

Genome engineering using the CRISPR/Cas system

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Core tip: This review introduces the latest information about the genome manipulation technology of the clustered regularly at interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) system to readers. We focus particularly on the application of CRISPR/Cas in mammalian cultured cells and mice. The problems of off-target effects and the prospects for therapeutic applications of CRISPR/Cas in the future are also discussed.

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Abstract

Recently, an epoch-making genome engineering technology using clustered regularly at interspaced short palindromic repeats (CRISPR) and CRISPR associated (Cas) nucleases, was developed. Previous technologies for genome manipulation require the time-consuming design and construction of genome-engineered nucleases for each target and have, therefore, not been widely used in mouse research where standard techniques based on homologous recombination are commonly used. The CRISPR/Cas system only requires the design of sequences complementary to a target locus, making this technology fast and straightforward. In addition, CRISPR/Cas can be used to generate mice carrying mutations in multiple genes in a single step, an achievement not possible using other methods. Here, we review the uses of this technology in genetic analysis and manipulation, including achievements made possible to date and the prospects for future therapeutic applications.

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INTRODUCTION

The recent development of site-specific endonuclease technologies for selective genome cleavage has been an important advance in mammalian genome engineering. Zinc-finger nucleases (ZFNs) consist of specific DNA-binding zinc-finger proteins and a nuclease domain of the *Fok I* endonuclease^[1-3]. Cleavage with *Fok I* requires dimerization of the protein; therefore, fusion to a pair of zinc-finger proteins provides target specificity, and allows cleavage of the target DNA locus, generating double-strand breaks (DSBs).

On the other hand, transcription activator-like effector (TALE) nucleases (TALENs) are fusions of DNA-binding domain TALE repeats with the cleavage domain of the *Fok I* restriction enzyme. TALE repeats are highly conserved 33-35 amino acid sequences found in naturally occurring TALEs encoded by *Xanthomonas* bacteria^[4]. Each TALE repeat binds to a single base pair of DNA and the identities of the amino acids at two posi-

was elucidated, many researchers expected that it functions in the cells of eukaryotic organisms such as yeast, plants, and even mammals. In January 2013, several papers using the CRISPR/Cas system in human cells were published in succession^[50-53]. Cho *et al.*^[52] showed that combination of Cas9 protein and artificial sgRNAs efficiently cleaved two genomic sites and induced indels with approximately 33% frequencies using human embryonic kidney (HEK) 293T-cells. Two papers published in *Science* used other cell types or targeting loci^[50,51]. For the endogenous AAVS1 safe harbor genomic locus, Mali *et al.*^[50] succeeded in gene targeting using 293T-cells (10%-25%), human chronic myelogenous leukemia K562 cells (8%-13%), and human induced pluripotent stem (iPS) cells (2%-4%). In addition, they also used HDR to integrate either a double-stranded DNA donor construct (SA-2A-Puro-pA + CAG-GFP-pA) or an oligo donor into the native AAVS1 locus, and obtained 293T or iPS clones showing HDR-mediated integration.

CRISPR/Cas also enables NHEJ- and HDR-mediated genome editing in mouse ES cells^[54,55]. The high efficiency of the CRISPR/Cas system coupled with the ability to easily create synthetic sgRNAs make it possible to target multiple genes simultaneously, which is not possible using previous methods^[54]. Wang *et al.*^[54] transformed embryonic stem cells using CRISPR/Cas system for three different genes (*Tet1*, *Tet2*, and *Tet3*), and found that > 20% (20/96) of ES cell clones had mutations in all six alleles. To further test the potential of multiplexed gene targeting using the CRISPR/Cas system, sgRNAs targeting five genes (*Tet1*, *Tet2*, *Tet3*, *Sry*, and *Uty*) were mixed and co-transfected with a Cas9-expressing vector into ES cells; of 96 clones screened using an restriction fragment length polymorphism assay, 10% carried mutations at all five loci.

The use of the CRISPR/Cas system in combination with haploid ES cells^[56-58] provides a powerful platform to manipulate the mammalian genome, because disruption of only one allele can cause loss-of-function phenotypes in haploid ES cells. We have recently reported that co-transfection of mouse haploid ES cells with vectors expressing Cas9 nuclease and sgRNAs targeting *Tet1*, *Tet2*, and *Tet3* results in the complete disruption of all three genes, causing a loss-of-function phenotype with higher efficiency (50%)^[59] than that previously reported using diploid ES cells^[54]. Thus, the CRISPR/Cas system used in the context of haploid cells will be useful for the efficient disruption of multiple genes.

ONE-STEP GENERATION OF GENOME-EDITED ANIMALS

Homologous recombination in mouse ES cells is the most popular method for targeted modifications of the mouse genome; however, generating gene-modified mice through germline chimeras is both time consuming and expensive. Therefore, alternative methods have been developed to accelerate the process of genome modification by the introduction of site-specific nucleases into

fertilized embryos to generate DNA DSBs at a target locus in various species. ZFNs and TALENs have been used to produce several gene-modified rodents^[60-62]. Although these technologies are widely used in other animals, their use in mice has been limited, principally because the ZFN and TALEN systems are labor-intensive and expensive techniques that do not perform substantially better than ordinary gene knockout technology. On the other hand, CRISPR/Cas-mediated genome editing has successfully demonstrated one-step generation of gene-modified mice, and this technology became widely used within only one year^[54,55,63-65]. To understand the functions of genes in families of two or more members, animals carrying multiple mutated genes are required; however, ZFNs or TALENs cannot be multiplexed to generate animals with several targeted loci. In contrast, the CRISPR/Cas system can be used to generate mice carrying mutations in multiple genes in one step^[54]. Co-injection of Cas9 mRNA and sgRNAs for *Tet1* and *Tet2* into fertilized embryos led to the generation of mice with biallelic mutations in both genes with an efficiency of 78% (22/28). Wang *et al.*^[54] also showed that co-injection of Cas9 mRNA and sgRNAs with mutant oligos generated precise-point mutations simultaneously in two target genes with an efficiency of 20% (2/10). Using this “one-step” procedure, Yang *et al.*^[55] produced mice carrying a tag or a fluorescent reporter construct in the *Oct4*, *Sox2*, and *Nanog* genes. In addition, *Mecp2* conditional mutant mice with two loxP sites were generated^[55]. These results show that a single step by CRISPR/Cas-mediated genome editing can generate mice having NHEJ- or HDR-mediated mutations in multiple genes.

OFF-TARGET MUTATIONS

Compared to ZFNs and TALENs, CRISPR/Cas technology has the advantages of a highly efficient mutation rate and the simplicity of the design of target-specific sgRNAs. It is difficult to compare the off-target effect risk among ZFN, TALEN, and CRISPR/Cas. Although the cleavage of off-target sites has also been observed in ZFN and TALEN systems^[66,67], it appears to be less likely because they require two adjacent recognition sites, while the CRISPR/Cas system requires only one. Therefore, it is important to pay careful attention to the specificity of CRISPR/Cas target sequences, because off-target mutations are detrimental to experimental results.

When genome-edited mice are produced using the CRISPR/Cas system, they are rarely influenced by off-target effects. For example, of seven double-mutant mice produced by injection with high RNA concentrations, none showed effects at potential off-target loci using the Surveyor assay^[54]. Mashiko *et al.*^[65] found only one off-target mutation in a total of 144 sites examined. In addition, Fujii *et al.*^[64] proposed that off-target effects are mostly avoided by the careful control of Cas9 mRNA concentration. Surprisingly, the optimized CRISPR/Cas system has a higher gene targeting rate and a lower occurrence of

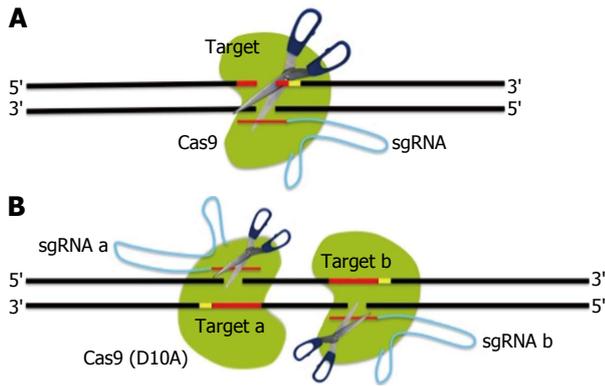


Figure 2 Improvement of site-specificity by double nicking. A: Double strand breaks (DSBs) using wild-type interspaced short palindromic repeats associated 9 (Cas9) endonuclease; B: DSBs using a pair of small guide RNAs (sgRNAs) guiding Cas9 D10A nickases. Using paired nicking can reduce off-target activity because individual nicks that unexpectedly occurred at off-target sites are predominantly repaired by the high-fidelity base excision repair pathway.

off-target effects compared to ZFN^[64]. Mutant mouse ES cells generated by the CRISPR/Cas system also showed a very low Cas9-mediated cleavage rate in off-target loci^[55]. These reports suggest that the CRISPR/Cas system is highly specific in the “one-step generation” of mutant mice and mouse ES cells.

By contrast, study of the CRISPR/Cas system in human cancer cell lines indicated a widespread occurrence of off-target mutations^[68,69]. Cas9-mediated cleavage can be abolished by single mismatches at the sgRNA-target site interface, particularly in the last 10-12 nucleotides located at the 3' end of the 20-nt sgRNA-targeting sequence^[48,51]. Using human cell lines (U2OS.EGFP, HEK293, and K562), Fu *et al.*^[68] found that one or two mismatches are tolerated to varying degrees, depending on their position along the sgRNA-DNA interface. In addition, they easily detected off-target alterations induced by 66% (4/6) of CRISPR/Cas experiments targeting endogenous loci by examination of partially mismatched sites. However, these mismatches were mainly located in the 5' region, with only one base mismatch detected in the last 12 nucleotides at the 3' end of one off-target locus.

Yang *et al.*^[55] considered several possibilities to explain the lower off-target cleavage rate observed in animals derived from manipulated zygotes compared to the results reported for CRISPR/Cas-treated human cell lines including the following: (1) the cells analyzed in mice and humans are clonal and heterogenous populations, respectively; (2) the transformed human cell lines may have different DNA damage responses, resulting in a different mutagenesis rate compared to normal one-cell embryos; and (3) introduced nucleotides are short-lived RNA or long-lived DNA plasmids in mouse and human systems, respectively, which lead to more extensive cleavage in human cells; however, a definitive explanation has not yet been found.

Several measures to improve the specificity of Cas9-mediated genome editing have been assessed. Firstly, it was hypothesized that cleavage specificity may be im-

proved by increasing the length of the region of base pairing between the sgRNA and its target locus. To test this, Ran *et al.*^[70] generated sgRNAs with 20 or 30 nucleotides guide sequences; however, they found that extension of the guide sequence did not improve Cas9 targeting specificity. Next, Ran *et al.*^[70] developed a strategy that combines the D10A mutant nickase version of Cas9^[48,51,71] with a pair of offset sgRNAs complementary to opposite strands of the target site (Figure 2A: DSBs using wild-type Cas9 endonuclease; B: DSBs using a pair of sgRNAs guiding Cas9 D10A). Whereas nicking of both DNA strands by a pair of Cas9 nickases leads to site-specific DSBs and NHEJ, individual nicks are predominantly repaired by the high-fidelity base excision repair pathway^[72]. As a result, this double nicking method can reduce off-target activity by 50- to 1500-fold and assisted gene knockout without reduction of on-target cleavage efficiency^[70,73]. Double nicking allows not only NHEJ-mediated indels but also insertion into the genome *via* HDR in human cells.

In the case of mutant animals produced by CRISPR/Cas, off-target mutations will be eliminated by backcrossing to wild-type animals. Therefore, if researchers do not use F0 pups obtained by CRISPR/Cas for experiments, off-target effects should not be a concern. RNA interference (RNAi) experiment to induce sequence-specific gene silencing is now a standard method for the functional analysis of genes. However, designed small RNA frequently repress translation from unexpected loci^[74-76]. To remove this off-target effect, two or more independent small RNAs are generally used in RNAi experiments. In CRISPR/Cas experiment, use of two or more independent sgRNAs for a gene will be also an effective control to remove off-target noise and improve the reliability of the obtained phenotype. Nevertheless, more detailed work will be necessary to determine the frequency of off-target mutations, and improve the specificity in CRISPR/Cas systems.

PROSPECTIVE APPLICATIONS OF THE CRISPR/CAS SYSTEM

Precise genome modifications by CRISPR/Cas system excite the interest of scientists working in both basic science and applied fields, including gene therapy. Undoubtedly, the CRISPR/Cas system is a strong candidate for application in human gene therapy. Several human iPS cell lines have been generated from patients for stem cell-based gene therapy by correction of gene mutations. But, gene targeting in human pluripotent stem cells including ES and iPS cells has been very difficult historically^[77]. Nevertheless, ZFNs and TALENs are capable of correcting gene mutations mediated by HDR repair mechanisms in human iPS cells^[78-82] and the CRISPR/Cas system has also recently been applied to the gene therapy model^[50, 83]. Of course, this application will require a highly efficient gene editing rate and no off-target mutations.

CRISPR/Cas is thought to be applicable for genome

editing based only on NHEJ or HDR; however, nuclease-null Cas9 (Cas9_N) can work as a transcriptional activator or silencer without changing DNA sequences^[84,85]. Mali *et al.*^[84] produced a Cas9_N directly fused with the VP64 activation domain to generate a Cas9_N-fusion protein capable of transcriptional activation. This Cas9_N-VP64 protein robustly activated transcription of reporter constructs and endogenous *REX1*, *OCT4*, *SOX2*, and *NANOG* genes when this fusion protein is combined with sgRNA-targeting sequences near the promoter^[84]. This is the example of RNA-guided transcriptional activation. By contrast, a Cas9_N-sgRNA complex is specifically able to interfere with transcriptional elongation, transcription factor binding, or RNA polymerase binding^[85]. This technology could be applied to genome-wide screens for gene function.

Prior genome-editing technologies, ZFNs and TALENs, suggest new applications for CRISPR/Cas. For example, Konermann *et al.*^[86] developed a light-inducible genome-editing system, using transcriptional effectors and the customizable TALE DNA-binding domain. They succeeded in transcriptional activation and epigenetic modification of endogenous genes using primary neurons as well as brain of living mice.

Bacterial DNA methyltransferases^[87-91] and human DNA methyltransferase 3a and 3b subunits^[92-94] have been fused to zinc-finger proteins and successfully demonstrated to perform targeted DNA methylation. Efficient targeting of DNA demethylation was also demonstrated using fusions of TALE repeat arrays and the TET1 hydroxylase catalytic domain (TALE-TET1)^[95]. These targeted methylation and demethylation technologies will be applicable for gene therapy of cancer and other epigenetic diseases such as Beckwith-Wiedemann and Angelman syndromes mediated by abnormal DNA methylation, or of Huntington disease, which is caused by extra repetitive DNA sequences. In addition, Jiang *et al.*^[96] inserted an inducible *XIST* transgene into chromosome 21 using ZFN in iPSCs derived from a Down's syndrome patient. In this system, chromosome 21 are coated with *XIST* non-coding RNA, followed by stable heterochromatin modifications, DNA methylation and chromosome-wide transcriptional silencing. This successful silencing of trisomy is the first step for chromosome therapy using genome engineering. These applications developed using ZFN and TALEN systems will be also applicable using the CRISPR/Cas technique.

In the future, CRISPR/Cas may be used to target the viral DNA that becomes integrated into the chromosomes of people with lifetime infections (*e.g.*, HIV). If this viral genetic material can be disrupted using the CRISPR/Cas system, this could negate the need for patients to continue taking antiviral drugs throughout their lives.

CONCLUSION

CRISPR/Cas has already been applied to many species in which genome engineering has been difficult, because this technology has the advantages of a highly efficient

mutation rate and a simple system for design of target-specific sgRNA. Although improvements in the specificity of CRISPR/Cas will be necessary to eliminate off-target effects, the technique will be indispensable for researchers in both basic and applied science.

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