional activities of the CK8e regulatory cassette in different adult mouse fiber types: 2b > 2d/x > 2a > 1. Given the important structural role of costameres in connecting the sarcolemma

to sarcomeres, we examined utrophin and μUtrn localization patterns and found endogenous utrophin localized in a costameric pattern adjacent to Z-line end points while abutting sarcolemma junctions. However, while μUtrn was also associated with a costameric lattice, its striations were unexpectedly only ~0.8 μm apart compared to ~2.2 μm for endogenous utrophin in untreated *mdx*^{4cv} muscles. How the presence of two different costameric lattice patterns may affect skeletal muscle function is not known. Since *mdx*^{4cv} mice are known to exhibit a fragmented synaptic phenotype, we also examined neuromuscular junctions (NMJs) in treated and untreated mice and found that μUtrn prevented synapse fragmentation and also restored the depth of synaptic folds. Interestingly, the NMJs in μUtrn-treated muscles exhibited a more highly branched architecture of the synaptic folds, and this may have compensated for the reduced number of fold openings observed in untreated mice. μUtrn treatment also provided partial restoration of the reduced Achilles myotendinous junction folds seen in *mdx*^{4cv} mice, and did not lead to either the myotendinous strain injury or the ringed fiber formation associated with micro-dystrophin$^{\Delta R4\text{-}R23/\Delta CT}$ mediated therapy. Importantly, physiological studies indicated that IM treated tibialis anterior muscles maintained peak force production and exhibited partially improved specific force production. Overall, rAAV6-CK8-μUtrn treatment of *mdx*^{4cv} mice provided major improvements in many muscle parameters. However, it did not overcome all dystrophic deficits, and it modified the normal costameric lattice structure. Further improvements might be achieved by expressing μUtrn via regulatory cassettes with higher relative activity in 1, 2a and 2d/x fibers, and by further modifications of μUtrn (ΔR4-R21/ΔCT)'s design.

381. Prohypertrophic Agents Enhance the Response to Gene Therapy in Pompe Disease

Sang-oh Han, Songtao Li, Dwight D. Koeberl
Department of Pediatrics / Medical Genetics, Duke University Medical Center, Durham, NC

Pompe disease causes a progressive myopathy resulting from acid α-glucosidase (GAA) deficiency in the heart and skeletal muscle. Enzyme replacement therapy (ERT) with recombinant human (rh) GAA has prolonged the survival of patients. However, complete reversal neuromuscular involvement has not been possible in Pompe disease by treating with ERT. The paucity of cation-independent mannose-6-phosphate receptor (CI-MPR) in skeletal muscle, where it is needed to take up rhGAA, correlated with a poor response to ERT by muscle in Pompe disease. Clenbuterol, a selective β2 receptor agonist, enhanced the CI-MPR expression in striated muscle, and increased the efficacy of either ERT or gene therapy in murine Pompe disease. The underlying mechanism of clenbuterol's therapeutic action is Igf-1 mediated muscle hypertrophy, which has correlated with increased CI-MPR (also the Igf-2 receptor) expression. In this study we have evaluated 4 new drugs in GAA knockout (KO) mice in combination with an adeno-associated virus (AAV) vector encoding human GAA. The dosage for each drug was selected to induce muscle hypertrophy with an associated increased expression of CI-MPR, analogous to clenbuterol's effects. Three alternative β2 agonists and dehydroepiandrosterone (DHEA) were evaluated in combination with gene therapy in GAA-KO mice. Mice were transgenic for a liver-specific human GAA transgene to induce immune tolerance to introduced GAA. The 3 new β2 agonists were chosen to be long-acting like clenbuterol. Furthermore, DHEA caused muscle hypertrophy similar to the β2 agonists, is available in the US, and was well-tolerated in rodent experiments. Mice were injected with AAV2/9-CBhGAA (1E+11 vector particles) at a dose previously found to be partially effective at clearing glycogen storage from the the heart. Heart GAA activity was significantly increased by either salmeterol ($p<0.01$) or DHEA ($p<0.05$), in comparison with untreated mice. Furthermore, glycogen content was reduced by treatment with DHEA ($p>0.001$), salmeterol ($p<0.05$), formoterol ($p<0.01$), or clenbuterol ($p<0.01$) in combination with the AAV vector, in comparison with untreated mice. Functional testing was performed subsequently, and the wirehang test at 18 weeks following vector administration revealed that the combination of salmeterol and the AAV vector significantly increased latency in comparison with untreated mice ($p<0.01$), AAV vector alone ($p<0.001$). Similarly, salmeterol with the vector increased latency significantly more than either DHEA ($p<0.001$), formoterol ($p<0.05$), fenoterol ($p<0.05$), or clenbuterol ($p<0.05$) with the vector. An important consideration with regard to adjunctive therapy is whether any effects are due to the adjuvant rather than the combined treatment. The most effective individual drugs were evaluated by themselves, and no significant effect upon GAA activity of heart, glycogen content of heart, or wirehang latency was observed, in comparison with untreated mice. Thus, salmeterol should be further developed as adjunctive therapy in combination with either ERT or gene therapy for Pompe disease.

382. Correction of Autophagic Dysregulation in Pompe Disease Following Gene Therapy

Angela L. McCall, Sylvia G. Stankov, Darin J. Falk, Barry J. Byrne
Pediatrics, University of Florida, Gainesville, FL

Pompe Disease (PD) is a fatal metabolic disorder caused by mutations in the *GAA* gene leading to a deficiency in acid alpha-glucosidase (GAA) and affects 1 in 40,000 births. Clinically, this lysosomal storage disorder presents with cardiomegaly and skeletal muscle weakness, leading to cardiorespiratory failure. GAA is responsible for the breakdown of glycogen in the lysosome, an important energy source for striated muscle and neurons. Currently, the only approved treatment for PD is enzyme replacement therapy (ERT). While ERT has increased patient survival, there are several limitations including treatment cost and the inability of the enzyme to cross the blood-brain barrier and breakdown lysosomal glycogen deposition within the central nervous system. ERT therapy is dependent upon receptor-mediated endocytosis of the exogenous enzyme; much of which remains in amphisomes resulting in insufficiently trafficking of GAA to the lysosome. These issues illustrate the need for an alternative treatment. We propose that endogenous production of GAA enzyme mediated by adeno-associated virus (AAV)-delivery of the *GAA* gene will improve targeting of GAA to the lysosome and reduce the overall dysregulation of vesicular systems.In this study, we performed intravenous delivery of AAV9-DES-coGAA to 12-week old *Gaa*$^{-/-}$ animals at three doses ($1x10^{11}$ vg/kg, $1x10^{13}$ vg/kg, and $1x10^{14}$vg/kg) and compared to AAV9-CMV-GFP ($1x10^{13}$ vg/kg) and vehicle-control groups. Previous research has revealed that cellular dysregulation due to lysosomal storage of glycogen is amassed by twelve-weeks of age prior to onset of the physiological phenotype of PD in the Gaa$^{-/-}$ murine model. One month after AAV or sham injection, cardiac and skeletal muscles were harvested for biochemical and histological analyses. GAA activity assays demonstrate that therapeutic levels of enzyme activity are not attained with doses $1x10^{11}$ vg/kg and $1x10^{13}$ vg/kg, but were when a dose of $1x10^{14}$ vg/kg is administered. Western Blot analysis of autophagy-associated proteins, such as Beclin, LAMP1, LC3-I, and LC3-II show a decrease in protein levels following gene therapy indicating an improvement in autophagic regulation. Hematoxalin & Eosin staining of tibialis anterior and gastrocnemius muscle sections show an inverse relationship between level of vacuolarization of muscle fibers and dose of AAV9-DES-coGAA vector. Additionally, preliminary data suggest increased endocytosis of AAVs without reaching the therapeutic threshold may activate autophagy. Further analysis by RT-qPCR and immunofluorescence will elucidate the mechanism by which treatment modifies autophagic flux.

383. Clinico-Pathological Correlation in the Earliest Stages of Muscular Dystrophy Suggests Sensitive Physiological Parameters as Novel Primary Endpoints for Systemic Gene Therapy

Benjamin W. Kozyak[1], Andy Mead[2], Yafeng Song[2], Leon Morales[2], Joe Kornegay[2], Hansell H. Stedman[2]
[1]*Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA,* [2]*Surgery, University of Pennsylvania, Philadelphia, PA*

The clinical progression of Duchenne Muscular Dystrophy (DMD) begins with locomotive symptoms in childhood but eventually includes symptoms of severe cardiorespiratory failure. Our studies in mdx mice and GRMD dogs have reavealed surprisingly large early deficits in physiological reserve, attributable to the deficiency of dystrophin. In young GRMD dogs, the findings of diaphragmatic fibrosis and myofiber shortening correlated strongly with non-invasively measured abnormalities in respiratory muscle recruitment. In juvenile GRMD dogs without clinical signs of heart failure, detailed studies of cardiac mechanics revealed profoundly abnormal Frank-Starling and inotropic responses. Thus, submaximal exercise loading has the capactiy to reveal very early, otherwise subclinical deficits in physiological reserve. We have utilized several non-invasive systems to measure integrative cardiopulmonary mechanics in animals trained to undertake submaximal volitional exercise. Our findings demonstrate the possibility of continuous non-invasive monitoring of cardiac output, stroke volume, systemic vascular resistance, and several ventilatory parameters in young dogs and children during treadmill exercise, as well as upright and recumbent bicycling at work outputs appropriate for low risk serial testing in the earliest stages of DMD. As the field contemplates primary endpoints for use in trials of systemic gene therapy, these metrics hold promise as the most sensitive measurements of the earliest functional deficits in organ systems eventually responsible for the lethality of DMD.

384. Abstract Withdrawn

385. Inhibiting the Myostatin Signaling Pathway using CRISPR/Cas9-Based Repressors

Pratiksha I. Thakore, Nishkala K. Shivakumar, Charles A. Gersbach
Department of Biomedical Engineering, Duke University, Durham, NC

Inhibition of myostatin signaling is a potential method to enhance skeletal muscle growth and has been proposed as a strategy to treat muscle weakness associated with sarcopenia, cachexia, and muscular dystrophies. Current approaches under clinical investigation for inhibiting the myostatin pathway rely on systemic administration of soluble factors, including myostatin blocking antibodies and soluble myostatin receptors. Treatment with these protein-based inhibitors has led to enhanced muscle mass in mouse models of muscular dystrophy and cachexia, but has demonstrated limited efficacy and some adverse side effects in subsequent clinical trials. We hypothesized that a targeted gene regulation strategy to localize inhibition of the myostatin pathway to skeletal muscle fibers may increase the effectiveness and safety of this therapy. The RNA-guided CRISPR/Cas9 system has emerged as a promising platform for targeted gene regulation. Fusion of catalytically inactive, "dead" Cas9 (dCas9) to the Kruppel-associated box (KRAB) domain generates a synthetic repressor capable of highly specific silencing of target genes. dCas9-KRAB repressors can be employed in gene therapy to silence detrimental gene products, repress oncogenes, inhibit viral replication, and treat dominant negative diseases. However, gene delivery of dCas9-KRAB to skeletal muscle *in vivo* is challenging because the size of the *S. pyogenes* dCas9 and KRAB domain fusion exceeds the packaging limit of standard AAV vectors. Recently, a smaller Cas9 protein derived from *S. aureus* was described for AAV delivery and *in vivo* gene editing. We generated a *S. aureus* dCas9-KRAB fusion and targeted the myostatin receptor, *Acvr2b*, for silencing. In cultured mouse myoblasts, *S. aureus* dCas9-KRAB potently repressed *Acvr2b* expression by qPCR and resulted in reduced myotube formation following differentiation compared to controls. The *S. aureus* dCas9-KRAB repressor was packaged into AAV vectors and expressed efficiently *in vitro* in cultured myoblasts and *in vivo* following direct injection into the mouse tibialis anterior muscle. Ongoing studies will determine the effects of transcriptional silencing of *Acvr2b* on myotube diameter *in vitro* and the efficiency of *Acvr2b* silencing achieved by engineered dCas9-KRAB repressors *in vivo*. These studies establish how transcriptional modulation with the CRISPR/Cas9 system can be used to investigate potential therapeutic gene targets for treating neuromuscular disorders.

386. Toxicology for DUX4-Targeted MicroRNAs

Lindsay M. Wallace, Jacqueline S. Domire, Danielle A. Griffin, Louise R. Rodino-Klapac, Scott Q. Harper
Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant disorder affecting 1 in 7500. Symptoms typically arise in young adulthood and most patients show clinical features before age 30. FSHD is characterized by progressive wasting and weakness of facial and shoulder-girdle muscles, though all skeletal muscle can be affected. Currently there is no treatment for FSHD. The pathogenic events leading to FSHD have only recently started coming into focus. Several studies now support an FSHD pathogenesis model involving aberrant expression of the DUX4 gene, which encodes a myotoxic transcription factor. The emergence of DUX4 enabled the development of FSHD therapies. Previously our lab demonstrated proof of principle for the use of a DUX4-targeted RNA interference (RNAi)-based gene therapy. We have pursued two engineered microRNAs that showed strong therapeutic efficacy determined by their ability to suppress pathogenic levels of DUX4, and prevent myopathy in mice. To continue down a translational path this study was designed to determine the safety of microRNA therapy to muscle (which has not been previously determined) by delivering high doses for preliminary non-GLP toxicity studies. Both local intramuscular injections and systemic isolated limb perfusions were performed to identify safe sequence and dosing parameters for lead DUX4-targeted microRNAs at acute (3 week) and long-term (5 month) timepoints. To further circumvent potential off-target organ toxicity we have optimized these vectors to restrict expression to skeletal muscle. The safety parameters along with the promoter optimization provide necessary data for translating an RNAi-based therapy for FSHD.

387. Transfer of Hyaluronic Acid Synthase-2 Gene into Canine Joints Using Adeno-Associated Viral Vectors

Monica D. Figueiredo[1], Patricia Berthelette[2], Cathy Sookdeo[2], Donna Armentano[2], Sirkka Kyostio-Moore[2]
[1]*Merial Inc, Athens, GA,* [2]*Genzyme, Framingham, MA*

Osteoarthritis (OA) is one of the more common causes of lameness in dogs and is estimated to affect approximately 20% of dogs >1 year old. OA is a progressive and degenerative disease that results in pain, inflammation and reduced joint mobility. Novel, safe and efficacious therapies that improve joint lubrication and reduce the extent of inflammation and pain are potential solutions for the management of canine OA. Here, we evaluated the efficiency of gene transfer to canine joint by intra-articular injections of recombinant

adeno-associated virus (rAAV) vectors encoding therapeutic genes. Specifically, we generated rAAV vectors (either AAV2 and AAV5 serotypes vectors) encoding canine codon-optimized hyaluronic acid (HA) synthase-2 (HAS2) to provide local and continuous synthesis of HA in the joint. Twenty-two adult healthy dogs that were seronegative for anti-AAV2 and -AAV5 antibodies were injected intra-articularly with rAAV2/HAS2 (1, 5 and $10x10^{11}$ vg/joint), rAAV5/HAS2 ($5x10^{11}$ vg/joint) or PBS (control). No adverse clinical signs were observed following the 28-day study period. Histopathological analysis showed minimal synovial inflammation in the joints of dogs treated with rAAV5/HAS2 and no significant changes in the rAAV2/HAS2 treated dogs. Vector genomes (VG) were detected in the synovium of all the rAAV-treated joints and in the majority of cartilage samples tested. The rAAV5/HAS2 vector resulted in findings of higher and more consistent detection of VG and transcripts compared to rAAV2/HAS2 in both the synovial and cartilage samples. Preliminary analysis also showed a trend towards increased HA levels in the synovial fluid of the treated joints. In summary, our study demonstrated that rAAV2 and rAAV5-mediated gene transfer to canine joint tissues is feasible and that both vectors present an acceptable safety profile following a single intra-articular injection.

388. Hypersensitivity of MYH16 Fibers (M Fibers) to Myodegeneration in Canine Muscular Dystrophy

Yafeng Song, Andy Mead, Mihail Petrov, Phoebe Ingraham, Leon Morales, Shira Rosenblum, Joe Kornegay, Hansell H. Stedman
Surgery, University of Pennsylvania, Philadelphia, PA

Golden Retriever Muscular Dystrophy (GRMD) dogs frequently develop severe restriction to mouth opening, a condition known as trismus, as one of their earliest physical signs of the disease. Trismus is generally due to fibrosis and/or tonic contraction of the muscles of mastication, the temporalis and the masseter muscle. These muscles are primarily composed of unique myofibers exhibiting a masticatory myosin heavy chain encoded by the MYH 16 gene (M fibers). M fibers have been shown to produce the highest specific force among mammalian myofibers, based on their atypical crossbridge kinetics. Here, we show for a first time a morphological analysis of M fibers for the purposes of their classification, distribution and proportion in the masticatory muscles in normal and GRMD dogs based on immunofluorescence staining by a highly specific anti-MYH16 antibody. Our data demonstrate that in both temporalis and masseter muscles, the predominant M fibers are larger in size when compared to any other striated myofiber. A comparative histological analysis of these and other muscles in GRMD dogs demonstrates a significant relative increase in the proportion of injured, hypercontracted M fibers, a highly significant relative abundance of regenerating fibers expressing the embryonic myosin isoform encoded by the MYH3 gene, and prominent fibrosis when compared to the other affected striated muscles. These results suggest that the higher specific force developed by M fibers, due to the MYH16 encoded MyHC isoform, accelerates the fibers' rate of degeneration in response to myonecrotizing sarcolemmal injury in the absence of dystrophin. Trismus is not seen in Duchenne MD, suggesting that the deletional frameshift in the human MYH16 gene (Nature 428: 373-4, 415-8) dramatically reduces the relative rate of sarcolemmal injury. These observations may offer novel insights into the physiological role of dystrophin in striated muscles, and provide an especially sensitive test for use in experimental therapeutics in the canine models for DMD.

389. Muscular Dystrophy Genes and the Early Metazoan Transition from Dynein- to Myosin-Powered Locomotion

Andy Mead, Alock Malik, Ranjith Krishnankutty, Hansell H. Stedman
Surgery, University of Pennsylvania, Philadelphia, PA

During early animal evolution, the primary source of locomotive mechanical power shifted from axonemal (flagellar) dynein to densely arrayed class II myosin. Although the emergence of muscle conferred the advantage of three-dimensional scalability, the transition brought with it the evolutionarily unprecedented problem of safely transmitting huge forces across rapidly deforming cell membranes. This task in vertebrates is accomplished in part through the dystrophin-dystroglycan-sarcoglycan complex (DGC), which is implicated in most forms of muscular dystrophy. We present the inferred earliest steps in the molecular evolution of these cell surface and cortical cytoskeletal proteins, using genome sequence data from all early branching metazoan phyla and a broad sampling of unicellular taxa. **Results**: Surprisingly, the phylogeny suggests that a DGC emerged before the "sarcomeric" clade of myosins, implying conserved function(s) among unicellular lineages closely related to the Metazoa. Furthermore, linkage of the DGC to the cytoskeleton occurred before the massive tandem-repeat expansions (>200) seen in sarcomeric scaffolding proteins of the titin superfamily. The rod-like domains of dystrophin and utrophin were coopted at their full length from a rapidly lengthening cytoskeletal protein with an unrelated function, that of connecting actin filaments to microtubules at large physical distances. Finally, intron positions in the inferred dystrophin gene in a common ancestor to all Bilateria provide clues to the molecular basis of Duchenne muscular dystrophy and to emerging therapies. **Conclusions**: Our reconstruction suggests that evolution of membrane-spanning "muscular dystrophy protein complexes" was an essential process as one geometric constraint on power transduction was traded for another. 80% of the length of dystrophin is attributable to tandem DNA duplications that pre-dated the evolutionary emergence of sarcomeres. This chronology implies that escalating power output from striated muscle played no role in providing selective pressure for the serial expansion of "spectrin-like" repeats. This finding has therapeutic implications, as it suggests that minimal length dystophins or utrophins may fully complement the physiological role of full length dystrophin in vertebrates as long as the deletional juxtaposition of triple helical repeats does not disrupt force transmission. This interpretation is consistent with the clinical findings of Becker Muscular Dystrophy in selected patients with in-frame exon duplications. In this view, the evolutionary stability of the length of the dystrophin rod is driven by the incompatibility, when placed in close physical proximity by deletion, of divergent triple helical repeats. The evolutionary translocation of the protein to the cytoskeletal cortex as part of the DGC may have greatly reduced the metabolic load associated with synthesis of the strong but scarce protein, also facilitating evolutionary drift to the enormous size of the gene in a common mammalian ancestor.

390. Impact of a Treatment with Antioxidant on Gene Transfer Efficiency After Recombinant Adeno-Associated Vector Injection in a Mouse Model of Duchenne Muscular Dystrophy

Jean-Baptiste Dupont[1], Benoît Tournaire[1], Romain Durand[1], Béatrice Marolleau[2], Emilie Bertil[2], Christophe Georger[2], Emilie Lecomte[1], Benjamin Cogné[1], Bernard Gjata[2], Laetitia van Wittenberghe[2], Alban Vignaud[2], Richard O. Snyder[3], Philippe Moullier[1], Adrien Léger[1]
[1]INSERM UMR 1089, Nantes, France, [2]Généthon, Evry, France, [3]Center of Excellence for Regenerative Health Biotechnology, University of Florida College of Medicine, Gainesville, FL

Recombinant adeno-associated virus (rAAV)-based vectors are promising tools for the treatment of Duchenne muscular dystrophy (DMD) by gene therapy. Following rAAV injection in murine and canine models of DMD, several groups have reported significant phenotype improvements without notable toxicity, raising hope for future clinical translations. However, the long term maintenance of therapeutic benefits is an important, and yet unresolved issue. In previous studies conducted in DMD mice, we and others have demonstrated that rAAV-mediated transgene expression decreases progressively, even when clinically relevant vector doses are injected. Our team described several "restriction" factors having a negative impact on rAAV transduction, notably a loss of vector genomes resulting from muscle cell necrosis but also an oxidative damage affecting transgene mRNA. These first results support the fact that the tissue context in which rAAV vectors are delivered is of critical importance and can significantly affect their efficiency. In addition, they open new avenues for improvement since we can now consider counteracting these restriction phenotypes prior to rAAV injection. In the case of DMD, oxidative stress seems to occupy a central position in both muscle cell pathophysiology and rAAV transgene mRNA degradation. Therefore, we designed an innovative strategy using a relevant antioxidant agent routinely used in human medicine: N-acetylcysteine. DMD mice, pre-treated or not with this compound, were subsequently injected with a rAAV vector carrying a reporter transgene. The transduction efficiency, together with the expression and activity of the transgene, were carefully monitored and compared two months later. The outcome of this innovative approach will certainly pave the way for future combinatorial protocols using pharmacological agents and rAAV vectors in DMD muscles.

Cancer-Immunotherapy, Cancer Vaccines II

391. Mutated Tumor Neoantigens Are Recognized by Tumor Infiltrating Lymphocytes from Metastatic Ovarian Cancer

Drew C. Deniger, Anna Pasetto, Todd D. Prickett, Jared J. Gartner, Mini Bharathan, Eric Tran, Paul F. Robbins, Steven A. Rosenberg
Surgery Branch, NCI/NIH, Bethesda, MD

Somatic mutations expressed by the tumor can serve as neoantigens for autologous T cells. Tumor infiltrating lymphocytes (TIL) with varying degrees of neoantigen-reactivity infused for the treatment of melanoma resulted in 50% overall objective response and 20% complete response rates. A largely oligoclonal population of ERBB2IP^{E805G} mutated neoantigen-specific T cells resulted in a long-term, ongoing partial regression of metastatic cholangiocarcinoma suggesting that infusion of selected mutation-reactive TIL could be efficacious in the treatment of common epithelial cancers. We now studied whether TIL obtained from metastatic ovarian cancer recognized tumor mutations. Exome and transcriptome sequencing was performed from resected metastatic ovarian cancer deposits in parallel with growth of TIL fragment cultures in interleukin-2. Long peptides and tandem minigenes encompassing all mutations were synthesized, introduced into autologous antigen presenting cells, co-cultured with individual TIL fragments and T-cell reactivity was determined by interferon-γ ELISPOT and surface expression of 41BB. In the 8 ovarian tumors evaluated, there was a median of 227 mutations (range: 63-332) and an average of 94% of 24 fragments initiated (range: 58% - 100%) available for testing. Six of eight (75%) patients had T cell responses to mutated neoantigens at >5% of the fragment culture. Antigens identified to date were unique to each patient, i.e. no overlapping mutations between patients, and both CD4 and CD8 responses have been detected. One patient had a CD4 T cell response to p53^{G245S} hotspot mutation, which opens opportunities for treatment of other cancer patients with TCR-transduced T cells because this mutation is present in 2.8% of all cancers. The average time from resection to identification of mutation-reactive T cell fragment culture was 8 weeks indicating that this strategy could be used for prospective therapy. In summary, mutation-specific T-cell responses were found in 6 of 8 patients with metastatic ovarian cancer, which opens the opportunity to use these cells for adoptive T cell treatment of advanced ovarian cancer.

392. Super-Resolution (STED) Imaging Reveals Simultaneous Co-Docking of Tandem Chimeric Antigen Receptors to Two Target Antigens Enhancing T Cell Functionality and Mitigating Antigen Escape

Malini Mukherjee[1], Shoba Navai[2], Daniel Landi[3], Kristen Fousek[3], Antonella Pignata[3], Kevin Bielamowicz[3], Tiara Byrd[3], Maksim Mamonkin[3], Meenakshi Hegde[2], Malcolm K. Brenner[3], Jordan S. Orange[1], Nabil Ahmed[2]
[1]Center for Human Immunobiology, Baylor College of Medicine, Houston, TX, [2]Baylor College of Medicine, Houston, TX, [3]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

Background: Tandem chimeric antigen receptors (CARs) are bispecific CAR molecules with two different target recognition exodomains joined in tandem. Tandem CARs mediate distinct T cell activation to either target antigen and super-additive functionality upon encounter of both targets simultaneously. Tandem CAR T cells successfully mitigate antigen escape and exhibit enhanced antitumor activity compared to their unispecific counterparts. Stimulated emission depletion (STED) microscopy can achieve a sub-diffractive lateral resolution of ~ 50 nm enabling imaging of the immunological synapse at near single molecule levels.

Hypothesis: Super resolution STED Imaging can differentiate heterodimers created by Tandem CAR molecules simultaneously co-docking to both target antigens at the immunological synapse (IS).

Methods: Human T cells were retrovirally transduced to express a Tandem CAR molecule specific for HER2(ErbB2) and IL13Rα2. Conjugates of Tandem CAR T cells and the human glioblastoma cell line U373 were fixed after 30 minutes of incubation then labelled with primary unconjugated monoclonal antibodies against HER2 and IL13Rα2 tumor ligands. The IS was examined at the T cell/ U373 interface using confocal and STED microscopy then confirmed with in situ proximity ligation assay (PLA). Unispecific CAR T cells, T cells co-expressing HER2 and IL13Rα2 CARs and non-transduced T cells were used as experimental controls.

Results: Confocal microscopy showed co-clustering of HER2 and IL13Rα2 at the IS in contrast to only HER2 or IL13Rα2 localized to the IS for the IL13Rα2 CAR and HER2 CAR T cell/ GBM conjugates, respectively. Using a fixed intensity threshold for all conjugates analyzed, quantification of receptor accumulation at the IS revealed that there was increased collective clustering of target molecules at the IS ($p = 0.0002$). Super-resolution STED microscopy was used to

interrogate the quality and quantity of co-localized Tan CAR target ligands at the immune synapse. Measurement of co-localized HER2 and IL13Rα2 aggregate diameters showed a distribution of 80-200 nm aggregate size. The aggregate size has high correlation to the predicted size of CAR-tumor ligand-antibody-antigen complex, indicating increased probability of co-docking of the target ligands at the Tan CAR synapse. Co-localization of the two tumor antigens was present with a very low frequency in the bi-specific T cell - tumor synapse with their average aggregate size ranging from 300-500 nm, suggesting that these ligands formed independent and not "co-docked" conjugates. Co-localization was absent in the single CAR -tumor synapse. In situ PLA with a maximum distance of 30-40 nm showed accumulation of HER2/IL13Rα2 heterodimer signals at the immunological synapse of Tandem CAR Tcell/ GBM IS.

Conclusion: Tandem CAR molecules simultaneously engage two target antigens mediating significantly enhances T cell activation through a bifunctional immunological synapse.

393. Engineering Armored T Cell Receptor-Mimic (TCRm) Chimeric Antigen Receptor (CAR) T Cells Specific for the Intracellular Protein Wilms Tumor 1 (WT1) for Treatment of Hematologic and Solid Malignancies

Sarwish Rafiq[1], Terence Purdon[1], Tao Dao[1], Chen Liu[2], David Scheinberg[1], Renier J. Brentjens[1]
[1]Memorial Sloan Kettering Cancer Center, New York, NY, [2]Eureka Therapeutics, Emeryville, CA

Adoptive therapy with chimeric antigen receptor (CAR) T cells specific for CD19 is clinically successful in a limited set of leukemias and most CARs studied are targeted against external antigens. Wilms Tumor Antigen 1 (WT1) protein (WT1) is an intracellular antigen overexpressed in many cancers, including leukemias and solid malignancies and is thus an appealing, broadly applicable target. We have engineered the first armored T cell receptor-mimic (TCRm) CAR against WT1. Derived from the ESK1 antibody, the second generation CAR, WT1-28z, is reactive with the RMFPNAPYL peptide of WT1 that is processed and presented on the surface of cells in the context of HLA-A*02:01. WT1-28z was further modified to secrete human IL-12 cytokine, thus creating the armored CAR WT1-28z/IL-12. T cells expressing WT1-28z or WT1-28z/IL-12 are cytotoxic against a range of both hematological and solid tumors. Importantly, both WT1-directed T cells are specific for the WT1-HLA-A*02:01 complex and are not reactive against cells that do not express both HLA-A*02:01 and WT1. In established SCID/Beige mouse models of either acute leukemia or ovarian cancer, one dose of WT1-28z T cells prolongs survival of mice over untreated or irrelevant antigen-specific CAR T cell treated mice. Furthermore, one dose of the armored WT1-28z/IL12 CAR T cells further significantly prolongs survival of mice in both models over WT1-28z CAR T cell treated mice, with a subset of mice whose disease was eradicated. The armored TCRm CAR T cells against WT1 are effective in eradicating disease in both hematologic and solid tumors and may hold great clinical potential to expand on the success of CAR T cell therapy.

394. Generation of a Novel Allogeneic CAR T Cell Platform Utilizing an Engineered Meganuclease and AAV Donor Template to Achieve Efficient Disruption of T Cell Receptor Expression and Simultaneous Homology-Directed Insertion of a CD19 CAR

Daniel T. MacLeod[1], Jeyaraj Antony[1], Keith J. Wetzel[1], Rachel Moser[2], Audrey E. Brown[1], Jo Ann Hux[1], Caitlin A. Turner[1], Armin Hekele[1], Janel Lape[1], Clayton W. Beard[1], Bruce McCreedy[1], Victor V. Bartsevich[1], Michael G. Nicholson[1], Jeff Smith[1], Matthew Hirsch[2], Derek Jantz[1]
[1]Precision BioSciences, Durham, NC, [2]UNC Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC

Chimeric antigen receptor (CAR) T cell therapies have achieved dramatic results treating hematological malignancies by redirecting the specificity of T cells to target CD19 expressing cancer cells. Clinical trials reporting these impressive results have used autologous therapies, which pose significant manufacturing, logistical, and cost issues, complicating the implementation of such therapies on a larger scale. Furthermore, CAR T cells are typically generated by using Lenti- or Retro- viral vectors, resulting in random integration of the CAR gene, heterogeneous expression of the CAR on the cell surface, and the potential for insertional mutagenesis. We have developed a platform to address these issues by using a streamlined process to insert a CAR gene into the T cell receptor (TCR) alpha chain locus of T cells obtained from healthy donors using a highly optimized engineered meganuclease. The resulting gene-edited allogeneic CAR T cells do not express an endogenous TCR and therefore should not be capable of eliciting graft versus host disease upon adoptive transfer. We first produced and validated an engineered meganuclease targeting TCRα, and developed a scalable process for electroporating T cells with meganuclease mRNA that routinely results in greater than 60% knockout of the TCR, as measured by flow cytometry. To test our gene knock-in approach, we treated cells with our meganuclease to induce a double-strand break (DSB) in TCRα. We then transduced these cells using an AAV6 vector containing a donor DNA template consisting of a second generation CD19 CAR driven by an exogenous promoter and flanked by homology arms to the target site in the TCRα sequence. Homology directed repair of the DSB utilizing this donor template results in insertion of the CD19 CAR at this site in the genome. Incorporating this process into a 14 day procedure for generation and expansion of TCR knockout CAR T cells, we successfully generated a large population of TCR knockout cells with >70% of those cells stably expressing the CD19 CAR. Importantly, these CAR T cells proliferated when co-cultured with $CD19^+$ target cells, released cytokines including IFN-γ and IL-2, and exhibited potent cytotoxic activity against $CD19^+$ target cells. Studies to confirm *in vivo* antitumor activity are currently being conducted. In summary, we describe a novel, highly efficient, and scalable method to produce allogeneic CAR T cells in a streamlined process using an engineered meganuclease in combination with an AAV6 donor template.

395. Development of the Novel Oral Tumor Vaccine Using *Bifidobacterium longum* Displaying Wilms' Tumor 1 Protein

Koichi Kitagawa[1], Tsugumi Oda[2], Ayame Araki[2], Reina Gonoi[3], Hiroki Saito[1], Yoshiko Hashii[4], Takane Katayama[5], Keiichi Ozono[4], Toshiro Shirakawa[1]
[1]Division of Translational Research for Biologics, Department of Internal Related, Kobe University Graduate School of Medicine, Kobe, Japan, [2]Department of International Health, Kobe University Graduate School of Health Sciences, Kobe, Japan, [3]Kobe University School of Medicine Faculty of Health Sciences, Kobe, Japan, [4]Department of Pediatrics, Osaka University Graduate School of Medicine, Suita, Japan, [5]Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kyoto, Japan

INTRODUCTION: The Wilms' Tumor 1 gene (WT1), firstly reported as a gene responsible for pediatric renal cancer, is highly expressed in leukemia and various types of solid tumors. Therefore, WT1 gene products are considered as ideal targets for the cancer immunotherapy. WT1 peptide-based immunotherapies are currently being developed for treatment of pediatric and adult cancers in Japan and other countries. However the efficacy of the peptide-based immunotherapy is restricted by patients' HLA types, and multiple injections of the peptides are painful especially in children and often cause scarring and swelling on the injection sites. In this study, we developed a genetically modified *Bifidobacterium longum* displaying a WT1 protein as an oral vaccine for HLA-nonrestrictive and less-invasive cancer immunotherapy, and confirmed its therapeutic efficacy in tumor bearing mouse model. Furthermore, we also demonstrated the synergistic anti-tumor effect of combination therapy of this oral vaccine and interleukin-2 immunotherapy. **EXPERIMENTAL SUMMARY:** For optimized expression of WT1 protein on bacterial cell surface, a shortened murine WT1 gene (a.a. 117-419) including several $CD8^+$ and $CD4^+$ T cell epitopes was fused to galacto-N-biose/lacto-N-biose binding protein (GLBP) gene that coded ABC transporter of *B. longum*. We transferred the WT1-GLBP fusion gene into *B. longum* 105A strain using our *E-coli-B. longum* shuttle vector system, and constructed the *B. longum* 420 vaccine strain, which is displaying GLBP-WT1 fusion protein. We confirmed that *B. longum* 420 displayed WT1 protein on cell surface and its stability by immunofluorescence staining and western blotting. For *in vivo* vaccination study, 1×10^6 of C1498 murine leukemia cells stably expressing full-length murine WT1 protein were inoculated subcutaneously into the right flanks of 8 week old C57BL/6N mice (n=15). Following tumor inoculation, mice were randomly assigned into 3 treatment groups; *B. longum* 420, *B. longum* 2012 which was transfected with control vector, and PBS. 100 μL of PBS with or without 1×10^9 CFU of each recombinant *B. longum* was orally administered every other day for 4 weeks (14 times totally). Furthermore, to enhance the anti-tumor effect, we added subcutaneous injections of 2000 IU of mouse IL-2 for five days a week for 4 weeks. As the result, *B. longum* 420 oral vaccination significantly inhibited the tumor growth compared with PBS group at the day 29 after tumor inoculation (5455±891 mm^3 vs 8953±777 mm^3; $p<0.05$). Also the additional IL-2 treatment significantly decreased the mean tumor volume of *B. longum* 420 group as early as day 19 (289±73 mm^3 vs 1063±252 mm^3; $p<0.01$), and at day 29 (1196±507 mm^3 vs 5455±891 mm^3; $p<0.05$). These results suggested that *B. longum* 420 could induce WT1-specific CTLs in mice and an additional IL-2 treatment boosted their anti-tumor effects. **CONCLUSION:** We confirmed that oral vaccination with *B. longum* displaying WT1 protein successfully induced WT1 specific anti-tumor immunity in mice, and it could be enhanced by IL-2 treatment. These findings guaranteed the feasibility of our oral tumor vaccine, which would create a new paradigm of cancer immunotherapy.

396. Development of GD2-Specific Immunoliposomes for Immunotherapy of Neuroblastoma

Wei Huang, Daofeng Liu, Linjie Guo, Ekaterina Marinova, Andras Heczey, Leonid Metelitsa
Baylor College of Medcine, Houston, TX

Tumor microenvironment (TME) impairs immune effector cell localization, persistence, or the execution of their anti-tumor function. This represents a major and as yet unsolved critical challenge to the development of effective adoptive immunotherapy of solid tumors. To selectively target TME in neuroblastoma and make it permissible for survival and function of tumor-specific effector cells, we have developed a novel nanoparticle (NP) delivery platform which consists of 150 nm immunoliposomes rendered specific for neuroblastoma cells using a single-chain variable fragment (scFv) from the anti-GD2 14g2a mAb. GD2-specific but not control NPs could specifically bind GD2-positive but not GD2-negative neuroblastoma cells in vitro as determined by FACS. To examine the in vivo biodistribution of NPs, DiR-labeled GD2-specific or non-specific NPs were injected to NSG mice implanted with human neuroblastoma xenografts. Tumor tissues and normal organs were analyzed after 72 hours using ex vivo fluorescence imaging. Up to 58% of GD2-specific NPs accumulated at the tumor sites. NPs loaded with human IL-15 effectively delivered cytokine to the tumor site. A combined immunotherapy using human NKT cells expressing a GD2-specific chimeric antigen receptor (CAR) and IL-15-containing NPs resulted in potent in vivo expansion of CAR NKT cells and tumor eradication. In contrast, soluble IL-15 failed to support long-term CAR NKT cell persistence that resulted in tumor recurrence. These results inform design of cancer immunotherapy in combination with TME-modifying NPs.

397. Allogenic CAR T-Cells Targeting CD123 Effectively Eliminate Myeloid Leukemia Cells

Roman Galetto[1], Céline Lebuhotel[1], Agnès Gouble[1], Alexander Bank[1], Gail J. Roboz[2], Monica L. Guzman[2], Julianne Smith[1]
[1]Cellectis, Paris, France, [2]Division of Hematology and Oncology, Department of Medicine., Weill Cornell Medical College, New York, NY

Chimeric antigen receptor (CAR)-redirected T-cells have given rise to long-term durable remissions and remarkable objective response rates in patients with refractory leukemia. At present, CAR technology is administered through the custom-made manufacturing of therapeutic products from each patient's own T-cells. However, this patient-specific autologous paradigm is a significant limiting factor in the large-scale deployment of CAR technology. We have developed a platform for the production of "off-the-shelf" CAR T-cells from unrelated third-party donor T-cells. This platform utilizes Transcription Activator-Like Effector Nuclease (TALEN) gene editing technology to inactivate the TCRα constant (TRAC) gene, eliminating the potential for T-cells bearing alloreactive TCR's to mediate Graft versus Host Disease (GvHD). We have previously demonstrated that editing of the TRAC gene can be achieved at high frequencies, obtaining up to 80% of TCRα negative cells. This allows us to efficiently produce TCR-deficient T-cells that have been shown to no longer mediate alloreactivity in a xeno-GvHD mouse model. Acute myeloid leukemia (AML) is incurable in the majority of patients. While allogeneic stem cell transplantation remains the most effective therapy for AML to date, other types of cellular therapy have not yet been successful in this disease. We have adapted this allogeneic platform to the production of T cells targeting CD123, the alpha chain of the interleukin-3 receptor, which is expressed in tumor cells of patients with AML. We will present both in vitro and in vivo data demonstrating specific anti-tumor activity of engineered CAR T cells against AML cells. The ability to carry out large scale

manufacturing of allogeneic, non alloreactive CD123 specific T cells from a single healthy donor will offer the possibility of an off-the-shelf treatment that would be immediately available for administration to a large number of AML patients.

398. Site-Specific Introduction of Chimeric Antigen Receptors to Primary Human T Cells

Malika Hale[1], Alexandra Grier[1], Yuchi Honaker[1], Guillermo S. Romano Ibarra[1], Karen Sommer[1], Iram F. Khan[1], Nicholas Hasle[1], Jaya Sahni[1], Baeckseung Lee[2], Jordan Jarjour[2], Alexander Astrakhan[2], Andrew M. Scharenberg[1], David J. Rawlings[1]
[1]Program for Cell and Gene Therapy, Center for Immunity and Immunotherapies, Seattle Children's Research Institute and Departments of Pediatrics and Immunology, University of Washington School of Medicine, Seattle, WA, [2]Bluebird Bio, Inc., Seattle, WA

In current clinical trials, delivery of an anti-cancer chimeric antigen receptor (CAR) to patient T cells is accomplished using randomly integrating retroviruses. Designer nucleases and AAV donor templates allow an alternative delivery method that introduces the CAR gene cassette at a target locus via homologous recombination, with concomitant disruption of the endogenous gene. Here we show high-efficiency introduction of anti-CD19 and anti-BCMA CAR expression cassettes in primary human T cells at two clinically relevant loci: *CCR5* and *TCRa*. These gene-targeted CAR$^+$ cells (tCARs) exhibit equivalent activity *in vitro* against CD19$^+$ and BCMA$^+$ cell lines compared to lentiviral-delivered CARs, and effectively clear tumor in a murine xenograft model. Advantages over lentiviral delivery include utilization of a defined integration site for the CAR construct linked with endogenous gene disruption. The use of specific nucleases and AAV for CAR delivery expands the possibilities for a precisely engineered cell therapy product in the clinic. For example, a *CCR5*-tCAR may be clinically useful in HIV$^+$ patients, where traditional delivery methods leave therapeutic cells vulnerable to HIV infection and elimination. Moreover, a *TCRa*-tCAR could allow for off-the-shelf CAR therapy, potentially removing limitations to current approaches. Finally, the approach described here is broadly applicable for targeted delivery of alternative therapeutic cassettes at translationally relevant sites across the human genome.

Reference: BCMA CAR construct from Carpenter *et al*., *Clin Cancer Res* April 15, 2013

399. Evaluation of CD123 Targeting CART Cells in Non-Human Primates

Linda Dong[1], Saar Gill[2], Vijay Bhoj[2], Olga Shestova[2], Lori Martin[3], Daher Ibrahim Aibo[3], Xiaorui Yao[3], LeeAnne Talbot[3], Elizabeth Skuba[3], Celeste Richardson[4], Regina Young[2], Michael C. Milone[2], Steve Busch[3], Jennifer Marlowe[5], Timothy MacLachlan[5]
[1]Preclinical Safety, Novartis Institutes for Biomedical Research, East Hanover, NJ, [2]University of Pennsylvania, Philadelphia, PA, [3]Preclinicial Safety, Novartis Institutes for Biomedical Research, East Hanover, NJ, [4]Exploratory Immuno-oncology, Novartis Institutes for Biomedical Research, Cambridge, MA, [5]Preclinical Safety, Novartis Institutes for Biomedical Research, Cambridge, MA

The CAR-T platform has provided an exceptionally potent means to treat cancers that have proven resistant to standard treatments. This potency, however, can work against normal tissue as well. Some clinical trials have identified adverse consequences as a result of CAR-T cell targeting of critical normal tissue. To enable as many targets as possible using the CAR-T cell approach, it is important to understand the potential liabilities of those targets in normal tissues prior to initiating clinical trials. CD123, the IL-3 receptor alpha subunit, is a viable target for treatment of acute myeloid leukemia, as it is expressed highly on primary AML blasts. We have previously shown that, in mice transplanted with human hematopoietic stem cells, CD123 targeting CAR T-cells eradicated these precursors and, in turn, normal hematopoiesis (Gill et al 2014). Here, we describe an animal model developed to address the potential effects of targeting CD123 on non-hematopoietic tissue, namely endothelial cells that are found to express significant levels of CD123. An scFv that bound cynomolgus monkey CD123 was identified and a chimeric lentivirus that efficiently transduced monkey PBMCs was developed. Cells were demonstrated to be active in vitro and dosed into monkeys, after which cellular expansion was observed. In vivo safety assessments and histopathology results will be discussed.

Saar Gill, Sarah K. Tasian, Marco Ruella, Olga Shestova, Yong Li, David L. Porter, Martin Carroll, Gwenn Danet-Desnoyers, John Scholler, Stephan A. Grupp, Carl H. June and Michael Kalos (2014): Preclinical targeting of human acute myeloid leukemia and myeloablation using chimeric antigen receptor-modified T cells. Blood 123: 2343-2354

400. CD269 (BCMA)-Specific CAR-Expressing T Cells Dramatically Eradicate Myeloma Cells from Bone Marrow of an Orthotopic Multiple Myeloma Mouse Model

Ryosuke Uchibori[1], Takeshi Teruya[2], Ken Ohmine[3], Masashi Urabe[4], Yasushi Saga[4], Hiroaki Mizukami[4], Junichi Mineno[2], Kazuto Takesako[2], Keiya Ozawa[5]
[1]Division of Immuno-Gene & Cell Therapy (Takara Bio), Jichi Medical University, Tochigi, Japan, [2]CDM Center, Takara Bio Inc., Shiga, Japan, [3]Division of Hematology, Department of Medicine, Jichi Medical University, Tochigi, Japan, [4]Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan, [5]The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Introduction: Multiple myeloma (MM) is an incurable hematological malignancy of plasma cells. During the past decade, overall survival rate of multiple myeloma have been improved. These improvements are linked to the induction of novel drugs with different mechanism of action such as proteasome inhibitors and immnomodulatory drugs. However, MM remains in most cases an incurable disease, and new therapeutic strategies are urgently required for radical cure or continued disease control. During recent years, some dramatic responses were reported using T cells expressing CD19-targeted chimeric antigen receptors (CAR). Therefore, CAR therapy could also be a promising new therapeutic strategy for MM. CD269 (also known as BCMA: B-cell maturation antigen) is a membrane protein that is selectively expressed on B-cell lineages and on plasma cells including myeloma cells. Our goal of this study is to verify whether treatment with CD269-specific CAR-expressing T cells can eradicate myeloma cells from bone marrow of tumor-bearing NOG mice. **Methods:** Firstly, we uniquely developed monoclonal antibodies against human CD269. Next, we designed and verified novel CD269-specific CAR. The CD269-CAR recombinant retroviral vector encodes the MoMLV (Moloney murine leukemia virus) retroviral backbone and the 3.3E-28z CAR. The 3.3E-28z CAR consists of an anti-human CD269 scFv that was derived from the 3.3E mouse hybridoma, a portion of the human CD28 molecule and the intracellular domain of the human CD3ζ molecule. **Results:** CD269-specific CAR-expressing T cells show redirected cytolysis toward CD269-positive U266 human MM-derived cells, but not CD269-negative K562 cells. Luciferase-expressing U266 cells that were injected into the cardiac chamber of NOG mice, selectively infiltrated to bone marrow. Six weeks after tumor inoculation, we injected saline, non-CAR gene-modified T cells, or CAR gene-modified T cells

(GMCs) into the cardiac chamber of tumor-bearing mice. In the group of GMC injection, U266 cells were dramatically eradicated from bone marrow. **Conclusion:** Although the efficacy of BCMA-CAR has been demonstrated by previous study using intradermal models of MM, our challenge is the first report that presents with orthotopic models of MM. Our results are more appropriate for predicting the efficacy of CD269-CAR in the treatment of MM. We conclude that adoptive transfer of CD269-CAR-expressing T cells could be a promising option for patient with MM.

401. *In Vivo* Expression of Plasmid Encoded IgG for PD-1 or LAG3 by Synthetic DNA as a New Tool for Cancer Immunotherapy

Kar Muthumani[1], C. Chung[1], S. Agarwal[1], R. Plyler[1], S. Kudchodkar[1], S. Flingai[1], K. E. Ugen[2], J. J. Kim[3], N. Sardesai[3], David B. Weiner[1]
[1]Dept. of Pathology and Molecular Medicine, Perlman School of Medicine, University of Pennsylvania, Philadelphia, PA, [2]Dept. of Molecular Medicine, University of South Florida Morsani College of Medicine, Tampa, FL, [3]Inovio Pharmaceutics, Inc., Plymouth Meeting, PA

Cancers employ various strategies to escape immune surveillance including the exploitation of immune checkpoint inhibitors. Checkpoint inhibitors are receptors found on immune and stromal cells whose function can impact the duration or potency of an immune response. Tumor cells often upregulate ligands for these receptors to protect themselves from the host immune response. Monoclonal antibody (MAb) therapeutics which block checkpoint inhibitor-ligand interactions restore T cell destruction of cancer cells in vivo. MAbs that target the inhibitory T cell signaling mediated by CTLA-4 and/or PD-1 checkpoint inhibitors have recently gained regulatory approval for the treatment of some cancers based on remarkable clinical outcomes.

Here we have focused on a new method to improve MAb delivery through direct engineering of MAb in the form of synthetic DNA plasmids. This technology would improve many aspects of such a therapy by lowering cost, increasing in vivo expression times and allowing for simple combination formulations in the absence of a host anti-vector immune response, possibly extending use of these groundbreaking therapies to disadvantaged patient populations. We report that "enhanced and optimized" DNA plasmid technology can be used to direct in vivo production of immunoglobulin heavy and light chains of established monoclonal antibodies which can target the immune checkpoint inhibitors LAG3 and PD-1 as determined in Flow cytometry, ELISA and Western blot assays. Both antibodies are produced at physiologically relevant levels in blood and other tissues of mice using electroporation-enhanced delivery of DNA plasmids encoding genes for each antibody. We report that serum antibodies from inoculated animals retain the ability to bind to their targets and are bioactive in vivo and exhibit immune stimulatory effects for host T cells. These studies have significant implications for prophylactic and therapeutic strategies for cancer and other important diseases and warrants further attention.

402. Bioengineering of Peripheral Blood Derived Gamma Delta T Cells in a Serum-Free Expansion Medium

Kathryn Sutton, Anindya Dasgupta, McCarty David, Christopher Doering, H. Trent Spencer
Pediatrics, Emory Unversity, Atlanta, GA

To date the main source of effector cells in preclinical and clinical immunotherapy trials has been αβ T cells. Although cellular products utilizing these cells have shown some promise, there is a need for better control of antitumor immunogenicity, especially in the context of chimeric antigen receptor technologies. Therefore, as an alternative, we are investigating the use of a T cell subset, γδ T cells. These are a potentially attractive cellular therapeutic as they can be expanded to clinically useful levels and have potent MHC independent intrinsic anti-tumorigenicity driven by their recognition of stress antigens on tumor cells. Our efforts have focused on development of a clinically reliable expansion and manufacturing process of genetically engineered γδ T cells. In the current study we describe the optimization of culture conditions in serum free media (SFM) that can support robust expansion followed by genetic modification of the expanded cells. Peripheral blood mononuclear cells (PBMC) isolated from healthy donors were cultured in several SFMs including: OpTmizer, X-Vivo, SCGM, and AIM V as well as commonly used media containing serum, RPMI1640 supplemented with either 10% FBS or human serum. Complete growth media also included 2 mM L-glutamine, zoledronic acid (5μM added only at the start of culture), and IL-2 (either 100 or 1000 IU/ml added twice weekly). Of the SFM cultures, robust γδ T cell expansion was only observed in OpTmizer supplemented with high-dose IL-2, which resulted in expansion and γδ T cell percentages to levels sufficient for clinical use. Specifically, in OpTmizer, the percentage of γδ T cells increased from a mean of 2.4% (0.8%-3.6%) in the starting product to 66.4% (35%-94.5%, 6 of 9 cultures were >70% γδ T cell), resulting in 80-fold expansion at 14 days. The SFM-expanded γδ T cells were evaluated for their anti-tumor cytotoxicity and shown to be equally effective at killing both hematologic and solid cancer cell lines as γδ T cells expanded in serum containing media. Transduction efficiency was tested using GFP encoding self-inactivating lentiviral vectors (LV). These studies demonstrated that SIV and HIV transduce expanded γδ T cells equally, and that compared to EF1α, cassettes employing an MSCV promoter provide greater transgene expression. The timing of LV addition was optimized with respect to i) γδ T cell expansion, ii) LDL Receptor (LDL-R) expression, and iii) LV dose (3-4 consecutive transductions over 48 hours). The greatest transduction efficiencies were achieved in cultures with the greatest expansion. As expected a range of transduction efficiencies was observed for various donors with a mean of approximately 20%, which is similar to LV transduction levels observed for αβ T cell transductions with similar multiplicity of infection and rates of expansion. Therefore, an optimized strategy to expand and transduce γδ T cells with demonstrated anti-cancer properties was developed. The utilization of SFM to expand these cells to clinically useful doses and redirection toward tumor cells via bioengineering can result in a clinically safe, widely applicable, and potentially more efficacious cellular immunotherapy.

403. Toca Retroviral Replicating Vector in Tumor and Blood from Clinical Trial Subjects with Recurrent High Grade Glioma

Daniel Hogan
Diagnostics, Tocagen Inc., San Diego, CA

Toca 511 (vocimagene amiretrorepvec) is a retroviral replicating vector (RRV) based on an amphotropic murine gammaretrovirus, that encodes a cytosine deaminase transgene that allows infected cells to selectively convert the antifungal drug 5-fluorocytosine (5-FC) into the antineoplastic drug 5-fluorouracil (5-FU). Toca 511 can be delivered by multiple routes, selectively infects and spreads in tumor cells and through multiple mechanisms can elicit an anti-tumor immune response. Thus, clinically, treatment with Toca 511 and extended-release 5-FC (Toca FC) is expected to selectively destroy tumor cells within the body, while leaving healthy cells unharmed. Toca 511 and Toca FC have been administered to >120 high grade glioma subjects in three phase I studies (NCT01156584, NCT01470794, NCT01985256) and, based on results from these

trials, a phase 2/3 trial (Toca 5) has recently started recruitment (NCT02414165). Although Toca 511 plus Toca FC has been well tolerated, we investigated potential off-target viral infection and integration, and whether such off-target events have potential toxicity; we also investigated the stability profile of the Toca 511 genome after infection of tumor targets and off-target tissue (blood). To date, few subjects (approximately 10-25%) show quantifiable viral signal (LOQ approximately 4000 copies/mL) in blood after initial clearance, following Toca 511 injection into the brain tumor or tumor bed. In all cases, quantifiable virus RNA and DNA signal was cleared from blood. In order to better understand interactions among Toca 511 and subjects' tumor and blood, we systematically mapped Toca 511 integration sites from subject samples with detectable virus and also sequenced, at high depth, integrated Toca 511 genomes, and in some cases RNA genomes, from these samples. Toca 511 integration profiles display a preference for integration near active transcription start sites, as has been seen generally for gammaretroviruses. There was no evidence for clonal expansion of Toca 511 integrated sites/cells or preferential retrieval of sites nearby oncogenes. Toca 511 sequences display an array of mutations in the tumor and blood, including transitions consistent with cytosine deaminase activity. The data provide a molecular correlate to the clinical safety profile of Toca 511 and Toca FC treatment as well as molecular characterization of RRV after infection, replication and therapeutic use in humans.

404. Development of Combinational Cellular Immunotherapy to Treat Pediatric Acute Myeloid Leukemia Patients

Niek P. van Til[1], Lea Knežević[1], Maud Plantinga[1], Colin de Haar[1], Rob C. Hoeben[2], Manuel A. Gonçalves[2], Stefan Nierkens[1], Jaap Jan Boelens[3]
[1]Laboratory for Translational Immunology, UMC Utrecht, Utrecht, Netherlands, [2]Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, Netherlands, [3]Pediatric Blood and Marrow Transplantation Program, UMC Utrecht, Utrecht, Netherlands

Hematopoietic stem cell transplantation is the standard of care for pediatric acute myeloid leukemia (AML) patients, but relapse still occurs in approximately 40% of AML patients. Hence there is an unmet need to improve disease control with fewer adverse acute and late effects. We therefore aim to develop 'HLA-matched' modified cellular therapies based on a platform of cord blood transplantation (CBT) to treat pediatric AML patients. In the majority of AML patients the tumor oncogene Wilms tumor 1 (WT1) is overexpressed and can serve as a target for tumor specific therapy. In addition to our previously developed cord blood-derived dendritic cell vaccine (CB-DC) we are currently developing gene modified T lymphocytes from the same CB-unit to specifically target WT1 expressing AML to support and increase the anti-leukemic effects of CBT. Lentiviral vectors were developed to express recombinant T-cell receptors (TCR) specifically recognizing HLA-A2+ restricted WT1 peptide. TCR expression was improved by codon-optimization of the cDNA sequence including cysteines in the alpha and beta chain constant domains. These WT1-specific TCR α/β-chains were incorporated in a single lentiviral vector linked by a 'self-cleaving' T2A peptide to improve efficiency. The order of the alpha and beta TCR chains, and inclusion of a furin cleavage site in the expression cassette did not enhance TCR expression in Jurkat and primary T cells derived from either peripheral blood or cord blood. Primary T cells were stimulated with T2 cells and CB-DCs pulsed with the specific peptide, which resulted in T cell activation as measured by CD69 and CD137 upregulation, as well as IFNgamma production. To reduce the risk of off-target antigen recognition of gene-modified T cells, and to further improve efficacy, CRISPR/Cas9 can be used to eliminate endogenous TCR expression. Cas9 protein and guide RNA (gRNA) were delivered to GFP positive reporter Jurkat cells using plasmid and mRNA electroporation (EP), integrating and non-integrating lentiviral vectors and adenoviral vectors with the highest efficiency of knockout being achieved by EP. Two gRNAs targeting the constant domains of the alpha chain and three targeting those of the beta chain resulted in similar knockout efficiencies in Jurkat cells (approximately 25%). Of note, combining the gRNAs did not result in higher knockout efficiencies. Gene-editing at the molecular level was confirmed by next generation sequencing. We envision that addition of TCR-engineered T cells (introduction of tumor-specific TCR and knockdown of endogenous TCR), derived from the same cord blood as used for the transplantation, will more efficiently and with improved safety prevent early relapse after CBT by targeting WT1 AML.

405. IL-2 and TNF-a Coding Adenoviruses Enable Adoptive T Cell Therapy in Metastatic, Solid Cancer by Systemically Activating Tumor-Reactive TILs

Siri Tähtinen[1], Carolin Blattner[2], Markus Vähä-Koskela[1], Dipongkor Saha[1], Mikko Siurala[3], Riikka Havunen[1], Suvi Parviainen[3], Jochen Utikal[2], Viktor Umansky[2], Akseli Hemminki[3]
[1]Department of Pathology, University of Helsinki, Helsinki, Finland, [2]Clinical Cooperation Unit Dermato-Oncology (G300), German Cancer Research Center (DKFZ), Heidelberg, Germany, [3]Department of Pathology, University of Helsinki; TILT Biotherapeutics Ltd, Helsinki, Finland

Adoptive T cell therapy (ACT) using genetically modified T cells has shown exceptional efficacy in the treatment of $CD19^+$ hematological cancers. However, far less impressive results have been achieved in the treatment of solid tumors due to local immunosuppression, rendering tumor-infiltrating T cells (TILs) hypofunctional. We have previously shown that adenovirus (Ad) infection can enhance the efficacy of ACT (Tähtinen et al, CIR 2015) and that intratumoral administration of immunostimulatory cytokines can result in favorable alteration of tumor microenvironment (Tähtinen et al, PLOS One 2015). To combine the benefits of both approaches, we studied if replication-deficient Ad5-vectors coding for interleukin-2 (IL-2) and tumor necrosis factor alpha (TNF-a) would affect the activity of adoptively transferred, TCR-transgenic $TRP\text{-}2_{(180\text{-}188)}$ specific T cells *in vivo*.

To gain clinically relevant mechanism-of-action data, we chose to use *ret* transgenic mouse model that develops spontaneous malignant skin melanoma which metastasizes into distant organs. Following ACT and intratumoral virus injection, a significant increase in activated $PD\text{-}1^+$ $CD8^+$ T cells was seen in both cutaneous lesions and in metastatic lymph nodes. Interestingly, a reverse correlation between tumor weight and the number of tumor-reactive $PD\text{-}1^+$ TILs ($p=0.0015$) was observed, indicating that T-cell hypofunction was overcome and successful tumor lysis was achieved. Local expression of cytokines did not affect the levels of immunosuppressive immune cell subsets such as myeloid-derived suppressor cells (MDSCs) or T regulatory cells (Tregs), latter of which has previously been associated with systemic IL-2 therapy (Ahmadzadeh and Rosenberg, Blood 2006). Instead, Ad5-IL2 treatment induced upregulation of IL-2 receptor α-chain (CD25) in conventional $CD4^+CD25^+Foxp3^-$ cells, suggesting that these helper T cells contributed to $CD8^+$ TIL activation. Finally, beneficial ratios between tumor-reactive $PD\text{-}1^+$ $CD8^+$ TILs and Tregs was observed in primary and secondary tumor sites, indicating that IL-2 and TNF-a coding adenoviruses can modify the cellular composition of the tumor microenvironment in favor of adoptively transferred T cells.

In conclusion, IL-2 and TNF-a coding adenoviruses can break tumor-associated immunotolerance and significantly increase the levels of active, tumor-reactive T-cells both in injected cutaneous lesions and in non-injected metastatic lymph nodes. Importantly, this triple modality may represent an efficient approach to achieve "CD19-like" clinical responses in the treatment of solid, metastatic cancers currently incurable by standard therapies.

406. Development and Optimization of PSCA-Specific CAR T Cells for the Treatment of Bone Metastatic Prostate Cancer

Dileshni Tilakawardane, Ethan A. Gerdts, Anthony K. Park, Wen-Chung Chang, Sarah Wright, Christine Brown, Stephen J. Forman, Saul J. Priceman
Beckman Research Institute, City of Hope National Medical Center, Duarte, CA

Prostate Cancer (PCa) is the third most common cancer type in the United States, with over 200,000 new cases projected to be diagnosed this year. In approximately 80% of PCa patients, tumor phenotype includes overexpression of prostate stem cell antigen, or PSCA. Furthermore, PSCA is expressed on nearly 100% of bone metastatic prostate cancers, making it an attractive immunotherapeutic target. We have genetically engineered T cells to express chimeric antigen receptors (CARs) which specifically target PSCA. Recent clinical trials with CARs targeting CD19 for B-cell malignancies have demonstrated impressive results, yet replicating this success with other antigen targets remains elusive. Immunotherapy against solid tumors poses a more difficult tumor challenge because of the immunosuppressive microenvironment that can significantly hinder CAR efficacy. Additionally, there have been instances of on-target, off-tumor toxicity due to low levels of antigen expression on normal tissue.

In the current project we have modified various components of our CAR constructs to improve specificity and overall therapeutic efficacy. Through various *in vitro* functional assays and *in vivo* xenograft models, we have evaluated and optimized a PSCA-targeting CAR. We first compared two single-chain variable fragments with different paratopes. While both show comparable potency, one of the scFvs showed nonspecific activity against PSCA-negative tumor lines. Similarly, our data suggest that the 28ζ-costimulatory domain, regardless of linker length, also shows non-specific activation and killing of PSCA-negative tumor lines as compared to the 4-1BB costimulatory domain. Finally, we have demonstrated differences between long, middle, and short linker lengths in intracellular cytokine production, activation, and killing capacities *in vitro* and *in vivo*. By modifying both the ectodomain and intracellular region, we are able to improve the specificity and functionality of our PSCA-CARs, which is essential to developing effective immunotherapies for this advanced disease.

407. A Novel Rabbit Antibody-Derived, Anti-CD123 LV/CAR Construct for AML Immunotherapy

Robyn A. A. Oldham[1,2,3], Elliot M. Berinstein[1,2], Jeffrey A. Medin[1,2,3,4]
[1]Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada, [2]University Health Network, Toronto, ON, Canada, [3]Medical College of Wisconsin, Milwaukee, WI, [4]Institute of Medical Sciences, University of Toronto, Toronto, ON, Canada

Chimeric antigen receptors (CARs) have emerged in the immunotherapy field as an exciting new option for cancer treatment, with clinical trials of CD19-directed CARs having already demonstrated long-lasting responses in patients with ALL and CLL. As a hematological malignancy, acute myeloid leukemia (AML) may be another viable target for CAR-mediated therapy. Furthermore, with a 5-year survival rate of just 5.5% for patients over 65, new treatments for AML are very much needed.

CD123 (the IL-3 receptor α-chain) is upregulated on AML blasts/stem cells and plays a role in proliferation and apoptotic resistance. This antigen demonstrates much lower expression levels on normal hematopoietic cells, where its expression is restricted to the myeloid progenitor subpopulation. Previous attempts to target CD123 in AML through several forms of immunotherapy have had variable success. Specifically, results of previous CD123 murine antibody-derived CARs have been mixed, with some results showing CAR-mediated eradication of normal myelopoiesis via targeting of HSCs with low CD123 expression.

We have developed a CD123 CAR, derived from a novel rabbit anti-CD123 mAb that we generated. Rabbit antibodies are reported to have a broader avidity and higher range of affinities than mouse mAbs; this may lead to a CAR with a unique binding profile. We will determine whether such a rabbit-derived CD123 CAR will lead to more specific binding, resulting in optimized killing of AML cells, while minimizing cytotoxic effects on HSCs.

To generate our CAR, human CD123 was purified as a GST-tagged protein and used for immunization of rabbits. Hybridoma cell lines were developed from the spleen cells of rabbits with positive immune responses, and novel antibodies were purified and screened for specificity to CD123 using a combination of ELISA, flow cytometry, and ADCC. A candidate antibody was selected, and the VL and VH chains were subcloned, sequenced, and assembled into an scFv. A second generation CAR was then designed that includes a CD8 hinge and transmembrane region, a 4-1BB costimulatory domain, and a CD3ζ signaling domain. This construct was then subcloned into a lentiviral backbone to facilitate expression in immune effector cells.

Our CD123 CAR has been transduced into primary T cells and the NK-92 cell line for in vitro testing. Flow cytometry demonstrates that our CARs are expressed at the cell surface. Furthermore, the expression of the CD123 CAR has been shown to be stable in the NK-92 cell line. Cytotoxicity assays are being performed in vitro in order to confirm binding specificity and cytotoxic potential of the CD123 CARs in both NK-92 and T cells. Future work will compare CAR T and CAR NK killing in vivo using NSG mouse models of AML.

AML may be an ideal target for CAR therapy, and we will exploit our novel CD123 CARs as therapeutic entities. We will examine whether the use of a novel rabbit anti-CD123 scFv in our LV/CAR construct will optimize the killing of AML cells while minimizing HSC eradication. This novel second-generation CAR has the potential to greatly impact the treatment of AML patients in the future.

408. Oncolytic Vaccines in Combination with PD-L1 Blockade for the Treatment of Melanoma

Cristian Capasso[1], Davide Cardella[2], Martin Muller[3], Mariangela Garofalo[1], Lukasz Kuryk[1], Karita Peltonen[1], Vincenzo Cerullo[1]
[1]University of Helsinki, Helsinki, Finland, [2]University of Naples "Federico II", Naples, Italy, [3]University of Munich, Munich, Germany

The Immunological escape of tumors represents one of the main obstacles to the treatment of malignancies. The approval of drugs able to disrupt the immune suppressive pathways through anti-CTLA-4 monoclonal antibodies represented a milestone in the history of immunotherapy. However, treatment with these immune checkpoint inhibitors (ICIs) seems to be effective only in small cohorts of patients. It has been proposed that the efficacy of ICIs relies on the presence of an undergoing immunological response. For this reason, we hypothesized that oncolytic vaccines, able to elicit a tumor specific response, would synergize with anti-PD-L1 therapy. B16 murine melanomas were established in immunocompetent C57 mice. Then mice were treated with anti-PD-L1 monotherapy,

PeptiCRAd (oncolytic vaccine) monotherapy or a combination of the two. The growth of the tumors was analyzed. At the end of the experiment, all the mice mice were euthanized and organs collected for immunological analysis. We investigated antigen-specific T-cell responses and immune suppressive background by flow cytometry and ELISPOT assays.

Cancer-Oncolytic Viruses I

409. Novel Recombinant Coxsackievirus B3 Infection Elicits Robust Oncolytic Activity Against Human Non-Small Lung Cancer and Triple-Negative Breast Cancer

Miyako Sagara[1], Yuto Takishima[1], Shohei Miyamoto[1], Hiroyuki Shimizu[2], Yoichi Nakanishi[3], Kenzaburo Tani[1]
[1]Project Division of ALA Advanced Medical Research, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, [2]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan, [3]Research Institute of Diseases of the Chest, Kyushu University, Fukuoka, Japan

Oncolytic virotherapy using enteroviruses emerges as a promising anticancer strategy. As therapeutic advantages, enteroviruses immediately induce robust oncolytic activity and do not have oncogenes that may lead to tumorigenesis. We recently showed coxsackievirus B3 wild type (CVB3-WT) infection elicited remarkably oncolytic activity against human non-small cell lung cancer cells (NSCLC). However, CVB3-WT infection caused adverse events of weight loss, pancreatitis, and myocarditis in mice. To overcome these pathogenicity, we engineered CVB3-WT genome for the development of microRNA (miRNA)-regulated oncolytic virus. We focused on two miRNAs (miR-1 and miR-217) expressed mainly normal muscle or pancreas. We successfully genetically constructed a novel recombinant CVB3-miR-1&217T (CVB3-miRT) by inserting 4 tandem target sequences complementary to two miR-1 and two miR-217 into the 3' UTR of CVB3-WT genome. Recently, we investigated whether an infection with CVB3-miRT displays oncolytic activities against NSCLC. We found that CVB3-miRT infection induced potent oncolytic activity comparable to CVB3-WT in human NSCLC *in vitro* and *in vivo*. Here, we attempted to explore the oncolysis to triple-negative breast cancer (TNBC) because TNBC are highly aggressive and intractable tumors with dismal prognosis. We performed in vitro crystal violet staining to examine the effect of CVB3-miRT on TNBC. These results showed that CVB3-miRT had potent oncolytic activity against TNBC cell lines in a MOI-dependent manner. Furthermore, consecutive administrations of CVB3-miRT into subcutaneous xenografts of human TNBC pre-established in athymic nude mice significantly suppressed the tumor growth with a prolonged survival rate. The intratumoral CVB3-miRT administrations into human TNBC xenograft tumor mice model displayed dramatically decreased side effects of CVB3-WT-induced pathogenicity. Collectively, we showed that CVB3-miRT infection indicated marked oncolytic activity against human NSCLC and TNBC cells *in vitro* and *in vivo* as well as CVB3-WT. This approach could be a promising new therapeutic modality to improve survival in patients suffering from NSCLC and TNBC in advanced stage.

410. Oncolytic Adenoviruses Armed with TNFa and IL-2 Induce Antitumor Immune Responses and Protection from Tumor Rechallenge

Riikka Havunen[1], Mikko Siurala[1], Suvi Parviainen[2], Michael Behr[1], Dirk Nettelbeck[3], Anja Ehrhardt[4], Akseli Hemminki[2]
[1]Medicum, Faculty of Medicine, University of Helsinki, Helsinki, Finland, [2]TILT Biotherapeutics Ltd., Helsinki, Finland, [3]German Cancer Research Center (DKFZ), Heidelberg, Germany, [4]Institute for Virology and Microbiology, University Witten/Herdecke, Witten, Germany

During the past few years, immune system-stimulating factors have emerged as promising means to treat malignant tumors. We aim to use cytokine-bearing oncolytic adenoviruses to improve and to extend the usage of adoptive T cell therapy, a treatment known to benefit a portion of melanoma and leukemia patients. Administered systemically or intratumorally the virus induces immune responses against the tumor by revealing tumor antigens, but immunostimulatory cytokines augment the effect further. We have shown that the most promising cytokines in this regard are interleukin (IL) -2 and Tumor Necrosis Factor alpha (TNFa). IL-2 is a common treatment for malignant melanoma and renal cell carcinoma, but systemic administration may lead to severe side effects. While IL-2 has a key role in recruiting and activating T cells, TNFa has prominent anti-immunosuppressive actions. Further, it directly promotes tumor cell death by apoptosis and necrosis. Armed oncolytic viruses accomplish local, long-lasting, high-level cytokine expression while systemic level remains low. Moreover, we have proven that adenoviruses enhance adoptive T cell therapy. In this study, we treated Syrian hamsters (*Mesocricetus auratus*) with Ad5/3-E2F-d24 virus bearing human IL-2, TNFa, or both in its *E3* region. Hamster cell lines are semipermissive for 5/3-chimeric adenoviruses and produce active transgenes from these viruses. In addition, human IL-2 and TNFa are evidently active in hamsters. Hamster pancreatic cancer (HapT1) was implanted subcutaneously and treated once with TILs extracted from syngenic tumors, and with five viral injections. We saw synergy between unarmed virus and TILs, while the armed viruses turned out to be even more effective. When the cured animals were rechallenged with the same cancer cells, previous treatment with cytokine-armed viruses protected the animals against new tumors. Also, splenocytes derived from animals treated with cytokine-bearing viruses proliferated more actively *ex vivo* than the controls. To conclude, our data demonstrates the immunological benefit gained with viruses bearing IL-2 and TNFa, and further supports combining oncolytic viruses with adoptive cell transfer.

411. Predicting Tumor Response to Oncolytic Virotherapy Using Dual Isotope SPECT/CT Imaging with NIS Reporter Gene and Duramycin

Kah-Whye Peng[1], Lianwen Zhang[1], Lukkana Suksanpaisan[2], Stephen J. Russell[1], Ming Zhao[3]
[1]Molecular Medicine, Mayo Clinic, Rochester, MN, [2]Imanis Life Sciences, Rochester, MN, [3]Northwestern University, Chicago, IL

Oncolytic virotherapy is a promising modality for cancer therapy and diverse viruses from various families have been genetically engineered to be tumor selective anticancer agents. To obtain an early readout on tumor susceptibility to the oncolytic virus, and potential toxicity from off target viral infection, we have engineered our oncolytic viruses to encode the thyroidal sodium iodide symporter gene (NIS) to enable noninvasive longitudinal imaging of the pharmacokinetics and sites of virus spread. The high resolution images obtained from the new generation of small animal SPECT/CT or PET/CT machines enable us to visualize increasing numbers of infectious foci daily within the subcutaneous tumors

and to establish a mathematical model to develop strategies that will improve the outcome of virotherapy. In this study, we used the oncolytic Vesicular Stomatitis Virus (VSV) encoding NIS to evaluate the kinetics of intratumoral viral spread, and the subsequent aftermath of tumor cell death by apoptosis. Through I-125 SPECT/CT and NIS imaging, we showed rapid VSV spread within the susceptible MPC-11 murine plasmacytoma tumors, and the infectious foci grew larger and increased in numbers over 5 days. Since SPECT imaging enables concurrent dual isotope imaging, we also gave the same animal Tc-99m duramycin that detects apoptotic cells. In this MPC-11 model, we found rapid onset of apoptosis within the tumor. The infectious foci of viral infection (NIS imaging) and cell death (duramycin imaging) were coincident, overlapping at the acute phase of infection. In contrast, the CT-26 tumor was significantly less responsive to VSV-mIFN-NIS therapy, and there was minimal positive NIS signals or apoptosis. We are keen to further evaluate the potential of using dual isotope SPECT imaging using NIS reporter gene and the duramycin apoptosis tracer, with the goal of using this twin set of imaging methods to give us an early prediction of tumor response to oncolytic virotherapy.

412. The Efficacy and Bio-Distribution of Oncolytic HSV-1 (G47Δ) in Mouse Orthotopic Esophageal Cancer Models

Shoh Yajima[1], Yasushi Ino[1], Hiroshi Fukuhara[1], Miwako Iwai[1], Yasuyuki Seto[2], Tomoki Todo[1]
[1]Division of Innovative Cancer Therapy, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, [2]Department of Gastrointestinal Surgery, Metabolic Care and Endocrine Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal among cancer, because it easily metastasizes to the mediastinal lymph nodes and invades adjacent organs. The majority of patients with ESCC are diagnosed at advanced stages. Conventional treatments often result in unsatisfactory clinical outcome, therefore a novel therapeutic approach is urgently needed. Oncolytic viruses emerge as promising therapeutic agents. G47Δ, a third generation oncolytic herpes simplex virus type 1 (HSV-1), shows high replication capability and robust cytopathic effects in a variety of cancer cells. In this study, we examined the efficacy and bio-distribution of G47Δ using mouse ESCC models. A panel of human ESCC cell lines (KYSE70, KYSE180, KYSE220, T.Tn, and TE8) was susceptible to G47Δ by cytotoxicity assays. G47Δ showed moderate to good replication capability in all cell lines tested. Athymic mice harboring subcutaneous tumors (KYSE180, TE8) or orthotopic abdominal esophageal tumors (TE8-luc) were used for *in vivo* studies. When the subcutaneous tumors reached approximately 6 mm in diameter, they were inoculated twice (days 0 and 3) with G47Δ ($4x10^4$, $2x10^5$, or $1x10^6$ pfu) or mock, and the tumor size was measured (n=10). G47Δ was significantly more effective in inhibiting the growth of subcutaneous tumors than mock in both models. The orthotopic TE8-luc tumors were inoculated once with G47Δ ($1x10^6$ pfu) or mock under laparotomy 18 days after tumor implantation (n=10). The tumor size was evaluated by measuring photons emitted from the tumors every 3 days using the IVIS and Living Image Software (Xenogen). Tumors treated with G47Δ were significantly smaller than those with mock ($p<0.01$, Wilcoxon's test). In order to validate the result, the tumor weight was measured on day 31. G47Δ treated tumors were significantly smaller than mock treated tumors ($p<0.01$, Student's t test). For bio-distribution evaluation, wild type HSV-1 strain F ($1x10^7$ pfu) or G47Δ ($1x10^7$ pfu) were administered to HSV-1 sensitive A/J mice via oral route or by direct injection to abdominal esophagus under laparotomy. Mice were sacrificed at each time point (1, 3, 5, and 7 days after administration, n=3) and the amount of viral DNA in major organs was examined by quantitative PCR. DNA copy numbers of Strain F remained high in esophagus or dramatically increased in the brain, spinal cord and adrenal gland through day 7. In contrast, G47Δ DNA copy number decreased rapidly in the esophagus and was mostly undetectable in other organs. These results indicate that G47Δ is highly efficacious and safe in both orthotopic and subcutaneous ESCC models. G47Δ may be useful as a new therapeutic approach for ESCC.

413. Effect of Adenoviral Death Protein on NIS-Based Iodine Therapy and Imaging for Pancreatic Cancer

Lisa Koodie[1], Eriko Kawakami[2], Ben Eidenschink[1], Jordan Sell[1], Chris LaRocca[1], Kari Jacobsen[1], Jessica Ryvlin[1], Ezequiel Tolosa[2], John C. Morris[2], Martin Fernandez-Zapico[2], Julia Davydova[1]
[1]Department of Surgery, University of Minnesota, Minneapolis, MN, [2]Mayo Clinic, Rochester, MN

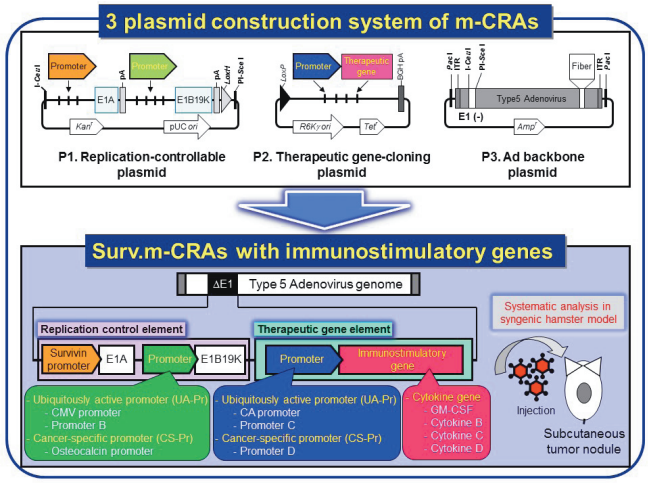
In this study, we assess our novel Oncolytic Adenovirus (OAd) expressing a dual function therapeutic and imaging transgene, the sodium-iodide symporter (NIS) as a promising alternative for pancreatic cancer treatment. We hypothesized that NIS expression in pancreatic cancer will induce uptake of radioiodine and allow noninvasive SPECT/CT imaging with ^{123}I. We designed our OAd-NIS to overcome the low efficacy of previous vectors. These viruses have a modified Ad5/Ad3 fiber-knob shown to improve the poor transduction of pancreatic cancer cells. Viral replication is controlled under the Cox2 promoter allowing specific delivery of viral genes, and most of adenoviral E3 genes are deleted and replaced with NIS and Adenovirus Death Protein (ADP) genes. We have previously demonstrated significantly improved ability of ADP-overexpressing OAds to enhance viral release and cytolytic activity. Although improved oncolysis enhances viral release and improve the therapeutic effect in solid tumors, it can potentially reduce radioiodide tracer uptake. To test this possibility we designed and compared identical vectors with ADP (OAd-NIS-ADP) and without ADP (OAd-NIS-noADP) *in vitro* and *in vivo*. We first compared virus killing ability in pancreatic cancer cell lines. The ADP+ vector was significantly more cytolytic that no-ADP counterpart and showed improved viral replication and spread. Cox2-controlled OAd did not produce cell death in Cox2-negative control, confirming selectivity. We next assessed OAds ability to induce NIS protein expression and radioiodine uptake. NIS-OAds efficiently produce glycosylated NIS multimers as early as 2-days post infection. Infection with no-ADP vectors resulted in a significantly greater radioiodine uptake (^{125}I) compared to ADP+ viruses. Importantly, *ex vivo* uptake test in human patient tissues confirmed the high level of NIS in pancreatic adenocarcinoma samples, and revealed no ^{125}I uptake in normal pancreas. We further assessed the ability of our vectors to visualize human pancreatic cancer xenografts in a mouse model by monitoring $^{99}mTc^{O4-}$ accumulation with SPECT-CT. The OAd-NIS-noADP showed an earlier and more sustained radioisotope uptake when compared to ADP+ supporting its use as a more sensitive diagnostic tool for pancreatic cancer. Of contrary, the ADP+ vector significantly outperformed OAd-NIS-noADP in tumor shrinkage. These results suggest that while OAd-NIS-noADP produces a greater radiotracer uptake and can be used as a diagnostic tool, its ADP+ counterpart results in a better therapeutic effect when applied as a monotherapy. It is now essential to estimate the therapeutic ability of both vectors upon combination with I-131 (ongoing study). Further investigation will include the imaging and therapeutic studies in patient-derived xenografts. Ultimately, the goal of our research is to design a multimodal therapy with radiation and oncolytic virus for diagnosis and therapy of cancers.

414. A Novel System to Systematically Analyze Diverse Types of Conditionally Replicating Adenoviruses Expressing Immunostimulatory Genes in Syngenic Hamster Models

Nobuhiro Ijichi, Hirotaka Kawakami, Yuki Obama, Ken-ichiro Kosai

Department of Gene Therapy and Regenerative Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan

We developed a method to efficiently construct diverse "conditionally replicating adenoviruses regulated with multiple cancer-specific factors" (m-CRAs) for an ideal gene-viro-therapy (Gene Ther 2005), after we had developed the immunological gene therapy, including granulocyte macrophage colony-stimulating factor (GM-CSF) (PNAS 1995, 1996, Cancer Res 1996). We then identified by systematic m-CRA analyses that survivin-responsive m-CRA (Surv.m-CRA), in which E1A is regulated by survivin promoter, could achieve stronger and more cancer-selective potentials than competing m-CRAs (Cancer Res 2005). The new Surv.m-CRA, of which an altered E1B promoter is regulated with another cancer-specific promoter (CS-Pr), further increased cancer specificity without reduced anticancer effects (Cancer Gene Ther 2011). Moreover, the unique feature of the increased effectiveness against cancer stem cells suggests that Surv.m-CRA is promising anticancer agent (J Trans Med 2014). In addition, we have developed a novel m-CRA strategy that specifically eliminates undifferentiated cells in pluripotent stem cell-based regenerative medicine (Mol Ther Methods Clin Dev 2015). Taken altogether, we are about to start its clinical trial in Japan. As the first-in-class oncolytic virus expressing GM-CSF was recently approved, m-CRAs expressing immunostimulatory genes should be promising. In general, the development of oncolytic adenoviruses with cytokines has been hampered by two technical limitations in the vector construction and the viral replication-permissive animals. Here we demonstrate a novel system to efficiently generate diverse types of m-CRAs expressing candidate immunostimulatory genes under candidate promoters, and systematically analyze their potentials in syngenic hamster models. We tested the feasibility of this methodology, and succeeded in the rapid generation of the new Surv.m-CRAs comprising 18 different therapeutic units, including a mouse or human cytokine gene downstream to a ubiquitously active promoter (UA-Pr) or CS-Pr. To test the utility of our experimental system, we first analyzed *in vivo* antitumor efficiencies and toxicities of Surv.m-CRA expressing GM-CSF under a strong UA-Pr in xenograft syngeneic tumor of syrian hamster. An intratumoral injection of the control Surv.m-CRA without GM-CSF inhibited a tumor growth and extended a survival to some degrees. Whereas Surv.m-CRAs expressing mouse GM-CSF more drastically reduced tumor growth, some of these animals died of excessive GM-CSF expressions (showing the remarkable splenomegaly) within 3 days. The results importantly suggest the necessity of systematical analyses to identify the best combination of cytokine genes and promoters to achieve the optimal cytokine levels (Int J Oncol 2004), and we are now analyzing 18 different Surv.m-CRAs, including those expressing GM-CSF at a moderate level and/or in the cancer-specific manner. In conclusion, we have developed a novel system to efficiently generate the candidate m-CRAs expressing diverse immunostimulatory genes under several promoters and to systematically analyze them in syngenic hamster models.



415. The Potency of a Histone Deacetylase Inhibitor and Reolysin in Head and Neck Squamous Cell Carcinoma

Matthew Old[1], Cristina Jamie-Ramirez[2], Jun-Ge Yu[1], Bhavna Kumar[1], Pawan Kumar[1], Quintin Pan[1], John Lee[3], Theodoros Teknos[1], Flavia Pichiorri[4], Balveen Kaur[2]

[1]Otolaryngology-Head and Neck Surgery, The Ohio State University, Columbus, OH, [2]Department of Neurological Surgery, The Ohio State University, Columbus, OH, [3]Otolaryngology-Head and Neck Surgery, Sanford Health, Sioux Falls, SD, [4]Comprehensive Cancer Center, The Ohio State University, Columbus, OH

Introduction: There is a clear and pressing need for novel therapies with activity against locally advanced head and neck cancers which still carry a dismal prognosis. Oncolytic viruses are powerful, targeted anti-cancer agents. Reovirus is a naturally occurring non-pathogenic virus that was isolated from the human respiratory and gastrointestinal tracts. Moreover, Reovirus type 3 Dearing (Reolysin; Oncolytics Biotech Inc., Calgary, AB, Canada) is currently being tested in phase I-III clinical trials in a variety of tumor types. Histone deacetylase inhibitors (HDACi) comprise a structurally diverse class of compounds that are targeted anticancer agents. The first FDA approved HDACi, vorinostat (suberoylanilide hydroxamic acid-SAHA), is highly effective in the treatment of cutaneous Tcell lymphoma. SAHA is currently being testing in head and neck cancer clinical trials. We previously found a synergistic combination of SAHA and Reolysin in a nude mouse model. Preclinical models of oncolytics are often in immunocompromised mice, negating the significant impact of the immune system. Mounting evidence demonstrates that the immune system is critically important in oncolytic viral response. In this study, we sought to investigate the impact of this combination in an immunocompetent model. **Methods:** Cell survival experiments were performed with reovirus and SAHA in shPTP-BL-Ras SCC cells. IC50 values were interpolated from a sigmoidal dose-response curve fit of the log-transformed survival data. JAM-1 surface levels were assessed via flow cytometric analysis. Cells were collected after 48 hours and cell death was assessed via Annexin V and PI staining. JAM-1 levels were assessed using anti-JAM-1-PE staining as compared to a control isotype-PE antibody. Whole splenocytes were isolated at the time of death for C57BL/6 mice bearing MTE tumors treated in 4 groups (control, SAHA, reovirus, and combination). Three independent mice were stained per group for the following: B cells (CD19+), NK cells (CD49b+), activated NK cells (CD49b+NKp46+), dendritic cells (CD11c+) and activated DCs (CD11c+MHCII+ or

CD11c+CD86+), CD4 T helper (CD3+CD4+), and CD8 cytotoxic cells (CD3+CD8+). **Results:** Experiments demonstrated significant efficacy of SAHA and Reolysin treatment in vitro and in the immunocompetent mouse model. Combination therapy exhibited a synergistic anti-tumor effect with a significant increased survival of mice compared to any of the agents alone. The Jam1 receptor was upregulated on tumor cells allowing enhanced reovirus uptake. There was marked and significant reduction in circulating B cells in combination treated mice versus all other groups. Activated NK cells were decreased in the combination group. T cells and dendritic cells (CD11c+MHCII+) were reduced in the SAHA groups. SAHA model withdrawal experiments show a significant synergistic response and immune system rebound after SAHA cessation. **Conclusion:** This data demonstrates that combination of reovirus plus SAHA therapy has significant activity in the treatment of SCCHN, even in an immunocompetent model. Immune inhibition due to SAHA as well as increased Jam1 receptor expression on tumor cells results in a synergistic effect of the combination therapy. Immune system rebound likely plays a significant role in the long-term anti-tumor response. This strong preclinical evidence supports the translation of this combination to phase-I clinical trials.

416. Enhanced Oncolytic Effect of Tumor Necrosis Factor Alpha Armed Engineered Oncolytic Measles Virus Therapy for Ovarian Carcinoma Cell Line

Ahmed M. Al-Shammari[1], Patrycja J. Lech[2], Stephen J. Russell[2]
[1]Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetic Research, Baghdad, Iraq, [2]Molecular Medicine, Mayo Clinic, Rochester, MN

Introduction. Tumor necrosis factor alpha (TNFa) has been recognized having anticancer properties, and proposed as a cancer treatment early in the 1980s, But its toxicity lead to limited use. Replication competent oncolytic viruses such as live attenuated measles virus (MV), targeting cancer cells selectively, have shown very promising results in both pre and clinical stages. one of its most useful aspects is their ability to produce high amounts of transgene products locally, resulting in high local tumor microenvironment versus systemic concentrations. Therefore, we developed Gene therapy approach to increase anti-tumor activity results from the combination of oncolytic activity, transgene-mediated direct cytotoxic effect such as TNFa-mediated apoptosis. **Methods**, we created novel oncolytic MV expressing murine TNFa by cloning TNFa gene into MV genome. the gene was amplified by PCR using primers that introduce restriction sites at either end of the coding sequence. The gene cloned into a plasmid encoding the entire measles genome. The insertion site for the TNFa gene was between H and L in the first version. In the second version, transgene was inserted upstream of N. The resulting full-length plasmids [MV-TNFa1 and MV-TNFa2] used to rescue the corresponding recombinant measles virus on 293-3-46 helper cells that was transfected with the MV plasmids. We evaluated the 2 recombinant versions of the MV expressing TNFa with 2 other viruses of MV, one having the sodium iodide symporter (NIS) between H and L, and the second has GFP upstream of N for oncolytic potential against ovarian carcinoma cell line SKOV-3. Infectivity, syncytium formation and cytotoxicity of recombinant MV-TNFs in SKOV-3 cells were evaluated by inverted microscopy and the MTT assay. Transgene expression in SKOV-3 cells after infection was assessed for TNFa functionality and concentration using cells that are genetically engineered for NF-κB Signaling Pathway and TNFa quantitative ELISA assay. **Results**, we were successful to generate the two versions of the virus with TNFa in different genome locations for each version. MV-TNFa2 shown to produce more TNFa than MV-TNFa1 (Figure-1a), but both viruses were producing functional TNFa by activating NF-κB Signaling Pathway (Figure-1b). Recombinant MV-TNFs viruses were efficiently infected SKOV-3 cell line, resulting in extensive syncytium formation followed by cell death. There was enhanced killing for the MV-TNFa1 infected cells comparing to all other viruses (Figure-1c). In conclusion, engineered MV-TNFs may be a potent and novel cancer gene therapy system. MV expressing TNFa elicited oncolytic effects in ovarian cancer cells, enhancing the killing effect which depend on the insert location.

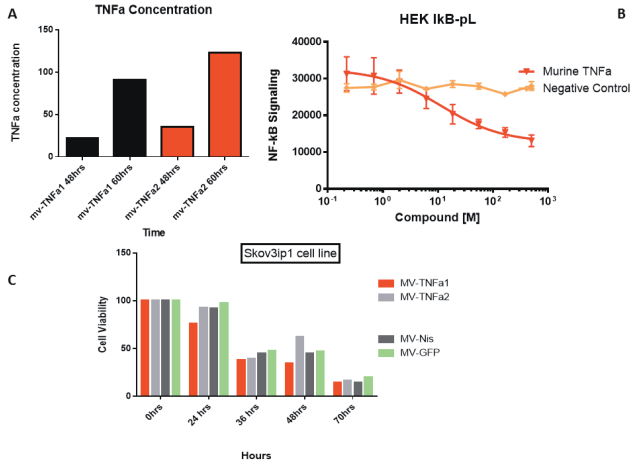


Figure-1, Experiments on MV-TNFs, A- shown MV-TNFa2 to produce more TNFa than MV-TNFa1. B- Secreted TNFa is activating NF-κB Signaling Pathway. C- enhanced killing for the MV-TNFa1 infected cells comparing to all other viruses.

417. Intratumoral Delivery of Oncolytic Adenovirus Expressing Decorin Inhibits Growth and Metastases of 4T1 Breast Tumors in Syngeneic Immune Competent BALB/c Mice Model

Weidong Xu[1], Yuefeng Yang[2], Thomas Neill[3], Hua Wang[2], Chi-Hsiung Wang[4], Chae-Ok Yun[5], Theresa Guise[6], Charles B. Brendler[4], Renato V. Iozzo[3], Lisheng Wang[2], Prem Seth[1]
[1]Gene Therapy Program, Department of Medicine, NorthShore Research Institute, an affiliate of the University of Chicago, Evanston, IL, [2]Beijing Institute of Radidation Medicine, Beijing, China, [3]Department of Pathology, Anatomy and Cell Biology and the Cancer Cell Biology and Signaling Program, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, [4]Department of Surgery, NorthShore Research Institute, Evanston, IL, [5]Department of Bioengineering, Hanyang University, Seoul, Korea, Republic of, [6]Department of Medicine, Indiana University, Indianapolis, IN

During the advanced stage of breast cancer, majority of the patients develop distal metastases that eventually lead to mortality. Unfortunately, conventional strategies generally invoke limited therapeutic responses in patients with tumor metastases. Therefore, the development of novel and highly effective therapies for the treatment of distal metastases of breast cancer is an unmet need in medicine. Decorin (DCN) is a small leucine-rich proteoglycan and is often down-regulated in tumor stroma of breast cancer patients. Our previous work demonstrated that systemic delivery of Ad.dcn, an oncolytic adenovirus expressing DCN, significantly inhibited skeletal metastases and the tumor induced bone destruction in MDA-MB-231 bone metastasis model. However, the therapeutic responses of Ad.dcn in immune-competent models have never been examined. In this study, we showed that non-replicated adenovirus Ad(E1-).dcn mediated high level expression of human DCN in 4T1 cells, and inhibited the expression of tyrosine kinase receptor MET, β-catenin

and vascular endothelial growth factor A. To examine the anti-tumor responses of Ad.dcn, we established subcutaneous 4T1 tumors in BALB/c mice. Ad.dcn was delivered intratumorallly (2.5×10^{10}VPs) on day 7 post-cell inoculation, and a repeat dose was given on day 10. Our data showed that Ad.dcn inhibited the growth of local tumors as measured by the caliper as well as by the Bioluminescence Imaging (BLI). At the terminal time point (day 25) we also observed a significant reduction in the tumor weight. Analysis of BLI in real time also demonstrated that Ad.dcn significantly reduced the tumor metastases to lung. On day 25 Ad.dcn group had fewer metastases (2/8 mice), compared to buffer treated group (7/8 mice). Similar reductions in lung metastases were observed by H&E staining. Moreover, Ad.dcn treatment reduced the expression of Th2 cytokines (IL-2, IL-4 and IL-10) in the tumors, which will potentially activate the antitumor immune responses. Furthermore, Ad.dcn also reduced the expression of N-cadherin and vimentin in the tumors, indicating Ad.dcn could also potentially reduce tumor metastases by inhibiting epithelial-mesenchymal transition (EMT) of the tumor cells. Taken together, our results suggested that Ad.dcn not only inhibited the growth of local tumor, but also prevented the distal metastases of tumor. Combining these results in the 4T1 tumor model, and previously examined MB-MDA-231 bone metastases model, we propose the following model of Ad.dcn-mediated inhibition of tumor growth and metastases. Ad.dcn is taken up by tumor cells after systemic or intratumoral delivery, and then replicates in tumor cells and kills them. Tumor cells produce and release high level of decorin protein into tumor microenvironment. Decorin then targets multiple tumor and stromal components to activate anti-tumor immune responses, decrease angiogenesis, and inhibit EMT. Therefore, Ad.dcn is a potential therapeutic strategy for the treatment of breast cancer and its distal metastases.

Y.Y. and W.X. made equal contribution. L.W. and P.S. are the corresponding Authors.

418. Peripheral Blood Leucocytes, Neutralizing Antibodies and Tumor Burden as Predictive and Prognostic Factors in Patients Treated with Oncolytic Adenoviral Immunotherapy

Suvi Parviainen[1], Kristian Taipale[1], Ilkka Liikanen[1], Anniina Koski[1], Anna Kanerva[2], Minna Oksanen[1], Akseli Hemminki[1]
[1]Cancer Gene Therapy Group, Medicum, Haartman Institute, University of Helsinki, Helsinki, Finland, [2]Helsinki University Hospital Comprehensive Cancer Center, Helsinki, Finland

Oncolytic immunotherapy has taken significant steps towards clinical availability for cancer patients, as the FDA recently approved Imlygic (T-Vec) for the treatment of melanoma. Despite the several oncolytic virus trials, there are no known biomarkers which could predict the treatment outcome of oncolytic virotherapy. Therefore, previous clinical experiences should be utilized as guidance for further treatment optimization and patient selection. The patients analyzed consisted of 246 patients treated with oncolytic adenovirus as a part of the Advanced Therapy Access Program which was ongoing in Helsinki 2007-2012. Taken together with the increasing understanding that immunological factors might determine the efficacy of oncolytic virotherapy, we studied whether neutralizing antibodies and peripheral blood cell counts would have prognostic and/or predictive significance in the context of oncolytic adenovirus treatments. Additionally, tumor burden was assessed both in terms of primary tumor and metastases. We observed presence of neutralizing antibodies before treatment to be correlated with shorter overall survival ($p=0.028$). Blood cell counts analyses revealed that patients who had high neutrophil to lymphocyte ratio at baseline had significantly shorter overall survival ($p<0.001$). After treatment patients demonstrated transient lymphopenia and increases in neutrophils, which varied between disease control and progressive disease groups. Patients with high total tumor load had significantly shorter overall survival compared to low tumor load group ($p=0.003$). Interestingly, we also found presence of liver metastases to be correlated with reduced rate of disease control ($p=0.021$). These data indicate possible reasons behind the nonuniform treatment outcomes frequently observed after virotherapy. Moreover, our results suggest potential approaches for treatment refinement and patient selection in the context of oncolytic immunotherapy. Given the similarity of biological effects seen with different oncolytic DNA viruses, it will be interesting to see if these results will be similar in the context of other oncolytic viruses and cancer immunotherapy approaches such as adoptive T-cell therapy and cancer vaccines.

419. Efficacy of Rambo for Sarcoma Therapy

Maninder Khosla
The Ohio State University, Columbus, OH

Soft Tissue Sarcoma (STS), a cancer which forms in the connective tissue, requires aggressive treatment. Metastatic soft tissue sarcomas are associated with a median prognosis of about twelve months accompanied frequently with many signs and symptoms leading to a poor quality of life. These tumors frequently have angiogenic properties. Angiogenesis is responsible for the formation of new blood vessels and is associated with local progression and increased metastatic disease. Strategies that work to block angiogenesis are currently being investigated as a treatment for STS. Oncolytic virus therapy is a novel treatment strategy that utilizes modified viruses to specifically replicate and lyse cancer cells. The combination of angiogenesis inhibitors with oncolytic virus treatment has not been tested for STS. Here we tested the effect of combining oncolytic viral therapy with antiangiogenic gene therapy. RAMBO is a HSV-1 derived oncolytic virus that codes for the production of Vstat120, a gene with potent antiangiogenic effects. We have tested a panel of sarcoma cells and shown effective infection and lysis of these cells by RAMBO and HSVQ-1 in vitro. In vivo, we compared the antitumor efficacy of RAMBO and HSVQ-1 against sarcoma tumors grown in mice. Briefly, mice were implanted with 150-300mm^3 A673 sarcoma tumors were injected with a PBS, HSVQ-1, or RAMBO ($5.5x10^6$ pfu). Mice were followed for tumor size over a period of time. Tumor volumes over time were logarithm (base 2) transformed for variance stabilization. The linear mixed model was used to compare the three groups while accounting for the variance-covariance structure due to repeated measures at different days from the same mouse. Statistically significant improvement in antitumor efficacy was observed in RAMBO treated tumors, compared to HSVQ-1 and control. H&E staining of tumor tissue reveals large areas of necrosis in RAMBO treated tumors. RAMBO and its inherent antiangiogenic gene therapy demonstrated significant efficacy in these sarcoma models and further investigations are underway with combination therapeutic strategies.

420. Improvement of Antitumor Activity in Intraperitoneal Ovarian Cancer Model by a Noble Cloned Carrier Cell Infected with Oncolytic Adenovirus

Katsuyuki Hamada, Kazuko Takagi, Takashi Sugiyama
Obstetrics and Gynecology, Ehime University, Toon, Japan

Infection inhibition of virus by antiviral antibodies is one of the major problems that cancer gene therapy has never shown the significant antitumor activity in human clinical trial. Oncolytic adenovirus-infected A549 carrier cells develop cell fragments and exosomes containing oncolytic adenoviral particles and could infect target cancer cells by overcoming antiadenoviral immunogenicity. A549 carrier cells induce complete tumor reduction in syngeneic subcutaneous mouse tumor model by direct antitumor activity of

oncolytic adenovirus, antiadenoviral cytotoxic T lymphocyte and antitumor immunity but not in syngeneic intraperitoneal ovarian cancer mouse model. In this study, we cloned and established a noble carrier cell by limiting dilution to induce the complete tumor reduction of the intraperitoneal ovarian tumor by increasing antitumor activity of carrier cells. Oncolytic adenovirus AdE3-*midkine* driven by *midkine* promoter and non-replicative adenovirus Ad-*mGM-CSF* were used in this study. We cloned a nobel carrier cell from EHMK adenocarcinoma cells by limiting dilution, which were established in our laboratory. EHMK cells were infected with AdE3-*midkine* and co-cultured with ovarian adenocarcinoma HEY cells with or without antiadenoviral antibodies. *In vitro* antitumor activity of cloned EHMK carrier cells was calculated by IC50 and compared with that of A549 carrier cells infected with AdE3-*midkine*. B6C3F1 mouse and cognate ovarian cancer OVHM cell line were used in this study. Mice were injected intraperitoneally with OVHM cells after immunization with adenovirus and treated by carrier cells infected with AdE-*midkine* with or without Ad-*mGM-CSF*. Antitumor activity of carrier cells was analyzed by Kaplan Meier survival curve. *In vitro* antitumor activity of EHMK carrier cells was 1.0±0.1 and 1.1±0.2 that of A549 carrier cells with or without antiadenoviral antibodies, respectively. *In vitro* antitumor activity of EHMK-51 carrier cells after limiting dilution of EHMK cells was 4.5±1.1 and 3.4±1.9 that of A549 carrier cells with or without antiadenoviral antibodies, respectively. EMHK-51 cells were further cloned by limiting dilution. *In vitro* antitumor activity of EHMK-51-35 carrier cells was 3.1±1.5 and 2.8±0.9 that of EHMK-51 carrier cells with or without antiadenoviral antibodies, respectively. In comparison with A549 carrier cells, *in vitro* antitumor activity of EHMK-51-35 carrier cells was 12.5±4.3 and 10.5±5.0 that of A549 carrier cells with or without antiadenoviral antibodies, respectively. In mouse intraperitoneal tumor model, A549 carrier cells infected with AdE3-*midkine* ± Ad-*mGM-CSF* did not show any antitumor effect but EHMK carrier cells infected with AdE3-*midkine* with or without Ad-*mGM-CSF* showed 40% and 70% complete tumor reduction, respectively. From these results, it is concluded that EHMK-51-35 carrier cells might be potent to treat intraperitoneally metastasized ovarian cancer

421. Evaluating the Efficacy of Combination Cancer Treatment: Oncolytic Adenovirus and 5FU Chemotherapy

Jordan Sell, Amanda Oliviera, Eric Jensen, Edward Greeno, Masato Yamamoto, Julia Davydova
Surgery, University of Minnesota, Minneapolis, MN

Previous studies have shown that Interferon(IFN)-based chemoradiation therapy can improve survival after resection of pancreas cancer. However, its clinical utility to this point has been limited due to the severe toxicity related to its use systemically. Gene Therapy represents a promising approach to deliver localized treatments that are effective and minimally toxic. Our aim in this study is to evaluate our group's novel oncolytic adenovirus (OAd) which allows targeting IFN treatment to cancer cells while sparing healthy tissue. The OAd, 5/3Cox2DE3ADPIFN, is selectively replicative in Cox2 (+) cell lines, allowing for specificity in therapy. It has also been modified to with a 5/3 chimeric knob to facilitate transduction, an overexpression of the adenoviral death protein, and expresses our IFN gene of interest. This study was conducted to analyze the combination of 5FU chemotherapy and our OAd in vitro in order to assess the interaction of treatments and determine the optimum combined treatment regimen. Treatments were analyzed in pancreatic cancer and esophageal adenocarcinoma cell lines S2013 and OE19. Recombinant OAds expressing luciferase rather than IFN were used in this study to isolate the combination of 5FU and the virus. Two viral models were evaluated: our selective therapeutic virus (Cox2) and a nearly identical but universally replicative virus (wild type). Cells were treated with 0, 1 or 10 viral particles per cell and 0, 5, 10, or 20 uM 5FU. Three timing regimens were used: simultaneous administration, 5FU 4 hours before virus, and virus 48 hours before 5FU. Crystal Violet and MTS Assays were used to measure cell death. Viral Copy number was used to assess viral replication using qPCR with a viral E4 primer. Cell death analysis showed an earlier killing effect from the wild type virus but otherwise similar patterns. 5FU and each virus produced dose dependent cell death independently. There was a significant additive effect seen in cell death from combining treatments when using *5FU before virus* in both S2013 and OE19. Simultaneous treatment showed an additive killing effect with the combination in S2013, but a reduced killing by the combination compared to virus alone in OE19. There was also reduced combination killing when using *virus before 5FU* in S2013. Viral copy experiments in S2013 using simultaneous treatment showed dose dependent inhibition of viral replication, with only the 20uM dose significantly limiting viral replication over time. Our Cox2 OAd shows a killing effect similar to wild type in multiple cancer cell lines. When combined with 5FU treatment the expected summation in overall cell death from the independent treatments varies by timing of administrations as well as by type of cancer cell line. The reduced killing of the combination treatment in the simultaneous and *virus before 5FU* regimens, as well as the replication inhibition shown by viral copy number, may suggest an inhibition of the virus by 5FU under certain conditions. This also suggests the possibility of a gene therapy treatment regimen with optimal therapeutic effect, which appears to be *5FU before virus* based on the results collected. Further studies investigating different chemotherapeutic drugs and regimens should be conducted to examine these trends.

422. Harnessing Immunogenic Cell Death to Potentiate the Oncolytic Effect of ORFV Infection in Murine Cancer Cells

Jacob P. van Vloten, Lisa A. Santry, Jondavid de Jong, Byram W. Bridle, Sarah K. Wootton
Pathobiology, University of Guelph, Guelph, ON, Canada

Immunogenic cell death (ICD) is a recently uncovered phenomenon in which cells committed to death undergo a distinct cascade of events that culminate in the release of factors that alert the immune system. This is in contrast to conventional apoptosis, which is immunologically silent. Studies investigating the effect of anthracycline-induced cancer cell death demonstrated that translocation of calreticulin (an endoplasmic reticulum protein) to the cell surface and extracellular secretion of ATP and high-mobility group box 1 (HMGB1; a nuclear DNA-binding protein) drive immunogenicity[1]. Surface calreticulin acts as a pro-phagocytic signal, leading to increased processing of tumor antigens by dendritic cells. ATP interacts with receptors on immune cells and has a strong chemotactic effect. Outside of the nucleus, HMGB1 functions as either a chemokine or pro-inflammatory cytokine depending on its oxidation state. In the context of ICD, calreticulin, ATP and HMGB1 enhance the immune response to tumor antigens and improve the efficacy of anti-cancer therapies in murine models. Current research is beginning to delineate the important cellular events that dictate the ICD response in cancer cells[2-4]. Importantly, several of these pathways overlap with the cellular response to viral infection[5] and numerous viruses have indeed been shown to induce ICD in infected cancer cells. *Parapoxivirus ovis* (ORFV) is a poxvirus and veterinary pathogen causing transmissible pustular dermatitis in sheep. ORFV is able to infect multiple cancer cell types and has demonstrated impressive efficacy in *in vivo* models, largely due to its high immunogenicity[6]. To elucidate the cause of ORFV immunogenicity, we investigate the relationship between ORFV infection and the ICD response in cancer cells by analyzing

the three hallmarks: calreticulin surface translocation and ATP and HMGB1 release. Multiple murine cancer cells lines were investigated, encompassing established models of mammary, ovarian, prostate and skin cancer. Additionally, the ability of ORFV to induce ICD was compared to that of other clinically relevant oncolytic viruses including vaccinia (JX-594), Maraba (MG1) and Newcastle disease virus (NDV/F3aa). Reference: 1. Kroemer G., Galluzzi L., Kepp O. and Zitvogel L. (2013). Immunogenic cell death in cancer therapy. *Annu. Rev. Immunol.* **31**:51-72. 2. Obeid M. *et al.* (2007). Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat. Med.* **13:**54-61. 3. Panaretakis T. *et al*. (2009). Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. *EMBO J.* **28:**578-590. 4. Kepp O. *et al*. (2013). Crosstalk between ER stress and immunogenic cell death. *Cytokine Growth Factor Rev.* **24:**311-318. 5. Kepp O. *et al.* (2009) Viral subversion of immunogenic cell death. *Cell Cycle*. **8:**860-869. 6. Rintoul J. *et al.* (2012). ORFV: a novel oncolytic and immune stimulating Parapoxvirus therapeutic. *Mol. Ther.* **20:**1148-1157.

423. Clinical Translation of Immunovirotherapy: Measles Virus and Anti-PD1

Christine E. Engeland, Rūta Veinalde, Christof von Kalle, Dirk Jaeger, Guy Ungerechts
National Center for Tumor Diseases, Heidelberg, Germany

After preclinical proof-of-concept that oncolytic virotherapy and immune checkpoint modulation have synergistic effects, we will conduct a phase I/II clinical trial combining a measles vaccine strain and an anti-PD1 antibody. Patients with hepatic metastases of pancreatic adenocarcinoma receive ultrasound-guided intralesional virus injections and systemic administration of anti-PD1. A main focus is the translational research program which accompanies the trial. In sequential biopsies we will quantify the type, density and distribution of immune effector cells. Cytokine and chemokine profiles of tumor and blood samples are analyzed to identify biomarkers predictive of response. Transcriptome and immunoreceptor sequencing are performed to predict neoepitopes and monitor T cell dynamics. This comprehensive data set will allow us to identify immune signatures associated with response and pinpoint mechanism of action in immunovirotherapy.

Immunological Aspects of Gene Therapy I

424. AAV Re-Administration Following Pediatric Application: Effect of Age at First Administration

Kimberly L. Pietersz[1], Sander J. van Deventer[2], Harald Petry[1], Valerie Ferreira[1]
[1]Uniqure, Amsterdam, Netherlands, [2]Gastroenterology & Hepatology, Leiden University Medical Center, Leiden, Netherlands

Gene therapy delivery in pediatric patients may prevent or reduce the deleterious impact of disease long-term. However, due to the cellular turn-over and growth of some target organs such as the liver re-administration of AAV vectors will have to be considered. The goal of our study is to determine the time frame after birth in which AAV can be delivered without the development of an immune response against capsid, but with a proper transgene expression, thereby permitting re-administration and a continuous transgene expression. Mice were administered with AAV1-hAAT-hSEAP intraperitoneally at 2, 7, 14, 21 days and adult mice were used as controls. Two weeks after administration SEAP concentration was measured in plasma as a measure for transgene activity. Initial transgene expression was higher in mice injected at two and seven days of age than in mice injected at 14 or 21 days; As expected, transgene expression decreased over time in all mice. At week 8 SEAP concentrations in plasma of all mice were similar. AAV1 neutralizing antibody (NAB) responses were assessed at 9 weeks following primary administration. Mice administered at 21 days of age had a NAB titer slightly above PBS level; while the other age groups of 2, 7, and 14 days were similar to PBS. Mice of all groups were re-administrated 9 weeks after primary administration with AAV1-LP1-hFIX in order to assess the effect of NAB induction on the expression of a novel transgene. Mice primed at 21 days of age had 67% less FIX than adult control mice. In the other groups that were primed at 2, 7 or 14 days of age the second administration generated the same FIX plasma levels as measured in adult controls. With this study, we show that AAV1 can be effectively used for pediatric application up to 14 days after birth without inducing an immune response. This strategy prevents the formation of neutralizing antibodies, and the same serotype can be used for re-administrated later in life.

425. Production of Gene Corrected T Cell Precursors for Therapy of SCID X1

Chantal Lagresle-Peyrou[1], Cécile Roudaut[2], Aurélie Gabrion[2], Laure Caccavelli[2], Hanem Sadek[2], Myriam Chouteau[1], Christian Reimann[3], Salima Hacein-Bey-Abina[2], Marina Cavazzana[1], Isabelle André-Schmutz[1]
[1]Imagine Institute - INSERM, Paris, France, [2]Biotherapy Department - Necker Hospital, Paris, France, [3]Universitatklinikum, Freiburg, Germany

Gene therapy for SCID and CID usually leads to a more efficient T-cell reconstitution as compared to HSCT from a mismatched related donor. However, as observed in ADA, SCID X1 and WAS gene therapy trials, T cell recovery is sometimes slow and associated with morbidity and mortality due to viral infections. In order to shorten post-transplant immunodeficiency and to reduce the frequency of these complications, one possible strategy is to transplant gene-corrected T-cell committed precursors. We have recently set up a protocol for transplanting *in vitro*-committed T-cell precursors able to seed the thymus and generate a wave of mature and polyclonal T-cells significantly faster than is usually observed. This protocol is based on a 3 to 7 day-culture of CD34+ cells in the presence of immobilized Notch ligand Delta-like-4 (DL-4), an adhesion molecule and a combination of cytokines; this culture system enables the *in vitro* generation of large amounts of T-cell precursor cells that (i) display the phenotypic and molecular signatures (i.e. gene expression pattern and TCR rearrangement pattern) of early thymic progenitor cells and (ii) have a high T-lymphopoietic potential *in vitro* and *in vivo* when transferred into NOD/SCID/γc knock-out (NSG) mice (Reimann C, Stem Cells, 2012). The goal of the present project was to combine exposure to DL-4 and gene therapy approaches (DL-4/GT). For this purpose, gene correction of CD34+ cells from SCID-X1 patients was performed on DL-4 coated plates and the *ex vivo* transduced cells were either seeded on OP9/DL-1 culture or injected into NSG mice to determine their *in vitro* and *in vivo* capacity to generate mature and diverse T cells. In the DL-4/GT condition, the frequency and number of CD7+CD5+commited T-cell precursors was increased as compared to GT condition alone. Experiments to assess if the DL-4/GT condition accelerate T cell differentiation of gene corrected cells are currently ongoing. Altogether, our results suggest that a short exposure to the Notch ligand DL-4 might be an useful tool to generate large amounts of corrected T-cell precursors.

426. Multi-Pathogen-Specific T Cells: A Single T-Cell Product Simultaneously Targeting Multiple Pathogens for Adoptive Immunotherapy

Anastasia Papadopoulou[1], Panayotis Kaloyannidis[2], Maria Alvanou[1], Stergios Papadimitriou[1], Ioannis Batsis[1], Ioanna Sakellari[1], Achilles Anagnostopoulos[1], Evangelia Yannaki[1]
[1]Gene and Cell Therapy Center, Hematology-BMT Unit, George Papanicolaou Hospital, Thessaloniki, Greece, [2]Adult Hematology &Stem cell Transplant, King Fahad Specialist Hospital Dammam, Dammam, Saudi Arabia

Viral infections, most commonly by cytomegalovirus (CMV), Epstein-Barr virus (EBV), polyoma virus type I(BK), andfungal infections, mainly by *Aspergillus Fumigatus* (Asp),are among the deadliest complications for patients undergoing allogeneic hematopoieticstem cell transplantation. Treatment with antiviral and antifungal pharmacological agents, which are today's standard therapy, is often ineffective or toxic whereas it can lead to the outgrowth of drug-resistant strains. Adoptive immunotherapy with the use of antigen-specific T-cells to restore antigen-specific immunity post-transplant, offers an attractive alternative approach to conventional drugs. We here, aimed to generate at large scale, multipathogen-specific T cells (mp-STs) that simultaneously target CMV, EBV, BK and Asp, from healthy donors, with a rapid, simplified, low-cost and minimally laborious protocol. A total of $1{,}5x10^7$mononuclear cells, derived from 15-20ml blood of eight CMV and EBV seropositive donors, were pulsed with viral pepmixes(CMV: IE1, pp65;EBV: EBNA1, LMP2, BZLF1;BK: Large T, VP1) combined with either Asp lysate or Asp pepmixes (Crf1, Gel1 and SHMT) and cultured in the presence of IL-4/IL-7 for 10 days in G-rex bioreactors. The cells were characterized immunophenotypically by flow cytometry and their specificity/functionality was assessed by IFN-γ Elispot assay. Cells stimulated with either Asp lysate (n=4) or Asp pepmixes (n=4), had a similarexpansion, reaching a mean of $170\pm36x10^6$and $206\pm46x10^6$ cells, respectively. All cell lines were polyclonal, comprised predominantly of CD4+ cells (70±5% and 72±3% respectively) and CD8+ cells (19±4% and 18±2% respectively) and expressed central (CD45RA-/CD62L+: 36±6%, and 35±2% respectively) and effector memory markers (CD45RA-/CD62L-: 53±5%, and 54±3% respectively). Importantly, allmp-STs lines (8/8) were specific against all targeted pathogens [mean±SEM spot forming cells (SFC)/$2x10^5$ input cells, with lysate:CMV:211±41; EBV:1137±165; BK:551±109; Asp: 143±8; with peptides: CMV: 251±111; EBV: 1031±238; BK: 616±119 SFC; Asp: 463±132).Given that all donors were CMV and EBV seropositive and BK or Asp prior donor exposure wasnot tested, our data suggest that BK or Aspspecificity can be obtained practically from all healthy individuals, due to the particularly high exposure of the general population to these pathogens. Notably, the combination of Asp targeted-proteins (Crf1, Gel1 and SHMT) induced stronger Th1 responses compared to Asp lysate (p=0,049). Overall, we established arapid and simple, optimizedprotocol of generating clinically relevant numbers of mp-STs from a small amount of donor blood. Should mp-STs move to the clinic and prove safe and effective, they will be an ideal treatment for patients suffering from life-threatening, post-transplant infections.

427. *In Vivo* Inhibition of HIV-1 in NSG Mice After Transduction of Primary Human T Cells with CXCR4 Conjugated to an HR2 Peptide

George J. Leslie[1], Max Richardson[2], Jianbin Wang[3], Philip Gregory[3], James Riley[2], Michael Holmes[3], James Hoxie[1]
[1]Medicine, University of Pennsylvania, Philadelphia, PA, [2]University of Pennsylvania, Philadelphia, PA, [3]Sangamo Bioscience Inc., Richmond, CA

Introduction: We previously reported that a 34 a.a. peptide from the C-terminal heptad repeat-2 domain (HR2) of HIV-1 gp41 (C34) when fused to the N-terminus of CXCR4 (C34-X4) inhibits HIV-1 infection in transduced cells *in vitro*. This construct provided protection irrespective of HIV viral tropism (i.e., C34-X4 could inhibit R5, X4 or dual-tropic isolates).

Methods: To assess the ability of C34-conjugated coreceptor to exert transdominant inhibition of R5- and X4-tropic HIV-1 *in vivo*, C34-X4 was transduced using a lentiviral vector into CD3/CD28-stimulated primary human CD4+ T cells with GFP-transduced T cells serving as a control. Cells were infused in NOD-scid-gamma (NSG) mice either undiluted or mixed (25% of total cells) with untransduced T cells. After 21 days, mice were challenged i.v. with R5 (US1, Clade B) and X4 (CMU02 Clade A/E) tropic HIV-1 and infection assessed by plasma viral RNA levels and by survival of C34-X4-expressing cells.

Results: In NSG mice receiving C34-X4 transduced cells, at Day 18 there was significant expansion of the $CD4^+/CD45^+/CXCR4^+$ T cell population, but not of GFP control cells (p=0.005). Viral loads were significantly lower in mice receiving C34-X4 transduced cells compared to those receiving GFP-transduced cells (p=0.047). At necropsy (Day 28) in splenocytes there was an approximate 2 log increase in the number of CD4 T cells in mice receiving C34-X4 transduced cells (p=0.001).

Conclusion: These studies demonstrate that the C34 peptide conjugated CXCR4 confers resistance of CD4 T cells to HIV-1 infection irrespective of viral tropism, providing a proof of concept that targeting an HR2 inhibitory peptide to a coreceptor exerts potent transdominant inhibition of HIV-1 in vivo. This novel genetic approach to confer HIV resistance to CD4 T cells, coupled with high barriers for viral resistance to C34-coreceptors, may provide a novel approach for HIV control in humans.

428. Generation of DNA Plasmid-Encoded Neutralizing Monoclonal Antibodies In Vivo

Seleeke Flingai[1], Emily Plummer[2], Ami Patel[1], Janess Mendoza[3], Yang Wang[4], Keith Riemann[4], Mark Klempner[4], Kate Broderick[3], Niranjan Sardesai[3], Kar Muthumani[1], David Weiner[1]
[1]University of Pennsylvania, Philadelphia, PA, [2]La Jolla Institute for Allergy and Immunology, La Jolla, CA, [3]Inovio Pharmaceuticals, Plymouth Meeting, PA, [4]MassBiologics, Boston, MA

The development of vaccines against arthropod-borne infectious diseases has been wrought with difficulties. Recent advances in human antibody isolation have uncovered neutralizing monoclonal antibodies (mAbs) that are capable of providing protection against pathogen challenge in various animal models. Yet generating and delivering biologically-relevant levels of such antibodies using conventional monoclonal antibody methodology is impractical, often requiring huge expenses and repeated administrations for clinical benefit. Creating new methods of delivering monoclonal antibodies could drastically tip the scale in the fight against a number of devastating pathogens.

Here, we describe an approach to delivering neutralizing mAbs in vivo using DNA plasmid-mediated antibody gene transfer. This approach, which we term DNA mAb (DMAb) delivery, generates biologically relevant levels of mAbs after a single intramuscular

injection of antibody-encoding DNA followed by in vivo electroporation (EP). First, we demonstrate the ability of DMAb technology to deliver cross-reactive neutralizing antibodies against DENV into the host circulation. Since this approach allows for genetic tailoring of the exact features of the desired antibody, we incorporated Fc region modifications to a naturally occurring human anti-DENV neutralizing antibody to enhance antibody function in vivo. We show that intramuscular delivery in mice of pDVSF-3 LALA, which encodes a human anti-DENV1-3 IgG1 neutralizing antibody modified with a mutation that abrogates FcγR binding, produces anti-DENV antisera capable of binding and neutralizing DENV1-3. Importantly, mice receiving pDVSF-3 LALA, but not the unmodified pDVSF-3 WT, were protected from both virus-only disease and antibody-enhanced lethal disease.

Using a similar, targeted genetic approach to antibody modifications, we also show that DMAbs encoding antibodies against Borrelia burgdorferi (the causative agent of Lyme disease) can undergo extensive amino acid modifications that substantially increase in vivo mAb production levels compared to wild-type DMAb sequences. These data illustrate a subset of the functional optimizations made possible with the DMAb platform.

This work was supported by grants funded to DBW through the National Institutes of Health, the DARPA-PROTECT award, and Inovio Pharmaceuticals Inc.

429. Antibody Delivery by Genetically Engineered Human Hematopoietic Stem Cells to Fight HIV

Anne-Sophie Kuhlmann, Kevin Haworth, Morgan Giese, Hans-Peter Kiem

Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA

The fight against HIV (Human Immunodeficiency Virus) has made considerable progress following antiretroviral therapy development. However, a cure has not been achieved yet due to the existence of replication-competent latent viral reservoirs in sanctuaries such as the Gut-Associated Lymphoid Tissue, lymph nodes or central nervous system. The elimination of these reservoirs appears as a key to eradicate HIV, and developing innovative delivery methods will be critical to reach this goal. Hematopoietic stem cells (HSCs) and derived lineages have been shown to migrate to these sanctuaries and represent a powerful way of delivering therapeutic molecules to target HIV. The recent identification of potent broadly neutralizing antibodies (bNAbs) directed against the Envelope of HIV holds new prospects. In addition to neutralizing various strains of HIV, a few reports have shown the ability of these antibodies to target HIV originating from the latent reservoirs. Recent efforts have focused on bNAbs delivery, but their *in vivo* half-life remains limited. Delivery by HSCs would provide a durable alternative. Based on these observations, we are genetically modifying $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) to give them the ability to express and secrete potent bNAbs targeting both circulating viral particles and latent viral reservoirs. To this end, cell lines or human HSPCs were transduced by lentiviral constructs encoding selected bNAbs and assessed for their ability to secrete functional antibodies. *In vitro*, 293T cells were able to release the bNAbs of interest in the supernatants at 4 and 7 days post-transduction. The secreted antibodies were also detected in modified human HSPCs supernatants starting at 9 days and for up to 21 days post-transduction. To assess the antibodies protective effect against HIV *in vivo,* we have utilized the NOD-SCID-gamma (NSG) mouse model. A single infusion of genetically modified HSPCs in this model allows for immune cell development and subsequent HIV challenge. Circulating human cells are detected in the peripheral blood as early as 8 weeks post-engraftment. Mice have now been challenged with HIV at 12 weeks and the infection is being tracked using quantitative PCR. This model will allow us to determine the ability of genetically modified HSPCs to secrete these bNAbs *in vivo* and prevent HIV infection as well as to track antibodies delivery to isolated sanctuaries such as the brain. The long-term secretion potency of these modified HSPCs and derived lineages will also be investigated. Here we show the development of an alternative delivery method for antibodies by genetically modified HSPCs. The ability of HSPCs to differentiate into multiple hematopoietic lineages and thus to cross physiological and anatomical barriers represents a valuable asset to target HIV latent viral reservoirs. Another advantage provided by the use of HSPCs is their long-term persistence that should support a long-term secretion and thus treatment. Future development will focus on controlling antibody expression. Importantly, this strategy could be applied to a broad variety of diseases for which antibodies have been shown to be efficient in patients, but require multiple injections for sustainable efficiency.

430. Antibodies to Recombinant Human α-L-Iduronidase Enhance Uptake into Macrophages in Murine Mucopolysaccharidosis Type I

Valentina Sanghez[1], Shih-Hsin Kan[1], Steven Q Le[1], Don Clarke[1], Kristen Vondrak[1], Moin U Vera[2], Michelina Iacovino[1], Mark S Sands[3], Patricia I Dickson[1]

[1]Dept. Pediatrics, LA Biomed, Torrance, CA, [2]Children's Hospital, Orange County, CA, [3]Department of Medicine, Washington University in Saint Louis, Saint Louis, MO

Mucopolysaccharidosis I (MPS I) is an inherited, and progressive lysosomal storage disease caused by lack or low level of α-L-iduronidase (IDU), altering the catabolism of glycosaminoglycans (GAG) in lysosomes. Some clinical improvement in patients has been observed by enzyme replacement therapy with recombinant human IDU (rhIDU), but studies suggested that anti-rhIDU antibodies reduce the effectiveness of treatment. Most MPS I treated patients develop a humoral immune response against rhIDU. To determine how anti-rhIDU antibodies alter the tissue and cellular distribution of rhIDU in vivo, we sensitized MPS I mice to rhIDU by administering rhIDU via tail-vein injection from 4 to 16 weeks of age. Mice that developed anti-rhIDU antibodies showed a shift in rhIDU uptake preferentially towards tissues with high reticuloendothelial (RE) content (liver, spleen, thymus) vs. low RE content (lung, kidney, heart, brain). Furthermore, in mice with anti-rhIDU antibodies, rhIDU was mainly localized in Kupffer cells, with relatively less available for hepatocytes. In order to further understand how the humoral immune response against rhIDU alters its distribution, we comparedin vitro uptake of rhIDU in the absence or presence of antibodies against rhIDU in MPS I fibroblasts and macrophages. Bone marrow monocytes were harvested from MPS I mice and derived into macrophages. MPS I macrophages were incubated with rhIDU with or without immunized rabbit serum, pre-immune serum, murine Fc receptor blocking agent and mannose 6-phosphate. We evaluated two types of immunized rabbit serum: one that completely abolished rhIDU uptake into human MPS I fibroblasts (inhibiting serum) and one that only partially (30%) inhibited rhIDU uptake into human fibroblasts (partially-inhibiting serum). The uptake of rhIDU in the presence of serum containing rhIDU antibodies was enhanced into MPS I mouse macrophages when both inhibiting and partially-inhibiting serum was applied. Uptake of rhIDU per cell was higher in macrophages when immune serum than was uptake of rhIDU into human fibroblasts normalized per cell. Treatment with mannose 6-phosphate or Fc receptor block partially inhibited the uptake in macrophages. Our study demonstrated that even a low-level humoral immune response against rhIDU, which only partially inhibits rhIDU uptake into fibroblasts in vitro, nevertheless might alter its tissue

distribution in vivo. At the cellular level, antibody-positive mice show reduced rhIDU distribution in hepatocytes, while distribution to tissue macrophages is maintained. In vitro, immunized serum reduced rhIDU uptake into human fibroblasts but increased uptake into murine macrophages. Our findings imply that the altered tissue distribution of rhIDU caused by anti-rhIDU antibodies is partly due to reduced uptake into fibroblasts and partly due to enhanced uptake into tissue macrophages. These results imply that functional immune assays of rhIDU uptake in vitro into fibroblasts may not completely predict the impact of the humoral immune response against enzyme replacement therapy.

431. Lung Antibody Factory for Passive Immunisation Against Influenza

Tiong Kit Tan[1], Pramila Rijal[2], Lee A. Davies[1], Ian A. Pringle[1], Alain R. Townsend[2], Stephen C. Hyde[1], Deborah R. Gill[1]
[1]Gene Medicine Group, Nuffield Division of Clinical Laboratory Science, RDM, University of Oxford, Oxford, United Kingdom, [2]Human Immunology Unit, Weatherall Institute of Molecular Medicine, RDM, University of Oxford, Oxford, United Kingdom

Influenza A is a major global health threat causing >500,000 deaths annually. Neither prior infections nor current vaccines provide lasting protection, mainly due to rapid antigenic evolution of the viral haemagglutinin (HA) protein. Broadly neutralising antibodies (bNAbs) isolated from vaccinated volunteers can provide passive immunity, but this approach is hindered by high antibody manufacturing costs and the relatively short half-life of antibody after delivery to the circulation. We hypothesise that using lentivirus pseudotyped with Sendai virus envelopes (rSIV.F/HN) to deliver genes encoding anti-influenza bNAbs to the lung could provide long-lasting passive immunity to widely divergent strains of influenza. We selected a novel bNAb that we isolated from vaccinated volunteers, namely T1-3B (V_H1-69 germline family) that cross-reacts with multiple group 1 influenza A strains including H1 (A/PuertoRico/8/34 & A/Brisbane/59/2007), pandemic H1 (A/California/07/2009) and H5 (A/Vietnam/1203/2004). Delivery of rSIV.F/HN expressing *Gaussia* luciferase (GLux) reporter protein to the mouse lung (1e7 TU/mouse) resulted in persistent secretion of GLux into both bronchoalveolar lavage fluid (BALF) (1,370,000 RLU/µl; $p<0.01$) and systemically into the serum (1,000 RLU/µl; $p<0.01$), representing 18,000-fold, and 25-fold over background, respectively. We then generated rSIV.F/HN vectors expressing T1-3B antibody and showed detection of antibody in the serum (715 ng/ml; $p<0.05$) for up to 28 days post intranasal delivery of rSIV.F/HN (5e7 TU/mouse). Animals treated with rSIV.F/HN expressing T13B were partially protected against a lethal challenge with either 1,000 or 10,000 $TCID_{50}$ (~10 and 100 LD_{50}) of A/PuertoRico/8/34 (H1N1) influenza. They experienced a ~2-3 days delay in symptoms and, significantly reduced weight-loss - less than 20% weight loss in 50% (1,000 $TCID_{50}$) and 20% (10,000 $TCID_{50}$) treated animals, while all control animals lost >20% weight irrespective of influenza dose ($p<0.001$ and $p<0.05$ for 1,000 and 10,000 $TCID_{50}$ respectively). One major advantage of rSIV.F/HN as a platform for bNAb passive immunization is the proven ability to achieve successful transgene expression after repeated lung vector administration (*Mol Ther* 18:1173, 2010; *Am J Respir Crit Care Med* 186:846, 2012), a major hurdle for all other viral vectors we have evaluated, including rAAV (*J Virol* 81:12360, 2007). Repeat administration of rSIV.F/HN vectors expressing alternative bNAbs, such as those that cross-react with both group 1 and 2 influenza, could provide a truly broad protection against a wide range of influenza strains. We speculate that during the next human influenza pandemic, prophylaxis provided by lung gene transfer may be feasible and more cost-effective and time-responsive than traditional vaccines or parenteral administration of therapeutic antibody.

432. Valproic Acid Treatment Enhances Hematopoietic Stem and Progenitor Cell Multipotency Ex Vivo for Enhanced Long-Term Engraftment of Gene-Modified Cells

Anthony Conway, Alisa Boyko, Jeremy Hardin, Michael C. Holmes, Gregory J. Cost
Sangamo BioSciences, Richmond, CA

The promising field of genome editing in hematopoietic stem and progenitor (HSPC) for use in autologous and allogeneic transplantation therapies relies on being able to engraft the edited cells into the bone marrow and to have those engrafted cells produce all the hematopoietic lineages necessary for proper immune and red blood cell function. Depending on the cell source to be used in the editing process, the fraction of CD34+ HSPCs can be quite low - approximately 0.0005%, 0.01%, or 0.1% for mobilized peripheral blood (mPB), bone marrow aspirate (BM), or cord blood, respectively. The fraction of long-term repopulating true stem cells (LT-HSCs) within these CD34+ cell populations, capable of long-term reconstitution of the entire hematopoietic lineage after transplantation, is even lower (<1%). In addition, it has recently been shown that LT-HSCs derived from cord blood can be much less permissive to homology-directed repair (HDR)-driven gene correction which may be essential for some types of therapeutic genome editing.

Several groups have discovered small molecules that promote expansion of cord blood-derived CD34+ HSPCs *ex vivo* while maintaining the stemness of the HSPCs, however these effects have not been reported in HSPCs from mPB or BM. Here we show that the small-molecule epigenetic modifier valproic acid (VPA) improves HDR-mediated targeted integration (TI) in mPB and BM HSPCs. In addition to increasing the fraction of LT-HSCs with TI by up to 100-fold, VPA also dramatically increases the overall number of cells expressing LT-HSC markers (CD34+CD133+CD90+CD49f+ or CD34+CD38-CD45RA-CD90+CD49f+) by up to 500-fold. VPA-treated gene-edited HSCs differentiate normally *in vitro* and retain consistent levels of TI in both erythroid and myeloid lineages. These results further the development of genome-edited mPB and BM-derived HSPC therapies.

433. DNA Monoclonal Antibodies Target Influenza Virus In Vivo

Sarah T. C. Elliott[1], Ami Patel[1], Megan C. Wise[1], Seleeke Flingai[1], Amelia Keaton[1], Jian Yan[2], Kate E. Broderick[2], Trevor Smith[2], Niranjan Y. Sardesai[2], Karrupiah Muthumani[1], David B. Weiner[1]
[1]Department of Pathology & Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, [2]Inovio Pharmaceuticals, Plymouth Meeting, PA

Despite promising innovations, influenza vaccines and antiviral drugs fail to provide full protection from seasonal infection, and provide little defense against novel and potentially pandemic viral strains. Broadly cross-protective monoclonal antibodies have been developed with the aim of providing protection against highly divergent influenza viruses. However, the utility of delivering purified protein antibody as therapy or prophylaxis against influenza is limited, especially in pandemic settings. Use of gene therapy to generate monoclonal antibodies in vivo provides a simplified, flexible, and relatively inexpensive alternative to protein antibody treatment.

In this study, we used intramuscular electroporation of plasmid DNA encoding immunoglobulin to express DNA monoclonal antibodies (DMAb) against influenza hemagglutinin (HA) surface protein in mice. Multiple aspects of plasmid construction, antibody design, and delivery were optimized to enhance expression of DMAb from muscle cells in vivo. We investigated multiple antibody clones, including the broadly-neutralizing anti-influenza-H1 antibody 5J8.

The 5J8 DMAb was expressed at μg/mL levels in serum of both nude and immune-competent mice. Serum DMAb produced from muscle in vivo were functional in vitro - with the ability to bind influenza HA, block hemagglutination of red blood cells, and neutralize influenza virus. Serum DMAb expression levels approximate those required for protection. Influenza challenge studies of mice treated with 5J8 DMAb are underway.

DMAb provide an important new approach to immune therapy. DNA has an excellent safety profile and averts challenges of pre-existing serology associated with many viral vectors. Here, we demonstrate that DNA can be used to deliver consistently high levels of potent monoclonal antibodies for protection against a viral pathogen.

434. Adoptive Treg Cell Therapy Using Factor VIII-Specific CAR Regulatory T Cells Regulates Anti-Factor VIII Immune Responses in Hemophilia A Mice

Richard Y. Fu, Meghan J. Lyle, Xuefeng Wang, Carol H. Miao
Immunity & Immunotherapies, Seattle Children's Research Institute, Seattle, WA

The immune response to factor VIII (FVIII; F8 in constructs) protein limits the effectiveness of treatments for hemophilia A (HemA) patients. Our previous studies demonstrated that regulatory T cells (Tregs) play a pivotal role in modulation of anti-FVIII immune responses. In particular, adoptive transfer of Tregs isolated from FVIII-primed HemA/Foxp3 mice attenuated anti-FVIII immune responses induced by gene transfer of FVIII plasmid in HemA mice. For developing adoptive Treg therapy, we successfully expanded FVIII-sensitized polyclonal Tregs using an FVIII-specific expansion protocol in vitro and showed that these cells had increased FVIII-specific suppressive activity compared with nonspecific Tregs. However, FVIII-specific Tregs in the polyclonal population are still in very small numbers. In this study, we explored the strategy to generate FVIII-specific Tregs using the chimeric antigen receptor (CAR) approach. Lentiviral vector (LV) incorporating a high-binding anti-FVIII antibody-derived variable region (scFv) linked to signaling and costimulatory moieties of immune receptors (third generation CAR) and fused with a murine Foxp3 cDNA (F8CAR-Foxp3-LV) was prepared and used to transduce murine $CD4^+$T cells. Flow cytometry analysis confirmed extracellular scFv and intracellular Foxp3 expression in transduced cells (F8CAR-Tregs). In vitro suppressive assay showed that transduced $CD4^+$T cells had significantly higher FVIII-specific suppressive activity than untransduced cells towards FVIII-specific $CD4^+$ effector T cells (Teffs). In addition, $1x10^6$ transduced cells and untransduced cells were adoptively transferred into HemA mice. One day after cell transfer, the treated mice were challenged with FVIII plasmid injected hydrodynamically. The anti-FVIII antibody titers are evaluated overtime. It is expected that F8CAR-Foxp3-LV transduced cells will prevent or decrease the production of anti-FVIII antibodies. We have also prepared a LV incorporating only the F8CAR region (F8CAR-LV). $CD4^+CD25^-$ and $CD4^+CD25^+$ cells isolated from HemA mice were transduced with F8CAR-LV to generate FVIII-specific Teffs and Tregs, respectively. In vitro FVIII-specific suppressive assays using $CD4^+$ Teffs from FVIII-primed HemA mice or F8CAR-LV transduced Teffs as responder cells are performed to compare the suppressive function of F8CAR-LV transduced $CD4^+CD25^+$ cells and F8CAR-Foxp3-LV transduced $CD4^+$ cells. $CD4^+CD25^+$ cells isolated from FVIII-primed HemA mice and untransduced $CD4^+CD25^+$ and $CD4^+$ cells from HemA mice are used as control cells. These experiments compare the extent of suppression towards specific F8CAR Teffs and polyclonal FVIII-specific Teffs as well as the potency of suppressive function between two differently engineered F8CAR Tregs. Finally, adoptive transfer experiments into HemA mice using the transduced and control cell populations are performed to evaluate their in vivo function to protect the HemA mice from anti-FVIII antibody production. We anticipate that compared with nonspecific and polyclonally expanded Tregs, FVIII-specific CAR Tregs will exert superior suppressive activity towards anti-FVIII immune responses without triggering systemic immune suppression.

435. Skin Delivery of a RSV Vaccine with Surface Electroporation Provides Full Protection from Lower Respiratory Disease in the Cotton Rat

Trevor Smith, Katherine Schultheis, Jay McCoy, Niranjan Sardesai, Kate Broderick
Inovio Pharmaceuticals, San Diego, CA

Respiratory syncytial virus (RSV) is a massive medical burden in infants and children worldwide, and an effective and safe RSV vaccine remains an unmet need. Here we report a novel vaccination strategy to deliver a pDNA vaccine encoding RSV-F using a surface electroporation device (SEP) to target epidermal cells in clinically relevant experimental models. We demonstrate the ability of this strategy to target epidermal Langerhans cells, and elicit robust cellular and humoral immune responses. In the cotton rat challenge model we demonstrate complete resistance to pulmonary infection after delivering a single low dose of vaccine. In contrast to the formalin-inactivated RSV (FI-RSV) vaccine there was no enhanced lung inflammation upon virus challenge after pDNA vaccination. In summary the data presented outlines the pre-clinical development of a highly efficacious, tolerable and safe non-replicating vaccine strategy against RSV.

436. Developing a Synthetic DNA Vaccine for an Emerging Pathogen - Middle East Respiratory Syndrome

Emma L. Reuschel[1], Karrupiah Muthumani[1], Kim Kraynyak[2], Colleen Tingey[1], Sagar B. Kudchodkar[1], Amir S. Khan[2], Niranjan Y. Sardesai[2], J. Joseph Kim[2], Joel Maslow[3], Young K. Park[3], Gary Kobinger[4], Darryl Falzarano[5], Heinrich Feldman[6], David B. Weiner[1]
[1]Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, [2]Inovio Pharmaceuticals, Inc., Bluebell, PA, [3]GeneOne Life Science, Seoul, Korea, Republic of, [4]Special Pathogens Program, University of Manitoba and Public Health Agency of Canada, Winnipeg, MB, Canada, [5]Vaccine and Infectious Disease Organization - International Vaccine Centre, University of Saskatchewan, Saskatoon, SK, Canada, [6]Laboratory of Virology, Division of Intramural Resesarch, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT

Background: Middle East Respiratory Syndrome (MERS) was first reported in 2012 in Saudi Arabia when a patient died from severe respiratory disease caused by a novel betacorona virus, MERS-CoV. Through November 2015, there have been 1618 confirmed global cases of MERS-CoV infection and 579 deaths reported to the World Health Organization (WHO). Currently, no vaccine or specific treatment is available and patients are treated with supportive care based on their clinical condition. While most MERS cases occur in and around Saudi Arabia, the recent outbreak in Korea highlights the potential for this disease to spread beyond the immediate region. A vaccine is needed to prevent future disease caused by MERS-CoV.

Methods: A synthetic DNA MERS vaccine was generated using a consensus sequence of the MERS spike protein. Mice, dromedary camels, and non-human primates (NHP) were immunized with MERS-vaccine by intramuscular injection followed by electroporation. Cellular immune responses were measured by flow

cytometry and IFNγ ELISpot. Humoral immune responses were measured by ELISA and neutralizing antibody (nAb) assay. Following immunization, NHPs were challenged with infectious MERS-CoV (EMC/2012) and monitored for signs of infection by clinical scoring and examinations. Viral load was measured by qRT-PCR and tissue sections were stained with H&E.

Results: Immunization of mice with MERS-vaccine induced strong humoral and cellular responses. Mice produced strong binding antibody (bAb) titers and nAb titers. A strong, polyfunctional, CD4 and CD8 T cell response was detected against multiple epitopes across the MERS spike protein. Immunization of dromedary camels induced the production of MERS spike specific antibodies and nAbs. Immunization of NHPs induced strong bAb titers and nAb titers and a strong CD4 and CD8 T cell response. NHPs immunized with multiple vaccination regimens were also protected from signs of disease upon challenge with infectious MERS-CoV and showed a greater than 3 log reduction in viral load after challenge compared to unvaccinated animals.

Conclusions: A consensus DNA MERS-vaccine was able to generate both a strong T cell and neutralizing antibody response in multiple animal models, including camels, a natural host for MERS-CoV and a probable source of human infection. MERS-vaccine was also able to protect NHPs from an infectious MERS-CoV challenge. These results demonstrate the promise of this consensus DNA MERS-vaccine as a candidate for vaccine development.

437. A Light-Producing Model of Infection-Related Preterm Birth

Natalie Suff[1], Rajvinder Karda[1], Dany Perocheau[1], Juliette M. Delhove[2], Tristan R. McKay[2], Mark Tangney[3], Suzanne M. K. Buckley[1], Donald Peebles[1], Simon N. Waddington[1]
[1]UCL, London, United Kingdom, [2]St. George's University, London, United Kingdom, [3]University College Cork, Cork, Ireland

Background:

Preterm birth is increasing in incidence and current therapies are relatively ineffective. It is responsible for >1million neonatal deaths per annum worldwide and long term complications in survivors. Approximately 50% of PTBs are preceded by microbial invasion of the intrauterine space; current clinical management centres on diagnosis of intrauterine bacterial presence by identifying the resultant inflammatory response. To investigate the relationship between intrauterine bacterial presence and inflammation we developed two separate gene technology approaches:

1. Intravaginal bioluminescent bacteria to measure bacterial ascent into the uterus which mimics the ascending vaginal infection seen in preterm birth.

2. Lentiviral gene transfer of an NFKB activated luciferase reporter construct to allow bioluminescent imaging of the subsequent systemic NFKB response.

Methods:

An NFκB response element was cloned into a lentivirus vector upstream of the genes encoding a codon-optimised firefly luciferase. High titred virus was injected intravenously at birth to neonatal female C57BL/6 J-Tyrc-2J mice to achieve luciferase expression predominantly in the liver (to monitor systemic inflammatory response). These mice received *Escherichia coli* (non-pathogenic K-12, MG1655 with integrated luxABCDE operon) intra-vaginally once reaching adulthood and intraperitoneal lipopolysaccharide (LPS) three weeks later. Luciferase expression was monitored by whole body bioluminescence imaging. Local inflammation was determined using H&E, ICAM-1 (Intracellular adhesion molecule 1) and Ly6g immunohistochemistry and enzyme-linked immunosorbent assays for serum and uterine TNF-α and IL1-β cytokines.

Results:

Bioluminescent imaging revealed that C57BL/6 J-Tyrc-2J mice were the most susceptible mice breeds for modelling of ascending vaginal infection with E.coli luxABCDE operon. Intraperitoneal LPS induced an NF-KB response in the liver by biosensing ($p<0.01$), however intravaginal E.coli administration induced no response. There was evidence of uterine inflammation with an upregulation of ICAM-1 and neutrophils.

Conclusion:

Although it is possible to detect LPS-induced NFKB inflammation in the liver by biosensing, ascending vaginal infection induced no response. This highlights the clinical challenge of identifying bacterial presence, confined to the uterus, using systemic markers. This model can be used to test new treatments for the prevention of PTB.

Figure 1. *E.coli* luxABCDE operon bioluminescence at 24h

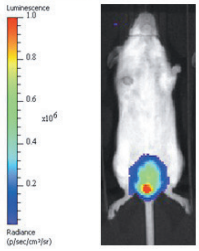
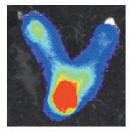
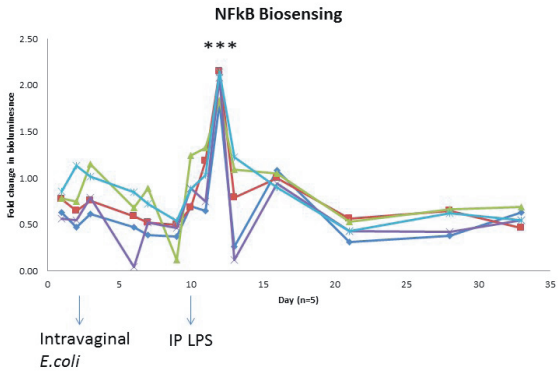


Figure 2. Tracking bacterial ascent



Ascending infection disease modelling using the : NF-Kb activated luciferase reporter construct - inflammation induces a bioluminescence response.



438. Human, Pig and Mouse IFITMs Partially Restrict Pseudotyped Lentiviral Vectors

Andrew L. Hornick, Ni Li, Mayumi Oakland, Paul B. McCray, Patrick L. Sinn
Pediatrics, University of Iowa, Iowa City, IA

Lentiviral vectors are increasingly used in clinical trials to treat genetic diseases. Our research has focused on strategies to improve lentiviral gene transfer efficiency in the airways. Previously we demonstrated that a feline immunodeficiency virus (FIV)-based lentiviral vector pseudotyped with the baculovirus envelope glycoprotein GP64 (GP64-FIV) efficiently transduced mouse nasal epithelia *in vivo* but transduced mouse intrapulmonary airways with ten-fold less efficiency. Here, we demonstrate that a family of proteins with antiviral activity, interferon induced transmembrane proteins (IFITMs), are more highly expressed in the mouse intrapulmonary airways as compared to the nasal airways. Using GP64 and VSV-G pseudotyped FIV, we show that expression of mouse IFITM1, IFITM2, and IFITM3 restricts gene transfer. Further we show that both

the nasal and intrapulmonary airways of IFITM locus knockout mice are more efficiently transduced by GP64-FIV than their heterozygous littermates. In anticipation of transitioning our studies into pig models of airway disease and clinical trials in humans, we investigated the ability of pig and human IFITMs to restrict lentiviral gene transfer. We observed that both human and pig IFITMs partially restricted both VSV-G-FIV and GP64-FIV transduction *in vitro*. These results implicate the IFITM proteins as restriction factors that can limit lentiviral gene transfer to airway epithelia. The findings are relevant to future pre-clinical and clinical airway gene therapy trials using lentiviral-based vectors.

439. Prolonged Expression of Secreted Enzymes in Dogs After Liver Delivery of *Sleeping Beauty* Transposons: Implications for Non-Viral Gene Therapy of Mucopolysaccharidoses Types I and VII

Elena L. Aronovich[1], Kendra A. Hyland[2], Bryan C. Hall[1], Jason B. Bell[1], Erik R. Olson[2], Myra Rusten[3], David W. Hunter[3], Chester B. Whitley[4], N. Matthew Ellinwood[5], R. Scott McIvor[1], Perry B. Hackett[1]

[1]Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, [2]Discovery Genomics, Inc, Minneapolis, MN, [3]Radiology, University of Minnesota, Minneapolis, MN, [4]Pediatrics, University of Minnesota, Minneapolis, MN, [5]Animal Sciences, Iowa State University, Ames, IA

The non-viral integrating vector the *Sleeping Beauty* (SB) transposon system is efficient in treating systemic monogenic diseases in mice including mucopolysaccharidosis (MPS) types I and VII caused by α-iduronidase (IDUA) and β-glucuronidase (GUSB) deficiency, respectively. More recently we have used modified approaches of the hydrodynamic procedure to deliver therapeutic transposons to dog liver. Reproducible delivery and transposition in dogs are about 1% the levels in mice. Using a transgenic canine reporter secreted alkaline phosphatase (cSEAP), in the absence of immune suppression we can detect transgenic protein for up to six weeks post infusion using catheter-mediated hydrodynamic delivery. A proof-of-principle immunomodulation using $GdCl_3$ to block macrophages in liver and spleen prolonged the presence of the cSEAP protein in circulation from 6 weeks to up to at least 5 months after a single vector infusion. We achieved stabilized activity in one dog at about 2-fold of baseline values. Durability of cSEAP in serum was inversely correlated with transient increase of liver enzymes ALT and AST in response to the vector delivery procedure, pointing to the deleterious effect of hepatocellular toxicity on transgene maintenance. However, $GdCl_3$ immunomodulation was ineffective for repeat vector infusions, suggesting a possibility of an alternative, more potent immunosuppression regimen. Evidence of transposition was obtained with the most efficient transposase SB100X but not with SB11. For transgenic IDUA and GUSB, therapeutic activity in serum peaked at 50-350% of wild-type at 2-4 days post-treatment, but lasted only a few days. The differences in levels and duration of detection of cSEAP in the blood compared to those of IDUA and GUSB may be in part due to the facilitated uptake of lysosomal enzymes into cells compared to cSEAP. Longer endurance of transgenic proteins at therapeutic levels may be possible in SB-treated dogs using alternative immunosuppressive regimens.

440. Extreme Polyvalency Induces Potent Cross-Clade Cellular and Humoral Responses in Rabbits and Non-Human Primates

Megan Wise[1], Kar Muthumani[1], Janess Mendoza[2], Jian Yan[2], Anna Slager[2], Kim Kraynyak[2], David Montefiori[3], Celia LaBranche[3], Kate Broderick[2], Matthew Morrow[2], Niranjan Sardesai[2], David Weiner[1]

[1]University of Pennsylvania, Philadelphia, PA, [2]Inovio Pharmaceuticals, Plymouth Meeting, PA, [3]Duke University, Durham, NC

There is a pressing need to determine possible unique vaccine combinations of Envs which induce enhanced breadth and functional antibody responses. Due to the ease of manufacturing and formulation, we sought to determine if increased breadth within a vaccine correlates with robust and broad responses. We have developed over 40 different DNA plasmids expressing consensus as well as primary Envs. We have shown that each of these plasmids are able to induce both cellular and humoral responses in mice. Different combinations of Envs were testing in rabbits to further characterize the humoral responses and explore neutralization. Rabbits immunized with clusters of clade A transmitter founder (TF) gp160 DNA induced cross-clade binding titers with limited neutralization. Including TF Envs from different clades, increased binding titers as well as neutralization breadth and potency. Formulating the gp160s to be administered to the same site induced quicker seroconversion than delivering the Envs at separate sites. The most potent combination was moved forward into NHPs, which were immunized with clusters of gp160 DNAs (14 different Envs in total) at weeks 0, 4, 8, 12 and boosted at weeks 48 and 85. The vaccine induced cross-clade cellular and humoral responses after two immunizations. These responses increased after each immunization and were maintained into memory. In addition to binding, vaccination also induced tier 1 neutralization. Boosting at week 48 and 85 further increased both responses. We show that DNA plasmids encoding consensus and TF Envs are expressed and induce a potent immune responses. We observed for the first time that exposure of the immune system to multiple Envs at one time can dramatically change the immune phenotype by inducing broader breadth of response which has significant implications for HIV vaccine development.

441. IL2 Plasmid Treated Hemophilia A Mice Show an Increase in Regulatory T Cell Population and Initial Tolerance to FVIII

Meghan J. Lyle, Richard Y. Fu, Carol H. Miao

Seattle Children's Research Institute, Seattle, WA

Hemophilia A is an inherited X chromosomal linked recessive disease. Hemophilia A patients lack or possess low levels of functional FVIII protein, which results in an inability for the blood to clot when injury occurs. This inability to clot can result in major blood loss and potential death. The current treatment for patients with hemophilia A is FVIII protein replacement, but this treatment is expensive and often results in anti-FVIII immune responses, neutralizing the clotting effect. In order to overcome this potential immune response patients undergo a high dose regimen of FVIII to induce tolerance. However, a third of patients still develop an immune response.

Regulatory T (Treg) cells help balance T effector (Teff) cells through their suppressive function during an immune response and keep autoimmunity in check. Both of these cell types bind to interleukin 2 (IL2), a cytokine which promotes the differentiation, expansion and activation of these cell populations. Treg cells possess a high affinity epitope of the IL2 receptor. With a low dose of IL2, the Treg population outcompetes the Teff cells, leading to an increase in activation and number. The increased suppressive activity may induce tolerance in hemophilia A mice treated with FVIII plasmid.

Hemophilia A mice were hydrodynamically injected with either 2 μg or 5 μg IL2 plasmid and 50 μg FVIII plasmid, either sequentially (1 week apart) or simultaneously. Both plasmids were driven by a liver-specific promoter (hAAT-HCR). Cell staining data showed a marked increase in Treg cell population and activation, demonstrating that a small amount of IL2 plasmid incorporated into liver cells is enough to dramatically increase the Treg population. A larger increase of Teff cells were observed in mice treated with 5 μg IL2 plasmid than in mice treated with 2 μg IL2 plasmid. Importantly, Treg/Teff ratio was significantly increased in the 2 μg IL2 plasmid group in the first 3 weeks and maintained at increased levels over several weeks afterwards. ELISA showed an initial higher level of IL2 production; IL2 expression dropped and maintained at low levels after one week. On Day 28-post FVIII plasmid injection, control mice had started to develop inhibitors associated with significant decrease of FVIII expression, while mice treated with IL2 plasmid showed no inhibitor development with persistent FVIII gene expression. The treated mice are monitored to determine the potential long term tolerance effects induced by low dose IL2 plasmid. Using gene therapy to slightly increase the amount of a cytokine in patients could provide a better treatment option than existing drug regimens. FVIII protein is also extremely expensive, especially in the amounts needed for treating hemophilia A patients. Combined gene transfer of FVIII and IL2 plasmids has the potential to produce therapeutic FVIII and simultaneously prevent inhibitory antibody formation, therefore reducing the amount of expensive reagents needed, the number of doctor visits required, and the overall cost, potential morbidity and stress to the patient.

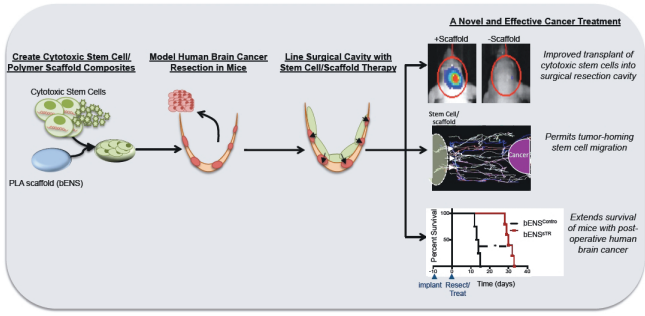
Cell Therapies II

442. Developing Polymeric Bio-Scaffolds That Increase the Efficacy of Stem Cell-Mediated Therapy for Brain Tumors

Juli Bago[1], Onyi Okolie[1], Mahsa Mohiti-Asli[2], Elizabeth Loboa[2], Shawn Hingtgen[1]

[1]Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]Department of Biomedical Engineering, North Carolina State University, Raleigh, NC

Engineered stem cell (SC)-based therapy holds enormous promise for treating the incurable brain cancer glioblastoma (GBM). Retaining the cytotoxic SCs in the surgical cavity after GBM resection is one of the greatest challenges to this approach. In this study, we describe a biocompatible electrospun nanofibrous scaffold (bENS) implant capable of delivering and retaining tumor-homing cytotoxic stem cells that suppress recurrence of post-surgical GBM. As a new approach to GBM therapy, we created poly(L-lactic acid) (PLA) bENS bearing drug-releasing human mesenchymal stem cells (hMSCs). We discovered that bENS-based implant increased hMSC retention in the surgical cavity 5-fold and prolonged persistence 3-fold compared to standard direct injection using our mouse model of GBM surgical resection/recurrence. Time-lapse imaging showed cytotoxic hMSC/bENS treatment killed co-cultured human GBM cells, and allowed hMSCs to rapidly migrate off the scaffolds as they homed to GBMs. *In vivo*, bENS loaded with hMSCs releasing the anti-tumor protein TRAIL (bENS^{sTR}) reduced the volume of established GBM xenografts 3-fold. Mimicking clinical GBM patient therapy, lining the post-operative GBM surgical cavity with bENS^{sTR} implants inhibited the re-growth of residual GBM foci 2.3-fold and prolonged post-surgical median survival from 13.5 to 31 days in mice. These results suggest that nanofibrous-based SC therapies could be an innovative new approach to improve the outcomes of patients suffering from terminal brain cancer.



443. Neural Stem Cell-Mediated Enzyme/Prodrug Therapy for Medulloblastoma

Margarita Gutova[1], Lusine Tsaturyan[1], Michael E. Barish[1], Karen S. Aboody[1], Robert Wechsler-Reya[2]

[1]Developmental and Stem Cell Biology, Department of Neurosciences, Beckman Research Institute of City of Hope, Duarte, CA, [2]Tumor Initiation & Mainenance Program, Sanford-Burnham Medical Institute, La Jolla, CA

Introduction: Medulloblastoma is the most common malignant brain tumor in children. Despite recent advances in treatment, these tumors continue to be associated with high morbidity and mortality, and new strategies for treatment of medulloblastoma are urgently needed. Major obstacles to successful treatment of pediatric brain tumors include the blood-brain-barrier (BBB), which prevents many systemically administered anti-cancer agents from entering the central nervous system. Neural stem cells (NSCs) will effectively cross the BBB and preferentially migrate to tumor cells throughout the brain.

Methods: We modified human NSCs to express and deliver the enzyme carboxylesterase (CE), which activates irinotecan (IRN; CPT-11) to the potent topoisomerase-1 inhibitor SN-38. For pharmacokinetic studies, tumor-bearing mice were injected intracranially with NSCs expressing carboxylesterase enzyme (CE-NSC) and followed by intravenous administration of IRN at dose 37.5 and 54.7 mg/kg after 2 days post NSCs administration. Mice were euthanized 1h after IRN injection. Brain tissues and mouse plasma were collected for quantitative LC/MS measurements of IRN and SN-38. The control group received IRN injections only.

Results: In vitro cytotoxicity experiments demonstrate a 2000-fold increase in sensitivity of medulloblastoma cells to IRN in the presence of CE-NSCs. We have quantitatively assessed NSC tumor distributions in MP (c-myc, DNp53) and patient-derived medulloblastoma xenografts (PDX) tumor models, and demonstrated that intranasally administrated NSCs migrate to medulloblastoma tumors. We also found that NSCs do not distribute to non-cancerous areas of the brain, or to other peripheral tissues, suggesting that this approach could selectively target SN-38 production to pediatric tumor sites while sparing normal tissues. Pharmacokinetic and therapeutic efficacy studies demonstrated that in both mouse models, CE-NSC cells mediate local tumor-specific conversion of irinotecan pro-drug to active drug SN-38 only at the tumor sites. Plasma levels of toxic SN-38 were low in both tumor models.

Conclusions: We provided proof-of-principle for a NSC.CE-mediated localized chemotherapy approach, which can potentially limit the severe cognitive and functional deficits associated with currently available therapies, improving clinical outcome and quality of life for patients with medulloblastoma.

444. Large Scale Culture and Differentiation of Induced Pluripotent Stem Cells for Neutrophil Replacement Therapies

Lisa Trump, Ramesh Nayak, Schroll Robin, Jose A. Cancelas, Carolyn Lutzko
Experimental Hematology, Cincinnati Childrens Hospital Medical Center, Cincinnati, OH

Neutrophils are a key component in the innate immune system and are crucial in the protection against bacterial and fungal infections. Patients with neutropenias are at high risk to develop serious life threatening bacterial and fungal infections. Transfusion of donor neutrophils into neutropenic patients may help alleviate disease burden, but difficulty in collecting and transfusing sufficient quantities of viable donor neutrophils has limited the clinical adaptation of neutrophil replacement therapies. Induced pluripotent stem cells (iPSC) are an attractive alternative source to donor derived neutrophils due to their ability to self-renew and differentiate into cells of the three embryonic germ layers, including neutrophils. Successful implementation of iPSC for neutrophil replacement therapies will require a) derivation of iPSC, b) large scale culture of iPSC, and c) efficient differentiation of these iPSC into functional neutrophils in xeno-fee, GMP-compliant conditions. First, normal human fibroblasts were reprogrammed in xeno-free, GMP compliant conditions though nucleofection of a non-integrating 3 plasmid system carrying OCT4, SOX2, KLF-4, l-MYC, Lin28 and shp53. The reprogrammed fibroblasts were cultured in defined conditions on Vitronectin XF in TeSR-E7 media until colony formation when they were transitioned to E8 medium. We generated 3 lines under these conditions and all iPSC derived and cultured under these conditions retain a normal karyotype, express pluripotency markers SSEA-4, Tra-1-60, Tra-1-81, and CD9 at >85% as determined by FACs analysis, and differentiate into all three germ layers *in vitro* . Once iPSC lines were established, cultures were adapted from adherent cultures to non-adherent, aggregate suspension cultures in spinner flask bioreactor systems in mTeSR or E8 medium. Briefly, adherent iPSC are dissociated into single cells and seeded into a spinner flask at a concentration of $2x10^5$ cells/ml and spun at 60 RPM. We evaluated 2 lines in 3 independent experiments and cells readily formed clusters and were able to achieve 4-log expansion (range: 2-4 log) within approximately 40 days (10 passages). All lines retained a normal karyotype, expressed pluripotency markers SSEA-4, Tra-1-60, Tra-1-81, and CD9 (>85%), and displayed a normal morphology when re-plated in adherent culture. We next adapted a neutrophil differentiation system for differentiation from iPSC from the defined media/spinnerflask system. Clusters of cells from spinner flask cultures were induced to form embryoid bodies (EBs) by transitioning the cultures to EB specification medium for 5 days, followed by a neutrophil specification medium containing IL-3 and G-CSF in adherent conditions. After 7-14 days in neutrophil specification conditions, EBs attached to the plate and CD45+CD11b+CD16+ neutrophils were shed into the medium and were harvested twice weekly for 30-60 days. In 10 experiments, we collected an average of 6.5x10^5 CD45+ cells per well of differentiation representing a 6.5 fold expansion (range: 3-10 fold). In conclusion, iPSC have been derived in GMP compliant conditions and can be cultured at a large scale using spinner flask bioreactors, and the iPSC from spinner flask bioreactors can then successfully be differentiated into CD45+ cells. Thus, iPSC represent an attractive, self-renewing resource of neutrophils for neutrophil replacement therapies.

445. cGMP Compliant Production for Human Embryonic Stem Cell Derived Retinal Pigment Epithelial Cells on a Synthetic Substrate for the Treatment of Non-Neovascular Age-Related Macular Degeneration for Phase I Clinical Study

P. M. Lopez[1], T. Aguilar[1], Rl Wu[1], A. Krishnan[1], D. Kong[1], J. Patel[1], H. Javier[1], W. Dang[1], D. Wong[1], D. Zhu[2], J. Dodd[1], Y. Shad[1], M. Humayun[2], D. R. Hinton[2], D. O. Clegg[3], L. V. Johnson[3], C. M. Lin[1], D. Hsu[1], L. Couture[1]
[1]City of Hope, Duarte, CA, [2]University of Southern California, Los Angeles, CA, [3]University of California Santa Barbara, Santa Barbara, CA

Non-neovascular age-related macular degeneration (dry AMD) is a leading cause of irreversible vision loss and is associated with retinal pigment epithelium (RPE) dystrophy. The RPE monolayer is integral for maintenance of healthy photoreceptors. Many studies have shown that AMD can be recovered by cellular therapy through transplantation of RPEs into the sub-retinal space to re-establish RPE functionality and halt neurological degeneration. Here we present a cGMP compliant manufacturing process to producing transplantable RPE cells on a synthetic substrate for clinical phase I study. The cGMP compliant process contains two major steps for generating the final product. The first step is to produce an intermediate cell bank (ICB) of RPE derived from human embryonic stem cell (hESC-RPE). The second step is to culture hESC-RPEs on a synthetic substrate as the final product. Analytical assays were also developed for the release testing of the final product and for evaluating transportation protocols of the final product. Three qualification runs were performed to assess the feasibility and the reproducibility of the manufacturing process. The results have shown a high success rate (88-98%) of the productions. 133 out of 144 hESC-RPEs seeded substrates met assay criteria for release including sterility, identity, purity, morphology, and viability. The assay results also showed the comparability of the final products between day 28 and day 43. In addition, 14 transportations of the non-cryopreserved products were successfully conducted by courier to the clinical site in a temperature regulated incubator. In conclusion, we have adapted and optimized laboratory protocols to establish a cGMP-compliant manufacturing process for generating the final product of hESC-RPE on a synthetic substrate for early phase clinical studies. A transportation protocol was also established to deliver the non-cryopreserved final product to clinical site. Moreover, analytical assays were developed and qualified for release testing of the final product.

446. Gender Differences in the Osteogenic & Chondrogenic Potential of Human Muscle-Derived Stem Cells In Vitro

Alex C. Scibetta[1], Andrea B. Liebowitz[1], Elizabeth R. Morris[1], Aiping Lu[2], Xueqin Gao[2], Johnny Huard[1,2]
[1]Steadman Philippon Research Institute, Vail, CO, [2]Department of Orthopaedic Surgery, McGovern School of Medicine, University of Texas Health Science Center at Houston, Houston, TX

Introduction: It has been shown that murine muscle-derived stem cells (MDSCs) are capable of multipotent differentiation[1]. These murine cells exhibit sexual dimorphism of osteogenic and chondrogenic potentials[1]. Previous studies have shown that male mouse MDSCs (M-MDSCs) differentiate into chondrocytes more effectively and display better cartilage regeneration potentials than female MDSCs (F-MDSCs)[2]. M-MDSCs have also been shown to be more osteogenic and demonstrate more rapid bone formation compared to F-MDSCs1 . *In vivo* murine studies have also demonstrated that M-MDSCs form more bone than F-MDSCs[1,3]. Adipose derived stem cells also display sexual dimorphism in their

Molecular Therapy Volume 24, Supplement 1, May 2016

multipotent differentiation potential[4]. This study further investigates this sexual dimorphism in human muscle-derived stem cells (hMDSCs). **Methods:** Two populations of human MDSCs, one male and one female, were used in these experiments. (1) Osteogenesis: M- and F-hMDSCs 3D pellets were cultured in osteogenic medium containing 100ng/ml bone morphogenic protein 2 (BMP-2) for 28d according to literature[1]. Cell pellets were fixed in neutral buffered formalin and subjected to micro CT scanning. After scanning, cell pellets were embedded in NEG50 freezing medium for sectioning. Von Kossa staining was used to detect mineralization. Osteocalcin immunohistochemistry (IHC) was used to detect osteogenic differentiation. (2) Chondrogenesis: M- and F-MDSC 3D pellets were cultured for 24d in chondrogenic medium using Stem Pro complete chondrogenic medium. After culture, all pellets were fixed and sectioned. Alcian blue and Col2A1 were used as indicators of chondrogenesis, and the percentage of each pellet stained with Alcian blue staining was quantified. **Results:** Osteogenesis: MicroCT analysis indicated that M-hMDSCs had significantly more pellet mineralization compared to F-MDSCs ($p<0.001$) (Fig.1A, 1B). Von Kossa staining showed stronger brown-black mineral deposition in male pellets than female pellets (Fig. 1C). Osteocalcin IHC indicated higher expression in male hMDSCs than female hMDSCs (Fig. 1D). Chondrogenesis: Alcian blue staining demonstrated that male cells have stronger blue cartilage matrices than female cells (Fig. 2A). Quantification of the blue matrix indicated significantly more matrix in male hMDSC pellets than female pellets (Fig. 2B). Col2A1 IHC showed stronger staining in male hMDSCs than female hMDSCs (Fig. 2C). **Conclusions:** Our results suggest that both gender hMDSCs can undergo osteogenic and chondrogenic differentiation, but male hMDSCs are more osteogenic and chondrogenic than female hMDSCs. Therefore, sex difference is an important factor to consider in the development and application of cell-based therapies for bone and articular cartilage repair. **References:** 1. Meszaros et al., 2012. 2. Matsumoto et al., 2008. 3. Corsi et al., 2007. 4. Jung et al., 2015

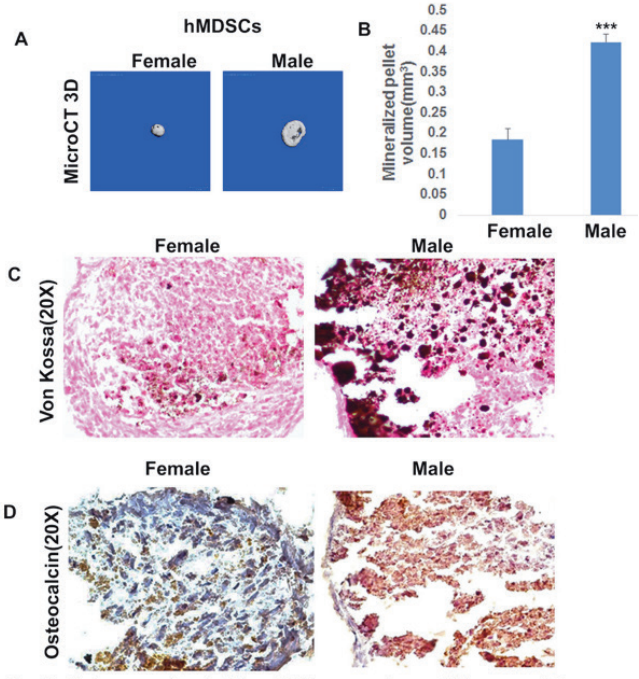


Fig. 1. Osteogenesis. A. MicroCT images showed bigger pellets formed by male hMDSCs than female hMDSCs. B. Mineralized pellet volume showed male hMDSCs have higher mineralized pellet volume than female hMDSCs ($p<0.0001$). C. Von Kossa staining showed more brown-black staining in male hMDSC group. D. Osteocalcin immunohistochemistry indicated more male cells expressed osteocalcin.

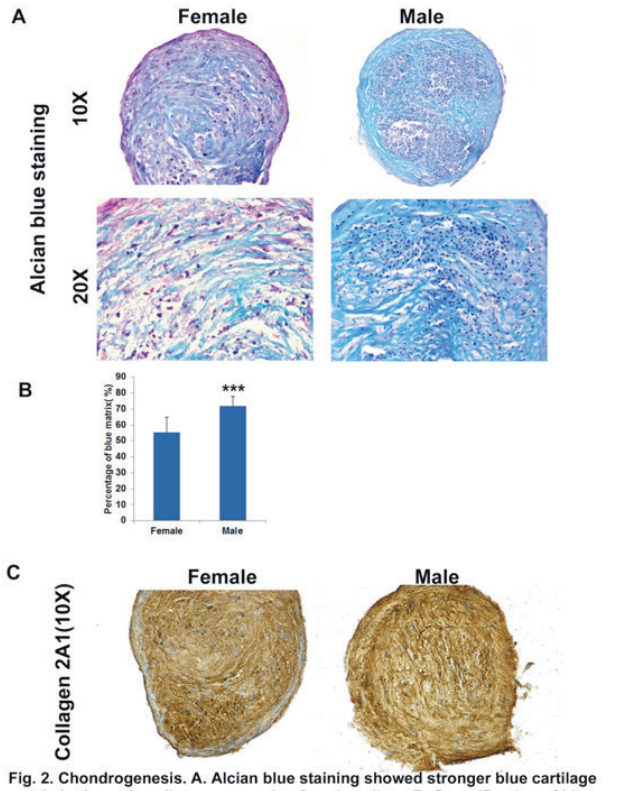


Fig. 2. Chondrogenesis. A. Alcian blue staining showed stronger blue cartilage matrix in the male pellets compared to female pellets. B. Quantification of blue cartilage matrix area indicated significantly more blue matrix in male hMDSC pellets compared to female hMDSCs. *** $p<0.001$. C. Col2A1 immunohistochemistry showed chondrogenic differentiation of both gender hMDSCs. Male hMDSCs pellets have stronger brown color than female hMDSC pellets.

447. Notch Activation Enchances Vascular Lineage Commitment of Cardiac Stem Cells

Konstantin Dergilev, Zoya Tsokolaeva, Marya Boldyreva, Irina Beloglazova, Ekaterina Zubkova, Yelena Parfyonova
Angiogenesis, Russian Cardiology Research and Production Complex, Moscow, Russian Federation

Notch signaling pathway is an evolutionary conserved system that control heart development and could be important for cardiac tissue renewal and regeneration after injury by controlling the maintenance and commitment of a cardiac stem cell compartment. However the precise cell targets of Notch signaling in the mammalian damaged heart remain poorly defined. OBJECTIVE: To investigate the functional role of Notch signaling in the regulation of cardiac stem cells (CSCs) commitment in ischemic mice myocardium. METHODS AND RESULTS: Notch 1 receptor, their ligands and markers of cardiomyocyte, SMC, and endothelial cell lineages were examined in undamaged and infarcted C57BL/6 mice hearts by immunofluorescence staining. We have found that the majority of c-kit+ CSCs were nested in the interstitium between cardiomyocytes, and expressed Notch 1 receptor. Ligand Jagged 1 is presented on cardiomyocyte and cardiac fibroblasts, localized near c-kit+Notch1+ CSCs. Conversely, Delta-like4 ligand expressed on the endothelial and smooth muscle cells of coronary vessels. No signs of Notch1 signaling activation (such as nuclear NICD localization) were detected in undamaged mice hearts. Acute myocardial infarction was accompanied by the increase in the total amount of c-kit + Notch1 + CSCs and the appearance of nuclear NICD localization in a number of CSCs showing an activation of Notch signaling. Cultivation of CSC in vitro on dishes coated with Jagged 1 ligand released

NICD and activated expression of Notch target genes (Hes, Hey). Activation of Notch signaling upregulated expression of endothelial cell transcription factors Gata 4 and Vezf1 in CSCs but γ-secretase inhibitor prevented Notch signaling activation and endothelial cell commitment of CSCs. CONCLUSIONS: These results revealed that Notch signaling activation promote CSCs commitment toward vascular cells. These findings suggest that modulation of Notch signaling can be promising therapeutic strategy for the treatment of myocardial ischemic damage.

448. Therapeutic Angiogenesis by Subcutaneous Cell Sheet Delivery Is Superior to Cell Injection: A Study of ADSC Efficacy in a Model of Hind Limb Ischemia

Pavel I. Makarevich[1], Maria A. Boldyreva[2], Anastasia Yu. Efimenko[1], Evgeny V. Gluhanyuk[3], Konstantin V. Dergilev[2], Julia O. Gallinger[1], Yu-Chen Hu[4], Yelena V. Parfyonova[2]
[1]Lomonosov Moscow State University, Moscow, Russian Federation, [2]Russian Cardiology Research and Production Complex, Moscow, Russian Federation, [3]Federal Research and Clinical Centre of Pediatric Hematology, Oncology and Immunology, Moscow, Russian Federation, [4]Tsing-Hua National University, Hsinchu, Taiwan

Engineering of cell sheets (CS) is an effective approach for delivery of cells to induce angiogenesis and tissue regeneration. Basis for increased CS efficacy is better engraftment and cell survival due to absence of anoikis and intact cell-to-cell interaction in the transplant. Still question to be addressed is whether CS are superior to injection route in terms of efficacy/cell engraftment and how can we improve therapeutic output of CS delivery for stimulation of tissue repair. We conducted a comparative study of adipose-derived stromal cells (ADSC) delivery in a model of hind-limb ischemia. C57/B6 male mice with unilateral limb ischemia (n=8-10/group) were injected with 10^6 of passage 3 syngeneic ADSC or transplanted with equivalent amount of cells in CS shape. After that animals were monitored for limb perfusion by laser-Doppler for 2 wks and then euthanized for histology studies of vessel density and ADSC detection using a PKH26 or CMFDA fluorescent label. Obtained samples were stained for macrophage invasion, endothelial cell markers and proliferation/apoptosis to evaluate cell fate. Our animal test data has revealed that by Day 14 delivery of ADSC by means of injection induced restoration of limb perfusion compared to negative control group (41.5±4.7% vs. 29.7±3.0% respectively; p=0.01) indicating well-known pro-angiogenic properties of these cells. Still, subcutaneous transplantation of CS was found to be superior to injection in terms of perfusion. CS-treated animals had the highest (55.3±7.3; p=0.03 vs. injected ADSC) perfusion by the end of experiment. This data was supported by vascular density assessment, which revealed increased capillary counts in both ADSC-treated groups with significantly higher values after CS delivery compared to injection (220.9±11.4 vs. 191.3±8.8 respectively; p=0.01). Analysis of necrotic tissue span in hematoxylin/eosin-stained section found a significant decrease of necrosis in ADSC-treated animals and also found CS to have better performance in terms of tissue protection compared to injection. Furthermore, we also evaluated ADSC engraftment and found that after injection pre-labeled cells reside as scattered mass and found their number to decrease over time by Day 14. Whilst after CS transplantation the cells were compactly localized in the site of application. CS were found to be vascularized by capillary vessels and infiltrated by CD68+ macrophage indicating graft-host interaction. Interestingly, certain cells within CS were found to show signs of proliferation (Ki67+) with sporadic apoptosis (cleaved caspase-3+) with overall transplant staying intact and viable by Day 14 after delivery. Overall, our data indicates that transplantation of CS is superior to injection of equivalent amount of cells. We may speculate that this is not limited to ADSC and can be utilized for novel treatment methods. In an attempt to enhance the CS efficacy we turned our attention to hybrid constructs consisting of ADSC and endothelial cells to generate pre-vascularized constructs. Our preliminary data revealed "tube-like" behavior of HUVEC seeded on top of a CS from ADSC and may be the way to overcome the diffusion distance issue and generate CS with vascularized structure, which have a closer resemblance to native tissue structures. Moreover, this unveils the possibility to generate multi-layered constructs and imitate cell-to-cell interaction for basic and applied studies.

449. IGF-1 Treatment Enhances the Myogenic Potential of Human Skeletal Muscle-Derived Stem Cells

Elizabeth Morris[1], Alex Scibetta[1], Aiping Lu[2], Xueqin Gao[2], Johnny Huard[2]
[1]Steadman Philippon Research Institute, Vail, CO, [2]University of Texas, Houston, TX

Human muscle-derived stem cells (hMDSCs) have been shown to promote the regeneration of a variety of tissues, including damaged heart, peripheral nerve, bone, articular cartilage and skeletal muscle[1-3]; however, without appropriate stimulation their differentiation potential remains limited.[4] In this study we examined whether insulin-like growth factor 1 (IGF-1) treatment could also enhance the myogenic potential of hMDSC, since it has been shown that IGF-1 treatment increased the myogenic potential of bone marrow-derived stem cells (BMSCs).[5] **Methods:** HMDSCs were isolated from human adult muscle biopsies via a modified preplate technique, as previously described.[6] Young female hMDSCs were plated in proliferation medium at a density of 1.58 x 10^4 cells/cm^2. After 24h in proliferation medium, the cells were cultured for an additional 7 days in myogenic medium containing various levels of IGF-1: 0ng/mL, 50 ng/mL, 100 ng/mL, or 200 ng/mL. To evaluate the extent of myogenesis, cells were fixed in MeOH and stained for fast myosin heavy chain (fMHC). Activity levels of creatine kinase, which is highly expressed in tissues like skeletal muscle that rapidly consume ATP, were measured according to the company's protocol. Additionally, RNA was extracted from cells, and cDNA was synthesized. Semi-quantitative PCR and qRT-PCR analyses were performed for myogenin, a late myogenic differentiation marker. **Results:** (1) fMHC staining indicated that IGF-1 treatment increased myotube formation (Fig. 1A). However, the mortality rates of the cells in all IGF-1 treatment groups were increased compared to the non-treated group. (2) hMDSCs treated with 50 ng/ml IGF-1 had significantly higher CK activity compared to all other groups when normalized to total protein. Furthermore, cells treated with 200 ng/ml IGF-1 also had significantly more CK activity than the untreated cells. This suggests that IGF-1 may enhance CK activity at low levels. At higher IGF-1 concentrations, CK activity may be reduced due to increased cell death (Fig. 1B). (3) Semi-quantitative analysis showed that IGF-1 treatment, at all concentrations, increased myogenin expression. The 100ng/mL concentration appeared to have the greatest effect. These results were corroborated by qRT-PCR analysis, which indicated that, compared to the non treated group, the 50ng/mL, 100ng/mL, and 200ng/mL groups had a 16.1 fold, 30.1 fold, and 14.3 fold increase in myogenin expression, respectively. **Conclusion:** These results indicate that IGF-1 enhances the myogenic potential of hMDSCs. However, we observed more cell death in IGF-1 treatment groups, which may be a result of overgrown cells during differentiation. In the future, we will determine whether IGF-1 treated hMDSCs display a higher regenerative potential in skeletal muscle,

after injury and disease, than non-treated hMDSCs. **References** 1. Sekiya et al., 2013. 2. Gao et al., 2014. 3. Distefano et al., 2013. 4. Chen et al., 2013. 5. Sacco et al., 2005. 6. Ogawa et al., 2015.

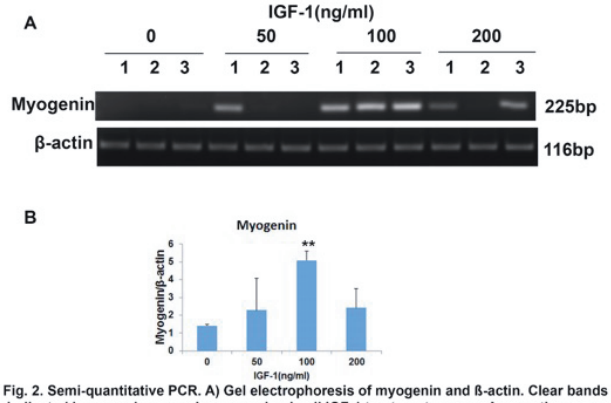


Fig. 2. Semi-quantitative PCR. A) Gel electrophoresis of myogenin and ß-actin. Clear bands indicated increased myogenin expression in all IGF-1 treatment groups. Among three replicates per group, the IGF-1 100ng/mL treatment group consistently had the strongest bands. B) Quantification of band density using ß-actin as an internal control. The IGF-1 100ng/mL treatment group showed a significant increase in myogenin expression, **p<0.01.

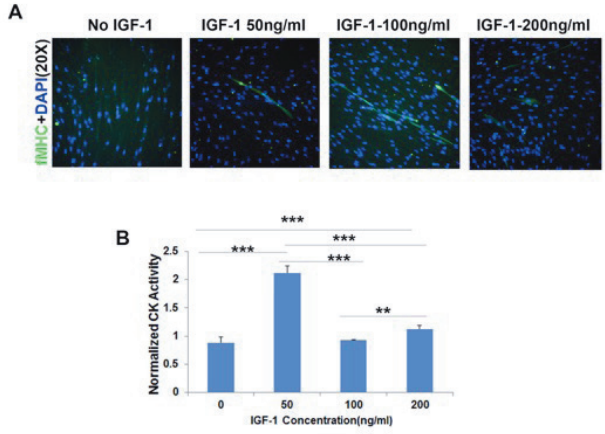


Fig. 1. Myogenic differentiation of hMDSCs. A) Fast myosin heavy chain (fMHC) staining, green, showed that more myotubes are present in all doses of IGF-1 treatment groups compared to the control group, which showed almost no myotubes. B) Creatine kinase activity in different groups. IGF-1 treatment at 50ng/mL demonstrated the highest CK activity, **p<0.01, ***p<0.001.

450. Abstract Withdrawn

451. Robust Manufacture of CAR-T Cells

Pradip Bajgain[1], John Wilson[2], Dan Welch[2], Helen E. Heslop[1], Cliona M. Rooney[1], Malcolm K. Brenner[1], Ann M. Leen[1], Juan F. Vera[1]

[1]Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children's Hospital, Houston Methodist Hospital, Houston, TX, [2]Wilson Wolf Manufacturing, New Brighton, MN

Although adoptive transfer of chimeric antigen receptor (CAR)-modified T cells has produced promising clinical responses, the broader application of this therapy has been hindered by prolonged and complicated cell production methods. In the current work, we have overcome this limitation through the incorporation of a gas permeable culture device (G-Rex) to support T cell expansion. This culture system consists of a suite of devices, all of which contain a gas permeable silicone membrane, which allows gas exchange to occur at the base. This configuration allows for the culture of T cells with an unconventionally large volume of media per unit of surface area (10ml of media/cm^2), thereby supporting uninterrupted cell growth without media exchange. Importantly, this system is simple to use and can be placed in a regular incubator as the G-Rex does not require active agitation or perfusion. To evaluate the utility of this system for the expansion of CAR T cells, we transduced healthy donor-derived primary T cells with a CAR targeting the prostate cancer antigen - PSCA (previously generated and characterized by our group). Three days after retroviral transduction, transgenic CAR T cells (transduction efficiency of 83.6±6%) from 3 donors were transferred to G-Rex100M devices (surface area of 100cm^2) in 1000ml of complete T cell media (10ml/cm^2) at low (total of 25E+06 CAR T cells), intermediate (50E+06 CAR T cells) and high (100E+06 CAR T cells) cell densities (250E+03 cells/cm^2, 500E+03 cells/cm^2 and 1000E+03 cells/cm^2, respectively). Subsequently, the cell cultures were monitored by glucose and lactic acid assessment. After 10 days of culture, the average fold-expansion was similar for all conditions (24.3±10.4, 35.5±7.8, 29.8±2.1 for low, intermediate, and high cell densities, respectively). Interestingly though, the donor-to-donor variability was decreased significantly at the higher cell density (SD of 10.4, 7.8, and 2.1 for cell densities of 250E+03 cells/cm^2, 500E+03 cells/cm^2 and 1000E+03 cells/cm^2, respectively), highlighting the importance of identifying the optimal seeding density to support robust manufacture. Notably, no media replenishment was required and the only culture manipulation performed was the addition of IL2 (50U/ml) 3 times/week. Importantly, T cells manufactured using this optimized method expressed higher levels of central memory and activation markers (CD62L and CD25) and demonstrated superior anti-tumor activity when compared to cells maintained in conventional tissue-culture plates. To further simplify the manufacturing process, we have now developed a semi-automated, closed system (GatheRex) for cell collection, which can be paired with G-Rex and allows collection of cells in a small volume (100ml) in under five minutes.

452. RegenVOX - Translational Exploitation Strategy for Stem Cell-Based Tissue-Engineered Laryngeal Implants Undergoing Phase I/II Clinical Trial

Emily J. Culme-Seymour[1], Carla Carvalho[2], Owen Bain[2], Eugene Omakobia[3], Sophie Wilson[3], Helen Knowles[4], Susan Tebbs[4], Kim Champion[4], Jeff Round[4], Gareth Ambler[5], Martin A. Birchall[3], Mark W. Lowdell[2], Chris Mason[6]

[1]LRMN, London, United Kingdom, [2]Centre for Cell, Gene & Tissue Therapeutics, Royal Free Hospital, London, United Kingdom, [3]Ear Institute, University College London, London, United Kingdom, [4]Clinical Trials Unit, University College London, London, United Kingdom, [5]Department of Statistical Science, University College London, London, United Kingdom, [6]Advanced Centre for Biochemical Engineering, University College London, London, United Kingdom

The larynx ('voicebox') regulates breathing, voice and airway protection during swallowing - all critical human activities. For patients who lose laryngeal function due to trauma or cancer, there are no satisfactory long-term solutions, hence quality of life would be dramatically improved if a living, tissue-engineered laryngeal replacement could be transplanted. Based on prior experience from 'first-in-man' successes, a Phase I/II clinical trial (NCT01977911) of these autologous cell-based, tissue-engineered laryngeal implants is now underway for ten UK patients with severe irreversible structural disorders of the larynx, unresponsive to conventional treatment. The trial has regulatory approval in the UK from the MHRA and the first patient has been enrolled for treatment. Safety and efficacy of the RegenVOX technology are the primary output for the Phase I/II trial. However, a considered commercialisation strategy is vital if the technology is to successfully translate to clinical application.

Alongside the clinical and manufacturing developments essential for treating the first patient enrolled for the trial, the RegenVOX team have thus given careful consideration to a number of aspects of business modelling, in order to product accurate data to use in planning how to deliver the product within a clinical setting. Economic modelling performed to date and planned for the future is described here, along with other aspects of translational and business model development. Further work on exploring the potential market for a RegenVOX technology, as well as understanding patient costs and implications for product uptake and delivery will be discussed, as well as additional considerations for onward commercialisation and translation into routine healthcare.

453. Tumorigenic Potential in Clones Derived from Rat Bone Marrow Cells

V. Arenas-Luna[1], L. Loredo-Mendoza[1], J. Navarro-Betancourt[1], C. Bazán-A[1], P. García-V[1], L. Lara-M[1], I. Gutiérrez-V[1], E. Rodríguez-Rivera[1], M. López-Martínez[1], A. Páez[2], F. Masso[2], S. Hernández[1]
[1]Universidad Panamericana School of Medicine, Mexico City, Mexico, [2]National Institute of Cardiology, Mexico City, Mexico

Therapy using different stem cell populations has recently emerged as a complementary treatment for several diseases, and a wide variety of stem cell lineages have been explored. Mesenchymal stem cells (MSCs) represent an attractive option for use in cellular therapy because of their accessible nature and abundance in various tissues, such as bone marrow (BM) and adipose tissue. While MSCs are routinely isolated from BM, it is now evident that MSCs can also be isolated from many other tissues. These cells can be divided, based on their source of origin, into two broad categories: marrow-derived and non-marrow-derived. Although MSCs isolated from different sources share similar surface antigens and exhibit similar classical differentiation potential (bone, fat, and cartilage), these cells still exhibit heterogeneity in their phenotype and biological properties, which apparently depends on their tissue of origin and microenvironmental niche. Self-renewal is a property shared among cancer cells and stem cells. Stem cells can certainly be genetically susceptible and acquire mutations through the cell-culture process; even so, the potential tumorigenic risk of using MSCs from a heterogeneous cellular mixture has not yet been properly evaluated. Some cells derived from BM can present some markers associated with cancer (e.g., CD117 and CD34, which are always found in a gastrointestinal stromal tumor (GIST) diagnosis, the most common mesenchymal tumor of the gastrointestinal tract. Nonetheless, the presence of both markers on BM with tumorigenic potential has not been sufficiently explored yet. Interestingly, some subpopulations isolated from BM supernatant express CD34 and CD117 (c-Kit). CD117 is a tyrosine-kinase receptor activated by stem cell factor. Upon stimulation, CD117 activates multiple signaling pathways, including ERK and mTOR, which ultimately lead the activation of NFk-B; this nuclear factor is coincidently upregulated in various tumors. The goal of this work was to identify a direct correlation between immunophenotype and possible tumorigenic potential in a specific clone of adherent c-kit+/ CD34+ cells isolated by cell sorting from the supernatant of unfractionated rat bone marrow (SNBMC). We evaluated SNBMC tumorigenic potential *in vivo* and *in vitro*. We subcutaneously injected SNBMC CD117/CD34-positive cells and heterogeneous SNBMC (unsorted) into the bilateral flank of 55 male nude mice. We measured tumor size weekly for a month, and we found that tumors were present in 20% of the mice treated with unsorted cells and in 100% of the mice that received SNBMC CD117/CD34-positive cells. All of the tumors were solid neoplasias with small, undifferentiated cells and frequent mitotic figures; intratumoral necrotic areas were abundant, suggesting a rapid proliferation rate. Immunohistochemistry staining revealed intense immunoreactivity for cytokeratin, and we suggested a diagnosis of anaplastic carcinoma. Flow cytometry analysis from tumor-isolated cells revealed a loss of MSC surface markers such as CD90 and CD73; however CD117 and CD34 were preserved. Finally the participation of NFk-B in cell proliferation was evaluated by transfection with an IkB-α adenovirus and a significantly reduced proliferation rate was found. Nonetheless, given the increasing interest in using MSCs for regenerative medicine applications, the safety of administering MSCs to humans remains to be carefully evaluated in light of the potential role of MSCs in driving tumorigenicity, particularly cells expressing CD117/CD34. Understanding the tumorigenic process in a future will be important to determining the role of these markers on the molecular level.

454. The Surface Marker Expression Changes in Monolayer Cultured Human Chondrocytes by TGF-β1

Hyun Joo Yoon
TissueGene Inc., Rockville, MD

Due to avascular environment, damaged or defective cartilage cannot be recovered voluntarily without intervention of a medical treatment. Among the treatments, autologous or allogenic chondrocyte transplantation is considered as one of promising methods. However, because the cell number that can be driven from a patient or a donor is limited, isolated chondrocytes should be in vitro cultured for amplification before being applied to a patient. This strategy was hindered again with dedifferentiation of monolayer cultured chondrocytes. Dedifferentiated chondrocytes change the phenotype and ECM profiles toward fibroblast-like ones, eventually resulting in insufficient recovery of cartilage. Previously, we showed that chondrocytes supplemented with TGF-β1 could be redifferentiated similar to articular chondrocytes. In this study, we investigated surface markers expressed on dedifferentiated chondrocytes utilizing flow cytometry analyses. The investigated markers include integrins and adhesion molecules like CD44, CD49a, CD49c, CD54, CD106, CD166, tetraspnins like CD9 and CD151, receptors like CD14 and CD105, and ectoenzyme, CD10. When compared to their expression pattern on chondrocytes cultured in TGF-β1 supplemented media, several markers showed differences in their expression levels, for example, such as up -regulation of CD49a and down-regulation of CD105 with TGF-β1 supplemented culture. The result seems supporting our previous study that showed chondrocyte redifferentiation by TGF-β1 and we speculate that the surface marker analysis would be applicable as a testing method to determine the potency and the comparability of chondrocytes originated from different donors and/ or from different manufacturing batches.

Vector and Cell Engineering/Manufacturing I

455. Automated Lentiviral Transduction of T Cells with CARS Using the CliniMACS Prodigy

Lauren Nickolay[1], Ulrike Mock[1], Brian Phillip[2], Gordon Weng Kit Cheung[2], Hong Zhan[1], Karl Peggs[2], Ian Johnston[3], Andrew Kaiser[3], Martin Pule[2], Adrian Thrasher[1], Waseem Qasim[1]
[1]Molecular and Cellular Immunology Unit, Institute of Child Health, London, United Kingdom, [2]UCL Cancer Institute, London, United Kingdom, [3]Research and Development, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

T lymphocytes are exhibiting enormous potential in early phase clinical trials in patients with haematological malignancies. However, the complex procedures involved in the ex-vivo modification of T cells is labour intensive and currently limited to a small number of centres with the required infrastructure and expertise. To simplify procedures and widen applicability for clinical therapies, we have adopted the CliniMACS Prodigy platform to automate these multifaceted cell

manufacturing processes. We found efficient lentiviral transduction of human T cells in a GMP compliant manner and demonstrate the feasibility of implementing this device in the manufacture of chimeric antigen receptor (CAR) based T cell immunotherapies. Eight automated T cell Transduction (TCT) processes have been performed using a self-inactivating third generation lentiviral vector encoding a CD19 specific CAR (CAR19), three of which using a clinical grade vector for final stage validation studies. Either fresh or cryopreserved peripheral blood mononuclear cells from non-mobilised leukapheresis from healthy donors were loaded onto the CliniMACS Prodigy using single use closed tubing sets. All cells were cultured in TexMACS media and activated with TransAct™. Transduction occurred 24-28hours post activation and cells were expanded for up to 8 days in the CentriCult-Unit enabling stable cell culture conditions and automated cell feeding. Finally, cells were harvested and cryopreserved to assess the functional capabilities of CAR19 T cells. Small scale comparison transductions were run in parallel to assess the efficiency of the automated T-cell modification process. The mean T cell expansion during automated cell cultivation was 16.2x (range 5.4-28.4x) with an average yield over 8 days of $14.5x10^8$ total lymphocytes from a starting lymphocyte count of $1x10^8$. This was comparable to cell expansion achieved in manual small scale experiments under the same activation conditions, 18.1x (range 11.5-27.5x). Successful transduction was also observed in the automated system with a mean transduction efficiency of 49.1% (range 23.9-64.9% CAR19+ T cells) which was again similar to transduction efficiencies achieved in manual small scale controls (mean of 51.8%). Flow cytometry analysis of the final product showed a high purity of CD45+CD3+ T cells (mean = 94%) with a relatively high frequency of CD8+ T cells (mean 48.9%). Further immunophenotyping revealed the bulk of the T cell product to be a mix of stem cell memory and central memory based on CD45RA, CD62L and CD95 expression with minimal expression of the T cell exhaustion marker PD-1. Additionally, CAR19 T cells generated using the automated procedure, were functional in cytotoxic activity both *in vitro* and in an *in vivo* mouse model. Importantly, these data are comparable to data generated from previous GMP manufacture of CAR19 T cells using the WAVE bioreactor with X-Vivo15 media and magnetic beads conjugated with anti-CD3/CD28 antibodies with the added advantage of increased simplicity in manufacture. In summary, we have demonstrated the feasibility of the CliniMACS Prodigy platform for the generation of CAR+ T cells for adoptive immunotherapy. Automated activation, transduction and expansion resulted in clinically relevant doses of CAR19 T cells with greatly reduced 'hands-on' operator time. Given the closed-system nature of the device, and automated features, the CliniMACS Prodigy should widen applicability of T-cell engineering beyond centres with highly specialised infrastructures.

456. Transgene Bioengineering Through Ancestral Protein Reconstruction

Philip M. Zakas[1], Harrison C. Brown[1], Kristopher A. Knight[2], Ernest T. Parker[2], H. Trent Spencer[2], Eric A. Gaucher[3], Christopher B. Doering[2]

[1]Graduate Program in Molecular and Systems Pharmacology, Laney Graduate School, Emory University, Atlanta, GA, [2]Aflac Cancer and Blood Disorders Center, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA, [3]School of Biology, Georgia Institute of Technology, Atlanta, GA

Bioengineering of the transgene often is a critical component of preclinical gene therapy R&D. Transgenes and their products represent the active agent in nucleic acid pharmaceuticals and similar to small molecule pharmaceuticals, they can be modified to possess improved pharmacological properties. However as they are significantly more complex than small molecules, the available strategies for bioengineering, such as in silico rational design, directed evolution and homolog/ortholog-scanning mutagenesis, are less robust. Herein, we propose combined ancestral sequence and protein reconstruction (ASR and APR, respectively) as newly accessible approaches to transgene/transgene product bioengineering. ASR is the prediction of ancient sequences from extant ones and well developed ASR methods and tools now exist. Furthermore, the availability of de novo custom DNA synthesis and recombinant protein expression systems now facilitates APR to complement and extend ASR findings. Previously through the study of extant FVIII orthologs, we discovered that differential molecular, biochemical and immunological properties with exist and could have a positive pharmacological impact upon engineering into human FVIII. For example, porcine FVIII was shown to display 10-100-fold more efficient biosynthesis than human FVIII in vitro and in vivo, while murine FVIII displays 5 - 10-fold greater stability following thrombin activation. Ovine FVIII displays intermediate biosynthesis and stability, but strikingly reduced cross-reactivity to anti-human FVIII inhibitory antibodies. APR provides a high-resolution mapping solution to these ortholog sequence-activity relationships and also takes advantage of the observation that ancient proteins often have unpredicted and/or expanded functionalities that can be efficiently mapped to specific amino acid residues through comparisons of ancestral proteins and genes within an evolutionary lineage. Therefore, we sought to validate APR as a FVIII discovery/bioengineering platform with the expectation that this approach can be successfully applied to essentially all hemostatic, as well as non-hemostatic, gene therapies. Initially, we employed ASR/APR to resurrect 14 ancestral (An) FVIII molecules. Each An-FVIII was shown to be active in standard coagulation assays using human plasma demonstrating evolutionary compatibility. To study biosynthetic efficiency, secreted An-FVIII activity and mRNA transcript levels were analyzed from stably transfected cells demonstrating that, An-53, an ancestral primate sequence with 95% identity to extant human FVIII, displayed the greatest biosynthetic efficiency equivalent to porcine FVIII and our lead bioengineered high expression FVIII, ET3. As a proxy for AAV gene therapy, hemophilia A mice were administered several doses of a liver-directed An-53 AAV plasmid DNA cassette via hydrodynamic injection resulting in peak plasma FVIII activity levels ≥12-fold higher than observed with the ET3 transgene. In addition to superior biosynthetic efficiency, we have identified An-FVIII variants with 2 - 3 fold improved specific activity and stability greater or equal to murine FVIII. Furthermore, we have identified An-FVIII molecules that display reduced immune reactivity and have used these constructs to define functional epitopes to the single amino acid level. Currently, we are refining this approach to identify the key functional residues responsible for each property with the goal of improving the pharmacology of the human FVIII transgenes.

457. A Characterization of Transgene-Specific "Proteomes" in Lentiviral Vector Clinical Production Lots Identifies Differences between Vectors with High and Low Infectious Titers

Scott Witting[1], Lilith Reeves[1], Frank Witzmann[2]

[1]Cincinnati Children's Hospital Medical Center, Cincinnati, OH, [2]Indiana University, Indiana, IN

Gene delivery using viral vectors is a powerful tool which can be used to correct genetic diseases and treat acquired illnesses. HIV-based lentiviral vectors (LVs) are advantageous in gene therapy applications for due to their genomic integration and ability to infect non-dividing cells. We have found that scale-up for is successful in retaining physical particle titers; however, the transduction efficiency of certain vectors, particularly in sensitive primary cells (e.g. human CD34+ cells), is significantly less than expected. Our

working hypothesis is that current large-scale production methods may concentrate proteins that modulate transduction. To address this possibility, we characterized the "lentiviral vector proteome" of several different clinical vector lots using an Orbitrap Velos Pro Hybrid Mass Spectrophotometer. Since X-VIVO 10 medium was the final product medium in all LV lots, proteins identified in a sample of unused medium were eliminated from the analysis. We first identified a subset of 24 proteins common to ALL vector lots. Not surprisingly, further analysis showed that 6 of these proteins are both "top 25" exosome markers in the ExoCarta database and known gag-pol interacting proteins listed in the HIV-1 Human Interaction Database. Independent of production size and tangential flow filtration brand, we next identified 57 proteins unique to a low titer LV expressing human beta-globin (BG). We similarly identified 47 proteins unique to a high titer LV expressing adenosine deaminase (ADA). BG lots were enriched in proteasome subunit proteins and ADA lots in ribosomal subunit proteins. Interestingly, the ADA lots were also enriched in several 14-3-3 family proteins that are known to modulate intracellular signaling pathways. It is possible that the mere presence or relative levels of specific proteins, such as 14-3-3 proteins, can affect transduction. We can speculate that the mechanism involves indirect effects of the vector transgene on the producer cells which yields a particle that is more stable and/or has an increased capacity to transduce cells.

458. Development of a Stable Producer Cell Line for Scalable Lentiviral Vector Production for Gene Therapy of Hemoglobinopathie

Sarah Slauson[1], Amelia Thomas[1], Byoung Ryu[2], Melissa Bonner[1], Gretchen Lewis[1], Gabor Veres[1], Janet Chung[1], Geoffrey B. Parsons[1]
[1]Bluebird Bio Inc., Cambridge, MA, [2]St. Jude Children's Research Hospital, Memphis, TN

Current manufacturing of clinical grade lentiviral vectors (LVVs) for gene therapy applications commonly relies on transient transfection of adherent 293T cells. Improvements in production efficiency and scalability would provide value in meeting the needs for increased amounts of vector required for clinical development. An inducible producer cell line grown in suspension culture represents a potentially more scalable manufacturing process for LVV production which eliminates the need for costly plasmid and transfection reagents. We have engineered a packaging cell line by introducing doxycycline-inducible Gag-Pol, Rev, and VSVG envelope genes into a suspension cell line. Packaging cell clones were isolated by single cell sorting and screened by qPCR for the presence of the delivered genes. Virus production was assessed by transient transfection of a lentiviral vector and doxycycline treatment. A titer of up to 5.0E6 transduction units per milliliter (TU/mL) was observed with packaging cell line stability demonstrated after greater than four months of continuous passage. Next, a self-inactivating lentiviral vector was excised from its plasmid backbone and ligated in vitro to a linear neomycin resistance cassette and used to transfect the lentiviral packaging cell line to generate a panel of producers. Following two weeks of G418 drug selection, adherent colonies were plucked and screened for viral production followed by single cell sorting to isolate individual producer clones. From five different plucked colonies with titers greater than 3.0E6 TU/mL, more than 100 clones were screened and 7 were identified that produced a harvest titer greater than 1.0E7 TU/mL. Four of these clones were successfully re-adapted to suspension and scaled up to 3L culture. LVV generated from individual producer clones was compared for the ability to transduce adult mobilized CD34+ hematopoietic stem cells (HSCs). Interestingly, the vector copy number (VCN) in CD34+ HSCs for the LVV derived from the producer cells varied between clones. Clones that produced the highest VCN have been chosen for further characterization in order to better understand the differences in HSC transduction. These lentiviral producer cell lines represent an important first step toward the creation of a manufacturing process that can better support clinical and commercial development of HSC lentiviral gene therapy.

459. Evaluation of Miltenyi ExpAct and TransAct CD3/28 Beads for CAR-T Cell Manufacturing

Xiuyan Wang, Jinrong Qu, Jolanta Stefanski, Oriana Borquez-Ojeda, Anniesha Hack, Qing He, Teresa Wasielewska, Fang Du, Michel Sadelain, Isabelle Rivière
Memorial Sloan Kettering Cancer Center, New York, NY

Adoptive transfer of chimeric antigen receptor (CAR) engineered T cells is a promising emerging strategy to treat cancer patients. Large-scale manufacturing of cGMP-grade CAR T cells using patient T cells selected and activated by CTS™ Dynabeads® CD3/CD28 (Dynabeads) followed by transduction with retroviral vectors is being used in the context of many clinical trials by our laboratory and others. Although we have established a robust protocol using Dynabeads, it is important to explore alternative sources to pre-empt supply chain limitations of this critical reagent. To this end, we evaluated T cell activation with either Miltenyi TransAct CD3/28 (TransAct) beads or Miltenyi ExpAct Treg (ExpAct) beads. In small-scale experiments, PBMCs were directly activated with TransAct or ExpAct beads and compared with our standard T cell selection and activation using Dynabeads. Overall, the transduction efficiency and expansion of T cells were comparable upon activation with all three reagents. The TransAct bead-stimulated cells exhibited comparable effector memory (EM)/central memory (CM) phenotype to that of the Dynabeads stimulated cells. In line with the EM/CM phenotype, CAR T cells stimulated with either TransAct or Dynabeads and tranduced with CD19-targeted CAR demonstrated robust and comparable antitumor activity in a systemic NSG/CD19+ NALM6 tumor mouse model. We further tested the efficacy of TransAct beads using positively or negatively selected T cells in a large-scale cGMP grade CAR-T cell manufacturing setting. Both the transduction efficiency and expansion of selected CD3+ cells activated with TransAct beads and Dynabeads were comparable. CD19-targeted CAR T cells activated by either TransAct or Dynabead were subjected to an in vivo stress test by using decreasing amount of CAR-T cells to treat systemic CD19+NALM6 tumors in NSG mice. In this experimental setting, T cells stimulated with TransAct beads demonstrated equivalent if not better anti-tumor activity than T cells stimulated with Dynabeads. In conclusion,our pre-clinical results suggest that TransAct beads support efficient transduction and expansion of CAR T cells. TransAct activated T cells exhibit antitumor activity equivalent to Dynabeads activated T cells in our NSG/CD19+NAML6 stress test. Therefore, Miltenyi TransAct beads can be used as an alternative to Dynabeads to stimulate T cells in clinical trials aiming at evaluating CAR T cell safety and antitumor activity.

460. Purification of AAV8 Using a Prototype Affinity Resin Compatible with cGMP Manufacturing

Colleen M. Dupuis, Amy A. Medeiros, Samuel C. Wadsworth, K. Reed Clark, Christopher J. Morrison
Downstream Process Development, Dimension Therapeutics, Cambridge, MA

Affinity interaction based resins have long been the foundation for various purification platforms of biological molecules. For the purification of rAAV, the affinity resin AVB Sepharose HP (AVB) has been used to successfully purify a number of different serotypes.

However, to date the use of AVB for the purification of the AAV8 serotype has been relatively unsuccessful due in part to modest binding capacity. In this work we investigate the use of a new, prototype affinity resin for the purification of AAV8 from crude feed streams. Performance of the AVB and prototype resins were directly compared for the purification of selected AAV8 feed streams. Differences in capacities, loading steps, wash step tolerances and elution steps will be discussed. In addition, the resulting performance of purifying AAV8 using the prototype resin is directly compared against the best case performance of the AVB resin purifying a different serotype more commonly used on AVB. Finally, the scale-up use of the prototype resin from bench to manufacturing will be discussed. These results demonstrate that the prototype affinity resin is a viable option for the scaleable, chromatographic purification of AAV8 from crude harvest streams.

461. The Testing Strategy to Determine the Pharmacological Activity of Gene Therapy Drug Product (rAd-IFN) to Treat Intravesical Non-Muscle Invasive Bladder Cancer

Minna M. Hassinen[1], Jenni Mykkänen[1], Hanna Lesch[1], Sara Paulo[2], Taina Koponen[2], Anna-Kaisa Lehtivarjo[2], Steve Parrish[2], Robert Shaw[2], Nigel R. Parker[3], Seppo Ylä-Herttuala[4]
[1]FKD Therapies OY/Finvector Vision Therapies OY, Kuopio, Finland, [2]Finvector Vision Therapies OY, Kuopio, Finland, [3]FKD Therapies OY, Kuopio, Finland, [4]A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, Finland

rAd-IFN is a recombinant adenoviral gene therapy vector encoding IFNα2b gene for the treatment of refractory non-muscle invasive bladder cancer. The vector transduces bladder wall cells where IFNα2b gene is expressed leading to death of cancer cells. The advanced testing strategy to determine the pharmacological activity of rAd-IFN drug product involves three key assays: 1. Infectious titer of the virus, quantitative assay 2. Expression of the transgene (IFNα2b), semiquantitative assay 3. Potency (IFNα2b mediated cell killing), quantitative assay The infectivity and transgene expression assays have been performed for batch release and stability monitoring of activity during Phase 2 and will remain unchanged in principle for Phase 3 and commercial use. In the infectivity assay the cells supporting adenovirus replication are infected with three concentrations of adenovirus and left to produce the virus for two days. The percentage of infected cells is then determined with a flow cytometer utilizing a fluorescently conjugated antibody against an adenoviral structural protein. Samples are analysed in parallel with a reference standard and infectivity is given as relative Infectious Units / ml. In expression assay, the IFNα expression capability of the virus preparation is determined by infecting IFN insensitive cells with the rAd-IFN virus and the concentration of produced IFNα is measured with a commercial IFNα ELISA (enzyme-linked immunosorbent assay) from cell culture supernatants For Phase 3 a new potency assay is developed and added to release and stability testing in order to provide evidence that batches of rAd-IFN are able to produce active IFNα2b which has a relevant pharmacological effect. In this assay cells are transduced using multiple dilutions of reference standard and test samples leading to expression of IFNα2b and subsequent cell death. Cell killing efficiency is determined using colorimetric method measuring dehydrogenase activity of the living cells. Relative potency of test sample is determined against reference standard response curve after testing parallelism by equivalence test. All activity assays will be fully validated according to ICH Q2 (R1) prior to release testing of Phase 3 clinical study material (Accuracy, Precision, Specificity, Linearity and Range, System Suitability and Robustness). The three validated assays will provide enhanced quantitative measure of biologic function of the rAd-IFN vector and thus demonstrate the quality and efficacy of drug product batches.

462. Serum Free Clinical Grade Large Scale Lentiviral Production System for Gene Therapy Application

Xin Yu, Mu Li, Xavier de Mollerat du Jeu
ThermoFisher Scientific, Carlsbad, CA

Recently, lentiviral vectors have become the center of attention for its use as gene transfer vectors in gene therapy. Current new generation of therapies CAR - T requires the lentiviral vectors as efficient gene transfer tool to express engineered Chimeric Antigen Receptors (CAR) on the surface of the T-cells to recognize and kill the cancer cells. One problem of developing CAR T cell therapies is the high cost associated with lentiviral viral production. Therefore preclinical and clinical researchers have demanded their lentiviral production on a much larger scale, high-titer and in animal serum free medium. Current lentiviral production system use mainly adherent cells which need fetal bovine serum to support cell growth in flasks or cell factories, the system is suitable for research purpose at small scale, but not at large scale which requires large incubators and large cell culture vessels. Such system is hard to operator and requires more effort to produce large amount of LVVs, and increase the cost. We have developed a new lentiviral system to produce vectors in a serum free suspension platform and at very high titers. This technology employs a newly developed propriety set of GMP reagents comprising of a new media, new cells, new Transfection reagent and enhancers. With this new system we are able to deliver >5x10^8 (TU/ml) of unconcentrated lentiviral vectors, which is at least 10 folds higher than any other published method of lentiviral production. In this report, we will describe the methods and DOE experiments that we used to identify this new suite of reagent and their application in the immune cell therapy field.

463. Suspension-Adapted HEK 293 Cells in Orbital Shaken Bioreactors for the Production of Adeno-Associated Virus Vectors

Daniel R. Blessing[1], Bernard Schneider[2], Florian M. Wurm[3], Nicole Déglon[1]
[1]Department of Clinical Neurosciences, University Hospital Lausanne (CHUV), Lausanne, Switzerland, [2]Faculty of Life Sciences, Brain Mind Institute, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland, [3]ExcellGene SA, Monthey, Switzerland

In recent years adeno-associated virus (AAV) has become one of the most popular vectors for gene therapy applications. AAV mediated gene therapy approaches offer the advantages of safety, broad tissue tropism and long-term transgene expression. The screening and engineering of novel recombinant AAV (rAAV) capsids has led to the generation of several promising candidates with highly interesting *in-vivo* characteristics. The evaluation of these vectors in pre-clinical studies and potential applications in the clinic require the production of considerable amounts of vectors. Even though much progress has been made in the last decade, AAV production remains a challenge for translating basic research into clinic. Here we describe the production of rAAV vectors comparing different protocols for the polyethyleneimine (PEI) based transfection of suspension adapted HEK 293 cells. Suspension cells were cultivated with serum-free medium in disposable, orbital shaken bioreactors (OSR), a technology which allows an easy scale-up of production. Using cultures from a milliliter to a liter scale, AAV6 and AAV9 vectors were produced and purified using gradient centrifugation, filtration and chromatographic

methods. Purified virus stocks were then analyzed and titrated to determine viral genomes and transducing units. Preliminary results indicate that our protocol for the production of rAAV particles using suspension cells grown in OSR yields infectious titers that are equal or higher to what has been reported for the production with human cell lines. Further work is ongoing to analyze and implement this OSR platform. The presented process offers a novel method to produce rAAV for pre-clinical and clinical trials, scalable, based on single-use material and compliant to Good Manufacturing Practice (GMP).

464. RCL-Pooling Assay: A Simplified Method for the Detection of Replication Competent Lentiviruses in Vector Batches Using Sequential Pooling.

Corre Guillaume[1,2,3], Michel Dessainte[4], Jean-Brice Marteau[4], Bruno Dalle[1], David Fenard[1,2,3], Anne Galy[1,2,3]
[1]Genethon, Evry, France, [2]INSERM UMR_S951, Evry, France, [3]University of Evry, Evry, France, [4]Genosafe, Evry, France

Non-replicative recombinant HIV-1 derived lentiviral vectors (LV) are increasingly used in gene therapy clinical trials for various genetic diseases, infectious diseases or cancer. Before they are used in man, preparations of LV must undergo extensive biochemical and biological quality control testing. In particular, the legislation stipulates that the absence of replication-competent lentiviruses (RCL) must be demonstrated with suitable methods, on representative fractions of batches. Current standard and widely used methods based on cell culture are challenging because high titers of vector batches achieved translate into high volumes of cell culture that have to be tested. Since vector batch titers and sizes are continuously-increasing due to the improvement of production and purification methods, it is necessary to modify the current cell culture method. Here, we propose a practical optimization of the p24-decrease-based culture assay developed by Escarpe et al. (2003) using a pairwise pooling strategy enabling the test of higher vector inoculum volumes. These modifications significantly decrease material handling, operator time, leading to a cost effective method, while maintaining optimal sensibility of the RCL testing. This optimized RCL-pooling assay ameliorates the feasibility of the quality control of large-scale batches of clinical-grade LV while maintaining the same sensitivity.

465. An Antibiotic-Free Strategy for Miniplasmid Production

Ram Shankar[1], Maurice Telaar[2], Karl Friehs[2], Martin Schleef[1], Erwin Flaschel[2]
[1]PlasmidFactory GmbH & Co. KG, Bielefeld, Germany, [2]Bielefeld University, Bielefeld, Germany

The segregational stability of plasmids in a recombinant bioprocess is of extreme significance. Although this is commonly achieved by the selection pressure from antibiotics, their application for the production of therapeutic DNA for gene therapy or for DNA vaccines would be undesirable. Similarly, the presence of antibiotic-resistance genes in the final product would have to be avoided. In addition to the minicircle approach, a type of miniplasmid is able to fulfil the regulatory requirements. The gene *tpiA* is responsible for the connection of the glycerol metabolic pathway with the essential glycolytic pathway in *Escherichia coli*. The knockout of genomic *tpiA* rendered cells completely auxotrophic in minimal medium with glycerol as sole carbon source while allowing growth at a reduced rate with glucose or in complex medium. This was advantageous for optimizing antibiotic-free cloning and selection of recombinant plasmid. Complementation of the auxotrophy by plasmid-borne *tpiA* led to high segregational and structural plasmid stability, resulting in stable production of a model recombinant enzyme under antibiotic-free conditions in a continuous cultivation. Thus, the complementation of *tpiA* represents a significant alternative to antibiotics as a selection principle and is therefore of interest for the recombinant production of biotherapeutics in the form of miniplasmids.

466. Manufacturing Solutions for Robust Cell Therapy Expansion and Harvest

Julie Murrell, Samantha Luther, Tristan Lawson, Anjali Verma, Susan Rigby, Sandhya Punreddy, Manjula Aysola, Tiffany Hood, Daniel Kehoe, Aletta Schnitzler, Martha Rook
MilliporeSigma, Bedford, MA

The long-term view of regenerative medicine therapies predicts an increased need for expansion solutions that ease scalability, utilize animal origin-free materials and are compatible with limited downstream processing steps. As more cell therapeutics progress through clinical testing, current *in vitro* culture methods are proving cumbersome to scale and lack robustness. Moreover, high quality animal origin-free reagents and downstream processing devices support the future implementation of large scale manufacturing solutions that will be required following clinical success. Here, we describe the implementation of single use bioreactors and high quality media for expansion of cell therapies. We include examples from allogeneic mesenchymal stem cells and autologous T cells. The presentation will review solutions addressing animal origin-free expansion of cells within the context of different upstream process development steps as well as scaling and downstream processing with good cell quality, high recovery, high viability and good activity. Start to finish solutions for expansion and harvest, including high quality reagents, are key enabling technologies for success in commercializing cell therapies.

Pharmacology/Toxicology Studies or Assay Development

467. Assay Conditions Significantly Alter AAV Neutralizing Antibody Determination

Heikki T. Turunen, Eric Zinn, Luk H. Vandenberghe
Grousbeck Gene Therapy Center, Ocular Genomics Institute, Schepens Eye Research Institute/Massachusetts Eye and Ear Infirmary/Harvard Medical School, Boston, MA

A major limitation of vectors based on current AAVs is the wide prevalence of memory B and T cells reactive to the AAV capsid in human populations arisen following natural AAV infection. The Neutralizing Antibody (NAB) assay guides experimentation and determines inclusion or exclusion of subjects in clinical studies in order to predict in vivo neutralization. Furthermore, evidence of a memory B-cell response to AAV may correlate with anti-capsid memory T-cell populations implicated in the safety and efficacy outcome of clinical AAV liver studies. Based on previously reported findings, NAB assay protocols in current use are unable to robustly predict in vivo neutralization in monkeys (Zinn et al. Cell Reports, 2015 & Wang et al. Mol Ther, 2010) leading to false negatives. In a clinical setting, the enrollment of a seropositive individual exposes the subject to undue risk, and generates outcome variability of the often small clinical studies. Here, we sought to permutate several of the NAB assay conditions in order to evaluate their impact on the NAB titer readout. Specifically, we varied the following variables: cell line, transgene detection, use of adenovirus, and AAV amount. Prior to evaluating these assay permutation, we sought to increase the sensitivity of the readout for transgene expression in the assay in order to evaluate at all conditions both high and low transducing AAV serotypes. This optimization increased our sensitivity by ~200-fold. With this increased sensitivity of readout, we first sought to evaluate

the compliance of standard AAV NAB assays with the percentage law, formulated by Andrewes and Elford in 1933, a standard for virological NAB assays, which seeks stoichiometric conditions of NAB and viral load that enables measurement of NAB titers independent of viral input in the assay. Our data shows that at the conditions in common use in the field the percentage law is not upheld, and NAB titers are highly dependent on input viral load of the assay, directly impacting sensitivity and specificity, and leading to the occurrence of false negatives. Indeed, quantitative assessment of seroprevalence in primate populations with assays that reduce the amount of AAV to within percentage law range affects assay outcome qualitatively and quantitatively. We also independently assessed other assay parameters to improve sensitivity, robustness and reproducibility, and to study whether they may also have qualitative effects on titer outcomes. Our studies highlight the importance of pre-existing immunity in AAV gene therapy and limitations of the current methodologies to measure it in a robust and predictive manner. Data indicates the potential for false positive and negative readout for commonly used assay protocols. An optimized protocol was developed for further validation and evaluation. These studies may impact translational and clinical AAV gene therapy studies.

468. GLP-Compliant Non-Clinical Safety and Biodistribution of a Recombinant AAV2/8 Vector Administered Intravenously for Treatment of Mucopolysaccharidosis Type VI

Rita Ferla[1], Jean-Brice Marteau[2], Severine Pouillot[2], Karen Kozarsky[3], James Brown[3], Stefania Galimberti[4], Maria Grazia Valsecchi[4], Vincent Zuliani[2], Alberto Auricchio[1]
[1]Telethon Institute of Genetics and Medicine, Pozzuoli (NA), Italy, [2]Genosafe, Evry Cedex, France, [3]REGENXBIO Inc., Rockville, MD, [4]Center of Biostatistics for Clinical Epidemiology, Department of Health Sciences, University of Milano-Bicocca, Monza (Mi), Italy

Mucopolysaccharidosis VI (MPS VI) is a lysosomal storage disorder caused by deficiency of the enzyme arylsulfatase B (ARSB), which results in widespread accumulation and excretion of toxic glycosaminoglycans. We recently developed a successful gene therapy approach based on a single systemic administration of AAV2/8 that targets liver of MPS VI animal models. In view of a gene therapy clinical trial for MPS VI, we performed GLP-compliant non-clinical studies to assess the safety and biodistribution of AAV2/8.TBG.*hARSB*, a recombinant AAV2/8 vector encoding human ARSB (hARSB) under the control of the thyroxine-binding globulin promoter (TBG). We used transgenic C57/BL6-Tg*ARSBC91S* mice that overexpress an inactive *hARSB C91S* mutant and are thus immune tolerant to hARSB. Mice were treated with either AAV2/8.TBG.hARSB or the vehicle alone, as control. Toxicity was evaluated on day 15 (D15) and 180 (D180) after systemic injection of $2x10^{13}$ gc/kg, which is 10X the highest dose proposed for the clinical study [20males(M)+20females(F)/treatment/timepoint]. No mortality, abnormal clinical signs and alteration in body weight, body temperature and food intake were observed through the study. Similarly, no clinically relevant changes in blood chemistry and hematology were found in treated mice compared to controls. Histopathology revealed thyroid epithelial hypertrophy in AAV-treated mice. AAV2/8.TBG.*hARSB* biodistribution and expression was evaluated on D15 and D180 at the dose of $2x10^{12}$ gc/kg, which is 1X the highest dose proposed for the clinical study (5M+5F/treatment/timepoint). Although vector DNA was present in all organs on D15, it was sequestered mainly in liver at levels at least 3 logs higher than those found in other organs. Vector DNA declined on D180, but remained high in liver. Accordingly, hARSB was mainly expressed stably in liver, supporting TBG tissue specificity. Vector DNA was found in gonads of both sexes at 3 logs lower than in liver. A robust reduction of vector DNA was observed on D180. A supportive study conducted in male rabbits showed that vector shedding in semen was only transient, which suggests that the risk of inadvertent germline transmission of AAV2/8.TBG.*hARSB* is minimal at least in male animals. An *in situ* hybridization study is ongoing in ovaries to elucidate AAV localization. Finally, AAV DNA was only transiently present in plasma, urine and stools of mice (up to D37, D2 and D14, respectively), which minimizes the potential risk associated with transmission to third parties and/or the environment. In conclusion, these studies show a safe profile of intravenous administrations of AAV2/8.TBG.*hARSB* and pave the way for the phase I/II clinical trial.

469. Improved Protocol and Use of Mitochondrial DNA as Reference for qPCR Quantification of Integrase-Defective Lentiviral Vectors (IDLVs)

Bala Sai Sundarasetty, Laura Gerasch, Renata Stripecke
Department of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Medical School Hannover, Hannover, Germany

Integrase defective lentiviral vectors (IDLVs) are attractive tools for genetic manipulation and are increasingly being tested as recombinant viral vaccines against infectious pathogens and cancer. IDLVs remain mostly as extrachromosomal/episomal DNA circles in the infected cells, hence with reduced risk of genotoxicity mediated by insertional mutagenesis. We have demonstrated in our previous work the production and characterization of a tricistronic IDLV (packaged with the D64V mutation in the integrase and co-expressing GM-CSF, IFN-alpha and the CMV pp65 antigen) and IDLV-mediated monocyte transduction achieved under Good Manufacturing Process (GMP) compliant conditions (Sundarasetty et al., JTM 2015). One of the main criteria for batch release of the cell vaccine is to confirm and quantify IDLV copies in the thawed cell product. Currently used DNA extraction methods are not efficient in isolating small molecular weight episomal DNA from cells. In addition, genomic DNA loci used as reference controls are not informative regarding quantification of episomal DNAs. Mitochondrial DNA (mtDNA) is an episomal small molecular weight circular DNA (16.5 kb) that can serve as a reference for IDLV quantification. In order to maximize recovery of episomal DNAs, we explored the total DNA (tDNA) extraction described for mtDNA isolation (Badralmaa et al., J Vir. Meth. 2013), based on dehydration and precipitation of proteins and subsequent tDNA precipitation from the supernatants by isopropanol. As a reference for the qPCR quantification, we constructed and validated a plasmid containing a sequence homologous to a regulatory region of transfer vector (wPRE), a sequence for a genomic house-keeping gene (PTBP2) and a sequence for a mitochondrial house-keeping gene (Cytochrome B). The amplification of three target regions was validated by generating a standard curve with the reference plasmid ranging from 5 x10 to $5x10^5$ copies (n=3). As a reference, we used an in-house generated 293T cell line (B5) containing three LV genomic copies. Total DNA extracted form B5 was serially diluted in non-transduced 293T DNA in order to result into 0.25, 0.5, 1, 2, 3 LV copies and the assay linearity was assessed in three independent runs. The reliable detection limit of the qPCR assay was 0.5 LV copies/cell and 0.9 copies/ ng of genomic DNA. Having assessed the linearity, we assessed if this method could be used to quantify IDLV in transduced monocytes. The tricistronic IDLV was used to transduce monocytes of different donors in triplicates at increasing multiplicities of infection (MOI: 1, 2.5, 5 and 10). After thawing each cryopreserved batch, tDNA was extracted and analyzed. Our results showed a direct correlation between the IDLV copies per cell and ng of DNA used for the assay and MOI, whereas detection of the genomic and mtDNA references remained constant. Thus, this simple

methodological adaptation provided an internal mtDNA control to better quantify episomal vector copies, which can be also used for qPCR quantification of other types of non-integrating vectors.

470. New Insights into rAAV Integration Mechanisms by Targeted Enrichment Sequencing

Stefan Wilkening[1], Saira Afzal[1], Elena Senís[2], Raffaele Fronza[1], Christof von Kalle[1], Dirk Grimm[2], Manfred Schmidt[1]
[1]Translational Oncology, NCT / DKFZ, Heidelberg, Germany, [2]Center for Infectious Diseases (Virology), Heidelberg University Hospital, Heidelberg, Germany

Comprehensive analysis of deep sequencing data originating from the newly introduced Targeted Enrichment Sequencing (TES) indicates so far undescribed recombinations within the Inverted Terminal Repeats of recombinant Adeno-Associated Viruses (rAAVs). For the detection of vector integration sites into the host genome we routinely apply LAM-PCR. However, TES, in which we enrich for genomic regions that include vector sequences, has major advantages over LAM-PCR, as it neither depends on the existence of a vector-specific primer binding site (often lost during rAAV integration) nor on a restriction site in the vicinity of the vector insertion site. Furthermore, regions that are captured together with the vector allow for relative quantification of vector copies per genome. As the entire vector can be sequenced by TES, mutations within the vector and transgene can be detected. In summary, we here provide new and so far unpublished information about rAAV integration patterns and show how we optimized TES to become an important complementary tool to primer-based approaches (like LAM-PCR) for mapping of vector/viral integration sites.

471. *In Vivo* Potency Assay for AAV-Based Gene Therapy Vectors

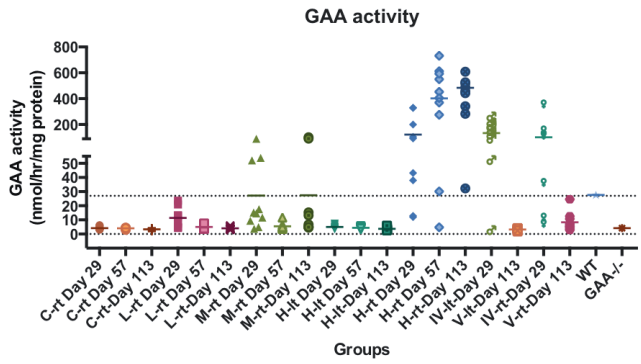
Bishnu P. De[1], Alvin Chen[1], Jonathan B. Rosenberg[1], Maria Chiuchiolo[1], Benjamin Van de Graaf[1], Odelya E. Pagovich[1], Dolan Sondhi[1], Carlo Russo[2], Stephen M. Kaminsky[1], Ronald G. Crystal[1]
[1]Weill Cornell Medical College, New York, NY, [2]Annapurna Therapeutics, Paris, France

One hurdle in the translation of adeno-associated virus (AAV) vectors for the treatment of human disease is the challenge in demonstrating drug function in a potency assay, a requirement in the clinical translation to product. *In vitro* measurements of AAV potency suffer from poor efficiency of infectivity and are insensitive measures of potency. To address this issue, we have developed a robust *in vivo* assay for the measurement of vector potency. Using as the model vector the AAVrh.10 serotype coding for human frataxin (FXN, a mitochondrial protein essential for cellular function and that is deficient in Friedreich's ataxia), and based on the knowledge that intravenous administration of all AAV vectors primarily transduce the liver, we have developed a reproducible *in vivo* potency assay that can be used to set quality control standards for vector production. The assay is based on administration of the AAV vector (2.5×10^{10} genome copies) administered intravenously to 6 to 8 wk old Balb/c male mice with the liver harvested 2 wk later following PBS perfusion. Liver homogenates are processed to assess vector genome copies, transgene mRNA, and expressed protein. In order to establish acceptance criteria for assays of vector genome and mRNA levels, separate specifications were set for DNA sample load using the mouse housekeeping gene Tfrc, and RNA sample load using mouse 18S RNA, both based on data from quantitative PCR analysis of mouse livers (n=35) assayed in duplicate. Specifications were set as the median ± 2 standard deviations based on these assay results; for the potency assay results to be accepted, each of these specifications must be met by the test sample. For the AAVrh.10 vector expressing FXN(AAVrh.10hFXN), liver vector genome levels (a measure of reproducibility of delivery) were $8.2 \times 10^4 \pm 2.2 \times 10^4$ genome copies/μg genomic DNA (n=10 mice, mean ± SD), liver human FXN mRNA levels (a measure of vector potency at the transcription level) were $2.2 \times 10^3 \pm 0.7 \times 10^3$ copies/μg total RNA (n=10 mice, mean ± SD) and liver human FXN protein levels (ELISA; a measure of vector potency at the protein level) were 54.8 ± 17.52 ng/mg protein (n=10 mice, mean ± SD). From this data, we established the following quality control specifications for AAVrh.10hFXN vectors: vector genome range 6×10^4 - 1×10^5 (copies/μg DNA), hFXN mRNA level range 1.6×10^3 - 2.9×10^3 (copies/μg RNA) and the hFXN protein levels 37 - 72 (ng/mg protein). This approach is adaptable to any AAV vector, providing an *in vivo* quality control for vector function.

472. Evaluation of Re-Administration of a Recombinant Adeno-Associated Vector Expressing Acid-Alpha-Glucosidase (rAAV9-DES-hGAA) in Pompe Disease: Preclinical to Clinical Planning

Manuela Corti[1], Brian Cleaver[1], Nathalie Clement[1], Thomas Conlon[1], Kaitlyn Faris[1], Gensheng Wang[2], Janet Benson[2], Alice Tarantal[3], Dave Fuller[1], Roland Herzog[1], Barry J. Byrne[1]
[1]University of Florida, Gainesville, FL, [2]Lovelace Respiratory Research Institute, Albuquerque, NM, [3]University of California, Davis, CA

A recombinant serotype 9 adeno-associated virus (rAAV9) vector carrying a transgene that expresses codon optimized human acid alpha-glucosidase (hGAA, or GAA) driven by a human desmin (DES) promoter (i.e. rAAV9-DES-hGAA) has been generated as a clinical candidate vector for Pompe disease. The rAAV9-DES-hGAA vector is being developed as a treatment for both early and late onset Pompe disease, in which patients lack sufficient lysosomal alpha-glucosidase leading to glycogen accumulation. In young patients, the therapy may need to be re-administered after a period of time to maintain therapeutic levels of GAA. Administration of AAV-based gene therapies is commonly associated with the production of neutralizing antibodies (NAb) that may reduce the effectiveness of the vector, especially if re-administration is required. Previous studies have demonstrated the ability of rAAV9-DES-hGAA to correct cardiac and skeletal muscle pathology in *Gaa*$^{-/-}$ mice, an animal model of Pompe disease. We describe the IND-enabling pre-clinical studies supporting the program for a phase I/II clinical trial in adult patients with Pompe. These studies were designed to evaluate the toxicology, biodistribution, and potential for re-administration of rAAV9-DES-hGAA injected intramuscularly into the tibialis anterior (TA) muscle using an immune modulation strategy developed for this study. In the proposed clinical study, six adult participants with Late-Onset Pompe Disease (LOPD) will be enrolled. The goal of the immune modulation strategy is to ablate B-cells prior to the initial exposure of the study agent in one leg and the subsequent exposure of the same vector to the contralateral leg four months after initial dosing. The dosing of active agent is accompanied by a control injection of excipient dosing in the contralateral leg to allow for blinding and randomization of dosing, which may also strengthen the approach to gene therapy studies in the future. Patients will act as their own controls. Repeated measures, at baseline and during the 3 months following each injection, will assess the safety, biochemical, and functional impact of the vector.



473. Qualification of a p24 ELISA Assay for Quantitation of Total Lentiviral Vector Concentration

Elisabeth Boucher[1], Pranali Buch[1], Melissa Buttimer[1], Josh Sorafine[2], Suzanna Melotti[1], Craig Fairchild[1], Mercedes Segura[1], James Warren[1]
[1]Vector Process Development and Manufacturing, Bluebird Bio, Inc., Boston, MA, [2]Quality Control, Bluebird Bio, Inc., Boston, MA

Lentiviral vectors (LVV) are efficient gene transfer vehicles used in a growing list of gene therapy applications that are currently being evaluated clinically. We are conducting clinical trials in subjects with β-thalassemia major, severe sickle cell disease, and childhood cerebral adrenoleukodystrophy, which employ autologous CD34+ cells transduced with LVV encoding a corrective transgene, βA-T87Q-Globin or ABCD1. Essential to LVV process development is the availability of reliable analytical tools to characterize the purified LVV product and in-process samples. Total lentiviral vector particle titers can be estimated by quantitation of HIV-1 p24 antigen concentration using commercial ELISA kits. In support of late-stage process characterization, it is essential to qualify the assay and demonstrate its suitability to test in-process samples in different matrices. Following ICH Q2 R1 guidelines, assay accuracy, repeatability, intermediate precision, quantitation limit, linearity, and linear range were evaluated. Additionally, qualification tests for specificity and dilutional linearity were performed on in-process samples and buffers to assess potential matrix interference. The results of these tests were used to define appropriate dilutions and acceptance criteria for p24 ELISA in-process samples. The assay has shown to be linear between 400 pg/mL and 25 pg/mL, while maintaining accuracy for p24 detection with the limit of quantitation being 25 pg/mL. To demonstrate assay repeatability, various concentrations within the linear range were tested resulting in coefficients of variation below 5%. The coefficient of variation across different days and different operators was below 8% for all concentrations tested, indicating consistent assay precision. Spike recovery studies have shown consistent recovery of spiked p24 antigen (73-126%), demonstrating assay specificity and lack of matrix interference in all in-process matrices tested while maintaining dilutional linearity. To conclude, the p24 ELISA assay used for LVV quantitation has shown precision, repeatability, specificity and accuracy, within an established linear quantitation range, adequate to support late-stage process characterization activities.

474. Consequences of Infusion Time on Efficiency of Intravenous Delivery of Vector Genomes to the Liver

Jonathan B. Rosenberg[1], Bishnu P. De[1], Lucy Quach[1], Carol Russo[2], Stephen M. Kaminsky[1], Dolan Sondhi[1], Ronald G. Crystal[1]
[1]Weill Cornell Medical College, New York, NY, [2]Annapurna Therapeutics, New York, NY

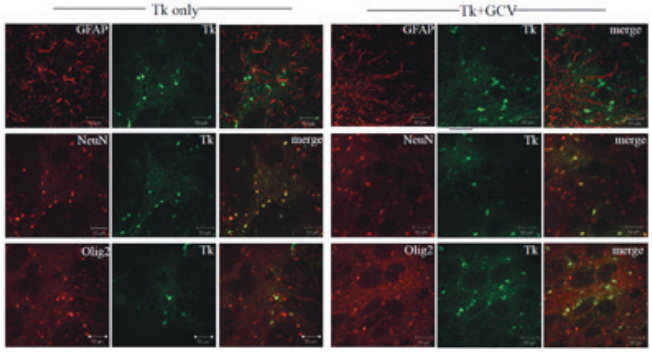
Based on the knowledge that, when administered intravenously (IV), 90% of adeno-associated virus (AAV) gene transfer vectors are transferred to the liver, the IV route has become the standard for gene therapy applications directed toward genetic modification of liver cells. To achieve maximum transfer of vector genomes to the liver, most attention has been focused on AAV serotype and dose, but little focus has been directed toward whether differences could be achieved by varying the timing of IV vector administration. To assess this variable, we administered rh.10 serotype AAV vectors intravenously to rodents using 2 variables: (1) single *vs* multiple administrations; and (2) length of time of infusion. To assess single *vs* multiple administration, mouse cohorts (C57Bl/6 males, n=5/group) were administered AAVrh.10hα1AT, encoding for human α1-antitrypsin (α1AT), IV at 10^{12} genome copies (gc)/kg, $4x10^{12}$ gc/kg and a 3rd cohort receiving the 10^{12} gc/kg dose on 4 consecutive days. Mice were bled at 4, 14, 28, and 112 days post-administration. Serum human α1AT levels showed the expected dose response. Mice administered 10^{12} gc/kg daily for 4 days had α1AT serum levels below those achieved by the single dose $4x10^{12}$ gc/kg group (day 112, $p<0.001$), but mice administered 4 days consecutively at 10^{12} gc/kg had significantly higher levels of serum α1AT levels than that of a single administration at the same 10^{12} gc/kg dose (day 112, $p<0.0003$). This could be an advantage when dosing for a single administration is limited by volume, vector concentration, or total dose. To assess whether single administration of the same dose over different times would achieve different levels of transfer of vector genomes to the liver, rats (Fischer 344 males) were administered IV AAVrh.10hFXN (an AAVrh.10 vector coding for the human frataxin cDNA), with a fixed volume (1 ml) over 1 min, 30 min, 4 hr and 24 hr, each infusion time tested at 3 doses (10^{11}, 10^{12}, and 10^{13} gc/kg). Rats were sacrificed after 7 days and liver vector genome levels evaluated by quantitative PCR. There was a dose-dependent increase in vector genomes in the liver for all infusion times ($p<0.0001$). However, there were no differences observed in vector genome copies in the liver between any of the 4 different rates of vector infusion, i.e., there was no advantage to administration over different lengths of times ($p>0.1$, for all time comparisons in each dosage group). We conclude that, at least in rodents, the use of multiple daily injections influences the expression patterns of the targeted gene; however, the rate of infusion does not appear to be a critical parameter for the design of intravenous infusion for targeting AAV vectors to liver.

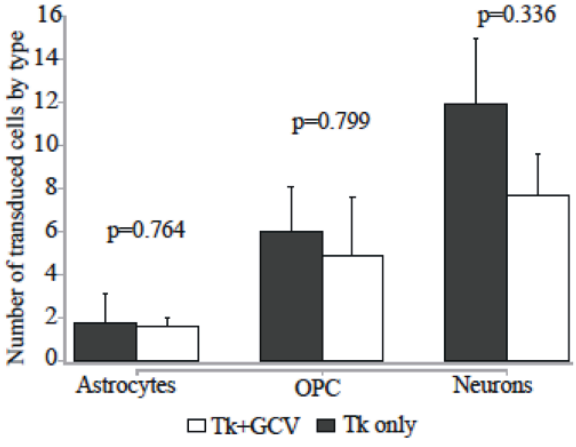
475. Effects of HSV-Tk Mediated Suicide Gene Therapy on Normal Brain Cells

Jubayer Al Hossain[1], Lars Romø Ystaas[1], Jelena Mrdalj[2], Kristjan Vaelk[1], Kristoffer Riecken[3], Boris Fehse[3], Rolf Bjerkvig[1], Janne Grønli[2], Hrvoje Miletic[1]
[1]K.G Jebsen Brain Tumor Research Center, Univesity of Bergen, Bergen, Norway, [2]Department of Biological and Medical Psychology, Univesity of Bergen, Bergen, Norway, [3]Research Department Cell and Gene Therapy, University Medical Center Hamburg, Hamburg, Germany

Malignant gliomas, the largest group of primary intracerebral tumours, are one of the most difficult-to-cure cancers. For glioblastoma, the most malignant form of glioma, the median survival time is generally less than one year. Lentiviral vector mediated suicide gene therapy has been reported to be a potential therapeutic

option for glioma and has been validated in clinically relevant animal models. At this end, specific targeting of tumor cells sparing normal brain tissue is a significant concern. Although several studies may suggest that HSV-Tk mediated suicide gene therapy is not likely to be toxic for normal brain cells, it has never been investigated which effect lentiviral vector mediated HSV-Tk suicide gene therapy exerts on the normal brain in a non-tumor setting. We addressed this important question by delivering the suicide gene HSV-Tk.007 to the brain of healthy, immunocompetent rats using lentiviral vectors pseudotyped with VSV-G. First, we studied behavior patterns and assessed any potential neurological symptoms in treatment and the control groups, during prodrug treatment with Ganciclovir. We did not observe any significant changes in their physical and behavioral patterns. Then, we carried detailed histological/ immunohistochemical analyses of the brains to analyze potential differences in the number of transduced brain cells between control and treatment groups. There was no significant difference in numbers of neurons, astrocytes and oligodendrocyte progenitor cells among the groups, indicating successful survival of these cells after pro-drug treatment. Furthermore, potential inflammation and apoptosis of brain cells were also investigated. No significant infiltration of immune cells was detected across the groups. We could not detect any sign of apoptosis or necrosis across all groups. In conclusion, our study which suggests that lentiviral suicide gene therapy mediated by HSV-Tk.007 is not toxic for normal brain cells.





476. No GVHD, but Human Inflammatory "Cytokine Storm" and Mouse Macrophage Activation Upon Accelerated Development of Human CD4+ Effector Memory T Cells in Long-Term Humanized Mice

Bala Sai Sundarasetty[1], Andreas Schneider[1], Katherina Sewald[2], Susanne Rittinghausen[2], Armin Braun[2], Constanca Figueiredo[3], Arnold Ganser[1], Renata Stripecke[1]

[1]Department of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Medical School Hannover, Hannover, Germany, [2]Department of Pathology, Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM), Hannover, Germany, [3]Department of Transfusion Medicine, Medical School Hannover, Hannover, Germany

Non clinical assessment of immunotoxicity of human cellular products in vivo is limited by the lack of availability of pharm-tox models enabling a full range of human innate and adaptive responses. We previously demonstrated improved development of human adaptive T and B cell responses in mice receiving human hematopoietic stem cell transplantation (HSCT) in combination with prime/boost immunization with induced dendritic cells (iDCs), i.e. monocytes derived the stem cell donor reprogramed with a lentiviral vector for co-expression of GM-CSF, IFN-alpha and the CMV antigen pp65 that self differentiate into DCs in vivo (Salguero et al, 2014 J. Immunology; Daenthanasanmak et al, 2015 Mol. Ther. Meth.). For these previous studies, mice were kept for 6-10 weeks after iDC administration, and we did not observe signs of tumorigenicity or graft-versus-host disease (GVHD). After consultation with the German regulatory authorities, we were requested to perform pharm-tox analyses 26 weeks after iDC immunization. We report here a pilot-feasibility study with humanized mice showing long-term (more than 33-36 weeks after HSCT) robust immune reconstitution with human T cells (control mice, n=2 Vs. iDC-immunized mice, n=6). Longitudinal analyses of human immune reconstitution in blood samples confirmed accelerated development of $CD4^+$ effector memory T cells 5-10 weeks after iDC immunization. No weight loss, GVHD or malignancies were observed after iDC immunization. 3/6 immunized mice developed skin erythema and inflammation around 23 weeks after iDC immunization and were sacrificed earlier for analyses. Analyses of all mice were performed at autopsy by macroscopic observations, H&E histopathology, immunohistochemistry and flow cytometry of target organs. Positivity for anti-human nuclei antibody, anti-human HLA-DR and anti-human CD3 leucocytes were detected in several tissues (spleen, bone marrow, brain, lungs, skin, eyelids, liver, and kidneys), confirming the long-term persistency of human T cells in the humanized mice. In inflamed skin, abundant human $CD4^+$ T cells were localized in the basal layers of the epidermis and hair follicles in the dermis. Human macrophages ($CD68^+$) could be detected in several tissues, but were rare in inflamed skin. However, in the inflamed dermis areas of ulceration, mouse macrophages ($F4/80^+$) were observed frequently with strongly positive cytoplasm. Mice with ulcerations had significantly higher levels of several human inflammatory cytokines in plasma (IFN-gamma, IL-6, GM-CSF and IL-8) than immunized mice with no skin inflammation or control mice. In summary, a long-term pilot study exploring a hybrid human-mouse model system was feasible and iDCs accelerated mature human T cell reconstitution with no signs of tumorigenicity or GVHD. However, accumulation of human $CD4^+$ memory T cells and activated mouse macrophages in inflamed skin was associated with elevated levels of human inflammatory cytokines resembling a "cytokine storm".

477. ANGPTL4 Limits the Extent of Colonic Inflammation

Terri Phua, Dickson Shao Liang Chee, Nguan Soon Tan
School of Biological Sciences, Nanyang Technological University, Singapore, Singapore

Gastrointestinal disorders such as inflammatory bowel disease, colitis and enteritis share signatures of an aggravated and protracted inflammatory response. As the first line of defense, epithelial cells play an important role in secreting chemoattractants and cytokines to launch or attenuate the immune response. However, little is known about the regulation of immune responses in the gastrointestinal tract. To understand the role of colonic epithelial cells during inflammation in C57BL/6J mice, we examine the effects of dietary C18 saturated (stearic acid), unsaturated (oleic acid) fat and dextran sulfate salt (DSS) in acute colonic inflammation. Microarray analysis was conducted comparing changes in global gene expression signatures between colon samples of three treatment groups. Preliminary studies suggest that the saturated diet resulted in a colonic gene expression profile that is more closely associated to that of DSS-treated. In addition, gene ontology analyses suggest that genes that overlap between all three treatment groups are mostly involved in immunological and metabolic diseases, as well as endocrine system disorders. Further interrogation revealed that an adipokine, angiopoietin-like 4 (ANGPTL4), modulates the degree and extent of colonic inflammation. We observed an elevated inflammation status, associated with massive immune cell infiltration, in ANGPTL4$^{-/-}$ mice compared to its ANGPTL4$^{+/+}$ littermates during DSS-induced colonic inflammation. Our preliminary studies highlights the importance of regulating the colonic expression of ANGPTL4 as an avenue to attenuate gastrointestinal inflammation.

Presidential Symposium

478. An Essential and Ubiquitous Protein Receptor for AAV; Glycans as Attachment Receptors

Nancy L. Meyer[1], Sirika Pillay[2], Qing Xie[1], Omar Davulcu[1], Andreas Puschnik[2], Jonathan Diep[2], Yoshi Ishikawa[1], Lucas Jae[3], Jonathan Wosen[2], Claude Nagamine[4], Alex Noble[5], Scott Stagg[5], Jan E. Carette[2], Michael S. Chapman[1]
[1]Dept. Biochemistry & Molecular Biology, Oregon Health & Sciences University, Portland, OR, [2]Dept. Microbiology & Immunology, Stanford University, Stanford, CA, [3]Netherlands Cancer Inst., Amsterdam, Netherlands, [4]Dept. Comparative Medicine, Stanford University, Stanford, CA, [5]Inst. Molecular Biophysics, Florida State University, Tallahassee, FL

Motivated by unsuccessful attempts to observe physical binding between AAV-2 and heterologously expressed domains of previously reported co-receptors, we set out to identify novel protein receptor(s) for AAV2 through an unbiased genome-wide knockout screen in human cells. Using an mCherry AAV vector, resistant cells were iteratively selected by FACS for gene trap screening in a library of mutagenized haploid cells. Upon deep sequencing, refractory cells had significantly high frequencies of mutation in genes encoding glycan synthesis and retrograde transport, but most significantly in a hitherto poorly characterized transmembrane protein, now termed AAVR. Genetic confirmation of AAVR's role in the entry of multiple AAV serotypes has come through CRISPR-Cas9 knockouts in multiple cell lines then restoration of susceptibility through complementation; infection of poorly permissive cells following AAVR transduction; and creation of a mouse knockout with greatly diminished susceptibility.

Various AAVR ectodomain constructs have been heterologously expressed and purified as fusion proteins, and these have been shown to inhibit *in vitro* viral transduction at concentrations consistent with effective nM binding constants (between AAV & AAVR) measured by surface plasmon resonance (SPR). Pre-incubation with antibodies to AAVR also inhibits infection or transduction. AAVR is transiently expressed on the plasma membrane. Expression of chimeric constructs suggests that AAV takes advantage of its trafficking to the *peri*-nuclear *trans* Golgi network as the dominant, but non-exclusive, entry pathway. Identification of AAVR and its apparently ubiquitous use has interesting implications for AAV's cell specificity. Progress towards structure of complexes will be reported.

AAVR exhibits the classic characteristics of a viral receptor, casting the roles ascribed to glycan "primary" receptors in new light. Electron microscopy has been used to visualize AAV-DJ in complex with various heparin analogs at increasingly high resolution. A structure at 2.8 Å resolution, as a pentasaccharide complex, shows some disorder in the glycan, but the side chains of viral amino acids are clearly resolved and in different conformations from those seen in a sucrose octasulfate complex. With little change to the backbone, the binding site accommodates diverse glycan sequences through adjustments to side chains, consistent with SPR binding assays of AAV-2 to a library of heparanoids. This, together with comparisons of heparan and AAVR cell knock-outs, indicates a more accessory role for glycans than is implied by the term "primary". As for several other viruses, in AAV-2 at least, the glycan is an attachment receptor that likely elevates the AAV concentration proximal to the membrane, improving the efficiency with which the virus then binds to AAVR.

479. An Optimized DNA Vaccine Formulation Protects Against Lethal Ebola Makona Virus Challenge in Non-Human Primates and Elicits Robust Immune Responses

Ami Patel[1], Emma L. Reuschel[1], Kimberly A. Kraynyak[2], Trina Racine[3], Daniel H. Park[1], Amelia A. Keaton[1], Muthumani Karuppiah[1], Devon Shedlock[1], Jian Yan[2], Amir Khan[2], Kevin Tierney[3], Niranjan Sardesai[2], Gary P. Kobinger[3,4,5], David B. Weiner[1]
[1]The Wistar Institute, Philadelphia, PA, [2]Inovio Pharmaceuticals, Plymouth Meeting, PA, [3]Public Health Agency of Canada, Winnipeg, MB, Canada, [4]University of Manitoba, Winnipeg, MB, Canada, [5]University of Pennsylvania, Philadelphia, PA

The West African Ebola hemorrhagic fever virus outbreak is the most wide-spread occurrence of the virus to-date. Over 28 000 cases have been confirmed, however the actual figures are likely much higher. There are currently no approved vaccines but several clinical trials of viral vector candidates are underway and evidence suggests that the VSV-ZEBOVGP vaccine can prevent further transmission of the virus. However, the considerable drawback of anti-vector immunity still remains to be addressed and reports from human clinical trials observe the presence of adverse events including fever, blistering, and development of arthritis/joint pain which may limit some applications of this vaccine. Therefore, investigating alternative immunization approaches is important. We designed a DNA vaccine formulation expressing 3 synthetic Zaire Ebola virus (EBOV) glycoproteins (GP): 2 designed based on GP sequence alignments (1976-2014) and a 3rd construct matched to a 2014 outbreak strain. Plasmid IL-12 (pIL-12) was also included as an adjuvant to further enhance cellular immune responses. We administered this multivalent GP DNA vaccine formulation in macaques following a DNA-DNA prime-boost immunization regimen. Macaques (n=3 or 4/group) received the multivalent GP DNA formulation + pIL-12 by intramuscular delivery followed by electroporation. We assayed differences in immunogenicity and monitored protection between

different doses, regimens (2, 3, 4, and 5 injections), and different spacing intervals between subsequent doses. Both antibody and T cell responses were observed in 83% of animals 2 weeks following the first injection and 100% of animals after the 2nd injection. The macaques were challenged with a lethal dose of the EBOV Guinea-Makona outbreak strain (1000pfu, 7-U virus) and monitored for 28 days following infection. 100% of animals receiving at least 3 injections at 4 week intervals survived lethal challenge. Animals were fully protected against signs of disease and did not exhibit elevated blood chemistry. Interestingly, 50% of animals receiving 2 injections survived lethal challenge. The surviving animals exhibited minimal signs of disease, suggesting that with further optimization complete protection with 2 injections is potentially achievable. In additional optimization studies in mice, single injections were found to be 100% protective and we observed that long-term immune responses 8 months post vaccination were induced. Further studies in NHP are now in progress.

480. Gene Therapy for Spinal Muscular Atrophy Type 1 Shows Potential to Improve Survival and Motor Functional Outcomes

Jerry R. Mendell[1], Samiah Al-Zaidy[1], Richard Shell[1], W Dave Arnold[2], Louise Rodino-Klapac[1], John T. Kissel[1], Thomas W. Prior[3], Carlos Miranda[1], Linda Lowes[1], Lindsay Alfano[1], Katherine Berry[1], Christopher Petek[1], Kathleen Church[1], Lyndsey Braun[1], Sarah Corcoran[1], Kathrin Meyer[1], Shibi Likhite[1], Arthur HM Burghes[1], Kevin D. Foust[1], Brian K. Kaspar[1]

[1]Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, OH, [2]Neurology, The Ohio State University, Columbus, OH, [3]Molecular Pathology, The Ohio State University, Columbus, OH

Spinal muscular atrophy (SMA) is the most common genetic cause of infant death with mutations of survival motor neuron 1 gene (SMN1) on chromosome 5q13 affecting 1 in 10,000 live births. SMA manifests as symmetrical weakness of proximal extremity, axial, intercostal and bulbar muscles. Longitudinal studies in SMA type I with 2 SMN2 copies show progressive weakness with a need for continuous non-invasive ventilation or death by a median age of 10.5 months. The mean rate of change over time in CHOP INTEND (CI) score is -1.27 points/year for all SMA type I infants. In this ongoing gene therapy trial, the criteria for enrollment for the first 10 patients was as follows: onset of symptoms before 6 months (m), homozygous loss of the *SMN1* gene, 2 copies of SMN2, and no c.859G>C exon 7 mutation. Two cohorts (Table 1) received intravenous AVXS-101(scAAV9.CB.SMN), Group 1 (n=3) $6.7x10^{13}$ vg/kg and Group 2 (n=7) $2.0x10^{14}$ vg/kg. The secondary endpoint for this safety study is death or need for >16 hours/day of noninvasive ventilation for at least 2 weeks. The minimal therapeutic dose cohort-1 had a mean age of 6.3±0.75m and a CI of 16.33±10.50 at baseline. Currently, this cohort is age 23.6±1.95mo with a CI of 22.3±13.65. The therapeutic dose cohort-2 was stratified by age at gene transfer. Group 2A (Pts 6 and 10) were treated at the earliest time points in the study 1.9m and 0.9 m, respectively with CI of 47 and 50 at baseline. Currently, this group is age 12.2m and 5.8m; and has a normal CI of 64. Group 2B (Pts 4, 5, 7, 9) had a mean age of 4.6±0.86m with a CI of 29.25±3.69 at baseline. Currently, this group is age 14.5±3.44m with a CI of 52±2.45. And Group 2C (Pt 8) was 7.9m with a CI of 12 at baseline. Currently, this patient is age 15.4m with a CI of 11. Electrophysiology studies showed increased compound motor action potentials (CMAPs) matching early treatment. It appears that an older age at enrollment (i.e. Pt 8 at 7.9m) with low CI results in stabilization with the therapeutic dose.

Gene delivery Dose	Groups based on Age at GT	Age* (m) at GT mean±SD	Current Age* (m) mean±SD	CI Score at GT mean±SD	Current CI Score mean±SD
$6.7 X 10^{13}$ vg/kg	Group 1	Pts 1,2,3 6.3 ± 0.75m	23.6 ± 1.9 m	16.33 ± 10.50	22.3 ± 13.65
$2.0 X 10^{14}$ vg/kg	Group 2A	Pts 6, 10 1.9m, 0.9m	12.2m, 5.8m	47, 50	64, 64
	Group 2B	Pts 4,5,7, 9 4.6±0.86m	14.5 ± 3.44m	29.25 ± 3.69	52 ± 2.45
	Group 2C	Pt 8 7.9m	15.4m	12	11

Treatment-related serious adverse events in this trial were limited to transient liver enzyme elevation without clinical manifestations and correlated with high level IFN-γ ELISpot assays to AAV9 capsid. This transaminasemia responded well to short-term prednisolone treatment. In summary, gene therapy for SMA type 1 appears to be neuroprotective allowing motor development to continue. At a higher CI score of 47- 50 with an enrollment age of 1-2 months, group 2A normalized to a score of 64. At a mid-level CI score with enrollment at age 4.6±0.86 m, improvement for group 2B appears to be equivalent to the SMA Type 2 phenotype. This trial provides evidence that gene therapy may provide patient benefit when compared to natural history. For example, natural history would predict that CI would decrease by 1.27 points per year and mortality of 75% of SMA Type 1 patients at 13.6 months, whereas 6 of 10 patients receiving gene therapy have passed the 13.6 month milestone. These results support the need for further studies of this promising therapeutic approach.

Targeted Genome Editing: In Vivo Genome Editing

481. CRISPR/Cas9-Mediated *In Vivo* Genome Editing to Correct the OTC *spf^{ash}* Mutation in Newborn Mice

Lili Wang[1], Yang Yang[2], Peter Bell[1], Deirdre McMenamin[1], Zhenning He[1], John White[1], Hongwei Yu[1], Chenyu Xu[3], Hiroki Morizono[3], Kiran Musunuru[4], Mark L. Batshaw[3], James M. Wilson[1]

[1]Gene Therapy Program, Department of Medicine, University of Pennsylvania, Philadelphia, PA, [2]State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, Chengdu, Sichuan, China, [3]Center for Genetic Medicine Research, Children's Research Institute, Children's National Health System, Washington, DC, [4]Division of Cardiovascular Medicine, Brigham and Women's Hospital, Boston, MA, Dept. of Stem Cell and Regenerative Biology, Harvard University, and Harvard Stem Cell Institute, Cambridge, MA

Many genetic liver diseases, including OTC deficiency, present in newborns with repeated, often lethal, metabolic crises. Adeno-associated virus (AAV) neonatal gene therapy in this setting would require multiple vector administrations to maintain the therapeutic effects because the non-integrating genome is lost as developing hepatocytes proliferate. As such, we reasoned that newborn liver may be an ideal setting for AAV-mediated gene correction using CRISPR/Cas9, a powerful genome-editing tool consisting of the Cas9 nuclease and a single-guide RNA (sgRNA). We developed a strategy using an AAV vector with high liver tropism (AAV8) to correct the point mutation in newborn *spf^{ash}* mice using Cas9 enzyme from Staphylococcus aureus (SaCas9). An animal model of OTC deficiency, the male sparse fur ash (*spf^{ash}*) mouse, has a G-to-A point mutation at the donor splice site at the end of exon 4 of the OTC gene, which leads to abnormal splicing and a 20-fold reduction in OTC mRNA and protein.

We developed a two-vector approach to incorporate all 3 components of CRISPR/Cas9 into AAV. Vector 1 expresses the SaCas9 gene from a liver-specific TBG promoter, while vector 2 contains both the sgRNA1 sequence expressed from a U6 promoter and the 1.8 kb donor OTC DNA sequence. *Spf^{ash}* pups were injected

intravenously on postnatal day 2 with mixtures of vector 1 and vector 2 and subsequently evaluated for indel (insertion and deletion) formation and functional correction of the *spf^{ash}* mutation. Following gene correction (3 and 8 weeks), indels were detected by deep sequencing in 31% of OTC alleles, and HDR-based correction of the G-to-A mutation was observed in 10% of OTC alleles. Liver sections were analyzed by immunohistochemistry for OTC expression, showing 15% OTC-positive cells at 3 weeks and 13% at 8 weeks. Direct measurements of OTC enzyme activity from liver homogenates and OTC mRNA from total cellular RNA from liver revealed similarly high levels of correction in treated animals. We further assessed the impact of gene correction on the clinical manifestations of OTC deficiency by evaluating the tolerance of *spf^{ash}* mice to a one-week course of high-protein diet. *Spf^{ash}* mice treated with the gene-editing vectors had significantly lower plasma ammonia levels and showed a survival improvement as compared with untreated *spf^{ash}* mice. This study provides convincing evidence for efficacy in an authentic animal model of a lethal human metabolic disease following *in vivo* genome editing.

482. Local and Systemic Gene Editing in a Mouse Model of Duchenne Muscular Dystrophy

Christopher Nelson[1], Matthew Gemberling[1], Chady H. Hakim[2], David G. Ousterout[1], Pratiksha I. Thakore[1], Ruth M. Castellanos[3], Sarina Madhavan[1], Xiufang Pan[2], F. Ann Ran[4], Winston Yan[4], Aravind Asokan[3], Feng Zhang[4], Dongsheng Duan[2], Charles A. Gersbach[1]

[1]Duke University, Durham, NC, [2]University of Missouri, Columbia, MO, [3]University of North Carolina, Chapel Hill, NC, [4]Broad Insitute of MIT and Harvard, Cambridge, MA

Duchenne muscular dystrophy (DMD) is a highly prevalent genetic disorder leading to muscle wasting, loss of ambulation, and premature death by the third decade of life. DMD is caused by gene deletions, duplications, or nonsense mutations leading to the loss of dystrophin, an essential musculoskeletal protein. Gene therapy has held tremendous promise for the treatment of monogenic disorders, yet an effective gene replacement therapy has been elusive. Genome editing has been established as a potential approach to correct the dystrophin gene in cultured human cells by excising non-essential exons from the dystrophin gene producing a shortened yet in-frame dystrophin protein (*1*). In contrast to gene replacement therapy, genome editing repairs the causative mutation in the native genomic context with the potential for permanent gene repair. Recently, we and others have demonstrated that CRISPR/Cas9 genome editing in neonatal and adult mouse models of DMD restores dystrophin expression, improves muscle biochemistry, and strengthens muscle force generation (*2-4*). However, further optimization of the approach for systemic gene correction is still needed.

To target the dystrophin gene in the mdx mouse, the 3.2kb *S. aureus* Cas9 and two guide RNAs (gRNA) targeting intronic regions surrounding exon 23 were packaged into an adeno-associated virus (AAV). Double stranded breaks created by Cas9 were repaired with the relatively efficient non-homologous end joining pathway leading to excision of the nonsense mutation in exon 23. AAV vectors were injected intramuscularly into the tibialis anterior muscle in adult mice and intravenously into neonatal mice and adult mice and characterized for gene deletions, dystrophin restoration, and improvements in muscle physiology.

Local correction restored overall dystrophin levels to 8% by western blot with 67% of muscle fibers positive for dystrophin by immunofluorescence. Repeated cycles of eccentric contraction showed 60% resistance to damage compared to sham-treated mice. Systemic correction was achieved through IP injection into P2 neonates with dystrophin restoration primarily in the cardiac muscle and skeletal muscle surrounding the peritoneal cavity. IV administration in adult mice restored dystrophin expression in the cardiac muscle. Improved systemic distribution and correction was achieved with intravenous administration into P2 neonates with AAV8 or AAV9 (**Fig. 1**).

This study establishes CRISPR/Cas9-based gene editing as a promising approach for the treatment of DMD. Ongoing work to improve the efficiency and safety of in vivo gene editing includes the incorporation of muscle specific promoters, minimization of vector packaging, and optimization of AAV serotype.

1. Ousterout et al. Nat Comm 2015. | 2. Nelson et al. Science 2015 | 3. Tabebordbar et al. Science 2015 | 4. Long et al. Science 2015

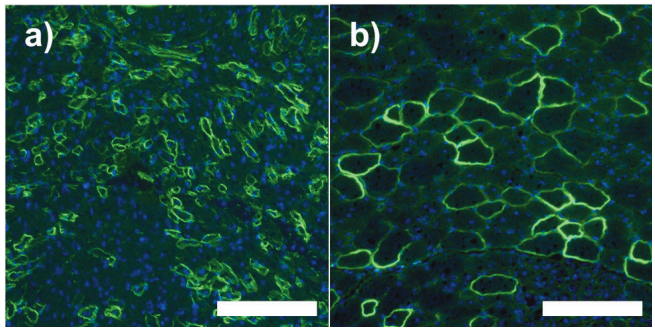


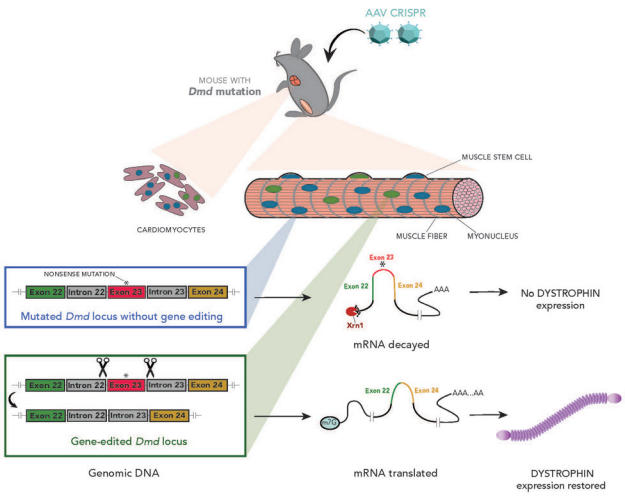
Figure 1 - Intravenously administered AAV restores dystrophin in cardiac and skeletal muscle in P2 neonates after 8 weeks of treatement. **a)** Cardiac muscle. **b)** Tibialis anterior muscle. Scale bar = 200μm, green - dystrophin, blue - DAPI.

483. *In Vivo DMD* Gene Editing in Muscles and Muscle Stem Cells of Dystrophic Mice

Mohammadsharif Tabebordbar[1], Kexian Zhu[1], Jason Cheng[1], Jeffrey Widrick[2], Winston Yan[3], Ru Xiao[4], Luk Vandenberghe[4], Feng Zhang[3], Amy Wagers[1]

[1]Harvard University, Cambridge, MA, [2]Division of Genetics and Program in Genomics, Boston Children's Hospital, Harvard Medical School, Boston, MA, [3]Broad Institute of MIT and Harvard, Cambridge, MA, [4]Grousbeck Gene Therapy Center, Schepens Eye Research Institute and Massachusetts Eye and Ear Infirmary, Boston, MA

Duchenne muscular dystrophy (DMD) is a X-linked genetic disorder that arises from frame-disrupting mutations in the *DMD* gene, encoding DYSTROPHIN. Lack of DYSTROPHIN expression destabilizes muscle fiber membranes, increases susceptibility to contraction-induced injury and drives muscle degeneration. Removing one or more exons from the mutated transcript can produce an in-frame mRNA and a truncated but still functional protein. In this study, we develop and test a direct gene editing strategy to recover DYSTROPHIN expression in the *mdx* mouse model of DMD. Coupling clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 endonucleases delivered via adeno-associated virus (AAV) with paired guide RNAs flanking the mutated *Dmd* exon 23, we demonstrate precise excision of intervening DNA and restoration of *Dystrophin* reading frame and protein expression *in vivo* in both skeletal and cardiac muscles following local or systemic delivery. DYSTROPHIN expression in AAV *Dmd*-CRISPR treated *mdx* mice was sufficient to partially recover functional deficiencies of dystrophic muscle. Finally, we demonstrate *in vivo* targeting of the *mdx* mutation in endogenous muscle stem cells, suggesting that AAV-CRISPR may provide a means to support ongoing repair of dystrophic fibers with corrected muscle precursors. This study provides proof-of-concept evidence supporting the feasibility and efficacy of *in vivo* genome editing to correct frame-disrupting mutations in DMD.



484. In Vivo Zinc-Finger Nuclease Mediated Iduronate-2-Sulfatase (IDS) Target Gene Insertion and Correction of Metabolic Disease in a Mouse Model of Mucopolysaccharidosis Type II (MPS II).

Kanut Laoharawee[1], Russell DeKelver[2], Kelly M. Podetz-Petersen[1], Susan Tom[2], Robert Radeke[2], Michelle Rohde[2], Amy Manning-Bog[2], Scott Sproul[2], Li Ou[1], Kathleen Meyer[2], Michael C. Holmes[2], Chester B. Whitley[3], Thomas Wechsler[2], R. Scott McIvor[1]

[1]Genetics Cell Biology and Development, University of Minnesota, Minneapolis, MN, [2]Sangamo BioSciences Inc., Richmond, CA, [3]Pediatrics, University of Minnesota, Minneapolis, MN

Hunter syndrome (Mucopolysaccharidosis Type II, MPS II) is a rare X-linked lysosomal disorder caused by lack of functional iduronate-2 sulfatase (IDS) enzyme and subsequent accumulation of glycosaminoglycans (GAG) in affected individuals. Manifestations include skeleton dysplasia, splenohepatomegaly, cardiopulmonary obstruction, and shortened life expectancy. In severe cases there is also neurologic impairment. Enzyme replacement therapy (ERT) is currently the only FDA-approved treatment to manage disease progression; however, ERT does not affect neurological aspects of the disease and requires that patients receive long and costly infusions of replacement factor on a frequent basis. We have developed a zinc-finger nuclease (ZFN) approach to insert the human IDS (hIDS) coding sequence into the albumin locus using AAV2/8 vectors. In this study IDS-deficient MPS II mice (n= 8-13 per group, age 7-8 weeks) were treated by intravenous infusion of a mixture of ZFN-encoding AAV vectors along with an AAV vector encoding the hIDS partial cDNA flanked by albumin sequence homology arms at three different vector doses. Wild-type littermates, untreated MPS II mice, and MPS II animals infused only with the hIDS donor vector (without ZFN-encoding vectors) were included as controls. Successful insertion of the hIDS coding sequence will result in hIDS expression regulated by the endogenous albumin promoter. Plasma and tissue IDS activities as well as urine and tissue GAG contents were monitored throughout the study to evaluate the effectiveness of the treatment. Sufficient animals were maintained for neurobehavioral testing at four-months post-injection to determine whether the treatment is neurologically beneficial. We found that IDS activities in the plasma of the treated groups were 10- to 100-fold higher than wild-type and stably expressed through the entire study duration in a dose-dependent fashion, while only very low levels of IDS activity were found in the animals infused with hIDS donor vector alone. At 4 weeks post-treatment IDS activities in peripheral tissues ranged from 1% to 200% wild-type in a dose-dependent fashion, while in the hIDS donor-only group enzyme activity was not detected in any tissue except liver (10% that of wild-type). We observed up to 2% of the wild-type IDS activity in the brains of animals administered the complete set of AAV vectors, while no IDS activity was observed in the brains of animals infused with the IDS donor vector alone. Urine GAGs were reduced in all of the ZFN + Donor treatment groups regardless of the vector dose. Tissue GAGs in the treatment groups were also decreased, but GAG content in the brain was not different from untreated MPS II litter mates at the initial analysis conducted four weeks post-treatment. No tissue GAG reduction was observed in animals infused with hIDS donor vector alone. Before conclusion of this study, animals from all six groups will be tested in the Barnes Maze as neurobehavioral assessment to determine the neurological effect of targeted hIDS expression in the liver. These results together with hIDS expression and GAG level data from final necropsy tissues will be presented. These results demonstrate that ZFN can be effectively used to mediate *in vivo* insertion of the hIDS coding sequence into the albumin locus with resultant stable and high-level hIDS enzyme expression and metabolic correction in MPS II.

485. ZFN-Mediated Liver-Targeting Gene Therapy Corrects Systemic and Neurological Disease of Mucopolysaccharidosis Type I

Li Ou[1], Russell DeKelver[2], Susan Tom[2], Robert Radeke[2], Michelle Rohde[2], Amy Manning-Bog[2], Scott Sproul[2], Michael J. Przybilla[3], Brenda L. Koniar[4], Kelly Podetz-Pedersen[3], Kanut Laoharawee[3], Renee D. Cooksley[1], Michael C. Holmes[2], Thomas Wechsler[2], R. Scott McIvor[3], Chester B. Whitley[1]

[1]Pediatrics, University of Minnesota, Minneapolis, MN, [2]Sangamo Biosciences, Inc, Richmond, CA, [3]Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, [4]Research Animal Resources, University of Minnesota, Minneapolis, MN

Mucopolysaccharidosis type I (MPS I) is characterized by progressive neurodegeneration, and premature death (<10 years). Caused by α-L-iduronidase (IDUA) deficiency and systemic accumulation of glycosaminoglycans (GAG), current therapies include stem cell transplant (with significant risk of morbidity and mortality), and enzyme replacement therapy (requiring costly and frequent long therapeutic infusion sessions). A promising alternative is genome editing by integration of a therapeutic hIDUA transgene using zinc finger nucleases (ZFN). We have previously demonstrated AAV-mediated *in vivo* targeting of the albumin locus as a "safe harbor" for coagulation factors, correcting clotting defects in hemophilic mice. Targeted insertion of a transgene into the genome offers multiple advantages. We can exploit the highly active albumin enhancer/promoter, such that stably modified hepatocytes demonstrate long-term transgene expression. Also, utilization of an endogenous promoter allows efficient packaging of the transgene donor into AAV. MPS I mice (n=8 per gender, 4-9 weeks old) were injected with a single dose of AAV2/8 encoding albumin-targeted ZFN and a donor encoding a partial hIDUA cDNA. MiSeq analysis showed that treated mice displayed significant levels of insertions/deletions (indels) (up to 56%) at the target locus, demonstrating efficient delivery and expression of the albumin ZFNs. Significant plasma IDUA activity was also observed in the ZFN+hIDUA donor treated mice, up to 10-fold of wild type levels, throughout the 120-day study. Urine GAG levels serving as a biomarker for hIDUA activity were reduced significantly (up to 90%). IDUA levels in these animals increased significantly in liver (up to 14 fold), heart, lung, muscle and spleen. Tissue GAG levels were significantly reduced in liver (by 91%), heart (85%), lung (86%), muscle (68%) and spleen (84%). Barnes maze

tests at the end of the study showed that ZFN+hIDUA donor treated MPS I mice achieved significant neurological benefits compared with untreated MPS I mice.

ZFN-mediated genome editing of hepatocytes *in vivo* thus resulted in high and stable levels of hIDUA expression in treated animals. This enzyme was secreted into plasma and then taken up by secondary tissues, leading to significant GAG reduction. The correction of the observable neurological impairment in MPS I mice suggests that a small but sufficient amount of hIDUA protein crossed the blood-brain barrier in these animals.

The study provides a general "proof-of-concept" for treatment of lysosomal diseases.

486. Abstract Withdrawn

487. Therapeutic Genome Editing by Combined Viral and Non-Viral Delivery of CRISPR System Components to the Mouse Liver

Hao Yin
MIT, Cambridge, MA

The CRISPR(clustered regularly interspersed short palindromic repeats)/Cas9 system has emerged as a transforming genome editing tool. Cas9/sgRNA recognizes the protospacer-adjacent motif (PAM) sequence and a complementary 20 nucleotide genomic sequence and induces double stranded DNA breaks (DSBs), which are repaired by error-prone non-homologous end-joining (NHEJ) or precise homology-directed repair (HDR). CRISPR/Cas9 genome editing has been applied to correct disease-causing mutations in mouse zygotes and human cell lines, but delivery to adult mammalian organs to correct genetic disease genes has not been reported prior to our study.

The liver disease hereditary tyrosinemia type I is a particularly suitable model for gene repair-based therapy because the repaired hepatocytes will expand and repopulate the liver. In tyrosinemia patients, mutation of fumarylacetoacetate hydrolase (FAH), the last enzyme catalyzing the tyrosine catabolic pathway, leads to accumulation of toxic metabolites and severe liver damage. The $Fah^{mut/mut}$ mouse model is caused by a G->A point mutation in the last nucleotide of exon 8. This causes splicing skipping of exon 8 and truncated Fah messenger RNA (mRNA). These mice can be treated with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), an inhibitor of an enzyme upstream of FAH, to prevent toxin accumulation in hepatocytes.

In our first proof-of-concept study, we demonstrate CRISPR/Cas9-mediated correction of a Fah mutation in hepatocytes in a mouse model of the human disease hereditary tyrosinemia. Delivery of components of the CRISPR/Cas9 system by hydrodynamic injection resulted in initial expression of the wild-type Fah protein in ~1/250 liver cells. Expansion of Fah-positive hepatocytes rescued the body weight loss phenotype. Our study indicates that for the first time CRISPR/Cas9-mediated genome editing is possible in adult animals and has potential for correction of human genetic diseases.

The combination of Cas9, guide RNA and repair template DNA can induce precise gene editing and the correction of genetic diseases in adult mammals. However, clinical implementation of this technology requires safe and effective delivery of all of these components into the nuclei of the target tissue. Here in our second study, we combined lipid nanoparticle—mediated delivery of Cas9 mRNA with adeno-associated viruses encoding a sgRNA and a repair template to induce repair of a disease gene in adult animals. We applied our delivery strategy to a mouse model of human hereditary tyrosinemia and show that the treatment generated Fah-positive hepatocytes by correcting the causative Fah splicing mutation. Treatment rescued disease symptoms such as weight loss and liver damage. The efficiency of correction was >6% of hepatocytes after a single application, suggesting potential utility of Cas9-based therapeutic genome editing for a range of diseases.

488. In Vitro and In Vivo Genome Editing of the RHO Gene to Downregulate Dominant Mutations

Maria C. Latella, Maria T. Di Salvo, Fabienne Cocchiarella, Daniela Benati, Valeria Marigo, Alessandra Recchia
Life Science, University of Modena and Reggio Emilia, Modena, Italy

Although progresses have been made in the understanding of the genetic basis for Retinitis Pigmentosa (RP), the development of therapeutic intervention is still lagging behind. Gene therapy was successfully applied to retina degeneration but only to recessive mutations. Rhodopsin (*RHO*) mutations represent a common cause of RP, accounting for 25% of adRP and 8 to 10% of all RP (Hartong et al., 2006) with more that 100 different mutations identified so far. RHO is a G-protein coupled receptor with localization restricted to rod outer segments where the phototransduction cascade initiates. Data on the pathogenic mechanism of mutant RHO are still controversial. Accumulation of mutant RHO in different subcellular compartments, and among those the ER, may trigger unfolded protein response (UPR) devoted to cytoprotective outputs to permit cells to reduce protein synthesis and up-regulate chaperons to cope with stress (Lin et al., 2007). In the autosomal dominant RP the mutation in only one allele is sufficient to the onset of retinal degeneration. To treat this kind of disorder a gene addition therapy is not suitable, as the mutant allele, mRNA, or protein product must be silenced beforehand. We aimed at developing genome editing tools to knock out the *RHO* defective alleles by introducing a double strand breaks (DSB) into the target gene. Two gRNAs were designed in the first exon on the RHO gene encompassing the P23H mutation. The two gRNAs were tested singularly or together *in vitro* on HeLa clones stably expressing P23H RHO. We demonstrated insertions or deletions (indels) in the genomic DNA specifically in the *RHO* gene by Cel I assay, TIDE and sequencing. Indels caused strong reduction of the *RHO* mRNA and of RHO protein up to 90%. The higher effect was obtained with the two gRNAs together. The two gRNAs were then *in vivo* expressed with Cas9 in photoreceptors of transgenic mice expressing the human P23H Rho gene by electroporation. Targeted cells were tracked by co-expression of EGFP. EGFP+ cells were FACS-sorted and indels in the human P23H *RHO* gene were analyzed by sequencing. We were able to detect up to 30% of genome editing *in vivo*. No editing was scored on murine *Rho* allele. We also detected reduction of *RHO* mRNA expression as well as RHO protein. Finally, we developed new tools to downregulate mutant RHO in dominant forms of RP. The CRISPR/Cas9 system reveled a high efficiency and should be tested for knock-down followed by gene replacement approaches.

Chemical and Physical Methods for Delivery of Gene Therapeutics

489. Tumor-Targeted Hursirna-Nanoparticle Delivery Inhibits Lung Tumor Growth In Vitro and In Vivo

Ranganayaki Muralidharan[1], Narsireddy Amreddy[2], Anish Babu[2], Akhil Srivastava[2], Janani Panneerselvam[2], Allshine Chen[3], Yan D. Zhao[3], Lichao Zhao[2], Uday B. Kompella[4], Anupama Munshi[5], Rajagopal Ramesh[2]
[1]Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, [2]Pathology, University of OKlahoma Health Sciences Center, Oklahoma City, OK, [3]Biostatistics and Epidemiology, University of OKlahoma Health Sciences Center, Oklahoma City, OK, [4]Department of Pharmaceutical Sciences, University of Colorado, Denver, CO, [5]Radiation Oncology, University of OKlahoma Health Sciences Center, Oklahoma City, OK

HuR, an mRNA binding protein regulates the stability of many oncoproteins associated with cell survival, proliferation, migration and angiogenesis. HuR overexpression is a marker for poor prognosis in patients diagnosed with cancer of lung, ovary, breast and colon. We hypothesized that the silencing of HuR using small interfering RNA (siRNA) could be a promising approach for lung cancer therapy. To test our hypothesis, we developed a tumor-targeted nanoparticle (NP) system that is targeted to transferrin receptor (TfR) for delivering HuRsiRNA (HuR-TfNP) in human lung cancer cells. Human lung cancer cells (A549, HCC827) and normal lung fibroblast (MRC-9) cell lines expressing varying levels of TfR were used in the present study. TfR expression was highest in A549, moderate in HCC827, and low to undetectable in MRC9 cells. *In vitro* studies demonstrated enhanced uptake of Tf-NP (51%) in TfR overexpressing A549 cells, compared to the non-targeted NP. Specificity studies using desferrioxamine (DFO; 100 μM), a stimulator of TfR, showed a two-fold increased uptake of Tf-NP whereas blocking TfR with exogenous transferrin (1 μg/well) reduced the uptake by 3 fold in A549 cells. Further, HuR-TfNP treatment reduced HuR expression and significantly suppressed cell proliferation at 24h and 48h compared to control siRNA containing NP (C-Tf-NP) in tumor cells but not in normal cells. Greatest inhibition was observed in A549 cells (23% and 30% inhibition at 24 and 48 h respectively) compared to 15% and 25% in HCC827. In MRC-9 cells, only 4% inhibition was observed. HuR-TfNP induced G1 cell-cycle arrest in tumor cells that correlated with marked reduction in Cyclin D1, and Cyclin E protein expression . Further, tumor cell migration and invasion was significantly inhibited in HuR-TfNP treated tumor cells compared to C-TfNP treatment ($p<0.001$). *In-vivo,* Tf-NP bio-distribution studies using indocyanine green (ICG) showed accumulation of the NP in tumor tissues over time with maximum accumulation at 24 h post NP injection. Efficacy studies in A549 tumor model demonstrated that systemic administration of HuR-TfNP significantly inhibited growth of both subcutaneous tumor growth and experimental lung metastasis compared to C-TfNP treatment ($P<0.05$). Further, tumor growth delay was sustained over 70 days when compared to control groups. A marked reduction in the expression of HuR and HuR-regulated oncoproteins (Bcl2, Cyclin D1 and Cyclin E) with a concomitant increase in p27 expression was observed in HuR-TfNP-treated tumors compared to control tumors. Our study results demonstrate HuR-TfNP therapy suppressed lung tumor growth both *in vitro* and *in vivo* and is therapeutic target for lung cancer treatment.

490. Electroporation-Mediated Gene Transfer of Caveolin-1 Protects from Bleomycin-Induced Pulmonary Fibrosis Through Regulating Activation of Inflammasome

Xin Lin, Michael Barravecchia, David A Dean
School of Medicine and Dentistry, University of Rochester, Rochester, NY

Pulmonary fibrosis (PF) is a progressive chronic interstitial lung disease associated with high morbidity and mortality. It is characterized by increased deposition of extracellular matrix proteins and the accumulation of scar tissue in the lung interstitium, resulting from alveolar epithelial injury and the accumulation of inflammatory cells. Currently, there is no effective therapy. Gene therapy is a promising approach to treat a variety of lung diseases, including pulmonary fibrosis. Caveolin-1 (Cav-1) is a major component of caveolae, and has been found greatly reduced expression in the lungs of PF patients. Moreover, Cav-1-deficient mice develop spontaneous pulmonary fibrosis. To investigate if Cav-1 can protect from bleomycin-induced pulmonary fibrosis, we delivered plasmids expressing Cav-1 driven by the ubiquitin promoter using electroporation. PF was induced 2 days after gene transfer by intratracheal administration of bleomycin (2 unit/kg). We found that electroporation-mediated gene transfer of Cav-1 to the lung significantly protected from subsequent bleomycin-mediated fibrogenesis by histological analysis and the expression of alpha-smooth muscle actin (alpha-SMA) and collagen measurement. We also found attenuated recruitment of neutrophils and monocytes and/or macrophages in bronchoalveolar lavage (BAL) fluid after gene transfer of Cav-1 compared with vector control. The underlying mechanism was associated with reduced activation of the inflammasome by determining the expression of cleaved caspase-1 and IL-1 beta in both mouse models and cultured cells after overexpression of Cav-1. These results demonstrate that Cav-1 might be a potential target for treatment of pulmonary fibrosis.

491. Enhanced Delivery of Plasmid DNA to the Skin Using Gene Electrotransfer

Richard Heller, Amy Donate, Siqi Guo, Chelsea Edelblute, Anna Bulysheva
Center for Bioelectrics, Old Dominion University, Norfolk, VA

The easy accessibility of skin makes it an excellent target for gene therapy applications. Gene electrotransfer (GET) of skin is a simple, direct, *in vivo* method to deliver genes for therapy and can be accomplished in a minimally invasive way. This approach has been tested in several animal models with varying skin thickness and delivery of plasmid DNA with GET resulted in significantly increased expression levels. Previously, we developed electrode arrays specifically for skin-based applications and designed applicators that can be easily applied and expanded. The limitations that we have encountered with our new designs are related to the depth of penetration of expression and occasionally cellular or tissue damage due to the required high applied voltage. GET requires a balance between efficient delivery and the maintenance of cell viability. In this current study, we have evaluated the addition of externally applied thermal energy to GET protocols to enhance delivery without increasing the applied voltage. We have determined both in vitro and in vivo that elevating the tissue temperature to between 42-45 ^{0}C was sufficient to enhance delivery when combined with GET. Administering GET to tissue with elevated temperature resulted in 5-8 fold higher expression compared to delivery with GET alone (ambient temperature). In addition, expression was obtained in the deep dermis and muscle utilizing a surface electrode. This deeper penetration was not consistently obtained when GET was used without a thermal component. Current work is evaluating approaches to achieve

more uniform heat distribution within the tissue and to potentially elevate temperatures in specific areas of tissue in order to target gene expression. GET is a powerful tool that allows for manipulation of expression levels and kinetics. Adjusting the delivery parameters enables maximum control of the expression profile enhancing the potential for a successful therapeutic outcome. The addition of the thermal component allows further control of delivery and reduces the potential for cellular damage.

492. Manipulation of Ultrasound Conditions for Effective Gene Delivery in a Porcine Model

James Harrang[1], Shuxian Song[1], Bryn M. Smith[1], Kyle Morrison[2], Carol H. Miao[1,3]
[1]Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, WA, [2]Sonic Concepts Inc., Bothell, WA, [3]Pediatrics, University of Washington, Seattle, WA

Our group has previously demonstrated that increasing pulse duration lowers the accompanying peak negative pressure (PNP) required for effective gene delivery (UMGD) in mouse and cell models. This effect allows selection of conditions with minimal associated tissue damage and enables tuning to maximize the capabilities of piezo-materials. To eventually extend this finding clinically, we investigated its scalability in a pig model. In parallel, we examined several spatial effects which are important scaling considerations.

According to our established UMGD protocol, the liver of each pig was exposed via a midline incision. Next, using contrast diagnostic ultrasound to confirm placement and perfusion, we catheterized a consistent branch of the portal vein. Just prior to therapeutic US exposure, the inferior vena cava was temporarily occluded. US exposure and infusion of a solution containing pGL4 plasmid and phospholipid MBs were initiated simultaneously. Therapeutic US was delivered via either H105, an unfocused 52 mm disc transducer, or H185D, a 49 mm disc transducer with three cylindrical focuses. Our US pulse durations spanned 19 μs-22 ms, with PNPs spanning 0.6-6.9 MPa. 24 hours after surgery, pigs were sacrificed to harvest treated and control liver lobes. After sectioning, spatially-mapped samples were analyzed for luciferase expression.

Our ongoing experiments have added further support for a species-generalized model that increasing pulse duration enables the use of lower PNP for effective UMGD. Notably, within a paired study, increasing pulse duration from 19 μs to 200 μs at a constant 6.9 MPa PNP yielded an up to 17-fold increase in sampled luciferase gene expression. Furthermore, in the same paired study, a 200 μs pulse duration at a lower 4.5 MPa PNP still maintained up to a 9-fold increase in sampled expression versus the 19 μs, 6.9 MPa group. Despite these increases, ALT and AST values remained consistent or lowered for both groups when moving from 19 to 200 μs. When comparing spatial effects, H105 yielded significantly increased expression relative to H185D at equivalent pulse duration and focal PNP. This result suggests that focal treatment volume is also an important consideration for UMGD in larger animal models. However, comparing expression relative to energy flux shows no such discrepancy in our data, suggesting that pressure-dependent cavitation dynamics still play a role in UMGD efficacy.

By manipulating US pulse durations, our group has successfully achieved increased expression, circumventing the peak power density limitations imposed by piezo-materials used in US transducers. Such tuning has also allowed us to achieve comparable expression at decreased PNPs. Since skin attenuation imposes a barrier to high PNPs in transcutaneous UMGD, this result has promising implications for advancing that modality. Our results demonstrate the advancement of UMGD technology for achieving efficient gene transfer in large animal models.

493. Nonviral Gene Transfer by Sequence-Defined Proton-Sponges with Combined Nucleic Acid Binding and Endosomal Buffering: Balancing Basicities

Ulrich Lächelt[1,2], Ana Krhac Levacic[1], Dominik Wendel[1,2], Ernst Wagner[1,2]
[1]Pharmaceutical Biotechnology, Center for System-based Drug Research and Center for NanoScience (CeNS), Ludwig-Maximilians-University, Munich, Germany, [2]Nanosystems Initiative Munich (NIM), Munich, Germany

The delivery of nucleic acids represents a therapeutic approach for the precise adjustment of cellular dysregulations on the genetic level. However, safe and efficient usage of therapeutic nucleic acids remains a challenging task since several barriers have to be overcome within the delivery pathway. Despite a lower efficiency compared to viral vectors, nonviral transfer systems also offer several advantages such as high control over structural design and properties, flexibility regarding cargo type and capacity and scalable manufacturing technologies. Polymers with protonatable amines are widely used as synthetic delivery systems since these compounds can fulfill several prerequisites such as nucleic acid binding, cellular uptake and endosomal release, as a result of their basic characteristics. To facilitate the individual delivery steps efficiently, sequential protonation in different pH ranges has to be provided by the transfecting agents. Herein solid-phase supported synthesis was used to set up a library of structurally related oligomers containing different oligoamino acid building blocks[1] and basic α-amino acids such as histidine and pyridylalanine. The impact of individual protonation characteristics on different stages of gene transfer, such as pDNA binding potency, endosomal buffering and mediation of transgene expression, was evaluated to identify favourable pK_a ranges and an optimal balance between high basicity for stable nucleic acid binding and residual buffer capacity for endosomal escape. Consistent with previous observations[2] the length of continuous diaminoethane motifs in oligoamino acid building blocks vastly determined the protonation characteristics having impact on the separate gene delivery steps. Oligoamine segments with only two protonatable amines exhibited low pDNA complexation capability but high endosomal buffering, whereas segments with three secondary amines provided good complexation but low buffer capacity. Since both parameters are important requirements, oligomers exclusively containing these building blocks as protonatable elements did not induce high transgene expression. Different basicities have to be combined and balanced in order to address all distinct barriers within the delivery pathway individually. Consequently, the combination of a potent pDNA binder with additional elements for endosomal buffering greatly enhanced the final transgene expression. Surprisingly, the exact location of maximal buffer capacity within the endosomal pH range seemed to be critical for successful gene transfer. We suggest that endosomal escape of pDNA polyplexes via the hypothesized proton-sponge effect is favoured only in a narrow window of the endosomal pH range.

References

1. Schaffert, D.; Troiber, C.; Salcher, E. E.; Fröhlich, T.; Martin, I.; Badgujar, N.; Dohmen, C.; Edinger, D.; Kläger, R.; Maiwald, G.; Farkasova, K.; Seeber, S.; Jahn-Hofmann, K.; Hadwiger, P.; Wagner, E., Solid-phase synthesis of sequence-defined T-, i-, and U-shape polymers for pDNA and siRNA delivery. Angew. Chem., Int. Ed. Engl. 2011, 50, (38), 8986-9.

2. Lächelt, U.; Kos, P.; Mickler, F. M.; Herrmann, A.; Salcher, E. E.; Rödl, W.; Badgujar, N.; Bräuchle, C.; Wagner, E., Fine-tuning of proton sponges by precise diaminoethanes and histidines in pDNA polyplexes. Nanomedicine (N. Y., NY, U. S.) 2014, 10, (1), 35-44.

494. Development of a pH Sensor to Probe Endosomal Buffering of Polymeric Nanoparticles Effective for Gene Delivery

David R. Wilson[1], Denis Routkevitch[1], Karl J. Wahlin[2], Don J. Zack[2], Alfredo Quinones-Hinojosa[3], Jordan J. Green[1]
[1]Biomedical Engineering, Johns Hopkins University, Baltimore, MD, [2]Opthalmology, Johns Hopkins University, Baltimore, MD, [3]Neurosurgery, Johns Hopkins University, Baltimore, MD

Introduction: Polymeric gene delivery suffers from low efficacy compared to viral gene delivery with one of the primary barriers to successful transfection being efficient endosomal escape. Cationic polymers have been hypothesized to facilitate endosomal escape via the proton sponge mechanism by buffering hydrogen ions in the endosomal compartment. Here we have created a nucleic acid based pH sensor and applied it using flow cytometry and confocal microscopy to investigate endosomal buffering of synthetic biodegradable cationic polymers for gene delivery, correlating the pH of delivered DNA with transfection. **Methods**: We created a nucleic acid pH sensor by conjugating pH sensitive (FITC, OG) and insensitive (Cy5) fluorophores to plasmid DNA. The fluorescence ratio of the sensor was calibrated to pH using flow cytometry and confocal microscopy following electroporation into cells. Cells were transfected with the plasmid pH sensor complexed with cationic polymers including poly(beta-amino ester)s (PBAEs) of variable transfection efficacy to investigate endosomal buffering. Additionally, confocal microscopy was used to assess colocalization of the plasmid pH sensor with a lysosomal dye. **Results**: PBAEs were demonstrated to effectively buffer endosomes and avoid lysosomal fate, whereas the negative controls poly-L-lysine (PLL) and polyethylenimine (PEI) were shown to accumulate in lysosomes by 24h post-transfection. The polymer molecular weight and weight-weight ratio to plasmid DNA was shown to have an effect on endosomal buffering as well as transfection efficacy in the case of PBAEs. From confocal microscopy analysis of endosomes, local pH was observed to be higher at the endosomal membrane than in the center, which may be indicative of polymer enrichment along the membrane due to excess soluble polymer. **Conclusions**: The constructed DNA sensor gave a linear relationship with intracellular pH. When investigating PBAE-induced buffering, results were consistent with the hypothesized proton sponge mechanism. These cationic polymers were demonstrated to affect endosomal pH and DNA lysosomal fate and lead to successful transfection.

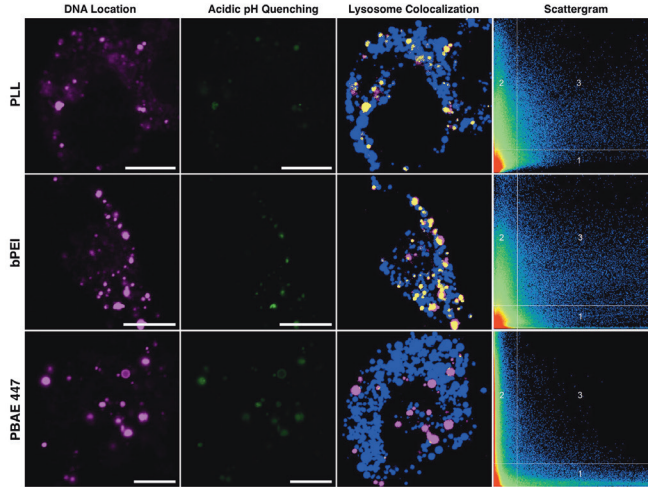


Fig 1: Confocal microscopy was used to assess the compartmental pH of endosomes containing nanoparticles formed with the pH sensor. Lysosome colocalization is shown in merged thresholded images with lysosomes (blue), DNA non-colocalized with lysosomes (pink) and DNA colocalized with lysosomes (yellow). Scattergrams show DNA fluorescence (horizontal) and lysosome fluorescence (vertical). Scale bar 10 μm.

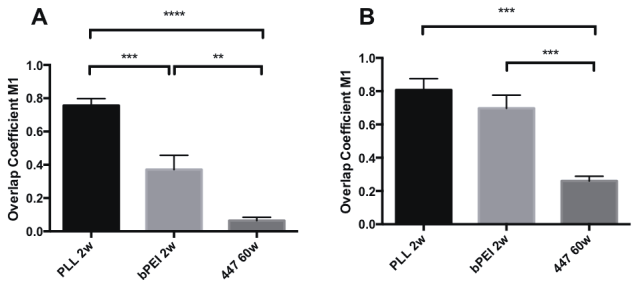


Fig 2. Confocal microscopy images were analyzed at (A) 1 hour and (B) 24 hours post-transfection using Pearson's correlation coefficient M1 to assess the fraction of DNA colocalized with lysosomal stain. When delivered via PBAE 447, the fraction of DNA colocalized with lysosomes was significantly different than both bPEI and PLL at both time points.

495. Activation of DNA Pattern Recognition Receptors After Plasmid Electrotransfer in Melanoma Cells and Tumors

Loree Heller[1], Masa Bosnjak[2], Katarina Znidar[3], Maja Cemazar[2]
[1]Old Dominion University, Norfolk, VA, [2]Institute of Oncology Ljubljana, Ljubljana, Slovenia, [3]University of Primorska, Izola, Slovenia

In vivo electroporation or electrotransfer, the application of controlled electric pulses, enhances delivery of plasmid DNA to a wide variety of healthy tissues as well as many tumor types. Electrotransfer of pDNA encoding therapeutic genes substantially increases gene expression, enhancing subsequent therapeutic effects. Delivery of therapeutic plasmid DNA has reached clinical trials in the US and in Europe, primarily for cancer therapies and infectious disease vaccines. In several preclinical tumor models, delayed tumor growth, increased survival time, and even complete tumor regression can occur with intratumoral electroporation, also known as electrotransfer, of DNA oligonucleotides or plasmid DNA devoid of a therapeutic gene (empty vector). In B16.F10 mouse melanomas, these effects are preceded by significant elevation of several proinflammatory cytokines and chemokines including IFNβ, implicating the binding and activation of intracellular DNA-specific pattern recognition receptors in response to DNA electrotransfer. The purpose of this study was to investigate whether melanoma tumors and cells express cytosolic DNA sensors and whether these sensors respond to pDNA electrotransfer. Histologically, tumor necrosis independent of caspase-3 was observed. Although the mRNAs for several DNA sensors were detected in tumors, none was significantly upregulated. In B16.F10 cells in culture, IFNβ mRNA and protein levels were significantly upregulated after pDNA electrotransfer. The mRNAs for several DNA sensors were present in these cells and DAI, DDX60, and p204 mRNAs were significantly upregulated after pDNA electrotransfer. DDX60 protein levels were coordinately upregulated. Mirroring the observation of tumor necrosis, cells underwent a significant pDNA concentration-dependent decrease in proliferation and survival. Taken together, increased IFNβ and DNA sensor expression accompanied by cell death and tumor necrosis indicate that pDNA electrotransfer activates intracellular DNA sensors in B16.F10 cells and tumors, producing both *in vitro* and *in vivo* effects. The absence of activation of DNA sensors *in vivo* could be due to the lower transfection efficiency compared to that *in vitro*

Molecular Therapy Volume 24, Supplement 1, May 2016

or to dilution by other tumor cell types. Electrotransfer is an efficient means of enhancing plasmid DNA introduction into tissues such as skin, muscle, and tumors for therapeutic application. Localized inflammation and induced cell death may contribute to cancer gene therapies but may impede gene therapies for which these effects are not desirable.

496. Elucidating Design Rules Governing Extracellular Vesicle-Mediated Therapeutic Protein Delivery

Michelle E. Hung, Joshua N. Leonard
Chemical and Biological Engineering, Northwestern University, Evanston, IL

Extracellular vesicles (EVs) are secreted biological nanoparticles that have great potential as therapeutic delivery vehicles - they are well-tolerated *in vivo* and naturally capable of transferring RNA and proteins between cells. Our ability to engineer EVs as therapeutic delivery vehicles is limited by an incomplete understanding of how EVs load biomolecular cargo and deliver it to recipient cells. In particular, the biophysical rules governing mRNA and protein delivery by EVs have not been elucidated. Open questions include: Does size limit mRNA loading efficiency into EVs? To what extent is EV mRNA cargo translated in recipient cells? What factors impact the degree to which RNA and protein cargo are delivered to the cytoplasm of recipient cells?

To quantitatively investigate the above questions, we leveraged our Targeted and Modular EV Loading (TAMEL) platform, which enables active loading of specific cargo RNA into EVs. TAMEL can enrich cargo mRNA loading into EVs up to 40-fold relative to passive loading. By directly comparing active loading efficiencies between mRNAs of different lengths, we characterized what type of RNAs *can* be loaded into EVs. While active loading of mRNA-length (> 1.5 kb) cargo molecules was significant, active loading was much more efficient for smaller (~0.5 kb) RNA molecules, providing the first direct evidence for the impact of cargo RNA size on loading into EVs. We next leveraged the TAMEL platform to elucidate the limiting steps in EV-mediated delivery of mRNA and protein to prostate cancer cells, as a therapeutically relevant model system. In this model system, we did not observe translation of EV-delivered mRNA in recipient cells, indicating this is a limiting step in functional delivery of EV cargo. In contrast, we observed robust EV-mediated delivery of dTomato reporter protein, and thus further explored EVs as therapeutic protein delivery vehicles.

To probe the efficacy of EV-mediated therapeutic protein delivery, we investigated using EVs to deliver the prodrug converting enzyme cytosine deaminase fused to uracil phosphoribosyl transferase (CD-UPRT), which converts the prodrug 5-FC to the toxic 5-FU. Importantly, CD-UPRT can function without without requiring endosomal escape because both 5-FC and 5-FU are membrane permeable. We also explored strategies for EV-mediated delivery of Cas9 nuclease, which must overcome the EV loading barrier imposed by its NLS sequence as well as escape the endosome in recipient cells. To address the loading challenge, we investigated a strategy for conditional NLS reconstitution to allow enhanced loading of Cas9 into EVs. We then used Cas9 delivery to assess the degree to which EV-delivered proteins can escape the endosomal/lysosomal pathways and traffic to other subcellular locations. Altogether, our investigations elucidated key design rules and central limiting steps that may guide the further development and utilization of EVs as therapeutic biomolecule delivery vehicles.

Musculo-Skeletal Diseases

497. Follistatin Gene Therapy Improves Six Minute Walk Distance in Sporadic Inclusion Body Myositis (sIBM)

Jerry R. Mendell, Zarife Sahenk, Mark Hogan, Samiah Al-Zaidy, Kevin Flanigan, Louise R. Rodino-Klapac, Markus McColly, Kathleen Church, Sarah Lewis, Linda Lowes, Lindsay Alfano, Katherine Berry, Natalie Miller, Igor Dvorchik, Melissa Moore-Clingenpeel, Brian K. Kaspar
Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, OH

Treatment of sIBM poses many challenges. The cause of this disease is enigmatic, and although considered to be an inflammatory myopathy, there is resistance to anti-inflammatory and immunosuppressive agents. sIBM muscle biopsies show vacuolated muscle fibers, widespread inflammation, and intracellular amyloid deposits. Follistatin is a potent inhibitor of the myostatin pathway and its potential as a therapeutic vehicle is enhanced by a pathway independent of the activin IIB receptor. We have demonstrated both safety and efficacy following direct intramuscular injection of follistatin in the quadriceps muscle in a previously reported gene therapy trial in Becker muscular dystrophy (Mendell JR, et al Mol Ther 2015). No off target effects were encountered attributed to the use of an alternatively spliced follistatin isoform, FS344, also used in the current sIBM gene therapy trial. Enrollment in the current gene therapy trial included 6 subjects with either definite or possible sIBM (Griggs RC, et al. Ann Neurol 1995). Pretreatment MRI's were obtained to determine areas of relative muscle sparing/lack of fibrosis. The intramuscular injections of AAV1.CMV.FS344 to 12 to 14 sites in the quadriceps muscle delivered $1.2X10^{12}$ vg/kg. Injections were performed with direct ultrasound guidance to target the most normal appearing muscle bundles, and intramuscular position was confirmed with simultaneous EMG. A three-patient, single limb, safety trial preceded the Phase I/IIA trial reported here. During the ongoing gene therapy trial, a control sIBM group (n=20) was prospectively studied by performance of the 6MWT with follow up from 9-28 months.

The 6MWT was the primary functional outcome (See table below). sIBM patients treated with AAV1.CMV.FS344 increased the 6MWT distance by 46.5m (457 to 503.5, p =0.001). Untreated sIBM controls lost 38.5m over a similar time period resulting in net difference of 85.0m between groups (p=0.0007). To validate findings and confirm the lack of selection bias we compared a subgroup of untreated sIBM controls (n=8), matched for age, gender, and 6MWD at baseline. Matched controls lost 39m (p=0.0036) in the 6MWD, a virtually identical loss to the larger control group.

The results of this study demonstrate that sIBM can benefit from follistatin gene therapy based on improvement in distance walked in the 6MWT. We did find a hierarchy of response based on muscle preservation and avoiding gene delivery to areas of fibrosis. In this study, gene delivery was limited to the quadriceps muscle, but in future trials more widespread delivery could potentially be more effective.

Six Minute Walk Distance Pre- and Post-Treatment* [median values (interquartile ranges provided)]

Group	Baseline (m)	Final Compared to Baseline (m)	Change from Baseline (m)	Change per Month (m)
sIBM Gene Therapy Pts (n =6)	457 (431,475)	Improved to 503.5 (443,573)	+46.5 (2,117)	+3.09 (0.39,8.9)
Untreated sIBM Controls (n =20)	393 (356.5,.451.5)	Declined to 354.5 (303.5,410.5)	-38.5 (-73,-22) p =0.0007	-2.3 (-4,-1.1) P =0.0032
Matched sIBM Controls for age, gender, and 6MWD (n = 8)	459 (439.5,469)	Declined to 420 (388.5,447.5)	-39.0 (-77, -8) p = 0.0036	-2.2 (-4.8,-0.7) P=0.0118

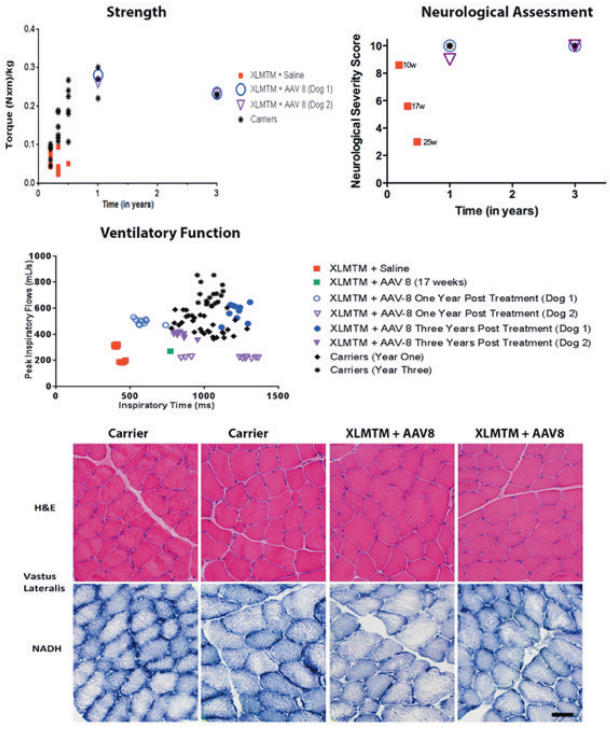
Data analysis used SAS 9.3 (SAS Institute, Cary NC) with two-sided p-values.

498. Prolonged Benefit from Systemic rAAV8 in a Canine Model of Myotubular Myopathy

Matthew Elverman[1], David Mack[2], Melissa Goddard[3], Jessica Snyder[4], Robert Grange[5], Karine Poulard[6], Virginie Latournerie[6], Philippe Veron[6], Laurine Buscara[6], Christine Le Bec[6], Samia Martin[6], Fulvio Mavilio[6], Federico Mingozzi[6,7], Hui Meng[8], Michael Lawlor[8], Alan Beggs[9], Anna Buj-Bello[6], Martin Childers[1,10]

[1]University of Washington Department of Rehabilitation Medicine, Seattle, WA, [2]University of Washington Institute for Stem Cell and Regenerative Medicine, Seattle, WA, [3]Department of Physiology and Pharmacology, School of Medicine, Wake Forest University, Winston Salem, NC, [4]Department of Comparative Medicine, University of Washington, Seattle, WA, [5]Department of Human Nutrition, Foods and Exercise, Virginia Polytechnic and State University, Blacksburg, VA, [6]Généthon, Evry, France, [7]University Pierre and Marie Curie, Paris, France, [8]Division of Pediatric Pathology, Department of Pathology and Laboratory Medicine, Medical College of Wisconsin, Milwaukee, WI, [9]The Manton Center for Orphan Disease Research, Boston Children's Hospital, Harvard Medical School, Boston, MA, [10]Institute for Stem Cell and Regenerative Medicine, School of Medicine, University of Washington, Seattle, WA

Body: Mutations in MTM1 result in X-linked myotubular myopathy (XLMTM), a rare neuromuscular disease that causes devastating muscular weakness in affected children, often fatal in the first years of life. A naturally occurring MTM1 mutation in Labrador retrievers provides unprecedented opportunity to assess MTM1 gene replacement strategies. Here, we report clinical and physiological outcomes more than 3 years after a single intravenous infusion of AAV8-MTM1 in two dogs. Readouts include neurological assessment, limb strength, gait, respiratory function, and histology. Results (Fig 1) are shown in comparison to age-matched littermates, and indicate that a single infusion of AAV8-MTM1 can markedly prolong life and achieve near normal levels of neurological function, strength, gait, and respiratory function for more than 3 years in MTM1 mutant dogs.



499. Intravenous Delivery of a Novel Micro-Dystrophin Vector Prevented Muscle Deterioration in Young Adult Canine Duchenne Muscular Dystrophy Dogs

Chady Hakim[1], Xiufang Pan[1], Kasun Kodippili[1], Thais Blessa[1], Hsiao T. Yang[2], Gary Yao[3], Stacey Leach[4], Craig Emter[2], Yongping Yue[1], Keqing Zhang[1], Sean X. Duan[1], Nalinda Wasala[1], Gregory Jenkins[1], Charles R. Legg[5], Joel S. Schneider[5], Jeffrey S. Chamberlain[6], Dongsheng Duan[1]

[1]Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO, [2]Department of Biomedical Sciences, College of Veterinary Medicine, University of Missouri, Columbia, MO, [3]Department of Bioengineering, College of Engineering, University of Missouri, Columbia, MO, [4]Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO, [5]Solid GT, LLC, Cambridge, MA, [6]Department of Neurology, School of Medicine, University of Washington, Seattle, WA

Duchenne muscular dystrophy (DMD) is a progressive, muscle wasting disorder that affects all muscles in the body. An effective gene therapy for DMD will require efficient whole body muscle transduction. It was recently demonstrated that a single intravenous injection of adeno-associated virus (AAV) can lead to safe, bodywide muscle gene transfer in adolescent dogs affected by the canine model of DMD (cDMD) (Yue *et al*. 2015 Hum Mol Genet). Here we evaluated systemic gene therapy in three 3.5-m-old cDMD dogs using a novel canine codon-optimized micro-dystrophin vector. Transcriptional regulation is controlled by the muscle-specific CK8 promoter and a synthetic polyadenylation signal. All experimental subjects received transient immune suppression. One dog was administrated with $5x10^{13}$ viral genome (vg) particles/kg of the vector. Two dogs received $1x10^{14}$ vg particles/kg of the vector. All dogs tolerated injection well. Blood biochemistry (weekly in the first four weeks and biweekly thereafter) was unremarkable. Growth curve was nominally disturbed during the immunosuppression regimen, but recovered thereafter. Biopsy at 1,3

and 6 months after injection revealed widespread micro-dystrophin expression in 50-80% myofibers. The dystrophin-associated glycoprotein complex, including neuronal nitric oxide synthase (nNOS), was restored. While limited in sample size, muscle damage usually seen in young adult untreated dogs (inflammation, fibrosis, calcification) were rarely observed. CD4+, CD8+, and regulatory T cells were minimally detected. Night activity monitoring showed a trend of improvement. Limb muscle force (both forelimb and hind limb) was significantly enhanced compared to that of pre-injection. Our data suggest that systemic AAV micro-dystrophin therapy may translate to large mammals afflicted by DMD (Supported by Solid GT, NIH, DOD, Jesse's Journey).

500. Gene Therapy Rescues Disease Phenotype in the Oculopharyngeal Muscular Dystrophy Mouse Model

George Dickson[1], Alberto Malerba[1], Pierre Klein[2], Susan Jarmin[1], Houria Bachtarzi[1], Arnaud Ferry[2], Gillian Butler-Browne[2], David Suhy[3], Michael Graham[3], Capucine Trollet[2]
[1]School of Biological Sciences, Royal Holloway - University of London, London, United Kingdom, [2]Centre de Recherche en Myologie, Sorbonne Universités UPMC Univ Paris 06, Paris, France, [3]Benitec Biopharma, Balmain, Australia

Among triplet expansion diseases, oculopharyngeal muscular dystrophy (OPMD) is an autosomal dominant, late-onset muscle disorder characterized by progressive eyelid drooping, swallowing difficulties and proximal limb weakness. OPMD is caused by a short trinucleotide repeat expansion in the polyadenylate-binding protein nuclear 1 (PABPN1) gene that results in an N-terminal expanded polyalanine tract. PABPN1 controls several biological processes such as the length of mRNA poly(A) tails, the mRNA export from the nucleus and the alternative poly(A) site usage. OPMD is characterized by nuclear aggregates of expanded PABPN1, fibrosis and muscle atrophy. Here we demonstrate that treating mice affected by OPMD over 4 months with an AAV gene therapy strategy based on DNA-directed RNA interference to silence the endogenous expPABPN1, combined with the re-expression of a healthy sequence-optimized human PABPN1 gene, significantly reduced the amount of nuclear aggregates in affected muscles, decreased the intramuscular fibrosis and reverted the muscle strength to the level of healthy wild-type muscles. Furthermore, although muscle atrophy was not reverted, the expression of a healthy PABPN1 markedly increased the cross sectional area of muscle fibres. These results obtained in a relevant mammalian animal model of OPMD pave the way for the clinical application of a gene therapy approach as a treatment for OPMD patients.

501. In Vivo Gene Editing for Duchenne Muscular Dystrophy

Niclas E. Bengtsson[1], John K. Hall[1], Guy L. Odom[1], James Allen[1], Joel R. Chamberlain[2], Jeffrey S. Chamberlain[1]
[1]Neurology, University of Washington, Seattle, WA, [2]Medicine, University of Washington, Seattle, WA

Gene replacement therapies utilizing adeno-associated viral vector delivery of truncated versions of dystrophin (mini- and microdystrophins) hold great potential for the treatment of Duchenne muscular dystrophy (DMD). However, these truncated dystrophins are not fully functional and episomal AAV vectors are not permanently retained following delivery to muscle. A more long-lasting and effective approach to gene therapy could result from methods to modify the dystrophin gene using gene editing strategies. The CRISPR/Cas9 system has been shown to be a highly specific method for genetic modifications, and recently has been proven applicable to correct mutations responsible for DMD. Here we present *in vivo* approaches for targeted correction of the dystrophin gene in the *mdx*^{4cv} mouse model of DMD using both single- and dual adeno-associated viral (AAV) vector delivery systems of Cas9 and DMD mutation specific targeting guide RNA's. Treated muscles demonstrated successful targeting of the dystrophin gene leading to a corrected open reading frame in the messenger RNA resulting in a restoration of near-to full-length dystrophin expression in up to 70% of treated muscle cross-sectional area. These results demonstrate that successful *in vivo* dystrophin correction can be achieved following AAV mediated delivery of CRISPR/Cas9, showing promise for the development of future clinical application of technologies for permanent correction of mutations leading to DMD and other genetic muscle disorders.

502. Biomaterial-Mediated Lentiviral Delivery of Anti-Inflammatory Genes in Cartilage-Derived Matrix Hemispheres

Katherine A. Glass[1], Christopher R. Rowland[2], Adarsh R. Ettyreddy[1], Jonathan M. Brunger[1], Nguyen P. T. Huynh[1], Charles A. Gersbach[1], Farshid Guilak[1,2]
[1]Biomedical Engineering, Duke University, Durham, NC, [2]Orthopaedic Research, Washington University in St. Louis, Shriner's Hospital, St. Louis, MO

Objectives: Cartilage tissue engineering seeks to provide a functional replacement for damaged or degenerated articular surfaces that develop in osteoarthritis (OA). Human bone marrow-derived mesenchymal stem cells (MSCs) seeded within cartilage-derived matrix (CDM) hemispherical scaffolds exhibit robust chondrogenic differentiation and extracellular matrix (ECM) deposition without evidence of a hypertrophic phenotype. However, inflammation within an OA joint may inhibit chondrogenesis of MSCs and induce degradation of both native and engineered cartilage, particularly through the action of interleukin-1 (IL-1). The goal of this study was to develop an anatomically-shaped, tissue-engineered cartilage capable of tunable and inducible expression of IL-1 receptor antagonist (IL-1Ra) in MSCs via biomaterial-mediated lentiviral gene delivery. **Methods:** IL-1Ra or eGFP coding sequences were cloned into doxycycline(dox)-inducible lentiviral vectors. Hemispherical scaffolds were fabricated from resuspended cartilage powder in hemispherical molds (outer radius 4.76 mm, inner radius 3.175 mm). Scaffolds were crosslinked and sterilized via dehydrothermal treatment. Dox-inducible eGFP or IL-1Ra lentivirus was immobilized to PLL-coated CDM scaffolds to transduce MSCs upon seeding. Non-transduced (NT), eGFP-expressing, or IL-1Ra-expressing constructs were treated with 0 or 0.1 ng/mL IL-1 during 28 days of chondrogenesis (+10 ng/mL TGF-ß3) with 1 μg/mL dox. **Results:** Transduction of MSCs occurred throughout the CDM hemispheres (Fig 1A) and the transduction efficiency of MSCs isolated from the construct was 57±5% eGFP+ by flow cytometry. Hemispherical constructs maintained their anatomical shape throughout chondrogenic culture (Fig 1B). IL-1Ra-expressing constructs produced over 200 ng/mL of IL-1Ra, a concentration expected to inhibit pathologic IL-1 concentrations, and IL-1 treatment did not affect IL-1Ra production (Fig 1C). IL-1 treatment reduced ECM deposition by NT and eGFP-expressing MSCs but not IL-1Ra-expressing MSCs, as evidenced by safranin-O/fast green staining for glycosaminoglycans and total collagen, respectively (Fig 1D). After 28 days of chondrogenesis, IL-1 treatment significantly increased matrix metalloproteinase (MMP) activity in the conditioned media of NT and eGFP-expressing constructs but not in IL-1Ra-expressing constructs ($p<0.05$) (Fig 1E). **Significance:** We engineered an anatomically and compositionally mimetic cartilage hemisphere that is inflammation-resistant and shows promise for total articular resurfacing in an arthritic environment.

Tunable production of IL-1Ra from an IL-1-resistant cartilage tissue may provide therapeutic effects to other joint tissues, such as the synovium or subchondral bone.

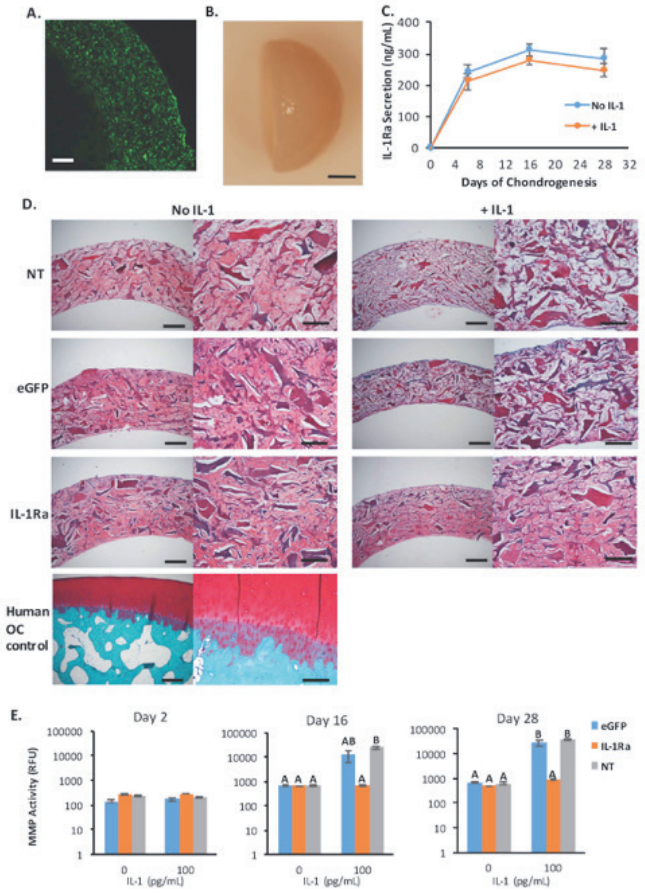


Figure 1. Results after 28 days of chondrogenesis of non-transduced (NT), eGFP-expressing, or IL-1Ra-expressing CDM hemisphere constructs cultured with or without IL-1 treatment. A. Representative confocal image of an eGFP-expressing construct. Cross-section, scale bar = 500 µm. **B.** Representative gross image of a CDM hemisphere construct, scale bar = 1 mm. **C.** IL-1Ra secretion (ng/mL) from IL-1Ra-expressing constructs into culture media over 48 hours at various time points. Mean +/- SEM, n=4. Baseline IL-1Ra in the absence of dox (day 0) was 1.66 ng/mL. There was no significant effect of treatment or time by repeated measures MANOVA. IL-1Ra secretion from NT and eGFP-expressing constructs was not detectable. **D.** Representative images of safranin-O/fast green/hematoxylin staining of CDM hemisphere constructs. The positive control is a human osteochondral tissue section. 4x images (left columns), scale bar = 500 µm. 10x images (right columns), scale bar = 250 µm. **E.** Total specific MMP activity (RFU) measured in 48 hours of conditioned media at various time points. Day 2 is the baseline prior to addition of IL-1. For Day 2, there was a significant effect of vector by ANOVA. For Day 16 and Day 28, groups not sharing the same letter are significantly different by two-factor ANOVA with Tukey's posthoc. Mean +/- SEM, n=5.

503. Adeno-Associated Virus Vector (AAV) Microdystrophin Gene Therapy Prolongs Survival and Restores Muscle Function in the Canine Model of Duchenne Muscular Dystrophy (DMD)

Caroline Le Guiner[1], Laurent Servais[2], Marie Montus[3], Thibaut Larcher[4], Bodvael Fraysse[5], Sophie Moullec[6], Taeyoung Koo[7], Alberto Malerba[7], Christine Le Bec[3], Matthias Hebben[3], Carole Masurier[3], Federico Mingozzi[3], Oumeya Adjali[5], Pierre Carlier[8], Jean-Yves Hogrel[9], Bernard Gjata[3], Yan Cherel[4], Takis Athanasopoulos[10], Fulvio Mavilio[3], Thomas Voit[11], Philippe Moullier[5], George Dickson[7], and the "AFM-sponsored Duchenne Consortium"[12]

[1]INSERM UMR 1089, Atlantic Gene Therapies & Genethon, Nantes, France, [2]Service of Clinical Trials and Databases, Institut de Myologie, Paris, France, [3]Genethon, Evry, France, [4]INRA UMR 703, ONIRIS, Atlantic Gene Therapies, Nantes, France, [5]INSERM UMR 1089, Atlantic Gene Therapies, Nantes, France, [6]Centre de Boisbonne, ONIRIS, Atlantic Gene Therapies, Nantes, France, [7]University of London, Royal Holloway, London, United Kingdom, [8]Laboratoire RMN, AIM and CEA, Institut de Myologie, Paris, France, [9]Neuromuscular Physiology and Evaluation Laboratory, Institut de Myologie, Paris, France, [10]University of Wolverhampton, Wolverhampton, United Kingdom, [11]Great Ormond Street Hospital, UCL Institute of Child Health, London, United Kingdom, [12]Atlantic Gene Therapies/Genethon/Institut de Myologie/Royal Holloway, Nantes, Evry, Paris, London, France

Duchenne Muscular Dystrophy (DMD) is a X-linked inherited muscle-wasting disease primarily affecting young boys with a prevalence of 1:5,000. The disease is caused by loss-of-function mutations in the gene encoding for the Dystrophin protein and is characterized by systemic, progressive, irreversible and severe loss of muscle function. Among vector systems that allow efficient *in vivo* gene transfer, recombinant Adeno-Associated Virus vectors (rAAV) hold great promise and allow very efficient transduction of skeletal and cardiac muscles. However, full-length dystrophin cDNA exceeds the packaging capacity for a single rAAV gene-delivery cassette. Therefore, truncated versions namely micro-dystrophins have been designed and optimized to contain few clinically important regions of the dystrophin protein. We have tested a rAAV2/8 vector encoding a sequence optimised canine micro-dystrophin transgene, driven by a muscle-synthetic Spc512 promoter (rAAV2/8-Spc512-µDys) in a total of 12 Golden Retriever Muscular Dystrophy (GRMD) dogs, the canine model of DMD. Isolated limb perfusion studies using a single administration of vector induced high levels of micro-dystrophin expression in the treated limb (up to 90% dystrophin positive fibres) with significant normalisation of histological, NMR imaging and spectroscopy parameters and muscle strength, without deleterious immune responses. Similarly, single-dose intravascular delivery of the same rAAV2/8-Spc512-µDys, in absence of immunosuppression, led to long-term transduction of distant muscle groups and extended lifespan (up to 2 years). Profound improvement of multiple clinical features was observed, including gait and respiratory parameters and no toxicity or deleterious humoral and/or cell-mediated immune responses were observed. This study demonstrates the safety and long term efficacy of rAAV2/8-Spc5.12-µDys gene therapy in a relevant large-animal models of DMD and paves the way towards human clinical gene therapy using systemic peripheral vein administration of vector, and applicable to all DMD patients regardless of their genotype.

Molecular Therapy Volume 24, Supplement 1, May 2016

504. Restoration of Dystrophin Expression by Gene Editing with *S. aureus* Cas9 in Models of Duchenne Muscular Dystrophy

Jacqueline N. Robinson-Hamm[1], Christopher E. Nelson[1], Ruth M. Castellanos Rivera[2], Annemieke Aartsma-Rus[3], Aravind Asokan[2], Charles A. Gersbach[1]
[1]Biomedical Engineering, Duke University, Durham, NC, [2]Genetics and Biochemistry & Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC, [3]Human Genetics, Leiden University Medical Center, Leiden, Netherlands

Duchenne muscular dystrophy (DMD) is the most common fatal genetic disease characterized by progressive muscle wasting, loss of ambulation, and typically death in the third decade of life due to respiratory and cardiac complications. DMD results from deleterious mutations in the dystrophin gene that disrupts the translational reading frame and causes a complete lack of dystrophin protein. Becker muscular dystrophy (BMD) is similar to DMD in that it is the result of deletions in the dystrophin gene. However these deletions maintain the translational reading frame and result in the production of an internally truncated but partially functional dystrophin protein. The BMD phenotype is typically less severe than DMD, and thus converting DMD to a BMD-like phenotype by restoring the dystrophin reading frame is a widely explored therapeutic strategy. CRISPR/Cas9 can target precise loci to make specific DNA sequence changes in the genome. We have previously used *S. pyogenes* Cas9 (SpCas9) to restore dystrophin expression in immortalized myoblasts from DMD patients by deleting dystrophin exon 51 to repair the disrupted reading frame. A promising therapeutic application of this strategy involves *in vivo* viral delivery of the CRISPR/Cas9 system by AAV, however AAV cannot efficiently package the large SpCas9 gene along with full size promoters. Therefore we have made use of AAV delivery of the smaller *S. aureus* Cas9 (SaCas9) to delete exon 23 in the mouse dystrophin gene *in vivo* in the *mdx* mouse model of DMD and showed restored dystrophin expression and functional recovery. Here we are continuing this work by preparing a SaCas9 system targeted to the human dystrophin gene. gRNAs were designed to target the intronic regions flanking exon 51 and tested *in vitro* in HEK293T cells as well as immortalized DMD patient myoblasts that are correctable by removal of exon 51. The expected deletion was confirmed by PCR and sequencing of the genomic DNA and dystrophin cDNA. Western blot of lysates from differentiated cells confirmed restoration of dystrophin protein expression. We have also demonstrated exon 51 deletion *in vivo* following AAV delivery of this CRISPR/Cas9 system to the muscles of a mouse model containing the full length human dystrophin gene. Ongoing work involves testing this approach in a novel dystrophic mouse model carrying a mutated version of the human dystrophin gene that is correctable by exon 51 deletion. This work is important to the continued development of a translational strategy for gene editing as a potentially curative treatment for DMD.

Cancer-Immunotherapy, Cancer Vaccines II

505. VGX-3100 Drives Regression of HPV16/18 CIN2/3 and Robust Cellular Immune Responses in Blood and Cervical Tissue in a Blinded, Randomized, Placebo-Controlled Phase 2B Study

Matthew Morrow[1], Connie Trimble[2], Xuefei Shen[3], Michael Dallas[1], David Weiner[4], Jean Boyer[3], Jian Yan[1], Kimberly Kraynyak[1], Albert Sylester[1], Mary Giffear[1], Kathleen Marcozzi-Pierce[1], Divya Shah[1], Kate Broderick[3], Amir Khan[1], Jessica Lee[1], Laurent Humeau[3], Niranjan Sardesai[1], Mark Bagarazzi[1]
[1]Inovio Pharmaceuticals, Plymouth Meeting, PA, [2]Johns Hopkins University, Baltimore, MD, [3]Inovio Pharmaceuticals, San Diego, CA, [4]University of Pennsylvania, Philadelphia, PA

Objectives: Assessment of the safety and efficacy and immunogenicity of VGX-3100 in women with biopsy-proven CIN2/3 with concurrent HPV16 and/or HPV18 infection. Methods: The randomized, placebo-controlled, double-blind study, which was stratified by age and severity of CIN, evaluated cervical tissue changes after three 6 mg intramuscular doses of VGX-3100 followed by electroporation with Inovio's CELLECTRA(r)2000 device at weeks 0, 4, and 12. Results: Among 167 vaccinated women, the study met its primary efficacy endpoint; the percentage of patients who had regression of CIN2/3 to CIN1 or no disease at 6 months post third dose was significantly higher in the VGX-3100 group compared to placebo (p=0.034). In addition, the trial demonstrated the ability of VGX-3100 to clear HPV infection concurrent with regression of CIN2/3 (p=0.003). Post-hoc immune analysis also revealed significantly elevated immune responses in treated patients who had CIN2/3 regression concurrent with HPV clearance when compared to those who did not. This included the presence of CD8+ T cells in the blood exhibiting CD137 expression concurrent with perforin (p=0.032) as well as perforin in addition to granzyme A (p=0.036) as well as an influx of CD8+ T cells into cervical tissue (p=0.008). Conclusion: The successful phase 2b results represent a significant milestone in the development of active immunotherapies to treat HPV-related dysplasia and cancer. The data generated from the trial reveal a significant clinical benefit afforded by treatment with VGX-3100 and underscore the mechanism of action of HPV specific T cells. Thus VGX-3100 has the potential to provide an important alternative or adjunct to surgery in treating CIN 2/3.

506. *In Vivo* Transduction of T Cells: The Future of Immunotherapy?

Inan Edes[1], Irene Schneider[2], Qi Zhou[2], Veit R. Buchholz[3], Christoph Kemna[1], Dirk H. Busch[3], Christian J. Buchholz[2], Wolfgang Uckert[1]
[1]Molecular Cell Biology and Gene Therapy, Humboldt University Berlin, Berlin, Germany, [2]Molecular Biotechnology and Gene Therapy, Paul-Ehrlich-Institute, Langen, Germany, [3]Institute for Medical Microbiology, Immunology and Hygiene, Technical University Munich, Munich, Germany

Genetically engineered T cells carrying antigen-specific chimeric antigen receptor (CAR) or T cell receptor (TCR) genes are highly effective in immunotherapy of leukemia and other solid cancers. At present, the modification of T cells requires an *ex vivo* gene transfer using viral or non-viral vector systems and the subsequent expansion of the engineered T cells to yield therapeutic numbers. However, extended periods of cell culture can lead to an alteration of the T cell phenotype due to antibody and cytokine-driven activation and may negatively influence T cell functionality. To counteract these drawbacks, efforts were undertaken to engineer naive or memory T cells *in vitro*. However, these T cell subsets are already

naturally available *in vivo* and could be used as a target for genetic engineering if it would be feasible to establish protocols that allow *in vivo* transduction of a sufficient number of these T cells to achieve tumor rejection or to control infectious diseases. We developed targetable, injectable gamma-retrovirus vectors capable of transducing specifically $CD8^+$ and $CD4^+$ T cells *in vivo*. We employed the measles virus (MV) envelope glycoproteins hemagglutinin (H) and fusion to generate retroviral vectors (RV), specific for either murine CD8α or murine CD4. To do so, we added single-chain antibody fragment (scFv) coding sequences derived from CD8α or CD4 hybridomas to the carboxy-terminus of the H-protein encoding sequence. We generated CD8α (MVm8) and CD4 (MVm4) targeted RV carrying fluorescence marker or TCR genes. When applying both targeted RV simultaneously in *in vitro* transduction experiments MVm8 and MVm4 exclusively transduced $CD8^+$ or $CD4^+$, respectively, both in T cell lines and primary mouse T cells. By intravenous injection of MVm8 RV, encoding an ovalbumin- (OVA) reactive TCR (OT-I TCR) linked to luciferase, into $Rag2^{-/-}$ mice previously repopulated with CD8 T cells, we demonstrate that MVm8 RV mediates selective *in vivo* transduction of $CD8^+$ T cells. The *in vivo* TCR engineered T cells showed antigen-specific functionality by homing towards sites of antigenic stimulation and persisted long-term, suggesting the formation of memory T cells. Upon *in vivo* T cell engineering, mice were challenged by OVA-expressing *Listeria monocytogenes* in an infection model. Notably, protective immunity could be demonstrated and was dependent on the number of *in vivo* OT-I TCR engineered T cells. In sum, we show that: (i) gamma-retroviruses in combination with a modified MV envelope can be employed as platform for the development of targeting vectors, (ii) these vectors are able to transduce subsets of T cells *in vivo* as shown for murine $CD8^+$ and $CD4^+$ T cells, and (iii) *in vivo* engineered T cells are functional and able to provide protective immunity. Currently, we are investigating the potential of *in vivo* engineered T cells to reject experimentally induced cancer in a mouse model.

507. Chondroitin Sulfate Proteoglycan 4 (CSPG4)-Redirected T Cells Eliminate Glioblastoma-Derived Neurospheres

Serena Pellegatta[1,2], Barbara Savoldo[2], Chuang Su[2], Soldano Ferrone[3], Gaetano Finocchiaro[1], Gianpietro Dotti[2]
[1]Unit of Molecular Neuro-Oncology, Fondazione IRCCS Istituto Neurologico C. Besta, Milan, Italy, [2]Department of Microbiology and Immunology, University of North Carolina, Lineberger Comprehensive Cancer Center, Chapel Hill, NC, [3]Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA

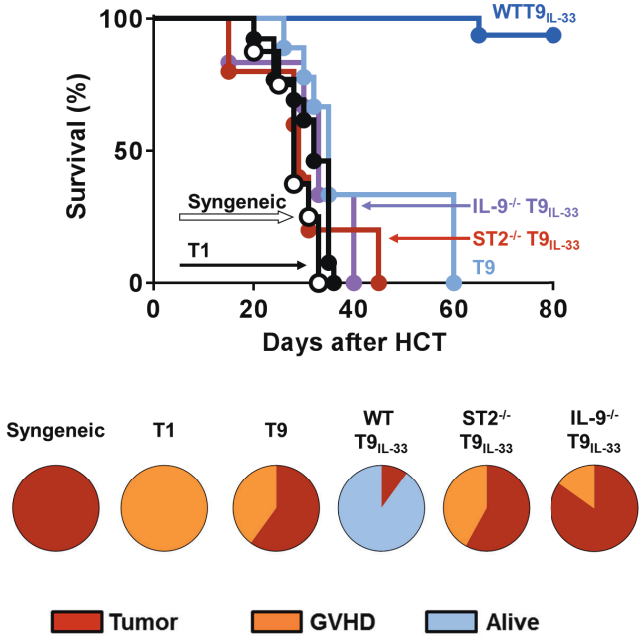
Adoptive therapy with chimeric antigen receptor-redirected T cells (CAR-Ts) remains challenging for the treatment of glioblastoma (GBM) because of the heterogeneous expression of targetable tumor antigens, which leads to the selection of antigen-loss variants. In addition, the emerging role of GBM-derived neurospheres (GBM-NS) as a critical cell subset in causing GBM recurrence highlights the need to eradicate these cells to achieve sustained responses. By exploiting a well-established culture system, we generated and expanded GBM-NS from 23 surgical samples, and tested them using flow cytometry for the expression of CSPG4, a membrane bound tumor antigen found to be overexpressed in GBM by mRNA profiling. We observed that 70% of GBM-NS displayed high expression of CSPG4 (from 71% to 99%), 17% moderate-high expression (from 51% to 70%), and 13% moderate-low expression (<50%). Based on these results, we hypothesized that CSPG4-specific CAR-Ts would represent a broadly applicable strategy for the treatment of GBM. We generated CSPG4.CAR-Ts, encoding the 4-1BB endodomain, from 6 healthy donors and tested them against 19 of the 23 generated GBM-NS that robustly grow in vitro. CSPG4.CAR-Ts efficiently eliminated all GBM-NS, with high to moderate-low CSPG4 expression, in co-culture experiments at E:T ratios ranging from 2:5 to 1:5 (0.2±0.5% and 0.6±0.9% residual GBM-NS, respectively). By contrast, GBM-NS continued to grow in the presence of control T cells (60.7±17.6% residual GBM-NS). CSPG4.CAR-Ts, but not control T cells, also rapidly proliferated in response to GBM-NS as evaluated by the CFSE assay. CSPG4.CAR-Ts showed a Th1 cytokine profile in response to GBM-NS, releasing significantly more IFN-γ (3593.8±1718.1 pg/ml/2x10^5 cells) and IL-2 (258.8±153.3 pg/ml/2x10^5 cells) than control T cells (1.8±2.5 and 0.9±1.2 pg/ml/2x10^5 cells, respectively). For the in vivo experiments we compared CSPG4.CAR-Ts encoding CD28, 4-1BB, or CD28-4-1BB co-stimulatory endodomains. Two GBM-NS with moderate-low and high CSPG4 expression, respectively were selected and transduced to express the FFluciferase gene to monitor the tumor growth by in vivo bioluminescence imaging. Both GBM-NS and T cells were intracranially injected in 5 wks old female nude mice. CSPG4.CAR-Ts were efficient in controlling tumor growth of both moderate-low and high CSPG4-expressing GBM-NS. We observed an early eradication of the tumor mass in high-CSPG4 expressing GBM-NS, and a significant improved survival in both mice bearing high or moderate-low CSPG4-expressing GBM-NS. CAR-Ts encoding 4-1BB were significantly more efficient than those encoding CD28 or CD28-4-1BB in prolonging tumor free survival (p=0.04). Our data suggest that CSPG4 is an attractive target for CAR-Ts in GBM and that the strategy we have shown to be effective in mice has the potential to be translated to a clinical setting.

508. IL-33/ST2 Triggering of IL-9-Secreting T Cells: From Proteomics to Therapeutics

Abdulraouf Ramadan, Jilu Zhang, Mohammad Abu Zaid, Lauren Taylor, Heather A. O'Leary, Reuben Kapur, Helmut Hanenberg, Hal E. Broxmeyer, Mark H. Kaplan, Sophie Paczesny
Indiana University School of Medicine, Indianapolis, IN

As one of the most validated immunotherapies to date, allogeneic hematopoietic cell transplantation (allo-HCT) is a potentially curative option for high-risk hematological malignancies, particularly acute myeloid leukemia (AML). The immunotherapeutic activity of allo-HCT is known as the graft-vs-leukemia (GVL) activity. However, GVL activity is often accompanied by T-cell reactivity to allo-antigens in normal host tissues, which leads to graft-versus-host disease (GVHD), another major cause of death after HCT. Therefore, there is a great unmet need to improve the current process of allo-HCT through increasing the GVL activity and decreasing GVHD. We have shown that an elevated plasma level of soluble (s)ST2 in HCT patients is a risk factor for severe GVHD. ST2 blockade reduces sST2-producing T cells while maintaining protective membrane (m)ST2-expressing T cells such as type 2 T cells and regulatory T cells during aGVHD. A novel IL-9 producing T helper subset, Th9, expresses mST2. Furthermore, Th9 cells and IL-9 producing CD8 cytotoxic (Tc9) cells have higher antitumor activity than Th1 and Tc1 cells in melanoma models. Interestingly, we found that the addition of IL-33 during T9 differentiation ($T9_{IL-33}$) increased expression of mST2 and PU.1, a transcription factor that promotes IL-9 production in both CD4 and CD8 T cells. Adoptive transfer of $T9_{IL-33}$ cells with bone marrow cells in a murine model of HCT resulted in less severe GVHD compared to transfer of $T9_{IL-33}$ cells generated from $ST2^{-/-}$ or $IL\text{-}9^{-/-}$ T cells. Furthermore, cytolytic molecules implicated in anti-leukemic activity (granzyme B and perforin) were upregulated in WT $T9_{IL-33}$ cells while $ST2^{-/-}$ $T9_{IL-33}$ cells did not. WT $T9_{IL-33}$ cells also exhibited higher anti-leukemic activity when cultured with a retrovirally transduced MLL-AF9 leukemic cells in comparison to $ST2^{-/-}$ $T9_{IL-33}$ in in vitro cytolytic assays. *In vivo* GVL experiments with MLL-AF9 AML and adoptive transfer of $T9_{IL-33}$ cells resulted in increased survival compared to syngeneic mice, allo-HCT mice

transferred with T1 cells, or T9 cells or $T9_{IL-33}$ cells generated from $ST2^{-/-}$ or $IL\text{-}9^{-/-}$ T cells (**Figure 1**). Human T9 cells are poorly studied. Here we demonstrate that IL-33 has the same impact on human T cells through enhancing IL-9 and Granzyme B production compared to T9 cells as well as demonstrated higher *in vitro* anti-leukemic cytolytic activity when incubated with MOLM14, an aggressive AML tumor cell line expressing FLT3/ITD mutations. Importantly, CD8α expression was upregulated in WT $T9_{IL-33}$ (both CD4 and CD8) cells in comparison to $ST2^{-/-}$ $T9_{IL-33}$ cells, and CD8α blockade with neutralizing antibody during allogeneic specific $T9_{IL-33}$ differentiation reduced cytotoxicity of both murine $T9_{IL-33}$, and human $T9_{IL-33}$ cells as compared to the cell blocked with isotype control, suggesting that CD8α was associated with MHC-restricted cytolytic activity in $T9_{IL-33}$ cells. Altogether, our observations demonstrated that adoptive transfer of $T9_{IL-33}$ cells represents a promising cellular therapy following HCT.



509. Regulated Expression of IL-12 as Gene Therapy Concomitant with Blockade of PD-1 for Treatment of Glioma

John A. Barrett[1], Hongliang Cai[1], John Miao[1], Margaret Murray[2], Paul Gonzales[2], Suma Krishnan[3], Francois Lebel[4], Laurence J. N. Cooper[4]

[1]Translational Research, Ziopharm Oncology, Boston, MA, [2]Research, Translational Drug Development, Scottsdale, AZ, [3]Research and Development, Intrexon Corporation, Germantown, MD, [4]Research and Development, Ziopharm Oncology, Boston, MA

The utility of immunotherapy in the treatment of glioma may be improved through combination therapies that enhance cytotoxic immune-activation while concomitantly reducing immunosuppression. We provide data in mice to support evaluation of combining controlled local interleukin 12 (IL-12) administration and blockade of programmed cell death protein 1 (PD-1) in humans. To circumvent challenges surrounding the uncontrolled activation, we have implemented clinical trials using a replication-incompetent adenovirus engineered to conditionally express IL-12 (Ad-RTS-IL-12), via our RheoSwitch Therapeutic System® (RTS®) gene switch. When directly injected intra-tumorally in pre-clinical or clinical studies, IL-12 expression is "off" when devoid of the activator ligand (veledimex, V) and IL-12 production is turned "on" (in a dose-dependent manner) by oral administration of veledimex. PD-1 inhibitors using therapeutic monoclonal antibodies (mAbs) demonstrate an ability to reverse tumor immunosuppression and are effective in the treatment of some cancers. In the present pre-clinical study we assessed the effects of Ad-RTS-mIL-12+veledimex (Ad+V) alone, Ad $5x10^9$ viral particles (vp) + V 10-30mg/m^2/day for 14 days or in combination with the antiPD-1-specific mAb RMP1-14 (antiPD-1, 7.5 & 15.0 mg/m^2 for 4/day for 5 days i.p.) in the orthotopic GL-261 mouse model. All mice without treatment succumb to disease progression by Day 35. Eighty days after immunotherapy, 70-80% receiving Ad +V monotherapy survived, 30-40% receiving antiPD-1 monotherapy survived and 100% receiving Ad +V 30 mg/m^2 + antiPD-1 15.0 mg/m^2 combination survived. There was an increase in tumor IL-12 (100 pg/mg) which was 15-times greater than that of plasma peak 5 days after Ad +V. Furthermore, the combination of Ad +V+antiPD-1 sustained peak IL-12 levels in tumor which was associated with a 100-150% increase of activated T cells in the spleen compared with the minimal changes observed with either immunotherapy alone. In addition, there was an additive reduction in regulatory T cells (FOXP3) compared with monotherapies. In summary we demonstrate that controlled local immunostimulation with IL-12 combined with inhibition of PD-1 is an attractive approach for the treatment of glioma. Since both Ad-RTS-IL-12 and mAb blocking PD-1 are clinically available, these data provide impetus for evaluating this combination immunotherapy in humans.

510. Treating Epithelial and Metastatic Malignancies with Glycoform-Specific Chimeric Antigen Receptors

Avery D. Posey, Alina Boestaneau, Laura A. Johnson, Carl H. June

Center for Cellular Immunotherapies, University of Pennsylvania, Philadelphia, PA

Cancer-specific glycosylation deficiencies generating Tn or sTn O-linked glycans are found in >80% of the most lethal cancers (lung, prostate, breast, colon, ovary, pancreas). These hypoglycosylated cancer glycoantigens decrease cell-cell interactions and increase the mobility and metastatic potential of tumor cells. We developed a CAR specific to the Tn/sTn antigen of the oncogene Mucin-1 (MUC1), which is highly upregulated in many epithelial cancers and is heavily O- and N-glycosylated. Our CAR uses the scFv sequence derived from the 5E5 mAb and the endodomains of CD3zeta and 4-1BB that promote T cell activation and co-stimulation, respectively. We have shown that the 5E5 mAb and the 5E5 CAR are specific to the cancer-specific glycoform of MUC1 but not normal MUC1 through *in vitro* binding and cytokine assays and that the 5E5 CAR is non-reactive to normal human primary cells. We demonstrated that the 5E5 CAR T cells are responsive to ovarian, breast, lung, pancreatic, and leukemia cancers and developed NSG mouse xenograft models using Jurkat leukemia cell line and Hs766T metastatic pancreatic cell line that show rapid clearance of established tumors by 5E5 CAR T cells and prolonged survival of tumor-bearing mice. Restoring normal glycosylation in Jurkat leukemia cells abolishes the expression of the hypoglycosylated form of Muc1 and also prevents 5E5 CAR T cell cytotoxicity, further highlighting the specificity of the 5E5 CAR for the cancer-specific glycoform of MUC1. Infusion of human MUC1-transgenic mice with murine 5E5 CAR T cells showed an exquisite safety profile, while infusion of normal glycoform MUC1-specific murine CAR T cells (HMFG1 CAR, a previously described MUC1-specific CAR) demonstrate extreme safety concerns. These results demonstrate that targeting the cancer-specific glycoform of MUC1

provides a safe, yet potent cancer-specific immunotherapy for treating most epithelial malignancies and holds the potential to eradicate metastatic disease.

511. Engineered Donor Marrow Macrophages Phagocytose Cancer Cells and Aggressively Shrink Solid Tumor Xenografts Compared to Tumor Associated Macrophages

Cory Alvey, Kyle Spinler, Manu Tewari, Dennis Discher
Systems Pharmacology and Translational Therapeutic, University of Pennsylvania, Phiadelphia, PA

Cell-based immunotherapies such as those based on engineered T-cells appear safe and often effective against liquid tumors. In solid tumors, macrophages are typically abundant, but the density of tumor associated macrophages (TAMs) correlates with poor clinical outcomes as they promote tumor growth, immunosuppression, and are nonphagocytic. In our studies, less differentiated donor marrow phagocytes are engineered to target tumors and selectively phagocytose cancer cells. Xenograft tumors were made on the flanks of NSG mice using a tdTomato human lung carcinoma cell line (A549). Systemic injections of anti-human IgG (anti-hum) with large tumors (~70 mm^2) showed no effect on tumor growth. However, systemic injection of bone marrow from donor NSG mice together with biweekly anti-hum treatments effectively stopped growth of the solid tumors. Replacing anti-hum with a non-specific antibody had no effect on tumor growth. Based on tdTomato signal intensity within macrophages isolated from tumors, 10-fold more donor macrophages are phagocytic compared to resident TAMs (2-3% are phagocytic). Since cancer cells express on their surface 'self' markers that limit the phagocytosis of these cells, we inhibited the 'self' receptors on the injected donor phagocytes prior to systemic injection of the donor marrow. This combination of 'self'-receptor inhibition with anti-hum causes a rapid decrease in tumor burden, shrinking tumors by ~40% in just 10 days compared to a similar growth of untreated tumors in the same time period. The anti-hum injection was again necessary as injection of a non-specific antibody failed to affect tumor growth. Tumor analysis showed that >85% of macrophages that were 'self'-receptor inhibited had phagocytosed the tdTomato A549 cells, which is ~30-fold greater than resident macrophages. Importantly, these cell therapy treatments appear safe with no significant decreases in hematocrit or platelets, which is unlike the anemia that has been reported upon systemic injection of 'self' inhibitors. Our results thus suggest that therapies based on engineered macrophages can be safe and effective against solid tumors if three requirements are met: a phagocytic phenotype, target opsonization, and inhibition of 'self' signaling.

512. The Cytokine Release Syndrome Crucially Contributes to the Anti-Leukemic Effects of CD44v6 CAR-T Cells

Margherita Norelli[1], Monica Casucci[2], Barbara Camisa[2], Laura Falcone[2], Aurore Saudemont[3], Luigi Naldini[1], Fabio Ciceri[1], Claudio Bordignon[1], Chiara Bonini[1], Attilio Bondanza[1]
[1]Università Vita-Salute San Raffaele, Milan, Italy, [2]Immunology, Tranplantation, and Infectious Disease, San Raffaele Scientific Institute, Milan, Italy, [3]Antony Nolan Scientific Institute, London, United Kingdom

Background: Despite the remarkable clinical results of CD19 CAR-T cells in B-cell leukemias, their long-term efficacy is limited by the emergence of CD19-loss escape variants. Moreover, whether the cytokine release syndrome (CRS) is necessary for durable remissions is a matter of debate. Currently available xenograft models in NSG mice are not suited for studying the antitumor effects of CAR-T cells beyond 3-4 weeks, because of xenograft-versus-host disease (X-GVHD). Moreover, since NSG mice lack functional myeloid cells, the CRS does not develop.**Aim**: To verify whether the CRS contributes to the antileukemic effects of CAR in an innovative xenotolerant mouse model.**Results**: NSG mice triple transgenic for human IL-3, GM-CSF and SCF (NSG-3GS) were sub-lethally irradiated and injected intra-liver with human HSCs soon after birth, enabling an accelerated and better balanced lympho-hematopoietic reconstitution compared with NSG mice. Reconstituting human T cells were single CD4+/CD8+ T cells, representing all memory sub-populations. After ex vivo isolation and activation with CD3/CD28-beads and IL-7/IL-15, NSG-3GS T cells were transduced with a CD44v6 CAR, retaining an early-differentiated (stem-cell/central-memory) phenotype and full antitumor functionality against acute myeloid leukemia (AML). NSG-3GS-derived CD44v6 CAR T cells were subsequently infused in tumor-bearing secondary recipients previously humanized with autologous HSCs. CAR-T cells persisted in vivo for at least 6 months and mediated durable leukemia remissions ($P<0.001$ vs controls) in the absence of X-GVHD. Tumor clearance associated with an acute malaise syndrome, characterized by high fevers and a surge in human IL-6 levels, which was lethal in 30% of the mice. Differently from CD19 CAR-T cells, the CRS by CD44v6 CAR-T cells was significantly anticipated (3 vs 8 days), coinciding with human CD44v6+ monocyte depletion. In humanized mice, previous myeloid-cell depletion by clodronate administration completely prevented this syndrome, but associated with late leukemia relapses. Conversely, mice developing the CRS entered a state of durable and profound remission, as demonstrated by prolonged observation times and secondary transplantation.**Conclusions**: By using an innovative xenotolerant mouse model, we have demonstrated that the CRS is needed for sustained antileukemic effects by CD44v6 CAR-T cells.

Cancer-Oncolytic DNA Viruses

513. Armed-Ad Gene Therapy Expressing PD-L1 Minibody Enhances the Anti-Tumor Effect of Adoptively Transferred Chimeric Antigen Receptor T-Cells for Solid Tumor Treatment

Amanda Rosewell Shaw, Kiyonori Tanoue, Norihiro Watanabe, Caroline Porter, Malcolm Brenner, Masataka Suzuki
Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

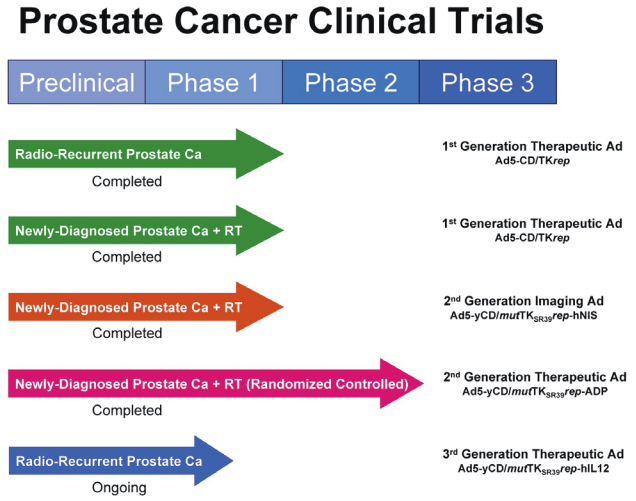
Intratumoral treatment with oncolytic adenoviral vectors expressing an immunomodulatory molecule (Armed Onc.Ads) is safe and has shown some clinical benefit in patients with solid tumors. However, local treatment with Armed Onc.Ad has limited anti-tumor effect against metastasized tumors. T cells modified with tumor-directed chimeric antigen receptors (CARs) have shown promise for the systemic treatment of hematological malignancies, but have been less effective in treating solid tumors. Major reasons for this failure include lack of T-cell migration into solid tumors and the inhibitory microenvironments (e.g. PD-L1) at the tumor site. Recent clinical trials with immune-checkpoint inhibitors (e.g. anti-PD-L1 antibody) have broadly enhanced antitumor immunity by improving tumor-specific T cell responses. We therefore hypothesized that an Armed Onc.Ad expressing anti-PD-L1 antibody could enable the blockade of PD-1:PD-L1 interaction between CAR T-cells and cancer cells at the tumor site, and that combining these treatment modalities may have potent and synergistic anti-tumor effect in solid tumors. In this study, we confirmed that PD-L1 is upregulated on squamous cell carcinoma (Pre: 20%, Post: 98%), and prostate cancer cells (Pre: 60%, Post: 100%), in the presence of co-cultured IFNγ producing HER2.CAR T-cells, a population of effectors that we have safely

administered to patients with solid tumors. Additionally, HER2.CAR T-cells also express PD-1 upon activation (Pre: 1%, Post: 30%) at 24 hr co-culture. Cancer cell killing by HER2.CAR T-cells was enhanced 2-fold in the presence of anti-PD-L1 IgG *in vitro*. We constructed a helper-dependent Ad (HDAd: no autonomous replication and no lytic effect) expressing PD-L1 minibody (fused anti-human PD-L1 scFv with human IgG1 constant region (HD*PDL1 mini*)) and confirmed by western blot that the minibody is secreted in a dose-dependent manner by cancer cells infected with HD*PDL1 mini*. The avidity of HDAd derived PD-L1 minibody for PD-L1 is similar to commercial anti-PD-L1 IgG, and the reagent enhanced HER2.CAR T-cell killing of target cells by 3-fold compared to HER2.CAR T-cells with control HDAd. Co-infection of Onc.Ad with HD*PDL1 mini* induces the same oncolytic effect as previously reported, but also induces replication of HD*PDL1 mini* and thereby markedly increases production of PD-L1 minibody *in vitro* and *in vivo*. Intratumoral co-administration of HD*PDL1 mini* with Onc.Ad induces 4-fold greater expansion of adoptively transferred HER2.CAR T-cells at the tumor site compared to combinatorial treatment of Onc.Ad (without HD*PDL1 mini*). Our therapy extended median survival from 35 to 70 days compared to HER2.CAR T-cell alone. Our "all-in-one" strategy also appears superior to infusion of anti-PD-L1 IgG, inducing 3-fold greater expansion of HER2.CAR T-cells at the tumor site. Hence, combining HD*PDL1 mini* with Onc.Ad overcomes a significant immune defense mechanism inhibiting CAR T-cell treatment of solid tumors, and we are now investigating its longer-term benefits.

514. Clinical Trials of Oncolytic Adenovirus-Mediated Gene Therapy

Svend O. Freytag[1], Benjamin Movsas[1], Hans Stricker[2]
[1]*Radiation Oncology, Henry Ford Health System, Detroit, MI,* [2]*Urology, Henry Ford Health System, Detroit, MI*

For the past 20 years we have been developing a multi-modal, gene therapy-based approach for the treatment of cancer. Our therapeutic platform utilizes an oncolytic adenovirus to deliver a pair of cytotoxic "suicide" genes to the tumor. The oncolytic adenovirus generates an anti-tumor effect by replicating in, and destroying, cancer cells (*oncolytic viral therapy*). The therapeutic effect of the oncolytic adenovirus is enhanced by invoking two cytotoxic gene systems, which render malignant cells sensitive to specific pharmacological agents (*cytotoxic gene therapy*) and sensitize them to ionizing radiation (*radiosensitization*). The combined effects of these three modalities result in significant tumor cell destruction and release of tumor antigens, which, when coupled with the robust immune response elicited by the oncolytic adenovirus, generate an environment suitable for tumor antigen processing and cross-presentation that may culminate in the development of anti-tumor immunity. Under four Investigational New Drug (IND) applications, we have evaluated the toxicity and efficacy of our multi-modal approach in six phase 1/2 trials of prostate cancer using four different oncolytic adenoviruses (Figure 1). These studies have demonstrated that our approach is safe and evidence of efficacy has been obtained. With respect to safety, there were no dose-limiting toxicities (DLTs) or treatment-related serious adverse events (SAEs) in over 100 patients treated to date. Only 6% of the adverse events were ≥ grade 3 with the vast majority being transient and asymptomatic. With respect to efficacy, in the locally recurrent setting following radiation failure, men who received the gene therapy exhibited a significant lengthening of PSA doubling time (PSADT), a surrogate endpoint that is highly prognostic for the development of distant metastases and prostate cancer-specific mortality. At 7 years, there was an improvement in disease-specific survival relative to well-matched historical controls. When combined with radiation therapy in the newly-diagnosed setting, the gene therapy reduced by 42% the percentage of men who had a positive prostate biopsy 2 years after treatment in a prospective, randomized, controlled phase 2 trial. An even greater treatment effect (i.e., 60% reduction) was observed in men with a low tumor burden at baseline. Together, the results demonstrate that oncolytic adenovirus-mediated gene therapy has the potential to improve tumor control in multiple settings of prostate cancer. To improve our approach further, we have generated a new oncolytic adenovirus expressing IL-12, which is now in the clinic. Updated results of our randomized controlled phase 2 trial (median follow-up 6 years) will be presented along with early results of our ongoing phase 1 trial with the oncolytic adenovirus expressing IL-12.



515. Oncolytic Adenovirus Armed with Cytokines Enhances CAR-T Cell Efficacy in Pancreatic Tumor Model

Keisuke Watanabe[1], John Scholler[1], Sonia Guedan[1], Shannon E. McGettigan[1], Yanping Luo[1], Siri Tähtinen[2], Suvi Parviainen[3], Riikka Havunen[2], Mikko Siurala[3], Akseli Hemminki[2], Carl H. June[1]
[1]*Abramson Cancer Center and the Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA,* [2]*Cancer Gene Therapy Group, Faculty of Medicine, University of Helsinki, Helsinki, Finland,* [3]*TILT Biotherapeutics Ltd, Helsinki, Finland*

Background: Chimeric antigen receptor T-cell (CAR-T) therapy has shown significant efficacy in hematological malignancies, however, the efficacy against solid tumors remains limited. Immunosuppression caused by tumor microenvironment or poor infiltration of transferred T cells can restrict T cell efficacy. We propose that oncolytic Adenovirus (O-Ad) armed with cytokines improves the efficacy of adoptive T cell therapy by modulating the tumor environment. **Aim:** Here we aimed to study if O-Ad armed with cytokines can 1) cause direct lysis of pancreatic cancer cells, 2) enhance killing by CAR-T cells. 3) enhance infiltration and persistence of CAR-T cells in the context of solid tumors. **Methods:** We targeted pancreatic tumor cell lines by mesothelin specific CAR-T cells (SS1-BBz CAR-T) in combination with O-Ad; Adv-5/3-d24-IL2 or Adv-5/3-d24-TNF-IL2, which consist of an adenovirus serotype 5 nucleic acid backbone, a serotype 5/3 chimeric fiber knob, a 24-bp deletion (d24) in the Rb binding constant region 2 of E1 promoter, an E2F tumor specific promoter and cytokines (interleukin 2 or tumor necrosis factor alpha or both). **Results:** Pancreatic tumor cell lines used in this study; ASPC1, BXPC3, Capan2 expressed the Adv-serotype 3 receptor, DSG2. We also confirmed that O-Ad does not have adverse effects on T cell viability and proliferation even at

high titer (1,000vp/cell) in an in *vitro* assay. To look at the efficacy of the O-Ad and CAR-T combination, we performed a killing assay. O-Ad clearly enhanced killing by CAR-T cells in luciferase based assay. We also used xCELLigence real time cell analyzer (RTCA) for kinetic analysis of killing. In combination with O-Ad, more rapid killing kinetics by CAR-T cells were observed especially in lower E:T ratio. To look at the impact of the combination in *vivo*, subcutaneous ASPC1-CBG-GFP in NSG mice were treated with CAR-T alone or in combination with intra-tumoral injection of O-Ad. O-Ad showed significant synergy with CAR-T in luciferase based photon assay. And higher number of T cells was observed in spleen in the O-Ad armed with IL2 combination group. **Conclusions:** These results suggest that combination therapy of O-Ad armed with cytokine(s) and CAR-T cells is effective against solid tumors by enhancing T cell activity.

516. A Novel Oncolytic Adenovirus Expressing Tumor Microenvironment Stimulators to Evoke and Facilitate Anti-Tumor Immune Responses

Jessica Wenthe[1], Emma Eriksson[1], Ioanna Milenova[1], Rafael Moreno[2], Ramon Alemany[2], Angelica Loskog[1,3]
[1]Dept of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden, [2]IDIBELL-Institut Català d'Oncologia, L'Hospitalet de Llobregat'Hospitalet de Llobregat, Barcelona, Spain, [3]Lokon Pharma AB, Uppsala, Sweden

Immunotherapy is becoming a cornerstone in cancer treatment of many indications. So far, pancreatic cancer has shown little response to so called checkpoint blockade antibodies. However, animal data suggests that activating immunotherapies releases the effect of checkpoint blockade also in pancreatic cancer. The tumor microenvironment (TEM) supports the growth of the tumor cells and consists of stroma cells, fibroblasts (stellate), blood vessels and immune cells. In some tumor lesions, such as those in pancreatic cancer, the TEM is dense and comprises most of the lesion. The TEM regulates immune activity via its high content of M2 macrophages, myeloid-derived suppressor cells and T regulatory cells. Further, the dysfunctional blood vessels in lesions are not optimal for recruiting lymphocytes. With these aspects in mind, LOAd703 was developed. LOAd703 is an oncolytic adenovirus carrying TEM modulators. LOAd703 contains E2F binding sites that control the expression of an E1a gene deleted at the pRB-binding domain to achieve replication conditional on a dysfunctional, hyperphosphorylated retinoblastoma pathway. The genome was further altered by removing E3-6.7K and gp19K, changing the serotype 5 fiber to a serotype 35 fiber to target CD46 expressed by most tumors, as well as by adding a CMV-driven transgene cassette with the human transgenes for a trimerized, membrane-bound (TMZ) CD40 ligand (TMZ-CD40L) and the full length 4-1BB ligand (4-1BBL). Hence, the transgenes will be expressed in both tumor and stroma while oncolysis is initiated in the tumor cells. We demonstrate herein that LOAd703 infection of a panel of pancreatic cancer cell lines efficiently induced tumor cell death within 48-72 hrs post infection as evaluated with MTS viability assay. However, LOAd703 infection of dendritic cells demonstrated an increased maturation and production of cytokines and chemokines as shown by flow cytometry and ProSeek. These DCs could in turn potently activate and promote expansion of both T- and NK cells. Further, LOAd703 infection of endothelial cells (HUVEC) induced upregulation of molecules involved in lymphocyte attachment, rolling and transmigration as shown by flow cytometry. Finally, stellate cells down regulated many factors that otherwise promote tumor growth such as Spp1, Gal-3, HGF, PlGF, TGFb and collagen type I in response to the transgenes (ProSeek). In murine xenograft and syngeneic models, LOAd703 was able to control tumor growth and could be combined with gemcitabine, which is standard of care treatment for pancreatic cancer. mLOAd703-infected tumor cells could induce immunity in a syngeneic model (Panc02/C57Bl6). In conclusion, LOAd703 is a novel oncolytic virus that targets both the tumor and its TME. A clinical trial is underway to elucidate its effect in pancreatic cancer.

517. Anti-Tumor Immunity Is Elicited by a Conditionally Replicative Adenovirus in Canine Osteosarcoma

Bruce F. Smith[1], Payal Agarwal[1], Elizabeth Gammon[1], David T. Curiel[2], Igor Dmitriev[3], Elena Kashentseva[4]
[1]Scott-Ritchey Research Center, Auburn University, Auburn University, AL, [2]Biologic Therapeutics Center, Washington University, St. Louis, MO, [3]Department of Radiation Oncology, Washington University School of Medicine, St Louis, MO, [4]Department of Radiation Oncology, Washington University School of Medicine, St. Louis, MO

Conditionally replicative viruses have been proposed as a potential therapy for a wide variety of tumors. Initially, these viruses were thought to exert their anti-tumor properties through the lysis of infected tumor cells. However, there is the potential that oncolytic viruses may also elicit anti-tumor immune responses in both the context of viral antigens and potentially tumor antigens. Canine osteosarcoma is an excellent intermediate animal model in which the ability of a conditionally replicative adenovirus (CRAd) to induce anti-tumor immunization can be uniquely ascertained. This tumor exhibits many of the same characteristics of the human disease, including metastasis to the lungs. A CRAd based on canine adenovirus type 2 with the E1 gene driven by the osteocalcin promoter was administered to canine patients with confirmed osteosarcoma of the appendicular skeleton. Cellular and humoral immune responses, as well as cytokine profiles and regulatory T-cells were assessed before and after treatment. The number of dogs surviving past one year was approximately 20%. No changes were identified in levels of cytotoxic T-cells after therapy, when using autologous tumor cells as targets. Likewise, no significant changes in a panel of cytokines were observed. However, evaluation of humoral immune responses by western blot and flow cytometry, using autologous tumor, indicated that all dogs in the study had pre-existing antibodies to their own tumors and that these antibodies increased post treatment. This increase consisted of both an increase in the amount of antibody present and the recognition of new antigenic determinants. Serum from treated dogs also cross-reacted with tumor cells from unrelated dogs as well as a canine melanoma cell line. These results indicate that treatment with a CRAd does result in active immunization against the tumor, however it is unclear if the observed immune responses play any role in patient outcomes. Based upon these findings, we can now hypothesize that manipulation of these immune responses by including immune modulating genetic constructs in the CRAd may serve to alter the type and efficacy of the patient's immune response and enhance survival.

518. Tumor Associated Macrophages Mitigate Oncolytic Herpes Simplex Virus Efficacy in Part Through TGF-β Signaling

Nicholas Denton[1], Chun-Yu Chen[1], Brian Hutzen[1], Jennifer Leddon[1], Pin-Yi Wang[1], Thomas Scott[1], Mark Currier[1], Tim Eubank[2], Timothy P. Cripe[1]
[1]Childhood Cancer, Nationwide Children's Hospital, Columbus, OH, [2]Pulmonary, Allergy, Critical Care & Sleep Medicine, The Ohio State University, Columbus, OH

Introduction: Ewing sarcoma is a highly aggressive bone tumor that is often lethal following recurrence or metastasis. Oncolytic viruses (OVs), such as the rRp450 herpes simplex virus, are promising

anticancer therapeutics designed to selectively replicate in cancer cells. While OV anti-tumor efficacy is partially caused by direct infection and lysis of cancer cells, stimulation of an anti-cancer immune response also contributes to virus-mediated efficacy. Immunologic responses to infections are known to be modulated by macrophages via various cytokines and chemokines and it is now appreciated that tumors are replete with tumor associated macrophages (TAMs). M2 alternatively activated macrophages in particular express pro-tumor immunosuppressive cytokines, such as IL-10 and TGF-β. We hypothesize that TAMs reduce therapeutic efficacy by producing an immunosuppressive tumor microenvironment via IL-10 and TGF-β signaling. **Research methods:** Human Ewing sarcoma xenografts were implanted into athymic nude mice and macrophages were depleted using liposomal clodronate (encapsula) prior to intratumoral injection of rRp450 oncolytic HSV. Tumors were allowed to grow for tumor progression. *In vitro* cytotoxicity was determined using MTS assay. *In vitro* and *in vivo* virus replication was determined through plaque assay. Bone marrow derived macrophages were cocultured with Ewing sarcoma cell lines and harvested for flow cytometry and PCR analysis of tumor inflammatory signaling and M1/M2 macrophage gene profiles. F4/80+ tumor associated macrophages were extracted from tumors using magnetic bead separation. TGF-β cytokine superfamily receptor signaling was inhibited with A83-01 (Sigma-Aldrich) small molecule treatment prior to intratumoral injection of rRp450. **Results:** Macrophage depletion significantly inhibited tumorigenesis and enhanced rRp450 anti-tumor efficacy in A673, but not 5838 Ewing sarcoma xenografts. No change in virus titer was observed in the macrophage depleted tumors, suggesting the effect isn't due to enhanced virus replication. Macrophages cocultured with A673 cells had higher expression of M2 pro-tumor macrophage genes than macrophages cocultured with 5838 cells. Macrophages in A673 tumors also demonstrated higher expression of IL-10 and TGF-β than 5838 tumor associated macrophages. Inhibition of TGF-β signaling enhanced rRp450 oncolytic virus anti-tumor efficacy in A673 tumors.**Conclusions:** Macrophages play a significant role in mitigating OV anti-tumor efficacy. Specifically, our tumor models that promote M2 macrophage polarization are significantly more resistant to oncolytic virus therapy, in part due to TGF-β signaling. Our results suggest that the combination of oncolytic virus therapy with a macrophage modulatory therapy will improve OV anti-tumor efficacy in patients with highly immunosuppressive tumors.

519. Intravenous Application of CXCR4 Targeted Conditionally Replicative Adenovirus with Fiber and Hexon Modifications to Pancreatic Cancer

Jing Li Huang[1], Christopher LaRocca[1], Kari Jacobsen[1], Sergey Kaliberov[2], David T. Curiel[2], Julia Davydova[1,3], Masato Yamamoto[1,3]
[1]Department of Surgery, University of Minnesota, Minneapolis, MN, [2]Department of Radiation Oncology, Washington University, St. Louis, MO, [3]Masonic Cancer Center, Minneapolis, MN

Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer-related death in the United States. Most newly diagnosed patients have unresectable disease due to local spread or early aggressive metastasis and have a medial survival of 6 months. Cancer stem cells (CSC) have been found to be involved in the development of metastatic disease. CSC markers such as C-X-C chemokine receptor 4 (CXCR4) and CD133 are overexpressed on the invasive front of PDAC. In this study, we tested a CXCR4-targeted conditionally replicative adenovirus (CRAd) with fiber and hexon modifications toward systemic treatment of PDAC. Due to poor infectivity of CRAd to PDAC cells, our vector contains a modified chimeric fiber which incorporates adenovirus 5 shaft and adenovirus 3 knob (Ad5/3) in order to target PDAC cells via an alternative receptor. The CXCR-4 targeted CRAd with Ad5/3 fiber modification showed strong replication in CD133(+) primary cells isolated from human PDAC tumors. The virus copy number in CD133(+) cells was more than 5 times higher than in CD133(-) cells ($p<0.0005$). Virus replication capability as measured by luciferase expression from Ad major late promoter was more than double in CD133(+) cells ($p<0.05$). This data indicates Ad5/3 modified oncolytic adenovirus shows enhanced replication in CSC-rich CD133(+) population.To overcome the issue of adenoviral sequestration upon systemic injection by non-target organs (ie liver, lung), our vector was additionally modified by replacing the Ad5 hexon hypervariable regions 5 and 7 with those from Ad3. We assessed the hexon modified adenovirus in a human Ad replication-permissive model that is most frequently used in IND-directed distribution/toxicology studies, the Syrian hamster. When the virus copy number was assayed after systemic injection via the saphenous vein, the hexon modified virus showed decreased liver sequestration, while showing no difference in the lungs. On the other hand, the major late promoter driven luciferase expression showed much more significant reductions in both liver and lung. The difference between the assays may indicate more contribution of macrophages to the copy number. Next, antitumor effect of the fiber and hexon modified CRAd was assessed in patient-derived xenografts. After intratumoral administration, the fiber-modified CRAd showed antitumor effect compared to the untreated group regardless of the presence (AdCXCR4 E1 F5/3 H5/H3) or absence of (AdCXCR4 E1 F5/3) hexon modification ($p<0.01$ and 0.05, respectively). On the contrary, with intravenous infection, only the fiber and hexon modified CRAd (AdCXCR4 E1 F5/3 H5/H3) showed significant antitumor effect. We believe that our CXCR4-targeted chimeric fiber and hexon modified CRAd may be employed in advanced PDAC, and that hexon modification of oncolytic adenovirus can be advantageous with systemic administration in the clinical setting.

520. Arming a Tumor Targeted Oncolytic HSV Vector with MMP9 for Enhanced Distribution and Killing Activity

Paola Sette[1], Aofei Li[2], Marco Marzulli[3], Daniela Leronni[1], Mingdi Zhang[3], William Goins[3], Balveen Kaur[4], Chelsea Bolyard[5], Nduka Amankulor[1], Joseph Glorioso[3], Paola Grandi[1]
[1]Neurosurgery, University of Pittsburgh, Pittsburgh, PA, [2]Tsinghua University School of Medicine, Beijin, China, [3]Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA, [4]Neurosurgery, Ohio State University, Columbus, PA, [5]Neurosurgery, Ohio State University, Pittsburgh, OH

Early phase human clinical trials using several versions of oncolytic herpes simplex virus type 1 (oHSV) have shown promise in the treatment of glioblastoma multiforme, but efficacy has been limited. Impediments to oHSV therapy include poor virus spread due in part to the tumor extracellular matrix, and insufficient replication in tumor cells as a result of attenuating mutations. Thus, our central goal was to improve oncolytic vector delivery, replication, and spread while maintaining safety and tumor specificity. We had already developed a new class of two stage tumor targeted oHSV combining (i) selective infection through tumor-specific receptors and (ii) selective replication based on differential expression of microRNAs (miRs) in tumor and normal cells. We further modified our vector by arming it with the matrix metalloproteinase 9 (MMP9) as a means to reduce vector trapping in the tumor extracellular matrix. MMP9 degrades collagen type IV, a major component of the extracellular matrix (ECM) and basement membranes of glioblastomas. Here we show that: (i) MMP9 expression improves vector spread in GBM neurospheres in vitro; (ii) MMP9 enhances the oncolytic therapeutic efficacy in animal model of human GBM without compromising vector safety.

Cell Therapies

521. Platelets Transfusion New Role as Brain Therapeutics for Acute Neuronopathic Gaucher Disease

Mei Dai[1], Xiaohong Wang[1], Benjamin Liou[1], Venette Inskeep[1], Gregory Grabowski[1,2], Ying Sun[1,2], Roscoe Brady[3], Dao Pan[1,2]
[1]Cincinnati Children's Hospital Medical Center, Cincinnati, OH, [2]Department of Pediatrics, University of Cincinnati School of Medicine, Cincinnati, OH, [3]National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesd, MD

There has no effective treatment for neuronopathic Gaucher Disease (nGD) due to the difficulty of therapeutics to cross the blood-brain-barrier. Platelets are blood elements that contain cytoplasmic secretory vesicles containing proteins involved in hemostasis, inflammation and angiogenesis. We have recently shown that platelets/megakaryocytes could serve as efficient and protective depots for lysosomal enzyme generation and distribution. In this study the potential therapeutic benefits of platelet transfusion in treating neuronopathic GD (nGD) was evaluated using a murine model of acute nGD (4L;C), which resembles types 2 and 3 Gaucher disease. Platelets containing significant amounts of wild type acid ß-glucosidase enzyme (GCase) were isolated from GFP mice and transfused weekly into 4L;C mice for four times beginning at 21 days of age, resulting in an average 10-13% of donor-derived GFP^+ platelets in circulation with half-life of 52 hr. Importantly, moderate platelet transfusion led to significantly increase of life-span in 4L;C mice from 47 days to 55 days with reduction of splenomegaly. The accumulation of glycosylsphingosin was significantly reduced in spleen, liver and brain. Moreover, the abnormal walk patterns with splaying of hindlimbs exhibited in 4L;C mice were improved significantly in platelet-treated mice, indicating amelioration of motor neuron dysfunction and hindlimb paresis. The improvement of short-term memory deficit in treated 4L;C mice was evidenced in repeated open-field test. Immunohistochemistry analysis showed that the elevated brain $CD68^+$ signals, a marker for activated microglia cells, were normalized in cerebellum folium and middle brain regions, and partially corrected in the cortex, cerebellum DCN, brainstem, thalamus and spinal cord of treated 4L;C mice, indicating the reduction of pro-inflammation by platelet transfusion. Notably, RIPK3-positive signals within Purkinje neurons in cerebellum of nGD mice were significantly reduced, suggesting therapeutic benefit of platelet transfusion on neuronal necroptosis. This study reveals an under-appreciated, unexpected role for platelets in treating CNS diseases. These results provide proof of concept that platelet transfusion can achieve CNS benefits with brain substrate reduction and improvement in neurological function, as well as reducing pro-inflammation and neuronal necroptosis. The findings will open a door for new clinical approaches with platelet transfusion in treating nGD and potentially other brain diseases.

522. Murine iPSC-Derived Macrophages Improve the *In Vivo* Disease Phenotype of Pulmonary Alveolar Proteinosis Due to *Csf2rb* Deficiency

Adele Mucci[1], Jessica Kunkiel[1], Mania Ackermann[2], Takuji Suzuki[3], Christine Happle[4], Sebastian Brennig[2], Elena Lopez-Rodriguez[5], Jens P. Bankstahl[6], Mark P. Kühnel[5], Miriam Hetzel[1], Axel Schambach[7], Gesine Hansen[4], Bruce Trapnell[3], Nico Lachmann[2], Thomas Moritz[1]
[1]RG Reprogramming and Gene Therapy, Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany, [2]JRG Translational Hematology of Congenital Diseases, Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany, [3]Translational Pulmonary Science Center, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, [4]Department of Pediatric Pneumology, Allergology and Neonatology, Hannover Medical School, Hannover, Germany, [5]Institute for Functional and Applied Anatomy, Hannover Medical School, Hannover, Germany, [6]Department of Nuclear Medicine, Hannover Medical School, Hannover, Germany, [7]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany

Induced pluripotent stem cells (iPSCs) have proven applicability to various areas including disease modeling and cell therapy. Hereditary pulmonary alveolar proteinosis (herPAP) is a rare lung disease caused by mutations within the α- (*CSF2RA*) or β-chain (*CSF2RB*) of the GM-CSF receptor gene (*CSF2R*), resulting in the inability of alveolar macrophages (Mφ) to clear the alveolar spaces from surfactant material. Given the limited treatment options in herPAP, we evaluated the suitability of murine iPSC-derived Mφ (iPSC-Mφ) for disease modelling and as a source for an innovative cell replacement therapy. To this point we first established an efficient and robust protocol to obtain mature and functional Mφ from healthy murine iPSCs. These Mφ closely resemble their counterparts generated *in vitro* from bone marrow cells with regard to morphology, surface phenotype and function. Furthermore, murine *Csf2rb*-deficient iPSCs (miPAP) were generated and submitted to our differentiation protocol. miPAP-derived Mφ faithfully recapitulated the defects in GM-CSF signaling and Mφ function present in herPAP patients. Of note, we also evaluated the feasibility, safety and clinical benefit of the intra-tracheal application of healthy iPSC-Mφ using a clinically relevant *in vivo* mouse model of herPAP. Following single pulmonary Mφ transplantation (PMT) of $4x10^6$ iPSC-Mφ, specific engraftment was observed in the alveolar spaces for up to 8 weeks as shown by tissue sections and PCR. Moreover, cells displayed donor-specific CD45.1 expression and typical Mφ morphology *in vivo* and upon re-isolation. No teratoma formation or tissue toxicity was detected in the organs of transplanted mice. Most importantly, following PMT a significant improvement of disease parameters such as reduced protein, M-CSF, GM-CSF and surfactant protein-D (SP-D) concentration was shown in the bronchoalveolar lavage fluid. We also observed a decrease in PAS-positive material in lung sections and a reduction in lung opacity in computer tomography scans. Thus, we here present an efficient differentiation protocol to obtain Mφ from iPSCs for disease modeling and introduce PMT of iPSC-Mφ as an innovative cell therapy for herPAP.

523. Human Umbilical Vein Endothelial Cell, HUVEC, Co-Culture Promotes Robust Expansion and Maintains Phenotypic Integrity of Rhesus Hematopoietic Stem and Progenitor Cells, HSPC, Prior to Autologous Transplantation

Sandhya R. Panch[1], Idalia Yabe[2], Diego Espinoza[3], Chuanfeng Wu[3], So gun Hong[3], Stephanie Sellers[3], Allen Krouse[3], Aylin Bonifacino[3], Robert Donahue[3], Cynthia Dunbar[3]
[1]Transfusion Medicine, NIH, Bethesda, MD, [2]Hematology, NHLBI, NIH, Bethesda, MD, [3]NHLBI, NIH, Bethesda, MD

The development of ex-vivo HSPC expansion techniques is particulary relevant for improving cord blood transplantation and gene therapy. Despite successful long-term, multilineage reconstitution of expanded human cord blood HSPC in immunodeficient mice, early phase clinical trials have failed to demonstrate improved outcomes. Thus, it is critical to develop a robust pre-clinical model to study ex-vivo expansion strategies, particularly function of long term HSPC and engrafment of all lineages, difficult in xenograft models. We have previously used retroviral insertion site retrieval and autologous competitive transplantation in rhesus macaques to track hematopoietic engraftment and ontogeny at a single HSPC level, in addition to comparing ex-vivo expanded and unexpanded HSPC (Gomes et al, Mol Ther). More recently we have developed retrieval of 31bp diverse barcodes as a more robust and quantitative in vivo HSPC tracking approach (Wu et al, Cell Stem Cell). Modified human endothelial cells (HUVEC) with the ability to be maintained in serum free media for prolonged periods have shown to support and expand human HSPC in murine Butler et al, Blood). We have developed a barcoded rhesus autologous transplant model to evaluate expansion of HPSC on HUVEC versus unexpanded or cytokine expanded rhesus HSPC. We first tested the feasibility of rhesus CD34+ cell expansion on HUVEC versus cytokines/fibronectin for 8 days. We also evaluated for the diversity of expanded HSPC by retrieving transduced viral barcode DNA tags from individual cells by low cycle PCR followed by Illumina sequencing. On average, rhesus CD34+ cells expanded 76 fold ($\pm$ 53) on HUVEC versus 13 fold ($\pm$7) in cytokines (n=4; p=0.03). By morphologic assessment, the HUVEC expanded cell fraction contained higher numbers of progenitors and differentiating cells, with up to 97% CD34+CD45+ cells in the HUVEC expanded fraction and 65% in the cytokine expanded fraction. The HUVEC expanded rhesus CD34+ cells were capable of multi-lineage colony formation after 8 days of expansion. Within the HUVEC expanded cell fractions, fold expansion was similar whether CD34+ cells were frozen after (C1) or before (C2) transduction-expansion. However, viability and percentages of CD34+CD45+ cells were higher in C2 compared to C1 (55% versus 26%). GFP transduction efficiency was also higher in C2 compared to C1 (43% vs. 28%). We demonstrated a slightly lower percentage but higher absolute number of CD34+CD38-CD45RA-CD90+CD49f+Rho-low putative long-term HSC after expansion on HUVEC compared to in cytokines over 26 days in culture (0.018% vs. 0.016% pre and post expansion, respectively). Retrieved barcode analysis and quantitation revealed that in the HUVEC expanded fraction, the top 20 clones constituted up 15% of the total valid reads by day 8, in contrast to only 3.9% of total clones in the cytokine expanded fraction. Cells have been transplanted into autologous macaques, in a competitive model comparing non-expanded, cytokine-expanded and HUVEC-expanded conditions, and in vivo expansion and barcode analysis will be presented. Our data thus far show that peripheral blood CD34+ cells from rhesus macaques expand more robustly in the presence of HUVEC and cytokines as compared to expansion in cytokines alone, and barcode analysis suggests that the HUVEC fraction selectively expanded a subset of highly proliferative cells, which may represent true HSCs within the CD34+ cell fraction.

524. Injectable BMP-2 Gene-Activated Scaffold for the Repair of Mouse Cranial Bone Defect

Hang Lin, Ying Tang, Kai Sun, Jingwen Xue, Weifeng Yin, Bing Wang, Rocky Tuan
University of Pittsburgh, Pittsburgh, PA

Introduction: A major requirement in bone tissue engineering is the persistent and robust promotion of osteogenesis, which requires sustained stimulation with osteoinductive factors, such as bone morphogenetic protein-2 (BMP-2). However, the direct application of these factors remains challenging due to their short half-life and rapid systemic clearance. Given the well demonstrated benefits of recombinant adeno-associated viral (rAAV) vector, including long-term gene transfer efficiency and relative safety, rAAV-based therapies have been developed in both basic and translational research. However, traditional *ex vivo* gene transduction process is time consuming and requires multiple steps of cell culture. In addition, rAAV shows inconsistent transfection efficacy in primary cells. In this study, we co-packed rAAV-BMP-2 and human bone marrow stem cells (hBMSCs) into a gelatin scaffolds through one-step visible light-based phtocorsslinking (PXL), and tested their application for bone regenerationi. We hypothesize that the concentrated and localized release of rAAV-BMP-2 from the scaffold matrix results in higher transfection efficacy and BMP-2 expression in hBMSCs seeded within the scaffolds, thus enhancing their osteogenic differentiation and bone formation upon implantation in bone defect. **Methods:** hBMSCs were obtained with IRB approval from total joint arthroplasty patient. The rAAV6-BMP-2 and rAAV6-GFP reporter (as control) vectors were designed and prepared using an established protocol in our laboratory. PXL was performed using 10% gelatin and 10×10^6 hBMSCs/ml with different amount of rAAV6-GFP. Transfection efficiency was determined by counting GFP-positive cells ratio as well as assessing cell viability. At an optimal ratio, rAAV-BMP-2 and hBMSCs were included into gelatin through PXL. At different time point, BMP-2 production and rAAV6 release kinetics were analyzed. The osteogenesis of hBMSCs was assessed at day 28 by real time PCR and histological staining. In animal study, a 0.5 cm diameter critical size of cranial bone defect model was generated in SCID mice and then the constructs described above were transplanted. Micro-CT imaging and histological staining were used to evaluate the skull bone healing process up to 6 weeks. **Results:** Both rAAV and hBMSCs survived during the scaffold fabrication process. 10×10^4 rAAV/hBMSCs was shown to be the optimal ratio in maintaining cell viability and achieving transduction efficiency. After *in situ* transfection within scaffolds, hBMSCs produced higher BMP-2 amount than those subjected to traditional *ex vivo* transduction process, and underwent robust osteogenesis as well. In animal study, micro-CT imaging results indicated that rAAV BMP-2 & hBMSCs-activated gelatin scaffolds effectively promoted bone regeneration in mice cranial bone defect, in a manner significantly superior to that of scaffolds loaded with hBMSCs/BMP-2 protein, and was remarkably compatible to scaffolds loaded with hBMSCs previously *ex vivo* transduced by rAAV-BMP-2. The histological staining further confirmed the efficacious bone formation from rAAV BMP-2 & hBMSCs loaded scaffold. The results shown here thus demonstrate the feasibility of combining gene and cell therapy with biomaterial engineering via a single-step fabrication and its application for the repair of bone defect.

525. Directed Molecular Evolution of Transcription Factors in Mammalian Cells for Enhanced Directed Cell Differentiation

Ami M. Kabadi, Tyler S. Klann, Malathi Chellappan, Jennifer Kwon, Timothy E. Reddy, Charles A. Gersbach
Duke University, Durham, NC

The advent of cellular reprogramming has introduced new possibilities for regenerative medicine, drug discovery, and controlling cellular behavior. Recent studies have identified many natural transcription factors that are capable of directing lineage-specific differentiation in adult somatic cells. However, genetic reprogramming is often inefficient and highly variable, making it difficult to obtain sufficient quantities of completely reprogrammed cells, and thus unrealistic as a reliable source for cellular therapies and regenerative medicine applications. Directed evolution presents a well-established and currently unexplored approach for improving the intrinsic properties of reprogramming transcription factors. MyoD is the master transcription factor that defines the myogenic lineage. Overexpression of MyoD in certain cell types upregulates the myogenic gene network and induces differentiation to a myogenic phenotype. This process is well characterized and therefore provides an ideal model for developing a directed evolution scheme for mammalian transcription factors. Here we present a mammalian-based directed evolution system that enhances transcription factor-mediated genetic reprogramming in mammalian cells. A quantitative and high-throughput selection system was engineered using a cell reporter assay to isolate enhanced MyoD variants from a library of mutated gene sequences. During skeletal myoblast differentiation, MyoD directly activates expression of the myogenin transcription factor. Myogenin is one of the first markers of muscle maturation and marks commitment to myocyte differentiation. It is also important for cell fusion into functional multinucleated myotubes. Therefore, we hypothesized that engineering MyoD variants with an enhanced ability to induce expression of myogenin would lead to more effective and efficient genetic reprogramming. For this purpose, we engineered an isogenic myogenin-eGFP reporter cell line. The library of MyoD mutants were packaged into a lentiviral vector and delivered to the reporter cell line for selection. Cells with high GFP expression are indicative of hyperactive mutants with an enhanced ability to activate the target promoter and were isolated using fluorescence activated cell sorting. The variants were recovered from the genomic DNA of these sorted cells by PCR. This new library of mutants was recloned into the lentiviral vector for subsequent rounds of selection. Following ten rounds of selection, the resulting libraries were evaluated for enrichment of amino acid substitutions through next generation sequencing. Variants of interests were isolated and tested individually for their ability to induce myogenic differentiation in human cells. In this study, we demonstrate that directed evolution can be used to identify single amino acid substitutions within the master transcription factor MyoD that improve the conversion of human fibroblasts to a skeletal myocyte phenotype. This study presents a generalizable approach for enhancing the activity of mammalian transcription factors.

526. Specific Companion Cells Enhance the Engraftment of Ex-Vivo Expanded HSCs ($CD34^+/CD38^-/CD90^+$)

Nikoleta Psatha[1], Thalia Papayannopoulou[2]
[1]*Medical Genetics, University of Washington, Seattle, WA,*
[2]*Hematology, University of Washington, Seattle, WA*

Efforts to expand in vitro the number of engraftable hematopoietic stem cells (HSCs) for transplantation, is an ongoing quest in stem cell biology. Success in this endeavor is critical for increasing insufficient donor cells for transplantation and/or for the in vitro manipulation of autologous stem cells in gene therapy approaches. Recent studies have revealed that a number of novel small molecules can efficiently enhance the HSC-like phenotype of cultured cells with Stem Regenin1 (SR1) and UM171 showing the most promising outcomes. However, most of the published studies were focused mainly on CB-derived HSPCs, whereas adult HSPC expansion has been largely understudied. Here, we explored the effect of some of the most successful small molecules (SR1,UM171,and Ly2228820 (Ly)) alone or in combination on mobilized adult CD34+ cells, all in the presence of the same 3 cytokines (SCF, Flt-3, TPO). Within a week the absolute number of nucleated cell and CD34+ cells was similarly increased in all conditions. Highly enriched HSCs ,as defined by CD34+/CD38-/CD90+ or CD34+/CD45RA-/CD133+ expression ,were comparable in UM171 alone or in SR-1+Ly (fold difference over control: 34/38/90: 6.54 and 5.94 34/45RA/133: 3.91 and 5.16, respectively), but the combination of all 3 rendered significantly higher numbers of these cells (fold difference over control: 34/38/90: 12.9, 34/45RA/133: 9.3). Apart from the phenotypically defined HSCs, however, other cell populations were distinctly different under these conditions. Under SR1+Ly stimulation there was a significant number of morphologically recognizable Megakaryocytes (CD41+/CD42+) and an increase of CFU-Meg even in the absence of TPO. This was not the case in UM171 stimulated cultures despite the presence of CD41+ cells (12.5±1.5%). The engraftment potential of ex vivo expanded cells under UM171 or SR1+Ly and UM171+SR1+Ly, was tested in xenogeneic animals (NSG) in vivo. Cells cultured in SR1+Ly (±UM171) displayed a transient benefit over the UM171 alone at 4 -8wks post transplantation, as higher proportions of human CD45+ cells (3- 4 fold) and higher numbers of human Platelets (2 fold) were detected in peripheral blood of transplanted mice. This early benefit was not related to the number of CFU present in the inoculum, as UM171 expanded cells had higher number of CFUs (1.7 fold). It is tempting to speculate that the presence of mature Megs in the SR1+Ly treated grafts is responsible for this outcome. Thus we conclude that a) the combinations of SR1+Ly or UM171 efficiently maintain and even enhance the engraftable HSCs from adult mCD34+ cells, b) presence of SR1+Ly in the expansion media favorably influence short term engraftment, especially platelet recovery, and c) highlight the fact that more differentiated companion cells (i.e Megs) under certain combinations of the right molecules can enhance transplant outcomes.

527. Improvement of Pre-Clinical Non-Human Primate Model for Pluripotent Stem Cell Based Therapies by Introducing Marker Genes in Safe Harbor Locus

Ravi Chandra Yada, So Gun Hong, Jizhong Zou, Kyujoo Choi, Arnaud Carpentier, T. Jake Liang, Randall Merling, Colin Sweeney, Harry Malech, Moonjung Jung, Marcus Corat, Youngshun Lin, Ilker Tunc, Xujing Wang, Maryknoll Palisoc, Stefania Pittaluga, Thomas Winkler, Cynthia Dunbar
National Institutes of Health, Bethesda, MD

Induced pluripotent stem cells (iPSCs) are being developed as sources for clinical cellular regenerative therapies, as well as valuable in vitro human disease models. Derivation of iPSCs from non-human primates (NHP) affords the opportunity to test the safety, feasibility and efficacy of proposed iPSC-derived cellular delivery in species with physiology, immunology and scale similar to humans. However, there is a need for stable and safe labeling methods for iPSCs and their differentiated progeny allowing analysis of survival, proliferation, tissue integration and biodistribution, *in vitro* and *in vivo*. Typically, marker genes have been inserted into target cells by transduction with randomly-integrating viral vectors. However, these methods raise concerns regarding genotoxicity and transgene silencing,

particularly in pluripotent stem cells, limiting their utility for tracking and eventual clinical applications. Targeted integration into genomic "safe harbors" offer a promising alternative approach to mark target cells, potentially circumventing these issues. The adeno-associated virus integration site 1 (AAVS1) has been proposed as a suitable safe harbor for human cells, and we now investigate its utility in our rhesus macaque NHP iPSC model. We have efficiently knocked-in both a truncated CD19 (hΔCD19) marker gene a non-immunogenic and clinical relevant marker, or green fluorescent protein (GFP) at the homologous AAVS1 site in rhesus iPSCs (RhiPSCs) using the clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9 (CRISPR-Cas9) system. PCR and Southern blot analyses demonstrated highly efficient knock-in into the AAVS1 locus, with over one third of clones screened containing only targeted but not random integrations. (Table 1). Edited RhiPSC-GFP/hΔCD19 clones retained a normal karyotype and pluripotency - as shown by teratoma formation. Directed differentiation of these clones to neutrophils, hepatocytes or cardiomyocytes was not hindered by the knock-in of marker genes into the AAVS1 sites. Notably, transgene expression was stable in undifferentiated RhiPSCs and differentiated cell types derived from the RhiPSC (Figure 1), in contrast to prior experience with viral vector delivery. We have established a computational platform to assess off-target effects of guide RNAs in the rhesus genome. Genetically marked RhiPSCs afford a unique opportunity to develop clinically relevant models for iPSC-based cell therapies.

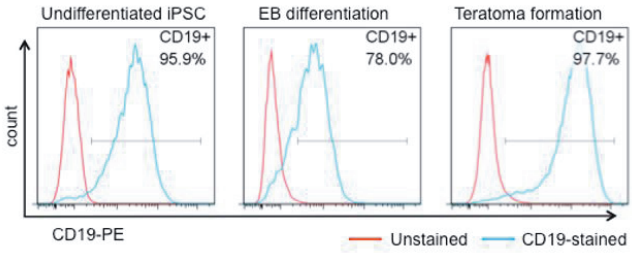


Figure 1 Stable transgene expression in CRISPR edited clones after *in vitro* and *in vivo* differentiation

RhiPSC-hΔCD19-derived cells maintained strong expression of hΔCD19 after in vitro spontaneous differentiation (15 days) and in vivo teratoma formation (6-8 weeks).

Table 1 Summary of CRISPR-mediated gene editing in rhesus iPSCs

Original iPSC clone	Reporter gene	Clones with TI/Clones screened[1]	Clones without RI/Clones with TI[2]
ZG15-M11-10	hΔCD19	4/4	2/8
	GFP	14/14	5/9
ZG32-3-4	hΔCD19	ND	1/4
	GFP	ND	2/4
ZH26-HS41	hΔCD19	ND	1/4
Total		18/18 (100%)	11/29 (37.9%)

RI: random integration, TI: targeted integration, ND: not determined
[1]based on by PCR analysis [2]based on Southern blot analysis

528. Towards Personalized Cell Therapy for Cancer: Tumor-Homing Human Induced Neural Stem Cells

Juli Bago[1], Onyi Okolie[1], Raluca Dumitru[2], Matt Ewend[3], Shawn Hingtgen[1]
[1]Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]UNC School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC, [3]UNC Department of Neurosurgery, The University of North Carolina at Chapel Hill, Chapel Hill, NC

Background: Engineered neural stem cells (NSC) are a promising new approach to treating glioblastoma (GBM). In clinical trials, the ideal NSC drug carrier should be easily isolated and autologous to avoid immune rejection. **Methods:** As a new approach to personalized NSC therapy for cancer, we directly transdifferentiated (TD) human fibroblasts in induced neural stem cells (h-iNSCs). The h-iNSCs were engineered to express optical reporters and either the pro-apoptotic agent TRAIL or thymidine kinase. The tumor-homing migration and therapeutic efficacy of cytotoxic h-iNSCs were then assessed in human-derived GBM models of solid and surgically resected disease. All statistical tests were two-sided. **Results:** Our new single-factor Sox2 strategy converted human skin fibroblasts into nestin+ h-iNSCs in only 4 days and the h-iNSCs survived in the brain of mice for 3 weeks. Time-lapse motion analysis showed h-iNSCs rapidly migrated to human GBMs cells and penetrated solid human GBM spheroids. h-iNSC delivery of TRAIL reduced solid human GBM xenografts 250-fold in 3 weeks and prolonged median survival from 22 to 49 days ($P<0.01$). h-iNSC prodrug/enzyme therapy regressed patient-derived GBM xenografts 20-fold and extended survival from 32 to 62 days ($P<0.01$). Mimicking clinical NSC therapy, intra-cavity h-iNSC thymidine kinase/ganciclovir therapy delayed the regrowth of post-surgical GBMs 3-fold and prolonged survival in mice from 46 to 60 days. **Conclusions:** Transdifferentiating human skin into h-iNSCs is a new platform for creating tumor-homing cytotoxic cell therapies for cancer. Translating this approach has the potential to avoid carrier rejection and maximize treatment durability in patient trials.

RNA Virus Vectors

529. Lentiviral Vectors with a Reduced Splicing Interference Potential Have a Significantly Improved Safety Profile *In Vivo*

Daniela Cesana[1], Pierangela Gallina[1], Laura Rudilosso[1], Andrea Calabria[1], Giulio Spinozzi[1], Ivan Merelli[2], Luciano Milanesi[2], Eugenio Montini[1]
[1]San Raffaele- Telethon Institute for Gene Therapy, Milan, Italy, [2]Italian National Research Council, Institute of Biomedical Technologies, Milan, Italy

Genotoxicity assays based on systemic vector injection into newborn tumor-prone *Cdkn2a*$^{-/-}$ and *Cdkn2a*$^{+/-}$ mice has shown that self-inactivating (SIN) lentiviral vector (LV) harboring strong or moderate enhancer/promoters in internal position caused acceleration in hematopoietic tumor onset compared to control mice. Integration site (IS) analysis in vector-induced tumors showed that oncogene activation or tumor suppressor inactivation occurs by mechanisms of aberrant splicing and/or enhancer-mediated overexpression of cellular genes. Although oncogene activation may be reduced by the use of SIN design, moderate cellular promoters and insulator sequences, how to reduce genotoxic splicing-capture events and aberrant transcript formation triggered by vector integration is still unclear. Here, we specifically designed SINLVs harboring sequences complementary to microRNAs (mirT sequence) which are active in hematopoietic cells (mir223 and mir142-3p) within the SIN LTR (mirsT-LTR.LV)

or in the vector backbone and outside the gene expression cassette (mirT-LV). In our rationale, the mirT sequences when incorporated in an aberrantly generated mRNA would be selectively degraded through the miRNA pathway. Thus, by taking advantage of our *in vivo* models, we assessed the genotoxicity of these LVs with mirT sequences. Systemic injection of mirsT-LTR.LV (N=34) and mirT-LV (N=39) in *Cdkn2a*$^{-/-}$ mice did not cause any significant acceleration in hematopoietic tumor onset compared to un-injected mice (N=37) or mice injected with a SINLV that does not harbor mirT sequences (N=24). Similar results have been obtained after injection of the same vectors in *Cdkn2a*$^{+/-}$ mice (N=29 mirsT-LTR.LV, N=25 mirT-LV, N=40 un-injected and N=15 injected control mice). To gain additional information on the safety profile of these vectors, we performed IS analysis (N>10,000) in tumor-derived DNA. By this analysis, we previously found that *Map3k8* activation by LV insertions was the major mechanism of genotoxicity when prototypical SINLVs were injected into *Cdkn2a*$^{-/-}$ mice. Now, we found that mice treated with mirsT-LTR.LV and mirT-LV did not show any *Map3k8* activating insertions, suggesting that the new vectors are efficient in preventing its activation and confirming their superior safety profile. Furthermore, as expected, *Pten* was the most frequently targeted gene in tumors derived from *Cdkn2a*$^{-/-}$ mice injected with the LVs harboring mirT sequences. *Pten* insertions mainly targeted exons, suggesting the potential inactivation of its transcription unit. Finally, we found that *Sfi1* was the major Common Insertion Site (CIS) in *Cdkn2a*$^{+/-}$ mice injected with LVs harboring mirT sequences. This CIS gene however appears to be the product of an intrinsic bias of LV integration, rather than the result of a selection process. Overall, our studies showed that these new advanced design LVs have a significantly improved safety profile and could represent the vector design of choice in future gene therapy applications.

530. Lentiviral Vector Particles Pseudotyped with Wild-Type Baboon Endogenous Retrovirus (BAEV) Glycoprotein Outperform VSV-G Particles in Transducing Human CD34+ Cells Isolated from Cytokine-Mobilized Peripheral Blood or Bone Marrow

Phillip Hargrove, Yoon-Sang Kim, Matthew Wielgosz, Arthur W. Nienhuis

Hematology, St. Jude Children's Research Hospital, Memphis, TN

Lentiviral vector gene therapy for hematopoietic disorders caused by single gene mutation or deficiency has demonstrated success in clinical trials. However, efficient gene delivery is still challenging, requiring high multiplicity of infection (MOI) to achieve average vector copy numbers of at least 1 per cell. Conditions utilizing high MOI result in improved transduction rates and adequate copy number but these benefits can coincide with potential insertional mutagenesis. Attempting to achieve optimal gene transfer at reduced MOI, we directly compared self-inactivating (SIN) lentiviral vector particles encoding eGFP under control of a murine stem cell virus (MSCV) promoter/enhancer pseudotyped with envelope glycoproteins for BaEV or vesicular stomatitis virus (VSV). BaEV proteins were either full-length (BaWT) or lacked the terminal R-peptide (BaR-less), a version previously reported to improve infectious titer (TU, transducing units per mL) using transient production procedures. Using a standardized four-plasmid transient production protocol, cells transfected with R-less expression plasmids produced syncitia; an issue that resolved using BaWT. Titers were significantly higher for virus packaged with BaWT compared to R-less (BaR-less: $3.6 \times 10^5 \pm 6.6 \times 10^4$ N=12; BaWT $2.4 \times 10^6 \pm 4.5 \times 10^5$; n=10; $p<0.001$) determined by flow cytometry analysis of GFP expression in transduced HEK-293T cells. However, these values were 10-to 20-fold less than those observed for VSV-G ($3.5 \times 10^7 \pm 4.5 \times 10^6$). Human CD34+ hematopoietic stem/progenitor cells isolated from bone marrow (BM) or cytokine-mobilized peripheral blood (mPB) of healthy donors were prestimulated and transduced overnight on retronectin-coated plates with BaWT particles at an MOI of 0.5, 1, 2, or 4. The following day, cells were either plated in methylcellulose to assess colony-formation (CFU), maintained in liquid culture (Bulk), or transplanted into NOD-*scid* IL2Rg^{null} (NSG) mice pre-conditioned with busulfan (n=10 mice/$1x10^6$ cells each). Gene transfer efficiency was gauged by expression of GFP following flow cytometry analysis of cells maintained in culture or visual inspection of colonies growing in methylcellulose using an inverted fluorescence microscope after 6 or 12 days, respectively. Transduction efficiency increased with viral MOI reaching peak levels using an MOI of 2 (Bulk, 93% GFP+; CFU, 98% GFP+). Six NSG mice survived to 14 weeks post-transplant and demonstrated engraftment of CD45+ cells ranging from 19 to 54% (39 ± 13%; mean ± SD) and GFP marking ranging from 32 to 59% (41 ± 10%) determined by flow cytometry. Finally, we compared GFP-encoding particles pseudotyped with VSV-G or BaWT normalizing for levels of p24 quantified by ELISA (BaWT, 1155.0 pg/ml, 3.3×10^6 TU; VSV-G, 1164.9 pg/ml, 5.1×10^7 TU) to account for differences in calculated titer. Prestimulated mPB CD34+ cells were transduced overnight and placed into methylcellulose or transplanted into 15 NSG mice each. Methylcellulose colonies were analyzed for GFP after 12 days of growth (BaWT, 95.6%; VSV-G, 76.6%). Transplanted mice will be analyzed at 16-18 weeks post-transplant to measure engraftment and GFP expression. We believe the stability of producer cells expressing BaWT, the high efficiency of transduction at low MOI, and the ability to transduce adequate numbers of cells with unconcentrated virus supernatant warrant continued optimization of transient production methods and development of a stable producer cell line utilizing BaWT envelope as an alternative to VSV-g.

531. Computational Pipeline for the Identification of Integration Sites and Novel Method for the Quantification of Clone Sizes in Clonal Tracking Studies

Lorena Leonardelli[1], Danilo Pellin[2], Serena Scala[1], Francesca Dionisio[1], Luca Basso Ricci[1], Davide Cittaro[3], Clelia Di Serio[2], Alessandro Aiuti[1], Luca Biasco[1]

[1]San Raffaele Telethon Institute for Gene Therapy (TIGET), Milan, Italy, [2]CUSSB, Vita-Salute University, Milan, Italy, [3]Center for Translational Genomics and Bioinformatics, San Raffaele Scientific Institute, Milan, Italy

Gene-corrected cells in Gene Therapy (GT) treated patients can be tracked in vivo by means of vector integration site (IS) analysis, since each engineered clone becomes univocally and stably marked by an individual IS. As the proper IS identification and quantification is crucial to accurately perform clonal tracking studies, we designed a customizable and tailored pipeline to analyze LAM-PCR amplicons sequenced by Illumina MiSeq/HiSeq technology. The sequencing data are initially processed through a series of quality filters and cleaned from vector and Linker Cassette (LC) sequences with customizable settings. Demultiplexing is then performed according to the recognition of specific barcodes combination used upon library preparation and the sequences are aligned to the reference genome. Importantly, the human genome assembly Hg19 is composed of 93 contigs, among which the mitochondrial genome, unlocalized and unplaced contigs and some alternative haplotypes of chr6. While previous approaches aligned IS sequences only to the standard 24 human chromosomes, using the whole assembled genome allowed improving alignment accuracy and concomitantly increased the amount of detectable ISs. To date, we have processed 28 independent human sample sets retrieving 260,994 ISs from 189,270,566 sequencing reads. Although, sequencing read counts at each IS have

been widely used to estimate the relative IS abundance, this method carries inherent accuracy constraints due to the rounds of exponential amplification required by LAM-PCR that might generate unbalances on the original clonal representation. More recently, a method based on genomic sonication has been proposed exploiting shear site counts to tag the number of original fragments belonging to each IS before PCR amplification. However, the number of cells composing a given clone could far exceed the number of fragments of different lengths that can be generated upon fragmentation in proximity of that given IS. This would rapidly saturate the available diversity of shear sites and progressively generate more and more same-site shearing on independent genomes. In order to overcome the described biases and reliably quantify ISs, we designed and tested a new LC encoding random barcodes. The new LC is composed of a known sequence of 29nt used as binding site for the primers upon amplification steps, a 6nt-random barcode, a fixed-anchor sequence of 6nt, a second 6nt-random barcode and a final known sequence of 22nt containing sticky ends for the three main restriction enzymes in use (MluI, HpyCH4IV and AciI). This peculiar design allowed increasing the accuracy of clonal diversity estimation since the fixed-anchor sequence acts as a control for sequencing reliability in the barcode area. The theoretical number of different available barcodes per clone (4^{12}=16,777,216) far exceeds the requirements for not saturating the original diversity of the analyzed sample (on average composed by around 50.000 cells). We validated this novel approach by performing assays on serial dilutions of individual clones carrying known ISs. The precision rate obtained was averagely around 99.3%, while the worst error rate reaches at most the 1.86%, confirming the reliability of IS quantification. We successfully applied the barcoded-LC system to the analysis of clinical samples from a Wiskott Aldrich Syndrome GT patient, collecting to date 50,215 barcoded ISs from 94,052,785 sequencing reads.

532. Stable Transcriptional Repression, Gene Excision, and Parastism of HIV by Conditionally Replicating Lentiviral Vectors

Uriel A. Lopez-Lemus[1], Amanda Ackley[2], Sheena Saayman[2], Heather Embree[3], Boro Dropulic[3], Ramesh Akkina[4], Paige Charlins[4], Marc S. Weinberg2[2], Kevin V. Morris[1]
[1]Hematological Malignancy and Stem Cell Transplantation Institute, The City of Hope, Duarte, CA, [2]The Scripps Research Institute, LA JOLLA, CA, [3]Lentigen Technology Inc, Gaithersburg, MD, [4]Colorado State University, Fort Collins, CO

Gene-based therapies represent a promising therapeutic paradigm for the treatment of HIV, as they have the potential for sustained viral inhibition via reduced treatment interventions. One innovative approach developed here involves using conditionally replicating vectors (CR-Vectors), as these vectors utilize HIV-expressed proteins to replicate. These vector payloads can spread along with HIV into the budding viral particles, and co-infect target cells, essentially disseminating to HIV infected cells. We have generated and characterized CR-Vectors carrying therapeutic payloads consisting of various non-coding RNA regulatory expression cassettes, which modulate HIV both transcriptionally and post-transcriptionally, as well as CRISPR directed excision machinery. Notably, we have followed both virus and vector expression in T-cells and *in vivo* in the presence and absence of mycophenolic acid (MPA) selection. We find here that CR-Vectors functionally suppress HIV expression in a long-term stable and potent manner in both the presence and absence of MPA; and that transcriptional targeting is more potent at modulating stable suppression of HIV than post-transcriptional targeting or CRISPR directed excision of HIV. This suppression may be physiologically relevant, as it appears to drive HIV to a sustained non-pathogenic set point. Our findings suggest that CR-Vectors with modulatory non-coding RNAs may be a viable approach to achieving long-term stable suppression of HIV leading ultimately to a functional cure.

533. Genomic Excision of *PiggyBac* Transposon Cassettes by Lentiviral Protein Transduction of GagPol-Fused, Excision-Only *PiggyBac* Transposase

Kristian A. Skipper[1], Sofie Andersen[1], Rasmus O. Bak[2], Yujia Cai[1], Jacob G. Mikkelsen[1]
[1]Department of Biomedicine, Aarhus University, Aarhus C, Denmark, [2]Department of Pediatrics, Stanford University, Stanford, CA

The *PiggyBac* (PB) transposon system is a potent nonviral gene delivery tool with relevance in both gene therapy and cell reprogramming for production of induced pluripotent stem cells (iPSCs). Moreover, by taking advantage of the ability of the PB transposase to excise transposon-embedded gene cassettes from the genome without leaving footprints, the PB system is unique in facilitating seamless genome editing. The need for intracellular production of the transposase, however, raises concerns related to delivery and to cytotoxicity caused by sustained transposase expression and insertional mutagenesis. Furthermore, transposon re-integration may decrease the overall efficiency of the PB-mediated excision as well as increasing the risk of adverse secondary insertions. Based on our previous work, we present a new approach for lentivirus-based delivery of PB transposase. By fusing the hyperactive PB transposase, hyPBase, to the C-terminus of the GagPol polyprotein, we show robust incorporation and subsequent release of the transposase in matured lentiviral particles. Furthermore, in an effort to limit transposon re-integration, we engineered a hyPBase variant carrying three missense mutations. This novel hyPBase variant, hyPBase$^{Exc+/Int-}$, demonstrates integration levels very close to background levels, thus limiting the risk of reintegration. Notably, the ability of hyPBase$^{Exc+/Int-}$ to excise transposons from plasmid-borne as well as from genomically integrated PB transposon cassettes is increased up to 6.3-fold relative to the original hyPBase transposase. By fusing the hyPBase$^{Exc+/Int-}$ to the C-terminus of GagPol, transposase protein can be efficiently delivered to cells by lentiviral protein transduction and, in our model system, performs seamless genomic excision of PB transposon cassettes in a copy number and dose-dependent manner, resulting in excision in up to 23.6% of virus-treated cells. Using a transposon containing the puro-deltaTK transgene cassette, we furthermore show that cells with successful excision can be enriched 17-fold by negative selection with FIAU. We believe that protein transduction of hyPBase$^{Exc+/Int-}$ may increase the applicability and safety of transposase-directed genomic excision in iPSCs and in hard-to-transfect cell types including hematopoietic cells.

534. Preparation for a First-in-Man Lentivirus Trial in Cystic Fibrosis Patients

Uta Griesenbach[1], Eric W. F. W. Alton[1], Jeffery M. Beekman[2], Christopher A. Boyd[3], Jane C. Davies[1], Lee A. Davies[4], Johanna F. Dekkers[2], Deborah R. Gill[4], Mamoru Hasegawa[5], Tracy E. Higgins[1], Takashi Hironaka[5], Makoto Inoue[5], Stephen C. Hyde[4], Alastair J. Innes[3], Ian A. Pringle[4], Kamila M. Pytel[1], Stephanie G. Sumner-Jones[4], Shu Tsugumine[5], Marguerite W. Wasowicz[1]
[1]Department of Gene Therapy, National Heart and Lung Institute, Imperial College London, London, United Kingdom, [2]Department of Pediatric Pulmonology, University Medical Centre, Utrecht, Netherlands, [3]Medical Genetics, Centre for Genomic and Experimental Medicine, University of Edinburgh, Edinburgh, United Kingdom, [4]Gene Medicine Group, NDCLS, John Radcliffe Hospital, University of Oxford, Oxford, United Kingdom, [5]ID Pharme Co. Ltd. (DNAVEC Center), Tsukuba, Japan

Background: We have recently shown that non-viral gene therapy can stabilise the decline of lung function in cystic fibrosis (CF) patients. However, the effect was modest, and it is important to develop more potent gene transfer agents in parallel. F/HN-pseudotyped lentiviral vectors are more efficient for lung gene transfer than non-viral vectors in pre-clinical models. In preparation for a first-in-man CF trial using the lentiviral vector we have undertaken key translational pre-clinical studies. **Methods:** Regulatory-compliant vectors carrying a range of promoter/enhancer elements were assessed in mice and human air liquid interface cultures to select the lead candidate; CFTR expression and function were assessed in CF models (knockout mice and human intestinal organoids) using this lead candidate vector. Toxicity was assessed and "benchmarked" against the leading non-viral formulation recently used in a Phase IIb clinical trial. Integration site profiles were mapped and transduction efficiency determined to inform clinical trial dose-ranging. The impact of pre-existing and acquired immunity against the vector and vector stability in several clinically relevant delivery devices was assessed. **Results:** A hybrid promoter consisting of the elongation factor 1α promoter and the CMV enhancer was most efficacious in both murine lungs and human air liquid interface cultures. The efficacy, toxicity and integration site profile supports further progression towards clinical trial and pre-existing and acquired immune responses do not interfere with vector efficacy. The lead rSIV.F/HN candidate expresses functional CFTR and the vector is stable in clinically relevant delivery devices. **Conclusions:** The data support progression of the F/HN pseudotyped lentiviral vector into a first-in-man CF trial due to start in Q2 2017. Regulatory-compliant toxicology studies are currently being performed.

536. The Viral Transduction Enhancer Vectofusin-1 Is a Nanofibrillar Peptide Capable of Increasing the Contact between Viral Vectors and Target Cells

Saliha Majdoul[1], Louic Vermeer[2], Loic Hamon[3], Justine Wolf[2], Ababacar K. Seye[4], Nathalie Holic[1], David Pastre[3], Daniel Stockholm[5], Burkhard Bechinger[2], Anne Galy[1], David Fenard[1]
[1]Genethon/INSERM UMR_S951, University of Evry, Evry, France, [2]Institut de Chimie, CNRS UMR_7177/ University of Strasbourg, Strasbourg, France, [3]University of Evry/ INSERM UMR_S1204, Evry, France, [4]Genethon/INSERM UMR_S951, Evry, France, [5]Genethon/INSERM UMR_S951, Ecole Pratique des Hautes Etudes, Evry, France

Gene transfer into hCD34+ hematopoietic stem/progenitor cells (HSPCs) using HIV-1-based lentiviral vectors (LVs) has several therapeutic applications ranging from monogenic diseases, infectious diseases and cancer. In such therapeutic context, the gene therapy could be improved by enhancing transduction levels of target cells and by reducing the amount of LVs used on the cells for greater safety and reduced costs. We recently identified a new cationic amphipathic peptide, Vectofusin-1, with viral transduction enhancing capacity, enabling higher transduction levels with low amounts of LV. Vectofusin-1 promotes the entry of several retroviral pseudotypes into target cells when added to the culture medium and is not toxic to HSPCs. Here, we present the first insights into the mechanism of action of this new transduction enhancer. First, a viral pull down assay showed that viral particles were easily pelleted by low speed centrifugation in presence of Vectofusin-1, suggesting that this latter may form unsoluble nanofibrils, trapping lentiviral particles. Atomic force (AFM) and electron microscopy (EM) of Vectofusin-1 confirmed that this peptide is rapidly forming annular aggregates and nanofibres in culture medium. Furthermore, these fibres were shown to be auto-fluorescent in medium with high-protein content (X-Vivo20), allowing the observation of a nanofibrillar network of Vectofusin-1 using confocal microscopy. Next, Vectofusin-1 was shown to strongly interact with Congo Red, especially in presence of lentiviral particles, but labeling with Thioflavin T was inefficient, suggesting that Vectofusin-1 fibres are not amyloid-type fibrils. Structural studies by circular dichroism confirmed this result. The capacity to form nanofibrils appears to be essential for the mechanism of action of Vectofusin-1 since a defective mutant called LAH2-A4, unable to promote lentiviral transduction (Majdoul S. *et al* (2016) J. Biol. Chem.), was also unable to form nanofibrils. In conclusion, biophysical, nanoscopic and microscopic observations have helped us to define Vectofusin-1 as a new nanofibrillar peptide capable of enhancing lentiviral transduction of target cells in conditions well-adapted to cGMP and scalable gene therapy protocols.

537. New Graph-Based Algorithm for Comprehensive Identification and Tracking Retroviral Integration Sites

Andrea Calabria[1], Stefano Beretta[2], Ivan Merelli[3], Giulio Spinozzi[1], Stefano Brasca[1], Fabrizio Benedicenti[1], Erika Tenderini[1], Alessandra Biffi[4], Eugenio Montini[1]
[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy, [2]University of Milano-Bicocca, Milan, Italy, [3]National Research Council, Milan, Italy, [4]Children Hospital, Boston, MA

Vector integration sites (IS) in hematopoietic stem cell (HSC) gene therapy (GT) applications are stable genetic marks, distinctive for each independent cell clone and its progeny. The characterization of IS allows to identify each cell clone and individually track its fate in different tissues or cell lineages and during time, and is required for assessing the safety and efficacy of the treatment. Bioinformatics pipelines for IS detection used in GT identify the sequence reads mapping in the same genomic position of the reference genome as a single IS but discard those ambiguously mapped in multiple genomic regions. The loss of such significant portion of patients' IS may hide potential malignant events thus reducing the reliability of IS studies. We developed a novel tool that is able to accurately identify IS in any genomic region even if composed by repetitive genomic sequences. Our approach exploits an initial genome free analysis of sequencing reads by creating an undirected graph in which nodes are the input sequences and edges represent valid alignments (over a specific identity threshold) between pairs of nodes. Through the analysis and decomposition of the graph, the method identifies indivisible subgraphs of sequences (clusters), each of them corresponding to an IS. Once extracted the consensus sequence of the clusters and aligned on the reference genome, we collect the alignment results and the annotation labels from RepeatMasker. By combining the set of genomic coordinates and the annotation labels, the method retraces the initial sequence graph, statistically validates the clusters through permutation test and produces the final list of IS. We tested

the reliability of our tool on 3 IS datasets generated from simulated sequencing reads with incremental rate of nucleotide variations (0%, 0.25% and 0.5%) and real data from a cell line with known IS and we compared out tool to VISPA and UClust, used for GT studies. In the simulated datasets our tool demonstrated precision and recall ranging 0.85-0.97 and 0.88-0.99 respectively, producing the aggregate F-score ranging 0.86-0.98 which resulted higher than VISPA and UClust. In the experimental case of sequences from LAM-PCR products, our tool and VISPA were able to identify all the 6 known ISs for >98% of the reads produced, while UClust identified only 5 out 6 ISs. We then used our tool to reanalyze the sequencing reads of our GT clinical trial for Metachromatic Leukodystrophy (MLD) completing the hidden portion of IS. The overall number of ISs, sequencing reads and estimated actively re-populating HSCs was increased by an average fold ~1.5 with respect the previously published data obtained through VISPA whereas the diversity index of the population did not change and no aberrant clones in repeats occurred. Our tool addresses and solves important open issues in retroviral IS identification and clonal tracking, allowing the generation of a comprehensive repertoire of IS.

538. Transient Non-Viral RNA Delivery Mediated by a Lentiviral Particle

Pascale Bouillé[1], Régis Gayon[1], Alexandra Iché[1], Christine Duthoit[1], Lucille Lamouroux[1], Jean-Christophe Pagès[2]
[1]Research & Development, Vectalys, Toulouse, France, [2]Faculté de Médecine, Inserm U966, Tours, France

Safe and efficient gene therapies including gene-targeting technologies are very challenging but very promising approaches nowadays. The scientific and clinical communities have been working for a long time together to encounter substantial clinical advances they have made possible thanks to numerous improvements in cell culture and gene transfer methods. Opportunities to improve gene transfer into primary or stem cells involve a better design of the vectors used. Such improvements must lead to an increase of the transduction efficiency including the percentage of positive cells, as well as a better level and duration of expression, cell phenotype preservation and the number of genes delivered··· Lentiviral vectors have seen their use largely increased in clinical protocols over the past few years but safety concerns have been highlighted. First, the permanent genetic modification remains a focus of significant regulatory oversight and even integrase- or reverse transcriptase-deficient lentiviral vectors leads to residual integration events. Moreover, all the gene-editing technologies entail a "hit-and-run" mechanism that requires only a transient expression of the nuclease complex. In parallel, mRNA delivery is a versatile, flexible, and safe mean for protein therapies but chemical or electroporation-based transfection protocols are known to induce cell toxicity and phenotype modifications of the target cells. Here, we describe a new chimeric lentiviral platform that allows mRNA delivery into the target cells without any genomic signature. The respective properties of the MS2 bacteriophage and the lentiviral vectors have been combined to build a non-integrative packaging system in which the wild type HIV packaging sequence is replaced by the MS2 stem-loop repeats and the MS2 Coat sequence is inserted into the NucleoCapsid sequence. The resulting lentiviral particle is able to deliver a non-viral RNA into the cytoplasm of target cells, directly available for protein translation. Transduction of immortalized cells but also of T cells and HSC with these RNA lentiviral particles (RLP) shows an efficient, fast and transient expression of both reporters and functional proteins such as genome editing enzymes. Particles structure and functionality, cell transduction and characterization of such engineered cells have been compared with those obtained with an integrative lentiviral vector. Particularly by recruiting the RNA independently of dimerization with more than four molecules per particles, RLPs allow the cotransfer of different species of RNA into target cells. This new delivery system is a great candidate handle the safe and clinically suitable delivery of the editing machinery, which can transiently act without inducing cellular toxicity or immunogenicity.

AAV Vectors III

539. Screening for Recombinant Adeno-Associated Viral Vectors That Selectively Transduce Hepatitis B Virus Infected Cells

Gustavo de Alencastro, Katja Pekrun, Mark Kay
Genetics and Pediatrics, Stanford University, Stanford, CA

Hepatitis B is a disease caused by infection with the hepatitis B virus (HBV), a small enveloped DNA virus which belongs to the *Hepadnaviridae* family. Even though a safe and effective vaccine is available, HBV represents the ninth most common cause of death worldwide with an estimated 350 million chronically infected individuals. HBV infection can cause cirrhosis and hepatocellular carcinoma and currently there are 7 approved drugs for the treatment of HBV infection in the USA. These drugs decrease the risk of liver damage from HBV by slowing but not eliminating viral replication in most patients, possibly related to the persistence of integrated HBV genomes. Gene therapy using recombinant adeno-associated viral (rAAV) vectors constitute a promising tool to combat HBV infection as rAAV vectors have the ability to transduce and establish long-term and stable transgene expression in liver cells. Preclinical studies have successfully utilized rAAV vectors to combat viral infections after delivering short-hairpin RNA (shRNA) expression cassettes for knocking down viral coding or host genes required for viral replication and spread. Because promiscuous targeting of host cellular genes in the majority of non-infected cells may be detrimental, specific targeting of HBV-infected cells adds a new layer of potential therapeutic targets and safety. Thus we set out to identify new AAV capsids that selectively transduce HBV-infected cells. To do this, we created new AAV shuffled capsid libraries and used these for multiple rounds of infection in HBV-infected hepatoma cells. Each library is co-infected with wild-type Adenovirus-5 (helper virus) allowing the rAAV genomes to replicate in the HBV-infected cells. Isolation of replicating rAAV over successive passages in cultured cells allows for a more stringent selection as all steps in AAV transduction must occur. We have varied the screens using various multiplicity of infections (MOIs) of the library and/or utilized a pre-clearing step using a HBV negative hepatoma cell line. Several selected capsids derived from multiple parental capsids were vectorized. One capsid (AAV-GK4) was found to transduce the infected cells approximately 6 times more robustly than uninfected cells (using an MOI of 100 and 1,000). Interestingly, the same result was observed with AAV-DJ, a variant previously selected on human hepatoma cells. Additional screens and candidates are pending further evaluation. The possibility of finding new rAAV capsids with an increased transduction efficiency and selective tropism for HBV-infected cells will provide a reagent or therapeutic that can deliver a transgene that will allow one to selectively modify or potentially kill infected but not the uninfected cells contained within the liver.

540. Identification of a 16 Amino Acid-Long Motif in the N-Terminal Half of the AAV9 Capsid Essential for Persistence in the Blood for an Extended Period of up to 3 Days

Kei Adachi, Hiroyuki Nakai
Department of Molecular & Medical Genetics, Oregon Health & Science University, Portland, OR

The distinctively prolonged half-life in the blood circulation is one of the key features of AAV9 vectors and is presumed to play an important role in efficient gene delivery to various target organs following systemic vector administration. We have so far analyzed blood clearance rates of nearly one thousand different AAV strains (serotypes and capsid-engineered mutants) in mice following intravenous vector injection, and have discovered that the wild-type AAV9 and many AAV9-derived capsid mutants are particularly unique in that they can persist in the blood circulation at significant levels when measured at 72 hours post-injection. Although non AAV9-derived capsid mutants exist that show a blood vector concentration at a level equivalent to or higher than that of the wild-type AAV9 at 24 hours post-injection, their blood vector concentrations surprisingly and without exception become substantially lower than the wild-type AAV9 concentration at 72 hours post-injection. In AAV vector development, long half-life should be an attractive feature of new vectors; however, the paucity of knowledge of the capsid amino acid sequences responsible for vector persistence in the blood has made it difficult to design novel AAV capsids with a prolonged half-life. Here we report the identification of a 16 amino acid-long motif in the N-terminal half of the AAV9 capsid essential for the vector persistence in the blood for an extended period of up to 3 days, which is the pharmacokinetic profile unique to AAV9. In our previous study, we performed double alanine scanning of the entire C-terminal half of the AAV9 capsids (amino acid positions 356 to 736), and identified approximately 40 amino acids that showed loss of vector persistence in the blood only at the 72-hour time point when mutated to alanine. Many of these amino acids are not unique to AAV9 or are scattered within the capsid amino acid sequence. In the present study, we performed double alanine scanning of the entire N-terminal half of the AAV9 capsids (amino acid positions 4 to 355), and determined their blood clearance rates in mice by the AAV Barcode-Seq approach. Among these 175 double alanine mutants, 52 mutants did not produce viral particles at levels required for the analysis; therefore, the pharmacokinetic data could be collected only from 123 of the 175 mutants. This analysis revealed 7 double alanine mutants that span a stretch of 16 consecutive amino acids and form a group that is clearly distinctive from the other 116 mutants (note: one mutant within this amino acid stretch could not be assessed due to the inability to produce viral particles). That is, the 116 mutants showed the same pharmacokinetic profile as that of the wild-type AAV9 while the distinctive 7 mutants persisted in the blood for only up to 24 hours post-injection at levels similar or superior to the wild-type AAV9 and showed substantially lower concentrations at 72 hours post-injection. Interestingly, this motif contains one of the most variable regions across different serotypes and the amino acid sequence of the identified motif is unique only to AAV9 and other Clade F AAV serotypes. We are currently investigating whether swapping this motif of AAV9 with other serotypes' could result in loss of persistence at 72 hours post-injection or whether replication of this motif in other non-AAV9 capsids could bestow long-term persistence. In summary, our study suggests that the 16 amino acid-long motif in the N-terminal half of the capsid that is unique to the Clade F AAV serotypes plays an essential role in maintaining the persistence of circulating viral particles in the blood for an extended period of several days.

541. Use of Endogenous Viral Elements to Deduce the Sequence of an Ancient Adeno-Associated Virus That Circulated Amongst Australian Marsupials ~30 Million Years Ago

Claus V. Hallwirth[1], Richard H. Smith[2], Michael Westerman[3], Nicola A. Hetherington[1], Yu-Shan Tseng[4], Sylvain Cecchini[2], Tamas Virag[2], Mona-Larissa Ziegler[1], Igor B. Rogozin[5], Eugene V. Koonin[5], Mavis Agbandje-McKenna[4], Ian E. Alexander[1], Robert M. Kotin[2]
[1]Gene Therapy Research Unit, Children's Medical Research Institute and The Children's Hospital at Westmead, Westmead, Australia, [2]National Heart, Lung and Blood Institute, Bethesda, MD, [3]La Trobe University, Bundoora, Australia, [4]University of Florida, Gainesville, FL, [5]National Center for Biotechnology Information, Bethesda, MD

Vectors based on adeno-associated viruses (AAVs) have been successfully applied in clinical trials and pre-clinical settings. AAV capsid proteins are the primary determinants of vector tropism and immunogenicity, and are continually being developed to impart desirable properties, such as immune evasion and specificity for selected target tissues in humans and those of model organisms. One approach that was recently adopted by at least two groups to generate novel capsid variants is the computational deduction of putative ancestral AAV sequences, based upon observable genetic diversity amongst closely related contemporary AAV isolates. Such inferred ancestral sequences potentially recapitulate the evolutionary history of AAVs for hundreds or possibly thousands of years. Germline endogenous viral elements (EVEs) can compensate for the lack of a viral fossil record by genetically preserving viral nucleotide sequences over a geologic time span. Here we describe an AAV-EVE (mAAV-EVE1) that is lineage-restricted to the germlines of members of the Australian marsupial suborder Macropodiformes (present-day kangaroos, wallabies, and related macropodoids), to the exclusion of other Diprotodontian marsupial lineages. Orthologous mAAV-EVE1 sequences from sixteen macropodoid species, representing a speciation history spanning an estimated 30 million years, facilitated *in silico* reconstruction of an inferred ancestral AAV sequence. This sequence represents the genome of an ancient marsupial AAV that circulated among Australian metatherian fauna sometime during the late Eocene to early Oligocene. The deduced ancient AAV bears remarkable resemblance to modern AAVs in its predicted structural and non-structural genes, underscoring the concept that despite their relatively rapid mutation rates, AAVs have evolved under tight constraints imposed by both form and function. Moreover, the evolutionary history of mAAV-EVE1 since its integration into the pro-macropodoid lineage reflects that of the respective host species. In addition to providing an inferred ancient AAV capsid sequence for future transduction studies, this sequence can serve as input for the generation of further novel capsids by approaches such as capsid shuffling and directed capsid engineering/evolution. Furthermore, by comparing it to contemporary AAV capsids, this sequence can inform rational capsid design approaches by providing insights into long-term capsid evolution.

542. Novel Barcode-Based *In Vivo* Screening Method for Generating *De Novo* AAV Serotypes for CNS-Directed Gene Therapy

Marcus Davidsson, Gang Wang, Patrick Aldrin-Kirk, Morgan Hartnor, Tomas Björklund
Molecular Neuromodulation, Lund University, Lund, Sweden

Capsid modification is a useful strategy to create adeno-associated virus (AAV) vectors with subtype specific neuronal targeting and enhanced retrograde transport. Incorporating known cell-specific

binding ligands is a rational method, but the creation of vectors without prior knowledge has the potential to reveal novel targets. Directed evolution and phage display are broadly utilized high-throughput methods, but are inefficient due to displaying random peptides wherein the vast majority will be non-functional.Here, we have developed a novel AAV library in which each virus particle display a peptide derived from known neuron-related proteins on the surface of an AAV2 capsid. The packaged viral genome encodes a unique barcode sequence to facilitate capsid identification. 92398 unique oligos encoding 14-amino-acid peptides derived from 135 proteins were synthesized using microarray. Four-fragment Gibson assembly and novel emulsion PCR was then used to generate a plasmid library by inserting oligos into the capsid gene and barcodes between the inverted terminal repeats. This plasmid library was then used to assemble a diverse library of AAV capsids, such that particles were composed of only peptide-modified capsid proteins which package an expression cassette containing RNA expressed barcodes providing post hoc identification of the capsid structure.In parallel, the plasmid library was sequences using Illumina paired-end sequencing to link the RNA expressed barcodes to the *de novo* capsid structures. The successfully generated AAV library efficiently infected neurons and astrocytes *in vitro* and displayed a subset of peptides that had efficient retrograde transport ability in neurons *in vivo* (*e.g.*, transported from striatum to substantia nigra). Functional peptides, which successfully promoted neuronal infectivity or retrograde transport, were identified by Illumina sequencing of RNA expressed barcodes both *in vitro* and *in vivo* and efficacy modeled through barcode counting. In conclusion, we developed a high-throughput combinatorial method to generate peptide-modified AAV libraries that are valuable for evaluation of receptor expression of neuronal populations and have the potential to generate novel vectors with unique properties for *in vivo* gene transfer in the CNS.

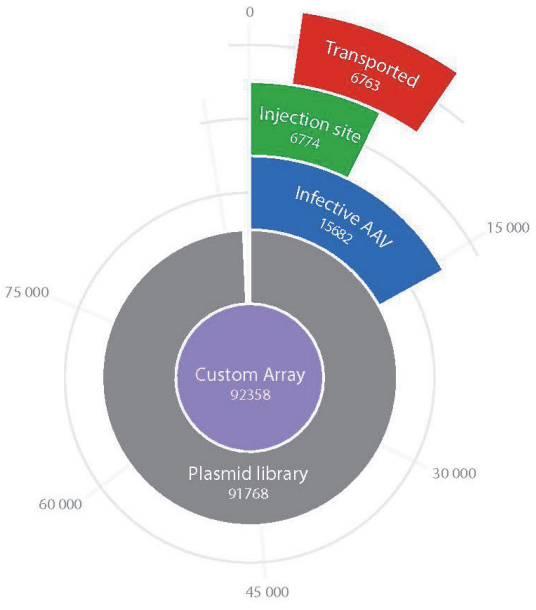


Figure 1. Polar plot showing the number of unique peptides found in custom array and plasmid library followed by the number found in infective AAV particles, at injection site and for transported AAVs.

543. In Vivo Beta-Cell-Targeted Gene Editing by AAV Vectors

Salma G. Morsy, Jason Tonne, Yaxi Zhu, Paul Belmonte, Yasuhiro Ikeda
Molecular Medicine, Mayo Clinic, Rochester, MN

The CRISPR/Cas9 and guide RNA system has emerged as a promising genome editing platform, due to its simplicity, versatility and efficiency, compared to prior techniques for genome modification. Although the original Cas9 gene is too long to be packaged by AAV vectors, Dr. Zhang and colleagues have identified a shorter Cas9 protein from S. aureus (saCas9), which allows generation of a single AAV vector carrying both saCas9 and a guide RNA. Previously, we and others have demonstrated that AAV serotypes 8 and 9-based vectors are highly pancreas-tropic. Our long-term goal is to develop efficient beta-cell- and acinar-cell-targeted gene editing systems based on the AAV-saCas9 system. We first assessed on-target effects of the AAV-saCas-gRNA vector system in vitro. We generated two GFP sequence-targeted gRNA vectors, and tested their genome editing effects using two HT1080 cell lines carrying a single copy of GFP. Infection of HT1080 lines with an multiplicity of infection (MOI) of $2x10^4$ resulted in 40-55% GFP knockout by two AAV-gRNA constructs. Gene editing efficiency was comparable in two HT1080 lines, and dose-dependent. Consecutive, multiple high-dose infections for 3 days achieved up to 80% GFP knockdown. To test whether a subset of cells are more resistant to the AAV-saCas9-mediated genome editing, we sorted the remaining 20% of GFP-positive cells and re-infected recovered GFP-positive cells with the two saCas9 vectors. Our data showed those cells were equally permissive to the saCas9 vectors, when compared to their parental line. To assess the off-target effects of the AAV-saCas9-gRNA system, we compared the exome sequences of the vector-treated cells. Our preliminary result identified multiple off-target deletions upon AAV-saCas9 treatments. We are currently analyzing whether those off-target effects were due to their similarity to the GFP targeting gRNA sequences, or independent from the gRNA sequences. We then tested the feasibility of the AAV-saCas9-gRNA vector system for beta-cell-targeted gene editing in vivo. We generated AAV8 vectors carrying Pdx1-targeting gRNA, with saCas9 driven by CMV promoter. Single intraperitoneal injection of AAV saCas9 vectors led to efficient knockout of Pdx1 expression in the pancreatic beta-cells. To limit potential off-target effects, we need to restrict saCas9 expression in beta-cells. However, our original beta-cell targeted vectors carry a relatively long, 1.1kb insulin promoter sequence. We therefore optimized the insulin promoter sequence and determined the minimum, essential promoter sequence, which facilitates robust transgene expression with high tissue-specificity. We are in the process of testing the AAV-saCas9-gRNA vectors with the optimized insulin promoter sequence for in vivo beta-cell gene editing.

544. Comparison of AAV Serotype2 Transduction by Various Delivery Routes to the Mouse Eye

Kenton T. Woodard[1], Melisa Vance[1], Brian Gilger[2], R. Jude Samulski[1], Matthew Hirsch[1]
[1]Neurobiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]North Carilina State University, Raleigh, NC

AAV gene therapy has demonstrated success for the treatment of several ocular diseases with the tropism and efficiency of AAV retinal transduction being a function of the route of administration. Subretinal injection has been the primary route to deliver AAV to the retina but this injection route carries surgical complications and transduction remains localized to the area of retinal detachment. Despite the use of suprachoroidal injections for ocular drug delivery in large animal models, this route has not been comprehensively

compared to AAV vector transduction following other administration routes in mice. AAV serotype 2 (AAV2) was used to deliver a self-complementary CMV-GFP reporter cassette via intrastromal, intracameral, intravitreal, subretinal, or suprachoroidal injections. These injections were validated by fundoscopy and optical coherence tomography at the time of injection. Transduction was assessed six weeks later by fundoscopy and whole globes were evaluated for histology. Transduction of the stroma, ciliary body, retinal ganglion cells, outer retina, and retinal pigment epithelium could be seen in the various routes of delivery to the eye. In particular, the transduction of multiple retinal layers throughout the retina without the damage of retinal detachment and widespread distribution makes suprachoroidal injections a better delivery route than to subretinal injection.

545. Syngeneic AAV Pseudo-Vectors Potentiates Full Vector Transduction

Qizhao Wang[1], Biao Dong[1], Katie A. Pokiniewski[1], Jenni Firrman[1], Zhongren Wu[1], Mario P.s. Chin[2], Yong Diao[3], Ruian Xu[3], Weidong Xiao[1]

[1]Temple University, Philadelphia, PA, [2]Huaqiao University, Xiamen, China, [3]Huaqiao University, Quanzhou, China

An excessive amount of empty capsids are generated during regular AAV vector production process. These pseudo-vectors often remain in final vectors used for animal studies or clinical trials. The potential effects of these pseudo-vectors on AAV transduction have been a major concern. In the current study, we systemically examined how the AAV pseudo-vectors interact with the full AAV vectors in the transduction processes. Three different types of AAV pseudo-vectors were used: syngeneic AAV pseudo-vector (sAAV, purified from the full AAV vector preparation with the same reporter gene), null AAV pseudo-vector (nAAV, produced by using AAV packaging plasmid and adenoviral helper plasmid), and allogeneic AAV pseudo-vector (aAAV, purified from the AAV vector preparation with a genome different to reporter gene). All three AAV2 pseudo-vectors exhibited inhibition effects on full AAV2 vectors particle transduction in a dose dependent manner *in vitro*. AAV2 pseudo-vectors not only inhibited full AAV2 vector transduction but also decreased gene expression from full AAV8 vectors. However, sAAV2 pseudo-vectors exhibited less inhibition effects on the transduction efficiency of full AAV2 or AAV8 vectors compared with nAAV2 or aAAV2 pseudo-vectors *in vitro*. In contrast, all three kinds of AAV8 pseudo-vectors did not show such inhibition effects on full AAV8 and AAV2 vectors *in vitro* or *in vivo*. Moreover, the sAAV8 pseudo-vector enhanced its full AAV8 vector transduction while nAAV8 and aAAV8 pseudo-vector did not have similar effects. Nine fold sAAV8 pseudo-vectors increased AAV8-TTR-hF8-X5 transduction by 2~5-fold in two different hemophilia A mouse models as measured the coagulation activity of factor VIII by aPTT assays and antigen levels by Elisa. Further characterization showed that sAAV8 and aAAV8 pseudo-vectors contain DNAs of varying sizes. In general, these DNAs still have AAV ITR sequences and partial vector genomes near ITRs. It is likely that such partial genome could anneal to the full AAV genomes and facilitate the second strand DNA synthesis thus increasing full AAV transduction.

546. Development of MFP-Inducible System for AAV5 Gene Therapy of Chronic Diseases in the Liver

Jolanda Liefhebber[1], Raygene Martier[2], Richard van Logtenstein[1], Harald Petry[1], Pavlina Konstantinova[1]

[1]uniQure n.v., Amsterdam, Netherlands, [2]uniQure n.v. and Leiden University, Amsterdam and Leiden, Netherlands

Introduction: Gene therapy offers long term solutions for chronic diseases, whereby the transgene is continuously expressed upon single vector administration. However in some cases it would be desirable to tightly regulate or switch off transgene expression. Methods: We are investigating regulated gene expression based on the mifepristone (MFP)-inducible GeneSwitch system. The GeneSwitch protein comprises yeast Gal4 DNA-binding domain, a human p65 activation domain and a MFP controlled domain derived from the human progesterone receptor. The classical GeneSwitch system consists of two expression cassettes on two separate vectors; one containing the GeneSwitch sequence and one containing the transgene. We compared this two-vector system to a single-vector system, where the two cassettes were put into one vector for efficacy *in vitro* and *in vivo*. Results: We show inducible expression of EPO, IGF and GNDF obtained *in vitro* upon addition of MFP to cells transfected with plasmids containing GeneSwitch and the gene expression cassette. The kinetics of EPO mRNA and protein expression followed a dose dependent fashion in the range of 0.1 to 10 nM MFP and reached a plateau at higher MFP concentrations. Surprisingly, the GeneSwitch protein expression decreased 48h after MFP induction. The indicibility of the single versus the two-vector system of GeneSwitch-EPO was compared. Both systems were equally inducible based on total amount of EPO produced in the presence of MFP and related to background expression in the absence of MFP. *In vivo* proof of concept was obtained for EPO in the liver. EPO is characterized by clear expression kinetics in plasma and raises blood hematocrit, hence provides a reliable in-life read-out for gene inducibility. Mice were injected with different doses of AAV5-AAT-GeneSwitch-EPO and gene expression was induced in two separate rounds at 4 and 8 weeks p.i. EPO plasma levels increased approximately 2-logs in the single or two-vector system-injected mice, compared to un-induced groups. Moreover in the absence of MFP background expression of EPO was lower in the single-vector system and hematocrit levels were unaffected. Measurements of MFP in tissue matrices and in plasma by mass spectrometry show the presence of MFP in plasma and liver, validating applicability of the GeneSwitch system in the liver. Conclusion: Overall, our data indicate that transgene expression can be repeatedly regulated in the liver using the GeneSwitch system and provides us with a novel AAV5 vector for further development.

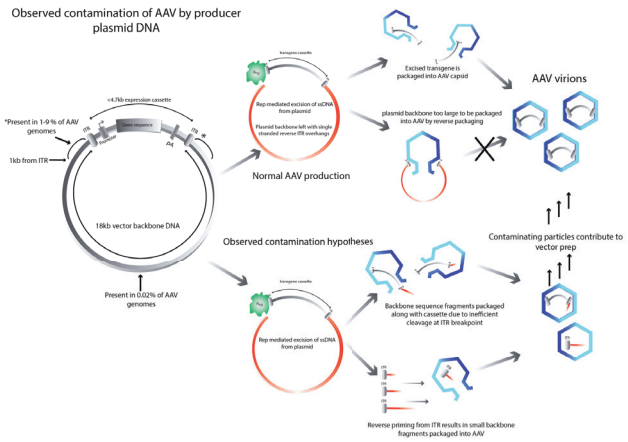
547. AAV Preparations Contain Contamination from DNA Sequences in Production Plasmids Directly Outside of the ITRs

Mark A. Brimble[1], Junfang Zhou[1], Christopher Morton[1], Michael Meagher[2], Amit C. Nathwani[3], John T. Gray[4], Andrew M. Davidoff[1]

[1]Surgery, St. Jude Children's Research Hospital, Memphis, TN, [2]Pathology, St. Jude Children's Research Hospital, Memphis, TN, [3]Haematology, University College London, London, United Kingdom, [4]Research and Development, Audentes Therapeutics, San Francisco, CA

Despite having the best safety profile of any current clinical viral vector, it is known that AAV preps contain contaminating sequences that are packaged alongside the expression cassette at a low rate. These sequences can originate from production plasmid DNA, or chromosomal DNA from producer cell lines. It has been reported

that sequences from the expression cassette producer plasmid are more likely to be packaged into AAV than DNA from other producer plasmids, or chromosomal DNA. We hypothesised that in the expression cassette plasmid, backbone sequences directly flanking the ITRs might be packaged into AAV at a higher rate than sequences further away from either ITR. We first confirmed the presence of these sequences via PCR amplification of non-expression cassette DNA flanking the ITR sequence up to 1.5kb in length in an AAV prep. Sequential qPCR assays showed that plasmid sequences at a range of distances up to 2kb from the ITR make up between 1% and 9% of AAV particles. Most significantly, there was an observable decreasing trend in contaminant titer as distance from the ITR increased. Contaminant sequences closer to the ITRs (within 1kb) are detected at a 100 fold greater rate than distal plasmid DNA (9kb from ITRs on the same plasmid). The disparity in the levels of ITR adjacent DNA sequences, compared to sequences 9kb from either ITR, suggest that the origin of this DNA is from within AAV particles rather than residual plasmid DNA remaining after purification procedures. ITR adjacent contamination is present at both a TRS mutated ITR (required for self-complementary vectors) and non TRS mutated ITRs. Contaminating plasmid sequences were present when the transgene was half of the packaging capacity (2.3kb FIX prep) and at the full capacity (5kb FVIII prep) at comparable levels, suggesting that increasing the transgene size with stuffer DNA to create a full genome will not solve this issue. Previous studies have concluded that increasing the size of the backbone with stuffer DNA reduces the level of plasmid backbone contamination, as the two ITRs are then not in range of each other to facilitate reverse packaging. However, the total plasmid size in our studies was >20kb. Therefore, the ITRs should not be in range for this to occur. We hypothesise that these ITR adjacent sequences are either a product of read-through from the expression cassette into flanking sequences, due to inefficient cleavage at the ITR breakpoint, or from reverse priming mediated by only 1 functional ITR. In the current expression cassette plasmids examined, the Kan^r gene and bacterial f1 origin of replication are within the range of the flanking sequences that could be packaged. With current, unsolved clinical challenges for AAV, including transaminitis post high dose infection, it is clear that clinical AAV vectors should be designed to contain as little contamination as possible. We conclude that newly designed AAV production plasmids should contain significant lengths of stuffer DNA flanking each ITR (at least 2kb) to ensure that bacterial sequences are not packaged into AAV preps. Further research into vector design is required to eliminate this source of non-functional DNA from AAV produced for the clinic.



548. Recombinant AAV3 Serotype Vectors Generated by Using AAV3 ITRs and AAV3 Rep Proteins Efficiently Transduce Human Liver Cancer Cell Lines *In Vitro* and Human Liver Tumors in a Murine Xenograft Model *In Vivo*

Chen Ling[1], Zifei Yin[2], Jun Li[1], Daniel Zhang[1], George Aslanidi[1], Arun Srivastava[1]
[1]Pediatrics, University of Florida College of Medicine, Gainesville, FL, [2]Traditional Chinese Medicine, Second Military Medical University, Shanghai, China

Our initial observation of the selective tropism of AAV3 serotype vectors for human liver cancer cell lines and primary human hepatocytes (*Mol Genet Metabol*., 98: 289-299, 2009; *Hum Gene Ther*., 21: 1741-1747, 2010; *Gene Ther*., 19: 375-84, 2012), led to renewed interest in this serotype, since AAV3 vectors and their variants have recently proven to be extremely efficient in targeting human and non-human primate hepatocytes *in vitro* as well as *in vivo* (*Nature*, 506: 382-386, 2014; *Hum Gene Ther*., 25: 1023-1034, 2014; *Mol Ther*., 23: 1867-1876, 2015; *Mol Ther*., 23: 1877-1887, 2015). Our previous studies also documented that the combined use of AAV3 ITRs, AAV3 Rep proteins, and AAV3 capsids led to the production of recombinant AAV3 vectors with higher titers than those produced using AAV2 ITRs, AAV2 Rep proteins, and AAV3 capsids. We also observed that the transduction efficiency of Rep3/ITR3 AAV3 vectors was ~4-fold higher than that of Rep2/ITR2 AAV3 vectors in a human hepatocellular carcinoma (HCC) cell line, Huh7, under identical conditions. In the present studies, we extended these observations to include capsid-optimized AAV3 vectors in which two surface-exposed residues (Serine 663 and Threonine 492) were mutated to generate a S663V+T492V double-mutant vector to examine whether the transduction efficiency of the Rep3/ITR3-S663V+T492V-AAV3 vectors could be further augmented. To this end, two human HCC cell lines, Huh7 and LH86, were transduced with WT-AAV3 and S663V+T492V-AAV3 vectors generated with ITR2/Rep2 and ITR3/Rep3, respectively, under identical conditions. Consistent with our previously published studies, the transduction efficiency of the S663V+T492V-AAV3 vectors was ~10-fold higher than its WT counterpart, and interestingly, there was a further 2-fold increase in the transduction efficiency of both vectors generated with ITR3/Rep3, compared with those generated with ITR2/Rep2 (Fig. 1A). We next evaluated the transduction efficiency of these vectors in a murine xenograft model bearing human liver tumors. AAV3-EGFP-Neo vectors generated with either ITR2/Rep2, or with ITR3/Rep3, were injected intra-tumorally at a dose of $1x10^{11}$ vgs/tumor. Forty-eight hrs post-vector administrations, transgene expression was determined in each tumor by Western blot assays. These results indicated that AAV3 vectors generated with ITR3/Rep3 transduced human liver tumors *in vivo* ~2-fold more efficiently than those generated with ITR2/Rep2 (Fig. 1B). It is anticipated that the combined use of ITR3/Rep3, and S663V+T492V-AAV3 capsids will further increase the transduction efficiency of these vectors. Taken together, our data suggest the transduction efficiency of AAV3 vectors can be significantly improved both by using homologous Rep proteins and ITRs as well as by capsid-optimization. The use of these modified AAV3 vectors should prove useful in liver-directed human gene therapy.

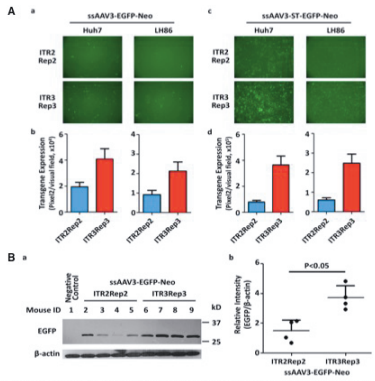


Figure 1. (A) Transduction efficiency of AAV3 vectors *in vitro*. WT-AAV3 (a and b) and S663V+T492V-AAV3 (b and d) vectors were generated with either ITR2/Rep2, or ITR3/Rep3 combinations. Human hepatocellular carcinoma cell lines, Huh7 and LH86, were transduced with the indicated vectors at an MOI of 5,000 vgs/cell. Transgene expression was determined by fluorescence microscopy 72 hrs post-transduction. Quantitation of the data (b and d). **(B) Transduction efficiency of AAV3 vectors *in vivo*.** Immune-deficient NSG mice (n=4) xenografted with human liver tumors were injected with $1x10^{11}$ vgs/tumor of AAV3-EGFP-Neo vectors, generated with either ITR2/Rep2 (lanes 2-5), or with ITR3/Rep3 (lanes 6-9) combinations. (a) EGFP expression in each tumor was determined by Western blotting 48 hrs post-vector administration. A tumor without vector injection (lane 1) was used as a negative control, and β-actin was used as a loading control. (b) Quantitation of EGFP expression in each tumor, normalized with β-actin expression.

549. Fine Tuning of Transduction Efficiency of rAAV Vectors via Modulation of Capsid Composition

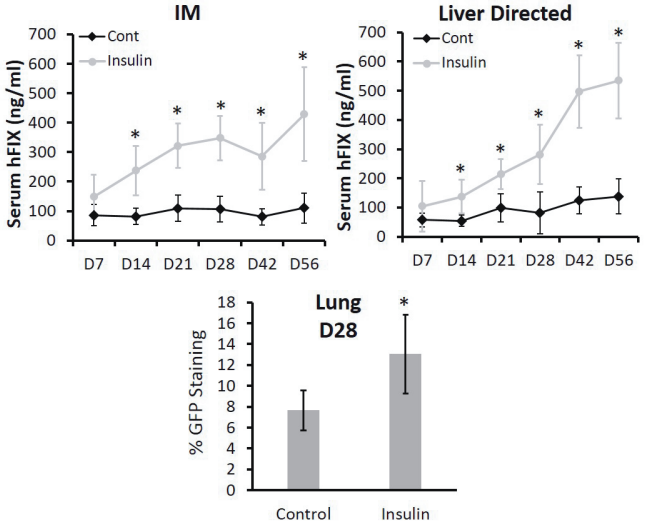
Oleksandr Kondratov[1], Damien Marsic[1], Mario Mietzsch[2], Regine Heilbronn[2], Sergei Zolotukhin[1]
[1]Dept. of Pediatrics, College of Medicine, University of Florida, Gainesville, FL, [2]Campus Benjamin Franklin, Charité Medical School, Institute of Virology, Berlin, Germany

Recombinant Adeno-associated virus (rAAV) vectors have emerged as one of the most versatile and successful gene therapy delivery vehicles. Even though the industry is poised for the expansion into several application areas represented by orphan diseases, a simple and scalable rAAV production technology is still lacking. We have recently developed the OneBac system to allow scalable, high-titer production of the full range of rAAV serotypes by infection of stable insect Sf9 cell lines with a single baculovirus. Some of the serotypes produced in this system, however, were characterized by a low transduction efficiencies compared to HEK 293-derived vectors. Here we describe an approach for resolving this drawback by modulating the ratios of VP1:VP2:VP3 capsid composition to derive particles with a higher VP1 content. This is accomplished by modifying a canonical Kozak sequence preceding VP1 ATG start codon. A range of Kozak sequences has been tested and the most favorable have been identified for AAV5 and AAV9 serotypes. These newly designed capsid genes were incorporated into Sf9 stable cell lines mediating packaging of AAV5 and AAV9 vectors which exceeded transduction efficiencies of HEK 293-derived counterparts by 2-3 folds. Curiously, the optimal ratios of VP1:VP2:VP3 were serotype-specific requiring fine-tuning to achieve a compromise between higher VP1 content mediating higher transduction potencies vs. lower packaging efficiencies impeding production yield. In summary, we have developed a novel approach of significantly enhancing biological potencies of AAV vectors derived from baculovirus system thus facilitating translational applications.

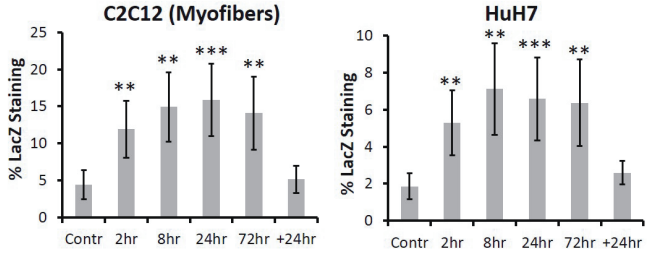
550. Developing an Optimal Insulin Augmentation Therapy to Improve AAV Gene Transfer to Skeletal Muscle and Liver in Mice

Sean Carrig, Mitchell J. Wopat, Ashley T. Martino
Pharmaceutical Sciences, St John's University, Queens, NY

We previously reported a novel finding that showed an insulin augmentation therapy can improve rAAV transduction of skeletal muscle and liver in cultured cells and mice. The insulin augmentation therapy in this earlier report was given just prior to AAV administration and was sustained for 28 days. The use of a long-term insulin augmentation was to determine if mice showed early signs of insulin resistance due to prolonged hyperinsulinemia. From the initial study we concluded that long-term insulin augmentation therapy improved AAV gene transfer to skeletal muscle and liver but that the mice showed a lack of elevated insulin activity and an increase in cortisol over time which are signs of insulin resistance. Additionally, our initial findings also suggested that an acute insulin augmentation therapy during the window of AAV administration would be sufficient without the concern for developing insulin resistance. The purpose of this new study was to investigate if an acute insulin augmentation therapy during the window of AAV delivery was sufficient to improve AAV transduction of skeletal muscle and liver. Additionally, we wanted to determine if an insulin augmentation therapy given after AAV infection would also enhance AAV gene transfer. Another aspect of this follow up study was to determine if an insulin augmentation therapy protocol would improve AAV transduction of lung tissue in mice. Finally, we wanted to investigate if fasting mice (which promotes reduced insulin activity) would result in sub-optimal AAV gene transfer. Our results showed an acute insulin augmentation therapy (4 hr treatment) given 30 minutes prior to intramuscular (IM) injection of AAV1-CMV-sc-hFIX or liver-directed delivery (via splenic capsule injection) of AAV8-CMV-sc-hFIX significantly improved (up to 5 Fold) sustained transduction for the length of the study (56 days).



The acute therapy showed a minor (but significant) 1.7 Fold increase in AAV1-CMV-EGFP transduction in lung. *In-vitro* AAV2-CMV-LacZ transduction of HuH7 (human liver) and differentiated C2C12 (mice myofibers) also showed improvement with an acute insulin treatment. A 2hr treatment (5 ug/ml) resulted in a 2.7 fold increase while 8hr, 24hr & 72hr treatments had a 3.5 fold increase and giving insulin 24 hrs after AAV infection had no impact of AAV transduction.



Finally, prolonged fasting will reduce insulin levels which could impact AAV gene transfer. Fasting mice for 12 hrs prior to and 4 hrs

after IM delivery of AAV1-CMV-sc-hFIX or at weekly intervals after administration timed around blood collection did not have an impact on AAV transduction of skeletal muscle.

551. Enhancement of Gene Therapy Treatment for Sandhoff Disease Through Complimentary Drug Therapy

Evan J. R. Woodley[1], Karlaina Osmon[2], Patrick Thompson[1], Subha Karumuthil-Melethil[3], Steven Gray[4], Jagdeep Walia[5]
[1]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada, [2]Centre for Neuroscience Research, Queen's University, Kingston, ON, Canada, [3]Gene Therapy Center, University of North Carolina, Chapel Hill, NC, [4]Department of Ophthalmology, University of North Carolina, Chapel Hill, NC, [5]Department of Pediatrics, Queen's University, Kingston, ON, Canada

GM2 gangliosidoses is a group of neurodegenerative lysosomal storage disorders caused by deficiency in the β-hexosaminidase A (HexA) enzyme. HexA is a heterodimer composed of 2 subunits; α- (encoded by the *HEXA* gene) and β-hexosaminidase β (Encoded by the *HEXB* gene). Mutations in either gene may cause inactivity of HexA leading to either Tay-Sachs disease (TSD, *HEXA* mutation) or Sandhoff disease (SD, *HEXB* mutation) respectively. TSD and SD are clinically indistinguishable phenotypes principally affecting infants and young children that are fatal before the age of 4 years; there is currently no effective available treatment. A mouse model for SD has been developed and these mice reach a humane end point at 14-17 weeks of age. Gene therapy can be an important primary therapeutic strategy, but may fall short of a complete rescue. Neuroinflammation and neurodegeneration have been identified as hallmark pathological mechanisms in GM2 gangliosidosis and can be adjunctive therapeutic targets. Neuroanti-inflammatory/neuroprotective agents like non-steroidal anti-inflammatory drugs (NSAIDs), Histone deacetylase inhibitors and pharmacological chaperones have shown ameliorating effects in SD and similar disease models when used alone. Adeno associated virus (AAV) based expression of Hexosaminidase isoenzymes has been shown to increase survival of Sandhoff mice for long term. We tested the combined role of gene therapy and neuroanti-inflammatory/neuroprotective agents in SD mice. Our methods include injecting neonatal SD mice with a relatively low dose ($2x10^{13}$ vg/kg) of a novel AAV9 vector expressing Hex A using both α and β subunits. A pilot study established the survival of these mice to be approximately 24 weeks, a 55% increase in life span over vehicle-injected controls. We observed a 47% increase in Hex A activity in the midbrain of treated mice as compared to the vehicle injected controls. Cohorts received treatment with neonatal gene therapy alone or in combination with indomethacin (a NSAID), pyrimethamine (a proven pharmacological chaperone for Hex A) and ITF2357 (a histone deactylase inhibitor) to elicit their combinational therapeutic potential. Each drug is administered daily via oral gavage starting the age of 6 weeks. All treatments have been completed and mice are being monitored for survival benefit and locomotor behaviour. Further analyses of enzyme activity, GM2 ganglioside levels and copy numbers will be done. The results of this study will establish a proof-of-concept for a novel combination gene therapy approach for treatment of GM2 gangliosidoses and similar disorders.

552. AAV9-Utrophin Restores Muscle Function in Dystrophic Mice

Shira T. Rosenblum[1], Hansell H. Stedman[2]
[1]University of Pennsylvania, Philadelphia, PA, [2]University of Pennsylvania Medical Center, Philadelphia, PA

The majority of mutations causing Duchenne muscular dystrophy (DMD) are multi-exon, frameshifting deletions, complicating therapy with recombinant dystrophin because of the potential for chronic immune recognition of the "non-self" protein. The paralogous protein utrophin is ubiquitously expressed at levels insufficient to prevent myonecrosis in animal models for DMD, but may confer central immunological tolerance through early developmental expression in the thymus. Here we show for a first time *histological* evidence for the complete prevention of myonecrosis in dystrophin-deficient striated muscles following systemic administration of an AAV9 vector carrying a 3.5 kb synthetic utrophin transgene (AAVμU). The cDNA was miniaturized by removal of domains least conserved in a comprehensive evolutionary comparison, and further optimized for maximal expression in striated muscle by using the codon bias of mammalian genes encoding contractile proteins. Administration of 10^{15} AAVμU vector genomes (vg) per kg to neonatal mice prevented centronucleation and saturated global recovery of the sarcoglycan complex, despite a subsequent tenfold increase in striated muscle mass with growth. In neonatal dystrophic dogs, intravenous injection of $10^{13.5}$ AAVμU vg/kg without immunosuppression restored sarcoglycan levels and normalized the myofiber size-distribution following a fourfold increase in muscle mass. Interferon-gamma ELISpot assays using utrophin-derived peptides revealed no reactivity in injected dogs, consistent with central immunological tolerance. Here we present for the first time results of a complex approach for the evaluation of *functional* rescue in mdx mice using non-invasive tests relevant to the clinically relevant symptoms of DMD through an open field cage system. The comparison between congenic wild type (C57Bl10) and mdx mice indicates similarity in parameters like rest time and the number of times entering and initiating use of the running wheel per day, but very significant differences with regards to running wheel associated locomotor activity characteristics including velocity, time, distance per run, and total distance per day. These data correlate with the differences in testing results between the two mouse strains observed in the force grip evaluation and serum levels of CK. Finally, we have shown significant enhancement of physiological performance using an open field running wheel system, and normalization of serum creatine kinase (CK) for the first time ever in treated dystrophic mice. These mice also demonstrated, improvements in both in vivo and ex vivo muscle strength, when compared with untreated mdx. The combination of these findings provide a rationale for high dose, neonatal gene therapy using utrophin as a "self" protein to forestall disability and mortality in DMD, while minimizing the risk of chronic immunotoxicity. These results also support the use of AAVμU as an experimental therapeutic for DMD based on a high level of functional reversal of muscular dystrophy in mdx mice. They demonstrate strong evidence in support of the running wheel open field system as a reliable and reproducible approach to assess the therapeutic efficacy in the mouse model of DMD. This approach also provides a platform for dissecting the physiological roles of dystrophin in supporting precisely measurable volitional activities in unrestricted animals, thereby offering potential improvements in the predictive power of preclinical studies of therapeutic efficacy to inform clinical trials.

553. Comparision of AAV Serotypes for Gene Delivery to iPSC Derived RPE

Vidyullatha Vasireddy, Thu T. Duong, Jeannette L. Bennicelli, Jean Bennett
University of Pennsylvania, Philadelphia, PA

Inherited retinal degenerations, such as retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA), are characterized by progressive impairment of visual function associated with degeneration of retinal pigment epithelium (RPE) and photoreceptors. These monogenic diseases are genetically heterogeneous due to mutations in genes expressed affecting those cells. Gene therapy holds great potential for the treatment of inherited retinal diseases for which there currently is none. Efficient retinal gene transfer has been achieved with several recombinant viral vectors, including those derived from adenovirus, retrovirus, herpesvirus, and adeno-associated virus (AAV). Among these, AAV vectors appear particularly amenable to retinal gene transfer. However the success of any preclinical study depends on the availability of relevant animal models. In situations where suitable animal models are unavailable recent studies have generated proof-of concept data using personalized cell models: induced pluripotent stem cells (iPSC) derived from affected individuals and un-affected controls. With the increasing number of retinal gene therapy paradigms and recombinant vectors, *in vitro* bioassays characterizing vector transduction efficiency and quality are becoming increasingly important. To date, most *in vitro* assays using recombinant vector transduction have targeted iPSCs. To elicit additional features relevant to the disease, we differentiated iPSCs to RPE and evaluated the transduction efficiencies of a panel of AAV serotypes. Characterization of iPSC-derived RPE by qRT-PCR, immunohistochemistry, western blot analysis and flow cytometry showed the expression of typical RPE markers, phagocytic ability and gene-expression patterns similar to those of native RPE. Comparison of transduction efficiencies of different AAVs in iPSC-derived RPE was carried out using an enhanced green-fluorescent protein (eGFP) reporter gene driven by the cytomegalovirus immediate-early (CMV) promoter. At 24 to 48 hours post transduction, cells expressing GFP were identified by Typhoon scanner, and flow cytometry. Relative GFP expression was evaluated by using the software, Image-J. Of the tested AAV serotypes, AAV2 transduced iPSC-derived RPE cells most efficiently, followed by AAV7m8, AAV1 and AAV6. Differentiation into retinal neuronal cells types may require use of alternative AAV serotypes in order to obtain transduction efficiencies relevant to determination of therapeutic efficacy.

554. Serotype Comparison of AAV Transduction in Adipose-Derived Stem Cells

Priyanka Sharma[1], Sunishka M. Wimalawansa[2,3], Gregory C. Gould[2,3], R. Michael Johnson[2,3], Katherine J. D. A. Excoffon[1,2]
[1]Biological Sciences, Wright State University, Dayton, OH, [2]Orthopaedic Surgery, Sports Medicine and Rehabilitation, Boonshoft School of Medicine, Wright State University, Dayton, OH, [3]Wright State Physicians Plastic Surgery, Miami Valley Hospital, Dayton, OH

Adipose-derived stem cells (ASC) demonstrate promising results in the treatment of many diseases. However, it is unlikely that a simple infusion of cells will provide the full range of desired treatment results. Transient genetic modification of ASC to turn them into drug-eluting depots is likely to enhance their reparative characteristics and accelerate healing. We hypothesized that adeno-associated virus (AAV), an approved gene therapy vector that has never been associated with disease, has several ideal characteristics needed for creating drug-eluting ASC. The most common recombinant AAV vectors were tested for transduction and duration of gene expression. rAAV5 demonstrated both the highest and longest term expression. The glycosylation profile of ASC was determined and we show that rAAV5 transduction requires plasma membrane associated sialic acid. Future studies will focus on rAAV5 as the vector of choice to deliver disease specific genes to drive biological drug delivery, engraftment, and disease correction.

555. Development of a Post-Exposure Treatment for Ebola Virus Infections Based on AAV Vectors and Zmapp Antibody Cocktail

Marc-Andre Robert[1], Amine Kamen[2], Gary Kobinger[3], Rénald Gilbert[4], Bruno Gaillet[1]
[1]Department of chemical engineering, Université Laval, Québec, QC, Canada, [2]Department of Bioengineering, Université McGill, Montréal, QC, Canada, [3]National Microbiology Labororatory, Public Health Agency of Canada, Winnipeg, MB, Canada, [4]National Research Council Canada, Montréal, QC, Canada

The recent Ebola outbreak in West Africa has been the deadliest in the history. To prevent future recurrence of such outbreak, better treatments and effective vaccines against Ebola virus are desirable. Among such promising treatments, the Zmapp cocktail containing neutralizing antibodies (13C6, 2G4 and 4G7) has successfully treated some patients. However, the feasibility of using it on large populations especially in developing countries is questionable. To address this potential issue, we propose to employ recombinant vectors derived from adeno-associated virus (rAAV). There are several advantages of using rAAV: because of 1) their safety profile; 2) only one injection (or a few) would be required; 3) the high stability of lyophilized rAAVs at ambient temperature and; 4) the panel of available serotypes. Because of these interesting features, we are currently developing a treatment based on three rAAVs to deliver the genes for the Zmapp cocktail of antibodies. We have already produced at small scale a rAAV expressing the 2G4 antibody. The DNA sequences for the heavy chain and light chains were codon-optimized for better expression in humans and were designed to be expressed from the same gene. A strong promoter (CAG) resistant to silencing in vivo was chosen to drive gene expression of the antibody. The rAAV were produced by transfection using our patented cGMP compatible HEK293 cell line. The production was performed in suspension culture in the absence of serum. Secretion of 2G4 antibody by rAAV transduced cells (HEK293 and CHO cells) was confirmed. The results demonstrated that rAAV-CAG-2G4 was functional and allowed for the correct assembly of the heavy and light chains of 2G4. Purification of 200 mL of rAAV-CAG-2G4 production was performed by ultracentrifugation on an iodixanol density-step gradient. Two other rAAVs coding 13C6 and 4G7 antibodies are in the processed of being constructed and produced in a similar manner. We are also in the process of comparing the efficacy of two serotypes of AAV (9 and DJ) in mice by intranasal delivery. Using the best serotype, the rAAVs will be produced and purified from a starting suspension culture of 20 L. Their efficacy for treating Ebola infections will then be evaluated in a mouse model infected by the virus.

556. Comparative *In Vitro* Transduction Efficiency of AAV Vector Serotypes 1-9 in Different Cellular Models

Thu T. Duong, Vidyullatha Vasireddy, Carrie E. Merkel, Jeannette L. Bennicelli, Jean Bennett
Department of Ophthalmology, University of Pennsylvania, Philadelphia, PA

Recombinant viral vectors can be useful tools for expressing transgenes in neuronal cells to study disease mechanisms and to test for therapeutic effect. Recombinant adeno-associated virus (AAV), generated from a nonpathogenic parvovirus, has become the most

widely used vector in gene therapy applications in human. However, lack of suitable animal models that accurately reflect the human disease phenotype often stalls the development of treatment strategies. To overcome this problem, research is focused on generating induced pluripotent stem cells (iPSCs) from affected human subjects as *in vitro* models to explore the potential of gene augmentation therapy. An advantage of iPSCs is that these cells can be differentiated along a retinal cell lineage. In this study, we differentiated iPSCs into Neural Progenitor Cells (NPC) and compared the *in vitro* transduction efficiency of AAV serotypes 1-9. Differentiated NPCs were characterized by immunocytochemistry with the expression of NPCs specific markers (Sox2, Nestin, Pax6). Experiments were carried out using different AAVs serotypes containing the same transgene cassette: an enhanced green-fluorescent protein (eGFP) reporter gene driven by the cytomegalovirus immediate-early (CMV) promoter. At 24 to 48 hours post transduction, positive GFP expressing cells are examined by laser scanning in Typhoon. Relative GFP expression was evaluated by using the software ImageJ. In addition to the laser scanning method, flow cytometry was used to evaluate GFP expression in the cell population. Live cell imaging and flow cytometry evaluation revealed that AAV2 is the most efficient vector for *in vitro* transduction in NPC followed by AAV1, AAV3 and AAV6 while AAV4, AAV5, AAV7, AAV8 and AAV9 showed no transduction or extremely low transduction efficiency.

Targeted Genome Editing III

557. Targeted *CYBB* Minigene Insertion into the *CYBB* Locus for Correction of X-CGD iPSCs Requires Intronic Elements for Expression

Colin L. Sweeney[1], Uimook Choi[1], Jizhong Zou[2], Randall K. Merling[1], Suk See De Ravin[1], Harry L. Malech[1]
[1]Laboratory of Host Defenses, Genetic Immunotherapy Section, NIAID, NIH, Bethesda, MD, [2]Center for Molecular Medicine, NHLBI, NIH, Bethesda, MD

X-linked chronic granulomatous disease (X-CGD) is an immune deficiency characterized by defective phagocyte production of microbicidal reactive oxygen species (ROS), resulting in recurring, life-threatening infections and hyper-inflammation. Mutations causing X-CGD span the entire 13 exons or intronic splice sites of the >30-kb *CYBB* gene encoding gp91phox, resulting in a loss of gp91phox protein expression. We previously tested a TALEN-mediated targeted gene therapy approach to insert a codon-optimized *CYBB* minigene into the start site of endogenous *CYBB*. Although targeted insertion into the endogenous start site was achieved in X-CGD patient iPSCs, little or no gp91phox expression or ROS activity was observed upon granulocyte differentiation, suggesting that downstream intronic or regulatory elements may be necessary for efficient gene expression from the *CYBB* promoter. To test this hypothesis, we tested CRISPR-mediated targeted insertion of a codon-optimized CYBB cDNA consisting of exons 2 through 13 (CYBB2-13) together with a puromycin-resistance gene cassette into exon 2 of the *CYBB* locus. In iPSCs from X-CGD patients with a *CYBB* mutation in exon 5, exon 7, or intron 10, the efficiency of targeted insertion of the CYBB2-13 plasmid donor without random inserts in puromycin-selected clones was 50-66%. Upon granulocyte differentiation of CYBB2-13 corrected X-CGD iPSCs, gp91phox expression and ROS production were restored to levels 64-100% (gp91phox) and 68-76% (DHR) of normal healthy donor controls. As expected for expression from the endogenous *CYBB* promoter, expression of gp91phox was specific to $CD13^+$ granulocytes, and was undetected in undifferentiated iPSCs. This targeted gene therapy approach should allow correction of ~90% of X-CGD patient mutations (those involving mutations in exons 2 through 13), to restore ROS activity while maintaining normal regulation of *CYBB* expression. Further, these findings demonstrate a key issue for the design of targeted gene insertion to capture expression from an endogenous promoter: for some endogenous promoters, the inclusion of intronic elements is necessary for efficient expression of the insert.

558. Targeted Gene Therapy in $CD34^+$ Cells from Healthy Donors and Fanconi Anemia Patients

Begoña Díez[1], Pietro Genovesse[2], Giulia Schiroli[2], Lara Álvarez[1], Francisco J. Román-Rodriguez[1], Philip D. Gregory[3], Michael Holmes[3], Angelo Lombardo[2], Luigi Naldini[2], Paula Rio[1], Juan A. Bueren[1]
[1]Hematopoietic Innovative Therapies, CIEMAT/CIBERER/IIS. Fundación Jiménez Díaz, Madrid, Spain, [2]San Raffaelle Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milano, Italy, [3]Sangamo BioSciences Inc., Richmond, CA

Gene targeting is rapidly progressing thanks to the development of improved nucleases and donor constructs, and represents a new gene therapy-based strategy for the treatment of inherited diseases, such as Fanconi anemia (FA). Because FA can be generated by multiple mutations in up to 19 different genes, we focused our interests in the insertion of therapeutic FA genes in *safe harbor loc*i, since this approach could serve as a platform for treating all FA subtypes and pathogenic mutations. In previous studies we demonstrated the feasibility of correcting the phenotype of hematopoietic progenitors through the genome editing and reprogramming of fibroblasts from FA patients. Because of the limited repopulating properties so far reported for iPSC-derived hematopoietic precursor cells, here we aimed to investigate the possibility of conducting a similar gene targeting approach using human primary $CD34^+$ cells. In a first set of experiments we investigated the efficiency of targeting the *AAVS1* locus in cord blood HSPCs. Pre-stimulated $CD34^+$ cells were transduced with integrase-defective lentiviral vectors (IDLV) encoding an *EGFP* reporter gene under the control of the PGK promoter, flanked by sequences homologous to the *AAVS1* target site. The cells were then nucleofected with *AAVS1*-specific ZFN mRNAs. The average targeting efficiencies ranged from 10-20% both in *in vitro* cultured cells and NSG mice-repopulating cells. In subsequent experiments, the *E*GFP reporter gene was replaced by the *hFANCA* therapeutic gene. Gene targeting experiments conducted in lymphoblast cell lines and primary $CD34^+$ cells from FA-A patients reverted the characteristic hypersensitivity of FA cells to mitomycin C (MMC), and also restored the formation of FANCD2 foci after DNA damage, evidencing the phenotypic correction of these cells. Our data confirm the efficacy of *AAVS1*-gene targeting in hHSPCs and demonstrate the feasibility of conducting these approaches in primary $CD34^+$ cells from FA patients.

559. Induction of Fetal Hemoglobin in Adult Erythroblasts by Genome Editing of the Beta-Globin Locus

Chiara Antoniani[1], Annalisa Lattanzi[2], Giulia Pavani[2], Vasco Meneghini[1], Elisa Magrin[1], Leslie Weber[1], Matthew Porteus[3], Marina Cavazzana[1], Fulvio Mavilio[2], Annarita Miccio[1]
[1]Imagine Institute, Paris, France, [2]Genethon, Evry, France, [3]Stanford University, Stanford, CA

Sickle cell disease (SCD) and β-thalassemia are severe anemias characterized by abnormal or reduced production of hemoglobin β-chains. Autologous transplantation of genetically corrected hematopoietic stem cells (HSC) is an attractive therapeutic alternative for patients lacking a compatible HSC donor. Naturally occurring, large deletions in the β-globin locus result in increased fetal hemoglobin (HbF) expression (HPFH, Hereditary Persistence

of Fetal Hemoglobin), a condition that mitigates the clinical severity of β-hemoglobinopathies. Here, we integrated BCL11A and GATA1 transcription factor binding site analysis and HPFH mutational data to identify potential HbF silencers in the β-globin locus. Based on this analysis, we designed a CRISPR/Cas9 strategy to disrupt a 13-kb genomic region commonly deleted in HPFH, which includes the δ- and β-globin genes and putative intergenic HbF silencers, and achieved efficient targeted deletion in erythroid cell lines by plasmid, RNA and lentiviral delivery of the CRISPR/Cas9 nuclease system. RT-PCR showed a dramatic increase in γ-globin mRNA levels in modified adult hematopoietic stem progenitor cells (HSPC)-derived erythroid cell lines (HUDEP-2). FACS and HPLC analysis demonstrated re-activation of HbF and a concomitant decrease in HbA expression. Cell morphology, erythroid marker profile, total Hb levels and erythroid maturation were unaffected, consistent with the asymptomatic phenotype of adult HPFH carriers. The same strategy was tested in primary human erythroblasts by lentiviral transduction of adult $CD34^+$ HSPCs followed by *in vitro* erythroid differentiation in liquid and clonogenic cultures. Deletion of potential HbF silencers resulted in a 35% increase in γ-globin expression compared to basal levels in primary human erythroblasts as measured by HPLC, suggesting that these sequences could serve as targets for therapeutic genome editing for HbF induction in β-hemoglobinopathies. We are currently testing the efficiency of our CRISPR/Cas9-based strategy in patient-derived HSPCs. Overall, this study contributes to the knowledge of the mechanisms underlying fetal to adult Hb switching, and provides clues for a therapeutic strategy for SCD and β-thalassemia.

560. Generation of Functional Regulatory T Cells by *FOXP3* Gene Transfer into CD4 T Cell from IPEX Patients

Emmanuelle Six[1], Nelly Sigrist[1], David Zemmour[2], Marie-Andrée Bessard[1], Fabienne Mazerolles[3], Samia Martin[4], Fulvio Mavilio[4], Frederic Rieux-Laucat[3], Christophe Benoist[2], Isabelle André-Schmutz[1], Marina Cavazzana[5]
[1]Laboratory of Human Lymphopoiesis, Imagine Institute, Paris, France, [2]Division of Immunology, Harvard Medical School, Boston, MA, [3]Laboratory of Immunogenetics of Pediatric Autoimmunity, Imagine Institute, Paris, France, [4]Genethon, Evry, France, [5]Biotherapy Clinical Investigation Center, Necker Children's Hospital, Paris, France

IPEX (Immunodysregulation Polyendocrinopathy Enteropathy X-linked) syndrome is the prototype of primary immunodeficiency with prevailing autoimmunity. The disease is caused by mutations in the gene encoding the transcription factor forkhead box P3 (*FOXP3*), which leads to the loss of function of thymus-derived CD4+CD25+ regulatory T (tTreg) cells. In IPEX patients, the absence of a functional Treg cell compartment leads to the development of multiple autoimmune manifestations (including severe enteropathy, type 1 diabetes and eczema) usually in the first months or years of life. The current treatments for IPEX syndrome include immunosuppressive, hormone replacement therapies. Unfortunately, immunosuppressive treatments are usually only partially effective and their dose is often limited because of the occurrence of infectious complications and toxicity. Currently, the only curative treatment for IPEX syndrome is allogeneic hematopoietic stem cell transplantation (HSCT). The absence of an HLA-compatible donor for all patients and their poor clinical condition particularly expose them to a risk of mortality when HLA partially compatible donors are used. For all these reasons, effective alternative therapeutic approaches are urgently needed. Various preclinical studies have shown that partial donor chimerism is sufficient for complete remission meaning that a small number of functional natural Treg is sufficient to restore immune tolerance. This suggests that T cell gene therapy approaches designed to selectively restore the repertoire of Treg cells is a promising potential cure for IPEX. We demonstrated that *FOXP3* gene transfer into CD4+ T cells from IPEX patients enable the generation of potent and stable regulatory T cells. Indeed we showed the functional suppressive properties of the generated $CD4^{IPEX}$-FOXP3 cells in an optimized flow-cytometry-based *in vitro* suppression assay. We are currently comparing the transcriptional profile of these regulatory $CD4^{IPEX}$-FOXP3 cells in comparison to natural Treg by RNA-seq analysis. Therefore, the introduction of a functional copy of the *FOXP3* gene into an IPEX patient's T cells may be enough to restore immune tolerance and thus avoid the complications of allogenic HSCT. We will also discuss the challenge of generating a large, homogenous and stable population of cells *in vitro* for adoptive transfer and whether it can ensure long-term disease correction without generating a context of generalized immunosuppression.

561. CRISPR-Induced Deletion (CinDel) Method Allows Permanent and Efficient Restoration of the DMD Gene Reading Frame in Duchenne Patient Myoblasts and Preserves Truncated Dystrophin Structure

Jean-Paul Iyombe-Engembe[1], Dominique L. Ouellet[1], Xavier Barbeau[2], Joël Rousseau[1], Pierre Chapdelaine[1], Patrick Lagüe[2], Jacques P. Tremblay[1]
[1]Médecine Moléculaire, Université Laval, Québec, QC, Canada, [2]Department of Chemistry, Université Laval, Québec, QC, Canada

The CRISPR/Cas9 system is a great revolution in biology. This technology allows the modification of genes *in vitro* and *in vivo* in a wide variety of living organisms. In most Duchenne Muscular Dystrophy (DMD) patients, expression of dystrophin (DYS) protein is disrupted because exon deletions result in a frame shift. Here we present CRISPR-induced deletion (CinDel), a new gene therapy approach to correct the DMD gene in Duchenne patients with one or more exons deletions. By using adequate pair of gRNAs targeting specifically the exons precede and follow the patient deletion in DMD gene, CinDel induces precise DSB in targeted sequences and allows an additional deletion. The remaining parts of the exons were fusioned by NHEJ to form a hybrid exon and restored the DMD reading frame in 62 % of hybrid exons *in vitro* in patient myoblasts and *in vivo* in electroporated muscle hDMD/mdx mice. Moreover, adequate pairs of gRNAs also restored the normal spectrin-like repeat of the dystrophin rod domain; such restoration is not obtained by exon skipping or deletion of complete exons. The expression of an internally deleted dystrophin protein was detected following the formation of myotubes by the **unselected** treated DMD myoblasts. Given that CinDel induces permanent reparation of the *DMD* gene this treatment would not have to be repeated as it is the case for exon skipping induced by oligonucleotides.

562. Fetal Hemoglobin Expression Can Be Increased by Targeted Disruption of the Gamma Hemoglobin Promoter in Human Peripheral Blood Stem Cells

Christopher T. Lux[1,2], Queenie Vong[1], Julia Yang[1], Brandon K. Hadland[3], Andy Scharenberg[1,4]
[1]Seattle Children's Research Institute, Seattle, WA, [2]Pediatric Hematology Oncology, Seattle Children's Hospital, Seattle, WA, [3]Fred Hutchinson Cancer Research Center, Seattle, WA, [4]Pediatric Immunology, Seattle Children's Hospital, Seattle, WA

Hemoglobinopathies such as sickle cell disease and β-thalassemia are both common and morbid inherited genetic disorders. Mutations in the β -globin (*HBB*) gene result in the expression of pathologic globin molecules or the reduction in expression of the wildtype allele.

Despite the high prevalence of these disorders, current treatment options are limited. Patients with a hereditary persistence of fetal hemoglobin (HPFH) have disease modifying mutations that result in the compensatory expression of gamma or delta hemoglobin and these patients tend to have a much less severe phenotype. A previously reported unique 13bp deletion identified in the gamma hemoglobin promoter of two sickle cell patients with HPFH (HbF >30%) offers a novel target for gene editing using targeted endonucleases. This region contains known binding sites for multiple regulatory factors including the DRED repressor complex and the CAAT-box binding factor (CBF). TALENs designed to target this 13bp site have been generated and the mRNA successfully transfected into human peripheral blood CD34 cells. Genomic analysis of TALEN transfected cells reveals the introduction of INDELs by NHEJ in both the γ1 (*HBG1*) and γ2 (*HBG2*) genes (33% and 8% respectively). TALEN edited human peripheral blood stem cells were then differentiated into erythroid progeny that express increased levels of fetal hemoglobin detected by both flow cytometry (>50% increase over control) and HPLC (>5-fold increase over control). CRISPR guides (traditional and chemically modified RNA guides) targeting the same locus are currently being compared to TALENs. These data suggest that the introduction of INDELs in this locus is capable of de-repressing the gamma globin gene in human CD34 cells. In vivo mouse xenografts are underway to further assess this approach as a means for a functional cure of a wide array of hemoglobinopathies.

563. Analysis of DNA Repair Pathway Choice Upon Induction of Double-Strand Breaks by Engineered Nuclease

R. Ileng Kumaran[1], Jingjing Li[1], William S. Dynan[2], Matthew H. Porteus[3], David L. Spector[1]
[1]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, [2]Emory University School of Medicine, Biochemistry, Atlanta, GA, [3]Stanford University, School of Medicine, Pediatrics - Cancer Biology, Stanford, CA

DNA double-strand breaks (DSBs) are among the most dangerous classes of DNA damage. Cells have evolved complex DNA damage response pathways to repair DSBs. In mammalian cells, two major and mechanistically distinct DSB repair pathways are non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ and HR can be regulated by multiple factors including position in the cell cycle. For single-copy loci, HR is thought to be restricted to late S/G2 phase, based on the need for a sister chromatid as a repair template. It remains unclear whether this is also true for multi-copy loci. Here, we have investigated DSB pathway choice by developing multi-copy and single-copy DSB reporter human cell systems. Using these systems we initiated a DSB with a specific zinc finger nuclease and then visualized DSB repair as it relates to cell cycle phase. We show that both the NHEJ and HR pathways can simultaneously repair DSBs at the reporter locus. The frequency of recruitment of the HR repair protein Rad51 is higher with the multi-copy DSB substrate than with the single-copy. This suggests that, availability of a homologous template near the DSB repair site influences repair pathway choice. Consistent with this, molecular analysis of repair at the single-copy reporter by SMRT sequencing revealed an increase in gene editing frequency by HR with increasing amounts of transfected exogenous donor template. Together, results indicate that HR can occur in G1 phase of the cell cycle if donor template is available for repair. Our findings suggest a competition between the HR and NHEJ repair machineries throughout interphase of the cell cycle, which could be a critical factor for the stability of mammalian genomes that are highly enriched with repeat sequences. Insights from these studies will help in understanding genomic instability disorders and to improve gene therapy approaches.

564. The Cytotoxic Effect of RNA-Guided Endonuclease Cas9 on Human Hematopoietic Stem and Progenitor Cells (HSPCs)

Kyung-Rok Yu[1], Marcus A. F. Corat[1,2], Jean-Yves Metais[3], Cynthia E. Dunbar[1]
[1]Hematology Branch, NHLBI, NIH, Bethesda, MD, [2]Multidisciplinar Center for Biological Research, University of Campinas, Campinas, Brazil, [3]Hematology, St. Jude Children Research Hospital, Memphis, TN

The CRISPR (clustered regulatory interspaced short palindromic repeats) - CRISPR-associated protein 9 (Cas9) system (CRISPR/Cas9) has recently emerged as an efficient and powerful approach widely used for targeted genetic engineering. RNA-guided endonuclease Cas9 in combination with a single guide RNA (sgRNA) targets the sequence-specific DNA binding domains and generates double strand breaks (DSBs), which are repaired through non-homologous end joining (NHEJ) or homologous recombination (HR). Although CRISPR/CAS9 system very efficiently disrupts target locations in the genome of multiple model organisms, editing in some primary cell types, especially hematopoietic stem and progenitor cells (HSPCs) has been more challenging, with lower efficiencies particularly in functional engrafting HSPC. However, the exact mechanisms have not been elucidated. To optimize editing via CRISPR/Cas9 in human HSPCs, we generated several different lentiviral vectors (Figure 1); a negative control GFP only non-editing vector (LeGO-GFP), U6 promoter-AAVS1 targeting sgRNA-EF1a promoter-GFP (AAVS1-GFP), U6-EFS promoter-Cas9-EF1a-GFP (Cas9-GFP), U6-AAVS1 targeting sgRNA-EFS-Cas9-EF1a-GFP (AAVS1-Cas9-GFP), U6-EFS-human codon-optimized Cas9-EF1a-GFP (hCas9-GFP), and U6-AAVS1 targeting sgRNA-EFS-human codon-optimized Cas9-EF1a-GFP (AAVS1-hCas9-GFP). These lentiviral vectors simultaneously deliver sgRNA targeting a "safe harbor" genome location (AAVS1 in these experiments), Cas9 and a fluorescent marker, however, we replaced P2A with EF1a promoter between Cas9 and a fluorescent marker, because we found dim or none fluorescent marker expression using P2A co-expression systems. Lentivirus-transduced HSPCs were analyzed by flow cytometry based on their GFP expression in a time-dependent manner (3, 6, 9, 12 days). All vectors resulted in a gradual decrease in the percentage of GFP+ cells over time, however, standard Cas9 containing vectors (Cas9-GFP and AAVS1-Cas9-GFP) showed most significant decreases (Figure 2). Vectors containing the human codon-optimized version of Cas9 (hCas9-GFP, AAVS1-hCas9-GFP) showed intermediate decreases, and vectors without Cas9, whether or not they expressed sgRNA (LeGO, AAVS1-GFP), showed the least decrease in GFP+ cells, with 30% of the cells still expressing GFP at 12 days following transduction. These results led us to investigate the cytotoxicity of Cas9 on HSPCs, using Vivid and Annexin V staining. Both standard Cas9 and human codon-optimized Cas9 expressing HSPCs showed strong Annexin V positivity and a gradual increase in Vivid+ cells, explaining the loss of GFP+ cells with Cas9-expressing vectors. On the other hand, HSPCs transduced with the non-Cas9-expressing vectors remained Vivid and Annexin V low until through 12 days. Our findings suggest that sustained expression of Cas9 in human HSPCs, for instance via an integrating vector, has significant toxicity, and that an alternative Cas9 delivery method for HSPCs is required. We have tried integrase-defective lentivirus (IDLV) and mRNA transfection to deliver Cas9 into HSPCs, and data will be presented. This approach will facilitate the use of CRISPR/Cas9 system to engineer genes of clinical significance in HSPCs with a therapeutically meaningful efficacy.

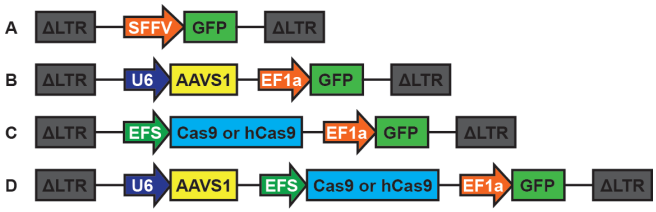


Figure 1. Schematics of lentiviral vectors. A. LeGO-GFP, B. AAVS1-GFP, C. Cas9-GFP or hCas9-GFP, D. AAVS1-Cas9-GFP or AAVS1-hCas9-GFP

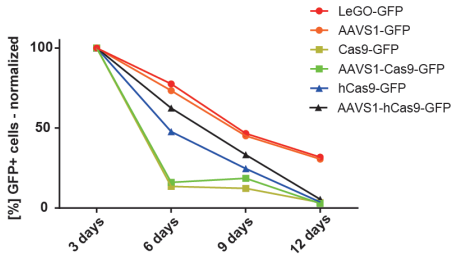


Figure 2. Percentage of GFP+ cells in sgRNA/Cas9 overexpressed HSPCs over time

565. Human Dystrophin Expression in Rag/Mdx Mice Muscles Following the Graft Off Genetically Corrected Dystrophic hiPSCs

William-Édouard Gravel[1], Dominique L.Oulette[1], Chantale Maltais[1], Catherine Gérard[1], Li Hongmei L.[2], Akitsu Hotta[2], Jacques-P. Tremblay[1]
[1]Neurosciences, Centre Hospitalier de l'Université Laval (CHUL), Québec, QC, Canada, [2]Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan

Human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) have shown self-renewal capacity and can potentially differentiate into all types of cell lineages. They represent an unlimited source of cells for the therapy of degenerative diseases, such as Duchenne Muscular Dystrophy (DMD), a disease characterized by a rapid degeneration of muscles that starts early in life. Dystrophic hiPSCs have been corrected by our collaborator, Dr. Hotta, by inserting of a single base pair in the exon 45 with Transcription Activator-Like Effector Nucleases (TALENs) to restore the reading frame of the gene. Our laboratory has developed a two-step procedure to differentiate hiPSCs into myogenic cells. We first used a myogenic culture medium especially developed in the laboratory (called MB-1) to promote the differentiation of hiPSCs into mesenchymal-like precursor cells. We next transduced them with a lentivirus expressing the myogenic transcription factor MyoD under the control of the composite CAG promoter, in order to induce their differentiation into myoblasts. Transduced cells have been grafted in the Tibialis anterior muscle of Rag/mdx mice where they fused with existing muscle fibers. The presence of the human dystrophin protein has been confirmed by immunohistofluorescence in muscles grafted with the genetically corrected cells and in a control graft with myoblasts of a healthy donor. Cell therapy shows great promises for DMD patients since it allows the expression of a normal gene capable of producing a functional dystrophin in muscle fibers and increases the regenerative capacity of the muscle and the muscle strength.

566. Targeted Lentiviral Vector Integration Mediated by the AAV Rep78 Protein

Pingjuan Li[1], Jizhong Zou[2], Michael T. Morreale[1], Michael P. Marino[1], Jakob Reiser[1]
[1]FDA/CBER, Silver Spring, MD, [2]NIH/NHLBI, Bethesda, MD

Our goal is to improve the safety of lentiviral vectors by shifting their integration preference to genomic "safe harbor" sites where therapeutic transgenes can integrate and function in a predictable manner. To do this, we have generated integrase-defective LV vectors (IDLVs) capable of inserting transgene sequences at predetermined genomic sites through homologous recombination (HR). To increase the frequency of HR, we employ genome editing approaches involving site-specific nucleases. A major drawback of this approach is safety concerns caused by inadvertent double strand breaks (DSBs) and non-homologous end-joining (NHEJ) events at unintended genomic (off target) sites. To bypass this risk, we are pursuing site-specific nucleases that do not involve DSBs as a potentially safer class of DNA-modifying agents for site-specific integration of transgenes in human cells. The sequence and strand-specific endonucleases Rep78 and Rep68 encoded by adeno-associated virus (AAV) have previously been reported to stimulate HR of plasmids bearing homology arms corresponding to the AAVS1 safe harbor locus on chromosome 19. We tested the integration efficiency of IDLVs containing short homology arms corresponding to the AAVS1 locus mediated by an engineered version of the AAV2-derived Rep78 protein in HEK293 cells. We compared the integration efficiency of IDLVs mediated by the Rep78 protein to that of an AAVS1-specific zinc finger nuclease (ZFN). Furthermore, we explored the potential of IDLV donor vectors for targeted integration mediated by Rep78 in human induced pluripotent stem cells (hiPSCs). The results obtained indicate that in the presence of the Rep78 protein, the integration efficiency of IDLVs was up to 8.3 fold higher compared to cells treated with the donor vector alone. Moreover, the efficiency of site-specific targeting mediated by Rep78 was higher than that of the AAVS1-specific ZFN. We also showed that Rep78 can successfully direct integration of IDLVs at the AAVS1 locus in hiPSCs. We anticipate that the approach used may ultimately contribute to the reduction of risks associated with LV vectors.

567. CRISPR-Cas9 Mediated Gene Editing in a Monogenic Form of Alzheimer's Disease

Bence Gyorgy[1], Martin Ingelsson[2], Camilla Loov[1], Shuko Takeda[1], Lars Lannfelt[2], Bradley T. Hyman[1], Xandra O. Breakefield[1]
[1]Neurology, Massachusetts General Hospital, Boston, MA, [2]Department of Geriatrics, Uppsala University, Uppsala, Sweden

Background

Individuals heterozygous for the Swedish mutation of the amyloid precursor protein (APPswe) display an increased β-secretase cleavage leading to higher Aβ levels - both in brain and peripheral tissues, such as fibroblasts and plasma. The mutation is a double base change adjacent to each other and has a dominant effect. We hypothesize that the CRISPR system would selectively disrupt the mutated allele without affecting the wild-type allele. Therefore we set out to use the CRISPR system to disrupt expression of mutant APP to counteract Aβ overproduction in Swedish patient fibroblasts.

Methods

Human APPswe fibroblasts and non-mutated control fibroblasts from subjects of the same family (n=3 for both mutated and control cell lines) were cultured in DMEM medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Approximately one million cells were used for transfection with the SpCas9 plasmid together with different guide RNAs (gRNAs) designed to bind either the mutated or non-mutated site with the mutation in the gRNA recognition sequence. Four days after transfection, the GFP positive cells were sorted by FACS, enabling separation of Cas9-transfected

Molecular Therapy Volume 24, Supplement 1, May 2016

from non-transfected cells. DNA was extracted from one fraction of the sorted cells, followed by Sanger sequencing and targeted deep sequencing. Another fraction of the cells was further cultured until they were confluent, after which they were kept for 48 h in FBS free media. ELISAs specific for Aβ40 and Aβ42 were used to measure peptide levels in conditioned media from the various cell populations.

Results

Sanger sequencing was performed on cells that had been successfully transfected with CRISPR plasmids. On such cells, both the APPswe mutant and wt alleles could be disrupted with gRNAs designed against the mutated and non-mutated sites, respectively. Moreover, these effects appeared to be highly specific as assayed by deep sequencing as we did not find any random mutations on the wt allele with the gRNA targeting the mutated site or vice versa. Most of the genomic changes were deletions, but there were some insertions as well. ELISA measurements indicated robustly decreased levels of both Aβ40 and Aβ42 in conditioned media from both mutated and wt cells treated with the respective gRNAs. Shortening the gRNA led to decreased on-target effectivity.

Conclusions

With the CRISPR/Cas 9 system we could selectively disrupt both the mutated and wt site in fibroblasts from subjects with and without the APPswe mutation. The adjacent double base change created a unique opportunity to selectively disrupt the mutant allele with one of our gRNAs. Furthermore, we could demonstrate a decreased generation of Aβ40 and Aβ42 in the conditioned media from cells treated with the specific gRNAs. This study provides the first experimental evidence that the CRISPR/Cas9 method could be used to develop a novel treatment strategy against familial forms of Alzheimer's disease caused by dominant mutations.

568. Transient Manipulation of DNA Damage Repair Pathway Choice Improves Homology-Directed Repair During CRISPR/Cas9-Mediated Genome Editing

Pankaj K. Mandal, Bruna S. Paulsen, Richard Frock, Paula Gutierrez-Martinez, Wataru Ebina, Suneet Agarwal, Frederick Alt, Derrick J. Rossi

Boston Children's Hospital, Boston, MA

CRISPR/Cas9 system allows efficient gene ablation through error-prone non-homologous end joining DNA repair. However, very low efficiency of homology-directed DNA repair (HDR) is the bottleneck in correcting genetic mutations of clinical relevance. Here we report that transient manipulation of DNA damage repair pathways increases the HDR frequency by 3-5 fold. Furthermore, we show that this approach is applicable to introduce precise genetic modifications at many genetic loci in multiple cell-types including human induced pluripotent stem (hiPS) cells. Furthermore, unbiased off-target mutational analysis using High Throughput Genome-wide Translocation Sequencing (HTGTS) suggests that transient manipulation of DNA damage repair pathways have no adverse effect on either CRISPR/Cas9 specificity or genomic integrity. Our data suggests that this approach could be used for correcting various genetic disorders in relevant cell types.

569. Precision Editing of the WAS Locus via Homologous Recombination in Primary Human Hematopoietic Cells Mediated by Either TALEN or CRISPR/Cas Nucleases

Iram F. Khan, Yupeng Wang, Courtnee Clough, Swati Singh, King Hung, David J. Rawlings

Center for Immunity and Immunotherapies and Program for Cell and Gene Therapies, Seattle Children's Research Institute, Seattle, WA

Wiskott-Aldrich Syndrome (WAS) is an inherited primary immunodeficiency caused by mutations in the *WAS* gene which encodes a protein (WASp) that regulates the actin cytoskeleton in multiple hematopoietic cell lineages. Currently, allogeneic stem cell transplantation constitutes the only available cure for WAS, although phase I/II clinical trials using lentiviral gene therapy of autologous stem cells are currently underway and have shown improvement in immune system defects. Although effective, the gene therapy approach carries a risk of insertional mutagenesis, and unpredictable expression due to promoter choice as well as influences of the random integration site, including epigenetic status and the presence of neighboring transcription regulatory elements. As a refinement, our goal is to develop gene editing methodologies that would allow the specific targeting of a WASp cDNA to a position within the *WAS* locus allowing transcriptional regulation by the endogenous *WAS* promoter. To this end, we first designed and tested the cleavage efficiency of several guide RNAs (delivered as self-complementary AAV along with Cas9, delivered as mRNA), as well as candidate TALEN pairs (delivered as mRNAs), in primary human T cells. Using the T7 endonuclease assay, we identified candidate nucleases from both platforms (CRISPR/Cas9 and TALEN) that achieved a high Indel frequency: 73 and 85%, respectively. We next created a synthetic AAV6 donor template for homology-directed repair (HDR) that contained an MND promoter driven GFP cDNA with 1kb of *WAS* homology arms flanking it. When delivered with the TALEN or CRISPR/Cas9 nucleases, we observed stable integration of the GFP reporter within ≥25% of primary human T cells. Subsequently, we utilzed the identical reagents to target integration of the reporter cDNA into the *WAS* locus in adult human mobilized CD34+ cells, albeit at lower efficiencies. The off-target cleavage sites for TALENs identified using the Prognos software were amplified and sequenced, with no evidence of off-target cleavage observed at any of the predicted loci. Thus, we have generated genome editing tools that possess a high degree of specificity, providing the foundation for site-specific modification of the *WAS* locus as a therapeutic option.

570. Transient Reconstitution of Cas9 Protein for Safer Gene Editing In Vivo - A Ferry Tale Starring rAAV Capsid

Dan Wang[1], Karen Tran[1], Blake Y. Wang[1], Guangchao Xu[1], Mengxin Li[1], Hong Ma[1], Ran He[1], Qin Su[1], Phillip D. Zamore[2], Guangping Gao[1]

[1]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA, [2]RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA

Transforming the gene editing technology into therapeutic uses encounters several obstacles including the concern over safety. These gene editing platforms, such as the Cas9/sgRNA system, have been shown to induce off-target DNA double-stranded breaks (DSBs) throughout genomes, which is associated with toxicity. Such off-target effects not only stem from the intrinsic ambiguity of DNA sequence recognition by nucleases, but also attribute to the prolonged presence of an active gene editing system in a given cell. As a result, off-target DSBs accumulate over time, and ultimately lead to genotoxicity.

To mitigate the potential toxicity due to prolonged expression of a gene editing system *in vivo*, we sought to transiently deliver the key component, i.e. the endonuclease protein, which will fulfill the task of inducing permanent gene editing followed by natural degradation. Specifically, we use the VP2 protein of AAV capsid as a protein delivery vehicle to ferry the Cas9 protein *in vivo*. We first constructed a sensitive gene editing reporter plasmid, and demonstrated that co-transfection of the reporter plasmid and a plasmid expressing the SpCas9-VP2 fusion protein induced gene editing in HEK293 cells. We then modified our rAAV packaging system to include a plasmid expressing VP1 and VP3, and another plasmid expressing either the SpCas9-VP2 fusion protein or the EGFP-VP2 fusion protein. We successfully produced EGFP-AAV2 (EGFP protein grafted on the AAV2 capsid). However, we were not able to produce rAAV particles carrying SpCas9 protein, likely because the large size of SpCas9 protein interfered with the AAV packaging process. Therefore, we split SpCas9 into halves aiming to utilize split intein-mediated protein trans-splicing (PTS) to transiently reconstitute the full-length SpCas9 **(Figure)**. When the two parts of a split intein (termed Int_N and Int_C, respectively) fused with two proteins, the split intein is able to mediate PTS, resulting in the generation of a fusion protein with the intein being spliced out. We generated plasmids expressing the fusion proteins $SpCas9_N$-Int_N and Int_C-$SpCas9_C$-VP2, respectively, and demonstrated productive intein-mediated reconstitution of SpCas9-VP2 protein in HEK293 cells by co-transfection. Importantly, co-transfection of plasmids expressing $SpCas9_N$-Int_N and Int_C-$SpCas9_C$-VP2 in HEK293 cells led to gene editing based on the reporter assay. Guided by structural analysis and prediction, we strategically screened and identified the SpCas9 split sites close to the C-term of SpCas9. Therefore, the Int_C-$SpCas9_C$ protein to be grafted on VP2 is equal or smaller than EGFP. Because we were able to produce EGFP-AAV2, we reasoned that the Int_C-$SpCas9_C$-VP2 will likely be amenable to the rAAV packaging process, which is underway. If successful, our novel off-target mitigating strategy described here will greatly facilitate the translational application of gene editing technology *in vivo*. In addition, this transient *in vivo* protein reconstitution approach is applicable to the delivery of therapeutic but highly toxic proteins for the treatment of cancer and other diseases.

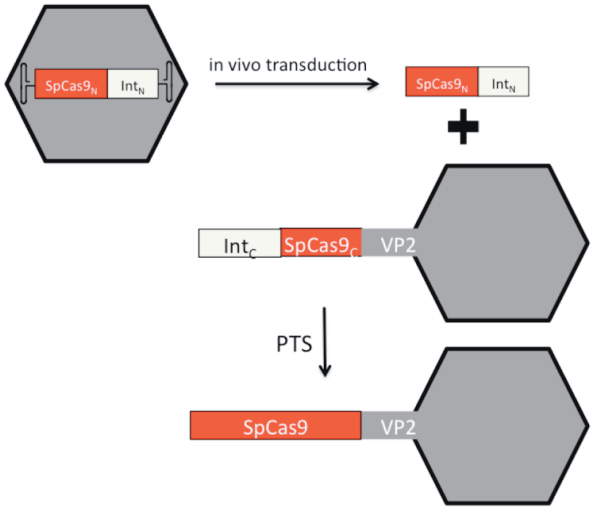


Figure. Reconstituting SpCas9 by intein-mediated protein trans-splicing (PTS). In the first AAV vector, the AAV genome encodes the N-terminal portion of SpCas9 ($SpCas9_N$) fused with Int_N. The second AAV vector carries Int_C and the C-terminal portion of SpCas9 fused to VP2. In vivo transduction of the first AAV vector produces the fusion protein $SpCas9_N$-Int_N, which is followed by delivery of the second vector. PTS occurs to reconstitute the full-length SpCas9 protein.

571. Safe Harbor Targeting of IL2RG Expression Cassettes for Gene Therapy of X-Linked Severe Combined Immunodeficiency (SCID-X1)

Sheng Zhou, Zhijun Ma, Taihe Lu, Mark A. Brimble, Yong-Dong Wang, Andrew M. Davidoff, Brian P. Sorrentino
St Jude Children's Research Hospital, Memphis, TN

SCID-X1 is caused by mutations in the IL2RG gene and results in severe defects in T, B, and NK-cell mediated immunity. Transduction of hematopoietic stem and progenitor cells with IL2RG-expressing gamma-retroviral vectors can be clinically effective but has also caused leukemia in several trials due to vector integrations that activate T-cell proto-oncogenes. One approach to eliminate these genotoxic integrations is to use genome editing techniques to insert an IL2RG transgene into a genomic safe harbor loci. The choice of a genomic safe harbor rather than the endogenous IL2RG locus offers several advantages including correction of a wide variety of mutations with a single therapeutic cassette, potentially higher editing efficiency in selected safe harbor sites, and the ability to adapt the specific nuclease reagents to other diseases and therapeutic templates. We are testing two different IL2RG-expression cassettes for targeting and expression into the AAVS1 locus. First, we generated a recombination template that contains a codon-optimized IL2RG cDNA driven by the short elongation factor alpha (EF1a) promoter based on clinical data with this cassette being transmitted via a lentiviral vector and showing immune correction in human SCID-X1 subjects in an ongoing trial at the NIH Clinical Center. We first tested whether a sufficient level of IL2RG expression can be achieved when a single copy of the EF1α-IL2RG-cDNA cassette was inserted into the AAVS1 locus in human ED7R T-cells. This transgene cassette was flanked by 500bp homologous regions from the AAVS1 locus and transfected into ED7R cells, along with a pair of TALEN endonucleases that cleave within the AAVS1 locus. Three weeks after transfection, a distinct 7% subpopulation of cells expressed IL2RG on the cell surface, which was subsequently sorted by flow cytometry. We analyzed 10 single cell subclones from this population and confirmed that homologous recombination and single allele targeting had occurred in each of these clones, demonstrating efficient homologous recombination at the AAVS1 site. Flow analysis showed that the EF1α-IL2RG-cDNA cassette was expressed at approximately the same level as that seen in cells transduced with a single copy of the clinical EF1α-IL2RG-cDNA lentiviral vector, verifying that the AAVS1 is permissive for therapeutic expression levels at the single copy level. One potential disadvantage of the safe harbor approach is the potential loss of precise regulation of gene expression resulting from the use of such synthetic cDNA constructs. For this reason, we are also currently targeting the full 5.3 kb genomic IL2RG locus and endogenous promoter to the AAVS1 safe harbor to compare their IL2RG expression level with that obtained from the cDNA construct. We are also comparing editing efficiency at AAVS1 using several TALEN pairs as well as 4 Staphylococcus Aureus Cas9 guide RNAs, which are deliverable by "all-in-one" single stranded AAV6 vectors. To date, the best guide has led to 37 % allele editing in 293T cells when transfected as plasmid. We are currently producing AAV vectors that contain this guide RNA and will test this AAV vector, along with IDLV vectors that will transfer either the cDNA or genomic IL2RG templates, for editing efficiency and IL2RG gene expression in ED7R and human CD34+ cells.

Molecular Therapy Volume 24, Supplement 1, May 2016

572. Successful Generation of CAR+PD-1- Primary T Cells Using Cas9-Mediated Genome Editing

Grant Welstead[1], Justin Fang[1], Chris Nye[2], Frank Buquicchio[1], McKensie Collins[1], Will Selleck[1], Hari Jayaram[1], Ruth Salmon[2], Alex Baturevych[2], Sherri Mudri[2], Jon Jones[2], David Bumcrot[1], Blythe D. Sather[2]
[1]Research, Editas Medicine, Boston, MA, [2]Research, Juno Therapeutics, Seattle, WA

Cancer immunotherapy is an exciting area of cancer treatment bolstered by recent success of anti-immune checkpoint antibodies in the clinic. Engineered T cells that are programmed to attack tumors via chimeric antigen receptors (CARs) have also shown promise in early clinical trials. It is speculated, however, that the PD-1/PD-L1 axis dampens the effectiveness of CAR T therapy in certain cancer types. In order to improve the function of transplanted CAR T cells in these contexts, we have deleted *PDCD1* using the genome editing system, CRISPR/Cas9. Delivery of the Cas9 protein and a *PDCD1* specific gRNA to primary T cells resulted in the deletion of PD1 expression in over 90% of cells. Additionally, we were able to achieve similar editing efficiencies in the context of a lentivirus CAR infection and successfully generated CAR^+/PD-1 knockout primary T cells with a very high level (>60%) of duel CAR expression and PD-1 editing. Utilizing a variety of assays, we have determined that the CAR^+/$PD\text{-}1^-$ primary T cells are viable, proliferate normally and can kill target expressing cells both in the presence and absence of PD-L1 on the target cells. In conclusion, we have successfully generated CAR^+/$PD\text{-}1^-$ primary T cells using Cas9-mediated editing that can be directed to kill PD-L1 expressing target cells.

573. Vector Free Genome Editing of Immune Cells for Cell Therapy

Tia DiTommaso, Jonathan Gilbert, Howard Bernstein, Armon Sharei
SQZ Biotech, Boston, MA

While the *ex vivo* manipulation of primary cells has signaled a new era in the application of cell-based therapies, common methods to manipulate primary cells have limitations. To overcome the limitations associated with conventional cell delivery and engineering systems, we have developed a microfluidic approach to delivery where cells are mechanically deformed as they pass through constricting channels. This deforms the cell membrane resulting in the diffusion of material from the surrounding buffer directly into the cytosol. This system has demonstrated efficacy in patient-derived cells, such as stem cells and immune cells and with a variety of target molecules that are difficult to address with alternative methods. Moreover, by eliminating the need for electrical fields or exogenous materials such as viral vectors and plasmids, it minimizes the potential for cell toxicity and off-target effects. Here, we present evidence detailing our ability to deliver functional material to primary human T cells via membrane deformation with little detectable perturbation in baseline gene expression, cell function, and viability. To determine effect of membrane deformation on gene expression and to compare to other delivery systems, human T cells were subjected to membrane deformation or electroporation and gene expression changes were compared to unmanipulated control cells using microarray analysis. Differential gene expression with respect to both methods of delivery was assessed by performing t tests on the coefficient of a linear mixed-effects model that treated delivery method as a fixed effect and donor as a random effect. Electroporation produced substantially more changes in gene expression than membrane deformation. Subsequently, we designed a series of experiments to manipulate gene expression with the CRISPR-CAS9 system using membrane deformation to deliver CAS9 ribonucleoproteins (RNPs; recombinant CAS9 protein complexed with a single-guide RNA) designed to edit a model locus, the B_2 microglobulin component of MHC class 1 (*B2M*). Here, we show that the delivery of the CRISPR-CAS9 system via membrane deformation results in a significant reduction in B2M surface protein expression by FACS analysis. Taken together, these data suggest that membrane deformation is a viable delivery method for genetic engineering of primary human cells with little off target effects on baseline gene expression. Indeed, the ability to deliver structurally diverse materials to difficult-to-transfect primary cells indicate that this method could potentially enable many novel clinical applications.

574. Lentivirus Pre-Packed with Cas9 Protein for Safer Gene Editing

Manjunath Swamy[1], Jang-Gi Choi[1], Ying Dang[1], Sojan Abraham[1], Hongming Ma[1], Hua Guo[1], Yujia Cai[2], Jacob Giehm Mikkelsen[2], Haoquan Wu[1], Premlata Shankar[1]
[1]TTUHSC, El Paso, TX, [2]Department of Biomedicine, Aarhus University, Aarhus, Denmark

The CRISPR/Cas9 system provides an easy way to edit specific site/s in the genome and thus offers tremendous opportunity for human gene therapy for a wide range of diseases. However, one major concern is off-target effects, particularly with long-term expression of Cas9 nuclease when traditional expression methods such as plasmid/viral vectors are used. To overcome this limitation, we pre-packaged Cas9 protein (Cas9P LV) in lentiviral particles for transient exposure and showed its effectiveness for gene disruption in cells, including primary T cells expressing specific sgRNAs. We then constructed an "all in one" lentivirus to express sgRNAs in association with prepackaged Cas9 protein (sgRNA/Cas9P LV). We successfully edited CCR5 in TZM-bl cells by this approach. Using an sgRNA targeting HIV LTR, we also were able to disrupt HIV provirus in the J-LAT model of viral latency. Moreover, we also found that pre-packaging Cas9 protein in LV particle reduced off-target editing of chromosome 4:-29134166 locus by CCR5 sgRNA, compared to continued expression from the vector. These results show that sgRNA/Cas9P LV can be used as a safer approach for human gene therapy applications.

575. High Content Analysis of CRISPR-Cas9 Gene-Edited Human Embryonic Stem Cells

Jared Carlson-Stevermer[1], Madelyn Goedland[2], Benjamin Steyer[2], Arezoo Movaghar[2], Meng Lou[2], Lucille Kohlenberg[2], Ryan Prestil[2], Krishanu Saha[2]
[1]Biomedical Engineering, Wisconsin Institute for Discovery, University of Wisconsin-Madison, Madison, WI, [2]Biomedical Engineering, University of Wisconsin-Madison, Madison, WI

Gene-edited human cells are important resources for drug target identification, regulatory science, regenerative medicine and basic biology. Recently, use of the CRISPR-Cas9 system drastically cut the time required to produce gene-edited cell lines down to a few months. Targeted gene disruption in a population of human cells followed by selection and next-generation sequencing can identify drug targets, however many of these methods destroy all mutant clones, so a subsequent gene-editing experiment is required to obtain living mutant cells for downstream analysis. Overall, there is a need to increase the speed, multiplexing and precision in generating CRISPR-Cas9 mutants. ArrayEdit, a simple approach utilizing surface-modified multiwell plates containing one-pot transcribed single-guide RNAs (see Figure), separates thousands of edited cell populations for automated, live, high-content imaging and analysis. The approach lowers the time and cost of gene editing and produces edited human embryonic stem cells at high efficiencies. Edited genes

can be expressed in both pluripotent stem cells and differentiated cells from all three major germ layers. This allows for the real-time observation of mutations causing phenotypic differences that can be measured in in vitro tissues and organoids during culture as opposed to defined end points. The live cell and tissue assays on ArrayEdit are compatible with standard screening platforms and a variety of human cell types, thus permitting robust and sensitive detection of differences in proliferation, differentiation and other downstream effects of gene editing. This preclinical platform adds important capabilities to observe editing and selection in situ within complex structures generated by human cells, ultimately enabling optical and other molecular perturbations in the editing workflow that could refine the specificity and versatility of gene editing.

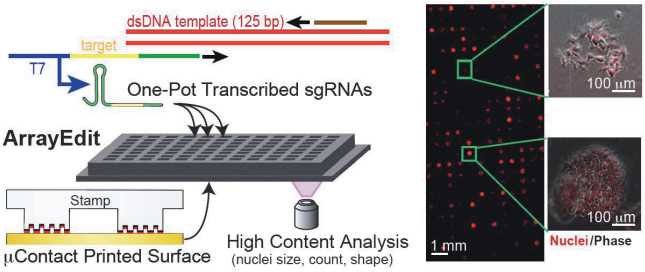


Figure. ArrayEdit provides a new window into the process of gene editing human cells. Overview of ArrayEdit assembly and key components. Top: Schematic of one-pot PCR and T7 transcription. Bottom: Surface modification to the bottom of multiwell plates generates cell-adhesive μFeatures on a glass bottom. Each μFeature can be tracked over time via high-content imaging and stitched together to form a time-lapse visualization of edited cell phenotypes.

576. DNA Ends Matter: The Impact of Using CRISPR/Cas9 Variants on DNA Repair Pathway Choices and Editing Profiles at the HBB Locus

Tanushree Phadke, Anne Bothmer, Christina Lee, Hayat Abdulkerim, Luis Barrera, S. Moss, Hari Jayaram, Cecilia Cotta-Ramusino
Editas Medicine, Cambridge, MA

Sickle Cell Anemia is an inherited recessive disorder caused by a single point mutation in the human beta globin (HBB) gene resulting in an abnormal type of hemoglobin. Here we report targeting the *HBB* locus using CRISPR/Cas9 technology for correction of Sickle Cell Disease, which affects nearly 1 million people worldwide. Cas9 and its variants can be used to introduce a variety of breaks including blunt double stranded break (DSB), single nicks, or dual nicks leaving either a 3' or 5' overhang. The type of cut and donor used can play a role in triggering different repair pathways, thus, resulting in various editing profiles. Using a single strand oligonucleotide (ssODN), we characterize different DNA repair outcomes including indel mutations resulting from Non Homologous End-Joining (NHEJ), Homology-Dependent Repair (HDR) using the donor as a template, and, finally, Gene Conversion (a kind of HDR event) using the closely related HBD gene as an endogenous template. Repair using homologous sequences from the HBD gene results in partial gene-conversion yielding a chimeric HBB-HBD gene that corrects the sickle cell point mutation. We observed that the Cas9 nickase/gRNA pair leaving a 5' overhang displayed a significantly higher frequency of gene conversion and gene correction than other Cas9-induced DNA end structures. We also provide evidence that overexpression or down-regulation of critical factors in the repair pathways influence the repair pathway balance.

In summary, we demonstrate that the frequency of various repair outcomes under different conditions offers insight into the mechanisms of repair of Cas9-induced DNA cleavage. The data support a therapeutic approach in which correction of the sickle-cell mutation is efficiently mediated through HDR using a donor template or by gene-conversion using the endogenous HBD gene.

577. Empower Multiplex CRISPR-Mediated Gene Manipulation with Self-Cleaving Ribozymes and tRNA

Li Xu, Mona El Refaey, Lixia Zhao, Jing Xu, Yandi Gao, Renzhi Han
Surgery, the Ohio State University Wexner Medical Center, Columbus, OH

Clustered regularly interspaced short palindromic repeat/Cas9 (CRISPR/Cas9) has been recently introduced as an efficient tool to edit the genome. Moreover, it can be targeted to specific gene loci by using single guide RNA (sgRNAs) for genetic manipulation and potential therapy. However, its targeting capability is often restricted by (gRNA) and generation of large deletions in genome by CRISPR requires expression of two distinct guide RNAs simultaneously. Here we report an innovative modification to increase the CRISPR/cas9 editing efficiency in the form of Ribozyme-flanked guide RNA/CRISPR system, which expresses two gRNA in equal molar amount. Our results show that this approach can be used to generate large DNA deletion with transfection of only one plasmid in human and mouse cells. Furthermore, we also used this system to target multiple transcriptional activators to a single promoter and the results showed that RNA level of targeting gene was statistically significantly up-regulated via Ribozyme gRNA/hCas9. Taken together our data, we prove that this strategy can be used in gene editing study broadly to enhance the targeting and the editing efficiency of CRISPR system.

578. Abstract Withdrawn

579. Meganucleases as an Efficient Tool for Genome Editing

Victor V. Bartsevich[1], John Morris[1], Ginger Tomberlin[1], Caitlin Turner[1], Wendy Sharer[1], Michael G. Nicholson[1], Jeff Smith[1], Nag Kollu[2], Rachel James[2], Robin Armstrong[2], Matthew Hirsch[2], Derek Jantz[1]
[1]Precision BioSciences, Inc., Durham, NC, [2]UNC Gene Therapy Center, University of North Carolina, Chapel Hill, NC

The field of genome editing has exploded in recent years. However, despite being the only nuclease naturally evolved for genome editing, meganucleases have largely remained behind the scenes compared to ZFNs, TALENs, and CRISPRs due to difficulties in modifying their DNA-recognition specificity. We have developed a next-generation meganuclease platform called "ARCUS" that overcomes these production difficulties and can produce nucleases with customized activity and specificity. Unlike some other genome editing technologies, ARCUS nucleases are capable of distinguishing target sites that only differ by 1 base pair. We will present the ARCUS platform and one such example of a meganuclease engineered to distinguish the dominant P23H point mutation in the rhodopsin gene from its wild type allele for a possible gene therapy of retinitis pigmentosa

Molecular Therapy Volume 24, Supplement 1, May 2016

Oligonucleotide Therapeutics II

580. Prevention of Airway Inflammation by Simultaneous Inhibition of NFkB and STST6 Using Chimeric Decoy Oligonucleotides in a Mouse Model of Asthma

Tetsuo Miyake, Takashi Miyake, Hizuki Hamada, Ryuichi Morishita
Clinical Gene Therapy, Graduate School of Medicine, Osaka University, Suita, Japan

Allergic asthma is a common inflammatory disease of the airways characterized by reversible airflow obstruction and airway hyperresponsiveness (AHR) to bronchoconstrictor stimuli. The pathophysiology in asthma mainly occurs as a consequence of overexpression of the TH2 cytokines. These inflammatory factors are tightly regulated by a transcriptional network system. Nuclear factor kappaB (NFkB) and STAT6 are well known to regulate a set of gene associated with inflammatory and immune responses, and is thought to play an important role in the induction of allergic asthma. Therefore, we focused on the simultaneous inhibition of these important transcription factors using a decoy strategy to develop a novel therapeutic approach for treating asthma. For increased efficacy of decoy oligodeoxynucleotides (ODN) *in vivo*, we employed chimeric decoy ODN containing consensus sequences of both NFkB and STAT6 binding sites. In addition, two strands of decoy ODN were combined by the chemical spacer to increase its resistance to endonuclease for intratracheal administration. The therapeutic effect of chimeric decoy ODN was investigated using OVA-induced experimental asthma in mice. Mice were sensitized by intraperitoneal injections of 20 ug of ovalbumin (OVA) and 2 mg aluminum hydroxide constituted in 0.1 ml of saline on days 0 and 14. On days 21, 22 and 23, mice were challenged with 1% OVA aerosol for 20 min. At 24 hours after the last challenge, AHR was measured by methacholine-induced airflow obstruction. Intratracheal administration of decoy ODN was performed in OVA-sensitized mice at 3 days before the first challenge with aerosolized OVA. FITC-labeled chimeric decoy ODN could be detected in macrophages and monocytes migrating into the lung and airway, and NFkB and STAT6 activity were simultaneously inhibited by chimeric decoy ODN. Twenty-four hours after the last OVA challenge, treatment with chimeric or single transfection of NFkB decoy ODN was protected from methacholine-induced AHR, while mice treated with scrambled decoy ODN or saline developed a significantly increase in airway reactivity to methacholine. Importantly, this inhibitory effect of chimeric decoy ODN on airway hyperresponsiveness was significantly greater than that of NFkB decoy ODN. Treatment with chimeric decoy ODN markedly suppressed airway inflammation after OVA sensitization and challenge as compared with control and scrambled decoy ODN treatment. Inflammatory infiltrate, such as macrophage, was significantly inhibited by chimeric decoy ODN through suppression of ICAM-1 and eotaxin expression. In addition, secretion of Th2 cytokines including IL-4, IL-5 and IL-13 in BALF, and histamine in the whole lung were reduced by chimeric decoy ODN. Furthermore, a significant reduction of mucin secretion was observed by chimeric decoy ODN treatment accompanied by a suppression of MUC5AC gene expression. However, intratracheal administration of chimeric decoy ODN did not affected IgE synthesis. The present study provides a novel strategy for treating bronchial asthma by the simultaneous inhibition of both NFkB and STAT6 using chimeric decoy ODN. Further modification of chimeric decoy ODN would be useful to treat asthma as a decoy-based therapy.

581. Correcting Exon Skipping Splicing Defects in *BTK* RNA by Using Bifunctional Oligonucleotides

C. I. Edvard Smith[1], Burcu Bestas[1], Janne J. Turunen[1], Jesper Wengel[2]
[1]*Laboratory Medicine, Karolinska Institutet, Huddinge, Sweden,* [2]*Physics, Chemistry and Pharmacy, University of Southern Denmark, Huddinge, Denmark*

An important percentage of disease causing mutations affects pre-mRNA splicing. The mechanism of pre-mRNA splicing is controlled by sequence elements within the exons and introns. These sequence elements recruit the spliceosome for directing the splicing process. Mutations disrupting the sequence elements commonly result in exon exclusion or cryptic exon inclusion named as pseudoexon. The result is typically a frame-shift in the pre-mRNA causing production of partially functional or a defective protein. A common way to correct splicing mutations is to use antisense oligonucleotides (AONs) that are short complementary sequences that bind to the pre-mRNA and re-direct the splicing.

X-linked agammaglobulinemia (XLA) is a primary immunodeficiency disease that is caused by the mutations in the gene named Bruton's Tyrosine Kinase (BTK). BTK has a crucial role in B cell development and the absence of BTK introduces a developmental block at the stage where the transition between pro-B and pre-B cells takes place. A prominent percentage of splicing mutations affect pre-mRNA splicing in XLA [1]. Previously, we have published a proof-of-concept study and shown that AONs can correct a cryptic exon mutation and restore a functional BTK in a humanized transgenic mouse model both in vitro and in vivo [2]. AONs in the previous study were designed to sterically block an intronic mutation and prevent the recruitment of the splicing factors around the cryptic exon.

We are here addressing a different scenario, where defective BTK is produced due to exon exclusion. This is typically due to mutations that weaken the splice sites or splicing enhancer sequences in their vicinity, and constitutes the most common type of splicing defect. Here we have designed bifunctional AONs that have a complementary binding site and a free tail region that is able to recruit splicing factors to the site in order to enhance exon inclusion. Initially, we constructed a library of *BTK* reporters with mutations known to cause XLA, and the bifunctional AONs were then tested in cultures of cells stably transfected with various *BTK* reporters for intronic mutations affecting the inclusion of *BTK* exons 16 and 17. These exons were selected owing to the rather small size of the corresponding introns simplifying the generation of relevant reporter cell lines. Screening of different tail sequences show that AONs designed to recruit TIA-1 or TDP-43 proteins can correct the splicing defects and give rise to wild-type *BTK* mRNA. Optimization of the antisense and tail parts of the AONs has also revealed the importance of chemical modifications (2'-*O*-methyl, phosphorothioate, and/or locked nuclei acid) for enhancing the efficacy of the AONs. We have also studied the influence of combining bifunctional AONs and found that this can profoundly enhance exon inclusion. Thus, depending on the exact type of mutation single or combinatorial approaches provide different outcomes. To the best of our knowledge, this is the first time that exon inclusion has been achieved in a hematopoietic setting and experiments are currently underway to further optimize and explore the design of bifunctional AONs for potential in vivo use.

1. Bestas, B., et al., Splice-correction strategies for treatment of x-linked agammaglobulinemia. Current Allergy and Asthma Reports, 2015. 15(3): p. 510.

2. Bestas, B., et al., Splice-correcting oligonucleotides restore BTK function in X-linked agammaglobulinemia model. The Journal of Clinical Investigation, 2014. 124(9): p. 4067-81.

582. Abstract Withdrawn

583. Translating RNAi Therapy for Spinocerebellar Ataxia 1 to the Clinic

Megan S. Keiser
The Children's Hospital of Philadelphia, Philadelphia, PA

Spinocerebellar ataxia 1 (SCA1) is among a group polyglutamine expansion diseases and is characterized by cerebellar ataxia and neuronal degeneration in the cerebellum and brainstem. Currently, there are no effective treatment strategies for this disease. RNA interference (RNAi) is a naturally occurring process that mediates gene silencing and is currently being investigated as a therapy for dominant diseases such as SCA1. Previously, we used AAV vectors to deliver RNAi triggers to transgenic and knock-in mouse models of SCA1 and noted improved neuropathological, motor phenotypes and transcriptional changes. We have also completed studies in non-human primates (NHPs) evaluating the biodistribution, safety and efficacy of vector delivery to the deep cerebellar nuclei (DCN) in NHPs. We next performed dosing studies in pre- and post-symptomatic mice to identify the lowest efficacious dose, the highest tolerated dose. For this, groups of pre-symptomatic mice were given 1 of 4 doses at 5 weeks of age and motor function assayed after symptom onset for untreated mice (34 weeks of age) and immediately sacrificed for post-necropsy analysis. We identified a ceiling dose that conferred toxicity, a low dose that had no effect, and two doses that prevented phenotypic rotarod deficits relative to control injected SCA1 littermates. Concurrently, post-symptomatic mice were injected at 12 weeks of age at 3 escalating doses. Motor function tests at rotarod at 20 weeks of age identified a dose that not only prevented further deficit but significantly improved performance relative to baseline performance. Thus, our AAV-mediated delivery of RNAi to the SCA1 model can reverse motor impairment in mice, and is scalable to nonhuman primates, two important considerations in advancing this therapy to the clinic.

584. Myoblast Fusion Mediates Morpholino Entry and Exon Skipping In Dystrophic Muscle

James S. Novak[1], Marie Nearing[1], Marshall W. Hogarth[1], Maria C. Vila[1], Jessica F. Boehler[1], Eric P. Hoffman[1], Kanneboyina Nagaraju[1], Sebahattin Cirak[2], Terence A. Partridge[1]
[1]Center for Genetic Medicine Research, Children's National Medical Center, Washington, DC, [2]Institute for Human Genetics, University of Cologne, Cologne, Germany

Duchenne muscular dystrophy (DMD) is a severe, progressive, X-linked myopathy involving cycles of muscle cell degeneration, regeneration, and inflammation. DMD results from mutations in the dystrophin gene that result in a consequent failure to translate dystrophin protein. Exon skipping is a promising therapeutic strategy employing antisense oligonucleotides (AO) to exclude exons that disrupt the open reading frame, so as to produce a truncated, partially functional dystrophin protein. Several different AOs, including the phosphorodiamidate morpholino (PMO) and the 2'O-methyl phosphorothioate (2'OMe), have been shown to induce dystrophin expression and are currently being investigated in clinical trials. However, drug uptake and efficacy is inconsistent and highly-variable between and within individual muscles. Our objective was to identify factors within dystrophic muscle responsible for the observed variability in myofiber penetration, exon skipping, and dystrophin expression following systemic AO administration. Specifically, we investigated the role of myofiber regeneration on exon skipping. By treating dystrophin-null *mdx* mice with a single high-dose of the PMO together with staggered pulses of bromodeoxyuridine (BrdU) we can precisely identify timeframes of myocyte proliferation and relate this to the efficiency of PMO delivery. Initially, we utilized a tagged-PMO to track its efficiency of entry into muscle fibers and determine how long after administration it persists. Intriguingly, we observed that the efficiency of entry of PMO into myofibers is strongly associated with the late stages of differentiation and fusion of satellite cells into regenerating dystrophic *mdx* myofibers. We also observed a specific co-localization of BrdU-positive myonuclei with dystrophin-positive myofibers when the pulse of BrdU immediately preceded PMO delivery, implying that satellite cell proliferation and fusion into regenerating myofibers play a crucial role in the effectiveness of PMO-mediated exon skipping. Our investigation of the mechanisms involved in PMO uptake and efficacy will provide valuable insights for optimizing protocols for this promising approach to therapy for DMD.

585. Long Non-Coding RNA NEAT1 Functions as a ceRNA to Regulate E2F3 Expression by Sponging miR-377-3p in Non-Small Cell Lung Cancer

Chengcao Sun
Department of Occupational and Environmental Health, Wuhan University, Wuhan, China

Recently, the long non-coding RNA (lncRNA) NEAT1 has been identified as an oncogenic gene in multiple cancer types and elevated expression of NEAT1 was tightly linked to tumorigenesis and cancer progression. However, the molecular basis for this observation has not been characterized in progression of non-small cell lung cancer (NSCLC). In our studies, we identified NEAT1 was highly expressed in NSCLC patients and was a novel regulator of NSCLC progression. Patients whose tumors had high NEAT1 expression had a shorter overall survival than patients whose tumors had low NEAT1 expression. Further, NEAT1 significantly accelerates NSCLC cell growth and metastasis in vitro and tumor growth in vivo. Additionally, by using bioinformatics study and RNA pull down combined with luciferase reporter assays, we demonstrated that NEAT1 functioned as a competing endogenous RNA (ceRNA) for has-miR-377-3p, antagonized its functions and led to the de-repression of its endogenous targets E2F3, which was a core oncogene in promoting NSCLC progression. Taken together, these observations imply that the NEAT1 modulated the expression of E2F3 gene by acting as a competing endogenous RNA, which may build up the missing link between the regulatory miRNA network and NSCLC progression.

586. Dimerization Inhibition of HIV-1 RNA by 2'-Deoxy-2'-fluoro-beta-D-arabinose Nucleic Acid (2'-FANA) Modified Antisense Oligonucleotides Results in Potential Inhibition of Viral Expression

Mayumi Takahashi[1], Haitang Li[1], Veenu Aishwarya[2], Masad J. Damha[3], John J. Rossi[1]
[1]Molecular and Cellular Biology, Beckman Research Institute, Duarte, CA, [2]AUM LifeTech, Philadelphia, PA, [3]Department of Chemistry, McGill University, Montreal, QC, Canada

Human immunodeficiency virus-1 (HIV-1) viral particle contains two copies of genomic RNA, which is known to form dimers via intermolecular interactions. The dimerization process is initiated by the formation of a kissing-loop dimer through base pairing of the palindromic loop sequence within dimerization initiation site (DIS). It has been shown that mutation or inhibitions of the DIS severely affect the viral infectivity. Antisense oligonucleotides (AONs) are single-stranded synthetic oligonucleotides that recognize target RNAs via Watson-Crick base pairing and cause post-translational inhibition. The mechanisms are believed to be RNase H cleavage of target RNA, steric hindrance of the translation machinery or

prevention of RNA-RNA or RNA-protein interactions. While AONs offer promising solutions for variety of human diseases in preclinical studies and many of these are currently in clinical studies, a number of challenges still hamper their translation from the bench to the bedside, the most significant of which include target accessibility, off target effects, poor extracellular and intracellular stability and effective delivery into target cells. 2'-deoxy-2'-fluoro-beta-D-arabinose nucleic acid (2'-FANA) modification significantly enhances chemical and intracellular stability, as well as binding to the target RNA, forming stable heteroduplex structures. Moreover, 2'-FANA modification has been shown to facilitate gymnotic cellular delivery, a carrier-free method of achieving cellular uptake. Thus, we hypothesized that 2'-FANA modified AONs that bind HIV-1 DIS can inhibit dimerization, and subsequently inhibit viral expression. Firstly, we tested gymnotic delivery of 2'-FANA modified AONs to peripheral blood mononuclear cells (PBMCs) freshly isolated from human blood. Cellular uptake of the 2'-FANA AONs without any transfection reagent were observed in live-cell confocal microscopy analysis as soon as an hour after adding AONs. Next, the HIV-1 inhibitory effect of the 2'-FANA AONs was tested in HIV-1 infected PBMCs. Cell supernatant was collected and HIV-1 expression was measured by HIV-1 p24 ELISA. The 2'-FANA AONs strongly inhibited HIV-1 expression as long as two weeks after 2'-FANA AONs treatment. A mechanism study showed that the 2'-FANA AONs work against HIV by steric blocking of dimerization. Unlike most oligonucleotide therapeutics, 2'-FANA AONs can be gymnotically delivered into PBMCs. This fact makes 2'-FANA AONs great drug candidates for antiretroviral therapy.

587. Genetic Delivery of a miRNA Cluster with Polycistronic siRNAs Reduces Expression of Epidermal Growth Receptor in Human Glioblastoma Cells

Martin J. Hicks, Imari Patel
Biology, Monmouth University, West Long Branch, NJ

Glioblastoma multiforme (GBM), the most common malignancy of the brain, is a grade IV astrocytoma. It is one of the most rapidly growing and invasive types of glial tumors. The standard therapy includes surgical removal, radiation and chemotherapy with a median survival of about 14 months. In addition, systemic therapies are limited by the blood-brain barrier. To bypass the barrier, we are developing a gene delivery strategy to inhibit the expression of tyrosine kinase receptors (TKR), which are commonly upregulated in GBM. One TKR, epidermal growth factor receptor (EGFR), is overexpressed in GBM leading to uncontrolled growth and proliferation. Our approach is to recruit the RNA interference pathway. Although small interfering RNAs (siRNAs) are often utilized to silence gene expression, exogenously expressed siRNAs are not an effective strategy to treat human disease due to both extracellular and intracellular nucleases as well as activation of cellular immunity against foreign nucleic acids. To bypass these degradatory mechanisms, we use a natural miRNA cluster genetic background to effectively deliver the DNA encoding multiple anti-EGFR siRNAs by inserting them into the tertiary structure of the miRNA cluster, miR-17-92. The anti-EGFR polycistronic miRNA cluster (pAAV-miR-IP1) expresses six siRNAs directed against EGFR, specifically targeting the extracellular ligand binding domain, transmembrane domain, intracellular tyrosine kinase domain and 3' untranslated region of the EGFR transcript. The vector, pAAV-miR-IP1, was transfected into the human GBM cell lines, A172 and U87MG. Results demonstrate that pAAV-miR-IP1 was expressed at high levels with a subsequent reduction in EGFR mRNA expression. Additional strategies include using the polycistronic delivery mechanism to target multiple TKRs in addition to EGFR.

588. MYCN Silencing Using RNA Interference Causes Apoptosis and Differentiation in MYCN Amplified Neuroblastoma Cell Lines

Ruhina Maeshima, Aristides D. Tagalakis, Andrew W. Stoker, Stephen L. Hart
UCL Institute of Child Health, London, United Kingdom

Neuroblastoma (NB) is the most common malignant solid tumour in childhood and accounts for 15% of childhood cancer deaths. High risk NB is strongly correlated with MYCN amplification and the survival rate is very low at approximately 30-50%. MYCN, a member of the MYC proto-oncogene family, is an evolutionarily conserved bHLHZip transcription factor. MYCN controls various target genes that regulate essential cellular processes such as proliferation, cell growth, apoptosis and differentiation in certain tissues in the embryo, while it is low or absent in adults. However, overexpressed MYCN in MYCN-amplified tumours induces proliferation and cell growth and suppresses apoptosis and differentiation pathways in NB tumour cells. Therefore, we hypothesise that MYCN is a promising target gene for NB therapy as its suppression may lead to apoptotic cell death, retarded proliferation or differentiation resulting in their terminal differentiation, and eventual senescence. Transcription factors are generally difficult targets to design drugs and thus we aim to silence MYCN in NB cells using RNA interference (RNAi). Our hypothesis is that MYCN silencing by RNAi triggers apoptosis and differentiation, and affects up/down-regulation of the genes downstream of MYCN, including p53. To observe the silencing efficiency and the biological downstream effects following the knocking down, we performed short interfering RNA (siRNA) transfections at 4different concentrations (50-5nM) using a commercial transfection reagent Lipofectamine RNAiMAX. These transfections were performed in different MYCN-amplified NB cell lines, Kelly and SK-N-BE(2), and the silencing efficiency measured at the mRNA and protein levels. SK-N-BE(2) cells have non-functional p53 and have shown resistance against cytotoxic therapy and conventional chemo/radio therapies and therefore, these cells were used as a NB model, that scarcely apoptose even when MYCN level goes down. Following transfections in both cell lines, approximately 40-50% silencing was obtained at the mRNA level. MYCN protein reduction was demonstrated in a dose dependent manner up to a maximum of 70% at a dose of 50nM in Kelly cells. Knockdown in protein levels were higher than that at the mRNA level. SK-N-BE(2) cells following MYCN siRNA silencing differentiated significantly after 6 days, and differed in their cell morphology when compared with control siRNA-transfected cells. Both, the number of cells with longer neurites and the total number of longer neurites, increased in 5 nM MYCN siRNA-treated cells when compared to control siRNA-transfected cells by approximately 21-fold and 2.4-fold, respectively. In conclusion, MYCN siRNA leads to the knockdown of MYCN mRNA levels, and that in turn induces downstream biological effects leading to apoptosis and differentiation. This suggests that MYCN silencing by RNAi might be applied as a therapy of NB with or without functional p53, in tumours that have resistance against chemotherapeutics. MYCN silencing by siRNA could be a promising therapy for a wide range of NB types with MYCN amplification.

589. Short Hairpin RNAs Based on miR-451 Show Potent Knockdown without Passenger Strand Activity

Erik Kaadt, Lars Aagaard
Department of Biomedicine, Aarhus University, Aarhus, Denmark

Based on the recent findings that RNAi designs with shortened shRNA stems bypass Dicer cleavage and rely on Ago2 for processing of the 'effector' guide strand, we have compared designs based on

'classical' shRNA structures with shortened stems and mimics of miR-451. Our results support the complete lack of passenger stands activity which is likely to improve safety in therapeutic applications. We report that miR-451 mimics are more potent than shortened shRNAs and our data further suggests that the U6 promoter is the preferred choice as compared to H1. We suggest that imprecise transcription initiation from H1 promoter makes it hard to predict the actual target site(s) and may thus be a safety concern. Tiling of U6-promoted miR-451 mimics supports this notion and demonstrate that knockdown efficacy vary substantially when shifting the target site one nucleotide.

590. Anti-EGFR Aptamer-Conjugated Lipoplexses, a Novel Gene Delivery Strategy for Triple Negative Breast Cancer Therapy

Min Woo Kim, Hwa Yeon Jeong, Seong Jae Kang, In Ho Jeong, Moon Jung Choi, Young Myoung You, Chan Su Im, Yong Serk Park
Yonsei University, Wonju, Korea, Republic of

Basal-like breast cancer, one of the subtypes of breast cancer, have been focused because of their pathologic features like absence of three common markers such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/neu). The basal subtype is also called triple negative breast cancer (TNBC) and has shown a higher rate of metastasis, poor prognosis, unresponsive to therapy. Especially, their lack of HER2 expression and aggressive progression make it difficult to utilize HER2-targeted therapeutics and diagnostics to the metastatic TNBC. In this study, theranostic cationic liposomes consisting of quantum dots (QDs) and O,O'-dimyristyl-N-lysyl glutamate (DMKE) were prepared. The quantum dot-incorporating cationic liposomes (QCLs) were then complexed with Bcl-2 and PKC- λ/ι small interfering RNA (siRNA) for silencing of genes inhibiting apoptosis, and promoting cell proliferation, and cell migrations. The cationic characteristics of the QCLs provided an effective siRNA transfection in vitro, but it had limitations in in vivo applications. To enhance cancer-directed delivery, epidermal growth factor receptor (EGFR) aptamer-polyethylene glycol conjugates were inserted into the prepared QCLs. The advantages of the two-step strategy of QCL preparation include effective QD formulation, in vivo long circulation, and TNBC specific gene silencing. This study suggests that EGFR aptamer-conjugated QCL can be utilized as a useful theranostic agent for TNBC subtype breast cancers.

591. The OTTC Plasmid Manager: A Comprehensive Database for Archiving Plasmid DNA Sequence

Emily J. Heathward[1], Giun Sun[2], Ron U. Domingo[2], Calvin A. Thomas[2], Christopher T. Richie[1], Brandon Harvey[1]
[1]Optogenetics and Transgenic Technology Core, National Institute on Drug Abuse, Baltimore, MD, [2]Center of Information Technology, National Institutes of Health, Bethesda, MD

A freely available plasmid database that is interoperable with popular freeware (such as *a Plasmid Editor*, or *ApE* [1]) has been developed for our research group. Our group has accrued a wealth of data that can no longer be efficiently analyzed without a comprehensive cataloging system. The OTTC Plasmid Manager provides an intuitive plasmid database with analytical and search capabilities, offering a versatile platform for the storage and organization of plasmid sequences and their associated metadata. The database has been designed with informatics and visualization support to enhance the efficiency and analytic capabilities of the user, with a user-friendly interface. Annotations performed in *ApE* can be imported directly into the database, and unlike most commercially available and free DNA editing software, the Plasmid Manager also includes a mechanism to standardize the annotations of uploaded sequences, facilitating the direct comparison of multiple plasmids at the feature level. The visualization component generates a graphic representation of the plasmid map together with its features and annotations, allowing the visual comparison of multiple plasmids side-by-side. The contents of the database can be browsed and searched using a variety of criteria, including plasmid name, database accession number and associated features. This innovative plasmid database presents a new platform for molecular biologists to catalog plasmids relevant to their laboratory, saving time and enhancing the analytical capabilities of researchers.

[1] Davis W. A Plasmid Editor. Retrieved September 9, 2014, from http://biologylabs.utah.edu/wayned/ape/

592. High Quality Grade Plasmid DNA for Indirect Clinical Applications

Marco Schmeer, Ram Shankar, Tatjana Buchholz, Martin Schleef
PlasmidFactory GmbH & Co. KG, Bielefeld, Germany

Amongst other applications, our plasmid DNA is used in the GMP-compliant production of recombinant viruses, antibodies and RNA, where these are the active pharmaceutical ingredients (API) used in clinical trials. Here, it is not always necessary to produce the plasmid DNA under GMP as well, in order to use it for such applications. An alternative is the so-called *High Quality Grade* plasmid DNA which is highly purified and well-characterised and, hence, meeting the requirements of most regulating agencies.

High Quality Grade plasmid DNA is produced in our facility based on a research cell bank (RCB) and the very effective, patented *ccc Grade* DNA technology. A number of quality controls, both to the cell bank and to the plasmid DNA product, ensure that the final result is a product designed especially for the intended application and that complies with the appropriate regulatory standards.

PlasmidFactory's new facility for the production of this high purity plasmid DNA is now operating successfully in new, modern labs where plasmid DNA of the highest quality is being produced in accordance with the EMEA guideline CHMP/BWP/2458/03. To ensure product safety, substances of animal origin are not used at any stage of the entire process, guaranteeing maximum possible product purity by reliable exclusion of contaminants such as bacterial chromosomal DNA or damaged plasmids. Only one plasmid is produced in each area - different plasmids are not produced in parallel in the same lab.

Non-Viral Gene Transfer and Therapy II

593. Anti-Angiogenic Therapy for Pancreatic Cancer by Systemic Delivery of Messenger RNA Using Polyplex Nano Micelle

Satoshi Uchida[1], Hiroaki Kinoh[2], Takehiko Ishii[2], Akitsugu Matsui[1], Theofilus A. Tockary[2], Kaori M. Takeda[2], Hirokuni Uchida[1], Kensuke Osada[2], Keiji Itaka[1], Kazunori Kataoka[2]
[1]Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, [2]Graduate School of Engineering, The University of Tokyo, Tokyo, Japan

In pancreatic cancer, the access of therapeutic reagents to cancer cells was restricted by thick fibrotic tissue, and thus anti-angiogenic therapy targeting endothelial cells, which are more accessible, is a promising strategy. Gene therapy based on messenger RNA (mRNA) delivery allows for sustainable introduction of anti-angiogenic factors without the risks associated with DNA delivery, such as insertion mutagenesis, and uncontrollably prolonged transgene expression.

Systemic intravenous injection is a proper delivery route for mRNA to access endothelial cells from blood vessels with a simple injection procedure. Because mRNA is highly susceptible to enzymatic degradation in the blood circulation, mRNA carrier with high stability is required. In this study, we used polyplex nanomicelles, prepared from mRNA and poly(ethylene glycol) (PEG)-polycation block copolymers (Adv Drug Deliv Rev 2001, 47, 113-131), and a cholesterol moiety (Chol) was introduced to the block copolymer to stabilize the nanomicelles by hydrophobic interaction. In the cationic segment of the block copolymer, a polyaspartamide with four aminoethylene repeating units in the side chain (PAsp(TEP)) was used, which has high endosomal escaping capability and mRNA stabilizing effect (J Am Chem Soc 2014, 136, 12396-12405). In quantitative PCR (qPCR) analysis of nuclease resistance after in vitro incubation in 50% serum, Chol introduction failed to increase mRNA stability. In contrast, after intravenous injection to mice, the nanomicelles with Chol showed significantly enhanced mRNA retention in the blood circulation in qPCR analysis, when compared to those without Chol. These results indicate that the stabilizing effect of Chol is obvious especially in the harsh environment of blood, where nanomicelles are exposed to large amount of polyanion, such as proteoglycans on the cell surface. Indeed, nanomicelles with Chol showed enhanced resistance to dissociation by dextran sulfate compared to those without Chol. Eventually, when luciferase mRNA was introduced to the mice subcutaneously inoculated with human pancreatic adenocarcinoma (BxPC3), the nanomicelles with Chol showed enhanced luciferase expression in the tumor tissue, compared to those without Chol. Then, anti-angiogenic treatment was performed to this BxPC3 model, using mRNA encoding sFlt-1, a soluble form of vascular endothelial growth factor (VEGF) receptor, which inhibits VEGF signaling by entrapping VEGF protein. In the evaluation of tumor volume, the nanomicelles with Chol exhibited significant growth inhibitory effect, whereas those without Chol failed to show detectable effect. In immunohistochemical staining of vascular endothelial cells, the nanomicelles with Chol induced significant reduction of vascular density in the tumor tissue, indicating that the anti-angiogenic effect contributed to the inhibition of tumor growth after injection the nanomicelles with Chol. In conclusion, we succeeded in anti-angiogenic treatment of intractable pancreatic cancer by systemic mRNA delivery using stabilized nanomicelles. This mRNA delivery system can also be applied to the treatment of various diseases in the future.

594. Exosome-Associated AAV Enhances Retinal Transduction Following Intravitreal Injection

Bence György[1], Sarah Wassmer[2,3], Livia Carvalho[2,3], Casey Maguire[1], Luk H. Vandenberghe[3,4]
[1]Massachusetts General Hospital, Charlestown, MA, [2]Department of Ophthalmology, Ocular Genomics Institute, Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA, [3]Grousbeck Gene Therapy Center, Boston, MA, [4]Ocular Genomics Institute, Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA

Introduction Adeno-associated virus (AAV) has been shown to be associated with cell derived exosomes (exo-AAV). Exo-AAV outperforms regular AAV in transduction efficacy and evades neutralizing anti-AAV antibodies. Here we investigate the retinal transduction profile of exo-AAV vectors when administered intravitreally in BL6 adult mice. **Methods** Exo-AAV vectors were isolated from the culture media of triple-transfected 293T cells (AAV2 rep/cap, GFP transgene and adenovirus helper plasmid) by differential centrifugation. Regular AAV vectors were isolated from the cell lysate by iodixanol density gradient ultracentrifugation. To assess the transduction ability of the regular AAV2 and exo-AAV2 , we performed intravitreal injections into mice (n= 12 eyes, $1x10^{12}$ VG/mL), using AAV2 as the control (n=14 eyes, $1x10^{12}$ VG/mL). Fundus imaging was performed at 2 and 4 weeks post-injection, at which point the eyes were collected for immunohistological processing. **Results** We found that the exo-AAV2 showed an early onset of robust GFP expression via fundus imaging at 2 weeks post injection, as compared to regular AAV2. This was further enhanced by larger spread and brightness of GFP expression at 4 weeks. Histological processing of retinal sections shows GFP expression after exo-AAV2 expression in the nerve fiber layer, retinal ganglion cell layer, inner nuclear layer, outer nuclear layer and photoreceptor inner and outer segments. Staining with ganglion cell and bipolar cell markers show co-localization of GFP in these cell types. AAV2 injected animals show GFP expression mostly restricted to the nerve fiber and ganglion cell layers. **Conclusion** Intravitreal injections are an ideal delivery route to the retina in humans as it is minimally invasive and performed routinely in the clinic. Repeated pre-clinical work by others and us using AAV2 shows that an intravitreal injection is limited to targeting the nerve fiber layer (consequently, optic nerve) and retinal ganglion cells. Here, we demonstrate that exo-AAV2 targets all cell layers of the retina at robust levels and successfully targets retinal bipolar cells. As a result, an intravitreal injection of exo-AAV with a cell specific promoter may be the vector of choice for future gene therapy.

595. Enhanced Liver Transduction and Efficient Protection from Pre-Existing Neutralizing Antibodies with Exosome-Associated AAV8 Vectors

Amine Meliani[1], Zachary Fitzpatrick[1], Florence Boisgerault[1], Giuseppe Ronzitti[1], Fanny Collaud[1], Severine Charles[1], Casey A Maguire[2], Federico Mingozzi[1]
[1]Genethon, Evry, France, [2]Massachusetts General Hospital, Boston, MA

Adeno-associated virus (AAV) based vectors are ideal tools for in vivo gene transfer. The excellent safety profile and the highly efficient targeting of hepatocytes *in vivo* have been demonstrated in both preclinical studies and clinical trials. Although AAV vectors are not strongly immunogenic, they can give rise to both cellular and humoral immune responses. Additionally, as a result of exposure to wild-type AAV, an important fraction of humans harbor pre-existing neutralizing antibodies against the viral capsid, which can prevent successful transduction by AAV vectors. This pose a serious obstacle to the widespread use of AAV vectors in gene therapy. Recently, we have shown that AAV can associate with exosomes (exo-AAV) ultracentrifuged media samples from 293T producer cells. Exosome-associated AAV1, 2, and 9 serotype capsids were tested and found to have enhanced transduction and antibody evasion capabilities compared to conventional AAV vectors. With the goal of developing an enhanced vector for liver directed gene therapy, we sought to characterize the *in vivo* transduction and biodistribution profile of exo-AAV8 compared to conventional cell-lysated harvested, iodixanol-gradient purified AAV8 vectors purified from the same preparation. The efficiency of liver targeting of conventional AAV8 vs. exo-AAV8 expressing human factor IX (F.IX) under the control of liver specific promotor were tested in naïve and pre-immunized animals. C57BL/6 mice (n=5/group) were passively immunized with intravenous human immunoglobulin (IVIg) intraperitoneally, followed 24h later by the intravenous administration of either conventional AAV8 or exo-AAV8 vectors expressing coagulation factor IX (F.IX, $5x10^{10}$ vg/mouse). exo-AAV8 completely shielded the capsid vector from neutralizing antibodies at IVIg doses between 0.5mg and 2mg/mouse (NAb titer ~ 1:3.16), resulting in equivalent

levels of F.IX transgene expression to naïve animals treated with conventional AAV8 vector. At the highest IVIg dose tested (8mg/mouse), residual levels of F.IX transgene expression were about 30% of naïve animals treated with conventional AAV8 vectors. We next evaluated the efficiency of exo-AAV8 vs. AAV8 vectors in naïve animals. In male mice, no statistically significant difference in F.IX transgene expression levels was observed between the two vector types; also indicated by the similar pattern of vector genome biodistribution. Interestingly, female mice (in which the efficiency of liver transduction with AAV is extremely low compared with male animals) treated with exo-AAV8 showed a dramatic increase in transgene expression, comparable to that of male mice receiving conventional AAV8 vectors. In conclusion, exo-AAV8 vectors present an enhanced liver transduction profile compared with conventionally purified AAV vectors, both in naïve female animals and in animals carrying anti-capsid neutralizing antibodies.

596. Effect of Chemokine/Cytokine Delivered by Nanoparticles on Tumor Migration of Neuroblastoma Targeting Chimeric Antigen Receptor T Cells

Mai T. Huynh
Center for Cell and Gene Tharapy, Baylor College of Medicine, Houston, TX

INTRODUCTION: Neuroblastoma is the most common extracranial solid tumor in children and morbidity and mortality of high risk disease remains significant. The ganglioside GD2 is overexpressed on neuroblastoma and T cells expressing chimeric antigen receptors (CARs) specific for GD2 (GD2.CAR T cells) have been shown to induce complete tumor remissions without toxicity in a clinical trial. However, insufficient migration of CAR T cells to solid tumors remains a major problem. XCL1 (lymphotactin) is a chemokine that attracts T cells and natural killer cells, however, like other chemokines, diffuses quickly when injected at the tumor site. Calcium Alginate Nanoparticles (CANs) have been used clinically to safely deliver vaccines and chemokines. We are now evaluating the use of CANs to deliver chemokines to the tumor microenvironment and improve tumor homing of GD2.CAR T cells. METHODS/RESULTS: In our *in vitro* experiments, XCL1 was released more gradually, with a change from 798 pg/µL detected after 1 hour to 1233 pg/ µL at 50 hours when loaded with CANs compared to a decrease from 1434 pg/ µL to 398 pg/ µL at 50 hours without CANs as measured by ELISA and was able to attract CAR T cells. For *in vivo* experiments, T cells obtained from fresh peripheral blood mononuclear cells were activated on CD3 and CD28 coated plates and transduced 3 days later with firefly luciferase and GD2. CD28-OX40z or GD2. 41BBz retroviral supernatants with 70-95% transduction rate. Immunodeficient NSG mice were subcutaneously injected with GD2 expressing LAN-1 tumor cells and divided into different treatment groups which received IL-2 intraperitoneally twice weekly (n=5). Mice that received CANs loaded with XCL1 showed a 6.6-fold increase in CAR T cell signal on day 10 while peritumorally injected XCL1 alone or empty nanoparticles had no such impact on T cell migration. We subsequently incorporated IL-15 in our experiments based on its reported enhancement of T cell expansion *in vivo*. Without other exogenous cytokines, mice receiving the combination of IL-15 and XCL1 with CANs on days 0, 7, 12, and 19 showed remarkable improvement in T cell signal at the tumor site: 8.19 and 4.62-fold increase compared to groups treated with CANs only and XCL1 loaded with CANs on day 7, respectively. As early as 6 days after T cell injection and the first CANs injections, mice in the control CANs had increased tumor burden, nearly double that of IL-15/XCL1/CANs group with intermediate tumor burden in the CANs/XCL1 group. The combination of both chemokine and cytokine delivered by CANs also had a positive impact on survival, which was lengthened to >45 days compared to 26 days in the CANs only control group. CONCLUSION: Calcium Alginate Nanoparticles loaded with a combination of both XCL1 and IL-15 achieved superior T cell expansion, tumor control and better survival outcome compared to controls. These findings indicate that CANs are a promising delivery vehicle for chemokines in combination with adoptive T cell therapies. In the future we will attempt to optimize dosing and plan to transduce T cells with selected phenotypes to further enhance T cell persistence.

597. Development of Non-Viral Gene Delivery Systems with Antibacterial Effects Deliverable via Aerosol for Lungs Gene Therapy

Angélique Mottais[1], Tony Le Gall[2], Mathieu Berchel[3], Yann Sibiril[1], Paul-Alain Jaffrès[3], Tristan Montier[1]
[1]Université Bretagne Occidentale, INSERM U1078, Brest, France, [2]INSERM U1078, Brest, France, [3]Université Bretagne Occidentale, CNRS UMR 6521, Brest, France

Objective: Cystic Fibrosis (CF) is a genetic disorder caused by the absence or malfunction of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR). CF gene therapy would consist in delivering a normal copy of the *CFTR* gene in the nucleus of respiratory epithelial cells. The presence of bacteria in the pulmonary tract can alter the transported DNA, the transfection efficiency as well as targeted cells. At present, the leading method to target the respiratory epithelium being the nebulisation, the goal of this research project is to develop a formulation that can be delivered as an aerosol exhibiting both a transfection activity and an antibacterial activity. **Methods:** The formulated complexe was composed of 50% (molar percentage) of cationic lipid (an arseno-phospholipid whose transfection efficiency and antibacterial activity against Gram-positive bacteria have already been demonstrated) and 50% of a silver salt (active against Gram-negative bacteria). The transfection activity was evaluated according to the expression of a luciferase reporter gene into human bronchial epithelial cells grown in liquid culture or at air-liquid interface. The antibacterial activity was determined according to the difference of growth in agar plate between a protected area and an exposed area to the aerosol. **Results:** Contrary to the aerosolization of the classical lipoplexe, the formulation developed containing a silver salt possesses an antibacterial activity against the Gram-negative bacteria. The transfection activity is maintained post-aerosolization with some similar levels of expression to those observed with the cationic lipid used alone, and this both in liquid culture and at air-liquid interface. **Discussion and Conclusion:** This study shows that an equimolar combination of two compounds: an arseno-phospholipid and a silver salt (i) is simultaneously effective on bacteria frequently isolated from patients, (ii) has a very low human bronchial epithelial cells toxicity, and (iii) exhibits a transfection efficacy under clinically relevant conditions. Unlike a gene transfer system devoid of an antibacterial activity, such a combination may therefore participate in the eradication of bacteria, thus reducing the stress on the bronchial epithelial cells while promoting the transfection process and finally, the expression of the transgene.

598. TARGT$_{CNS}$ for the Treatment of Central Nervous System Disorders

Nir Shapir[1], Reem Miari[1], Cathryn A. Sundback[2], Irina Pomerantseva[2], Joshua P. Aronson[3], Husam A. Katnani[3], Olive Mwizerwa[3], Herman Tse[3], Haim Goltsman[1], Yaron Fuerst[1], Yonit Moraly H[1], Shany Blum[1], Sagi Harnof[4], Garry A. Neil[5]
[1]R&D, Medgenics Medical Israel Ltd., Misgav, Israel, [2]Center for Regenerative Medicine/Department of Surgery, Massachusetts General Hospital, Boston, MA, [3]Department of Neurosurgery, Massachusetts General Hospital, Boston, MA, [4]Department of Neurosurgery, Sheba Hospital, Ramat Gan, Israel, [5]Medgenics, Inc., Wayne, PA

Diseases and disorders of the central nervous system (CNS) represent a large field of unmet need that significantly impacts the lifespan and quality of life of many patients. The ability to deliver therapeutic proteins from the systemic circulation to the CNS is hampered by the blood-brain barrier (BBB). A number of strategies have been developed to allow therapies to cross the BBB, but to date with limited success. We have developed a novel localized approach to deliver protein and peptide therapeutics to the CNS based on our proprietary **T**ransduced **A**utologous **R**estorative **G**ene **T**herapy system (TARGT™) platform. TARGT is based on autologous dermal micro-organs (MO) harvested and transduced *ex-vivo* to produce a therapeutic protein and then re-implanted in the patient. In this study we assessed whether TARGT can survive and deliver sustained localized protein post implantation into the CNS. In rats, MOs, 2x1mm each, were harvested from Lewis rat skin, characterized *in-vitro,* and then implanted in the Lewis rats' cisterna magna. The procedure was well-tolerated and without observed behavioral change in all implanted rats. Histopathology of MOs excised several weeks post implantation, demonstrated viable and integrated MOs without signs of implant rejection. In the second phase, rat MOs were processed *ex-vivo* into TARGT secreting erythropoietin (TARGT$_{EPO}$) by transduction with Helper Dependent Adenoviral vector encoding human *erythropoietin* (HDAd-HuEPO). Rat TARGTs secreting human EPO were then implanted into Lewis rats' cisterna magna. Post implantation, cerebrospinal fluid (CSF) and serum were collected and EPO levels were measured by HuEPO ELISA. Results obtained suggest that implantation was well tolerated with no observed signs of rejection. Measurable human EPO levels were detected in rat CSF for the duration of the experiment. Detectable but low levels of Hu-EPO were also measured in the rat serum, as expected, since CSF drains into the peripheral blood. In pigs we have tested the viability and monoclonal antibody secretion from TARGTs into the CNS. Humira, an established antibody drug, was selected as a proof of concept. Pig MOs were harvested and transduced with the HDAd vector carrying the Humira sequence. Two TARGT$_{Humira}$ were implanted in the pig subdural space at the parietal convexity region and the pig was followed for one week post implantation. Results obtained post implantation suggests no observed pig's behavioral change. Humira was measured in CSF samples taken from the implantation area and the lumbar space. One week post implantation, TARGTs were excised out of the pig brain when they are viable and no signs of inflammation or damage to the brain tissue were observed. These results demonstrate that TARGT is a promising novel therapeutic platform with the potential for treatment of CNS disorders. We are now actively studying TARGT$_{CNS}$ treatment of lysosomal storage diseases and brain malignancies.

599. Microfluidic Based Synthesis of Folate-Targeted Monomolecular siRNA-Lipid Particles

Rafał Krzysztoń[1], Bässem Salem[2], Gerlinde Schwake[1], Joachim O. Rädler[1]
[1]Department of Physics, Ludwig-Maximilians-Universität (LMU), München, Germany, [2]Department of Chemistry and Biochemistry, Ludwig-Maximilians-Universität (LMU), München, Germany

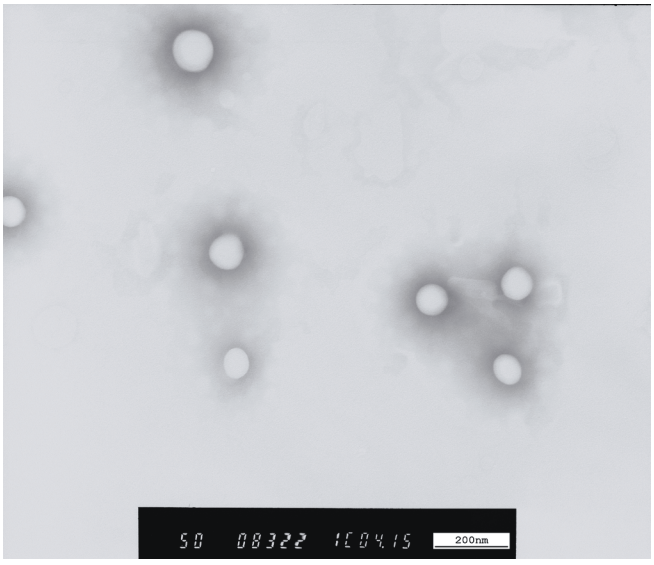
The limiting factors of the effective therapies based on RNA interference are the design and synthesis of efficient transfection agent. Optimal extra- and intracellular transport demands particle to be small, stable and functional in terms of selective cell uptake and triggered release of encapsulated cargo. Strong relation between structure and function of non-viral transfection nanoagents leads to approaches based on tailored design where control over composition and molecular order of nanoparticle is needed. In this context, control over the particle synthesis and its efficiency are the bottlenecks on the way to successful pharmaceutical performance. Here the characterization of small (~30nm) mono-nucleic acid / lipid particles (mNALP) based on rational design of particle structure is made. mNALP particle consists of single 21bp double-stranded oligonucleotide covered by asymmetric lipid bilayer and PEG shielding layer. Bilayer composition of DOTAP:DOPE:DOPC:DSPE-PEG(2000) in molar ratio of 1:5:6:1,2 is an effect of considerations, aiming on a tight coverage of single oligonucleotide in order to minimize the size of whole particle structure. We emphasize the controlled formation of particles by solvent exchange method performed in hydrodynamic focusing microchip. This micromixing method shows significant improvement in physicochemical parameters e.g. reduced polydispersity, negligible aggregate formation and 20% increase in encapsulation efficiency when compared with standard bulk mixing approaches. Furthermore, mNALPs show high stability in blood plasma and serum due to the shielding PEG layer on the particle surface. Also addition of small amounts of folic-acid-conjugated PEG-lipid to the mNALP structure results in specific binding and uptake by epithelial carcinoma KB cells overexpressing folate receptor. Small size, high stability and receptor-specific cell uptake makes the mNALP a highly promising system for systemic delivery of short double stranded oligonucleotides like siRNA.

600. Development and Characterisation of Polymeric Nanocapsules Containing Macromolecule

Omar S. Abu Abed, Amal A. Elkordy, Ching Chaw, Lee Williams, Marc Carlile
Pharmacy and Well-Being, University of Sunderland, Sunderland, United Kingdom

Oral drug delivery systems are the most common route of administration. However, development oral delivery system containing macromolecules is still challenging due to different hurdles, including, digestive enzymes degradation, and physiochemical properties of the molecules. Currently, several techniques have been applied to deliver the macromolecules orally e.g. polymer conjugation, excipients such as cyclodextrin, and entrapment inside carriers, such as liposomes, and nanocapsule systems. Accordingly, the aim of this study is to overcome these challenges by encapsulating macromolecule inside polymeric nanocapsules, trypsin as a protein was used as a model macromolecule. The polymeric nanocapsules were prepared by double emulsion method s/o/w or w/o/w based on the quality by design (QbD) concepts. Critical quality attributes (CQA), were determined in order to achieve the quality target product profile (QTPP). The formulations were developed by using Poly(DL-lactide-co-caprolactone) copolymers in two different ratios (86:14, and 40:60)for lactide and Ɛ-caprolactone blocks, respectively. Trehalose was encapsulated with some formulations. Table1shows the

experimental design consisting of eight formulations. Nanocapsules morphology and particle were investigated by Transmission Electron Microscope, and Dynamic Light Scattering, respectively. The entrapment efficiency of nanocapsules, the effect of the polymer and preparation procedures on trypsin activity and the release profile in simulated gastric fluid SGF and simulated intestinal fluid SIF were assessed.Applying QbD concepts saved the resources, and provided huge useful results within short time. Nanocapsules were spherical with no distortion, Figure 1. The encapsulation efficiency has reached up to 80.7%, and it has been affected significantly (P 0.005) by the copolymer blocks ratio. Moreover, the drug release profile in SIF over 24 hours was also affected by the copolymer ratios, with released drug up to 64.01% in a triphasic pattern concluded in the following three steps; the first burst phase with 8% release within 15 minutes, then a plateau for 8 - 10 hours, finally, trypsin started to release in a sustained rate over the rest of 24 hours, whilst the release in SGF reached 8% in average; which can protect the macromolecules from the gastric enzymes degradation. Trypsin biological activity was affected by the materials and process parameters and retained only 18% of the original activity. However, adding trehalose and encapsulation of solid protein helped the protein to retain up to 84.65% of the original activity. In conclusion, polymeric nanocapsules showed promising results to potentially deliver active proteins orally in the presence of trehalose especially when it was prepared by s/o/w, and when the copolymer blocks ratio was optimised. The applied rationale could be applicable to further macromolecules including monoclonal antibodies, genes, and cells, in order to obtain close quality attributes.



1			
Formulation	caprolactone ratio	Core physical state	Trehalose mM
F1	14%	Liquid	0
F2	60%	Liquid	0
F3	14%	Solid	0
F4	60%	Solid	0
F5	14%	Liquid	10
F6	60%	Liquid	10
F7	14%	Solid	10
F8	60%	Solid	10

601. An Optimized Approach to Non-Viral Transfection of Brain Tumor Initiating Cells Derived from Embryonal Tumor with Multilayered Rosettes Using Cationic Reagents

Charlie Y. Hsu[1], Myra J. Chen[2], Derrick E. Rancourt[1], Jennifer A. Chan[2]

[1]Biochemistry and Molecular Biology, University of Calgary, Calgary, AB, Canada, [2]Pathology and Laboratory Medicine, University of Calgary, Calgary, AB, Canada

Embryonal tumor with multilayered rosettes (ETMR) is an aggressive type of primitive neuroectodermal brain tumors of the central nervous system that occur primarily in infants and young children. Despite intensive therapy, prognosis is extremely poor with typical survival outlook of <1 year after diagnosis. Resistance to conventional chemo/radiation therapy among ETMR patients has been attributed to the resilience and resurgence of a sub-population of "brain-tumor initiating cells" (BTIC) within the tumor mass. These BTICs are a type of cancer stem cell with the capacity for long-term self-renewal, multi-lineage differentiation, and tumor formation. To better understand the pathogenesis of ETMR and develop effective therapies against this disease, we have previously established and characterized a BTIC cell line (BT183) with molecular signatures and tumor-initiating properties that are hallmarks of ETMR. However, BTICs have so far proven difficult to transfect at high efficiency, making them refractory to genetic manipulation approaches. Here, we describe an optimized approach to transfect BTICs using cationic reagent. We initially tried transfecting BT183 with Lipofectamine-2000 according to manufacturer's suggested protocol, but were not able to get any GFP+ cells. Because Lipofectamine is formulated with a multivalent cationic lipid, one rate-limiting factor could be its susceptibility to serum inactivation. While BTICs are cultured in a defined, serum-free neural stem cell-promoting media, the presence of growth factors and additives (e.g. EGF, bFGF, heparin) may interfere with the maturation and stability of lipoplexes. To test this hypothesis, we incubated lipoplexes in different preparations of the NeuroCult™-XF Proliferation media, each devoid of one or more of the required additives. We then assayed for complex stability using SYBRSafe, an intercalating dye that binds to the minor grooves of free DNA more efficiently than the condensed form of complexed DNA. We noted that the fluorescent intensities of the complexes were significantly higher (1.2x) in media with EGF, bFGF, heparin, suggesting DNA may be partially dissociated in the presence of these additives. We then transfected cells in the different media preparations and saw that only transfection carried out in media devoid of heparin/growth factors had GFP+ cells. While the number of GFP+ cells were too few to quantitate at this stage, we noted that there were more GFP+ at lower seeding densities, but the GFP+ cells were mostly buried among non-transfected cells as neurospheres. We postulated that the tendency for these BTIC to aggregate in a density-dependent manner may interfere with the efficient binding and uptake of lipoplexes; hence methods to prevent them from sticking to each other during transfection might enhance efficiency. We therefore tried attaching them using a number of cell attachment methods (i.e. gelatin, PEI, CELLSTAR). Only CELLSTAR was able to facilitate uniform cell attachment without causing significant cytoxicity. When we eventually transfected BTIC attached to CELLSTAR-treated tissue culture plates in media without heparin or growth factors, we saw a remarkable increase in transfection efficiency with up to 24% of the cells being GFP+. In summary, we showed here that BTICs can be efficiently transfected using cationic reagent by temporarily remove media components that are otherwise inhibitory to transfection and by adapting cell culture format to one that is more conducive to the binding and uptake of DNA complexes. The approaches outlined here may be adapted to other hard-to-transfect suspension cultures such as lymphocytes and leukemic cells.

602. Stable In Vitro Reprogramming of Lentiviral Vectors for Target Delivery of Genetic Cargo

Zhilei C. Chen, Nagarjun Kasaraneni, Ana M. Chamoun-Emanuelli
Microbial Pathogenesis and Immunology, Texas A&M University Health Science Center, College Station, TX

Progress in gene therapy has been impeded by the absence of a suitable delivery vector that is both easy to produce and delivers genetic payloads efficiently and specifically to the targeted disease cells. Cell-targeting proteins, primarily monoclonal antibodies, already exist in abundance but there is currently no robust and reproducibly effective way to functionalize viral vectors with these proteins. In the case of antibodies, non-covalent approaches to incorporate antibody onto the lentivirus surface leaves the linkage vulnerable to interference from serum immunoglobulins in immune-competent individuals. In this study, we developed two *in vitro* chemical biological approaches for facile reprogramming of lentivirus to deliver genetic payloads to specific cell type through covalent functionalization *in vitro* with cell-binding proteins. Two covalent-bond forming protein-protein pairs were explored to conjugate a HER2-binding protein to lentivirus pseudotyped with a binding-deficient, fusion-competent Sindbis virus envelope protein. Both strategies produced high infectious titers (>10^6 IU/mL) of HER2-specific lentiviruses. Our new virus reprogramming strategy should be applicable to other viral vectors as well, enabling facile creation of new disease-specific gene delivery vectors.

603. Feldan Shuttle - Advanced Protein Delivery Technology for Cell Therapy

Thomas Del'Guidice[1], Jean-Pascal Lepetit-Stoffaes[2], Joannie Roberge[1], Louis-Jean Bordeleau[1], Mario Harvey[1], Alain Garnier[2], David Guay[1]
[1]Research, Feldan Therapeutics, Quebec, QC, Canada, [2]Chemical Engineering, Laval University, Quebec, QC, Canada

Methods for maximizing the therapeutic value of human cells often involve the transfer of genetic material and use of viruses, both raising important safety and economical concerns. One promising solution to improve cell therapy processes consists on the direct delivery of active proteins into human cells. Regenerative medicine studies established powerful proofs of concept using delivery of transcription factors for cell reprogramming and differentiation; however, the lack of efficiency of current protein delivery methods slowed down the transfer of these methods toward human clinical researches. In the last years, the CRISPR/Cas9 revolution brought forward the possibility of using diverse transfection approaches to deliver Cas9 protein complexed with guide RNA, a method yielding interesting results with cationic lipid agents and electroporation systems. In a human therapy context, lipidic agents are generally not suitable because of their high cellular toxicity. As for electroporation, few systems have been approved for cell therapy; in addition, this process represents a serious concern because of its high cost, inconsistency, associated cell mortality and the fact that some cells are simply refractory to electroporation. In all, these characteristics accentuate the need for a protein delivery process suitable for human cell therapy. In order to efficiently use transcription factors, Cas9 or any other intracellular proteins in cell therapy, Feldan Therapeutics is developing a protein delivery agent, the Feldan Shuttle, explicitly designed for cell therapy. Feldan Shuttle is entirely protein-based, thus allowing cells to naturally degrade it and the delivered protein after their active use. With this novel method, Feldan Therapeutics successfully delivered fluorescent proteins as well as functionally active transcription factors and Cas9/RNA complex with high efficiency and low toxicity into multiple cell lines and human primary cells. With this new technology, Feldan Therapeutics offers an innovative method designed to efficiently and safely deliver native proteins for cell therapy applications.

Delivery Questions for Neural Applications

604. Comparison of Intra-Cisterna Magna and Lumbar Puncture Intrathecal Delivery of scAAV9 GeneTherapy for Giant Axonal Neuropathy

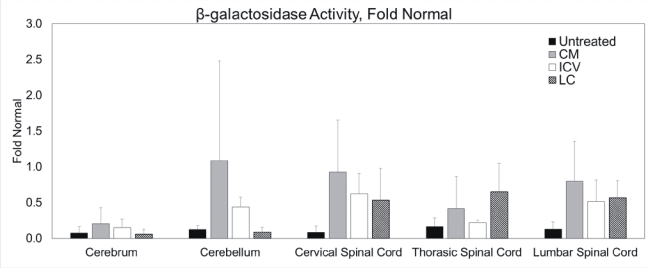
Rachel M. Bailey[1], Alejandra Rozenberg[1], Caleigh Toppins[1], Steven J. Gray[1,2]
[1]Gene Therapy Center, UNC Chapel Hill, Chapel Hill, NC, [2]Department of Ophthalmology, UNC Chapel Hill, Chapel Hill, NC

Giant axonal neuropathy (GAN) is a rare pediatric neurodegenerative disorder characterized by progressive neuropathy that presents as early as 3 years of age and with ultimate mortality during the second or third decade of life. GAN is caused by autosomal recessive loss-of-function mutations in the *GAN* gene that encodes for the gigaxonin protein. Gigaxonin plays a role in the organization/degradation of intermediate filaments (IFs) and GAN patients are pathologically characterized by large axonal swellings filled with disorganized aggregates of IFs. While GAN is primarily described as a progressive peripheral neuropathy, diffuse pathology from disorganized IFs is apparent throughout the central nervous system, enteric nervous system and other organ systems. An NIH-sponsored Phase I study is underway to test the safety of intrathecal lumbar puncture (LP) administration of scAAV9/JeT-GAN to treat the most severe aspects of GAN, namely the motor and sensory neuropathy. Gigaxonin gene transfer through a single LP injection is the first proposed therapy for GAN. Intra-cisterna magna (ICM) delivery of AAV9 vectors shows high transduction of the brain and spinal cord of animals; however, this method of vector delivery has not yet been tested for the treatment of GAN. This study compared the efficacy of using ICM or LP delivery of the scAAV9/JeT-GAN vector to treat GAN KO mice. GAN KO mice were injected with scAAV9/JeT-GAN at 15 months of age using ICM or LP delivery and motor performance was tested monthly. In agreement with our previous studies, we found that GAN KO mice have impaired motor performance around 20 months of age and that this deficit is attenuated with LP delivery of scAAV9/JeT-GAN. In contrast, GAN KO mice receiving ICM-delivered scAAV9/JeT-GAN did not have significantly improved motor function as compared to vehicle treated GAN KO mice. Analyses of disease-relevant pathologies in the brain, spinal cord, and peripheral nerves of these mice are ongoing. To date, antibodies are not available to reliably detect gigaxonin protein expression via immunohistochemical (IHC) analysis, so to directly compare the transduction of the two intrathecal delivery methods, we injected wild-type mice with ~4 x 10^{11} vg scAAV9/GFP via an ICM or LP injection, which is a higher dose than has been reported in any publication. Mice were harvested 4-weeks post-injection and the biodistribution of scAAV9 was analyzed via semi-quantitative PCR and IHC analysis of GFP. Compared to LP-injected mice, GFP expression was notably higher and more wide-spread in the brains of ICM-injected mice, while higher amounts of GFP were detected in the sciatic nerves of LP-injected mice as compared to ICM-injected mice. IHC analysis is ongoing and we will present a detailed map of the transduction of ICM- and LP-delivered scAAV9/GFP across the central and peripheral nervous systems and different organ systems. In conclusion, we report here that ICM-delivery of scAAV9/JeT-GAN does not attenuate motor deficits in GAN KO mice. Furthermore, the vector spread varies between ICM and LP delivery, suggesting that the site of intrathecal injection may have a critical impact on the therapeutic benefit of gene vectors for a given disease.

605. Cerebrospinal Fluid for Delivery of AAV Gene Therapy in GM1 Gangliosidosis

Amanda L. Gross[1], Heather Gray-Edwards[1], Bryan Murdock[1], Amanda Taylor[2], Brandon Brunson[3], Ashley N. Randle[1], Lorelei Stocia[4], Sophia Todessa[4], Jaclyn Lata[4], Miguel Sena-Esteves[4], Douglas R. Martin[1,3]
[1]Scott-Ritchey Research Center, Auburn University, Auburn University, AL, [2]Department of Clinical Sciences, Auburn University, Auburn University, AL, [3]Department of Anatomy, Physiology, & Pharmacology, Auburn University, Auburn University, AL, [4]Department of Neurology and Gene Therapy Center, University of Massachusetts Medial School, Worcester, MA

GM1 gangliosidosis, a hereditary lysosomal storage disease caused by a deficiency of lysosomal β-galactosidase, is characterized by rapidly progressing and fatal neurologic disease. The most common form of GM1 affects children and is fatal by approximately 4 years of age. Currently, there is no effective treatment for GM1 gangliosidosis beyond palliative or supportive care. There are several naturally occurring models of GM1 gangliosidosis, including a well-characterized feline model that closely reproduces human disease progression and facilitates testing of experimental therapies for clinical application. Adeno-associated viral (AAV) therapy has proven effective in GM1 cats, with a greater than 6 fold increase in lifespan after thalamic and deep cerebellar nuclei injections. Prior to initiation of human clinical trials, CSF delivery was evaluated to circumvent the risk of cerebellar parenchymal injection and to improve cortical biodistribution. AAVrh10 was delivered at a total dose of 1e12 vector genomes/kg body weight via 3 routes: cisterna magna (CM), bilateral intracerebroventricular (ICV), or lumbar cistern (LC). Cats were followed for 4 weeks post injection, after which biodistribution of enzyme and vector were assessed. After LC injection, enzyme was limited to the spinal cord, where it reached a maximum of 0.7 fold normal. As expected, the highest vector concentration was limited to the lumbar spinal cord. This indicates limited cranial flow of vector from the lumbar cistern of injected cats, minimizing the efficacy of lumbar delivery. Delivery by the CM led to 0.4 fold - 1.5 fold normal levels of enzyme activity in the spinal cord and up to 2.1 fold the cerebellum and thalamic regions. Following CM injection, vector distributed well to the caudal region of the cerebrum, all of the cerebellum, and throughout the spinal cord. Enzyme activity after ICV injections showed increases from 0.2 fold - 0.8 fold normal in the spinal cord and 0.1 fold - 0.5 fold in the brain. ICV injections led to vector distribution throughout the cerebrum as well as the spinal cord. CSF delivery via cisterna magna, ICV, or lumbar injection is substantially less invasive than cerebellar injection. Based on the results presented here, CM or ICV injection may prove beneficial in clinical trials. Additionally, combining cisterna magna and ICV injection of vector could further enhance therapeutic effect.



606. Use of a Bicistronic Vector to Silence SOD1 in Motoneurons and Astrocytes for the Treatment of Familial Amyotrophic Lateral Sclerosis

Julianne Bobela-Aebischer, Cylia Rochat, Nathalie Bernard-Marissal, Patrick Aebischer, Bernard L. Schneider
Brain Mind Institute, EPFL, Lausanne, Switzerland

Mutations in the superoxide dismutase 1 (SOD1) gene account for 20% of the familial cases of Amyotrophic Lateral Sclerosis (ALS), a fatal neurodegenerative disorder mainly characterized by motoneuron degeneration. Mutant SOD1 exerts multiple pathogenic effects through the gain of toxic properties in both neurons and glial cells. While the expression of mutant SOD1 in motoneurons mainly determines disease onset, SOD1 pathology in non-neuronal cell types plays a key role in disease progression. In this setting, silencing mutant SOD1 expression by gene therapy in the key cell types appears as a logical approach to counteract its toxicity. To design the most effective approach for SOD1 silencing, we have evaluated the respective therapeutic contribution of AAV-miRNA-based targeting of mutated SOD1 either in motoneurons or in astrocytes. In this set of experiments, we used two separate vectors to target either cell type. Following vector administration to both newborn and adult ALS mice, most complete protection of motor units was obtained when targeting human SOD1 principally in motoneurons. Suppressing SOD1 in astrocytes also showed neuroprotective effects, which were clearly different from those observed with the motoneuron-targeting vector. Although we observed only partial protection of spinal motoneurons when suppressing SOD1 in astrocytes, muscle innervation was nearly completely preserved, indicating a possible effect on the ability of the remaining motoneurons to re-innervate the muscle. Therefore, while SOD1 silencing in motoneurons provides most effective neuroprotection in this ALS mouse model, targeting SOD1 in astrocytes may have complementary effects in restoring neuromuscular function. Based on these complementary effects, we tested if therapeutic efficacy could be improved by targeting SOD1 in both motoneurons and astrocytes using a single vector. We therefore designed a bicistronic AAV9 vector, allowing expression of miRNA against SOD1 in motoneurons and astrocytes using cell-type specific promoters. This vector contains an expression cassette under the control of the neuronal promoter synapsin, and a second cassette based on the astrocytic GFAP promoter. Compared to vectors targeting SOD1 in a single cell type, the bicistronic AAV9-miRNA SOD1 vector showed a superior therapeutic efficacy on the motor performance, following intrathecal injection in adult $SOD1^{G93A}$ mice. These results demonstrate the synergistic effect of targeting SOD1 in motoneurons and astrocytes by gene therapy. A further main advantage of this bicistronic vector is that it promotes the safety of this gene therapy approach. Indeed, the use of cell-type specific promoters can avoid undesired expression of the artificial miRNA in periphery. This new vector system has the potential to drastically improve the therapeutic benefits obtained by gene therapy in ALS patients.

607. Organotypic Culture of Adult Porcine Retina as an *In Vitro* Screening Model for AAV-Mediated Gene Therapy in Ophthalmology

Diana Cepeda, Tawny Neal, Rangoli Aeran, Mehdi Gasmi, Annahita Keravala
Avalanche Biotechnologies Inc, Menlo Park, CA

Introduction: Gene therapy studies *in vivo* using animal models provide critical information regarding vector specificity, therapeutic efficacy, and safety. However, long durations to observe transgene expression, high degree of variability between animals, and reduced control of experimental conditions often leads to large sample sizes and expense. Organotypic cultures of the retina can provide an

efficient *in vitro* model with a high degree of throughput and control that can be used for pharmacological screening. These explants preserve complex intercellular processes and communications among the neural retinal layers with minimal manipulation of tissue architecture and molecular pathways, making it a useful model in target-tissue validation of adeno-associated virus (AAV) vector variants. Porcine retina has similar anatomical and physiologic features to the human retina and is therefore a suitable surrogate for preclinical testing in ophthalmology. Methods: We have developed a porcine explant culture system from retinas isolated from adult pigs as a model to screen novel rAAV vectors for transduction efficiency and cellular tropism. Pig eyes were enucleated and the neural retina dissected away from the pigment epithelium within 1-2 hours post-mortem. Tissue was cut into 4 mm pieces and placed photoreceptor layer down on polycarbonate transwells, and cultured in Neurobasal-A medium supplemented with B-27. Viability of the explant cultures was examined over time by gross morphology, quantification of glucose-6-phosphate dehydrogenase release from dying cells, and TUNEL assay. Transductions with AAV vectors were performed on retinal explants on the day the cultures were started, and transgene expression was analyzed by live cell imaging and immunofluorescence (IF) staining, or ELISA from tissue supernatants. Results: Retinal explants were viable for up to 4 weeks and IF staining of retinal protein markers including β-III-tubulin, Chx10, glutamine synthetase, rhodopsin and L/M opsin suggested that most retinal cell types were preserved *in vitro,* with tissue architecture closely resembling that of intact *in vivo* retina. Transduction with different rAAV serotypes encoding GFP displayed protein expression at 3 days, with gradually rising levels during the first 2 weeks following transduction. Furthermore, IF staining using retinal cell markers revealed differential cellular transgene expression from the AAV vectors encoding various cell-specific or ubiquitous promoters under investigation. Finally, high levels of secreted soluble VEGFR-1 protein expressed from rAAV were detected from explant culture media samples with expression increasing over a period of 3 weeks. Conclusions: Our results demonstrate viability of retinal explants for up to 4 weeks and efficient rAAV transduction with various serotypes. Our organotypic porcine retinal explant model facilitates the evaluation of efficacy, cellular tropism, and promoter selectivity of rAAV vectors in a fast, reproducible, and economical manner, contributing to a reduction in the use of animals for the development of therapies in ophthalmology.

608. In Silico Reconstructed Ancestral Adeno-Associated Viruses Transduce Mouse Anterior Segment

Ru Xiao, Li Wang, Livia Carvalho, Sarah Wassmer, Luk H. Vandenberghe
Grousbeck Gene Therapy Center, Ocular Genomics Institute, Schepens Eye Research Institute/Massachusetts Eye and Ear Infirmary/Harvard Medical School, Boston, MA

Adeno-associated virus (AAV) has been widely used as a gene delivery vehicle in ocular gene therapy, especially for the treatment of inherited retinal diseases. It has been proven in animal models and humans that many AAVs can efficiently transduce different cell types in the retina. However, there are very few reports showing that single strand AAVs can successfully transduce the anterior segment of the eye. Some self-complementary AAVs, such as self- complementary AAV2 (scAAV2), have been shown to have robust transduction in the anterior segment cells of mouse, rat, and monkey. Unfortunately, the maximum package genome size of the self-complementary AAV is approximately 2.2kbp, which is less than half the package capacity of single strand AAV vectors. In previous studies, we have shown that an ancestral AAV, Anc80, which was developed in our lab by in silico ancestral sequence reconstruction, can achieve fast onset and long-term stable expression in retinal cells. In the present study, we have explored the tropism and transduction efficiency of the ancestral AAV vector (Anc80) in the anterior segment of mice eyes and compared it with some other contemporary AAVs. Three different AAV vectors (single strand AAV2-ssAAV2, scAAV2 and Anc80) carrying eGFP reporter gene driven by CMV promoter. The viral vectors were injected into wild type C57Bl/6 mouse eyes either via anterior chamber injection at a dose of 1×10^9 particles, or via intravitreal injection at a dose of 2×10^9 particles. At day 28 post- injection, mice eyeballs were collected and sectioned to examine GFP expression in the anterior segment by using confocal microscopy. Our data shows all three AAV vectors can transduce cornea, trabecular meshwork, and ciliary body by anterior chamber injection, as determined by GFP expression level and expression area in the cornea, Anc80 > ssAAV2 > scAAV2, and in the trabecular meshwork and ciliary body, Anc80 > scAAV2 > ssAAV2. Via intravitreal injection, GFP expression was detected in cornea and ciliary body in the Anc80 group, while no GFP expression in cornea or ciliary body was found in the ssAAV2 group. **In Conclusion,** anterior chamber or intravitreal injections of Anc80 efficiently transduce all components of the mouse anterior segment. These results will inform future studies that aim to deliver therapeutics in such diseases as glaucoma, which affect the cornea, iris, or ciliary body.

609. Transduction of the Central Nervous System with the LTR1 Lentiviral Backbone

Rajvinder Karda[1], John R. Counsell[1], Conrad A. Vink[2], Dany P. Perocheau[1], Suzanne M. K. Buckley[1], Joanne Ng[1], Simon N. Waddington[1], Steven J. Howe[2]
[1]Gene Transfer and Technology, Institute for Women's Health, UCL, London, United Kingdom, [2]GSK, Hertfordshire, United Kingdom

A novel HIV-1-based lentiviral vector has been generated that eliminates 1.7kb of wild-type HIV-1 elements from the vector provirus. This vector, known as LTR1, potentially offers several advantages over current lentiviral technology, such as increased transgene-carrying capacity, resistance to remobilisation in HIV-1 particles and a reduced risk of splicing interactions with host genes. In this study we investigated the persistence and strength of LTR1-derived expression within the central nervous system (CNS). We began by developing LTR1 vectors containing the spleen-focus forming virus (SFFV) promoter driving either green fluorescent protein (GFP) (LTR1-SFFV-GFP), or a bicistronic luciferase-GFP construct (LTR1-SFFV-Luc-GFP). Both LTR1 vector preparations were pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) and administered intracranially to newborn outbred CD1 mice. Those that received the LTR1-SFFV-Luc-GFP virus were imaged continually by whole body bioluminescence imaging. From this, we observed long term luciferase expression within the CNS for 32 days post-administration, at which point the mice were sacrificed for further analysis. Mice receiving the LTR1-SFFV-GFP virus were euthanized at day 35 and the brain tissues were collected, sectioned and stained for GFP by free-floating immunohistochemistry, which showed GFP-positive neurons predominately within the cortex and hippocampus. In this research, we demonstrated long-term expression within the CNS, specifically the cortex and the hippocampus, after a single neonatal administration of the LTR1 virus. We have therefore highlighted its potential use as a gene therapy vector for neurological disorders. This, combined with LTR1's potential to incorporate large transgenes, highlights a possible application in gene therapy of channelopathies, which would benefit from viral delivery of the relatively large sodium ion channels.

610. An Inducible AAV Vector Mediating GDNF Signal Transduction at Clinically-Acceptable Sub-Antimicrobial Doxycycline Doses

Liliane Tenenbaum[1], Abdelwahed Chtarto[2], Marie Humbert-Claude[1], Olivier Bockstael[2], Atze T. Das[3], Sébastien Boutry[4], Ludivine Breger[5], Bep Claver[6], Robert N. Müller[4], Olivier De Witte[2], Marc Levivier[1], Cecilia Lundberg[7], Ben Berkhout[6]
[1]Clinical Neuroscience Department, Lausanne University Hospital, Lausanne, Switzerland, [2]Experimental Neurosurgery, Université Libre de Bruxelles, Bruxelles, Belgium, [3]Laboratory of Experimental Virology, University of Amsterdam, Amsterdam, Netherlands, [4]Centre for Microscopy and Molecular Imaging, Université Mons-Hainaut, Mons, Belgium, [5]CNS Gene Therapy team, Wallenberg Neuroscience Centre, Lund University l, Lund, Sweden, [6]Laboratory of Experimental Virology, Amsterdam University, Amsterdam, Netherlands, [7]CNS Gene Therapy team, Wallenberg Neuroscience Centre, Lund University, Lund, Sweden

Preclinical and clinical data stress the importance of pharmacologically-controlling glial cell line-derived neurotrophic factor (GDNF) intracerebral administration to treat Parkinson's disease. The main challenge is finding a combination of a genetic switch and a drug which, when administered at a clinically-approved dose, reaches the brain in sufficient amounts to induce a therapeutic effect. We describe a highly-sensitive doxycycline-inducible AAV vector (AAV-Dox-ON). This vector allowed for the first time a longitudinal analysis of inducible transgene expression in the brain using bioluminescence imaging. To evaluate the dose range of GDNF biological activity, AAV-Dox-ON-GDNF was injected in the rat striatum and increasing doxycycline doses administered orally. ERK signaling activation was induced at a plasmatic doxycycline concentration of 140 ng/ml which is known not to increase antibiotic-resistant microorganisms in patients. In these conditions, GDNF covered the majority of the striatum. No behavioral abnormalities or weight loss were observed. Motor asymmetry resulting from unilateral GDNF treatment only appeared with a 2.5-fold higher vector and a 13-fold higher inducer doses. Our data suggest that using our new sensitive doxycycline-inducible AAV vector, beneficial effects of GDNF can be obtained in response to sub-antimicrobial doxycycline doses without eliciting adverse effects.

611. Promoter Evaluation of AAV Gene Therapy in the Central Nervous System for Feline Niemann-Pick Type C Disease

Brittney L. Gurda[1], Gary Swain[1], Jessica H. Bagel[1], Maria Prociuk[1], Steven J. Gray[2], Caitlin Fitzgerald[1], Patricia O'Donnell[1], Charles H. Vite[1]
[1]Clinical Studies, University of Pennsylvania, Philadelphia, PA, [2]Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC

Niemann-Pick type C1 (NPC1) disease is characterized by progressive cerebellar ataxia, dementia, and death in adolescence. It is caused by mutations that result in deficient function of lysosomal, membrane-bound NPC1, resulting in the intralysosomal accumulation of cholesterol and sphingolipids. There are no FDA-approved therapies for NPC1 disease, however, we have previously shown that administration of 2-hydroxypropyl-beta-cyclodextrin (HPßCD) via the cerebromedullary cistern (CBMC) in presymptomatic cats with NPC1 disease prevented the onset of cerebellar tremor and resulted in Purkinje cell (PC) survival and near normal concentrations of cerebral cortex and cerebellar cholesterol and sphingolipids. Remarkably, these cats remained alive for greater than three years of age, in contrast to untreated NPC1 cats that died before six months of age. This therapy has advanced to clinical trials where patients receive biweekly HPßCD. In an attempt to both reduce the number of HPßCD injections and to treat uncorrected brain regions, we studied the feasibility of viral gene therapy, providing a one-time injection, in NPC1 cats. Preliminary studies utilized administration of an adeno-associated virus serotype 9 carrying the feline NPC1 transgene (fNPC1) via the CBMC of pre-symptomatic NPC1 cats. These treated cats showed reduced ataxia compared to untreated cats. Histologic evaluation of the brains revealed that NPC1 expression had been restored to some PCs and that this was associated with increased PC survival. This initial success prompted us to further explore the potential of various promoters to express functional NPC1 protein in the brain. In this study we evaluated the ability of four different promoters, three ubiquitous (CB7, GUSB, and JeTi) and one neuron-specific (CamKII), to drive GFP expression in the central nervous system of normal cats. Our preliminary therapeutic studies used the GUSB promoter (pGUSB) to mediate fNPC1 expression. Expression of both fNPC1 and a GFP reporter under pGUSB were low in PCs, and in cerebrocortical neurons and astrocytes. Overall, the CB7 promoter drove the highest level of expression in similar regions; however, this large promoter (~970bp) limits transgene size (where NPC is ~4.3Kbp) and led us to examine a smaller exogenous promoter based on CB7, termed JeTi. This promoter was similar in expression levels to the pGUSB, leading to low-level expression at our limit of detection using DAB-based immunohistochemistry. Interestingly, a six-month therapeutic study using the JeTi promoter had a similar outcome as pGUSB where treated cats had reduced clinical signs at the end of study. Expression driven by the neuron-specific promoter CamKII produced levels similar to the CB7 promoter, although PCs of the cerebellum had low to undetectable expression levels. These data and preliminary therapeutic data in AAV-treated NPC1 cats will be further explained in this presentation.

612. Systemic AAV Injection in Guinea Pigs and Non-Human Primates Targets the Enteric Nervous System

Sara E. Gombash[1], Christopher J. Cowley[1], Julie A. Fitzgerald[1], Kathryn Hook[2], Emily Armstrong[1], Brian K. Kaspar[3], Andrew J. Fischer[1], Levi Todd[1], Eric C. Bielefeld[4], Kevin D. Foust[1]
[1]Neuroscience, Ohio State University, Columbus, OH, [2]Belmont University, Nashville, TN, [3]Nationwide Children's Hospital, Columbus, OH, [4]Speech and Hearing Science, Ohio State University, Columbus, OH

We previously reported that intravenous delivery of AAV8 or AAV9 produces robust transduction in the enteric nervous system (ENS) of mice that is capsid dependent. The current proof-of-concept studies were to determine if intravenous AAV delivery transduces the ENS of two higher species, specifically guinea pigs, a commonly used species for ENS physiology studies, and cynomolgus macaques. Neonatal guinea piglets (P2) were intravenously injected with either of scAAV8 ($2.8\text{-}3.1x10^{10}$ vg/g) or scAAV9-CB ($2.8\text{-}3.4x10^{10}$ vg/g) expressing green fluorescent protein (GFP) which was 3-10x lower than our previous mouse studies. Twenty-one days post-injection, piglets were euthanized and tissues were harvested for vector biodistribution with special focus on the ENS. Overall, AAV8 more efficiently transduced guinea pig tissues than AAV9. scAAV8 produced robust transgene expression in the ENS, reaching 23-39% submucosal plexus (SMP) neurons and 3-21% of myenteric neurons with higher percentages of transduction in upper gastrointestinal tract regions. Intravenous scAAV9 produced GFP expression in 0.5-4% of SMP neurons and 1-4% of myenteric neurons. AAV9 transduction was spread evenly through the GI tract. Capsid had limited effect on the types of neurons targeted in the myenteric plexus of the guinea pig. Biodistribution in injected animals revealed GFP expression in the liver, spleen, kidneys, cardiac muscle, skeletal muscle, brain and spinal cord of AAV8 and

AAV9 injected guinea pigs, with AAV8 always producing higher expression. Cochlea and eyes were negative for GFP expression in all animals. To determine the translational potential of ENS gene delivery, gastrointestinal tissues from cynomolgus macaques previously IV injected with scAAV9-CB-GFP (1-3 x 10^{14} vg/kg) at P1 or P30 were dissected and examined for GFP expression. GFP was detected in the myenteric plexus of the small intestine and distal colon myenteric plexus, the lone regions examined. Together these data indicate that intravenous delivery of AAVs for ENS transduction is translatable across species.

613. A Comparison of CNS Transduction After Systemic versus Cranial Delivery of an AAV2/9 CamKII Promoter-eGFP Vector in Mice

Alana L. Gibson[1], Randy J. Chandler[1], Ian M. Williams[2], Arturo A. Incao[1], Forbes D. Porter[2], William J. Pavan[1], Charles P. Venditti[1]
[1]National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, [2]National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD

Niemann-Pick type C (NPC) disease is a rare but fatal lysosomal storage disorder characterized by accumulation of unesterified cholesterol and other lipids within the lysosome. Clinical manifestations include ataxia, dementia and hepatosplenomegaly. 95% of cases are caused by an *NPC1* gene mutation resulting in a lack of functional NPC1 protein, a putative cholesterol transport protein found in the lysosomal limiting membrane. The neuronal pathology seen in *NPC1*$^{-/-}$ mice begins with cerebellar axonal swelling, progresses to gliosis and visible lipid accumulation within lysosomes, then is followed by a marked loss of Purkinje cells. Weight loss and ataxia accompany disease progression. We have previously demonstrated that systemic delivery of an AAV9 vector designed to express the human *NPC1* gene under the control of the neuronal-specific promoter, mouse calcium/calmodulin-dependent protein kinase II (CamKII), to *Npc1*$^{-/-}$ mice resulted in a modest but significant increase in survival. Transgene expression, assayed by immunohistochemistry (IHC), revealed widespread *NPC1* expression within the brain, broadly correlating with the endogenous CamKII expression pattern. Delayed loss of Purkinje cells in the AAV treated *Npc1*$^{-/-}$ mice was also observed. Despite the increase in lifespan, expression of *NPC1* in the Purkinje cells of the AAV treated *Npc1*$^{-/-}$ mice was limited, and the mice eventually succumbed to NPC1 disease. This result is notable due to the association between Purkinje cell loss and NPC1 disease progression. In an effort to improve neuronal transduction, particularly in Purkinje cells, we have explored various central nervous system (CNS) delivery routes with an AAV9 reporter, configured to express eGFP under the CamKII promoter. Three groups of mice (n=3 mice per group) received stereotactic-guided AAV9 CamKII-eGFP injections into either the lateral ventricles (dose: 3.8 x 10^{11} GC), cisterna magna (dose: 3.1 x 10^{11} GC), or a combination of both (dose: 3.8 x 10^{11} GC). After two weeks, eGFP expression was assessed using IHC and microscopy. Lateral ventricle and combination injections yielded widespread neuronal transduction throughout the brain, but expression in the cerebellum was limited and sporadic. Injections into the cisterna magna - expected to target the cerebellum - did not improve cerebellar expression compared to the other routes. Overall, aside from a heightened intensity of transgene expression, cranial injections did not appear to increase neuronal targeting compared to systemic delivery by retro-orbital injections (dose: 1x10^{12} GC). Based on preliminary evidence from a limited number of mice, gross differences in transduction between *Npc1*$^{-/-}$ and control mice were not observed, suggesting that the disease state at the time of assessment did not influence vector tropism. Our results will help define the optimal delivery route for future NPC1 vector optimization studies, and may be informative for others who seek to correct neurodegenerative mouse models using AAV9-mediated gene therapy.

614. Microfluidic Manufacture of RNA-Lipid Nanoparticles Leads to Highly Efficient Delivery of Potent Nucleic Acid Therapeutics for Controlling Gene Expression

Grace Tharmarajah[1], Eric Ouellet[1], Oscar Seira[2,3], Jie Liu[2], Anitha Thomas[1], Timothy Leaver[1], Andre Wild[1], Yuping Li[4,5], Yu Tian Wang[4,5], Wolfram Tetzlaff[2,3], Carl Hansen[6], Pieter Cullis[7], James R. Taylor[1], Euan Ramsay[1]
[1]Precision NanoSystems Inc., Vancouver, BC, Canada, [2]Department of Zoology, University of British Columbia, Vancouver, BC, Canada, [3]International Collaboration on Repair Discoveries (ICORD), University of British Columbia, Vancouver, BC, Canada, [4]Department of Medicine, University of British Columbia, Vancouver, BC, Canada, [5]Brain Research Center, University of British Columbia, Vancouver, BC, Canada, [6]Physics and Astronomy, University of British Columbia, Vancouver, BC, Canada, [7]Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada

Lipid nanoparticles (LNPs) have demonstrated efficient nucleic acid delivery in vitro and in vivo, as well as in clinical development. They exploit endogenous delivery pathways, by co-opting apolipoprotein E (apoE), to mediate effective delivery of the encapsulated nucleic acids into cells via the low-density lipoprotein receptor (LDLR). However, use of LNPs from the bench to the clinic has been considerably limited by challenges in manufacturing at both small and large scales. Here, we bridge that gap by describing the robust manufacture and use of clinical-grade lipid-based nanoparticles for highly efficient delivery of nucleic acids at scales suitable for both in vitro screening and in vivo applications.

RNA-LNPs manufactured using an optimized microfluidic platform enables efficient encapsulation of nucleic acids (e.g. siRNA, mRNA, pDNA) into biocompatible "solid-core" nanoparticles (~50 nm). The resultant nanoparticles can then be applied to cell cultures in vitro or administered in vivo. The following reports a comprehensive set of studies conducted to evaluate the merits of the technology and further provide insights for delivering short interfering RNA (siRNA) and mRNA in difficult-to-transfect cells both in vitro and in vivo.

RNA-LNPs were formulated to encapsulate a potent siRNA directed against PTEN - a clinically relevant gene associated with neural regeneration. Exceptional cellular uptake (>98%) with minimal toxicity was observed in both primary rat hippocampal and mixed cortical cell cultures. High transfection efficency (>95%) of the encapsulated material resulted in concomitant high-level (>85%) PTEN knockdown within the first 4 hours of a low dose (100 ng/ml) treatment; that level of knockdown was further sustained for 21 days. Similarly, RNA-LNPs encapsulating mRNA were also found to mediate early (< 4 hours) and sustained gene expression (>75% for 7 days) following a single (500 ng/ml) treatment in primary rat mixed cortical cultures.

Strategies for locally administering RNA-LNPs into the brain and spinal cord of adult Sprague Dawley rats were also investigated. Controlled localized injections of PTEN-encaspulated siRNA into the motorcortex resulted in significant and sustained (7 days) knockdown. Similarly, local administration at the site of a cervical spinal cord injury significantly reduced target PTEN expression, 10 days later. Visible uptake of RNA-LNPs characterized by their presence in the soma of neurons found in the red nucleus provides further insights into a regtrograde transport mechanism involving the axons.

Collectively, these studies reflect the simplicity and efficacy of this commercially available technology in presenting a cost-effective and advantageous avenue for screening and validating new targeted nucleic acid therapies.

615. Choroid Plexus-Targeted Viral Gene Therapy for Lysosomal Storage Diseases

Eun-Young Choi[1], Shih-hsin Kan[2], S. Q. Le[2], Patricia I. Dickson[2], Stephen G. Kaler[1]
[1]NIH, Bethesda, MD, [2]Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA

Lysosomal storage diseases (LSDs) represent a category of approximately 60 inherited neurometabolic illnesses with considerable morbidity and mortality in children and adults. The burden of LSD is high due to the chronic progressive decline in the neurocognitive function of affected individuals that profoundly limits their societal integration. Current therapy for LSDs has focused on recombinant enzyme replacement therapy (ERT), which while promising, is not ideal for numerous reasons, including the short half-lives of ERT with weekly or monthly administration, potential immune responses against ERT may also reduce the effectiveness for the treatment and inefficient passage through the blood-brain barrier. The choroid plexuses are vascularized structures that project into the cerebrospinal fluid (CSF) and feature specialized polarized epithelia derived from neuroectoderm that are post-mitotic, i.e., do not undergo turnover, and produce CSF by transporting water and ions into the brain ventricles. We hypothesized that remodeling these epithelia to secrete missing lysosomal enzymes by one-time administration of a recombinant AAV vector with selective tropism for choroid plexus (e.g., serotype 5) would be an attractive strategy for long-term treatment of LSDs. Potentially this approach would result in steady secretion of the missing enzyme into the CSF, which normally carries molecules throughout the ventricular system into the subarachnoid space, and ultimately deliver enzyme to the entire brain. The cross-correction phenomenon in many LSDs would provide a further advantage. To evaluate this hypothesis in preclinical animal models, we chose two prototypical LSDs, α-mannosidosis and mucopolysaccharidosis type IIIB (MPS IIIB or Sanfilippo B syndrome). We cloned the respective cDNAs (human (h) *LAMAN* and *NAGLU*) into rAAV shuttle plasmids and generated high titer of rAAV5 expressing the enzymes. We administered viral particles to the lateral cerebroventricles sof homozygous mutant mice on day 2 or 3 of life at doses of 5×10^9 or 5×10^{10} vector genomes. In the LAMAN deficient mice, we documented dose-dependent transduction and h*LAMAN* mRNA expression confined to the choroid plexuses of rAAV5-treated animals. Brain biochemical analyses at 1, 2 and 6 months post-treatment documented sustained, highly statistically significant increases of LAMAN enzyme activity in the brain globally (olfactory bulb, cerebral cortex, cerebellum, brainstem). By 8 months of age, untreated mutant mice showed prominent lysosomal vacuoles in hippocampal neurons, in contrast to rAAV5-hLAMAN treated mutants for which brain histopathology was comparable to wild-type. Lysosomal associated membrane protein 1 (Lamp1) levels were normalized in AAV5-hLAMAN treated mutant brain. In MPS IIIB mutant mice, levels of NAGLU enzyme activity were 2-8 fold higher in brain sections six weeks after rAAV5-hNAGLU treatment compared to normal controls. β-Hexosaminidase activity, which is elevated in MPS IIIB, was reduced to heterozygote carrier levels. Tissue evaluations by immunohistochemistry showed robust hNAGLU expression in the choroid plexus epithelia. Lamp1 expression was significantly reduced in hippocampus and frontal cortex of treated mice. The approach outlined herein challenges existing treatment modalities for LSDs, by exploiting the ability of choroid plexus-targeted gene therapy to restore missing or defective lysosomal enzymes at concentrations and distributions in CSF suitable for disease correction. The potential impact on clinical practice in the field of LSD is high since, if these results extend to larger animal disease models, nonhuman primates, and human subjects, the largest current barriers to health for affected patients would be circumvented.

616. Tailored Expression of a Transgene to Specific Cell Types in the Central Nervous System After Peripheral Injection of AAV9

Eloise Hudry, Jonathan Dashkoff, Paul Lerner, Shuko Takeda, Nhi Truong, Casey Maguire, Bradley T. Hyman
Massachusetts General Hospital -Harvard Medical School, Charlestown, MA

Because of their efficient tropism for the neural tissue and their well-established safety and tolerability profile, adeno-associated vectors (AAV) hold great potential to express therapeutic genes in the context of various neurodegenerative disorders. Importantly, the characterization of novel AAV serotypes (AAV9; rhAAV10, etc…) that can cross the blood brain barrier after intravenous delivery now offer an opportunity for non-invasive delivery to the brain. However, in absence of a well-tailored system, the use of a peripheral route injection may lead to undesirable transgene expression in various cell types of the nervous system as well as in other organs. In order to refine this approach, the present study characterizes the transduction profiles of newly engineered self-complement AAV9 (scAAV9) expressing the Green Fluorescent Protein (GFP) either under an astrocyte (GFA') or neuronal (Synapsin, Syn) promoter, after a single injection in the lateral tail vein of adult mice (5x10e11vg/animal). We report that our scAAV9-GFA'-GFP and scAAV9-Syn-GFP respectively led to robust and long-lasting transduction of astrocytes (10%) and neurons (8%) throughout the entire cerebral tissue, in the absence of aberrant expression leakage in other cell types of the brain or in the liver. GFP positive excitatory as well as inhibitory neurons could be observed in the CNS, while motor neurons were also efficiently transduced in the PNS. In addition, both activated (GFAP positive) and resting astrocytes expressed the reporter gene. Quantitative stereological assessment demonstrated an increase in reactive astrocytes after transduction with scAAV9 but did not lead to any obvious detrimental phenotype in any animals. These data suggest the potential interest of tailoring AAV to drive expression of therapeutic genes specifically in astrocytes or neurons of the CNS after intravenous delivery, thus preventing adverse side-effects associated with peripheral expression.

617. Identification of Novel AAV Capsid Variants with Enhanced Tropism for the Canine Outer Retina Following Intravitreal Delivery

Leah C. Byrne[1], Meike Visel[2], Valerie Dufour[3], Felipe Pompeo Marinho[3], Gustavo D. Aguirre[3], William A. Beltran[3], John G. Flannery[2], David V. Schaffer[2]
[1]Department of Clinical Studies, School of Veterinary Medicine/ Helen Wills Neuroscience Institute, University of Pennsylvania/ University of California Berkeley, Berkeley, CA, [2]Helen Wills Neuroscience Institute, University of California Berkeley, Berkeley, CA, [3]Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA

The delivery of AAV vectors across physical barriers to target the outer retina is key to the success of retinal gene therapies. Subretinal injection of AAV vectors is the most common approach to deliver genes to photoreceptors and RPE; however, this route of administration presents surgical challenges and potential complications, as well as mediates transfer to only a limited region of the retinal surface. Intravitreal administration may represent a safer way of targeting larger areas of the outer retina; physical barriers such

as the internal limiting membrane significantly limit efficiency of natural AAVs through this route. In a prior murine proof of concept study, we showed that directed evolution is promising method for engineering new AAV capsid variants with enhanced permissivity for the outer retina. Here, we show results from a screen performed in the canine retina, a large animal model with eye structure and size similar to the human eye. A library of $>10^7$ distinct capsid variant AAV vectors was injected intravitreally into the eye of a wildtype dog. A subset of the AAV vectors injected was recovered from samples of outer retina by PCR amplification. Isolated variants were then pooled and re-injected in additional wildtype canine eyes, and following 5 total rounds of selection the pool had converged to a relatively small number of variants. High throughput sequencing was used to characterize the convergence of variants over all rounds of selection. The final candidate variants were then evaluated by packaging a GFP reporter controlled by a ubiquitous CAG promoter. Retinal distribution and cellular tropism of the variants was examined by cSLO (AF mode) imaging, histology and immunocytochemistry. Nineteen variants were identified as candidate vectors for targeting the canine outer retina. Intravitreal injection of a mixture of equal amounts of these variants carrying GFP fused to a barcode resulted in efficient targeting of all retinal layers including photoreceptors and RPE in 6 eyes of 3 wildtype dogs. Thus, directed evolution performed in a large animal model, with an eye structure similar to that of humans, resulted in new AAV capsid variants with improved abilities to bypass significant structural barriers and efficiently transduce the outer retina. High throughput sequencing allowed for identification of promising variants and monitoring of convergence. These vectors show promise for gene therapy delivery via the intravitreal route of administration, potentially providing a safer approach for targeting a larger area of outer retina than currently achieved by the more traumatic and focal subretinal injection method.

618. Directional Transduction of AAV-5 Vectors in the NHP Brain

Bas Blits[1], Lluis Samaranch[2], John Bringas[2], Waldy San Sebastian[2], Krystoff Bankiewicz[2], Harald Petry[3]
[1]Neurobiology Research, uniQure, Amsterdam, Netherlands, [2]Neurological Surgery, University of California San Francisco, San Francisco, CA, [3]Research, uniQure, Amsterdam, Netherlands

This study was performed to investigate the delivery of AAV vectors into the putamen and thalamus of nonhuman primates. The vectors were delivered to the targeted brain regions by MRI-guided convection enhanced delivery. Special attention was given to the analysis of axonal transport and levels of transduction. Recombinant AAV is an excellent candidate for delivery of therapeutic molecules to the central nervous system to target neurodegenerative diseases. UniQure has succeeded in developing a proprietary platform manufacturing technology that allows safe, effective, cGMP-compliant, economically feasible and commercially scalable manufacturing of AAV. UniQure's novel approach is based on the use of a combination of recombinant baculoviruses and insect cells. Using our production platform, two AAV stocks encoding GDNF or GFP were generated. At eight weeks following infusion into the thalamus, for instance, massive transduction of the thalamus, cortex, striatum and substantia nigra was observed indicating both anterograde and retrograde transduction. At the site of injection, transduction was both glial and neuronal, whereas off site transduction was mainly neuronal. Following injection of a lower AAV volume into the putamen, transduction was limited to areas within the putamen and substantia nigra suggesting only anterograde transport of viral particles. These data indicate a dose dependent anterograde or retrograde transport mechanism. This data set confirms that production of AAV using the (scalable) baculovirus-based platform results in an effective vector that is able to mediate expression patterns that can be used to develop an AAV-mediated therapeutic strategy to treat neurodegenerative diseases.

619. AAV9 Transduction Is Similar in Adult and Aged Mouse Brains Following Intraparenchymal Injection

Sara E. Gombash[1], Julie A. Fitzgerald[1], Christopher J. Cowley[1], Mitchell G. Neides[1], Emily Armstrong[1], Diana M. Norden[2], Jonathan P. Godbout[1], Kevin D. Foust[1]
[1]Neuroscience, Ohio State University, Columbus, OH, [2]Physical Therapy, Ohio State University, Columbus, OH

We previously reported that systemic injection of AAV9 in aged mice (P550) results in significantly fewer transduced CNS cells compared to adult mice (P49). This was not due to the presence of neutralizing antibodies in the aged mice. To determine whether aging in the brain and/or the periphery is responsible for this reduction, we quantified CNS transduction following stereotaxic brain injection in aged or adult mice. Aged male Balb/c mice (n = 6, P550) and adult mice (n = 7, P50) received direct injections into the striatum and ipsilateral hippocampus with scAAV9-CB expressing green fluorescent protein or scAAV9-CB expressing glial cell line derived neurotrophic factor (GFP or GDNF). All mice were euthanized three weeks post-injection. Brains of aged and adult mice injected with scAAV9-CB-GFP were sliced and processed for near-infrared imaging to quantify vector spread and GFP intensity. GFP expression was similar in directly injected aged and adult brains. Vector spread along the rostrocaudal axis was approximately 2 mm from either the striatal or hippocampal injection site and did not vary with age. Brains of mice directly injected with scAAV9-CB-GDNF were hemisected and processed for ELISA to quantify GDNF protein levels. GDNF levels in the striatum and hippocampus reached 2x those of uninjected controls and no differences in GDNF expression were detected between aged and adult injected mice. Together, we found that AAV9 CNS transduction is similar in aged and adult mouse brains following direct injection. These results suggest that reduced CNS transduction in aged mice following AAV9 systemic injection is due to alterations in the periphery. Future studies include investigation of altered viral blood brain barrier penetration and viral retention within peripheral tissues of aged mice.

620. Use of Transgenic Mice to Quantify Transduction Efficiency and Specificity of Novel AAV Vectors in Retina

Miranda L. Scalabrino, K. Tyler McCullough, Shreyasi Choudhury, James J. Peterson, Seok-Hong Min, Sanford L. Boye, Shannon E. Boye
Ophthalmology, University of Florida, Gainesville, FL

Modifications to surface-exposed residues of recombinant Adeno-associated virus (AAV), incorporation of novel regulatory elements into the vector cassette, alternative injection routes, and combinations thereof are being explored as strategies to enhance gene delivery to retina. We previously showed that intravitreal injection of a novel AAV capsid was capable of transducing all neuronal layers of the retina, including photoreceptors (PRs). We also demonstrated that this capsid, when combined with a novel promoter ("Ple155") was capable of driving therapeutic nyctalopin (*Nyx*) expression exclusively in ON bipolar cells (ON BCs) and partially restoring retinal function to a mouse model of complete Congenital Stationary Night Blindness (CSNB1) following early (postnatal day 2) treatment. In order to achieve more efficient transduction of PRs and ON BCs, further modifications will be required. Methods with which to quantify relative transduction efficiencies of novel variants *in vivo* are

warranted. The purpose of this study was to develop robust methods for quantifying transduction efficiency or transgene expression in PRs and ON BCs, respectively by rAAVs *in vivo*. Specifically, we sought to develop methods that don't require time consuming subjective analysis. To do so, we employed two transgenic mouse models: *Nrl*-GFP and *Grm6*-GFP mice which have constitutively fluorescent rod PRs and ON BCs, respectively. Adult mice (~postnatal day 30) were injected either subretinally (SR) or intravitreally (Ivt) with rAAVs containing the mCherry transgene. Fundoscopy was performed at 1 month post injection. Immediately thereafter, retinas were dissociated and evaluated by FACS for GFP and mCherry fluorescence, the overlap of which indicated targeted transduction of either ON BCs or rods, depending on mouse model. A subset of retinas was also evaluated with immunohistochemistry. In agreement with previous experiments, Ivt and SR delivered AAV2(quadY-F+T-V) and AAV2(4pMut) transduced a relatively large percentage of retina, including PRs. However, ON BC transduction was minimal, with ~6% transduced by Ivt AAV2(quadY-F+T-V)-CBA-mCherry delivery. AAV2 weakly transduced retina via both delivery routes. AAV2(4pMut)ΔHS, which lacks canonical HSPG binding residues, failed to transduce retina by Ivt injection, but efficiently transduced rods (75%) following SR delivery, indicative of significant 'lateral spread' from the injection bleb. Incorporation of the ON BC-specific promoter ("Ple155") used in the CSNB1 gene replacement study abolished non-ON BC retinal expression. Experiments are ongoing to assess the extent to which rAAVs containing Ple155 can increase expression efficiency in ON BCs relative to CBA, and to determine whether the addition of other transcriptional and post transcriptional regulatory elements can further increase rAAV-mediated expression in these cells. In summary, we have developed a method for quantifying transduction of PRs and ON BCs by novel rAAVs *in vivo*. As the AAV vector toolkit expands, such a method will be highly useful for scoring relative performance of novel vectors. We also conclude that while the ubiquitous CBA promoter effectively drives transgene expression in PRs, it drives relatively poor transgene expression in ON BCs and caution that CBA should not be considered as a default universal promoter for retinal expression.

621. Widespread Gene Delivery to the Nonhuman Primate Brain for the Treatment of Huntington's Disease

Piotr Hadaczek[1], Lisa Stanek[2], Agnieszka Ciesielska[3], Philip Pivirotto1[1], Catherine O'Riordan[4], Bryan Mastis[2], Waldy San Sebastian[1], John Forsayeth[1], Seng H. Cheng[5], Krystof S. Bankiewicz[1], Lamya S. Shihabuddin[2]

[1]Interventional Neuro Center, Department of Neurological Surgery, University of California San Francisco, San Francisco, CA, [2]Neuroscience, Sanofi Genzyme, Framingham, MA, [3]Neuroscience, University of California San Francisco, San Francisco, CA, [4]Gene Therapy, Sanofi Genzyme, Framingham, MA, [5]Rare Diseases, Sanofi Genzyme, Framingham, MA

Huntington disease (HD) is an autosomal dominant neurodegenerative disease caused by a CAG-trinucleotide repeat expansion in a coding exon of a single allele in the *HTT* locus. In HD, the resulting polyglutamine (polyQ) expansion confers a toxic gain-of-function to the mutant huntingtin protein (mHTT). Reduction of expression of mHTT using gene silencing by RNA interference (RNAi) may confer transformative disease modifying therapeutic approach for HD. Adeno associated vectors (AAV) provide an ideal delivery system for nucleic acid therapeutics and have the potential to allow for long lasting and continuous expression of these huntingtin lowering RNAi in the brain. Despite this promise, global delivery of AAV to the adult brain remains an elusive goal. Furthermore, the appropriate brain areas to target for achieving transformative therapeutic benefit in HD patients remain to be defined. Postmortem analyses of HD patient brains reveal extensive medium spiny neuronal loss in the striatum, in addition to loss of pyramidal neurons in the cerebral cortex and hippocampus. Recent studies in rodent models suggest that simultaneous targeting of striatum and cortex is more efficacious than targeting either individually. Thus, available evidence suggests that delivery of Htt-lowering therapeutics to both striatal and cortical regions may provide optimal therapeutic efficacy.The current study demonstrate for the first time the successful use of an AAV targeting strategy that leads to viral transduction in key brain areas considered to be important for HD pathology. The study compared the efficiency of transduction of AAV1 and AAV2 vectors in the rhesus monkey brain following intra-striatal injection. Both vectors encoded green fluorescent protein (GFP) under control of a hybrid CMV enhancer/chicken beta-actin promoter. One month following injection, brains were analyzed for distribution of GFP-positive cells. We found that the AAV1 vector provided extensive delivery to the majority of the primate striatum, and additionally transduced large numbers of cells within the cerebral cortex, thalamus, and hippocampus. In summary, the data suggest that intrastriatal delivery maybe sufficient for the delivery of nucleic acid-based therapeutics to multiple areas of the human brain relevant in HD.

Musculo-Skeletal Diseases II

622. Systemic β-Sarcoglycan Gene Therapy for Treatment of Cardiac and Skeletal Muscle Deficits in LGMD2E

Eric Pozsgai[1], Danielle Griffin[2], Kristin Heller[2], Jerry Mendell[1], Louise Rodino-Klapac[1]

[1]The Ohio State University/Nationwide Children's Hospital, Columbus, OH, [2]Nationwide Children's Hospital, Columbus, OH

Limb-girdle muscular dystrophy type 2E (LGMD2E) results from mutations in the beta-sarcoglycan (SGCB) gene causing loss of a sarcolemmal structural protein component of the dystrophin-associated protein complex (DAPC). This leads to a progressive dystrophy with numerous histopathological features, resulting in deteriorating muscle function. This occurs not only in limb muscle but also in the diaphragm and the heart. Consequences include respiratory failure and cardiomyopathy in 50% or more of LGMD2E patients. SGCB knockout mice share many of the phenotypic deficiencies of LGMD2E patients. In this mouse model we quantified dystrophic histopathology, fibrosis, and functional outcomes in lower limb, upper limb, and torso skeletal muscles, as well as the diaphragm and cardiac muscles. Diaphragms of SGCB-/- mice demonstrated reduced specific force output (116.24 mN/mm^2) compared to wild-type (WT) mice (236.67 mN/mm^2), and hearts from SGCB-/- mice had lower ejection fraction (58.19%) compared to WT mice (75.04%) as determined by MRI. Additionally, radiographic imaging defined the degree of kyphoscoliosis in SGCB-/- mice. Laser monitoring of open-field cage activity showed a reduction of ~55% in both total ambulation as well as hindlimb vertical rearing in SGCB-/- mice compared to WT. For treatment, we designed a self-complementary AAVrh74 vector containing a codon optimized human SGCB transgene driven by a muscle specific promoter. We next demonstrated efficacy of vector delivery by intramuscular (IM) injection to the tibialis anterior muscle, as well as isolated-limb perfusion (ILP) to the lower limb muscles of SGCB-/- mice. Along with restoration of SGCB expression in treated muscle, we saw histological and functional improvements and a reduction in fibrosis as indicated by reduced collagen deposition. These regional gene delivery studies were followed by systemic delivery of scAAV.hSGCB through the tail vein of SGCB-/- mice to provide a potential rationale for gene delivery in clinical trial that would lead to clinically meaningful results. Tail vein injection

of scAAV.hSGCB resulted in nearly 100% transgene expression in numerous muscles throughout the hindlimbs, forelimbs, torso, and the heart, that was accompanied by improvements in histopathology including reduction in central nucleation and increased fiber diameter. Kyphoscoliosis of the spine was also improved, and total ambulation increased in scAAV.hSGCB treated mice by ~22% while hindlimb vertical rearing increased by ~77% in treated mice compared to KO. We also saw complete restoration of diaphragm function following treatment with specific force output improved to 226.07 mN/mm^2. In this well-defined mode of LGMD2E, we have demonstrated that systemic delivery of scAAV.hSGCB normalizes histologic and functional outcome measures in limb, diaphragm, and heart. These findings have established a path for AAV mediated gene therapy for LGMD2E that we are currently pursuing.

623. *Dystrophin* Exon 52-Deleted Pigs as a New Animal Model of Duchenne Muscular Dystrophy: Its Characterization and Potential as a Tool for Developing Exon Skipping Therapy

Yusuke Echigoya[1], Nhu Trieu[1], Kana Hosoki[1], William Duddy[2], Terence Partridge[3], Eric Hoffman[3], Joe Kornegay[4], Christopher Rogers[5], Toshifumi Yokota[1]

[1]Department of Medical Genetics, University of Alberta, Edmonton, AB, Canada, [2]Therapies for striated muscle disease, Institute of Myology, Paris, France, [3]Center for Genetic Medicine Research, Children's National Medical Center, Washington, DC, [4]Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, TX, [5]Exemplar Genetics, LLC, Sioux Center, IA

Duchenne muscular dystrophy (DMD) is a lethal X-linked recessive disorder primarily affecting boys, which is caused by mutations in the *dystrophin* (*DMD*) gene and the subsequent lack of dystrophin protein. Patients exhibit progressive degeneration and weakness in bodywide muscles, leading to death due to respiratory or cardiac failure. Currently, a most promising therapeutic approach is exon skipping with antisense oligonucleotides (AOs), which can correct the reading frame of mutant dystrophin mRNA and restore truncated-yet-functional dystrophin protein. Dystrophic mouse and dog models have been widely used for developing exon skipping therapies, as well as for improving scientific understanding of DMD pathogenesis; however, currently available animal models have a few limitations: First, they do not always share similar disease phenotypes with DMD patients (e.g., milder symptoms). Second, their available mutation patterns are limited, reducing applicability for research (importantly, AO drugs for exon skipping need to be developed according to mutation patterns). Lastly, mice and dogs are far from humans in terms of their anatomy, physiology, and genetics, which could prove a hurdle to interpreting and extrapolating treatment effects from animal to human. Although the wide variety of currently available animal models may partially compensate for some drawbacks, a more suitable animal model is most desirable to overcome them. Here, we generated a new DMD animal model of miniature pigs. Exon 52 deletion, one of the most common mutation patterns in the human *DMD* gene, was created in the pig *dystrophin* gene using a combination technique involving somatic cell nuclear transfer and gene targeting. The pig model systemically produced out-of-frame dystrophin mRNA transcripts lacking exon 52. Accordingly, no expression of dystrophin protein was observed in bodywide muscles, including the heart. Serum creatine kinase levels were dramatically increased, accompanied with histological deterioration in the muscles as observed in patients with DMD. We also tested AO-mediated exon skipping targeting exon 51 or 53 in primary skeletal muscle cells derived from the dystrophic pig model. The exon 52 deletion mutation is correctable by either exon 51 or 53 skipping which approach is theoretically applicable to the largest proportion of DMD patients (14% and 10%, respectively). AO sequences for skipping porcine exon 51 or 53 were designed as analogs of human AO sequences identified with our *in silico* AO design tool (Echigoya et al, *PLoS One* 2015 Mar 27;10(3):e0120058) and current antisense drugs under clinical trials. We successfully induced exon 51 or 53-excised transcripts *in vitro* with primary skeletal muscle cells derived from the exon 52-deleted transgenic pigs. We are currently planning *in vivo* exon skipping in the new pig model. Therapeutic outcomes derived from the DMD pig model could be more reliably extrapolated to human patients, facilitating development of novel AO drugs and translation into human clinical trials.

624. A Single Neonatal Delivery of an Exon 2 Directed AAV9.U7snRNA Vector Results in Long-Term Dystrophin Expression That Prevents Pathologic Features in the Dup2 Mouse

Nicolas Wein[1], Tabatha R. Simmons[1], Felecia Gumienny[1], Jacqueline Yurkoski[1], Nianyuan Huang[1], Francesco Muntoni[2], Kevin M. Flanigan[1]

[1]Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH, [2]Developmental Neuroscience, UCL Institute of Child Health, London, United Kingdom

We recently identified an internal ribosome entry site (IRES) within exon 5 of the DMD gene. Mutations that truncate the reading frame 5' of this exon can result in use of the IRES for alternate translational initiation beginning within exon 6 that results in expression of an N-truncated isoform. Despite lacking the calponin homology domain 1 (CH1) of the actin binding domain 1 (ABD1), this isoform is highly functional, as demonstrated by the minimal symptoms in patients who express it. Consistent with genotype-phenotype correlations in DMD patients, the IRES is not active in the presence of exon 2 duplication but is active when exon 2 is deleted. We developed an AAV9.U7snRNA vector to that truncates DMD reading frame by skipping of exon 2, and have shown that in a Duchenne muscular dystrophy (DMD) mouse model carrying a duplication of exon 2 (the Dup2 mouse), postnatal intramuscular (IM) or intravascular (IV) treatment results in functional and pathologic improvement in skeletal muscle. Relevant to efforts to identify and treat DMD patients at an earlier age, we sought here to determine whether earlier gene transfer might slow down or even prevent the development of pathology. Dup2 mice were injected via facial vein at postnatal day 1 (P1) with 1E12 total vector genomes of the AAV9.U7snRNA vector and sacrificed at either 1, 3, 6, or 12 months post-injection for evaluation of exon 2 skipping by RT-PCR, quantification of dystrophin expression, and characterization of histopathology. To model the applicability of this approach beyond exon 2 duplication patients, the same vector was used to treat 6 human patient fibroblast-derived transdifferentiated myoblasts (FibroMyoD cells) harboring various mutations within exons 1 to 4. In the Dup2 mouse, efficient skipping and abundant dystrophin expression were present up to one year following the single AAV injection. Dystrophic pathology was absent at all-time points; at one year, less than 1 % of fibers showed central nucleation, in comparison to ~70% in untreated Dup2 mice. Two tests on the ex vivo diaphragm preparations: isometric force (providing assessment of strength), and eccentric contractions (evaluating sarcolemma stability) were performed at 3 and 6 months following P1 injection. Both tests demonstrated little to no difference between treated animals and wild type mice. In all FibroMyoD cultures, abundant exon 2 skipping and dystrophin expression were detected in myotubes at 14 days of culture after treatment. These results suggest that this exon-skipping vector offers a therapeutic approach not only to patients with exon 2 duplications but with all mutations within the first four DMD exons (~6% of patients), an area of the gene largely ignored

by the current therapeutic approaches. This work strongly supports the idea that early treatment of these patients will have longstanding and significant benefit resulting in a better outcome.

625. RNAi Therapy for Dominant Limb Girdle Muscular Dystrophy Type 1A

Lindsay M. Wallace[1], Carlee R. Giesige[2], Danielle A. Griffin[1], Louise R. Rodino-Klapaca[1], Scott Q. Harper[1]
[1]Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH, [2]Biomedical Sciences Graduate Program, The Ohio State University, Columbus, OH

Limb Girdle Muscular Dystrophy (LGMD) refers to a group of disorders characterized by progressive wasting and weakness of pelvic and shoulder girdle muscles. Patients commonly require wheelchair assistance, and individuals with some forms of LGMD may have cardiac and respiratory muscle involvement. Modest improvements in a limited set of muscles may dramatically improve patients' quality of life, but currently no effective treatment exists. There are 8 dominant forms of LGMD (LGMD1) that would benefit from gene therapy strategies to reduce their pathogenic alleles. We previously showed the first RNAi-based pre-clinical treatment for LGMD1A, which is caused by dominant mutations in one allele of the myotilin (MYOT) gene. Our strategy involved delivering MYOT targeted artificial microRNAs (miMYOT) to muscles of the T57I transgenic mouse model of LGMD1A using AAV6 vectors. Both 3 months (short-term) and 9 months (long-term) time points showed significant reduction of mutant myotilin mRNA and soluble protein expression. MYOT gene silencing resulted in histological improvements that were accompanied by significant functional correction including increased muscle weight and improved specific force. While the initial study showed proof-of-principle for this therapy, translation of miMYOT requires additional work. Mouse knockout studies suggested MYOT is a non-essential gene, as animals lacking MYOT develop normally. In the case of LGMD1A this would suggest the mutation is harmful but complete loss would be tolerated. However, we do not currently know if haploinsufficiency of wild-type MYOT is tolerated in other organisms, including humans. To address this question, we delivered morpholinos or AAV.miRNAs to multiple species. We found knockdown of endogenous MYOT to be safe in developing Xenopus, adult mice and non-human primates, suggesting that a non-allele-specific silencing approach for MYOT would be tolerated in LGMD1A-targeted treatments. We also performed dose escalations to assess the safety parameters of AAV.miMYOT vectors and utilized microarray data to identify potential biomarkers for clinical outcome measures. We then validated that some of our lead biomarkers were significantly normalized in miMYOT-treated T57I mice. Together these data provide additional data to support the further translation of miMYOT therapy for LGMD1A.

626. Directing Skeletal Myogenic Progenitor Cell Lineage Specification with CRISPR/Cas9 Transcriptional Activators

Jennifer B. Kwon[1], Charles A. Gersbach[2]
[1]Duke University, Durham, NC, [2]Biomedical Engineering, Duke University, Durham, NC

Induced pluripotent stem cells (iPSCs) are a promising source for autologous cell-based therapies, disease modeling, and drug discovery in pathologies of muscular disease and wasting. The field of regenerative medicine has made significant strides in the successful reprogramming of iPSCs into various specialized cell types, including skeletal muscle cells. Genetic reprogramming of iPSCs into skeletal muscle progenitors and myocytes has been previously demonstrated by cDNA expression of exogenous myogenic transcription factors, including Pax7 and MyoD, to achieve varying levels of conversion toward the myogenic lineage.

Recent advances in genome engineering technologies have established the CRISPR/Cas9 system as a programmable transcriptional regulator capable of targeted activation or repression of endogenous genes. The nuclease-deactivated dCas9 protein can be fused to a variety of transcriptional activation domains, such as the histone acetyltransferase p300 and the transactivation domain VP64, to potently activate genes in their natural chromosomal context. In contrast to ectopic expression of transgenes, activation of endogenous genes can facilitate chromatin remodeling and can also capture the full complexity of transcript isoforms, mRNA localization and stability, and other effects of non-coding regulatory elements.

Here, we use ^{VP64}dCas9^{VP64} and dCas9-p300 constructs for targeted activation of the endogenous myogenic transcription factor Pax7 in human iPSCs to direct differentiation into skeletal muscle progenitors. We hypothesize that Pax7 activation will be sufficient to induce the myogenic program, as it plays a key role in myogenesis through regulation of muscle regeneration and the function of muscle progenitor cells. Lentiviral transduction of the dCas9 transcriptional activators under a constitutive promoter along with gRNAs targeted to the Pax7 promoter resulted in increased Pax7 transcript levels as assessed by qRT-PCR at 4 days after transduction. Additionally, widespread expression of Pax7 protein was detected by immunofluorescence staining by 13 days post transduction, indicating a high efficiency of endogenous gene activation by dCas9 effector constructs. Current efforts aim to achieve transient induction of high levels of Pax7 to examine progressive differentiation into mature myotubes expressing downstream myogenic markers. Future experiments will focus on assessing the *in vivo* skeletal muscle regenerative potential of Pax7+ human iPSC-derived myogenic progenitor cells by examining engraftment and self-renewal in injured muscle tissues of immunodeficient mice. These studies aim to introduce a novel method for the ex vivo derivation and expansion of myogenic precursor cells from patient-derived iPSCs, which will create new opportunities for disease modeling and cell therapies in disorders of skeletal muscle regeneration including muscular dystrophies, sarcopenia, and cachexia.

627. Concerted Evolution of Pseudogenes Facilitated Contiguous Deletion of the Canine Dystrophin and TMEM47 Genes by Homologous Recombination

Daniel H. VanBelzen[1], Alock Malik[1], Joe Kornegay[2], Hansell H. Stedman[1]
[1]Surgery, University of Pennsylvania, Philadelphia, PA, [2]College of Veterinary Medicine, College Station, TX

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive disorder caused by sporadic mutations in the 2.4 Mb dystrophin gene. The large size of the gene is responsible for one of the highest loss-of-function mutation rates in the entire human genome. The majority of dystrophin mutations that cause DMD are deletions that span multiple exons. As the resulting absence of central immunological tolerance has the documented potential to complicate gene therapies in which recombinant dystrophin is expressed, there is an urgent need for well characterized, deletional null animal models for DMD. Preliminary characterization of a naturally occurring mutation in a German shorthaired Pointer (GSHPMD) suggested that the canine dystrophin gene was disrupted by a single, large deletion. Here, we further characterize the GSHPMD deletion and determine the breakpoints to be within short, highly homologous DNA elements spaced over 5.6 Mb apart, suggesting homologous recombination as the most plausible mechanism. The deletion generously flanks the

entire dystrophin gene, and additionally encompasses the TMEM47 gene. This is reminiscent of the famous B.B. deletion in the DMD patient whose DNA was used for subtractive hybridization in the cloning of the DMD and nearby CGD loci. Further investigation revealed that the homologous canine DNA elements are ferritin-like pseudogenes, and surprisingly that there are several additional copies present on the X-chromosome. Phylogenomic comparison of the physical map positions of the orthologous pseudogenes in proximity to the dystrophin gene shows a pattern of positional synteny, consistent with the expansion of this pseudogene family prior to the mammalian radiation. Unexpectedly, only in the canine lineage have the pseudogene sequences remained virtually identical to one another, suggesting the existence of a heretofore uncharacterized process of concerted molecular evolution spanning large physical distances. The fully characterized deletional null animal model represents an invaluable resource for the evaluation of the immunological consequences of recombinant dystrophin expression via gene therapy, as it provides the only mammalian system in which the complete absence of dystrophin prevents the development of central tolerance, as would be expected in most cases of DMD. Thus the GSHPMD model can be uniquely leveraged to address a major safety concern as the field pursues strategies for systemic gene delivery in DMD. Of note, published studies have revealed evidence for deleterious immunological responses following administration of vectors expressing recombinant HUMAN dystrophin in both DMD and the Golden Retriever canine model (NEJM 363: 1429-1437 and Mol. Ther. 18:1501-1508).

628. Transposons Expressing Full-Length Human Dystrophin Enable Genetic Correction of Dystrophic Mesoangioblasts and iPS-Derived Mesoangioblast-Like Cells

Mariana Loperfido[1], Susan Jarmin[2], Sumitava Dastidar[1], Mario Di Matteo[1], Ilaria Perini[3], Marc Moore[2], Nisha Nair[1], Ermira Samara-Kuko[1], Takis Athanasopoulos[2], Francesco Saverio Tedesco[4], George Dickson[5], Maurilio Sampaolesi[3], Thierry VandenDriessche[1], Marinee K. Chuah[1]
[1]Department of Gene Therapy & Regenerative Medicine, Free University of Brussels, Brussels, Belgium, [2]Royal Holloway, University of London, London, United Kingdom, [3]Translational Cardiomyology Laboratory, University of Leuven, Leuven, Belgium, [4]University College London, London, United Kingdom, [5]Royal Holloway, University of London, Brussels, United Kingdom

Duchenne muscular dystrophy (DMD) is a genetic neuromuscular disorder caused by the absence of dystrophin. We developed a novel gene therapy approach based on the use of the piggyBac (PB) transposon system to deliver the coding DNA sequence (CDS) of either full-length human dystrophin (DYS: 11.1 kb) or truncated microdystrophins (MD1: 3.6 kb; MD2: 4 kb). PB transposons encoding microdystrophins were transfected in C2C12 myoblasts, yielding 65±2% MD1 and 66±2% MD2 expression in differentiated multinucleated myotubes. A hyperactive PB (hyPB) transposase was then deployed to enable transposition of the large-size PB transposon (17 kb) encoding the full-length DYS and green fluorescence protein (GFP). Stable GFP expression attaining 78±3% could be achieved in the C2C12 myoblasts that had undergone transposition. Western blot analysis demonstrated expression of the full-length human DYS protein in myotubes. Subsequently, dystrophic mesoangioblasts from a Golden Retriever muscular dystrophy dog were transfected with the large-size PB transposon resulting in 50±5% GFP-expressing cells after stable transposition. This was consistent with correction of the differentiated dystrophic mesoangioblasts following expression of full-length human DYS. Alternatively, dystrophic mesoangioblast-like cells were generated from iPS of DMD patients. These iPS-derived mesoangioblasts, constitute an essentially unlimited supply of stem/progenitor cells that could be genetically corrected using PB transposons expressing dystrophin. These results pave the way toward a novel non-viral gene therapy approach for DMD using PB transposons underscoring their potential to deliver large therapeutic genes.

629. Intravenous Delivery of a MTMR2-Encoding AAV9 Vector Extends Lifespan and Improves Muscle Function in Mice with X-Linked Myotubular Myopathy

Nathalie Danièle, Laura Julien, Christelle Moal, Thibaut Jamet, Alban Vignaud, Ana Buj Bello
Genethon, Evry, France

X-linked myotubular myopathy is a rare genetic disease affecting the skeletal musculature. Male patients present profound neonatal muscle hypotonia and weakness, respiratory insufficiency, and in most cases have a very severe reduction in lifespan. At the pathological level, muscle fibers are hypotrophic and contain central nuclei with disorganized mitochondria and triads. The disease is caused by mutations in the *MTM1* gene, which encodes myotubularin, the founder member of a family of 15 homologous proteins in mammals (including MTMR1 to 14). We recently demonstrated the therapeutic efficacy of intravenous delivery of rAAV vectors expressing MTM1 in murine and canine models of myotubular myopathy. In the present study, we tested whether *Mtmr1* and *Mtmr2* overexpression, *Mtm1* closest homologs, could also rescue the XLMTM phenotype. Recombinant serotype-9 AAV vectors encoding either MTM1, MTMR1 or MTMR2 under the control of the desmin promoter were compared by injection into the tibialis anterior muscle of two-week-old *Mtm1* deficient mice. Two weeks after vector delivery, a therapeutic effect was observed with *Mtm1* and *Mtmr2*, but not *Mtmr1*, with *Mtm1* being the most efficacious transgene. We further explored a systemic route of administration, intravenous injection of a single dose of rAAV9-*Mtmr2* in XLMTM mice ameliorated muscle histology and strength, and extended lifespan throughout the 3-month period of the study. Even though *Mtmr2*-treated mutant mice remained smaller than their wild-type counterparts, with partial increase in body weight and myofiber size, most importantly, the contractile force of myotubularin-deficient muscles improved strongly. Altogether, these results establish the proof-of-concept that overexpression of MTMR2 in skeletal muscle represents a novel therapeutic approach for myotubular myopathy.

630. AAV Transduction of a Truncated Dysferlin Improves Dysferlinopathy

Telmo Llanga[1], Bryan Sutton[2], Nadia Nagy[1], Matthew Hirsch[1]
[1]University of North Carolina, Chapel Hill, NC, [2]Texas Tech University, Lubbock, TX

Dysferlinopathy is a progressive muscular dystrophy caused by the absence of functional Dysferlin. Dysferlin cDNA is over the packaging capacity of adeno-associated viral vectors (AAV), complicating potentially therapeutic gene addition strategies. The use of dual or fragmented AAV vectors, while genetically intriguing, is undesired as these formats demonstrate decreased transduction efficiencies. Therefore, an alternative approach using a panel of smaller dysferlin-like molecules was executed in vitro and in vivo in an AAV vector context. Three of the four "hybrid" dysferlin reading frames produced protein which behaved similar to full length dysferlin in localization studies. Upon AAV vector production, only 1 variant (341) demonstrated intact genome packaging despite final cassettes sizes of approximately 5kb. Intramuscular administration of vectors encoding 341 in dysferlin deficient mice resulted in increased muscle

integrity without indications of toxicity. Dysferlin deficient mice receiving AAV9-341 through intravenous injection demonstrated increased rearing activity that was sustained 6 months post-injection. Consistently a trend of decreased muscle damage was observed in these mice. Our data suggest that 341, and additional hybrid dysferlin candidates under evaluation, may find relevance for the treatment of dysferlinopathy.

631. Rankl Knock-Out Mesenchymal Stromal Cells Have an Unexpected Osteogenic Differentiation Defect Which Is Improved by a RANKL-Expressing Lentiviral Vector

Ciro Menale[1,2], Francesca Schena[3], Lorenzo Diomede[1,2], Lucia Sergi Sergi[4], Lucia Susani[1,2], Eleonora Palagano[1,2,5], Elisabetta Traggiai[6], Cristina Sobacchi[1,2], Anna Villa[1,2]
[1]CNR-IRGB, Milan Unit, Milano, Italy, [2]Humanitas Clinical and Research Institute, Rozzano, Italy, [3]Istituto Gaslini, Genoa, Italy, Italy, [4]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milano, Italy, [5]BIOMETRA Dept, University of Milan, Milano, Italy, [6]Novartis Institute for Research in Biomedicine, Basel, Switzerland

Osteoclast-poor RANKL-dependent Autosomal Recessive Osteopetrosis (ARO) is a rare bone disease characterized by an increase in bone density due to the failure of bone resorption by impaired osteoclast formation. Hematopoietic stem cell transplantation is not an effective therapy for this ARO form, since in bone RANKL is produced mainly by cells of mesenchymal origin. Therefore Mesenchymal Stromal Cells (MSC) transplantation together with a gene-therapy strategy to correct RANKL defect in MSC could represent a possible effective therapy. Of note, whether also MSC, besides the osteoclasts, are affected by RANKL deficiency is unknown. To verify this, we established and characterized bone marrow derived MSC (BM-MSC) lines from the Rankl$^{-/-}$ (KO) mouse model, which recapitulates the human disease, and from wild type (WT) mice. No differences were found between KO and WT MSC in terms of morphology, immunophenotype and proliferation capacity. However, KO MSC displayed a reduced clonogenic potential with a decrease in stemness genes expression. KO MSC were able to normally differentiate towards the adipogenic and chondrogenic lineages, while showed a significantly impaired osteogenic differentiation capacity compared to WT MSC, as demonstrated by reduced Alizarin Red staining (ARS) and expression of osteogenic genes. To confirm that this alteration was due to the lack of functional RANKL, we developed a third generation lentiviral vector expressing human soluble RANKL (hsRL) for the genetic correction of KO MSC. We first investigated lentiviral transduction in 293T cells to optimize transduction efficiency at different multiplicity of infection (MOI) ranging from 1 to 100. hsRL production increased proportionally to the MOI and was stable over time. However, the higher the MOI the higher the cytotoxicity observed. Based on these data, we performed a lentiviral hsRL transduction in KO MSC at 20 and 50 MOI, to define the optimal transduction conditions. After transduction 99.5% of MSC were GFP^{+}. While in Rankl$^{-/-}$ control cells the cytokine was not detected, in corrected cells hsRL production and secretion was measurable and comparable to sRL levels in WT mouse. KO MSC stably expressing hsRL showed an improved osteogenic differentiation capacity compared to untransduced KO MSC, as demonstrated by increased ARS and expression of osteogenic genes. Moreover, the expression of RANK receptor in both MSC suggested an autocrine role of sRL as possible mechanism. Our data suggest that restoration of RANKL production in lentiviral-transduced KO MSC might not only allow osteoclast differentiation in Rankl$^{-/-}$ mice upon transplantation, but also improve the osteogenic differentiation defect of KO MSC.

632. Alpha-1 Antitrypsin Gene Therapy Prevented Bone Loss in an Ovariectomy Induced Osteoporosis Mouse Model

Mohammad A. Akbar[1], Jay J. Cao[2], Yuanqing Lu[3], David Nardo[1], Mong-Jen Chen[1], Ahmed S. Elshikha[1], Rubina Ahamed[1], Mark Brantly[3], Lung-ji Chang[4], Lexie Shannon Holliday[5], Sihong Song[1]
[1]Pharmaceutics, University of Florida, Gainesville, FL, [2]USDA, ARS, Grand Forks Human Nutrition Research Center, Grand Forks, ND, [3]Medicine, University of Florida, Gainesville, FL, [4]Molecular Genetics and Microbiology, University of Florida, Gainesville, FL, [5]Orthodontics, University of Florida, Gainesville, FL

Osteoporosis is a major healthcare burden affecting mostly postmenopausal women characterized by compromised bone strength and increased risk of fragility fracture. Although pathogenesis of this disease is complex, inflammation is clearly involved in bone loss at menopause. Therefore, anti-inflammatory strategies hold great potential for the prevention of postmenopausal osteoporosis. Human alpha-1 antitrypsin (hAAT) is a multifunctional protein that has anti-inflammatory and cytoprotective properties. In this study, we investigated the protective effect of hAAT against bone loss. In vitro studies showed that hAAT significantly inhibited osteoclast formation and function in a dose-dependent manner. Treatment of hAAT inhibited M-CSF (macrophage colony-stimulating factor) induced cell surface RANK receptor expression by downregulating cFos mRNA expression. To test the protective effect of hAAT in an osteoporosis mouse model, we treated ovariectomized (OVX) mice with rAAV8-CB-hAAT, or mesenchymal stem cells (MSCs) infected with a lentiviral vector expressing hAAT (MSC-Lenti-hAAT) or phosphate buffer saline (PBS). Sham operated age-matched animals were used as controls. Eight weeks after the treatment, animals were sacrificed and subjected to μCT scanning for the evaluation of vertebral bone microarchitecture. Gene and stem cell-based hAAT therapies significantly increased bone volume density, trabecular number and decreased structure model index compared to PBS injection in OVX mice. Gene therapy also increased connectivity, density and trabecular thickness compared to PBS injection in OVX mice. We also observed that both therapies inhibited RANK gene expression in bone, which is consistent with the results of our *in vitro* study. These results demonstrate that hAAT gene and MSCs based therapies mitigate ovariectomy-induced bone loss in a mouse model, possibly through inhibition of osteoclast formation by reducing RANK gene expression. Considering the safety profile of the hAAT and rAAV vector in human, our results provide a new insight for the treatment of osteoporosis.

633. A Reproducible and Reliable Non-Invasive Approach to Assess the Efficacy of Gene Therapy in mdx Mice with Relevance to Clinical Trials

Yafeng Song, Mihail Petrov, Shira Rosenblum, Leon Morales, Hansell H. Stedman
Surgery, University of Pennsylvania, Philadelphia, PA

The mdx mouse carries a naturally occurring nonsense mutation in the dystrophin gene and has become a critical animal model for research on Duchenne Muscular Dystrophy (DMD). Despite the extensive published studies on muscle structure and function in this model, we still lack appropriate protocols for evaluation of functional rescue in mdx mice using non-invasive tests relevant to the clinically relevant symptoms of DMD. Here we present for the first time results of a complex approach for the evaluation of locomotor and behavioral patterns of mdx mice during running wheel performance in a modified open field monitoring system. These results are examined in the same mice with force grip measurements and serum enzyme biomarker

creatine kinase (CK) measurement. The comparison between juvenile congenic wild type (C57Bl10) and mdx mice indicates similarity in parameters such as rest time and the number of times entering and initiating use of the running wheel per day, but highly significant differences with regard to running wheel-associated locomotor activity characteristics including velocity, time, distance per run, and total distance per day. This suggests that exploratory behavior at low work rate is unaffected by dystrophin deficiency, but that the animals respond to as yet uncharacterized sensory inputs that limit their running speed and duration without preventing the desire to re-initiate after rest. This may be viewed as analogous to the early loss of function and metabolic reserve in the juvenile period of DMD disease progression, where boys are described by parents as frustrated at first being unable to keep pace with their friends. As presented in another poster by our group, blinded studies of the response to gene therapy indicates that it is unlikely that this phenotypic difference reflects the mislocalization of neuronal nitric oxide synthase (nNOS) in dystrophin deficiency. In further blinded studies, the open field data correlate with the differences in testing results between the two mouse strains observed in the force grip evaluation and serum levels of CK, offering possible leads for a further mechanistic dissection. These results demonstrate strong evidence in support of the running wheel open field system as a reliable and reproducible approach to assess the therapeutic efficacy in the mouse model of DMD. This approach also provides a platform for dissecting the physiological roles of dystrophin in supporting precisely measurable volitional activities in unrestricted animals, thereby offering potential improvements in the predictive power of preclinical studies of therapeutic efficacy to inform clinical trials.

634. Abstract Withdrawn

635. Effects of Mechanical Strain on Zmpste24-Deficient Prematurely Aged Muscle-Derived Stem Cells

Andrea Liebowitz[1], Elizabeth Morris[1], Alex Scibetta[1], Xiaodong Mu[2], Johnny Huard[1,2]
[1]CRSM, Steadman Philippon Research Institute, Vail, CO, [2]University of Texas Medical Center, Houston, TX

Mice deficient in Zmpste24, a metalloproteinase involved with formation of lamin A, are a model for aging and for Hutchinson-Gilford Progeria Syndrome, a human disorder caused by a similar defect. Z24-/- muscle-derived stem cells (MDSCs) are defective in proliferation and differentiation potential. Stretching stem cells prior to implantation has been shown to improve outcomes. Based on these results, stretching may be able to rejuvenate aged stem cells. Therefore, examining the response of stretched and non-stretched Z24-/- MDSCs to stress can show if stretching rescues some of the negative processes involved with aging.

Z24-/- MDSCs were seeded on BioFlex plates (Flexcell Inc) at a density of 50,000 cells per well. Cells were stretched for 24 hours with 10% uniaxial elongation and a 0.5Hz frequency. Z24-/- mice had increased cell senescence and muscle stem cell depletion in the skeletal muscle. Muscle stem cells isolated from Z24-/- mice have increased activation of ROS (reactive oxygen species). Cells mechanically strained for 24 hours appeared to be more resistant to oxidative stress and express higher levels of RhoA, which is a mediator of cell stress: more senescent cells were observed in the non-flexed vs. flexed groups under standard conditions and under oxidative stress. The flexed cells also had a better survival rate following oxidative stress for 24h. These results indicate that flexing compensates for some of the progeria phenotype. Flexing appears to prime the cells so they are more resistant to stressors like H_2O_2. The lower number of senescent flexed vs. non-flexed cells seems to indicate that the flexing protects the cells from entering senescence. Mechanical strain also appears to give the cells greater resistance to reactive oxidative species. PCR results of TNF-α showed that non-flexed cells expressed the pro-inflammatory cytokine 1.33-fold compared to flexed cells. This indicates that mechanical stimulation may improve the cells' response to stressors and reduce inflammation. These preliminary results indicate that mechanical strain may rejuvenate and rescue the cells from defects in cell proliferation and differentiation. Based on these results, flexing cells *in vitro* may improve the quality and outcome of stem cells implanted into patients, especially in older donors whose cells may behave similar to the Z24-/- cells.

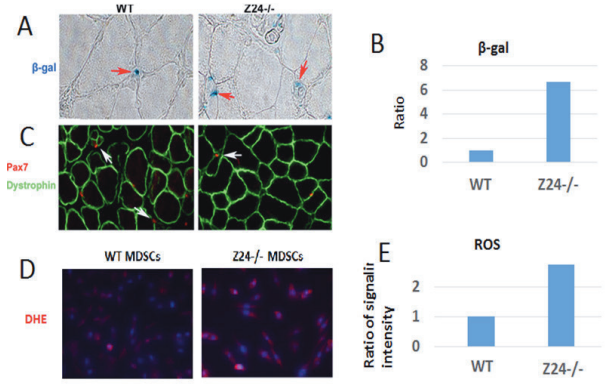


Figure 1: Senescence-associated beta-galactosidase staining showed increased number of senescent cells (**A, B**) but decreased number of Pax7+ muscle stem cells (**C**) in the Z24-/- muscle, compared to WT muscle. Dihydroethidium (Hydroethidine) staining of ROS in the cells indicated that Z24-/- MDSCs have higher level of ROS activation compared to WT MDSCs (**D**).

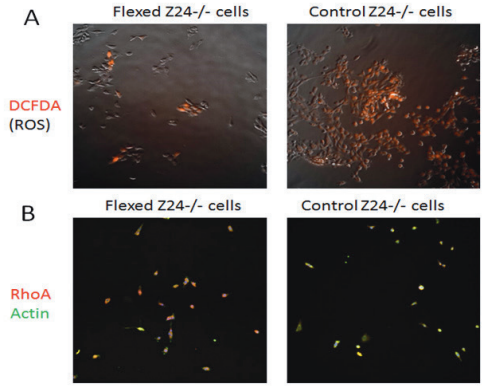


Figure 2: DCFDA staining for reactive oxidative species (ROS) indicates that flexed cells have a greater resistance oxidative stress or scavenging capacity of ROS induced by H_2O_2 **(A).** Immunostaining of RhoA showed that Flexed Z24-/- MDSCs have greater levels of RhoA expression than non-flexed control cells **(B).**

636. Enhanced Skeletal Muscle Regeneration in Mice After Multiple Pregnancies

Alex C. Scibetta[1], Aiping Lu[2], Johnny Huard[1,2]
[1]Steadman Philippon Research Institute, Vail, CO, [2]Department of Orthopaedic Surgery, McGovern School of Medicine, University of Texas Health Science Center at Houston, Houston, TX

Introduction: It has been recently shown that the reduction in the regenerative potential of various tissues during aging is potentially attributed to the depletion of stem cells[1]. Interestingly, aged progenitor cells can be rejuvenated when exposed to a young systemic environment. Pregnant women transiently acquire a population of

cells from their fetuses[2], and recent studies have shown that fetal cells persist in maternal blood and organs decades after delivery[3,4]. Pregnancy is believed to represent a natural form of parabiosis, which joins two organisms so that they share a circulatory system and a systemic environment[5]. Fetal cells have the potential to differentiate and repair injured tissues and organs in the mother, including skeletal muscle[6,7]. Previous studies have demonstrated that pregnant mice have improved musculoskeletal tissue healing after injury compared to non-pregnant controls[2]. In this study, we wanted to determine if the effect of pregnancy on the regenerative potential of skeletal muscle is only transient during the pregnancy period or can persist after pregnancy. **Methods**: This study used 5 retired breeders and 5 virgin heterozygous Zmpste24 mice (normal phenotype) that we received from Dr. Carlos López Otín. Mice were 8 months of age at the time of cardiotoxin (CTX) injury, and all breeders had been pregnant at least 5 times. To create muscle injury, 4uM of CTX was injected intramuscularly into the gastrocnemius muscle of mice. The mice were sacrificed 7 days after injury and muscles were harvested, flash frozen, and sectioned. H&E staining was performed according to the manufacturer's instructions. Cryosections from the gastrocnemius were stained for mouse IgG using a Mouse on Mouse (M.O.M) kit to analyze necrosis. Muscle regeneration was evaluated using an eMyHC antibody, and the extent of muscle tissue inflammation was analyzed using an F4/80 antibody (macrophage marker). **Results**: (1) H&E showed histology improvement in the muscles of the breeder mice 7 days post-injury compared to virgin mice. Damaged areas and mononuclear infiltration are two indicators of muscle damaged. We observed that virgin mice had significantly larger damaged areas ($p=0.016$) and more mononuclear infiltration ($p=0.016$) compared to breeder mice, which suggested a slower muscle regeneration in the virgin mice. There were no differences in centrally nucleated myofibers, an indicator of early regenerating muscle, between the two groups ($p=0.069$). (2) Based on immunostaining there was no difference in macrophage infiltration between the two groups. We also did not find significant differences for eMyHC and mouse IgG between breeder and virgin mice. **Conclusion**: During pregnancy, the fetus may supply the mother with rejuvenating factors and cells that alter the maternal microenvironment. This study demonstrated that mice that have experienced multiple pregnancies repair their muscle faster than virgin mice do, and the rejuvenating effects of the fetus on maternal muscle repair may continue after childbirth. Future studies comparing breeder and virgin mice will increase the number of animals used. These studies may provide insight into the long-lasting effects of pregnancy and have applications for gene and cell therapy. The ultimate goal is to identify beneficial factors from the fetus that can be purified and eventually used to delay aging related disease and conditions. **References**: 1. Conboy et al., 2003. 2. Lapaire et al., 2007. 3. Bianchi et al., 1996. 4. Khosrotehrani et al., 2004. 5. Slavin, 2002. 6. Nguyen et al., 2007. 7. Zeng et al., 2010.

Cancer-Immunotherapy, Cancer Vaccines III

637. Targeting of Myeloid Leukemia by IL-10-Engineered Human CD4$^+$ Tr1 Cells

Grazia Locafaro[1], Grazia Andolfi[1], Fabio Russo[1], Barbara Camisa[2], Fabio Ciceri[3], Angelo Lombardo[1], Attilio Bondanza[2], Maria Grazia Roncarolo[4], Silvia Gregori[1]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milan, Italy, [2]Innovative Immunotherapies Unit, Division of Immunology, Transplantation and Infectious Diseases, IRCCS San Raffaele Scientific Institute, Milan, Italy, [3]Hematology and Bone Marrow Transplantation Unit, Division of Regenerative Medicine, Stem Cells and Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy, [4]Division of Stem Cell Transplantation and Regenerative Medicine, Department of Pediatrics, Stanford School of Medicine, Palo Alto, CA

T regulatory type 1 (Tr1) cells, characterized by the co-expression of CD49b and LAG-3 and the ability to secrete high amounts of IL-10, control immune responses by IL-10 and TGF-beta production and by killing of myeloid cells *via* a Granzyme B-dependent mechanism. Tr1 cells are induced *in vitro* in the presence of recombinant human IL-10 or tolerogenic dendritic cells secreting high amounts of IL-10 (DC-10). Proof-of-principle clinical trials suggest that Tr1 cells can modulate Graft-versus Host Disease (GvHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, their ability to mediate anti-leukemic activity or their effects of Graft versus Leukemia is largely unknown. We previously showed that enforced IL-10 expression converts human CD4$^+$ T cells into Tr1-like (CD4$^{IL\text{-}10}$) cells that suppress effector T cells *in vitro* and prevent xenogeneic-GvHD in humanized models. We now demonstrate that these CD4$^{IL\text{-}10}$ cells selectively kill myeloid cell lines and myeloid blasts *in vitro* in HLA-class I-dependent but antigen-independent manner. Moreover, cytotoxic activity of CD4$^{IL\text{-}10}$ cells is Granzyme B-dependent, is specific for CD13$^+$ cells, and requires CD54 and CD112 expression on target cell lines or primary leukemic blast. Adoptive transfer of CD4$^{IL\text{-}10}$ cells in humanized models mediates direct anti-leukemic activity, and does not compromise the anti-leukemic effect of allogeneic T cells while inhibits xeno-GvHD. These findings provide a strong rationale for designing personalized immunotherapy approaches using CD4$^{IL\text{-}10}$ cells after allo-HSCT to cure myeloid malignancies.

638. TGFBeta Signaling Blockade within PSMA Targeted CAR Human T Cells for the Eradication of Metastatic Prostate Cancer

Christopher Kloss, Jihyun Lee, Carl June

University of Pennsylvania, Philadelphia, PA

The recent efficacies demonstrated using Chimeric Antigen Receptor (CAR) mediated immunotherapy to treat hematological malignancies have been met with great enthusiasm as holding great potential for the eradication of hematological malignancies. Unfortunately, the ability to target metastatic solid tumors like prostate cancer with CAR T cells has been less successful. The major parameter to achieve in using CAR T cells to treat prostate cancer is overcoming the immunosuppression that is created by the tumors to inhibit CAR T cells. Our efforts have aimed to create PSMA specific CAR T cells that are resistant to the TGFBeta induced suppression extensively demonstrated to exist in prostate cancer. Upon binding PSMA, our second-generation CAR supplies 4-1BB and CD3zeta signaling previously demonstrated to allow for long-term T cell persistence and eradication of leukemia when targeted to CD19. We therefore created anti-PSMA CAR T Cells that coexpress the dominant negative TGFBeta receptor II (dnTGFRBetaII).

Having focused on safety and efficacy of these anti-PSMABBz CAR T cells that express dnTGFRBetaII (dnTGFRBetaII-T2A-PBBZ), we demonstrate evidence of dnTGFRBetaII functionality. The dnTGFRBetaII functions to prevent SMAD signaling induced by TGFBeta, therefore resisting upregulation of CD25 and CTLA-4 by T cells. When co-cultured with tumor cells in vitro, efficient antigen specific lysis is induced by the PBBZ CAR and the dnTGFRBetaII-T2A-PBBZ CAR T cells exhibit up to 15 fold overall proliferation than PBBZ alone CAR T cells over 42 days. This allows for superior levels of T cell persistence in the peripheral blood of NSG mice after 3-4 weeks post infusion when compared to T cells expressing the anti-PSMA CAR alone. Most importantly, these CAR T cells are very effective at eradicating systemic PSMA+ PC3 prostate cancer cells in vivo demonstrated in two animal experiments at three different doses of CAR T cells. These studies suggest proper resistance to TGFBeta by CAR modified T cells that show great promise to eradicate metastatic prostate cancer in the clinic.

Disclosure of Conflict of Interest:

C.C.K. reports having ownership interest in patents owned by Memorial Sloan-Kettering Cancer Center and licensed to Juno Therapeutics and is a consultant for 121 Bio, LLC. J.L. reports no conflicts. C.H.J. reports receiving commercial research grants from Novartis and has ownership interest in patents owned by University of Pennsylvania and licensed to Novartis.

Funding Acknowledgments:

This study was supported by the Prostate Cancer Foundation All-Star Killer T-Cell Special Challenge Award 2014 - 2016.

639. Oncolytic Adenovirus Expressing IFN Alpha Works Synergistically with Chemoradiation

Amanda O. Salzwedel, Christopher LaRocca, Joohee Han, Julia Davydova, Masato Yamamoto

Department of Surgery, University of Minnesota, Minneapolis, MN

Aside from curative resection, there is no curative treatment against pancreatic adenocarcinoma (PDAC) at present. Late diagnosis and high recurrence results in five-year survival of 6%. Notably, Phase II trials based on adjuvant therapy combining systemic IFN Alpha (IFN) and chemoradiation reported 30-50% increase in two-year survival and impressive 35% increase in five-year survival of PDAC patients. Despite promising results, trial drawbacks included high patient dropout due to IFN systemic toxicity and low IFN levels in tumors. Low intratumoral IFN hampered the full potential of the therapy while IFN is known to induce tumor apoptosis, chemoradio sensitization, and decreased tumor neo-vasculatization.

Aiming to improve efficacy and tolerability of IFN therapy, we have developed an oncolytic adenovirus expressing human IFN (OAd-IFN). Vector has Ad5/3 fiber modification and overexpresses Adenoviral Death Protein respectively contributing to increased infectivity and oncolysis. Taking advantage of Cox-2 up-regulation in PDAC, the Cox-2 promoter was included in the upstream of Adenovirus E1 region, restricting vector replication to cancer cells. Human IFN-alpha gene was placed in the Adenovirus E3 region in the way that its expression is controlled by the adenovirus major late promoter. Therefore, IFN expression in this vector is replication dependent. To test the vector in an immunocompetent syngeneic hamster model of pancreatic cancer, OAd-IFN expressing hamster IFN was generated. Vector contains RGD fiber modification enhancing its infectivity in hamster cells.

MTS and crystal violet assays demonstrated sensitization of PDAC cells to chemotherapy (5-FU, Cisplatin, and Gemcitabine), and radiation (4 and 8Gy) by OAd-IFN. Comparison between OAd-IFN and control vector not expressing IFN (OAd-LUC) indicated that IFN expressed by OAd-IFN is functional in combination therapy sensitizing PDAC cells to chemoradiation. Colony formation assay showed that combinations of OAd-IFN with chemotherapy, radiation, or chemoradiation are synergistic and exhibit superior killing effect compared to groups without OAd-IFN.

In vivo studies using immunocompetent syngeneic hamster model of pancreatic cancer showed that combinations including OAd-IFN resulted in augmented tumor shrinkage and survival compared to groups treated with chemotherapy, radiation, chemoradiation, or OAd-LUC + radiation. Hexon staining and viral DNA quantification by qPCR show OAd-IFN effectively replicates and spreads in tumors.

Our data suggests OAd-IFN synergistically improves chemoradioation in PDAC cells, and shows superior therapeutic effect when treating immunocompetent model of pancreatic cancer. Vector capacity to express high levels of IFN intratumorally and the strong synergism between OAd-IFN and radiation, chemotherapy, and chemoradiation indicates OAd-IFN will contribute to more olerable and effective IFN therapy. Strong synergism between OAd-IFN and chemoradiation combined with focal expression of IFN can help to reduce not only IFN toxicity, but also dose-dependent toxicity of chemoradiation. Considering systemic IFN injection combined with chemoradiation is one of the few therapies to effectively treat pancreatic cancer, usage of OAd-IFN in combination with chemoradiation has great potential to result in more effective and tolerable therapy agaisnt pancreatic cancer.

640. Ex Vivo AKT Inhibition Promotes the Generation of Potent CD19CAR T Cells for Adoptive Immunotherapy

Ryan Z. Urak, ChingLam W. Wong, Elizabeth Budde, Christine E. Brown, Sandra Thomas, Stephen J. Forman, Xiuli Wang

Hematology, City of Hope, Duarte, CA

Insufficient persistence and effector function of chimeric antigen receptor (CAR) re-directed T cells in vivo has been a challenge for adoptive T cell therapy. Generation of long-lived potent CAR T cells is an increasing demand in the field. AKT activation triggered by convergent extracellular signals evokes a transcription program that enhances effector functions. However, sustained AKT activation severely impairs T cell memory and protective immunity because AKT drives differentiation of effectors, diminishing T cell potential to survive and differentiate into memory cells. We now investigate whether inhibition of AKT signaling can prevent terminal differentiation of T cells that are genetically modified to express CD19-specific chimeric antigen receptors (CD19CAR), as well as increase the number of memory CD19CAR T cells, which would enhance the antitumor activity following adoptive therapy. CD8+ T cells from healthy donors were isolated, activated with CD3/CD28 beads, and transduced with a lentiviral vector encoding a second-generation CD19CAR containing a CD28 co-stimulatory domain, which carries mutations at two sites (L235E; N297Q) within the CH2 region on the IgG4-Fc spacers to block diminshed potency and persistence due to Fc receptor binding. The lentiviral vector also expressed a truncated human epidermal growth factor receptor (huEGFRt) for selection and ablation purposes. IL-2 (50U/mL) and AKT inhibitor (1uM/mL) were supplemented every other day. Transduced CD19CAR T cells without AKT inhibitor treatment were used as controls. The engineered CD19CAR T cells were expanded in vivo for 21 days before in vitro and in vivo assays. We found that AKT inhibitor did not compromise the CD19CAR T cell proliferation and survival in vitro; comparable CD19CAR T cell expansion was observed after culturing in the presence or absence of AKT inhibitor. Functionally, AKT inhibitor did not dampen the effector function of CD19CAR T cells as indicated by equivalent levels of interferon gamma production and CD107a expression upon CD19 antigen stimulation. Memory-like phenotype such as CD62L and CD28 expression on CAR T cells is associated with better antitumor activity

in vivo. We therefore characterized the CD19CAR T cells after ex vivo expansion. We found that 40% of AKT-inhibited CD19CAR T cells expressed CD62L and co-expressed CD28. In contrast, only 10% of control untreated CD19CAR T cells expressed CD62L and they were CD28 negative, indicating that AKT-inhibited CD19CAR T cells may have superior anti-tumor activity following adoptive transfer. To test the potency of the AKT inhibitor treated CAR T cells, $0.5x10^6$ CD19+ acute lymphoid leukemic cells (SupB15) that were engineered to express firely lucifierase were inoculated intravenously into NOD/*Scid* IL-2RgammaCnull (NSG) mice. Five days post tumor engraftment, $2x10^6$ CD8+ CD19CAR T cells were intravenously injected into tumor bearing mice. Control mice received either no T cells, non-transduced T cells (Mock), or CD19CAR T cells that were not treated with AKT inhibitor during in vitro expansion. Tumor signals post T cells infusion were monitored by biophotonic imaging. In contrast to the untreated CD19CAR T cells, which exhibited lower and transient anti-tumor activity, AKT-inhibited CD19CAR T cells completely eradicated the $CD19^+$ tumor in all mice (Figure 1) 21 days post CD19CAR T cell infusion. In conclusion, inhibition of AKT signaling during the ex vivo priming and expansion gives rise to a CD19CAR T cell population that possesses superior antitumor activity. These findings suggest that therapeutic modulation of AKT might be a strategy to augment antitumor immunity for adoptive CAR T cell therapy, which could easily be transitioned into the clinic with the availability of pharmaceutical grade AKT inhibitor.

641. Highly Efficient, ZFN-Driven Knockout of Surface Expression of the T-Cell Receptor and HLA Class I Proteins in Human T-Cells for Enhancing Allogeneic Adoptive Cell Therapies

Gary K. Lee, Pei-qi Liu, Nimisha Gandhi, Lynn Truong, Andreas Reik, Stephen Lam, Sarah J. Hinkley, Jeffrey C. Miller, David E. Paschon, Lei Zhang, Edward J. Rebar, Michael C. Holmes
Sangamo BioSciences, Richmond, CA

While adoptive transfer of T-cells modified to express a chimeric antigen receptor or tumor antigen specific T-cell receptor (TCR) has shown great promise for the treatment of malignant cancers, most current clinical applications are limited by the use of autologous T-cell products. Targeting of the TCR and HLA Class I genes in primary T-cells thus represent attractive targets for genome editing in order to produce universal T cells from allogeneic donors. Elimination of the native TCR and HLA class I proteins on T-cells would, respectively, reduce the risk of graft-versus-host disease and host-versus-graft clearance mediated by the adaptive immune system. We have developed clinical grade zinc finger nuclease (ZFN) reagents that can efficiently target the T-cell receptor alpha constant (TRAC) and beta-2-microglobulin (B2M) loci. ZFN encoding mRNAs were introduced into purified T-cells by electroporation. Without selection, T-cells treated with TRAC specific ZFNs showed modification of up to 89% of alleles as gauged by deep sequencing. Flow analysis showed 91% of treated cells were negative for CD3 expression. Results from ZFN mRNA dose titration studies showed that the level of TRAC gene modification by deep sequencing was highly correlated with the percentage of cells that stain negative for CD3 expression by FACS (Spearman rho = 0.96; $p < 0.0001$). B2M is a subunit in all HLA class I molecules, and represents a conserved target for eliminating HLA class I presentation in cells from different donors. Analysis of T-cells transfected with mRNAs encoding B2M specific ZFNs showed up to 94% of alleles were modified, as determined by deep sequencing. FACS analysis showed that 89% of treated cells were negative for expression of HLA-A, B, C. Similar to TRAC modified cells, a marked correlation was observed between the percentage of B2M alleles modified and percentage of cells lacking surface expression of HLA-A,B, and C (Spearman rho = 0.57; $p < 0.0001$). ZFN mediated genome editing was well tolerated. T-cells treated with TRAC or B2M specific ZFNs showed similar viability and growth characteristics as mock transfected cells. Together, these highly efficient ZFN reagents permit the highly efficient double knockout of TCR and HLA class I surface expression in primary human T-cells for potential use in the development of allogeneic cell therapies.

642. Oncolytic Vaccines with Modified Tumor Epitopes for Cancer Immunotherapy

Cristian Capasso[1], Aniket Magarkar[2], Victor Cerveca Carrascon[3], Martin Muller[4], Mariangela Garofalo[2], Lukasz Kuryk[2], Davide Cardella[5], Karita Peltonen[2], Alex Bunker[2], Vincenzo Cerullo[2]
[1]Laboratory of Immunovirotherapy, University of Helsinki, Helsinki, Finland, [2]University of Helsinki, Helsinki, Finland, [3]Faculty of Pharmacy, University of Madrid, Madrid, Spain, [4]University of Munich, Munich, Germany, [5]University of Naples "Federico II", Naples, Italy

Oncolytic adenoviruses (OAds) are capable of killing tumor cells while activating the immune system due to their immunogenicity. Hance, they are an excellent platform for oncolytic vaccine. We previously demonstrated that the injection of peptide-coated conditionally replicating adenoviruses (PeptiCRAd) is capable of reducing the growth of established aggressive melanomas (murine B16).

Oncolytic vaccines, like PeptiCRAds, often rely on inducing an immune response against specific tumor antigens. However, many tumor antigens are also self-antigens, hence the peripheral tolerance might impair the activity of tumor-reactive T-cells. Therefore, mutated epitopes represent an optimal tool to break the tolerance, exploiting the cross-reactivity of T-cells. To this end we developed an Epitope Discovery and Improvement System (EDIS) framework to study native epitopes and predict, *in silico,* mutated forms suitable for cancer therapy. The novel aspect of EDIS is the ability to interrogate different prediction servers, integrate the different results and validate these by molecular dynamics simulations.

We started by studying the model epitope *SIINFEKL*. According to our *in silico* predictions, two mutated variants were suggested to be more immunogenic than the native *SIINFEKL* peptide. To test whether this prediction would reflect in enhanced vaccine effect we studied the immune response against these peptides in B16-OVA bearing mice. Mice were challenged with B16-OVA and treated with three different PeptiCRAds coated with *SIINFEKL* and the two predicted derivates. By ELISPOT assay we assessed the anti-peptide response and demonstrated that the two mutated forms were in fact more effective in reducing the growth of established B16OVA tumors.

Finally, we studied the native epitope *SVYDFFVWL* from the tyrosinase-related protein 2 (TRP2), a melanoma antigen in clinical evaluation. By using the EDIS framework we selected two mutated variants that show increased MHC-I binding affinity and we tested them by treating aggressive B16F10 tumors. As expected, treatment with the native TRP2 reduced the growth of the tumors compared to the controls. Suprisingly, one of the two analogues improved significantly the survival of mice and reduced the growth of their tumors compared to the group treated with the native TRP2 epitope. In conclusion, we demonstrated that the integration of different in silico methods increases the accuracy when predicting mutated epitopes for cancer immunotherapy.

643. Various Forms of CD40L Encoded as an Immune Plasmid Adjuvant Generate Unique Anti-Cancer DNA Vaccine Induced Responses

Megan Wise[1], Daniel Villarreal[1], Lumena Louis[1], Jian Yan[2], Matthew Morrow[2], Niranjan Sardesai[2], David Weiner[1]
[1]University of Pennsylvania, Philadelphia, PA, [2]Inovio Pharmaceuticals, Plymouth Meeting, PA

The use of immune plasmid adjuvants encoding cytokine, chemokine or immune modulators to tailor the vaccine induced response is a strength of DNA vaccine approaches. This has been established through the recent HVTN080 trial which demonstrated the potency of pIL-12 in increasing cellular responses above that of DNA only vaccination. Due to its role in both innate and adaptive immunity, the co-delivery of plasmid encoded CD40 ligand (CD40L) could increase DNA vaccine responses. In its natural form, CD40L can occur as either a surface bound form or a cleaved/solubilized form. Thus, we sought to determine if different forms of pCD40L can influence cellular and humoral responses when co-delivered with a HPV16 DNA vaccine expressing the oncogenic proteins E6 and E7. Mice were immunized with HPV DNA with or without synthetic optimized plasmids expressing various forms of CD40L. Mice which received the soluble form of CD40L (sCD40L) exhibited significantly higher antigen specific $CD8^+$ T cell responses including IFN-γ, IL-2 and TNF-α expression. These responses were maintained into memory. On the other hand, the surface bound as well as the wild type form blunted the vaccine induced responses compared to vaccine alone. Time course analysis revealed that 11 days after primary immunization, $CD8^+$ tetramer specific ($H\text{-}2D^b$ HPV16 E7 (RAHYNIVTF)) T cells in mice immunized with sCD40L averaged around 18% compared to vaccine alone at 4%. These responses were partially dependent on $CD4^+$ T cell help. Upon therapeutic tumor challenge, mice immunized with sCD40L displayed significant tumor regression compared to vaccine alone or naïve animals. We have also seen similar vaccine induced immune responses when sCD40L was combined with an HIV Envelope expressing DNA vaccine as well as an H1N1 flu DNA vaccine. These results demonstrate the power of using an immune plasmid adjuvant encoding a synthetic optimized sCD40L in a DNA vaccine. Additional studies in other models are important for this approach to be considered for possible clinical development.

644. In Vivo Expression of Functional DNA Monoclonal Antibodies Targeting IL-6 and CD126

Sarah T. C. Elliott, Ami Patel, Seleeke Fllingai, Megan C. Wise, Karuppiah Muthumani, David B. Weiner
Department of Pathology & Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Pro-inflammatory cytokine interleukin-6 (IL-6) and its receptor (CD126) are multi-faceted immune therapy targets. IL-6 plays a substantial role in innate inflammation and sepsis. Elevated levels of IL-6 are clinically linked to poor cancer prognoses; numerous studies demonstrate an association between IL-6 signaling and tumor development in multiple myeloma, lung cancer, colorectal cancer, renal cell carcinoma, cervical cancer, breast cancer, and ovarian cancer. Currently, therapeutic antibodies targeting IL-6 (Siltuximab) and CD126 (Tocilizumab) are approved for treatment of multicentric Castleman disease and rheumatoid arthritis, respectively. Manufacture and delivery of these purified protein antibodies are cost-prohibitive. Furthermore, anti-IL-6 and anti-CD126 protein antibodies must be re-administered weekly-to-monthly, an especially challenging consideration in treatment of chronic conditions such as cancer and auto-immune disease. Use of gene therapy to deliver antibodies, with enhanced potency and long-term expression in vivo, provides an economical and practical alternative to protein-based therapy. DNA is relatively inexpensive to manufacture, has an excellent safety profile, and lacks complications of pre-existing serology associated with some viral vectors.

Here, we demonstrate functional DNA monoclonal antibodies (DMAb) targeting IL-6 and CD126 are expressed in vivo. We constructed codon-optimized variable region DNA sequences from four anti-IL-6 and two anti-CD126 monoclonal antibodies on the human IgG1 constant domain. Plasmid DNA encoding each antibody was delivered intramuscularly with electroporation to nude and immune-competent mice. We optimized multiple aspects of DMAb delivery - including antibody sequence, plasmid heavy and light chain arrangement, and formulation - to enhance in vivo expression.

Anti-IL-6 and anti-CD126 DMAb were expressed in serum with levels ranging from 1.5 μg/mL to 7.1 μg/mL in BALB/c mice. We also observed long-term DMAb expression in nude mice. Serum DMAb retained functional binding to purified IL-6 and CD126. Serum DMAb also blocked downstream IL-6 cell signaling in vitro. Studies are underway to investigate anti-IL-6 and anti-CD126 DMAb for their role in controlling sepsis, limiting inflammation during acute viral infection, and slowing tumor progression. These studies not only provide a novel method to further define the role of in vivo IL-6 signaling in immune pathologies, but also define DMAb as an alternative to protein antibody therapies.

645. T-Cell Activating Mesenchymal Stem Cells as a Biotherapeutic for HCC

Arpad Szoor, Zhuyong Mei, Adrian Gee, Andras Heczey, Stephen Gottschalk
Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

Background: The outcome for advanced stage hepatocellular carcinoma (HCC) remains poor, highlighting the need for novel therapies. Mesenchymal stem cells (MSCs) are actively being explored as cancer therapeutics due to their inherent ability to migrate to tumor sites and to deliver 'toxic payloads'. We have recently shown that cells can be genetically engineered to secrete bispecific T-cell engagers (ENG), which activate T cells in an antigen-dependent fashion. The goal of this project was now to determine if it is feasible to genetically modify MSCs to secrete ENG molecules that are specific for the HCC-associated tumor antigen Glypican-3 (GPC3), and to evaluate their ability to redirect T cells to HCC. **Methods:** Bone-marrow derived MSCs were genetically modified with lentiviral vectors encoding GPC3-ENG or an irrelevant ENG (EGFRvIII-ENG) and GFP. We performed coculture assays with GPC3-ENG-secreting MSCs (GPC3-ENG.MSCs, +/- freshly isolated T cells, and GPC3+ (HUH7 and G401) or GPC3- (A549) tumor cells. MSCs expressing an irrelevant ENG (EGFRvIII-ENG) served as control. We used standard immunological techniques for measuring cytokine production, proliferation, and cytolytic activity of T cells. **Results:** Transduction of MSCs resulted in a median transduction efficiency of 95% (range 90-97%) as judged by GFP expression. GPC3-ENG. MSCs activated T cells only in the presence of GPC3+ targets as judged by IFNγ production, and their ability to T-cell proliferation and killing of GPC3+ targets. EGFRvIII-ENG.MSCs did not activate T cells, confirming specificity. However, GPC3-ENG.MSCs did not induce IL2 secretion by T cells indicating lack of costimulation. To evaluate if provision of costimulation could overcome this obstacle, ENG.MSCs were further genetically modified to express CD80, 41BBL, or both. GPC3-ENG.MSCs expressing CD80 and 41BBL induced robust IL2 production in the presence of GPC3+ tumor cells in contrast to GPC3-ENG.MSCs expressing only CD80 or 41BBL. **Conclusions:** We have successfully generated MSCs that secrete GPC3-ENG and express costimulatory molecules on their cell

surface. These MSCs induced potent anti-HCC T-cell responses in vitro, warranting further exploration of our MSC-based cell therapy approach for HCC.

646. Development of a Novel Chimeric Antigen Receptor as a Therapy Against Solid Tumors

John M. Szarejko[1], Thomas M. Yankee[1], Gary D. Myers[2]
[1]Department of Microbiology, Molecular Genetics, and Immunology, University of Kansas Medical Center, Kansas City, KS, [2]Division of Pediatric Hematology/Oncology, Children's Mercy Hospital, Kansas City, MO

The use of chimeric antigen receptor (CAR) T cells as a therapy for cancer is gaining traction as they potentially target tumors with high specificity and minimal off-target toxicities. Current CAR designs consist of an antigen-specific single chain antibody fragment (scFv) fused to the transmembrane and signaling domains of receptors capable of activating T cells. In CAR T cell therapy, patients' T cells are transduced with cDNA encoding the CAR allowing CAR expressing T cells to target tumor cells. Clinical trials using anti-CD19 CAR T cells to target B cell malignancy have demonstrated the potential of this therapy with numerous patients achieving partial or full remission. In the treatment of solid tumors, however, CAR T cell therapy has not proven as effective. Evidence supporting T cell persistence is sparse, suggesting that currently used CARs may not provide optimal signals for memory cell generation. In addition, some patients have experienced life-threating side effects including cytokine release syndrome and on-target off-tumor activity, indicating a need for better modulation of CAR T cell activity. In line with these clinical observations, data from our laboratory and others demonstrate that currently used CARs can activate T cells in the absence of antigen, which promotes toxicity and T cell exhaustion while preventing differentiation into memory T cells. We propose that this antigen-independent activation is a result of high expression of CAR molecules that lack the regulatory context of the endogenous T cell receptor (TCR) complex. To more effectively mimic the temporal regulation of endogenous TCR signaling pathways, we developed a novel TCR-based CAR in which a scFv is fused to the constant region of TCRβ, thereby becoming incorporated into the native complex. We hypothesize that incorporation of our CAR into the TCR complex will mimic endogenous T cell activation and regulation by including contributions from all CD3 members (CD3δ, CD3ε, CD3γ, and CD3ζ). Furthermore, we propose that endogenous regulation of CAR signaling will prevent the constitutive T cell activation observed with currently used CAR constructs and allow for the formation of memory T cells. We have demonstrated that surface expression of our TCR CAR is modulated by endogenous CD3 expression levels and mirrors normal TCR surface expression. In contrast to both first and second generation CARs, T cells expressing our novel CAR lack constitutive activation, are specific for tumor cells expressing high levels of antigen, and retain higher proliferative potential in culture. Moreover, TCR CAR T cells are capable of signaling through the TCR complex and activating T cells in an antigen specific manner. Currently, we are examining the effector potential of our TCR CAR T cells compared to first and second generation CARs. Additionally, we are developing TCR CARs capable of signaling not only through the TCR complex but also through co-stimulatory receptors. In summary, we are designing the next generation of CARs by incorporating the tumor recognition component of a scFv into the endogenous TCR complex.

647. Efficient Targeting of T Cell Malignancies *In Vitro* and *In Vivo* Using CD4-Specific Chimeric Antigen Receptor (CAR)-Engineered NK Cells

Kevin G. Pinz[1], Elizabeth Yakaboski[1], Alexander Jares[2], Hua Liu[2], Amelia E. Firor[1], Masayuki Wada[1], Lai-Han Leung[3], Xun Jiang[1], Yupo Ma[2]
[1]iCell Gene Therapeutics, LLC, Stony Brook, NY, [2]Pathology, Stony Brook Medicine, Stony Brook, NY, [3]Institute for Applied Research in Medicine, Macau University of Science and Technology, Macau, China

Chimeric antigen receptor (CAR) immunotherapy has shown exceptional promise in targeting otherwise untreatable hematologic and solid tumor malignancies, providing new hope to both pediatric and adult patients. Although remarkable progress has been achieved in clinical trials for patients with relapsed/refractory B cell malignancies, CAR immunotherapy for patients with T cell leukemias and lymphomas has not yet been developed, despite a generally poorer prognosis. In light of this unmet clinical need, we engineered natural killer (NK) cells to express a third-generation CAR directed against CD4. Indeed, most aggressive peripheral T-cell lymphomas are CD4-positive with uniform expression of this surface molecule. Therefore, CD4 is potentially an ideal target for CAR. Furthermore, in contrast to donor T cells, CAR NK cells have the advantage of mediating anti-cancer effects without the risk of inducing graft-versus-host disease (GvHD). Also, their shorter lifespan relative to T cells may limit off-target events and thus eliminate the need for a "suicide switch" that would ablate the modified cells in the event of off-target effects. Other potential advantages of CAR NK cells over CAR T cells include the opportunity to be an off-the-shelf therapy, and simpler manufacturing. We generated a third generation CD4-specific CAR (CD4CAR) containing CD28, 4-1BB and CD3zeta signaling domains. This CAR was introduced into the NK-92 cell line, which has used in multiple clinical studies, resulting in CD4CAR NK cells. When assayed in co-culture, these CD4CAR NK cells had a profound ability to kill CD4 positive tumor cells *in vitro* using both CD4+ cell lines (Karpas 299, HL60, and CCRF-CEM) and two primary patient samples from pediatric and adult T cell leukemia and lymphomas (Figure 1). To address any potential CD4CAR NK cell impact on the hematopoietic compartment's ability to repopulate, we also confirmed by CFU assay that CD34+ cells co-cultured with CD4CAR NK cells were able to differentiate into BFU-E and CFU-GM colonies at ratios statistically similar to CD34+ cells co-cultured with non-CAR NK cells. We then confirmed *in vivo* anti-CD4 positive tumor activity using xenogeneic mouse models. Together, our encouraging results of this preclinical study support the further development of anti-CD4 CAR-engineered NK cell immunotherapy for patients with T cell malignancies.

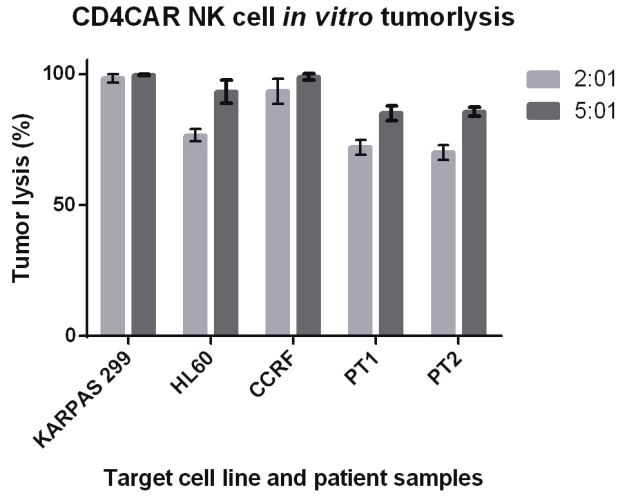


Figure 1. CD4CARNK cells kill both peripheral T cell lymphoma cell line and primary patient malignant cells at effector to target ratios of 2 to 1 and 5 to 1. Peripheral T cell lymphoma cell lines and primary patient T cell leukemia lymphoma samples were co-cultured for 24 hours with CD4CAR NK cells. The percent of malignant cell killing was determined by comparison to vector control transduced NK cells via flow cytometry analysis of cell survival.

648. Mitigating Tumor Escape: Tandem Anti-CD20- and CD19 SCFV-Based Chimeric Antigen Receptors (CARs) in Leukemia/Lymphoma

Ying Xiong, Darong Wu, Boro Dropulic, Rimas Orentas, Dina Schneider
Lentigen Technology Inc., Gaithersburg, MD

In recent years, adoptive cell therapy using T cells engineered with chimeric antigen receptors (CAR T) specific for CD19 has shown marked clinical efficacy. Nevertheless, cases of tumor escape due to tumor escape in B cell malignancies could be mitigated by CAR T the loss of CD19 antigen have been reported. We hypothesized that approaches that target the CD19 and CD20 tumor antigens simultaneously. Second generation CARs containing scFv specific for CD19 and CD20 were generated by lentiviral transduction of human primary T cells. The configurations were: a) single targeting domain, b) tandem targeting domains, or c) two full-length CAR receptors co-expressed bicistronically in the same T cell. CAR T expression for all constructs confirmed by Western blotting and protein L flow cytometry. CAR T cytolytic activity and IFN gamma secretion were evaluated by killing assays and ELISA, respectively. Single, tandem, and bicistronic CAR T cells exhibited CAR surface expression of 50-70%. All anti- CD19, anti-CD20 CAR T cells demonstrated target-specific cells lysis and induction of IFN-gamma. Interestingly, bicistronic CAR19, CAR20 construct yielded high CAR T surface expression and IFN-gamma production, but inferior *in vitro* cytolytic activity. To confirm CAR T specificity, K562 $CD19^+$ and K562 $CD20^+$ cell lines were generated. In flow-based 1h killing assays, CAR19 and CAR20 lysed their respective target lines only, whereas tandem CAR constructs lysed both K562 $CD19^+$ and K562 $CD20^+$ cells, but not K562 control. In a subsequent series of co-culture experiments, Raji target cells were combined with very low ratios of CAR T cells, to allow for the evolution of escape variants. On day 5 of co-incubation in the presence of CAR19 T cells, the surviving Raji cells numbers were comparable to control (85,000 cells/well vs 77, 000 cells/well in sham transduction). In comparison, viable Raji numbers decreased drastically post co-incubation with CAR20-, CAR19_20- and CAR20_19-T cells (546, 1,355 and 1,543 cells/well, respectively). Among the surviving Raji population, CD19 surface expression was reduced to 1.6%, vs 93.29% for sham control, whereas the expression of CD20 and CD22 remained high (97% and 78%, respectively). By contrast, the CD20 surface marker was less prone to down-regulation by CAR-T cells expressing anti-CD20 scFv. Similar results were seen when experiments were of longer, 7 days, or shorter, 1 day, duration. Thus, targeting two tumor antigens simultaneously using tandem CAR T cells is a promising new strategy for mitigation of tumor escape via antigen down-regulation. Moreover, expression of two scFv in a single CAR format is superior to expression of two independent CARs from a bicitsronic vector. It also appears that the order of the targeting domains within the tandem CAR impacts anti-tumor activity. These findings are currently being explored further in animal model systems.

649. Developing FGFR4 Chimeric Antigen Receptor CAR T Cell Therapy Against Rhabdomyosarcoma

Nityashree Shivaprasad[1], Ying Xiong[2], Marielle Yohe[1], Dina Schneider[2], Jack Shern[1], Sivasubramanian Baskar[1], Dimiter Dimitrov[1], Paul Sorenson[3], Rimas Orentas[2], Javed Khan[1]
[1]National Institute of Health, Bethesda, MD, [2]Lentigen Technology Inc., Gaithersburg, MD, [3]BC Cancer Agency, Vancouver, BC, Canada

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in pediatrics with an annual incidence of 4.5 cases per 1 million. Patients with high-risk metastatic disease have dismal prognosis and newer treatments are needed. We identified, fibroblast growth factor receptor 4 (FGFR4) as an overexpressed cell surface protein in RMS by mRNA expression analysis. Furthermore, activating mutations in FGFR4 are associated with metastatic disease. FGFR4 protein overexpression in RMS provides a specific target for immune-based therapy of RMS. We are developing T cells genetically modified to express chimeric antigen receptors (CARs) that target FGFR4.

To verify FGFR4 RNA expression at the protein level we performed both immunohistochemistry (IHC) and electrochemillumescence (ECL) ELISA assays. Using IHC analysis we measured FGFR4 protein levels on tissue microarrays (TMA) of normal tissue and primary tumor from RMS patients, increased staining for FGFR4 protein on RMS primary tumors, compared to normal tissues was demonstrated. FGFR4 expression measured using ECL ELISA assay, shows a range of 300 - 800pg FGFR4 per 1mg of total lysate in RMS cell lines. The range for normal tissues was 30 - 40 pg/mg for all tissues with the exception of liver, which expressed 70 pg/mg. A single-chain variable fragment (scFv) cDNA library derived from a human B cells was screened, and clones that showed binding to recombinant FGFR4 extracellular domain (ECD) selected. We identified ten specific human anti-FGFR4 scFv binders. The scFvs were cloned into prokaryotic expression vector containing the human IgG1 Fc region. Anti-FGFR4 scFv-Fc were expressed in 293FT cells by transient transfection and purified using Protein A affinity chromatography. The binding of scFv-Fcs to recombinant FGFR4 ECD was verified by ELISA. scFv-Fc binders were then assayed for binding to cell surface FGFR4 on RMS cell lines using flow cytometry. Anti-FGFR4 scFv-Fc bound to 293T cells transfected to express FGFR4 but not 293T control cells. Anti-FGFR4 scFv-Fc also bound FGFR4 on three RMS cell lines. The first two anti-FGFR4 scFv binder sequences evaluated, M410 and M412, were used to make short (S, extracellular scFv only) and long format (L, scFv with a CH2CH3 domain of IgG1) CAR constructs. Activated T cells were transduced with lentiviral CAR expression vector (LV) encoding M410-L, M412-S and M412-L CAR constructs and cell surface

expression of FGFR4 CAR on transduced T cells was measured using flow cytometry. The M410 and M412 FGFR4 CARs, both short and long constructs, were tested for cell-mediated cytotoxicity against RMS cell lines. M410-L showed higher cytotoxic activity compared to M412-L. M412-S showed greater cytotoxic activity compared to M412-L CAR. Thus, overall CAR structure format may be important for its functional activity. The remaining scFv will be further analyzed in various CAR formats for its functional activity including cytotoxicity and interferon-gamma production. In summary, the overexpression of FGFR4 protein in RMS versus normal cell lines demonstrates that FGFR4 may be a suitable target for immune-based therapy. FGFR4 CAR-T cell therapy offers the potential of a novel therapeutic intervention for high-risk, refractory and relapsed RMS.

650. Development of a Manufacturing Process Using Monte Carlo Simulations to Support KTE-C19 (Anti-CD19 CAR T Cells) Studies in Leukemia

Kenny Choi, Marianna Sabatino, Vijay Chiruvolu, Marc Better
Product Sciences, Kite Pharma, Santa Monica, CA

Introduction: ZUMA-3 and -4 (NCT02614066 and NCT02625480) are ongoing phase 1-2 multicenter trials evaluating KTE-C19 in subjects with relapsed/refractory acute lymphoblastic leukemia (r/r ALL). We designed a process suitable for CAR-T manufacturing in the presence of circulating leukemic cells using risk-based Monte Carlo simulations. The result is a robust process capable of generating the target subject dose with high certainty, and minimal operational complexity and variability.

Methods: Process design was initiated with the development and classification of unit operations within a sequential model. Rather than defining each process step with a large multivariate array of parameters, probability density functions (PDF) were utilized to represent all potential operational states. Best-fit values for the scale and shape of each PDF, with most units being Weibull and Johnson SU distributions, were computed from empirical data. The values were subsequently utilized to perform a Monte Carlo simulation (n=10,000 iterations) for the generation of a cumulative distribution function for each unit operation, thus characterizing ranges of operational confidence as a function of varying process inputs.The manufacturing success rate for the model was evaluated based on random and independent modifications of key process parameters, and each value was graded based on their relative impact on the process. This method of identifying high-risk conditions throughout the manufacturing design provided a basis for process optimization activities and experimental design. Furthermore, the number of T cells required to initiate the manufacturing process was minimized by performing an inverse mass balance of the simulation. Once optimal ranges were identified for each unit operation, a master protocol was prepared for testing the entire process at full-scale.

Results: In preparation for clinical manufacturing, healthy-donor GMP runs (n=8) initiated with no more than 4 x 10^8 T cells have successfully manufactured the target cell dose of 2 x 10^6 CAR^+ T cells/kg body weight within ≤7 process days. The upfront T cell isolation process from leukapheresis material achieved a target cell recovery with nominal variance (median 54%, range 46%-65%), and %$CD3^+CD45^+CD56^-$ cell enrichment (median 87%, range 78%-94%). Transduction efficiency was measured using a direct identity assay of anti-CD19 surface expression (median 54%, range 38%-66% CAR^+ T cells), and stable rates of fold expansion (FE) and doubling time (T_d) from day 2 to 6 were achieved (median 9.0 FE, range 6.6-11 FE), (median 27 hours, range 26-35 hours T_d). The products contained predominantly naïve (median 52%, range 18%-92%) and central memory (median 46%, range 2.6%-64%) T cells, with the remaining population containing effector memory (median 4.0%, range 1.0%-17%) and effector phenotypes (median 0.9%, range 0.3%-5.1%).

Conclusion: Using principles of risk-based Monte Carlo simulations, a robust and optimized process has been developed to address the potential challenges of manufacturing an autologous T cell product for leukemic subjects. Performance data generated from healthy-donor material provide a range of confidence for work with patient apheresis material, and this process which was designed to minimize the manufacturing failure rate will be utilized in ZUMA-3 and -4. The process model can be reassessed with data from relevant subject populations to further improve modeling activities.

651. Integration of Dual Signal Inputs Strategies in Novel Chimeric Antigen Receptors to Control the CAR T-Cell Functions

Alexandre Juillerat[1], Alan Marechal[2], Jean Marie Filhol[2], Yannick Valogne[2], Valérie Guyot[2], Anne-Sophie Gautron[2], Julien Valton[1], Aymeric Duclert[2], Laurent Poirot[2], Philippe Duchateau[2]
[1]Cellectis Inc, New York, NY, [2]Cellectis, Paris, France

Adoptive immunotherapy using engineered T-cells has emerged as a powerful approach to treat cancer. The potential of this approach relies on the ability to redirect the specificity of T cells through genetic engineering. Novel specificities in T cells have been typically implemented through the genetic transfer of the so-called chimeric antigen receptors (CARs). CARs are synthetic receptors composed of an extracellular targeting moiety and one or more intracytoplasmic signaling domain derived from lymphocyte activation receptors. Present CAR architectures are designed to combine all relevant domains within a single polypeptide, thereby; they combine advantages of MHC unrestricted target recognition to the potent native effector mechanisms of the T cell. Although adoptive transfer of CAR T cells is proven to be an effective cancer therapy, potential adverse effects such as cytokine release syndrome (CRS) and/or the risk of on-target off-tumor targeting are still a major concern.

Synthetic biology applies many of the principles of engineering to the field of biology in order to create biological devices which can ultimately be integrated into increasingly complex systems. Our ability to engineer synthetic systems in primary T-cells that function as Boolean logic gates responding to multiple inputs would benefit adoptive immunotherapy using engineered T-cells. Exogenous or endogenous environmental signal integration by a modular AND gate may represent an important advancement in improving our control of the safety of the CAR T-cell technology.

Here, we describe the development of novel CAR designs that integrate new components directly within the CAR architecture to improve our capacity to spatiotemporally control and switch the CAR T-cells functions between on and off states. In particular, we showed that such a system can be engineered to control the CAR through addition of an exogenous small molecule (Rapamycin or synthetic rapalogs) ultimately inducing the cytolytic properties of the engineered T-cell. Alternatively, properties of the tumor microenvironment can also be used as additional endogenous input to the target antigen recognition. We showed that low oxygen levels can be used to trigger the CAR surface presentation, creating a so called "self-decision making" CAR T-cell.

652. Culture and Characterization of Tumor-Infiltrating Lymphocytes of the Syrian Hamster

Mikko Siurala[1], Markus Vähä-Koskela[1], Riikka Havunen[1], Siri Tähtinen[1], Simona Bramante[1], Suvi Parviainen[2], Akseli Hemminki[1]
[1]Cancer Gene Therapy Group, University of Helsinki, Helsinki, Finland, [2]TILT Biotherapeutics Ltd, Helsinki, Finland

Adoptive transfer of tumor-infiltrating lymphocytes (TIL) has developed into a promising treatment modality for metastatic cancer. However, preclinical animal models for studying TIL therapy are limited to mice and therefore we set out to culture and characterize TIL from another rodent species, the Syrian hamster. First, we examined the presence of CD4+ and CD8+ cells in six implantable syngeneic hamster tumors representing various histologies. A tumor type-dependent pattern of lymphocyte infiltration was observed, with CD8+ cells ranging from 0.08% to 0.93% (of all tumor cells) and CD4+ cells ranging from 0.17% to 3.25%. To establish TIL cultures, excised tumor fragments were cultured in high-dose human interleukin-2 (IL-2) for 10 days. Flow cytometric analyses of Day 10 TIL cultures revealed a predominance of CD4+ cells over CD8+ cells in all tumor types studied. In effector/target assays the TIL cultured from HapT1 (pancreatic adenocarcinoma) and RPMI 1846 (melanoma) exhibited tumor-specific cytolytic activity. In addition, MHC Class I blocking abrogated the cell killing of RPMI 1846 TIL, suggesting that cytotoxic CD8+ T-cells were responsible for the observed cytolytic activity. Only partial abrogation was seen with HapT1 TIL suggesting the presence of cytotoxic CD4+ T-cells in the TIL population, which is supported by the observation that HapT1 tumor cells express MHC Class II. To our knowledge, this is the first time tumor-infiltrating lymphocytes of the Syrian hamster have been cultured and characterized. Thorough immunophenotyping of hamster tumors and TIL cultures requires the development of new hamster-specific antibodies in addition to the currently available ones used in this study. In conclusion, our data supports the use of the Syrian hamster as a novel platform for testing TIL protocols in a species other than mouse or human.

653. Sialyl Glycolipid Stage-Specific Embryonic Antigen 4 (SSEA4) - A Novel Target for CAR T Cell Therapy of Solid Cancers

Rita Pfeifer[1], Dominik Lock[1], Andrea Aloia[2], Andreas Bosio[1], Andrew Kaiser[1], Olaf Hardt[1], Ian C. D. Johnston[1]
[1]Miltenyi Biotec GmbH, Bergisch Gladbach, Germany, [2]Institute of Molecular Health Sciences, ETH, Zurich, Switzerland

Owing to their capacity to eradicate tumors, T cells represent an attractive means for immunomodulation in cancer immunotherapy. In this context, chimeric antigen receptor (CAR) - based therapies are receiving increasing attention. The combination of antibody-derived specificity with T cell effector function renders the immune cells MHC-independent and even enables targeting of antigens for which there is immunological tolerance. T cells, genetically modified with CAR, have shown impressive success in the treatment of leukemia. However, the application of CAR T cells to the treatment of solid tumors remains challenging due to the lack of truly cancer-specific targets and an immunosuppressive tumor microenvironment hostile to T cells.

We have identified the sialyl glycolipid stage-specific embryonic antigen 4 (SSEA4) as an epitope whose expression strongly correlates with metastasis and chemoresistence in triple negative breast cancer cells (TNBC). Single chain antibody fragments (scFv) were derived from an antibody that specifically recognizes this sialyl-glycolipid and were cloned into a lentiviral expression vector encoding a CAR containing an IgG1 spacer domain with CD137 and CD3z signaling domains. Healthy donor T cells were enriched by magnetic cell sorting and activated with TransAct™ Reagent, a colloidal nanomatrix-based activation reagent, before lentiviral transduction of the anti-SSEA4 CAR expression construct. Engagement of SSEA4 by CAR expressing T cells induced T cell degranulation, secretion of inflammatory cytokines and resulted in an effective killing of SSEA4 expressing target cells.

As TNBC patients are exposed to multiple rounds of chemotherapy and SSEA4 expression is found enriched in residual tumor cells surviving chemotherapy, a combinatorial approach using chemotherapy followed with CAR T cell therapy holds great promise to improve treatment outcome and overall survival of TNBC patients. Having assessed the performance of different anti-SSEA4 CAR constructs in vitro, current studies are focusing on in vivo functionality using mouse models.

Cancer-Oncolytic Viruses II

654. Oncolytic Vesicular Stomatitis Virus Retargeted to the Tumor Stem Cell Marker CD133

Dina Kleinluetzum[1], Alexander Muik[1], Julia Hanauer[1], Sarah-Katharina Kays[1], Camilo Ayala-Breton[2], Kah-Whye Peng[2], Christian J. Buchholz[1]
[1]Paul-Ehrlich-Institut, Langen, Germany, [2]Mayo Clinic, Rochester, MN

Therapy resistance and tumor recurrence are often linked to cancer stem cells (CSCs) which represent a minor proportion of highly refractory cancer-propagating cells within a tumor. A putative marker of CSCs is CD133 (prominin-1) which has been suggested to define the tumorigenic population in glioma and hepatocellular cancer (HCC). Moreover, poor prognosis correlates with CD133 expression in cancer biopsies. CD133 is therefore a prime target for novel anti-tumoral strategies. We have previously developed a strategy by which the envelope protein complex of oncolytic measles virus (MV) can be engineered to use CD133 as receptor for cell entry. Notably, MV-CD133 was more effective in killing tumors than non-targeted MV when treating tumors with a high percentage of CD133-positive cells (Bach et al., 2013; Can Res 73, 865). We have meanwhile extended the receptor usage of MV-CD133 to CD46 to effectively infect both CD133-negative and -positive tumor cells. Moreover, to take advantage of the faster replication kinetics and superior cytotoxic activity of the vesicular stomatitis virus (VSV), we used the MV envelope glycoproteins to develop hybrid viruses. The resulting oncolytic virus VSV-CD133 is deleted for its glycoprotein gene G and instead equipped with the mutated MV hemagglutinin (H) and fusion (F) glycoproteins displaying a single-chain antibody (scFv) specific for CD133. The data show that VSV-CD133 infects CD133-positive tumor cells as selectively as MV-CD133, while MV-CD133/CD46 in addition infected $CD133^-/CD46^+$ tumor cells. We further studied the cytotoxic activity of VSV-CD133 compared to its MV counterpart. Even though both were effective at killing tumor cells, VSV-CD133 induced cell death more rapidly and at lower MOI than MV-CD133. In a mouse tumor model with subcutaneously transplanted HuH7 cells VSV-CD133 reduced the tumor burden more efficiently than MV-CD133 or MV-CD133/CD46. Most importantly, this increase in efficacy did not come at the expense of safety, as $CD133^+$ human hematopoietic stem cells (HSCs) were neither susceptible to infection nor impaired in growth and differentiation into hematopoietic lineages. Ongoing studies will determine whether the superior oncolytic effects of VSV-CD133 translate into prolonged survival in a mouse model with orthotopically transplanted human primary glioblastoma cells.

655. CD133-Targeted Oncolytic Adenovirus Exhibits Anti-Tumor Effect by Attacking Colon Cancer Stem Cells

Mizuho Sato-Dahlman[1], Yoshiaki Miura[2], Masato Yamamoto[1]
[1]*Surgery, University of Minnesota, Minneapolis, MN,* [2]*Internal Medicine (3), University of Toyama, Toyama, Japan*

Colorectal cancer is the third most common cancer in the world and about 50% of patients relapse after treatment. Cancer stem cells (CSCs) have contribution to recurrence, metastasis and and chemotherapy resistant of colorectal cancers. CD133 (Prominin-1), a member of the transmembrane glycoprotein family, is a marker of CSCs in several cancers and is also generally accepted as a colorectal cancer stem cell marker. CD133 expression was correlated with recurrence, metastases and chemotherapy resistance, as well as poor prognosis in colorectal cancer. It is therefore reasonable to develop CSCs-directed therapeutic strategies by employing CD133 as a target molecule. Recently, we have established a method for isolating transductionally-targeted adenovirus by high-throughput screening.

In this study, the CD133-specific Oncolytic Adenovirus (OAd) was isolated by using this novel system, and the resultant virus was tested for the oncolytic activity both *in vitro* and *in vivo*. CD133-targeted OAd (AdML-TYML) showed strong binding to CD133-positive cells (293-CD133 and LoVo (CD133 (+) human colon carcinoma)), not to CD133-negative cells (parental 293 and LS174T (CD133 (-) human colon carcinoma)) and this virus also showed strong oncolysis selectively in LoVo cells, whereas there was no effect on LS174T cells. In the analyses of the effect of AdML-TYML on colorectal cancer stem cells, CD133-targeted OAd treatment leads to inhibition of tumor establishment of CD133-positive colorectal cancer cell lines in nude mice. 100% of the mice inoculated with non-infected LoVo cells had developed tumors, while 0% of the mice inoculated with AdML-TYML infected LoVo cells had done so. In addition, when the anti-tumor effect of the CD133-targeted OAd was analyzed in established tumors of CD133(+) colorectal cancer subcutaneous xenografts, intra-tumor (i.t.) administration of AdML-TYML exhibited significantly stronger antitumor effect compared to its counterpart with wild type fiber.

We focused on the CD133 as a target molecule of CSCs in colon cancer and successfully identified potent CD133-targeting OAd using high-throughput screening system. The CD133-targeted OAd exhibited selective infectivity and oncolysis to CD133-positive cells and anti-tumorigenic activity against colon cancer cells. The ability of CSCs-targeted Ad such as CD133-targeted OAd to infect and kill CSCs has important implications for the prevention of metastases and relapses in a variety of cancers.

656. Effects of STAT3 Inhibition on the Innate Immune Response to OV Therapy

Alexander Weiss, George Koutras, Chelsea Bolyard, Balveen Kaur
Neurological Surgery, The Ohio State Wexner Medical Center, Columbus, OH

Objective. Our lab has established that an alteration in STAT3 signaling affects the replicative ability of oncolytic virus (OV) (Okemoto 2013). However, STAT3 inhibitors are currently in clinical trials for treating several cancers, including glioblastoma (NCT01904123). One STAT3 inhibitor, WP1066, has been shown to modulate the immune response to tumors (Hussein 2007). The innate immune response to OV is known to limit its therapeutic efficacy (Meisen 2015, Han 2015) This study examines the effects of STAT3 inhibition on the innate immune response to OV therapy. **Methods**. *In vitro*, we examined the effect of STAT3 inhibition on immune cell response to OV-infected glioma. We evaluated changes in migration of immune cells towards OV-infected tumor cells, using a modified Boyden chamber. Macrophages or microglia +/- STAT3 inhibitor pretreatment were seeded on the upper chamber, and tumor cells +/- OV infection were plated on the lower chamber, acting as a migration stimulus. Migration was quantified after 6 hours. To evaluate the effect of STAT3 inhibition on the direct immune cell response to OV-infected GBM cells, we utilized a co-culture. Herein, we infected tumor cells with OV, and then overlaid them with immune cells +/- drug pretreatment. Virus replication was visualized through fluorescent imaging, and quantified via plaque assay. We utilized murine models of glioblastoma (GBM) to evaluate the effect of combining STAT3 inhibitors with OV therapy. Female nude mice were implanted intracranially or subcutaneously with human GBM cells, and then treated with PBS, pharmacologic STAT3 inhibitor, luciferase-expressing OV, or combination. Using bioluminescent *in vivo* live imaging, we monitored viral replication by measuring luciferase activity. We also monitored mice for survival. **Results**. Pharmacologic inhibition of STAT3 signaling in macrophage and microglial cell lines (RAW264.7 and BV2) reduced their migration towards OV-infected glioma cells. Furthermore, pre-treating immune cells with STAT3 inhibitor reduced their reactivity to OV-infected glioma cells, permitting increased replication. *In vivo*, combination therapy with STAT3 inhibitor and OV resulted in a significant increase in viral replication. Survival studies are ongoing. **Conclusions**. We hypothesize STAT3 inhibitors may alter the immune response to oncolytic virus, permitting increased viral replication and prolonged survival *in vivo*. Further research into this area may prove useful in developing new treatments to be used in combination with OV for treatment of GBM.

657. Enhancing Therapeutic Efficacy of an Oncolytic Virus with a Beta-1 Integrin Blocking Antibody, OS2966

Tejaswini Reddy Nallanagulagari
Neurological Surgery, The Ohio State University, Columbus, OH

Glioblastoma Multiforme (GBM) is a destructive cancer of the central nervous system with poor outlook for its patients, which prompts for development of novel therapeutic approaches such as oncolytic viruses (OVs). OVs are genetically engineered viruses with tumor specific replication and lead to lysis and destruction of cancer cells. Our lab has developed a novel oncolytic Herpes Simplex Virus (oHSV), 34.5ENVE, which expresses the anti-angiogenic protein Vasculostatin. This virus has shown unparalleled antitumor efficacy against intracranial glioma in mice. OV therapy is limited by the activation and infiltration of monocytic cells that promote virus clearance and inhibit tumor destruction. β1 integrins are cell surface molecules involved in cellular proliferation, invasion and inflammation. We revealed that oHSV-induced cysteine-rich 61 protein (CCN1) binds with integrins on the surface of glioma cells and macrophages, triggering antiviral type-I interferon and chemokine responses that increase macrophage infiltration and activation, which causes increased virus clearance in glioblastoma cells and limits oncolytic virus efficacy. To improve OV therapeutic efficacy, we combined oHSV with OS2966, a β1 integrin neutralizing antibody, which has been humanized in anticipation of clinical trials. We hypothesized that OS2966 would mitigate CCN1- β1 integrin induced inflammation and increase oHSV replication. Combination treatment with OS2966 increased oHSV replication and glioma cell killing. Migration assays revealed that treatment with OS2966 strongly inhibited oHSV-induced raw246.7 macrophage cell migration toward infected glioblastoma cells. From our results, we demonstrate that β1 integrins limit oHSV replication. Furthermore, we concluded that OS2966 could rescue CCN1- β1 integrin mediated macrophage and microglia migration toward oHSV infected GBM cells. Our findings show how a β1 integrin neutralizing antibody (OS2966) inhibits the

innate immune response triggered upon oHSV treatment. This work suggests that β1 integrin inhibition may be utilized in the development of a novel oHSV therapeutic strategy.

658. Bortezomib Treatment Sensitizes Oncolytic Virus Treated Tumors to NK Cell Immunotherapy

Ji Young Yoo
The Ohio State University, Columbus, OH

Background: Bortezomib, a proteasome inhibitor and oncolytic herpes simplex virus-1 (oHSV) are currently FDA-approved and continue to be evaluated in several human cancers. Various combinatorial treatment modalities are being investigated to enhance the efficacy of each treatment. In this study, bortezomib-mediated oHSV killing sensitization and anti-tumor immunity were evaluated. **Experimental Design:** The synergistic interaction between oHSV and bortezomib was calculated using Chou-Talalay analysis. Western blot, flow cytometry, and caspase 3/7 activity assays were used to evaluate the induction of necroptotic cell death, JNK activation, and apoptosis. Production of reactive oxygen species (ROS) was measured. Inhibitors/shRNA targeting ROS, JNK and RIP1 kinase (RIPK1) were utilized to investigate the mechanism of cell killing. Natural killer (NK) cells isolated from normal human blood and co-cultured with tumor cells at an Effect/Target ratio of 2:1. Q-PCR, ELISA, and FACS analysis were used to evaluate NK cell activation. Intracranial tumor xenografts were utilized to evaluate anti-tumor efficacy. **Results:** Combination treatment with bortezomib and oHSV induced necroptotic cell death with increased production of mitochondrial ROS and phosphorylation of JNK. Inhibitors/shRNA of RIPK1 and JNK rescued synergistic cell killing. Combination treatment also increased HMGB1 and IL-1α secretion and significantly enhanced NK cell activation and tumor cell killing. Moreover, combinatorial therapy enhanced NK cell therapy. **Conclusions:** This study provides a significant rationale for triple combination therapy of bortezomib, oHSV, and NK cells to achieve synergistic efficacy, leading to future clinical testing of oHSV with bortezomib in patients.

659. Oncolytic Adenovirus Loaded with Bioactive Modified Peptide as a Novel Approach to Treat Cancer

Mariangela Garofalo[1], Barbara Iovine[2], Lukasz Kuryk[1], Cristian Capasso[1], Mari Hirvinen[1], Andrea Vitale[2], Marjo Yliperttula[1], Maria Assunta Bevilacqua[2], Vincenzo Cerullo[1]
[1]University of Helsinki, Helsinki, Finland, [2]University of Naples, Naples, Italy

Cancer is still a leading cause of death worldwide. Although many kinds of treatment have been developed during the past decades, there is still a lack of effective therapy for advanced cancer. Currently treatments such as surgery, chemotherapy and radiotherapy can help to improve patient prognosis and increase patient life expectancy. Therefore new treatment strategies against cancer are in high demand. Efficient anticancer agent and its targeted delivery into the tumor mass is a key prerequisite for the successful cancer therapy. Oncolytic virotherapy is emerging as a potential approach to treat cancer, using viruses, which are specifically engineered to selectively infect, replicate in and kill cancer cells without causing damage to normal cells. Their combination with chemotherapeutic agents have shown promising results due to the synergistic effect of viruses and drugs; therefore the combinatorial therapy is considered a beneficial approach for cancer treatment. Taken into account these considerations we optimized a strategy to conjugate peptides on the viral capsid, based on electrostatic interaction and used this strategy to deliver an active anti-tumor dipeptide. We used L-carnosine, a naturally occurring histidine dipeptide with anti-proliferative activity. A modified L-carnosine, positively charged was absorbed onto the viral capsid of an oncolytic adenovirus to generate a virus-carnosine complex. The complex showed enhanced anti tumor efficacy *in vitro* and *in vivo* and higher infectious titer compared to a naked oncolytic adenovirus in colorectal and lung cancer cells. The *in vivo* efficacy of the complex was analyzed in lung and colon cancer xenograft models, displaying a significant reduction in tumor growth and synergistic effect between virus and dipeptide. Moreover, we studied the molecular mechanisms underlying the effects of complex on tumor growth reduction. Complex can induce apoptosis in both cells lines, by using two different mechanisms, enhancing viral replication and affecting the expression of Hsp27. Our system could be used in further studies also for specific delivery of other active drugs.

660. Enhance Antitumor Effect by Combining Oncolytic Virus HF10 and Bevacizumab in the Treatment of Human Breast Cancer Xenograft

Gewen Tan[1], Hideki Kasuya[2], Zhiwen Wu[2], Tsutomu Fujii[2], Maki Tanaka[3], Saori Fukuda[2], Toyone Kikumori[4], Nengquan Sheng[1], Zhigang Wang[1], Yasuhiro Kodera[2]
[1]Department of General Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China, [2]Department of Surgery II, Nagoya University Graduate School of Medicine, Nagoya, Japan, [3]TAKARA BIO INC, Shiga, Japan, [4]Department of Breast and Endocrine Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan

Background: The high prevalence and poor prognosis of breast cancer provides a strong rationale for developing new treatment strategies. Oncolytic herpes simplex viruses has a promising prospect because of its selectivity, and the replicating and tumor killing ability. In our study, the antitumor effect by combining oncolytic virus HF10 and Bevacizumab in the treatment of human breast cancer xenograft is evaluated. Methods: The VEGFA gene transcription and protein expression were measured in candidate cell lines (MCF-7,T47D and MDA-MB-231) by RT-PCR, Western blot and ELISA. The MTT analysis was applied to evaluate the efficiency of the combination therapy in vitro. Viral replication was assessed by PCR and plaque assay. Animal models were formed by implanting MDA-MB-231 tumor in the flank site of female BALB/c nude mice. The HF10 group of advanced tumor model received two injections of 10^6 pfu/dose intratumorally on Day 1 and Day 14. The HF10 group of single tumor model received single injection of 10^6 pfu/dose intratumorally on Day 1. The Bevacizumab group received 5μg/g Bevacizumab intra-peritoneally twice a week for two weeks. The combination group received both intratumoral HF10 and intraperitoneal Bevacizumab at the same dose of single treatment groups. The tumor diameter was measured twice a week. On Day 3 and Day 36, the tumors were collected and observed respectively. Histopathological parameters were HIF1α, VEGFA, CD31 driven microvascular density, Caspase 3 and HSV-1 antigen. Results: MDA-MB-231 cells have the highest level of VEGFA expression, while T47D cells have the lowest level. The cytotoxic effect of HF10 is time- and dose- dependent in vitro. The combination therapy does not affect viral replication in vitro. The combination group has the smallest tumor volume comparing with other groups in both animal models ($P<0.05$). The combination therapy induces synergistic antitumor effect in both animal models. Viral distribution is significantly enhanced in the combination group compared to the HF10 group on both Day 3 and Day 36. Enhanced tumor hypoxia and the up-regulation of angiogenesis gene as well as enlarged population of apoptotic cells in the combination group are also demonstrated in the tumor sample on Day 3. Conclusions: Increased angiogenesis effect and limited viral distribution remain obstacles of oncolytic viral therapy. Anti-angiogenesis reagent is considered to be effective to achieve better antitumor effect of

oncolytic virus. Our results show that Bevacizumab enhances viral distribution as well as tumor hypoxia and enlarges the population of apoptotic cells, and therefore induces a synergistic antitumor effect. It can be a promising virus-associated agent in the anticancer treatment.

661. Synergistic Anti-Tumor Efficacy of Immunogenic Adenovirus ONCOS-102 and Standard of Care Chemotherapy in Preclinical Mesothelioma Model

Lukasz Kuryk[1,2], Elina Haavisto[1], Mariangela Garofalo[2], Cristian Capasso[2], Mari Hirvinen[2], Sari Pesonen[1], Tuuli Ranki[1], Lotta Vassilev[3], Vincenzo Cerullo[2]
[1]Targovax Oy, Helsinki, Finland, [2]IVT lab, University of Helsinki, Helsinki, Finland, [3]Merck, Helsinki, Finland

Malignant mesothelioma is a rare cancer type with no effective treatment therapies so far. Most of the mesothelioma cases are caused due to asbestos exposure. The median survival time of mesothelioma cancer patient is short, around one year from diagnosis and conventional treatments such as surgery, chemotherapy and radiotherapy can help only to improve mesothelioma prognosis and finally increase patient's life expectancy. Unfortunately, currently there is no cure for mesothelioma, and new treatment strategies against mesothelioma are in high demand. ONCOS-102 is a double targeted, chimeric oncolytic adenovirus, coding for human GM-CSF. Its potency as an antitumor agent has been reported in pre-clinical and clinical studies. In this study we evaluated the anticancer activity of combined therapy: first-line chemotherapy (Pemetrexed, Cisplatin, Carboplatin) with ONCOS-102 in a human malignant mesothelioma xenograft model in BALB/c mice. ONCOS-102 was able to kill all human mesothelioma cell lines *in vitro* and exhibited anti-tumor activity in treatment refractory H226 mesothelioma xenograft model. Current standard of care chemotherapy regimens for malignant mesothelioma presented no anti-tumor efficacy in tested mesothelioma model. Importantly, a synergistic anti-tumor effect was shown when ONCOS-102 was combined with chemotherapy regimens. Our findings support the clinical use of ONCOS-102 with first-line chemotherapy against malignant mesothelioma.

662. An Oncolytic Adenovirus Gene Therapy Targeting Both Tumor Cell Survival and Desmoplasia in Pancreatic Cancer

Ioanna Milenova[1], Emma Eriksson[1], Rafael Moreno[2], Ramon Alemany[2], Angelica Loskog[1,3]
[1]Dept of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden, [2]IDIBELL-Institut Català d'Oncologia, L'Hospitalet de Llobregat, Barcelona, Spain, [3]Lokon Pharma AB, Uppsala, Sweden

The tumor microenvironment (TEM) supports the growth of tumor cells. In pancreatic cancer, the tumor lesions consist mostly of stellate cells, immune cells and extracellular matrix, a condition referred to as desmoplasia. The tumor cells can be less than twenty percent of the lesion. IL6 is overexpressed in pancreatic cancer and drives STAT3 activation leading to transforming growth factor (TGF) beta production and, hence, production of collagen type 1. TGF beta in the TEM also promotes immunosuppression by direct inhibition of T cells and expansion of T regulatory cells (Tregs). Hence, IL6 is one of the regulators of desmoplasia and is associated to poor prognosis of pancreatic cancer. To address the IL6 pathway as a target as well as the ongoing immunosuppression in pancreatic cancer, we developed a novel oncolytic adenovirus gene therapy expressing both an anti-IL6 receptor antibody single chain fragment (aIL6R scFv) and a trimerized membrane-bound CD40 ligand (TMZ-CD40L). LOAd713 is an Ad5/35 virus containing E2F binding sites that control the expression of an E1a gene deleted at the pRB-binding domain to achieve replication conditional on a dysfunctional, hyperphosphorylated retinoblastoma pathway. The genome was further altered by removing E3-6.7K and gp19K, changing the serotype 5 fiber to a serotype 35 fiber to target CD46 expressed by most tumors, as well as by adding a CMV-driven transgene cassette with the transgenes for TMZ-CD40L and anti-IL6R scFv. The activity of LOAd713 was compared to the activity of an empty LOAd(-) virus, a virus containing only TMZ-CD40L or uninfected cells. LOAd viruses including LOAd713 had oncolytic capacity in a panel of pancreatic cancer cell lines as shown by performing a standard MTS viability assay. Human pancreatic stellate cells were infected with LOAd viruses and analyzed by ProSeek proteomics. LOAd713 significantly decreased the expression of hepatocyte growth factor (HGF), TGF beta, fibroblast growth factor-5 (FGF-5) and collagen type I, all connected to stellate cell function and desmoplasia as well as to a poor prognosis. However, the LOAd713-infected stellate cells increased their expression of the cytokines IL1 alpha, IL6 and IL8 as well as the chemokines CXCL10 and CCL20. Infection of immature human dendritic cells (DCa) showed an increased level of maturation markers such as CD83 and IL12 as shown by flow cytometry and MesoScale, and such DCs could expand antigen-specific T cells. In conclusion, LOAd713 is an oncolytic virus that also targets the IL6/IL6R pathway resulting in reduction of factors that drive desmoplasia. Further, via TMZ-CD40L, LOAd713 can activate DCs to produce IL12, which in turn can activate T cell responses.

663. Can Oncolytic Measles Virus Targeted to CD20 Recapitulate Any of the Effects of Rituximab in the Treatment of Acute Lymphoblastic Leukemia?

Katharine Bailey[1], Aditi Dey[1], Sarah Aref[1], Lucile Broyer[2], Adele K. Fielding[1]
[1]Cancer Institute, UCL, London, United Kingdom, [2]Novimmune SA, Geneva, Switzerland

The Edmonston-B-derived, vaccine strain of measles virus (MVNSe) has oncolytic activity in numerous preclinical tumor models; safety and some efficacy has been demonstrated in phase 1 clinical trials for myeloma and ovarian carcinoma. Our work focuses on developing MV for the treatment of adult acute lymphoblastic leukaemia (ALL) - we have shown MV to be highly effective in preclinical models. An overall goal of our work is to determine how best to combine MV with other relevant, effective agents in ALL therapy. The antiCD20 monoclonal antibody rituximab improves outcome in adult ALL. Overall, we aim to determine whether targeting the CD20 antigen with a modified MV - wherein a single chain Fv antibody against CD20 is expressed as a C-terminal extension of the H glycoprotein [MVHαCD20, Bucheit et al, 2003] can recapitulate the effects of exogenously administered rituximab. A particular question in this study is whether neutrophils - which we have shown are important in mediating some of the MV-oncolytic effects and are definitely important as effector cells in rituximab antibody-dependent cellular cytotoxicity - can be as efficiently recruited by MVHαCD20 as by rituximab. We retrovirally transduced CD20 negative B-precursor ALL cell line NALM-6 cells to express human CD20. Cells were flow sorted to give 'high', and 'low' expressing populations, based on mean fluorescence intensity. Raji, a Burkitt lymphoma cell line with very high CD20 expression, was used as a positive control. We compared the oncolytic activity of MVNSe and MVHαCD20 by quantifying syncytia formation and cell death. Characteristic syncytia appeared earlier and were larger in CD20+ NALM6 cells when infected by MVHαCD20 as compared to MVNSe. However there was no significant difference between the conditions when cell death was measured after 5 days. Next, we carried out FACS-based neutrophil

phagocytosis assays in which target cells were labelled with PKH67. Percentage phagocytosis was determined by the frequency of dual expressing CD15+/PKH67+ cells, relative to total CD15+ cells. We established that the'high'CD20NALM6 cells generated a significantly greater neutrophil-mediated phagocytic response to rituximab alone than the 'low'CD20NALM6 cells with a maximum of 57% at E:T ratio of 1:5. Next, we investigated neutrophil-mediated phagocytosis following MV infection. Complement was not added to the medium. At 24 hours post infection with MVNSe, there was only a background level of phagocytosis (<5%) suggesting that neutrophil-mediated phagocytosis is not stimulated by direct microbial recognition in response to MV infection. MVHαCD20 showed similar results, suggesting that the scFVantiCD20 was also unable to stimulate neutrophil-mediated phagocytosis, possibly due to lack of Fc receptor. Studies in the presence of complement are ongoing. Despite the lack of neutrophil-mediated phagocytosis, the overall cell death, as assessed by DAPI staining, was significantly greater after MVHαCD20 infection than after rituximab administration. Future studies into the differential utility of MVHαCD20 over MVNSe in ALL therapy will include work with monocytes and NK cells and co-administration of corticosteroids.

664. Therapeutic Effect of Oncolytic Herpes Simplex Virus Type 1 (G47Δ) in Combination with Chemotherapy on Colorectal Cancer

Shinya Abe[1], Yasushi Ino[1], Hiroshi Fukuhara[2], Miwako Iwai[1], Toshiaki Watanabe[3], Tomoki Todo[1]
[1]Division of Innovative Cancer Therapy, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, [2]Department of Urology, The University of Tokyo, Tokyo, Japan, [3]Department of Surgical Oncology, The University of Tokyo, Tokyo, Japan

One of the reasons for the difficulty of curing unresectable advanced stage colorectal cancer is that cancer cells acquire resistance to anticancer drugs. Oncolytic virus therapy is a potent candidate for developing a new therapeutic approach that counteracts drug resistance, in which an oncolytic virus kills cancer cells in the course of tumor cell specific viral replication. G47Δ is a third-generation oncolytic herpes simplex virus type 1 with triple mutations in the γ34.5, ICP6, and α47 genes. G47Δ not only destroys cancer cells directly, but also induces systemic antitumor immunity efficiently. In the present study, we investigate the efficacy of G47Δ in combination with 5-fluorouracil (5-FU) or oxaliplatin for colorectal cancer. A human colorectal cancer cell line HCT116 and a murine colorectal cancer cell line CT26 were used *in vitro* to examine cytotopathic effects and replication capabilities of G47Δ. Combination index analyses demonstrated additive or synergistic effects of G47Δ when used in combination with chemotherapeutic drugs. Importantly, cytotoxic anticancer drugs did not affect the replication capability of G47Δ *in vitro*. *In vivo* experiments were performed using BALB/c mice bearing syngeneic subcutaneous CT26 tumors or athymic mice bearing subcutaneous HCT116 tumors. Each established tumor was treated with 2-time intratumoral injections with G47Δ (1×10^6 pfu) or mock, with concomitant intraperitoneal injections with chemotherapeutic drugs (3mg/kg 5-FU three times or 5mg/kg oxaliplatin four times) or vehicle. Combination therapy inhibited tumor growth significantly better than each therapy alone. Next, we established a 5-FU-resistant CT26 cell line (CT26FuR) by continuously exposing the naive CT26 cells to increasing concentrations of 5-FU. The tolerability of the CT26FuR against 5-FU was confirmed *in vitro* and *in vivo*. Cytotopathic effect and replication capability of G47Δ in CT26FuR cells were comparable to those in naive CT26 cells *in vitro*. Also *in vivo*, intratumoral administration of G47Δ was as efficacious in subcutaneous CT26FuR tumors as in CT26 tumors. In conclusion, G47Δ was shown efficacious for colorectal cancer both *in vitro* and *in vivo*. G47Δ may be especially useful for the treatment of multi-drug resistant colorectal cancer.

665. Therapeutic Efficacy of Third Generation Oncolytic HSV-1 (G47Δ) for Glioma Cells with Stem Cell Property

Hirotaka Ito, Yasushi Ino, Tomoki Todo
Division of Innovative Cancer Therapy and Department of Surgical Neuro-Oncology, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Glioblastoma is the most malignant brain tumor with highly dismal prognosis. Recent studies suggest that glioblastoma cells form a subpopulation with stem cell properties, termed glioma initiating cells (GICs). GICs grow less rapidly but are resistant to standard therapies, and are deemed to be a major reason for the disease to be incurable. In this study, the efficacy of G47Δ, a third generation oncolytic herpes simplex virus type 1, was evaluated using GICs established from surgically removed clinical specimens. The GICs grew in vitro as CD133-positive, nestin-positive neurospheres in serum-free medium supplemented with growth factors, and the stemness was confirmed by sphere forming assay. As few as 5x103 GICs implanted in the brain of athymic mice were capable of forming intracerebral tumors that retained histopathological appearances consistent with glioblastoma. On magnetic resonance imaging, the GIC-derived intracerebral tumors appeared as iso intensity in T1 weighted images, high intensity in T2 weighted images, and non contrast-enhanced mass lesions. In in vitro cytotoxicity assays, G47Δ killed 60 to 80% of GICs by day 4 when cells were infected at MOI of 0.01. Infecting GIC spheres with G47Δ caused significant inhibition in secondary sphere formation when compared with mock. In mice harboring GIC-derived intracerebral tumors, a single G47Δ inoculation (8 x 104 pfu, 10 days after tumor implantation) significantly prolonged the survival when compared with mock. Immunohistochemical study of G47Δ injected tumors demonstrated widespread distribution of G47Δ within the tumor but selective to tumor cells. These results indicate that G47Δ efficiently kills GICs as well as non-GIC glioma cells. Taken into account that a phase 2 clinical study with G47Δ in glioblastoma patients is ongoing in Japan, G47Δ may soon become a new therapeutic option for glioma patients with a potential of curing glioblastoma.

666. Oncolytic Herpes Virotherapy Induces a Paracrine Death Signal Causing Synergistic Antitumor Efficacy with Aurora A Kinase Inhibition

Leslee Sprague[1], Mark Currier[2], Tilat Rizvi[3], Brooke Nartker[2], Chun-Yu Chen[2], Pin-Yi Wang[2], Brian Hutzen[2], Meghan Franczek[2], Jeffrey Ecsedy[4], Joe Conner[5], Nancy Ratner[3], Timothy Cripe[2]
[1]Ohio State University, Columbus, OH, [2]Nationwide Children's Hospital, Columbus, OH, [3]Cincinnati Children's Hospital, Cincinnati, OH, [4]Takeda Pharmaceuticals International Co., Cambridge, MA, [5]Virttu Biologics, Glasgow, United Kingdom

Aurora A Kinase (AURKA) inhibition with the investigational agent alisertib results in abnormal mitotic progression and the induction of apoptosis in multiple tumor cell lines. Cell cycle arrest is a strategy employed by a variety of viruses, including herpes simplex virus (HSV), to bolster viral reproduction. We previously demonstrated the anti-tumor efficacy of oncolytic HSV and alisertib as monotherapies in models of malignant peripheral nerve sheath tumor (MPNST). We then proceeded to test alisertib and the oncolytic HSV Seprehvir in combination, finding a synergistic anti-tumor effect between the two therapies. Considering this, we hypothesized that alisertib potentiates Seprehvir infection, leading to more rapid

and robust viral proliferation with concomitant tumor regression. However, our efforts to quantify enhanced viral infection with combination therapy have uncovered no significant differences in Seprehvir viral growth kinetics witih or without alisertib therapy. Therefore, the synergistic tumor response of Seprehvir combined with alisertib in vitro or in vivo is not due to augmented viral infection. We then hypothesized that Seprehvir infection potentiates the anti-tumor effect of alisertib. In line with this hypothesis, there is a signficant decrease in tumor cell proliferation in alisertib-treated tumor cells that are exposed to soluble factors released by Seprehvir-infected cells. Furthermore, by UV inactivation of Seprehvir conditioned media, we demonstrate that these soluble death signals are not replication competent virus released by infected cells. Taken together, our results suggest a novel mechanism through which Seprehvir-infected cells release a paracrine signal to surrounding cells promoting cell death and increasing the anti-tumor activity of alisertib therapy.

667. Natural Selection of a Neurovirulent Herpes Simplex Virus Type1 Strain via Passage in Human Glioma Xenografts

Kentaro Fujii
Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

The strategy of using oncolytic viruses (OVs) in cancer therapy is based on their replicative selectivity in tumor compared to normal tissue. In general, these tumor-tropic OVs are genetically engineered to be attenuated to minimize toxicity and for retargeting towards tumors. Directed genetic manipulation of viruses depends on knowledge of the host-virus interactions at the molecular level, which can be highly complex and context-dependent. Another approach is to utilize natural selection and evolution to adapt a virus to be more selective for tumor cells. We thus obtained a wild-type HSV1 strain harvested from a human patient with HSV1 encephalitis patient. This HSV1 was repeatedly passaged in nude mice with human glioblastoma xenografts. After 10 rounds of serial passage, we propagated and purified the passaged virus for subsequent experiments. To assay for phenotypic differences, the parent virus and passaged virus were compared in their replicative efficiency in glioma cells *in vitro* and *in* vivo, as well as in toxicity by injection in nude mouse brains. There was a statistical significant increase in the replication yield of the passaged virus compared to the parental virus. Although there was also a trend for enhanced anticancer efficacy of the paasaged virus compared to the parental virus, it did not reach a p value less than 0.05. There was no difference in the toxicity of either virus in nude mouse brains. These results suggest that natural evolution by serial passage may lead to a more efficacious oncolytic HSV1, but that more passages may be required to further evolve this strain.

Cancer-Targeted Gene and Cell Therapy II

668. Platelets Engineered to Express Interleukin-24 Inhibited Melanoma Tumor Growth in Mice

Juan Fang[1], Ming Yao[2], Weiqing Jing[1], Beicheng Sun[2], Bryon D. Johnson[1], David A. Wilcox[1]
[1]Pediatrics, Medical College of Wisconsin, Milwaukee, WI, [2]Liver Transplant Center, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

Background: Activated platelets secrete agents (e.g., cytokines) that can promote solid tumor growth & cancer metastasis. Previous studies showed that hematopoietic stem cells (HSC) can be genetically modified to induce platelets to express & secrete proteins to establish hemostasis in animal models of bleeding disorders. Interleukin-24 (IL-24) is a cytokine (normally produced by activated monocytes, macrophages & Thelper cells) with cytotoxic & anti-angiogenic activity preferentially towards cancer cells. Thus, we hypothesized it may be feasible use HSC gene transfer to target platelet synthesis, storage & secretion of IL-24 to inhibit tumor growth. **Aims:** To investigate if HSC gene transfer targeting IL-24 synthesis, storage & secretion from platelets can inhibit melanoma in mice. **Methods:** C57BlL/6 mice were transplanted with bone marrow transduced with a lentiviral construct encoding a megakaryocytespecific *ITGA2B* gene promoter driving synthesis of IL-24 gene. Four weeks after transplant, IL-24 protein was characterized in platelets by flow cytometry. Murine melanoma cells ($1x10^6$) were implanted in mice at 5 weeks after transplant. Tumor size was measured in IL24 & control groups using a digital caliper periodically for 4 weeks. Then mice were sacrificed to record tumor mass. **Results:** Flow cytometry showed that HSC gene transfer of murine bone marrow led to synthesis & storage of IL-24 in platelets. An elisa assay showed that activated platelets could secrete IL-24 in vitro. At 30 days after implant of melanoma cells, platelet IL24 mice displayed significantly smaller (≈50%) tumor size ($550 \pm 101 mm^3$) & weight (599 ± 120 mg) compared to control mice with larger tumor size (1120 ± 114 mm^3) & weight (1391 ± 134 mg) n≈5 mice/group ($p<0.05$) indicating that platelet IL24 inhibited tumor growth *in vivo*. **Conclusion:** HSC gene transfer can be utilized to induce synthesis, storage and secretion of anti-oncogenic agent IL24 in platelets. Melanoma tumor challenge in mice with platelet IL24 showed a significant decrease in tumor growth suggesting that platelets may serve as an ideal therapeutic vehicle to treat cancer.

669. rAAV-Based and Intraprostatically Delivered miR-34a Therapeutics for Efficient Inhibition of Prostate Cancer Progression

Jianzhong Ai[1], Yi Lu[2], Jia Li[2], Qin Su[2], Qiang Wei[1], Hong Li[1], Guangping Gao[2]
[1]Department of Urology, West China Hospital, Sichuan University, Chengdu, China, [2]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA

Prostate cancer (PCa) is the second most common diagnosed cancer and the fifth cause of cancer-related mortality for males worldwide. At present, there is no effective treatment for PCa. Towards further understanding molecular mechanism and developing therapeutics for PCa, we investigated the role of miR34a in PCa progression. Previous studies have reported that miR-34a was significantly downregulated in PCa cells; therefore, modulation of miR34a expression might be a promising therapeutic approach for PCa treatment. To this end, we first verified the downregulation of miR34a in prostate tumor from transgenic adenocarcinoma mouse prostate (TRAMP) model. We found that overexpression of miR-34a could significantly inhibit the cell cycle of PC3 cells (a human PCa cell line) by prolonging G_1 and shortening S phases through targeting cyclin D1 (*CCND1*). To investigate if *in vivo* gene delivery of pri-miR34a to the prostates of TRAMP mice can inhibit PCa progression, we opted to screen 12 serotypes of rAAVs, an efficient and safe *in vivo* gene delivery platform with serotype dependent tissue tropisms for efficient prostate *in vivo* and PCa cell *in vitro* transduction. Among several leading candidate vectors (i.e. AAV6.2, AAV7 and AAV9) identified, we first tested the concept of intraprostatic injection of rAAV9·pri -miR34a ($4x10^{11}$ GCs/prostate) to 8-week old TRAMP mice for inhibition of PCa progression. We observed that the treatment lowered body weights significantly ($p < 0.05$) as compared to the control group starting from 24 weeks after injection, likely resulted from the higher tumor burden in the control group. rAAV9·pri -miR34a treatment also significantly extended the lifespan of TRAMP mice ($p < 0.05$). Moreover, we confirmed that the proliferation and neoplasia in the

treated prostates were significantly diminished when compared to those in the control group. Currently, the experiments are under the way to elucidate the molecular mechanism of overexpressed miR34a to slow down PCa progression. Taken together, our results demonstrated, for the first time, the potentials of rAAV-mediated efficient modulation of miRNA expression in the prostate for inhibiting PCa progression and studying molecular mechanisms in PCa development.

670. A Novel Approach to Expand Antigen Recognition in Chimeric Antigen Receptors

Robert Moot[1], Sunil Raikar[1], Lauren Fleischer[1], Melissa Querrey[1], Christopher Doering[2], Trent Spencer[2]
[1]Emory University, Atlanta, GA, [2]Pediatrics, Aflac Cancer and Blood Disorders Center, Atlanta, GA

Chimeric antigen receptor (CAR) technology, although very promising, is limited in its application by the availability of target antigens. We propose to expand the pool of potentially targetable cell-surface antigens using the variable lymphocyte receptor (VLR) of the sea lamprey as the antigen recognition region of the CAR. VLRs represent the functional region of the lamprey and hagfish adaptive immune system. They are single chained and variable length, crescent shaped proteins that are produced by assembly of leucine-rich repeat cassettes to form a gene encoding region capable of exceeding 10^{15} unique variations. Due to their difference in structure compared to Ig based antibodies, VLRs bind antigen in a geometrically dissimilar manner. This unique property of VLRs allows them the ability to bind antigen epitopes that may not typically be bound by scFvs, the result being a potentially expanded repertoire of tumor cell target antigens that may be used in CAR design and application. In our studies, VLRs have been successfully developed from immunized lampreys and target cell specific VLRs have been cloned for several different antigens including VLRs specific for cancer cell lines and purified proteins. This is accomplished using yeast surface display combined with flow sorting and results in monoclonal VLRs specific for the cell line with which the lampreys were immunized. The functionality of the VLR was initially demonstrated in Jurkat cells transduced with a previously generated VLR specific for the B-cell receptor of the mouse tumor line, BCL. CAR protein expression from whole cell lysates of transduced Jurkat cells showed expression of CAR protein. CAR cell surface expression was also confirmed in Jurkat cells using a construct co-expressing GFP preceding a P2A sequence with >90% cells GFP positive. Transduced CAR-Jurkat cells showed upwards of 85% activation in co-culture assays with target cells. In transduced but not co-cultured cells, activation was <5% and activation in naïve cells co-cultured with BCL cells was <2%. These results establish the ability of these cells to effectively produce and express VLR-CAR protein. The BCL VLR-CAR construct was also shown to be functional in several different types of cytotoxic effector cells including gamma delta T-cells and NK-92 cells, where target cell killing was increased significantly over non-transduced cells, indicating that target cell specific toxicity is mediated through the VLR-CAR. From these results, showing that VLRs function effectively as the antigen recognition region of the CAR construct we have concluded that VLRs can serve as a unique alternative for directing CAR activity toward specific effector cells.

671. Potent Anti-Tumor Effects of ApDCs (Aptamer Drug Conjugates) for Targeted Therapeutics in Pancreatic Cancer

Sorah Yoon[1], Kai-Wen Huang[2], Nagy Habib[3], John Rossi[1]
[1]Molecular and Cellular Biology, Beckman Research Institute of City of Hope, Duarte, CA, [2]Department of Surgery and Hepatitis Research Center, National Taiwan University College of Medicine, Taipei, Taiwan, [3]Department of Surgery and Cancer, Imperial College London, London, United Kingdom

Aptamer **D**rug **C**onjugates (**ApDCs**) are a potential class of targeting therapeutics to improve therapeutic index over traditional chemotherapy. As most existing chemotherapies are absorbed into cells non-specifically through lipophilic interaction, to improve the therapeutic index, the drugs were attached to pancreatic cancer specific RNA aptamer, P19, as targeting moieties that preferentially delivered the payload to tumor cells. Active metabolites of gemcitabine (dFdCMP) and 5FdU (5FdUMP) were incorporated P19 intrinsically to make conjugates. The conjugates of P19-dFdCMP or P19-5FdUMP kept their structural functionality getting internalized in their target cells, PANC-1. P19-dFdCMP and P19-5FdUMP was incorporated into replicating DNA, resulting in chain termination and stalling of replication forks in nuclear. They induced the phosphorylation of histone H2AX on Ser139 (γ-H2AX), markers of double-strand DNA breaks, and increased the forms of nuclear foci at the sites of DNA damage. In consequence, the cell proliferation was significantly inhibited 51-53% in PANC-1 and 54-34% in gemcitabine resistance AsPC-1. Targeted anti-mitotic therapies have emerged as a new concept of cancer drugs. Chemotoxine, monomethyl auristatin E (MMAE) is a very potent antimitotic agent that inhibits cell division by blocking the polymerization of tubulin. However, it can't be used by drug itself because of high toxicity. Herein, toxic molecule, MMAE, was conjugated to P19 for the targeted anti-mitotic therapies. P19-MMAE caused mitotic G2/M phase arrest, consequently inhibited 56% of cell proliferation on dose-dependent manner comparing control. *In vivo* assay, P19-MMAE induced the tumor regression *via* tail vein injection. In this study, the drug attached aptamer significantly decreased non-specific uptake of the drug and increase specific uptake of the conjugate in tumor cells. Our approaches suggest guiding cytotoxic drugs into malignant cancer cells specifically without causing significant harm to the normal cells.

672. Hybrid AAV/Phage Vector Enhances Chemotherapy Efficacy Against Cancer

Keittisak Suwan, Effrosyni Tsafa, Justyna Przystal, Paladd Asavarut, Amin Hajitou
Medicine, Imperial College London, London, United Kingdom

Chemotherapy has been widely used for cancer treatment, both in early and late stages. However, chemotherapy does not selectively target tumor cells, as normal cells are also harmed by the drugs resulting in several side effects. Another major obstacle to the success of chemotherapy in cancer treatment is the development of tumor drug resistance by cancer cells. In order to avoid these problems, the combination of chemotherapy with other therapeutic strategies has been used in order to lower the chemotherapeutic drug dose. Gene therapy is one of the therapeutic strategies that can be combined with chemotherapy. Mammalian viruses are well recognized vehicles for gene therapy, but major drawbacks of these viral vectors are broad tissue tropism following systemic administration (low specificity for the target cells/tissues), their fragility to harsh environments and difficulty for large scale production. Therefore, our group has developed a vector from bacteriophage, a bacteria virus also named phage. This novel engineered phage, called AAV/Phage, displays the RGD4C peptide to target a specific receptor ($\alpha_v\beta_3$ integrin) on cancer cell surface, while the phage genome is merged with recombinant

rAAV2 virus genome carrying the transgene to deliver. In comparison with mammalian viral vectors, the production of AAV/Phage is quicker, simpler and more economical. In addition, the vector is stable at 4°C for many years. The AAV/Phage vector efficiently targeted, delivered, and expressed transgene in cancer cells *in vitro*. We also proved that the vector selectively targeted gene delivery to tumors after intravenous injection in animal models of cancer. Moreover, in brain tumor models, the vector can penetrate through blood brain barrier and selectively delivers transgene expression to brain tumors. We used our vector as combination therapies with some well-known cancer drugs, such as doxorubicin, and temozolomide. Combination treatment of AAV/Phage vector carrying the *Herpes Simplex virus thymidine kinase* gene (AAV/Phage-*HSVtk*) with doxorubicin increased the targeted cancer cell killing in 2D tissue cultures and 3D tumor spheroids of rat gliosarcoma (9L) and human melanoma (M21) cells. We found that this increase in tumor cell killing was associated with a synergistic effect of doxorubicin on enhancing gene expression by AAV/Phage. We then combined AAV/Phage carrying short hairpin RNA to suppress *mTOR* gene expression (AAV/Phage-*shmTOR*) with temozolomide to treat medulloblastoma cells (DAOY). The results exhibited that treatment of medulloblastma with the vector alone efficiently suppresses the expression of *mTOR* gene, but has no effect on cell killing. Treatment of temozolomide at low dose (500 uM) did not have effect on cell killing, but combination therapies of temozolomide with AAV/Phage-*shmTOR* significantly increased cell death. Altogether, our results demonstrate that combination of AAV/Phage carrying therapeutic genes with cancer chemotherapeutic drugs is an effective strategy for cancer treatment. In future work, we plan to investigate the efficacy of AAV/Phage and cancer drug combination treatment in pre-clinical models of cancer.

673. Therapeutic Efficacy of Retroviral Replicating Vector (RRV) -Mediated Prodrug Activator Gene Therapy for Pancreatic Cancer

Kazuho Inoko[1], Kei Hiraoka[1], Akihito Inagaki[2], Mizuna Takahashi[1], Toshihiro Kushibiki[1], Koji Hontani[1], Hironobu Takano[1], Shoki Sato[1], Toru Nakamura[1], Takahiro Tsuchikawa[1], Toshiaki Shichinohe[1], Douglas J. Jolly[3], Noriyuki Kasahara[2], Satoshi Hirano[1]
[1]Department of Gastroenterological Surgery II, Hokkaido University Graduate School of Medicine, Sapporo, Japan, [2]Department of Cell Biology, University of Miami, Miami, FL, [3]Tocagen Inc., San Diego, CA

Pancreatic ductal adenocarcinoma is one of the most lethal cancers, thus new therapeutic strategies for this disease are urgently needed. Retroviral replicating vectors (RRV)-mediated prodrug activator gene therapy with Toca 511 (*vocimagene amiretrorepvec*), an optimized RRV encoding yeast cytosine deaminase (yCD) which converts the prodrug 5-fluorocytosine (5-FC) to the anticancer drug 5-fluorouracil (5-FU), is showing promising clinical activity in patients with recurrent high grade glioma, and is now being evaluated in a multi-center Phase II/III clinical trial. In the present study, we evaluated the therapeutic efficacy of RRV-mediated prodrug activator gene therapy in preclinical models of pancreatic cancer. We first examined the replication kinetics of RRV expressing the GFP reporter gene (RRV-GFP) in murine (Pan02) and human (MIAPaCa-2, BxPC-3, PANC-1 and SUIT-2) pancreatic cancer cell lines by flow-cytometric analysis and quantitative PCR. In all of these pancreatic cancer lines, RRV-GFP inoculated at MOI=0.05 (~5% initial transduction levels) showed rapid viral replication subsequently resulting in high levels of transduction, with the majority of pancreatic cancer lines reaching >90% GFP-positive cells over time. Next, we tested *in vitro* cytotoxicity by MTS assay after prodrug treatment of pancreatic cancer cells (Pan02 and MIAPaCa-2) transduced with Toca 511. In RRV-transduced pancreatic cancer cells, significant (~90%) cytotoxicity was induced by exposure to 0.1-1.0 mM 5-FC prodrug for 4 days, compared to untransduced and RRV-GFP transduced controls. We then evaluated *in vivo* therapeutic efficacy of Toca 511/5-FC prodrug activator gene therapy in Pan02 pancreatic tumor models established subcutaneously in immunocompetent syngeneic C57BL/6 mice. While 5-FC treatment alone showed no obvious inhibition of tumor growth as well as the nontreated control group, the majority (n=7/8) of Toca 511-transduced tumors showed complete regression after 5-FC treatment. Notably, systemic biodistribution studies showed no detectable RRV signals in genomic DNA from normal tissues of treated mice. In orthotopic models using luciferase-marked MIAPaCa-2 tumors established in the pancreas in nude mice, significant inhibition of bioluminescent signals was observed by optical imaging after 5-FC administration in mice with Toca 511-infected tumors, as compared to untreated control tumors. Thus, RRVs are highly efficient vehicles for delivering prodrug activator genes such as yCD to pancreatic cancer cells, thereby achieving significant cell killing upon pro-drug administration. Further preclinical studies are ongoing toward translating this novel therapeutic strategy into clinical trials for patients with pancreatic cancer.

674. Insertional Mutagenesis to Identify Mechanisms of Cetuximab Resistance in Colorectal Cancer

Riccardo Biavasco[1,2], Valentina Pirazzoli[1], Giulio Spinozzi[1], Andrea Calabria[1], Erika Tenderini[1], Fabrizio Benedicenti[1], Eugenia Zanella[3], Andrea Bertotti[3], Livio Trusolino[3], Eugenio Montini[1]
[1]San Raffaele Telethon Institute for Gene Therapy, Milano, Italy, [2]Università Vita-Salute San Raffaele, Milano, Italy, [3]Candiolo Oncology Institute, Candiolo, Italy

Anti-cancer drugs designed to target specific molecular pathways have shown an excellent therapeutic potential but also very poor long-term durability of tumor responses, mainly due to the outbreak of resistant clones among the residual neoplastic cell population. For that reason, understanding the molecular mechanisms underlying the onset of anti-cancer drug resistance (ACDR) is one of the major goals of clinical research. ACDR has been widely studied by DNA/RNA sequencing of primary human samples and several culprits identified. We have previously developed an approach based on lentiviral vector (LV)-induced insertional mutagenesis that allowed to identify the genes involved in lapatinib and erlotinib resistance on HER2+ human breast cancer cell lines and EGFR+ pancreatic cell line respectively. Here we took advantage of this platform to investigate ACDR genes in colorectal cancer (CRC). Cetuximab, anti-EGFR monoclonal antibody, is used as first line therapy in metastatic CRC, which results in prolonged survival of treated patients. However, nearly all patients relapse due to ACDR. We thus selected CRC cells sensitive to cetuximab deriving either from five microsatellite stable cell lines or from eight Patient Derived Xenografts (PDX), primary human CRC cells implanted subcutaneously into immunodeficient mice (NSG). To induce insertional mutagenesis we generated a luciferase-expressing LV harboring the SFFV enhancer/promoter in the long terminal repeats able to perturb the expression of genes nearby the integration site. As control, we used a non-genotoxic SIN-LV. We set up a collagenase IV-based disaggregation protocol that allows single-cell suspension and a serum-free culture condition to maintain the stemness of *in vitro* cultured cells. This protocol allowed to efficiently disaggregate and expand CRC cells *in vitro* as well as reach a LV copy number per cell ranging from 0.25 to 5.6. Luciferase gene expression was stable and allowed live-animal monitoring for up to 30 weeks after transplant. CRC-0069 and -0077 PDXs and NCI-H508 and HDC82 cell lines were transduced ex vivo and kept in vitro and/or

transplanted in NSG mice. After in vitro or in vivo expansion of the transduced CRCs cetuximab treatment was applied. After an initial shrinking of the tumor mass in mice we observed ACDR in 3 out of 10 mice transplanted with NCI-H508 cells transduced with SFFV-LV and in none of the controls. Genomic DNA from resistant cells is being used for insertion site (IS) analysis to identify common IS, ACDR gene candidates. IS obtained from SIN-LV groups will be used to filter LV integration biases, whereas IS from SFFV-LV transduced cells but not treated with cetuximab will be used to filter mutations that provide a proliferative advantage unrelated to cetuximab treatment. We will validate the most promising candidates by LV-mediated overexpression and knockdown techniques. This approach could pave the way to perform insertional mutagenesis-based forward genetics studies on primary human samples.

675. Exploring the Effects of Stroma-Derived PPARB Signaling in Colorectal Tumorigenesis

Eddie H. P. Tan[1], Walter Arthur Wahli[2], Nguan Soon Tan[1]
[1]School of Biological Sciences, Nanyang Technological University, Singapore, Singapore, [2]LKC School of Medicine, Nanyang Technological University, Singapore, Singapore

Peroxisome Proliferator Activated Receptors (PPARs) are members of the superfamily of nuclear receptor transcription factor. While the roles of isotypes PPARα and PPARγ in colorectal cancer have been well studied, the role of PPARβ/δ still remains controversial. The reason for the discrepancies remains unclear and several explanations have been put forth. One explanation for the contrary view is the heterogeneous tumor microenvironment. A tumor is made up of the cancerous epithelia, as well as stromal elements such as cancer-associated fibroblasts (CAFs). The role of PPARβ/δ in CAFs is not well understood, and concomitantly on tumor progression is not easily determined. We first examined mRNA expression of PPARβ/δ in murine intestinal tumor stroma. Laser-capture microdissected tumor stroma showed a statistical significant change in stromal PPARB expression upon tumor formation. Mouse carrying a stromal-specific deletion of PPARβ/δ showed that these mutant mice demonstrated increased cancer survivability, with reduced tumor size and numbers compared to their wild-type littermates, confirming the importance of stromal-derived PPARβ/δ in colorectal tumorigenesis. Preliminary observations also suggest that stromal PPARβ/δ may be a potential target for adjunctive anti-tumor treatment.

676. An Engineered CAR T Cell Platform for Allogeneic Combination Immunotherapy

Philippe Duchateau[1], Julien Valton[2], Alexandre Juillerat[2], Laurent Poirot[1]
[1]Cellectis, Paris, France, [2]Cellectis Inc, New York, NY

The adoptive transfer of CAR T cell represents a highly promising strategy to fight against multiple cancers. The clinical outcome of such therapies is intimately linked to the ability of effector cells to engraft, proliferate and specifically kill tumor cells within patients. When allogeneic CAR T cell infusion is considered, host versus graft and graft versus host reactions must be avoided to prevent rejection of adoptively transferred cells, host tissue damages and to elicit significant antitumoral outcome. This work proposes to address these three requirements through the development of multidrug resistant TCR-deficient CAR T cells. We demonstrate *in vitro* and in an *in vivo* xenograft mice model, that these engineered T cells displayed efficient antitumor activity and proliferated in the presence of single or muliple nucleotide analogues, currently used in clinic as preconditioning lymphodepleting regimens and antineoplastic agents. The absence of TCR at their cell surface along with their nucleotide analogues-resistance properties could prevent their alloreactivity and enable them to resist to lymphodepleting regimens that may be required to avoid their ablation via HvG reaction. By providing a basic frame work to develop a universal T cell compatible with allogeneic adoptive transfer, this work is laying the foundation stone of the large scale utilization of CAR T cell immunotherapies.

677. Evaluation of Tumor Specific Promoters for Use in Conditionally Replicating Adenovirus Mediated Virotherapy of Canine Lymphoma

Abdul Mohin Sajib, Samantha Morici, Maninder Sandey, Payal Agarwal, Bruce Smith
Pathobiology, Auburn University, Auburn, AL

Conditionally Replicating Adenoviruses (CRAds) are genetically modified therapeutic viruses that incorporates transcriptional targeting of replication to promote selective killing of tumor cells. Transcriptional targeting utilizes tissue/tumor-specific promoters driving the expression of genes in a tissue- or tumor-specific manner to allow replication of the virus in tumor cells while sparing normal cells. Selection of appropriate intermediate animal models is a basic-requirement for successful cancer virotherapy. The dog is an outstanding animal model of cancer and other complex human diseases. The outbred nature of the dog, the heterogeneity of their tumors, their genomic similarity to humans and similar disease causation, progression and pathology support the use of this animal model. Previous studies have shown high levels of expression of several promoters, including human telomerase reverse transcriptase (hTERT), survivin, chemokine receptor 4 (CXCR4) and progression elevated gene 3 (PEG3) in a variety of human cancers and murine models. The exogenous promoter PEG3 from rats has not only shown tumor-specificity in the human model, but has also shown pan-tumor properties with active transcription occurring in almost all tumor cells. None of these promoters have been tested for their potential as a transcriptional targeting tool for canine cancers. Non-Hodgkin lymphoma accounts for 83% of all hematopoietic cancer and 6 % of all malignancies in the dog. Resistance to current treatments has emerged as a critical challenge for lymphoma treatment due to the presence of genetic diversity among tumor cells. These studies explore tumor-specific activity of these promoters with the goal of identifying a suitable canine lymphoma specific promoter to generate transcriptionally targeted CRAds facilitating viral replication in canine lymphoma, but not in normal cells. In this regard, a GFP reporter gene driven by the rat PEG3 promoter was evaluated for activity after transfection into canine lymphoma cells as well as normal canine cells. The activity of the endogenous canine promoters CXCR4, cTERT, and cSurvivin were examined using quantitative reverse transcriptase PCR. Results showed negligible expression differences between normal and lymphoma cells for cTERT and PEG3 whereas cSurvivin and cCXCR4 showed markedly higher expression in tumor cells when compared with most normal cells and tissues. However, cCXCR4 also showed a high level of expression in normal peripheral blood mononuclear cells (PBMC) cells. In contrast, cSurvivin showed increased expression in canine lymphoma cells, along with other canine tumors, with reduced expression in normal canine cells/tissues and canine PBMCs. These findings will be used to generate a canine lymphoma specific CRAd.

678. Neural Stem Cell-Based Gene Therapy for Malignant Brain Tumor by Doubl-Stranded Adeno-Associated Viral Vectors

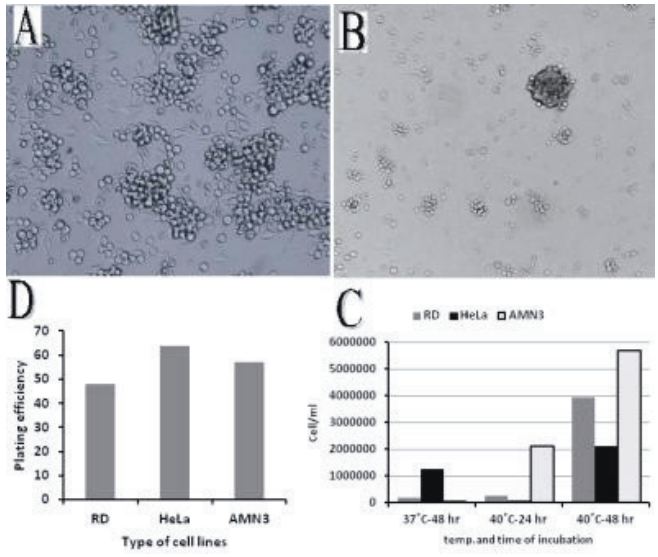
Li-juan Wang, Hsin-I Ma
Neurological Surgery, National Defense Medical Center, Taipei, Taiwan

Despite intensive efforts exploring gene therapeutic approaches for human cancers, the clinical results in treating of malignant brain tumors are still poor. We demonstrated that the combined therapeutic strategy by using an adenoviral vector carrying the thymidine kinase (suicidal) gene and an adeno-associated viral vector carrying the angiostatin gene achieved better therapeutic results. However, the malignant brain tumor could not be completely eradicated. Therefore, novel vectors and strategies are urgently needed. Neural stem cells (NSCs) may be used either for cell replacement or for gene delivery vehicles in neurodegenerative diseases. The expression of therapeutic proteins by NSCs can be enhanced by viral-mediated gene transfer, though the effects of several common recombinant viruses on primary progenitor cell populations have not been widely tested. The goal of this study is to identify and develop a safe and effective NSCs-based gene therapy protocol to treat malignant glioma. We examined what seral type of the novel double-stranded recombinant adeno-associated viral (AAV) vector can efficiently deliver anti-angiogenic genes and suicidal gene into the rat NSCs. Meanwhile, we investigated the gene product delivered by the genetically engineered NSCs in the adult normal brain. Then, we injected genetically engineered NSCs into different sites of the animal model of human malignant glioma. Flowcytometry data showed that AAV2 had higher transduction efficiency in neural stem cells. The NSCs were engineered to express Decorin and Angiostatin by AAV2, and then injected into the hemisphere contralateral to tumor cell implantation. We demonstrated that genetically-modified NSCs have the capacity to migrate into brain tumors. The therapeutic efficacy of genetically-modified NSCs is remaining explored.

679. Heat Stress Elevate Cell Population That Express Cancer Stem Cell Markers in Different Types of Cancer Cell Line

Amer T. Tawfeeq Al-Saeedi
Molecular Biology, Iraqi Center for Cancer and Medical Genetics Research, Baghdad, Iraq

Discovery of cancer stem cells (CSC) as driving force behind cancer cells tumorigenesis and heterogeneity in solid tumors led to a paradigm shift. The recognition of non-CSC to CSC transient introduces the cancer cell plasticity concept. This behavior appeared to be brought into existence majorly by the hostility of tumor microenvironment and stress imposed by hypoxia, pH, and nutrient depletion in the tumor niche. This recognition draw attention to the correlation between cancer hallmarks such as metastasis, chemotherapy, radiotherapy resistant and the transition of non-CSC to CSC by determination of CSC markers in cells subpopulations. Different types of cancer cell lines were shown to have a subset of cells expressing CSC markers such as CD44, ALDH, C133, Oct 3/4 with tumorigenicity *in vivo*. Recently, metabolic stress in a long-term nutrient deprivation showed to induces conversion of non-CSC to CSC like state. Additionally the hypoxic microenvironment was shown to upgraded stem-like properties of gastric cancer cells. Since it was recognized that metabolic alterations in tumors often combined by hyperthermia, we hypothesized that hyperthermia could be one of the stress components imposed by microenvironment that may manipulate non-CSC to CSC status and we asked if this elevation in temperature would affect phenotypic characteristics or up-regulate the genes responsible for stress resistant and the genes of cancer stem cell markers. To answer this, three different types of cancer cell lines were investigated by incubating in elevated temperature without CO_2 supplement. They were rhebdomyosarcoma cell line (RD), cervical carcinoma cell line (HeLa), and mice mammary adenocarcenoma (AMN3). In all experiments cell lines were seeded in 12 well tissue culture plates and incubated at 37°C with 5% CO_2 until they reach absolute confluent monolayer, after that subsets of plates incubated either at 40°C or 37°C without CO_2 for 24 or 48 hr. Cultivation of cell lines in RPMI1640 at 37°C for 24hr without CO_2 served as control untreated experiments. We followed changes in cells viability, phenotypic alterations, cologencity, and genetic markers of heat resistant (HSP90-beta and HIF), genetic markers of cancer stem cells (CD44, ALDH, Oct4, and CD133) by RT- qPCR . Results showed that upon incubation at 40°C for 24 hr or 48 hr without CO_2 supplement cells were detached floated and acquired spherical shapes and aggregated. Noticeably the formed aggregation resembled to some extent the cell spheres that induced in cancer stem cells at sphere forming assay (fig.1,A,B). The count of viable floated cells for RD, HeLa, and AMN3 cells were 3.93E−6, 2.1E−6, and 5.7E−6 cell/ml at 40°C for 48 hr respectively, and generally it was temperature and time dependant (Fig 1,C). All viable floated cells from the cell lines investigated were capable to form colonies in colony forming assay, the plating efficiency for RD, HeLa, and AMN3 cells were 48%, 64%, and 57% after incubation at 37°C for 10 days with CO_2 supplement respectively (Fig.1,D). Up regulation of heat stress gene HSP90-beta and HIF was detected in floated cells derived from all experiment and time of incubation. This was combined by up regulation of CSC markers CD44, ALDH, Oct4 and CD133 in floated cells derived from all experiment. These results may indicate a shift of non-CSC to CSC during heat stress and the increment of cancer stem cell population in the cell line under investigation which need more careful investigation.

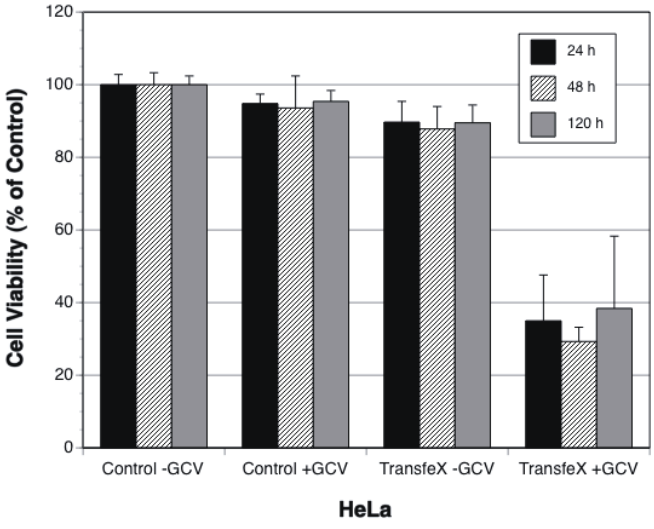


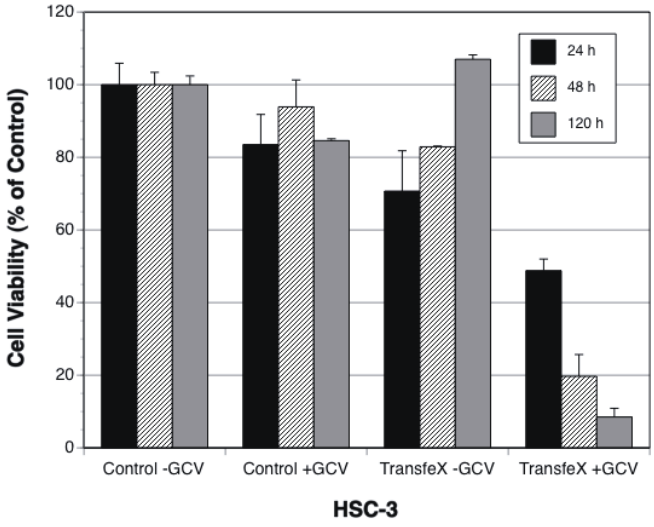
680. TransfeX-Mediated HSV-tk/Ganciclovir Suicide Gene Therapy in HeLa Cervical Carcinoma and HSC-3, FaDu, and H357 Oral Cancer Cells

Jennifer G. Cheung, Krystyna Konopka, Nejat Düzgüneş
Biomedical Sciences, University of the Pacific, Arthur A. Dugoni School of Dentistry, San Francisco, CA

INTRODUCTION: Herpes Simplex Virus thymidine kinase/ganciclovir (HSV-tk/GCV)-induced suicide gene therapy has been used to successfully treat various cancers. TransfeX-mediated

transfection of pCMV.Luc into HeLa cervical carcinoma and HSC-3, FaDu, and H357 oral squamous cell carcinoma (OSCC) cell lines in the presence of 10% serum has proved to be highly efficient, with low non-specific cytotoxicity. The plasmid pNGVL1-tk encoding HSV-tk under the control of the CMV promoter was delivered to the cells *in vitro* via the novel cationic liposomal reagent, TransfeX, followed by treatment with ganciclovir. METHODS: HeLa, HSC-3, FaDu, and H357 cells were seeded in 48-well culture plates one day prior to transfection, at a density of 1.5 x 10^5 cells per well in 1 ml of complete DMEM medium containing 10% serum (DME/10). Lipoplexes for transfection were prepared by mixing 1 µg pCMV.Luc or pNGVL1-tk per 100 µl of serum-free DMEM followed by the addition of 2 µl of TransfeX. Lipoplexes were added directly to cells grown in the presence of 10% serum. Twenty-four hours after transfection, 20 µg of ganciclovir was added per well and cells were incubated an additional 24 h. The Alamar blue assay was used to determine cell viability after 24, 48, and 120 h. Fresh medium and ganciclovir were added after each of these readings. RESULTS: After 24 h, 65% and 50% cytotoxicity were seen in HeLa cells and HSC-3 cells, respectively. FaDu and H357 cells were more resistant to treatment, showing 15% and 5% cytotoxicity, respectively, after 24 h. After 120 h, cytotoxicity remained the same in HeLa, and increased to 90% in HSC-3. In FaDu and H357, 65% and 30% cytotoxicity, respectively, were observed after 120 h. CONCLUSIONS: Suicide gene therapy is most effective against HSC-3 OSCC among the cells examined. Longer periods of incubation with ganciclovir or prolonged gene expression via enhanced episome vectors may be necessary to increase cytotoxicity in FaDu and H357 cells.





Hematologic & Immunologic Diseases II

681. HIV-1 Mediated Insertional Activation of STAT5B and BACH2 Promotes the Formation of a Viral Reservoir in T Regulatory Cells

Daniela Cesana[1], Francesca R. Santoni de Sio[1], Laura Rudilosso[1], Pierangela Gallina[1], Andrea Calabria[1], Laura Passerini[1], Silvia Nozza[2], Elisa Vicenzi[3], Guido Poli[3], Silvia Gregori[1], Giuseppe Tambussi[2], Eugenio Montini[1]

[1]San Raffaele- Telethon Institute for Gene Therapy, Milan, Italy, [2]Department of Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy, [3]AIDS Immunopathogenesis Unit, San Raffaele Scientific Institute, Milan, Italy

Since its identification, HIV-1 remains a global health threat responsible for a world-wide pandemic. The introduction of Anti-Retroviral Therapy (ART) greatly extended patients survival; however ART can control but not cure HIV infection. It has been recently suggested that HIV-1 by integrating near cancer-associated genes could promote the expansion and persistence of infected cells in patients under ART. However, the molecular mechanism/s of insertional mutagenesis used and the physiological impact on the cells harboring these integrations are completely unknown.Here, we found that in peripheral blood mononuclear cells (PBMC) from 54 HIV-1 infected patients under ART, *BACH2* and *STAT5B* were targeted by a significantly higher number of integrations and with the same orientation of gene transcription compared to other lentiviral integration (LV) datasets ($p<0.0001$). Furthermore, aberrant chimeric transcripts containing viral sequences fused to the first protein coding exon of *BACH2* or *STAT5B* and predicted to encode for unaltered full-length proteins were found in PBMC of 34% (30/87) of HIV-1 patients under ART. Tracking the expression of HIV-1/*STAT5B* and HIV/*BACH2* transcripts by droplet digital PCR in T cell subpopulations purified from the blood of patients under ART (N=6), we found a significant enrichment (higher than 10 fold) of the chimeric mRNAs in T-regulatory cells in all patients tested. The absence of any reported cases of T cell leukemia induced by HIV insertions in patients (despite several years of infection) and the role of these transcription factors suggest that cell clones harboring this type of activating insertions acquire a selective advantage possibly by altering the T-cell identity and/or homeostasis rather than triggering cell transformation. In vitro experiments performed on T regulatory cells purified from healthy donors demonstrated that the LV-mediated expression of these transcription factors significantly increased their proliferation

ability ($p<0.001$) without impacting on their immune-suppressive function. Overall, our findings provide novel evidences that HIV-1 takes advantage of insertional mutagenesis to favor its persistence in the host by activating *STAT5B* and *BACH2*. Indeed, the HIV-mediated deregulation of these transcription factors could confer a selective advantage to T regulatory cells, which being able to diminish the immune surveillance against infected cells contribute to promote long-term viral persistence. Hence, new targeted therapies aimed at interfering with *BACH2* and *STAT5B* regulated pathways could provide the general means for the immunological re-sensitization towards HIV-1 infected cells to reduce long lived cellular reservoirs and the eradication of the viral infection in HIV-1 patients.

682. Correction of CTLs Cytotoxic Function Defect by SIN-lentiviral Mediated Expression of Munc13-4 in Type 3 Familial Hemophagocytic Lymphohistiocytosis

Tayebeh-Shabi Soheili[1], Ida Ricciardelli[2], Julie Riviere[1], Amandine Durand[1], Els Verhoeyen[3], Chantal Lagresle[1], Geneviève de Saint Basile[1], Persis Amrolia[2], Isabelle André-Schmutz[1], Marina Cavazzana[4]

[1]U1163, Inserm, Imagine Institute, Université Paris Descartes, Sorbonne Paris Cité, Paris, France, [2]Molecular and Cellular Immunology Unit, Institute of Child Health, London, United Kingdom, [3]Ecole Normale Supérieur de Lyon, Lyon, France, [4]U1163 Inserm, Département de Biothérapie, AP-HP, Hôpital Universitaire Necker-Enfants Malades, Paris, France

Patients with mutations in UNC13D gene, coding for Munc13-4 protein, suffer from type 3 Familial hemophagocytic lymphohistiocytosis (FHL3), a life-threatening disorder of the immune system. Munc13-4 controls docking of lytic granules before they fused with the plasma membrane in cytotoxic T and NK lymphocytes and it's defect results in defective cytotoxic function of these cells. Hematopoietic stem and progenitor cell (HSPC) transplantation, which is the only curative treatment for FHL3 to date, is partially successful even when a compatible donor is available because of the important inflammatory background of patients. In this context gene therapy could be a promising therapeutic option especially for those patients without any compatible donor. As Munc13-4's function is to allow proper cytotoxic activity in mature cytotoxic CD8+ T cells we proposed that these laters may constitute target for gene correction. We constructed a self-inactivating HIV-1 derived lentiviral vector encoding human Munc13-4 in two different pseudotypes, the high tropism VSV-G and the measles virus glycoproteins (H/F) envelope which target more efficiently lymphoid cells through the signaling lymphocyte activation molecule (SLAM) as described by Verhoeyen et *al*. 2011. We demonstrated that both vectors are able to stably transduce FHL3 CD8+ T cells resulting in correction of defective degranulation capacity of these cells. However comparative analysis showed that H/F pseudotyped vector was more efficient than VSV-G vector to transduce FHL3 T cells. Adoptive transfer of the gene-corrected FHL3 T cells in SCID mice bearing autologous B-LCL lymphoma led to significant tumor regression due to an efficient homing into the tumor mass and long persistence of corrected T cells in peripheral blood as compared to non-corrected T cells receiving mice. Our study shows for the first time that a lentiviral mediating gene transfer in T cells could be proposed to treat a hemophagocytic lymphohistiocytosis disorder.

683. Foamy Virus Gene Therapy Significantly Restores Lymphocyte Development and Function in SCID-X1 Mice

Matthew MacQuivey[1], Gabrielle Curinga[1], Nick Hubbard[1], Iram Khan[1], Courtnee Clough[1], Daniel Humphrys[2], Jennifer Adair[2], Grant Trobridge[3], Troy Torgerson[1], Andy Scharenberg[1], Hans-Peter Kiem[2], David J. Rawlings[1]

[1]Program for Cell and Gene Therapy, Center for Immunity and Immunotherapies, Seattle Children's Research Institute and Departments of Pediatrics and Immunology, University of Washington School of Medicine, Seattle, WA, [2]Fred Hutchinson Cancer Research Center, Seattle, WA, [3]Pharmaceutical Sciences, Washington State University, Pullman, WA

X-linked severe-combined immune deficiency (SCID-X1) results from inactivating mutations in the gene encoding the common gamma chain (γc), a cytokine receptor subunit required for lymphoid development and function. In humans, SCID-X1 is characterized by the absence of T cells and natural killer cells, non-functional B cells, and is fatal within the first year of life if left untreated. Although HLA matched allogenic stem cell transplants yield survival rates exceeding 90%, such donors are often unavailable. Thus, gene replacement therapy offers a promising alternative treatment for patients lacking suitable donors. Clinical trials using gamma-retroviral vectors demonstrated efficacy; however, adverse events highlighted the need for improved safety. While adverse events have been eliminated using SIN-gamma-retroviral vectors, we hypothesized that foamy viruses (FV) that lack native pathogenicity and boast an integration site profile much less focused on either active genes or promoter regions than lentiviruses or gamma-retroviruses might provide an improved and safer therapeutic platform. We determined whether a candidate clinical FV vector containing the human phosphoglycerate kinase 1 (PGK) promoter driving human γc expression could safely and effectively rescue lymphocyte development and function in SCID-X1 mice. Our promoter choice was based upon ongoing studies using FV vectors in parallel in a canine model of SCID-X1 where the PGK promoter outperforms the EF1α promoter currently in use in SIN-retro and lentiviral-based SCID-X1 clinical trials. We report here the combined results of 66 primary and 130 secondary transplant mice that demonstrate significant and sustained immune reconstitution. SCID-X1 mice were transplanted with 2x10^6 FV- or LV-transduced, or WT or SCID-X1 lineage negative HSCs. Animals were sacrificed at ~25 weeks post-transplant; bone marrow, spleen and thymus were collected for analysis and secondary recipients were also established. Primary FV gene therapy recipients showed significantly greater B and T lymphoid numbers compared to SCID-X1 controls, with output viral copy numbers of ~1-4 in all tissues. Splenocytes from primary treated animals proliferated in response to CD3/CD28 and also demonstrated γc dependent intracellular pSTAT signaling in response to IL-7 and IL-21. FV and LV gene therapy-treated mice displayed comparable lymphocyte reconstitution and output copy number. Secondary recipients revealed sustained partial rescue of lymphoid compartments and viral marking, indicating the transduction of long-term repopulating HSCs. RIS analysis demonstrated polyclonal marking in splenic lymphoid populations with 3508 unique FV and 2441 unique LV integrations detected (3 experiments/3 animals per experiment). Further, analysis of individual mice from a primary transplant experiment demonstrated polyclonal marking with 588-1060 and 651-895 unique integration sites in FV- and LV-treated animals, respectively. Analysis to determine detailed integration site profile and potential proximity to protooncogenes is ongoing. Together, these data suggest FV gene therapy may provide an effective alternative treatment option for SCID-X1.

684. Enhanced FVIII AAV Vector Cassette Produces Improved Virus Yields and Supraphysiological FVIII Levels *In Vivo*

Brigit Riley, Jeff Boonsripisal, Alicia Goodwin, Eudean Garces, Stephen Ballaron, Hung Tran, Andrea Kang, Lei Zhang, Judy Greengard, Richard Surosky, Kathleen Meyer, Dale Ando, Geoff Nichol, Michael C. Holmes
Research, Sangamo BioSciences, Inc., Richmond, CA

Hemophilia A is the most common inherited bleeding disorder in humans and is caused by a deficiency of blood coagulation factor VIII (FVIII). This disease is an ideal candidate for liver-directed gene therapy, as even modest increases in FVIII activity (>1% of normal) can ameliorate the severe bleeding phenotype. Adeno-associated viral (AAV) vectors have shown great promise in both preclinical and clinical trials to efficiently deliver therapeutic transgenes to the liver. Suboptimal virus yields of AAV comprising human FVIII (hFVIII) for use in human therapy have hampered clinical scale manufacturing, both from mammalian (HEK293) and insect cells (Baculovirus system). We optimized an hFVIII AAV vector cassette to improve both virus yields and liver-specific hFVIII expression. Compared to historical hFVIII AAV vector cassettes that routinely produce 20% of standard, non-hFVIII containing AAV2/6 virus preparations, the improved hFVIII AAV vector cassette produced yields 100% of standard AAV2/6, both from HEK293 cells at the cell factory (CF) scale, but also in the Baculovirus system at large scale. Improved yields were also observed using other AAV serotypes including hFVIII AAV2/8 and AAV2/9. Using virus produced from the improved hFVIII AAV cassette we transduced mice at 6E+10 vg/mouse (~2E+12 vg/kg). Peak levels of hFVIII protein were achieved in mouse plasma at day 14 and represented supraphysiological levels of normal hFVIII in humans with serotypes AAV2/8 and AAV2/9; up to 337% and 516% of normal hFVIII plasma levels respectively (1U = 200 ng/mL = 100%). Transducing mice with serotype AAV2/6, known to be inefficient at transducing mouse liver, at 6E+10 vg/mouse (~2E+12 vg/kg) we achieved a mean peak value of 91.9 % +/- 15.5 SEM (n=6) of normal hFVIII plasma levels in humans. At a higher dose representing ~6E+12 vg/kg we achieved a mean peak value of hFVIII plasma over six independent *in vivo* mouse studies of 169.2 % +/- 10.1 SEM (n = 36). Supraphysiological hFVIII plasma levels were also achieved in non-human primates at a dose of 2E+12 vg/kg using serotype AAV2/6, representing up to 840% of normal hFVIII levels (8.4U). The robust production of hFVIII from the enhanced hFVIII AAV vector could significantly reduce the dose required to achieve therapeutic levels in human subjects. Additionally the improved hFVIII AAV vector cassette will enable clinical scale manufacturing.

685. Restoration of Antiviral Immunity Following Hematopoietic Stem Cell Transplantation with Virus-Specific T-Lymphocyte Therapy

Michael D. Keller[1], Jamie C. Hoover[2], Patrick J. Hanley[2], Sarah McCormack[3], Cecilia Barese[3], Elizabeth Williams[3], Neha Joshi[3], Maria Martin-Manso[3], Lauren Roesch[3], Conrad R. Cruz[3], Allistair Abraham[2], Kirsten Williams[2], Blachy Davila[2], Evelio Albuerne-Perez[2], David Jacobsohn[2], Catherine M. Bollard[3]
[1]Division of Allergy & Immunology, Children's National Health System, Washington, DC, [2]Division of Blood and Marrow Transplantation, Children's National Health System, Washington, DC, [3]Center for Cancer and Immunology Research, Children's National Health System, Washington, DC

Background: Adoptive immunotherapy using virus-specific cytotoxic T-lymphocyte (VST) products has been successful in restoring antiviral immunity after hematopoietic stem cell transplantation (HSCT). VST may be produced from a stem cell donor in 10-14 days using current rapid protocols, or banked, partially-HLA matched VST may be used as an "off the shelf" treatment. **Objective**: To evaluate the clinical efficacy of HSCT donor-derived VST and third-party VST for prevention or treatment of CMV, EBV, and adenovirus following HSCT. **Design/Methods**: VST were cultured from HSCT or third-party donors using a GMP-compliant rapid-manufacture protocol. VST were tested for specificity and non-alloreactivity via IFN-γ ELISpot and cytotoxicity assays. Patients were monitored for 45 days following infusion for toxicity and for up to 22 months for immune reconstitution. **Results**: Nineteen patients received VST on existing protocols, of which 15 products were derived from HSCT donors, and 6 were from partially matched, third-party donors. Sixteen patients were treated for CMV (N=11), EBV (N=5), and/or adenovirus infections(n=3), of which fourteen (88%) had partial or complete antiviral responses. Immune reconstitution against targeted viruses was seen using IFN-g ELISpot at a median time of 28 days following infusion. Three patients developed grade I-II GVHD within 45 days of VST infusion, all of which was treatment responsive. Sixteen of the 19 patients remain alive and free of active viral infection up to 22 months post-VST infusion. **Conclusions**: VST are effective for the restoration of antiviral following HSCT. Expansion of targeted viruses may further extend the utility of this therapy.

686. Forced Fetal-Globin Reactivation Mediated by the TALE-ldb1 Fusion Protein

Jun Zhan, Irudayam Maria Johnson, Arthur Nienhuis
Hematology, St. Jude Children Hospital, Memphis, TN

Sickle cell disease (SCD) and thalassemia are very severe inherited disorders of the red cell and are amongst the world's most common genetic diseases. Thalassemia is caused by a serious lack of normal β-globin production in red cells whereas, sickle cell disease reflects a mutant hemoglobin that produces protein aggregates. The production of fetal-globin (HbF) may compensate for the reduction of β-globin and also it functions to inhibit sickling by participating in formation of mixed tetramers including both the γ and β^S chain. Thus the induction of HbF holds tremendous promise to ameliorate the clinical symptoms of β -thalassemia and sickle cell disease (SCD). For this reason, the adult-to-fetal hemoglobin switch has been an area of long-standing interest among hematologists and basic scientists. The recent revolution in gene editing technology has provided new opportunities for high efficiency fetal-globin gene activation and provides strong potential for clinical benefit. Recently, it was reported that zinc finger artificial proteins that bind to the Ɣ globin gene promoter could reactivate fetal-globin expression, via tethering the globin enhancer LCR to the Ɣ globin gene promoter. We have designed Transcriptional Activator Like Effectors (TALE) targeting the Ɣ globin gene promoters to reactivate Ɣ globin gene expression. The EF1 short promoter was used initially to drive TALE gene expression. The TALEs were fused with a ldb1 dimerization domain, followed by a T2A GFP cassette. The various elements were then assembled into a lentiviral vector. To prevent deletions caused by repeats of TALEs during the lentivirus packing process, we changed the TALE's DNA coding sequence by using codon-optimized DNA for their expression. Both PCR and Southern blot analysis confirmed that TALEs remained as full-length intact DNA without deletion. We employed HUDEP-2 (human umbilical cord blood derived erythroid progenitor cells) to examine the function of TALEs with respect to fetal-globin gene reactivation. Quantitative RT-PCR was performed to determine the relative mRNA level of fetal-globin which was normalized to the alpha-globin mRNA content. Three of eight TALEs were found to activate fetal-globin expression in HUDEP-2 cells with increases in mRNA levels from several fold to more than 400-fold. Furthermore, we also observed a symmetrical reduction of β-globin mRNA by more than 50%. The fetal-globin level determined by HPLC

was up to 40% of total globin, compared to 0.4% in control cells. To define the ability of the vector to transduce primitive CD34 cells, we performed methylcellulose culture after transduction of CD34 cells with TALE encoding lentiviral vectors at an MOI of 5. Individual red colonies were picked up to determine the vector copy number (VCN) by quantitative real time-PCR. Forty to fifty percent of the colonies were positive for vector insertion. In the future we are planning to do human CD34 cell erythroid differentiation to evaluate how the vector activates fetal-globin expression in red cells. To promote strong expression of TALE in the erythroid linage, we have replaced the EF1 short promoter with strong MND promoter or one erythroid specific ankrin promoter.

687. Development of a Safe and Effective Combinatorial Foamy Virus Vector for HIV Gene Therapy

Arun K. Nalla, Theodore Williams, Athena Lemon, Rahaf Qamou, Elizabeth Everson, Grant D. Trobridge
Department of Pharmaceutical Sciences, Washington State Univeristy, Spokane, WA

The Berlin patient and successes in retroviral hematopoietic stem cell (HSC) gene therapy suggest that gene therapy may provide a functional cure for HIV. However, retroviral-HSC gene therapy has resulted in serious adverse side effects due to vector mediated genotoxicity, including leukemia. Foamy virus (FV) vectors have a promising integration profile and may be safer than retroviral vectors. FV vectors also can efficiently deliver anti-HIV transgenes that can reduce the titer of HIV-1 based lentiviral vectors. We report a novel combinatorial anti-HIV FV vector that uses a housekeeping elongation factor 1 alpha (EF1α) promoter but still potently blocks HIV infection. We first evaluated the relative potency of various previously described anti-HIV transgenes, including the C46 fusion inhibitor, the F12-Vif derivative Chim3, lens epithelium derived growth factor-integrase binding domain (LIBD) and TRIM5α-CyclophilinA fusion (TCypA) in a FV vector background. We found that C46 was the most potent anti-HIV transgene, followed by TCypA and LEDGF-IBD. Next, we hypothesized that using a less-genotoxic internal promoter to drive the transgenes would reduce vector-mediated genotoxicity. Therefore, a direct comparison was made between the efficacy of housekeeping gene promoters (EF1α and Ubiquitin C; UbC) and a highly genotoxic SFFV promoter in expressing the C46 transgene and subsequently blocking HIV replication. We observed that C46 EF1α had ~2 to 4 fold higher anti-HIV effect than C46 driven by either SFFV or UbC promoters, respectively. Based on these results, we designed a novel combinatorial FV vector expressing three anti-HIV transgenes: C46, TcypA and LIBD. The gene cassette was driven by an EF1α promoter and also has mCherry as a reporter gene (FV-E C46TLmC-W). This vector can be produced at high titer, 1.4 x 10^7 transducing units/ml which is critical for clinical translation. This novel combinatorial anti-HIV FV vector showed a higher potency in blocking HIV replication than C46 alone at a late time point, 21 days post HIV infection (Figure 1). An *in vitro* competitive survival advantage assay indicated that cells transduced with our novel combinatorial anti-HIV FV vectors are highly resistant to HIV infection compared to cells transduced with FV expressing a control EGFP reporter gene alone (FV-EG-W) (Figure 2). Further studies will be focused on the efficacy of our novel combinatorial anti-HIV FV vector in human $CD34^+$ cells transplanted in a mouse xenotransplant *in vivo* model. Our goal is to develop a safe and potent combinatorial FV vector for clinical studies.

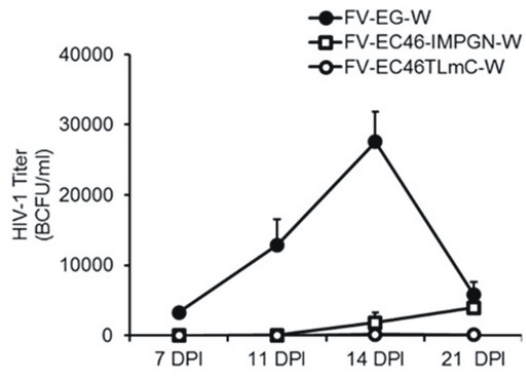


Figure 1. Efficacy of combinatorial anti-HIV FV (FV-E C46TLmC-W) in inhibiting HIV replication compared to FV expressing only C46 (FV-EC46 IMPGN-W) in 174xCEM cells.

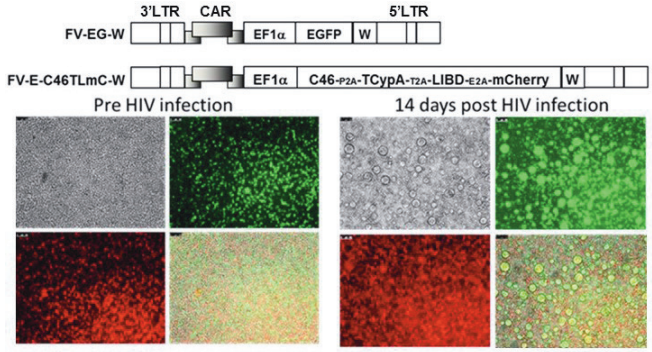


Figure 2. Vector map of control (FV-EG-W; with EGFP reporter gene) and combinatorial anti-HIV FV (with mCherry reporter gene) vectors. In vitro HIV challenge assay evaluating the anti-HIV combinatorial anti-HIV FV vector. 174xCEM cells transduced with either control or anti-HIV FV vector were mixed at 1:1 ratio and challenged with HIV. Syncytia, due to the cytopathic effect of HIV infection, was observed specifically in control EGFP expressing cells.

688. Supplemental Immune Suppression Is Required for AAV-F8 ITI in Hemophilia A Mice with Pre-Existing Inhibitors

David Markusic, Brett Palaschak, Irene Zolotukhin, Roland Herzog
Pediatrics, University of Florida, Gainesville, FL

Hemophilia is an inherited coagulation disorder resulting in the loss of functional clotting factors VIII (hemophilia A) or IX (hemophilia B). Patients with the severe hemophilia (less than 1% residual clotting factor activity) develop spontaneous bleeds into the joints and closed spaces and have a high incidence of morbidity if not treated. Current treatment involves frequent infusion of short-lived exogenous plasma derived or recombinant clotting factors. A major complication with protein therapy is the development of inhibitory antibodies in 25-30% hemophilia A and 3-5% hemophilia B patients that prevent normalization of hemostasis with continued protein therapy. Inhibitor patients are treated with either bypassing agents or immune tolerance induction (ITI) therapy requiring daily infusions (months to years) of supraphysiological levels of clotting factor protein. ITI therapy is effective in up to 70% of hemophilia A patients, while it often fails in hemophilia B patients due to the development of pathogenic antibodies that induce anaphylaxis and nephrotic syndrome. Both treatment options for inhibitor patients have high associated financial

and health related costs for the patient. We have previously shown that hemophilia B mice with pathogenic inhibitory antibodies are effectively treated with a novel immune tolerance induction protocol using a liver directed adeno-associated virus expressing the gene encoding for human factor IX protein (AAV8-F9). AAV8-F9 ITI rapidly eliminates pre-existing inhibitors and provided sustained therapeutic levels of FIX protein even following repeated exposure to recombinant FIX protein. Based on our success with hemophilia B, we sought to develop a complementary protocol for hemophilia A mice with pre-existing inhibitors. Our initial studies were conducted using a codon-optimized F8 (coF8) gene, which has been shown to enhance the levels of transcribed FVIII protein. In the first set of experiments we tested two different vector doses ($1x10^{11}$ and $1x10^{12}$ vg) of an AAV8-cohF8 vector for factor VIII expression levels and tolerance induction in hemophilia A mice on two different genetic backgrounds (BALB/c-F8$^{-/Y}$ and 129/BL6-F8$^{-/Y}$). Since BALB/c-F8$^{-/Y}$ mice develop lower titer FVIII inhibitors following intravenous hFVIII protein challenge compared to 129/BL6-F8$^{-/Y}$ mice we expected better outcomes with AAV8-cohF8 vector treated BALB/c-F8$^{-/Y}$ mice. Surprisingly BALB/c-F8$^{-/Y}$ mice spontaneously developed FVIII inhibitors approximately 4 weeks following vector delivery. In contrast,129/BL6-F8$^{-/Y}$ mice expressed vector dose dependent therapeutic levels FVIII protein without inhibitors and 75% of the mice remained inhibitor free following four weekly IV FVIII protein challenges. Subsequent studies for AAV8-cohF8 ITI were conducted in 129/BL6-F8$^{-/Y}$ mice. We generated a group of inhibitor mice and treated a subset of these mice with $1x10^{12}$ vg AAV8-coF8. AAV8-cohF8 ITI alone was not sufficient to eliminate inhibitors. Therefore we designed two additional studies to determine if $1x10^{12}$ vg AAV8-coF8 ITI would be more effective if combined with transient immune suppression using oral rapamycin (4mg/kg) or anti-CD20 (10mg/kg) mediated B cell depletion. While rapamycin adjunct therapy failed to lower inhibitors, preliminary data show that anti-CD20 combined with AAV8-coF8 ITI leads to a sharp reduction in high titer inhibitors. Given that FVIII protein invokes a stronger immune response in mice and patients, our data suggests that successful gene therapy for hemophilia A may require supraphysiological levels of FVIII protein to prevent inhibitor formation and transient immune suppression to be effective as an ITI therapy.

689. The Content of the More Immature Hematopoietic Stem Cells (HSCs) Is Dependent from the Underlying Genetic Diseases: Consequence on Transduction Efficiencies

Laure Caccavelli[1], Cécile Roudaut[1], Maeva Joigneaux[1], Johanna Blondeau[1], Fabien Touzot[1], Salima Hacein-Bey-Abina[2], Marina Cavazzana[3]

[1]Biotherapy, Hôpital Necker-Enfants Malades, AP-HP, Paris, France, [2]Immunologie Biologique, Groupe Hospitalier-Universitaire Paris-Sud, AP-HP, Le Kremlin-Bicêtre, France, Paris, France, [3]CIC- Biotherapy, Groupe Hospitalier Universitaire Ouest, INSERM/AP-HP, Paris, France

Ex vivo gene transfer into hematopoietic CD34+ cells provides therapeutic benefits in primary immunodeficiencies. However, the CD34+ cell population is heterogeneous and is mainly composed of progenitors (CD34+lin-CD38+) and multilineage progenitors (CD34+lin-CD38-). This latter population can be divided in 3 subtypes regarding to the CD45RA and CD90 expression. CD45RA+ define cells with lymphoid potential, CD45RA-CD90- defined cells with myeloid potential and CD45RA-CD90+ define cells with long term repopulating capability (HSCs) which constitutes the relevant target for gene correction. We found that in bone marrow (BM) CD34+linneg, addition of CD133 helps to delimitate CD38neg population and we have analysed HSC, MLP and MPP population in BM from SCID-X1 (n=4), Wiskott Aldrich Syndrome (WAS) (n=2), Artemis (n=2), Chronic Granulomatous Disease (CGD) patients (n=3), sickle cell disease (SCD) (n=3) and age matched healthy controls (n=6).As expected, mean percentage of HSC represent 1% of CD34+ in healthy donors and similar results are obtained in CD34+ from SCID-X1 (1.76% ± 0.72), Artemis (1.98% ± 0.15), and SCD (2.16% ± 0.6) patients. On the contrary HSC percentage are lower in CD34+ from WAS (0.35% ± 0.15) and nearly undetectable in CGD patients. Moreover in multilineage progenitors, proportion of HSC, MLP and MPP varies from one pathology to another as shown in table1.

Table 1: Mean percentage of HSC, MLP and MPP in the linnegCD133+CD38neg CD34+ cells

	HSCs	MLP	MPP
Healthy Donor	42%	33%	24%
SCIDX1	37%	45%	28%
WAS	35%	35%	30%
Artemis	30%	48%	20%
SCD	36%	19%	44%

Our aim was then to determine the ability of vectors used for gene therapy to transduce these different progenitors. We first analysed transduction of bulk CD34+ and HSCs by SIN retroviral vector for IL2RG in 2 SCIDX-1 patients enrolled in the clinical trial. After transduction, the 3 cell types were purified by flow cytometry and transduction determined in CFU after 14 days of culture in methyl cellulose in order to avoid detection of non-integrated vector. We show that in both patients, transduction efficiency was lower in HSCs than in bulk CD34+ cells. We were not able to recover enough CFU to determine VCN from MPP fraction and as expected, no CFU were obtained from MLP fraction. These results will be extended to transduction of CD34+ from WAS and SCD patients and efforts will be concentrated on transduction protocols in order to improve transduction of HSCs. Nevertheless low HSCs contents observed in CD34+ from CGD patients also represents a major difficulty to obtain long term reconstitution after gene therapy.

690. Development of a Clinical Lentiviral Vector for Gene Therapy of SCID-X1

Sabine Charrier[1], Valentina Poletti[1], Samia Martin[1], Bernard Gjata[1], Matthias Hebben[1], Alban Vignaud[1], Fang Zhang[2], Karen Buckland[2], Michael Rothe[3], Axel Schambach[3], Bobby Gaspar[2], Adrian Thrasher[2], Fulvio Mavilio[1]

[1]Genethon, Evry, France, [2]University College London, London, United Kingdom, [3]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany

X-linked severe combined immunodeficiency (SCID-X1) is caused by mutations in the gene encoding the interleukin-2 receptor γ chain (*IL2RG*), and is characterized by profound immunological defects caused by a partial or complete absence of T and NK cells and the presence of non-functional B cells. To overcome the safety issues raised by the use of MLV-based retroviral vectors in previous gene therapy clinical trials, we designed a SIN lentiviral vector (LV) carrying the codon-optimized human *IL2RG* cDNA under the control of the human EF1αS promoter and a mutated WPRE. Replacement of the native IL2RG open reading frame by a codon-optimized sequence resulted in a 3-fold increase in mRNA expression and a 1.5-fold increase in IL2RG protein expression per integrated vector copy. The vector was VSV-G-pseudotyped and produced by a new manufacturing process based on quadri-transfection of suspension-adapted 293T cells grown in serum-free conditions in 50- to 200-L bioreactors, purified by ion-exchange chromatography and concentrated by tangential-flow filtration. The efficacy of this vector was demonstrated *in vitro* by the restoration of a normal level

of IL2RG mRNA or protein in a human IL2RG-deficient T-cell line at a VCN of 1 to 3 and by high efficiency (81±7%) transduction of human mobilized CD34$^+$ hematopoietic stem/progenitor cells with no impact on viability or clonogenic capacity. A biosafety evaluation study of the IL2RG LV in the murine model of the disease showed biodistribution of the transgene in hematopoietic organs only, restoration of T, B and NK cell counts, normalization of lymphoid organs (thymus and spleen) and a low frequency of hematopoietic malignancies, comparable to that of untreated animals. An in vitro assay (IVIM) showed a safe genotoxic profile, while insertion site analysis in transplanted mice revealed a standard lentiviral integration profile and no signs of clonal dominance. These studies will enable a multicenter phase-I/II clinical trial aimed at establishing the safety and clinical efficacy of lentiviral vector-mediated gene therapy for SCID-X1.

691. Abstract Withdrawn

692. Genome Editing for Personalized Gene Therapy of IVSI-110 Beta-Thalassemia

Petros Patsali[1,2], Claudio Mussolino[3], Constantinos Loucari[1,4], Coralea Stephanou[1,2], Michael Antoniou[2], Toni Cathomen[3], Carsten W. Lederer[1,4], Marina Kleanthous[1,4]
[1]Molecular Genetics Thalassemia, Cyprus Institute of Genetics and Neurology, Nicosia, Cyprus, [2]King's College London, London, United Kingdom, [3]Institute for Cell and Gene Therapy, University Medical Center Freiburg, Freiburg, Germany, [4]Cyprus School of Molecular Medicine, Nicosia, Cyprus

Thalassemia is amongst the commonest single-gene disorders worldwide, caused by deficient production of α- or β-globin. Of particular clinical relevance is β-thalassemia, which as a severe monogenic disease of the hematopoietic system is an ideal target for gene therapy, either by gene addition or gene correction. Problems inherent to the gene-augmentation approach for β-thalassemia, including insertional mutagenesis or low expression of the therapeutic transgene, may be avoided using targeted gene correction of mutations with site-specific designer nucleases, such as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 or transcription activator-like effector nucleases (TALENs). Our study is focused on the development of a personalized gene-correction therapy of the common β-thalassemia mutation, HBB$^{IVS1-110}$, which in most Mediterranean and many Western countries has a frequency of above 20% (with 80% on the island of Cyprus) amongst β-thalassemia carriers. HBB$^{IVS1-110}$ creates an abnormal splice-acceptor site in intron I of the β-globin gene, leading to a pre-mature in-frame stop codon in the aberrantly spliced mRNA and to early transfusion dependence of homozygotes. We have developed and evaluated designer nucleases targeting the site of the HBB$^{IVS1-110}$ β-thalassemia mutation, including direct comparison of targeted disruption efficiency and toxicity for CRISPR/Cas9 and TALEN tools in human embryonic kidney cells and in murine erythroleukemia (MEL) cells carrying a HBB$^{IVS1-110}$mutant transgene based on the GLOBE (MA821) vector. Using the latter, we have assessed the therapeutic efficiency and cleavage properties of these designer nucleases. Analyses included immunoblots, absolute quantification of correctly and aberrantly spliced HBB mRNAs using multiplex RT-qPCR, sequencing and characterization of the resulting genome-editing events. We noted superior performance of several TALEN pairs compared to a single CRISPR/Cas9 nuclease suitable for the target site. Moreover, we demonstrate a significant increase of correct splicing and β-globin chain synthesis for genome-edited transgenic MEL MA821 HBB$^{IVS1-110}$ cells. For the two most efficient TALEN pairs, β-globin protein levels in pools of edited cells reach over 20% of that detected in MEL cells harbouring a wild-type MA821 HBB control, up from 1% for the mock-treated MA821 HBB$^{IVS1-110}$ control. Our findings validate our approach and indicate its suitability also for the correction of other intronic disease-causing mutations.

693. Single Cell Dynamics Causes Pareto-Like Effect in Stimulated T Cell Populations

Alice Moussy[1], Jérémie Cosette[2], Fanny Onodi[3], Adrien Auffret-Cariou[3], Thi My Anh Neildez-Nguyen[1], Andras Paldi[1], Daniel Stockholm[1]
[1]Genethon/INSERM UMR_S951/EPHE, Evry, France, [2]Genethon, Evry, France, [3]Genethon/INSERM UMR_S951, Evry, France

The host's immune response is a frequent obstacle to successful gene therapy. The suppressive activity of regulatory T cells can be used to get round the problem. To do this, understanding the mechanism of proliferation and differentiation of CD4 + T cells is necessary. Here, we used time-lapse microscopy of individual murine CD4 + T cells to investigate the dynamics of proliferation and fate commitment. We observed highly heterogeneous division and death rates between individual clones resulting in a Pareto-like dominance of a few clones at the end of the experiment. Commitment to the regulatory T (Treg) fate was monitored using the expression of a GFP reporter gene under the control of the endogenous Foxp3 promoter. All possible combinations of proliferation and differentiation were observed and resulted in exclusively GFP-, GFP+ or mixed phenotype clones of very different population sizes. We simulated the process of proliferation and differentiation using a simple mathematical model of stochastic decision-making based on the experimentally observed parameters. The simulations show that a stochastic scenario is fully compatible with the observed Pareto-like imbalance in the final population. These observations may help to develop new strategies for the amplification of Treg cells for therapeutic use.

Immunological Aspects of Gene Therapy II: AAV Vectors

694. Anti-Transgene Cellular Immune Repsonses Can Be Induced by Subretinal Gene Transfer with rAAV in a Dose-Dependent Manner

Julie M. Vendomele[1], Quentin Khebizi[1], Mirella Mormin[2], Sabrina Donnou[1], Catherine Poinsignon[1], Anne Galy[1], Sylvain Fisson[1]
[1]Généthon, UMR_S951, Université d'Evry Val d'Essonne (UEVE), Evry, France, [2]Généthon, UMR_S951, Evry, France

From animal experiments to the first human clinical trials in 2007, recombinant adeno-associated virus (rAAV)-mediated ocular gene therapy has shown successful results which have been attributed in part to the immune-privileged situation of the eye. Recently, some ocular gene therapy clinical trials have reported that visual acuity returned to baseline 6 to 12 months after therapy. The involvement of anti-transgene immune responses may lead to loss of therapeutic efficacy. This prompted us to evaluate in a murine model if rAAV gene transfer leads to the expected immune ignorance of the transgene or perhaps to an immuno-modulatory mechanism already described with subretinal peptide injection (*eg*. Anterior Chamber Associated Immune Deviation / ACAID), or to anti-transgene immunization. In this study, we characterized the CD4 and CD8 T cell responses specifically directed toward the transgene product in a murine model following rAAV2/8-mediated subretinal gene transfer. An rAAV2/8 encoding for the GFP-HY fusion protein under the phosphoglycerate kinase (PGK) promoter was used. The transgene expresses the HY male antigen which contains MHC class I and MHC class II-restricted T cell epitopes (UTY and DBY, respectively) that are immuno-dominant in female mice. For the study, 2μL of endotoxin-free,

PBS-formulated rAAV2/8 PGK GFP-HY were injected subretinally in C57Bl/6 female mice. At day 7, mice were challenged subcutaneously with the UTY and DBY peptides adjuvanted in CFA, and the immune response was analyzed at day 14 by IFNγ ELISpot, cytokine titration and proliferation assays. Our results revealed that: ***(i)*** The subretinal injection of 10E8 to 2.10E9 vg/mouse of rAAV2/8 PGK GFP-HY did not induce a significant HY-specific peripheral immune modulation in contrast to the ACAID obtained after subretinal injection of UTY and DBY peptides (50µg each); ***(ii)*** Higher doses of rAAV2/8 PGK GFP-HY (5.10E10 vg/mouse) triggered increased Th1 and Tc1 cellular immune responses against the transgene product in peripheral lymphoid organs. Lower doses of vector did not increase peripheral immune responses toward the transgene product following the challenge. We have thus far failed to demonstrate the induction of ACAID by rAAV2/8 subretinal gene transfer. We show that rAAV2/8 vector-mediated subretinal gene transfer is not necessarily ignored at the immunological level. High doses of vector can effectively trigger anti-transgene T-cell responses with the potential for elimination of transgene-expressing cells. Clearly, anti-transgene-specific immune monitoring should be refined at least in preclinical models, to improve the biosafety and the long-term efficacy of rAAV-mediated ocular gene transfer.

695. An AAV8 Mutant with Better Transduction in Murine Muscle and Nasal Airway Than AAV8

Qiang Wang[1], Peter Bell[1], Hongwei Yu[1], Christine Draper[1], Deirdre McMenamin[1], Maria P. Limberis[2], James M. Wilson[1]
[1]Gene Therapy Program, Department of Medicine, University of Pennsylvania, Philadelphia, PA, [2]Gene Therapy Program, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Adeno-associated virus (AAV)-based gene therapy is showing increasing promise, stimulated by encouraging results from clinical trials in recent years. To advance the platform, we have been exploring AAV capsid manipulation. By combining three mutations at hyper-variable regions (HVRs) I, IV, and VIII of the AAV8 capsid - mutations isolated from saturation mutagenesis followed by several rounds of *in vivo* selection - we created an AAV8 mutant, referred to as AAV8.Triple. *In vitro*, the AAV8.Triple mutant showed resistance to various antisera of monkey and human, as well as human IVIG (titers that are at levels 2 to 4 fold that of AAV8, with respect to human IVIG). All three mutations contributed to the observed resistance. In mice, the liver transduction efficiency of AAV8.Triple was reduced compared with AAV8, however its muscle transduction was higher than that of AAV8 by approximately 10 fold. In addition, AAV8.Triple demonstrated a higher heparin affinity than AAV8. Interestingly, reducing the positive charges of the HVR.IV mutation decreased the vector's heparin affinity while liver transduction was partially restored. Similar to the trend observed in muscle, intranasal administration of AAV8.Triple resulted in a transduction efficiency 2 to 3 fold greater than that of AAV8, which was further improved to levels approximately 10 fold greater than AAV8 by swapping the VP1 unique region of AAV8.Triple with that of another AAV serotype. Overall, our findings indicate that AAV capsid manipulation was able to generate an AAV8 mutant with improved transduction in both murine muscle and nasal airway compared with wild-type AAV8. These findings may be relevant to disease models where high-efficiency intramuscular or intranasal gene delivery and resistance to pre-existing neutralizing antibodies are desired.

696. TLR9 Signaling Mediates Transgene Antibody Formation

Scott Ashley, Deirdre McMenamin, Christine Draper, James M. Wilson
Gene Therapy Program, Department of Medicine, University of Pennsylvania, Philadelphia, PA

Recent success in gene therapy clinical trials have shown great promise towards developing efficacious treatments, however antibody formation to the therapeutic protein resulting in treatment attenuation still remains a concern. Driven by the immunologic stimuli of vector administration, transgene expression can elicit a humoral response against "non-self" protein, as these antigens were not presented during the process of central tolerance induction. Previously, we have shown that TLR9 signaling is necessary to mount a CD8+ T cell response that reduces expression of non-secreted transgene product. Here, we show that TLR9 signaling is also involved in driving the activation of B cells to secrete transgene-specific neutralizing antibody. Delivery of the viral vector and the effect of TRL9, route of administration, and promoter on the immune response to transgene expression was studied using the human IDUA gene, which is mutated in the lysosomal storage disorder, mucopolysaccharidosis type I (MPS I). IV administration of C57Bl/6 mice with AAV9.hIDUA under the control of a universal CMV promoter resulted in the production of neutralizing IgG antibodies and loss of transgene activity measured in serum. Surprisingly, when administered to TLR9 knockout mice, antibody formation was very modest to not detectable and IDUA activity maintained at a steady state. Next, we administered AAV9 vectors with expression driven by different promoters, including the universal promoters, CMV and CB7, and the liver-restricted promoter, TBG. Mice that received vectors containing universal promoters demonstrated elevated IgG to hIDUA, while those with liver-specific expression did not. Co-administration of the TBG- and CMV-containing vectors did not inhibit IgG formation, demonstrating that expression in non-liver tissue drives the immune system to a humoral response, and overcomes the potential tolerogenic effect of liver-specific expression. In ongoing experiments, hIDUA constructs containing CpG motifs that are known to bind efficiently to TLR9 will be used to assess the impact of TLR9 signaling on transgene-specific neutralizing antibody production. Overall, our results suggest that TLR9 signaling is important for the development of anti-transgene antibodies.

697. Phenotypic and Functional Characterisation of Human Anti-AAV CD8+ T Cells Using MHC Class I Tetramer-Associated Magnetic Enrichment

Celine Vandamme[1], Marie Devaux[1], Nicolas Jaulin[1], Mickaël Guilbaud[1], Johanne Le Duff[1], Leslie Hesnard[2], Hanane Djamai[1], Marc Bonneville[2], Philippe Moullier[1], Xavier Saulquin[2], Oumeya Adjali[1]
[1]Atlantic Gene Therapies, INSERM UMR 1089, Nantes, France, [2]CRCNA, INSERM UMR 892, Nantes, France

Introduction: Recombinant Adeno-Associated Vectors (AAV) represent nowadays the most largely used platform for *in vivo* gene therapy. Unfortunately, despite promising results in preclinical and clinical studies, pre-existing immunity against the viral capsid remains one of the major hurdles to the safety and efficiency of AAV-based gene transfer in Humans. Particularly, the impact of pre-existing anti-AAV $CD8^+$ T lymphocytes on gene transfer remains poorly defined. This gap in knowledge can, in part, be attributed to the scarcity of AAV-specific $CD8^+$ T cells among the peripheral blood mononuclear cells (PBMCs), and to the lack of relevant animal models. ***Experimental approach***: In order to set up a more sensitive

and comprehensive method to detect and characterise AAV-specific $CD8^+$ T cells among PBMCs, we used Tetramer-Associated Magnetic Enrichment (TAME) to analyse the frequency and phenotype of AAV-specific $CD8^+$ T cells by flow cytometry. To this end, we previously generated AAV-loaded tetramers using the UV-mediated peptide exchange technology. ***Results***: We were able to detect AAV8 and AAV2 capsid-specific $CD8^+$ T cells among PBMCs in all healthy donors tested (n>40), without any amplification, at frequencies ranging from 1.10^{-6} to 1.10^{-4} $CD8^+$ T cells. Phenotypic assessment of the detected cells revealed a small proportion of memory $CD45RO^+$ cells, a population that expectedly could have emerged after primary infection with wild-type AAV. Though AAV-specific $CD8^+$ T cells were detectable by TAME in all donors tested, only few of them (11/42) had a positive response when cellular responses in PBMCs were assessed by anti-AAV IFN-γ ELISpot assays. Interestingly, we registered higher *ex vivo* frequencies and more frequent positive ELISpot responses in HLA-B7 donors then in HLA-A2's. Evaluation of anti-AAV antibody and neutralizing factor titres in the sera of the same donors revealed no correlation between humoral and cellular responses, as has been previously described. To further the functional assessment of the detected cells, we sorted AAV8 capsid-specific $CD8^+$ T cells after TAME and expanded human primary T cell lines. Purity of the cell lines was checked using tetramer staining. We succeeded in generating several functional AAV-specific $CD8^+$ T cell lines that upregulated CD107 and secreted IFN-γ, TNF-α, Granzyme B and Perforin when faced with AAV8-loaded target cells. Interestingly, some cell lines seemed to display a lack of reactivity that could not be attributed to a generally compromised functionality, suggesting that a fine tuning of AAV-specific $CD8^+$ T cells' activation might come into play, and is still under investigation. ***Conclusions and Perspectives***: The dissimilarities observed *ex vivo* between humoral and cellular responses, as well as the different activation patterns registered *in vitro* highlight the need to gather multiple insights on capsid immunogenicity to better understand the onset of pre-existing anti-AAV immunity on recombinant AAV-based gene transfer and its impact on clinical outcome.

698. Effective Depletion of Pre-Existing Anti-AAV Antibodies Requires Broad Immune Targeting

Haiyan Fu[1], Aaron S. Meadows[1], Victoria Velazquez[2], Marybeth Camboni[1], Ricardo Pineda[1], F. Jason Duncan[1], Christopher M. Walker[2], Douglas M. McCarty[1]

[1]Center for Gene Therapy, Research Institute at Nationwide Children's Hospital, Columbus, OH, [2]Center for Vaccines and Immunity, Research Institute at Nationwide Children's Hospital, Columbus, OH

Pre-existing antibodies (Abs) poses a critical challenge for the translation of gene therapy approaches using viral vectors, especially AAV, which is widespread in humans. It is known that very complex anatomical niches, molecular signals and cellular components are required for the development, priming and survival of Ab producing plasma cells (PCs), and the maintenance of Ab production. We therefore hypothesize that overcoming pre-existing Abs requires broad immune targeting. In preliminary studies, we generated a mouse model of pre-existing AAV9-Abs by an IP injection of 1e10vg rAAV9 vector, and tested multiple immunosuppressants (IS), including proteasome inhibitor Bortezomib (Bort), m-TOR inhibitor rapamycin (Rap) and the classical broad IS agent prednisolone (Pred), individually or in combination at routine clinical doses for humans. As a result, we have identified an effective approach combining Rap and Pred, that led to significant reduction in serum AAV9-Abs, by 70-80% at 4wk and 85-93% at 8wk of treatment. Flow cytometry and ELISpot showed that Rap+Pred treatment led to significant decreases in bone marrow B cell and PC frequency, and in the frequency of IgG-secreting and AAV9-specific Ab-producing PCs. Splenocyte analyses of Rap+Pred treated mice showed significantly reduced frequencies of class-switched (IgD^-IgG^+) B cells, and of $CD4^+$ T cells expressing the co-inhibitory receptor programmed death 1 (PD-1). While the mechanisms remain unclear, our data indicate that Rap+Pred may have selective inhibitory effects on both T helper type 2 support of B cell activation in the spleen, and on PC survival in the bone marrow, leading to effective depletion of pre-existing AAV9-Abs. This promising immunomodulation approach for depleting pre-existing Abs is highly translatable to clinical applications. Ongoing efforts are to identify the optimal Rap+Pred regimen for maximal effect and minimal risk of potential side effects.

699. Effective AAV9 Vector Delivery to Nasal Mucosa for Protection Against Airborne Challenge with Influenza A and B

Marco Crosariol[1], Ghazal Stity[1], Omua Ahonkhai[1], Erik Wielechowski[1], Angelica C. Medina-Jaszek[1], Arbans Sandhu[1], Julie Johnston[1], Martin Lock[1], Anna Tretiakova[1], James M. Wilson[1], Maria P. Limberis[2]

[1]Gene Therapy Program, Department of Medicine, University of Pennsylvania, Philadelphia, PA, [2]Gene Therapy Program, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

To transition the AAV-mediated passive immunization strategy for protection against influenza A and B from mice to humans, an efficient vector delivery strategy is needed. Here, we demonstrate the ability of AAV9 expressing anti-influenza A and B antibodies (FI6 and CR8033, respectively) coupled with an intranasal mucosal atomization device (IMAD) to effectively protect mice against challenge with pandemic and seasonal influenza. First, we determined that an equimolar combination of AAV9.FI6 and AAV9.CR8033 vectors delivered intranasally at a dose of $5x10^{10}$ GC/KG per mouse effectively protected mice against lethal intranasal challenge with influenza A (PR8) or influenza B (B/Lee/40), respectively. In an effort to progress AAV9 into the clinic, we next determined vector compatibility with the IMAD for loss in volume, vector titer and vector potency. Groups of mice were intranasally administered a mixture of AAV9.FI6 and AAV9.CR8033 as well as a mixture of AAV9.FI6 and AAV9.CR8033 that was processed through the IMAD. Seven days later, the mice were challenged with 5LD50 of PR8 or B/Lee/40. No difference in effectiveness of protection against influenza A or B was observed when the vector mixture was processed through the IMAD. We next translated this approach to the nose of macaques, a model more closely related to the human nose that is the target tissue. In this model, we assessed the kinetics of the onset of transgene expression and stability of expression when vector was delivered via the IMAD compared with traditional, direct liquid delivery. At the conclusion of the study, vector biodistribution was also examined. Overall, no significant differences in the kinetics and profile of gene expression or vector biodistribution were observed between the two delivery methods. In conclusion, we demonstrate the safety and effectiveness of AAV-mediated prophylaxis against influenza A and B when vectors are delivered via the IMAD, an easy-to-use device that localizes transduction to the site of influenza infection, the nasal mucosa.

700. Successful Repeated Hepatic Gene Delivery in Mice and Non-Human Primates Achieved by Sequential Administration of AAV5 and AAV1 Vector Serotypes

Anna Majowicz[1], David Salas[2], Nerea Zabaleta[2], Estefania Rodríguez-Garcia[2], Gloria Gonzalez Aseguinolaza[2], Harald Petry[1], Valerie Ferreira[1]
[1]Research, uniQure N.V., Amsterdam, Netherlands, [2]Gene Therapy and Regulation of Gene Expression Program, CIMA, University of Navarra, Pamplona, Spain

The major challenge in AAV-based gene therapy is the presence of circulating neutralizing antibodies (NAB) against AAV vector capsids. NAB can be present in patient's blood prior to AAV treatment due to naturally acquired infections with the wild type AAV virus (pre-existing NAB). Anti-AAV NABs are also raised after first administration of AAV in the course of gene therapy treatment. There is a need to develop strategies that would permit a repeated AAV gene delivery not only to be able to treat the patients that have pre-existing NAB but also for the patients previously treated with recombinant AAV, that might experience overtime a decrease in therapeutic protein expression due to the natural turnover of transduced cells. To address those issues, we explored the feasibility of using the AAV5 and AAV1 serotypes for repeated, liver-targeted gene delivery in non-human primates (NHPs). Sequential AAV-based gene delivery with AAV5 and AAV1 proved to be successful as an expression of the two reporter transgenes used in the study was observed (hSEAP and hFIX). In contrast, the re-administration of the same serotype (AAV5-hSEAP followed by AAV5-hFIX) was unsuccessful due to the total inhibition of secondary AAV5 transduction by anti-AAV5 NAB. In order to determine the long-term stability of transgenes activity/expression, the NHP cross administration experiment was continued up to 1 year after primary injection (AAV5- hSEAP or PBS). All NHPs cross administered with AAV5-hSEAP followed by AAV1-hFIX showed hSEAP and hFIX protein expression in plasma. In a long-term follow-up, decreases of transgenes activity/expression were observed in 5 out of the 12 NHPs injected. The reductions of transgenes activity/expression were associated with specific immune responses directed against hSEAP or hFIX. Interestingly, DNA and mRNA levels in the animals presenting transgene directed immunity were comparable to those observed in the other animals that did not lose the transgene activity/expression. Additionally, fluorescent in situ hybridization (FISH) was performed on the liver sections of the animals to localize AAV vector DNA and transgene mRNA. In summary, our data demonstrates that a successful re-administration can be achieved in AAV-based gene therapy when combining AAV5 and AAV1 in NHPs.

701. Successful *In Vivo* Re-Administration of AAV with the Use of Two-Step AAV Injection

Anna Majowicz, Harald Petry, Valerie Ferreira
Research, uniQure N.V., Amsterdam, Netherlands

Introduction: The major challenge for a successful re-administration of AAV vectors is the presence of neutralizing antibodies (NABs) directed against AAV developed after the first administration of AAV vectors. Those serotype-specific neutralizing antibodies directed towards the viral capsid proteins prevent efficient gene transfer with AAV of the same serotype. The generation of a humoral immune response does not permit the use of "vector of choice" more than once which is a concern for life-long disorders for which re-administration has to be considered. Currently different methods are being explored in order to circumvent the presence of anti-AAV NABs at re-administration. One of those methods is based on the principle of antibody adsorption in AAV-based gene delivery. It was first observed in mice by Scallan et al. who reported that the administration of AAV vectors in the presence of empty AAV capsids significantly reduced the neutralization of AAV vectors by anti-AAV circulating NAB (Scallan et al. 2006). Additionally, Mingozzi et al. have also shown in mice and non-human primates that injection of therapeutic AAV vector together with empty AAV capsids allows liver transduction in the presence of high titers of anti-AAV NABs (Mingozzi et al. 2013). Goal: The goal of our study was to decrease the anti-AAV NABs to a level that would allow successful repeated gene delivery with the same AAV serotype. Study design and results: We have designed an approach to decrease the levels of circulating NABs *in vivo* by mean of anti-AAV NABs adsorption. In our study, for the re-administration of AAV in the presence of anti-AAV NABs, a two-step AAV vector injection was used. The first dose of AAV vector was used as a "decoy" AAV that captured circulating anti-AAV NABs. To allow a proper binding of circulating anti-AAV NABs by "decoy" AAVs, we delayed the second AAV injection. In our proof of concept experiments, mice were initially immunized with AAV5-GFP. Two weeks later the re-administration of AAV5 was performed. The re-administration procedure consisted of a first "decoy" AAV5-GFP injection followed by an injection with AAV5-hSEAP. In a first experiment, mice were injected with a "decoy" AAV5-GFP followed by AAV5-hSEAP at 30, 60 or 120 minutes after the first injection. A significant decrease of total anti-AAV5 antibody levels after injection of the "decoy" AAV5-GFP vector was observed. In the second study, we used a similar procedure with a 30 min time frame between "decoy" AAV5-GFP and AAV5-hSEAP injection, using three different doses of the "decoy" AAV5-GFP. The decrease of total anti-AAV5 antibodies 30 min after "decoy" AAV5-GFP injection was inversely proportional to the dose of the "decoy" AAV5-GFP used and to the hSEAP activity levels that were achieved after AAV5-hSEAP injection. Conclusions: Based on hSEAP transgene activity levels, we were able to determine a cut-off level of anti-AAV5 antibodies that allow a successful re-administration of the AAV5. Overall, the two-step AAV injection approach as such is promising for AAV re-administration.

702. Detection and Isolation of Immune Cells After Intramuscular AAV Injection in Mouse and Human

Gwladys Gernoux, Alisha Gruntman, Terence R. Flotte, Christian Mueller
Gene Therapy Center, UMass Medical School, Worcester, MA

Recombinant adeno-associated viral (rAAV) vectors are promising tools for gene transfer to the skeletal muscle. However, studies on mouse and large animal models as well as humans demonstrate that intramuscular (IM) rAAV delivery can trigger immune responses to AAV capsid and/or transgene. Recently, IM delivery of rAAV1 in humans induced a tolerogenic response to rAAV characterized by the presence of capsid-specific regulatory T cells (Tregs) in the periphery. The aim of our study was to develop an assay to allow detection and characterization of immune cells *in situ* after AAV gene transfer. Mice were injected intramuscularly with an AAV vector. Seven days after injection, muscles were collected and enzymatic dissociation was performed. Our results show that we are able to detect immune cells (monocytes/macrophages, lymphocytes) in mouse muscle by flow cytometry. This assay was also used on a human muscle biopsy collected 5 years after AAV1-AAT gene transfer and revealed the presence of infiltrated Tregs and CD8+ T-cells at the site of injection. This method appears as a new tool in understanding immune responses in the muscle after AAV gene transfer.

Vector and Cell Engineering/Manufacturing II

703. Development of RD-MolPack-trLNGFR/dCK.DM.S74E Stable Packaging Cells

Claudia Piovan[1], Erica Giuliani[1], Stefano Corna[1], Cinzia Scavullo[1], Sergio Bossi[1], Monika Pema[1], Jeffrey A. Medin[2], Monty McKillop[2], Bryan Au[2], Claudio Bordignon[1], Gian Paolo Rizzardi[1], Chiara Bovolenta[1]
[1]MolMed S.p.A., Milano, Italy, [2]Medical College of Wisconsin, Milwaukee, WI

The efficacy of several clinical approaches of adoptive T cell therapy (ACT) can be compromised by the insurgence of severe unfavorable events as graft-versus host disease (GvHD) secondary to bone marrow transplantation unless *ex-vivo* manipulation endow transplantable T cells with safety system. Introduction of drug-induced suicide genes, as for example the herpes simplex virus thymidine kinase (HSV-TK) transgene in T cells during bone marrow transplantation for leukemia and lymphoma treatment, represents a valid approach to control gene-modified cell proliferation and survival overtime. Transplanted modified cells are selectively deleted *in vivo* by specific activation of an otherwise inactive prodrug in those cells. Despite the well-documented utility of this approach, some limitations arose in terms of poor prodrug activation kinetics, escape from drug selection and immunological rejection.Engineered variants targeting the active site of the human deoxyCytidine Kinase (dCK) suicide enzyme were described as a powerful alternative to current strategies being able to activate a large variety of nucleoside analogue-based prodrugs, having low immunogenicity and permitting in vivo imaging of genetically modified cells. Lentiviral vector (LV)-mediated delivery of the triple mutant dCK.R104M.D133A.S74E (dCK.DM.S74E) fused to the truncated low density nerve growth factor receptor (LNGFR) selection marker renders human cells highly sensitive to the prodrugs bromovinyl-deoxyuridine (brivudine or BVdU), l-deoxythymidine (LdT), and l-deoxyuridine (LdU) via induction of apoptosis. MolMed has developed the proprietary RD-MolPack technology for the stable production of both second and third generation LVs, pseudotyped with the RD114-TR envelope that, in contrast to inducible systems based on VSV-G envelope, permits the generation of a simple, constitutive, no-toxic LV packaging system. We thus exploited the RD-MolPack technology to obtain several colonies of RD-MolPack-trLNGFR/dCK.DM.S74E producer cells by stable transfection of a SIN transfer vector plasmid carrying the trLNGFR/dCK.DM.S74E transgene followed by zeocin selection. The average titer calculated on CEM A3.01 target cells of several colonies is $1.9 \times 10^5 \pm 8.15 \times 10^4$ TU/10^6 cells/day, in line with the previous results obtained with the previously established RD2-MolPack-Chim3 and RD3-MolPack-GFP producer cells. These preliminary data suggest that the human suicide gene trLNGFR/dCK.DM.S74E can be constitutively produced in LV stable packaging cells without toxicity and its use can be therefore considered a valid option in the scenario of prodrug convertase enzymes.

704. Enhanced Lentiviral Production Through Rational Design of Mammalian Host Cells

Jean-Francois Gelinas, Deborah R. Gill, Stephen C. Hyde
Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom

The development of lentiviral-based therapeutics is hindered by the high costs of cGMP manufacturing, which is a particular concern for applications focused on *in vivo* delivery where both high titres and volumes are typically required. The key cost component of viral vector manufacturing is the production titre, which is typically several log-orders lower for lentiviral vectors than for non-enveloped vectors such as rAd or rAAV. Some progress has been made towards the generation of producer cell lines to reduce costs. However, the majority of effort to increase lentiviral production has focused on optimisation and scale-up of transient transfection-based processes, including: the selection of efficient gene transfer reagents, the ratio of plasmid DNA components, the composition of cell culture media, supplementation with growth substrates and bioreactor growth conditions (e.g. pH, O_2, lactate, etc). Intriguingly, little attention has been paid to the optimisation of the mammalian host cell, with nearly all reported processes relying on commonly available and, readily transfectable, HEK 293T derivatives. We hypothesised that HEK 293T cells were not necessarily optimal for lentiviral production and have embarked on a rational design process to establish cell lines with enhanced manufacturing properties. General reviews of the HIV infectious life cycle typically describe only four host factors that limit lentiviral replication (APOBEC3G, SAMHD1, Tetherin and TRIM5α). We conducted an extensive literature review and identified a further 130 putative restriction factors active in the late phase of the lentiviral life cycle (from transcription to maturation) that are possibly relevant to lentiviral vector production. We have evaluated the effect of siRNA knock-down of these genes (3 days production, 2e5 HEK 293 cells/well, 100 nM siRNA/well in quadruplicate) on lentiviral vector production titre (3 days transduction, 5e4 HEK 293 cells/well, eGFP detection via Perkin Elmer Operetta) in a high-throughput 96-well format. Control studies with a non-target siRNA revealed that the assay variability was less than 0.2-fold (n=40). We showed that siRNA knockdown of the four canonical host restriction factors had no significant effect on lentiviral production (1.06 to 0.68-fold, n=4-23, $p > 0.05$). We identified 9 host factors, the knockdown of which significantly increased lentiviral production by 1.4 to 2.1-fold (threshold > 2 SD over control; $p < 0.05$, power > 80%). To extend the utility of these findings we are employing CRISPR/Cas9-mediated genetic disruption of these host factors. We anticipate that permanent disruption, either singly or synergistically in combination, will yield novel mammalian cell lines with a greater ability to support lentiviral production, which will be of considerable value to ongoing efforts to reduce lentiviral production costs and increase the speed of clinical development.

705. Enhancement of CAR Expression of *piggyBac* Transposon-Engineered T Cells by Stimulation with Viral Antigens

Daisuke Morita[1], Miyuki Tanaka[1], Nobuhiro Nishio[2], Nozomu Kawashima[3], Yusuke Okuno[2], Matthew H. Wilson[4], Cliona M. Rooney[5], Yoshiyuki Takahashi[3], Yozo Nakazawa[1]
[1]Department of Pediatrics, Shinshu University School of Medicine, Matsumoto, Nagano, Japan, [2]Center for Advanced Medicine and Clinical Research, Nagoya University Hospital, Nagoya, Aichi, Japan, [3]Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan, [4]Vanderbilt University School of Medicine and VA Tennessee Valley Health Care, Nashville, TN, [5]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

Introduction We previously showed that the *piggyBac* transposon system is a promising genetic tool for stable, non-viral gene engineering of primary human T cells. Our initial culture strategy for expanding *piggyBac*-modified CD19 CAR-T cells required magnetic cell isolation due to low CAR expression and 21 days of culture and our CAR included an IgG1-Fc spacer that may limit CAR-T cell expansion *in vivo*. We have now improved these process and construct to avoid magnetic cell isolation, shorten the culture period, and increase *in vivo* efficacy. **Methods** We used a 4D-Nucleofector device to electroporate 1×10^7 peripheral blood mononuclear cells from 9 healthy donors with a CD19.CD28.ζ−CAR transposon plasmid, with

or without an IgG1-CH2CH3 spacer and a *piggyBac* transposase plasmid. We compared 2 new culture conditions with our original method for expansion of CD19 CAR-T cells. All cells were cultured in serum-free medium (TexMACS) containing IL-7 and IL-15 in 24-well plates. Electroporated cells were immediately transferred to irradiated autologous activated T-cells (ATCs), either pulsed with 4 viral peptide pools (PepTivator; AdV5 Hexon, CMV pp65, EBV EBNA-1, and BZLF1) (ACE), or unpulsed (non-ACE). The next day, non-ACE cells were transferred to CD3 and CD28 antibody-coated plates for 5 days. In the original method, electroporated cells were transferred to CD3/CD28-coated plates one day after electroporation without ATCs. On day 7, all cells were transferred into G-Rex10 culture flasks with ACE-pulsed or unpulsed irradiated ATCs or no ATCs for ACE, non-ACE and original cells respectively. On day 14, we collected CAR-T cells from all conditions. **Results** We obtained $4.7 \pm 3.0 \times 10^7$ ACE CAR-T cells, $6.7 \pm 2.1 \times 10^7$ non-ACE CAR T cells, and $5.3 \pm 2.3 \times 10^7$ original CAR T cells after 14 days of culture. ACE CAR-T cells showed significantly higher expression of the CAR transgene (33.0 ± 9.7% and >50% for CD19.CAR with and without the CH2CH3 spacer respectively) than non-ACE (10.7 ± 7.8%) or original CAR-T cells (4.6 ± 3.2%). All CAR-T-cells inhibited the growth of CD19+ tumors (95%, 60% and 51% for ACE, non-ACE and original CAR-T at an E:T ratio of 1:50; ACE CAR-T cells contained 29.6 ± 17.3% CD4+ cells and 64.3 ± 16.8% CD8+ cells, with 79.9 ± 5.9% cells co-expressing CD45RA and CCR7 and 62.6 ± 20.9% cells co-expressing CD45RO and CD62L. In an *in vivo* mouse model, ACE CAR-T cells delayed tumor growth by 30 days compared to no T-cells. **Conclusions** We markedly increased CAR expression from *piggyBac*-mediated CD19 CAR-T cells without cell selection, reducing the culture period to 14 days using viral antigen stimulation. Deletion of IgG1-Fc spacer led to further increase of CAR expression. Together with the simple manufacturing process and cost-effectiveness of DNA transposon technology, *piggyBac*-based gene transfer provides an alternative to retro- or lentiviral gene transfer for CAR T-cell therapy.

706. Consistent Viral Vector Manufacturing for Phase III Using iCELLis 500 Fixed-Bed Technology

Minna Karhinen[1], Elisa M. Nurminen[1], Piia Valonen[1], Outi Rautsi[1], Vesa Huusko[1], Kati Heikkilä[1], Janne Weisell[1], Achim Muller[1], Hanna P. Lesch[1], Outi Närvänen[1], Minna M. Hassinen[1], Minna Pollen[1], Steve Parrish[1], Robert Shaw[1], Nigel R. Parker[2], Seppo Ylä-Herttuala[3]

[1]Finvector Vision Therapies, Kuopio, Finland, [2]FKD Therapies, Kuopio, Finland, [3]AIV Institute for Molecular Science, University of Eastern Finland, Kuopio, Finland

Single use disposable technology faces further challenges in producing viral vectors in sufficient large quantities especially where adherent cells are needed. Scaling up the adherent system has proven to be troublesome. The PALL iCELLis® disposable fixed-bed bioreactors offer a novel option for viral vector manufacturing in large quantities in an adherent environment. We have made process development in iCELLis Nano, where the cultivation area varies between 0.53 - 4 m^2, after which we went forward to iCELLis 500, where the cultivation volume can be upgraded to 500 m^2 (66 - 500 m^2). iCELLis 500 has proven to be ideal to satisfy upstream demand and large-scale downstream purification process was developed to supply high quality recombinant adenovirus based gene products in our fully-licensed GMP manufacturing facility for pre-clinical and clinical trials The process is initiated by HEK293 cell mass expansion in suspension mode using Biostat® CultiBag RM (Sartorius Stedim Biotech S.A.) bioreactor. The expanded suspension cell mass is inoculated into iCELLis 500 for further expansion in adherent mode. This is infected by Working Viral Seed Stock and subsequent virus is released from the infected cells by chemical lysis. Downstream process contains Benzonase digestion, clarification, concentration and conditioning by crossflow ultrafiltration, capture and polishing by anion exchange chromatography, and final concentration and formulation is achieved using crossflow filtration. All product contact parts are fully disposable. Several batches have been produced with consistent results. Further validation of the process for commercial manufacturing is currently ongoing. We established a scalable, large-scale manufacturing process to supply high quality recombinant adenovirus based gene products in our fully-licensed GMP manufacturing facility for clinical trials. Alongside this we have validated assays which are providing a relevant quantitative measure of biologic function of the vector and demonstrating the quality and comparability of drug product batches in Phase 3 and commercial use.

707. Attenuated HIV Does Not Amplify in Primary Human T-Lymphocytes During a Model *Ex Vivo* Gene Therapy Procedure

Pratima Cherukuri[1], Scott Witting[2], Lilith Reeves[2]

[1]Indiana Univeristy, Indianapolis, IN, [2]Cincinnati Childrens' Hospital Medical Center, Cincinnati, OH

Current regulatory guidelines require RCL testing of lentiviral vector preparations used in human clinical trials. Cells transduced *ex vivo* and cultured four days or more must also be tested before administering to the patient. Attenuated HIV (R8.71) can be considered as a theoretical RCL that could develop from a recombination event with wild-type HIV. Importantly, R8.71 lacks accessory proteins (*vip, vif, vpr and nef*) that are known to be essential for viral replication in T-lymphocytes. Based on this fact, the risk of a recombinant RCL being amplified in T-lymphocytes during an *ex vivo* transduction and expansion protocol is questionable. To test this possibility, we transduced human T-lymphocytes with one of two RCL-negative lentiviral vector preparations (eGFP or a chimeric antigen receptor (CAR)) that were spiked or not with R8.71 or wild-type HIV. C8166 cells served as a positive control for cells permissive to R8.71. The transduction and subsequent expansion lasted 10 days total and the experiment was performed on two independent occasions. Using day 10 supernatants from all groups, a full RCL assay (with amplification and indicator phases) was performed. Product enhanced reverse transcriptase (PERT) and P24 ELISA assays served as readouts for the RCL assay. Transduction rates were determined by flow cytometry and found to be 57-60% and 10-37% for the eGFP and CAR vector, respectively. The RCL assay results showed that all T-lymphocyte groups exposed to R8.71 were negative but those exposed to wild-type HIV were positive. C8166 cells exposed to R8.71 or wild-type HIV were positive in the RCL assay. These results suggest that a recombinant RCL that possess the wild-type HIV envelope would NOT amplify in human T-lymphocytes during a model *ex vivo* transduction procedure. The amplification of an arguably more dangerous VSVG-enveloped recombinant RCL is still possible; however, it could be reliably detected by quicker and less expensive assays such as qPCR to VSVG sequences.

708. Process Development of Lentiviral Vectors for Large Scale Production Using a HEK293 Producer Cell Line

Alexandre Audy[1,2], Marc-André Robert[1,2], Parminder Chahal[2], Rénald Gilbert[2], Bruno Gaillet[1]

[1]Laval University, Québec, QC, Canada, [2]National Research Council, Montréal, QC, Canada

Lentiviral vectors (LVs) are promising vectors for gene therapy. Most often, they are used to deliver a functional copy of a gene to sustainably replace a defective or missing gene. However, processes for LVs must be improved to increase the yield, facilitate the scale

up and satisfy Health regulatory agencies. For these reasons, we have developed and optimized a LVs production process in serum-free medium using an inducible HEK293 producer cell line which possesses the capacity to grow in suspension culture. By adding two inducing molecules, (cumate and doxycycline) this cell line produces LVs pseudotyped with the protein G of the vesicular stomatitis virus without the need of any transfection. Our tested LV carried an expression cassette for GFP to facilitate LV quantification. To optimize the process, a design of experiment (DoE) was prepared which included the study of different culture media, high cell density production using six cell boosts commercially available and the addition of sodium butyrate, caffeine and valproic acid. We found that two cell boosts were outperforming the other cell boosts tested. At the present time, two commercial media (Hycell TransFx-H and SFM4TransFx-293 media) were our best candidates to maximize viral titer by achieving high cell density culture. In parallel, a LV carrying the cDNA for a shorter version of dystrophin (mini-dystrophin) was constructed. The truncated version of the dystrophin was produced by transient transfection in 293A cells and its presence was confirmed by western blot. We are planning to evaluate if the optimal conditions for the production of LV-GFP will be also applicable to LV-mini-dystrophin, a LV encoding a much longer transgene than GFP (0.7 kb vs 5.8 kb). This LV could be first evaluated for cell therapy in animal models and later, in patients suffering from Duchenne muscular dystrophy, where the dystrophin gene is defective and the protein is absent.

709. Characterization of Nanoparticles in Lentiviral Vector Preparations

Erik Hansen, Kristen Lee, Suzanna Melotti, Jesse Milling, Maya Miatkowski, James Warren, Mercedes Segura
Process Development Vector Analytics, Bluebird Bio, Cambridge, MA

The potential of lentivirus-based gene therapy vectors for the treatment of severe genetic diseases using genetically modified CD34+ cells and hematological malignancies using chimeric antigen receptor T-cells (CAR-T) is supported by recent positive data in clinical trials showing promising therapeutic benefits and safety. The progress from early-to-late stage clinical development requires enhanced characterization of the purified lentivirus vector product. Lentiviral vector preparations are complex in nature and contain a heterogeneous mixture of transduction-competent as well as transduction-deficient virus particles. In addition, lentiviral vector production utilizes host cells that can produce not only the viral particles of interest, but a variety of closely-related impurities that include exosomes and microvesicles. These cell-derived impurities can overlap key biophysical and biochemical attributes of the lentiviral vector, including size, net charge and composition, making them challenging to analyze. We used a variety of analytical tools to further characterize lentiviral vector preparations in terms of size distribution, particle counts and to determine the total particle (viral or non-viral) to infectious particle ratio. These tools included Nanoparticle Tracking Analysis (Nanosight), Field-Flow Fractionation coupled to Multi-Angle Light Scattering (FFF-MALS), and p24-ELISA. Particle protein composition was evaluated by several standard orthogonal protein quantitation assays. Characterization of nanoparticle subpopulations is important as it can support the refinement of manufacturing processes. The impact of variation of these parameters on lentivirus vector performance still remains largely unknown, so interpretations of the results must be carefully assessed and further study is warranted.

710. Surface Adsorptive Loss of rAAV on Materials Used in cGMP Manufacturing

Monica Jiang, Colleen M. Dupuis, Amy A. Medeiros, Samuel C. Wadsworth, K. Reed Clark, Christopher J. Morrison
Downstream Process Development, Dimension Therapeutics, Cambridge, MA

Continued interest in using rAAV vectors as therapies for a range of indications with varying prevalence often translates to increased manufacturing scales. A significant challenge that is encountered is the use of different manufacturing materials during the transition between early research and development and the clinical manufacturing scale. For example, manufacturing components, ranging from bioprocessing bags and hard plastics to bioprocess tubing and filters can be constructed of different materials and are not often engineered for use with rAAV. In this work we investigate the loss of rAAV to non-specific surface adsorption on various materials of construction. In addition, the effect of different background matrices was also investigated. These data demonstrate that surface adsorptive loss of rAAV can be significant, and that losses can vary significantly even for materials of similar construction. To mitigate adsorptive loss, several surfactants and other components were examined for their ability to reduce surface adsorption of rAAV. A number of these agents were identified as viable options for cGMP manufacturing. These results provide insight for the development of scaleable, more robust and higher yielding manufacturing processes to meet the continued demand for rAAV therapeutics.

711. Development of a Clinically-Acceptable Lentiviral Vector for Cystic Fibrosis Airway Gene Therapy

Alexandra McCarron[1], Chantelle McIntyre[2], Martin Donnelley[2], Patricia Cmielewski[3], David Parsons[3]
[1]School of Medicine, University of Adelaide, Adelaide, Australia, [2]Robinson Research Institute, University of Adelaide, Adelaide, Australia, [3]Respiratory and Sleep Medicine, Women's and Children's Hospital, North Adelaide, Australia

Background: Lentiviral (LV) vectors are a promising gene delivery vehicle for cystic fibrosis airway gene therapy. However, the development of a clinically-acceptable method suited to large-scale lentivirus production remains a barrier to the translation of gene therapy to the clinic. Current virus production methods have limited-scalability and often result in contamination of the vector preparation with potentially immunogenic components such as bacterially-derived plasmid DNA, and animal sera products. Accordingly, a scalable process for the production of clinical-grade vector is required.

Methods: LV vector preparations were treated with Deoxyribonuclease I (DNase I) to remove residual plasmid DNA, with the effectiveness measured by PCR using primers to detect the presence of the cytomegalovirus promoter sequence. The potential for scalable LV vector production in serum-free conditions was investigated by performing transient transfection of HEK 293FT cells adapted to grow in serum-free, suspension culture. To determine the efficiency of the virus production system RNA titering was performed using a commercial qPCR kit, and reporter gene expression of GFP and Luciferase were quantified using FACS and IVIS bioluminescence imaging.

Results: Plasmid DNA was detected in the vector preparations following DNase treatment, indicating that DNase I was ineffective at eliminating the residual plasmid DNA. Lentivirus was successfully produced using transient transfection of serum-free, suspension growing HEK 293FT cells, and was scaled-up to a one-litre culture capacity in shake flasks. Titres of up to 10^8 TU/mL were achieved in the suspension cell culture system, which is comparable to the yields obtained in the standard adherent culture system.

Conclusions: The protocol developed in this study for serum-free suspension culture virus production resulted in high vector titres that were comparable to those routinely achieved using adherent cells. Furthermore, relative to traditional virus production methods, this protocol is easily scalable, less labour intensive, and has less potential for contamination of the final vector preparation. DNase I treatment was ineffective at removing residual plasmid DNA from the vector preparation, indicating that enzymatic removal of plasmid DNA may not be a feasible approach.

Funding: The University of Adelaide; Cure4CF Foundation.

712. Challenges of Production of NanoPlasmid with Large Gene insert using the HyperGRO™ Fermentation Process

Neha Tiwari[1], Jill Beilowitz[1], Carlos Sampson[1], Dorothy Peterson[1], Aaron Carnes[2], Jim Williams[2]
[1]VGXI.Inc, The Woodlands, TX, [2]Nature Technology Corporation, Lincoln, NE

Plasmid based DNA vaccines are emerging as a promising alternative to traditional vaccines due to several advantages. One of these advantages is faster production of DNA plasmids using *E.coli* host cells. However, production of plasmids containing large inserts coding for antigen may have various challenges. Bacterial cell machinery may not be able to produce high cell growth during fermentation due to a large gene insert in the plasmid. A Nanoplasmid™ is a plasmid backbone designed by our collaborators, which contains reduced bacterial sequence and produces a smaller plasmid with the gene insert. With the years of expertise in Plasmid production, our company has successfully implemented the HyperGRO™ Fermentation process (an antibiotic-free, fed-batch system) for Nanoplasmid production. Plasmid pNano1, a Nanoplasmid™ with a gene insert of 3762bp, was produced using a modified HyperGRO™process. The fermentation process was successfully scaled up to 10L scale with high end cell density (OD_{600}) of 90.1 and volumetric yield of 0.696g/L.

713. Optimization and Scale-Up of a Manufacturing Process for Clinical-Grade Adenovirus-Based Vectors

Maria Fe C. Medina[1], Shabnam Shoaebargh[2], Natasha Kazhdan[1], Uma Sankar[1], David R. Latulippe[2], Brian Lichty[1]
[1]Pathology and Molecular Medicine, Robert E. Fitzhenry Vector Laboratory, McMaster Immunology Research Centre, McMaster University, Hamilton, ON, Canada, [2]Chemical Engineering, McMaster University, Hamilton, ON, Canada

We have developed a manufacturing protocol for clinical-grade Adenovirus-based vectors utilizing suspension 293 cells grown with chemically defined media in disposable spinner flasks. We have manufactured four batches of clinical-grade Adenovirus vectors utilizing this protocol, and some vectors have been administered in TB or cancer vaccine clinical trials in Canada. The maximum volume for a batch was from a 30-liter infected cell culture consisting of three lots with 10 liters. Each 10-liter lot was purified by cesium chloride density gradient ultracentrifugation and desalted by a Biogel column. The purified vectors were then pooled, filtered and filled into cryovials.

In an effort to increase manufacturing capacity, we have investigated the use of a Pall XRS-20 Bioreactor with a maximum cell culture capacity of 20 liters. We assessed cell growth kinetics/infection kinetics and found that at the minimum, we can achieve triple the maximum cell density compared with the use of spinner flasks. We have observed virus yields similar to what can be achieved with spinner flasks, and are currently optimizing the infection protocol to assess if virus yields can still be improved.

In order to address the scale-up concerns associated with the cesium chloride density gradient ultracentrifugation method, we are also developing a chromatography-based virus purification protocol. We are using a high-throughput test protocol to identify the critical process parameters (e.g. specific buffer species, pH, ionic strength) in resin binding experiments that achieve the required removal of impurities but also maintain immunogenic activity. A design-of-experiment strategy is being used to minimize the overall number of experiments.

714. Use of a Modular Workstation for Reduced Biosafety Risks Also Increased Cellular Yields

Alicia D. Henn, Shannon Darou, Randy Yerden
BioSpherix, Parish, NY

Cell culture processing in a room-air biologic safety cabinet (BSC), even in a cleanroom, creates multiple risks for both cell cultures and users. Humans can be exposed to genetically-modified cells, viruses, and other biological hazards during suboptimal (open) cell handling in a room air BSC. The cell cultures can also be exposed to suboptimal separation of air during cell handling in the BSC which creates a contamination hazard. In addition, even in the cleanest clean room, cells are exposed to room air temperatures and gas levels. Until cells re-equilibrate with the incubator after handling, cells are chilled, hyperoxic, and hypocapnic compared to physiologic conditions. A barrier isolator (closed cell processing workstation) creates a constant physical barrier between cells and users. This barrier reduces the risks of air mixing between cells and people without dependence upon laminar air flow in a BSC or a costly whole-room HVAC system. We undertook the following study to test the hypothesis that the use of a barrier isolator, during cell handling as well as incubation, could improve cell growth by providing unbroken physiologic conditions to cells. We used K562 cells, an undifferentiated leukemic cell line that has been used for T cell bioprocess optimization. We divided a single culture into two sets of triplicate cultures in T-75 flasks, three for growth in a barrier isolator and three for growth in a standard room-air incubator equipped with an inner chamber (C-chamber) for control of gasses. Both sets of cultures were incubated at 5%CO_2, 5%O_2, 37 C, and tracked for cell growth and viability over two weeks. For routine subculturing twice weekly, the cultures in the isolator were handled in the processing chamber of the isolator in physiologic conditions identical to the incubator. The cultures in the room-air incubator and C-chamber were handled in a standard room-air BSC (open). While cell viability was high in both sets of cultures, we found that handling cells under continuous physiologic conditions produced statistically higher cell yields over time ($p=0.00086$, Day 14, two-tailed paired T test, assuming unequal variances). No contamination events occurred and particle count data indicated no breach of functional separation between room air and isolator cell culture workspace. We concluded that cell handling in unbroken physiologically relevant conditions produced better cell growth over time while reducing risks to both cells and cell culturists. We predict that as cells become increasingly valuable, controlled workspaces for better cell growth and lower biosafety risks will become increasingly valuable as a part of a total quality approach to cell culture.

Clinical Protocol Development, Regulatory Interactions, and Ethics

715. Giant Axonal Neuropathy - The Role of Natural History Studies in Gene Transfer Therapy Trial Design

Diana Bharucha-Goebel[1,2], Minal Jain[3], Melissa Waite[3], Tanya Lehky[4], Reghan Foley[1], Jonathan D. Marra[5], Wadih Zein[3], Carsten G. Bonnemann[1]
[1]Neurogenetics Branch, NIH/ NINDS, Bethesda, MD, [2]Children's National Health System, Washington, DC, [3]NIH, Bethesda, MD, [4]NIH/ NINDS, Bethesda, MD, [5]Columbia University Medical Center, New York, NY

OBJECTIVE: Giant axonal neuropathy (GAN) is a rare childhood onset autosomal recessive neurodegenerative disorder affecting the central and peripheral nervous system. Mutations in the *GAN* gene cause loss of function of gigaxonin, a cytoskeletal regulatory protein, clinically leading to progressive sensorimotor neuropathy and neuropathy, reduced coordination, slurred speech, seizures, and progressive respiratory failure leading to death by the 2nd to 3rd decade of life. We are in the midst of a first-in-human intrathecal AAV9 mediated gene transfer trial for GAN. Given the ultra-rare nature of the disease, it is critical to build disease appropriate outcome measures and have lead in safety and outcome data to feed into the clinical trial. Our aim here was to identify and develop targeted quantitative markers of disease severity in GAN. METHODS: This natural history study evaluated measures of motor, neurophysiologic, and ophthalmologic function as well as exploratory neuroimaging markers in genetically confirmed GAN patients seen at the National Institutes of Health (NIH). The primary aim was to correlate a quantitative motor scale (MFM32) with a semi-quantitative Neuropathy Impairment Score (NIS) in cross sectional analysis of GAN patients. Secondary aims included evaluation of strength (myometry) and motor nerve amplitude (nerve conduction) compared to motor function (MFM32). Quantitative ophthalmologic testing included retinal nerve fiber layer thickness analysis correlated to visual acuity, MFM32, and motor nerve amplitude. Additional exploratory measures of spinal cord volume, brain DTI, quantitative MR spectroscopy, and biochemical (in serum and CSF) measures are underway. RESULTS: 15 GAN patients were evaluated at the NIH. Data analysis (Pearson correlation) showed the following: 1) NIS and MFM32 are significantly correlated ($p<0.0001$); 2) Muscle strength of knee extension, knee flexion, hip abduction, and hand grip each correlate significantly with MFM32 (p values from <0.0001 to 0.039) ; 3) Median motor nerve amplitude correlates significantly with MFM32 ($p=0.0016$). There was no significant correlation between elbow extension or elbow flexion strength and MFM32. Retinal nerve fiber layer corresponds strongly with MFM32 and the median motor nerve amplitude ($p<0.0001$ and $p=0.005$, respectively). CONCLUSIONS: The phase I intrathecal gene transfer study for GAN is a novel clinical trial, and will set a precedent by proof of principle for future gene transfer trials for neurodegenerative disorders. The primary outcome is safety, but given that the trial entails administration of a presumed effective dose, careful selection to outcome measures was essential in the protocol design. Through this natural history study, we have identified markers such as the MFM32, NIS, median motor amplitude, and retinal nerve fiber layer thickness, as well as spinal cord volumetric data that clearly correlate with disease progression. These markers are being followed longitudinally in natural history and were designed into the phase I trial. Such broad data capture allows for the incorporation of more targeted disease specific clinically meaningful outcome measures and data on natural rate of progression of such markers over short durations of follow up. Such models of trial design will be crucial to the development of genetic therapeutic trials for rare neurodegenerative disorders.

716. Abstract Withdrawn

717. Revising the Common Rule: Changes to the Federal Policy Protecting Human Subjects

Bambi Grilley
Baylor College of Medicine, Houston, TX

The protection of human subjects involved in research in the United States (U.S.) is based on the Belmont Report. The current regulations governing these protections were published in 1991 and are followed by 18 federal agencies. These regulations are referred to as the Common Rule. In July of 2011 the U.S. Department of Health and Human Services (HHS) issued an Advance Notice of Proposed Rulemaking (ANPRM) asking for public comment on proposed changes to the Common Rule. The comment period for the ANPRM ended in October 2011. In September 2015 HHS released a Notice of Proposed Rulemaking (NPRM) that included clarifications and modifications based on the feedback they received from the ANPRM. The NPRM also asked for public comment on the now modified proposed changes to the Common Rule. The comment period for the NPRM ended on January 2016. The NPRM document is quite long and detailed however a summary of the changes can be categorized into three major categories. The first category is increasing subject ability and opportunity to make informed decisions through modifications of requirements for informed consent documents. The second category is reducing the potential for harm and increasing justice for all subjects by increasing requirements for privacy protections inclusive of biospecimen research, expanding coverage of the protections to be inclusive of all clinical trials regardless of funding source, and expanding requirements for compliance with the Common Rule to all Institutional Review Boards (IRBs) inclusive of those not affiliated with an assurance holding institution. The third major category is facilitating current/evolving research that offers promising approaches by reducing ambiguity in the interpretation of the regulations, increasing efficiencies in the performance of the review system most specifically in the area of continuing review requirements and reducing burdens on researchers that do not appear to provide commensurate protections to human subjects (inclusive of re-defining exempt research categories and proposing a new research category identified as excluded research). Implementation of the Final Rule will be one year following publication allowing for voluntary application of provisions that provide regulatory flexibility to occur as early as 90 days after publication while other categories allow for implementation to occur as long as 3 years after publication. Many of the proposed changes relate to the use of biospecimens which will have significant impact on cell and gene therapy researchers. The regulatory community anticipates that the Final Rule will be released in 2016. If the Final Rule is released prior to the ASGCT meeting this presentation will focus on the Final Rule. If the Final Rule is not release prior to the ASGCT meeting this presentation will provide more in-depth information regarding the NPRM.

718. Cell Therapy Commercialization: An Assessment of Translational Barriers

David Pettitt[1], Georg Hollander[2], David Brindley[3]
[1]The Oxford – UCL Centre for the Advancement of Sustainable Medical Innovation (CASMI), University of Oxford, Oxford, United Kingdom, [2]Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom, [3]Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Science / Said Business School, University of Oxford, Oxford, United Kingdom

Cellular based therapies represent a platform technology within the rapidly expanding field of regenerative medicine and are distinct from conventional therapeutics - offering a unique approach to managing what were once considered untreatable diseases. Despite a significant increase in basic science activity within the cell therapy arena, alongside a growing portfolio of cell therapy trials and promising investment, the translation of cellular based therapeutics from "bench to bedside" remains challenging, and the number of industry products available for widespread clinical use remains comparatively low. This systematic review identifies unique intrinsic and extrinsic barriers in the cell based therapy domain. Key electronic databases were searched and manuscripts subjected to pre-defined inclusion and exclusion criteria. Two independent reviewers examined the retrieved publications, and performed data extraction. 3374 unique publications were identified. 138 of these qualified for full assessment and subsequent data extraction. A number of key themes were identified, enabling examination of current challenges and opportunities facing cell therapy development, including manufacturing, regulatory, reimbursement, ethical and clinical adoption issues. In addition to an up-to-date analysis of the current landscape, we discuss a number of pragmatic solutions to facilitate future development and translation.

719. Stability Could Be a Weak Point for Gene Therapy Sponsors

Dominique Kissick
Anges Inc, Bethesda, MD

So far, no gene therapy product has been approved by the Food and Drug Administration (FDA). One gene therapy for an extremely rare disease was approved in Europe in 2012, but questions about its effectiveness remain. Gene therapy refers to products that introduce genetic material into a person's DNA to replace faulty or missing genetic material, thus treating a disease or abnormal medical condition. There have been hundreds of trials of gene therapy in humans since 1990, and few gene therapies have reached advanced stages of development and have been submitted for authorization. Therefore, regulators have not yet worked out how best to assess gene therapy. While the same requirements as other medicinal products generally apply to the clinical development of gene therapies, regulators recognize that there might be cases where the principles might not apply and have issued guidance specific to gene therapy in order to facilitate product development and marketing approval. The shelf lives of gene therapy products may vary widely, depending on the nature of the product and its storage conditions. The design of stability testing should be based on a comprehensive understanding of the final product and its intended use. Testing should be based on real-time, real-temperature studies and should include a measure of product integrity, sterility, identity, purity, quality and other applicable assays. Additionally, potency assays should measure a relevant biological functionality either in vitro or in vivo. Interaction with the FDA however indicates that, beyond what is provided in the current guidelines, the regulators will likely scrutinize the results from the stability program for gene therapy during submission for marketing approval. Therefore, establishing shelf life and storage conditions of gene therapy products would most likely be a weak point for gene therapy sponsors. Even if the stability protocols follow the current guidelines, because the gene therapy territory is new and innovative, it is difficult to gauge whether an unexpected impact on product quality can occur years after stability has been completed. Based on this feedback, the recommendation is that the stability protocol should be established conservatively, maximizing time points, lot testing and with a careful evaluation of quality parameters and critical product attribute. In conclusion, the regulatory environment for gene therapy is still being established and commonly accepted principles guiding current product development should be thoroughly evaluated and most likely reassessed. Because of the biological complexities of these products, a conservative approach should be followed with regards to the stability program.

720. Data-Driven Development to Deployment of Recombinagenic to Reprogramming Drives

Faisal Reza
Yale University, New Haven, CT

We consider an approach to addressing health and well-being disparities on large scales by the development and deployment of recombinagenic and reprogramming drives. Among these are, for example, "gene drives" which "drive" themselves and proximally involved genetic elements through space, i.e. (sub)populations of individuals, and time, i.e. successive generations of these (sub) populations. In doing so, they can be engineered to propagate through (sub)populations despite reducing individual fitness, since most all offspring harboring drives are affected through inheritance, rather than fractional numbers of progeny. Fitness costs are thus compensated by this inheritance advantage. This, too, has spatial, e.g. ecological, as well as temporal, e.g. evolutionary, consequences for the (sub) populations being driven, as well as the network of interactions among the (sub)populations and the shared living environment.

We analyze and extend the data in our research and that of others to present technically possible types of drives, and the epidemiological contexts in which they can be applied to recombine genetic components and reprogram cellular chasses. Transient behavior during these modifications can give way to new equilibria in which, for example, disease transmission is mitigated, or disease resistance is bolstered, thus ultimately benefiting human health and agricultural well-being, respectively.

We combine the aforementioned and conclude with the technological and environmentally amenable consequences of drives as applied to contexts. As a powerful synthetic technology operating in the living world as intimately and broadly as natural evolutionary drives, potential unintended and off-targeting issues as well as safeguards and optimization of the development to deployment of drives are discussed. Simultaneously, as a technology dependent on its successful interplay with the environment, natural and man-made, drives are limited in their applicability due to their own inherent requirements, e.g. suitable for mating organisms, and brief generational timespans, as well as those external to them, e.g. ethics and safety for the target for the drive and others in the ecosystem, and immediate and long-term effects on other interactions in the network.

AAV Vectors and Pre-Clinical Analysis

721. Long-Term (2-8 years) Body-Wide Expression of AAV9-Minidystrophin Gene in Golden Retriever Muscular Dystrophy Dogs After Regional Limb Vein Injection

Juan Li[1], Joe Kornegay[2], Chunping Qiao[1], Hui Zheng[1], Jianbin Li[1], Ruhang Tang[1], Yi Dai[1], Xiao Xiao[1]
[1]UNC Eshelman School of Pharmacy, Chapel Hill, NC, [2]College of Veterinary Medicine, Texas A&M University, College Station, TX

Previously we have shown that AAV9-minidystrophin gene delivery by isolated hindlimb vein injection with pressure was able to obtain widespread gene expression, not only in injected limb but also in muscles body-wide. This phenomenon was attributed to both leaky tourniquet blockade and/or AAV9 vector retained in the vasculature space that later entered systemic circulation. Here we report the results of long-term study up to 8 years. **Methods**: Five GRMD dogs were treated with the same vector dose of 1 x 10^{13} v.g./kg. Dog Jelly (2.5 months old; 6.3 kg) was given AAV9-CMV-opti-hMinidys (codon-optimized human minidystrophin gene); Dogs Jasper and Peridot (4 mon old; 12.2 kg & 12.5 kg) were given AAV9-CMV-cMinidys (canine minidystrophin gene). Dogs Rutela and Laredo (14 mon old; 15.5 kg & 15.3 kg) were given AAV-CK-opti-cMinidys. No immuno-suppressant was used. The vectors were injected via the great saphenous with a tourniquet positioned at the proximal pelvic extremity (first 3 dogs) or above the knee (last 2 dogs) to block the blood circulation for a total of 10 minutes. MRI imaging after vector injection in Jelly confirmed vector fluid and muscle enlargement in the injected limb. Muscle biopsy and final necropsy were performed at various time points (from 2 months to 8 years) and analyzed for gene expression and immune responses. **Results**: Immunofluorescent (IF) staining showed that all 5 dogs obtained long-term minidys expression in a majority of muscle samples examined up to final necropsy. Contractile force measurement showed partial improvement when compared to the untreated dogs. While the percentages of minidys positive myofibers varied among different muscles, certain muscles had greater than 90% of myofibers positive upon necropsy. Importantly, the human minidys expression persisted for 8 years in Jelly despite initial inflammation in injected limb. Overall gene expression was largely stable. For example, positive myofibers in cranial sartorius muscle remained comparable throughout the 5 time points, from 2, 7 months to 1, 4 and 8 years. Minidys was also observed in approximately 20% of the cardiomyocytes. Interestingly, injected limb had lower expression than non-injected limb, suggesting procedure related inflammation and partial CMV promoter shut-down. Jelly remained ambulant throughout the more than 8-year post treatment study and was euthanized due to cardiomyopathy in the final year. DNA sequencing showed that Jelly did not carry the recently reported disease-modifying Jagged 1 mutation found in two phenotypically mild GRMD dogs. However, it is not possible to attribute Jelly's long-term survival to gene therapy because in the past a few GRMD dogs in the same colony survived to 5 years without treatment. For the other 4 dogs who were larger at the age of treatment, the injected limb consistently showed higher expression and no procedure related inflammation throughout the time course of 2 years. Moreover, no tumors were found in any of the treated dogs. Final analyses of necropsy samples on vector distribution and Western blot are underway to evaluate the efficiency of gene transfer and expression. **Conclusions: 1**) AAV9 can render long-term and significant systemic minidys gene expression without immune suppression upon isolated limb vein injection. **2**) The human minidys gene and pressurized perfusion might have caused inflammation and innate immune responses in the injected limb in Jelly. **3**) Muscle-specific promoter is preferable for reducing expression in non-muscle cells. **4**) These findings support the feasibility of AAV9-CK-minidys gene therapy in human DMD patients.

722. AAV Capsid Evolution for Enhanced Antibody Delivery to Human Skeletal Muscle for Use in Next-Generation HIV Vaccines and Muscle Gene Therapies

Nicole K. Paulk, Gregory Charville, Katie Maguire, Katja Pekrun, Yue Zhang, Matthew Tiffany, Jose Vilches-Moure, Gordon Lee, Joseph Shrager, Thomas Rando, Mark A. Kay
Stanford University, Stanford, CA

Our goal is to develop an AAV vector that can transduce human skeletal muscle after intramuscular administration at levels sufficient to express therapeutic levels of antibodies with broad-spectrum protection against HIV. AAV has begun being used in passive vaccines for HIV and influenza. Yet for many indications, greater human skeletal muscle transduction is needed than has been achieved with existing AAV serotypes. To bioengineer novel capsids with unparalleled human skeletal muscle transduction, we utilized primary human skeletal muscle cells from surgical resections to screen libraries of replication-competent shuffled AAV capsids. Two screens were performed in pools of primary human skeletal muscle stem cells or myotubes from six patients. Six rounds of replicating selection were carried out and the five most highly selected variants from each screen were vectorized and validated against existing serotypes with muscle tropism (AAV1, 6 and 8). In primary human muscle stem cells, variants NP22, NP66 and NP94 had significantly increased transduction that ranged from a 65 to 284-fold improvement over AAV1, 27 to 118-fold over AAV6, and 10 to 45-fold over AAV8. In primary human myotubes, NP22, NP66 and NP94 again showed significantly increased transduction that ranged from a 13 to 464-fold improvement over AAV1, 25 to 871-fold over AAV8, and NP94 showed a 15-fold improvement over AAV6. To assess human skeletal muscle transduction capabilities *in vivo*, we injected xenografted humanized muscle NSG mice with shuffled or control capsids expressing luciferase by intramuscular injection (1E9/leg) and assessed transduction weekly by live imaging in time course studies over two months. AAV6 was the first to uncoat but NP66 produced the highest sustained transduction levels. To control for species-specific transduction, we performed the same time course experiment in strain- and gender-matched non-transplanted mice. Strikingly, AAV6 outperformed shuffled variants when no human muscle fibers were present, highlighting the importance of performing preclinical studies in human cells and xenograft systems whenever possible to prevent misleading results which are mouse-specific. To further demonstrate the superiority of our shuffled variants and more accurately predict eventual muscle transduction in humans, we transduced human skeletal muscle explants from surgical resections *ex vivo*. Four adult patients (two male and two female) had healthy skeletal muscle tissue removed for *ex vivo* transduction analyses with luciferase. In all 4 patients, NP22 and NP66 (and NP94 in 3/4 patients) had significantly increased transduction by live fiber luciferase imaging as well as luciferase assays on lysed fibers that ranged from a 4 to 116-fold improvement over all control serotypes. To support preclinical vaccine testing in non-human primates, we assessed transduction in rhesus macaque skeletal muscle explants *ex vivo*. Here again we showed that NP22 and NP66 showed a 30 to 57-fold improvement in transduction over control serotypes. Taken together, our results demonstrate that capsid variants NP22 and NP66 (and in some cases NP94), have highly significant increased human skeletal muscle fiber transduction when assessed *in vitro*, *in vivo* and *ex vivo*. These capsids represent powerful tools to express

therapeutic quantities of human monoclonal antibodies for use in passive vaccines, or transgenes for various muscle disorders in gene therapy, specifically in humans.

723. AAV Mediated Cancer Targeting: Systemic Trafficking to Tumor Is More Important Than Vector Tumor Cell Interaction

Yuan Lu[1], Damien Marsic[2], Chen Ling[2], Maria V. Guijario[1], Padraic P. Levings[1], Glyn D. Palmer[1], Anthony Dacanay[1], Rachael S. Watson-Levings[1], Viktoria Hyddmark[1], Margaret E. White[1], Sergei Zolotukhin[2], Steven C. Ghivizzani[1]
[1]Department of Orthopaedics and Rehabilitation, University of Florida, Gainesville, FL, [2]Department of Pediatrics, University of Florida, Gainesville, FL

Adding tumor specific ligands to enhance vector tumor cell interaction is the conventional concept to generate tumor targeting adeno-associated viral vector (AAV). However, it remains poorly proved whether high AAV tumor cell interaction contributes to high tumor localization *in vivo* following systemic delivery. Here, we conducted directed evolution selections on patient derived xenograft models using a complex AAV capsid library. Uniquely, we compared the pressure for AAV tumor cell interaction alone (intratumoral library injection) and multi-layer pressure including traveling to the tumor and infection (intravenous library injection). Distinct patterns of AAV capsid motifs were identified after intratumoral and intravenous screenings. Motifs isolated from intratumoral screenings were named tumor specific motifs and those isolated from intravenous screenings were named systematic trafficking motifs. AAVs with tumor specific motifs but not systemic trafficking motifs showed significantly increased tumor cell transduction *in vitro,* indicating enhanced vector target cell interaction after intratumoral-based selections. Interestingly, following systemic delivery, AAVs with systemic trafficking motifs mediated hundreds of folds higher transgene expression than those with tumor specific motifs and wild type AAV *in vivo*. The combination of both motifs further increases the tumor tropism but not the transduction efficiency *in vivo*. When analyzing the AAV genome biodistribution by quantifying the genome copy number, the systemic trafficking motifs greatly reduced the native AAV tropism, which potentiated higher accessibility of AAV to the tumor. In contrast, AAVs only bearing tumor specific motifs maintained native AAV tropism and failed to mediate increased genome localization in tumor. Furthermore, in two independent patient derived xenograft models and two different tumor types, our novel AAV vector armed with combined motifs all showed hundreds of folds increase in transduction efficiency with no detectable off-targeting expression. In conclusion, contradictory to the prevailing theory, our study demonstrated that the AAV tumor cell interaction did not contribute to increased tumor localization but just specificity *in vivo*. Therefore, targeting is not only depending on AAV tumor cell interaction but also, and more importantly, depending on the accessibility of AAV to the tumor cells following systemic delivery. Future cancer directed AAV vector design should take into account the complex processes during systemic delivery as well as the vector tumor cell interaction.

724. A Comprehensive Map of CNS Transduction by Eight Recombinant Adeno-Associated Virus Serotypes upon Cerebrospinal Fluid Administration in Pigs

Nicolina C. Sorrentino[1], Veronica Maffia[1], Sandra Strollo[1], Vincenzo Cacace[1], Noemi Romagnoli[2], Anna Manfredi[1], Domenico Ventrella[2], Francesco Dondi[2], Francesca Barone[2], Massimo Giunti[2], Anne-Renee Graham[3], Yan Huang[3], Susan L. Kalled[3], Alberto Auricchio[1], Maria L. Bacci[2], Enrico M. Surace[1], Alessandro Fraldi[1]
[1]Telethon Institute of Genetics and Medicine, Tigem, Pozzuoli, Italy, [2]Department of Veterinary Medical Sciences (DIMEVET), University of Bologna, Bologna, Italy, [3]Discovery Biology and Translational Research, Shire, Lexington, MA

Gene transfer of recombinant adeno-associated virus (rAAVs) holds promises to treat neurological disorders. Studies on different recombinant vector serotypes and modes of delivery to the central nervous system (CNS) showed that the combination of both rAAV serotype used and delivery routes play a key role in CNS transduction properties and thus in disease phenotype rescue outcome. However, one of the major hurdles to developing an effective clinical protocol for neurological disorders is the efficiency of vectors to reach the specific cell types in disease-specific CNS subdomains. An attractive vehicle to reach the CNS is represented by the cerebral spinal fluid (CSF) and different injection techniques (intrathecal cisterna magna, intra-ventricular and intrathecal lumbar injections) actually allow the central nervous system targeting by using the CSF flux. Along these years different AAV serotypes have been tested by intrathecal injections and some of these displayed a very specific tropism for the central nervous system. However, a quantitative and qualitative analysis of transduction patterns of the most promising rAAV serotypes for brain targeting in large animal models is missing. Here, we characterize distribution, transduction efficiency and cellular targeting of rAAV serotypes 1, 2, 5, 7, 9, rh.10, rh.39 and rh.43 delivered into the cisterna magna of wild type pigs. Despite the rAAV9 showed the highest transduction efficiency and the widest distribution capability among the vectors tested, the other serotypes showed a specific distribution pattern from the rostral to the caudal part of the CNS. rAAV9 robustly transduced both glia and neurons, including the motor neurons of the spinal cord. Relevant cell transduction specificity of the glia was observed after rAAV1 and rAAV7 delivery. rAAV7 also displayed a specific tropism to Purkinje cells. Evaluation of biochemical and hematological markers suggested that all rAAV serotypes tested were well tolerated. This study provides a comprehensive CNS transduction map in a useful pre-clinical large animal model enabling the selection of potentially clinically transferable rAAV serotypes based on disease specificity. Therefore, our data are instrumental for the clinical evaluation of these rAAV vectors in human neurodegenerative diseases.

725. Assessment of CSF Route for Gene Delivery in Sandhoff Mice Using AAV9 Expressing an Hexosaminidase Isoenzyme.

Patrick Thompson[1], Karlaina Osmon[1], Evan Woodley[1], Subha Karumuthil-Melethil[2], Steven J. Gray[3], Jagdeep S. Walia[4]
[1]Medical Genetics/Departments of Pediatrics and Pathology and Molecular Medicine, Queen's University, Kingston, ON, Canada, [2]Gene Therapy Center, University of North Carolina, Chapel Hill, NC, [3]Gene Therapy Center, Department of Ophthalmology, University of North Carolina, Chapel Hill, NC, [4]Medical Genetics/Departments of Pediatrics and Pathology and Molecular Medicine, Department of Biome, Queen's University, Kingston, ON, Canada

G_{M2} gangliosidoses are a group of neurodegenerative disorders, characterized by the malfunctioning Hexosaminidase A (HexA) enzyme. HexA is formed by heterodimerization of two subunits, α and β. Hex A, in interaction with GM2 activator protein (GM2AP), is the main isoenzyme able to hydrolyze GM2 gangliosides. HexA deficiencies result in Tay-Sachs (α-subunit deficiency) or Sandhoff disease (SD, β-subunit deficiency). In the recent work by Tropak et al. (Mol Ther Methods Clin Dev, *in press*), a hybrid human α-subunit, named "μ" and coded by *HEXM*, was created (patent pending) by incorporating the dimer stabilization and GM2AP binding sites of the β-subunit while maintaining the catalytic properties of the α-subunit. The μ-subunit is able to homodimerize to form a stable and functional enzyme, named HexM, which can interact with the human GM2AP and effectively hydrolyse G_{M2} gangliosides. Another advantage of this subunit (~1.6 kb) is that it can be packaged in a self-complementary adeno-associated virus (scAAV/*HEXM*). An intravenous route of scAAV administration has been shown to be successful, but brings with it translational issues including large scale viral preparation and relatively high vector uptake by liver. We tested the cerebrospinal fluid (CSF) route, as an alternative, for delivering scAAV9/*HEXM*. We injected 2.5E+11 vector genomes per mouse of scAAV9/*HEXM* via the *cisterna magna* at 6 weeks of age (n=13). Our controls include treatment with scAAV9/*GFP* (n=3) and vehicle (n=8). One additional cohort received an IV injection of 25% mannitol (2g/kg) post-AAV9 injection (n=10). We sacrificed part of the cohorts (n=4 each group) at 16 weeks of age (humane end-point of untreated SD mice) for tissue analysis. The remainder are being monitored for long-term survival. The parameters for analyses are survival benefit, locomotor behaviour, Hexosaminidase activity, GM2 ganglioside accumulation, and vector genome biodistribution. The preliminary results from this ongoing study show a significant survival advantage to the humane endpoint in HexM treated (average 28 weeks to date) as compared to negative controls (~16 weeks). The behaviour tests showed improved locomotor activity in HexM-treated mice as compared to negative controls. Results were similar when mannitol was administered in conjunction with scAAV9/*HEXM*. These preliminary results indicate that the intra-CSF route of administration of AAV9/*HEXM* is a tractable translatable approach for SD worth further exploration. Additional studies are focused on increased dosage and methods to improve distribution.

726. Prevention of Muscular Dystrophy by an AAV Vector Encoding a Nonimmunogenic Protein Based on the Evolution of Utrophin and Dystrophin

Yafeng Song[1], Alock Malik[1], Andy F. Mead[1], Marilyn Mitchell[1], Mihail Petrov[1], Leonard Su[1], Leon Morales[1], Shira Rosenblum[1], Tejvir Khurana[1], Joe N. Kornegay[2], Hansell H. Stedman[1]
[1]Surgery, University of Pennsylvania, Philadelphia, PA, [2]School of Veterinary Medicine, Texas A&M, College Station, TX

The majority of mutations causing Duchenne muscular dystrophy (DMD) are multi-exon, frameshifting deletions in the dystrophin gene. Expression of recombinant dystrophin in DMD therefore risks chronic immune recognition of the "non-self" protein. Early developmental expression of the paralogous protein utrophin in the thymus may confer central immunological tolerance to its peptide sequence. Here we address previously unanswered questions about the therapeutic efficacy and immunogenicity of "μutrophins" encoded by synthetic minigenes suitable for use in AAV. First we used a comparative phylogenomic approach to address a deceptively simple question with critical implications for this field: "which came first-dystrophin or the sarcomere". This analysis provided evidence that the long rod-like domain of utrophin and dystrophin was "coopted" from a higher molecular weight protein in dynein-propelled animals lacking striated muscle. When considered in light of recent crystalographic studies, these results suggest that the full functionality of native dystrophin may be conferred by recombinant mini-utrophins with internally deleted rod domains that preserve inter-repeat folding and hence maximal tensile strength. We maximized recombinant μUtrophin (μU) expression from synthetic open reading frames by using the codon bias of striated myosin. Our blinded studies of mdx mice injected with a μU transgene are the first to show COMPLETE recovery of peripheral myofiber nucleation following a single systemic injection of an AAV vector. Sarcoglycan expression, as measured by western blot in the μU-treated mice, are restored to control levels throughout growth to skeletal maturity. To investigate the correlation between our histological findings and functionality, we validated a novel open field cage and attached running wheel system. Our blinded data show remarkable *functional* rescue in μU-treated mdx mice using this non-invasive test with relevance to the clinical assessment of disease progression in DMD. These data also strongly correlate with those derived from in vivo force grip and ex vivo force transducer measurements. Remarkably, a majority of mdx mice show complete normalization of serum creatine kinase (CK), a first for single-dose treatment by any clinically translatable modality. In dystrophic puppies, intravenous injection of a 30-fold lower relative dose of AAV9U fully restored sarcoglycan levels and normalized the myofiber size-distribution following a threefold increase in muscle mass. Interferon-gamma ELISpot assays using utrophin-derived peptides revealed no reactivity in injected dogs, consistent with central immunological tolerance. These findings suggest a rationale for neonatal gene therapy using utrophin as an internally deleted "self" protein in DMD to minimize the risk of chronic immunotoxicity. We outline a rigorous translational approach using escalating vector doses in GSHPMD dogs harboring a newly defined 5 megabase deletion encompassing the dystrophin ORF.

727. Characterizing the Adeno-Associated Virus 1 Sialic Receptor Binding Site and Its Overlap with Antigenic Epitopes

Lin-Ya Huang[1], Ami Patel[1], Robert Ng[1], Edward B. Miller[1], Sujata Halder[1], Robert McKenna[1], Aravind Asokan[2], Mavis Agbandje-McKenna[1]
[1]Biochemistry and Molecular Biology, University of Florida, Gainesville, FL, [2]Genetics and Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC

Adeno-associated viruses (AAVs) are being development as gene delivery vectors and have shown promise in several clinical trials. And an AAV1-based vector has been approved by the European Commission for the treatment of lipoprotein lipase deficiency disease. However, limitations in tissue transduction specificity as well as neutralization by pre-existing antibodies remain are two major obstacles. To overcome these problems, it is important to characterize functional capsid regions that dictate virus binding to cellular receptors during infection and the potential overlap(s) of these regions with capsid antigenic epitopes. In this study, the sialic acid (SIA) binding site on AAV1 was determined by solving the structure of the AAV1-SIA complex using X-ray crystallography. Residues that form the SIA binding pocket are identical between AAV1 and AAV6; hence, structurally predicted SIA binding sites were mutated on both serotypes to confirm their role in the SIA interaction. Binding and transduction assays with these mutants in CHO cell lines Pro5, Lec2, Lec8, and Lec1, which display different terminal glycans, confirmed the structurally mapped binding pocket. Furthermore, native dot blot showed that several of the AAV1 SIA binding mutants can escape from antibody recognition. This finding is consistent with the overlap between the SIA binding site and the previously mapped AAV1 epitopes. Significantly, a glycan binding mutant with slightly reduced transduction ability is able to escape from antibody recognition. This study identifies an overlap between receptor recognition and antibody reactivity and provides amino acid level information for rational capsid engineering of AAV vectors for improved therapeutic efficacy.

728. Tissue-Directed Transgene Engineering for AAV and Lentivector Gene Therapy Approaches

Harrison C. Brown[1], Phillip M. Zakas[1], Stephan N. George[2], Ernest T. Parker[2], H. Trent Spencer[2], Christopher B. Doering[2]
[1]Laney Graduate School, Molecular and Systems Pharmacology Program, Emory University, Atlanta, GA, [2]Aflac Cancer and Blood Disorders Center, Emory University School of Medicine, Atlanta, GA

Clinical gene therapy frequently is encumbered by low transgene product biosynthesis at predictably safe vector doses. It has been hypothesized that the presence of rare codons may regulate transgene product expression through depletion of the available cognate tRNA pool. Codon optimization is the prominent strategy utilized to overcome this hypothesized limitation and involves replacing rare, presumably translation rate-limiting, codons with the most frequent ones. Typical algorithms attempt to match the codon usage frequency of the transgene coding sequence to that of target organism's total mRNA pool, which has been shown to approximate the overall available tRNA concentrations. Upon closer examination, it appears that both codon frequency and tRNA content vary between tissue types. Therefore, we hypothesize that codon-optimization can be improved by tailoring to the codon-frequency of the most highly expressed genes present in target cell types. Our tissue-directed codon optimization algorithm utilizes novel codon usage indices generated from target cell gene expression data. As proof of concept, we developed tissue-codon optimized variants of coagulation factor VIII (FVIII) to be utilized in lenti- and adeno-associated viral (LV and AAV, respectively) vectors. These two vectors are the leading platforms for clinical gene therapy of hemophilia A. LV is utilized to target autologous hematopoietic cell types ex vivo while AAV is delivered intravenously to genetically-modify hepatocytes. However to date, development-stage LV and AAV gene therapy products for hemophilia A have been characterized by low-level FVIII transgene product biosynthesis. Initially, we designed human hepatocyte-, monocyte- and standard overall human-optimized FVIII constructs (LCO, MCO and HCO, respectively) to be compared to wild-type FVIII. Upon initial examination, expression of LCO-FVIII was shown to be 3-fold greater than either MCO- or wild-type FVIII from the human hepatoma cell line, HepG2, following transient transfection. In contrast, LCO-FVIII and MCO-FVIII expression was diminished 12 and 4-fold, respectively, compared to wild-type in the 'neutral' human embryonic kidney 293T cell line. Furthermore, following hydrodynamic injection of naked plasmid DNA into hemophilia A mice, LCO-FVIII exhibited a sustained 10-fold increase in FVIII expression relative to the HCO-FVIII comparator. In attempt to generate a lead candidate for clinical translation, we utilized several of our most promising vector components to construct a liver-optimized AAV8 vector consisting of LCO-ET3, our previously described high-expression bioengineered FVIII variant, transcriptionally driven by a novel 146bp liver-directed promoter and adjacent MVM intron. Following intravenous delivery into hemophilia A mice, vector doses of 1e12 and 1e11 vector genomes per kg achieved sustained, predictable curative plasma FVIII levels of 200% and 20% of normal human levels, respectively. These initial results support the utility of our novel approach of clinical tissue-directed transgene optimization.

Targeted Genome Editing: Methods and Technology

729. Inheritable Silencing of Endogenous Gene by Hit-and-Run Targeted Epigenetic Editing

Angelo Amabile[1,2], Alessandro Migliara[1,2], Paola Capasso[1], Mauro Biffi[1], Luigi Naldini[1,2], Angelo Lombardo[1,2]
[1]San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, Italy, [2]Vita-Salute San Raffaele University, Milan, Italy

Gene silencing holds great promise for the treatment of several diseases and can be exploited to investigate gene function and activity of the regulatory genome. Here, we develop a novel modality of gene silencing that exploits epigenetics to achieve stable and highly efficient repression of target genes. To this end, we generated Artificial Transcriptional Repressors (ATRs), chimeric proteins containing a custom-made DNA binding domain fused to the effector domain of chromatin-modifying enzymes involved in silencing process of Endogenous RetroViruses (ERVs). By performing iterative rounds of selection in cells engineered to report for synergistic activity of candidate effector domains, we identified a combination of 3 domains (namely KRAB, DNMT3A and DNMT3L) that, when transiently co-assembled on the promoter of the reporter cassette, recreate a powerful embryonic-specific repressive complex capable of inducing full and long-term (>150 days) silencing of transgene expression in up to 90% of the cells. The ATR-induced silencing was cell type and locus independent, and resistant to metabolic activation of the cells. Importantly, these findings were holding true also for endogenous genes embedded in their natural chromatin context, as shown for the highly and ubiquitously expressed *B2M* gene. Here, transient co-delivery of TALE-based ATRs resulted in loss of surface expression of B2M and, consequently, of the MHC-I molecules in up to 80% of the cells. This phenotype was associated with a drastic switch in the epigenetic and transcriptional state of the constitutively active *B2M* promoter, which become highly decorated with *de novo*

DNA methylation and deprived of RNAP II. Importantly, silencing was sharply confined to the targeted gene and resistant to INF-γ, a potent natural activator of *B2M*. We further extended these studies by showing that our silencing approach is portable to the CRISPR/dCas9 DNA binding technology. In this setting, comparable levels of *B2M* silencing (up to 80%) were achieved using either pools or even individual sgRNAs coupled to dCas9-based ATRs. Yet, adoption of this technology allowed performing simultaneous, highly efficient multiplex gene silencing within the same cell, as shown for *B2M*, *IFNAR1* and *VEGFA*. Finally, we assessed resistance of the silenced gene to activity of potent artificial transcription activators and chromatin remodelers, and found that only targeted DNA demethylation was able to reawaken the silent gene. This allowed performing iterative cycles of silencing and reactivation of the same gene in the same cell population. Overall, these data provide the first demonstration of efficient and stable epigenetic silencing of endogenous genes upon transient delivery of ATRs. This was accomplished by repurposing the ERVs silencing machinery, which instructs self-sustaining repressive epigenetic states to the target gene. While silencing of *B2M* might be used to generate universally transplantable allogeneic cells, our hit-and-run strategy provides a powerful new alternative to conventional gene silencing for both basic and translational research.

730. Permanent Correction of Diverse Muscular Dystrophy Mutations in Human Cardiomyocytes by Myoediting

Chengzu Long[1], Hui Li[1], Viktoriia Kyrychenko[1], Huayu Zhou[1], Samadrita Bhattacharyya[1], Cristina E. Rodriguez[1], John M. Shelton[2], Pradeep Mammen[2], Jay Schneider[2], Rhonda Bassel-Duby[1], Eric N. Olson[1]
[1]Department of Molecular Biology, UT Southwestern Medical Center, Dallas, TX, [2]Internal Medicine, UT Southwestern Medical Center, Dallas, TX

Dilated cardiomyopathy (DCM) is one of the most common lethal features of Duchenne muscular dystrophy (DMD), caused by diverse mutations in the X-linked dystrophin gene (*DMD*). Without dystrophin, a large cytoskeletal protein, muscles degenerate, causing myopathy. Although several gene therapies have been tested, there is no curative treatment so far. In 2014, we first used CRISPR/Cas9-mediated genome editing (termed Myoediting) to correct the mutation in the germ line of *mdx* mice, the mouse model of DMD [Long *et al.* ***Science*** 345:1184-8]. Recently, we applied Myoediting to postnatal muscle tissues in mice by delivering gene editing components via a harmless adeno-associated virus. Cardiac and skeletal muscle showed progressive rescue of dystrophin protein [Long *et al.* ***Science*** doi: 10.1126/science.aad5725]. These studies paved the way for novel genome editing-based therapeutics in DMD. We have now advanced Myoediting to cells from human DMD patients by engineering the permanent skipping of mutant or out-of-frame exons in the genome of DMD patient-derived induced pluripotent stem cells (iPSCs). We have optimized Myoediting of DMD mutations using pools of sgRNAs to target the top 12 hot spot mutant exons. We targeted the conserved RNA splicing acceptor/donor sites of each exon. NHEJ-mediated indels efficiently abolished the splicing sites and skipped the mutated or out-of-frame exons. Based on the known DMD mutations, we established a publicly available online resource (**Duchenne Skipper Database**) for selecting the optimal target DMD sequences for Myoediting, which will rescue dystrophin function in 60-80% of DMD patients. We performed Myoediting on representative iPSC-derived cardiomyocytes from multiple patients with point, deletion or duplication mutations and efficiently restored dystrophin protein expression in cardiomyocytes. Rescued DMD cardiomyocyte shows enhanced function. Opportunities and obstacles in the path toward efficient and permanent elimination of the genetic cause of DMD and other DCM will be discussed.

731. High-Fidelity CRISPR-Cas9 Nucleases with No Detectable Genome-Wide Off-Target Effects

Benjamin P. Kleinstiver, Vikram Pattanayak, Michelle S. Prew, Shengdar Q. Tsai, Nhu T. Nguyen, J. Keith Joung
Molecular Pathology, Massachusetts General Hospital, Charlestown, MA

CRISPR-Cas9 nucleases are widely used for genome editing but can induce unwanted off-target mutations at genomic locations that resemble the intended target. These so-called off-target effects can confound research applications and are important considerations for potential therapeutic use. Existing strategies for reducing genome-wide off-targets of the broadly used *Streptococcus pyogenes* Cas9 (SpCas9) have thus far proven to be imperfect by possessing only partial efficacy and/or other limitations that constrain their use. Here we describe a high-fidelity variant of SpCas9, called SpCas9-HF1, that contains alterations in the amino acid sequence designed to reduce non-specific contacts to the target strand DNA. SpCas9-HF1 retains on-target activities comparable to wild-type SpCas9 with >85% of the 37 single-guide RNAs (sgRNAs) tested in human cells. Strikingly, with eight different sgRNAs targeted to standard non-repetitive sequences in human cells, SpCas9-HF1 rendered all or nearly all off-target events imperceptible by genome-wide break capture and targeted sequencing methods. Even for atypical, repetitive target sites, the vast majority of off-targets induced by SpCas9-HF1 were not detected. With its exceptional precision, SpCas9-HF1 provides an important and easily employed alternative to wild-type SpCas9 that can eliminate off-target effects when using CRISPR-Cas9 for research and therapeutic applications. Our findings also suggest a general strategy for improving or optimizing the genome-wide specificities of other Cas9 orthologues and engineered variants.

732. CRISPR/Cas9 Mediates Highly Efficient Gene Editing in Long-Term Engrafting Human Hematopoietic Stem/Progenitor Cells

Jack M. Heath, Aditi Chalishazar, Christina S. Lee, William Selleck, Cecilia Cotta-Ramusino, David Bumcrot, Jennifer L. Gori
Editas Medicine, Cambridge, MA

Transplantation of genetically corrected autologous hematopoietic stem/progenitor cells (HSPCs) is an effective treatment for patients with inherited hematologic disease. Genome editing with CRISPR/Cas9 ribonucleoprotein (RNP) precisely modifies gene targets in human cells, including HSPCs. In order to translate this technology to a clinical setting, gene editing must be reproducible and efficient across multiple patient donors without compromising HSPC viability, multipotency, and long-term engraftment capability. To determine the reproducibility of Cas9 RNP mediated gene editing in HSPCs, human $CD34^+$ cells obtained from 15 different patient donors (cord blood n=12, mobilized peripheral blood [mPB] n=3) were electroporated with *S. pyogenes* Cas9 RNP targeting the β-hemoglobin (*HBB*) or *AAVS1* genetic locus. DNA sequence analysis of 15 separate experiments demonstrated that Cas9 supported up to 72% gene editing in cord blood $CD34^+$ cells and up to 61% gene editing in adult mPB $CD34^+$ cells (mean % editing by DNA sequencing: 57% ± 8%). Cas9 induced multiple modifications that comprise insertions and deletions at the *HBB* locus, and some of the lesions were repaired through an HDR repair mechanism that used the homologous sequences from the endogenous *HBD* gene as a template (Gene Conversion). Gene edited $CD34^+$ cells retained viability and hematopoietic colony forming cell (CFC) activity *ex vivo*, with no significant differences

between treatment groups or across donors. Indels were detected in one (BFU-E/GEMM: 50%-75%, CFU-GM/M: 50-66%) or both (BFU-E/GEMM: 12-38%, CFU-GM/M: 12-50%) alleles, at high frequencies in clonal erythroid and myeloid colonies (range of 63-100% of individual colonies assayed across experiments). To evaluate engraftment potential, both Cas9 modified (unsorted) and untreated control human $CD34^+$ cells were transplanted into immunodeficient mice. Twelve weeks after transplantation, up to 34% human $CD45^+$ cell engraftment was detected in the peripheral blood of recipients of Cas9 RNP treated $CD34^+$ cells. There was no significant difference in the engraftment of Cas9 RNP treated and untreated control $CD34^+$ cells which were detected in blood, spleen, and bone marrow. Human lymphoid, myeloid, and erythroid cells were detected in blood and hematopoietic organs with no difference in lineage distribution between cohorts. Analysis of the bone marrow from both groups revealed an average of 20% human $CD45^+$ and 13% $CD34^+CD45^+$ cell engraftment long-term. Analysis of human gDNA revealed that up to 20% gene editing in the marrow of treated mice and edited cells were also detected in the blood and spleen. In summary, these data show that Cas9 RNP gene edited primary human $CD34^+$ HSPCs retained the ability to engraft long-term and reconstitute hematopoiesis *in vivo* at levels higher than reported previously. These findings also show that electroporation of HSPCs with Cas9 RNP mediated highly reproducible gene editing levels across multiple donors and did not alter hematopoietic reconstitution or differentiation properties in comparison to untreated control $CD34^+$ cells.

733. Somatically Repairing Compound Heterozygous Recessive Mutations by Chromosomal Cut-and-Paste for In Vivo Gene Therapy

Dan Wang[1], Haiwei Mou[2], Karen Tran[1], Jia Li[1], Blake Y. Wang[1], Dominic J. Gessler[1], Phillip WL Tai[1], Qin Su[1], Wen Xue[2], Guangping Gao[1]

[1]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA, [2]RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA

Patients affected by monogenic recessive genetic disorders often carry two different mutated alleles of the same gene, which is known as compound heterozygous. Theoretically, exchanging the genetic material between the two mutated alleles will reconstitute a mutation-free allele that can be therapeutic **(Figure)**. We hypothesized that generating DNA double-stranded breaks at the same location on both mutant alleles can induce allele exchange, reconstitute a mutation-free allele, and therefore yield therapeutic benefit. We first tested this hypothesis in a targeted knock-in mouse model that carries $GFP^{N\text{-term}}$-intron-$tdTomato^{C\text{-term}}$ and $tdTomato^{N\text{-term}}$-intron-$GFP^{C\text{-term}}$ expression cassettes, respectively, at the same genomic location on each copy of Chr 11. Therefore, allele exchange at the intronic region will reconstitute the full-length GFP and tdTomato, serving as a reporter system. We injected recombinant AAV (rAAV) vectors expressing SpCas9 and sgRNA targeting the intron into adult mice by tail vein injection. Five weeks later, we observed GFP and tdTomato fluorescence in cryosections of peripheral tissues including liver and heart, whereas there was no such fluorescence observed in the tissue samples from untreated mice, demonstrating that allele exchange occurred, and that the reconstituted alleles yielded protein expression. Furthermore, we generated mice that carry two different mutations of the *Aspa* gene as a compound heterozygous mouse model of Canavan disease. We treated these mice with rAAV vectors expressing SpCas9 and sgRNA targeting an intron between the two mutation sites. Three weeks after treatment, we detected reconstituted, mutation-free *Aspa* DNA sequence by allele-specific PCR and single-molecule, high-throughput DNA sequencing in the liver. The reconstituted *Aspa* allele carried insertion at the predicted SpCas9 cleavage site, indicating that the DNA allele exchange was mediated by the non-homologous end joining DNA repair pathway. We also observed allele exchange in mouse liver using the SaCas9 system. Evaluation of the therapeutic benefit following Cas9/sgRNA-mediated allele exchange in various compound heterozygous mouse models and patient cell lines is underway. The gene repairing strategy described here is a novel approach to tackling a broad range of autosomal recessive genetic disorders.

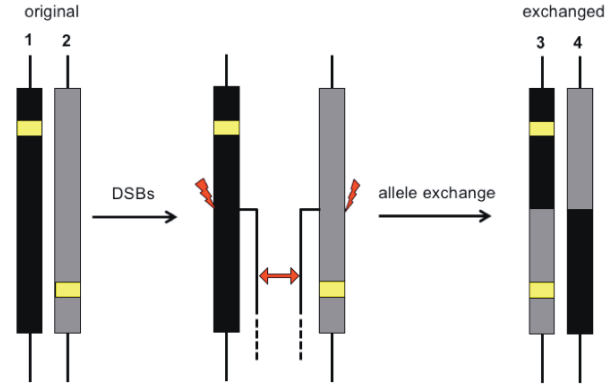


Figure 1. Concept of repairing compound heterozygous mutations by allele exchange. Each of the two original alleles (1 and 2, black and gray bars) carries a mutation (yellow boxes). Targeted DNA double-stranded breaks (DSBs) at a site between the two mutations are induced by nucleases (red lightning) such as Cas9. The homologous chromosomes exchange material (red double-head arrows) at the break points. The exchanged alleles (3 and 4) are a mixture of the original alleles. Allele 3 carries two mutations and allele 4 is mutation-free.

734. Pre-Clinical Development and Qualification of ZFN-Mediated Disruption of CCR5 in Human Hematopoietic Stem and Progenitor Cells

Rodica Stan[1], David DiGiusto[1], Paula Cannon[2], Colin Exline[2], Lijing Li[1], Anitha Rao[1], Jianbin Wang[3], Gary Lee[3], Philip D. Gregory[3], Nancy Gonzalez[1], Victoria Bedell[4], Michael C. Holmes[3], John A. Zaia[1]

[1]Center for Gene Therapy, City of Hope, Duarte, CA, [2]Keck School of Medicine, University of Southern California, Los Angeles, CA, [3]Sangamo Biosciences, Richmond, CA, [4]City of Hope, Duarte, CA

INTRODUCTION: Gene therapy for HIV-1 infection is a promising alternative to lifelong combination anti-viral drug treatment. When deleted, chemokine receptor 5 (CCR5), the co-receptor required for R5-tropic HIV-1 infection of human cells, renders cells resistant to HIV-1 infection. This has formed a strategy for the potential cure of HIV-1 infection by disrupting CCR5 genomic sequences in hematopoietic stem/progenitor cells developed using Zinc Finger Nucleases (ZFN). We demonstrate that delivery of CCR5-specific ZFN mRNA (SB-728mR) to HSPC via electroporation results in efficient disruption of CCR5 resulting in a high percentage of cells possessing biallelic modification and yields clinical-scale numbers of CCR5-modified HSPC with minimal cellular cytotoxicity, while maintaining their hematopoietic potential both *in vitro* and *in vivo*. **METHODS**: Pre-clinical experiments were performed at scale using donor HSPC with different concentrations of SB-728mR to optimize the electroporation-mediated CCR5 disruption in CD34+ HSPC for clinical studies in HIV. SB728mR-HSPC were evaluated *in vitro* for: a) mono- vs bi-allelic disruption and off-target ZFN cutting using MiSeq deep sequencing and the surveyor nuclease assay; b) total yield and viability; and c) hematopoietic function in colony forming unit (CFU) assays. Tumorigenicity studies were performed in *NOD-scid-IL2Rgamma^{null}* (NSG) mice irradiated with 150 cGy and IV injected with 1e6 SB-728mR-HSPC. equivalent of one clinical

dose (150e6 CD34+ cells).Engraftment efficiency was analyzed by %CD45 cells in PBMC at intervals for 5 months. **RESULTS**: We have demonstrated the specificity and efficacy of ZFN-based CCR5 disruption in HSPC: a) 40-60% CCR5 disruption with 50 to 150 µg/mL mRNA, with off-target modification observed at CCR2 and in 3 additional sites that occur in non-coding (either at intergenic or intronic) sequences; and b) effective disruption of ≤70% CFU from adult mobilized HSPC with maintenance of hematopoietic potential *in vitro* and *in vivo*. The tumorigenicity study demonstrated the safety of the gene modified cell product. SB-728mR-HSPC and untransfected control HSPC successfully engrafted as shown by human hematopoietic progeny measured in blood and bone marrow in all animals. Gene modified cells were observed in blood and bone marrow during the course of the study and were generally well tolerated. No ZFN treatment related tumor formation was observed in any animals. **CONCLUSIONS**: We have developed a method to genetically modify the CCR5 locus in human CD34+ HSPC. This approach is safe, efficient and reproducible. A combination of *in vitro* and *in vivo* studies evaluated ZFN specificity and potential tumorigenicity of ZFN modified cells in NSG mice. The established process complies with the regulatory requirements and results of the preclinical studies were used to support the filing of an IND with the FDA. The clinical trial testing SB-728mR-HSPC (NCT02500849@clinicaltirals.gov) is currently enrolling patients with the support of the California Institute for Regenerative Medicine.

735. Functional Restoration of Dystrophin Protein in HiPSC-Derived Skeletal Myotubes and Cardiomyocytes After CRISPR/Cas9-Mediated Deletion of 530-725kb of *DMD*

Courtney S. Young[1], Michael R. Hicks[2], Natalia V. Ermolova[1], Haruko Nakano[3], Majib Jan[2], Shahab Younesi[2], Atsushi Nakano[3], Stanley F. Nelson[4], M. Carrie Miceli[2], Melissa J. Spencer[1], April D. Pyle[2]

[1]Neurology, UCLA, Los Angeles, CA, [2]Microbiology, Immunology, and Molecular Genetics, UCLA, Los Angeles, CA, [3]Molecular, Cell, and Developmental Biology, UCLA, Los Angeles, CA, [4]Human Genetics, UCLA, Los Angeles, CA

Duchenne muscular dystrophy (DMD) is typically due to frame-shifting mutations in the *DMD* gene encoding dystrophin. Loss of dystrophin protein results in progressive muscle degeneration and premature death. Approximately 60% of DMD patients have frame-shifting mutations in a hotspot region within exons 45-55 in the rod domain of dystrophin. Genotype/phenotype assessments have revealed that in-frame deletion of exons 45-55 leads to the milder, allelic disease, Becker muscular dystrophy. This finding suggests that restoration of the reading frame by targeting exons 45-55 could treat ~60% of DMD patients to greatly reduce disease severity. We have developed a platform using clustered regularly interspaced short palindromic repeats (CRISPR) and- associated protein (Cas9) gene editing to achieve this purpose. We have utilized CRISPR/Cas9-mediated deletion and rejoining of up to 725kb to restore the reading frame in DMD human induced pluripotent stem cells (hiPSCs). This is the largest deletion shown to date in *DMD*. Clonal hiPSC lines containing the exon 45-55 deletion were differentiated to disease relevant types, such as cardiomyocytes and skeletal muscle myotubes, which had restored dystrophin protein. We demonstrated, for the first time, that the internally deleted dystrophin generated by CRISPR/Cas9 was functional and improved membrane integrity, reduced miR31 expression, and restored the dystrophin glycoprotein complex *in vitro* and after engraftment of skeletal muscle cells *in vivo*. This gene editing platform restores the reading frame for the majority of DMD patients and offers potential as an *ex vivo* correction for stem cell therapy or for use *in vivo*.

736. Silencing Substituting the RHODODPSIN Gene to Treat Blinding Disorders

Salvatore Botta[1], Elena Marrocco[1], Nicola de Prisco[1], Fabiola Curion[1], Mario Renda[1], Martina Sofia[1], Mariangela Lupo[1], Maria Laura Bacci[2], Carlo Gesualdo[3], Settimio Rossi[3], Francesca Simonelli[3], Enrico Maria Surace[1]

[1]Tigem, Naples, Italy, [2]Department of Veterinary Medical Sciences (DIMEVET), University of Bologna, Bologna, Italy, [3]Multidisciplinary Department of Medical, Surgical and Dental Sciences, Eye Clinic, Second University of Naples, Naples, Italy

Silencing and replacement strategy is a promising strategy to overcome mutational heterogeneity of genetic defects. In autosomal dominant retinitis pigmentosa (adRP) approximately 200 different mutations have been associated with rhodopsin gene (RHO). We recently described a system based on the delivery of a synthetic transcriptional repressor (DNA-binding-mediated silencing; ZF6-DBD), which upon Adeno-associated viral (AAV) vector delivery to the porcine retina, demonstrated selective somatic ablation of RHO in mutation independent manner, thus silencing both the wild-type and mutated alleles. To determine the actual therapeutic impact of DNA-binding-mediated silencing, we carried out the silencing-replacement strategy by coupling ZF6-DBD with RHO replacement (human RHO, hRHO CDS). To warrant simultaneous photoreceptor transduction of both ZF6-DBD and hRHO, we enclosed two expression cassettes into a single vector (DNA-binding repression and replacement, DBR-R). To generate the construct we evaluated key variables for balanced simultaneous RHO repression and replacement, which are the vector dose and promoter strength. To ensure high and rod-specific hRHO replacement, we opted for a high vector dose and the strength of the GNAT1 promoter elements. To decrease ZF6-DB expression levels at high vector dose, while keeping rod-specificity, we both shortened the human RHO promoter and deleted the 5' sequence of the ZF6-DB target. We used 1x1012 gc of vector of DBR-R (AAV8-RHOΔ-ZF6-DB-GNAT1-hRHO) to administer to porcine retina. Administration of the DBR-R vector resulted in concomitant rod-specific transcriptional repression of the porcine Rho (35%) and in balanced replacement with the exogenous hRHO (45%), as assessed by transcripts levels, protein expression and integrity of photoreceptor outer segments. The data support pre-clinical development including a dose-response and long-term efficacy and toxicity.

Oligonucleotide Therapeutics II

737. RNAi Induced Hepatotoxicity Results from a Functional Depletion of the First Synthesized Isoform of miR-122

Paul N. Valdmanis[1], Shuo Gu[2], Kirk Chu[1], Lan Jin[2], Feijie Zhang[1], Elizabeth M. Munding[1], Yue Zhang[1], Yong Huang[1], Huban Kutay[3], Kalpana Ghoshal[3], Leszek Lisowski[1], Mark A. Kay[1]

[1]Pediatrics, Stanford University, Stanford, CA, [2]GRCBL, Center for Cancer Research, National Cancer Institute, Fredrick, MD, [3]Pathology, The Ohio State University, Columbus, OH

To ensure success of RNA interference (RNAi) therapeutics, small hairpin RNAs (shRNAs) must co-opt sufficient quantities of the endogenous microRNA machinery to elicit efficient gene knockdown without impeding normal cellular function or causing liver toxicity. Using several recombinant adeno-associated viral (rAAV) vectors expressing shRNAs followed by small RNA sequencing, we determined that hepatic toxicity arises when exogenous shRNA levels exceed 12% of liver microRNAs. High shRNA expression specifically reduced miR-122-5p without affecting any other microRNAs ultimately resulting in functional de-repression of miR-122 target mRNAs. Furthermore, we found that only one isoform of miR-122-5p,

22 nucleotides in length, is displaced in toxic liver samples and that this isoform is the first to be synthesized from miR-122. A causative link between miR-122 reduction and toxicity was established when delivery of an AAV-shRNA expressing miR-122-5p could circumvent toxicity despite reaching 70% of microRNA reads. Consistent with these results, toxicity was not observed in miR-122 knockout mice - which in part adapt to an absence of miR-122 reduction - regardless of the level or sequence of shRNA. Together these results establish the limit to expendable miRNA/RNAi machinery and providing new paradigms for the role of miR-122 in liver homeostasis.

738. Engineered MicroRNA Silences C9ORF72 Variants in BAC Transgenic Mouse

Gabriela Toro Cabrera[1], Owen M. Peters[2], Tania F. Gendron[3], Alisha Gruntman[4], Robert H. Brown[2], Christian Mueller[1]
[1]Horae Gene Therapy Center, UMASS Med, Worcester, MA, [2]Neurology, UMASS Med, Worcester, MA, [3]Neuroscience, Mayo Clinic, Jacksonville, FL, [4]Gene Therapy, UMASS Med, Worcester, MA

Amyotrophic Lateral Sclerosis (ALS) is a fatal adult-onset neurodegenerative disease that affects upper and lower motor neurons causing progressive muscle weakening. Respiratory failure is ultimately the cause of death approximately 2-5 years after symptom onset. The recent discovery of an expanded hexanucleotide repeat located in chromosome 9 open reading frame 72(C9ORF72) now accounts for the majority of familial ALS cases as well as frontotemporal dementia (FTD) cases. Analysis of patient brain samples have shown that in the presence of the expansion, C9ORF72 mRNA is reduced and RNA sequences complimentary to the expansion aggregate into nuclear foci. Interestingly this RNA can translate by non-ATG repeat associated (RAN) translation into long dipeptide chains. These findings have led to the three following hypothesis on the pathogenies of C9ORF72: 1) An RNA gain-of-function, in which the expanded RNA foci sequester RNA binding and/or splicing proteins. 2) RAN translation from the repeat expansion generates inclusions of toxic poly-dipeptide proteins. 3) Haploinsufficiency, where decreased levels of mRNA lead to insufficient gene product. Our group has generated mice containing a bacterial artificial chromosome (BAC) composed of exons 1-6 of the human C9ORF72 gene and a 500 repeat hexanucleotide expansion. This mouse model recapitulates the major histopathological features seen in human ALS/FTD patients such as: lower levels of C9ORF72 mRNA, RNA nuclear foci, and the RAN translation products. We have used this mouse as a platform in which to test RNAi therapeutic strategies. To this end we have designed artificial microRNAs that target the human C9ORF72 gene with the purpose of decreasing the mRNA levels to determine if we can reduce the toxic RNA foci and/or RNA dipeptide proteins. We packaged one of the microRNAs into a recombinant adeno-associated virus (rAAV) serotype 9 to use with primary neuron cultures and *in vivo* experiments. Silencing was initially validated in primary cortical neurons from the C9ORF72 mouse model. Our results suggest that AAV9-mediated microRNA not only reduced the mRNA levels of C9ORF72 but also decreased the presence of the most abundant poly dipeptide (GP). Currently, experiments are underway to silence C9orf72 *in vivo* via intra cranial ventricular (ICV) injection of this microRNA in C9ORF72 transgenic pups and the results will be presented at the meeting.

739. rAAV Delivered MicroRNA Therapeutics Towards Efficacious Treatment of Corneal Neovascularization

Yi Lu[1], Jianzhong Ai[2], Phillip Tai[2], Dominic J. Gessler[2], Qin Su[2], Qiang Zheng[3], Phillip D. Zamore[4], Xun Xu[1], Guangping Gao[2]
[1]Department of Ophthalmology, Shanghai First People's Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China, [2]Horae Gene Therapy Center, University of Massachusetts, Medical School, Worcester, MA, [3]Research and Development Department, Chengdu Kanghong Pharmaceuticals Group Co. Ltd, Chengdu, China, [4]RNA Therapeutics Institute, University of Massachusetts, Medical School, Worcester, MA

Corneal diseases are the second major cause of blindness worldwide. Corneal neovascularization (NV) is one of the most common pathologies in corneal diseases, leading to vision loss or even blindness. However, even though different treatments for corneal NV are available, a safe and effective therapy remains to be an unmet medical challenge. MicroRNAs (miRNAs) play roles in regulating more than half of all protein-coding genes in mammals including those involved with angiogenesis. We hypothesize that modulating expression of angiogenesis related miRNAs might be an effective approach for treating corneal NV. To this end, we first set out to identify target miRNAs that play roles in corneal NV using Nanostring technologies and the classic alkali-burn induced corneal NV mouse model. Among 618 mouse miRNAs profiled, we found 35 up-regulated and 3 down-regulated miRNAs in the neovascularized corneas, which were confirmed by qRT-PCR. We selected miR-184 and miR-204, two miRNAs that were down-regulated > 10-folds in neovascularized corneas, as our candidate targets to test the concept of *in vivo* gene delivery for therapeutic miRNA to treat corneal NV. As recombinant adeno-associated virus (rAAV) holds promise for highly efficient and safe *in vivo* gene delivery to different target tissues in a serotype dependent manner, we opted to evaluate 14 serotypes of rAAV.EGFP for gene transfer efficiency in mouse corneas by eye drops (ED), intra-stromal (IS) and subconjunctival (SC) injections to search for the suitable vector(s) for delivering miRNA therapeutics to the corneas. Among the 4 leading serotype vectors (i.e. rAAVrh.8, rh.10, rh.39 and rh.43) identified, we chose rAAVrh.10 for overexpressing pri miR-184 and pri miR-204 in the corneas with alkali-burn induced corneal NV. rAAVrh.10-mediated overexpression of miR-184 or miR-204 by IS and SC injections but not ED efficiently inhibited angiogenesis and was efficacious and safe for either prevention (IS) or treatment (SC) of corneal NV. We further invested potential target genes and pathways to elucidate the anti-angiogenic mechanism of these two miRNA therapeutics. We revealed that the anti-angiogenic effect of rAAV.pri-miR-184 was achieved by targeting *Fzd4* gene, thus suppressing the canonical Wnt signaling, a well-known pathway playing vital roles in angiogenesis; while rAAV.pri-miR-204 exerted its anti-angiogenic effect by targeting angiopoietin-1 gene (*Angpt1*), a well-acknowledged pro-angiogenic factor. Meanwhile, the transcriptome analysis is under the way to search for novel gene targets of the miRNA therapeutics. Our study has great clinical relevance and demonstrated, for the first time, that miRNA-targeted novel therapeutics that can be delivered as either rAAV or synthetic nucleic acid drugs (i.e. miRNA mimics and antagomir) might offer an additional efficacious and safe clinical option for treating corneal NV and other angiogenesis-related diseases including age-related macular edema (AMD) and cancer.

740. The TLR9 Agonist EnanDIM® - Evaluation of New Enantiomeric Oligonucleotides for Cancer Immunotherapy In Vitro and In Vivo

Kerstin Kapp[1], Matthias Schroff[1], Burghardt Wittig[2], Manuel Schmidt[1]
[1]Mologen AG, Berlin, Germany, [2]Foundation Institute Molecular Biology and Bioinformatics, Freie Universitaet, Berlin, Germany

Introduction The use of TLR9 agonists as immunomodulators is supported by preclinical and clinical studies, showing their anti-tumor effect by enhancing both cellular and humoral immune responses. So far, two different families of DNA molecules containing non-methylated CG-motifs for TLR9 activation have been established: Dumbbell-shaped dSLIM(R) molecules are protected against exonucleolytic degradation by their covalently-closed, natural phosphodiester (PO) backbone. In contrast, single-stranded, oligodeoxynucleotides (CpG-ODN) are most commonly chemically-stabilized by phosphorothioates (PTO) in their phosphate moieties. PTO modification, however, produce off-target effects in immune cell populations and have resulted in an unfavorable risk-to-benefit ratio.

Methods To avoid the off-target effects of PTO-modified CpG-ODN, linear single-stranded ODN were synthezised using L-deoxyribonucleotides at their 3'-ends, which are the natural enantiomers of Ddeoxyribonucleotides. The vast majority of deoxyribose in present organisms are D-deoxyribose, thus co-evolved nucleases are blind for L-deoxyribose thereby leaving L-protected ODN intact. We selected nucleotide sequences of such L-protected, CG-motif containing, single-stranded ODN, EnanDIM(R), for high secretion of IFN-alpha and IP-10 from human peripheral blood mononuclear cells (PBMC). In a maximum feasible dose approach in CD-1 mice EnanDIM(R) doses of 10 to 50 mg per mice were injected subcutaneously to evaluate the acute toxicity and immunomodulatory properties of EnanDIM(R) molecules *in vivo*.

Results EnanDIM581 and EnanDIM532 were chosen since they caused high secretion of IFN-alpha and IP-10 from human PBMC and resulted in a strong activation of monocytes, NK cells and plasmacytoid dendritic cells (pDC) i*n vitro*. Notably, both showed a distinct immune activation pattern, with the highest secretion of IFN-alpha by EnanDIM581 and the strongest maturation of TLR9-bearing pDC by EnanDIM532. EnanDIM744, comprising EnanDIM581 with additional 5'-end L-nucleotide protection and exhibiting an immune activation pattern similar to EnanDIM581, was selected as third EnanDIM(R) for *in vivo* studies. In the maximum feasible dose approach, safety assessments were performed throughout the study period and no mortality, clinical signs and body weight changes were observed, despite the fact that extremely high doses of app. 300 to 1700 mg/kg were used. A gross necropsy consisting of a macroscopic organ evaluation at day 15 revealed also no toxicity. Regarding immune activation, increased levels of IP-10 in serum were observed 24 hours after injection but not after 15 days confirming that L-nucleotides in EnanDIM(R) do not change the kinetic profile known from other DNA-based TLR9 agonists.

Conclusions EnanDIM(R), a new family of TLR9 agonists with conformation-mediated nuclease-resistance, broadly activates the immune system *in vitro*. Maximal feasible doses of EnanDIM(R) resulted in no signs of toxicity and confirmed immunomodulatory effects *in vivo*. Therefore EnanDIM(R) has the potential for clinical development in the treatment of cancer.

741. Development of LNA Gapmer Oligonucleotide Based Therapy for FTD/ALS Caused by the C9orf72 Repeat Expansion

Yoshitsugu Aoki[1], Raquel Manzano[2], Shin'ichi Takeda[1], Kevin Talbot[3], Matthew Wood[4]
[1]Department of Molecular Therapy, National Center of Neurology and Psychiatry, Tokyo, Japan, [2]Department of Molecular Therapy, National Center of Neurology and Psychiatry, Tokyo, United Kingdom, [3]Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, United Kingdom, [4]Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom

A hexanucleotide repeat expansion mutation in the C9orf72 locus is the commonest genetic cause of amyotrophic lateral sclerosis and frontotemporal dementia (C9ALS/FTD). Currently, the biological function of C9orf72 remains unknown. However, bioinformatic analysis has revealed that the C9orf72 gene product has a differentially expressed in normal and neoplasia (DENN)-like domain and might regulate membrane trafficking as a Rab-GTPase GDP/GTP exchange factor. Here, using comprehensive GST-Rab pulldown analysis, we show that C9orf72 physically interacts with the Rab29 GTPase (also known as Rab7L1), suggesting that C9orf72 is a Rab29 effector. Further, we show that loss of the C9orf72/Rab29 interaction leads to dramatically reduced trafficking of extracellular vesicles (EVs), impaired intracellular trans-Golgi trafficking, and a consequent deficiency of autophagy in fibroblasts and iPSC-derived neurons from patients with C9ALS/FTD. Both overexpression of C9orf72 and antisense oligonucleotides targeting repeat-containing C9orf72 transcripts to upregulate normal variant 1 transcript levels are able independently to rescue the defective EV, trans-Golgi vesicle trafficking and basal autophagy phenotype. Our findings identify C9orf72 haploinsufficiency as a major contributing factor in ALS/FTD neurodegenerative disorders and directly link ALS/FTD to EV biogenesis. Furthermore, we demonstrate that this novel ALS/FTD pathogenic mechanism can be rescued by an antisense-based intervention.

742. A Therapeutic miRNA for Brain Disorders

Kai C. Sonntag, Woori Kim, Yenarae Lee, Donna L. McPhie, Kwang-Soo Kim, Gordana Vitaliano, Bruce M. Cohen
Psychiatry, Division of Basic Neuroscience, McLean Hospital, Harvard Medical School, Belmont, MA

One of the main hurdles in developing novel therapeutics for age-related disorders is the still limited understanding of both the biology of normal aging and the pathologic mechanisms of the diseases seen in old age. Brain aging and age-related brain disorders are characterized by slow progressive deterioration or death of neurons, and are influenced by age- and disease-specific neurotoxicity, and compensatory neuroprotective mechanisms that are important in cell survival. If the cellular defense and restorative mechanisms are compromised, the penetrance of neurotoxic factors becomes higher and cell survival less likely. Among the key regulatory factors that govern gene and protein networks and, consequently, influence neuronal health and function are small non-coding RNA molecules such as miRNAs. It is increasingly appreciated that even modest disturbances of these regulatory factors can have profound effects on cell survival in response to stress. We have identified a novel mechanism in neurons, mediated by miR-126, which regulates the effects of numerous neurotrophic and neuroprotective growth factors (GF). Specifically, we found that elevated levels of this miRNA are neurotoxic and increase the vulnerability of neurons to a variety of non-specific and disease-specific toxic factors, including Staurosporine (STS), Alzheimer's disease (AD)-associated amyloid beta 1-42 oligomers ($A\beta_{1-42}$), and 6-OHDA, which induces oxidative

stress in dopamine (DA) neurons, thereby mimicking Parkinson's disease (PD) pathology. Mechanistically, miR-126 targets a series of factors in PI3K/AKT/GSK-3β and MAPK/ERK signaling pathways and small increases of this miRNA cause a downregulation of these signaling cascades, impairing the effects of neurotrophic and neuroprotective GF, such as IGF-1, NGF, BDNF, and soluble amyloid precursor protein α (sAPPα). In turn, inhibiting miR-126 enhances the actions of GF without disturbing normal neuronal cell function. Our data indicate that miR-126 may play a profound role in neuronal cell survival, at least in part by regulating GF/PI3K/AKT and MAPK/ERK signaling. While its elevation is neurotoxic, its inhibition is neuroprotective, suggesting that targeting this miRNA may have therapeutic potential for neurological and age-related disorders. To experimentally address this concept, we have developed a therapeutic strategy using a nanotechnology approach to prevent disease onset in a mouse model of AD.

743. Minicircles Are Similar to Plasmids in Providing High Level, Long-Term Expression in the Lung

Deborah R. Gill[1], Reto P. Bazzani[1], Mary M. Connolly[1], Martin Schleef[2], Stephen C. Hyde[1], Ian A. Pringle[1]
[1]Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom, [2]Plasmid Factory, Bielefeld, Germany

Background: Many gene therapy applications require persistent transgene expression to treat chronic disease. We have previously developed CpG-free plasmids capable of robust, long-term transgene expression in the mouse lung (Hyde *et al*; 2008 Nat. Biotech. 26:549) and confirmed their utility in clinical trials, where monthly aerosol delivery of plasmid/liposomes to patients with Cystic Fibrosis resulted in a stabilisation of their lung function over a 12-month period (Alton *et al* 2015 Lancet Respir. Med. 3:684). The removal of all CpGs from a plasmid, including the selection of a CpG-free promoter, can be time-consuming, therefore we evaluated which aspects of plasmid design were crucial for long-term transgene expression in the lung, including the use of minicircles instead of conventional plasmids. Results: First, we measured luciferase (Lux) expression in the lungs of BALB/c mice from a CpG-free transgene expression cassette (hCEFI-soLux) compared with a standard CpG-rich (hCEFI-Lux) cassette. Plasmid DNA was complexed with 25 kDa branched polyethylenimine (PEI) and delivered as an aerosol to the lungs of BALB/c mice (n=6 per time-point). Lux activity from the CpG-free hCEFI-soLux cassette was greater than its CpG-rich counterpart ($p < 0.005$) at every time-point (up to 28 days), with no loss of activity over the course of the study. Expression from the expression cassette containing CpGs, however, was not persistent, but declined to between 30% and 2% of respective day 1 levels by day 28 ($p < 0.05$). Next, we performed similar studies using minicircles, where the CpG-free and CpG-rich expression cassettes were manufactured as minicircles with minimal backbone sequences (209bp with 11 CpGs). When the CpG-free cassette was used there was no significant difference between Lux activity obtained from the plasmid and minicircles; in both cases robust expression persisted beyond the duration of the study (28 days). Finally, we investigated whether the detection of CpGs by the Toll-like receptor-9 (TLR9) signalling pathway played a role in the loss of expression from the CpG-rich cassette. The experiments were repeated in TLR9-deficient mice and the results showed that Lux levels were similar to those obtained in BALB/c mice. This indicated that the effect of CpGs on the *in vivo* expression profile (whether in the transgene cassette or backbone) is independent of the TLR9 pathway. Conclusion: These studies indicate that a CpG-free transgene cassette is crucial to achieving high levels of persistent expression in the murine lung, when delivered as a plasmid or minicircle. Minicircles have not yet been evaluated clinically, but they have several advantages over plasmids, including their reduced overall size such that a higher effective dose is delivered. These findings could also be applicable to other organs, such as muscle and liver.

744. Optimizing Conditions for Aptamer Folding Using a High-Throughput Aptamer Fluorescence Binding and Internalization (AFBI) Assay

David D. Dickey, Paloma H. Giangrande, William H. Thiel
Department of Internal Medicine, University of Iowa, Iowa City, IA

Aptamers are small structured RNA or DNA oligonucleotides that bind target molecules with high avidity and affinity. Aptamer selection begins with a hugely complex library of aptamer sequences that are enriched for a specific target using the SELEX process, Systematic Evolution of Ligands by EXponentional enrichment. An important consideration in the SELEX process is the protocol used to fold aptamers into their active conformations. The folding conditions include multiple variables, such as temperature, buffer components, incubation time and aptamer concentration. Aptamer folding protocols vary widely across the aptamer field, and most published folding conditions primarily describe only temperature and folding time. To understand how variations in folding conditions impact aptamer function, we developed a novel high-throughput assay to interrogate the optimal folding conditions of several published aptamers. The Aptamer Fluorescence Binding and Internalization (AFBI) assay is a cell-based platform that uses a 96-well plate format to rapidly and efficiently screen multiple fluorescent-labelled aptamers against hundreds of conditions. The AFBI assay can be applied to rapidly determine aptamer binding constants (Kd) on cells, time course of aptamer internalization and cross reactivity of aptamers against different cell types.

Using the AFBI assay, we screened several different folding parameters against published aptamers. We found that the buffer components contributed significantly more to an aptamer's function than any other examined factor. The concentration of an aptamer during folding was important for some aptamers but not others, including aptamers that originated from the same selection. Most surprising was that most temperature protocols had little impact on aptamer function after folding, the exception being that high temperature (95°C) often attenuated aptamer function. In summary, our data using the AFBI assay revealed that aptamer folding is more dependent on buffer components than the temperature protocol. Furthermore, aptamers derived from the same selection may have different optimal folding conditions. These data demonstrate that optimal aptamer folding protocols need to be more carefully interrogated on a per aptamer basis and reported in detail to allow for efficacious and reproducible results.

Cancer-Immunotherapy, Cancer Vaccines III

745. Updated Phase 1 Results from ZUMA-1: A Phase 1-2 Multi-Center Study Evaluating the Safety and Efficacy of KTE-C19 (Anti-CD19 CAR T Cells) in Subjects with Refractory Aggressive Non-Hodgkin Lymphoma (NHL)

Frederick L. Locke[1], Sattva S. Neelapu[2], Nancy L. Bartlett[3], Tanya Siddiqi[4], Julio C. Chavez[5], Chitra M. Hosing[6], Armin Ghobadi[3], Lihua E. Budde[4], Adrian Bot[7], John M. Rossi[7], Marika Sherman[7], Lynn Navale[7], Meg Elias[7], Jeff Wiezorek[7], William Y. Go[7]
[1]Blood and Marrow Transplantation, Moffit Cancer Center, Tampa, FL, [2]Department of Lymphoma/Myeloma, The University of Texas MD Anderson Cancer Center, Houston, TX, [3]Siteman Cancer Center, Washington University School of Medicine, St. Louis, MO, [4]Department of Hematology & Hematopoietic Cell Transplantation, City of Hope National Medical Center, Duarte, CA, [5]Department of Malignant Hematology, Moffit Cancer Center, Tampa, FL, [6]Department of Stem Cell Transplantation and Cellular Therapy, The University of Texas MD Anderson Cancer Center, Houston, TX, [7]Kite Pharma, Santa Monica, CA

Introduction: Anti-CD19 CAR T cells with CD3-zeta and CD28 signaling domains showed durable remissions in subjects with relapsed/refractory advanced B cell malignancies at the NCI (Kochenderfer et al. Blood 2012, JCO 2014, ASH 2014). KTE-C19 utilizes the same anti-CD19 CAR construct as investigated in the NCI study in a 6-8 day manufacturing process (Better et al. ASCO 2014). ZUMA-1 is a phase 1-2 multicenter, open-label study evaluating the safety and efficacy of KTE-C19 in subjects with refractory aggressive B-cell NHL. **Methods:** Subjects received KTE-C19 at a target dose of 2 x 10^6 (minimum 1 x 10^6) anti-CD19 CAR T cells/kg after a fixed dose conditioning chemotherapy regimen of cyclophosphamide 500 mg/m^2/day and fludarabine 30 mg/m^2/day for 3 days. The primary objective of phase 1 was to evaluate the safety of KTE-C19 as determined by the incidence of dose-limiting toxicities (DLT). Key secondary objectives were overall response rate, duration of response, levels of CAR T cells in the blood, and levels of serum cytokines. Key inclusion criteria were ≥ 18 years old, ECOG 0-1, and chemotherapy-refractory disease defined as progressive disease or stable disease as best response to last line of therapy, or disease progression ≤ 12 months after autologous stem cell transplant (ASCT). **Results:** As of 20 Nov 2015, 7 subjects were dosed in the phase 1 portion of the study. All subjects were evaluable for safety and 6 were evaluable for efficacy with a median follow up time of 13 weeks post KTE-C19 infusion. One subject experienced a DLT of grade (gr) 4 encephalopathy and gr 4 cytokine release syndrome (CRS) and died due to an intracranial hemorrhage deemed unrelated to KTE-C19 per the investigator. Key safety and efficacy findings are summarized in the table. CRS and neurotoxicity were managed with supportive care, tocilizumab and systemic steroids. 5 of 7 subjects (71%) had an objective response including 4 complete remissions (57%). Three subjects have ongoing complete remission at 3 months. CAR T cells peaked within two weeks post infusion were detectable 1-3+ months post infusion. Updated clinical and biomarker results will be presented. **Conclusions:** The KTE-C19 regimen evaluated was safe for further study. The predominant toxicities include CRS and neurotoxicity which are generally reversible. Complete and partial responses have been observed in subjects with refractory disease. KTE-C19 can be centrally manufactured and administered in a multicenter trial. The potentially pivotal phase 2 portion of the study is ongoing. Clinical trial: NCT02348216.

Subject	Sex/ Age/ ECOG	Disease Type	Treatment History	Gr 3 or Higher KTE-C19-Related Adverse Events	Best Response
1	M/59/0	DLBCL	Relapse ≤ 12 mo after ASCT	Gr 3 encephalopathy (resolved)	Partial Response
2	M/69/1	DLBCL	Refractory to 2nd line or later line chemotherapy	Gr 3 tremor (resolved) Gr 3 delirium (resolved) Gr 3 agitation (resolved) Gr 3 restlessness (resolved) Gr 3 somnolence (resolved)	Complete Remission*
3	M/69/0	DLBCL	Refractory to 2nd line or later line chemotherapy	Gr 3 encephalopathy (resolved)	Stable Disease
4	M/67/1	DLBCL	Relapse ≤ 12 mo after ASCT	None	Complete Remission Ongoing 3 mo+
5	F/34/0	DLBCL	Relapse ≤ 12 mo after ASCT	Gr 3 hypoxia (resolved)	Complete Remission Ongoing 3 mo+
6	M/40/0	DLBCL	Relapse ≤ 12 mo after ASCT	None	Complete Remission Ongoing 3 mo+
7	F/29/1	DLBCL	Refractory to 2nd line or later line chemotherapy	Gr 4 CRS Gr 4 encephalopathy	NE

mo - months, M - male, F - female, NE - not evaluable, *relapsed and retreated with an ongoing PR

746. Go-TCR: Inducible MyD88/CD40 (iMC) Enhances Proliferation and Survival of Tumor-Specific TCR-Modified T Cells, Increasing Anti-Tumor Efficacy

Tsvetelina P. Hoang[1], Aaron Foster[1], Tania Rodriguez[1], David Torres[1], An Lu[1], Jeannette Crisostomo[1], Lorenz Jahn[2], Mirjam H. M. Heemskerk[2], David M. Spencer[1]
[1]R&D, Bellicum Pharmaceuticals, Inc, Houston, TX, [2]Hematology, Leiden Univ Med Ctr, Leiden, Netherlands

Introduction: Use of tumor antigen-specific T cell receptors (TCRs) to refocus T cell killing has shown tantalizing clinical efficacy; however, durable responses have been limited by poor T cell persistence and expansion *in vivo*. Also, MHC class I downregulation in tumors further reduces therapeutic efficacy. Therefore, we co-expressed in human T cells a small molecule dimerizer (rimiducid)-dependent "activation switch", called inducible MyD88/CD40 (iMC), along with tumor-targeted TCRs to regulate T cell expansion and activation, while affecting upregulation of MHC class I on tumors.

Methods: Human T cells were CD3/CD28-activated and transduced with αβTCR-encoding γ-retroviruses recognizing either the CT antigen, PRAME (HLA-A*0201/SLLQHLIGL), or the B-cell-specific transcriptional co-activator, Bob1/OBF-1 (HLA-B*0702/APAPTAVVL). Parallel "Go-TCR" vectors co-expressed iMC,

comprising MyD88 and CD40 signaling domains along with rimiducid-binding FKBP12-V36. Proliferation, cytokine production and cytotoxicity of modified T cells was assessed using peptide-pulsed T2 cells (PRAME only) or against PRAME$^+$/Bob1$^+$, HLA-A2$^+$ -B7$^+$ U266 myeloma cells +/- 10 nM rimiducid. MHC class I induction was measured using transwell assays and flow cytometry. *In vitro* tumor killing was analyzed by T cell and tumor coculture assays at various effector to target ratios over a 7-day period. Finally, *in vivo* efficacy was determined using immune-deficient NSG mice engrafted i.v. with U266 cells and treated i.v. with 1x10^7 transduced T cells. iMC was activated *in vivo* by weekly i.p. rimiducid injections (1-5 mg/kg). Tumor size and T cell expansion was measured using *in vivo* BLI imaging and flow cytometry.

Results: All vectors efficiently (~85%) transduced activated T cells and showed antigen-specific IFN-γ production and cytotoxicity against peptide-pulsed T2 cells and/or PRAME$^+$Bob1$^+$ U266 cells. However, both iMC signaling and TCR ligation of PRAME peptide-pulsed T2 Cells were required for IL-2 production. Coculture assays with U266 cells showed that tumor elimination, IL-2 secretion and robust (~ 50-fold) T cell proliferation (vs TCR signaling alone) was optimized with concurrent rimiducid-driven iMC activation in both "Go-PRAME" and "Go-Bob1" constructs. Further, iMC activation produced TCR-independent IFN-γ that increased (~100-fold) MHC class I expression on tumor cells. In NSG mice engrafted with U266 tumors, iMC-PRAME TCR-modified T cells persisted for at least 81 days post-injection and prevented tumor growth, unlike other T cell groups. Importantly, weekly rimiducid injection dramatically expanded iMC-PRAME TCR-expressing T cell numbers by ~1000-fold on day 81 post-injection vs T cells expressing only the PRAME TCR ($p \sim 0.001$).

Summary: The novel rimiducid-regulated "Go" switch, iMC, greatly augments activation and expansion of TCR-engineered T cells while sensitizing tumors to T cells via cytokine-induced MHC class I upregulation. iMC-enhanced TCRs are prototypes of novel "Go-TCR" engineered T cell therapies that increase efficacy, safety and durability of adoptive T cell therapies.

747. Towards the Clinical Application of BCMA CAR T Cells: The Importance of Reduced Tonic Signaling and Methods to Enhance Memory T Cells

Kevin Friedman, Holly Horton, Molly Perkins, Shannon Grande, Tracy Garrett, Richard Morgan
bluebird bio, Cambridge, MA

B cell maturation antigen (BCMA) is expressed on most multiple myeloma (MM) and some lymphoma cells, yet normal tissue expression is limited to plasma and some B cells. Here we describe the development of a CAR T cell that targets BCMA. We tested 19 BCMA-specific single chain variable fragments (scFv) linked to CD137 (4-1BB) and CD3zeta T cell signaling domains. The activity of each CAR T cell candidate differed despite targeting the same antigen. One anti-BCMA CAR T cell candidate (bb2121) was selected based on strong surface CAR expression, superior biological activity to multiple BCMA+ cell lines, and low antigen-independent activity. In vitro analysis demonstrated that antigen-independent activity was associated with T cell differentiation characterized by significantly lower CD62L expression (p=0.003). To determine the impact on tumor control, CAR T cells with antigen-independent activity, or bb2121 CAR T cells, were used to treat an NSG mouse model of MM (RPMI-8226). Immunohistochemical analysis found MM-infiltrated bb2121 T cells within 5 days and mice showed complete regressions by 12 days post treatment. In contrast, mice that received CAR T cells with antigen-independent activity had delayed regressions that occurred 20 days post treatment. Robust recognition of as few as 220 BCMA molecules/cell (compared to >10,000 molecules on MM cell lines) permitted reactivity to primary chronic lymphocytic leukemia (CLL) and other CD19+ B cell tumor lines. Indeed comparable tumor regressions were observed after administration of bb2121 or anti-CD19 CAR T cells into an NSG mouse model of disseminated Burkitt's lymphoma (Daudi: 1,170 BCMA and 267,000 CD19 molecules). Prior investigators have shown improved therapeutic efficacy by enriching for memory CAR T cells, yet current antibody-based selection methods are expensive and difficult to scale. Unexpectedly, we found bb2121 manufacture in the presence of a PI3-kinase inhibitor enriched for memory-like CAR T cells without a complicated cell sorting procedure. bb2121 CAR T cells cultured with a PI3K inhibitor expressed markers associated with T cell memory including CD62L, CD127, CD197, and CD38. In a first "stress test," of advanced disseminated lymphoma (Daudi), NSG mice were administered an amount of bb2121 CAR T cells that failed to control tumor outgrowth. Mice administered the same number of bb2121 CAR T cells cultured with a PI3K inhibitor resulted in complete tumor regressions. A defining property of memory T cells is durability despite multiple antigen encounters. In a second "stress test," bb2121 CAR T cell durability was evaluated after initial MM (RPMI-8226) clearance. Two weeks post-tumor elimination, mice were re-challenged with tumor on the opposite flank without an additional dose of CAR T cells. None of the mice treated with bb2121 CAR T cells prevented tumor outgrowth in this model. In contrast, all mice treated with bb2121 CAR T cells cultured with a PI3K inhibitor were able to control the tumor re-challenge. These data demonstrate that a potent, antigen-dependent, memory-like BCMA CAR T cell produced with an industrially scalable manufacturing process has promise for robust tumor regressions in clinical applications.

748. A Clinical Trial Using Third Generation CD19 Targeting CAR T Cells for Relapsed Lymphoma and Leukemia

Gunilla Enblad[1], Hannah Karlsson[2], Jessica Wenthe[2], Kristina I. Wikstrom[3], Magnus Essand[2], Barbara Savoldo[4], Gianpietro Dotti[4], Martin Hoglund[5], Helene Hallbook[5], Malcolm K. Brenner[4], Hans Hagberg[1], Angelica Loskog[2]
[1]Dept of Immunology, Genetics and Pathology, Uppsala University and University Hospital, Uppsala, Sweden, [2]Dept of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden, [3]VECURA, Karolinska Hospital, Huddinge, Sweden, [4]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, [5]Dept of Medical Sciences, Uppsala University and University Hospital, Uppsala, Sweden

Chimeric antigen receptor (CAR) T cells have shown promising results in patients with B cell malignancy. Herein, we report initial results from a phase I/IIa study (NCT:02132624) using CD19-targeting (CD28/4-1BB) CAR T cells. Patients with refractory CD19+ B cell malignancy were eligible, provided there was no other curative treatment available. Of 18 enrolled, 14 patients have received CAR T cells. Eleven had lymphoma and 3 patients had acute lymphoblastic leukemia (ALL). Autologous CAR T cells were manufactured by expansion using aCD3/aCD28 and IL2, and engineered using a gamma retrovirus encoding the CAR. During T cell manufacture, all lymphoma patients received conventional treatment to control tumor burden. Prior to T cell infusion, day -5 to -1, patients #6-17 received cyclophosphamide (500mg/m^2) and fludarabine (25mg/m^2) as preconditioning to decrease immunosuppressive cells. The patients received one infusion of CAR T cells ranging from 2x10^7 to 2x10^8 cells/m^2. Six of 14 patients had initial complete responses (CR) but 5 progressed later and 2 of those had another CR to additional chemotherapy to which they were previously refractory. Six patients are still alive of which 2 have been alive for >1.5 years post CAR therapy, 1 >10 months, and the 3 patients >3 months. The two last

patients received anti-PD1 antibody therapy post CAR infusion. Blood samples from the patients have been analyzed for CAR T cells (PCR) and immunosuppressive cells such as T regulatory cells (Treg), myeloid-derived suppressor cells (MDSCs) and M2 macrophages (CD163+). The CAR gene could be detected at the highest levels 1w post infusion and then the copy number varied over time. The levels were not correlated to ongoing response in terms of CR or PD but patients with higher levels commonly had a response to treatment and/or an ongoing cytokine release syndrome (CRS). Mild CRS or signs of neurological toxicity were noted in several patients but only 3 CRS and 1 neurological toxicity required hospitalization. The level of suppressive cells at enrollment did not correlate to response but a decline of suppressive cells over time was more often noted in responding patients. Proteomic analysis (ProSeek platform) has been done on patient plasma and is under evaluation to define response biomarkers. In summary, 14 patients have been treated with increasing doses of CAR T cells in Sweden. The conditioning has been relatively mild as compared to previous published studies and no lethal toxicity occurred. Six of the 14 treated patients had an initial CR, two of them and four other patients are still alive.

749. Escape from Tumor Dormancy Following Gene- or Viro-Therapies Is Mediated by Acquisition of a Phenotype in Which Innate Immune Surveillance Actively Drives Tumor Cell Recurrence

Tim Kottke[1], Diana Rommelfanger[1], Kevin G. Shim[1], Jose Pulido[1], Jill Thompson[1], Karishma Rajani[1], Oleg Bobr[1], Peter Selby[2], Elizabeth Ilett[2], Alan Melcher[2], Richard Vile[1]
[1]Mayo Clinic, Rochester, MN, [2]University of Leeds, Leeds, United Kingdom

When established (~0.3-0.5cm) primary murine B16 tumors are treated with either cytotoxic gene therapy (HSVtk/GCV), oncolytic virotherapy (intra-tumoral reovirus) or adoptive T cell therapy, a proportion of mice will become apparently tumor free. However, if the animals are observed for prolonged periods of time (>60-100 days), the majority of mice will develop aggressively growing recurrent tumors. Excision of the site of tumor injection during the period of tumor dormancy (no palpable tumor) indicated that residual tumor cells are readily detectable in most mice by histology. Unexpectedly, it was extremely difficult to re-grow the tumor cells from these specks of minimal residual disease *in vitro*. However, by screening multiple cytokines for their ability to support re-growth of recurrent B16 cells, we identified TNF-α as a major growth factor for recurrent cells. Conversely, treatment of primary B16 populations with TNF-α led to highly significant reductions in tumor cell viability. Similarly, depletion of NK cells, or antibody blockade of TNF-α, from C57Bl/6 mice led to significantly increased tumorigencity of primary B16 tumors. In contrast, depletion of NK cells allowed for decreased tumorigenicity of TNF-α stimulated recurrent B16 cells recovered from mice in a state of minimal residual disease. These data suggest that tumor recurrence may be mediated by a distinct phenotypic switch *in vivo*. Thus, initially, primary tumor cells are highly sensitive to innate immune cell surveillance through NK cells and TNF-α. However, escape from dormancy in vivo is associated with acquisition of a phenotype in which such TNF-α-mediated immune surveillance actively drives tumor growth. TNF-α growth promoted recurrent cells also expressed high levels of PD-L1 compared to primary tumor cells, suggesting that this may be a further mechanism by which recurrences could emerge *in vivo*, even in the presence of anti tumor T cell responses. Consistent with this hypothesis, recurrence was significantly inhibited *in vivo* following gene, viro- or adoptive T cell therapies, by treatment with TNF-α blockade or systemic checkpoint inhibitor therapy.

750. Anti-Tumor Effects of Anti-CD20 Chimeric Antigen Receptor Therapy in Canine B Cell Lymphoma Patients

M. Kazim Panjwani, Josephine S. Gnanandarajah, Martha A. MaloneyHuss, Nicola J. Mason
University of Pennsylvania, Philadelphia, PA

Chimeric antigen receptor (CAR) T cell therapy has shown great promise in treating human leukemias. However, for CAR T technology to achieve its full potential against solid malignancies, better pre-clinical models are needed that recapitulate the immune suppressive tumor microenvironment and the distribution of target antigens. Pet dogs are an outbred population that have a close phylogenetic relationship with man and frequently develop naturally occurring, spontaneous malignancies. We explored the feasibility of evaluating CAR T cell technologies in dogs with spontaneous cancers by assessing CD20-targeting CAR T cells in dogs with relapsed CD20+ B cell lymphoma.

We have developed standard procedures for optimal *ex vivo* canine T cell activation, expansion, and lentiviral transduction. Canine PBLs were activated *in vitro* using artificial antigen presenting cells (aAPCs) loaded with anti-canine CD3, in the presence of rhIL-2 and rhIL-21, and the time of peak T cell activation and disappearance of aAPCs from the culture were determined. Peak CD25 expression was observed 3-4 days post-stimulation, when less than 5% of aAPCs remained in culture, eliminating potential competitor cells for viral infection. Transduction of canine T cells, 4 days post-stimulation using VSVg-pseudotyped lentivirus encoding a second-generation CD20-targeting CAR (CD20-28-ζ) at an MOI of 20 resulted in up to 26% surface expression of the CAR on T cells grown from a healthy dog. *In vitro* co-culture of CD20-specific canine CAR T cells derived from a lymphoma patient with CD20+ target cells resulted in antigen-specific CAR T cell expansion and effective killing.

To determine whether canine CD20 CAR T cells could survive, expand and exhibit cytotoxic activity *in vivo*, three pet dogs with relapsed CD20+B cell lymphoma were treated with autologous CD20-28-ζ CAR T cells in an IACUC-approved protocol. PBLs were transduced (1.5-6.5%), expanded (22-153 fold) and infused into the patients either IV or IV plus intra-nodal injection. A 52% decrease in tumor volume was observed in the first patient 4 days post-IV infusion, coinciding with an 82% increase in T cell frequency within the malignant lymph node. CAR T cells were administered via intra-nodal injection into the largest malignant node of the second dog, which stayed stable in volume over two weeks while all other non-injected malignant nodes doubled in size. The third dog received the largest number of CAR T cells administered IV. In this dog, CAR T cells have expanded and persisted for three weeks and malignant nodes have remained below the limit of accurate measurement.

These data demonstrate the ability of canine CAR T cells to function in vitro and to halt and even temporarily reverse solid tumor progression in canine patients, mirroring what has been observed in human disease and treatment. This work shows the feasibility of this approach in dogs and offers a spontaneous cancer model for pre-clinical testing of novel CAR targets and next generation CAR design that aims to inform human clinical trials.

751. Improving CAR T Cell Function by Reversing the Immunosuppressive Tumor Environment of Pancreatic Cancer

Sujita Sukumaran, Somala Mohammed, Norihiro Watanabe, Pradip Bajgain, Salma Ansari, Usanarat Anurathapan, Helen E. Heslop, Cliona R. Rooney, Malcolm K. Brenner, Ann M. Leen, Juan F. Vera
Baylor College of Medicine, Houston, TX

Adoptive transfer of T cells redirected to tumor-associated antigens (TAAs) by expression of chimeric antigen receptors (CARs) can produce tumor responses, even in patients with resistant malignancies. To target pancreatic ductal adenocarcinoma (PDAC), we generated T cells expressing a CAR directed to the TAA prostate stem cell antigen (PSCA). T cells expressing this CAR were able to kill PSCA(+) tumor cell lines CAPAN1 and K562-PSCA but not PSCA(-)293T cells (74±4%, 73±6% and 9±3% specific lysis, respectively, 10:1 E:T, n=3). Although these CAR-T cells had potent anti-tumor activity, pancreatic tumors employ immune evasion strategies such as the production of inhibitory cytokines, which limit in vivo CAR-T cell persistence and effector function. Indeed, when we examined the serum of patients with pancreatic cancer (n=8) we found the levels of the immunosuppressive cytokine IL4 to be elevated relative to patients with benign pancreatic disorders or normal healthy controls (14.25±19.48 pg/mL vs 7.28±9.03 vs 1.13±1.42 pg/mL). Thus, to protect our CAR-PSCA T cells from the negative influences of IL-4, we generated a chimeric cytokine receptor in which the IL4 receptor exodomain was fused to the IL7 receptor endodomain (IL4/7 ChR). Transgenic expression of this molecule in CAR-PSCA T cells can invert the inhibitory effects of tumor-derived IL4 to instead promote the proliferation of the effector CAR T cells. In preliminary experiments, we successfully co-expressed both CAR-PSCA and IL4/7 ChR (47.5±12.3% double-positive cells, n=4) on primary T cells. These T cells retained their tumor-specific activity (80±8% specific lysis against CAPAN1, 10:1 E:T, n=3) and when cultured in conditions that mimic the tumor milieu (IL4 12.5 ng/ml), CAR-PSCA 4/7R ChR-modified T cells continued to expand (increase from 2x10e6 cells on day 0 to 5.53±8.46x10e10 cells on day 28), unlike unmodified CAR-PSCA T cells which plateaued at 3.84±5.43x10e8 cells (n=4). Indeed, in the presence of IL4, transgenic cells had a selective advantage (comprising 44.8±11.0% of the population on day 0 and 87.6±10.0% on day 28; n=4). However, even after prolonged cytokine exposure these T cells remained both antigen- and cytokine-dependent. In conclusion, CAR-PSCA 4/7 ChR-modified tumor-specific T cells can effectively target pancreatic cancer cells and are equipped to expand, persist, and retain their cytotoxic function even in the presence of high levels of IL4 in the tumor microenvironment.

752. Single Chain TCR Gene Editing in Adoptive Cell Therapy for Multiple Myeloma

Sara Mastaglio[1], Pietro Genovese[1], Zulma Magnani[1], Elisa Landoni[1], Barbara Camisa[1], Giulia Schiroli[1], Elena Provasi[1], Angelo Lombardo[1], Andreas Reik[2], Nicoletta Cieri[1], Martina Rocchi[1], Giacomoq Oliveira[1], Giulia Escobar[1], Monica Casucci[1], Bernhard Gentner[1], Antonello Spinelli[1], Anna Mondino[1], Attilio Bondanza[1], Luca Vago[1], Maurilio Ponzoni[1], Fabio Ciceri[1], Micheal Holmes[2], Luigi Naldini[1], Chiara Bonini[1]
[1]San Raffaele Scientific Institute, Milan, Italy, [2]Sangamo Biosciences, Richmond, CA

Transfer of T cell receptors (TCR) specific for tumor-associated antigens is a promising approach for cancer immunotherapy. The TCR gene editing technology, based on the knockout of the endogenous TCR alpha and beta genes, followed by the introduction of exogenous tumor-specific TCR genes, proved safer and more effective than conventional TCR gene transfer in vitro and in animal studies. While successful, complete editing requires multiple manipulation steps and four transduction procedures. To reduce the duration and complexity of the procedure, we developed and tested the 'single TCR editing' (SE) approach, based on the disruption of the endogenous TCR alpha chain only, followed by the transfer of the tumor specific TCR genes. We validated SE using an HLA-A2 restricted TCR specific for NY-ESO-$1_{157-165}$, a cancer testis antigen expressed by numerous solid tumors and hematological malignancies, including high-risk multiple myeloma. Conventional TCR gene transfer (TR) and SE cells were compared in terms of efficacy and safety in vitro (phenotypical analysis, gamma-IFN EliSPOT, ^{51}Cr release and co-culture assays) and in vivo (adoptive T cell transfer in an experimental humanized-mouse model of multiple myeloma). SE rapidly produced high numbers of tumor specific T cells, with an early differentiation phenotype. When tested *in vitro*, SE T cells showed a high killing activity against the U266 multiple myeloma cell line, similar to that of T cells redirected with conventional TCR gene transfer; however, while TR cells proved highly alloreactive, SE cells showed a favorable safety profile. The SE and TR cells were then compared in vivo by infusing the cells into NSG mice previously engrafted with myeloma. In contrast to the TR cells, SE cells mediated tumor rejection without inducing xenogeneic graft versus host disease, thus promoting a significantly higher survival than that observed in mice treated with TR cells. The detrimental alloreactive effect mediated by TR cells led to a xenogeneic GvHD rate comparable to that of untransduced allogenic T cells. This finding was confirmed by histopathological examination which revealed TR cell infiltrations and GvHD-like lesions in several murine organs and tissues. Conversely, the abrogation of the endogenous TCR in the SE tumor-redirected lymphocytes prevented off-target reactivity that normally leads to GvHD in mice, potentially providing a safer T cell therapy against cancer. Overall, the single TCR gene editing procedure provides a rapid and efficient method for generating primary T cells that highly express a tumor specific TCR and are devoid of their endogenous TCR, and thus represents a protean platform for the effective and safe adoptive transfer of allogeneic and autologous T lymphocytes redirected towards any desired tumor associated antigen.

Hematologic & Immunologic Diseases II

753. Lentiviral-Mediated Gene Correction of Mobilized Peripheral Blood Progenitors and Repopulating Cells from FA-A Patients

Paula Río[1], Susana Navarro[1], Guillermo Guenechea[1], Anne Galy[2], Rebeca Sánchez[1], María L. Lamana[1], Rosa Yañez[1], José A. Casado[1], José C. Segovia[1], Jordi Surrallés[3], Ricardo López[4], Nagore García de Andoaín[5], Pedro Ruiz[6], Albert Catalá[7], Cristina Díaz de Heredia[8], Julián Sevilla[9], Juan A. Bueren[1]
[1]Hematopoietic Innovative Therapies, CIEMAT/CIBERER/IIS. Fundación Jiménez Díaz, Madrid, Spain, [2]Genethon, Evry, France, [3]Autónoma University of Barcelona/CIBERER, Barcelona, Spain, [4]Hospital de Cruces, Bilbao, Spain, [5]Hospital de Donostia, San Sebastián, Spain, [6]Hospital Materno Infantil, Málaga, Spain, [7]Hospital San Joan de Deu, Barcelona, Spain, [8]Hospital Vall d'Hebron, Barcelona, Spain, [9]Hospital del Niño Jesús, Madrid, Spain

Fanconi anemia (FA) is an inherited disease mainly characterized by congenital abnormalities, progressive bone marrow failure and cancer predisposition. Based on previous FA gene therapy studies we aimed at improving the therapeutic efficacy of gene therapy in FA-A patients using optimized protocols of hematopoietic stem cell (HSC) harvesting and transduction. The mobilization regimen consisted on the administration of G-CSF (neupogen; 12 µg/Kg/12 hours) and plerixafor (mozobil; 240 µg/kg body weight/day). Two out of six

patients with 15 and 16 years old did not reach the threshold level of $CD34^+$ cells in PB, and therefore, apheresis was not conducted. In the four youngest patients, the total number of collected $CD34^+$ cells/kg ranged between $1.6x10^6$ to $7.6x10^6$ ($8.6x10^5$ to $5.1x10^6$ $CD34^+$ cells/Kg after purification), and were cryopreserved for clinical use. The short transduction of small aliquots of mPB $CD34^+$ samples from these patients with a GMP-produced lentiviral vector that harbors the *FANCA* therapeutic gene showed transduction efficacies between 20-40%, measured by the survival of transduced colony forming cells (CFCs) to mitomycin C (MMC). To assess the repopulating ability of transduced FA-A $CD34^+$ cells, aliquot samples were transplanted into NSG mice conditioned with 1.5 Gy. Remarkably, most of the transplanted samples engrafted into the NSG mice (1-10% of the BM cells were $hCD45^+/mCD45^-$). Moreover, a selection advantage of corrected $CD34^+$ FA-A cells was observed in engrafted mice, as deduced from the high MMC-resistance of hCFCs obtained from recipients' BM. Our results show for the first time that clinically applicable transduction protocols can correct the phenotype of human FA-A hematopoietic repopulating cells. Currently a gene therapy trial of FA-A patients based on the *ex vivo* transduction of mPB $CD34^+$ cells with lentiviral vectors is open in our Institutions.

754. Exploring the Human Hematopoietic Hierarchy Through Retroviral Integration Sites Tracking in the Wiskott Aldrich Syndrome Gene Therapy Trial

Emmanuelle Six[1], Laure Caccavelli[2], Cecile Roudaut[2], Frances Male[3], Yinghua Wu[3], Nicolas Cagnard[4], Alessandra Magnani[2], Fabien Touzot[2], Elisa Magrin[2], Anne Galy[5], Isabelle André-Schmutz[1], Salima Hacein-Bey Abina[6], Jean-Philippe Jais[7], Charles C. Berry[8], Frederic D. Bushman[3], Marina Cavazzana[2]

[1]Laboratory of Human Lymphopoiesis, Imagine Institute, Paris, France, [2]Biotherapy Clinical Investigation Center, Necker Children's Hospital, Paris, France, [3]Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA, [4]Plateforme de Bioinformatique, SFR Necker, Paris, France, [5]Généthon, Evry, France, [6]Immunology Laboratory, Groupe Hospitalier Universitaire Paris-Sud, Le Kremlin Bicetre, France, [7]Service d'Informatique Médicale et de Biostatistiques, Necker Children's Hospital, Paris, France, [8]Division of Biostatistics and BioInformatics, University of California at San Diego, San Diego, CA

The historical model of hematopoiesis, mainly derived from murine studies, is based on the existence of a single hematopoietic stem cell (HSC) capable of generating all blood cell lineages. However this model has been challenged by the proposal of four types of murine HSC, which differ by their contribution to the myeloid and lymphoid lineages. Here we have used data from a gene therapy trial to treat Wiskott-Aldrich syndrome (WAS) to explore hematopoiesis in humans. In the trial, the therapeutic vector (lentivirus) integrates into the genome at unique positions in each hematopoietic stem and progenitor cell and is consequently transmitted to all its progeny. Thus hematopoietic ontogeny in humans can be inferred by tracking the appearance of unique integration sites in fractionated blood cell populations. This provides a unique opportunity to model the developmental complexity of the human haematological system. Considerable effort over the last 15 years has been devoted to optimizing retroviral integration sites (RIS) analysis using ligation mediated PCR (LM-PCR), combined with acoustic shearing and high-throughput Illumina sequencing. Acoustic shearing enables more precise quantification of RIS abundance through the enumeration of the various sizes of shear fragments containing a given RIS, which correspond to individual cells in the starting blood cell populations. In addition, the great diversity of these clonal populations results in very sparse samples for many clones, necessitating the development of statistical methods to account for unsampled cell fractions. In four WAS patients treated by gene therapy, we have sorted peripheral blood samples for 5 cell types: myeloid (granulocytes and monocytes) and lymphoid subpopulations (T, B and NK cells), and analysed their RIS profile. Each RIS corresponds to a particular stem/progenitor cell clone, with a particular pattern defined by its presence or absence in each of the 5 lineages. These data are then use to reconstruct aspects of the hematopoietic hierarchy. In order to face the challenging issue of cell sorting contamination we have been using a stringent sort precision mode and we treat residual contamination explicitly in downstream statistical models. Using these approaches, we have characterized up to tens of thousands RIS per patient with a follow up of 4 years. We showed that a large fraction of RIS clones are detected in a single lineage, while other RIS clones are characterized by different levels of contribution to the myeloid and lymphoid lineages, highlighting the heterogeneity of human HSCs. Clones contributing to all 5 lineages are readily recovered but do not constitute the majority of the population. Longitudinal analysis of clonal dynamics is ongoing. These new findings provide unique data on human hematopoiesis in gene corrected WAS patients.

755. One-time Gene Therapy for Hereditary Angioedema

Odelya E. Pagovich[1], Ting Qiu[1], Adele S. Whaley[1], Anthony R. Russo[1], Jonathan Rosenberg[1], Bishnu P. De[1], Carlo Russo[2], Maria J. Chiuchiolo[1], Dolan Sondhi[1], Stephen M. Kaminsky[1], Ronald G. Crystal[1]

[1]Weill Cornell Medical College, New York, NY, [2]Annapurna Therapeutics, Paris, France

Hereditary angioedema (HAE) is a potentially life-threatening autosomal dominant deficiency affecting 1 in 50,000 individuals from all ethnic groups worldwide. More than 99% of HAE cases are caused by a deficiency in functional plasma C1 esterase inhibitor (C1E-INH, a serine protease inhibitor) due to mutations in the SERPING1 gene. Low plasma C1E-INH activity dysregulates the contact, complement, and fibrinolytic systems, resulting in unpredictable, recurrent submucosal edema of cutaneous tissues, gastrointestinal and respiratory tracts. If not treated in a timely manner, laryngeal edema can result in death by asphyxiation. Current HAE treatments consist of management of acute attacks, repeated prophylactic therapy with C1E-INH or long term prophylaxis with attenuated androgens, each complicated by a high economic burden, limited compliance, drug side effects and contraindications. To circumvent this challenge, we hypothesized that a one-time administration of an adeno-associated virus (AAV) gene transfer vector expressing the genetic sequence of C1 esterase-inhibitor (serotype 10 expressing the human CIE-INH coding sequence, AAVrh.10hC1EI) would provide sustained C1E-INH activity levels in plasma, sufficient to prevent angioedema episodes. To study the efficacy of AAVrh.10hC1EI, using CRISPR/Cas9 technology we created a novel C1E-INH deficient mouse model analogous to human HAE disease, and characterized the resulting SERPING1 gene mutations by genome sequencing. The heterozygous mouse model shares characteristics associated with HAE in humans including decreased C1E-INH and C4 levels in plasma and increased vascular permeability. Administration of AAVrh.10hC1EI to the heterozygous mice resulted in sustained human C1E levels above the predicted therapeutic levels. In order to demonstrate that the increased vascular permeability observed in heterozygote *C1E-INH+/-* mice was a direct result of C1E-INH deficiency, Evans blue dye was injected intravenously and extravasation of dye from the vasculature evaluated. Compared to wild type mice under baseline conditions, the *C1EINH+/-* mice had increased extravasation of the dye into the hind paws quantitated by optical absorbance at 600 nm. Strikingly, AAVrh10.hC1EI-treated (10^{11} gc) mice displayed a

marked decrease in dye extravasation, whereas non-treated *C1E-INH +/-* mice had markedly increased dye extravasation. These results demonstrate that a single treatment with AAVrh.10hC1EI has the potential to provide long term protection from angioedema attacks in the affected population, representing a paradigm shift in current therapeutic approaches.

756. Stable Amelioration of Hemophilia B in Dogs by Intravenous Administration of Lentiviral Vectors Expressing Hyper-Functional Factor IX

Alessio Cantore[1], Michela Milani[1,2], Tongyao Liu[3], Patrizia Della Valle[4], Armando D'Angelo[4], Thierry VandenDriessche[5], Marinee Chuah[5], Haiyan Jiang[3], Timothy Nichols[6], Luigi Naldini[1,2]
[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy, [2]Vita-Salute San Raffaele University, Milan, Italy, [3]Biogen, Cambridge, MA, [4]Coagulation Service, San Raffaele Scientific Institute, Milan, Italy, [5]Free University of Brussels, Brussels, Belgium, [6]University of North Carolina, Chapel Hill, NC

Lentiviral vectors (LVs) are attractive vehicles for liver-directed gene therapy by virtue of their ability to stably integrate in the genome of target cells and the low prevalence of pre-existing immunity against HIV in humans. Over the past years, we have developed a LV platform that can achieve stable transgene expression in the liver, induce transgene-specific immune tolerance and establish correction of hemophilia in mouse models upon systemic administration. This LV is designed to stringently target transgene expression to hepatocytes through transcriptional and microRNA-mediated regulation. We then investigated the efficacy and safety profile of portal vein administration of LVs expressing canine factor IX (FIX) in a canine model of hemophilia B. We produced large-scale batches of LVs qualified for in vivo administration and treated adult hemophilia B dog by portal vein administration. We observed long-term stable reconstitution of canine FIX activity up to 1% of normal and significant amelioration of the clinical phenotype in 3 treated dogs with 6, 3.5 and 2.5 years of follow up. LV infusion was associated with transient signs of inflammatory response and mild hepatotoxicity, which could be abrogated by pretreatment with anti-inflammatory drugs. There was no detectable long-term toxicity or development of FIX inhibitors. In the perspective of clinical translation and to increase therapeutic efficacy, we next treated two 10-kg hemophilia B dogs by peripheral vein administration of LVs expressing a codon-optimized and hyperfunctional canine FIX at a 5-fold higher dose than those previously administered. Intravenous LV administration was well tolerated with mild and self-limiting elevation of aminotransferases in one dog. In the dog that reached more than 1 year of follow up FIX activity ranged between 4-8% of normal. Treatment of two more dogs at a higher dose is underway. Overall, our studies position LV-mediated liver gene therapy for further pre-clinical development and clinical translation. LVs may thus complement other available vectors to address some of the outstanding challenges posed by liver gene therapy of hemophilia and conceivably other diseases.

757. Stable *In Vivo* Transduction of Primitive Hematopoietic Stem Cells After Mobilization and Intravenous Injection of an Integrating Gene Transfer Vector

Maximilian Richter[1], Roma Yumul[1], Donna Palmer[2], Philip Ng[2], Zsuzsanna Izsvák[3], Roberto Cattaneo[4], Jing Liu[5], Anja Ehrhardt[5], Wolfgang Uckert[3], Thalia Papayannopoulou[1], André Lieber[1]
[1]University of Washington, Seattle, WA, [2]Baylor College of Medicine, Houston, TX, [3]Max-Delbrück-Center for Molecular Medicine, Berlin, Germany, [4]Mayo Clinic, Rochester, MN, [5]Universität Witten/Herdecke, Witten, Germany

Current protocols for hematopoietic stem cell (HSC) gene therapy involve the collection of HSCs from donors/patients, in vitro culture, transduction with retrovirus vectors, and retransplantation into myelo-conditioned patients. Besides its technical complexity, disadvantages of this approach include the necessity for culture in the presence of multiple cytokines which can affect the pluripotency of HSCs and their engraftment potential. Furthermore, the requirement for myeloablative regimens in patients with non-malignant disorders creates additional risks.

We therefore explored the potential for in vivo transduction of HSCs. We developed an approach that involves GCSF/AMD3100-mediated mobilization of HSCs from the bone marrow into the peripheral blood stream, followed by intravenous injection of a Sleeping Beauty transposase-based integrating helper-dependent adenovirus (HD-Ad5/35++) vector system. These vectors target CD46, a receptor that is expressed at higher levels on HSCs than on more differentiated bone marrow and blood cells. We demonstrated in human CD46 transgenic mice and immunodeficient mice with engrafted human CD34+ cells that HSCs transduced in the periphery home back to the bone marrow where they persist and stably express the transgene long-term. In the CD46 transgenic mouse model we showed that our in vivo HSC transduction approach allows for the stable transduction of primitive HSCs, i.e. cells capable of forming multi-lineage progenitor colonies. At 12 weeks after in vivo transduction, we detected GFP marking in bone marrow HSCs in the range of 1 to 2%. Importantly, the proportion of transduced primitive HSCs increased over time. Furthermore, in vivo transduced HSCs were able to repopulate the hematopoietic system of lethally irradiated C57BL/6 mice, showing the functionality of the modified HSCs. Our in vivo HSC transduction approach did not result in innate toxicity or significant transduction of non-hematopoietic tissues. Genome-wide integration site analysis in in vivo transduced HSCs revealed a close-to-random integration pattern without preference for genes and the absence of integration into or near to cancer-associated genes.

In conclusion, our novel in vivo transduction approach allows for stable genetic modification of primitive HSCs without the need of ex vivo culture, myelo-conditioning, and transplantation. Thus, our method is relevant for a broader clinical application of gene therapy of inherited diseases as well as infectious diseases and cancer.

758. A Nonhuman Primate Transplantation Model to Evaluate Gene Editing Strategies Aimed at Inducing Fetal Hemoglobin Production for the Treatment of Hemoglobinopathies

Olivier Humbert, Hans-Peter Kiem
Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA

Fetal hemoglobin (HbF) is the major form of hemoglobin present in newborns but is almost completely replaced by adult hemoglobin after birth, where it constitutes less than 1 percent of total hemoglobin. Hereditary persistence of HbF is linked to mutations at multiple genetic loci that regulate the switch from fetal to adult hemoglobin. The

targeting of these genes using site-specific nucleases thus constitutes a promising approach for the treatment of hemoglobinopathies by increasing HbF production.We have developed a nonhuman primate (NHP) model to investigate gene editing strategies aimed at inducing HbF production following hematopoietic stem cell (HSC) transplantation. As proof of principle, we focused on the transcription factor B-cell lymphoma/leukemia 11A (BCL11A), which functions as suppressor of HbF in humans. We disrupted the Bcl11a coding region using Transcription Activator-Like Effector Nucleases (TALENs) and achieved on average 30% gene editing by electroporation of mRNA in NHP CD34+ cells. Erythroid differentiation of these cells in culture confirmed that HbF expression was increased in Bcl11a-edited cells as compared to control cells. To determine if Bcl11a-edited HSCs could engraft and give rise to HbF-producing erythrocytes, we transplanted a NHP with autologous CD34+ electroporated with Bcl11a TALEN mRNA following conditioning by total body irradiation. Using next generation sequencing, we detected about 1 % disruption in vivo one week after transplant, to reach a set point of about 0.3% over the course of the experiment. We were able to track several clones that persisted at least 200 days post transplantation based on their mutation signatures, suggesting engraftment of Bcl11a-modified cells. HbF production was monitored in this animal by flow cytometry analysis of peripheral blood and was compared with three transplanted controls and one untransplanted control. In all transplanted animals, we observed a rapid increase in the frequency of F cells, reaching 10% to 40%, and lasting for about 140 days. In contrast, F cell production in the untransplanted control remained constant and minimal (<0.5%). After returning to basal levels, we found significantly higher HbF levels (1-1.5%) in the animal transplanted with Bcl11a-edited cells as compared to all other transplanted animals. These findings were confirmed by real-time PCR analysis of hemoglobin transcripts, which showed a 5-to 10-fold increase in gamma to beta globin ratio in the animal transplanted with Bcl11a-edited cells as compared to all controls. We also initiated work demonstrating the targeted integration of the chemoselection cassette P140K/MGMT at the Bcl11a locus in NHP HSCs by co-delivery of TALEN mRNA with a donor template carried on an adeno-associated viral vector, offering the potential for in vivo selection of modified cells. In summary, our experiments establish the NHP as pre-clinical model to evaluate therapeutic gene editing strategies for the treatment of hemoglobinopathies.

759. Reduction of HLH-like Manifestations in Murine Model of Munc13-4 Deficiency Following Lentiviral Gene Transfer into Hematopoietic Stem and Progenitor Cells

Tayebeh-Shabi Soheili[1], Fernando Sepulveda[1], Amandine Durand[1], Julie Rivière[1], Samia Martin[2], Geneviève de Saint Basile[1], Marina Cavazzana[3], Isabelle André-Schmutz[1]
[1]U1163, Inserm, Imagine Institute, Université Paris Descartes, Sorbonne Paris Cité, Paris, France, [2]Genethon, Evry Cedex, France, [3]U1163 Inserm, Département de Biothérapie, AP-HP, Hôpital Universitaire Necker – Enfants Malades, Paris, France

Patients with mutations in *UNC13D* gene, coding for Munc13-4 protein, suffer from type 3 Familial hemophagocytic lymphohistiocytosis (FHL3), a life-threatening disorder of the immune system which represents 25% of all FHLs. Munc13-4 controls docking of lytic granules before they fused with the plasma membrane in cytotoxic T and NK lymphocytes and it's defect results in defective cytotoxic function of these cells. Hematopoietic stem and progenitor cell (HSPC) transplantation, which is the only curative treatment for FHL3 to date, is partially successful even when a compatible donor is available because of the important inflammatory background of patients. In this context gene therapy could be a promising therapeutic option especially for those patients without any compatible donor. In this study, we took advantages from a murine model of FHL3, the Jinx mice, to investigate the feasibility of HSPC gene therapy for this pathology. Jinx mice do not spontaneously develop clinical features of hemophagocytic lymphohistiocytosis (HLH), but do so when infected with lymphocytic choriomeningitis virus (LCMV). We generated and used a self-inactivated lentiviral vector to complement HSC from *Unc13d* -/- (Jinx) mice and transplanted them back into the irradiated Jinx recipients. This transplantation led to the complete reconstitution of the immune system at levels comparable to that of control mice. The recipients were then challenged with LCMV. While Jinx mice reconstituted with GFP expressing HSPC developed leukopenia, anemia and body weight loss, characteristic of HLH in this murine model, gene corrected Jinx recipients developed only mild or no HLH manifestations. This reduction in HLH manifestation correlated with a significant reduction of virus titer in the liver and serum level of IFN-g and inflammatory cytokines. All these ameliorations might be explained by the restoration of cytotoxic function of CTLs as demonstrated in an in-vitro degranulation assay. Overall, this study provides data supporting the potential of HSC gene therapy in a FHL immune dysregulation such as *UNC13D* deficiency.

760. Optimized AAV-Mediated Human Factor VIII Gene Therapy in Hemophilia A Mice and Cynomolgus Macaques

Jenny A. Greig[1], Qiang Wang[1], Amanda L. Reicherter[1], Erin Bote[1], Deirdre McMenamin[1], Christine Draper[1], Shu-Jen Chen[1], Alexandra L. Hanlon[2], Tamara Goode[1], K. Reed Clark[3], Samuel Wadsworth[3], Lili Wang[1], James M. Wilson[1]
[1]Gene Therapy Program, Department of Medicine, University of Pennsylvania, Philadelphia, PA, [2]School of Nursing, University of Pennsylvania, Philadelphia, PA, [3]Dimension Therapeutics, Cambridge, MA

In an effort to optimize expression of human coagulation VIII (hFVIII) for the treatment of hemophilia A, an extensive study was performed combining liver-specific promoter and enhancer elements with a codon-optimized human B-domain-deleted hFVIII transgene. Due to the large size of the FVIII coding sequence, there is a strong requirement for gene expression control elements to be as short as possible while retaining hepatocyte-restricted transcription. Several strong liver-specific promoters were shortened and combined, with combinations of up to three liver-specific enhancer sequences, to generate 42 enhancer/promoter combinations.

These 42 liver regulatory gene cassettes were packaged into the AAVrh10 capsid and were tested in FVIII KO mice. Following intravenous (IV) administration of 10^{10} genome copies (GC), mice were bled every two weeks to follow hFVIII activity and antibody generation to the transgene. At week 2 post-injection, mice showed a range in hFVIII activity from 0.12-2.12 IU/ml. FVIII KO mice developed antibodies to hFVIII at week 4, and by week 8, mice in most of the 42 vector groups had detectable anti-hFVIII IgG levels.

Based on the FVIII KO mouse studies and a small pilot rhesus macaque study, two of the original 42 enhancer/promoter combinations were selected for further evaluation in cynomolgus macaques, using two different Clade E capsids for expression. Each of the four vector combinations were administered IV at a dose of $1.2x10^{13}$ GC/kg into five macaques per group. With one capsid plus enhancer/promoter combination, peak expression of 37% of normal FVIII levels was seen at week 2 post-vector administration, which then plateaued at 20% of normal. While antibodies to the hFVIII were detected in the majority of macaques by week 8, antibodies remained undetectable in two animals at week 30 post-vector administration.

Immunological Aspects of Gene Therapy II

761. Targeted Killing of HIV Infected Cells Using *CCR5*-Disrupted Anti-HIV-CAR T Cells

Guillermo S. Romano Ibarra[1], Malika Hale[1], Karen Sommer[1], Iram Khan[1], Jaya Sahni[1], Christopher Peterson[2], Hans-Peter Kiem[2], Jordan Jarjour[3], Alexander Astrakhan[3], Andrew M. Scharenberg[1], David J. Rawlings[1], Thor A. Wagner[1]
[1]Program for Cell and Gene Therapy, Seattle Children's Research Institute, Seattle, WA, [2]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, [3]Bluebird Bio, Seattle, WA

A cure for HIV remains an important treatment goal for 30 million HIV-infected individuals worldwide. Long term control of HIV following a single treatment will require a mechanism to eradicate the latent reservoir of HIV infected cells that are capable of reactivating. A previous phase II clinical trial using an anti-HIV chimeric antigen receptor (αHIV-CAR) targeting the CD4-binding site on HIV envelope was partially effective. To optimize this approach we have developed a series of CARs based on the scFV of broadly neutralizing HIV antibodies targeting four different structural regions of the HIV envelope: the V1/V2 loop, the V3 loop, the CD4 binding site, and the membrane-proximal external region. αHIV-CAR T cells targeting different epitopes were compared and were able to kill > 80% of HIV-infected cells grown in the presence of ART. However, a limitation of αHIV-CAR T cells is that the αHIV-CAR also serves as a receptor for HIV and allows HIV infection of αHIV-CAR T cells. Therefore we disrupted the major co-receptor required for HIV cell entry, CCR5, using a megaTAL nuclease, as a means of protecting the CAR-expressing cells from HIV infection. We used two strategies for achieving these dual modifications in primary human T cells. For both strategies, the CCR5 megaTAL nuclease was delivered by mRNA electroporation, which has previously been shown to induce a high rate of bi-allelic NHEJ-mediated gene disruption. αHIV-CAR expression cassettes were delivered into the host genome via lentiviral vectors (LV), or were targeted to the megaTAL nuclease cleavage site in CCR5 using an adeno-associated virus (AAV) that included CCR5 homology arms. Both strategies resulted in stable expression of the CAR construct and specific activation of CAR+ T cells in the presence of an HIV+ cell line. In the presence of actively replicating virus, CCR5-megaTAL treated CAR+ T cells out-performed CAR+ T cells generated by LV delivery alone as measured by reduction in HIV capsid protein. To enable testing in non-human primates (NHP), we re-optimized the cell-editing protocol with NHP lymphocytes. Primary T cells from pigtail macaques were successfully transfected with CCR5 megaTAL mRNA and achieved a CCR5 disruption rate of 50%. Cells were subsequently transduced with αHIV-CAR LV resulting in ~70% of manipulated cells with αHIV-CAR. Expansion of the cells in vitro resulted in 60-fold expansion over 8 days. CAR+ NHP cells specifically killed HIV infected human cells demonstrating that NHP-derived αHIV-CAR+ T cells retained killing function. In conclusion, it is feasible to construct αHIV-CAR+ T cells that are protected from HIV infection in human and NHP cells, and warrants further study in vivo.

762. A Quantitative Imaging Tool Box for the Functional Analysis of Chimeric Antigen Receptor - T Cell Immune Synapse Helps Identify Novel Structural and Functional Features of CAR T Cells

Malini Mukherjee[1], Maksim Mamonkin[2], Nabil Ahmed[2], Malcolm Brenner[2], Jordan Orange[3]
[1]Pediatrics, Baylor College of Medicine, Houston, TX, [2]Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, [3]Pediatric Immunology Allergy and Rheumatology, Baylor College of Medicine, Houston, TX

Introduction: The cytolytic immunological synapse (IS) in T cells is a discrete structural entity formed after the ligation of specific activation receptors and leading to the destruction of a cancerous cell through the targeted release of the contents of T cell lytic granules. Although the "native" T cell IS formation where the TCR binds to antigenic peptide-MHC complex has been intensely studied, the "engineered" CAR T cell synapse and its variations based on single chain variable fragment (scFv) design and co-stimulatory domain arrangements, still remains a black box. Our work focuses on the quantitative evaluation of the CAR T cell lytic IS using highly quantitative high and super resolution imaging techniques. Specifically, by combining quantitative interrogations of CAR synapse formation kinetics and dynamics at the cellular level, we are able to obtain an unprecedented level of insight into the effectiveness of therapy cells relative to the traditional measures of cytotoxicity and effector assays like chromium release, ELISPOT and flow cytometry. Super-resolution microscopy enables us to further "see" subtle variations in therapy cell quality at a near molecular level and can be used as a very precise functional readout of CAR T cell rational design. **Methods**: High and super resolution fluorescence microscopy platforms are used to image CAR T cell-target conjugates in the x, y and z dimensions. Imaging parameters include direct evaluation of CAR engagement at the IS by measuring mean fluorescence intensity (MFI) and volume of synaptic aggregates of CAR specific ligands as well as their relative distribution at the synapse. **Results**: We report here the quantitative measurement and comparison of single and dual CAR targeted ligand aggregation at the IS, lytic machinery re-arrangement and time to target cell killing. Our studies using this predictive imaging tool box establishes the superiority of T cells expressing a bispecific (tandem) CAR to those expressing one or two monospecific CARs by the presence of significantly increased accumulation of F-Actin (> five fold), greater convergence and polarization of the lytic granule machinery. Dual engagement of the targeted ligands by a tandem CAR also significantly offsets antigen escape variants in tumors predicting reduced tumor relapse. Furthermore, application of our imaging tool box reveals that tonic signaling of CARs containing a 4-1BB co-stimulatory domain can result in an almost three fold upregulation of Fas on plasma membrane and co-localization with Fas L leading to enhanced apoptosis and lower persistence of CAR T cells. **Conclusions**: Using our quantitative imaging tool box we are able to define a specific list of parameters to measure CAR engagement and lytic function at the IS. These imaging parameters can be directly applied to interrogate and predict functionality outcomes upon CAR design variations. By doing this we hope to provide crucial early insight in CAR T cell design that can guide improvements in the efficacy, safety and, ultimately, clinical effectiveness of CAR T cell therapies.

763. HIV-Specific T Cells Can Be Expanded from Virus-Naive Donors to Target a Range of Viral Epitopes: Implications for a Cure Strategy After Allogeneic HSCT

Shabnum Patel[1], Sharon Lam[2], Conrad Russell Cruz[1], Kaylor Wright[2], Christina Cochran[1], Richard Ambinder[3], Elizabeth Shpall[4], Catherine Bollard[1]

[1]The George Washington University & Children's National Health System, Washington, DC, [2]Children's National Health System, Washington, DC, [3]Johns Hopkins University School of Medicine, Baltimore, MD, [4]The University of Texas, MD Anderson Cancer Center, Houston, TX

Background: Adoptive T cell therapy has been successful in boosting viral-specific immunity post-hematopoietic stem cell transplant (HSCT), preventing viral rebound of CMV and EBV. However, the therapeutic use of T cells to boost HIV-specific T cell immunity in HIV+ patients has been met with limited success. Despite multiple attempts to eradicate HIV infection with allogeneic HSCT, the Berlin patient remains the only case of functional HIV cure. Previous infusions of HIV-specific T cells have resulted in immune escape from single epitope specificity and limited persistence of the T cell product. Our approach to address these limitations is to expand HIV-specific T cells derived from virus-naïve donors including umbilical cord blood, employing a non-HLA restricted approach for HIV+ patients receiving allogeneic HSCT for HIV-associated hematologic malignancies. **Design:** We have developed a robust, reproducible platform that can expand HIV-specific T cells (HXTCs) from the naïve pool in the allogeneic setting. Peripheral blood mononuclear cells isolated from virus-naïve donors are used to generate dendritic cells and T cells. T cells are stimulated with antigen presenting cells pulsed with HIV-pepmix and a combination of cytokines that promote proliferation and differentiation. T cells were tested for: (1) specificity against HIV antigens and individual peptides, (2) pro-inflammatory cytokine secretion in response to stimulation with HIV peptides, and (3) ability to suppress HIV replication *in vitro*. **Results:** We successfully expanded (75.705 mean fold expansion) HXTCs recognizing HIV antigens from virus naïve donors. IFNg ELISPOT showed HXTCs (n=8) were specific against Gag (mean=331.25 SFC/1e5 cells) and Nef (mean=242.63 SFC/1e5 cells) vs Irrelevant (mean=13 SFC/1e5 cells). HXTCs produced significantly pro-inflammatory responses (p less than 0.05) to stimulation by gag/nef, as determined by levels of TNF-alpha, IL-2, IL-6, IL-8, and perforin (n=3). Importantly, HXTCs (n=4) were able to suppress HIV replication more than non-specific CD8+ T cells when co-cultured with autologous CD4+ T cells infected with HIV SF162 (HXTC 78.62% viral suppression compared to CD8+ T cell 34.19% viral suppression). HXTCs showed both HLA Class I or II specificity for individual HIV epitopes, as determined by HLA blocking and IFNg ELISPOT. **Conclusion:** This is the first report demonstrating generation of functional, multi-HIV antigen specific T-cells from HIV-negative donors, which has implications for using allogeneic HSCT as a functional HIV cure. The low frequency of circulating HXTCs post-infusion suggests these HXTCs could have a significant effect on preventing viral rebound. The generation of HXTCs from cord blood could provide a further advantage to increase the donor pool.

764. Dosing and Re-Administration of Intravenous Lentiviral Vector for Liver-Directed Gene Transfer in Young Rhesus Monkeys and ADA-Deficient Mice

Denise A. Carbonaro-Sarracino[1], Alice F. Tarantal[2], Chang I. Lee[2], Michael L. Kaufman[1], Michele Martinez[2], Danielle N. Clark[1], Colin Koziol[1], Donald B. Kohn[3]

[1]Microbiology, Immunology and Molecular Genetics, UCLA, Los Angeles, CA, [2]Depts Pediatrics and Cell Biology and Human Anatomy and California National Primate Research Center, UC Davis, Davis, CA, [3]Microbiology, Immunology and Molecular Genetics and Pediatrics, UCLA, Los Angeles, CA

Liver-directed gene transfer with lentiviral vectors (LV) is a treatment modality being investigated for a number of genetic diseases. To study re-administration of LV, rhesus monkeys and Adenosine deaminase (ADA) knockout mice were repeatedly dosed with vesicular stomatitis virus (VSV) pseudotyped LV. In some studies the LV were modified with polyethylene glycol (PEG) since others showed that PEG modification of lysine residues of VSV increases LV half-life in serum and reduces compliment-mediated inactivation. ADA-deficiency is a defect in purine metabolism that results in severe combined immunodeficiency due to substrate accumulation. Unlike humans, ADA-/- mice do not survive to day 21 if not treated, however, when treated with intravenous ADA LV, high ADA expression in the liver was associated with prolonged survival and immune reconstitution. Liver vector copy number (VCN) was determined by qPCR. Anti-vector IgG concentration in plasma was determined by ELISA, using VSV LV as the capture antigen and a mouse anti-VSV IgG standard. Untreated animals have 10 µg/ml of reactive IgG. Direct competitive ELISA was used to determine the PEG concentration on PEG LV. ADA-/- mice treated with 5x10e10TU/kg of ADA LV at birth did not develop anti-vector antibodies (n=12) and showed 0.3±0.23 liver VCN whereas ADA-/- mice immune reconstituted with ERT and treated with 1.5x10e10 TU/kg at 4 months (m) produced 100-fold more anti-vector IgG (n=5; 6890±1143µg/ml; p=0.01) with no difference in VCN when adjusted for dose. Similar results were found in rhesus monkeys treated with 2.0x10e9 TU/kg of LV at 1 month postnatal age (n=2; 4030±10µg/ml) compared to rhesus monkeys treated at 4 m postnatal age (n=2; 17800±300µg/ml). In immune competent ADA+/- mice treated with ADA LV at birth followed by unmodified enhanced green fluorescent protein (eGFP) LV (n=3; 0.05 VCN; 1390µg/ml) or PEG modified eGFP LV (n=3; 0.05 VCN; 1330µg/ml) at 8 m, the liver VCN and the anti-vector responses were not different. However, if mice were treated with an unmodified eGFP LV or PEG modified eGFP LV at 4 m and an unmodified ADA LV at 8 m, anti-vector IgG concentration was increased 10-fold with the unmodified eGFP LV (n=3; 114666±666µg/ml) compared to the PEG modified eGFP LV (n=3; 9733±333µg/ml); however, the second LV was inactivated and no vector was detected from mice in either group. When ADA+/- mice were treated with an unmodified ADA LV at birth and again at 5 m (booster), followed by an unmodified (n=5) or PEG modified (n=5) eGFP LV at 8 m, the third injection was associated with acute toxicity and reduced probability of survival in both groups (40%; p=0.01). In immune competent rhesus monkeys dosed with an unmodified LV carrying a non-expressed transgene at 3 m of age and followed by an unmodified LV or a PEG modified LV carrying a different non-expressed transgene at 6 m, the second vector was not detected in either group and both the unmodified LV (n=2; 15600±1,200µg/ml) and the PEG-modified LV (n=2; 15500±900µg/ml) had similar anti-vector responses. In conclusion, LV dosing at birth is associated with reduced anti-vector responses compared to dosing at ~3 m of age. Repeat LV dosing can result in inactivation of a second vector administration that is associated with higher anti-

vector IgG responses and a higher probability of acute toxicity when dosed months apart. PEG modification does not appear to protect the second dose from inactivation or prevent toxicity.

765. Detection of Treatment-Resistant Infectious HIV After Genome-Directed Antiviral Endonuclease Therapy

Daniel Stone[1], Harshana S. De Silva Feelixge[1], Harlan L. Pietz[1], Pavitra Roychoudhury[1], Alex L. Greninger[2], Joshua T. Schiffer[1], Martine Aubert[1], Keith R. Jerome[1]
[1]Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA, [2]Dept of Laboratory Medicine, University of Washington, Seattle, WA

Incurable chronic viral infections are a major cause of morbidity and mortality worldwide. One newly emerging approach to cure persistent viral infections is via the use of targeted endonucleases. To date, endonucleases have been used as therapeutic agents targeting the genomes of viruses including EBV, JCV, HBV, HCV, HIV, HPV, HSV and HTLV, and have been shown to inhibit viral replication and subsequently persistence. Nevertheless, a potential concern for endonuclease-based antiviral therapies is the emergence of treatment resistance. Here we detect for the first time an endonuclease-resistant infectious virus that is found with high frequency after antiviral endonuclease therapy. While testing the activity of a panel of four HIV *pol*-specific zinc finger nucleases (ZFNs) we identified a provirus encoding a treatment-resistant and infectious mutant virus that was derived from a ZFN2-mediated disruption of reverse transcriptase (RT). Although ZFN-mediated disruption of HIV protease (ZFN1), RT (ZFN2 & ZFN3) and integrase (ZFN4) coding sequences could inhibit viral replication, a RT mutant provirus was detected that produced a replication competent and ZFN2 cleavage resistant HIV. Mutant virus ZFN2(+3) contained a single amino acid insertion that introduced a Leucine-Leucine (LL) motif in the thumb domain of RT. The RT of mutant virus ZFN2(+3) likely remained functional since a LL motif is also found in the RT of several hominid, old world monkey or equine spumaviruses. In ZFN2-treated HEK293 and CD4+ SupT1 T cells, we found that up to 25% and 6% of all ZFN2 HIV *pol* target site mutations detected by Illumina sequencing encoded the ZFN2(+3) mutant respectively. We found that ZFN2(+3) mutant virus could replicate at levels comparable to wild type HIV in primary $CD4^+$ T cells, but importantly remained susceptible to treatment with both NRTi and NNRTi antiretroviral inhibitors. When secondary ZFN-derived mutations were introduced into other RT or integrase domains of mutant virus ZFN2(+3), replication could be abolished. Our observations suggest that caution should be exercised during endonuclease-based antiviral therapies; however, combination endonuclease therapies may prevent the emergence of resistance.

766. Genetic Correction of Ifnγr1 Deficiency in Hematopoietic Cells Repairs the Cellular Phenotype of Mendelian Susceptibility to Mycobacterial Disease

Nico Lachmann[1], Adele Mucci[2], Miriam Hetzel[2], Robert Meineke[2], Olga K. Halle[3], Mark P. Kühnel[4], Ralph Goethe[5], Sebastian Brennig[1], Daniel Brand[6], Reinhold Förster[3], Axel Schambach[6], Thomas Moritz[2]
[1]Translational Hematology of Congenital Diseases, Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany, [2]Reprogramming and Gene Therapy, Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany, [3]Institute of Immunology, Hannover Medical School, Hannover, Germany, [4]Institute of Functional and Applied Anatomy, Hannover Medical School, Hannover, Germany, [5]University of Veterinary Medicine, Foundation Hannover, Hannover, Germany, [6]Hannover Medical School, Hannover, Germany

Mendelian Susceptibility to Mycobacterial Disease (MSMD) is a rare primary immunodeficiency characterized by recurrent severe infections with otherwise only weakly virulent mycobacteria. MSMD is associated with mutations in different genes, all leading to an impaired activation of macrophages by T-cells and a defective innate immune response. Specifically mutations in the interferon-gamma (IFNγ)-receptor-1 or-2 (*IFNγR1 /2*) genes result in a life-threatening disease phenotype with most patients dying in early childhood. We here introduce a novel gene therapy approach for IFNγR1 deficiency. Thus, we have designed a 3rd generation SIN lentiviral vector expressing the murine cDNA of Ifnγr1 from a spleen focus forming virus (SFFV) promoter element coupled by an IRES to GFP (Lv.SFFV.Ifnγr1.iGFP). Transduction of hematopoietic stem/progenitor cells from $Ifn\gamma r1^{-/-}$ mice showed expression of Ifnγr1 by FACS and qRT-PCR and no abnormalities in clonogenic growth when compared to WT control cells. Moreover, differentiation of transduced cells towards macrophages (MΦ) by M-CSF was normal as determined by morphology on cytospins and surface marker expression of CD11b, CD200R, CD115, CD45 and F4/80. When subjecting corrected MΦ to stimulation with IFNγ, corrected cells were able to build a functional IFNγR1-Ifnγ-IFNγR2 and internalize IFNγ as efficiently as WT cells, as suggested by the clearance of IFNγ from the medium within 24h of stimulation. Consequently, Lv.SFFV.Ifnγr1.iGFP corrected MΦ revealed restored up-regulation of HLA-DR and CD86 (B7.2) comparable to WT-MΦ. IFNγ dependent T-cell activation was evaluated using T cell receptor- transgenic T cells recognizing ovalbumin (OVA). Both WT and corrected MΦ were unable to activate T-cells in the presence of IFNγ and OVA, whereas MΦ from $Ifn\gamma r1^{-/-}$ mice induced significantly stronger proliferation of T cells. This observation was accompanied by the induction of indoleamine 2,3-dioxygenase (IDO) in WT and corrected MΦ, suggesting that IDO enables MΦ to deplete tryptophan from the medium and interferes with T-cell proliferation. Finally, Ifnγr1 downstream signaling showed restored phosphorylation of STAT1 in corrected MΦ, consistent with induction of *iNos* and *Irf1* upon stimulation with IFNγ. Moreover, correction of $Ifn\gamma r1^{-/-}$ MΦ led to a significantly improved anti-mycobacterial activity as measured by comparable killing of *Mycobacterium Avium* in corrected and WT MΦ. Thus, we here introduce a novel gene therapy approach for MSMD in the context of Ifnγr1 deficiency.

767. A Novel Humanized Mouse Model for Testing Anti-HIV T-Cell Gene Therapy Strategies

Kevin G. Haworth, Christina Ironside, Hans-Peter Kiem
Fred Hutchinson Cancer Research Center, Seattle, WA

Despite major advances in the therapeutic treatment of individuals infected with HIV using anti-retroviral therapies (ART), patients are not cured. Deviation from standard drug administration can lead to rapid rebound of viremia, and the emergence of drug-resistant variants. New approaches for treatment of HIV include a range of techniques from gene therapies to vaccine studies. Many of these methods utilize animal models to test the efficacy of treatment prior to advancing to clinical settings. Humanized mouse models have been invaluable for such strategies. Immune compromised mice can engraft with human hematopoietic stem cells (HSC), give rise to a functional human immune system, and maintain an HIV infection. A novel concept for treatment involves engineering a specific immune response against infected cells using chimeric antigen receptors (CAR) to viral epitopes on infected cells. These CAR modified T cells have been used in the preliminary treatment of some cancers, where modified cells lead to the elimination of target cancer cells. A similar approach is being investigated now for HIV. While progress has been made on creating engineered receptors, there has not been a good pre-clinical animal model to test them in. We address this problem by building on the well characterized NOD-scid-gamma (NSG) animal model of HIV treatment, and adapted it to test the functionality of T cell-based immunotherapies. To better model the clinical setting, we use adult apheresis HSCs from healthy mobilized donors as the source for engraftment instead of cord blood or fetal tissue. Neonatal NSG mice injected with these harvested HSCs give rise to over 90% CD3 T-cells in the human fraction circulating in the blood unlike other models which don't readily develop T-cells. Instead of using additional sources for T-cell collection, we harvested CD3 cells directly from the mice, cultured, expanded, and transduced them using GFP expressing lentiviral vectors, and re-infused them back into the mice. The modified CD3 cells were detected in the peripheral blood with frequencies reaching 50% of total CD3 cells without the symptoms of graft-vs-host disease. These cells persisted for over two months and were also found in all lymphoid tissues analyzed at necropsy. To assess safety of vector modified cells, we utilized integration site (IS) analysis to measure the clonal repertoire of injected cells. While over 500 unique clones were detected, we did not observe indications of clonal outgrowth in animals over the course of the experiment. Mice receiving the engrafted HSCs and transduced CD3 cells were capable of sustaining HIV infection with titers between 10^5-10^7 viral copies/mL and demonstrated significant CD4 depletion in both blood and lymphoid tissues. With the establishment of this clinically relevant model for testing the effectiveness of T-cell based therapies to treat HIV, we are beginning to test anti-HIV engineered cells. By building upon the existing animal models and combining T-cell gene therapy approaches with IS to monitor for vector safety, we have established a novel method for testing new anti-HIV cure therapies.

768. The CRISPR/Cas9 System as an Anti-Viral Treatment to Prevent Primary Infection by HCMV Positive Hematopoietic Stem Cells

Janina Gergen, Flora Coulon, Alison Creneguy, Jean-Marie Heslan, Ignacio Anegon, Tuan Huy Nguyen, Franck Halary, Fabienne Haspot
INSERM U1064; Université de Nantes; Centre Hospitalier Universitaire Hôtel Dieu, Nantes, France

HCMV is a beta-herpes virus, which induces a life long latency in hematopoietic stem cells after an asymptomatic primary infection. In hematopoietic stem cell transplantation, donor hematopoietic stem cells will engraft and differentiate in the recipient. This differentiation induces the reactivation of HCMV in the myeloid compartment and the release of new virions from the donor cells. The HCMV spread leads a primary infection of the immunocompromised HCMV negative recipient and can cause severe end organ diseases. The common treatments of HCMV in immunosuppressed patients are viral DNA replication inhibitors, which cause strong side effects, such as inhibition of hematopoiesis, kidney toxicity, and are likely to promote resistant strain emergence. As a consequence, none of the available drugs can target the HCMV in latent state.

To circumvent this, we aim to directly alter the HCMV genome by using the CRIPSR/Cas9 system to knock-out the immediate early gene (IE) encoding essential viral proteins for lytic replication as well as the end of latency. We transduced the low HCMV-permissive U373-MG cells with a lentiviral vector encoding the Cas9 and tree different single gRNA targeting the IE gene. We FACS sorted those cell lines based on their Cas-9-GFP expression and then infected them with a HCMV laboratory strain. We detected mutations at the target site in up to 70% of the viral genomes. We observed a concomitant reduction of 50% less infected cell by FACS staining of the IE protein. Moreover, we set up an assay to analyze the virion release of infected U373-MG cells, which will allow us to address the decrease or inability to release new virions form U373-MG-gRNA-Cas9 HCMV infected cells. We are currently investigating a multiplex strategy with 3 gRNA targeting different parts of the IE gene which are delivered within the same lentiviral vector. The multiplex strategy is auspicious to show higher efficiency than a single gRNA. This strategy will be also tested on HCMV latently infected CD34+ hematopoietic stem cells to prevent viral reactivation.

The CRISPR/Cas9 system anti-HCMV is a promising tool to block viral replication. With this anti-HCMV tool we will be able to treat HCMV positive donor cells in order to prevent the primary infection of the immunosuppressed recipient.