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REVIEW

Recent Progress in CRISPR/Cas9 Technology

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ABSTRACT

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, a simple and efficient tool for genome editing, has experienced rapid progress in its technology and applicability in the past two years. Here, we review the recent advances in CRISPR/Cas9 technology and the ways that have been adopted to expand our capacity for precise genome manipulation. First, we introduce the mechanism of CRISPR/Cas9, including its biochemical and structural implications. Second, we highlight the latest improvements in the CRISPR/Cas9 system, especially Cas9 protein modifications for customization. Third, we review its current applications, in which the versatile CRISPR/Cas9 system was employed to edit the genome, epigenome, or RNA of various organisms. Although CRISPR/Cas9 allows convenient genome editing accompanied by many benefits, we should not ignore the significant ethical and biosafety concerns that it raises. Finally, we discuss the prospective applications and challenges of several promising techniques adapted from CRISPR/Cas9.

KEYWORDS: CRISPR/Cas9; Genome editing; Epigenome editing; RNA editing

INTRODUCTION

Genome editing technologies, including chemical- and UV-induced mutagenesis, DNA recombinase-mediated gene replacement, zinc-finger nucleases (ZFNs), and transcriptional activator-like effector nuclease (TALEN) systems, have profoundly contributed to both fundamental and clinical advances in biological research (Urnov et al., 2005; Bedell et al., 2012). Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system, derived from the adaptive immune system of the bacterium *Streptococcus pyogenes* (Sapranauskas et al., 2011), has dramatically increased our ability to edit the genomes of diverse species, such as *Drosophila* (Bassett et al., 2013, 2015; Gratz et al., 2013; Yu et al., 2013; Xu et al., 2015), *Caenorhabditis elegans* (Dickinson et al., 2013; Friedland et al., 2013), zebrafish (Chang et al., 2013; Hwang et al., 2013; Jao et al., 2013), mouse (Cong et al., 2013), rat (Li et al., 2013a), *Bombyx mori* (Ma et al., 2014) and humans (Cong et al., 2013; Mali et al., 2013).

The CRISPR/Cas system, which is an important component of bacterial adaptive immunity, consists of the Cas nuclease and two individual RNA components, a programmable crRNA (CRISPR RNA) and a fixed tracrRNA (trans-activating crRNA). Cas protein is able to cut the invading phage DNA into small fragments, which are then integrated into the CRISPR array as a spacer. Subsequently, the CRISPR array is transcribed to generate crRNA and a complementary

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR associated; crRNA, CRISPR RNA; tracrRNA, trans-activating crRNA; PAM, protospacer adjacent motif; sgRNA, single guide RNA; gRNA, guide RNA; ssODN, single-stranded DNA oligonucleotide; DSB, double-strand break; NHEJ, non-homologous end joining; HDR, homology-directed repair.

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tracrRNA, which form a double-stranded RNA structure that recruits Cas proteins for cleavage (Datsenko et al., 2012; Jinek et al., 2012). Adjacent to the crRNA-targeted sequence on the invading DNA, a short sequence named as the protospacer adjacent motif (PAM) plays an essential role in the adaptation and interference stages, which the CRISPR/Cas complex recognizes during target DNA binding. The absence of a PAM sequence can alter the affinity between Cas and the target DNA, since specific PAM sequence recognition serves to discriminate non-self target sequences from non-target sequences (Westra et al., 2013).

To date, three types of CRISPR/Cas systems (I, II, and III) have been identified. Compared with other systems that necessitate multiple Cas proteins, the type II system only requires a single Cas protein: Cas9. The Cas9 protein functions in both RNA-guided DNA recognition relying on sgRNA (single guide RNA) formed by crRNA and tracrRNA fusion and cleavage, making it potentially useful for genome engineering applications (Cong et al., 2013; Wu et al., 2014). In the past two years, the efficient and simple manipulation enabled by the CRISPR/Cas9 system, together with emerging variants of the natural type II CRISPR/Cas9 system and its growing capacity for multiplexed target recognition, has led to profound advances in related biological fields. As a result, the relevant applications and prospects of the CRISPR/Cas9 system have been extensively reviewed. In this review, we will describe the progress in the elucidation of the molecular mechanisms underlying CRISPR/Cas9 genome editing, focusing on the continuous development to improve the accuracy and efficiency in gene targeting up till the present. Moreover, we will discuss changes in the application of the CRISPR/Cas9 system in genome and RNA editing, and methods for modifying, regulating, and marking genomic loci of cells and organisms in detail.

DEVELOPMENT IN CRISPR/CAS9 MODIFICATIONS

Components of the CRISPR/Cas9 system

In the CRISPR/Cas9 system, the Cas9 protein functions in nucleic acid cleavage. Crystal structure analysis has revealed that the Cas9 protein contains one recognition (REC) lobe and one nuclease (NUC) lobe. The REC lobe, which consists of a long α helix, an REC1 domain and an REC2 domain, is a Cas9-specific functional domain, interacting with the repeat:anti-repeat duplex, whereas the NUC lobe is composed of the RuvC, HNH, and PAM-interacting (PI) domains (Nishimasu et al., 2014). The nomenclature of RuvC and HNH is based on their homology to known nuclease domain structures: RuvC is similar to the *Escherichia coli* RuvC domain, which resolves Holliday junctions, and HNH is similar to phage T4 endonuclease VII, which functions in DNA binding and cleavage. During the DNA cleaving by Cas9, the RuvC and HNH domains cleave the non-complementary and complementary strands of the target DNA through a two- or single-metal mechanism, respectively. The PI domain is a unique structure in the Cas9 family, which is required for recognition of the PAM sequence on the non-complementary strand (Hsu et al., 2014), and thus provides evidence for a critical determinant function of Cas9, with respect to its PAM specificity.

The crRNA and tracrRNA can be fused to generate sgRNA, which enables recruitment of Cas9 that is essential for cleavage of double-stranded DNA (dsDNA). This makes the CRISPR/Cas9 system a manageable genome-editing tool for the reason that sgRNA can be designed to recognize the sequence preceding a PAM sequence. In particular, the crRNA contains a 20-nt guide RNA (gRNA) and a 12-nt repeat region, whereas the tracrRNA is divided into a 14-nt anti-repeat region and three stem loops, named loop 1, loop 2, and loop 3 (Fig. 1). Thus, the unique structure of the sgRNA bridges Cas9 and the target sequence.

Crystal structure analysis has illustrated the mechanism of CRISPR/Cas9. The CRISPR/Cas9 components form a T-shaped configuration, which is composed of the gRNA:-target sequence heteroduplex, the repeat:anti-repeat duplex, a tetraloop, three stem loops, and a linker between stem loop 1 and 2. First, the gRNA:target sequence heteroduplex forms when gRNA binds to the target sequence *via* Watson−Crick base pairing. Second, in the same manner, the repeat and anti-repeat regions form the repeat:anti-repeat duplex, which is essential for Cas9 function. Concurrently, the remaining tracrRNA bases form stem loops 1, 2, and 3 *via* three, four, and six Watson−Crick base pairs, respectively (Fig. 1). Stem loop 1 plays a critical role in the formation of the functional Cas9:sgRNA complex, while stem loop 2 and 3 affect the stability and activity of the CRISPR/Cas9 system. Importantly, the repeat:anti-repeat duplex, the gRNA:target sequence, and stem loop 1 can be recognized by both the REC and NUC lobes of the Cas9 protein, whereas the linker, stem loop 2, and stem loop 3 are specifically recognized by the NUC lobe (Nishimasu et al., 2014).

Through the above interactions, a Cas9-sgRNA binary complex is formed. Simultaneously, the complex recognizes the target sequence, which is complementary to the gRNA, and the PI domain recognizes the PAM sequence on the non-complementary strand to form an R loop configuration. At this point, the Cas9-sgRNA-target DNA ternary complex is formed, which then initiates the subsequent cleavage of the complementary and non-complementary strands, performed by the HNH and RuvC domains, respectively. The growing understanding of the CRISPR/Cas9 system underlies unforeseen potential for its modification and application.

Modifications of Cas9 based on its functional domains

The rapid progress in development of CRISPR/Cas9 system has largely revolved around genetic engineering of the system, especially modifications of Cas9 and sgRNA to achieve more precise editing. Originally, human codon-optimized versions of the Cas9 and RNaseIII genes, derived from *S. pyogenes,* are accompanied by nuclear localization signals (NLSs) for nuclear compartmentalization to synthesize the first generation of CRISPR/Cas9 plasmids for genome editing (Cong et al., 2013). The modified Cas9 protein can generate a double-

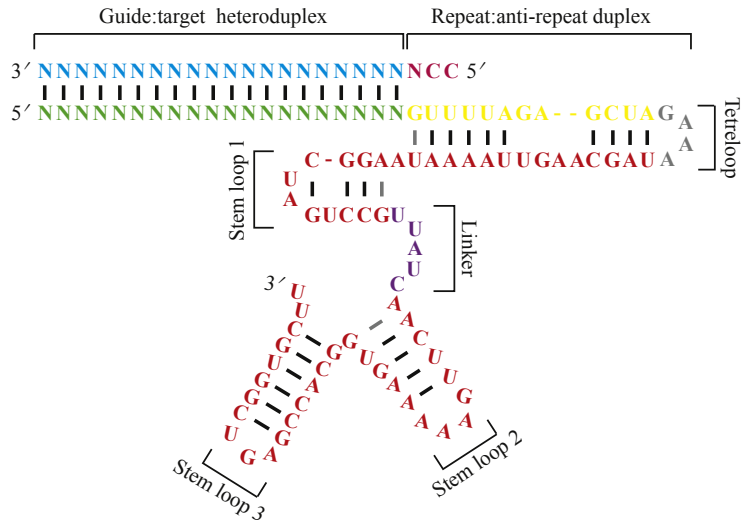

Fig. 1. The overall structure of sgRNA:target DNA complex.
The guide and repeat region of crRNA sequence are colored green and yellow, respectively. The tracrRNA sequence is colored red, with the target DNA sequence colored blue. The linker region and the tetraloop are colored violet and gray, respectively. Watson−Crick and non-Watson−Crick base pairs are represented by black and gray lines, respectively, with disordered nucleotides indicated by dashed lines.

strand break (DSB) at the targeted region (Fig. 2A), thereby activating the DNA repair mechanism to induce insertions or deletions (Heintze et al., 2013). Significantly, with the modifications of nuclease function of REC and NUC, several different versions of Cas9 have been applied in editing genomes of both mammalian cells and transgenic animals.

Cas9 nickase (Cas9n)

Cas9n consists of an inactivating mutation in the endonuclease cleavage domains (Fig. 2B and C). For example, a D10A mutation can abolish the endonuclease activity of the RuvC domain; in the same manner, an H847A mutation can eliminate the function of the HNH domain. By combining Cas9n with two separate sgRNAs, a staggered DSB can be produced to activate the DNA repair process, through which the efficiency and specificity of the CRISPR/Cas9 system can be further improved (Ran et al., 2013a).

Dead Cas9 (dCas9)

Nuclease-null deactivated Cas9 (dCas9), which is generated by preventing the enzymatic activities of both the RuvC and HNH domains in the Cas9 nuclease (Wu et al., 2014), has the ability to direct transcriptional perturbation of target genes without modifying the DNA, due to the blockade formed by the Cas9-sgRNA complex. The CRISPR system with a catalytically inactive dCas9 is also referred to as CRISPR interference (CRISPRi) (Fig. 2D). This system provides two different functions, dCas9 fusion-mediated inhibition (CRISPRi) and dCas9 fusion-mediated activation (CRISPRa). It is noteworthy that selection of gRNA is crucial for successful target sequence silencing (Lawhorn et al., 2014). For example, the efficiency of CRISPRi repression can reach 99.9%, using a 20-nt gRNA sequence complementary to the target gene (Larson et al., 2013). With expanded application of CRISPRi, multiple sgRNAs can be delivered simultaneously to silence multiple genes. Strikingly, after being fused to a repressor (such as Krüppel-associated box (KRAB)) (Lawhorn et al., 2014) or an activator (such as VP16 and VP64) (Gilbert et al., 2013), dCas9 can block or activate gene expression, respectively (Fig. 2E). Moreover, dCas9-SunTag transcriptional system can greatly enhance the activation or silence of single or multiple endogenous gene expression, and then potentially result in complex genetic re-writing, which can replace traditional overexpression of transfected plasmids in cells or organisms to a great extent (Tanenbaum et al., 2014). These evidences suggest that dCas9 shows great potentialities by fusion to the appropriate enzymes or transcription factors in other fields, such as epigenetics, cancer or neurologic disorders.

Light-activated Cas9

Recently, light-activated Cas9 has emerged as a promising technology for spatial- and temporal-specific genome editing (Fig. 2G) (Polstein and Gersbach, 2015). It has been reported that the presence of multiple lysines (K), an important caging site, could inhibit CRISPR/Cas9 system. Two sites have been identified for optical control of Cas9 function: 1) K163, which is highly conserved and located near gRNA binding sites, might play a role in Cas9-gRNA interactions; 2) K886, which could alter the Cas9 structure to expose the lysine residue during the gRNA binding process, might be involved in positioning the non-target DNA strand (Hemphill et al., 2015). Two independent research groups developed a light-activated transcriptional activation CRISPR/Cas9 system. This system contains two fusion proteins: 1) a cryptochrome-interacting basic-helix-loop-helix 1 (CIb1), which can be fused with dCas9 to target the genome sequence by using sgRNAs as the genomic anchor probe; 2) a light-sensitive cryptochrome 2

(CRY2), either full-length CRY2 (CRY2FL) or a truncated CRY2 containing a photolyase homology region (CRY2PHR), functioning as an activator probe combined with a transcriptional activator domain. With the heterodimerization of CRY2 and CIb1, the modified Cas9 can be recruited to the target site to activate gene transcription with blue light stimulation (Nihongaki et al., 2015; Polstein and Gersbach, 2015). Moreover, by integrating technologies such as fluorescence

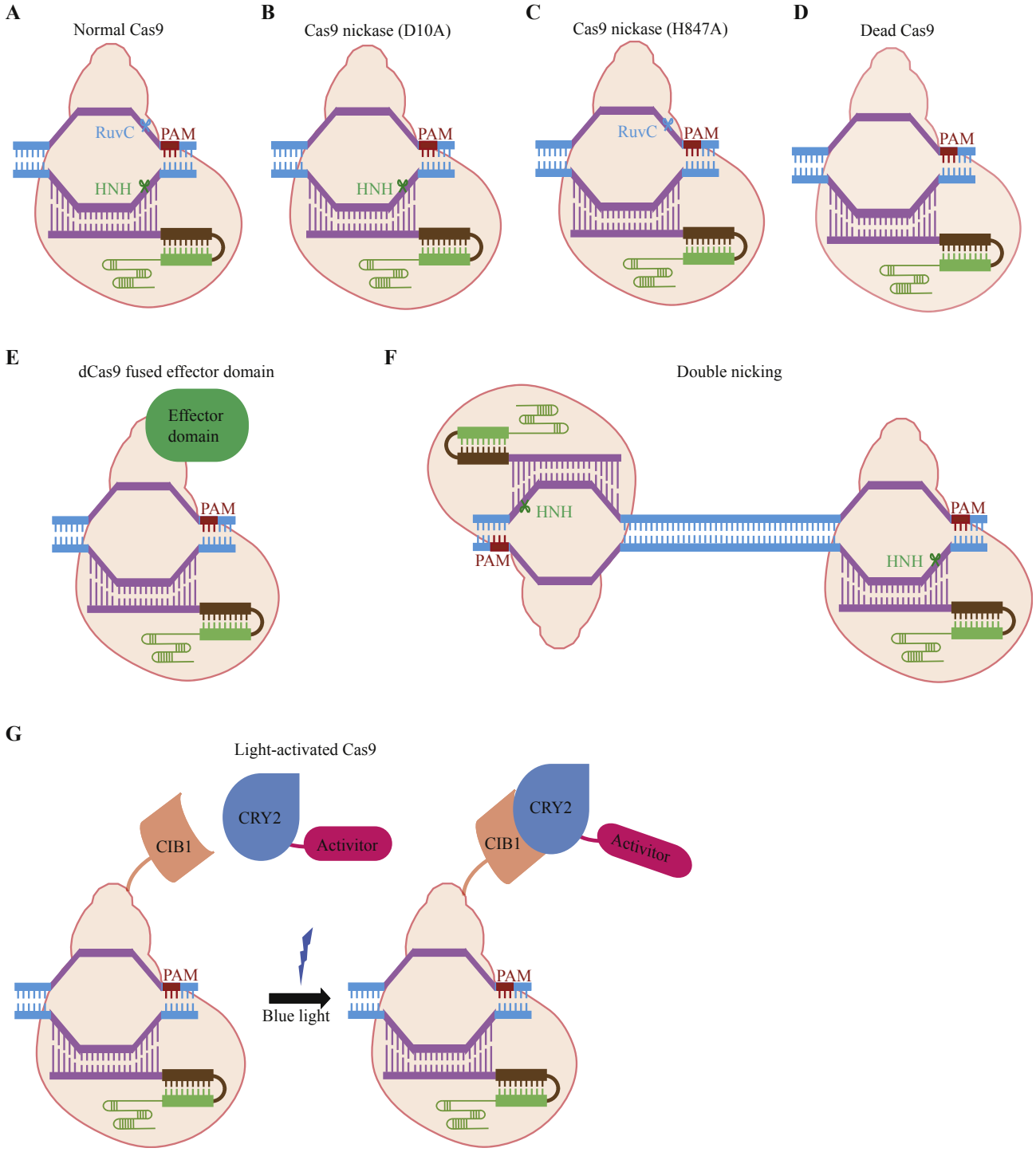

Fig. 2. The modifications of Cas9 based on its functional domains.

A: The Cas9 nuclease cleaves the complementary and non-complementary strands of the target DNA *via* HNH and RuvC domains, respectively. **B:** Cas9 can function as a nicknase containing an inactivating mutation D10A in RuvC domain. **C:** Cas9 can function as a nicknase containing an inactivating mutation H840A in HNH domain. **D:** Cas9 can function as an RNA-guided DNA binding protein with inactivating mutations in both RuvC and HNH domains. **E:** dCas9 can be fused to activator or repressor (effector) domain to activate or block gene expression. **F:** Schematic representation of double nicking. **G:** Light-activated Cas9 enables optical control of spatial- and temporal-specific genome editing.

microscopy (Ratz et al., 2015) and flow cytometry (Rojas-Fernandez et al., 2015), several reporters and tags, such as GFP and puromycin, have been fused to Cas9 to achieve screening and selection of stable genome-edited cell lines (Rojas-Fernandez et al., 2015).

CRISPR/Cas9 system specificity and the selection of sgRNA

As specific target site cleavage of Cas9 is mainly determined by the 20-nt gRNA sequence within the sgRNA (Ran et al., 2013b), selection of the gRNA sequence poses a critical step for which the following standards should be met: 1) To minimize the off-target activity of Cas9, the gRNA sequence should be highly matched with the target sequence; 2) The gRNA-targeted sequence should immediately precede a 5′-NRG PAM (R can be G or A) (Heintze et al., 2013; Wang et al., 2015a); 3) A guanine (G) nucleotide is essential at the 5′ end of the sgRNA when the human U6 RNA polymerase III promoter serves as the promoter of the CRISPR plasmids, since it prefers a G at the first base to initiate sgRNA expression. Therefore, an additional G residue should be added to the 5′ end of the sgRNA if the initiation start site does not begin with a G.

Interestingly, it has been confirmed that extending the length of the gRNA region cannot improve the cleavage specificity of Cas9. Moreover, compared with 20-nt sgRNA, the truncated RNA containing 17- or 18-nt of complementarity not only functions with high efficiency at target site, but also shows improved specificities to minimize the off-target effect (Fu et al., 2014). However, the sgRNA region can tolerate no more than three mismatches to its target sequence (Ran et al., 2013b), implying that binding of sgRNA to the target DNA region is structure-dependent.

Notably, double-nicking with Cas9n and two separate sgRNAs are necessary and sufficient for the break in each DNA strand (Fig. 2F), which minimizes the unwanted cleavage of high-fidelity base excision repair caused by single-strand nicks. However, even though this reduced the chances of off-target cleavage, the double-nicking system is not completely accurate, and off-target events may still occur (Ran et al., 2013a).

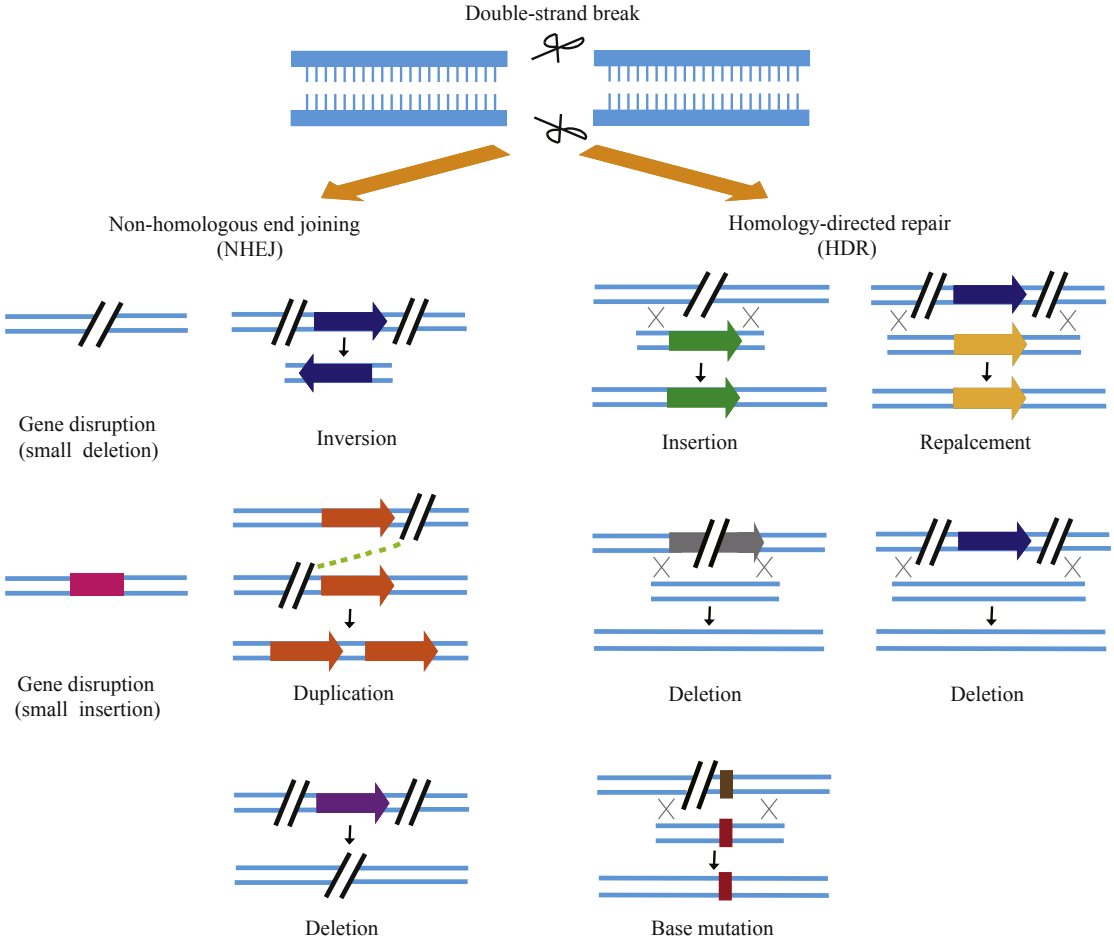

Fig. 3. Overview of potential genome editing outcomes based on NHEJ and HDR.
With either single or two double-strand breaks (DSBs), random non-homologous end joining (NHEJ) can induce unpredictable insertions, deletions, inversion and duplication, while homology-directed repair (HDR) can induce precise base mutations, insertions, deletions and replacement.

Design of the template ssODN

Although CRISPR/Cas9 system enables high efficient and specific cleavage activity, it does not necessarily relate to the efficiency and accuracy of genome editing dependent on DNA repair mechanism. Specifically, the DSB cleaved by Cas9 could stimulate at least two distinct DNA repair processes, including homology-directed repair (HDR) and non-homologous end joining (NHEJ) repair (Fig. 3). The NHEJ pathway, which rejoins both end of DSB, induces unpredictable insertions, deletions, or substitutions with the efficiency of 20%−60%, while the HDR pathway produces precise base mutations, insertions or deletions through the employment of homologous donor DNA sequences with a low frequency. Compared to the traditional large homologous arms that perform HDR, the CRISPR/Cas9 system only requires a ~90-nt single-stranded DNA oligonucleotide (ssODN), which functions as a repair template. The ssODN, which contains the complementary sequence of the target region and extends at least 40-nt in the 5′ and 3′ directions, can be arranged in either the sense or antisense strand (Ran et al., 2013b). Meanwhile, the choice of pathway is governed by the phases of cell cycle, in which NHEJ is restricted to G1, S and G2 phases, while HDR prefers S and G2 phases (Lin et al., 2014).

It is demonstrated that due to the competitive relation, the inhibition of NHEJ can improve the frequency of HDR. NHEJ comprises two types of pathways: a canonical ligase IV-dependent NHEJ (C-NHEJ) and the alternative NHEJ (alt-NHEJ) relying on ligase I/III, and C-NHEJ occurs in most cases. The efficiency of HDR-mediated genome editing is increased up to 19-fold under Scr7 treatment, for that Scr7 displayed the ability to suppress C-NHEJ pathway by inhibiting DNA ligase IV (Maruyama et al., 2015).

Besides substitutions, insertions and deletions, CRISPR/Cas9 system can also produce duplications and inversions with a pair of sgRNAs. The duplication can be generated by trans-allelic recombination between two DSBs induced by CRISPR/Cas9 cleavage in homologous chromosomes. Meanwhile, the inversions mediated by microhomology-mediated end joining (MMEJ) through short inverted repeats shows higher efficiency (Li et al., 2015).

Efficiency verification of the CRISPR/Cas9 system

A few bioinformatic tools are available for the prediction of the efficiency of the designed gRNA. Considering that the indels (insertions and deletions) or mutations can be generated through the DNA repair mechanism initiated by sgRNA-guided cleavage of Cas9, several technologies can be utilized to determine the efficiency of the CRISPR/Cas9 system, such as the SURVEYOR assay (Swiech et al., 2015) and deep sequencing (Levy et al., 2015). In the SURVEYOR assay, the reannealed heteroduplexes of 200−400 bp target region can be identified and cleaved by the SURVEYOR nuclease T7E1, leaving the homoduplexes intact. Through gel electrophoresis, the intensity of the gel bands shows the cleavage efficiency to be mediated by Cas9. The deep sequencing technology, based on the use of the Illumina Miseq and Hiseq 2500 machines, allows the indels to be crosschecked against the reference sequence in the 100−200 bp size range.

Table 1
Application of CRISPR/Cas9 system in genome, epigenome and RNA editing

Application	Targeting system and epigenetic modification	Model	Gene	Technology	Reference
Genome	*In vivo*	Mouse model	*KRAS, p53, LKB1*	Cre-LoxP technology, AAV, lentivirus, particle	Platt et al., 2014
			Tyr	Microinjection	Seruggia et al., 2015
		Liver	*Pten, p53*	Cre-LoxP technology, hydrodynamic injection	Xue et al., 2014
		Brain	*Mecp2, Dnmt1, Dnmt3a, Dnmt3b*	AAV, hydrodynamic injection	Swiech et al., 2015
	In vitro	Human hepatoma cell lines	*HBV* cccDNA	Transfection	Dong et al., 2015
		ESCs	*Tet1, Tet2, Tet3*	Microinjection	Wang et al., 2013
		Human cells	*HIV*	Transfection	Liao et al., 2015a
Epigenome	DNA methylation	Human ESCs	*DMNTs*	Transfection	Liao et al., 2015b
	Histone acetylation	HEK293T	*p300*	Transfection	Hilton et al., 2015
RNA	RNA	HeLa		Transfection	O'Connell et al., 2014
	LncRNA, eRNA	ESCs		Microinjection	Pefanis et al., 2015

AAV, adeno-associated virus; ESC, embryonic stem cell.

Table 2
Summary of CRISPR/Cas9-mediated GM mouse models

Gene	Number of CRISPR-targeted mice/total mice	Delivery site	Used nuclease and mode of action	Reference
Tet1	10/12	Cytoplasm	Cas9 RNA/DSB	Wang et al., 2013
Tet2	19/22	Cytoplasm	Cas9 RNA/DSB	
Tet3	11/15	Cytoplasm	Cas9 RNA/DSB	
Tet1, Tet2	28/31	Cytoplasm	Cas9 RNA/DSB	
Tet1	6/15	Cytoplasm	Cas9 RNA/DSB	Yang et al., 2013
Tet2	8/15	Cytoplasm	Cas9 RNA/DSB	
Tet1, Tet2	3/15	Cytoplasm	Cas9 RNA/DSB	
Sox2	12/35	Cytoplasm	Cas9 RNA/DSB	
Nanog	7/86	Cytoplasm	Cas9 RNA/DSB	
Oct4	3/10	Cytoplasm	Cas9 RNA/DSB	
Pouf5	1/5	Not available	NLS-flag-linker-Cas9 mRNA/DSB	Shen et al., 2013
CAG	1/7	Not available	NLS-flag-linker-Cas9 mRNA/DSB	
Th	8/9	Cytoplasm	Cas9 RNA/DSB	Li et al., 2013a
Th	1/11	Pronucleus	SP6-Cas9 plasmid/DSB	
Rheb	3/4	Cytoplasm	Cas9 RNA/DSB	
Uhrf2	11/12	Cytoplasm	Cas9 RNA/DSB	
Mecp2	61/61	Cytoplasm	Cas9n (D10A) RNA/double nicking	Ran et al., 2013a
Mecp2	34/37	Cytoplasm	Cas9 RNA/DSB	
Rosa26	7/7	Cytoplasm	Cas9 RNA/DSB	Fujita and Fujii, 2013
Hprt	17/18	Cytoplasm	Cas9 RNA/DSB	
Crygc	13/23	Cytoplasm	Cas9 px330/DSB	Wu et al., 2013
Cetn1	10/17	Pronucleus	Cas9 px330/DSB	Mashiko et al., 2014
Cetn1	5/20	Cytoplasm	Cas9 RNA/DSB	
Prm1	2/3	Pronucleus	Cas9 px330/DSB	
Prm1	4/4	Cytoplasm	Cas9 RNA/DSB	
Agbl1	5/13	Cytoplasm	Cas9 RNA/DSB	Zhu et al., 2014
Agbl2	4/13	Cytoplasm	Cas9 RNA/DSB	
Agbl3	9/14	Cytoplasm	Cas9 RNA/DSB	
Agbl5	3/5	Cytoplasm	Cas9 RNA/DSB	
Nmi	26/32	Cytoplasm	Cas9 RNA/DSB	
Them2	16/19	Cytoplasm	Cas9 RNA/DSB	
Rosa26	8/10 (insertion) 2/10 (deletion)	Pronucleus	Cas9n (D10A) RNA/DSB	Fujii et al., 2014a
Fah	7/9	Pronucleus	Cas9 RNA/DSB	Li et al., 2014
Fah	3/10	Pronucleus	Cas9n (D10A) RNA/DSB	
Eml4, Alk	52/52	Lung	Adenovirus with sgRNA/Cas9 cassette	Maddalo et al., 2014
Tyr	9/9	Pronucleus	Cas9 RNA/DSB	Fujii et al., 2014b
Gdf8	8/9	Pronucleus	Cas9 RNA/DSB	
Hprt	8/9	Pronucleus	Cas9 RNA/DSB	
Tyr, Gdlf8, Hprt	7/9	Pronucleus	Cas9 RNA/DSB	
Fah		Tail	Cas9 px330/DSB	Yin et al., 2014
Tyr	19/64	Pronucleus	Cas9 RNA/DSB	Seruggia et al., 2015
Tyr	1/7	Pronucleus	Cas9 RNA/DSB	Parikh et al., 2015
Tyr	2/17	Pronucleus	Cas9n (D10A) RNA/double nicking	

(*continued on next page*)

Table 2 (*continued*)

Gene	Number of CRISPR-targeted mice/total mice	Delivery site	Used nuclease and mode of action	Reference
Tet2	14/18	Pronucleus	Cas9 RNA/DSB/electroporation	Qin et al., 2015
Cd69	0/23	Pronucleus	Cas9 RNA/DSB/electroporation	
Cd226	4/19	Pronucleus	Cas9 RNA/DSB/electroporation	
Clec16a	0/57	Pronucleus	Cas9 RNA/DSB/electroporation	
Cyp27b1	12/23	Pronucleus	Cas9 RNA/DSB/electroporation	
Fut2	7/25	Pronucleus	Cas9 RNA/DSB/electroporation	
Ormdl3	17/19	Pronucleus	Cas9 RNA/DSB/electroporation	
Rgs1	8/18	Pronucleus	Cas9 RNA/DSB/electroporation	
Tlr7	6/22	Pronucleus	Cas9 RNA/DSB/electroporation	
Tlr8	1/15	Pronucleus	Cas9 RNA/DSB/electroporation	
Tnfsf9	15/21	Pronucleus	Cas9 RNA/DSB/electroporation	
Cd69	0/16	Pronucleus	Cas9 RNA/DSB/microinjection	
Cd226	0/13	Pronucleus	Cas9 RNA/DSB/microinjection	
Clec16a	2/16	Pronucleus	Cas9 RNA/DSB/microinjection	
Cyp27b1	0/20	Pronucleus	Cas9 RNA/DSB/microinjection	
Fut2	9/25	Pronucleus	Cas9 RNA/DSB/microinjection	
Ormdl3	2/15	Pronucleus	Cas9 RNA/DSB/microinjection	
Rgs1	5/19	Pronucleus	Cas9 RNA/DSB/microinjection	
Tlr7	0/15	Pronucleus	Cas9 RNA/DSB/microinjection	
Tlr8	3/22	Pronucleus	Cas9 RNA/DSB/microinjection	
Tnfsf9	0/14	Pronucleus	Cas9 RNA/DSB/microinjection	
Dip2a	3/14	Pronucleus	Cas9 px330/DSB	Zhang et al., 2015a
LacZ	2/18	Pronucleus	Cas9 px330/DSB	
Actb	8/8	Pronucleus	Cas9 RNA/DSB	Aida et al., 2015
Actb	5/19	Pronucleus	Cas9 protein/sgRNA/DSB	
Actb	9/11	Pronucleus	Cas9 protein/crRNA:tracrRNA/DSB	
Kcnj13	3/38	Cytoplasm	Cas9 RNA/DSB	Zhong et al., 2015

Off-target avoidance

Off-target mutation remains a main challenge in the application of the CRIPSR/Cas9 system in genome editing. Therefore, designing an optimized gRNA, employing a functional Cas9, and choosing a unique target sequence are critical for improving efficiency and reducing off-target mutations.

Because not all sgRNA can induce efficient and precise editing, a unique target sequence is required. The results of comprehensive experiments have revealed that the CRISPR system can tolerate less than three mismatched bases between the target DNA and the sgRNA, in general, especially when the mismatched bases occur on the 3′-end of the gRNA (Ran et al., 2013b). However, it has been reported that a seed, defined as position 1−5 of the sgRNA, is sufficient for Cas9 binding both *in vivo* and *in vitro*, followed by sequence-tag NGG rather than NAG (Wu et al., 2014). Furthermore, a 14-nt long region containing a 12-nt portion of the sgRNA and 2-nt of the PAM is a key component for determining target specificity (Larson et al., 2013).

However, the PAM specificities can be modified by mutagenesis and selection of SpCas9 variants for substantial elimination of off-target. Compared to wild-type SpCas9, the VQR variant (D1135V/R1335Q/T1337R) displays the highest activity on an NGAN PAMs (NGAG > NGAT = NGAA > NGAC) and lower efficiency on NGNG PAMs, while VRER variant (D1135V/G1218R/R1335E/T1337R) robustly cleaves the sites bearing NGCG PAMs. Moreover, D113E variant shows better discrimination between NGG and NAG PAMs than wild-type SpCas9 (Kleinstiver et al., 2015). Meanwhile, similar to SpCas9, most Cas9 orthologues from other bacteria can successfully cleave 3-bp upstream of the putative PAM under the guidance of sgRNA, while only SaCas9 (derived from *Staphylococcus aureus*) and StlCas9 (derived from *S. thermophiles*) have been confirmed to be comparable with SpCas9 in highly efficient genome editing. For StlCas9, the PAM has been characterized as NNAGAA. And for SaCas9, three PAMs containing NNGGGT, NNGAGR, and NNGAAT, were considered as potential targets (Kleinstiver et al., 2015).

As mentioned above, a functional Cas9 can generate two separate single-strand breaks (SSBs), whereas Cas9n with D10A or H847A mutation only creates a nick in each strand (Shen et al., 2014). On basis of this, which can be detected by deep sequencing, Cas9n was found to produce much fewer off-target changes than Cas9 nuclease (Cho et al., 2014). Recently, three deep sequencing methods for the detection of off-target cleavage events have been reported, termed high-throughput, genome-wide, translocation sequencing (HTGTS), genome-wide, unbiased identification of DSBs enabled by sequencing (GUIDE-seq), and *in vitro* Cas9-digested whole genome sequencing (Digenome-seq), all of which can capture the DSBs induced by Cas9. Compared with the other two methods, Digenome-seq can profile the genome-wide off-target effects of the CRISPR/Cas9 system, using either Cas9 nuclease or modified Cas9 for genome editing, in a more unbiased manner, and is not limited by chromatin accessibility. Importantly, the off-target can be detected with a frequency of 0.1% or lower (Kim et al., 2015). Predictably, the growing development and modification of the CRISPR/Cas9 system will facilitate a broader range of its applications.

APPLICATIONS OF CRISPR/CAS9 SYSTEMS

With the capacity for easy and convenient genome, epigenome, and RNA editing, CRISPR/Cas9 provides immense capacity to make remarkable progress in biotechnological, basic biological, and medical research fields (Table 1).

Genome editing using the CRISPR/Cas9 system

Generation of genetically modified (GM) mouse model of human diseases

Traditional GM mouse models are generated by transgenesis or gene targeting in embryonic stem cells (ESCs), which is time-consuming and expensive. Utilizing the CRISPR/Cas9 system to investigate gene function in development and disease pathology, however, GM mice, with either null, conditional, precisely mutated genes, or reporter, or tagged alleles, can be generated rapidly and efficiently (Table 2) (Singh et al., 2015). An one-step approach has been successfully set up to generate mice carrying mutations by using CRISPR/Cas9 system (Cong et al., 2013; Wang et al., 2013; Yang et al., 2013; Matsunaga and Yamashita, 2014; Qin et al., 2014; Zhou et al., 2014; Hyun et al., 2015). Specifically, fertilized oocytes at the pronuclei stage were directly injected with Cas9 mRNA, sgRNA, and donor oligos to knock-in a specific mutation in the target genes, such as *Tet1*, *Tet2*, and *Tet3*, and then cultured *in vitro* up to the blastocyst stage (Wang et al., 2013). Subsequently, the blastocysts can be delivered into the uterus of a surrogate female to produce mutant offspring. Moreover, mice which contain multiple gene alterations can be established either by a one-step approach (Cong et al., 2013; Matsunaga and Yamashita, 2014; Qin et al., 2014; Hyun et al., 2015) or by site-directed mutagenesis (SDM) (Hyun et al., 2015; Zhang et al., 2015b). Meanwhile, mouse models for lung adenocarcinoma with *KRAS, p53,* and *LKb1* mutations were established by using distinct CRISPR/Cas9 systems, in which gRNA could be delivered into neurons and immune cells with a variety of reagents, such as adeno-associated virus (AAV), lentivirus, and chemicals (Platt et al., 2014).

Impressively, the CRISPR/Cas9 system can be used to disrupt infection of human immunodeficiency virus 1 (HIV-1) in human cells, where the expression and replication of the virus are interrupted by altering coding or non-coding sequences during the pre-integration or provirus stages, suggesting that the CRISPR/Cas9 system may be a novel therapeutic strategy against HIV (Liao et al., 2015a).

Furthermore, injection of plasmids containing Cas9 and sgRNA targeting the conserved regions of hepatitis B virus (HBV) to tail vein, can inhibit replication of HBV and result in the down-regulation of covalently closed circular DNA (cccDNA) and HBV protein, which suggests that the CRISPR/Cas9 system may offer a new strategy for the treatment of HBV infection (Dong et al., 2015).

Genome editing in specific tissues

Using the CRISPR/Cas9 system, researchers can edit the genome of specific tissues, such as the liver and brain tissues, directly and efficiently using hydrodynamic injection and AAV vectors (Rodriguez et al., 2014; Senis et al., 2014). Specifically, the application of the CRISPR/Cas9 system can generate a cancer model with *Pten* and *p53* mutations by delivering a plasmid containing Cas9 and sgRNAs to the liver *via* direct hydrodynamic tail-vein injection. Deletion or insertion mutations at the *Pten* locus were confirmed by deep sequencing, suggesting that the CRISPR/Cas9 system can directly induce loss-of-function mutations in somatic cells *in vivo*. In addition, a long-term influence has been identified by harvesting and examining the livers of mice injected with CRISPR plasmids four months later, and the resulting loss of Pten expression suggested that the CRISPR/Cas9 system produced a long-term phenotype in knockout mice (Xue et al., 2014).

Recently, the CRISPR/Cas9 system was successfully and efficiently applied to the mammalian nervous system, where a mix of AAV-spCas9 and AAV-spGuide plasmids tagged with GFP were injected into the hippocampal dentate gyrus of adult male mice *in vivo* (Swiech et al., 2015). It is expected that such applications will be more extensively employed in the cancer and neuroscience communities.

Simultaneous generation of multiple gene mutations

Increasing number of evidence confirmed that CRISPR/Cas9 system could generate mutants for multiple target genes in numerous organisms. Dating back to 2013, multiple gene mutations have been achieved in rat through simultaneously delivering six sgRNAs targeting six genomic sites encoding *Tet1 (sgTet1-1, sgTet1-2)*, *Tet2 (sgTet2-1, sgTet2-2)*, *Tet3 (sgTet3-1, sgTet3-2)*, together with Cas9 mRNA into the cytoplasm of one-cell stage rat embryos. It turned out that 59% newborn pups were identified with mutations of all three Tet genes concurrently (Li et al., 2013b). Similarly, it was also confirmed in other organisms. For instance, the indel

mutations at *golden/gol* and *tyrosinase/tyr* genes, which take participate in pigment formation, and *s1pr2* and *spns2* genes, which are related to the development of cardiac, were detected simultaneously, after co-injection sgRNAs and Cas9 mRNA into one-cell stage zebrafish embryos (Ota et al., 2014). Meanwhile, in *Arabidopsis*, the multiple gene mutants can not only be produced by CRISPR/Cas9 system for mosaics T1 generation, but also be achieved with non-mosaics T1 generation by specifically expressing Cas9 and sgRNAs tergeting *CPC, TRY*, and *ETC2* in egg cells and one-cell stage embryos (Wang et al., 2015b).

Flexible manipulation in epigenome

The CRISPR system not only provides the capacity to directly modify target DNA sequences, but also has the ability to alter the epigenome, for regulating the expression of specific genes. For example, the CRISPR/Cas9 system has been used to suppress DNA methyltransferases (DNMTs) in human cells by disrupting their catalytic domains, which leads to a rapid and global loss of DNA methylation and causes cell death (Liao et al., 2015b). Additionally, using dCas9 fused to the catalytic histone acetyltransferase core domain of p300 to form a dCas9$^{p300\ core}$ fusion protein (Hilton et al., 2015), it is feasible to regulate gene expression by modifying acetylation at the target sites, such as promoters or proximal and distal enhancers. Meanwhile, the dCas9$^{p300\ core}$, after activated by light or chemicals, can be applied more broadly to regulate the dynamic variation of gene expression in a space- and time-dependent manner. Furthermore, the core domain of p300 can be fused to other programmable DNA-binding proteins to alter the acetylation level at a targeted site. Thus, similar to the genome-editing model using the CRISPR/Cas9 system, gRNA and PAM sequences need to be developed for epigenome editing as well.

Moreover, the CRISPR/Cas9 system has been utilized in long non-coding RNA (lncRNA) and enhancer RNA (eRNA), which can regulate gene expression and epigenetic processes. For instance, in CH12F3 cell line, a subclone of the lgM$^+$ CH12.LX lymphoma cell line, the eRNA-expressing element (lncRNA-CSR) and the lncRNA-expressing enhancers were downregulated by the deletions induced by the CRISPR/Cas9 system (Pefanis et al., 2015). In addition, the large fragment deletions of lncRNA, Rian, induced by CRISPR/Cas9 system, disturbed the expression of nearby genes (Han et al., 2014). Over the long term, CRISPR/Cas9 system might provide more chances for people to edit epigenome at different levels.

Novel potentiality in RNA editing

In addition to double-stranded DNA, RNA sequences can be edited by using the CRISPR/Cas9 system. Such an RNA editing CRISPR/Cas9 system consists of a PAM-presenting DNA oligonucleotide (PAMmer), ssRNA (single strand RNA), gRNA, and a Cas9 protein. The PAMmer functions as a PAM, which is specifically recognized by Cas9 and directs Cas9 to bind or cleave the target ssRNA. The 5′-extended PAMmers, which contain several ssRNA-matched bases and immediately precede the PAM, are required for the specific binding of the target ssRNA. Furthermore, dCas9-gRNA has a higher affinity for binding the target ssRNA than wild-type Cas9 (O'Connell et al., 2014). As RNA has more varied functions than DNA, it could make the CRISPR/Cas9 system more flexible than other applied genome editing tools.

ETHICAL CHALLENGES

Although the CRISPR/Cas9 has brought increasing convenience and efficiency, several concerns regarding CRISPR/Cas9 ethical and biosafety have emerged. Recently, the mutagenic chain reaction (MCR) based on the CRISPR/Cas9 system, has provided a novel and prospective way to generate homozygous loss-of-function mutations by autocatalysis, as confirmed in *Drosophila* (Gantz and Bier, 2015). The core cassette, carrying a Cas9, a gRNA, and two homology arms adjacent to the target site, can be inserted into the target locus by HDR. Subsequently, the inserted cassette expresses Cas9 and gRNA to cleave the allele in the complementary strand, leading to a conversion from heterozygous to homozygous *via* HDR. As expected, the phenotype of the offspring, generated from hybridization of a wild-type and a homozygote individual containing the CRISPR/Cas9 system, is all homozygous, in accordance with theoretical MCR-based inheritance. However, due to poor understanding of the mechanism, the possible severe consequences to trigger an entire change of spices, and the potential effects on the ecological balance (Bohannon, 2015), some arguments against use of this technology have been raised.

Recently, a paper reporting gene editing in human embryos was published in the journal *Protein & Cell*, which raised concerns about the ethics of employing the CRISPR/Cas9 system (Liang et al., 2015). Thereafter, both the editorial team of *Nature* and *Science* announced that although the CRISPR/Cas9 system shows huge potential for genome editing, its use for modifying human germline cells should be considered very seriously, and progressive policy on this issue should be developed (Baltimore et al., 2015; Zhang, 2015). At the same time, however, Emilie Marcus, the editor-in-chief of *Cell*, stated that the journal would consider publication of manuscripts describing human germline modification, if they met high technical and ethical standards. The acceptance of this article should not be considered as endorsement or encouragement of modifying human germline cells, but should be viewed as a point to start the discussion about the human germline editing (Zhang, 2015). Thus, embryo editing or engineering of human fetuses is becoming increasingly controversial among scientists. Especially, some countries have already restricted CRISPR/Cas9 technology, with some completely banning its use in humans. To address such a complicated debate, positive and negative aspects of germline editing should be weighed by an authoritative national agency, and both the scientific and social ethical concerns should be taken into consideration simultaneously (Baltimore et al., 2015).

CONCLUDING REMARKS

The CRISPR/Cas9 genome editing system, with its accelerated development and expanded applications, is an indispensable tool for precise and efficient genome editing, but some related problems need more attention.

First, the current knowledge of the CRISPR/Cas9 system at the biochemical and crystal structural levels is insufficient and requires additional research, including a deep analysis of the Cas9 protein, one of the main components in the CRISPR/Cas9 system. The natural variation in Cas9 proteins isolated from different species might provide new Cas9 proteins with higher efficiency and thereby broaden the choices available for precise genome editing (Ran et al., 2015).

Moreover, specific modes for delivering Cas9, gRNA, and donor oligos to cells and tissues have been developed in numerous species, such as mice, *Drosophila*, zebrafish, worms, and humans. For example, the CRISPR/Cas9 system being used to create transgenic mice could be fused with other proteins or effectors to control or stimulate the expression or initiation of the CRISPR/Cas9 system *in vivo*. The off-target mutation rates of diverse CRISPR/Cas9 systems, nevertheless, remain a challenge.

Last, but certainly not least, the direct and precise genome editing raises ethical concerns, such as gene modification of human germline cells using the CRISPR/Cas9 system to create "engineered babies" (Liang et al., 2015), which initiates arguments and queries among scientists and the public. In addition, the invention of MCR also creates intense concern regarding environmental balance and species safety (Gantz and Bier, 2015). It is urgent that the government and related social organizations formulate and enact a series of laws and regulations to enable the safe and ethical application of the CRISPR/Cas9 system in basic research and clinics. We envisage a bright future in which the CRISPR/Cas9 system will facilitate revolution and improvement of genome, RNA, and epigenome editing.

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