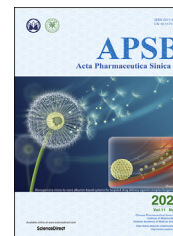




Chinese Pharmaceutical Association
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Acta Pharmaceutica Sinica B

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REVIEW

CRISPR-Cas9: A method for establishing rat models of drug metabolism and pharmacokinetics



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Received 23 September 2020; received in revised form 25 October 2020; accepted 16 November 2020

KEY WORDS

CRISPR-Cas9;
Drug metabolism;
Pharmacokinetics;
Animal model;
Gene editing

Abstract The 2020 Nobel Prize in Chemistry recognized CRISPR-Cas9, a super-selective and precise gene editing tool. CRISPR-Cas9 has an obvious advantage in editing multiple genes in the same cell, and presents great potential in disease treatment and animal model construction. In recent years, CRISPR-Cas9 has been used to establish a series of rat models of drug metabolism and pharmacokinetics (DMPK), such as *Cyp*, *Abcb1*, *Oatp1b2* gene knockout rats. These new rat models are not only widely used in the study of drug metabolism, chemical toxicity, and carcinogenicity, but also promote the study of DMPK related mechanism, and further strengthen the relationship between drug metabolism and pharmacology/toxicology. This review systematically introduces the advantages and disadvantages of CRISPR-Cas9, summarizes the methods of establishing DMPK rat models, discusses the main challenges in this field, and proposes strategies to overcome these problems.

Abbreviations: AAV, adeno-associated virus; ADMET, absorption, distribution, metabolism, excretion and toxicity; BSEP, bile salt export pump; CRISPR-Cas, clustered regularly interspaced short palindromic repeats-CRISPR-associated; crRNAs, CRISPR RNAs; DDI, drug–drug interaction; DMPK, drug metabolism and pharmacokinetics; DSB, double-strand break; HBV, hepatitis B virus; HDR, homology directed repair; HIV, human immunodeficiency virus; HPV, human papillomaviruses; KO, knockout; NCBI, National Center for Biotechnology Information; NHEJ, non-homologous end joining; PAM, protospacer-associated motif; pre-crRNA, pre-CRISPR RNA; RNP, ribonucleoprotein; SD, Sprague–Dawley; sgRNA, single guide RNA; SREBP-2, sterol regulatory element-binding protein 2; TALE, transcriptional activator-like effector; TALEN, transcriptional activators like effector nucleases; tracrRNA, trans-activating crRNA; T7E I, T7 endonuclease I; OATP1B, organic anion transporting polypeptides 1B; OTS, off-target site; WT, wild-type; ZFN, zinc finger nucleases.

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Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

<https://doi.org/10.1016/j.apsb.2021.01.007>

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1. Introduction

Clustered regularly interspaced short palindromic repeats-CRISPR-associated (CRISPR-Cas) system is an adaptive immune system existing in bacteria and archaea¹. CRISPR-Cas9 technology is the third-generation artificial nuclease technology based on the CRISPR-Cas system, and has become a powerful gene editing tool^{2,3}. In particular, the 2020 Nobel Prize in Chemistry was awarded to Emmanuelle Charpentier and Jennifer A. Doudna for their development of the CRISPR-Cas9 for genome editing. CRISPR-Cas9 technology is widely used in the construction of gene editing models both *in vitro* and *in vivo*. In recent years, scientists have constructed and reported a series of gene-edited animal models for the drug metabolism and pharmacokinetics (DMPK) research based on this technology. These models provide excellent tools for the study of drug metabolism and gene functions *in vivo*.

The aim of this review is not only to introduce the basic information of CRISPR-Cas9 technology, but also to discuss the difficulties and challenges of this technology in the construction of ADME-related animal models. Thus, this review firstly introduces the basic information of CRISPR-Cas system and technology, and then compares the advantages and disadvantages of CRISPR-Cas9 with other artificial nuclease technologies. Furthermore, we describe the application of CRISPR-Cas9 technology in the construction of animal models, especially in the generation of DMPK-related animal models. Finally, taking the DMPK animal model as an example, this review summarizes the basic steps of constructing a gene-edited animal model based on CRISPR-Cas9 technology. At the same time, difficulties and challenges in model construction are pointed out, and the corresponding solutions are also proposed.

2. Basic information of CRISPR-Cas9 system

2.1. CRISPR-Cas system

CRISPR-Cas adaptive immune system is a natural defense system adopted by bacteria and archaea to defend against phage infection^{2,3}. It provides microbes with RNA-guided adaptive immunity to foreign genetic elements by guiding nucleases binding and cleaving specific nucleic acid sequences. The discovery of this excellent system has completely changed modern molecular biology. Now it has been engineered as an RNA-guided endonucleases system for genome editing, providing an incredible method for the treatment of genetic diseases. It introduces desirable engineering genetic characteristics, including live cell imaging, functional gene high-throughput screening and nursing diagnosis⁴.

These systems consist of two key components. One is a genomic locus called CRISPR array, which contains a series of short sequences of foreign DNAs called spacers^{5,6}. These spacers enable recognition of specific mobile genetic elements encountered previously. The spacer region is separated by some repetitive regulatory sequences, named repeats. These spacers, together with repeats and the leader sequence (AT-rich), constitute the CRISPR

array^{7,8}. When infected by a new phage, the CRISPR array is expanded by adding a new spacer from the phage genome. Further, the invading foreign DNA can be recognized and inserted into a genome locus to form CRISPR region. The other key component of CRISPR-Cas systems is Cas protein, which is encoded by *Cas* genes on both sides of the CRISPR arrays⁹.

As shown in Fig. 1, CRISPR-Cas system can be divided into six types according to different “signature genes”, and additionally distinguished into two classes based on the structure of the “effector complex”. Class 1 ribonucleoprotein (RNP) consists of multiple subunits, while class 2 system consists of a single unit RNP complex¹⁰. Each class includes three types. Types I, III, and IV systems are included into class 1 system, and types II, V, VI systems are grouped into class 2 system. Types I–III systems are distinguished with the differences of signature proteins: Cas3 for type I, Cas9 for type II and Cas10 for type III. Different from the above systems, type IV system has Csf1, an uncharacterized protein. Type V system also contains a Cas9-like single nuclease, either Cpf1, C2c1, or C2c3, depending on the subtype, and type VI system has C2c2¹¹. Finally, according to additional signature genes and characteristic gene arrangement, each type is divided into multiple subtypes¹⁰. Type I contains subtypes A–F and U. Type III contains subtypes A–D. Type II contains subtypes A–C. Type V contains subtypes A, B and U. Type VI contains subtypes A–C^{12,13}.

The immune mechanism of CRISPR includes three stages: adaption, maturation and interference. During the adaption phase, foreign genome elements are recognized, acquired, and integrated into the CRISPR array as a new spacer. The maturation phase includes the transcription of CRISPR array, which contributes to the formation of pre-CRISPR RNA (pre-crRNA). And the pre-crRNA is further processed into mature CRISPR RNAs (crRNAs), each of which contains a single spacer¹⁴. Subsequently, crRNAs are incorporated into RNP complexes as effector complexes along with Cas proteins¹⁵. This effector complex recognizes the nucleic acid encoded by crRNA, and then triggers the interference stage, resulting in the degradation of the recognized nucleic acid complementary to the crRNA spacer sequence^{16–18}. These steps are performed by Cas proteins^{11,19}.

2.2. CRISPR-Cas9 technology

CRISPR-Cas9 is a type II CRISPR-Cas system, which has been developed into a gene editing technology. The technology involves two key components: single guide RNA (sgRNA) matching the target gene and Cas9 protein causing double strand DNA break²⁰. Two components are necessary for the sgRNA: a target specific crRNA and a trans-activating crRNA (tracrRNA). These two parts work together to guide Cas9 protein to bind to specific genomic sites through the principle of complementary base pairing between crRNA sequence and target sequence.

After binding to the crRNA/tracrRNA duplex, Cas9 begins to bind and scan the foreign DNA containing the protospacer-associated motif (PAM) located at the 5' end of the protospacer sequence, where Cas9 protein binds and mediates a specific double-strand break (DSB)²¹. The PAM consists of a canonical

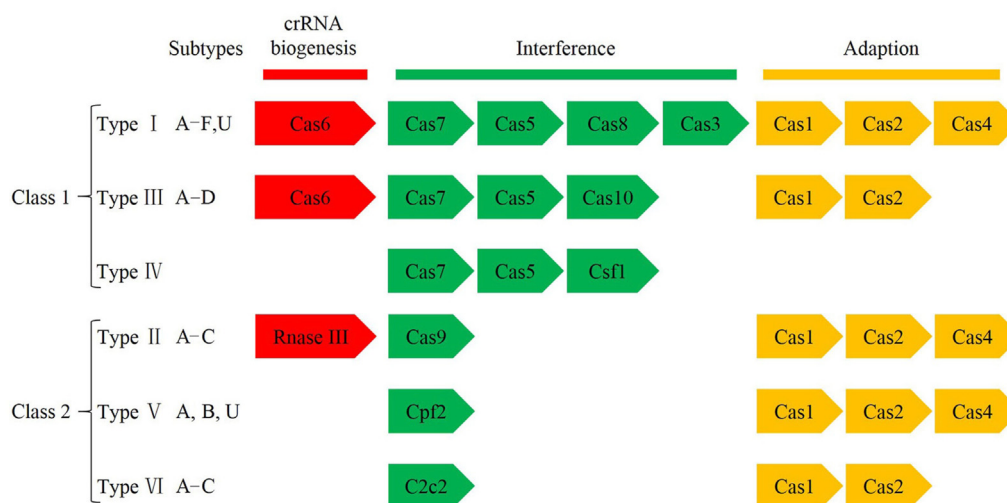


Figure 1 Classification of CRISPR-Cas systems.

Table 1 Technical characteristics of three kinds of artificial nuclease.

Characteristics	ZFN	TALEN	CRISPR
Components	DNA binding and cleavage domain	TALE protein DNA cleavage domain	sgRNA Cas9 protein
Recognition sites	(9–18 bp)*2	(8–31 bp)*2	20 bp+NGG*1
Disadvantages	High cost Cytotoxicity Cumbersome operation	Cytotoxicity Large size Prokaryotic origin	Poor specificity PAM limitation
Advantages	Simple design High specificity	High success rate Design flexibility	High efficiency Easy construction Low off-target

NGG or NAG sequence²². The DNA strand is then unwound, allowing sgRNA to perform complementary verification. A DSB is produced by positive recognition and can be repaired by either non-homologous end joining (NHEJ), typically leading to random insertion/deletion, or by homology directed repair (HDR), in which the homologous section of DNA is used as a repair template²².

As a reliable and adaptable tool, CRISPR-Cas9 system has greatly accelerated the pace of research and has been applied in a wide range of fields. Because of its practicability, simplicity and efficiency²³, it has been engineered for genome editing, and then applied to the establishment of animal models, screening of functional genomes and correction of genetic disorders¹⁹. It is worth noting that with the deepening understanding of CRISPR component transmission, more opportunities in the CRISPR-Cas9 genome editing *in vivo* will appear. Therefore, CRISPR-Cas9 has developed into a general technical tool for gene editing, especially for genome editing *in vivo* to generate powerful animal models.

2.3. Advantages and disadvantages of CRISPR-Cas9 technology

Artificial nuclease is a revolutionary improvement in biological research, including zinc finger nucleases (ZFN), transcriptional activators like effector nucleases (TALEN) and CRISPR (Table 1). In fact, these emerging technologies have greatly expanded the ability to generate animal models, which are used in disease research and DMPK.

In 2009, the first knockout (KO) rat was reported by using ZFN²⁴. ZFN consists of two functional components: the DNA binding domain of zinc-finger protein and the DNA cleavage domain of Fok I endonuclease. ZFN works in dimetric form, and its target site size is (9–18 bp)*2. The zinc-finger protein region includes three to six Cys2–His2 fingers, which recognize a triplet nucleotide code. After recognizing the binding site, it dimerizes and activates Fok I endonuclease, and then cleaves DNA^{25–27}. Similar to ZFN, TALEN consists of two parts: transcriptional activator-like effector (TALE) protein and DNA cleavage domain. The TALEN is a modular DNA recognition code secreted by the plant pathogenic bacteria genus *Xanthomonas*, which can identify a target with (8–31 bp)*2. And TALE protein contains DNA-binding domains composed of repeated 33–35 amino acid sequence^{26,28}. Just like ZFN, the DNA cleavage of TALEN is coupled to the nonspecific nuclease Fok I. As mentioned above, CRISPR-Cas9 system contains two key components: sgRNA and Cas9 protein, enabling it to recognize targets with a size of 20 bp+NGG*1.

ZFN, TALEN and CRISPR-Cas9, as genome editing tools, have great potential in biomedical research. However, there are still many challenges to be addressed in these three technologies. One of the main challenges is the specificity of gene editing methods. Thus, it is necessary to study new methods to control off-target effects. In addition, the existing delivery methods should be optimized to achieve an adequate level of efficiency²³. Although ZFN is special, it needs highly skilled experts along with screening of ZFN library to engineer it, while TALEN is

easier to construct. However, compared with ZFN, TALEN has the disadvantages of large size, prokaryotic origin, and cytotoxicity. The large size of TALEN may limit its delivery through size-restricted vectors such as adeno-associated virus (AAV), which has been shown to be adaptive to ZFN genes²⁹. At the same time, TALEN is a complicated system due to its repetitive sequences, which leads to its complex delivery system and structure³⁰. CRISPR-Cas9 is a simplified genome editing tool, which has aroused the interest of the scientific community. Its main advantage is that it works through Watson-Crick base-pairing between sgRNA and target DNA²⁹. Unlike ZFN and TALEN technologies, CRISPR does not rely on protein engineering. Furthermore, CRISPR-Cas9 is simple and efficient. It only needs to synthesize a small fragment of RNA to recognize the target²³. Technical characteristics of three kinds of artificial nuclease are summarized in Table 1. In short, all these systems have some shortcomings, but the comparison with the other two systems highlights the characteristics of CRISPR-Cas9. Therefore, CRISPR-Cas9 system is a feasible genome editing method, and has been widely used in animal model construction.

3. Application of CRISPR-Cas9 technology in animal model generation

3.1. Animal models for disease research

One of the most exciting applications of CRISPR-Cas9 is the generation of animal models for the study of a variety of diseases. Direct injection of Cas9 mRNA and sgRNA for gene editing of single cell embryos is a new method for rapid establishment of animal models²³. This approach has been successfully applied to the generation of animal models, such as mice³¹, rats³², monkeys³³, zebrafish³¹ and cattle³⁴. In particular, transgenic animals can be changed more easily, faster and more efficiently³⁵. These animal models may be important *in vivo* models for diseases, such as cancer, bone disease, immunodeficiency disease and many other inherited human diseases. A good example in the establishment of animal models for tumor research was done on lung cancer by establishing a Cre-dependent Cas9 knock in mice³⁶. A prominent example in the study of cardiovascular disease is the generation of transgenic mice with severe heart failure by using AAV9 to transfer sgRNA targeting *Myh6* locus of cardiomyopathy³⁷. Furthermore, CRISPR-Cas9 system has also been used to establish animal models of infectious disease like human immunodeficiency virus (HIV), human papillomaviruses (HPV)^{38,39}, and chronic hepatitis B virus (HBV)^{40–42}.

3.2. Animal models for DMPK research

Apart from disease models, CRISPR-Cas9 system is also widely used for the generation of DMPK-related animal models. DMPK mainly studies the absorption, distribution, metabolism, excretion and toxicity (ADMET) of drugs⁴³. It is necessary to understand the properties of ADMET for drug discovery and development^{44,45}. The CRISPR-Cas9 system, as a powerful genetic engineering tool, provides an effective method for DMPK and drug–drug interaction (DDI) research⁴³. Now, CRISPR-Cas9 system enables scientists to generate more reliable and predictable animal models of ADMET *in vivo*. This system has been applied to the study of drug metabolism genes, drug transporters, nuclear receptors and ADMET gene regulatory elements¹. Rat

models for DMPK research, generated by using CRISPR-Cas9, are summarized in Table 2^{46–52}.

3.2.1. Animal models for drug metabolizing enzyme research

Currently, the animal models for drug metabolizing enzyme mainly focus on CYP enzymes. These models are majorly based on Sprague–Dawley (SD) or Wistar rats, and the targeted genes code proteins in CYP families 1, 2 and 3, such as *Cyp2c11*⁴⁹, *Cyp2e1*⁴⁶, *Cyp2j3/10*⁵⁰, *Cyp3a1/2*⁴⁸. We have reported the first CRISPR-Cas9 mediated gene KO rat model of *Cyp2e1*⁴⁶. Then, the *Cyp3a1/2* double KO rat model is also generated by CRISPR-Cas9 system⁴⁸. The *in vitro* (substrate: midazolam and nifedipine) and *in vivo* (substrate: nifedipine) metabolism studies of *Cyp3a1/2* demonstrated the functionally inactivity of *Cyp3a1/2* in KO rats⁷², which provided a powerful tool for the study of ADMET. Moreover, we investigated the potential effect of CYP3A on the pharmacokinetic interaction between erlotinib and docetaxel using *Cyp3a1/2* double KO rat model⁵³. Our data suggest that CYP3A is a key factor in docetaxel inhibiting erlotinib biotransformation *in vitro* and *in vivo* in rats. It is also concluded that *Cyp* KO rat model is a powerful animal model for studying DDI. In 2018, the *Cyp2c11*-null rat model was also successfully generated by using the CRISPR-Cas9 method. Using tolbutamide as probe substrate, the metabolic function of CYP2C in KO rats was detected, but there was no significant difference between WT and KO rats⁴⁹. In the further study, this *Cyp2c11*-null rat model was used to explore not only the effects of CYP2C11 on the pharmacokinetics and pharmacodynamics of warfarin⁵⁴, but also the effects of CYP2C11 on vasorelaxation *in vivo*⁵⁵.

3.2.2. Animal models for drug transporter research

Drug transporter KO model is a useful tool to study the pharmacokinetics based on DDI. It has been reported that quizartinib (a second-generation FLT3 inhibitor) is transported through multidrug efflux transporters, such as ABCB1 and ABCG2, by using *Abcb1a/1b*^{-/-}, *Abcb1a/1b* and *Abcg2*^{-/-} mice. It provides a possible rationale for using ABCB1/ABCG2 inhibitors together with quizartinib to increase its concentration in the brain to treat malignant lesions situated behind the blood–brain barrier⁵⁶. To date, the research on gene editing of drug transporters by CRISPR-Cas9 has focused on the ATP-binding cassette transporters¹. A Madin-Darby canine kidney II cell line was established *in vitro* by using CRISPR-Cas9 to knock out endogenous canine MDRI⁵⁷. And various *in vivo* transporter models for ADMET studies have also been developed by using CRISPR-Cas9 system. These models allow the study of the role of specific drug transporters in DMPK

Table 2 DMPK-related rat models generated by using CRISPR-Cas9.

Gene	Strain	Modification	Year
Drug metabolizing enzyme			
<i>Cyp2e1</i>	SD rats	KO	2016 ⁴⁶
<i>Cyp2d1/2/3/4/5</i>	Wistar rats	KO and <i>CYP2D6</i> knock in	2016 ⁴⁷
<i>Cyp3a1/2</i>	SD rats	KO	2017 ⁴⁸
<i>Cyp2c11</i>	SD rats	KO	2018 ⁴⁹
<i>Cyp2j3/10</i>	SD rats	KO	2020 ⁵⁰
Drug transporter			
<i>Abcb1a/b</i>	SD rats	KO	2019 ⁵¹
<i>Slc10b2</i>	SD rats	KO	2020 ⁵²

research. For example, we generated a novel *Abcb1* (*Abcb1a/b*) double KO rat model by the CRISPR-Cas9 system, which can be used as a powerful tool to study the function of MDR1 in drug absorption, tumor multidrug resistance and drug target validation⁵¹. In addition, the *Slco1b2* KO rat model was also successfully constructed by CRISPR-Cas9 technology⁵². We further applied this model to explore the role of organic anion transporting polypeptides 1B (OATP1B) in the interaction between paclitaxel and sorafenib⁵⁸.

3.3. Animal models for physiological function research of DMPK genes

3.3.1. Animal models for physiological function research of CYP isoforms

CYP isoforms (CYPs) play an important role in chemical metabolism, toxicity, and carcinogenicity. Most drugs are inactivated by CYPs, either directly or by promoting excretion from the body. At the same time, various endogenous substances are bioactivated by CYPs to form active compounds. Therefore, the loss of CYPs has a profound impact on the metabolism and physiological functions of some endogenous substances. Now, the CYP KO animal models established by CRISPR-Cas9 have been a powerful tool to study the physiological function of CYPs. Members of the CYP3A subfamily are the most abundant CYP enzymes both in the liver and small intestine^{59,60}. *Cyp3a* KO (*Cyp3a*^{-/-}) mice were used to investigate the effects of CYP3A deficiency on gene expression responsible for cholesterol influx, efflux, metabolism, and biosynthesis⁶¹. Compared with wild-type (WT) mice, *Cyp3a*^{-/-} mice had higher mRNA levels of cholesterol biosynthesis and bile acid synthesis related enzyme genes, higher nuclear levels of sterol regulatory element-binding protein 2 (SREBP-2), and lower liver cholesterol levels. These results suggest that decrease of hepatic total cholesterol in *Cyp3a*^{-/-} mice may be the result of increased bile acid synthesis. However, the concentrations of cholesterol and bile acids in *Cyp3a1/2* KO rat model (generated by CRISPR-Cas9) did not change⁴⁸. This may reflect the species differences in the metabolism of cholesterol and bile acids mediated by CYP3A. As a powerful tool, *Cyp3a*^{-/-} mice have also been used to study the relationship between CYP3A and testosterone in the prostate⁶². The subsequent study reported a significant increase in plasma free testosterone levels in *Cyp3a*^{-/-} mice and a decrease in the activity of testosterone 6 β -hydroxylation in liver microsomes. Based on remarkably increased mRNA expression levels of the AR target genes and more abundant AR bindings to the promoter region in the prostate, *Cyp3a* deficiency promotes *Scap* expression by activating AR. The increase of testosterone was also observed in *Cyp3a1/2* KO rats. However, the mechanism and role of elevated testosterone need to be further explored⁴⁸. Interestingly, the number of sperm in testis of *Cyp2c11* KO rats decreased and puberty was delayed for 20 days, indicating that CYP2C11 is related to the hydroxylation of testosterone and prostaglandin⁴⁹. In addition, CYP2C11 also plays an important role in the regulation of blood pressure mediated by epoxyeicosatrienoic acid^{49,55}.

3.3.2. Animal models for physiological function research of drug transporters

Drug transporters, which allow drugs to enter and leave cells through carrier-mediated pathways, are now considered to be an important determinant of some endogenous substance⁶³. With the rapid development of CRISPR-Cas9 gene editing technology,

animal models of drug transporter gene deletion have been generated and applied to the study of physiology and pathology.

OATP is an important family for pharmacokinetics formed by influx transporters expressed in different tissues⁶⁴. In *Oatp1a/1b* and *Abcc3* deficient mice, ABCC3 secretes bilirubin conjugates into the blood, and the OATP1A/1B transporter mediates liver resorption⁶⁵. In the contrast, the *Oatp1b2* KO rats generated by CRISPR-Cas9 show the phenotypes of Rotor syndrome and hyperbilirubinemia, and show the characteristic of significantly increased bilirubin concentration in blood⁵². Therefore, the *Slco1b2* KO rat model may be an important disease model for the study of Rotor syndrome, hyperbilirubinemia, and some hyperbilirubinemia-related diseases.

CRISPR-Cas9 gene editing of drug transporters has also focused on ATP-binding cassette transporter family, where it has been applied in the KO of *Abcb1* homologs in the zebrafish and rat^{1,51}. Ellis et al.⁶⁶ applied CRISPR-Cas9 genome editing technology to delete *Abcb11b* in zebrafish and found that these mutants died prematurely. By histological and ultrastructural analyses, they also found that hepatocytes of *Abcb11b* KO zebrafish failed to excrete bile acid with fluorescent tag, which is a substrate of human bile salt export pump (BSEP). We used CRISPR-Cas9 system to construct *Abcb1a/b*^{-/-} rats, and found the serum levels of direct bilirubin, indirect bilirubin, total bilirubin, HDL-cholesterol, total cholesterol, and total bile acid were significantly increased in KO rats. In addition, the expression of *Abcg5* and *Abcg8* in the liver of KO rats was significantly lower than that in WT rats, indicating that cholesterol and bile acid may be deposited in the body, and bilirubin may be the endogenous substrate of MDR1⁵¹.

4. Challenges of CRISPR-Cas9 animal model construction: A case study of DMPK model

Although the CRISPR-Cas9 system is widely used in the construction of gene editing animal models, there are still some problems worthy of attention. In this part, we will briefly introduce the general process of CRISPR-Cas9 mediated gene KO. The flowchart of gene editing animal model construction based on CRISPR-Cas technology is summarized in Fig. 2. In addition, this part will take the DMPK model construction as an example to illustrate the challenges in the model construction.

4.1. Target sites prediction and selection

The gene sequence is obtained from the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/pubmed/>). According to the annotation (under the format of 'Gene Bank') of the gene, the cDNA sequence can be obtained. The "CRISPR DESIGN" online system (<http://crispr.mit.edu/>) can predict potential target sites by submitting the cDNA sequence of the gene. Here are some tips for target selection. First, in order to knock out the gene of interest more completely, the target site is preferably selected in the exon near the start codon. For example, target sites for the KO of *Cyp3a1* and *Cyp3a2* in rats are selected in the second exon, which are just downstream to the initiation codon⁴⁸. If the exon near the start codon is too short to find a suitable target site, the target site can be searched downstream. For the targeting of *Abcb1a* and *Abcb1b*, the target is selected on the third exon because the first two exons are less than 70 bp⁵¹.

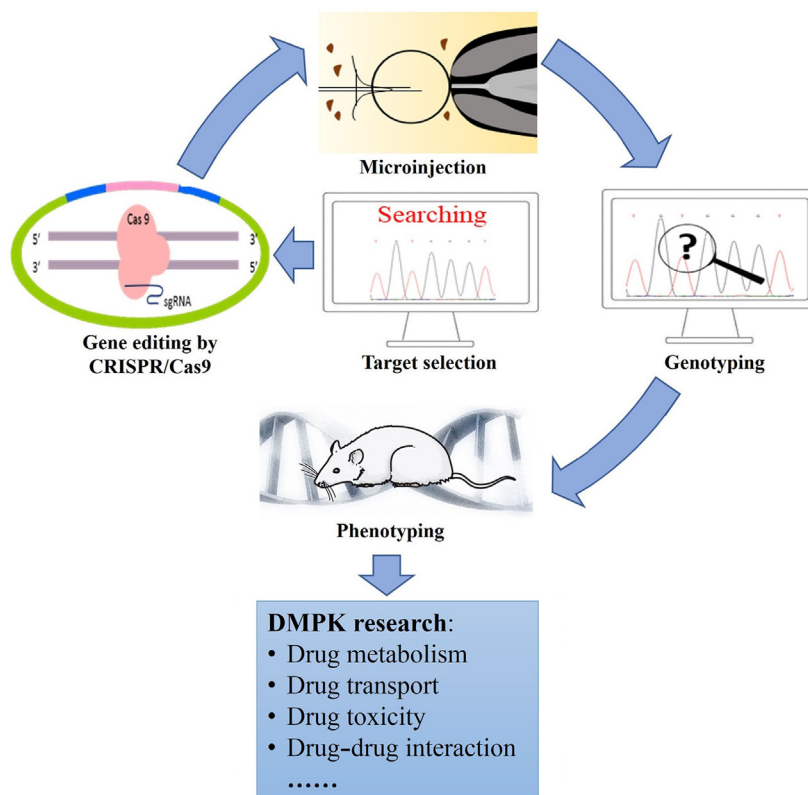


Figure 2 Flowchart of gene editing animal model construction based on CRISPR-Cas9 technology.

Second, in order to improve the targeting efficiency, multiple target sites can be designed for the same gene of interest.

4.2. Construction of sgRNA *in vitro*

RNA injection method is chosen to generate double DNA strand breaks. To synthesize the sgRNA *in vitro*, the T7 *in vitro* Transcription Kit was employed. The procedure is summarized as follows:

- i. Single-stranded DNA (60 bp) containing T7 promoter sequence, the target site sequence (18 bp) and partial sequence homologous to the sgRNA backbone, successively from 5' end to 3' end, was synthesized.
- ii. The single-stranded DNA and sgRNA backbone were spliced by overlap PCR to form the sgRNA transcription precursor corresponding to the target.
- iii. The transcription products of sgRNA were synthesized from its precursor by using the T7 *in vitro* Transcription kit.
- iv. The sgRNA was purified by RNA purification kit, and then stored at -80°C after being tested by agarose gel electrophoresis.

4.3. Construction of Cas9 mRNA *in vitro*

The Cas9 expression vector is available on the market. To synthesize Cas9 mRNA *in vitro*, the Sp6 *in vitro* transcription kit can be used. The procedure is summarized as follows:

- i. The expression vector of Cas9 was linearized by endonuclease and purified by phenol chloroform extraction.
- ii. Using the linearized plasmid as a template, Cas9 mRNA was transcribed through Sp6 *in vitro* transcription kit.
- iii. The Cas9 mRNA was purified by RNA purification kit, and then stored at -80°C for further use, after being tested by agarose gel electrophoresis.

4.4. Co-microinjection of sgRNA and Cas9 mRNA into zygotes

The sgRNA and Cas9 mRNA need to be delivered into cytoplasm of the zygotes to perform their gene editing function. Therefore, co-microinjection technology is used to achieve this purpose. The general steps of co-microinjection are summarized as follows:

- i. Superovulation induction. Eight-week-old female rats were intraperitoneally injected with horse pregnant serum gonadotropin and human chorionic gonadotropin to induce superovulation.
- ii. Mating. The super ovulated female rats mated with normal male rats to form fertilized eggs.
- iii. Obtaining fertilized eggs. Female rats with vaginal suppository (indicating successful mating) were euthanized. The fertilized eggs were removed from the fallopian tubes, placed in the medium and incubated at 37°C for 2 h.
- iv. Microinjection. The sgRNA and Cas9 mRNA were mixed and co-injected into the fertilized eggs by microinjector.

- v. Pseudo-pregnant rat preparation. Male rats ligated the fallopian tubes, and then mated with female rats to generate the pseudo-pregnant rat. On the next day, the vaginal suppository was checked. The detection of vaginal suppository indicated the pseudo-pregnant rats were successfully prepared.
- vi. Fertilized eggs transfer. The surviving fertilized eggs were transplanted into the oviduct of the female pseudo-pregnant rats for natural implantation and development.

Both Cas9 mRNA and Cas9 protein can be injected into zygote, and each method has its own advantages and disadvantages. For Cas9 sgRNA, the preparation process is relatively simple, while the relatively long-term transcription activity of sgRNA may increase the risk of off-target effects. For Cas9 protein, it can work directly as a functional protein without the need for transcription, and the short half-life may reduce the possibility of off-target occurrence. However, the synthesis of Cas9 protein *in vitro* is relatively complex and the experimental period is long.

4.5. Genotyping of founders and progenies

T7 endonuclease I (T7E I) assay can be applied for the genotyping of F₀ chimeras preliminarily. The genomic DNA of founders is usually extracted from the toes of newborn rats with a standard extraction procedure. The DNA sequences flanking the target site should be amplified for genotyping of founders and progenies. An anneal program can be conducted with PCR products to generate mismatched heteroduplexed DNA. Then resulting products were digested with T7E I for 30 min, and detected by agarose gel electrophoresis. If T7E I digestion produces a small band, it indicates that the offspring may have a gene editing at the target site.

To identify the modification details of F₀ founders, PCR products amplified from each rat toe genome can be cloned into pMD-18T vectors according to the instructions of TA cloning kit. Then the precise sequence around the target site can be obtained by gene sequencing. For the genotyping of F₁ or F₂ progenies, PCR products can be directly sequenced.

4.6. Off-target site validation

Target-sites can be submitted to the Cas9 online designer (<http://cas9.wicp.net/>), which contains a genome-wide sgRNA off-target searching tool. Off-target site (OTS) with relatively high score should be selected for further PCR analysis. The off-target effects should be evaluated by T7E I digestion or gene sequencing. Furthermore, to evaluate off-target effects more comprehensively, the deep sequencing is generally used⁶⁷.

4.7. Expression detection of target gene

In order to further confirm the success of gene deletion, it is necessary to detect the mRNA and protein expression levels of the target gene in KO rats. In general, the mRNA level of target gene is detected by Q-PCR or agarose gel electrophoresis. The protein level of target gene is usually measured by Western blot or immunohistochemical analysis.

In order to verify whether the knocked-out gene will produce a truncated protein, it is better to use an antibody that specifically recognizes the N-terminus of the protein for protein expression

analysis. However, this may be limited by commercial antibodies. In some cases, the protein band or spot may also be detected in the KO model. For example, we have reported a *Cyp3a1/2* double KO rat model, but the CYP3A band can be detected in the liver tissue of KO rats by Western blot analysis⁴⁸. This may be because the antibody used for protein detection can recognize the truncated protein produced by the deleted gene. In addition, it may be due to the existence of homologous subtypes, which has a similar sequence to the target gene, and the antibody used can also recognize this homologous subtype. To solve this problem, it is necessary to develop and prepare more specific antibodies. With these customized antibodies, some specific isoform or region (such as C-terminus) of the target gene can be detected.

4.8. Function evaluation

The method of function evaluation depends on the role of the KO gene. For DMPK-related genes, their functions mainly include drug metabolism and transport. CYP is an important phase I drug metabolizing enzyme, and the probe substrates are generally used to verify the function of KO animal model^{46,48,49}. For example, nifedipine and chlorzoxazone are used to detect the metabolic function of *Cyp3a1/2* and *Cyp2e1* KO rats *in vitro* and *in vivo*, respectively. Although gene KO can significantly reduce the metabolism of probe substrate, it does not mean that the substrate is not metabolized in KO rats. Sometimes gene KO may not change the metabolism of certain probe substrates⁴⁹. The main reason is that the so-called probe substrate may be metabolized by other enzymes at the same time. Therefore, different substrates should be selected to verify the function of KO model.

4.9. Compensatory changes in the expression of other ADME related genes

To explore whether the deletion of one gene will lead to the expression changes of other ADME related genes, the compensatory expression of other specific proteins can be measured. The ADME related proteins to be detected include absorption transporters (such as OATP1B2), efflux transporters (such as MDR1), phase I enzymes (such as CYP) and phase II enzymes (such as UGT). The compensatory changes can be measured at the mRNA level, while it is better to perform compensatory expression testing at the protein level if commercial antibodies are available or easy to be prepared. KO of the ADME gene may lead to the change of genes with similar or opposite functions. These changes need to be considered in the subsequent experimental design and results analysis. In addition to expression, the most direct way to verify compensation is to detect the function of compensated genes, which is generally applicable to genes with expression compensation.

4.10. Physiological and pathological evaluation

More and more studies have found that ADME related genes not only participate in the clinical drug disposition, but also participate in the disposition of many endogenous substances^{68,69}. The latter may affect the concentration of endogenous substances, thereby changing the physiological or pathological state of the body. Therefore, it makes sense to evaluate the physiological and pathological changes of the KO rat. The methods to study the

physiological and pathological changes of KO rats generally include the detection of blood biochemical index, tissue and organ sections and staining. Furthermore, other tests related to the potential function of KO genes can also be used to further improve the assessment of the physiology and pathology of KO animals. For example, the testosterone concentration of CYP3A KO model and the cholesterol concentration of the *Abcb1* KO model should be included in physiological and pathological evaluation^{48,51}. In addition, the physiological and pathological evaluation of KO animal model can not only fully characterize the animal model, but also discover the new functions of ADME related genes.

5. Expectations

Due to its simplicity and ease of use in genome editing, CRISPR-Cas9 has been widely used in animal construction. In order to improve the efficiency and accuracy of the system, various strategies have been explored to increase the delivery efficiency and reduce the off-target effect. The introduction of Cas9 protein into nucleus is a major obstacle to the application of CRISPR-Cas9. It can be delivered in a variety of formats, including viral/plasmid-based approaches, Cas9-encoded RNA and direct Cas9 protein delivery⁷⁰. In the future, some novel CRISPR delivery methods may be explored to improve the delivery efficiency. In fact, the off-target effect is an unavoidable issue of CRISPR-Cas9 mediated gene targeting technology. The off-target problem of CRISPR-Cas9 system not only affects its application in clinical gene therapy, but also affects the construction of experimental animal models to a certain extent. With the development of off-target detection technology (including deep sequencing, GUIDE-seq and BLESS), the off-target effect of the CRISPR-Cas9 system will be evaluated more comprehensively. Various approaches have been taken to reduce off-target mutations, including SpCas9-HF1, a highly-fidelity variant harboring alternations designed to reduce non-specific DNA contacts. In addition, the discovery of new Cas genes that can recognize different PAM sequences may be a potential solution to the off-target problem. Despite these limitations, CRISPR can cut the time and cost of making new animal models by one-third, thus putting tailor-made models within reach of a wide range of researchers⁷¹. With the increase of delivery efficiency and the decrease of off-target effect, the targeting efficiency of CRISPR-Cas system will be further improved.

Both rats and mice are powerful model animals for medical research, and the rat model is more important for pharmacological research, especially for DMPK studies. The larger size of the rats enables the study of distance effects of drug administration to specific anatomical areas and enables serial blood draws for drug analysis⁴⁶. In addition, rats have biological characteristics that are more similar to humans in certain diseases, such as diabetes and breast cancer, compared to mice^{43,46,48}. Therefore, the rat model may be the preferred model of drug metabolism in some cases.

CRISPR-Cas9 technology can be used not only to construct gene KO rat models, but also to construct humanized rat animal models. The humanized rat model of ADME genes is a potential tool to overcome the difference in drug metabolism between rats and humans⁴³. First of all, CRISPR-Cas9 technology breaks through the limitations of gene editing technology in rat species.

Secondly, CRISPR-Cas9 technology has the characteristics of high efficiency, simple construction process, and low cost. These features make it the best choice for constructing humanized rat models of ADME-related genes. However, different from the construction of gene KO rat model, the construction of humanized rat model faces the challenges of low integration efficiency and low efficiency of exogenous gene expression. In particular, it is a challenging task to introduce large gene segments (>5 kb in length) into the human genome, which is often desired for humanization. Therefore, it is a promising research direction to explore the effective construction of ADME-related gene humanized rat model.

Although CRISPR-Cas9 technology is widely used in the construction of ADME-related animal models, there is still much more in-depth work to be done. DMPK-related gene editing animal models can be extensively used in the study of metabolism, transport and toxicity of drugs or compounds, and can also be used to explore the mechanism of DDI. Moreover, the physiological and pathological functions of DMPK-related genes are still an important research direction. With the further study of DMPK-related gene editing animal models, we believe that more abundant animal model types, such as humanized model, will be generated and reported. At the same time, more influential achievements will appear in the field of drug metabolism and pharmacokinetics.

Acknowledgments

This work was supported in part by grants from the National Natural Science Foundation of China (No. 81773808) and the Science and Technology Commission of Shanghai Municipality (Nos. 17140901000, 17140901001 and 18430760400, China).

Author contributions

Xin Wang was responsible for the conception and design of the review. Jian Lu, Jie Liu and Yeye Xu collected literatures. Jian Lu, Jie Liu and Yuanqing Guo analyzed literatures and summarized results. Jian Lu and Jie Liu drafted the manuscript. Yuanjin Zhang and Xin Wang revised the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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