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Opinions and Hypotheses

Rabbit models for biomedical research revisited via genome editing approaches

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Abstract. Although the laboratory rabbit has long contributed to many paradigmatic studies in biology and medicine, it is often considered to be a "classical animal model" because in the last 30 years, the laboratory mouse has been more often used, thanks to the availability of embryonic stem cells that have allowed the generation of gene knockout (KO) animals. However, recent genome-editing strategies have changed this unrivaled condition; so far, more than 10 mammalian species have been added to the list of KO animals. Among them, the rabbit has distinct advantages for application of genome-editing systems, such as easy application of superovulation, consistency with fertile natural mating, well-optimized embryo manipulation techniques, and the short gestation period. The rabbit has now returned to the stage of advanced biomedical research.
Key words: Gene targeting, Genome editing, Knockin, Knockout, Rabbit

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Characteristics of the rabbit as an experimental model

Generation of gene knockout (KO) by embryonic stem (ES) cells in animals requires stable and germline-competent ES cells [1]. Besides the quality of ES cells, it is also important that the methods for manipulating embryos should have been well established in the animals of choice. Furthermore, it is desirable that they should be relatively inexpensive, easy to maintain, easy to breed, and less restricted by ethics compared with primates. So far, only mice and rats have fulfilled all these requirements. Meanwhile, rabbits have a longer history of use in embryology research than mice or rats, although no germline-competent ES cells are available yet. The cost of purchasing sexually mature rabbits is less than that of other animals of similar size (e.g., mini-pigs or small primates). Rabbits become sexually mature at 4–5 months of age and give birth to 6–7 litters each time with a 29–31-day gestation period. Using a conventional superovulation technique, 30–50 oocytes/embryos can be recovered from a single female. A total of 15–20 early embryos can then be transferred into the fallopian tube, and 30–50% of them will grow to offspring. They are gentle, and can be handled easily by experienced persons. The rabbits' short reproductive cycle and large litter size are important advantages for breeding animals carrying genotypes or phenotypes of interest. Therefore, rabbits are very suitable for experiments using gene KO studies, once any gene-targeting strategy becomes practical (Table 1). Although generation of KO rabbits by somatic cell nuclear transfer (SCNT) is possible [2], SCNT in rabbits is generally very inefficient unlike that in pigs [3, 4].

Table 1. Reproductive system values of the rabbit

Biological parameter	Typical value
Body weight (kg)	2.5–4.0
Superovulation (oocytes/rabbit)	30–50
Gestation length (days)	29–32
Litter size (kits/litter)	7–9
Sexual maturity (weeks)	18–24
Developmental bioengineering	**Typical value**
Embryo transfer	easy
Transgenic rabbits	easy
Chimeric rabbits	very difficult
Somatic cell nuclear transfer	very difficult

History of gene KO techniques in rabbits

ES cells typically take on a primed state or a naïve state [5]. Naïve-state ES cells contribute to the development of chimeras and can readily differentiate into the germ line *in vivo*; however, there is little reported evidence on the use of ES cells to generate KO in animals other than mice and rats [6, 7]. As rabbit ES cells are known to be in a primed state, the generation of KO rabbits via ES cells might be challenging [8]. We have demonstrated conversion from a primed state into a naïve-like state; however, true naïve-state ES cells that would enable the generation of chimeric rabbit embryos and adults have yet to be established [9,

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Table 2. Production of knockout rabbits using genome editing

Nucleases	Genes	Nucleic acids (concentration)	Injection type	Pups obtained (% transferred)	Pups with mutation (% pups)	Germline transmission	Mosaicism	Off-target mutation	Reference
ZFN	*IgM*	mRNA (3–9 ng/μl)	Cytoplasmic	52/526 (9.9)	16/52 (30.1)	yes	yes	N.D.	[11]
	APO CIII	mRNA (5 ng/μl)	Pronuclear	21/145 (14.5)	5/21 (23.8)	yes	N.D.	N.D.	[12]
TALEN	*RAG1*	mRNA (50 ng/μl)	Cytoplasmic	18/40 (45)	17/18 (94)	yes	yes	no	[13]
	RAG2			4/24 (18)	4/4 (100)				
CRISPR/Cas9	*APOE* *CD36* *LDLR* *RyR2*	mRNA (Cas9, 150 ng/μl) (sgRNA, 6 ng/μl)	Cytoplasmic	68/301 (22.6)	38/68 (55.9)	yes	N.D.	no	[16]
	TYR	Plasmid DNA (5 ng/μl)	Pronuclear	9/77 (11.7)	2/9 (22.2)	yes	yes	no	[26]
	IL2rγ *Tikil* *Il2rg+RAG1*	mRNA (Cas9, 200 ng/μl) (sgRNA, 20 ng/μl)	Cytoplasmic	18/163 (11.0)	18/18 (100)	yes	N.D.	yes	[17]
	GJA8	mRNA (Cas9, 180 ng/μl) (sgRNA, 40 ng/μl)	Cytoplasmic	11/110 (10.0)	11/11 (100)	yes	N.D.	no	[18]
	MSTN	mRNA (Cas9, 180 ng/μl) (sgRNA, 40 ng/μl)	Cytoplasmic	20/158 (12.7)	16/20 (80.0)	yes	N.D.	no	[19]
	PHEX	mRNA (Cas9, 200 ng/μl) (sgRNA, 40 ng/μl)	Cytoplasmic	52/262 (19.8)	38/52 (73.1)	yes	yes	no	[20]
	TYR	mRNA (Cas9, 100 ng/μl) (sgRNA, 25 ng/μl)	Cytoplasmic	17/169 (10.1)	15/17 (88.2)	yes	N.D.	no	[21]

N.D.: not determined.

10]. While we have been struggling with improving methods for generating rabbit ES cells, genome editing techniques have enabled generation of gene KO in several living organisms including vertebrates. The speed of technical improvements in genome editing and their applications to new genes and organisms has been extremely rapid. That was not exceptional in the study using rabbits.

Zinc finger nuclease (ZFN)

Given the difficulties of generating KO rabbits via ES cells, genome editing has been used worldwide as an alternative method for generating KO rabbits (Table 2). Gene KO in rabbits was reported for the first time by Flisikowska *et al*., who used ZFN to KO the gene for rabbit immunoglobulin M (IgM) [11]. In that study, mutations of the targeted gene were detected in 30.1% of the offspring obtained, and the mutant alleles were transmitted through the germline. Thus, the study demonstrated that this genome editing technique was suitable for establishing gene KO rabbits.

Transcription activator-like effector nuclease (TALEN)

The TALEN system was developed following the ZFN system. This was first applied to the generation of immunodeficient rabbits by deleting the *RAG1* and *RAG2* genes, which function to activate or catalyze the V(D)J recombination in primary lymphoid tissues. The efficiency of KO in founder offspring was extremely high, reaching 94% for *RAG1* TALENs and 100% for *RAG2* TALENs [13]. In peripheral blood from the RAG-deficient rabbits, no CD4/CD8 double-positive T cells or mature CD4/CD8 single-positive T cells were detected. Furthermore, only a very small population of leukocytes expressed IgM. Although the usefulness of these RAG-deficient rabbits has not been determined, mice lacking *Rag* genes are known to be effective for allogeneic or xenogeneic transplantation research [13, 14]. It is noteworthy that given the high efficiency of genome editing by TALEN, the number of embryos for transfer was reduced compared with that using the ZFN system [12]. More recently, a rabbit model that developed arteriosclerosis has also been established by applying TALEN [15].

Clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9 (CRISPR/Cas9)

As demonstrated in several animal species, the CRISPR/Cas9 system is simple and highly efficient, and can serve as the core genome editing technique in rabbits. In 2014, Yang *et al.* reported the use of the CRISPR/Cas9 system to develop gene KO in rabbits for the first time, and they demonstrated the ability to KO four genes to develop models of hyperlipidemia [16]. The average efficiency of KO for these four genes was 55.9%. They also demonstrated germline transmission with no evidence for off-target mutation. In the same year, Yan *et al.* successfully performed

simultaneous KO of multiple (three and five) genes [17]. The genes targeted in this study were those involved in the development of an immunodeficient rabbit model, overwhelming the *RAG1* and *RAG2* KO models that were generated previously using the TALEN system [13]. This success could have arisen from several factors, including the increased concentration of Cas9 mRNA from 150 to 200 ng/μl, and the increased concentration of gRNA from 6 to 20 ng/μl. However, the resulting proportion of KO offspring was reduced from 22.6 to 11.0%, and off-target events were identified. These findings indicate the importance of selecting target sequences using the CRISPR/Cas9 system. Lai *et al.* have since developed disease models [18–20] and techniques for deleting a large sequence (105 kb) using the CRISPR/Cas9 system [21]. In those studies, they achieved a genome editing efficiency of 73–100% using 180–200 ng/μl of Cas9 mRNA and 40 ng/μl of gRNA. Thus, the conditions required for genome editing in rabbits using the CRISPR/Cas9 system are being established.

Our group has developed a technique for inactivating genes in rabbits by pronuclear injection of plasmid DNA containing Cas9 and gRNA, which was originally developed by Mashiko *et al.* [22] in mice. We first selected the gene for rabbit tyrosinase (*TYR*) as a target to establish the gene KO system in rabbits. *TYR* KO has previously been achieved in mice, rats, and zebrafish, and is characterized by the variety of coat colors in offspring [23–25]. We successfully generated KO *TYR* rabbits with the Dutch-belted rabbit strain genetic background [26] (Fig. 1).

Fig. 1. CRISPR/Cas9-mediated modification of the TYR gene in founder rabbits. Photograph of F1 pups that showed white coat color and red eyes (open arrows).

Future perspectives for genome editing in rabbits

To use rabbits in genome editing, the effects of inbreeding depression, mosaicism, and the efficiency of generating gene knockin (KI) rabbits must be considered. First, previous studies on KO rabbits demonstrated that both alleles are often mutated; hence, the resultant phenotype of the biallelic mutations can be analyzed in the F0 generation without intercrossing of the monoallelic mutant founders. Reproduction between heterozygous KO siblings could cause inbreeding depression, which would hinder the proliferation and practical use of the KO rabbits established. Second, mosaicism might occur during preimplantation embryo development in rabbits as in other species. The first cleavage of rabbit embryos occurs approximately 24–32 h after fertilization, while the second and third divisions occur within the next 8 h to form 8-cell embryos. This brief period during the 2-cell and 8-cell stages might cause more complex patterns of mosaicism than in other species. Indeed, we have observed that the injection of plasmid DNA into the cytoplasm instead of the pronucleus results in mosaicism in most embryos (data not shown). Last, the use of the Cre/loxP system is limited in mammals other than mice and rats because of the lack of ES cells for generating chimeric embryos and animals. To this end, Yang *et al.* recently generated KI rabbits using the CRISPR/Cas9 system [27]. Aida *et al.* further demonstrated that the pronuclear injection of Cas9, gRNA, and trans-activating crRNA (tracrRNA) results in the efficient KI of a double-stranded DNA cassette in mice [28]. The 2 Hit-2 oligo method proposed by Mashimo *et al.* might also be effective for generating KI rabbits [29]. To this end, several studies have investigated methods to improve KI efficiency in rabbits. For example, one study demonstrated that the addition of a compound named RS-1, which enhances homology-directed DNA repair, increased KI efficiency in rabbits to 17.6 and 26.3% with the TALEN and CRISPR/Cas9 systems, respectively [15] (Table 3). The use of such a system might facilitate conditional gene targeting in a tissue-specific and time-controlled manner using transgenic rabbits that contain LoxP sites and Cre recombinase transgenes, enabling complex genome editing.

Rabbits have been used as models for human diseases, including hyperlipidemia and arteriosclerosis, as well as in several fields such as ophthalmology and orthopedics. Moreover, rabbits are suitable for genome editing studies thanks to their high reproductive performance. Further improvements in genome editing techniques for rabbits would make them appropriate models that can overcome some of the limitations associated with other animal models— such as mice, rats, pigs, and monkeys— to enable the investigation of human diseases.

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Table 3. Production of Knock-in rabbits by genome editing

Nucleases	Genes	Nucleic acids (concentration)	Injection type	Pups obtained (% transferred)	Knock-in efficiency (% pups)	Germline transmission	Mosaicism and off-target mutation	Reference
CRISPR/Cas9	*ROSA26*	Cas9 mRNA (150 ng/μl) sgRNA mRNA (6 ng/μl) donor DNA (100 ng/μl)	Cytoplasmic	20/100 (20.0)	7/20 (35.0)	yes	no	[27]
TALEN (with RS-1)	*ApoAI RLL*	TALEN mRNA (50 ng/μl each) donor DNA (50 ng/μl)	Cytoplasmic	17/145 (11.7)	3/17 (17.6)	yes	N.D.	[15]
CRISPR/Cas9 (with RS-1)	*ApoAI RLL*	Cas9 mRNA (100 ng/μl) sgRNA mRNA (6 ng/μl) donor DNA (100 ng/μl)	Cytoplasmic	38/146 (26.0)	10/38 (26.3)	yes	N.D.	[15]

N.D.: not determined.

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