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Perspective

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Genome-edited rabbit, a prospective alternative model for neurological diseases

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Abstract

Animal models have great importance in the research of human neurodegenerative diseases due to their value in symptom mimicking, mechanism investigation, and preclinical tests. Although non-human primate and large animal models have good performance in disease modeling due to their high maintenance cost and critical ethical standards, rodent models are commonly used. Rodent models have been successfully applied in modeling many neurological diseases; however, their genetic background, neuroanatomical features, and nervous system development are different from those of humans. Moreover, the short lifespan and small body size of rodent models also limit the monitoring of disease progression and observation of clinical symptoms in studying neuronal disorders that are late-onset or have a long course of progression. In comparison with rodents, rabbits are phylogenetically closer to humans and have closer similarities to humans in brain development, thus are an alternate animal model for human neurological diseases.

Keywords: Neurodegenerative diseases, genome editing, animal model, rabbit, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis

INTRODUCTION

Neurodegenerative diseases (NDDs) are associated with progressive neuron losses, most of which are linked with genetic disorders[1]. At present, neurological disorders are considered as one of the major causes of mortality and disability worldwide[2]. Unfortunately, many NDDs are late-onset and hard to detect in the early stage; for instance, Parkinson's disease (PD) and Alzheimer's disease (AD) have high morbidity in elderly patients, while amyotrophic lateral sclerosis (ALS) does not exhibit clear symptoms at the early stage of the disease. Moreover, due to the irreversibility of neuronal death, no effective therapeutic approaches are available at present. Therefore, a thorough investigation of these diseases is essential for the development of disease-specific and effective prognostic, diagnostic, and therapeutic strategies. Model organisms are essential platforms for the above research; cell lines and animal models are frequently used. Although cell models can be used for the investigation of pathological pathways and molecular mechanisms of disease pathogenesis[3], due to the limitation in modeling organogenesis and human physiology[4], they cannot mimic histological, morphological, and behavioral changes in human diseases. Therefore, animal disease models that partially recapitulate the aspects of human diseases are essential. Additionally, animal models have irreplaceable value in preclinical tests; they are also important for the development of prognostic and therapeutic strategies.

In comparison with non-mammalian animals such as zebrafish and *Drosophila melanogaster*, mammalian models have greater similarities to humans in genetics, metabolism, and physiology and can therefore mimic some of the biological and clinical features of human disease[5,6]. In practical research, large animal models are often used because of the biological characteristics of these animals for modeling human NDDs[7]. With the development of genome editing tools and somatic cell nuclear transfer (SCNT) techniques, genetically modified large animal models can be effectively produced, which can promote the utilization of these models[8]. Unfortunately, maintenance costs, space requirements, and ethical standards are still problems for the use of large animal models in biomedical research. Compared with large animal models, rodents have a small body size, low maintenance cost, and can be easily handled, making them cost-efficient models. Moreover, rodents have a relatively high genetic identity and physiological similarity to humans, and genetic modification capabilities can facilitate the modeling of genetic disorders[9]. Therefore, currently, rodents are the main experimental animal for biomedical research and disease modeling. From 1950 to 2010, approximately 80% of animal-based biomedical studies were performed on rodents (59% on mice and 18% on rats)[10].

Rodents have been applied in modeling many neurological diseases and have adequate precision in mimicking the pathology and physiology in some cases. However, due to factors such as lifespan, genetic differences from humans, and small body size, rodent models have some limitations in studying neuronal disorders[9]. For example, they cannot replicate the exact pathological hallmarks in some human diseases because of physiological and genetic differences. PD mouse models (α-synuclein transgene or knockout of *LRRK2*, *PRKN*, and *PINK1*) do not show degeneration of nigrostriatal dopaminergic neurons[11] and striatal neurons remain viable, which is different from the pathological features in human disease[11]. The absence of intranuclear inclusion body formation in neuronal cells of ALS mice overexpressing mutant *hSODl* is inconsistent with the phenotype of human ALS disease. Thus, finding an alternative animal species for modeling is needed to produce better models for diseases that cannot be recapitulated in mice.

Rabbits are docile and easy to handle; their short reproductive cycle and high reproductive performance can guarantee an abundant sample size for experiments; and the efficiency of model production and the low demand for rearing and surgical operation equipment make rabbits easy to maintain and handle[12]. Moreover, rabbits have an intermediate lifespan (longer than rodents but shorter than large animals such as

non-human primates), and, compared with rodents, rabbits are phylogenetically closer to humans[13] and are more similar to humans in brain development[14]; therefore, they may have better precision in disease modeling. With the development of targeted genome editing tools, producing targeted genome-edited rabbit models for human neuronal disorders has become attainable.

ADVANTAGES OF RABBITS AS ANIMAL MODELS OF NEUROLOGICAL DISEASES

Rabbits are phylogenetically closer to humans than mice

Genetic similarity to humans is linked with the identity of protein structure and function, and high genetic similarity could increase the precision of disease modeling. Compared with rodents, rabbits are phylogenetically closer to primates[13], suggesting they may have better precision in disease modeling. Notably, some human genes do not have orthologs in mice: approximately 1% of human genes cannot find orthologs in mice's genomes. For instance, caspase 10, a gene that is linked to neurodegeneration via the extrinsic apoptosis pathway[15], is absent in mice but has orthologs in rabbits[11]. However, the phylogenetic similarity between rabbits and humans does not guarantee rabbits would be a better model for all human diseases; the performance of disease modeling is still dependent on the type of mutant gene, and the mechanism involved in the pathogenesis should also be considered.

The development of the central nervous system of rabbits has greater similarity to humans compared with rodents

Neurological features are critical for NDD modeling, and the CNS development of rabbits is highly similar to that of humans compared with that of rodents. Specifically, the phase of brain development and myelination in rabbits is more similar to humans than that of rodents, since such a process happens during the perinatal period in humans and rabbits but postnatally in rodents[14]. Moreover, rabbits have a higher brain volume and cerebral surface area than mice. The time point of morphological configuration of major CNS structures of rabbits is closer to humans. The development of structures such as primitive streak, neural tube closure, and primary brain vesicles in rabbits is chronologically similar to that in humans[14]. In addition, the development of white matter in rabbits is closer to that of humans[14], and rabbits have a higher white matter ratio than mice (approximately 20% *vs.* 10%)[16,17] [Table 1]. Moreover, rabbits have larger brain volume, cortex surface area, and number of neurons compared with rodents [Table 1], suggesting that rabbits may exhibit better cognitive, learning, and memory abilities.

Rabbits can be trained to learn basic skills (e.g., recall signals) through positive reinforcement[25]. Rabbits also have both short- and long-term memory[26,27] and can exhibit memory losses when mimicking NNDs such as AD[27]. Specifically, in an AD rabbit model constructed by drug induction, the results of novel object recognition (NOR) and object location memory (OLM) tests suggest that the model can track cognitive impairment[28]. In other studies, the results of conditional and unconditional response tests also suggest that the AD rabbit model has reduced learning ability[29,30].

Additionally, axon degeneration is a common pathological feature of NDDs, and neurons with longer projections have a higher vulnerability to axon degeneration, which can be easily affected in NDDs[31]. Some mice models of motor neuron diseases exhibit molecular pathological features in neurons but only exhibit mild or even no behavioral symptoms[32,33]; vulnerability to axon degeneration might be the explanation for this phenomenon, since the axon length in rodents is shorter than that in larger animals. Collectively, rabbit models might have better accuracy in mimicking human neuronal diseases compared to rodents.

Rabbits have a relatively large body size for handling and sampling

In addition to the genetic and neurological features, rabbits also have a bigger body size compared with rodents, which can facilitate better animal handling and symptom observation. The relatively large body size

Table 1. Major differences in size and structures between rabbits and mice

	Human	Rabbit	Mice
Brain volume[18]	1300-1400 g	10-13 g	0.4-0.5 g
Spinal cord length[18]	43 to 45 cm	18 cm	7.5
Gray-white matter ratio[16,17]	40:60	80:20	90:10
Duration to reach adult brain volume[19,20]	20 years	4 months	2 months
Glia-neuron ratio (GNR)[21]	1.66	0.32-0.49	0.29-0.42
Number of neurons[22-24]	86,000 million	494.2 million	71 million

of the rabbits is also associated with larger organ size and blood volume (45-75 mL per kg body weight versus 1.5-2.5 mL)[10,34], which can be beneficial for diagnostic investment, surgical operation, and sampling for pathological analysis. Cerebrospinal fluid (CSF) and blood biomarker analyses are commonly used in the diagnosis of NDDs such as AD and FTD[35-37]. Such assays are hard to perform in mice due to the poor sample size, especially for experiments that need continuous monitoring; in contrast, for larger animals such as rabbits, an adequate amount of sample can be collected with minimal harm to the animal.

In addition, medical imaging approaches are usually needed for the prognosis and diagnosis of NDDs[38,39]. However, it is hard to perform high-definition medical imaging on small animals due to the limitation of the equipment. High-resolution magnetic resonance imaging (MRI) in mice requires a scanner with an ultra-high field strength of 7 T or higher[40], which is inaccessible for most researchers. Compared with rodents, rabbits have larger CNS [Table 1]; thus, a normal MRI scanner with a 3.0 T field strength is adequate for CNS imaging in rabbits[41]. The large body size of rabbits can also benefit electromyography tests, which are commonly used in the diagnosis of neuromuscular diseases such as ALS. Additionally, the scale of the central neuron system also affects the maneuverability of tissue sampling and the intraparenchymal or epidural injection of therapeutic vectors such as AAV in future translational medical research.

Collectively, the larger body size of rabbits makes it easier to handle and sample compared with small animals, which largely facilitate phenotype observation and surgical operation.

The lifespan of rabbits is long enough for the observation of disease progression

The onset of neurodegenerative disease and the speed of progression are affected by both genetic and environmental factors[42]. For pathological mutations that induce late onset and slow progression, the lifespan of animal models should also be considered, since the effect of aging can interfere with the observation of clinical symptoms[9]. Generally, NDDs are progressive diseases that last from years to decades. For instance, the median survival time of ALS patients is 20-48 months[43], while that of AD patients can reach up to 30 years[44]. Moreover, late-onset NDDs such as AD and PD develop late in life; both diseases usually begin at age 60 or older in human patients[45]. However, the normal lifespan of mice is 12-36 months[10], which means that for diseases that begin late or have a slow rate of progression, mice may not fully exhibit the whole course of the disease. For mutations that can only induce late-onset symptoms or slow progressive disease, mice models may not exhibit observable symptoms in their lifetime without extra administration, such as drug stimulation[46]. Furthermore, due to the short lifespan of mice, it is hard to identify whether a symptom (e.g., vision loss) is caused by pathological neuronal death or age-related reasons[9]. In the adult phase, 2.6 mice days is equivalent to one human year, while, in the post-senescence phase, 2.069 mice days is equivalent to one human year[10]. Such fast senescence processes can largely limit the progression of disease and interfere with the observation of disease-related clinical symptoms. In contrast, the maximum lifespan of laboratory rabbits can reach up to 10 years under proper conditions, and

one human year is equivalent to 18.25 and 50.34 rabbit days in the adult and post-senescence phases, respectively[34]. Thus, for most NDDs, the lifespan of rabbits is long enough for the observation of disease progression.

PRODUCTION OF GENOME-MODIFIED RABBIT DISEASE MODELS VIA CRISPR-CAS SYSTEM

The production of disease models that recapitulate the pathological features of human disease is an important approach to investigating the pathogenesis of the disease. Artificially induced disease models can exhibit clinical features of some NDDs. For instance, hydroxydopamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, rotenone, and paraquat are commonly used in the induction of Parkinson's disease[47]. However, many NDDs are caused by pathological mutation of the disease-related gene, and an induction model cannot fully recapitulate the whole pathological pathway of diseases caused by genetic disorders[1]. Therefore, to elucidate the whole pathogenesis process of neuron degeneration, the production of animal models that carry pathological mutations that mimic human disease is necessary.

With the development of gene-editing tools, efficient and accurate genome modification has become achievable. To date, various genome-edited rabbits have been constructed, as shown in Table 2. In 2013, the CRISPR-Cas9 system was harnessed for efficient targeted genome editing in eukaryotic cells[99,100]. Moreover, with the further development of research on CRISPR-Cas systems, the CRISPR-Cas systems and their derivates can facilitate targeted gene knockout (KO), knockin (KI), activation, suppression, and single-base substitution. Presently, various genome editing tools based on CRISPR-Cas systems are widely used in multiple species, including non-human primates, large non-primate animals, rodents, and rabbits[101-103].

The first CRISPR-Cas-mediated gene KO in rabbits was successfully generated in 2014[101] [Table 2]; however, full-length gene KO can only recapitulate diseases caused by loss of function. To mimic diseases caused by gain-of-function mutation due to point mutation, more accurate gene manipulation is needed. Furthermore, more than 50,000 disease-causing mutations in humans are point mutations; therefore, a novel system that can mediate single base substitution is needed. Since 2017, the development of cytosine and adenine base editing systems can facilitate efficient C to T and A to G base substitutions, which can facilitate precise gene manipulation[104]. Such systems were identified as having ideal editing efficiency in rabbits [Table 2]; the efficiency of cytidine base editor (CBE) and adenine base editor (ABE) in rabbits after co-microinjection of base editor mRNA and sgRNA are 53%-88% and 44%-100%, respectively[90]. The following refinement of base editors has overcome or reduced the limitations of PAM sequences and the incidence of bystander activities[92,105]. At this stage, base editing systems are capable of inducing disease causative missense and nonsense mutations in rabbits to generate disease models.

Although base editing systems can induce four transversion mutations, it is impossible for such systems to induce the other eight transversion mutations. Moreover, the generation of bystander mutations cannot be completely avoided when there are multiple C or A in the editing window. Importantly, conventional gene editing systems cannot induce efficient single base or oligonucleotide insertions and deletions. Therefore, it is hard to generate disease models with fragment shift mutations. Fortunately, the development of prime editing systems solved such problems in 2019. The system, which is based on the target binding capacity of the CRISPR-Cas9 system and the retro-transcription activity of retrotrancripsase, can facilitate the whole genome "search and replace" activity in organisms. Prime editor was successfully used in generating a Tay-Sachs disease (TSD) rabbit model in 2021 [Table 2], which is a model of neurological disease generated by prime editor-mediated four base insertion[98].

Table 2. Summary of genetically modified rabbits

System	Genes	Modification	Application	Refs.
ZFN	*IgM*	KO	Immunodeficiency	[48]
ZFN	*APOC3*	KO	Lipid metabolism and atherosclerosis	[49]
ZFN	*APOE*	KO	Lipid metabolism and atherosclerosis	[50]
ZFN	*CETP*	KO	Lipid metabolism and atherosclerosis	[51]
TALENs	*RAG1; RAG2*	KO	Immunodeficiency	[52]
TALENs	*FAH*	KO	Hereditary tyrosinemia type 1	[53]
CRISPR/Cas9	*FBN1*	KO	Marfanoid progeroid lipodystrophy syndrome	[54]
CRISPR/Cas9	*DMD*	KO	Duchenne muscular dystrophy	[55]
CRISPR/Cas9	*ANO5*	KO	Muscular dystrophy	[56]
CRISPR/Cas9	*α-Crystallin*	KO	Congenital cataracts	[57]
CRISPR/Cas9	*GJA8*	KO	Congenital cataracts	[58]
CRISPR/Cas9	*LDLR*	KO	Lipid metabolism and atherosclerosis	[59]
CRISPR/Cas9	*MSTN*	KO	Muscle hypertrophy	[60,61]
CRISPR/Cas9	*SRY*	KO	Sex reversal syndromes and hermaphroditism syndromes	[53,54]
CRISPR/Cas9	*PHEX*	KO	X-linked hypophosphatemia	[62]
CRISPR/Cas9	*LMNA*	KO	Premature aging syndrome	[63]
CRISPR/Cpf1	*WRN*	KO	Werner syndrome	[64]
CRISPR/Cas9	*TYR*	KO	Oculocutaneous albinism	[65,66]
CRISPR/Cas9	*DMP1*	KO	Mineralization defects	[67]
CRISPR/Cas9	*GADD45G*	KO	Congenital cleft palate	[68]
CRISPR/Cas9	*HOXC13*	KO	Hair and nail ectodermal dysplasia	[69]
CRISPR/Cas9	*GCK*	KO	Maturity-onset diabetes of the young type 2	[70]
CRISPR/Cas9	*HBB2*	KO	β-thalassemia	[71]
CRISPR/Cas9	*WAS*	KO	Wiskott-Aldrich syndrome	[72]
CRISPR/Cas9	*CBS*	KO	Congenital hyper-homocysteinemia	[73]
CRISPR/Cas9	*LDLR; APOE*	KO	Lipid metabolism and atherosclerosis	[74]
CRISPR/Cas9	*APOC3*	KO	Lipid metabolism and atherosclerosis	[75]
CRISPR/Cas9	*CFTR*	KO	Cystic fibrosis	[76]
CRISPR/Cas9	*CFTR*	KO ΔF508	Cystic fibrosis	[77]
CRISPR/Cas9	*CLPG*	KO	Muscular hypertrophy syndrome	[69]
CRISPR/Cas9	*FGF5*	KO	Long hair	[78]
CRISPR/Cas9	*IL2RG*	KO	X-linked severe combined immunodeficiency	[79]
CRISPR/Cas9	*MC1R*	KO	Block the synthesis of eumelanin and create a novel pale-yellow coat color	[80]
CRISPR/Cas9	*XIST P1*	KO	X-chromosome inactivation	[73]
CRISPR/Cas9	*MSTN*	KO	Muscle hypertrophy	[74]
CRISPR/Cas9	*PCSK9*	p.S386A	Lipid metabolism and atherosclerosis	[75]
CRISPR/Cas9	*ATP7B*	p. R778L	Wilson Disease	[81]
CRISPR/Cas9	*TYR*	p. T373K	Oculocutaneous albinism	[82]
CRISPR/Cas9	*TYR*	KO	Oculocutaneous albinism	[83]
CRISPR/Cas9	*RAG; RAG2; TIKI1; ALB; IL2RG*	Multiplex gene KO	Immunodeficiency	[84-86]
CRISPR/Cas9	*FUT1; FUT2; SEC1*	KO	Fucosyltransferases enzymes activity	[87]

CRISPR/Cas9	*ROSA 26*	KI	Safe harbor gene	[88,89]
BE3	*MSTN*	p.Q93stop	Muscle hypertrophy	[90]
	TYR	p.Q68stop	Oculocutaneous albinism	
ABE7.10	*DMD*	p.T279A	Duchenne muscular dystrophy	
eAID-BE4max	*TYR*	p.R299H	Oculocutaneous albinism	[91]
YFE-BE4max	*TYR*	p.Q68Stop	Oculocutaneous albinism	[92]
nNme2-CBE	*FGF5*	p.Q79Stop	Long hair	[93]
eA3G-BE	*TYR*	p.Q48stop	Oculocutaneous albinism	[94]
NG-ABEmax	*HOXC13*	p.Q271R	Hair and nail ectodermal dysplasia	[95]
BE4max	*FGF5*	Start Codon Disruption	Long hair	[96]
ABE8.17	*TYR*	p.T325A	Oculocutaneous albinism	[97]
	LMNA	p.L530P	Emery-Dreifuss muscular dystrophy	
PE3	*HEXA*	p.Y427fs	Tay-Sachs disease	[98]

PROSPECTS AND LIMITATIONS FOR EVALUATING RABBIT DISEASE MODELS

The observation of the clinical phenotypes of diseases is important for the evaluation of animal models. However, unlike the well-developed testing platforms for rodent models, currently, the evaluation criteria for rabbit NDD models are not well established.

In general, the diagnosis, prognosis, and autopsy criteria in human NDDs can be used in animal models. Such investigations can provide data that are comparable to human clinical reports and have better referential value. Indeed, commercialized analysis platforms, such as serological testing, enzyme-linked immunosorbent assay, MRI, electromyography, and histological analysis, are versatile and authentic tools for the assessment of both humans and animals including rabbits. However, it is impossible to apply the whole set of human diagnostic criteria to animals. For example, the investigation methods for behavioral and cognitive analyses in humans are hard to apply in animal models. Standardized and species-specific behavioral analysis platforms can support the assessment of animal disease models. For rodents, systematic behavioral analysis systems are well established and standardized; systems such as multivariate concentric square field and cylinder test are used to investigate traits such as sensory-motor function[106]. In contrast, the behavioral and cognitive analysis platforms for rabbits are not well developed at present, and further development of these systems is necessary for the future use of rabbits in neurological disease modeling.

CONCLUSION

Collectively, rabbits are more similar to humans in brain development, with more genetic similarities than rodents, and longer lifespan and larger body size, suggesting that rabbits can perform well in human neurological disease modeling in addition to traditional non-human primates, large animals, and rodent models. Therefore, it is expected that, in the near future, with the further development of genome editing technology and the establishment of phenotype assessment platforms for rabbit models, the value of rabbits in the research of neurological diseases can be maximized, not only for the understanding of pathological mechanisms but also for innovation of therapeutic approaches.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Zhang Z, Song Y

Performed data acquisition, as well as provided administrative, technical, and material support: Li Z, Lai L

Availability of data and materials
Not applicable.

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All authors declared that there are no conflicts of interest.

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Not applicable.

Consent for publication
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