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Mouse Models in Hematopoietic Stem Cell Gene Therapy and Genome Editing

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Abstract

Gene therapy has become an important treatment option for a variety of hematological diseases. The biggest advances have been made with CAR T cells and many of those studies are now FDA approved as a routine treatment for some hematologic malignancies. Hematopoietic stem cell (HSC) gene therapy is not far behind with treatment approvals granted for beta-hemoglobinopathies and adenosine deaminase severe combined immune deficiency (ADA-SCID), and additional approbations currently being sought. With the current pace of research, the significant investment of biotech companies, and the continuously growing toolbox of viral as well as non-viral gene delivery methods, the development of new *ex vivo* and *in vivo* gene therapy approaches is at an all-time high.

Research in the field of gene therapy has been ongoing for more than 4 decades with big success stories as well as devastating drawbacks along the way. In particular, the damaging effect of uncontrolled viral vector integration observed in the initial gene therapy applications in the 90s led to a more comprehensive upfront safety assessment of treatment strategies. Since the late 90s, an important read-out to comprehensively assess the quality and safety of cell products has come forward with the mouse xenograft model. Here, we review the use of mouse models across the different stages of basic, pre-clinical and translational research towards the clinical application of HSC-mediated gene therapy and editing approaches.

Graphical Abstract

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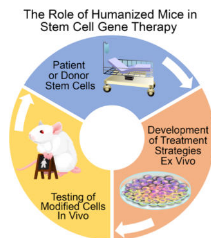

Keywords

Gene Therapy; Hematopoiesis; Stem Cells; Mouse Models; Review

Introduction - The use of mouse models for gene therapy

Development and clinical translation of HSC-mediated gene therapy and editing approaches requires the comprehensive assessment of cell products in order to guarantee quality and safety. This assessment includes detailed analysis of cells regarding 1) their maintenance of multilineage differentiation potentials after *ex vivo* modification and culture, 2) the capability of human hematopoietic stem and progenitor cells (HSPCs) to efficiently engraft into the bone marrow (BM) stem cell niche, and 3) the safety of cell products by longitudinal monitoring for potential side/off-target effects due to the gene modification. All three requirements are nowadays routinely addressed in the mouse xenograft model throughout the different phases of basic, translational, and pre-clinical development of gene therapy approaches (Table 1).

Here we review key literature that involves the mouse model to address fundamental questions on basic stem cell biology as well as genetically-engineered and humanized mouse strains to model *ex vivo* and *in vivo* HSC gene therapy-based strategies. The review has been organized in 4 main chapters. **Chapter 1:** Immunocompromised mice have been incredibly valuable for many gene therapy studies to demonstrate the maintenance of long-term multilineage engraftment potentials, confirm the therapeutic benefits brought by the gene modification, and validate the safety of *ex vivo* generated infusion products. **Chapter 2:** Humanized mouse strains have been developed to overcome limitations of the current models that have entered the field and are expected to replace the "classically" cross-bread strains providing improved multilineage differentiation of human HSCs. **Chapter 3:** A great variety of genetically-engineered murine disease models have also been generated to demonstrate efficacy of treatment and lay the foundation for clinical translation of novel HSC-based therapeutic strategies. **Chapter 4:** Lastly, the engraftment of neonatal mice with human HSCs enables the development of *in vivo* matured human T cells for the evaluation of HSC-mediated immunotherapy strategies as well as gene therapy approaches directly targeting human stem cells *in vivo.*

"Classical" mouse models as a read-out for HSCs

The first use of mouse xenograft models for the assessment of human HSCs started in the 1980s. Research in this field continuously increased over the last four decades as

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comprehensively reviewed by others [1–3]. Here, we focus on some key literature from the last 40 years specifically relevant to the field of HSC gene therapy.

First studies engrafting human fetal liver (FL) $CD34^+$ cells into immunodeficient SCID (severe combined immunodeficiency) mice were performed in the late 80s by McCune et al. demonstrating successful development into functional human T and B cells [4]. However, the level of human engraftment in these SCID mice was low, differentiation restricted to these two lymphoid lineages, and administration of human cytokines required throughout the entire follow-up. A few years later, Shultz *et al.* described the non-obese diabetic (NOD)/ SCID mouse [5]. Lack of an adaptive as well as innate immune system permitted human multilineage engraftment without external administration of cytokines. In the following two decades, several groups continued to improve the mouse model by cross-breeding new strains (e.g. the NSG mouse) to accommodate higher levels of human chimerism, increase the immune-tolerance of the graft, and enhance the support for multilineage differentiation [1, 2].

Availability of the mouse xenograft model triggered the idea to model human gene therapy protocols *in vivo* and pre-evaluate experimental strategies for clinical translation [6]. Particularly with the serious setback encountered by HSC gene therapy in the treatment of X-linked SCID patients in the 90s [7], countless publications utilized various mouse strains to test safety and efficacy of gene therapy approaches in order to avoid more adverse events [8, 9]. Ever since, the NOD/SCID and NSG mouse model developed during this time period became by far the most frequently used strains and widely accepted gold standard read-out to determine the multilineage engraftment potential and safety of candidate human HSCs from different stem cell sources [10, 11] after *ex vivo* expansion [12], undergoing gene-modification [13–15], as well as for ESC (embryonic stem cells)-/IPSC (induced pluripotent stem cell)-derived human HSPCs [16–18].

Attempts to model and improve gene therapy in these early mouse models (here SCID and NOD/SCID) was accompanied by the discovery of new cell surface antigens for the purification of human HSCs. Of special interest for HSC gene therapy, the identification of cell surface marker for human HSCs would allow improved targeting and at the same time potentially reduce unwanted side-effects. In 1992, enrichment of human HSCs with SCID engraftment potential in the $Lin^-CD34^+CD90^+$ subset was reported [19]. Bhatia et al. 5 years later associated the lack of CD38 expression ($CD34^+CD38^-$ cells) with primitive human HSCs capable of multilineage repopulation potential in NOD/SCID mice [20]. Majeti et al. combined previous marker and refined the HSC-enriched subset in umbilical cord blood (UCB) and BM as Lin^- $CD34^+CD38^-CD90^+CD45RA^-$ using newborn NOG mice [21]. Setting the current standard for the identification of highly purified human HSCs, Notta *et al.* reported human HSCs in UCB as lin^- $Rho^{lo}CD38^{low/-}CD34^+CD135^+CD45RA^-CD90^{+/-}CD49f^+$ [22] (Figure 1). Intra-femoral transplantation of only a single cell from this phenotype was sufficient to reconstitute hematopoiesis in sublethally irradiated NSG mice and display multi-lineage chimerism [22].

While the analysis of such complex phenotypes has become the standard for gene-modified cell products in basic and pre-clinical research, isolation and gene-modification of these

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highly-defined subsets in the clinical routine is technically challenging and hard to translate. Due to these mostly technical limitations, most if not all currently available HSC gene therapy approaches still modify CD34^{+} cells, a heterogeneous mix of >99% committed progenitor cells and only very few "true" HSCs with long-term multilineage engraftment potential (Figure 1). As a result, current gene therapy strategies inefficiently target true long-term engrafting HSCs [23–26], are costly [27–29], and may cause unwanted side effects [30–36]. Attempts to reduce the target cell number, improve targeting efficiency, and enhance feasibility are currently ongoing [37]. Approaches with translational potential currently aim to purify HSC-enriched CD34 subset using only one additional cell surface marker. Examples are the CD34^{+}CD38$^{low/-}$ [14, 38], CD34^{+}CD133^{+} [39], or CD34^{+}CD90^{+} [40, 41] cell fractions. The potency of the different subsets identified in these studies relies on mouse xenograft experiments to evaluate stem cell features such as homing, multilineage differentiation, and long-term reconstitution in serial transplants of gene-modified cells.

First attempts to enrich for a phenotypically defined, HSC-enriched CD34-subpopulation for stem cell transplantation in humans date back into the late 1990s. Myeloma, breast cancer, and Non-Hodgkin lymphoma patients received autologous flow-sorted lin^{-}CD34^{+}CD90^{+} or CD34^{+}CD90^{+} cell fractions which are enriched for primitive long-term engrafting HSCs and phenotypically depleted for malignant cells [42–45]. Rapid and sustained hematopoietic recovery was seen in patients with myeloma and breast cancer [43–45]. These initial studies showed that the purification of HSC-enriched CD34-subpopulations for transplantations and consequently for HSC-mediated gene therapy is technically possible and at the same time safe. While the very first purification and gene-modification of an HSC-enriched CD34^{+} subset in SCID patients is currently in a phase-1 trial at Stanford University (trial identifier: NCT02963064), proof-of-concept studies in the NSG mouse [14] and the nonhuman primate (NHP) [46, 47] have already demonstrated improved efficiency and feasibility of HSC gene therapy with this purified CD34 subset. Enrichment of HSCs reduced the target cell number, improved the efficiency of gene-modification in long-term engrafting HSCs, and significantly reduced expenses making HSC gene therapy a more accessible treatment option for patients.

Another hurdle currently limiting the efficiency of gene therapy has been associated with the quiescence and therefore inherent protection of long-term engrafting HSCs from gene modification [48, 49]. Particularly problematic for gene editing approaches, the low activity of the homology-directed repair (HDR) pathway in HSCs due to the quiescent state limits the ability to stably integrate genetic material at precise genomic loci [25, 50, 51]. Current attempts therefore focus on the short-term exposure of HSCs to small molecules or other chemical compounds to temporarily stimulate them, make them permissive to the gene modification, and shortly after either set them back to a primitive state or expand them without exhaustion or differentiation. Reported approaches include the use of compounds such as UM171 [52], PGE2 [14, 53], rapamycin [54], cyclosporine [54], or inhibitors such as i53 to favor HDR after CRISPR/Cas9 cutting [55]. In the majority of studies, assessment of *ex vivo* gene-modified and expanded cells was performed in the NSG mouse model [14, 52, 53] to demonstrate multilineage long-term engraftment of human HSPCs after the exposure of HSCs to these novel compounds.

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“Humanized” mouse models for improved multilineage engraftment of HSCs

Although classically cross-bred mouse strains (NOD/SCID, NSG, etc.) can harbor human B cells, T cells, and granulocytic/myeloid $CD33^+$ cells [56, 57], the maturation of some lineages is only partly supported. Furthermore, the frequency of most lineages is not representative of human blood composition. For example, the frequency of fully mature and functional monocytes and macrophages is typically low [58, 59], long-term erythropoiesis, full erythroid maturation, and megakaryopoiesis barely supported [11, 56, 60], and the function as well as homeostasis of NK cells defective [61, 62]. In addition, detailed discrimination of granulocyte subsets (basophils, eosinophils, neutrophils), monocyte/ macrophage subtypes (M1, M2), detection of human mast cells, or the assessment of dendritic cells (DCs) in mouse xenografts is rarely performed [56, 63].

In an effort to overcome these restrictions and improve the hematopoietic lineage output, various groups have genetically engineered and humanized existing mouse strains to overexpress human cytokines, increase their immune tolerance, and reduce the rejection of human blood cells [64–66]. These novel humanized mouse strains (e.g. NSG-S, NSG-W41, MISTRG) demonstrate significantly improved levels of human cell engraftment in the peripheral blood (PB) and BM, a more realistic lympho-myeloid composition of WBCs, and better development of functional monocyte subsets as well as NK cells [64, 67–69].

Even though these genetically modified mice demonstrate improved support for human engraftment, most research labs still rely on older stains such as NOD/SCID and NSG while not taking advantage of the novel humanized models. To promote this transition, we recently compared the ability of next-generation humanized mouse models regarding their ability to support the BM engraftment of phenotypically and functionally primitive human HSPCs [67]. Comparison of multiple mouse strains showed high levels of human chimerism in the PB as well as HSCs in the BM of NSGW41 and MISTRG mice, whereas HSC exhaustion was observed in NSG mice. Most importantly, MISTRG mice supported the development of lymphoid (B, T, NK cells) as well as myeloid (granulocyte, monocyte) lineages providing an improved multilineage read-out for transplanted human HSCs over the classical mouse strains.

Designed to support improved engraftment of human cells, the MISTRG mouse was further shown to accommodate multilineage engraftment of nonhuman primate (NHP) HSPCs [41]. While NOD/SCID and NSG mice do not support engraftment of NHP HSPCs [41, 70], knocked-in human SIRPA along with other human cytokines in MISTRG mice enabled monocytes, granulocytes, NK cells, B cells, and $CD4^+$ and $CD8^+$ T cells to engraft in the spleen, BM, thymus, and PB. Most importantly, only $CD34^+CD90^+CD45RA^-$ NHP HSPCs were capable of engrafting, consistent with our recent findings using autologous NHP transplantation [46, 47]. Availability and similarity of this monkey-mouse xenograft model with the autologous transplant setting in the NHP transplantation and gene therapy model is closing a gap in between basic and translational stem cell research. Virtually all HSC gene therapy studies currently evaluated in the NHP can be pre-tested in this new ‘monkeynized’ MISTRG mouse, hopefully enhancing the thorough testing of new gene therapy approaches.

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The use of these next-generation mouse strains will likely gain traction with the currently growing field of *in vivo* gene therapy, where HSPC transduction takes place in situ, thus bypassing the need to purify and manipulate cells ex vivo. Robust levels of BM CD34 HSPCs in MISTRG mice and the generation of more complete and mature human hematopoiesis makes this model highly attractive for the modelling of *in vivo* HSC gene therapy. Proof-of-concept studies in C57BL/6 mice stably overexpressing the human CD46 receptor have shown successful mobilization of BM-resident HSPCs into the PB to make them accessible to the modifying vehicle that is ideally delivered intravenously. GCSF/ AMD3100 efficiently mobilized the murine HSCs into PB to enable *in vivo* transduction with an adenoviral vector targeting the CD46 transgenic murine HSCs [71]. This approach has also been more recently used in the context of beta-thalassemia where *in vivo* delivery of the hemoglobin transgene via adenoviral vectors resulted in a near complete phenotypic correction of the disorder [72].

Despite the advantages we highlighted of these new mouse strains, their implementation in pre-clinical and translational research is not guaranteed and may take significantly longer since this field requires more time to adapt new tools replacing old standards. Until then and due to the ease of use and availability, NOD/SCID and NSG mice will very likely remain the *in vivo* model of choice for many researchers.

HSC-mediated gene-therapy in the mouse xenograft model

After facing an initial setback in the 90s, the field of stem cell gene therapy incorporated more stringent regulations and additional safety features in its pre-clinical development. The mouse xenograft model has been instrumental to establish new guidelines and assess engraftment of gene-modified HSPCs. In addition, transgenic mouse models were created to recapitulate human disorders by the knockout of the disease-causing genes. These murine disease models have proved extremely valuable to demonstrate correction by gene therapies employing viral vectors or more recently by gene-editing-based technologies. As a result, major milestones have been reached with several of these approaches now being approved for use in patients. Below, we provide a few examples of studies that built upon the mouse model for pre-clinical testing of new therapies specifically focusing on the treatment of primary immunodeficiencies, hematopoietic and hemoglobin disorders.

Stem cell gene therapy for primary immunodeficiencies.

Primary immunodeficiencies (PIDs) define rare monogenic disorders that cause a severe impairment in the development of a normal immune system, resulting in immune dysregulation, autoimmunity and susceptibility to opportunistic infections. PIDs include a large number of distinct genetic disorders [73], which are estimated to occur only in 1:10,000 birth [74] but can be fatal in infants, particularly in SCID. The introduction of newborn screening [75] and advances in gene therapy, now allow for earlier detection and treatment to improve the prognosis of these diseases.

The first clinical trials of stem cell gene therapy for PIDs began in the late 1990s with adenosine deaminase (ADA)-SCID patients using gamma-retroviral vectors [76]. This clinical trial was based on comprehensive upfront studies demonstrating successful

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engraftment of gene-modified human T lymphocytes in immunocompromised BNX mice [77]. Despite the limitations of this mouse model at that time, the researchers were able to demonstrate successful restoration of enzyme activity, full maturation, and long-term engraftment of functional human T cells from ADA-SCID patients in the spleen and PB of xenotransplanted mice [78]. To further improve safety, lentiviral vectors for ADA-SCID were later generated and tested *in vivo* in ADA-deficient (−/−) mice. Since ADA−/− mice die perinatally, further genetic engineering was necessary to restore ADA expression in trophoblast cells, to prolong survival [79]. The resulting mice retained many features associated with ADA deficiency in humans, including a combined immunodeficiency, severe pulmonary insufficiency, as well as bone and kidney abnormalities, leading to postnatal death within the first 3 weeks [80, 81]. This model allowed the validation of ADA activity restoration leading to normal immune function after *ex vivo* transduction and transplantation of BM cells modified with an ADA-encoding lentiviral vector.

Gamma-retroviral vectors were also used initially for the treatment of X-linked SCID (X-SCID), a disease caused by deficiency of the common gamma chain (γc), also known as interleukin 2 receptor subunit gamma or *IL-2RG,* resulting in a failure of both cellular and humoral immune responses. As discussed earlier, gamma-retroviral vector gene therapy demonstrated clear clinical benefits but also resulted in leukemogenesis with monoclonal blast expansion due to the activation of a proto-oncogene following viral vector integration [82, 83]. Safer vectors were subsequently generated and tested including self-inactivating (SIN) gamma retroviral and lentiviral vectors. For X-SCID, SIN lentiviral vectors were optimized and tested by transduction and transplantation of BM cells in the X-SCID (γC−/−; Rag2−/−) mouse model to evaluate reconstitution of a functional immune system [84]. While restoration of the disease phenotype was established in this model, its relevance to assess vector safety is however limited since it lacks the sensitivity necessary to measure vector-mediated oncogenesis. Tumor-prone mouse models generated by knocking out of *Cdkn2a,* a major regulator of cell proliferation, senescence and apoptosis, could alternatively be used with the drawback that they show high background rate of tumor formation independent of insertion events [85, 86]. More recently, the NSG model was employed to assess CRISPR/Cas9-based gene correction strategies of $CD34^+$ HSPCs obtained from multiple human donors carrying different types of X-SCID-causing mutations [87]. Beyond verifying the adequate engraftment of HSPCs edited by the HDR repair pathway for correction of the mutations, this model was also useful to demonstrate rescue of lymphopoiesis and thus validated this novel and precise gene correction strategy as treatment of X-SCID. In an alternative approach to assess safety and efficacy of gene editing based treatment for X-SCID, the Naldini group developed a new X-SCID mouse model derived from NSG mice by substituting the murine *Il2rg* locus with the human *IL2RG* counterpart that contained a disease-causing mutation [88]. These animals showed comparable immunophenotypical and histological phenotypes with NSG mice and enabled the validation of gene editing strategies that are directly translatable to the correction of human HSPCs. Notably, in this study, mouse HSPCs that were corrected for the human *IL2RG* gene rescued the mouse X-SCID defect indicating cross reactivity of the human γc chain function with other subunits of the mouse pathway.

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Similar to ADA-SCID and X-SCID, gammaretroviral vectors were initially used and shown to be effective in 10 patients suffering from Wiskott-Aldrich syndrome (WAS), a primary immunodeficiency characterized by eczema, thrombocytopenia, infections, and a high risk of developing autoimmunity and cancer. However, long-term follow up studies showed expansion of clones with insertions in proto-oncogenes, some of which progressed to leukemias [89]. SIN vectors were subsequently generated for WAS and safety was assessed in primary transplantation experiments in WASP-deficient mice (BL6-*was*^{null}) and also in secondary transplantation using a different background, the 129-*was*^{null} mouse model, which has a shorter lifespan due to colitis exacerbation [90]. Additional preclinical data was also later generated from engraftment studies of lentiviral vector-modified human $CD34^+$ from healthy and WAS patients in sublethally irradiated $Rag2^{-/-}/\gamma c^{-/-}$ neonate mice generated in the BALB/c background [91], which demonstrated a safe and polyclonal distribution of vector integration profile.

Hematopoietic stem cell gene therapy for hematological disorders.

Fanconi anemia (FA) is a hereditary disease characterized by cellular hypersensitivity to DNA crosslinking agents, resulting in BM failure and aplastic anemia during early childhood. Since more than half of FA patients demonstrate nucleotide mutations in the *FANCA* gene, therapies that deliver a corrected copy of the *FANCA* cDNA to HSCs have been developed and tested in a *FANCA* knockout model developed by Noll et al. [92], which was generated in both the $129S_4$ and C57/BL syngeneic background. This model recapitulates certain phenotypes of the human disease, such as sensitivity to genotoxic agents that cause DNA double-stranded cross-links such as mitomycin C, a potent DNA-damaging agent used to assess functionality of the DNA damage repair pathway. Other phenotypes such as anemia or tumor development have however not been reported in these mice. This model proved instrumental for the validation of viral vectors used for delivery of the corrected gene [93, 94] as well as for the establishment of short transduction protocols that promote engraftment of corrected HSPCs [95]. Complementary to these studies, NSG mice were used to test engraftment of transduced $CD34^+$ cells obtained from Fanconi anemia patients and to determine if the gene therapy could restore *in vivo* repopulating activity as well as mitomycin C resistance in these cells [96]. This work ultimately provided protocols to successfully treat several FA patients using autologous lentiviral gene therapy in HSPCs with no prior conditioning [97].

Hematopoietic stem cell gene therapy for β-thalassemia/sickle cell disease.

Efforts to develop gene therapy treatments for hemoglobinopathies were initiated over 3 decades ago. β-hemoglobinopathies are the most common monogenic disorder worldwide that affect the normal production of adult hemoglobin due to mutations in the β-globin gene. The two most common diseases are β-thalassemia with low or absent β-globin production and sickle cell disease (SCD) with the production of a mutant form of β-globin causing polymerization of globin molecules and sickling of red blood cells. Pioneering work used a gamma-retroviral vector with an intact copy of the β-globin gene that was validated in transduction and transplantation of mouse BM cells [98]. The subsequent use of the newly discovered locus control region (LCR) for expression of the β-globin transgene [99] in conjunction with the development of safer lentiviral vector platforms helped overcome many

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of the initial limitations of retroviral vectors. Consequently, the first cell gene therapy trial in humans was performed in the early 2000s with results made public in 2010 [100].

This long journey towards clinical translation would have not been possible without decades of research in the mouse model. A number of models have been created over the years to closely recapitulate the human disease phenotype, which have been the subject of a recent review [101]. Transgenic methodologies permitted the introduction of the entire human β-globin locus while replacing the murine counterpart to mimic human globin gene expression. SCD models including the so-called Berkeley, Birmingham or San Francisco models were constructed and exhibited faithful sickle cell pathology. Fully humanized β-thalassemia strains were produced with different degrees of β-thalassemia intermedia named *th1, th2* and *th3.* The latest model involving deletion of both the β^{major} and β^{minor} genes was particularly useful for the validation of lentiviral vectors used in the first human clinical trial. Transduction and primary/secondary transplantation of gene-modified BM cells in syngeneic, lethally irradiated C57BL/6 Hbbth3−/+ mice rescued anemia, abnormal red cell morphology and splenomegaly that characterize these animals [102]. Rescue of the disease phenotypes was also confirmed in homozygote Hbbth3−/−/th3 animals suffering from severe thalassemia [103]. Recently, non-viral gene editing strategies for the correction of the underlying disease-causing mutation or for the reactivation of fetal hemoglobin have been investigated in mouse models. In the majority of studies, NSG or NBSGW animals were employed to assess engraftment of HSPCs engineered using a strategy aimed at the correction of the SCD mutation [51, 104, 105], at inactivating the repressor of fetal hemoglobin BCL11A [106, 107], or at recapitulating mutations associated with hereditary persistence of hemoglobin [108]. In particular, Wu and colleagues demonstrated the engraftment and in vivo persistence of $CD34^+$ HSPCs obtained from SCD patients and corrected for the mutation using CRISPR/Cas9-mediated HDR [105]. Together, these studies served as launching platform for several clinical trials such as CTX001, which started to enroll patients suffering from SCD and β-thalassemia in February 2019.

HSC-mediated gene therapy in humanized mice

While most HSC-mediated gene therapy protocols successfully transplant gene-modified cell products into adult mice to assess multilineage engraftment and safety, immunotherapy-centered strategies frequently face severe graft-versus-host disease (GvHD) symptoms in the existing mouse models due to HLA incompatibility between infused donor T cells and recipient cells. Maturation of either human T cells from gene-modified HSPCs or the direct infusion of human CAR T cells into immunocompromised and conditioned adult mice often results in GvHD-mediated death anywhere in between 14 to 60 days limiting the ability of this model to follow the cells long-term or even test their response to their target [109, 110].

The idea to mature human T cells in mice and increase tolerance in the host originates in the 1980s with McCune et al. surgically transplanting human fetal liver and thymus tissue fragments into SCID mice (hu-SCID). The human tissue supports the engraftment of human fetal liver HSPCs and the generation of functional T cells [4]. While this early model has been successfully used in multiple studies with the primary focus on HIV [111], hu-SCID mice lack the support for the development of normal adaptive immune responses of human T

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cells *in vivo.* To solve this problem, Lan et al. performed identical human tissue transplants in NOD/SCID mice demonstrating engraftment of a fully functional human immune system [112]. So-called BLT (BM, liver, thymus) mice and their derivatives were successfully used in countless studies predominantly associated with viral infections [111, 113–115]. However, BLT mice still developed GvHD symptoms and generation of this model is labor-intensive. A recent report replacing human tissues with biomaterial-based scaffolds, so called BM cryogel (BMC), was able to mitigate GvHD symptoms, enhanced the seeding of the murine thymus, and promoted a greater T cell repertoire diversity in the murine model [116].

Another promising strategy to circumvent limitations of classical mouse strains is to engraft human HSPCs into the fetal liver of neonatal NSG mice within the first 3 days post-birth. Human HSPCs home together with murine HSCs into the BM, human T cells develop and mature with constant exposure to the foreign environment, thus building tolerance and reducing GvHD symptoms [117]. Making this animal model especially attractive to HSC-mediated immunotherapy approaches, human HSPCs can be gene-modified before transplantation and HSC-derived CAR-T cells are stably produced throughout the lifetime of the animal inducing tolerance without causing unspecific and deadly side-effects. Once engrafted, these mice can be followed long-term, challenged with target cells (e.g. tumor cell lines, primary cancerous tissue) to verify efficacy. Mimicking autologous immunotherapy approaches *in vivo* matured CAR-T cells can even be transplanted into other syngeneic mice engrafted with human HSPCs from the same donor [117].

HSC-mediated immunotherapy approaches are currently far less frequently performed than regular T cell-based strategies and in most cases performed *ex vivo* [118, 119]. In comparison to T cell-based strategies, successful engraftment of gene-modified HSCs can provide a potentially life-long supply of T cell receptor (TCR)- or CAR-modified T lymphocytes. Similar to previously discussed gene therapy approaches, pre-evaluation of gene-modified HSPCs and the successful generation of engineered T cells is commonly analyzed in BLT [120, 121] and NSG mice [122]. Humanized mouse strains have not yet entered this field of research either. Instead, classical mouse strains such as the NSG strain are getting modified to achieve an HLA-restricted human immune response of *in vivo* HSC-derived T cell [123]. Other improvements for T cell function include the expression of human cytokines specifically for the development and maturation as well as the modification of the environment to closely mimic either lymphoid tissues or the tumor microenvironment as comprehensively reviewed before [124, 125].

Despite their promising features, genetically humanized mouse strains such as the NSGW41 and MISTRG are only slowly entering the field of HSC-mediated immunotherapy. Forward-looking, the field of immunotherapy is evolving significantly faster than other HSC-mediated gene therapy approaches discussed above and pioneering work in this field may actually help to facilitate the adaptation of novel humanized mouse models throughout the field of HSC gene therapy.

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Limitations of the mouse model of HSC gene therapy

Immunocompromised and genetically engineered mouse strains have been extremely instrumental in the field of HSC gene therapy and editing. Easily outcompeting other USDA-covered species due to the ease of use, accessibility, cost, and availability of reagents, the mouse model has manifested its central role in the field. However, every model has its own limitations and not all questions can be addressed in the mouse. General differences in the physiology of humans and mice, incompatibility of several cytokines, and the relatively short lifespan still dampen the use of this model for some applications. For example, the lack or incomplete disease phenotype in some genetically engineered strains precludes the assessment of clinically-relevant levels of gene-modification required for a functional cure. Similarly, the lack of support for certain human lineages (particularly platelets and erythrocytes) in classical as well as more recently developed strains makes the research on hemoglobinopathies and thrombocytopenia less attractive.

However, a less frequently discussed and obvious limitation of the mouse model is actually disease phenotype-independent and associated with the lack of standardization. As a consequence, interpretation and especially comparison of data obtained from different research groups focusing on similar or even identical approaches is getting increasingly complicated due to the enormous variety of experimental parameters. Variables include different mouse strains (SCID, NOD/SCID, NOG, NSG, NSGS, NSGW41 MISTRG, etc.), modes of donor cell injection (in utero, intrahepatic, intravenous, intrafemoral), age of mice (neonatal, adult), human stem cell source (fetal liver, UCB, BM, granulocyte colony-stimulation factor [GCSF]-mobilized PB stem cells), cell dose, purity/composition/ phenotype of the HSPC infusion product ($CD34^+$, $CD34+CD38^{low/-}$ etc.), type/dose of conditioning (total body irradiation, chemical, none), duration of follow-up, mode of PB sampling (orbital sinus, submandibular), frequency of PB draws, engraftment acquisition in PB (staining panel, lineage coverage), tissue harvest for final necropsy (BM, spleen, thymus, liver, gut, lung), and performance of human cells in sequential transplants (secondary, tertiary).

In addition to these experimental parameters, assessment of human engraftment and interpretation of data has significantly changed in the last few decades. Affected by the discovery of novel hematopoietic progenitor cells and the revision of the classical model of human hematopoiesis, multilineage engraftment in transplanted mice seems to be not exclusively limited to HSCs [63, 126]. As demonstrated by Hogan *et al.,* lineage-committed $CD34^+CD38^+$ human HSPCs from UCB show high levels of engraftment giving rise to lymphoid ($CD19^+$) and myeloid ($CD33^+$, $CD13^+$, $CD14^+$) cells in the PB [127]. This CD34 subset is further capable of lympho-myeloid, erythroid ($CD45^-CD71^+CD235a^+$), and $CD34^+$ engraftment in the BM up to 12 weeks post-transplant [127]. Confirming these findings, Majeti *et al.* reported robust myeloid, lymphoid, erythroid and megakaryocytic engraftment of “short-term” repopulating human multipotent progenitors (MPPs) from UCB ($CD34^+CD38^-CD90^-$) with reduced but not fully absent secondary repopulation potential [21]. Furthermore, culture-expanded $CD133^+CD34^+$ HSPCs lacking erythro-megakaryocytic *in vitro* differentiation potential demonstrate robust lympho-myeloid differentiation as well as BM CD34-engraftment in sublethally irradiated NSG mice [128].

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The described engraftment of human progenitor cells in the mouse model is actually contradictory to findings from autologous transplants performed in the nonhuman primates (NHPs), demonstrating that committed progenitor cells are not contributing to the short-term recovery after transplantation for more than a week [46, 47]. While the reason for this discrepancy remains unknown and research in this particular field is lacking, the support of engraftment for committed progenitor cells is particularly beneficial for studying the maturation of lymphoid and myeloid lineages that are otherwise complicated to generate from culture of human HSPCs *ex vivo*. Providing a less artificial environment, maturation of functional human blood cells is supported to a certain extend despite the incomplete cross-reactivity of several cytokines and signals between both species.

Closing this gap and complementing the features of the murine xenograft model, many gene therapy approaches are tested in large animal models such as the dog, swine, or NHP. Major advantages of large-animal models include the ability to perform autologous/allogeneic transplants with full multilineage support for HSC differentiation, the ability to track long-term engraftment over several years or even decades post-transplant, and an intact immune system for most models. In addition, closer genetic relatedness, physiology, size and HSC biology relative to humans, as well as the cross-reactivity of reagents offer unique opportunities for the translation of experimental and pre-clinical gene therapy protocols into actual treatment strategies and clinical applications [129, 130]. While these large animal models have successfully been used for the development of novel gene therapy strategies for multiple hematological diseases, they do not permit a high throughput, cost-efficient, and timely assessment of new gene therapy approaches. Financial limitations and lower availability of large animal models make researchers favor the mouse model at least for the initial screening of conditions that will later on advance into more elaborate testing within these models.

Outlook

Mouse models will very likely remain the primary and most frequently used *in vivo* read-out for the development of HSC-mediated gene therapy approaches. The ease of use, low cost, widespread availability, and multifaceted aspect of this model offer considerable advantages as compared to most large-animal models. Far less predictable is the use of specific strains and their future acceptance in the community. Despite the undisputable advantages of some genetically-humanized mouse strains, the transition proves to be surprisingly slow in the field of HSC gene therapy. While cross-breed strains combining various naturally occurring mutations into new strain backgrounds were easily accepted by researchers in the 90s and early 2000s, mutations generated by means of genetic engineering may require more thorough upfront validation.

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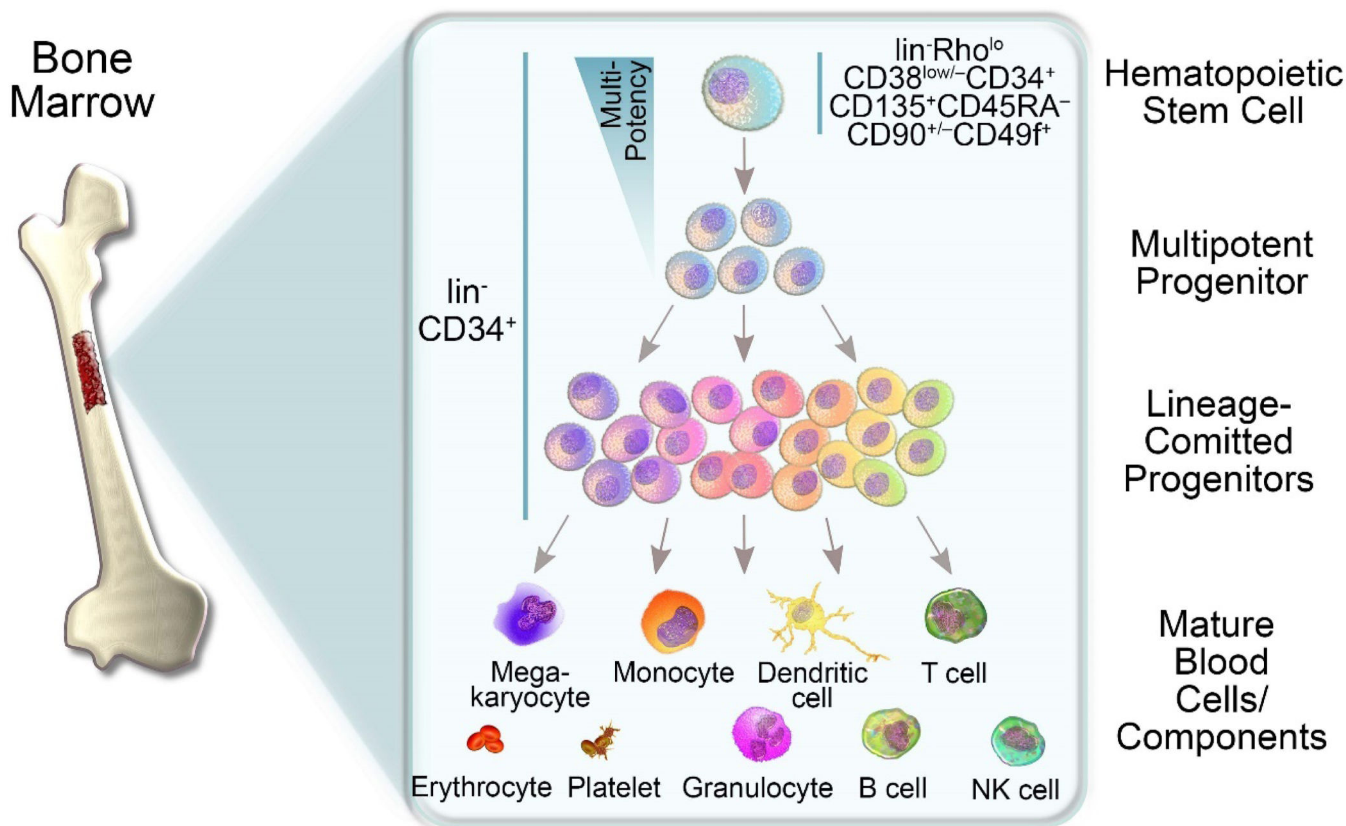

Figure 1: Development of hematopoietic lineages.

The formation of blood cells originates in the bone marrow containing $lin^{-}CD34^{+}$ hematopoietic stem and progenitor cells (HSPCs) with the most primitive human hematopoietic stem cells (HSCs) enriched in the $lin^{-}Rho^{lo}CD38^{low/-}$ $CD34^{+}CD135^{+}$ $CD45RA^{-}CD90^{+/-}CD49f^{+}$ phenotype [22]. HSCs gradually lose their multipotency giving rise to multipotent progenitors (MPPs) followed by a variety of lineage committed progenitor cells and ultimately mature blood cells.

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Table 1:

Overview of mouse models and examples of their use in gene therapy

Mouse model	Genotype	Field of research
BNX	NIH-Beige-Nude-XID	Adenosine deaminase (ADA)- severe combined immunodeficiencies (SCID)
C57BL/6	B6(Cg)-Tyrc-2J/J	Human HSC research, Immunotherapy
NBSGW	NOD.Cg-Kit^{W-41J} Tyr^{+} $Prkdc^{scid}$ $Il2rg^{tm1Wji}$/ThomJ	β-thalassaemia/sickle cell disease
NOD/SCID	NOD.CB17- $Prkdc^{scid}$/J	Human HSC research
NOG	NOD/SCID/IL-2Rγ-nuN	Human HSC research
NSG	(NOD.Cg-B2mtm1Unc Prkdcscid Il2rgtm1Wjl/SzJ)	Human HSC research X-linked SCID (X-SCID) β-thalassaemia/sickle cell disease Fanconi anemia (FA)
SCID	B6.CB17-$Prkdc^{scid}$/SzJ	Human HSC research
Humanized strains		
BLT	NOD/SCID with human bone marrow, liver, thymus	Immunotherapy
MISTRG	C;129S4-$Rag2^{tm1.1Flv}$ $Csf1^{tm1(CSF1)Flv}$$Csf2/Il3^{tm1.1(CSF2,IL3)Flv}$ $Thpo^{tm1.1(TPO)Flv}$ $Il2rg^{tm1.1Flv}$Tg(SIRPA)1Flv/J	Human HSC research NHP HSC research
NSG-S	NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$ Tg(CMV-IL3, CSF2, KITLG)1Eav/MloySzJ	Human HSC research
NSG-W41	NOD.Cg-Kit^{W-41J} $Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/WaskJ	Human HSC research
Hu-SCID	Humanized with fetal liver and thymus tissue fragments	Human HSC research Immunotherapy
Disease-specific models		
FANCA knockout	129S4 and C57/BL	Fanconi anemia (FA)
ADA knockout	NIH-Beige-Nude-XID ADA−/−	Adenosine deaminase (ADA)- severe combined immunodeficiencies (SCID)
HBB deficient	C57BL/6 Hbbth3−/+	β-thalassaemia/sickle cell disease
WASP knockout	BL6-was^{null}	Wiskott-Aldrich syndrome (WAS)
X-SCID knockout	gC−/−;Rag2−/−	X-linked SCID (X-SCID)