



ABSTRACTS

# Program and Abstracts of the 16th Transgenic Technology Meeting (TT2020)

Virtual meeting, October 26–29, 2020

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מכון ויצמן למדע  
WEIZMANN INSTITUTE OF SCIENCE

ISTT

TT 2020

THE 16<sup>th</sup> TRANSGENIC TECHNOLOGY MEETING

October 26–28 | 2020

TT2020 ONLINE

ISTT [www.transtechsociety.org](http://www.transtechsociety.org)  
International Society for Transgenic Technologies

The TT2020 Meeting is hosted by The Weizmann Institute of Science, Israel



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## TT2020 Scientific Program

### Monday, October 26th

Time (Israel time)	Session/Lecture info
15:00–15:15	<b>Opening Address TT2020-Virtual</b>
15:15–16:30	<b>Session 1: Panel Discussion—Animal Models: From Bench to Bedside (sponsored by GEMM-Genetic Engineered Mouse Models; National Israel Facility)</b> Moderators, Karen Avraham, Jan Parker-Thornburg
15:15–15:30	Paula Rio, CIEMAT, Spain Gene therapy for Fanconi anemia: From the lab to the clinic
15:30–15:45	Eital Galun, The Hebrew University of Jerusalem, Israel Better Bedside-to-bench than Bench-to-bedside, why?
15:45–16:00	Aris Economides, Regeneron, USA Inhibition of Activin A by a monoclonal antibody as a disease-modifying therapy for fibrodysplasia ossificans progressiva (FOP)
16:00–16:15	Tom Wishart, Roslin Institute, UK How should we model neurodegenerative conditions? Using livestock to bridge the translational gap
16:15–16:30	Discussion and Q&A
16:30–16:45	<b>Break</b>
16:45–18:20	<b>Session 2: Epigenetics</b> Chair: Janet Rossant
16:45–17:20	Keynote: Howard Cedar, The Hebrew University of Jerusalem, Israel TBA
17:20–17:35	Wei Li, Chinese Academy of Sciences, China Balancing embryonic development through “imbalance”

17:35–17:50	Eran Meshorer, The Hebrew University of Jerusalem, Israel NFIX, which is differentially methylated between modern and archaic humans, regulates vocalization in mice	15:15–15:30	Swathi Arur, MD Anderson Cancer Center, USA Transgenic and CRISPR analysis in <i>C. elegans</i>
17:50–18:05	Yonatan Stelzer, Weizmann Institute of Science, Israel Modeling mouse gastrulation at single embryo and single-cell resolution	15:30–15:45	Tessa Montague, Columbia University, USA The neural basis of cuttlefish camouflage
18:05–18:20	Nissim Benvenisty, The Hebrew University of Jerusalem, Israel Defining human pluripotency and gastrulation utilizing genome wide screening in haploid ES cells	15:45–16:00	Tim Corbin, Stowers Institute, USA Development of methodologies for the generation of genetically modified snails, <i>Pomacea canaliculatae</i> (Gastropoda, Ampullariidae), a novel, non-mammalian research organism
18:20–18:35	<b>Break</b>	16:00–16:15	<b>Break</b>
18:35–19:20	<b>ISTT Prize Presentation and Lecture (Sponsored by Genoway)</b>	16:15–17:15	<b>Session 4: Panel Discussion—Ethics of Gene Editing in Animals</b> Chair: Lluís Montoliu
18:35–18:45	ISTT Prize Presentation	16:15–16:30	Lluís Montoliu, Centro Nacional de Biotecnología (CNB), Spain Gene editing in mice can easily lead to an increase of animals used
18:45–19:20	Alexandra Joyner (ISTT Prize Recipient), Sloan Kettering, USA Genetic exploration of neural stem cell self-renewal and plasticity	16:30–16:45	Kirk Leech, The European Animal Research Association Animal Research: Time to talk!
19:20–20:20	<b>Social hour, chat with ISTT Prize winner, breakout social rooms</b>	16:45–17:00	Andy Greenfield, MGU, Harwell, Oxford, UK Genome editing and farmed animals: ethical considerations Ethics discussion by panelist

**Tuesday, October 27th**

<b>Time</b>	<b>Session/Lecture info</b>
15:00–16:00	<b>Session 3A and 3B: CONCURRENT SESSIONS 3A: LARGE ANIMAL TRANSGENESIS 3B: NON-MAMMALIAN TRANSGENESIS</b>
15:00–16:00	<b>Session 3A: Large Animal Transgenesis</b> Chair: Bruce Whitelaw
15:00–15:15	Jun Wu, UT Southwestern Medical Center, USA Derivation of intermediate pluripotent stem cells amenable to primordial germ cell specification
15:15–15:30	Hui Yang, Chinese Academy of Sciences, China Interspecies blastocyst complementation generates functional rat cell-reconstituted forebrain in mice
15:30–15:45	Alison Van Eenennaam, UC Davis, USA Agricultural animal transgenesis for food applications
15:45–16:00	Nathan Buzzell, LFB, USA Transgenic goats expressing high levels of recombinant Trastuzumab in milk over multiple generations
15:00–16:00	<b>Session 3B: Non-mammalian Transgenesis</b> Chair: Richard Behringer
15:00–15:15	Claude Desplan, New York University, USA Manipulating aging

17:00–17:15	Ethics discussion by panelist
17:15–17:20	<b>Break</b>
17:20–18:20	<b>POSTER SESSION 1</b>
18:20–19:00	<b>Social hour, breakout social rooms</b>

**Wednesday, October 28th**

<b>Time</b>	<b>Session/Lecture info</b>
15:00–16:00	<b>ISTT GENERAL ASSEMBLY</b>
16:00–16:15	<b>Break</b>
16:15–17:15	<b>Session 5: Germ Cells, IPS and Trophoblast Cells</b> Chair: Yonatan Stelzer
16:15–16:30	Nicolas Rivron, Austrian Academy of Science, Vienna Blastoids: modeling blastocyst development and implantation with stem cells
16:30–16:45	Yaqub Hanna, Weizmann Institute of Science, Rehovot, Israel TBA
16:45–17:00	Yossi Buganim, Hebrew University-Hadassah Medical School, Jerusalem, Israel Comparative Parallel Multi-Omics Analysis of Cell Undergoing Reprogramming to Pluripotent and Trophoblast States

17:00–17:15	Nitzan Gonen, Bar-Ilan University, Israel Testis formation in XX individuals resulting from novel pathogenic variants in Wilms' tumor 1 (WT1) gene	16:35–16:50	Stephan Rosshart, University Medical Center Freiburg, Germany Born to be wild—utilizing the common link among mammals to create better translational research models
17:15–17:50	<b>Sponsored by the Czech Centre for Phenogenomics</b>	16:50–17:05	Daniel Schramek, Mount Sinai Hospital, Canada <i>In vivo</i> CRISPR technology to rapidly screen function of hundreds of genes within a single mouse
17:15–17:50	<b>ORBIS PICTUS LECTURE</b> Elizabeth Hillman Visualizing in vivo systems at the speed of life	17:05–17:20	Jessica Woodley—IDT Sponsored talk, IDT, USA Improved methods for CRISPR HDR using Alt-R modified dsDNA donors and Alt-R HDR Enhancer V2
17:50–18:00	<b>Break</b>	17:20–17:35	<b>Break</b>
18:00–19:00	<b>POSTER SESSION 2</b>	17:35–18:05	<b>Session 9: Animal models for COVID-19 Research</b>
19:00–20:00	<b>Session 6: Roundtable—Running a Transgenic Unit</b> Chair: Peter Hohenstein	17:35–17:50	Chair: Lauryl Nutter Benjamin tenOever, Icahn School of Medicine at Mount Sinai, TBA Speakers to be announced
19:00–19:15	Ben Davies, Oxford University, UK	17:50–18:05	<b>INTERACTIVE COFFEE BREAK—coffee rooms with discussion topics</b>
19:15–19:30	Lisbeth Ahm Hansen, Aarhus University, Denmark	18:05–18:35	<b>EMBO KEYNOTE LECTURE</b> Janet Rossant, Sick Kids, Canada Dynamics of early development: from embryos to stem cells
19:30–19:45	Karen Brennan, Australian National University, Australia	18:35–19:10	<b>CONCLUDING REMARKS, TT2022 PRESENTATION</b> by Ernst-Martin Fuechtbauer, Peter Hohenstein
19:45–20:00	Philip Damiani, Charles River, US		
20:00–20:45	<b>Social hour, breakout social rooms</b>		

#### Thursday, October 29th

<b>Time</b>	<b>Session/Lecture info</b>
15:00–15:45	<b>Session 7: 3R's</b> Chair: Branko Zevnik
15:00–15:15	Ivo Huijbers, Netherlands Cancer Institute, Netherlands Rapid generation of versatile cancer models with minimal breeding effort
15:15–15:30	Sara Wells, MRC Harwell, UK Refining Phenotyping for Refined GA strains
15:30–15:45	3Rs Prize - Sponsored by Janvier Labs. Lydia Teboul, MRC Harwell, UK Evolving the generation by genome editing of complex alleles and their validation
15:45–16:05	<b>YOUNG INVESTIGATOR AWARD</b> This activity is supported by an independent medical education grant from Regeneron Pharmaceuticals Inc.
15:45–16:05	Young Investigator Award—Hiromi Miura, Tokai University School of Medicine, Japan Development of targeted transgenic technologies before and after the CRISPR era
16:05–16:20	<b>Break</b>
16:20–17:20	<b>Session 8: Emerging Technologies</b> Chair: Soren Warming
16:20–16:35	Kimberly Cooper, UC San Diego, USA Meiotic Cas9 expression mediates genotype conversion in the germline of male and female mice

#### Oral Abstracts Session 1: Panel Discussion— Animal Models: From Bench to Bedside

**Inhibition of Activin A by a monoclonal antibody as a disease-modifying therapy for fibrodysplasia ossificans progressiva (FOP)**

##### **Aris Economides**

Fibrodysplasia ossificans progressiva (FOP) is an ultra-rare genetic disorder characterized by episodic but cumulative heterotopic bone formation in select skeletal muscles, tendons, ligaments, and fascia. An additional phenotype of FOP edematous and painful swellings of soft tissue that are referred to as flare-ups. FOP is caused by mutations in ACVR1 (type I Bone Morphogenetic Protein (BMP) receptor), with ~ 97% of patients carrying the variant ACVR1[R206H]. The predominant explanation on how heterotopic bone forms in FOP had been that it results from overactive signaling of FOP-mutant ACVR1 in response to osteogenic BMPs. Using a genetically and physiologically accurate mouse model of FOP we demonstrated this not to be the case. In contrast to the prevailing view, we showed that the ligand that is drives the formation of heterotopic bone in FOP is Activin A (Hatsell, Idone et al., 2015; Upadhyay, Xie et al., 2017). Outside of FOP, Activin A engages wild type ACVR1 to form a non-signaling complex, and effectively compete with BMPs for ACVR1 (Aykul et al.,

2020). (The physiological role of this non-signaling complex is currently not understood.) Unlike what occurs in the wild type setting, in FOP Activin A uniquely activates Smad1/5/8 signaling through FOP-mutant ACVR1. More importantly, inhibition of Activin A (using the anti-Activin A monoclonal antibody REGN2477) completely blocks the initiation of heterotopic bone lesions as well as the growth and expansion of nascent heterotopic bone lesions in FOP mice. Encouraged by this data, we embarked on a clinical trial—LUMINA-1—to test whether inhibition of Activin A using REGN2477 would be a safe and efficacious treatment for blocking heterotopic bone formation in adult FOP patients. The top line results of LUMINA-1 demonstrate that REGN2477 substantially reduces the formation of new HO lesions and flare-ups and displays a favorable benefit/risk profile for adults with FOP. These results support that activin A is a key driver of HO in FOP.

## **Session 2: Epigenetics**

### **Balancing embryonic development through “imbalance”**

**Wei Li**

Imbalanced allelic gene expression has been widely observed and plays important roles in mammalian development and disease progress. How it is established, regulated and functioning, remains to be explored. Here by combining the haploid stem cell and genome editing technologies, we identified the imprinting-related factors necessary for crossing same-sex reproduction barriers in mammals, and then established a unisexual reproduction method for producing normally growing bimaternal mice and live bipaternal mice. We also identified the aberrantly expressed non-canonical imprinting genes in cloned mice, which can significantly increase the animal cloning efficiency up to 14% after being rescued.

### **NFIX, which is differentially methylated between modern and archaic humans, regulates vocalization in mice**

**Eran Meshorer**

Changes in regulatory elements are thought to be key drivers of phenotypic divergence. However, identifying changes to regulatory elements that underlie human-specific traits has proven very challenging. Using reconstructed and experimentally measured DNA methylation maps of ancient human, present-day humans, and chimps, we detected differentially methylated regions that likely emerged in modern humans after the split from Neanderthals and Denisovans. We find that genes associated with face and vocal tract anatomy went through particularly extensive methylation changes. Specifically, we identify widespread hypermethylation in a network of face- and voice-affecting genes, including NFIX, suggesting higher expression levels in archaic humans. To test whether the effects of NFIX on voice box anatomy extend also to vocalization, we generated NFIX-over-expressing transgenic (Tg) mice by zygote injections. Vocalization recordings demonstrated significant changes in vocalizations in both male and female

NFIX Tg mice compared with WT littermates. Taken together, our results demonstrate that comparative epigenetics is a powerful tool in revealing the genetic basis of human-specific traits.

### **Modeling mouse gastrulation at single embryo and single-cell resolution**

**Yonatan Stelzer**

Mouse embryonic development is a canonical model system for studying mammalian cell fate acquisition. Recently, single-cell atlases comprehensively charted embryonic transcriptional landscapes, yet inference of the coordinated dynamics of cells over such atlases remains challenging. Here we introduce a temporal model for mouse gastrulation, consisting of data from 153 individually sampled embryos spanning 36 h of molecular diversification. Using new algorithms and precise timing we infer differentiation flows and lineage specification dynamics over the embryonic transcriptional manifold. Rapid transcriptional bifurcations characterize the commitment of early specialized node and blood cells. However, for most lineages, we observe combinatorial multi-furcation dynamics rather than hierarchical transcriptional transitions. In the mesoderm, dozens of transcription factors combinatorially regulate multi-furcations, as we show using time-matched chimeric embryos of Foxc1/Foxc2 mutants. Our study rejects the notion of differentiation being governed by a series of binary choices, providing an alternative quantitative model for cell fate acquisition.

## **ISTT PRIZE LECTURE**

### **Genetic exploration of neural stem cell self-renewal and plasticity**

**Alexandra Joyner**

Development of the brain is fueled by neural stem/progenitor cell (NSC) populations that produce distinct neuron types and glia in a strict developmental progression from the ventricular zone lining the ventricle. The cerebellum is unique in the brain as it not only has a protracted period of neural production, but has two NSC populations that leave the ventricular zone and proliferate near the surface of the brain for 2 weeks after birth in mice (several months in humans). The late development of the cerebellum makes it sensitive to injury at birth, which has been associated with autism, but our work has also shown that it has an unusual ability to recover from injuries. One cerebellar NSC population is dedicated to making excitatory granule neurons, the most numerous cell type in the brain (more than half of all neurons). The other NSC population, nestin-expressing progenitors (NEPs), produces inhibitory interneurons and astrocytes after birth. We uncovered that the NSC populations communicate to ensure that the proportions of different neuron types are maintained, even when one cell type is depleted due to genetic mutations. Most recently we used single cell RNA-seq and genetic inducible fate mapping (GIFM) to define subpopulations of NEPs based on their gene expression profiles and lineage propensities. We further studied the plasticity of NEPs following loss of granule cell progenitors at birth and discovered that one subpopulation of NEPs undergoes adaptive

reprogramming to produce new excitatory granule neurons. scRNA-seq, GIFM and conditional mutagenesis revealed that the neurogenic protein ASCL1 is required during adaptive reprogramming to shut down the gliogenic program of the NEPs and allow them to initiate an excitatory neuron program. Thus, the different NSC populations of the cerebellum not only have distinct lineage propensities, but also communicate during development and upon injury to ensure robust neural circuits are generated.

### **Session 3A: Large Animal Transgenesis**

#### **Derivation of intermediate pluripotent stem cells amenable to primordial germ cell specification**

**Jun Wu**

Dynamic pluripotent stem cell (PSC) states represent in vitro adaptations of the in vivo pluripotency continuum. Decades of studies have generated a number of PSCs with distinct molecular and phenotypic features. To date, however, no known PSCs have demonstrated direct primordial germ cell (PGC) specification responsiveness, a property unique to the embryonic day 5–6 (E5–6) epiblast in mice. Here, we developed a method that enabled the derivation of intermediate PSCs from mouse blastocysts, which not only shared transcriptional similarity with E5–6 epiblast but were also capable of efficient PGC specification in vitro and germline transmission in vivo. The same culture system supported the derivation of embryonic stem cells (ESCs) from horse blastocysts and transgene-free induced pluripotent stem cells (iPSCs) from horse and human fibroblasts, which could also be directly induced into PGC-like cells in vitro. PGC responsive PSCs are invaluable for studying mammalian pluripotency and early PGC development, and our method may be broadly applicable for the derivation of analogous stem cells from other mammalian species.

#### **Interspecies blastocyst complementation generates functional rat cell-reconstituted forebrain in mice**

**Hui Yang**

Blastocyst complementation has been considered to be a promising approach for generating xenogeneic organs 1–7. Exogenous embryonic stem cells (ESCs) are injected into blastocysts of a host species deficient in a critical gene for developing specific organs, allowing donor ESCs to populate the vacated niche and form desired organs 8–12. Previous studies showed that the mouse pancreas, lung, brain and other organ or cell lines could be generated in mice via blastocyst complementation and prevented neonatal lethality 8, 13, 22–26, 14–21. By contrast, interspecies blastocyst complementation (IBC) is more challenging due to the nature of xenogeneic barrier 27–33. While rat pancreas and thymus have been successfully generated in the mouse 8, 9, forebrain, the most complex organ in the body, has not yet been generated in the mouse via IBC. Here, we developed an improved blastocyst complementation method that enables rapid interspecies organogenesis by injection of embryonic stem cells into the blastocyst in which organogenesis-relevant gene was

completely deleted by CRISPR editing with multiple guide RNAs. Among 7 head formation-associated genes, we found only mouse blastocysts with gene X deletion allowed forebrain generation via mouse ESCs injection. Furthermore, injecting rat ESCs into mouse blastocysts with deletion of gene X generated adult forebrain structurally and functionally similar to the mouse brain but composed of a large proportion of rat cells, as shown by fluorescently marking ESCs and forebrain single-cell genotyping. Thus, our blastocyst complementation method enables rapid screening of organogenesis-relevant genes and allows efficient interspecies organogenesis.

#### **Agricultural animal transgenesis for food applications**

**Alison Van Eenennaam**

The year 2020 marks 35 years since the first genetically engineered (GE) livestock were reported, and yet only a single GE food animal, the fast-growing AquAdvantage salmon, has been commercialized. There are a number of reasons for the slow progress in this field, including technical issues, the structure of livestock industries, lack of research funding and investment, regulatory obstacles, and concerns about public opinion. It is sobering to consider the tremendous scientific progress that has been made in the production and utility of millions of transgenic rodents, and even in producing transgenic models for biomedical research, as compared to transgenic livestock for food applications. There are considerable opportunity costs associated with precluding the adoption of useful GE livestock applications in animal agriculture. The GE mastitis-resistant dairy cow, first reported in the scientific literature in 2005, could have helped to avoid over \$10 billion in losses from *Staphylococcus aureus* mastitis in the USA and EU dairy industries alone. Delays in the commercialization of GE livestock, beyond the normative 10-year GE product evaluation period, are also costly. Delays in the diffusion of the AquAdvantage salmon into the world market of salmon from 2002, 10 years after the initial publication of this transgenic event in 1992, to 2020 were estimated at more than \$25.5 billion. These costs are the baseline against which the value of information acquired as a result of such delays, if any, must be weighed. A cost-benefit analysis should be incorporated into regulatory decisions about transgenic and gene edited livestock.

### **Session 3B: Non-mammalian Transgenesis**

#### **Manipulating aging**

**Claude Desplan**

Aging is a process of progressive decline in intrinsic physiological functions. A molecular and genetic understanding of aging has recently emerged. For instance manipulation on the insulin pathway and of epigenetic marks in *C. elegans*, *Drosophila* and mice can significantly extend their life span. There is also a trade-off between lifespan and reproduction as higher reproductive activity in females is normally associated with shorter lifespan. We are studying *Harpegnathos*, an ant species that represents a powerful system to study lifespan

extension. In this ant, individuals that are not exposed to queen pheromones can undergo a reversible switch from non-reproductive workers to reproductive pseudo-queens ('gamergates') that exhibit fully developed ovaries and, importantly, a 5–10 × increase in lifespan: this means that an individual born to live for months can now live for years, presumably by changing its epigenetic profile. Furthermore, gamergates can be reverted to workers when placed in a colony with a queen and then revert to a shorter lifespan. Therefore, this species provides a unique system to understand environment-induced aging and rejuvenation processes. We have developed genetics in *Harpegnathos* ants to investigate the regulatory system underlying the plasticity of longevity. We have identified a group of differentially expressed genes (DEGs), including dramatic changes in genes implicated in the regulation of longevity, insulin and IGF signaling pathway components. We have generated CRISPR mutants for the *orco* gene, which encodes the obligatory co-receptor of all Olfactory Receptors, rendering the ant unable to perceive pheromones that allow communication in the ant society. Strikingly, these *orco* mutant ants become asocial and fail to become reproductive. We have analyzed the dynamics of insulin signaling during the transformation to gamergates. Insulin is increased systemically, presumably to support the heavy metabolic load for egg production; however, the fat body (aka adipose tissue where insulin needs to be low in flies and mammals to extend lifespan) and ovary appear to have decreased insulin response because of anti-insulins produced locally, hence potentially explaining how social ants can increase reproduction while extending their lifespan. We have in hand a unique system that exhibits extreme forms of reversible aging and rejuvenation and have successfully developed the genomic and genetic tools to study the process at the molecular levels.

### Transgenic and CRISPR analysis in *C. elegans*

**Swathi Arur**

*C. elegans* is an excellent model system for in vivo analysis of cellular, molecular and developmental processes, in part because of its completely sequenced genome, a small genome size (~ 100 MB) and a transparent body. Using transgenic and CRISPR based technologies we study the intersection of environmental and nutrient signaling in regulation of oocyte development and the transition of oocyte to a zygote. During the talk, I will cover some of the key transgenic and CRISPR based technologies used in *C. elegans* and in my lab to understand processes that determine oocyte development and quality. Oocyte quality is a key determinant that dictates progeny health. Because of the deep conservation between *C. elegans* and human genomes, much of our work has been translated to mammalian biology.

### The neural basis of cuttlefish camouflage

**Tessa Montague**

To navigate the visual world, animals create an internal representation of the environment and extract salient features, permitting the generation of appropriate behaviors. Cuttlefish

present a unique system for studying the internal representation of visual stimuli. Cuttlefish dynamically change their skin pigmentation and texture to camouflage to their surroundings, creating a physical readout of what they see. This is achieved by expanding and contracting pigment-filled saccules called chromatophores using motor neurons projecting from the brain. Thus, the skin of cuttlefish can be likened to a digital display in which the chromatophores ("pixels") create a physical manifestation of neural activity. We are using this system to understand how the physical properties of the visual world are represented by patterns of neural activity in the brain, and how this representation is transformed into an approximation of the physical world on the skin. Cuttlefish provide an ideal system to study this transformation because the brain utilizes just 3 hierarchical lobes during camouflage and the motor output is measurable. We are generating transgenic cuttlefish that express genetically-encoded calcium indicators and channels, to permit the live imaging and manipulation of neural activity. By establishing a behavioral paradigm in which changes in the visual environment evoke simple changes in skin patterning, we will simultaneously record neural activity and measure behavior to uncover how visual information is deconstructed in the brain, and then reconstructed into an image of the physical world on the skin.

## **Session 4: Panel Discussion—Ethics of Gene Editing in Animals**

### **Animal Research: Time to talk!**

**Kirk Leech**

Animal research remains a contentious issue. There is strong and vocal opposition to the use of all animal models in scientific research. Activist campaigns have targeted both private and public research. It is clear that researchers must be ready to counter opposition to their work. Unfortunately, public engagement by many European researchers and institutions has often remained hesitant, and defensive. This lack of positive communication allows the voices of those opposed to animal research to dominate public discourse; with the result that public and political opinion is often uncertain on the use of animals in research. This disposition has the potential to lead to further restrictions on research, to the detriment of science, medical progress and society. For too long, the scientific community has allowed the fear of anti-animal research campaigns to prevent its members from speaking publicly about animal research. Today, this fear, although understandable, is increasingly unfounded. While some researchers and institutions may encounter campaign groups, involved in vocal but lawful activities, very few will ever come across extremists. These campaign groups are often large, well-funded organizations with professional advocates, lobbyists, and media consultants, who can successfully command public discussion on the subject of animal research (often with misinformation, and unfounded opinion); particularly in the absence of public communication from the scientific community. Countering these successful global PR campaigns necessitates the delivery of comprehensive and balanced information to the public on the benefits for humans and animals of the use of animal models in scientific research. Pro-active

communication, and openness on animal research will encourage public trust, and allow the scientific community to speak with a united voice. In doing so, this prevents individuals and organizations from being isolated. Non-communication will only prolong opposition and mistrust. We all need to play a role in illuminating the complex social issues involved with animal research. The scientific community should not allow those who are opposed to animal research to set the public agenda. The aim of this will be to discuss why and how we can encourage and practice greater openness about animal research.

### **Genome editing and farmed animals: ethical considerations**

#### **Andy Greenfield**

Genome editing is a disruptive technology that has revolutionized research in genetics, biotechnology and synthetic biology. But its impact can be seen beyond research. Potential applications in farmed animals promise improved food security and higher standards of animal welfare. However, these proposed interventions in farming practices exist in a social, ethical and political space that is highly contested. Over the last 18 months, I have been a member of a working group of the UK Nuffield Council on Bioethics\* that is examining the ethics of genome editing in farmed animals. I will discuss progress in this project. In particular, I will discuss a number of the central questions that this topic raises, which include:

- Does genome editing raise any distinctive ethical concerns not already raised by selective breeding of naturally occurring genetic variants?
- Might improvements to the welfare of farmed animals using genome editing simply further entrench contentious systems of intensive farming?
- Do such interventions threaten to harm ‘animal nature’ or ‘animal dignity’?
- What should be the role of public opinion in making policy in this area?

\* <https://www.nuffieldbioethics.org/publications/genome-editing-and-farmed-animals>.

## **Session 5: Germ Cells, IPS and Trophoblast Cells**

### **Blastoids: modeling blastocyst development and implantation solely from stem cells**

#### **Nicolas Rivron**

The blastocyst, the early mammalian conceptus, forms all embryonic and extra-embryonic tissues. It consists of a spherical thin-walled layer, the trophoblast, that surrounds a fluid-filled cavity sheltering the embryonic cells. From mouse blastocysts, both trophoblast and embryonic stem cell lines can be derived, which are in vitro analogues of the trophoblast and embryonic compartments, respectively. Our lab showed

that trophoblast and embryonic stem cells self-organize in vitro to form structures that morphologically and transcriptionally resemble blastocysts (‘blastoids’). Blastoids are permissive to the formation of primitive endoderm-like cells, the second extra-embryonic lineage thus comprising the 3 founding lineages, and implant upon in utero transfer. Like blastocysts, blastoids form via inductive signals originating from the inner embryonic cells and driving outer trophoblast development. The nature and function of these signals are largely unexplored. Genetically and physically uncoupling the embryonic and trophoblast compartments, along with single cell transcriptomics, revealed an extensive list of inductive signals. We specifically show that the embryonic cells maintain trophoblast proliferation and self-renewal, while fine-tuning trophoblast epithelial morphogenesis. Altogether, these embryonic inductions are paramount to form a trophoblast state that robustly implants and triggers a genuine decidualization in utero. Thus, at this stage, the nascent embryo fuels the development and implantation of the future placenta. Overall, the blastoid is a powerful tool that can be reproducibly generated in large numbers, finely tuned, contains all the cell types to form the conceptus, and implants in utero. Our lab now investigates how synergies and self-organization processes occurring between the different compartmented cell types (epiblast, primitive endoderm, and trophoblast) regulate implantation and post-implantation development.

### **Comparative Parallel Multi-Omics Analysis of Cell Undergoing Reprogramming to Pluripotent and Trophoblast States**

#### **Yossi Buganim**

Following fertilization, totipotent cells divide to generate two compartments in the early embryo: the inner cell mass (ICM) and trophoblast (TE). It is only at the 32–64-cell stage when a clear segregation between the two cell-types is observed, suggesting a ‘T’-shaped model of specification. Here, we examine whether the acquisition of these two states in vitro by nuclear reprogramming share similar dynamics/trajectories. We conducted a comparative parallel multi-omics analysis on cells undergoing reprogramming to Induced pluripotent stem cells (iPSCs) and induced trophoblast stem cells (TSCs), and examined their transcriptome, methylome, chromatin accessibility and activity and genomic stability. Our analysis revealed that cells undergoing reprogramming to pluripotency and TSC state exhibit specific trajectories from the onset of the process, suggesting ‘V’-shaped model. Using these analyses, not only we could describe in detail the various trajectories toward the two states, we also identified previously unknown stage-specific reprogramming markers as well as markers for faithful reprogramming and reprogramming blockers. Finally, we show that while the acquisition of the TSC state involves the silencing of embryonic programs by DNA methylation, during the acquisition of pluripotency these specific regions are initially open but then retain inactive by the elimination of the histone mark, H3K27ac.



## **Testis formation in XX individuals resulting from novel pathogenic variants in Wilms' tumor 1 (WT1) gene**

**Nitzan Gonen**

Sex determination in mammals is governed by antagonistic interactions of two genetic pathways, imbalance in which may lead to disorders/differences of sex development (DSD) in human. Among 46, XX individuals with testicular DSD (TDSD) or ovotesticular DSD (OTDSD), testicular tissue is present in the gonad. Although the testis-determining gene SRY is present in many cases, the etiology is unknown in most SRY-negative patients. We performed exome sequencing on 78 individuals with 46, XX TDSD/OTDSD of unknown genetic etiology and identified seven (8.97%) with heterozygous variants affecting the fourth zinc finger (ZF4) of Wilms' tumor 1 (WT1). The introduction of ZF4 mutants into a human granulosa cell line resulted in up-regulation of endogenous Sertoli cell transcripts and Wt1 Arg495Gly/Arg495Gly XX mice display masculinization of the fetal gonads. This talk will focus of the genome editing strategy used to generate the mice carrying the human mutation.

## **ORBIS PICTUS LECTURE**

### **Visualizing in vivo systems at the speed of life**

**Elizabeth Hillman**

Living things change over time. Harnessing the dynamics of life can permit interrogation of movements, cellular function, blood flow changes and physiological responses to perturbations and drugs. While many imaging systems are optimized for resolution at the expense of imaging speed, we have focused on developing imaging methods that enable holistic imaging of biological systems at higher and higher speeds. One method that we have developed, swept confocally aligned planar excitation (SCAPE) microscopy is a single-objective light sheet approach that can image living samples in 3D at cellular resolution at up to 300 volumes per second, with high signal to noise and minimal photodamage. We have applied SCAPE microscopy to a wide range of living samples, including small organisms such as *C. elegans* worms, zebrafish larvae and fruit flies, but also intact mouse olfactory epithelium and in vivo mouse brain. In these systems, we have leveraged the power of genetically encoded fluorescent indicators of cellular activity, as well as tracking and extracting 4D movements and tissue deformations to uncover new physiological processes. We have also developed a clinical version of SCAPE, for high-speed in situ histopathology, as well as systems capable of high-throughput and high-content imaging of fresh, fixed, cleared and expanded samples. In further work, we have developed high-speed meso-scale and multi-spectral methods for imaging biological dynamics, permitting extraction of a wide range of valuable spatial and temporal features of physiological systems using novel dynamic unmixing image analysis approaches. Our work demonstrates the power of high-speed imaging for biomedical applications, opening up new ways to interrogate the real-time and functional features of physiology and disease across scales.

## **Session 7: 3R's**

### **Rapid generation of versatile cancer models with minimal breeding effort**

**Ivo Huijbers**

Human cancer modeled in Genetically Engineered Mouse Models (GEMMs) has provided important mechanistic insights into the molecular basis of tumor development and enabled testing of new intervention strategies. Many existing GEMMs have complex genetic background with often multiple modified tumor suppressor genes and oncogenes. Modifying these GEMMs to validate a potential cancer gene or drug target has been challenging due to the extensive intercrossing required to obtain the desired genetic background. We have developed a strategy to overcome this hurdle by re-deriving Embryonic Stem Cells (ESCs) from existing GEMMs. These GEMM-ESCs are subsequently used for genetic engineering to create cohorts of chimeric animals or F1's in which tumors can be induced and tested. This GEMM-ESCs approach has been successfully applied to generate a broad array of new small cell lung cancer models and breast cancer models. Recently, we combined the GEMM-ESC approach with expression of Cas9 or Cas9-base editors to enable flexible somatic cancer modeling. This powerful combination enables the rapid generation of versatile cancer models with minimal breeding effort.

### **Refining Phenotyping for Refined GA strains**

**Sara Wells**

The prolific use of genome editing in the mouse has allowed the development of refined and sophisticated new mouse strains, as well as accelerating the generation of knock out alleles. The utility of these new alleles will only truly be realised if progress in phenotyping keeps pace. Continuous automated home cage assessment over several light:dark cycles has been shown to be a robust method to assess multiple biologically relevant phenotypes. Where variability due to factors such as experimenter intervention, time of experiment and testing order can be almost completely overcome. In this presentation I will show how this approach is particularly relevant to investigating novel models and progressive conditions where the phenotypes are unpredictable, subtle or their onset is hard to reliably observe through traditional out of cage phenotyping methods.

## **Young Investigator Award**

### **Development of targeted transgenic technologies before and after the CRISPR era**

**Hiromi Miura**

In order to produce an ideal mouse model by genetic modification, I have been involved in the development of a few breakthrough transgenic technologies called (1) Pronuclear Injection-based Targeted Transgenesis (PITT), (2) Efficient additions with ssDNA inserts-CRISPR (Easi-CRISPR), and (3)

improved-Genome-editing via Oviductal Nucleic Acids Delivery (i-GONAD). PITT method, allowing single-copy transgene integration into the predetermined genomic locus by site-specific recombination, overcomes major problems of the traditional transgenesis method such as “transgene silencing” and “variegated transgene expression” which is caused by random transgene insertion with multiple copies. The PITT method was successfully applied to generate transgenic mice and knockdown mice showing reliable transgene expression before the CRISPR era. After the recent development of CRISPR genome editing technologies, I demonstrated that long single-stranded DNA molecules (synthesized via *in vitro* transcription and reverse transcription; ivTRT) serve as highly efficient donors for creating knock-in mouse models. In the first proof-of-principle work on this concept, I applied the concept for developing knockdown mouse models by inserting artificial microRNA sequences into intronic region of eEF2 locus. Now, the method is called Easi-CRISPR and hundreds of mouse models consisting of conditional knockout (floxed) and knock-in (e.g. reporter genes) lines have been created all over the world. The Easi-CRISPR method was also adapted in another breakthrough technology called i-GONAD developed in our laboratory. The i-GONAD method does not depend on the complex mouse transgenesis steps, such as isolation of zygote from pregnant females, microinjection of zygote and transfer of injected zygotes into recipient females, and genome edited mice can be created by directly electroporating genome editing components into zygotes *in situ*. In the TT2020 meeting, I would like to present my research history related to the development of these technologies as well as recent update of our research.

## **Session 8: Emerging Technologies**

### **Meiotic Cas9 expression mediates genotype conversion in the germline of male and female mice**

**Kimberly Cooper**

Super-Mendelian inheritance systems have potential to overcome obstacles to studying complex genetic traits using laboratory mice and to limit loss of biodiversity and disease transmission caused by wild rodent populations. We previously showed that such a system mediated by genotype conversion after a sequence targeted CRISPR/Cas9 double strand DNA break is feasible in the female mouse germline. In the male germline, however, no double strand breaks were repaired by interchromosomal homology directed repair to copy a heterozygous transgene to homozygosity. Almost all offspring inherited an ‘insertion/deletion’ mutation formed instead by non-homologous end joining or micro-homology mediated end joining. We interpreted these data to suggest that timing Cas9 expression to coincide with meiosis I is critical to favor conditions when homologous chromosomes are aligned and homology directed repair mechanisms predominate. Here, using a Cas9 knock-in allele at the Spo11 locus, we show that meiotic expression of Cas9 does indeed facilitate genotype conversion in the male germline. However, the low frequency of both HDR and indel mutation in both male and female germlines suggests that Cas9 may be expressed from the Spo11 locus too late and/or at levels too low for a double strand DNA break to form frequently and

for repair to complete prior to segregation of homologous chromosomes. We suggest that further refinement to early meiosis I and more robust expression may improve the efficiency of genotype conversion and increase the rate of super-Mendelian inheritance from both male and female mice.

### **Born to be wild: utilizing the common link among mammals to create better translational research models**

**Stephan Rosshart**

Laboratory mice are a mainstay of biomedical research and have been instrumental for many important discoveries in the field of immunology. However, there are also major limitations, including conflicting results rooted in divergent microbiota among research facilities and the limited ability to predict the complex immune responses of humans. We demonstrated that ultra-clean lab mice are too far removed from natural, microbially diverse, “wild” environmental conditions to faithfully mirror the physiology of free-living mammals like humans. Mammals and their immune systems evolved to survive and thrive in a microbial world and behave differently in a sanitized environment. This distorts how the immune system of ultra-clean lab mice develops/functions, leading to false assumptions of how our own “wild” immune system works. To address these shortcomings, we transferred C57BL/6 embryos into wild mice, creating “wildlings.” These mice combine the natural microbiota of wild mice with the tractable genetics of lab mice. The natural microbiota as well as the phenotype of wildlings were not only stable over multiple generations, but also resilient against antibiotic, dietary, and microbial challenges thereby providing an excellent model for long-term work and reproducible experimentation. Wildlings, but not conventional laboratory mice, phenocopied human immune responses in two preclinical studies. A combined natural microbiota- and pathogen-based model may enhance the reproducibility of biomedical studies and increase the bedside safety and success of immunological studies.

### **Improved methods for CRISPR HDR using Alt-R modified dsDNA donors and Alt-R HDR Enhancer V2**

**Jessica Woodley**

CRISPR-based homology-directed repair (HDR) is an invaluable tool for researchers looking to make precise, specific mutations in a genomic region of interest. Unfortunately, HDR is a challenge for many as efficiency is often low. HDR repair outcomes with CRISPR/Cas systems are most efficient with single-stranded DNA (ssDNA) templates when small insertions (up to ~ 120 bp), deletions, or SNP changes are desired edits. For these applications, synthetic oligonucleotides (ssODN) are commercially available with modifications for enhanced efficacy in HDR. Larger insertions can be incorporated via HDR using enzymatically generated ssDNA or double-stranded DNA (dsDNA) donor templates. Here, we present work demonstrating that improved efficiency in HDR rates for large insertions is obtained when dsDNA donor templates include novel end-modifications. These modifications improve the frequency of HDR and reduce homology-independent (blunt)

insertion events that can occur at both on- and off-target CRISPR edits. Using Alt-R modified dsDNA templates, we observe more than a threefold increase in the ratio of HDR:blunt repair outcomes as compared to an unmodified dsDNA template. In addition, we demonstrate improved HDR rates when using Alt-R HDR Enhancer V2, a novel small molecule solution that increases the rate of HDR in varied cell types including iPSCs. Together the use of Alt-R modified dsDNA donor templates and the Alt-R HDR Enhancer V2 allows researchers to maximize HDR rates for their large knock-in experiments.

## **EMBO KEYNOTE LECTURE**

### **Dynamics of early development: from embryos to stem cells**

**Janet Rossant**

By studying the mouse blastocyst and its derived stem cells, we have been able to identify signaling pathways and transcription factors that specify pluripotent versus extraembryonic cell fate. New tools of single cell genomics and live imaging and are providing insights into the dynamics of these processes in unprecedented detail.

## **POSTER ABSTRACTS**

### **CRISPR/Cas9 Mice Genome Editing to Study New Human Deafness Genes**

**Amal Abu-Rayyan<sup>1,2</sup>, Mor Bordeynik-Cohen<sup>1</sup>, Shahar Taiber<sup>1</sup>, Tal Koffler-Brill<sup>1</sup>, Einat Rosen<sup>1</sup>, Ryan Carlson<sup>3</sup>, Rebecca Haffner<sup>4</sup>, Moien Kanaan<sup>2</sup>, Mary-Claire King<sup>3</sup>, Karen B. Avraham<sup>1</sup>**

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CRISPR/Cas9 technology has rapidly emerged as one of the most powerful tools to characterize the functions of newly discovered genes and potentially to treat genetic disorders. We have applied this tool to our studies of human deafness. Hearing loss is the most prevalent human sensory deficit, affecting 466 million people worldwide. It can be caused by genetic factors and environmental factors, such as noise, trauma and drugs. Many genes responsible for deafness have been identified, but others remain unknown and may lie in pathways about which little is known. Our approach for studying deafness is to identify candidate causal mutations in human families, then to create mice with the exact human mutations using CRISPR/Cas9 gene editing. With this approach, we can study the function of novel genes and regulatory networks in the inner ear. We design a guide RNA (gRNA) to direct a double-strand break in the desired region of the mouse genome, and a donor DNA repair template. The guide, donor DNA and Cas9 protein are then

electroporated into single-cell embryos to induce genomic double-strand breaks and homology directed repair by donor DNA. After mice are born, they are genotyped and potential off-target sites are assessed. For recessive traits, founder mice are bred to homozygosity. Mutant mice are phenotyped using auditory brainstem response (ABR) to assess hearing function and various imaging techniques and molecular assays to dissect the mechanisms underlying the pathology. This work will expand understanding of the biology of hearing and provide new paths in the development of management and treatment of hearing loss. Research funded by NIH/NIDCD R01 DC011835 to KBA, MK, and MCK.

### **A new and versatile allele for overexpression**

**Alasdair Allan, Elke Malzer, Gemma F. Codner, Gene Delivery Team, Christopher Godbehere, Janet Kenyon, Jorik Loeffler, Matthew Mackenzie, Sara Wells and Lydia Teboul**

MRC Harwell Institute, The Mary Lyon Centre, Harwell Campus, Oxon, OX11 0RD, UK

Over expression mouse models are valuable tools to understand gene function, genetic diseases and to develop and test new therapeutic treatments *in vivo*. The ability to achieve precise expression pattern is essential and can be ensured by employing an appropriate cre driver. However, the optimal expression level of the over-expressed coding sequence is difficult to predict at the time of a new allele design. Here, we present a new tuneable over-expression allele that combines different transgenic cassettes in a modular fashion to achieve a large range of expression levels from a single targeting event.

### **On-target DNA-writing is improved with DNA ministrings**

**S. R. Bischoff<sup>1,2</sup>, L. W. Vaccaro<sup>1</sup>**

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Digital genome engineering is a branch of synthetic biology which accelerates combinatorial genome modification of cells or organisms using DNA-writing and DNA-editing technologies and aims to increase the range of edit types, to simultaneously edit at multiple genomic loci, and to improve the speed and accuracy of on-target gene editing. Strategies to improve knock-in gene editing include chemical complementation, cell-cycle synchronization, extension of isogenic homology arm lengths and marker-assisted co-selection among others; nonetheless, there still is considerable room for improvement of seamless replacement by homology-directed repair. We and others have documented multi-copy head-to-tail targeted arrays, which present themselves as concatemers at the intended knock-in site using standard gene targeting reagents: double-stranded DNA plasmids as donor vectors that encode a repair template for knock-in with Cas endonucleases in ribonucleoprotein formats. We observe these multi-copy arrays at the intended target site regardless of cell type such as human or mouse pluripotent stem cells or human cancer lines, of

animal model species including mouse, rat, pig and cow, of developmental stage such as zygotic or 2-cell pre-implantation embryos, or of delivery method including nucleofection, slide or cuvette electroporation or microinjection. We explore the hypothesis that DNA-end topology can improve on-target gene editing outcomes using linear covalently closed DNA repair templates that we refer to as DNA ministrings. Finally, we outline strategies for detecting and collapsing precisely targeted multiple integration events and for improving on-target DNA-writing to endeavor digital genome engineering.

### Transgenic goats expressing high levels of recombinant Trastuzumab in milk over multiple generations

S. Blash, N. Buzzell, G. Allard, W. Gavin

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At LFB USA, the ultimate use of transgenic goats is for the production of recombinant human protein therapeutics in the milk of these dairy animals through LFB USA's proprietary and proven rPRO Technology<sup>®</sup> as evidenced by the commercial approvals of both ATryn<sup>®</sup> and Sevenfact<sup>®</sup>. Trastuzumab is a recombinant monoclonal antibody therapeutic approved for treating both breast and stomach cancer. LFB USA produced transgenic goats containing both heavy and light chains for trastuzumab by pronuclear microinjection. One high expressing transgenic male founder was selected for trastuzumab production. The Trastuzumab expression level in milk was determined by an HPLC Protein A assay which specifically isolates human IgG from all other host proteins, including goat IgG. An F1 female's mean expression level over the entire 200 day lactation was 66.9 g/L. This resulted in the production of over 16,500 grams (16.5 kg) of Trastuzumab from a single animal in her 1st lactation. This line has been subsequently bred out to the F3 generation with all animals and generations maintaining similar milk production and continuing to average 60 + g/L. This transgenic line represents the highest expressing recombinant human protein level that has been produced in the milk in published literature. These transgenic animals from this primary line demonstrated both transgene and expression stability over multiple lactations and generations. In summary, this demonstrates that LFB USA's rPRO Technology<sup>®</sup> platform is a robust and cost effective system for the production of human recombinant Trastuzumab as evidenced here and could be utilized for countless other recombinant proteins.

### Targeted locus amplification (TLA) applications on a multi-integration-site founder transgenic goat produced from pronuclear microinjection

L. Chen<sup>1</sup>, N. Buzzell<sup>1</sup>, S. Blash<sup>1</sup>, G. Allard<sup>1</sup>, W. Gavin<sup>1</sup>, M. Kelder<sup>2</sup>, J. Bergboer<sup>2</sup>

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At LFB USA, transgenic goats are developed for producing recombinant human therapeutic proteins in their milk. For this

project, a transgenic male was generated via pronuclear microinjection (PMI). PMI may produce founders with multiple integration sites that can complicate the selection process for subsequent propagation. The TLA assay identified three sites of integration on chromosomes (chr) 5, 19, and 23 for this founder male. A homozygous male carrying a single chr 5 integration site was desired for further large-scale herd expansion. TLA was performed on one of his female offspring to confirm a single integration on chr 5. Homozygous males were subsequently generated by breeding the founder male to his female offspring with the chr 5 site only. From this breeding, 26 offspring were produced and analyzed for detection of the transgene, integration sites, and homozygosity using TLA-derived breakpoint sequence data. Twenty-three of the offspring were positive for the transgene and two were homozygous for chr 5 only. Additionally, TLA did not detect any single nucleotide polymorphisms (SNP) and/or insertions/deletions (indels) of the transgene in the founder male or F1 female, confirming transgene integrity. In summary, we demonstrate the bene-fits of TLA application through; (a) identification of 3 integration sites at the nucleotide level in a founder transgenic male produced by PMI, (b) insight into assay development to determine the state of zygosity and (c) identification of the lack of SNPs within the transgene, thereby demonstrating construct integrity, and hence protein fidelity.

### Development of methodologies for the generation of genetically modified snails, *Pomacea canaliculatae* (Gastropoda, Ampullariidae), a novel, non-mammalian research organism

Timothy Corbin<sup>1</sup>, Alice Accorsi<sup>1,2</sup>, Michael Durnin<sup>1</sup>, Kym Delvanthal<sup>1</sup>, Alejandro Sánchez Alvarado<sup>1,2</sup>

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The freshwater snail, *Pomacea canaliculata*, has complex camera-type eyes anatomically comparable to vertebrate eyes that can regenerate *de novo* after complete amputation. These features make this snail an excellent example of a novel, non-mammalian animal in which CRISPR based genome editing could significantly advance the study of camera-type eye regeneration. Protocols for harvesting and culturing snail embryos *ex ovo* were initially established. As transgenic methodologies for the generation of genetically modified mollusks are limited, we optimized and developed techniques to overcome the challenging and unique characteristics of the *P. canaliculata* embryos, making them amenable for micro-manipulation and microinjection. Delivery of mRNA and CRISPR/Cas9 reagents was examined via conventional microinjection, utilization of the WPI MICRO-ePORE pinpoint cell penetrator, and in vitro electroporation. Here we report our results on embryo survival rate and genome modification in *P. canaliculata* embryos for the three tested techniques. We can conclude that microinjection into one-cell fertilized *P. canaliculata* embryos successfully delivers functional exogenous mRNA and CRISPR/Cas9 reagents capable of inducing gene knockouts. Increased survival rates were

achieved using the MICRO-ePORE. Further experimentation and modification of our current in vitro electroporation protocol is required to determine if this is a viable method to produce genetically modified snails.

### Efficient generation of functional null alleles using HDR instead of NHEJ repair pathways

Jade Desjardins<sup>1</sup>, Nobuko Honma-Yamanaka<sup>1</sup>, Erzsebet Nagy-Kovacs<sup>1</sup>, Mitra Cowan<sup>1</sup>, Marie-Pier Cloutier<sup>1</sup>, and Yojiro Yamanaka<sup>1,2,3</sup>

MICAM, McGill University

Non-homologous end joining (NHEJ) mediated mutagenesis using CRISPR/Cas9 is the most common strategy to generate a functional null allele. Although the NHEJ repair pathway is easy to use, the randomness of the repair mechanism does not always efficiently generate the desired null allele. Two common strategies where we have encountered problems are using a single gRNA to generate indels or using 2–4 gRNAs flanking the target sequence to generate deletions. We observed that using one gRNA can generate up to 90% indels in the founder mice, unfortunately many of these mice do not have mutations leading to a functional null allele. In the second strategy, using 2–4 gRNAs to create a deletion, we found primer sequences might be disrupted or large deletions might not be observable, leading to a loss of heterozygosity and misinterpretation of the genotype. To circumvent these problems, we now use a short single-stranded donor (ssODN) template in conjunction with the homology-directed repair (HDR) pathway to insert a specific mutation such as a stop codon, a frame shift, or a predetermined deletion. We successfully generated 8 knock-out models in the past year using this approach including deletions of up to 5 kb. This approach can also be used with a variety of delivery methods such as microinjection and electroporation at 1-cell or 2-cell stage or Oviductal Nucleic Acids Delivery (iGONAD).

### Genome Editing Mice for Medicine: Facilitating access to in vivo models for validating mutation causative of human disease and for developing therapeutic strategies

Alex Fower, Gemma F. Codner, Alasdair Allan, Daniel Archer, Rosie K. A. Bunton-Stasyshyn, Adam Caulder, James Cleak, Skevoulla Christou-Smith, Charlotte Davis, Gene Delivery Team, Christopher Godbehere, Marie Hutchison, Janet Kenyon, Jorik Loeffler, Matthew Mackenzie, Elke Malzer, Edward O'Neill, Peter Price, Jan Rainier Sidiangco, Michelle Stewart, Anna Thamm, Susan Varley, Sara Wells and Lydia Teboul

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The increasing availability of large datasets from both patient genome sequencing and genome-wide functional genomics programmes is yielding many candidate mutations causative of human diseases. Thus, it increases the requirement for in vivo models for candidate validation and testing potential therapeutic

strategies. The Medical Research Council has established the Genome Editing Mice for Medicine (GEMM) programme to facilitate the generation and access to such mouse models. Mutations are nominated by the scientific community and selected by a scientific panel. The genome engineering service at the MRC Harwell Institute is continuously taking advantage of new genome editing techniques and has already generated and validated 67 mouse models, all of which are available for distribution as part of the GEMM programme. A minimum of two calls per year over a period of 5 years will ensure that models are prioritised in a timely fashion, so the programme answers the current requirements of the community. The majority of alleles are point mutations, but other allele types include conditional alleles, tag KIs, cre drivers and over-expression models. We will present the process for generation and validation of these animals and examples of new alleles.

### Is a reduction in embryo lysis possible using the micro-pore (MEP)?

W. Gardiner, J. Kenyon, S. Atkins, T. Bell, H. Swash

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CRISPR-Cas9 gene editing technology has increased our ability to produce Genetically Modified mice not only in a highly specific manner but also quickly and on demand. Establishments can easily design and produce the CRISPR guides and donors needed to engineer their mice. However despite these advances in the field there is still the need for the highly technical task of Pronuclear or Cytoplasmic microinjection to deliver the reagents. These methods can require a large number of embryos in order to reach the required number of founders, one factor contributing to this high embryo usage is lysis due to damage to the embryo by the injection needle. We have used the MEP to aid cytoplasmic and pronuclear microinjection of one and two cell embryos and to see if it helped to reduce embryo lysis rates. We also used the MEP for Crispr-CAS9 targeted deletion, point mutations (oligo donors) and more complex projects (long single stranded donors). Our data shows intermittent reduced lysis rates, no difference in birth rates, mutation rates and on-target mutation rates when using the MEP for both pronuclear and cytoplasmic delivery but it is still a useful tool to try to reduce lysis rates particularly for people who are new to microinjection.

### The quality of pre-implantation mouse embryo development is impacted by the mode of euthanasia

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Cervical dislocation (CD) without prior anesthesia is a conditionally acceptable method of euthanasia that requires scientific justification (American Veterinary Medical Association Panel on Euthanasia, 2000). We performed a study to

determine if the recommended use of Isoflurane (Iso) before CD affects the viability of mouse embryos and their development in vitro. We collected zygotes from superovulated and plugged CD-1 female mice euthanized by two methods, Iso + CD and CD, cultured the embryos in vitro for 96 h, scored blastocyst development and counted the number of cells in the blastocysts to assess embryo quality. We observed that the rate of embryo development to blastocyst was significantly reduced in the Iso + CD group (36%) compared with the CD group (67%) at 72 h. By 96 h the proportion of blastocysts was more similar between the two groups (75% vs 89%, respectively). However, the quality of embryos from the Iso + CD group was substandard as determined by the lower total cell number in Iso + CD group compared to the CD group (63.64 vs 83.5) accompanied by an increased cell death rate (cell death index of 7.66% vs 4.46%, respectively). We concluded that anesthesia of mice with isoflurane prior to euthanasia by cervical dislocation negatively impacts embryo development and quality supporting the use of cervical dislocation for euthanasia of pre-implantation stage mouse embryo donors.

#### **Production of Conditional Knockout Alleles in Mice by CRISPR/Cas9-Mediated Genome Editing Using Two Guides/Two Oligos Approach**

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CRISPR-Cas9 technology represents a significant improvement of genome editing tools, reaching a new level of targeting, efficiency, and ease of use, thereby relieving many steps of traditional mouse ES cell technology when it comes to generation of mouse knockout alleles. However, production of conditional knockouts remains an important challenge. Several studies provided contradictory reports regarding efficiency in generating conditional knockout alleles in mice using 2 single guide RNAs (sgRNA) and 2 single-stranded oligonucleotides (ssODN). We assessed the efficiency of using this method in creating conditional targeted alleles in a set of mouse genes. Even though overall success rate was low—about 2.5%—and not all genes have been correctly targeted, we show that it's possible to generate conditional knockout alleles using the CRISPR/Cas9 two guides/two oligos approach on regular basis.

#### **“Bred but not used” animals in transgenic activities: lessons from the report of EU commission on implementation of Directive 2010/63/EU**

**Emmanuel Gomas**

EGS Conseil

On February 2020, the European Commission released a report on the implementation of Directive 2010/63/EU in European

State Members. For the first time, the number of animals born in animals' facilities but not used for experiments was recorded. Analyzing these data provides lot of insights about the “Bred but not used” animals. These animals represent up to 68% of the overall animals entering in Genetically Modified (GM) model's creation, maintenance and experiments, not less than 7,4 million individuals (83% are mice) in 2017. The responsibility of the transgenic technologies community is then to understand the origin of these bred but not used animals (improper genetic background, improper/useless WT littermates, unused lines mating, improper colony management, Mendel laws...) as well as training the scientific community to proper use of colony management tools (cryopreservation, backcross, colony management, production calculation tools...) to make sure reduction of bred but not used animals become a priority on day to day GM models breeding.

#### **Understanding the role of histone chaperone FACT in mammalian cells using a conditional FACT knockout mouse model**

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Histone chaperone FACT (Facilitates Chromatin Transcription) is known to be essential for the survival of human and mouse tumor cells but not normal cells and can be selectively targeted by small molecule curaxin for anti-cancer effect. It is highly expressed in mouse and human undifferentiated stem cells but not in fully differentiated adult tissues. Although previously considered to be a factor required to facilitate transcription, depletion of FACT does not cause significant change in levels of transcription in cells. In addition, how FACT interacts with nucleosomes during transcription to maintain chromatin organization is yet to be fully elucidated. Thus, the role of FACT in normal mammalian cells remains poorly understood. In order to fully exploit the potential therapeutic value of FACT in cancer, it is critical to understand its role in normal cells and whether its targeting causes adverse effects. Previous studies attempting to study effects of FACT knockout have shown early embryonic lethality in mice. To circumvent this problem, we developed a tamoxifen inducible conditional FACT Knockout mouse model and showed that FACT loss causes rapid weight loss, sickness and death in these mice within a few weeks. Using data obtained from tissues, fibroblasts and stem cells derived from these mice we hereby propose that FACT is necessary for preventing histone loss during transcription in a cell-type specific manner.

### Simplified cloning of donor DNA constructs by prolonged overlap extension (POE)

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The relative ease to create complex genetic modifications using CRISPR/Cas has increased the demand for generation of complex donor DNA constructs. Here we describe the application and optimization of simple cloning by prolonged overlap extension (POE) PCR (Appl. Environ. Microbiol. 78:1593–1595 (2012)) for fast and easy scarless assembly of sequences from several plasmids with the possibility to introduce multiple site-directed mutations. Simple cloning by POE utilizes overlapping PCR fragments to create large repetitive multimers, which can be used to directly transform bacteria. Internal circularization generates the desired plasmid. We show that several mutations can be introduced in a plasmid simultaneously using this technique. Simple cloning by POE-PCR is a fast and economic method to assemble different fragments seamlessly into one plasmid. Furthermore, we expanded the range of POE-PCR to an insert size of only 70 bp, considerably smaller than the 200 bp reported previously. Technical aspects of this method have been described in (Biotechniques, vol. 68, no. 6). The plasmids can then be used to generate single or double strand donor DNA. We will discuss advantages and potential problems in using this simple cloning technique and show some application of gene editing in murine ES cells.

### Establishing an optimal method to deliver a Y chromosome-derived Eif2s3y transgene to mouse iPSC

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Mammalian reproduction conventionally requires oocytes provided by a female and sperm provided by a male. We hypothesize that male gametes can be developed from a female, and that these gametes are functional in assisted fertilization (ART). The overall strategy includes establishment of a somatic cell line from an adult female mouse, reprogramming to induced pluripotent stem cells (iPSCs), transgenic addition of the Y chromosome-derived spermatogenesis driver Eif2s3y, identifying clones that lost one X chromosome, differentiation into primordial germ cell-like cells, transplantation into testes of males lacking endogenous spermatogenesis, and using ART with resulting male gametes to generate offspring. To establish strong and sustained expression of Eif2s3y, we compared four gene-targeting strategies: (1) lentiviral transduction and (2) transfection by electroporation with random integration, and CRISPR/Cas9-mediated knock-in into (3) a genomic safe harbor H11 locus and (4) into germline-specific Ddx4 locus. Female somatic cells were successfully

reprogrammed to iPSCs and targeted with the Eif2s3y transgene (Eif2s3y-tg). After transfection/transduction, the iPSCs were assessed for Eif2s3y-tg copy number/cell, and tested for normalcy. Male iPSC express endogenous Eif2s3y at 100–200 copies/cell. Transfection by electroporation was not successful yielding 6–8 Eif2s3y-tg copies/cell. Both lentiviral transduction and knock-in into H11 locus established strong Eif2s3y-tg expression (~ 750 copies/cell and ~ 1200 copies/cell, respectively). The data for Ddx4 knock-in is pending. The iPSC-Eif2s3y-tg cells retained proper iPSC characteristics and are now used for the subsequent steps of the strategy. We conclude that the optimal method of Eif2s3y-tg delivery into iPSC is CRISPR/Cas9-mediated knock-in. Funded by NIH R01 HD07238 to MAW.

### Cooperative apoptotic—necroptotic cell death signaling in stem cells

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Apoptotic versus necroptotic cell death signaling has traditionally been viewed as competitive in nature, in which members of these pathways act to inhibit activation of the alternative path in a ‘winner take all’ form of signal activation. This interaction has traditionally focused on caspase 8-mediated RIPK inhibition. Intriguingly however we observe in embryonic stem cells exposed to programmed cell death-inducing agent cisplatin a form of cooperative interaction between apoptotic and necroptotic signaling involving RIPK1, RIPK3 and caspase-3 seen by both genetic inhibition and pharmacologic small molecule approaches, in which a non-additive enhancement of cell survival is conferred by inhibition of these agents when compared sister wild-type lineages. Molecular analysis of this interaction reveals that RIPK1 and RIPK3 regulate caspase-3 activation, suggesting that they lie upstream of caspase-3 with respect to signal activation. Consistent with this, cisplatin-induced cell death of wild-type stem cell populations demonstrates unique morphologic features distinct from classical apoptosis or necroptosis; features lost upon ablation of RIPK1, RIPK3 or caspase-3. Thus, these studies describe for the first time a cooperative form of apoptotic/necroptotic signaling in stem cells, which may have important implications for the regulation of stem cell death in primary tissues under both physiologic and pathologic conditions.

### CRISPR-mediated rapid generation of humanized alleles in mouse embryos with complex genetic backgrounds

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Regeneration

The delivery of CRISPR/Cas9 (CC9) nucleases to model systems has enabled rapid and facile manipulation of target genomes. Direct modification of mouse embryos can accelerate the generation of murine models harboring genetic modifications and circumvent the need to derive and validate new mESC lines. Here, we describe an *in vitro* workflow for strategic optimization of injection conditions allowing simultaneous evaluation of gene targeting approaches. Optimized conditions for mouse embryo gene editing enabled successful replacement of genomic regions up to 6.1 kb in naïve or previously targeted lines. This has enabled us to create robust models to study diseases such as diabetes and neurodegenerative diseases. Looking forward, we aim to increase the size of knock-ins (KIs) by exploring different targeting approaches. The combination of these novel technologies has greatly improved our ability to generate cohorts in high-throughput production to support drug discovery and provide a robust foundation for future technology development.

### Zygote electroporation of Cre and Dre proteins enables efficient recombination in mouse embryos

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Zygote electroporation is an efficient technique for mouse genome engineering using CRISPR/Cas system. Here, we demonstrate that the same procedure can be used for efficient delivery of Cre and Dre recombinases to mediate allele conversions in 1-cell stage mouse embryos. The method is simple, inexpensive, and enables highly efficient allele conversion in newborn animals. Proof of concept experiments were performed with Rosa26-tdTomato-EGFP and Rosa26-VFRL-EGFP reporter lines, carrying a transgenic cassettes for Cre/Dre -dependent expression of GFP protein. We have achieved 91% and 98% of fully converted animals after Cre and Dre protein zygote electroporation, respectively.

Moreover, the technique can be combined with *in vitro* fertilization leading to fast generation of tm1b converted animal models with the efficiency 67–100% in relation to the tm1a allele. This dramatically reduces time and costs needed for generation of desired models, especially when compared with traditional methods based on breeding with Cre-deleter mouse lines. Furthermore, we believe that simple and accessible option to utilize less common recombinases such as Dre, can lead to generation of more complex and versatile mouse models in the future.

### Successful knock-in of large DNA sequences in mice and rats using Easi-CRISPR

Kempston T

Van Andel Research Institute

CRISPR facilitates genome editing by creating a double strand break at a target DNA locus. Even before CRISPR it was well established that, left alone, such a break often resulted in a frameshift mutation able to knock out a gene, and that further addition of an exogenous DNA molecule could act as a template to introduce a desired insertion. CRISPR was first used in mice in 2013 by microinjection into zygotes. Shortly afterwards it was shown that addition of a small, single-stranded DNA molecule could efficiently introduce mutations up to ~ 100 bp, a size limitation based on contemporaneous single-stranded oligo synthesis ability. Despite promising initial reports, knock-ins of more than 100 bp using doublestranded plasmid templates were largely ineffective; practically, this made routine floxing of genes or insertion of large reporters difficult. In 2017 Easi-CRISPR was developed in consideration of the two types of knock-ins' disparate success rates. Dependent on generation of longer single-stranded DNA for use as template, the strategy's results showed that regardless of insertion size, when paired with functional guides, single-stranded DNA was an effective repair molecule at the zygote stage. We have performed 8 Easi-CRISPR experiments to knock in large stretches of DNA: 6 in mice and 2 in rats. In mice, 5/6 experiments were successful, inserting sequences from 656 to 1134 bp. In rats 0/2 experiments were successful, though in each case useful intermediary insertions were generated.

### Overcoming Unpredictability in Microinjection

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Genetically altered (GA) mice are an essential tool in biomedical research enabling us to elucidate the function of genes and the pathways involved in biological processes. Moreover, the similarities between mouse and man make the mouse a good model to determine the mechanisms underlying human disease. Traditionally GA mice were produced through the genetic modification of particular loci in embryonic stem (ES) cells that are reintroduced into blastocysts, incorporating



themselves into the growing embryo. More recently genome-editing tool like CRISPR-associated protein 9 (Cas9) have been developed enabling us to directly modify 1-cell embryos, creating highly defined mouse mutants which previously would have been costly both in terms of time and financially. This CRISPR-Cas9 technology has had a number of draw backs; the high animal usage required to produce the 1-cell embryos used, specialized equipment and training needed to inject into pronucleus and an inability to cope with unpredictable embryo harvest used to feed a mouse production pipeline. We present here work in our lab to overcome these difficulties including: embryo electroporation removing the need for specialized equipment, embryo vitrification allowing us to work flexibility to changes in harvest and IVF to feed the delivery pipeline and reduce the number of females used in superovulation.

### Dissecting functional contributions of microglia and CNS border associated macrophages using a binary Cre transgenic approach

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Brain macrophages are major players in central nerve system (CNS) physiology and pathophysiology. Much of our recent insight derives from fate mapping, intra-vital imaging, cell ablation and targeted mutagenesis using respective Cre/loxP system-based mouse models. Advances in cytometry and single cell transcriptomics have revealed a profound complexity of the brain macrophage compartment. Specifically, the latter comprises parenchymal microglia and nonparenchymal, CNS border-associated macrophages (BAM) located in perivascular and meningeal niches, as well as the choroid plexus. In depth understanding of specific functional contributions of these distinct CNS macrophage populations will require the development of Cre approaches that allow the study in physiological context. Here we report the development of a novel binary transgenic mouse model that relies on co-expression of split Cre fragments to target specific CNS macrophage populations. Following crossing to animals harboring conditional reporter alleles or a 'Ribo-tag' allele that allows translome profiling (Haimon, Z. et al. (20198) NI 159:1312), we confirmed differential targeting of microglia and BAM. Specifically, a combination of Cx3cr1Ccre and Sall1Ncre transgenes allowed for the first time for a specific targeting of microglia. Conversely, Cx3cr1Ccre:Lyve1Ncre transgenic mice were found to specifically target non-parenchymal macrophages located in meningeal and perivascular regions. We are currently using these animals in combination with the RiboTag approach to define distinct functional contributions of microglia and BAM to CNS pathologies.

### The role of XAB2 in pre-mRNA splicing and DNA repair

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Accumulating evidence indicate that pre-mRNA processing factors are key players in preserving genome stability, by participating in the DNA damage response and DNA repair. Indeed, individual RNA processing factors either regulate the expression of DDR genes at multiple post-transcriptional levels or are directly involved in DNA repair. XAB2 is a ubiquitously expressed essential protein that has been implicated in premRNA processing and Nucleotide Excision Repair, however its function remains elusive. To identify its role in mammalian physiology we have generated a new mouse model that expresses biotinylated XAB2, providing a powerful tool for the identification of protein–protein interactions and XAB2-bound gene/RNA targets at any developmental stage and from any tissue. Here we show that XAB2 is part of a pre-mRNA splicing complex that regulates the proper maturation of DDR genes. Moreover, the absence of XAB2 results in defective pre-mRNA splicing and impaired DDR and NER. Together these findings indicate that XAB2 plays an essential role in genome stability by regulating the expression of DDR genes. In addition we show that XAB2 interacts with DNA repair factors suggesting a possible role in the regulation of DNA repair per se.

### BLiSC Mouse Genome Engineering Facility (MGEF): An Indian Resource for Laboratory Mouse Model Design, Cryo-Archiving and Repository Management

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MGEF is a young multifunctional, national facility at the National Centre for Biological Sciences (NCBS) of the Bangalore Life Science Cluster (BLiSC) campus. With substantial support from the Indian Department of Biotechnology National Mouse Resource (NaMoR) grant, the MGEF was set up in 2014, as a state-of-the-art facility. Our mission is to

offer a wide range of services such as mouse stock cryopreservation/vitrification, cryo-recovery, in vitro fertilization, rederivation and generation of novel genome engineered mice for the Indian scientific community. As part of our mission to enhance access to these resources and expertise nationwide: we also conduct regular hands-on workshops and individual training to scientists from all over India and neighboring countries. Acting as a major Indian national repository of over 280 different mouse stocks, MGEF has over the last 6 months of COVID19 pandemic been instrumental in helping cryo-archive and save dozens of additional precious mouse stocks by reorganizing all its logistical operations. In response to immediate needs of researchers nationwide, we also have designed 3 different sets of ACE2 mouse models to help investigators in their SARS-CoV/COVID-19 related vaccine research and therapeutics projects. Our preliminary ACE2 mice characterization results are presented here. We would also like to present here how we are expanding our portfolio of services and national and international collaborations with the scope to consolidate and facilitate access to local and international expertise in Mouse Model based research and technologies in India.

#### The fibrodysplasia ossificans progressiva-causing ACVR1[R206H] and ACVR1[R258G] mutations exhibit distinct skeletal phenotypes in neonatal mice

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Gain of function mutations in the intracellular domain of the type I BMP receptor ACVR1 are associated with fibrodysplasia ossificans progressiva (FOP), a rare but catastrophically debilitating disease of ectopic intramuscular bone formation. Although the vast majority of FOP cases are caused by a R206H mutation the GS domain of ACVR1 (ACVR1[R206H]), FOPcausing mutations occur throughout the GS and the kinase domain of ACVR1. Of particular note, all three reported cases of a R258G mutation in the ACVR1 kinase domain exhibited more severe developmental defects than are observed for ACVR1[R206H] bearing FOP patients. In an effort to model this phenotypic variance, we engineered a Cre-regulated ‘conditional-ON’ allele of ACVR1[R258G] in the mouse (Acvr1[R258G]FIEx/+), similar to the previously described Acvr1[R205H]FIEx/+ mouse (Hatsell, Idone et al. 2015). As observed in the previously published Acvr1[R206H]FIEx/+;Gt(ROSA26)SorCreERT2/+ mouse, body-wide activation

of the FOP allele in adult Acvr1[R258G]FIEx/+;Gt(ROSA26)SorCreERT2/+ mice resulted in heterotopic ossification, which was entirely ameliorated by treatment with an Activin A-blocking antibody. When recombination was driven by early-embryonic expression of Cre-recombinase, both ACVR1[R206H] and ACVR1[R258G] muteins exhibited skeletal deformities and neonatal lethality. Detailed skeletal and soft-tissue imaging via iodine-contrast micro-CT confirmed that the skeletal phenotype presented more severely in ACVR1[R258G] mice than ACVR1[R206H] mice, consistent with the increased rarity and severity of ACVR1[R258G] relative to ACVR1[R206H] within the human FOP patient population. Experiments are underway to determine whether the increased severity of the ACVR1[R258G] phenotype is due to enhanced ligand dependent signaling, ligand independent signaling, and/or neoresponsiveness to TGF- $\beta$  or BMP family ligands.

#### Using CRISPR-Cas9 gene editing system in mouse embryonic stem cells to generate new relevant tools for the study of Trp73 gene isoforms

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The p53 family, constituted by p53, p63 and p73, has been widely studied for its role in various biological processes -such as proliferation, differentiation, senescence or apoptosis-involved in cell fate pathways. These transcription factors have multiple isoforms that are generated by alternative promoters and/or splicing variants. In particular, the Trp73 gene has two promoters that give rise to TA and DNp73 isoforms. It is generally accepted that TA-isoforms can perform p53-like tumour suppression functions, while DN-isoforms can act as a dominant negative of TAp73 and p53. The existence of these opposing isoforms gives p73 a bimodal function that will depend on the cellular context. Most of the studies regarding p73 function have been performed in tumor cell lines. Therefore, the development of physiological cell models that would help to decipher the role of the individual isoforms has become of great relevance. Embryonic stem cells (ES) and induced pluripotent stem cells are two key tools in Biomedicine and Developmental Biology, allowing the generation of differentiation models that recapitulate complex biological processes in vitro. The aim of this study was to generate and characterize p73-deficient mouse ESC lines lacking total p73 or either isoform (TAp73, DNp73). Using the CRISPR-Cas9 gene editing system, we have reproduced the genetic modifications created by gene targeting in the Trp73<sup>-/-</sup> mouse or the specific isoform-knockout mice. For this purpose, we have followed an editing strategy involving two sgRNAs surrounding the target gene regions. Efficient cleavage resulted in the expected deletions and subsequent loss of gene function.

## Generation of SARS-CoV-2 infection pre-clinical models on defined genetic backgrounds

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Pre-clinical models of SARS-CoV-2 infection are of obvious value. The transgenic Tg(K18-ACE2)2Prln mouse line is highly sought-after for its ability to confer sensitivity to viral infection through the expression of the receptor involved in infection in human. However this initial line was originally generated employing (C57BL/6 J x SJL/J)F2 embryos, a mixed background that is not optimal for many *in vivo* studies. We will describe the generation and early characterisation of new lines obtained by the microinjection of the same DNA construct in several inbred genetic backgrounds. We will show genotyping results obtained employing droplet digital PCR for founder animals and their offspring. We will discuss how this method allows for the precise assessment of transgene copy numbers.

## Inversion through an artificial intron, a tool for conditional mutagenesis

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The use of the Cre/loxP system in conditional mutagenesis is widespread in transgenic studies. The commonly used Lox-STOP-lox (LSL) method is often leaky, and after Cre activity a loxP site is left near the translational start site that may result in decreased protein expression in the CNS, probably due to its AT rich sequence. To overcome this problem we designed an artificial intron containing the lox66/lox71 mutant loxP sites for a unidirectional inversion based conditional system (coin technology), and we placed this intron into the coding region of the eGFP and other cDNAs. A great advantage of this system is that the cDNA's start codon is fused into the endogenous regulation region's ATG site, and only the second half of the cDNA is inverted between the lox66/lox71 sites. Transgenic constructs presented here are BAC based, and utilize the Sleeping Beauty transposase system for increased integration efficiency. For the integration of the necessary transposase IR/DR arms into the BAC vector we developed a plasmid construct which can deliver both arms in a single recombination step. Cholecystokinin expresses in wide range of neurons, such as principal cells and some basket cells, and the differentiation between subpopulations of CCK positive cells is important for neurobiological studies. A Cre dependent transgene family, based on an artificial intron combined with the coin system may play an important role in successful targeting of specific neuron populations.

## Floxed Mice Using a Novel DECAI CRISPR System

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Although inserting one loxP site using CRISPR is relatively easy, getting proper insertion of the two loxP's required for tissue specific knock-out is much more difficult. Several novel methods for generating floxed mice with CRISPR have been developed to improve the low efficiency of correct dual loxP insertion but are either technically challenging or still relatively inefficient. We adapted a new method called DECAI (DEgradation based on Cre-regulated-Artificial Intron, <https://doi.org/10.1038/s41598-017-16931-z>) by inserting a 201 bp artificial intron via CRISPR into an exon found in all isoforms of the Phf21a mouse gene. This intron is floxed around its branch point and carries 3 STOP codons, one in each frame, that will be transcribed upon deletion of the branch point. To determine whether the method is useful for floxing mice, we need to show that (1) the intron can be efficiently inserted into the gene, (2) without Cre recombinase, the gene is expressed at wild-type levels and spliced appropriately, and (3) in the presence of Cre, all of the gene's mRNA is degraded in the homozygote. The artificial DECAI intron was efficiently inserted without accompanying mutation into the Phf21a gene both in blastocysts (~ 36%) and founder mice (~ 17%). When these mice were crossed with wildtype, half their offspring contained the DECAI intron. We are now generating homozygotes to examine gene expression levels and to cross with Cre-expressing mice to test for Phf21a knock-out. Thus far, this adapted method for making floxed mice appears significantly easier and more efficient than previous methods.

## CD4+ and CD8+ Treg characterization in the rat using a new rat Foxp3- GFP model

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It is well established regulatory T cells (Treg) play a central role in controlling immune effector mechanisms and tissue integrity. The FOXP3 transcription factor is essential for the function of canonical natural Treg (nTreg) and is also expressed in induced Treg (iTreg). nTreg and iTreg can be both CD4+ and

CD8+ FOXP3 + but there are also FOXP3- iTreg. The respective roles of each of these Treg subsets in different pathophysiological situations is subject of intense research. In mice, *in vivo* Treg lineage identification has been obtained by placing eGFP under the control of the *Foxp3* promoter but this has not yet been performed in rats. Also, rat Treg are in general less well defined than human and mouse Treg and comparison of Treg among different species are rare and only on the T CD4+ lineage. We generated by KI line using microinjection of CRIPR/Cas9 elements and a donor DNA a rat line with a *Foxp3* allele that expresses functional FOXP3 and eGFP. We show that eGFP expression is restricted to subsets of T CD4+ and CD8+ Treg as defined by membrane phenotypic markers and segregates with T suppressor activity. Using CD4+ and CD8+ eGFP- cells we show generation of iTreg associated with expression of eGFP. Further studies are underway to fully characterize rat CD4+ and CD8+ FOXP3 + Treg.

### Generating new CRISPR-based mouse models for different types of albinism

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Albinism is a human rare genetic condition affecting 1:10,000–20,000 newborns world-wide, characterized by a poor vision and altered pigmentation. The visual deficit appears in all known 22 types of albinism, whereas the hypopigmentation is not present in all of them. Traditionally, mouse models have been generated with genetic alterations on the homologous genes in the murine genome, corresponding to the same genes in the human genome. With the advent of CRISPR genome-editing tools it has become easier to reproduce in mice equivalent mutations diagnosed in patients. In our lab we have been generating numerous mouse models for the different types of albinism, including oculocutaneous (OCA) type 1 (OCA1), OCA2, OCA4, OCA5, OCA6 and OCA7; ocular albinism (OA1) and FHONDA. In particular, we have generated diverse commonly found (in human beings) tyrosinase gene (*Tyr*) alleles for which their pathogenicity was not clear or established. We will update on our progress generating and initially characterizing the phenotype associated with these newly created CRISPR-based mouse edited mutations.

### The role of *Trabd2* metalloproteinase in mouse brain and head development

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Wnt signalling pathways are crucial for proper cell differentiation and proliferation during both embryonic development and adult tissue maintenance. Thus, they are tightly regulated by plethora of interacting proteins on multiple levels. One of

such potential Wnt-regulating proteins is *Trabd2*, a transmembrane manganese-dependent metalloproteinase, previously described to cleave some of the Wnt proteins regardless of their involvement in canonical or non-canonical pathway. However, the precise mechanism of its action has been described only *in vitro*. To examine its function *in vivo*, we generated *Trabd2*-deficient mouse model using CRISPR/Cas9 technology. *Trabd2b*-deficient mice display pathological effects on head and brain development whereas the level of damage differs between individual embryos. Some of the embryos remind of WT embryos, while others have hydrocephaly, and some are exencephalic lacking developed facial area. This suggests a wide range of *Trabd2* penetrance. To identify the connection of *Trabd2* with Wnt regulatory networks, we used Wnt signalling challenged model of nucleoredoxin (*Nxn*) knockout mice (*Nxn* acts as a negative regulator of Wnt signalling) crossed with the *Trabd2* mutants. Similarly to *Trabd2* mutants, *Nxn* heterozygous embryos show also wide range of phenotypes. Observed phenotypes result from defects in neural tube closure during early embryonic development which requires proper balance of Wnt signalling activity, important for molecular definition of brain development field, neural tube closure and later cranial neural crest cells migration. By model of embryonic development, we propose that *Trabd2* metalloproteinase is a novel Wnt signalling regulator during mammalian development.

### Development of remote teaching system for reproductive technology using online digital technology

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In consequence of COVID-19 pandemic, researchers and technicians were restricted to go to their laboratory and animal facility. The restriction hindered the animal experiments and breeding animals. In addition, the number of mice that could be kept in animal facility was limited. Therefore, there was a growing demand for technical support for cryopreservation of sperm and embryos in order to maintain mouse lines. Till now, we have continuously developed mouse reproductive technologies, and educate researchers and technicians in CARD Mouse Reproductive Technology Workshop to share the latest knowledge and techniques of mouse reproductive technologies in domestic and overseas institutes. At the last TT2019 meeting in Kobe, we also had the opportunity to provide a hands-on workshop about mouse reproductive technology. However, under the situation of COVID-19 pandemic, researchers and technicians cannot attend the workshops. Therefore, we are developing a remote teaching system which enables us to provide technical training using online digital technology. We hope we can contribute to promote the spread of reproductive technologies by incorporating online digital technology.

## Supercoiled plasmids from 1.0 to 11.5 kb can efficiently drive homology directed repair in mouse embryos

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Investigators at MD Anderson Cancer Center often require complex mouse models that are generated using CRISPR/Cas9 technology. Based on work by Kuhn (presented at TT2016), we chose to use supercoiled plasmids as donor DNA for homology driven repair (hdr) of large DNAs. The Genetically Engineered Mouse Facility (GEMF) has performed 25 projects (24 unique projects) using DNA donor constructs of 1 kb and greater. Injections were performed using supercoiled DNA plasmid donors and modified sgRNAs that were combined with enhanced-specificity spCas9 for gene targeting. For plasmids of 1.0–1.9 kb, we averaged 16.25 pups, from which we averaged 2.5 founders (N = 4). For constructs from 2 to 2.9 kb, pup and founder averages were similar (17.66 pups, 1.5 founders, N = 6), as well as for constructs from 3 to 3.9 kb (15 pups, 2.4 founders, N = 10) and 4–4.9 kb (9.5 pups, 3 founders, N = 2) While we generally produced over 10 pups per project, there were rare occasions where we only obtained 3–4 pups per project. However, with one exception, even for low numbers of pups, we were able to generate at least one founder. Interestingly, we were also able to obtain founder animals after injection of an 8.5 kb plasmid (1 founder) and a mix of 4 plasmids of 11.5 kb for ROSA targeting (9 pups, 3 founders, all with the same insertion). We conclude that supercoiled plasmids are an effective DNA source for efficient hdr of large DNAs.

## Modeling Left Ventricular non Compaction (LVNC) in vitro

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Left Ventricular non-Compaction (LVNC) is a cardiomyopathy characterized by prominent trabeculae into the lumen of the ventricles and a reduced thickness of ventricular walls. We previously identified two point mutations in the MIND BOMB1 gene MIB1, a type 3-ubiquitin ligase of the NOTCH pathway. The V943F and R530X MIB1 mutations cause LVNC with autosomic dominant inheritance. Conditional inactivation of Mib1 in the mouse produces LVNC. In order to understand the mechanisms underlying ventricular development and maturation, in vitro we generated human induced pluripotent stem cells (hiPSC) from skin fibroblast of MIB1V943F and MIB1R530X LVNC patients. Using CRISPR/Cas9 genomic edition we have reverted these mutations obtaining hiPSC isogenic controls. These hiPSC lines have been differentiated in vitro into cardiomyocytes. V943F and R530X hiPSC-derived cardiomyocytes (hiPSC-CM) showed higher proliferation, lower percentage of multinucleation, and predominant nuclear versus cytosolic cardiac Troponin I (cTNI) compared to wild type hiPSC-CM, indicating that mutant and edited hiPSC-CM remain immature. RNA from V943F and R530X hiPS lines has been collected at different time points of cardiac differentiation to assess a global gene expression analysis that would help us to understand the mechanism by which MIB1 affects the maturation of these cardiomyocytes.

## Creating genetically complex mouse models using embryos with existing modifications

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GEM Lab, Genentech

Embryonic stem (ES) cells enable generation of genetically engineered mouse models (GEMMs). However, the unavailability of robust ES cell lines for specific backgrounds, the lengthy timeline of the ES cell workflow, and the inability to generate distant cis modifications on the same chromosome are a few of the issues that limit the application of the technology. CRISPR technology provides an alternative approach to generating GEMMs that enables efficient targeting of loci of interest for KO and KI models. Although an abundance of publications describe genetic modifications using CRISPR in the wild-type embryo (e.g. B6N), reports using GEMM embryos (harboring one or more modifications) to generate genetically complex models are lacking. Here, we report results for three different types of projects utilizing embryos obtained from extant GEMMs: (1) KO on a BALB/c KO colony background, (2) double KO on a B6N colony background (2 KO genes and 1 KI gene), (3) KO on a B6N colony background (1 KO gene and 1 KI gene). Targeted mice were successfully produced for all three projects.

## Optimization of workflow for the establishment of novel CRISPR/Cas-generated mouse alleles

Ryzhova L

Maine Medical Center Research Institute

CRISPR/Cas methodology has increased our ability to generate novel genomic modifications in the mouse. However, CRISPR/Cas-mediated genome modification can yield relatively complex results, particularly when donor sequences are used for precise gene editing. Issues of mosaicism, frequency of genomic modification, mutational events, or unexpected recombination events can confuse molecular analyses. We developed a systematic approach on how to determine a F0 mouse with desired genomic modifications. In one experiment, we demonstrated how simple routine genotyping techniques in optimum order helped us to identify potential founders and chose the best candidates to establish new mouse strain. We sequentially implemented several different genotyping techniques: routine PCR genotyping, restriction fragment length polymorphism (RFLP), PCR sequencing, and computational analysis including Inference of CRISPR Edits (ICE). The CRISPR/Cas9 mediated C/T replacement was intended in the coding region of *Cryab* mouse gene. Routine PCR genotyping and RFLP identified 25 out of 46 born mice carried a genomic modification. Manual analysis of *abl* traces from these mice identified 6 carriers of desired SNP. ICE analysis identified 16 mice with Knockin (KI) score above 1, or 10 mice with KI score above 5. 3 mice with highest KI score were chosen to breed and establish the colony. 3 mice with KI score above 5 were kept as second line of founders. Simple established methods implemented sequentially enabled us to make informed decisions for identifying correctly targeted founder mice.

## A Streamlined Approach for Generating Conditional Knockout Mice Using Dual CRISPR/Cas9 and Single-Stranded Donor Oligos

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The generation of conditional knockout (cKO) animal models using CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats)-Cas9 remains technically challenging. The requisite need of homology directed repair—a relatively low frequency event, to create a floxed allele, is challenging but surmountable with the use of in vitro validated reagents. Here, we report our efficiencies across 40 cKO mouse models using validated CRISPR/Cas9 gRNAs and single-stranded donor oligonucleotides (ssODNs)—with two CRISPR/Cas9 ribonucleoprotein complexes (RNPs) and two ssODNs. We show that 86% of mouse models with at least one putative founder (positive for the both loxP sites) had germline transmission of a

floxed allele. Importantly, we describe a retargeting strategy for instances of only single site loxP integration in F0s that adds 2 weeks to the overall timeline of cKO animal generation. We show that the combination of high quality, validated reagents, efficiently delivered through electroporation, can achieve a high success rate for the dual CRISPR/Cas9 RNP and ssODN approach.

## How easy is Easi-CRISPR—more of Glee, less of Gloom

Tripti Sharma, Yu Zhang, Hao Zhu

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Crispr-Cas system has proven to be a nifty tool for precise genome editing, especially in generation of knockout and point mutation models. But generation of large insertion mouse models like Cre-ER or Fluorescent protein knock-in have been inefficient with the use of dsDNA repair template. This constraint was overcome with Easi-Crispr (Efficient additions with ssDNA inserts-CRISPR)1,2 using long ssDNA as repair template for homology directed repair. Here, we show the results of implementing this technological advancement in generation of mouse models. Reference: (1) Quadros et al., 2017 (2) Miura et al., 2018.

## Embryo electroporation—'shocking' advances in the generation of mouse models of human disease

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High quality, accurate genetically altered mice are essential for advancing our understanding of human disease and gene function. Prior to the application of the CRISPR/Cas system to genetic manipulation the production of precise, true mouse mutants was; limited, financially costly, time inefficient and required the use of many animals. Electroporation of mouse zygotes offers an attractive alternative to the traditional microinjection technique for gene editing. Electroporation can target a larger number of zygotes within the same time window and with greater simplicity, due to its user-friendly procedure. The procedure requires skills that are more widespread within the scientific community; basic microscopy and embryo handling skills. Electroporation data collated from the Gene Delivery Team show a reduction in physical damage and zygote lysis post CRISPR/Cas9 delivery, with greater viability and consequently a reduction in number of animals used for model generation. Mouse zygote electroporation offers versatility as the technique does not require the visibility of pronuclei, non-accessible zygotes can be targeted; removing this developmental constraint also supports its application with IVF embryos. Electroporation has been successful with producing accurate on target founders at MRC Harwell. Advancing to embryo electroporation supports the 3R's ethos; reducing animal usage, improving the efficiency in which zygotes are used and transgenic zygotes are created for the generation new mouse models.

## AAV Gene Therapy Rescues Hearing in a Mouse Model of SYNE4 Deafness

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The auditory system is responsible for translating sound into neural signals. Mechanosensory hair cells are the receptors of this system, and they are found in the cochlea, a spiral shaped structure in the inner ears of mammals. Damage to hair cells leads to hearing loss, a common and often disabling condition. Genetic variants in SYNE4 are known to cause deafness in humans (DFNB76) in Israel, Turkey and the UK, and in mice, to deafness and degeneration of hair cells in the cochlea. SYNE4 encodes nesprin 4, a component of the linker of nucleoskeleton and cytoskeleton (LINC) complex that connects the cytoskeleton to the nucleus and is responsible for nucleus positioning. We used a synthetic adeno associated virus, named AAV9-PHP.B, to deliver the coding sequence of Syne4 into hair cells of Syne4 knock-out- mice and prevent their degeneration. AAV9-PHP.B transduced hair cells with high efficiency following local delivery into the inner ears of neonatal mice and a single treatment was sufficient to preserve hair cells and prevent hearing loss. Treated mice showed no evidence of toxicity from the treatment. While some mice showed unexplained deterioration of hearing function over time, others maintained wild-type auditory function up to 12 weeks after treatment. Humans with SYNE4 deafness show a postnatal onset of hearing loss with gradual progression, suggesting that a wide therapeutic time window in humans may exist. Our results provide an encouraging proof-of-concept for gene therapy in SYNE4 deafness.

## Evolving the generation by genome editing of complex alleles and their validation

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Mouse models are valuable tools to understand gene function, genetic diseases and to develop and test new therapeutic treatments *in vivo*. The CRISPR/Cas9 system has brought new perspectives for the generation of mouse models in a more efficient and precise fashion, at reduced price, all within a shorter time scale. We are evolving protocols for the production of increasingly complex alleles. Alongside the generation of mutants, their validation represents a new challenge that is

essential to meet to ensure research reproducibility. We will present our recent developments of processes for genome engineering, including a new process for robust identification of positive mosaic founders and validation of models that employs long-read sequencing. We will also discuss standards for validation of new genome edited alleles. Early identification of founders that bear correct complex prevents the production of unnecessary animals through breeding of false-positive founders.

## Multilayer insulation of mouse sperm and immersion in liquid nitrogen for cryopreservation. A novel all-in-one method for pre-freezing packaging, sorting, insulation and cooling rate control

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Cooling rate and cryoprotectant are two critical factors required for success of animal sperm cryopreservation. Currently, animal sperm cryopreservation is performed in two primary ways. Samples are cryopreserved in a simple container (such as a foam box), in which the cooling rate is poorly controlled. Samples are also cryopreserved in a program-controlled freezer, requiring an expensive device. After cooling, the containers need to be packaged inside liquid nitrogen for long term storage. This is dangerous and burdensome, especially when preserving thousands of samples. I developed a novel method (Patent No. US 10,660,327 B2) to overcome the drawbacks of current methods. Sperm is insulated, packaged and sealed in multilayer rigid and/or flexible containers before freezing, and directly immersed into liquid nitrogen to freeze at a controlled ideal cooling rate. The cooling rate is controlled by the thermal conductivity of the walls of the containers, the layer(s) of the walls and the quantity of the containers at each layer. For mouse sperm, a prototype device uses four layers of insulation. (1) Sperm is insulated in 0.25 mL mini straws. (2) 10–12 sealed mini straws are arranged in two layers, insulated and packaged into a (4) 10 mm goblet lined with (3) standard printer paper label, sealed, placed on a cane and immersed into liquid nitrogen. 449 mouse strains have been successfully cryopreserved using this method over 7 years at Yale. When shorter straws (20 mm length) are used, the storage capability increases four-fold compared to standard length straws (133 mm).

## Efficient transport and storage system for genetically engineered mice using refrigeration and cryopreservation of sperm

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Due to the spread of the new coronavirus, breeding staff and researchers have restricted to enter animal facilities and had to reduce the number of laboratory mice. Cryopreservation of sperm and embryos in mouse banks has been effective in backing up valuable mice. However, it did not work in some facilities due to difficulties in transporting living mouse organisms during the corona disaster. On the other hand, in the CARD mouse bank, we have developed a technology to transport cold-stored mouse sperm as an alternative to biological transport. And we were able to cryopreserve the sperm and make fertilized eggs using the sperm in the CARD mouse bank using this technology. In the technique of cold-transport of mouse sperm, sperm preservation solution supplemented with dimethyl sulfoxide (DMSO) and quercetin was used to maintain the fertilization ability of cold-stored sperm for up to 10 days. Furthermore, the DMSO and quercetin concentrations were optimized to allow cryopreservation of cold-stored sperm. In this study, we will report data on the optimal concentrations of DMSO and quercetin for refrigeration and cryopreservation of sperm. In addition, we will show result on the refrigerated transport and cryopreservation of sperm from Japan and abroad using this technology. In summary, these findings are useful as an efficient transport and storage system for genetically engineered mice that can be used in the event of a corona pandemic.

#### **Advanced Technologies and Automation in mES Cell Workflow Increase Speed and Efficiency for Mouse Model Creation in the GEM Lab at Genentech**

**Charles Yu, Roger Caothien, Lucinda Tam, Anna Pham, Merone Roose-Girma**

Genentech

The Genetically Engineered Mouse (GEM) Lab generates many custom mouse models at Genentech. These models may involve complex and multi-allelic mutations, constitutive or conditional knock-outs, humanized or point mutation knock-ins, and random transgenesis. Though CRISPR technology enables fast and efficient generation of knock-out and simple knockin mouse models. Mouse embryonic stem (mES) cell technology is still the preferred route for more complex alleles. By incorporating new technology and automation to our pipeline, the GEM Lab is streamlining processes and increasing efficiency to our mES cell workflow. Automation, such as liquid handling robots and a Biomek i5 high-throughput automated DNA purification workstation, coupled with new technologies such as the QIAcuity multiplex digital PCR system from Qiagen, magnetic activated cell sorting (MACS) feeder cells removal from Miltenyi Biotec and the long-read third generation sequencing platform from Oxford Nanopore Technologies (ONT) platform has enabled the GEM Lab to increase screening throughput, reduce timelines and improve ergonomic issues in the workplace enhancing the reproducibility of genetic screening results, yielding a fully characterized mouse model up to 10 weeks sooner than traditional methods.

#### **High-throughput electroporation of CRISPR/Cas9 components into one-cell zygotes for simplified and efficient production of knockout mice**

**Jade Zhang, Charleen Hunt, Jadine Vallelunga, Michael Kelley, Clarissa Herman, Timothy Hanna, Craig Grant, Jianchun Chen, John Nuara, Heather Brown, Jarrell Wiley, Katie Huling, Christopher Simao, Virginia Hughes, Samer Nuwayhid, Sharon McDowell, Jennifer Schmahl, Suzanne Hartford, Derek White, Lynn Lee, Yajun Tang, Joseph Hickey, William Poueymirou, Marine Prissette, Jean Siao, Nick Gale, Eric Chiao, Brian Zambrowicz, Guochun Gong**

Regeneron

The CRISPR/Cas9 system (CC9) is a powerful tool for the production of genetically modified animals. The delivery of CC9 to one-cell zygotes bypasses standard embryonic stem cell manipulation and microinjection to enable rapid knockout (KO) mouse production. Here, we evaluate electroporation as a high throughput alternative to time-consuming microinjection by a skilled technician. Using *in vitro* and *in vivo* approaches, we test various permutations of KO strategies with respect to (1) concentration and timing of CC9 component delivery, (2) number and placement of single guide RNAs (sgRNAs), and (3) optimal timing of embryo transfer to surrogates. In addition, we characterize the F0 mice resulting from one-cell zygote electroporation to determine the penetrance of expected phenotypes and the extent of mosaicism in tissues. Our results suggest that design and electroporation conditions can be optimized to minimize the production of mosaic animals and may support generation F0 bi-allelic null mice for direct phenotyping. Moreover, we demonstrate that a tail-biopsies may be sufficient to screen for mosaicism and can be predictive of the alleles observed in mouse tissues. We aim to continue the optimization of one-cell zygote electroporations with a goal of establishing a high-throughput *in vivo* KO screening platform to quickly validate drug discovery targets and provide a robust foundation for future technology development.

#### **Sperm fertility in young B6N mice is enhanced *in vitro* by D-Aspartate treatment**

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We previously reported that D-aspartate (D-Asp) administration in drinking water for 2–4 weeks to 7-week-old mice resulted in higher sperm quality, *in vitro* fertilisation (IVF) and



systemic increases of luteinizing hormone and testosterone. We investigated the effects of *in vitro* treatment of cryopreserved-thawed C57BL/6NTacNm (B6N) spermatozoa from males aging 9, 11, 13, and 16 weeks with 4 mM D-Asp on the IVF rate, embryo transfer, and sperm parameters. After treatment of spermatozoa with D-Asp during capacitation, the capacitation rate, IVF ability, and embryo transfer efficiency were analysed. The kinetic activity of the treated spermatozoa and the acrosome reaction were measured after 1 h, 2 h, and 5 h of incubation. D-Asp-treated spermatozoa from 9- and 11-week-old mice led to significantly increased IVF rates than controls (50% vs. 29% and 55% vs. 39%, respectively), in contrast to spermatozoa from 13- and 16-week-old mice (51% vs. 49% and 55% vs. 56%, respectively). No differences were observed in the birth rate (72% to 78%) and kinetic parameters. The capacitation rate and the acrosome reaction were significantly higher with D-Asp-treated spermatozoa from 9-week-old (67.5% vs. 41% and 14.5% vs. 10.5%, respectively) and 11-week-old mice (78.5% vs. 41.1% and 21.0% vs. 3.8%, respectively). Our results demonstrate, for the first time, a direct role of D-Asp in the capacitation process and acrosome reaction. D-Asp treatment of spermatozoa *in vitro* led to significantly higher IVF rates in young compared to older mice. Less mice (21%) can be used to generate embryos without affecting birth rate, thereby contributing to the 3Rs.

#### Customized transgenesis via modification of spermatogonial stem cells

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Spermatogonia mediated transgenesis is becoming an increasingly popular method of genetic modification in animals. It guarantees direct germline transmission because spermatogonial stem cells are the unipotent type of stem cell and can differentiate only into the mature spermatozoa. Additionally, modified spermatogonial stem cells can be clonally expanded. Clone or several clones can be specifically identified for the presence of the desired alteration, expanded *in vitro* and transplanted *in vivo*. Since expression of DAZL (Deleted In Azoospermia Like) gene as well as DAZL promoter driven transgene is restricted to germ cell line, we expected that DAZL promoter-driven transgenic tamoxifen inducible ERT2-CreERT2 (ERT2, mutated ligand-binding domain of estrogen receptor (ER)) can be utilized for the germline-specific controllable Cre mediated recombination of targeted DNA flanked by loxP sites. We conducted a transposon-mediated transformation of spermatogonial stem cells and generation of transgenic rat line, DAZLERT2CreERT2 facilitated by transplantation of modified cells into testis of DAZL deficient recipients-producers. In obtained animal line tamoxifen inducible engineered ERT2CreERT2 recombinase is specifically expressed in germ cells at a level sufficient for liganddependent efficient recombination. Using male germ cells from a generated animal and its cross to GCS-EGFP

transgenic rat, several reporter spermatogonial cell lines were prepared to test the performance of the transgene *in vitro* and *in vivo*. This transgenic rat line can be utilized not only for analysis of the genetic makeup of spermatogenesis but potentially for germline activation and transmission of desirable ERT2CreERT2 mediated rearrangements of tissue-specific genetic elements flanked by loxP sites located in the part of the spermatogonial genome, accessible for the recombinase.

#### SPRINT-CRISPR: S-Phase Pronuclear Injection for Targeting with Large DNA Donor in Mouse Zygotes

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Center for Biosystems Dynamics Research

While understanding of mechanisms underlying targeted gene modifications in zygotes has gradually progressed in recent years, techniques to routinely achieve high efficiencies of large (kb-sized) DNA cassette KI by zygote manipulation are yet to be generalized. In CRISPR/Cas9-mediated KI strategy, DNA insertion occurs thorough DNA repair pathways after Cas9-induced double strand DNA breaks, and distinct DNA repair pathways could engage depending on the cell cycle. However, the optimal timing of pronuclear stage zygotes used for microinjection for large DNA cassette knock-in has not been examined systematically. Here, we focused on the timing of pronuclear injection and established a new method for KI, SPRINT (S-Phase Pronuclear Injection for Targeting) - CRISPR. This method showed that pronuclear injection of homologous recombination-based DNA donors with CRISPR into mouse zygotes during S-phase yielded efficient and accurate large DNA KI at the ROSA26 locus (up to 70%) and other loci (up to 89.3%). We further applied SPRINT-CRISPR method to efficiently obtain biallelic KIs by sequential injection into both maternal and paternal pronuclei. Our results suggest that delivery of genome editing components and donor DNA during S phase is critical for efficient large DNA KI in mouse zygotes.

#### Disruption of the candidate tumour suppressor ING3 in the murine prostate does not lead to malignant transformation.

Viktor Lang, Lisa Barones, Karen Blote, Karl Riabowol, Thomas Rüllicke, Dieter Fink.

Institute for Laboratory Animal Sciences

The inhibitor of growth, family member 3 (ING3) acts as an epigenetic reader and is involved in various cellular functions, such as cell cycle control, cell growth, and apoptosis. Although ING3 has been assigned a tumour suppressor candidate status in many types of cancers, its role in the initiation and progression of prostate cancer remains to be elucidated. The aim of this work was to establish a murine prostate-specific and ubiquitous *Ing3* knockout model based on Cre-mediated excision of a critical exon and assess the recombination efficiency in the prostatic glands at the DNA and protein level. Paternal inheritance of the probasin-Cre was used to generate

the prostate-specific *Ing3* knockout, while maternal inheritance was utilised for global deletion. A duplex probe-based digital PCR assay capable of counting undisrupted *Ing3* copies was designed and a calibration curve with 0% (+/+, wild type), 50% (+/–, heterozygous), and 100% (–/–, homozygous) recombination efficiency was prepared. The impact of DNA recombination at the protein level was investigated by immunohistochemistry. In the prostate-specific knockout, mosaic gene deletion of approximately 25% was detected in the anterior, ventral, and dorsolateral prostate lobes. ING3 staining in the anterior and dorsolateral prostate was weak and no differences in signal intensity between wildtype and knockout animals were detected, suggesting low levels of ING3 protein expression. Altogether, this study reveals that disruption of *Ing3* in the murine prostate does not lead to malignant transformation in prostatic glands and we conclude that *Ing3* is not a tumour suppressor in prostate cancer.

### Finding Rat Models

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Rats have been used in biomedical research for over 150 years. These models arise from naturally occurring mutations, selective breeding and, more recently, direct genome manipulation. The wealth of data produced from rat models is manifested in the recent comprehensive review by Szpirer (2020) where more than 500 references were cited. The Rat Genome Database (RGD), foreseeing the importance of capturing and organizing rat data, started standardizing the rat records and their associated data using controlled vocabularies in 2000. To better represent the use of rat strains in disease research, the RGD curation team has revisited strain curation to update annotations with direct experiment evidences and highlight the established human disease models. Rat strains are curated from published literature and annotated with ontology terms. Several qualifiers are used in conjunction with disease/phenotype terms to specify which aspect of the disease/phenotype is studied in rats. As examples, ‘sexual dimorphism’ indicates that there is a differential response between males and females; ‘treatment’ indicates that a treatment had an effect against the curated disease. An

additional set of weighted qualifiers with the prefix ‘MODEL’ is used to mark strains which are established models of human disease, and when available, their disease resistant controls are also captured using the ‘MODEL: control’ qualifier. These curated strain data are searchable at RGD (<https://rgd.mcw.edu/>) and the specialized tool ‘Find Rat Models’ (<https://rgd.mcw.edu/rgdweb/models/findModels.html>). The tool leverages the improved annotations to facilitate finding strains by diseases, phenotypes, affected genes or conditions used in experiments.

### Validation of CRISPR Reagent Activity in Mouse Cells and Embryos

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We have adopted the strategy of assessing CRISPR reagent activity prior to initiating a mouse production experiment. This validation is particularly important when undertaking a complex genetic alteration, such as those requiring long DNA templates. Current software algorithms to predict CRISPR reagent target activity are a poor predictor of function. Our group has developed procedures to validate CRISPR reagents in the system in which they will be used to generate a gene-edited model: reagents to be used to modify embryonic stem cell (ES cell) models are validated in ES cells, and CRISPR reagents used to generate gene-modified mouse models are validated in mouse embryos. To assess CRISPR activity, electroporated zygotes were cultured to the blastocyst stage and the target regions were PCR-amplified. These PCR products were subjected to Sanger sequencing to detect the presence of insertions and deletions (indels) generated by nonhomologous end joining resolution of the target site double strand break. The activity of a large number of CRISPR reagents in mouse embryos were compared to their activity in mouse E14 ES cells, N2A cells, and mouse fibroblasts. Seven guides were active in all four models tested; one guide was inactive in all four models; and 10 guides differed in their activity between the models. Eight of these guides showed little or no activity in embryos, despite creating indels in cell models. These results demonstrate the need for validating CRISPR guides within the model system you wish to modify, especially when generating gene-edited mice.

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