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Humanized Mice as a Model to Study Human Hematopoietic Stem Cell Transplantation

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Hematopoietic stem cell (HSC) transplantation has the potential to treat a variety of human diseases, including genetic deficiencies, immune disorders, and to restore immunity following cancer treatment. However, there are several obstacles that prevent effective HSC transplantation in humans. These include finding a matched donor, having a sufficient number of cells for the transplant, and the potency of the cells in the transplant. Ethical issues prevent effective research in humans that could provide insight into ways to overcome these obstacles. Highly immunodeficient mice can be transplanted with human HSCs and this process is accompanied by HSC homing to the murine bone marrow. This is followed by stem cell expansion, multilineage hematopoiesis, long-term engraftment, and functional human antibody and cellular immune responses. As such, humanized mice serve as a model for human HSC transplantation. A variety of conditions have been analyzed for their impact on HSC transplantation to produce humanized mice, including the type and source of cells used in the transplant, the number of cells transplanted, the expansion of cells with various protocols, and the route of introduction of cells into the mouse. In this review, we summarize what has been learned about HSC transplantation using humanized mice as a recipient model and we comment on how these models may be useful to future preclinical research to determine more effective ways to expand HSCs and to determine their repopulating potential in vivo.

Human hematopoietic stem cell transplantation (HSCT) is used to treat a variety of human diseases, including genetic disorders that affect the immune system, rescue following irradiation or chemoablation as a cancer treatment, autoimmune disorders, and chronic infectious diseases [1]. Gene therapies are also currently under evaluation in conjunction with cellular therapies, thus greatly expanding the diseases that could be potentially treated by HSCT. Human HSCs used for transplantation can be obtained from several sources, including umbilical cord blood (UCB), mobilized peripheral blood (MPB), or direct extraction from bone marrow. Advantages of the various sources of HSCs are reviewed elsewhere [2]. Although UCB is the most readily available source of HSCs, these samples typically have insufficient numbers of HSCs for successful transplantation in adult humans [3] although they may still be useful for pediatric patients.

HSCT in humans has a relatively high mortality rate, which varies depending upon several factors such as the severity of the disease being treated, similarity of donor cells to the recipient, and the carrier status of the donor and recipient for pathogens such as human herpesviruses. Rejection of transplanted cells and graft versus host disease (GVHD) are common outcomes when the major histocompatibility complex (MHC) types differ between the donor and recipient. The availability of experimental models to evaluate these various parameters can provide insight into how to perform HSCT with minimal risk to patients.

Immunodeficient mice can be engrafted with various types of human cells to produce what are referred to as "humanized mice." Current humanized mouse models are excellent recipients for human HSCT because they exhibit high rates of HSC engraftment and multilineage hematopoiesis, migration of HSCs and their progeny cells to lymphoid and nonlymphoid tissues occurs, and functional human innate and adaptive immune responses are detected in vivo. It is useful to understand the history of how current humanized mouse models were developed to better understand improvements that are still needed or those that are currently under development.

The original humanized mouse models were introduced in 1988 using severe combined immunodeficiency (SCID) mice [4] or *bg/nu/xid* mice [5]. [*Many references are made to mouse strains in this review. See Table 1 for technical names of these strains.*] SCID mice are unable to produce B or T lymphocytes due to a gene mutation that prevents DNA rearrangement steps required to generate the genes encoding B- and T-cell receptors. However, these mice do go on to produce a limited repertoire of mature B and T cells as they age [6], and thus, different/additional genes involved in lymphocyte development are now commonly targeted. *Bg/nu/xid* mice lack the ability to produce a thymus due to the nude

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Table 1. Mouse Strains Commonly Used to Produce Humanized Mice

Common name	*Technical name*	*Comments*
SCID or CB17-*scid*	CB17-*Prkdc*^{scid}	
NOD/SCID	NOD.CB17-*Prkdc*^{scid}	
NOD/SCIDγc$^{-/-}$ or NOG	NOD.Shi.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug}	Truncation of γc receptor
NOD/SCIDγc$^{-/-}$ or NSG	NOD.Cg-*Prkdc*^{scid}*Il2*^{tm1Wjl}	Entire deletion of γc receptor
Balb/c-Rag2$^{-/-}$γc$^{-/-}$	C.129-*Rag2*^{tm1Fwa}*Il2rg*^{tm1Sug}	See references 38 and 39 for additional similar strains
Rag1$^{-/-}$γc$^{-/-}$	C.129-*Rag1*^{tm1Mom}*Il2rg*^{tm1Wjl}	

mutation, have a reduced number of natural killer (NK) cells due to the beige mutation, and also harbor the *scid* mutation to prevent lymphocyte maturation [7]. One original humanized mouse model (pioneered by Mosier) uses human peripheral blood leukocytes (PBLs) and is usually referred to as the SCID-hu-PBL model [8]. PBL populations contain very low levels of human HSCs and thus that model was not useful to study human HSC engraftment or multilineage hematopoiesis [9]. A second model uses human fetal thymic and liver tissues, which are transplanted under the kidney capsule to produce a thymic organoid. This model was developed by McCune and is referred to as the SCID-hu *thy/liv* model [10]. A third model used human HSCs originally obtained from unpurified bone marrow and was developed by Dick (human/immune-deficient or HID mice) [5]. Of the original models, the SCID-hu *thy/liv* and HID models resulted in transplantation of a significant number of human HSCs. Although HSCT can be accomplished in the SCID-hu *thy/liv* model, HSC homing to the bone marrow cannot be studied. Human T cells are the major product of hematopoiesis in these mice and these cells also remain largely restricted to the graft [11]. In the original HID model, human bone marrow was injected directly into the murine bone marrow and thus HSC trafficking was largely unnecessary [12].

As our understanding of HSCs has grown, new humanized mouse models have been sought that can recapitulate the human immune system more faithfully. A groundbreaking study was published in 2004 when Traggiai et al. [13] showed that highly immunodeficient Rag2$^{-/-}$γc$^{-/-}$ mice (C.129-Rag2^{tm1Fwa}Il2rg^{tm1Sug} strain) can be engrafted intrahepatically with human HSCs (CD34^{+} cells) isolated from UCB. They demonstrated multilineage hematopoiesis, a broad distribution of human immune cells, and functional human antibody and CD8^{+} T-cell responses [13]. Many other studies have been published using similar protocols, and a wide variety of human hematopoietic cell types have been detected in these models, including strong production of B and T lymphocytes, monocytes/macrophages, dendritic cells, and typically a weak production of granulocytes, erythrocytes, and platelets [13–17]. The precursor cells for production of granulocytes, erythrocytes, and platelets are detectable in the bone marrow of humanized mice, but murine macrophages appear to prevent the proper development of erythrocytes and platelets as evidenced by increased detection following murine macrophage depletion [18,19]. These human cells are in many cases widely dispersed throughout the lymphoid organs (bone marrow, thymus, lymph nodes, and spleen) as well as other organs (brain, lungs, gut mucosa, reproductive tracts, etc.) [13,15,20–23].

A broad diversity of humanized mouse models is currently in use. Models differ based upon many variables, but successful engraftment of human HSCs has been detected under many different experimental conditions. These differing conditions include the mouse strain used as a recipient, the conditioning protocol used to prepare mice for transplantation, the source of human HSCs used for engraftment, the phenotypes of HSCs used for engraftment, the culture and/or expansion of HSCs with various cytokines or no culturing at all, the number of cells used for engraftment, the use of fresh or frozen cells, the use of coinjected non-HSC support cells, and the method or site of inoculation of cells into the host. Although the vast array of conditions used to make humanized mice makes it difficult to directly compare the results of these various studies to determine which method is most effective, they also indicate that a wide variety of engraftment protocols can be successfully carried out in immunodeficient mice. Thus, factors that influence the efficacy of HSCT can be compared to discover methods which are more effective and carry fewer risks. The use of well-controlled experiments to compare single variables and their individual effects on the efficacy of HSCT is an area that is still underdeveloped in the humanized mouse field.

The definition of the phenotype of a true HSC is currently not well defined, but nearly always includes the CD34 marker. A review of the capacity of CD34-negative cells to act as HSCs is available [24]. Interestingly, one common way to define HSC populations is actually in terms of their ability to engraft immunodeficient mice; in this case, the HSCs are referred to as SCID repopulating cells. As mentioned previously, Traggiai et al. showed that intrahepatic (i.h.) injection of UCB CD34^{+} cells into Rag2$^{-/-}$γc$^{-/-}$ mice resulted in the development of human B, T, and dendritic cells [13]. Since then, experiments have been performed with various types of cellular populations that revolved around the CD34 marker. Results of these various studies are summarized in Table 2 as a function of the cellular phenotype and the source of HSCs. Notta et al. recently showed that a single purified human HSC is capable of producing detectable engraftment in highly immunodeficient mice, and their work sheds further light on the phenotype of true HSCs [25]. There are several methods of isolating human HSCs to engraft humanized mice, including from UCB, fetal liver, MPB, and from adult human bone marrow. UCB is a common source of HSCs because it is readily available and has a high concentration of HSCs. Mononuclear cells from human UCB are isolated by Ficoll separation and then enriched using CD34^{+}-specific magnetic beads. The cells can either be used immediately for engrafting or they can be cultured. Culturing these cells requires specific cytokines to stimulate growth/expansion without differentiation.

Table 2. Phenotypes and Sources of Hematopoietic Stem Cells Successfully Used to Produce Humanized Mice

Phenotype	*Source of cells*	*References*
$CD34^+$	UCB	[9,38,41,47,48,61,62]
$CD34^+CD7^{++}$	UCB	[47]
$CD34^+CD38^-$ $CD90^+$	UCB	[44]
$CD34^+CD38^-$	UCB	[12,63,64]
$CD34^+CD133^+$	UCB	[43,65]
$CD34^+$	Fetal liver	[11,14,25,32,37,66–69]
$CD34^+CD38^-$	Fetal liver	[70–73]
$CD34^+$	Derived from human embryonic stem cells	[24]
$CD34^+$	Mobilized peripheral blood	[19,20,62]
$CD34^+CD38^-$	Adult bone marrow	[64]
$CD34^+$	Adult bone marrow	[18,62]

UCB, umbilical cord blood.

Fetal liver is another source of the HSCs for engraftment. Tissues are minced and a single-cell suspension is created, then $CD34^+$ cells are isolated as above. These samples are more difficult to obtain, but contain much higher numbers of $CD34^+$ cells as compared to UCB [15]. HSCs can also be obtained by direct extraction from bone marrow, followed by similar methods to obtain the $CD34^+$ fraction [26].

Murine engraftment can also be accomplished using human MPB as a source of cells. A human patient is injected with cytokines such as the granulocyte-colony stimulating factor (G-CSF), which increases the number of circulating HSCs. A blood sample is drawn and leukapheresis is performed. $CD34^+$ cells are then purified out of the sample [27]. It has been reported that 50-fold more cells are required to achieve the same level of mouse engraftment when comparing hMPB cells to UCB cells [28], but it is unclear why these cells require a higher dose.

Human embryonic stem cells (hESCs) or induced pluripotent stem cells can also be used to obtain $CD34^+$ cells for engraftment and multiple types of human blood cells have successfully been produced from these sources [29,30]. One way to do this is to culture the hESCs with irradiated murine cell lines. This coculture allows hESCs to differentiate into HSCs without additional cytokines [31]. These differentiated cells are injected into irradiated mice to produce human immune cell engraftment. Since samples containing primary HSCs can be difficult to obtain due to scarcity, the ability to derive HSCs from a replenishable source is highly desirable. In addition, cells from a replenishable source can be better characterized as compared to those obtained from cord blood or fetal liver where each donor is unique. hESCs can be maintained in their undifferentiated state indefinitely if they are passaged regularly [31] and this suggests their utility as a HSC source. Human HSCs themselves cannot currently be expanded indefinitely in culture without losing their potency for long-term engraftment and multilineage hematopoiesis. Tian et al. demonstrated successful murine engraftment when hESC-derived HSCs were injected into the bone marrow or intravenously [32].

Highly immunodeficient mice are critical for success in the engraftment of human HSCs, and many such mouse strains are currently in use. Common strains include nonobese diabetic/SCID mice (NOD-SCID), NOD-SCID $\gamma c^{-/-}$ (NSG or NOG; see Table 1), $Rag2^{-/-}\gamma c^{-/-}$, $Rag1^{-/-}\gamma c^{-/-}$, among others and have all been used to make humanized mice and study HSC transplantation [15,33–36]. These mutations impair the ability to produce functional T and B lymphocytes (*SCID*, *Rag1*, and *Rag2*) or mature NK cells (γc). γc is the signaling subunit of both the interleukin-2 (IL-2) and IL-15 receptors, thus preventing expansion/maturation of T cells and NK cells, respectively. When the HSC donor and the recipient MHCs do not match, then myeloablative conditioning and use of highly immunodeficient mice are required for effective engraftment [37]. For the above mouse strains, conditioning is always required to achieve human engraftment levels higher than a low fraction (1%–5%) of chimerism in peripheral blood. Waskow et al. [37] created a mouse model that they termed a "universal" HSC recipient because it can accept allogeneic grafts without earlier conditioning. The mouse strain used ($Rag2^{-/-}\gamma c^{-/-}Kit^{W/Wv}$) was generated in a $Rag2^{-/-}\gamma c^{-/-}$ background and additionally has a *Kit* knockout, which prevents sustained self-renewal of HSCs [37]. Excellent reviews of the types of immunodeficient mouse strains currently in use to make humanized mice are available [38–40].

There is another type of humanized mouse model, referred to as the bone marrow, liver, thymus (BLT) mouse, which utilizes these immunodeficient mouse strains to make an effective model of HSC engraftment. In this model, immunodeficient mice (NOD/SCID, NSG, or $Rag2^{-/-}\gamma c^{-/-}$) are sublethally irradiated and the following day 1 mm^3 human fetal liver and thymus tissue fragments are inserted under the kidney capsule of the mouse. These tissues develop into a human thymic organoid. After the thymic organoid develops, the mice are then injected intravenously with autologous human fetal liver-derived $CD34^+$ cells to create the BLT model. This model also shows good engraftment and has the added advantage of the selection of human T cells on human MHC-I-expressing stromal cells in the thymic graft, which leads to better human T-cell responses in vivo [41–43].

HSC homing to the bone marrow in humanized mice occurs rapidly and engraftment can be a long-lasting and stable phenomenon. Human HSC homing to the murine bone marrow has been seen in as little as 20 h following intravenous (i.v.) injection of $CD34^+$ HSCs into NOD/SCID mice [44,45]. Humanized $Rag2^{-/-}\gamma c^{-/-}$ mice injected with human fetal liver $CD34^+$ cells showed evidence of human cell engraftment up to 63 weeks later [46]. Various other studies have shown engraftment lasting for at least 6 months [15,35,47–49]. Takahashi et al. showed that $CD34^+$ cells were present in NOG mice at a statistically constant level over 4 months [45].

The two most common methods for engraftment of human HSCs into immunodeficient mice are i.h. injection and i.v. injection. Other methods of injection include intraperitoneal, intracardial, intrasplenic, or directly into the bone marrow. It is currently unknown if one of these methods is significantly better than the others because of multiple variables across the various studies. However, a comparison of i.h. and i.v. injection showed an insignificant difference in the effectiveness of human HSC engraftment [50]. I.h. injection of cells is commonly used in newborn mice; this method may be effective because HSCs are primarily located in the liver of newborn mice and traffic to the bone marrow within the first

weeks after birth. It is possible that human HSCs respond to the same trafficking signals as murine HSCs, thus explaining the effectiveness of this method. However, i.v. injection is also successfully used in newborn mice, and it is clear that a variety of injection routes are successful in both newborn and adult mice. In the literature, Rag2$^{-/-}\gamma c^{-/-}$ mice are commonly engrafted as newborns [15,50,51], while mice on the NOD/SCID background are commonly engrafted as adults [44,45], although animals with the NOD/SCID background can be engrafted as newborns as well [16,52].

Secondary transplantation can be accomplished in immunodeficient mice, thus providing additional evidence for true HSC engraftment. Human CD34$^+$ cells can be taken from the bone marrow of engrafted mice and serially transplanted to other immunodeficient mice. Eighteen weeks after initial engraftment of NOD/SCID/$\gamma c^{-/-}$ mice, cells obtained from the bone marrow were successfully transplanted into secondary recipients to achieve engraftment. This is another demonstration that human CD34$^+$ cells do home to the engrafted mouse bone marrow [53]. CD34$^+$CD133$^+$ cells have also been proven effective for secondary transplantation and this population appears to also have long-term repopulating HSCs [54]. Furthermore, NSG mice are able to undergo serial transplants of bone marrow with as few as 10 HSCs [55]. Genetically modified human CD34$^+$ cells were also capable of secondary transplantation [56].

One of the problems of engrafting human patients with UCB-derived HSCs is that it usually takes 6 months or more to detect donor-derived immune cells (B and T lymphocytes) in the recipient [53]. A way to induce a quicker recovery and repopulation of immune cells would be valuable in increasing the effectiveness of UCB transplantation. The dose of progenitor cells given in a transplant is correlated with the successful outcome of the graft in humanized mice, with the mice receiving the highest amount of transplanted cells showing detectable levels of HSCs in the peripheral blood just 4 weeks postengraftment [53]. These findings suggest that higher UCB HSC doses may be more effective in terms of the kinetics of reconstitution in humans.

The dose of HSCs required for stable engraftment in humans is not well defined, although 2×10^8 bone marrow cells/kg is considered adequate [2]. Use of at least 3×10^6 CD34$^+$ cells/kg showed a higher efficacy than lower doses [57]. Similarly, the number of HSCs required to achieve engraftment in an immunodeficient mouse is not entirely understood, although a broad range of cell doses has been used. Since a 2–3-day-old mouse (age at time of engraftment) weighs about 0.002 kg, a similar dose of CD34$^+$ HSCs for mice (using the 3×10^6 CD34$^+$ cells/kg amount cited above) would be only 6,000 cells per animal. Whereas a dose this low has not been reported in humanized mice, it is not clear if it has been attempted. Traggiai et al. [13] reported human nucleated cells in the bone marrow and spleen of the mice engrafted with as few as $3.8–12\times10^4$ CD34$^+$ cells from human UCB. The peripheral blood engraftment ranged from ~5% to ~85% at time periods of 4–26 weeks [13]. Human leukocytes have also been found in the thymus of mice engrafted with $2.5–5.0\times10^5$ CD34$^+$CD7^{++} cells and $1.5–2.5\times10^5$ CD34$^+$ cells harvested from human UCB [58]. Lang et al. reported successful engraftment after injecting mice with a range of human CD34$^+$ UCB cells from 5×10^4 to 2×10^6 [50].

Although the current generation of humanized mice is superior in many ways to the original models, there are still improvements in development. One such improvement is in seeking ways to increase the number and/or potency of HSCs available for engraftment. Since UCB samples typically do not contain sufficient cells for human adult HSCT, these findings are highly relevant to methods that can improve human HSCT outcomes. Some mouse humanization protocols call for the CD34$^+$ cells to be expanded in vitro before engraftment, whereas others use the cells for engraftment shortly after purification, without expansion. Expansion of HSCs in vitro increases the number of mice that can be engrafted by increasing the total number of CD34$^+$ cells. Cytokines are commonly used to culture CD34$^+$ cells in an effort to increase the number of HSCs and also to prevent HSC differentiation in vitro and a variety of cytokine cocktails have been shown to be effective [15,41,50,59]. Following are examples that illustrate some specific experimental protocols and their effects on engraftment levels.

In one study, human fetal liver-derived CD34$^+$ cells were cultured for 7 days with stem cell factor (SCF), thrombopoietin (TPO), Flk2/Flk3 ligand, and IL-3. This combination of cytokines for HSC culture led to higher peripheral blood engraftment levels in Rag2$^{-/-}\gamma c^{-/-}$ mice as compared to other cytokine growth cocktails examined [41]. Another group reported that CD34$^+$CD133$^+$ cells purified from UCB and supplemented with fibroblast growth factor 1, SCF, TPO, insulin-like growth factor binding protein 2, angiopoietin-like 5, and heparin were cultured for 10 days and then used to engraft NOD/SCID$\gamma c^{-/-}$ mice. These mice showed ~21-fold increase in SCID repopulating activity as opposed to the untreated cells and showed good reconstitution in both neonates and adults that received the transplant, providing the option of engraftment in older mice. This method of ex vivo expansion minimizes the number of cells needed for injection and thus provides a way to maximize the amount of mice engrafted from a single cord blood sample [54].

When CD34$^+$ cells from UCB were cultured short term (1–8 days) in IL-6, SCF, and Flt3-Ligand, and T cell-depleted CD34$^-$ support cells were engrafted into Rag2$^{-/-}\gamma c^{-/-}$ mice, these samples showed higher levels of human IgM and IgG as compared to mice that received fresh/uncultured CD34$^+$ cells or long-term cultured cells (9–28 days). Human leukocyte levels were significantly higher in peripheral blood as well as lymphoid tissue in mice receiving these short-term cultured cells, indicating that culturing cells short term in the presence of appropriate cytokines and support cells is beneficial to successful humanization of mice [50]. Further, engraftment with autologous T cells promotes more effective B-cell maturation in HSC-humanized mice [60]. It has also been shown that clearing the space for human donor HSCs to populate by eliminating the recipient mouse's own HSCs using ACK2, an antibody that blocks c-kit function, can provide much higher engraftment in treated mice [61].

Sangeetha et al. observed that UCB-derived CD34$^+$ cells show increased levels of apoptosis in vitro when treated with cytokines to promote expansion [59]. Treatment of expanding CD34$^+$ cells with apoptotic inhibitors resulted in increased expansion of the cells. Additionally, higher engraftment levels in mice were detected in animals that received cells treated with apoptotic inhibitors during expansion in vitro. In a recent study, a screen was carried out for novel agents that induce

effective expansion of UCB-derived $CD34^+$ cells followed by successful engraftment into immunodeficient mice. The screen was carried out with SCF and TPO, accompanied by other chemicals. They found that the chemokine CCL28 both enhanced cellular proliferation and decreased rates of apoptosis, and these findings were replicated in the fetal liver and bone marrow-derived cells [62]. Such findings illustrate the utility of humanized mice as a model to study methods to produce larger numbers of potent HSCs.

As mentioned above, culturing cells in the presence of cytokines and chemokines can lead to enhanced engraftment. Several research groups have also shown that supplementing humanized mice with human cytokines in vivo results in enhanced engraftment. Use of a lentiviral vector to stably produce human IL-7 (hIL-7) resulted in enhanced levels of human T cells in humanized mice [63]. Similarly, enhanced levels of hIL-15 resulted in the production of NK cells in humanized mice [17,64,65]; NK cells are very rare without introduction of hIL-15. Administration of other human cytokines leads to enhanced reconstitution of T and B lymphocytes, dendritic cells, erythrocytes, and monocytes/macrophages [17], and improved T- and B-lymphocyte production and dendritic cell maturation lead to better human antibody responses [66].

A recent article demonstrates the utility of humanized mice to study complications associated with HSCT. They showed recapitulation of human GVHD in humanized mice, indicating that this common complication of HSCT can be studied in a mouse system. They also demonstrated that $CD8^{hi}$ regulatory T cells were able to control GVHD by reducing proliferation of alloreactive T cells and by decreasing production of inflammatory cytokines and chemokines [67]. It should be noted that HSCs were not used to engraft these animals. Rather, they used mature human peripheral blood mononuclear cells (PBMCs) for the initial graft, followed by a second graft of allogeneic PBMCs.

Viral infections are common risk factors for complications associated with HSCT, and humanized mice have been shown to support viral replication and associated pathogenesis for a variety of human viruses of blood cells, including the human cytomegalovirus (hCMV) and the Epstein-Barr virus [68–71]. One such study showed that G-CSF treatment of humanized mice latently infected with hCMV induced reactivation of the virus, indicating that the use of G-CSF to mobilize HSCs from humans may also reactivate the virus and potentially lead to hCMV-associated disease in donors and/or recipients [68]. However, relatively few studies have been performed to examine complications associated with HSCT, and this area warrants further investigation in humanized mouse models.

In summary, humanized mice are a useful tool to perform preclinical studies aimed at increasing our understanding of the mechanisms of HSC expansion, homing, and engraftment. These models can be effectively engrafted with human HSCs under a large variety of experimental parameters, and have proven to be a useful preclinical testing ground for the repopulating potential of human HSCs expanded by novel methodologies. Whereas strides have been made to discover the phenotype of a true human HSC and new techniques to expand human HSCs without differentiation are being reported regularly, there is still much to learn in these areas. We expect that HSCT engraftment of immunodeficient mice will continue to be an important tool as we seek to improve the efficacy of HSCT in humans.

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