ZOOLOGICAL RESEARCH

The big bang of genome editing technology: development and application of the CRISPR/Cas9 system in disease animal models

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ABSTRACT

Targeted genome editing technology has been widely used in biomedical studies. The CRISPR-associated RNA-guided endonuclease Cas9 has become a versatile genome editing tool. The CRISPR/Cas9 system is useful for studying gene function through efficient knock-out, knock-in or chromatin modification of the targeted gene loci in various cell types and organisms. It can be applied in a number of fields, such as genetic breeding, disease treatment and gene functional investigation. In this review, we introduce the most recent developments and applications, the challenges, and future directions of Cas9 in generating disease animal model. Derived from the CRISPR adaptive immune system of bacteria, the development trend of Cas9 will inevitably fuel the vital applications from basic research to biotechnology and bio-medicine.

Keywords: CRISPR/Cas9; Animal models; Gene therapy

INTRODUCTION

With more and more patient genomes have been sequenced, a large number of mutations were identified. It has been a primary task to determine the relationship between these mutations and diseases. Genome editing refers to the manipulation of the specific gene loci to gain genome modifications, such as insertions, deletions or point mutations (Cong et al., 2013; Gaj et al., 2013). The appearance of the DNA recombination technology opened the door of molecule biology in the 1970s. Biologists can, for the first time, directly manipulate the DNA molecules and perform some simple genome editing. These molecule biology tools are essential for elucidating the function of targeted genes and regulatory factors. Precise genome editing can help us to generate disease-associated animal models (Dow, 2015). What's more, genome editing technology is sparking a new revolution on drug development and gene therapy (Gori et al., 2015).

Recent advances in genome editing technologies based on programmable nucleases are fruitful, especially the birth of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated enzyme 9 (Cas9) system. Just a few years ago, meganucleases (Thermes et al., 2002), zinc fingers nucleases (ZFN)(Geurts et al., 2009) and transcription activator-like effector nucleases (TALEN) (Cermak et al., 2011) play leading roles in genome editing. Although ZFN and TALEN were demonstrated to be successful gene editing techniques, many drawbacks including time-consuming, low efficiency and specificity significantly restrict their application (Gaj et al., 2013). As a new genome editing technique, CRISPR/Cas9, derived from bacteria or archaea adaptive immune system, can edit genome much more efficiently and specifically (Garneau et al., 2010; Jansen et al., 2002; Jinek et al., 2012). CRISPR/Cas9 has proven to be a powerful and versatile tool for genome engineering in multiple cell types and organisms. Up to now, CRISPR/Cas9 has been successfully applied in bacteria (Bikard et al., 2013; Selle et al., 2015), yeast (DiCarlo et al., 2015; Lee et al., 2015; Mans et al., 2015; Tsai et al., 2015), *C. elegans* (Chen et al., 2014, 2015c), *Drosophila* (Bassett et al., 2013; Gratz et al., 2013), rice (Xu et al., 2014, 2015b; Zhang et al., 2014), zebrafish (Varshney et al., 2015), mouse (Shen et al., 2013), monkey (Niu et al., 2014) and human beings (Mali et al., 2013).

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In this review, we first introduce the latest developments and applications of CRISPR/Cas9 in the past several years. Following that, the origin, formation and functional mechanism of CRISPR/Cas9 are summarized. Next, we focus on the applications of this technique in generating disease animal models. Finally, challenges and future directions are discussed.

The history of CRISPR/Cas system

The history of CRISPR/Cas9 system development is short and rapid (Figure 1). Ishino et al. (1987) first discovered a group of 29-nucleotide repeats divided by non-repetitive short sequences in *E. coli*, which is now known as spacers. The following researches found that the similar interspaced repeats widely exist in the genomes of bacteria and the archaea (Mojica et al., 2000). Jansent et al. (2002) further studied the short repetitive DNA sequences in prokaryotes. The authors named the family as the clustered regularly interspaced short palindromic repeats (CRISPR). Meanwhile, they identified four CRISPR-associated (Cas) genes, suggesting that CRISPR/Cas system might play an important role in biological processes. In 2005, three groups linked this kind of element to the immune system of defending against invading DNA (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). Marakova et al. (2006) predicted that CRISPR might function as immunity defenders through the mechanism analogous to eukaryotic RNA interference (RNAi).

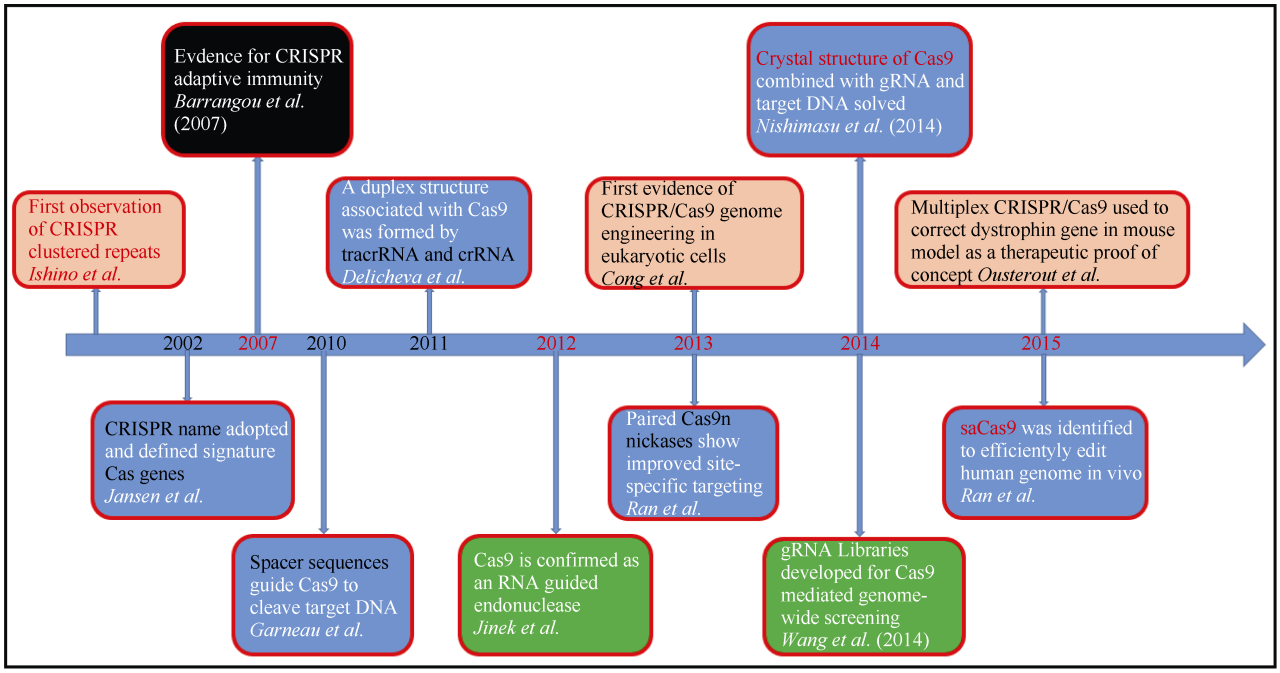

Figure 1 Time line of the CRISPR/Cas system development

Important scientific events happened in the history of CRISPR/Cas9 over the past 30 years.

A key turning point came in 2007, Barrangou et al. (2007) first determined that CRISPR-induced immunity was used to protect bacteria from phage. Marraffini & Sontheimer (2008) did an experiment on *S. epidermidis* CRISPRs and found that the bacteria CRISPR system could prevent the transfer of exogenous plasmid. Because of these important findings, biologists opened the door of elucidating the function mechanism of CRISPR/Cas system. The development of CRISPR/Cas has been dramatically accelerated. Garneau et al. (2010) reveled that spacer sequences guided Cas9 to cleave target DNA. Deltcheva et al. (2011) found that a duplex structure was formed by tracrRNA and crRNA and associated with Cas9. Jinek et al. (2012) demonstrated that Cas9 was an RNA guided endonuclease. Cong et al. (2013) first used CRISPR/Cas9 in eukaryotic cells and successfully achieved efficient and specific genome editing. Nishimasu et al. (2014) addressed the crystal structure of Cas9 and characterized the interactions between Cas9 and gRNA and target DNA. Then Wang et al. (2014) developed gRNA libraries and combined with Cas9 for genome-wide screening. The true value of technology is application, in early 2015, Ousterout et al. (2015) used multiplex CRISPR/Cas9 to cure Duchenne muscular dystrophy in mouse model. Meanwhile, Ran et al. (2015) from MIT identified a smaller Cas9, saCas9, which was proved to be more efficient and specific in mammal genome editing.

Recently, Zetsche et al. (2015) found a new gene editing system, CRISPR/Cpf1. The researchers compared Cpf1 from 16 different bacterial enzymes and found two Cpf1 to shear human DNA. They demonstrated that Cpf1 mediates robust DNA interference with features distinct from Cas9. Cpf1 is a single RNA-guided endonuclease lacking tracrRNA and utilizes a T-rich protospacer-adjacent motif. Moreover, Cpf1 cleaves DNA via a staggered DNA double-strand break (DSB). All these features broaden our understanding of CRISPR/Cas systems

and extend their genome editing applications. With further investigation, more specific and efficient genome editing system may be found to substitute the traditional CRISPR/Cas9.

Structure of CRISPR/Cas9

To date, three types (1-3) of CRISPR systems have been identified in a wide range of bacteria and archaea (Chylinski et al., 2014; Garneau et al., 2010; Makarova et al., 2011). They share three similar construction elements: a group of CRISPR-associated (Cas) genes, CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) (Bolotin et al., 2005; Garneau et al., 2010; Ran et al., 2013a). CRISPR/Cas9 belongs to the type 2 CRISPR system and has been widely used in genome editing of cells and organisms. In type 2 CRISPR system, Cas9 is a critical component, which is an RNA-guided DNA endounuclease enzyme associated with the CRISPR adaptive immunity system in *Streptococcus pyogenes* and other bacteria (Jinek et al., 2012; Chylinski et al., 2014). SpCas9, the most common used Cas9, was acquired from *S. pyogenes*. In the host, it is produced to degrade the invading plasmids or virus with the guidance of crRNA and tracrRNA (Garneau et al., 2010; Jinek et al., 2012).

From the structure of CRISPR-Cas9, scientists uncovered that the Cas9 protein had two functional domains: RuvC and HNH (Figure 2), each nicks one strand of the target DNA and generates a DSB together (Gasiunas et al., 2012). Specificity of cleavage is determined by the matured crRNA, which targets complementary DNA, flanked by a short protospacer adjacent motif (PAM). Additionally, tracrRNA is essential to recognize and cleavage target genes when forms loop with crRNA (Jinek et al., 2012; Upadhyay et al., 2013). Structurally, the native crRNA and tracrRNA duplex can be fused together to create an engineered chimeric, single guide RNA (sgRNA). Typical sgRNA consists of a 20-nt sequence determining the target DNA recognition according to Watson-Crick base pairing (Jinek et al., 2012). CRISPR/Cas9 can be guided to any target sequence adjacent PAM by changing the sgRNA sequence. A key feature of Cas9 recognition and cleavage is based on the PAM near the 3' terminal of the target sequence (Jinek et al., 2012; Sternberg et al., 2014). Different Cas9 orthologs have different PAM sequences. For example, the widely used spCas9 has a common 5'-NGG-3', or at a low frequency of 5'-NAG-3' PAM. To determine the function of Cas9 nuclease, researchers analyzed the crystal structure of Cas9 protein in detail by X-ray crystallography (Nishimasu et al., 2014) and determined that the core structure of Cas9 was consist of two major lobes, a Cas9 recognition (REC) domain and a nuclease (NUC) lobe (Figure 2), both of this lobes are essential for site-specific gene editing (Anders et al., 2014; Jinek et al., 2014).

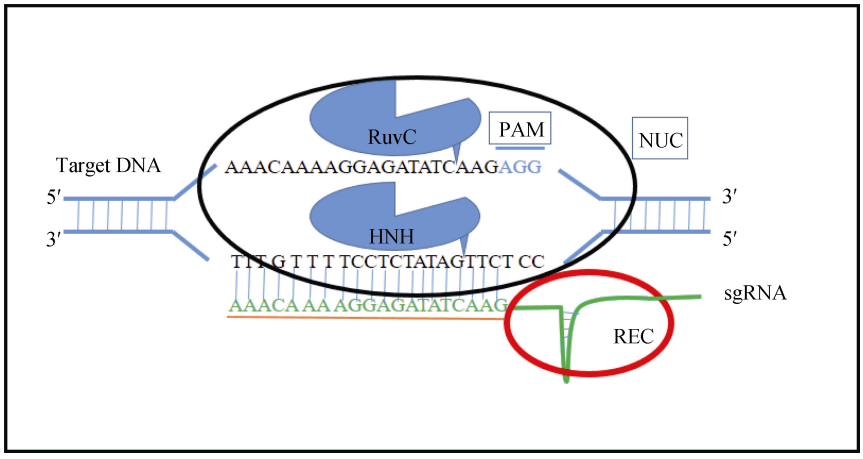

Figure 2 Schematic of the structure of the RNA-guided Cas9 nuclease

CRISPR/Cas9 contains two functional lobes, REC (red) and NUC (black). REC lobe is the Cas9 recognition domain interacting with the sgRNA (green), while the NUC lobe consisting of two nuclease domains (RUVC and HNH) drives the interaction with the PAM and target sequence, leading to a DSB after each nick one DNA-strand.

The mechanism of Cas9-mediated genome editing

The CRISPR/Cas9 system in nature is used to protect bacteria or archaea from invading genetic elements by recognizing and degrading them (Bikard et al., 2013; Garneau et al., 2010). Scientists utilize this feature and reconstruct some programmable engineered Cas9 nucleases from bacteria or archaea. As an example, human codon-optimized Cas9 and the requisite sgRNA are widely used in genome editing of animal cells and organisms. The mechanism of Cas9-mediated genome editing can simply divided into three steps.

Recognition

Accurate recognition of target sequence is critical for precision cleavage. The REC domain of Cas9 played an important role in the interactions between sgRNA and Cas9 (Jinek et al., 2014). After sgRNA-Cas9 complex formation, the Cas9 nuclease recognizes and binds to the target sequence (Anders et al., 2014). spCas9 could be directed to any target of interest upstream of a requisite 5'-NGG PAM through RNA-DNA base pairing (Cong et al., 2013). PAM is very important for sgRNA-

Cas9 binding to right locus of target gene. When Cas9 recognizes PAM, sgRNA-Cas9 utilize complementary base pairing reactions to read out and capture the DNA of interest, gain site-specific binding and avoid unexpected self-mutilation (Sternberg et al., 2014). Anders et al. (2014) reveled that target DNA unwinding and recognition by Cas9 are PAM-dependent. The CRISPR-Cas9 nuclease selectively bonds a target DNA containing a canonical 5'-NGG-3' PAM and unzips DNA complementary to the seed sequence of sgRNA to generate a sgRNA-target DNA heterodupex and trigger R-loop formation.

Cleavage

CRISPR/Cas9-mediated genome editing depends on the generation of DSB and subsequent cellular DNA repair process (Figure 3). Once the target sequence is recognized and created a RNA-DNA heteroduplex, the target dsDNA destabilized at the PAM motif. These actions catalytically activate the two functional nickase domains (HNH and RuvC) of Cas9. The double-stranded endonuclease activity of Cas9 also requires PAM motif. In fact, even fully complementary sequence are ignored by Cas9-sgRNA in the absence of PAM sequence (Anders et al., 2014; Nishimasu et al., 2014; Sternberg et al., 2014). During the cleavage of target DNA, Cas9 functions like a scissor, with the HNH nuclease domain nicking the DNA strand complementary to the guide RNA, the RuvC domain cutting the displaced strand, yielding a site-specific DSB (Gasiunas et al., 2012; Jinek et al., 2012).

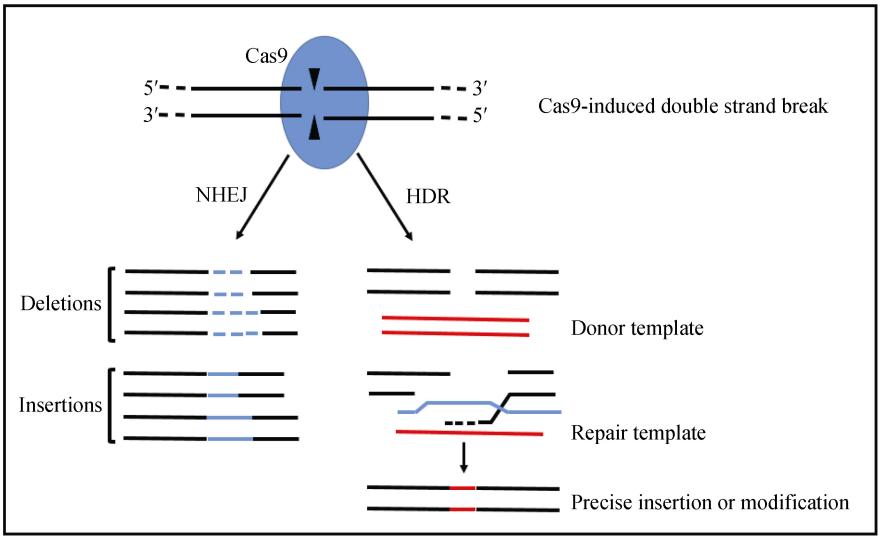

Figure 3 The mechanism of CRISPR/Cas9-mediated genome editing

When Cas9 induces DSB, two repair approaches, NHEJ and HDR can be activated. The error-prone NHEJ pathway can result in random deletions or insertions at the site of junction, while HDR pathway can be used within a repair template, leading to precise genome editing.

Repair

The presence of nuclease-induced DSBs in the DNA activates two mainly repair machineries, including non-homologous end joining (NHEJ) pathway and homology-directed repair pathway. In the NHEJ-mediated error-prone DNA repair process, both ends of a DSB are processed by endogenous DNA repair machinery and rejoined, which can generate random indel (insertion and deletion) mutations at target sites. If indel mutations occur within the coding region of a gene, it may result in frame shifts and the generation of a premature stop codon, leading to gene disruption or knockout. In addition, DSB can also initiate HDR-mediated DNA repair, which requires a homology-containing donor dsDNA sequence or ssDNA as a repair template. The HDR pathway allows high fidelity and precise editing. What is more, single-base substitution mutation or long target sequence insertion can be easily achieved through HDR (Anders et al., 2015; Chen et al., 2015f; Chu et al., 2015; Cong et al., 2013).

The strategy to generate CRISPR/Cas9 system for genome editing

As a new promising genome editing technology, RNA-guided CRISPR/Cas9 technique was rapidly developed. Based on the principle of the RNA-guided CRISPR/Cas9 system, the two essential components, Cas9 and sgRNA expression cassettes, are designed (Figure 4). Typically, the *Cas9* gene has been codon optimized for expression in a variety of cell types and organisms and tagged with a nuclear localization signal. To date, over 300 kinds of Cas9 plasmids have been deposited to the Addgene database. Among these plasmids, most of them belong to spCas9, which has been extensively studied.

Biologists initially utilized Cas9 and sgRNA expression vectors separately (Cong et al., 2013; Gilbert et al., 2014). Some began to use simple all-in-one expression system (Sakuma et al., 2014). In fact, both strategies have been successfully applied in many cell types and organisms. The

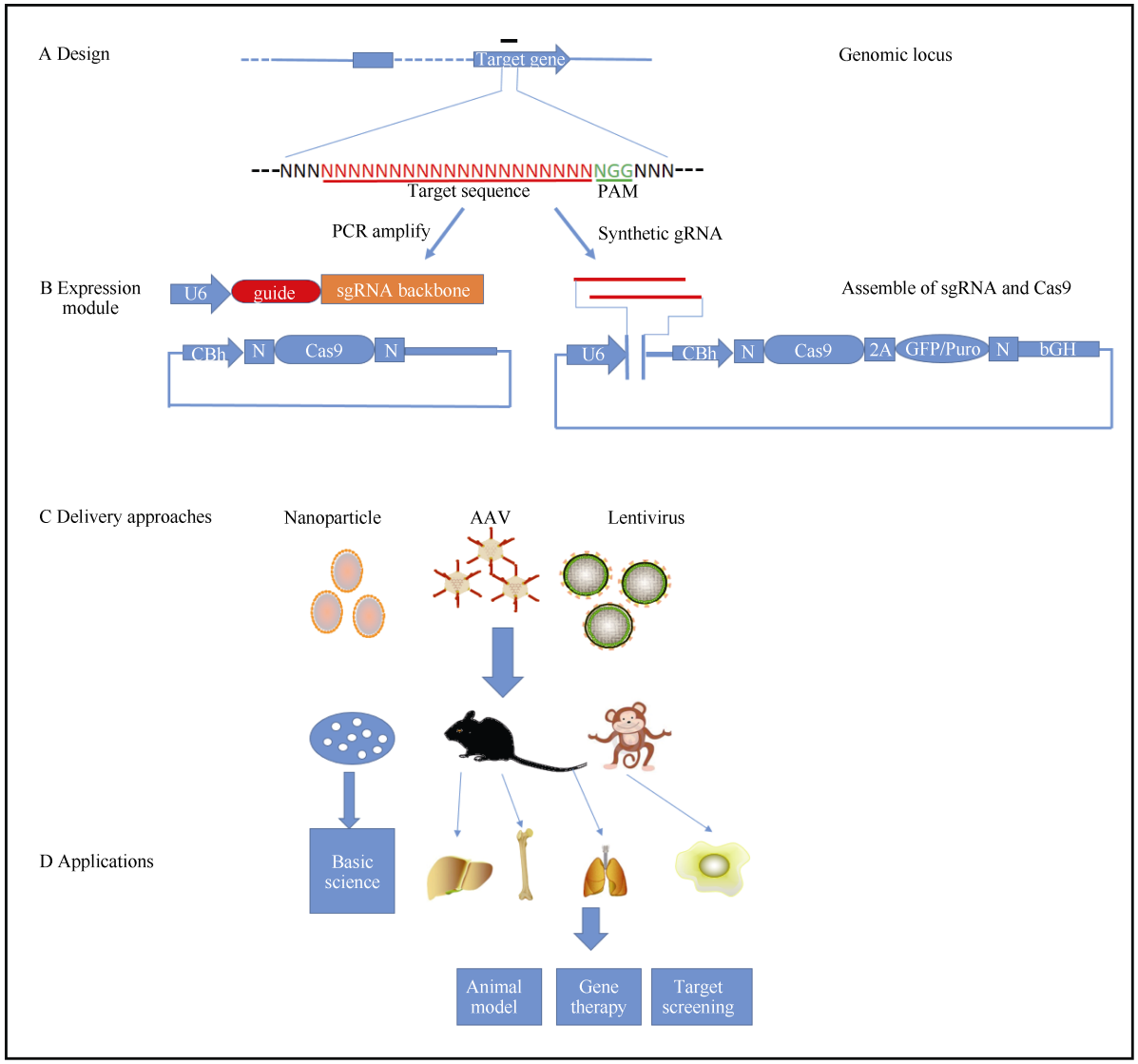

Figure 4 The overview of CRISP/Cas9 generation and application

A: Target sequence slection and sgRNA design. Based on the PAM site, 20-nt target sequences can be easily choosed on on-line softwares, and this method can also evaluate and minimize off-target effects; B: sgRNA and Cas9 expression. Left: The Cas9 expression plasmid and a U6-driver sgRNA expression cassette are separately delivered. Right: sgRNA and Cas9 are expressed in the same vector; C: Deliver to cells or organisms. Viral and no-viral approaches are widely used to facilitate the delivery of CRISPR/Cas9 system; D: Application of CRISPR/Cas9. This system has successfully applied in many cell types and organisms, whatever in vivo or invitro, Cas9-mediated genome editing greatly promotes the development of basic science to clinical research.

all-in-one expression plasmids of Cas9 and sgRNA can be purchased from many biological companies. Users just need to design appropriate sgRNA according to the position of PAM sequence. Several groups developed and provided online CRISPR design tools, such as the ZiFiT Targeter software (Sander et al., 2007) and the CRISPR DesignTool (http: // crispr.mit.edu/) (Hsu et al., 2013).

The promoter used to initiate the sgRNA expression can affect the Cas9-mediated genome editing. It's worth noting that the widely used U6 promoter prefers a guanine (G) nucleotide as the first base of its transcript, an extra G is appended at the 5' of the sgRNA when the 20-nt guide sequence does not begin with G (Ran et al., 2013a). More than two sgRNAs for a target locus should be designed simultaneously.

Minimization of off-target activity

Cas9 nucleases have been widely adopted for simple and robust targeted genome editing but also have the potential to induce high frequency off-target mutations (Fu et al., 2013). It is

well understood that sgRNA can tolerate certain mismatches to the DNA targets and thereby promote undesired off-target mutagenesis (Cong & Zhang, 2015). To minimize the off-target effects, the following standards should be kept when designing sgRNAs (Hsu et al., 2013): (1) Minimization of pairing bases between sgRNA and the predicted off-target site sequence; (2) At least two bases can not match between sgRNA and the predicted off-target site adjacent to PAM; (3) Avoid four continuous or interspaced base pairing between sgRNA and off-target sequence.

Over the last few years, engineered Cas9 nucleases and optimized guide RNA have been greatly improve the off-target activity of CRISPR/Cas9. The D10A mutant Cas9 nickase (Cas9n) significantly increases the specificity of genome editing (Ran et al., 2013b). Combination Cas9n and certain truncated sgRNAs could further reduce the off-target effects (Ren et al., 2014). Cas9 can also be transformed into a catalytically inactive, "dead" Cas9 (dCas9) and fused to the catalytic domain of the FokI nuclease (Guilinger et al., 2014). FokI-dCas9 can generate DSBs at the target site after dimmerization (Wyvekens et al., 2015). Although these methods significantly improved Cas9 cleavage specificity, there are various drawbacks, such as a decreased number of potential target sites, the need of multiple guides delivery, and increasing the nuclease protein size.

Increase of targeting efficiency

Identification of novel Cas9 orthologs, or chemical modification of the sgRNA may further improve this cutting edge technology. In early 2015, a small Cas9 from *S. aureus* subsp. was identified through a metagenomic screen of Cas9 orthologs. Comparing to spCas9, aureus Cas9 (saCas9) cleaves mammalian endogenous DNA with higher efficiency. Because of smaller protein size, saCas9 can be easily packaged into adeno-associated virus for expression (Ran et al., 2015). Recently, Zhang F and his colleagues created an "enhanced specificity" SpCas9, called eSpCas9, showing robust on-target cleavage (Slaymaker et al., 2016).

The function and application of the engineered CRISPR/ Cas9 system

In 2013, Zhang F et al. first successfully applied the CRISPR/ Cas9 system in mammalian genome editing (Cong et al., 2013). The CRISPR/Cas9 system could be used for gene functional identification, generation of animal models and gene therapy (Figure 4).

Gene functional identification

Gene knockout is a fundamental strategy for investigating physiological and pathological functions of a defined gene. Functional genomic screening has been extensively used to identify functional genes. The mostly used RNAi has been mainly applied for large-scale genome screening (Kamath & Ahringer, 2003; Yu et al., 2015). However, RNAi has severe off-target effects (Jackson & Linsley, 2010). In addition, RNAi could not completely silence the target gene. Partial suppression of gene expression is often insufficient to generate remarkable changes in phenotype, which leads to high false-positive and false-negative rates (Echeverri et al., 2006; Vu et al., 2015). The CRISPR/Cas9 system has been rapidly developed into a large-scale function-based screening in 2014 (Gilbert et al., 2014; Shalem et al., 2014; Wang et al., 2014). It has been successfully used in various genome-scale loss-of-function screening.

Wang et al. (2014) used a lentiviral sgRNA library targeting approximately 7 000 human genes to perform a positive selection screening based on cell viability resistance to 6-thioguanine. All expected genes related to the DNA mismatch repair pathway have been identified. Similarly, Shalem et al. developed a genome-scale CRISPR/ Cas9 knockout library and successfully used it to screen genes associated with the resistance to vemurafenib, a cancer therapeutic agent for late-state melanoma (Shalem et al., 2014).

In addition, CRISPR-mediated repression (CRISPRi) and activation (CRISPRa) have been demonstrated as robust tools for functional genome screening in gene expression modulation. Rather than inactivating genes by the introduction of indels after DSBs, CRISPRi, consisting of a catalytically inactive Cas9 (dCas9) and a guide RNA, has been show to specifically and efficiently inhibit the transcription of target genes in *E. coli* and mammalian cells when the dCas9 is recruited to a transcriptional inhibitory domain (Gilbert et al., 2013), whereas a dCas9 tethered to a transcriptional activation domain has been used to activate the expression of target endogenous genes (Gilbert et al., 2014; Kampmann et al., 2015). Genome-scale CRISPRi/a libraries has been successfully used to identify mediators for cellular sensitivity to a cholera-diptheria fusion toxin, as well as essential genes for proliferation, differentiation, tumor suppression and so on. What's more, CRISPRa offers the ability for gain-of-function, which provides a good way to study a novel gene. It has been shown that the dCas9 activation complexes, with a sgRNA library, can activate multiple genes simultaneously, upregulate long non-coding RNA transcripts and identify genes conferring resistance to a BRAF inhibitor in melanoma (Konermann et al., 2015). These results indicate that the CRISPR/Cas9 technology is a versatile functional genomic screening tool for discovering crucial genes in various biological processes.

Generation of animal models

Animal models are potent tools for understanding human disease pathogenesis and developing novel therapeutics (Ohtori et al., 2015; Stewart & Kalueff, 2015). Traditional gene targeting strategy depends on homologous recombination of embryonic stem cells. However, low efficiency severely limits its application. The CRISPR/Cas9 technology greatly improves the efficiency of generating gene targeting animal model (Gaj et al., 2013). CRISPR has been used to generate genetically modified mouse models such as KO/KI models, somatic genome editing models (Dow, 2015; Flynn et al., 2015; Mou et al., 2015). Moreover, Fujii et al. (2013) successfully generated large-scale genome-modified mice using the CRISPR/Cas9 system. Both KI and KO mouse models could be effectively gained with the added benefit of reduced levels of off-target effects by application of mutated Cas9n nucleases. One advantage of this

technology is to target multiple genes at the same time, which greatly promotes the study of multiple gene interactions. For example, Wang et al. (2013) simultaneously targeted five genes in mES cells using this technology.

Rapid progress of CRISPR/Cas9-mediated genome engineering enables rapid functional identification of putative human disease genes in different models via somatic genome editing *in vivo* (Table 1). Xue et al. (2014) used hydrodynamic injection to deliver a CRISPR plasmid DNA expressing Cas9 and sgRNAs to liver, and CRISPR-mediated pTEN mutation alone or in combination with p53 mutation phenocopies the effects of pTEN and p53 knockout using Cre-LoxP technology. Furthermore, they delivered an activated mutant β-catenin gene into hepatocytes through co-injection of Cas9 plasmids expressing sgRNAs targeting β-catenin and a single-stranded DNA oligonucleotide donor carrying activating point mutations (Xue et al., 2014). This study demonstrates that CRISPR/Cas9 can be used, feasibly and directly, to modify tumor suppressor genes and oncogenes in somatic tissues, providing a new approach for rapid development of disease models. Furthermore, Sá nchez-Rivera et al. (2014) developed pSECC, a lentiviral-based delivery system that delivers both CRISPR system and Cre recombinase selectively to lung and other tissues. Using this system, they demonstrated that CRISPR-induced genome editing of tumor suppressor genes combined with Cre-dependent somatic activation of oncogenic $Kras^{G12D}$ lead to lung adenocarcinomas. This rapid somatic genome engineering approach further expands the application of CRISPR/Cas9 in generation of animal models. Using Cas9 KI mice, Platt et al. (2014) easily create lung adnocarcinomas models by simultaneously delivering a single AAV vector carrying mutations in p53, Lkb1 and $Kras^{G12D}$ in the lung.

Cas9-mediated target genome editing could be used for rapidly generating genome modification in various organisms beside mouse. Other traditional animal models with heritable germline modification, high-efficiency of specific mutations, and transgenic, tissue-specific, inducible editing have been efficiently and rapidly produced with this next generation genome editing technology, including *C. elegans* (Long et al., 2015), *D. melanogaster* (Lin et al., 2015) and *Danio rerio* (Li et

Table 1 CRISPR/Cas9-mediated generation of animal models and application in human health

Organism	Disease type and application	Strategy	References
Zebrafish	Pfeiffer Syndrome	HDR induces Pro252Arg gain of function	Tomaszewski et al., 2015
Xenopus	Albinism	NHEJ-induced simultaneous disruption of two X. laevis tyrosinase homeologs	Wang et al., 2015b
Mouse	Liver and lung carcinoma	p53, pTEN, KRAS and β-catenin	Xue et al., 2014; Sánchez-Rivera et al., 2014; Platt et al., 2014
	Pancreatic cancer	Lkb1 deletion and Kras modification	Chiou et al., 2015
	Medulloblastoma and glioblastoma	Somatic gene modification of Ptch1 or multiple genes (p53, pTEN, Nf1)	Zuckermann et al., 2015
	Genome-wide screening for tumor genes	Knock-in mice with gRNA are used to screen oncogenes	Chen et al., 2015b
	Rett syndrome	Generation of MeCP2-dificient mice	Tsuchiya et al., 2015
	Cataracts	HDR-induced correction of mutant Crygc gene	Wu et al., 2013
	Acute myeloid leukemia	Lentivirus-delivered sgRNA: Cas9 is used to modify several cancer driver genes together	Heckl et al., 2014
	HBV	Cleave HBV DNA	Ramanan, et al., 2015; Lin et al., 2014
	Alveolar Rhabdomyosarcoma	Pax3-Foxo1 chromosome translocation	Lagutina et al., 2015
Rat	Duchenne muscular dystrophy (DMD)	Edit two exons in the rat *Dmd* gene	Nakamura et al., 2014
Rabbit	Tyrosinase disorders	Zygote direct injection of Cas9 and sgRNA to tyrosinase gene	Honda et al., 2015
Pig	Gene-modified pigs	One-stage-embryo injection of Cas9/sgRNA	Wang et al., 2015c
	Generation of B Cell-Deficient Pigs	IgM-targeting Cas9 delivery to produce B cell-deficient mutant pigs by somatic cell nuclear transfer (SCNT) technology	Chen et al., 2015a
Monkey	Gene-modified cynomolgus monkey	Coinjection of one-cell embryos with Cas9 mRNA and sgRNAs	Niu et al., 2014
	Human AHC-HH	Dax1-null mutations	Kang et al., 2015
	Duchenne muscular dystrophy (DMD)	Monkey dystrophin gene disruption	Chen et al., 2015e

al., 2015b). CRISPR/Cas9-mediated multi-gene targeting and conditional allele modifications have been successfully applied in generation of new transgenic rat models (Nakamura et al., 2014). Furthermore, the CRISPR/Cas9 technology has been proved to be effective in large mammalian animal models, such as pigs and non-human primates. Wang et al. (2015c) demonstrated that co-injection Cas9 mRNA and target gene sgRNA into one-cell stage embryos is an efficient and reliable approach for generation of gene-modified pigs. Recently Yang L's work caused great attention because she successfully knockout all copies of the PERV *pol* gene in pigs and trigger a 1000-fold reduction of PERV infectivity of human cells, which made pigs almost perfect animal for generating transplantation organs for human beings (Yang et al., 2015). Non-human primates are the best animal model for studying human neurodegenerative diseases. In 2014, Chen Y. et al. first achieved precise gene targeting in cynomolgus monkeys by co-injection of Cas9 mRNA and sgRNAs into one-cell-stage embryos (Chen et al., 2015d). Tree shrew has been proved to be a suitable animal model for breast cancer (Ge et al., 2016; Xia et al., 2014; Xu et al., 2013), it is worth to use the CRISPR/Cas9 system to generate breast cancer tree shrew models in the future.

CRISPR–Cas9 based therapeutics

CRISPR/Cas9-mediated genome editing provides a promising strategy for gene therapy of human diseases through correcting disease-causing mutations or inserting new protective genes. In 2013, Wu et al. (2013) first corrected a dominant *Crygc* gene mutation in a cataracts mouse model by co-injection of Cas9 mRNA and sgRNA targeting the mutant *Crygc* allele into zygotes, together with a *HDR* template. A study in 2014 showed that CRISPR/Cas9-based gene modification can be used to correct the dystrophin gene (*Dmd*) mutation in the germline of mdx mice, a model for Duchenne muscular dystrophy (DMD), providing a promising approach for correction of disease-causing mutations in the muscle tissue of patients (Long et al., 2014).

Considering that the CRISPR/Cas9 system was initially discovered as an adaptive immune system against virus and phages in bacteria and archaea, it is a natural idea to use the system as an anti-viral therapeutic for treating infectious diseases by eliminating pathogen genomes from infected individuals, such as HBV and HIV. Although current technologies can inhibit the covalently closed circular viral DNA template (cccDNA) of HBV, it is still difficult to destroy the virus. Nevertheless, Ramanan et al. (2015) found that sustained expression of Cas9 and sgRNA targeting the cccDNA in cell culture can dramatically reduce the cccDNA and other parameters of viral gene expression and replication by directly cleavage of cccDNA, demonstrating that directly targeting viral episomal DNA is a novel anti-viral therapy approach for completely eradicating infectious diseases. In addition, HIV-1 remains to be cured in spite of application of antiretroviral therapy (ART). In fact, the persistence of HIV reservoirs can be controlled but not be completely cleared by current ART (Archin et al., 2012). CRISPR/Cas9-based gene therapy offered a new tool to modify the targeted intervention points, such as $CD4^+$ receptor and the CCR5 (Ebina et al., 2013; Li et al., 2015a). Hu et al. (2014) showed that CRISPR/Cas9 can be used to disrupt latent HIV infection and also block new HIV infection. Similarly, Liao et al. (2015) recently demonstrated that engineered human IPS stably expressing Cas9 and HIV-targeted sgRNA was able to be efficiently differentiated into HIV reservoir cell types and keep their resistance to HIV-1 challenge, providing long-term adaptive defense against new viral infection, expression and replication in human cells. All these results reveal that the CRISPR/Cas9 system is a new effective therapeutic strategy against viral infections and holds the great promise of eradicating infectious diseases.

CHANLLENGES AND PROSPECTS

CRISPR/Cas9, as the robust, specific and efficient genome editing tool, has greatly enhanced the development of biology. CRISPR/Cas9 opens the door from basic research to clinical applications. The rapid progress of development and application of the CRISPR/Cas9 technology has been boosted now. In spite of the enormous potential of CRISPR/Cas9 in genome editing from basic biology to translational medicine, the challenges still exist and need to be addressed.

The development of CRISPR/Cas9 technology

In the future, efforts should be made to increase the specificity, reduce the off-target effects and develop efficient delivery methods of CRISPR/Cas9.

Although Cas9 from *S. pyogenes* is the enzyme most extensively used, there are some limitations because the spCas9 gene size is quite large (>4 000 bp) and is difficult to be efficiently packaged into many virus vectors. In addition, spCas9-dependent PAM is just NGG or NAG, which highly restricts the selective cleavage sites of target. It is a challenge to explore more efficient Cas9 enzymes from native bacteria or archaea, as well as recombinant Cas9. Besides Cas9D10A and dCas9 of spCas9, saCas9 has been identified as a more efficient enzyme (Nishimasu et al., 2015; Ran et al., 2015). SaCa9 holds a great promise to expand this technology in genome editing and *in vivo* therapy. It is possible that thousands of spCas9 orthologs could be identified from other species. Some innovative improvements, such as photoactivatable CRISPR/Cas9 and CRISPR/Cpf1 (Nihongaki et al., 2015; Zetsche et al., 2015), has been developed. The former makes it possible for optogenetic control of targeted genome editing, which facilitates better understanding of complex gene networks and could precisely control genome modification in biomedical applications. The latter is a new genome editing system in which Cpf1 orthologs exhibit robust nuclease activity in human cells. Recently more exciting achievement of CRISPR/Cas9, named self-cloning CRISPR/Cas9 (scCRISPR), was developed to more economic and time saving in specific and efficient target genome editing (Arbab et al., 2015).

Although more suitable Cas9 enzymes are coming into

being, the off-target effects remain a big problem. Other than modified Cas9, highly specific sgRNA design is crucial for minimization of off-target effects. Specific PAM sequence directs sgRNA design (Hsu et al., 2013). Based on the features of CRISPR/Cas9 recognition and cleavage, it's necessary to design sgRNA targeting a highly unique genomic region (Hsu et al., 2013). To efficiently and quickly design high specific sgRNAs, *in silico* prediction tools are commonly used, such as CRISPR Design (Hsu et al., 2013), CRISPRdirect (Naito et al., 2015), ZiFit Targeter (Sander et al., 2007), Cas9 designer (Park et al., 2015), E-CRISP (Heigwer et al., 2014), CHOPCHOP (Montague et al., 2014), sgRNACas9 (Xie et al., 2014) and Protospacer Workbench (MacPherson & Scherf, 2015). These online or off-line software has their unique characteristics. It's still a big challenge to develop better approaches to design high specific and efficient sgRNA for targeting genome modification.

Additionally, the methods for delivery of Cas9 and sgRNA need to be optimized for application in diverse cell types or organisms, especially for human gene therapy. Transient expression of Cas9-mediated genome editing permits consideration of a range of delivery choices for therapeutic application. Viral delivery methods, including self-inactivating lentivirus, adenovirus, and AAV, provide an efficient Cas9/sgRNA delivery approach for somatic genome editing (Chiou et al., 2015; Sánchez-Rivera et al., 2014). However, delivery of plasmid DNA, Cas9 mRNA, *in vitro* synthesized or transcribed sgRNA, Cas9/sgRNA complexes and donor nucleic acid templates can be achieved through other approaches, including electroporation (Hashimoto & Takemoto, 2015), lipid-based transfection (Hendriks et al., 2007), hydrodynamic delivery (Wang et al., 2015a) and induced osmocytosis (Liu et al., 2015). Recently, a novel and simple microinjection-independent technique, called genome-editing via Oviductal Nucleic Acids Delivery, was established to effectively deliver Cas9 mRNA/sgRNAs to pre-implantation embryos within the intact mouse oviduct (Takahashi et al., 2015). All these methods have been broadly used both *in vitro* and *in vivo* for genome editing. Nevertheless, it still remains a big challenge to efficiently and specifically deliver Cas9/sgRNA into cells or tissues with minimizing side-effects. More attentions should be paid to develop novel robust delivery methods for CRISPR/Cas9.

The ethical problems and safety

CRISPR/Cas9 has been triggering revolution in biomedicine. It shows great potential in application for human genetic diseases, including drug target validation (Housden et al., 2015; Shi et al., 2015) and gene therapy (Lu et al., 2015; Xu et al., 2015a). As described above, CRISPR/Cas9 can be used to cure genetic diseases, including brain genetic disorders, immune diseases, cancers and some infectious diseases in animal models, providing promising platform for developing efficient and specific therapies for human diseases. However, it is still a long way to apply CRISPR/Cas9 in clinic. In April 2015, geneticists from Guangzhou, China firstly published the use of CRISPR/Cas9 in human embryos attempting to eradicate the disease causing gene of HBB (Human β-globin) directly from the germ line (Liang et al., 2015). As the study was first manipulated in human zygotes, which shows great promise in generation of hereditable human genome editing. However, This study also raised a debate concerning the ethical considerations and application safety in human diseases. Whether we have the right to produce desired babies should be seriously considered. As a matter of fact, we almost can not completely avoid off-target effects at present.

Additionally, whether we should delete a disease gene should be seriously considered. Recently, Rossi et al. (2015) found that deleterious mutations but not gene knockdowns can induce genetic compensation when they compared CRISPR-mediated *egfl7* mutants with *egfl7* knockdown of zebrafish. We should think twice before we decide to delete or block deleterious genes when using genetic engineering technologies.

Although CRISPR/Cas9 has been proved to be an efficient and site-specific approach for gene therapy, it is still a long way to apply this technology in patients. We must ensure the high-specificity of CRISPR/Cas9 for target sites and avoid possible off-target effects by choosing effective therapeutic targets, designing high-specific sgRNAs and using delivery system with high efficiency and low toxicity. With the development of this cutting edge technology, we believe that CRISPR/Cas9 opens up exciting possibilities for applications across basic science, biotechnology, and medicine.

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