Immune-deficient mouse models for analysis of human stem cells

Todd E. Meyerrose, Phillip Herrbrich, David A. Hess, and Jan A. Nolte

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The field of murine models of xenotransplantation has grown immensely over the past two decades. The explosive growth in this field is in part due to the fact that good in vitro methods do not exist yet to allow examination of human stem cell homing into the bone marrow compartment versus other tissues, long-term survival of human stem cells, or differentiation into tissues outside of the hematopoietic system. Since these important aspects of human stem cell biology can be examined in vivo using immune-deficient mice, the number of different strains and models is constantly increasing. The current review discusses the merits and drawbacks of each immune-deficient mouse xenograft system as it stands to date and reviews how each immune-deficient mouse model has been used to further our knowledge of human hematopoietic stem cell biology.

SEVERE COMBINED IMMUNODEFICIENT MICE

Excellent large animal models exist for accepting human stem cell xenografts in utero (1–6) and for studying engraftment of nonhuman primate (7–16) and canine stem cells (17–19). However, the use of the immune-deficient mouse as a recipient of the human stem cell graft is the most common and cost-effective strategy available to most laboratories. One of the first strains of immune-deficient mice to be used as a recipient of human hematopoietic cells was the severe combined immunodeficient (SCID) strain (20,21). SCID mice have defects in T and B cell development due to a mutation in the gene for DNA-dependent protein kinase (DNA PK) on chromosome 16 (22). The DNA PK gene is required for successful rearrangement of T cell receptor (TCR) and immunoglobulin gene segments, and without it, murine T and B lymphocytes cannot develop. The SCID mutation also confers extreme radiosensitivity, due to the inability of affected cells to mediate repair of double-strand breaks (22–26). A drawback to the use of SCID mice is that, in spite of their lack of T and B cells, they do have high natural killer (NK) cell levels. Therefore, this strain is acceptable for implantation of solid tissues, such as fetal thymus or sections of tumor, but single-cell suspensions can be rapidly recognized and lysed by the murine NK cells.

The hu-PBL-SCID xenograft model was created by transplanting human peripheral blood into SCID mice (21,27–29). In spite of the high NK cell levels in SCID mice, a portion of the human cells did engraft. However, the human cells that had undergone prior thymic selection in the human do-

nor became profoundly anergic in the mice (27). Human T lymphocytes recultured from hu-PBL-SCID mice could not respond to antigen stimulation for as long as 15–40 days after removal from the mice (27). Tary-Lehman et al. proposed that in the hu-PBL-SCID model, the continuous stimulation of antimouse-reactive human T lymphocytes leads to the exhaustion of the response, resulting in peripheral tolerance or anergy (28,29). The induction of anergy may also be due to the fact that murine β -2 microglobulin (β 2M), associated with the type 1 major histocompatibility complex, cannot be recognized as a stimulatory signal by human T cells (30–32). Thus, the human T cells may exist in a state of partial activation resulting in anergy in the mice, when supporting human thymic tissue and antigen-presenting cells are not present.

To allow studies of functional immunity, an excellent model, the SCID/hu mouse, was then developed by implanting fragments of human fetal liver and fetal thymus under the renal capsule of SCID mice (33,34). The fetal liver acts as a source of hematopoietic stem cells that mature in the human thymic fragments. In contrast to the hu-PBL-SCID system, SCID/hu mice have continued production of human T lymphocytes from stem cells within the human fetal liver (34–36). The human T lymphocytes do not cause graft-vs.-host disease (GVHD), perhaps because they have undergone positive and negative selection in the human thymus and see the murine tissue antigens as “self,” since murine dendritic and other antigen-presenting cells colonize the human thymus. Human CD3⁺ cells recovered from SCID/hu thy/liv mice can be readily stimulated by mitogens or anti-CD3 antibodies, suggesting tolerance in vivo rather than anergy (34).

Adult human CD34⁺ cells can also be infused into the fetal thymus fragment in SCID/hu mice and can subsequently mature to functional T cells (37). The SCID/hu thy/liv system is the best xenograft model of human T cell development that can be used currently to study functional immunity. However, drawbacks to the use of the SCID/hu model are the need to obtain human fetal tissues, which are not available to all laboratories, and the requirement for surgical skill to place the fetal tissues under the murine kidney capsule. In preliminary experiments, the transplantation of postnatal, rather than fetal, thymus sections into the muscles of immune-deficient mice was not as successful (X. Wang, R. Hong, and J. Nolte, unpublished data). In addition, the lack of large numbers of developing human T cells precludes the performance of some types of studies in SCID/hu mice. Therefore, enhanced models for human T lymphocyte generation or engraftment in immune-deficient mice are still sought.

NOD/SCID MICE

An improved xenograft recipient, the nonobese diabetic SCID [(NOD/LtSz-SCID/SCID (NOD/SCID))] mouse, was initially derived from the SCID strain by Shultz and colleagues at The Jackson Laboratory (Bar Harbor, ME, USA). Now the most prevalently used strain of immune-deficient mouse, NOD/SCID mice have an excellent capacity to engraft with human cells due to additional defects in NK cell and macrophage function and circulating complement (38,39). The mice must be kept in a clean barrier facility because of their extreme immune deficiencies but are very easy to breed, with large robust litters. Transplanted human stem cells primarily generate B lymphocytes in the NOD/SCID microenvironment, CD34⁺ cells are retained, and myeloid cells develop to a lesser extent than B lymphocytes. Human T cells are not generated from highly purified stem cells in adult NOD/SCID mice. Mature human T cells can survive in some cases and undergo peripheral expansion, especially if extremely high cell numbers are transplanted (40).

There have been reports that human T cells can be found in the spleen, thymus, and lymph nodes of NOD/SCID mice one month after transplantation with unseparated umbilical cord blood cells (41). These cells may have arisen from peripheral expansion. NOD/SCID mice are an excellent system in which to study human B lymphocyte development, but there are few reports of human T cell *development* from purified stem cells in NOD/SCID mice, under normal transplant conditions. We and others have been unable to achieve T cell development in NOD/SCID mice, using 99.5% pure CD34⁺/CD38⁻ cells from human bone marrow or umbilical cord blood, potentially due to the presence of residual NK cells in NOD/SCID mice (30). Another problem might be that the acellular murine thymic microenvironment could be too atrophied to allow cell seeding and development, by the time that human stem cells are injected into the NOD/SCID recipients. These questions remain to be answered.

Civin's group recently described a model for GVHD using NOD/SCID mice as recipients for mature human T cells (40). Human T cells were recovered, and a GVHD-like disease

was observed only when high cell doses were transplanted (40). In many cases, however, even mature T lymphocytes in transplanted human PBL or cord blood samples can be rapidly cleared from NOD/SCID mice by unknown mechanisms. Leakiness for NK cells appears to be a prime factor in human T cell rejection in xenograft models, particularly the NOD/SCID (42). In their study, the treatment of NOD/SCID mice with an antimurine interleukin-2R beta (IL-2R β) antibody, previously shown to decrease NK cell activity, resulted in functional human T cells recovered from 60% of the treated mice, in contrast to sporadic low levels in the untreated mice (42).

Although human T cells do not develop well following transplantation of purified hematopoietic stem cells into untreated NOD/SCID recipients, interestingly, the fetal thymus organ culture (FTOC) works well when the thymic lobes are harvested from fetal NOD/SCID mice. FTOC is a method to develop T lymphocytes from human stem and progenitor cells, in murine fetal thymic lobes grown at the liquid/air interface. Traditionally, the murine fetal thymic lobes must be depleted of murine thymocytes by extended culture prior to introduction of the human cells. Laure Coulombels' group described a more simplified technique, in which NOD/SCID fetal thymic lobes are used immediately after harvest, due to the absolute lack of murine thymocytes (32). This system has proven to be very reproducible for generating human T lymphocytes from small numbers of highly purified human pluripotent stem cells, which rules out peripheral expansion of mature T cells.

The NOD/SCID strain is by far the most widely used xenograft recipient to date. However, one drawback to the use of NOD/SCID mice is their shortened lifespan (often 4–6 months) due to the presence of a provirus, *Emv-30*, on chromosome 11, that causes thymoma (39,43). The duration of the experiments that can be done to examine human stem cell engraftment using NOD/SCID mice is therefore limited. Some of the mice do live to old age, but others in each arm of the experiment are lost to early thymoma. Therefore, if many mice are initially transplanted per arm of each experiment, it is possible to obtain some long-term survivors for analysis out to 6 months posttransplantation. Many groups also use NOD/SCID mice as recipients for human stem cells for 1–2 months, followed by a secondary transplant into the same strain, for several more months (41,44–46).

The NOD/SCID strain was the first to be used to show that human CD34⁺ cells could have engraftment capacity (47,48). This field has been interesting yet controversial. Osawa et al. initially reported a murine CD34⁺ cell that had full engrafting capacity (49). In the same year, Goodell et al. characterized a primitive "side population" (or SP) cells in mouse, monkey, and man, that excluded the Hoechst 33342 dye, lacked CD34 expression, and had reconstitution capacity (50,51). Bhatia et al. next demonstrated that there was a low level of engraftment and hematopoietic capacity in human CD34⁺ cells injected into NOD/SCID mice (52), and Fujisaka et al. confirmed this finding (53).

In those initial studies, the levels of engrafting CD34⁺ cells were extremely low in the NOD/SCID mice. Nakamura et al. later demonstrated that preculture of the human CD34⁺ cells

with cytokines in vitro prior to transplantation greatly increased their capacity for homing and engraftment (54). Other groups who infused unstimulated human CD34⁺ cells saw little to no engraftment (55). Sato et al. hypothesized that the CD34⁺ stem cells are extremely quiescent, and that some type of activation is required to cause up-regulation of CD34 expression, and to induce the capacity to engraft a recipient mouse (56). In the studies by their group, that activation was promoted by in vivo treatment of donor mice with 5-FU. These studies demonstrated that CD34⁺ cells can generate CD34⁺ cells, and that stimulation of the quiescent CD34⁺ stem cells was necessary to allow engraftment into recipient mice.

More recently, our group used the NOD/SCID strain as secondary recipients in studies of human CD34 reversibility. We initially identified and functionally characterized a population of human CD45⁺/CD34⁺ cells that was recovered from the bone marrow of the long-lived immune-deficient beige/nude/xid (BNX) mice 8–12 months after transplantation of highly purified human bone marrow-derived CD34⁺/CD38⁺ stem/progenitor cells (57–59). The human CD45⁺ cells were devoid of CD34 protein and mRNA when isolated from the mice (60). However, significantly higher numbers of human colony-forming units (CFU) and long-term culture-initiating cells (LTCIC) per engrafted human CD45⁺ cell were recovered from the marrow of BNX mice than from the marrow of human stem cell-engrafted NOD/SCID mice, where 24% of the human graft maintained CD34 expression. In addition to their capacity for extensive in vitro generative capacity, the human CD45⁺/CD34⁺ cells recovered from the BNX bone marrow were determined to have secondary reconstitution capacity and to produce CD34⁺ progeny following retransplantation into the NOD/SCID recipients (60). These studies demonstrate that the human CD34⁺ population can act as a reservoir for generation of CD34⁺ cells, which can, in turn, regenerate CD34⁺ cells. Expression of CD34 was therefore shown to be reversible on reconstituting human hematopoietic stem cells.

In follow-up experiments, it was determined that human CD34⁺/CD38⁺ cells do retain CD34 expression for up to 4 months in BNX recipients. Therefore, it appears that the expression of CD34 may have been lost over time in the mice, perhaps as the human cells in the murine marrow became very quiescent. This data would agree with a seminal report from Makio Ogawa's group, who showed that in murine stem cells, CD34 expression reflects the activation state of hematopoietic stem cells and that this is reversible. Ogawa's group hypothesized that up-regulation of CD34 expression in the murine system is an "activation event" (56). The activation to which Ogawa and colleagues refer is not likely to be simply cell cycle induction, since both murine and human hematopoietic cells can be identified that express high levels of CD34, yet are very highly quiescent (59,61). The molecular nature of the activating factors that regulate induction of CD34 expression in the 5-fluoruracil (5-FU)-treated mice described by Sato et al. (56) remain unknown at the current time. Whether CD34 is up-regulated by an increase in metabolic activity as the quiescent cells progress toward cell cycle by down-regulation of cyclin-dependent kinase (CDK) inhibitors and induction of cyclins and cell cycle regulatory

proteins (62), or whether one or both progeny from the initial divisions of a CD34⁺ cell acquire CD34 expression, is not yet clearly defined. This question is currently a subject of investigation in several laboratories, using both in vitro and immune-deficient mouse xenograft systems. As with many other areas of research requiring engraftment of primitive hematopoietic stem cells, the NOD/SCID mouse is the strain most commonly used in studies of CD34 expression.

NOD/SCID/ β -2 MICROGLOBULIN NULL MICE

NOD/SCID/ β 2M null mice have a more absolute immunodeficiency than the NOD/SCID strain and have virtually no NK cell function (63). They are very permissive for xenograft acceptance (64,65). Committed progenitor populations, as well as primitive stem cells, can engraft in NOD/SCID/ β 2M null mice (63,66,67). Unfortunately, although it is the best and most consistent murine xenograft model, in our experience, the NOD/SCID/ β 2M null strain also has a lifespan that is even shorter than the NOD/SCID strain, due to the early development of thymoma (39,43). The lifespan may be even shorter than in the NOD/SCID parental strain, potentially due to duplication of the lethal provirus to additional chromosomes, or perhaps the more profound NK cell deficiency. However, this strain appears to be permissive for human T cell development, in addition to B and myeloid lineages, and can accept more mature progenitor populations in addition to stem cells, so is well worth working with. Breeding is difficult because the female mice often die from thymoma before they can rear their first batch of pups. To combat the problem of female breeders dying before the first or second litter can be raised to maturity, we and other groups routinely foster pups to clean but immune-competent mothers that consistently live long enough to raise the pups to the weaning stage.

A xenograft assay based on "conditioned newborn" NOD/SCID or NOD/SCID/ β 2M null mice was developed that allows longer term in vivo engraftment from human umbilical cord blood cells than the standard assays, in which mice are transplanted at 6–12 weeks of age (68). Four to five months after transplantation, multilineage engraftment was demonstrated by flow cytometry and by the growth of multilineage colonies in methylcellulose culture. Secondary transplantation of the isolated human CD45⁺ cells, also performed at 4–5 months posttransplantation, showed that human stem cells had durably engrafted the newborn pups, following conditioning by busulfan and cyclophosphamide, but no radiation (68). This method, combined with fostering the newborn and transplanted pups to allow survival of the nursing mother while the pups mature, is an excellent example of modifying experimental strategies to compensate for the limitations of the xenograft model. The NOD/SCID/ β 2M null strain allows the highest and most reproducible engraftment with human cells to date, and is only limited by its short lifespan.

BEIGE/NUDE/XID MICE

The BNX mouse was the immune-deficient strain first

reported to accept a human hematopoietic progenitor xenograft, by Kamel-Reid and Dick in 1988 (69). The BNX mice are triple recessive mutant immune-deficient mice with a 2-year lifespan and deficiencies in T, B, and NK cells. Previous studies had examined human cell lines and solid tumors in nude mice, but since nude mice have high NK cell levels to compensate for their lack of T cells, a transplantation of a single-cell suspension of human hematopoietic cells would be rapidly rejected. Another problem that had prevented the field of human-to-murine xenotransplantation from progressing prior to 1988 had been the lack of cytokines cross-reactive to human stem cells in the mice. Kamel-Reid et al. described the BNX/hu model, in which the immune-deficient mice were injected with human interleukin 3 (IL-3), and human progenitors could survive for several months, with a primarily myeloid differentiative capacity (69).

To avoid costly injections of cytokines every 48 hours as previously described (69), our group co-transplanted human mesenchymal stem cells (MSCs) engineered to secrete human IL-3, in addition to their endogenous human growth factors (70). We had previously referred to the CD45⁺ cell-depleted, expanded, adherent myofibroblastic layer from the marrow as "stroma," but the better term, mesenchymal stem cells, reflects their capacity to differentiate into multiple tissues (71–73). It is also of interest that MSCs have been reported to enhance engraftment of hematopoietic stem cells (70,74–76), and to suppress an immune reaction (77,78). Therefore, in retrospect, the co-transplantation of human mesenchymal stem cells with the hematopoietic stem cells in our BNX/hu xenograft system may have been important for reasons other than IL-3 production, since the BNX mice can be leaky in their B cell deficiency and can also generate T cells through extrathymic production. Yet, in spite of the leakiness of their immune systems, BNX mice have been quite useful as xenograft recipients because they provide one of the few models in which transplanted human hematopoietic cells or tumors can be followed long term (79–83), due to the normal lifespan of the mice, in contrast to the short lifespan of NOD/SCID mice.

BNX mice are useful for detecting loss or alteration of specific lineages or engrafting stem cell populations, following *in vitro* manipulation, due to their normal lifespan. Sustained multilineage human hematopoiesis in BNX mice can be achieved from transplanted CD34⁺ and CD34⁺/CD38[−] progenitors. The presence of human myeloid/erythroid colony-forming progenitors, mature myeloid B cells, and T lymphoid cells can be demonstrated in the marrow of the mice for up to 18 months. Our group capitalized on this extended engraftment capacity to examine methods to optimize transduction and *ex vivo* culture of primitive human stem cells. We demonstrated that engrafting cells are best cultured with the ability to engage integrins on a stromal monolayer (76) or a purified fibronectin (FN) fragment (84), to sustain the capacity for long-term hematopoiesis. In more recent studies, we used the BNX/hu system to show that the engrafting human stem cell pool is maintained on FN and has sustained expression of GATA-2, c-Myb, and CD34, as opposed to cells that have only short-term engraftment capacity following suspension in culture (84a). We used the BNX system to determine

that FLT3 ligand could partially overcome the late graft failure that occurred in human bone marrow-derived hematopoietic stem cells cultured in suspension (85). Our group also used the BNX system to study effects of cytokines on altering lineage production *in vivo* (86,87). Therefore, the BNX/hu xenograft system is a sensitive method to assess the capacity of marrow and umbilical cord blood-derived stem cells to undergo multilineage, long-term differentiation *in vivo*.

Transplantation of human hematopoietic progenitor cells from mobilized peripheral blood mononuclear cells, adult bone marrow, and fetal bone marrow, were done by Turner et al. to evaluate the capacity of each progenitor source to produce multilineage human hematopoietic engraftment in BNX mice (88). Fetal progenitors were found to have the best engraftment capacity, and all lineages of blood cells, including T lymphocytes, were produced from the transplanted stem/progenitor cells in the BNX mice.

Human T lymphocytes develop from transplanted hematopoietic stem cells in the bone marrow and livers of athymic BNX mice. The T cells are of the phenotypes CD3⁺/CD4⁺, CD3⁺/CD8⁺, or "double positive" CD3⁺/CD4⁺/CD8⁺. They all express TCR $\alpha\beta$, and the V β repertoire is severely skewed (86). The levels of CD3⁺/CD8⁺ cells almost always exceed the levels of CD3⁺/CD4⁺ cells (86,87,89). These data are in agreement with that of Mackall et al., who demonstrated that regeneration of murine CD8⁺ cells occurs more readily than CD4⁺ cell regeneration after transplantation in athymic mice (90). Human T lymphocytes that develop by extrathymic mechanisms are normally anergic following recovery from the bone marrow of the BNX mice (87). The cells do not respond well to stimulation *in vitro* after recovery from the marrow. Co-transplantation of human interleukin 7 (huIL-7)-secreting human MSCs in BNX mice significantly increased the levels and activation status of human CD4⁺ cells that regenerated in the bone marrow of athymic BNX mice, to the levels seen in normal human PBL (87).

Our group used the capacity of BNX mice to develop T lymphocytes from transplanted purified human hematopoietic stem cells to document that stem cells capable of generating both T lymphoid and myeloid cells could arise from the same, retroviral vector-transduced hematopoietic stem cell (89). This data was obtained using the inverse PCR method of clonal integration analysis before the more popular NOD/SCID strain, discussed below, was widely available. Now, a more common strategy using the NOD/SCID strain, which generates human B but not T lymphocytes from purified stem cells with normal transplantation methods, is to document production of both B lymphoid and myeloid progenitors arising from the same stem cell (91).

One advantage to the use of BNX mice, in addition to their 2-year lifespan, is that they lack the radiation sensitivity that is a constant problem with the most commonly used xenograft strain, the NOD/SCID mice. NOD/SCID mice cannot repair double-strand DNA breaks due to a mutation in the gene for DNA PK. BNX mice do not have the SCID mutation in DNA PK and recover from sublethal radiation as quickly as normal mice, if kept on low-dose antibiotics in strict barrier conditions. However, BNX mice are difficult to breed, since only females heterozygous at the nude allele can

be used, and their B cell defect is sometimes leaky, so each recipient mouse must be screened prior to use. To avoid these problems, we developed a novel immune-deficient mouse strain, the nude/NOD/SCID mouse.

NUDE/NOD/SCID MICE

As discussed earlier in the review, the NOD/LtSz-SCID/SCID (NOD/SCID) strain (38) is the most common immune-deficient mouse xenograft strain used to date. The mouse is relatively hardy, breeds well, and can be engrafted with human hematopoietic cells to very high levels, with up to 90% of the marrow replaced, if human umbilical cord blood is transplanted. However, a major drawback to the use of the NOD/SCID strain, in addition to their radiation sensitivity, is that their average lifespan is short, due to the presence of the provirus *Emv-30* on chromosome 11. Our group hypothesized that, by bringing two copies of the nude gene (also on chromosome 11) into the NOD/SCID background, the provirus, and thymic tissue where the thymoma arises would be eliminated from homozygous *nu/nu* NOD/SCID mice. We therefore extensively backcrossed nude and NOD/SCID mice over a period of three years, monitoring for immunodeficiency and carrying the nude gene. Nude progeny were backcrossed to NOD/SCID females for seven generations, resulting in a mouse that has the characteristics of NOD/SCID mice, but lacks fur and the life-shortening provirus. The resulting nude/NOD/SCID mice have a 2-year lifespan. Engraftment of human hematopoietic stem cells occurs efficiently in these mice, with levels reaching 82% marrow replacement (91a, 91b), as can be achieved with the NOD/SCID parental strain. As with many of the newer strains of immune-deficient mice, this strain is currently only available from the investigators that developed it (Nolta laboratory).

Nude/NOD/SCID mice do not require systemic human IL-3 to allow survival of the transplanted human cells, as our group and John Dick's group have reported to be necessary for the BNX strain (69,70,89). It is interesting that different cytokine and chemokine levels likely exist in the various strains of immune-deficient mice and can

differentially support development of human hematopoietic lineages. We compared engraftment of human umbilical cord blood CD34⁺ progenitors in three strains of immune-deficient mice (nude/NOD/SCID, NOD/SCID, and BNX) and obtained highly different outcomes in human blood cell lineage survival or development, in support of this theory (Figure 1). The proportion of each hematopoietic lineage that develops from transplanted human hematopoietic stem and progenitor cells in all three strains differs. NOD/SCID mice generate primarily human B lymphocytes from transplanted human CD34⁺ cells, while human cells of all myeloid lineages and B and T lymphocytes develop in the bone marrow of BNX mice co-transplanted with human bone marrow CD34⁺ and CD34⁺/CD38⁻ progenitors and IL-3 producing MSCs, as described above. Nude NOD/SCID mice are athymic. Therefore, as with the BNX mice described above, only extrathymic human T cell development is possible. These extrathymically developed human T cells are abnormal, as we have shown in the BNX strain (91c). Nevertheless, the nude/NOD/SCID is one of the few immune-deficient mouse strains where mature human T cells survive following injection.

In recent studies, we used the nude/NOD/SCID strain in comparison to the NOD/SCID strain to study tracking of adipose-derived mesenchymal stem cells (AMSCs) into various tissues. There was no significant difference in the tissue distribution observed when cells were introduced into either

Figure 1. Lineage development from human CD34⁺ cells in three strains of immune-deficient mice. CD34⁺ progenitors were isolated from human bone marrow and one million cells were transplanted into mice of three different strains after sublethal irradiation. Between 2 and 7 months later, the mice were harvested, and lineage analyses were performed. The percentages of each human blood cell lineage that make up the total human CD45⁺ compartment are shown in the pie chart for each strain. BNX, beige nude xid; NOD/SCID, nonobese diabetic severe combined immunodeficient.

nude/NOD/SCID or NOD/SCID mice via intravenous, intraperitoneal, intramuscular, or subcutaneous routes. This suggests that AMSCs have the ability to navigate either through intravenous or extravascular routes. The nude/NOD/SCID strain allowed a longer follow-up and more reproducible data at day 75 posttransplantation, when many of the NOD/SCID mice had to be removed from the study due to the development of thymoma. We have not yet observed thymoma developing in nude/NOD/SCID mice, out to 2 years of observation. Nude/NOD/SCID mice therefore represent a significant advance over the traditional NOD/SCID in that they have the same engraftment capacities but their extended lifespan will allow long term in vivo xenograft studies that were previously available only in the relatively leaky BNX strain. The combination of favorable aspects of the NOD/SCID background crossed with the nude mouse has resulted in a mouse with a profound immune deficiency but not a shortened lifespan.

NOVEL STRAINS OF IMMUNE-DEFICIENT MICE

Several new strains have been reported recently and show great promise for the xenograft field, although they are not yet widely and commercially available. The newer strains are currently available only from the investigators who have developed them. Shultz et al. generated the NOD/LtSz-Rag-1 null mouse, which lacks the DNA PK mutation in the SCID strain, and is therefore radioresistant. Instead, it has a mutation in the *Rag1* gene, important for lymphocyte gene rearrangement. *Rag1*-deficient mice cannot initiate V(D)J recombination in immunoglobulin and TCR genes, and therefore lack functional T and B lymphocytes. NOD/LtSz-Rag1null mice were also documented to be devoid of mature T or B cells and had low NK cell activity. They supported high levels of engraftment with human hematopoietic stem cells and also allowed lymphoid engraftment. Of interest, transplanted human T cells could be readily infected with human immunodeficiency virus. The mice still did develop lymphoma, like the NOD/SCID strain, but the onset was significantly delayed (92). The NOD/LtSz-Rag1null strain may therefore be suitable as xenograft recipients for experiments in which engraftment with human cells must progress longer than the short duration possible with NOD/SCID mice.

The Rag-2^{-/-}γC^{-/-} mouse combines the Rag-2 knock-out with a null mutation for the common gamma chain receptor, essential for signaling through

the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors, which are important in lymphoid development (93,94). The mice were reported to have a complete absence of all T, B, and NK cell function and to live to a normal lifespan (under barrier conditions), so should allow longer-term experimentation than the NOD/SCID strain (95). Goldman et al. demonstrated that intravenous injection of human B-lymphoblastoid cells resulted in earlier fatal metastatic lymphoproliferative disease than in NOD/SCID controls, possibly due to the fact that the Rag-2^{-/-}γC^{-/-} strain is more defective in NK cell function (93). Levels of engraftment with human lymphocytes were also increased, following injection of peripheral blood. This new strain of immune-deficient mice therefore has advantages over other existing strains for engraftment of human cells, and the ready acceptance of human T cells may be useful for study of adoptive immunotherapy and other studies of the human immune system.

The most recent immune-deficient mouse strain to be developed is the perforin (Pfp)-deficient nonobese diabetic (NOD)-Rag1null (NOD/LtSz-Rag1nullPfpnull) mouse (96). These mice are devoid of an immune system and have an average lifespan of 37 weeks. They support better engraftment by human peripheral blood mononuclear cells and umbilical cord blood cells than the NOD/LtSz-Rag1null controls. Hu-

Figure 2. Tissue localization of human adipose-derived mesenchymal stem cells (MSCs) in NOD/SCID/MPSVII mice. Mice were transplanted intravenously with one million human adipose-derived MSCs following sublethal irradiation. Thirty days later, mice were anesthetized and perfused prior to tissue harvest. Samples of each tissue to be studied were embedded in Tissue-Tek® Optimal Cutting Temperature (OCT) freezing media (Sakura, Torrance, CA, USA) and frozen on liquid nitrogen. Sections were made on a cryotome and stained for β-glucuronidase enzyme activity using naphthol-AS-BI-β-D-glucuronide as the substrate. A brilliant red color was deposited over each donor-derived cell. Slides were then counterstained with methyl green, to allow visualization of the host cells that lack the β-glucuronidase enzyme. An example of a kidney tissue slide stained using this method is shown. In this relatively radioresistant tissue, low MSC seeding efficiency is observed. NOD/SCID/MPSVII, nonobese diabetic severe combined immunodeficient mucopolysaccharidosis type VII.

man engraftment is multilineage and includes progenitors and CD4⁺ T cells. Since the lack of murine NK activity seems to be an important factor in allowing human T cell development in immune-deficient mice, this new strain may be important for studying human immune function in a xenograft setting.

Another recently reported new immune-deficient mouse strain is the NOD/SCID/MPSVII mouse (97), developed to study the *in vivo* localization of xenotransplanted human cells. To create the strain, the β -glucuronidase (GUSB) mutation of the mucopolysaccharidosis type VII (MPSVII) mouse was backcrossed onto the NOD/SCID strain. This new strain allows donor human or murine cells transplanted into the immune-deficient mice to be readily visualized by a bright red substrate stain for the enzyme β -glucuronidase, of which they have normal levels. The mutant mice give no background for this staining, due to their enzyme deficiency. Therefore, any donor cell with normal β -glucuronidase levels can be quickly tracked in tissue slides (or a fluorescence-activated cell sorting-based enzyme assay) without the need for *in situ* hybridization to verify donor status. The donor cells can be counterstained with an immunohistochemical reaction to verify tissue or lineage type, after the enzymatic staining (97). This model will greatly facilitate studies of human and murine stem cell homing, tracking, and differentiation.

Our group recently used sublethally irradiated NOD/SCID/MPSVII immune-deficient mice as recipients for human AMSCs. Figure 2 shows the low efficiency of the AMSC seeding process into kidney, examined 1 month after intravenous transplantation of normal human AMSCs into NOD/SCID/MPSVII mice. The use of this strain of mice allows more rapid quantitation of the cells than was previously possible. In spleen tissue, 3–4 AMSCs were detected per 10 $\times$ field from the spleens of all mice tested. MSCs are known to remain in the spleen and liver for up to 6 months and release the protein products from retroviral vector integrants into the bloodstream (86). Using the rapid tracking system in NOD/SCID/MPSVII mice, it is now possible to obtain seeding efficiencies into different tissues, without the potential artifacts of fluorescence *in situ* hybridization analyses.

NEW DIRECTIONS FOR THE XENOTRANSPLANTATION FIELD

The field of human to mouse xenotransplantation is constantly evolving and improving. There are many aspects of stem cell biology that can only be studied *in vivo*, and therefore the need to make these models better and more physiologically relevant for human stem cells is ongoing. A major current challenge is to develop a xenogeneic host that allows robust human T cell development from stem cells, without the need for implantation of human fetal tissues. This would allow reproducible examination of human immune function in laboratory mice. Another challenge is to extend the lifespan of the most robustly engrafting strain, the NOD/SCID/B2M null mouse, and/or to develop methods that allow better breeding efficiency for this short-lived strain. New strains such as a NOD/RAG/ γ C/WW^v mouse can be envisioned, which might not require damaging radiation to allow human

stem cell engraftment. As mentioned, new strains are being constantly generated, with The Jackson Laboratory at the forefront of the effort.

The new field of “stem cell plasticity” opens an entire new realm of investigation, using xenograft models. Human hematopoietic and MSCs can be transplanted into immune-deficient mice, allowing tissues that develop through transdifferentiation or fusion to be analyzed *in vivo*. Xenograft systems will therefore assist in allowing the signals that regulate stem cell recruitment and subsequent differentiation to eventually be understood. A few of the studies already done have demonstrated highly purified human hematopoietic stem cell differentiation into hepatocyte-like cells, following liver injury (98,99); have shown that rat liver oval cell-derived islet cell-like clusters or total murine bone marrow displayed the ability to reverse hyperglycemia in diabetic NOD-SCID mice (100,101), and also that donor murine marrow can reverse hyperglycemia in the same recipients (101); have demonstrated that human fetal-derived neurosphere cells transplanted into neonatal immunodeficient NOD-SCID mice proliferated, migrated, and differentiated in a site-specific manner (102); and finally, have demonstrated that human multipotent adult progenitor cells can engraft to multiple tissues following transplantation into NOD/SCID mice (103). Therefore, xenotransplantation systems pave the way for future studies using immune-deficient mice to more fully understand mechanisms of homing and differentiation of purified human stem cells of different types and from different tissues to repair injury of not only the hematopoietic system, but potentially also to contribute to the repair of damaged organs.

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Address correspondence to:

Jan A. Nolta
 Washington University School of Medicine
 Department of Internal Medicine, Division of Oncology
 Section of Hematopoietic Development and Malignancy
 660 South Euclid, Box 8007
 St. Louis, MO 63110, USA
 e-mail: jnolta@im.wustl.edu