ARTICLE ADDENDUM

No irradiation required: The future of humanized immune system modeling in murine hosts

Brian E. McIntosh^{a,#} and Matthew E. Brown^{b,#}

^aCovance Laboratories Inc., Madison, WI, USA; ^bDepartment of Surgery, University of Wisconsin – Madison, Madison, WI, USA

ABSTRACT

Immunocompromised mice are an essential tool for human xenotransplantation studies, including human haematopoietic stem cell (HSC) biology research. Over the past 35 years, there have been many advances in the development of these mouse models, offering researchers increasingly sophisticated options for creating clinically relevant mouse-human chimeras. This addendum article will focus on our recent development of the “NSGW” mouse, which, among other beneficial traits, is genetically modified to obviate the need for myeloablative irradiation of the animals. Thus, the complicating haematopoietic, gastrointestinal, and neurological side effects associated with irradiation are avoided and investigators without access to radiation sources are enabled to pursue engraftment studies with human HSCs. We will also discuss the topics of transgenics, knock-ins, and other mutants with an overarching goal of enhancing chimerism in these animal models.

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Introduction

Small animal models are essential tools for studying a wide range of biological processes relevant to humans, including human haematopoietic stem cell (HSC) biology, cancers and lymphopoiesis. In particular, immunocompromised mouse models offer a means of studying these by providing a tractable *in vivo* environment allowing researchers to undertake experiments that would not be possible *in vitro*, in immunocompetent animals or with human subjects. Over the past 35 years, technological achievements in the manipulation of mouse genetics and creative utilization of these animals have allowed researchers to successfully study complex human diseases and biology.

A number of important milestones in the development of murine models have facilitated human xenotransplantation/chimerism research, including studies of human HSC development. Our 2015 Stem Cell Reports article, “Nonirradiated NOD, B6.SCID *Il2ry*^{−/−} *KitW41/W41* (NBSGW) Mice Support Multilineage Engraftment of Human Haematopoietic Cells”,¹ detailed our research conducted at the University of Wisconsin-Madison, which describes the

integration of 4 murine models into one optimized strain—the NBSGW or “NSGW” (JAX Stock# 026622). In this addendum article we will discuss a short background of the previous scientific work that opened the door to the creation of the NSGW, as well as future applications of this novel mouse model.

Development of the NSGW

In the early 1980s researchers working with the CB-17 strain at the Fox Chase Cancer Center discovered a spontaneous mutation in the *protein kinase, DNA activated, catalytic polypeptide* gene (*Prkdc*). The natural occurring mutation yielded a severe combined immunodeficiency (*scid*) phenotype which resulted in significant impairment of B and T cell development within the animals.^{2,3} Subsequent work with irradiated CB-17.*Prkdc*^{*scid*} murine hosts indicated positive engraftment of human hemato/lymphoid cells in this strain.⁴ While a lack of murine B and T cell immunosurveillance in theory bodes well for xenotransplanted cells and tissues, the CB-17.*Prkdc*^{*scid*} still possesses a robust innate immune system correlating with very low observed human chimerism in this model.⁵

A second model was built on advances in mouse strain development. Researchers at the National Institutes of Health crossed the CB-17. *Prkdc^{scid}* with different backgrounds to find a more permissive host. Selecting for mice with the *Prkdc^{scid}* mutation, the non-obese diabetic (NOD)-*scid* (NOD.*Prkdc^{scid}*) mice were shown to support the engraftment of human haematopoietic cells, with irradiation, much better than other tested strains.^{6,7} More recently it became appreciated that the enhanced permissiveness of the NOD background is driven by polymorphisms in the *Signal-regulatory protein α* (*Sirpa*) allele. The transmembrane protein *Sirpa* is one portion of an innate immune activation program residing on splenic macrophages and dendritic cells seeking foreign material, including donor haematopoietic cells.^{8,9} Although the NOD.*Prkdc^{scid}* strain, with its reduced innate function, allows for increased human engraftment with irradiation conditioning, limitations such as short life span and remnants of the host's innate immune system left room for a better model.

The third breakthrough occurred when researchers reported that normal innate and adaptive immune system development is dependent on a functional interleukin 2 (IL2) receptor.^{10,11} When targeted mutations in *Il2 receptor, gamma chain* (*Il2rg*) allele were generated in mice, defects in T-cell, B-cell and natural killer (NK) cell development were observed.¹⁰⁻¹² Researchers crossing the *Prkdc^{scid}* to *Recombination activating gene 1* (*Rag1*) and 2 (*Rag2*) mutants observed a higher level of human chimerism.^{13,14} However, researchers at the Jackson Labs demonstrated that the highest levels of human chimerism were observed when crossing the *Il2rg* mutant with a complete absence of the receptor to the NOD.*Prkdc^{scid}* strain and they subsequently generated the NOD.*Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ* (NSG) strain to capitalize on this positive attribute.¹⁵ The NSG mouse has gone on to become one of the most commonly used immunocompromised mice in scientific research.

Obviating irradiation

A central issue of the NSG strain, and other strains carrying *Il2rg* null alleles, is that they require conditioning (i.e. myeloablation via irradiation) in order to achieve higher levels of human cell engraftment, a property that is vividly illustrated in HSC studies.¹⁶ Although irradiation is shown to enhance hematopoiesis and survival

through increased expression of stem cell factor (SCF), the radiation also acts to destroy actively dividing cells, including some host HSCs, thereby generating a space for the donor graft in the bone marrow niche.¹⁷ Side effects of sublethal irradiation can include severe damage to the hemato-lymphoid system, gastrointestinal, neural and muscle.¹⁸⁻²⁰ In turn, these side effects may ultimately lead to wasting, infection, enhanced graft versus host progression, and premature death of the animals.

The recent development of the NSGW strain by our group and others²¹ allows for robust engraftment potential in the absence of irradiation with its associated side effects. In our paper we demonstrate a high degree of human chimerism and the serial transplantation of injected HSCs, both of which are crucial attributes necessary for HSC development research and other xenotransplantation studies, such as the creation of humanized mouse models.

“Genetic myeloablation” via kit mutation

In short, the NSGW model was developed by adding *Kit^{W41/W41}* alleles to the NSG. *Kit* (i.e., c-Kit, stem cell factor receptor (SCFR)) encodes for a type I membrane protein belonging to the type III tyrosine kinase growth factor receptor family.²² When its ligand, SCF, binds to c-Kit, it induces receptor homodimerization and signal transduction.²³ In the adult and during embryogenesis, SCFR is expressed on haematopoietic, melanocyte, neural, glia, mast and germ cells. To this end, mutations in the phosphorylation sites of the tyrosine kinase motif of SCFR manifest themselves as developmental defects observed in the listed cellular targets.²⁴ For the haematopoietic system, *Kit* is required for normal hematopoiesis with mutations most closely resembling those of aplastic anemias.²⁵ Of the 10-reported white spotting murine *Kit* (*W*) mutants, 4 have been further characterized for homozygous manifestations.²³ Two of the viable homozygous mutants, *Kit^{Wv}* and *Kit^{W41}*, are successful recipients of both isogenic mouse grafts, and human xenografts. However strains possessing *Kit^{W41}* (e.g. NSGW), are superior to *Kit^{Wv}* strains in their longevity and breeding prowess.^{21,26} Thus, mouse strains with mutations in *Kit* provide a competitive advantage to donor cells and are amenable to accepting a HSC graft in the absence of irradiation. The NSGW strain, which is considered to be “genetically-myeloablated”

through the dual *Kit*^{W41} mutations, appears to maintain all of the beneficial features of the NSG strain with additional benefits owing to the lack of the irradiation requirement and is thus ideal for researchers who do not have access to irradiation sources and/or who wish to study hematopoiesis in the absence of the numerous side effects of irradiation.

Future directions

NSGW transgenics: Rebalancing the chimeric immune system

One common theme that persists across all human to murine xenotransplantation studies is the trend of engrafted mice to be lymphoid-biased; i.e. the resultant human immune systems in these animals, while containing all the relevant immune cell types, have a skewed frequencies of those cell types compared to what is typically seen in humans.²⁷ This is observed in a wide variety of studies utilizing differing approaches, cell numbers and cell types engrafted into NSG mice. It can thus be postulated that the mouse microenvironment predisposes human HSCs to a lymphoid B-cell fate.

Investigators have incorporated a multitude of transgenic modifications into immunocompromised animals in an attempt to more accurately mimic the immune repertoire observed in the humans. Of particular interest are animals harboring transgenes encoding for various cytokines. Two animals of interest to this topic are the NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} Tg(PGK1-KITLG*220)441Daw/SzJ (NSG-Tg(hu-mSCF)) and NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ (NSG Tg(Hu-IL-3, GM-CSF, SCF)).^{28,29} The first strain, NSG-Tg(hu-mSCF), combines all the beneficial attributes of the NSG along with expressing an isoform (220 amino acids) of the human membrane-bound SCF. A recent study indicated limited success in xenografting HSCs into non-myeloablated newborn SCF NSG-Tg(hu-mSCF) hosts.³⁰ Although the murine model illustrated rejection of HLA mis-matched skin grafts and an increase in the level of human mast cells, the reported human chimerism was low in this strain, accompanied by large variances. This prompted us to explore whether it is possible to attain human chimerism levels greater than those reported at the time in the absence of myeloablation.

The second transgenic strain, NSG Tg(Hu-IL-3, GM-CSF, SCF) also possesses the positive attributes of

the NSG while also expressing measurable circulating soluble forms of human interleukin 3 (IL-3), colony stimulating factor 2 (CSF2) and SCF. When this strain is xenografted with human HSCs (bone marrow or fetal liver derived), the expression of the soluble human cytokines support enhanced human myelopoiesis, while suppressing human erythropoiesis, and human B-cell lymphopoiesis in mice.³¹ The rebalancing of the human blood cell percentages in the mouse makes this murine model appear to be more tractable, yet the animal requires myeloablation and doesn't generate circulating human red blood cells (RBCs). The model's main benefit, however, is found in its utility in generating better patient-derived xenografts (PDX) as observed with success in evaluation of acute myelogenous leukemia patient samples.^{29,32} Our limited preliminary work intercrossing both the NSGW and the NSG Tg(hu-IL-3, GM-CSF, SCF) and engrafting human cord blood CD34+ cells indicates a rebalancing of the human blood cell percentages toward human-like percentages, in the absence of irradiation conditioning. Further work with this strain will be of interest to both the PDX and red blood cell (RBC) research communities. Further incorporation of beneficial transgenic modifications to the NSGW mouse strain, such as the expression of human cytokine and human leukocyte antigen molecules^{33,34} may result in an animal with the ability to develop a more clinically-relevant human immune system, in addition to the above-mentioned benefits of not having to use irradiation.

Knock-in humanization: Potential path to circulating human RBCs?

Recent work from a variety of labs has culminated in the production of 2 very promising murine strains; MITRG and MISTRG. MITRG mice possess 3 humanized knock-in alleles (*M-CSF*, *IL-3/GM-CSF* and *TPO*) incorporated into the NOD.*Rag*^{-/-} *IL2rg*^{-/-} (NRG) strain.³³ In addition to the knockin alleles of the MITRG, the MISTRG animals possess the additional enhancement of a human SIRPa transgene (hSIRPA^{tg}). When these strains are grafted with HSCs from various sources (bone marrow or fetal liver) the strains exhibit development and function of NK cells as well as cells of the myeloid lineage. The strains are highly permissive; albeit still require irradiation to obtain the most efficient humanization. These strains are of great interest

to biologists as this is the first success in which a strain carrying *Rag*^{-/-} alleles has outcompeted the NSG strain in engraftment.^{35,36} Therefore, it will be of particular interest, once the strains are available to the scientific community, to observe the humanized alleles incorporated into the NSG strain to observe if the NSG allows greater humanization. Given the large number of Glycophorin A+ (erythrocyte marker) cells detected in the chimeric marrow, it will also be of interest to observe if crossing either animal with the NSGW allows for the detection of circulating human RBCs. If accomplished, this would be a significant breakthrough for hematopoiesis research, with the resultant animals having a wide variety of downstream research applications.

Humanized mice with human thymus tissue

As previously described, the NSG strain has a poor thymic microenvironment; i.e., as the mice age to early adulthood the thymus rapidly atrophies. By the time of T cell development in most HSC transplantation experiments the mouse thymus is not typically grossly identifiable. This aspect is even more pronounced in males. [unpublished data, Burlingham Lab] This characteristic also applies to the NSGW strain. While some degree of T cell development is seen in human HSC humanized mice,¹ the T cells in these animals are low in frequency and poorly suited to human studies due to their positive and negative selection on mouse epithelial cells, with mouse MHC molecules, rather than human.

In 2006, the blood-liver-thymus (BLT) mouse was developed, which involved surgically implanting a human fetal thymus and liver fragment under the mouse kidney capsule thereby allowing for proper development and selection of human T cells on human MHC molecules.³⁷ The BLT and a more recent iteration utilizing cryopreserved thymus tissue without an added liver fragment³⁸ have proven beneficial for a wide area of research interests, including transplantation medicine and human immunodeficiency virus (HIV) studies. Mold et al.³⁹ showed that the developmental status of the HSC source in fetal thymus humanized animals impacted the gene expression profile of the resultant T cells. Further research is warranted to determine whether the developmental status of the thymus tissue in these animals contributes to the T cell repertoire phenotype, independent of the HSC source. Additionally, production of thymic

epithelial progenitor cells from pluripotent cells has made possible an attractive additional source of patient-specific thymic epithelial cells with which to create humanized mouse models.⁴⁰ Our lab is currently exploring these above mentioned areas of focus with the NSGW strain. Of additional particular interest is the minimization of graft-vs.-host disease (GVHD) symptoms, which shortens the experimental time window in traditional humanized mouse models.

The future of xenotransplantation studies

It may also be beneficial to combine different human tissues into a mouse model at different time points (based on cellular development and cellular growth dynamics); either those produced *in situ* or *in vitro*. A recent report indicates that immunocompromised *Fumarylacetoacetate hydrolase* (*Fah*) deficient mice are able to accept human primary hepatocytes without rejection.⁴¹ The *Fah* gene produces an enzyme that breaks down the amino acid Tyrosine in the liver. Without proper Tyrosine metabolism, the hepatocytes of the liver suffer toxic insult and die. The *Fah*^{-/-} model is similar to the NSGW, in that the 2 models take advantage of endogenous murine defects, and rescue the animals by engrafting human cells. One could imagine the power of combining the NSGW with the *Fah*^{-/-} and the far-reaching benefit this model could have for science and human health. The ideal hypothetical NSGW *Fah*^{-/-} mouse would possess a humanized haematopoietic system, transplanted thymus, and hepatocytes, all made from *in vitro* produced pluripotent stem cells. A small animal model of this type may allow for a new pre-clinical standard in safety testing of molecules/biologics to treat human diseases, vaccine development to combat hepatotropic pathogens (e.g., malaria, HBV or HCV) or human cellular therapies.

Disclosure of potential conflicts of interest

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