**Name of journal: World Journal of Medical Genetics**

**ESPS Manuscript NO: 8499**

**Columns: Review**

Genome engineering using the CRISPR/Cas system

Horii T *et al*. Genome engineering using the CRISPR/Cas system

Takuro Horii, Izuho Hatada

**Takuro Horii, Izuho Hatada,** Laboratory of Genome Science, Biosignal Genome Resource Center, Institute for Molecular and Cellular Regulation, Gunma University, Gunma 371-8512, Japan

**Author contributions:** Horii T and Hatada I solely contributed to this paper.

**Supported by** Grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Ministry of Health, Labour and Welfare of Japan; the National Institute of Biomedical Innovation; the Asahi Glass Foundation; the Ichiro Kanehara Foundation; and the Program for Cultivating Global Leaders in Heavy Ion Therapeutics and Engineering

**Correspondence to: Izuho Hatada, PhD,** Laboratory of Genome Science, Biosignal Genome Resource Center, Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15 Showa-machi, Maebashi, Gunma 371-8512, Japan. hatada@gunma-u.ac.jp

**Telephone**: +81-27-2208057 **Fax:** +81-27-2208110

**Received:** December 28, 2013 **Revised:** May 13, 2014

**Accepted:** May 16, 2014

**Published online:**

**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.

© 2014 Baishideng Publishing Group Inc. All rights reserved.

**Key words:** Clustered regularly at interspaced short palindromic repeats; Cas9; Genome engineering; Double-strand breaks; Non-homologous end joining; Homology-directed repair

**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.

Takuro Horii, Izuho Hatada. Genome engineering using the CRISPR/Cas system. *World J Med Genet* 2014; In press

**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 *Xanthamonas* bacteria[4]. Each TALE repeat binds to a single base pair of DNA and the identities of the amino acids at two positions have been associated with specificities for different nucleotides[5, 6].

These chimeric nucleases enable genome editing by inducing targeted DNA DSBs that are repaired by error-prone, non-homologous end joining (NHEJ) or homology-directed repair (HDR)[7-10]. NHEJ-mediated repair induces small insertions or deletions (indels) at the cleavage site, and results in disruption of gene function by frame-shift mutations. In the presence of a single- or double-stranded DNA template containing homology to the sequences flanking the DSB, mutant alleles with precise-point mutations or DNA inserts can be produced by HDR. However, both ZFNs and TALENs require the design of DNA-binding proteins and the construction of complicated plasmids for expression of these, making these methods time-consuming and laborius.

Recently, a new efficient genome manipulation technology, CRISPR/Cas system, which uses the RNA-guided nuclease, Cas9, and is derived from the immune system of bacteria and archaea, has been developed. CRISPR/Cas technology has the advantages of a highly efficienct mutation rate and simple-to-design target-specific RNA molecules, compared to the complex ZFN and TALEN systems. Therefore, CRISPR/Cas has been rapidly adopted and applied to many species in a short period of time[11-40].

Several reviews about CRISPR/Cas have already been published[41-44]; however, this technology is progressing rapidly, with new reports published weekly. Here, we introduce recent research made possible by CRISPR/Cas technologies and discuss the application of these reagents for genetic analysis and manipulation. We also show the therapeutic potential of CRISPR/Cas and make discussion of future prospects for the field.

**THE CRISPR/CAS SYSTEM**

CRISPR/Cas is the RNA-based acquired immunity system in bacteria and archaea[45, 46]. CRISPR RNA-guided Cas9 nucleases use short RNAs to target and cleave DNA elements captured from foreign invaders (termed “spacers”) in a sequence-specific manner. In the type II CRISPR/Cas system, a single gene encoding the Cas9 protein and two RNAs, a mature CRISPR RNA (crRNA) which is transcribed from spacers, and a partially complementary transacting RNA (tracrRNA) are sufficient for RNA-guided cleavage of foreign DNAs. For maturation of crRNA, RNase III and tracrRNA are necessary[47]; however, this process can be simplified by an engineered small guide RNA (sgRNA) containing a hairpin that mimics the tracrRNA-crRNA complex and short guide sequence[48] with a protospacer-adjacent motif (PAM) (with the sequence NGG, Figure 1)[49]. Thus, the Cas9 endonuclease can generate sequence-specific DSBs of target DNAs bound to sgRNAs (Figure 1). DSBs generated by the Cas9 endonuclease are repaired by NHEJ or HDR[7-10]. NHEJ-mediated repair leads to the generation of small indels at the targeted site, which results in disruption of gene function via frame-shift mutations. In the presence of a single- or double-stranded DNA template with homology to the sequences flanking the DSB, mutant alleles with precise-point mutations or DNA inserts can be produced by HDR.

**GENOME EDITING IN CULTURED CELLS**

When the CRISPR/Cas system in bacteria and archaea 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] suceeded 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 AAVS1locus, 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 ES 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 (RFLP) 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 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 improved 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*[48, 51, 71] developed a strategy that combines the D10A mutant nickase version of Cas9 (Cas9N) 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 nickases (Cas9N)]. 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 (BER)[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 efficienct 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 (Cas9N) can work as a transcriptional activator or silencer without changing DNA sequences[84, 85]. Mali *et al*[84] produced a Cas9N directly fused with the VP64 activation domain to generate a Cas9N – fusion protein capable of transcriptional activation. This Cas9N-VP64 protein robustly activated transcription of reporter constructs and endogeneous *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, aCas9N-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 (LITEs) and the customizable TALE DNA-binding domain. They suceeded in transcriptional activation and epigenetic modification of endogeneus genes using primary neurons as well as brain of living mice.

Bacterial DNA methyltransferases[87-91] and human DNA metyltransferase 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 by extra repetitive DNA sequences. In addition, Jiang *et al*[96] inserted an inducible *XIST* transgene into chromosome 21 using ZFN in iPS cells 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.

**REFERENCES**

1 **Kim YG**, Cha J, Chandrasegaran S. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc Natl Acad Sci U S A* 1996; **93**: 1156-1160 [PMID: 8577732]

2 **Smith J**, Berg JM, Chandrasegaran S. A detailed study of the substrate specificity of a chimeric restriction enzyme. *Nucleic Acids Res* 1999; **27**: 674-681 [PMID: 9862996]

3 **Bibikova M**, Carroll D, Segal DJ, Trautman JK, Smith J, Kim YG, Chandrasegaran S. Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. *Mol Cell Biol* 2001; **21**: 289-297 [PMID: 11113203 DOI: 10.1128/MCB.21.1.289-297.2001]

4 **Bonas U**, Stall RE, Staskawicz B. Genetic and structural characterization of the avirulence gene avrBs3 from Xanthomonas campestris pv. vesicatoria. *Mol Gen Genet* 1989; **218**: 127-136 [PMID: 2550761]

5 **Boch J**, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A, Bonas U. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 2009; **326**: 1509-1512 [PMID: 19933107 DOI: 10.1126/science.1178811]

6 **Moscou MJ**, Bogdanove AJ. A simple cipher governs DNA recognition by TAL effectors. *Science* 2009; **326**: 1501 [PMID: 19933106 DOI: 10.1126/science.1178817]

7 **Barnes DE**. Non-homologous end joining as a mechanism of DNA repair. *Curr Biol* 2001; **11**: R455-R457 [PMID: 11448783]

8 **Lieber MR**. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu Rev Biochem* 2010; **79**: 181-211 [PMID: 20192759 DOI: 10.1146/annurev.biochem.052308.093131]

9 **van den Bosch M**, Lohman PH, Pastink A. DNA double-strand break repair by homologous recombination. *Biol Chem* 2002; **383**: 873-892 [PMID: 12222678 DOI: 10.1515/bc.2002.095]

10 **Wyman C**, Kanaar R. DNA double-strand break repair: all's well that ends well. *Annu Rev Genet* 2006; **40**: 363-383 [PMID: 16895466 DOI: 10.1146/annurev.genet.40.110405.090451]

11 **Friedland AE**, Tzur YB, Esvelt KM, Colaiácovo MP, Church GM, Calarco JA. Heritable genome editing in C. elegans via a CRISPR-Cas9 system. *Nat Methods* 2013; **10**: 741-743 [PMID: 23817069 DOI: 10.1038/nmeth.2532]

12 **Bassett AR**, Tibbit C, Ponting CP, Liu JL. Highly efficient targeted mutagenesis of Drosophila with the CRISPR/Cas9 system. *Cell Rep* 2013; **4**: 220-228 [PMID: 23827738 DOI: 10.1016/j.celrep.2013.06.020]

13 **Yu Z**, Ren M, Wang Z, Zhang B, Rong YS, Jiao R, Gao G. Highly efficient genome modifications mediated by CRISPR/Cas9 in Drosophila. *Genetics* 2013; **195**: 289-291 [PMID: 23833182 DOI: 10.1534/genetics.113.153825]

14 **Hwang WY**, Fu Y, Reyon D, Maeder ML, Kaini P, Sander JD, Joung JK, Peterson RT, Yeh JR. Heritable and precise zebrafish genome editing using a CRISPR-Cas system. *PLoS One* 2013; **8**: e68708 [PMID: 23874735 DOI: 10.1371/journal.pone.0068708]

15 **Jao LE**, Wente SR, Chen W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc Natl Acad Sci U S A* 2013; **110**: 13904-13909 [PMID: 23918387 DOI: 10.1073/pnas.1308335110]

16 **Li D**, Qiu Z, Shao Y, Chen Y, Guan Y, Liu M, Li Y, Gao N, Wang L, Lu X, Zhao Y, Liu M. Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. *Nat Biotechnol* 2013; **31**: 681-683 [PMID: 23929336 DOI: 10.1038/nbt.2661]

17 **Li W**, Teng F, Li T, Zhou Q. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. *Nat Biotechnol* 2013; **31**: 684-686 [PMID: 23929337 DOI: 10.1038/nbt.2652]

18 **Shan Q**, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL, Gao C. Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat Biotechnol* 2013; **31**: 686-688 [PMID: 23929338 DOI: 10.1038/nbt.2650]

19 **Xie K**, Yang Y. RNA-guided genome editing in plants using a CRISPR-Cas system. *Mol Plant* 2013; **6**: 1975-1983 [PMID: 23956122 DOI: 10.1093/mp/sst119]

20 **Feng Z**, Zhang B, Ding W, Liu X, Yang DL, Wei P, Cao F, Zhu S, Zhang F, Mao Y, Zhu JK. Efficient genome editing in plants using a CRISPR/Cas system. *Cell Res* 2013; **23**: 1229-1232 [PMID: 23958582 DOI: 10.1038/cr.2013.114]

21 **Chiu H**, Schwartz HT, Antoshechkin I, Sternberg PW. Transgene-free genome editing in Caenorhabditis elegans using CRISPR-Cas. *Genetics* 2013; **195**: 1167-1171 [PMID: 23979577 DOI: 10.1534/genetics.113.155879]

22 **Katic I**, Großhans H. Targeted heritable mutation and gene conversion by Cas9-CRISPR in Caenorhabditis elegans. *Genetics* 2013; **195**: 1173-1176 [PMID: 23979578 DOI: 10.1534/genetics.113.155754]

23 **Tzur YB**, Friedland AE, Nadarajan S, Church GM, Calarco JA, Colaiácovo MP. Heritable custom genomic modifications in Caenorhabditis elegans via a CRISPR-Cas9 system. *Genetics* 2013; **195**: 1181-1185 [PMID: 23979579 DOI: 10.1534/genetics.113.156075]

24 **Waaijers S**, Portegijs V, Kerver J, Lemmens BB, Tijsterman M, van den Heuvel S, Boxem M. CRISPR/Cas9-targeted mutagenesis in Caenorhabditis elegans. *Genetics* 2013; **195**: 1187-1191 [PMID: 23979586 DOI: 10.1534/genetics.113.156299]

25 **Dickinson DJ**, Ward JD, Reiner DJ, Goldstein B. Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination. *Nat Methods* 2013; **10**: 1028-1034 [PMID: 23995389 DOI: 10.1038/nmeth.2641]

26 **Jiang W**, Zhou H, Bi H, Fromm M, Yang B, Weeks DP. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic Acids Res* 2013; **41**: e188 [PMID: 23999092 DOI: 10.1093/nar/gkt780]

27 **Miao J**, Guo D, Zhang J, Huang Q, Qin G, Zhang X, Wan J, Gu H, Qu LJ. Targeted mutagenesis in rice using CRISPR-Cas system. *Cell Res* 2013; **23**: 1233-1236 [PMID: 23999856 DOI: 10.1038/cr.2013.123]

28 **Kondo S**, Ueda R. Highly improved gene targeting by germline-specific Cas9 expression in Drosophila. *Genetics* 2013; **195**: 715-721 [PMID: 24002648 DOI: 10.1534/genetics.113.156737]

29 **Seth A**, Stemple DL, Barroso I. The emerging use of zebrafish to model metabolic disease. *Dis Model Mech* 2013; **6**: 1080-1088 [PMID: 24046387 DOI: 10.1242/dmm.011346]

30 **Hisano Y**, Ota S, Kawahara A. Genome editing using artificial site-specific nucleases in zebrafish. *Dev Growth Differ* 2014; **56**: 26-33 [PMID: 24117409 DOI: 10.1111/dgd.12094]

31 **Upadhyay SK**, Kumar J, Alok A, Tuli R. RNA-guided genome editing for target gene mutations in wheat. *G3 (Bethesda)* 2013; **3**: 2233-2238 [PMID: 24122057 DOI: 10.1534/g3.113.008847]

32 **Blitz IL**, Biesinger J, Xie X, Cho KW. Biallelic genome modification in F(0) Xenopus tropicalis embryos using the CRISPR/Cas system. *Genesis* 2013; **51**: 827-834 [PMID: 24123579 DOI: 10.1002/dvg.22719]

33 **Nakayama T**, Fish MB, Fisher M, Oomen-Hajagos J, Thomsen GH, Grainger RM. Simple and efficient CRISPR/Cas9-mediated targeted mutagenesis in Xenopus tropicalis. *Genesis* 2013; **51**: 835-843 [PMID: 24123613 DOI: 10.1002/dvg.22720]

34 **Sebo ZL**, Lee HB, Peng Y, Guo Y. A simplified and efficient germline-specific CRISPR/Cas9 system for Drosophila genomic engineering. *Fly (Austin)* 2013; **8**: 52-57 [PMID: 24141137]

35 **Wang Y**, Li Z, Xu J, Zeng B, Ling L, You L, Chen Y, Huang Y, Tan A. The CRISPR/Cas system mediates efficient genome engineering in Bombyx mori. *Cell Res* 2013; **23**: 1414-1416 [PMID: 24165890 DOI: 10.1038/cr.2013.146]

36 **Daimon T**, Kiuchi T, Takasu Y. Recent progress in genome engineering techniques in the silkworm, Bombyx mori. *Dev Growth Differ* 2014; **56**: 14-25 [PMID: 24175911 DOI: 10.1111/dgd.12096]

37 **Auer TO**, Duroure K, De Cian A, Concordet JP, Del Bene F. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome Res* 2014; **24**: 142-153 [PMID: 24179142 DOI: 10.1101/gr.161638.113]

38 **Frøkjær-Jensen C**. Exciting prospects for precise engineering of Caenorhabditis elegans genomes with CRISPR/Cas9. *Genetics* 2013; **195**: 635-642 [PMID: 24190921 DOI: 10.1534/genetics.113.156521]

39 **Sung YH**, Kim JM, Kim HT, Lee J, Jeon J, Jin Y, Choi JH, Ban YH, Ha SJ, Kim CH, Lee HW, Kim JS. Highly efficient gene knockout in mice and zebrafish with RNA-guided endonucleases. *Genome Res* 2014; **24**: 125-131 [PMID: 24253447 DOI: 10.1101/gr.163394.113]

40 **Hruscha A**, Krawitz P, Rechenberg A, Heinrich V, Hecht J, Haass C, Schmid B. Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. *Development* 2013; **140**: 4982-4987 [PMID: 24257628 DOI: 10.1242/dev.099085]

41 **Gaj T**, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 2013; **31**: 397-405 [PMID: 23664777 DOI: 10.1016/j.tibtech.2013.04.004]

42 **Menke DB**. Engineering subtle targeted mutations into the mouse genome. *Genesis* 2013; **51**: 605-618 [PMID: 23913666 DOI: 10.1002/dvg.22422]

43 **Wei C**, Liu J, Yu Z, Zhang B, Gao G, Jiao R. TALEN or Cas9 - rapid, efficient and specific choices for genome modifications. *J Genet Genomics* 2013; **40**: 281-289 [PMID: 23790627 DOI: 10.1016/j.jgg.2013.03.013]

44 **Gratz SJ**, Cummings AM, Nguyen JN, Hamm DC, Donohue LK, Harrison MM, Wildonger J, O'Connor-Giles KM. Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. *Genetics* 2013; **194**: 1029-1035 [PMID: 23709638 DOI: 10.1534/genetics.113.152710]

45 **Wiedenheft B**, Sternberg SH, Doudna JA. RNA-guided genetic silencing systems in bacteria and archaea. *Nature* 2012; **482**: 331-338 [PMID: 22337052 DOI: 10.1038/nature10886]

46 **Horvath P**, Barrangou R. CRISPR/Cas, the immune system of bacteria and archaea. *Science* 2010; **327**: 167-170 [PMID: 20056882 DOI: 10.1126/science.1179555]

47 **Deltcheva E**, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 2011; **471**: 602-607 [PMID: 21455174 DOI: 10.1038/nature09886]

48 **Jinek M**, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012; **337**: 816-821 [PMID: 22745249 DOI: 10.1126/science.1225829]

49 **Marraffini LA**, Sontheimer EJ. Self versus non-self discrimination during CRISPR RNA-directed immunity. *Nature* 2010; **463**: 568-571 [PMID: 20072129 DOI: 10.1038/nature08703]

50 **Mali P**, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. *Science* 2013; **339**: 823-826 [PMID: 23287722 DOI: 10.1126/science.1232033]

51 **Cong L**, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013; **339**: 819-823 [PMID: 23287718 DOI: 10.1126/science.1231143]

52 **Cho SW**, Kim S, Kim JM, Kim JS. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat Biotechnol* 2013; **31**: 230-232 [PMID: 23360966 DOI: 10.1038/nbt.2507]

53 **Jinek M**, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. *Elife* 2013; **2**: e00471 [PMID: 23386978 DOI: 10.7554/eLife.00471]

54 **Wang H**, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 2013; **153**: 910-918 [PMID: 23643243 DOI: 10.1016/j.cell.2013.04.025]

55 **Yang H**, Wang H, Shivalila CS, Cheng AW, Shi L, Jaenisch R. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* 2013; **154**: 1370-1379 [PMID: 23992847 DOI: 10.1016/j.cell.2013.08.022]

56 **Leeb M**, Wutz A. Derivation of haploid embryonic stem cells from mouse embryos. *Nature* 2011; **479**: 131-134 [PMID: 21900896 DOI: 10.1038/nature10448]

57 **Yang H**, Shi L, Wang BA, Liang D, Zhong C, Liu W, Nie Y, Liu J, Zhao J, Gao X, Li D, Xu GL, Li J. Generation of genetically modified mice by oocyte injection of androgenetic haploid embryonic stem cells. *Cell* 2012; **149**: 605-617 [PMID: 22541431 DOI: 10.1016/j.cell.2012.04.002]

58 **Elling U**, Taubenschmid J, Wirnsberger G, O'Malley R, Demers SP, Vanhaelen Q, Shukalyuk AI, Schmauss G, Schramek D, Schnuetgen F, von Melchner H, Ecker JR, Stanford WL, Zuber J, Stark A, Penninger JM. Forward and reverse genetics through derivation of haploid mouse embryonic stem cells. *Cell Stem Cell* 2011; **9**: 563-574 [PMID: 22136931 DOI: 10.1016/j.stem.2011.10.012]

59 **Horii T**, Morita S, Kimura M, Kobayashi R, Tamura D, Takahashi RU, Kimura H, Suetake I, Ohata H, Okamoto K, Tajima S, Ochiya T, Abe Y, Hatada I. Genome engineering of mammalian haploid embryonic stem cells using the Cas9/RNA system. *PeerJ* 2013; **1**: e230 [PMID: 24432195 DOI: 10.7717/peerj.230]

60 **Carbery ID**, Ji D, Harrington A, Brown V, Weinstein EJ, Liaw L, Cui X. Targeted genome modification in mice using zinc-finger nucleases. *Genetics* 2010; **186**: 451-459 [PMID: 20628038 DOI: 10.1534/genetics.110.117002]

61 **Geurts AM**, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, Vincent A, Lam S, Michalkiewicz M, Schilling R, Foeckler J, Kalloway S, Weiler H, Ménoret S, Anegon I, Davis GD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jacob HJ, Buelow R. Knockout rats via embryo microinjection of zinc-finger nucleases. *Science* 2009; **325**: 433 [PMID: 19628861 DOI: 10.1126/science.1172447]

62 **Sung YH**, Baek IJ, Kim DH, Jeon J, Lee J, Lee K, Jeong D, Kim JS, Lee HW. Knockout mice created by TALEN-mediated gene targeting. *Nat Biotechnol* 2013; **31**: 23-24 [PMID: 23302927 DOI: 10.1038/nbt.2477]

63 **Shen B**, Zhang J, Wu H, Wang J, Ma K, Li Z, Zhang X, Zhang P, Huang X. Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell Res* 2013; **23**: 720-723 [PMID: 23545779 DOI: 10.1038/cr.2013.46]

64 **Fujii W**, Kawasaki K, Sugiura K, Naito K. Efficient generation of large-scale genome-modified mice using gRNA and CAS9 endonuclease. *Nucleic Acids Res* 2013; **41**: e187 [PMID: 23997119 DOI: 10.1093/nar/gkt772]

65 **Mashiko D**, Fujihara Y, Satouh Y, Miyata H, Isotani A, Ikawa M. Generation of mutant mice by pronuclear injection of circular plasmid expressing Cas9 and single guided RNA. *Sci Rep* 2013; **3**: 3355 [PMID: 24284873 DOI: 10.1038/srep03355]

66 **Szczepek M**, Brondani V, Büchel J, Serrano L, Segal DJ, Cathomen T. Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nat Biotechnol* 2007; **25**: 786-793 [PMID: 17603476 DOI: 10.1038/nbt1317]

67 **Grau J**, Boch J, Posch S. TALENoffer: genome-wide TALEN off-target prediction. *Bioinformatics* 2013; **29**: 2931-2932 [PMID: 23995255 DOI: 10.1093/bioinformatics/btt501]

68 **Fu Y**, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* 2013; **31**: 822-826 [PMID: 23792628 DOI: 10.1038/nbt.2623]

69 **Hsu PD**, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* 2013; **31**: 827-832 [PMID: 23873081 DOI: 10.1038/nbt.2647]

70 **Ran FA**, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 2013; **154**: 1380-1389 [PMID: 23992846 DOI: 10.1016/j.cell.2013.08.021]

71 **Gasiunas G**, Barrangou R, Horvath P, Siksnys V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci U S A* 2012; **109**: E2579-E2586 [PMID: 22949671 DOI: 10.1073/pnas.1208507109]

72 **Dianov GL**, Hübscher U. Mammalian base excision repair: the forgotten archangel. *Nucleic Acids Res* 2013; **41**: 3483-3490 [PMID: 23408852 DOI: 10.1093/nar/gkt076]

73 **Cho SW**, Kim S, Kim Y, Kweon J, Kim HS, Bae S, Kim JS. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res* 2014; **24**: 132-141 [PMID: 24253446 DOI: 10.1101/gr.162339.113]

74 **Jackson AL**, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS. Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* 2003; **21**: 635-637 [PMID: 12754523 DOI: 10.1038/nbt831]

75 **Saxena S**, Jónsson ZO, Dutta A. Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. *J Biol Chem* 2003; **278**: 44312-44319 [PMID: 12952966 DOI: 10.1074/jbc.M307089200]

76 **Snøve O**, Holen T. Many commonly used siRNAs risk off-target activity. *Biochem Biophys Res Commun* 2004; **319**: 256-263 [PMID: 15158470 DOI: 10.1016/j.bbrc.2004.04.175]

77 **Zwaka TP**, Thomson JA. Homologous recombination in human embryonic stem cells. *Nat Biotechnol* 2003; **21**: 319-321 [PMID: 12577066 DOI: 10.1038/nbt788]

78 **Hockemeyer D**, Wang H, Kiani S, Lai CS, Gao Q, Cassady JP, Cost GJ, Zhang L, Santiago Y, Miller JC, Zeitler B, Cherone JM, Meng X, Hinkley SJ, Rebar EJ, Gregory PD, Urnov FD, Jaenisch R. Genetic engineering of human pluripotent cells using TALE nucleases. *Nat Biotechnol* 2011; **29**: 731-734 [PMID: 21738127 DOI: 10.1038/nbt.1927]

79 **Choi SM**, Kim Y, Shim JS, Park JT, Wang RH, Leach SD, Liu JO, Deng C, Ye Z, Jang YY. Efficient drug screening and gene correction for treating liver disease using patient-specific stem cells. *Hepatology* 2013; **57**: 2458-2468 [PMID: 23325555 DOI: 10.1002/hep.26237]

80 **Hockemeyer D**, Soldner F, Beard C, Gao Q, Mitalipova M, DeKelver RC, Katibah GE, Amora R, Boydston EA, Zeitler B, Meng X, Miller JC, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jaenisch R. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat Biotechnol* 2009; **27**: 851-857 [PMID: 19680244 DOI: 10.1038/nbt.1562]

81 **Zou J**, Maeder ML, Mali P, Pruett-Miller SM, Thibodeau-Beganny S, Chou BK, Chen G, Ye Z, Park IH, Daley GQ, Porteus MH, Joung JK, Cheng L. Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. *Cell Stem Cell* 2009; **5**: 97-110 [PMID: 19540188 DOI: 10.1016/j.stem.2009.05.023]

82 **Soldner F**, Laganière J, Cheng AW, Hockemeyer D, Gao Q, Alagappan R, Khurana V, Golbe LI, Myers RH, Lindquist S, Zhang L, Guschin D, Fong LK, Vu BJ, Meng X, Urnov FD, Rebar EJ, Gregory PD, Zhang HS, Jaenisch R. Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. *Cell* 2011; **146**: 318-331 [PMID: 21757228 DOI: 10.1016/j.cell.2011.06.019]

83 **Horii T**, Tamura D, Morita S, Kimura M, Hatada I. Generation of an ICF syndrome model by efficient genome editing of human induced pluripotent stem cells using the CRISPR system. *Int J Mol Sci* 2013; **14**: 19774-19781 [PMID: 24084724 DOI: 10.3390/ijms141019774]

84 **Mali P**, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L, Church GM. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat Biotechnol* 2013; **31**: 833-838 [PMID: 23907171 DOI: 10.1038/nbt.2675]

85 **Qi LS**, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 2013; **152**: 1173-1183 [PMID: 23452860 DOI: 10.1016/j.cell.2013.02.022]

86 **Konermann S**, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Platt RJ, Scott DA, Church GM, Zhang F. Optical control of mammalian endogenous transcription and epigenetic states. *Nature* 2013; **500**: 472-476 [PMID: 23877069 DOI: 10.1038/nature12466]

87 **Xu GL**, Bestor TH. Cytosine methylation targetted to pre-determined sequences. *Nat Genet* 1997; **17**: 376-378 [PMID: 9398832 DOI: 10.1038/ng1297-376]

88 **McNamara AR**, Hurd PJ, Smith AE, Ford KG. Characterisation of site-biased DNA methyltransferases: specificity, affinity and subsite relationships. *Nucleic Acids Res* 2002; **30**: 3818-3830 [PMID: 12202767]

89 **Carvin CD**, Parr RD, Kladde MP. Site-selective in vivo targeting of cytosine-5 DNA methylation by zinc-finger proteins. *Nucleic Acids Res* 2003; **31**: 6493-6501 [PMID: 14602907]

90 **Smith AE**, Ford KG. Specific targeting of cytosine methylation to DNA sequences in vivo. *Nucleic Acids Res* 2007; **35**: 740-754 [PMID: 17182629 DOI: 10.1093/nar/gkl1053]

91 **Nomura W**, Barbas CF. In vivo site-specific DNA methylation with a designed sequence-enabled DNA methylase. *J Am Chem Soc* 2007; **129**: 8676-8677 [PMID: 17583340 DOI: 10.1021/ja0705588]

92 **Li F**, Papworth M, Minczuk M, Rohde C, Zhang Y, Ragozin S, Jeltsch A. Chimeric DNA methyltransferases target DNA methylation to specific DNA sequences and repress expression of target genes. *Nucleic Acids Res* 2007; **35**: 100-112 [PMID: 17151075 DOI: 10.1093/nar/gkl1035]

93 **Rivenbark AG**, Stolzenburg S, Beltran AS, Yuan X, Rots MG, Strahl BD, Blancafort P. Epigenetic reprogramming of cancer cells via targeted DNA methylation. *Epigenetics* 2012; **7**: 350-360 [PMID: 22419067 DOI: 10.4161/epi.19507]

94 **Siddique AN**, Nunna S, Rajavelu A, Zhang Y, Jurkowska RZ, Reinhardt R, Rots MG, Ragozin S, Jurkowski TP, Jeltsch A. Targeted methylation and gene silencing of VEGF-A in human cells by using a designed Dnmt3a-Dnmt3L single-chain fusion protein with increased DNA methylation activity. *J Mol Biol* 2013; **425**: 479-491 [PMID: 23220192 DOI: 10.1016/j.jmb.2012.11.038]

95 **Maeder ML**, Angstman JF, Richardson ME, Linder SJ, Cascio VM, Tsai SQ, Ho QH, Sander JD, Reyon D, Bernstein BE, Costello JF, Wilkinson MF, Joung JK. Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins. *Nat Biotechnol* 2013; **31**: 1137-1142 [PMID: 24108092 DOI: 10.1038/nbt.2726]

96 **Jiang J**, Jing Y, Cost GJ, Chiang JC, Kolpa HJ, Cotton AM, Carone DM, Carone BR, Shivak DA, Guschin DY, Pearl JR, Rebar EJ, Byron M, Gregory PD, Brown CJ, Urnov FD, Hall LL, Lawrence JB. Translating dosage compensation to trisomy 21. *Nature* 2013; **500**: 296-300 [PMID: 23863942 DOI: 10.1038/nature12394]

**P-Reviewers:** Guo ZS, Vargas FR, Wei L, Xuei X  **S-Editor:** Wen LL  **L-Editor:**  **E-Editor:**



**Figure 1 Schematic of the principles of** c**lustered regularly at interspaced short palindromic repeats /Cas-mediated genome editing.** The Cas9 endonuclease can generate sequence-specific double strand breaks (DSBs) of target DNAs bound to small guide RNAs (sgRNAs). The binding site of a target DNA requires a protospacer-adjacent motif (PAM) (with the sequence NGG). DSBs generated by the Cas9 endonuclease are repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR).



**Figure 2 Improvement of site-specificity by double nicking.** A: Double strand breaks (DSBs) using wild-type 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.