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Review

Immunodeficient Rabbit Models: History, Current Status and Future Perspectives

Jun Song [1], Brooke Pallas [1,2], Dongshan Yang [1], Jifeng Zhang [1], Yash Agarwal [3], Y. Eugene Chen [1], Moses Bility [3,*] and Jie Xu [1,*]

1 Center for Advanced Models and Translational Sciences and Therapeutics, University of Michigan, Ann Arbor, MI 48109, USA; songjun@med.umich.edu (J.S.); bpallas@med.umich.edu (B.P.); doyang@umich.edu (D.Y.); jifengz@umich.edu (J.Z.); echenum@umich.edu (Y.E.C.)

2 Unit for Laboratory Animal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109, USA

3 Department of Infectious Diseases and Microbiology, Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Public Health, Pittsburgh, PA 15261, USA; YAA30@pitt.edu

* Correspondence: mtbility@pitt.edu (M.B.); jiex@med.umich.edu (J.X.)

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Abstract: Production of immunodeficient (ID) models in non-murine animal species had been extremely challenging until the advent of gene-editing tools: first zinc finger nuclease (ZFN), then transcription activator-like effector nuclease (TALEN), and most recently clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR)/Cas9. We and others used those gene-editing tools to develop ID rabbits carrying a loss of function mutation in essential immune genes, such as forkhead box protein N1 (FOXN1), recombination activating gene 1/2 (RAG1/2), and interleukin 2 receptor subunit gamma (IL2RG). Like their mouse counterparts, ID rabbits have profound defects in their immune system and are prone to bacterial and pneumocystis infections without prophylactic antibiotics. In addition to their use as preclinical models for primary immunodeficient diseases, ID rabbits are expected to contribute significantly to regenerative medicine and cancer research, where they serve as recipients for allo- and xeno-grafts, with notable advantages over mouse models, including a longer lifespan and a much larger body size. Here we provide a concise review of the history and current status of the development of ID rabbits, as well as future perspectives of this new member in the animal model family.

Keywords: immunodeficient rabbits; gene editing; animal models

1. The Pre-Gene-Editing Age Where Mice Dominated

The development of germline transmitting mouse embryonic stem cells (mESCs) in the 1980s enabled versatile gene targeting manipulations, especially gene knockout (KO), in this species [1–4]. Many mouse models have been generated by utilizing mESCs. Unfortunately, except for rats, no germline transmitting ESCs have been generated in any other mammalian species despite over two decades of effort. Consequently, mouse models have been and remain the dominant animal model in biomedical research, representing greater than 70% of all mammalian models to date [5].

Immunodeficiencies (IDs), caused by mutations in genes affecting lymphocyte development or function such as interleukin 2 receptor subunit gamma (*IL2RG*) (OMIM# 300400), recombination activating gene 1 (*RAG1*) or recombination activating gene 2 (*RAG2*) (OMIM# 601457), and *PRKDC* (OMIM# 600899), are deadly human diseases in which patients are highly susceptible to bacterial, viral, and fungal infections in early infancy and are subject to opportunistic infections in the lungs and gastrointestinal tracts, due to their impaired immune system [6].

Mouse models carrying loss of function mutations in essential ID-causing genes have been established [7]. In addition to their use as models for primary ID and acquired ID (e.g., AIDS) diseases, ID mice are widely used for allo- and xeno-graft experiments, taking advantage of their compromised immune rejection responses. The following are several generations of ID mouse models [8], each with increasing immunodeficiency:

i. Nude mice (*Foxn1*$^{-/-}$). Forkhead box protein N1 (*Foxn1*) encodes a transcription factor required for both hair follicle and thymic development. KO of *Foxn1* leads to a hairless phenotype and a failure of thymus development;
ii. SCID (severe combined immunodeficiency) mice (*Prkdc*$^{-/-}$) or Rag deficient (*Rag1/2*$^{-/-}$) mice. *Prkdc* encodes a polypeptide that is essential for the repair of double-strand breaks (DSBs) in the DNA; the breaks occur during somatic recombination of T cell receptor (TCR) and immunoglobulin (Ig) genes. The loss of *Prkdc* leads to T and B cell deficiency. *Rag1* and *Rag2* cluster closely in the genome and are critical elements for somatic recombination of TCR and Ig genes. The absence of Rag1 or 2 also results in both T and B cell deficiency.
iii. *Il2rg* deficiency on either the *Prkdc* or *Rag* deficient background. *IL2rg* mediates IL2, IL4, IL7, IL9, and IL15 high affinity signaling, which promotes the development of a myriad of immune cells.
iv. Additionally, the NOD/ShiLtJ strain exhibits deficiency in innate immune response, specifically, NK cells and antigen-presenting cells (i.e., macrophages). Coupling the previously described *Prkdc* or *Rag* mutation along with the *Il2rg* deficiency on a NOD background results in multigenic immunodeficiency, and a severely immunodeficient mouse model. Examples include NOD-*Prkdc*^{scid} *IL2rg*^{Tm1Wjl} (NSG) and NOD-*Rag1/2*^{scid} *IL2rg*^{Tm1Wjl} (NRG) lines.

ID mouse models have made invaluable contributions to basic and translational research in biomedicine. However, as in any other field, "no model is perfect", and ID mouse models have their limitations, specifically due to their relatively short lifespan (1 y) and small size (25 g). Their short lifespan prohibits experiments greater than 1 y, such as those following long term efficacy and safety parameters in regenerative medicine. Their small size makes organ transplantation and other experimental technical manipulations extremely challenging, if not impossible. Prior to the gene editing age, unfortunately, researchers had no other choice but to use what was available (i.e., mouse models) rather than what was clinically relevant.

2. The Gap between Mice and Humans

Nevertheless, it has long been realized that there is a preclinical research gap that is unmet by rodent models [9], including ID mouse models, especially when it comes to long term studies and those that involve sophisticated manipulations.

Interventional studies that require long-term follow-up are not feasible in ID mice. For example, gene therapy offers tremendous hope for a permanent cure for SCID patients. In the late 1990s, gene therapy for X-SCID was conducted using a gamma-retroviral vector [10]. Correction of the disease phenotype and clear clinical benefit proved the principle of using this strategy to treat X-SCID effectively. However, the reported success was tempered by the demonstration of leukemic proliferation in 5 of 20 patients 2.5–5.5 y after the gene therapy [11,12]. In all cases, evidence pointed to the integration of the vector in the vicinity of oncogenes (LMO2 or CCND2) [13]. Such long-term safety risks are undoubtedly impossible to reveal by a mouse model.

Sophisticated manipulations are limited by the small size of anatomical structures and the frailty of ID mice. Vascular bypass grafting, for example, is an effective treatment for ischemic heart disease and peripheral vascular disease. In the US, there are more than 200,000 arterial bypass operations performed annually. However, many patients who require arterial bypass procedures do not have suitable vessels for use. In recent years, patient-specific stem cells (i.e., induced pluripotent stem cell)-derived blood vessels (SDBV) represent a potential solution to this problem. To advance SDBV to clinical applications, robust efficacy and safety data must be collected in preclinical animal models,

where ID animal models are preferred to minimize immune rejections associated with this xeno-graft procedure. One major technical challenge using ID mice for this purpose is the tiny size of their blood vessels, making surgical transplantation very difficult.

3. Gene Editing Tools and the Rise of Non-Murine Immunodeficient Animal Models

The development of efficient gene editing tools eventually enabled the production of ID animal models in non-murine species. The past decade witnessed the advent of three generations of gene editing nucleases: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and, most recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9).

ZFN is the first-generation programmable gene-editing nuclease. In 2009, ZFN was successfully used to achieve efficient gene targeting in mammalian cells [14]. ZFN consists of the zinc finger DNA-binding domains and the DNA cleavage domain of a Type II restriction enzyme Fok1. The zinc finger domains are programmable, and a unique set of zinc fingers can be constructed to target a unique DNA sequence. The DNA cleavage domain Fok1 only works when two Fok1s are paired. Therefore, in a typical ZFN-mediated gene targeting, a pair of ZFNs are designed to flank the target locus such that the two Fok1 domains (one from each ZFN) will meet and induce site-specific double-strand breaks (DSBs) in the DNA, which leads to targeted modification of the genome.

TALENs were first reported in 2011 [15,16]. Like ZFNs, TALENs also work in a pair, where two Fok1s generate DSBs at the target locus in a scissors-like manner. Instead of using zinc fingers, TALENs use TAL DNA binding domains targeting the desired sequence. The TALEN DNA binding domain contains a repeated highly conserved 33–34-amino-acid sequence, except for the 12th and 13th amino acids, which are highly variable (repeat variable diresidue, RVD) and show a strong correlation with specific nucleotide recognition [17,18]. The simple relationship between the amino acid sequence and DNA recognition has made it easy to design and it is relatively practical compared with ZFN technology.

Only two years after TALEN was adopted, the third-generation tool, CRISPR/Cas9, emerged and quickly dominated the field [19,20]. Originally discovered as the bacterial adaptive immune machinery, CRISPR/Cas9 contains two endonuclease domains by itself (i.e., no need to fuse an exogenous nuclease such as Fok1). The most significant difference between CRISPR/Cas9 and its predecessors is that it uses nucleic acid complementation for target recognition. In this way, the tedious protein engineering work to construct sequence-specific zinc fingers or TALs is bypassed. Instead, a complement 20 nucleotide RNA sequence is used, which can be quickly and easily designed and constructed.

These programmable nucleases were quickly adapted in animal model development to bypass the need for germline transmitting ESCs [21]. The technical procedure is similar to that of the pronuclear DNA microinjection, which is used for the production of conventionally transgenic animals. Briefly, targeting elements (ZFN, TALEN, or Cas9) are microinjected into pronuclear stage animal embryos in the format of plasmid DNA, mRNA, or protein. The gene-editing nuclease would generate DSBs in the embryo genome; however, the efficiency is very much sequence-dependent. Many of the offspring animals carry mutations at the target locus, which can be used to establish mutant animal lines through routine herd expansion strategies.

The production of ID non-murine animal models boomed after 2009. Prior to that, only naturally occurring ID horses and dogs were established through selective breeding [22–27]. In the past ten years ID rabbits, rats, pigs, hamsters, and non-human primates have been reported from different laboratories across the world (Tables 1 and 2). Among these animal models, ID rats, pigs, and rabbits represent the majority, consistent with the impact of the corresponding model species in the biomedical research community.

Table 1. Production of immunodeficient (ID) non-murine animal models (except rabbits). The "Method" row indicates the debut year of the method. G: gene targeting refers to the conventional gene editing nuclease-free, homologous recombination-based gene targeting method. M: meganuclease refers to an I-CreI based nuclease.

Year	Pre 2009	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
Metho	B: breeding	Z: ZFN		T: TALEN		C: Cas9							
	G: gene targeting												
	M: meganuclease												
IL2RG													
Dog	B [24,25]												
Rat			Z [28]		Z [29]						C [30]	C [31]	
											T [32]		
Pig					G [33]	Z [34,35]			G [36]				C [37]
									T [38]				
									C [39,40]				
NHP									Z/T [41]				
Rag1													
Rat					Z [42]	M [43]						C [31]	
Pig							G [44]						
							T [45]						
Hamster											C [46]		
Rag2													
Rat								T [47]			T [48]	C [31]	
Pig							T [45,49]		G [36]				
									C [40]				
IgM													
Rat			Z [50]								C [51]		
Pig								C [52]					
Prkdc													
Dog	B [26,27,53]												
Horse	B [22,23]												
Rat					Z [29]						C [30]		
Foxn1													
Rat									C [54]				
Artemis													
Pig						B [55]		B [56]					

IL2RG: interleukin 2 receptor subunit gamma; Rag1: recombination activating gene 1; Rag2: recombination activating gene 2; IgM: immunoglobulin M; Prkdc: protein kinase, DNA-activated, catalytic subunit; Foxn1: forkhead box protein N1. Artemis: a protein coded by the *DCLRE1C* (DNA cross-link repair 1C) gene.

Table 2. Production of immunodeficient rabbits. The "Method" row indicates the debut year of the method. Tg: transgenic, refers to the conventional method used in transgenic animal production, without the use of gene editing nucleases or any other gene targeting methods.

Year	Pre 2009	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
Method	Tg: transgenic	Z: ZFN		T: TALEN		C: Cas9							
VDJ-Cmu	Tg [57]												
IL2RG										C [58]			C [59]
Rag1						T [60]				C [58]			
Rag2						T [60]				C [58]			
IgM				Z [61]									
Prkdc										C [58]			
Foxn1										C [58]			

VDJ-Cmu: a transgene that codes the V, D, J and the constant region of the mu chain of immunoglobulin; IL2RG: interleukin 2 receptor subunit gamma; Rag1: recombination activating gene 1; Rag2: recombination activating gene 2; IgM: immunoglobulin M; Prkdc: protein kinase, DNA-activated, catalytic subunit; Foxn1: forkhead box protein N1.

The five significant immune genes, *Il2rg*, *Rag1/2*, *Prkdc*, and *Foxn1* that are deficient in ID mouse lines, have all been targeted in rats and pigs (Table 1). The phenotypes (i.e., immunodeficiency) of these animals are in line with those observed in ID mice with the same gene knockout. In addition to these five genes, IgM is targeted in rats and pigs, likely part of an effort to destroy the endogenous immunoglobulin system to introduce human immunoglobulin genes in order to humanize these animals for antibody discovery practices. Artemis, a protein coded by the *DCLRE1C* (DNA cross-link repair 1C) gene that plays an essential role in V(D)J recombination, was also targeted to produce Artemis KO pigs [55,56].

There is a clear pattern regarding the specific nuclease that is used for producing ID rats and pigs: ZFN dominated from 2010 to 2012 (Table 1), then was replaced by TALEN from 2013 to 2015, and was replaced by CRISPR/Cas9 from 2016 and thereafter. The first reports of any given nuclease used in ID animal production typically occurred 1 to 2 years later than the original introduction of that nuclease, which is expected considering the time that is required to adopt the technology, to produce the animals, and to publish the paper. Nevertheless, this pattern clearly reflects the research community's preference regarding the type of nuclease used in gene editing work over the past decade.

4. The Birth of Immunodeficient Rabbits

The rabbit (*Oryctolagus Cuniculus*) is a classic laboratory animal model species and is increasingly becoming a translational model of choice for bridging the gap between rodent models and larger animal models [62–71]. The method to produce transgenic rabbits by pronuclear microinjection is well-established and comprehensively described [72].

The significant advantages of rabbit models over ID mouse models are their longer lifespan and larger size. A New Zealand White (NZW) rabbit housed in a specific pathogen-free (SPF) animal facility can live beyond six years, and adult NZW rabbits are similar in weight and size to human infants. In the context of ID, rabbit models would extend studies past one year, such as those required for gene therapy development, and allow sophisticated xenotransplantation procedures. We also note that the rabbit is a classic animal model for the study of immunology [73–75]. They are used for the study of viral [76–78] and bacterial infectious diseases and in particular *S. aureus* infections in the lungs, which is a significant pathogen in human SCID patients [79]; therefore an ID rabbit model may better reflect *S. aureus* infection-related pathologies in immunodeficient patients than do mouse models. Compared to other large animals such as pigs and non-human primates, the rabbit is a more affordable

lab animal species that can be easily housed in most research institutes. In addition, rabbits possess a short gestational period of 30–31 days, and routinely produce relatively large litters of 4 to 12 kits, allowing efficient herd expansion.

Although these features make ID rabbits a promising model to be developed, the production of gene-targeted transgenic (GTT) (e.g., knockout, knock-in) rabbits has been a challenge. Efforts to produce GTT rabbits date back to the 1980s, but unfortunately, all gene targeting attempts failed until ZFN became available in 2009. This is mainly due to the lack of germline ESCs [80], and the extremely low efficiency of somatic cell nuclear transfer (SCNT) in rabbits [81]. ESCs are the primary tool to generate GTT mice, and SCNT has been successfully applied to generate KO animals in species lacking germline transmitting ESCs such as pigs [82–84], sheep [85,86] and cattle [87], but not in rabbits. As a result, no ID rabbits have yet been produced by the ESC or SCNT approach.

Interestingly, the laboratory of Katherine Knight at Loyola University (Chicago, IL, USA), who first established a working protocol to establish rabbit monoclonal antibodies, generated the first ID rabbit line accidentally [57]. They initially worked to produce IgH-transgenic rabbits carrying a productive VDJ-Cμ sequence. Instead of obtaining B-cells producing the immunoglobulins coded by the recombinant VDJ-Cμ, they found that these transgenic rabbits were B-cell deficient, with a 50–100% reduction in serum IgM and IgG levels. Nevertheless, the production of this ID rabbit line was accidental, unexpected, untargeted, and not reproducible.

The first report of ID rabbit production through targeted gene editing, and also the first report of GTT rabbit production in a broader scope, came in 2011, two years after ZFN appeared in the scientific toolbox. Flisikowska and colleagues from the Technical University of Munich, Roache Diagnostics GmBH, and Sangamo BioSciences reported the production of immunoglobulin M (IgM) knockout rabbits by microinjecting ZFN mRNAs to pronuclear stage rabbit embryos [61]. They demonstrated that ZFN efficiently disrupted the target gene, in ex vivo cultured embryos, and ultimately in offspring kits born after embryo transfer. The IgM knockout rates were high, ranging from 23 to 35%, calculated as the number of positive KO founders per born kits. The KO rates were magnitudes higher than those of prior efforts of gene targeting in rabbit cells, which were in the range of < 0.1% to < ~1% (unpublished data and personal communications). They further demonstrated that the ZFN-introduced mutant alleles transmitted to the next generation animals, satisfied the golden standard of a successful GTT method. Lastly, they showed that gene knockout of IgM, as predicted, led to serum IgM and IgG deficiency and lack of IgM+ and IgG+ B lymphocytes in homozygous KO animals.

The success of Flisikowska et al. opened a new possibility in biomedical research, i.e., efficient production of GTT rabbits. The entire research community is no longer bound by the lack of rbESCs to achieve this goal. Nevertheless, it did not take long before the second-generation gene-editing tool TALEN came into play. Compared to ZFN, TALEN has several significant advantages: design and construction are easier, and the efficiency is comparable and often higher.

In 2013 Dr. Liangxue Lai's group in Guangzhou China obtained *Rag1/2* KO rabbits by embryo microinjection of TALENs mRNA, the first (and the only to date) report using TALEN for ID rabbit production [60]. The KO efficiency in founders was exceptionally high, reaching 94% for *Rag1* and 100% for *Rag2*, representing a 3 to 4-fold higher efficiency than those in the ZFN IgM work. The authors found that a high percentage of founder animals carried bi-allelic modifications (61% for *Rag1*; 100% for *Rag2*). As a result, the founder-generation of RAG deficient rabbits displayed typical ID phenotypes with defective T and B cell populations.

Only one year after the first TALEN report, Lai's group used the third-generation tool CRISPR/Cas9 and first produced *Il2rg* knockout rabbits [88]. Again, the KO efficiency reached 100% such that all kits born after microinjection/embryo transfer carried mutant alleles. The authors then demonstrated the multiplex capacity of CRISPR/Cas9 by producing *I2lrg/Rag1* double knockout rabbits in one round of microinjection/embryo transfer. Comparing to TALEN, the CRISPR/Cas9 system is easier to set up, faster, cheaper, and has similar or higher targeting efficiencies. These favorable traits soon made it the dominant tool in ID rabbit production.

In 2017, Xu's group at the University of Michigan produced multiple lines of ID rabbits, including FOXN1, RAG2, IL2RG, and PRKDC knockouts by multiplex sgRNA microinjection and multiplex embryo transfer [58]. In the multiplex sgRNA microinjection approach, different sgRNAs targeting different genes were pooled and microinjected to the same embryo, leading to the production of founder rabbits carrying multiple gene knockouts. One such animal named "NuSRG" had mutations in all these genes: Foxn1 (nude), Prkdc (SCID), Rag2 and Il2Rg. This NuSRG rabbit was hairless and severely immunodeficient. In the multiplex embryo transfer approach, embryos injected with different individual sgRNAs were pooled and transferred to the same recipient, leading to the production of different gene knockout founders in one litter. The authors were able to produce four single-gene knockout founders (i.e., *Foxn1, Rag2, Il2rg*, and *Prkdc* KOs) in one embryo transfer litter. The same group later established *Foxn1, Rag2*, and *Il2rg* KO rabbit lines; all displayed typical ID phenotypes. In a comprehensive characterization report by this group [79], Song et al. showed that IL2RG-null rabbits demonstrated severe combined immunodeficiency, characterized by the absence of pronounced hypoplasia of the thymus and splenic white pulp, and absence of immature and mature T and B-lymphocytes in the peripheral blood. Without prophylactic antibiotics, the ID rabbits universally succumbed to lung infections caused by *Bordetella bronchiseptica* in several animals, but by *Pneumocystis oryctolagi* in all animals. Their findings were recently confirmed by Hashikawa et al. from Osaka University, Japan, where they also produced *Il2rg* KO rabbits by CRISPR/Cas9 and demonstrated that these animals are immunodeficient [59].

5. Where the ID Bunnies (Could) Rule

Although five labs have reported the successful production of ID rabbits (Table 2), the application of this animal model has not been firmly established. The models, which included all significant ID genes that are targeted in mice (i.e., Foxn1, Rag1/2, Prkdc, and Il2rg), are not yet commercially available.

One challenge that is limiting the scale-up of the ID rabbit supply is their housing. There is no economically affordable germ-free caging system for rabbits yet. The team at the University of Michigan has developed a working protocol [89], in which a HEPA filtered, positive pressure, flexible film isolator cleanroom (BioBubble) is used to house ID rabbits. One bioBubble ($10 \times 12 \times 9$ feet) can house up to 18 rabbits. Enhanced personal protective equipment (PPE) and sanitation practices are implemented and necessary to limit environmental contamination. ID rabbits also receive oral prophylactic sulfamethoxazole/trimethoprim antibiotics to reduce bacterial and *Pneumocysis* infections. These practices have proven effective; an ID rabbit colony with a daily census of greater than 30 is routinely housed at the facility. A broad adaption of these practices or similar ones is needed to eventually enable the use of ID rabbits in biomedical research. In addition, a reliable shipping method is yet to be developed to distribute ID rabbits. Alternatively, cryopreserved sperm [90] or embryos [91] can be used to rederive the animals at the destination institutes.

On the consumer end, ID rabbits are expected to help researchers fill the gap between mice and humans in basic and translational research. The larger size and longer lifespan would make immediate contributions. Examples include having a non-rodent ID model for FDA drug development studies, long term testing immunotherapies, increased ease of blood collection, the ability to collect larger tissue samples, and a larger platform for advanced surgical manipulation, and implantation of tissue-engineered constructs and medical devices. Other opportunities will undoubtedly arise, taking advantage of other unique features of rabbits; for example, an ID rabbit reconstituted with human lymphocytes for antibody or T-cell receptor (TCR) discovery. Below we provide our perspective on the future use of ID rabbits and describe how they could "rule", by elaborating upon several specific examples that we consider high priority projects to be developed in the next five years.

5.1. Humanized Rabbits for Infectious Diseases

Several infectious agents are human-specific, and animal models do not recapitulate many human diseases in part due to differences in biological response in major organ systems, such as the immune

system [92,93]. Therefore, animal models that incorporate human organ systems could provide a means to bridge that species gap and enable the evaluation of the safety and efficacy of novel therapeutics [93]. Immunodeficient mouse models support human cell and tissue engraftment and development and are termed, humanized mice [94,95]. Humanized mouse models provide a means for studying human-specific infectious agents and human-specific biological effects. However, the limited lifespan and size of mice create a barrier for their use in preclinical studies [93]. To address these challenges, we are working to develop humanized ID rabbits by implanting human hematopoietic stem cells (HSC) and lymphoid tissues (Figure 1), a process which is similar to a procedure we reported in mice [96]. Preliminary data indicates that ID rabbits with only a single-gene knockout background (i.e., IL2RG KO), can be implanted with human immune cells and lymphoid tissues and can support their development (up to >30% human CD45+/total PBMC). We anticipate that the efficiency can be improved on a multigenic background (e.g., Rag2 KO + Il2RG KO). Furthermore, transgenic integration or precise knock-in of human genes such as *SIRP-alpha* is expected to enhance the engraftment rates of human cells/tissues. Of note, the gene editing nuclease mediated precise gene knock-in has been reported in generating rabbit models [97] but has not yet been applied in ID rabbit engineering.

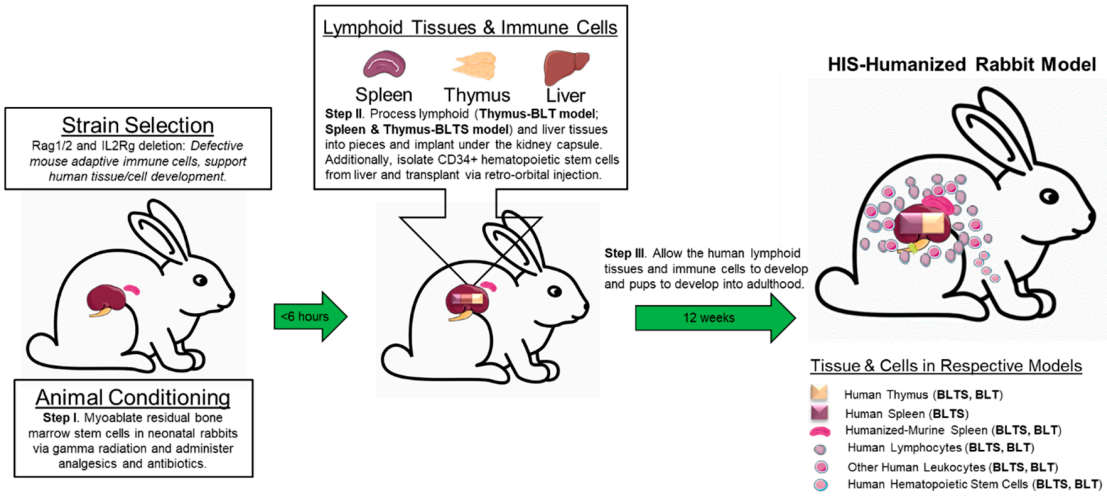

Figure 1. Illustration of the construction of the human immune system (HIS)-humanized rabbit models. First, immunodeficient rabbits, for example the ones with defects in recombination activating gene 1/2 (Rag1/2) and interleukin 2 receptor subunit gamma (IL2Rg), are developed and myoablated via gamma irradiation or busulfan, followed by the administration of antibiotics and analgesics at 1 to 2 weeks after birth. Second, to generate human lymphoid tissue xenografts along with autologous immune cell reconstitution in the rabbits, human fetal lymphoid tissue(s) and liver are processed into 1 mm^3 pieces, and autologous CD34+ hematopoietic stem cells (HSCs) are isolated from the fetal liver via immunomagnetic selection of $CD34^+$ cells. $CD34^+$ HSCs are then transplanted into the neonatal rabbit via intrahepatic injection following renal capsule transplantation of the lymphoid tissues. Third, the transplanted rabbit is maintained under specific pathogen-free conditions and the lymphoid tissue(s), and/or immune cell reconstitution in the peripheral blood, are allowed to develop over a period of 3 months, resulting in the HIS-humanized rabbit model. BLTS: bone marrow stem cells, liver, thymus, and spleen. BLT: bone marrow stem cells, liver, and thymus.

5.2. CDX and PDX

Tumor cell line-derived xenotransplant (CDX) and patient-derived tumor xenotransplant (PDX) are widely utilized procedures in cancer studies, where currently ID mice are used as the dominant host species [98]. It is realized, however, that the small size of the mouse sets a limitation on the size of tumor growth. More importantly, orthotopic transplantation, such as transplanting breast cancer cells to the host animal's mammary gland duct, is very challenging in ID mice. The availability of ID rabbits

would provide a solution to these challenges and enable the study of more clinically applicable and large sized tumors in vivo.

5.3. Testing of Stem Cell-Derived Blood Vessels

Aortic aneurysm is a major threat to human health, for which aorta transplantation is the primary treatment option. Advances in patient-specific stem cell-derived blood vessels (SDBV) have made artificial vasculature a promising source of tissue for clinical procedures. However, studying SDBV transplantation in mouse models is technically challenging due to the differences in the size of the vasculature. ID rabbits, whose size is >100 times that of ID mice and comparable to that of a human infant, may permit practical evaluation of SDBVs.

5.4. Preclinical Development of Gene Editing Therapy for SCID

Gene-editing therapy (GETx) has emerged as a new therapeutic option for many human diseases, including primary immunodeficiency [99]. Preclinical testing of the efficacy and safety in ID rabbit models offers several advantages over the use of ID mouse models. For example, rabbits allow for relatively easy bone marrow aspiration through a survival procedure. After gene correction of the HSCs ex vivo, the cells can be transplanted back to the donor animal (i.e., autologous transplantation), a procedure that is not feasible in mice. Furthermore, long term efficacy and safety profiles of GETx can be documented in ID rabbits (but not in ID mice), which is essential given the substantial tumorigenesis risks observed in the clinical trials of conventional gene therapy strategies.

5.5. Hepatocytes Regeneration

The supply of primary hepatocytes represents a limiting factor in the study of liver biology and medicine due to the technical limitations of culturing primary human hepatocytes ex vivo. In this regard, an ID rabbit on the background of the *Fah* knockout may serve as an efficient bioreactor to produce human hepatocytes. This approach has been proven to work in mice, where the *Fah* gene deficiency leads to hepatocyte death, while the immunodeficiency allows engraftments of human hepatocytes [100]. The much larger size of the ID rabbit liver would make this production strategy attractive.

Rome wasn't built in a day; neither will be the "kingdom" of ID rabbits. It took a decade to produce the major lines, it is now imperative to robustly demonstrate the advantages of these new models over ID mice, a necessity before the broader scientific community adopts them in basic and translational research. Table 3 presents many examples of applications of immunodeficient rabbits. Work to continue to develop new lines and to optimize existing lines is also urgent. For example, the lack of the none-obese-diabetic (NOD) equivalent in rabbits calls for the generation of human *Sirp-alpha* transgenic (Tg) to minimize macrophage mediated immune-rejection [101]. Multigenic ID gene knockout, such as *Rag2/Il2rg* KO (RG) should be developed as well to improve the engraftment efficiencies in xenotransplant experiments. Ultimately, the *Sirp-alpha* Tg line should be crossed with RG or the Prkdc/Il2rg KO (SG) line to establish NSG or NRG equivalent lines in rabbits. Challenging as they are, many of these goals are within reach, given that we are now armored with powerful gene editing tools and the completed rabbit genome project. It is not an overstatement that ID rabbits will contribute greatly to biomedical research, especially in the fields of regenerative medicine and cancer research in the near future.

Table 3. Example applications of immunodeficient rabbits.

1. Transplantation of stem cell-derived blood vessels, and examine long term safety and efficacy.
2. PDX/CDX for drug development and screening for cancer and other diseases.
3. Humanized livers for pharmacokinetics and hepatotoxicity studies.
4. Long term efficacy and safety evaluation of neuronal stem cell-based therapy.
5. A bioreactor for production of human hepatocytes.
6. A bioreactor for production of human T-cells for CAR-T cell expansion.
7. A large animal model with the human immune system for the study of infectious diseases.
8. A large animal model with the human immune system for antibody discovery.
9. A large animal model with the human immune system for TCR discovery.
10. Development and validation of imaging and diagnostic tools.
11. Study graft-versus-host disease (GVHD) in a large animal model.
12. Development of gene therapy and/or gene editing therapy for primary immunodeficient diseases.

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