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REVIEW ARTICLE

Application of genome editing technologies in rats for human disease models

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Abstract

Laboratory rats and mice are representative experimental animals for models of human disease. The emergence of genome editing technologies has enabled us to produce a variety of genetically modified animals, including rats, as a means of elucidating the in vivo functions of the gene of interest and characterizing the molecular mechanisms of human disease. Several advanced techniques for knock-in methodologies in rats are currently in development, which permit researchers to introduce precise nucleotide modifications at target sites in the rat's genome. Furthermore, recent studies with knock-out rats have revealed that observed disease phenotypes are often more similar than mouse models to those of humans. In this article, we introduce the methodologies for efficient gene manipulation in rats using genome editing technologies, and describe the advances made using rats for human disease models. We also discuss the importance of gene manipulation in animal models for the better understanding of fundamental processes among different species.

Introduction

In vivo experiments using laboratory animals are indispensable for the elucidation of pathological and physiological mechanisms in human diseases, which can lead to the development of new treatments and prevention approaches. Altogether with the mouse, the laboratory rat (*Rattus norvegicus*) is a representative experimental mammal that has been widely used for the past century as a human disease model for hypertension [1], diabetes [2, 3], epilepsy [4, 5], inflammation [6], and cancer [7, 8]. Indeed, rats are often a better choice than mice because of their larger body size and ease of manipulation. They are useful not only for longitudinal drug efficiency tests and toxicity assessments but also for surgical manipulations in neurological research such as electrode insertions in the brain and optogenetic neural stimulation [9]. Moreover, comprehensive database platforms related to laboratory rats have been recently developed, for example, the National BioResource Project-Rat in Japan and The Rat Genome Database in the US [10, 11]. These platforms provide valuable resources and updated datasets of genomic elements including gene annotations, transcripts, sequence information, and variations associated with phenotypes and disease.

While rats have contributed greatly to the development of therapeutic agents and regimens as human disease models, they have lagged behind mice in the genetic research field. Mouse reproductive technologies such as in vitro fertilization and embryo manipulation have been established since the 1970s and gene knock-out (KO) mice using embryonic stem (ES) cells were developed before 1990 as critical tools for understanding gene functions [12–14]. Knock-in (KI) mice, including reporter tagging of endogenous genes and conditional knock-outs, are widely used for spatial or temporal observation to control gene activation, avoiding early lethal phenotypes [15, 16]. Despite these advances, ES cell-mediated gene targeting technologies remain critical tools for understanding gene functions in mice. Although fundamental reproductive technologies have also been developed in rats, it has proved more difficult to produce KO rats using gene targeting technologies because of the lack of optimal culture conditions for germline-competent ES cells until the mid-2000s.

Therefore, before the availability of ES cell-mediated gene targeting and genome editing technologies for rats,

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transgenic techniques involving random mutagenesis were used instead. Transgenic rats can be generated by the microinjection of donor DNA derived from another species into a male pronucleus of a fertilized egg. This enables the random integration of foreign genes into a rat genome which permits investigation of the in vivo function of the gene of interest. These methods contributed to the past acquisition of important biological knowledge about human diseases such as inflammation [17], neurological disorders [18], and cancer [19]. Random mutagenesis can induce mutations throughout the rat genome by introducing a transposon such as Sleeping Beauty or a chemical mutagen such as N-ethyl-N-nitrosourea (ENU) [20, 21]. However, these methods require large populations of rats and a high-throughput screening strategy to identify mutations in a targeted gene. Thus, several strategies have been developed to identify ENU-induced mutations in animals, including yeast-based screening [21], CelI-based enzymatic cleavage [22], sequencing-based screening [23], and Mu transposase-based heteroduplex identification [24].

Mouse ES cells have facilitated the application of genome engineering technologies to create precise genetic modifications in vitro via homologous recombination (HR). Transplantation of these cells into a host embryo can then generate a chimeric mouse carrying genetic modifications. Although several methodologies have been attempted to establish authentic rat ES cells, there is no robust evidence of chimera generation because rat ES cell stability can be greatly influenced by culture conditions and the genetic background of rat strains. A new strategy to maintain rat-derived ES cells was finally developed in 2008 using the 3i/2i culture system [25]. This method lead to the generation of a p53 gene KO rat via HR in 2010, almost 20 per s after the emergence of KO mice [26]. Around this time, other genome editing milestones were developed such as zinc finger nucleases (ZFNs), transcription activator-like (TAL) effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nucleases (Cas). These groundbreaking technologies are major approaches for gene modification that avoid the need for germline-competent ES cells. Furthermore, their simplicity, efficiency, and reliability facilitate KO and KI production in a variety of animals including mice and rats [27]. Here, we introduce recent methods of these innovative technologies that have been used to manipulate a rat genome, and describe genetically modified rats that have been developed as models of human disease.

Generation of genetically modified rats using ZFN, TALEN, and CRISPR/Cas9

ZFNs are artificial fusion proteins carrying DNA binding domains, which consist of tandem zinc finger motifs with customized specificity and a non-specific nuclease domain from the endonuclease FokI [28, 29]. FokI domains can introduce a double-strand break (DSB) at a precise genomic locus following recognition of the targeted DNA sequence

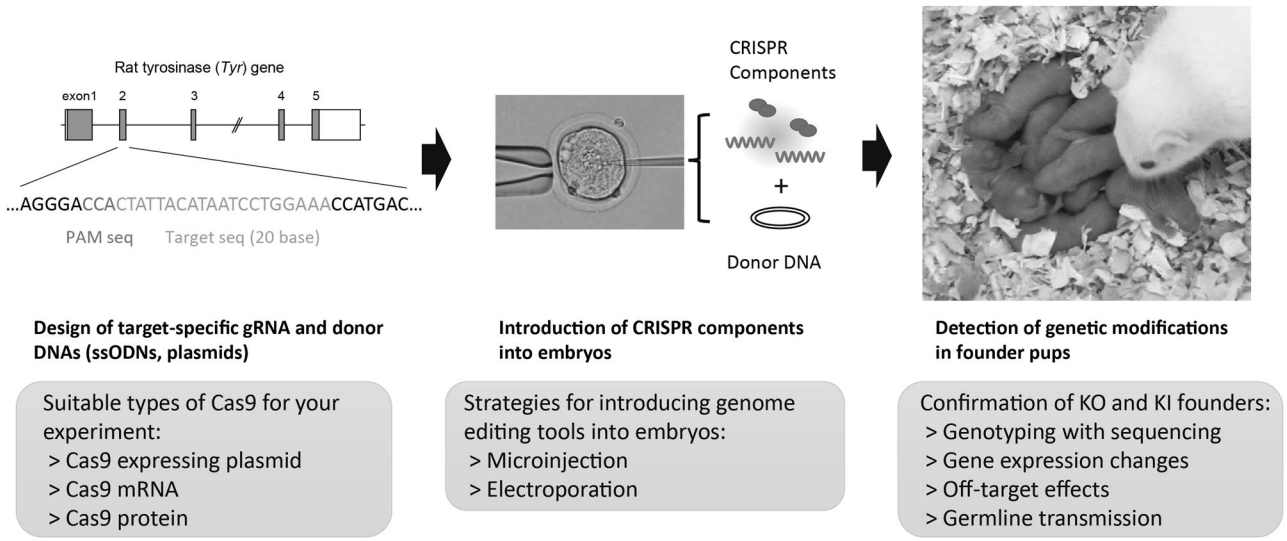

Fig. 1 Experimental procedures for generating genetically modified rats with the CRISPR/Cas9 system. gRNA recognizing the target sequence in the genome is designed using web tools to avoid off-target effects. The target-specific gRNA and Cas9 mRNA or protein are then introduced into rat embryos. It is also possible to introduce an all-in-one plasmid expressing Cas9 and gRNA. Donor DNAs such as ssODN and plasmid DNA should be mixed and co-introduced into embryos. While microinjection is a popular choice for this, electroporation and lentiviral infection are suitable alternatives. About 3 weeks after transfer of the eggs to a pseudopregnant females, founder pups are obtained. Genetic mutations can be identified in founder individuals by DNA sequencing analysis. The off-target effect and germline transmission to the next generation can then be confirmed

by paired ZFNs. Inserted or deleted mutations often occur at the DSB site via the non-homologous end joining (NHEJ) repair process. Introduction of a DSB at the coding region can cause a frameshift mutation of the targeted gene, which creates a KO mutation. The process of generating KO rats using ZFNs is facilitated by the microinjection of DNA or RNA coding for ZFN components into fertilized rat embryos (Fig. 1). Moreover, KO founder rats with a KO efficiency >20% can be generated in the G0 generation within 3–4 months, which is quicker than the ES cell-based method for mice that usually takes 12–18 months. In addition, gene targeting with artificial nucleases is not strain-dependent and accordingly can be performed with any inbred strains. Therefore, numerous research articles about KO rats generated using ZFN technology have been published since the first report of GFP-KO rats in 2009 [30–33]. However, despite the great contribution of ZFNs to KO rats, several limitations remain associated with this technique. These include the difficulty of producing suitable ZFNs for the targeted sequence, the potential to produce off-target cleavage and mutations, and the high expense of custom-designed ZFNs [27, 34].

TALENs are an alternative tool for genome engineering [35–37]. They are also fusion proteins of tandem repeats of a TAL effector protein derived from plant-pathogenic *Xanthomonas* bacteria and the FokI nuclease. While the targeted sequences are severely limited because of the recognition pattern of ZF domains, TAL effector motifs can recognize any sequence except when thymine is at the first position. TALENs cleave the targeted region in pairs, similar to ZFNs, which increases their efficiency as a tool for KO animals and cells [38, 39]. To provide a cost-effective targeted nuclease platform at the general laboratory level, several strategies have been established for the simple and rapid construction of TALENs [40–45]. For example, Sakuma et al. [42] showed that TALENs with periodically patterned repeat variants harboring non-repeat-variable di-residue (non-RVD) variations (Platinum TALENs) have higher activities than TALENs without non-RVD variations. We applied the Platinum TALEN to the rat *Il2rg* gene to produce KO rats at a higher efficiency compared with ZFNs and original TALENs.

Although ZFNs and TALENs have been applied to various species, CRISPR/Cas9, the newest genome editing tool, is widely used for generating KO and KI mutations in many cell types of different species including mice. The CRISPR/Cas9 system was first identified as a gene-targeting technology in mammalian cells [46–48]. Cas9 nucleases are combined with synthetic single-guide RNAs (gRNAs) that are complementary to the 20 bases of target sequence beside the NGG sequence. The complex then recognizes the target sequence and introduces DSBs. The system enables the navigation of Cas9 to any genomic locus through the design of synthetic gRNA, which can be readily generated. The precision of this mechanism has made the CRISPR/Cas system more renowned as a robust genome editing tool than ZFNs and TALENs [49–52]. Several studies have reported the generation of targeted KO in rats using CRISPR/Cas9 [53–56], and we also generated a targeted KO rat at the tyrosinase (*Tyr*) locus by microinjecting target-specific gRNA and Cas9 mRNA into fertilized eggs [57]. Crossing founders demonstrated that the CRISPR/Cas9-mediated mutations were faithfully transmitted to the next generation.

In addition to producing a gene KO rat, we used the target-specificity of CRISPR/Cas9 to perform allele-specific genome editing [57]. An albino allele-recognizing gRNA:Tyr^c was introduced into F1 rat embryos from a DA strain×F344 strain cross containing wild-type *Tyr* and single-nucleotide polymorphisms (SNPs) for each albino type, leading to the occurrence of KO mutations only at the albino allele. Conversely, introduction of a wild allele-recognizing gRNA:Tyr^c caused mutations to occur only at this allele. This suggested that the CRISPR/Cas9 system can be used for highly accurate allele-specific genome editing, which is applicable to the highly heterogenized human genome. In the near future, CRISPR/Cas9 is predicted to be used as gene therapy for destroying genetic mutations that cause human disease and restoring mutated regions to normal alleles [58].

Cas9 and gRNA expression vectors have been used for genome editing in rodent embryos [59, 60]. These vectors can be readily prepared at low cost and express high levels of Cas9 and gRNA over several days, leading to more complete embryonic modifications. However, the potential for off-target mutations and random integration of plasmids into genomic DNA may also be increased. We used Cas9 mRNA for genome editing in rodents to avoid these limitations. Although it is necessary to transcribe them in vitro, the toxicity level is very low and the mRNA in a fertilized egg can express Cas9 protein rapidly and transiently. Recently, highly active purified Cas9 protein has been made commercially available, and is ready for use in genome editing of embryos as soon as it is mixed with gRNAs. The Cas9 and gRNA complex can efficiently modify the rodent genome with a high turnover that induces fewer off-target effects. Several researchers have also reported rodent genome editing using Cas9 protein introduced by microinjection and electroporation [61–63].

Methodologies to introduce CRISPR components such as Cas9 mRNA and gRNA into embryos have also improved the efficiency of gene modification leading to time and cost savings (Fig. 1). Microinjection by manipulating a glass needle under a microscope into fertilized eggs is the main strategy for producing genetically modified rats, as well as the conventional method for producing transgenic animals. However, laboratories routinely

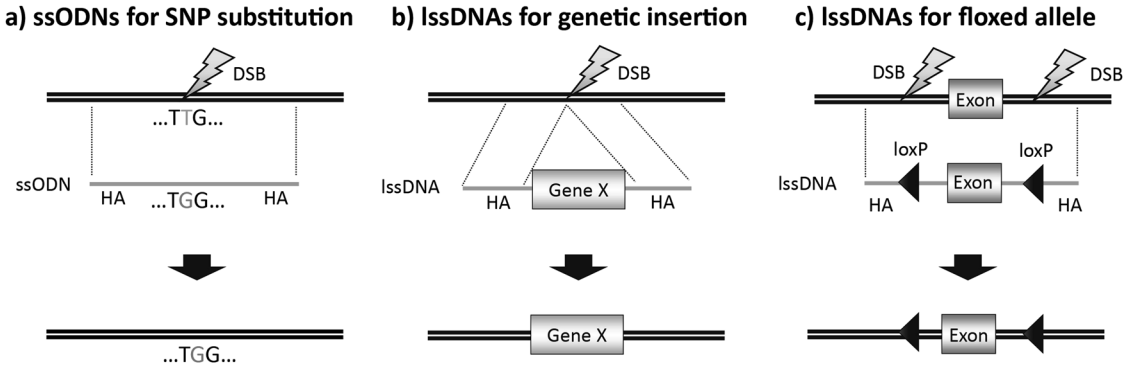

Fig. 2 Strategies for single strand DNA-mediated KI with CRISPR/Cas9. **a** Single-stranded oligodeoxynucleotide (ssODN)-mediated KI for SNP substitution. The introduction of ssODNs with short homology arms can generate SNP substitutions or short fragment insertions at the targeted site of genome editing. **b** Long single-stranded DNA (lssDNA)-mediated KI for large genetic insertions. LssDNAs including exogenous gene sequences with homologous arms can produce genetic insertions at the targeted site, as well as ssODN-mediated KI. The length of homology is typically 50–300 bases, although this should be optimized fort each targeted region. **c** LssDNA-mediated KI for floxed alleles. The co-introduction of two gRNAs and lssDNAs carrying an exon between two loxP sites can induce the genetic replacement of targeted exons with the floxed sequence. *DSB* double strand break, *HA* homology arm

performing this technique are limited because of the requirement for advanced skills and expensive equipment. Recently, several electroporation methods for mouse and rat embryos have been developed to overcome these limitations [64–67]. During electroporation, DNA or RNA can be introduced through fine holes in the egg membrane that have been generated by a series of electric pulses. We successfully optimized electric pulse conditions for fertilized rat and mouse eggs, which led to the highly efficient generation of KO rats [64, 65]. This method can simultaneously introduce DNA and RNA into about 100 cells in one experiment by arranging fertilized eggs in line into the medium. It requires no complicated skills, and can also be applied to fertilized eggs from various species. In addition, not only Cas9 mRNA but also Cas9 protein can be available for gene modification by electroporation [68]. This technique is expected to become a popular method for the introduction of CRISPR components into fertilized eggs because of its high level of convenience.

Efficient methodologies for precise KI

The development of effective genome editing technologies to generate KI models is one of the major research subjects in laboratory animal science. KI animals may carry insertions in their genomes such as additional genes, amino acid tags, fluorescent reporters, or conditional knockout alleles, which aid our study of human disease and provide more sophisticated information about targeted gene functions. The KI strategy using the CRISPR/Cas9 platform is based on the co-introduction of CRISPR/Cas9 components with double-stranded DNA or single-stranded oligodeoxynucleotides (ssODNs). CRISPR/Cas9 induction of a DSB at the target site is restored via homology directed repair using a DNA template homologous to both ends of the DSB site. Utilization of this mechanism enables researchers to insert, delete, or replace genetic material of choice into the genome [55, 69, 70]. However, the KI efficiency depends on the frequency of the HR pathway. Several small molecules, such as RS-1 or Scr7, that enhance the HR pathway or inhibit NHEJ pathways can increase the HR efficiency in cell lines [71–73]. However, researchers still face difficulties with KI in cell lines and animals with low HR frequencies. Furthermore, it is necessary to construct targeting vectors by adding two homology arms of appropriate length to both sides of the inserted DNA sequence.

Several laboratories including our own have reported on targeted KI such as an SNP or small size integration with ssODN donors in mice and rats (Fig. 2) [51, 57]. Although high-grade ssODNs are readily designed and synthesized by several companies, their maximum length is only ~200 bases. Therefore, it is difficult to use this system for integrating long sequences such as green fluorescent protein (GFP) gene cassettes into the genome. To overcome this, laboratory-based strategies have generated long single-strand DNAs (lssDNAs) by PCR-mediated methods including magnetic separation by streptavidin-coated beads [74], lambda exonuclease enzymatic digestion [75], and asymmetric PCR using unequal concentrations of primers [76]. Recently, we demonstrated a novel method to generate highly purified lssDNAs using double nicking endonucleases [77] that digest DNA plasmids including lssDNA sequences between two nickase-specific sites; lssDNAs are then purified by electrophoresis and gel extraction. We microinjected lssDNAs with CRISPR/Cas9 components to generate several types of KI, such as GFP cassette insertions

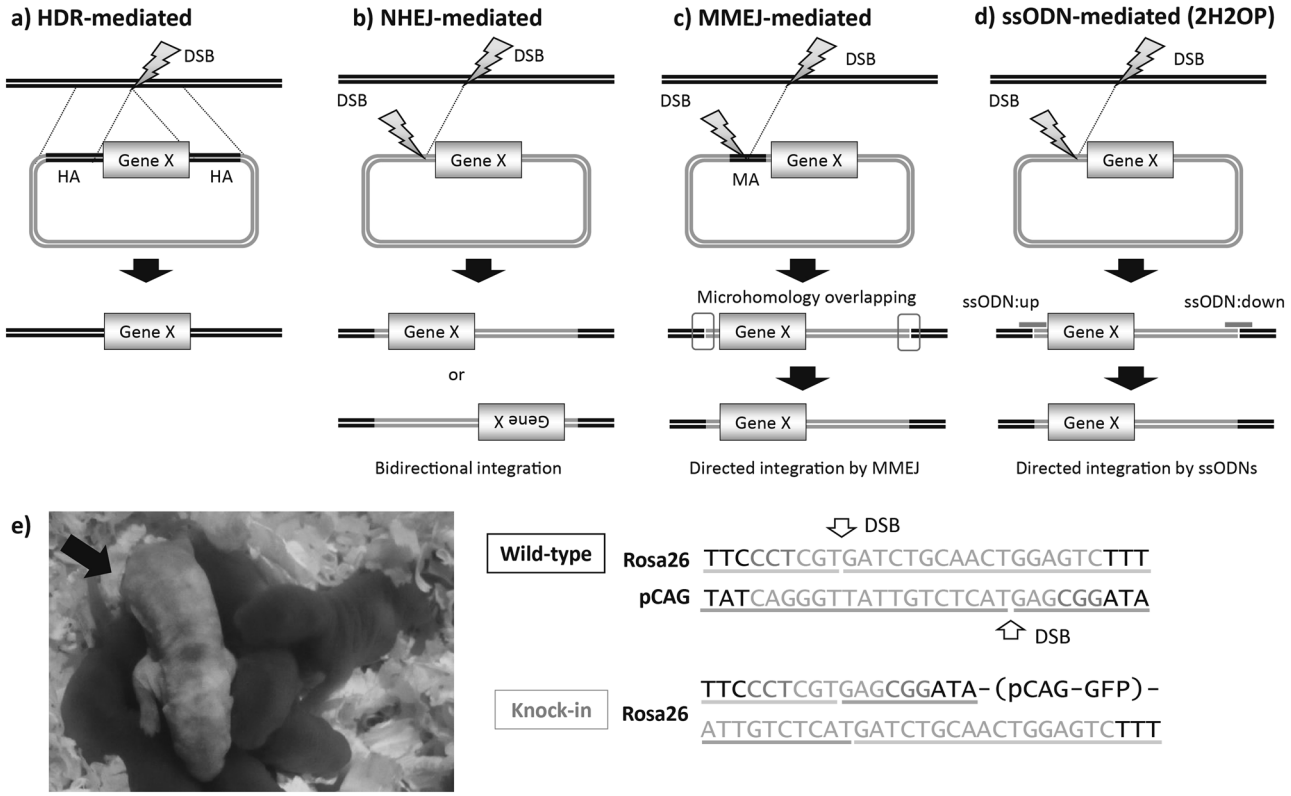

Fig. 3 Strategies for plasmid KI with CRISPR/Cas9. **a** Homologous recombination (HR)-mediated KI. Exogenous genes can be inserted via HR without backbone integration, as well as into ES cells. The KI efficiency of this technique is low and requires the inclusion of 1–2 kb homology arms in the plasmid. **b** Non-homologous end joining (NHEJ)-mediated KI. This technique integrates the plasmid into the cleaved region with a higher efficiency than random integration, but there are several risks including bidirectional and multiple copy insertion. **c** Microhomology-mediated end-joining (MMEJ)-mediated KI. The design of several bases of microhomology allows the generation of directional plasmid integration via the MMEJ repair system. Design and development of the plasmid carrying microhomology arms of cleaved genomic regions must be done with precision. **d** ssODN-mediated KI that we developed as a 2H2OP method. Plasmids can be integrated directionally via ssODN-mediated ligation with no plasmid modification, though the plasmid backbone is also integrated. **e** CAG-GFP knock-in rat (arrow) prepared by the 2H2OP method, and its sequencing analysis. The CAG-GFP plasmid was inserted into an intron of the rat Rosa26 locus. *DSB* double strand break, *HA* homology arm, *MA* microhomology arm

and conditional KO alleles in mice and rats (Fig. 2). Another research group generated KI mice with lssDNAs synthesized by reverse transcription and the digestion of an RNA template [61, 78]. LssDNAs can also be used in electroporation-mediated KI in embryos as a simpler, more rapid, and efficient technique than microinjection-mediated KIs (Miyasaka, unpublished data). In the near future, nucleotide synthesis technology is expected to allow us to synthesize even longer lssDNAs of several kilobases which will extend the possibilities of generating more productive animal KIs.

Alternatively, NHEJ and the microhomology-mediated end-joining (MMEJ) pathway can assist the targeted integration of donor DNAs into DSB sites (Fig. 3). Improved KI strategies using these systems have been reported as ObLiGaRe and CRIS-PITCh methods [79, 80]. When microhomologies from several bases to several tens of bases in length exist at both DNA ends, they can be combined and ligate together via the MMEJ pathway. This enables the directional integration of linearized donor DNA into the DSB site. MMEJ-assisted genome editing strategies have also been reported for the generation of cassette KI in mammalian cells and zygotes [81, 82].

We have developed another strategy for efficient KI in zygotes using the NHEJ system assisted by two ssODNs; this is known as the two-hit by gRNAs and two oligos with the targeting plasmid (2H2OP) method (Fig. 3) [77]. In this technique, the co-introduction of two target-specific gRNAs excises target sites in genomic and plasmid DNA, producing cutting edges without homology arms between the genome and the plasmid. Two short ssODNs carrying homology arms between the genome and the plasmid then assist in ligating the ends cut by CRISPR/Cas9, resulting in the integration of an extrinsic targeting vector into the endogenous targeted region. This method can modify both small and large genomic regions, resulting in the generation of bacterial artificial chromosome KI including the replacement of whole genes, gene clusters, and endogenous promoters [77]. This enables evaluation of the intact in vivo function of targeted genes.

Genetically modified rats elucidate the mechanisms of human disease

While genome editing technologies are progressing rapidly, gene functional analysis using laboratory mice is still at a fundamental level. The characterization of gene function using multiple species is nevertheless vital to clarify universal in vivo functions and species-specific functions of the targeted gene. However, some experiments with genetically modified mice found no significant difference in predicted disease phenotype compared with wild-type. Recent studies with genetically modified rats reported closer phenotypes to human disease than mice; for example, rat models have contributed more to colorectal cancer research. Adenomatous polyposis coli (*Apc*) mutant mouse strains such as the Min mouse demonstrate tumorigenesis in their intestinal tract, so have been used as a human colorectal cancer model [83]. However, their main site of polyp formation is in the small intestine rather than the large intestine where tumors develop in humans with familial adenomatous polyposis. Therefore it is difficult to observe the real-time sequential changes of these tumors in live animals. On the other hand, the *Apc* KO rat developed by ENU mutagenesis spontaneously developed colorectal tumors in the large intestine, similar to humans [84]. It was also possible to observe and manipulate specific tumors using an endoscope, providing us with a useful model of colon cancer development [7].

In immunology research, a KO mouse with severe combined immunodeficiency (SCID) was generated by interrupting the *Prkdc* gene which is crucial for T cell and B cell function. However, homozygous KO mice showed neither fetal lethality nor the decrease in cell proliferation seen in humans with SCID. On the other hand, SCID rats that are deficient in the *Prkdc* gene following the use of TALEN demonstrated significant weight loss and decreased cell proliferation, as well as immunodeficiency [33, 85]. Furthermore, SCID mice with the "leaky" phenotype sometimes recovered their defect immune system through the production of immunoglobulins such as IgG in the blood, whereas SCID rats showed no immune system recovery [86]. This suggests that the rat model could be used for the xenotransplantation of human cancer cells, stem cells, and tissues [87].

KO rats are also useful models for human neurological disorders. For example, *Atm* KO rats showed clear signs of neurodegeneration in the spinal cord and developed hind limb paralysis, as early as 4 months of age, which is likely caused by the loss of motor neurons in the lumbar region of the spinal cord [88]. Moreover, although there are discrepancies between rats and humans, the KO rat can be useful for providing insights into the disease mechanisms of ataxia telangiectasia. In another example, *Bscl2* KO rats showed several abnormalities associated with nervous systems that were not seen in mice [89]. For instance, *Bscl2* associated with fat atrophy was strongly expressed in mouse adipose tissue and testes, whereas rats also expressed it in the brain, similar to humans. This likely explains the reduction of spatial task memory seen in the KO rat model.

In addition to the physiological similarities between KO rat models and human systems, recent studies have demonstrated that the gut bacterial communities of humanized models achieved by fecal microbiota transplantation in rats more closely reflect the gut microbiota of human donors than those of mice. Hence, rats can also be used as representative models of digestive system disorders [90]. These examples show that the choice of animal model depends on the purpose and aim of the study. Indeed, the intensity and distribution of gene expression can be dependent on the species. Therefore, the influence of the species should be carefully considered to obtain reliable and reproducible findings.

Conclusion

In this review, we have described the history and the recent developments in cutting-edge genetic modification technologies. We have also explained the utility of genetically modified rats as models for human diseases. Such rats can be developed quickly with a high level of efficiency compared with ES cell-mediated modification. Moreover, new techniques such as nucleic acid introduction are rapidly progressing alongside in vivo genome editing methods using fertilized eggs. KI generation is one of the most informative approaches for gene functional assessment. It can be used to establish humanized animals as models by introducing SNP or mutations identified in human diseases, and replacing the genomic region of animals with human genomic sequences. These humanized animals are expected to contribute to the elucidation of novel gene functions and to be beneficial in translational medicine.

Taken together, rats are powerful animal models because their biological background and systems are more similar than mice to those of humans. The use of genetically engineered rats in biomedical research can therefore provide fundamental knowledge to understand human physiological and pathological systems and to help develop therapeutic strategies for human disease.

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Compliance with Ethical Standards

Conflict of interest The authors declare no competing financial interests.

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