

## CRISPR/Cas9 for Genome Engineering: the Next Genomic Revolution

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### ABSTRACT

Genetically modified cells and animals represent a crucial tool for understanding gene function in development and diseases. The recently developed simple-to-design, easy-to-use and multiplexing CRISPR/Cas9 system is an efficient gene-targeting technology that has already stimulated innovative applications in biology and enabled researchers to make changes in the sequence or expression of any gene in virtually any cell type or organism of interest. When combined with large libraries of sgRNAs, CRISPR/Cas9 enables facile comprehensive forward genetic screens both *in vitro* and *in vivo*. Although challenges still remain regarding the off-target mutations, delivery methods as well as the frequency of homology-directed repair, the rapid advance in CRISPR/Cas9 technology opens the door for gene function revealing and genome and epigenome editing. It can be optimistically anticipated that, in the future, CRISPR/Cas9 technology may revolutionize gene therapy research and become a convenient and versatile tool to treat a wide variety of human diseases.

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**Key words:** CRISPR/Cas9; Genome Engineering; Mechanism; Application

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### EDITORIAL

The last two decades have witnessed a rapid progress in genetic sciences. Despite tremendous advances in high-throughput sequencing technology and the rapid generation of whole-genome sequencing data for large numbers of all types of organisms, elucidation of the underlying molecular mechanism of genes influencing individual phenotypes remains a major challenge facing the researchers. A rational way to elucidate the function of a gene or a gene mutation is to silence or overexpress it in living organisms. Conventional genetic engineering methods have generally been limited to random addition of new pieces of DNA into the host genome without any control on the insertion loci of interest and insertion copy numbers, thus not allowing the editing of specific DNA sequence in their natural context. New genome editing approaches that built on engineered, programmable and highly specific nucleases, which can induce site-specific changes in the genome of cellular organisms through a sequence-specific DNA-binding domains and a nonspecific DNA cleavage domain have emerged and are widely used in the studies of functional genomics, transgenic organism and gene therapy.

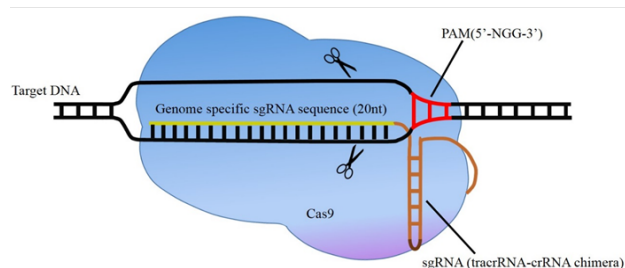
The first well studied artificial nuclease system is zinc-finger nucleases (ZFNs)<sup>[1-4]</sup>. Zinc fingers are small protein structural motifs that are typically 25-30 residues long and possess two cysteine and two histidine residues that coordinate a zinc ion. Zinc finger DNA binding proteins typically contain two or more zinc fingers with each finger interacting with 3 base pairs of DNA, typically interacting with contiguous 3-base pair recognition sites. By fusing a zinc-finger

DNA-binding domain with a nonspecific cleavage domain from the type IIS restriction endonuclease FokI, ZFNs can be engineered to target desired DNA sequence and induce DNA double strand break. The nuclease domain requires dimerization to be active, and thus two individual ZFNs must bind opposite strands of DNA with their C-termini a certain distance apart to facilitate dimerization of the nuclease domain<sup>[1,2,5-11]</sup>. Although ZFNs are effective genome editing tools and numerous innovations to improve the utility of ZFNs have occurred, the technical challenges inherent in designing and validating engineering zinc-finger proteins for a specific DNA locus of interest have limited their use beyond by experts in the field. In 2009, the genome editing field has further expanded with the development of transcription activator-like effector nucleases (TALENs)<sup>[12,13]</sup>. TALEs are transcriptional activators that specifically bind and regulate plant genes during pathogenesis. Within the TALE structure, a repeated highly conserved 33-34 amino acid sequence with the exception of the 12th and 13th amino acids that mediate DNA recognition. These two locations are highly variable (Repeat Variable Di-residue, RVD) and show a strong correlation with specific nucleotide recognition. Each unit of a TALE protein recognizes only a single base pair. This simple relationship between amino acid sequence and DNA recognition has allowed for the engineering of specific DNA binding domains by selecting a combination of repeat segments containing the appropriate RVDs, which is much easier than zinc fingers to produce and validate, enabling much more widespread applications in genome editing<sup>[14-20]</sup>. The recent emergence of the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system, which depends on small RNA guided sequence-specific cleavage, however, has transformed the genome engineering fields by removing the need for any expertise in protein engineering<sup>[21-26]</sup>.

CRISPRs were firstly discovered from the genome of *Escherichia coli* in 1980s and had been described as a series of direct repeats interspaced with short sequences<sup>[27]</sup>. Later studies have shown that CRISPR/Cas exists in nearly 40% genomes of sequenced bacteria and nearly 90% genomes of sequenced archaea<sup>[28]</sup>. Based on the observations that many spacer sequences within CRISPRs derive from plasmid and viral origins, CRISPRs loci can be transcribed and that *cas* (CRISPR-associated gene) encode proteins with putative nuclease and helicase domains, CRISPRs were predicated as an adaptive defence system that use antisense RNAs as memory signatures of past invasions<sup>[29]</sup>. However, it was not until in 2007 that infection experiments with lytic phages provided the first experimental evidence of CRISPR/Cas-mediated adaptive immunity. In those studies, bacteria integrated new spacers derived from phage genomic sequences. Removal or addition of particular spacers modified the phage-resistance phenotype of the cell. Thus, CRISPR, together with associated *cas* genes, provided resistance against phages, and resistance specificity is determined by spacer-phage sequence similarity<sup>[21]</sup>. In 2008, Brouns *et al* found that mature CRISPR RNAs (crRNAs) served as guides in a complex with Cas proteins to interfere with viral proliferation<sup>[22]</sup>. Functional CRISPR-Cas loci consist of a series of conserved repeated sequences interspaced by distinct non-repetitive sequences named spacers that encode the crRNA components and an operon of *cas* genes encoding the Cas protein components. After the first infection of viruses or phages, invading foreign DNA is processed by Cas nucleases into small DNA fragments, which are then incorporated into CRISPR locus of host genomes as the spacers. In response to virus or phage infections, the spacers are used as transcriptional templates for producing precursor crRNA (pre-crRNA) that undergoes maturation to generate individual crRNAs, each composed of a repeat portion

and an invader-targeting spacer portion, which guides Cas to cleave foreign nucleic acid by Cas proteins at sites complementary to the crRNA spacer sequence. More than 40 different Cas protein families have been reported, which are primarily classified into three types (I, II, and III)<sup>[30-32]</sup>. The type II CRISPR/Cas system utilizes Cas9, a large multifunctional protein with two putative nuclease domains (HNH<sup>[29,30]</sup> and RuvC-like<sup>[29]</sup>), to cleave dsDNA, whereas Type I and Type III systems require multiple distinct effectors acting as a complex<sup>[33,34]</sup>. CRISPR/Cas9 has been demonstrated to be a simple and efficient tool for genome engineering.

In endogenous CRISPR/Cas9 system, mature crRNA is combined with trans-activating crRNA (tracrRNA), a small RNA that is trans-encoded upstream of the type II CRISPR-Cas locus and is essential for crRNA maturation by ribonuclease III and Cas9<sup>[35]</sup>, to form a tracrRNA:crRNA complex that guides Cas9 to a target site. Cas9 unwinds the DNA duplex and searches for sequences matching the crRNA to cleave. Target recognition occurs upon detection of complementarity between a “protospacer” sequence in the target DNA and the remaining spacer sequence in the crRNA. Importantly, Cas9 cuts the DNA only if a correct protospacer-adjacent motif (PAM) is also present at the 3' end. PAM is a short sequence motif adjacent to the crRNA-targeted sequence on the invading DNA, playing an essential role in the stages of adaptation and interference in type I and type II systems<sup>[36,37]</sup>. Different Type II systems have differing PAM requirements. The *S. pyogenes* system requires an NGG sequence<sup>[34]</sup>, *S. thermophilus* Type II systems require NNAGAA for CRISPR1<sup>[23,25]</sup> and NGGNG for CRISPR3<sup>[33,38]</sup>, while *Neisseria meningitidis* requires NNNNGATT<sup>[39]</sup>. After binding to the target site, the DNA single-strand matching crRNA and opposite strand are cleaved, respectively, by the HNH nuclease domain and RuvC-like nuclease domain of Cas9, generating a double-strand break (DSB) at the target site<sup>[33,34]</sup>. The CRISPR/Cas9 induced DSB will trigger cellular DNA repair processes, including nonhomologous end-joining (NHEJ)-mediated error-prone DNA repair and homology-directed repair (HDR)-mediated error-free DNA repair, both of which can be used to achieve a desired editing outcome. In NHEJ-mediated DNA repair, the break ends are directly ligated without the need for a homologous template, generating small insertion and deletion mutations at target sites. These mutations can help us to disrupt or abolish the function of target genes or genomic elements. If the insertion or deletion occurring within a coding exon can lead to frameshift mutations and premature stop codons, gene knockouts are achieved<sup>[40]</sup>. In contrast to NHEJ-mediated DNA repair, HDR-mediated error-free DNA repair requires a homology-containing donor DNA sequence as repair template. The repair template can either be double stranded DNA with homology arms flanking the insertion sequence, or single-stranded DNA oligonucleotides (ssODNs). ssODNs provides an effective and simple method for making small edits in the genome, such as the introduction of single nucleotide mutations<sup>[41]</sup>. However, HDR is generally active only in dividing cells and typically occurs at lower frequencies than NHEJ<sup>[42]</sup>. For easy application in genome editing, the dual tracrRNA:crRNA was then engineered as a single guide RNA (sgRNA), which was a chimeric RNA containing all essential crRNA and tracrRNA components (Figure 1)<sup>[34]</sup>. In this two-component system, by simply changing the guide sequence of the sgRNA, it theoretically enables CRISPR/Cas9 to target any DNA sequence of interest as long as it is adjacent to a PAM. Furthermore, when Cas9 is coupled with several sgRNAs targeting on different sites, CRISPR/Cas9 is able to simultaneously induce genomic modifications at multiple genes or multiple independent sites of the same gene<sup>[25,36]</sup>, accelerating the study of gene function and epistatic



**Figure 1** Illustration of CRISPR/Cas9-mediated genome editing. The dual tracrRNA:crRNA is engineered as a single guide RNA (sgRNA), which is a chimeric RNA containing all essential crRNA and tracrRNA components. sgRNA guides Cas9 to a target site when detects complementarity between a “protospacer” sequence in the target DNA and the remaining spacer sequence in the crRNA. After binding to the target site, the DNA single-strand matching crRNA and opposite strand are cleaved by the HNH nuclease domain and RuvC-like nuclease domain of Cas9 respectively, generating a double-strand break (DSB).

relationships<sup>[43-45]</sup>. For this reason, the CRISPR/Cas9 has been rapidly and widely adopted by the scientific community to target, edit, or modify the genomes of a vast array of cells and organisms.

As a robust genome editing tool, CRISPR/Cas9 enables researchers to precisely manipulate specific genomic elements not only in mammalian genomes<sup>[25,46-49]</sup> but also in the genomes of rat<sup>[50]</sup>, mice<sup>[43,51]</sup>, zebrafish<sup>[37,52-54]</sup>, *Drosophila*<sup>[55-60]</sup>, *C. elegans*<sup>[61-64]</sup>, *Bombyx mori*<sup>[65-68]</sup>, bacteria<sup>[69-71]</sup> as well as in crop plants<sup>[72-77]</sup>. Targeting with multiple sgRNAs (multiplexing) was also successfully achieved<sup>[25,48]</sup>. When combined with lentiviral sgRNA library, a pooled, loss-of-function genetic screening approach suitable for both positive and negative selection was developed, establishing Cas9/sgRNA screening as a powerful tool for systematic genetic analysis in mammalian cells<sup>[78,79]</sup>. Recently, the genome-wide CRISPR/Cas9-mediated loss-of-function screen was further developed to systematically assay gene phenotypes in cancer evolution *in vivo*<sup>[80,81]</sup>. It can be anticipated that this high-throughput sequencing tool will not only facilitate the rapid identification of genes that participate in certain biological processes but will also enable large-scale screening for drug targets and other phenotypes. Another important application of CRISPR/Cas9 system is in the research of transcriptional regulation. By targeting on the transcription-related functional sites, CRISPR/Cas9 can regulate the transcription of specific genes. However, this process is irreversible due to permanent DNA modifications. To develop a CRISPR inference (CRISPRi) system for RNA-guided transcription regulation beyond permanent modification of DNA, sgRNA was co-expressed with a catalytically defective Cas9 mutant (dCas9) to form a recognition complex, which could interfere with transcriptional elongation, RNA polymerase and transcription factor binding<sup>[82-84]</sup>. Demonstrated first in *E. coli*, whole-genome sequencing showed that there were no detectable off-target effects<sup>[83]</sup>. CRISPRi has also been proved to repress multiple target genes simultaneously, and its effects are reversible<sup>[83,85]</sup>. After fusion with repressive or activating effector domains, dCas9 together with sgRNA, could implement precise and stable transcriptional control of target genes, including transcription repression (CRISPRi) and activation (CRISPRa) with high specificity<sup>[82,86]</sup>. Similar to those performed using RNAi, dCas9 can be used as a modular and flexible DNA-binding platform for the recruitment of proteins to a target DNA sequence, laying the foundation for future experiments involving genome-wide screening. The CRISPR/Cas9 system has also been employed for efficient correction of genetic disease<sup>[87,88]</sup>. A dominant mutation in the *Crygc* gene responsible for cataracts

was successfully corrected in mice by co-injection of CRISPR/Cas9 with an exogenously supplied oligonucleotide into zygotes<sup>[88]</sup>. The CRISPR/Cas9 system can be further developed as an imaging tool for imaging of specific loci, by fusing dCas9 with fluorescent proteins, in live cells<sup>[89]</sup>, and developed as a new therapeutic strategy against viral infections, disrupting proviruses, eliminating viral genomes and thus curing viral infections<sup>[90-93]</sup>.

A few careful studies, however, duly raised concerns that CRISPR/Cas9 had tolerance to base pair mismatches between gRNA and its complementary target sequence. Cas9 can cleave the target DNA both *in vitro*<sup>[34]</sup> and in mammalian<sup>[25]</sup> and bacterial cells<sup>[70]</sup> when complexed with a crRNA that contains a one-base mismatch with the target sequence. More recently, three group independently showed that CRISPR/Cas9 can induce off-target mutations even the mismatch up to 5 nt<sup>[26,94,95]</sup>. Since CRISPR/Cas9 tolerates mismatches especially in the 5' upstream region, but not in the seed region of 6-11 nt that is immediately upstream of the PAM sequence, target sites should be carefully selected so that none or very few of the seed region sequence of the designed sgRNA exist at any other location of the genome<sup>[26,94,96]</sup>. Another important factor in tolerance to mismatches is the amount of Cas9 enzyme expressed in the cells. Usually, high concentration of the enzyme increases off-site targeting, whereas lowering the concentration of Cas9 increases specificity while diminishing on-target cleavage activity<sup>[94,95]</sup>. As mentioned previously, the DNA single-strand matching crRNA and opposite strand are cleaved by the HNH nuclease domain and RuvC-like nuclease domain of Cas9 respectively<sup>[33,34]</sup>, mutating either domain in Cas9 generates a variant protein with single-stranded DNA cleavage (nickase) activity. With the principle that two adjacent off-target binding events and subsequent cleavage are less likely to occur than a single off-target cleavage, Cas9 nickase with paired gRNAs properly positioned on the target DNA exhibits low off-target mutagenesis compared to wild-type Cas9<sup>[97-102]</sup>. Also there are studies use an sgRNA-guided dCas9 fused to the FokI nuclease where two fused dCas9-FokI bind target sites at a defined distance apart, inducing DNA double strand break after dimerization of the two monomers<sup>[103,104]</sup>. Additionally, following the reasons that the 5'-end nucleotides of the sgRNAs are not necessary for their full activity, however, they may compensate for mismatches at other positions along the guide RNA-target DNA interface, leading to off-target mutations, shorter sgRNAs truncated by two or three nucleotides at the distal end relative to the PAM can be used in the double nicking strategy to further reduce off-target activity<sup>[105]</sup>.

## CONCLUSION

In conclusion, the RNA-guided, two component CRISPR/Cas9 system offers several advantages over the previous protein guided counterparts. The simple-to-design, easy-to-use and multiplexing technology has already stimulated innovative applications in biology and enabled researchers to make changes in the sequence or expression of any gene in virtually any cell type or organism of interest. When combined with large libraries of sgRNAs, CRISPR/Cas9 enables facile comprehensive forward genetic screens both *in vitro* and *in vivo*. Furthermore, this gene editing tool has shown therapeutic potentials for genic diseases, infectious diseases and cancer. Despite the great potential of CRISPR/Cas9 in genome editing, challenges still remain regarding the off-target mutations, delivery methods as well as the frequency of homology-directed repair. However, with the rapid advance in CRISPR/Cas9 technology, it opens the door for gene function revealing and genome and

epigenome editing. It can be optimistically anticipated that, in the future, CRISPR/Cas9 technology may revolutionize gene therapy research and become a convenient and versatile tool to treat a wide variety of human diseases.

## CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

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