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STATE-OF-THE-ART REVIEW

Genome modification by CRISPR/Cas9

Yuanwu Ma[1], Lianfeng Zhang[1] and Xingxu Huang[2]

1 Key Laboratory of Human Disease Comparative Medicine, Ministry of Health, Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, Beijing, China

2 MOE Key Laboratory of Model Animal for Disease Study, Model Animal Research Center of Nanjing University, Nanjing Biomedical Research Institute, National Resource Center for Mutant Mice, China

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Correspondence

X. Huang, MOE Key Laboratory of Model Animal for Disease Study, Model Animal Research Center of Nanjing University, Nanjing Biomedical Research Institute, National Resource Center for Mutant Mice, Nanjing 210061, China

Fax: +86 25 58641500

Tel: +86 25 58641517

E-mail: xingxuhuang@mail.nju.edu.cn

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Clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein (Cas)9-mediated genome modification enables us to edit the genomes of a variety of organisms rapidly and efficiently. The advantages of the CRISPR/Cas9 system have made it an increasingly popular genetic engineering tool for biological and therapeutic applications. Moreover, CRISPR/Cas9 has been employed to recruit functional domains that repress/activate gene expression or label specific genomic loci in living cells or organisms, in order to explore developmental mechanisms, gene expression regulation, and animal behavior. One major concern about this system is its specificity; although CRISPR/Cas9-mediated off-target mutation has been broadly studied, more efforts are required to further improve the specificity of CRISPR/Cas9. We will also discuss the potential applications of CRISPR/Cas9.

Introduction

Precise modification of specific sites within a gene of interest is considered to be a standard approach to elucidate gene function, to create disease animal models, and to improve desired characteristics of animals and plants. Targeted gene modification also provides the potential for therapeutic applications. In the past decades, strategies for precise genome modifications using embryonic stem cell-mediated modification by homologous recombination were limited to certain organisms. Recently, engineered nucleases, including zinc finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein (Cas) have provided a much simpler and more economic method for gene-targeted modification [1]. These engineered nucleases generate a DNA double-strand break (DSB) at the targeted genome locus. The break activates repair through error-prone nonhomologous end joining

Abbreviations

Cas, clustered regularly interspaced short palindromic repeats-associated protein; CRISPR, clustered regularly interspaced short palindromic repeats; crRNA, clustered regularly interspaced short palindromic repeats RNA; dCas9, dead clustered regularly interspaced short palindromic repeats-associated protein 9; DSB, double-strand break; HDR, homology-directed repair; NHEJ, nonhomologous end joining; PAM, protospacer adjacent motif; RGEN, RNA-guided endonuclease; sgRNA, single guide RNA; SpyCas9, *Streptococcus pyogenes* clustered regularly interspaced short palindromic repeats-associated protein 9; tracrRNA, *trans*-activating clustered regularly interspaced short palindromic repeats RNA.

(NHEJ) or homology-directed repair (HDR). In the absence of a template, NHEJ is activated, resulting in insertions and/or deletions (indels) that disrupt the target loci. In the presence of a donor template with homology to the targeted locus, the HDR pathway operates, allowing for precise mutations to be made.

The CRISPR/Cas system was first described as an adaptive immune system in bacteria and archaeons, and has now been engineered as RNA-guided endonucleases (RGENs) for genome editing. Typically, a type II CRISPR system functions by the CRISPR RNA (crRNA) interacting with a *trans*-activating crRNA (tracrRNA) to form a crRNA·tracrRNA duplex [this RNA duplex could be replaced with a single guide RNA (sgRNA)], which directs *Streptococcus pyogenes* Cas9 (SpyCas9) to specific sites, thereby generating a DNA strand break [2]. Unlike zinc finger nucleases and transcription activator-like effector nucleases, the CRISPR/Cas9 system does not require the engineering of specific protein pairs for each target site, and introduces Cas9 to the target sequence based on RNA·DNA base-pairing rules. The simplicity of the CRISPR/Cas9 system has revolutionized genome engineering in a variety of cells and organisms.

In this review, we describe how the CRISPR/Cas9 system can be engineered for genome editing in different kinds of organisms. We will also discuss applications of CRISPR/Cas9 beyond genome editing, the improvement of the specificity of this system, and the challenges still remaining. Finally, we will highlight the bright future of this fascinating system in basic research and therapeutic applications.

Engineering CRISPR/Cas9 for genome editing

RNA-guided DNA cleavage systems protect bacteria and archaeons against invading DNA contaminants, serving as an adaptive immune system. The process is quite complicated. The invading foreign DNA can be recognized and inserted into a genome locus to form a CRISPR region; the captured foreign DNA sequences are termed protospacers. In type II CRISPR systems, the CRISPR locus is transcribed into a pre-CRISPR RNA, and processed to a matured crRNA with the assistance of tracrRNA. Interaction between crRNA and tracrRNA directs SpyCas9 to recognize the specific DNA sequence complementary with the protospacer. This RGEN target site is usually 20 bp in length and must be immediately adjacent to the NGG motif, or sometimes NAG (with much lower cleavage efficiency) [3], which are known as protospacer adjacent motifs (PAMs). The programmable crRNA and fixed tracrRNA are fused to form an sgRNA, which directs Cas9 to the desired site and catalyzes the cleavage of both DNA strands effectively.

Cas9 contains the RuvC and HNH nuclease domains (Fig. 1). The HNH domain is a single domain, whereas the RuvC domain consists of three subdomains. Single-particle electron microscopy reconstructions of SpyCas9 showed an sgRNA-guided structural change forming a central channel for the RNA·DNA heteroduplex [2]. Later, the high-resolution structure of SpyCas9 in complex with guide RNA and target DNA showed a bilobed architecture including a target recognition lobe and a nuclease lobe [4]. The nuclease lobe is composed of an HNH nuclease domain, a RuvC nuclease domain, and a C-terminal region. The HNH and RuvC nuclease domains are

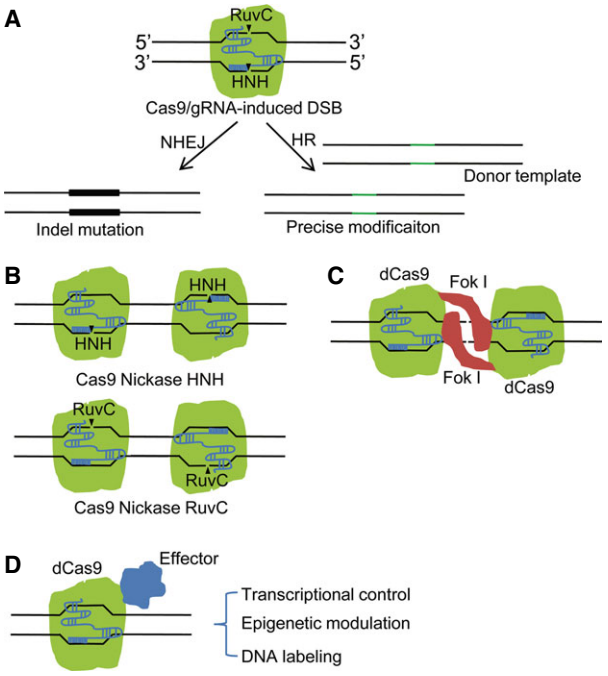

Fig. 1. CRISPR/Cas9-mediated genome editing. (A) Cas9·sgRNA-induced DSBs can be repaired by either NHEJ or by HDR pathways. Cas9 contains RuvC and HNH nuclease domains, each of which is responsible for cleavage of one DNA strand. (B) Paired nickases were used to improve the specificity in the genome editing. Cas9 nickase (HNH) cleaves only the DNA strand (complementary strands of the target DNA) recognized by the sgRNA. Cas9 nickase (RuvC) cleaves the DNA strand (noncomplementary strands of the target DNA) not interacting with the sgRNA. (C) dCas9 (both HNH and RuvC nuclease domains are inactivated by mutation) is fused with Fok I nuclease to improve the specificity of genome editing. (D) dCas9 fused with an effector domain, such as DNA methylases, demethylases, histone acetylases, deaceylases, and kinases, to provide the specific chromatin modifications for desired effects.

responsible for the cleavage of the complementary and noncomplementary DNA strands of the target sites (Fig. 1A) [5].

In general, Cas9 and sgRNA are the essential components for genome editing of the CRISPR/Cas9 system: sgRNA is responsible for the site targeting, and Cas9 contributes to the DNA cleavage at the target site. The PAM is required for target site recognition [2]. Thus, any DNA sequence that contains the N_{20}-NGG motif could be recognized as a target site. This system can be widely used in bacteria and many other kinds of organisms. Also, codon-optimized Cas9 with an appropriate nuclear localization signal showed high activity in eukaryotes such as human cells, mice, and rats [6–8]. Since its first description 2 years ago, the sgRNA-guided Cas9 system has been applied to modify endogenous genes in a wide range of cells and organisms, including bacteria [9], yeast [10], plants [11–13], roundworms [14], silkworms [15], fruit flies [16], zebrafish [17], frogs [18], rabbits [19], mice [7,20], rats [8,21], pigs [22], monkeys [23], and different human cells [6,24–26].

By providing multiple sgRNAs and programming the DNA-cleaving activity of Cas9, it has proved possible to simultaneously modify multiple genes in cells or animals. Four genes, i.e. *ApoE*, *B2m*, *Prf1*, and *Prkdc*, have been disrupted simultaneously in rats at an efficiency of 24% [27], five genes, i.e. *B2m*, *Il2rg*, *Prf1*, *Prkdc*, and *Rag1*, have been targeted in mice at an efficiency of 75% [28], five genes, i.e. *Tet1*, *Tet2*, *Tet3*, *Sry*, and *Uty*, have been targeted in mouse embryonic stem cells at an efficiency of 10% [20], and five genes, i.e. *Ddx*, *Egfp*, *Gol*, *Mitfa*, and *Tyr*, have been targeted in somatic cells of zebrafish, also at high efficiency [29]. The capacity to simultaneously disrupt multiple genes is especially useful for studying adjacent genes. Moreover, by exploiting the advantages of Cas9 used with multiple sgRNAs, large-fragment deletion or inversion between sgRNA targeting sites has been successfully achieved [30]. When supplied with plasmid DNA or single-strand oligonucleotide as templates, Cas9-assisted HDR operated and successfully generated site-specific mutation and *loxP* or reporter gene insertion in mice [31], and *loxP* or reporter gene insertion in rats [32,33] and zebrafish [34].

The simplicity of the CRISPR/Cas9 system has also facilitated the generation of lentivirus-based sgRNA libraries covering almost all mouse and human genes, which can be used for high-throughput functional screening [35,36]. Genome-wide loss-of-function screens have already been applied to robust negative and positive selection screens in human cells [35,37]. The sgRNA can be designed to target nearly any DNA sequence. Therefore, sgRNA libraries may also be used to study the function of noncoding genetic elements. Fusion of a nonfunctional or 'dead' Cas9 (dCas9; see below) with different effector domains has been used for studies beyond loss-of-function phenotypes [38,39]. For example, dCas9 fused to an epigenetic modifier was applied to elucidate the methylation effect and certain chromatin states in defined conditions.

In addition, the CRISPR/Cas9 system has been used to correct the genetic disease in mice and in intestinal stem cell organoids of cystic fibrosis patients [40,41]. Other evidence showed that CRISPR/Cas9 corrected the *Fah* mutation in an adult mouse model of human hereditary tyrosinemia disease, and alleviated the symptoms of this disease [42].

The simplicity and high DNA cleavage efficiency have made CRISPR/Cas9 an increasingly popular genome-editing tool. Nevertheless, potential users should pay attention to several points.

The first is the delivery of sgRNA and Cas9. Both sgRNA and Cas9 are required for efficient target site recognition and subsequent cleavage, and delivering both of them simultaneously is more difficult than delivering either one alone. Different approaches, such as electroporation, nucleofection, and lipofectamine, have been used for the delivery of Cas9 and sgRNA expression plasmids into mammal cells. Lentiviral vectors have been used to construct large-scale sgRNA expressing libraries covering nearly all human and mouse genes [35,36]. Meanwhile, *in vitro*-transcribed Cas9 mRNA/plasmid DNA and sgRNA/plasmid DNA have been microinjected into the one-cell embryos of zebrafish [17], fruit flies [16], mice [7, 20], rats [8,21,32], pigs [22] and monkeys [23] to generate gene-modified animals. The purified Cas9 protein complex together with sgRNAs has also been microinjected into one-cell embryos to generate knockout mice and zebrafish [43]. Time course experiments showed that the Cas9 protein·sgRNA complex works earlier than Cas9 mRNA plus sgRNA [43]. Polyethylene glycol) and *Agrobacterium* have been used for Cas9 and sgRNA delivery into wheat, rice, sorghum, tobacco and thale cress for genomic editing of these plants [11–13,44]. The selection of a proper delivery system depends on the cells or organisms used.

The second point concerns target site selection and sgRNA design. In theory, any DNA sequence that contains the sequence NGG (or NAG, a some lesser extent) is a potential target site. The RNA polymerase III-dependent U6 promoter and T7 promoter are most commonly used for sgRNA expression. These promoters require a G or GG at the 5′-end of the RNA to be transcribed. Therefore, a G or GG at the 5′-end of the

target site is required to start the transcription. A recent study reported a new vector using the U6 promoter to drive sgRNA expression without these limitations. In the vector, the U6 promoter drives the expression of multiple sgRNAs, each flanked by two Csy4 RNase cleavage sites from *Pseudomonas aeruginosa* [45]. The expressed tandem multiple sgRNAs will be separated when Csy4 RNase is present [45]. This strategy allows any sequence containing NGG at the 3′-end to be used as the potential targeting site [45]. In addition, a group reported that truncated sgRNAs with 17 or 18 nucleotides complementary with the target site also induce DNA cleavage efficiently. Therefore, sites in the form of $GN_{16–19}NGG$ or $GGN_{15–18}NGG$ can serve as the potential targeting sites. Such potential targeting sites will theoretically be found within the whole genome every 1 in 32 bp or 1 in 128 bp, respectively.

The third point concerns the genotyping of CRISPR/Cas9-induced modifications. One pair of primers flanking the target site were designed to detect the modification in the target site with the T7 endonuclease I assay [7,32]. Also, the same PCR products can be used for restriction fragment length polymorphism assay to detect the mutation [20,31]. Detailed modifications were detected by sequencing analysis.

Finally, off-target effects must be considered, as Cas9 induces both mutations in its target and off-target mutations. As the off-target events may cause unwanted modifications, it is very important to evaluate these in gene-modified cells. However, for animals, the side effects can be diluted by crossing with wild-type animals.

Improving the specificity

The CRISPR/Cas9-mediated off-target effects could be useful for bacteria and archaeons to recognize and destroy invading hypervariable viral DNA or plasmid DNA. However, for biological research or gene therapy, the off-target events will generate unwanted mutations beyond the target site and result in side effects. Thus, increasing attention has been given to improving the specificity of CRISPR/Cas9 systems.

A number of studies on potential off-target effects have shown that mismatches at the 5′-end of the target site, but not the 'seed' (8–12 bp upstream of the PAM) region, are generally better tolerated [2,25]. However, mismatches of fewer than three nucleotides and outside the 5′-end also induce off-target events in human cells and rats [27,32,46]. Sometimes, the off-target mutation occurs at a rate almost as high as that of the on-target cleavage [46]. Indeed, by analyzing the off-target events described in previously published papers, we found that mismatches of up to five nucleotides cause off-target mutations [46].

Deep sequencing assays were used to detect the off-target effects *in vivo* [45,47–49]. Interestingly, most reported potential off-target sites were located at the noncoding regions in the host genomes [49]. A whole exome sequencing assay showed no off-target events in CRISPR/Cas9-modified human cells [49]. Several CRISPR/Cas9 engineering online design and off-targeting search tools have been developed, such as WTSI Genome Editing (http://www.sanger.ac.uk/htgt/wge/), E-CRISP (http://www.e-crisp.org/E-CRISP), Genome engineering resources (www.genome-engineering.org/crispr/), RGEN tools (http://www.rgenome.net/), ZiFiT TARGETER software (http://zifit.partners.org/ZiFiT/), GT-SCAN (http://gt-scan.braembl.org.au/gt-scan/), and CHOPCHOP (https://chopchop.rc.fas.harvard.edu) [1,50–52]. However, a more comprehensive evaluation of the off-target effects mediated by CRISPR/Cas9 is still expected. For example, how many mismatches are tolerated for a given target site, and why do some of the potential off-target sites cause mutations whereas others do not? These differences may be caused by genomic/epigenomic context and/or chromatin structure. We believe that a better understanding of the sgRNA target site screen is required for further improvement of the specificity of the CRISPR/Cas9 system.

Great efforts have already been made to explore different strategies to reduce off-target effects, as follows.

- Select good target sites. Some guidelines for target site selection and sgRNA design are helpful for reducing the off-target effects. For example, it has been reported that high GC contents (up to 70%) in the target site could improve hybridization and allow more mismatches to be tolerated [53], whereas a high rate of off-target sites was observed with low a GC content ($< 30\%$) [46,54]. Mismatches that form DNA bulges at the 5′-end, the 3′-end or 7–10 bp away from the PAM [53] should be avoided, as should potential sgRNA with bulges beyond the seed region [53].
- Reduce the concentrations of Cas9 and sgRNA. This strategy decreases the off-target effects, but may also affect on-target cleavages [55].
- Use Cas9 mRNA/protein and sgRNA instead of Cas9 and an sgRNA expression plasmid. Cas9 mRNA/protein and sgRNA work for a shorter time, whereas plasmids keep expressing Cas9 and sgRNA, which may increase the off-target effects and cause

possible random integration of Cas9 and sgRNA into the genome [1].

- Truncate sgRNA at the 3′-end of tracrRNA and add two extra GG to the 5′-end beyond the complementary region. Those two strategies decreased the ratio of off-target to on-target effects [54,56].
- Use paired sgRNAs and Cas9 nickases. A Cas9 variant with a D10A or an H840A mutation in Cas9 nucleases induces DSBs between the paired sites by two sgRNAs (Fig. 1B) [57]. Target sites on opposite DNA strands separated by 4–100 bp would be recognized and cleaved by paired Cas9 nickases [50,57]. Although several studies have shown that Cas9 nickases with a single sgRNA induce indel mutations at a very low level, the paired sgRNAs and Cas9 nickase do reduce the off-target effects in human cells and mice dramatically [58].
- Truncate sgRNAs at the 5′-ends of prospacer regions. The truncated sgRNAs have 17 bp or 18 bp of complementary sequence, which functions as effectively as full-length sgRNA with improved specificity [47].
- Use the dimeric CRISPR guide Fok I nuclease. Fusion of dCas9 to Fok I nuclease generated fCas9 (Fig. 1C) [45,48]. DNA cleavage induced by fCas9 requires two sgRNAs targeted on opposite site of the DNA strands separated by 15–25 bp in the 'PAM-out' orientation. This modified fCas9 system showed comparable DNA modification efficiency to the Cas9 nickases, which is approximately two-thirds of the efficiency of the wild-type Cas9, but dramatically increased the specificity [45,48]. The studies on Spy-Cas9 structure also provide useful information for reconstruction of Cas9 and sgRNA to improve the specificity [4,5].

Expanding the applications

As a versatile genetic engineering tool, CRISPR/Cas9 has been exploited beyond genome editing. The Cas9 variant containing D10A and H840A mutations is catalytically inactive Cas9 or dCas9 (Fig. 1D). This dCas9 can be directed to the target site by sgRNA as effectively as the wild-type, but it cannot function as a nuclease for genome editing. This mutated CRISPR–dCas9 has been applied for promoter targeting to repress gene expression in *Escherichia coli* and human cells, and also to recruit heterogeneous functional domains to a specific locus to repress/activate gene expression or label specific genomic loci in living cells or organisms (Fig. 1D). For example, dCas9 has been fused to transcriptional activation domains such as VP64 or the p65 subunit to increase gene expression in human cells, and has also been fused to the Krüppel-associated box domain to decrease gene expression [38,39,59–61]. Such gene regulation can also be amplified in a synergistic way by using multiple sgRNAs. In a recent study, the fusion protein of transcription activator-like effector domains and LSD1 histone demethylase was used to regulate enhancer-associated chromatin modifications [62]. Although no attempt has yet been successful, it will be very interesting to test whether dCas9 could be used for target epigenome editing by fusing it with chromatin modification domains, such as DNA methylases, demethylases, histone acetylases, deacetylases, and kinases.

An enhanced GFP-tagged dCas9 has been used for imaging of the repetitive elements of telomeres and coding genes in living cells [63]. The labeling of specific genomic loci in living cells or organisms is a powerful strategy for the study of the spatiotemporal organization and dynamics of chromatin in regulating genome function [58]. The capability to regulate any endogenous gene will help us to pinpoint the factors responsible for cell differentiation and other cellular processes.

Conclusions and prospects

In summary, the simplicity and high efficiency of the CRISPR/Cas9 system allows affordable genome editing. In addition, the large sgRNA library will make both drug target identification and function screening more efficient. This RNA-guided genome-editing tool also gives rise to the potential to change the genetic landscape of animals and plants around us to obtain the desired genotypes at will.

The application of this system has been expanded beyond genome editing, to areas such as gene expression regulation and specific chromatin labeling with fluorescent protein. Although great advances have been made in improving the specificity and expanding the application of this technology, there is still plenty of room for improvement and extension.

The specificity of this system still needs improvement. A major concern with the application of this system is off-target mutagenesis. In the past 2 years, many papers have reported off-target events of this system. Many entities, such as paired nickases, truncated sgRNAs, and dimeric FokI–dCas9 nucleases, have been exploited to reduce off-target effects. Although these modifications have reduced off-target events significantly, further improvement is needed, especially for the more precise modifications or therapeutic applications. Further optimization of this system needs unbiased strategies for more comprehensive evaluation of off-target effects. Meanwhile,

the mechanisms underlying the target search remain unclear. Learning more about the mechanisms of the target search and cleavage will provide the basis for improvement of Cas9 and/or sgRNA to increase the specificity.

Another area for improvement is to shift the balance of HDR/NHEJ from NHEJ-mediated indels towards HDR-mediated modifications. Although the site mutation or knockin events can be achieved at high rates in cells and animals by providing a single-stranded DNA or a double-stranded DNA plasmid as template, how to reduce the NHEJ-mediated indels and improve the HDR-mediated precise modification are still interesting issues. Colocalization of the template DNA with the Cas9·sgRNA complex to the target site to enhance HDR or use of the small interfering RNA or inhibitor of the NHEJ-mediated repair pathway to reduce the competing NHEJ should be very helpful.

Another, but not the last, important problem is to efficiently deliver CRISPR/Cas9 into those cell types or tissues that are hard to transfect and/or infect. The development of safe gene delivery vehicles is necessary for the versatile use of the CRISPR/Cas9 system.

Undoubtedly, the basic research will make its way into clinic practise. Further optimization and development of next-generation CRISPR/Cas9 tools for genome and epigenome editing is expected to satisfy the requirements for therapeutic applications.

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Author contributions

X. H., Y. M. and L. Z. wrote the manuscript.

References

1 Kim H & Kim JS (2014) A guide to genome engineering with programmable nucleases. *Nat Rev Genet* **15**, 321–334.
2 Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA & Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821.
3 Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L & Church GM (2013) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat Biotechnol* **31**, 833–838.
4 Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, Ma E, Anders C, Hauer M, Zhou K, Lin S *et al.* (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science* **343**, 1247997.
5 Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F & Nureki O (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* **156**, 935–949.
6 Jinek M, East A, Cheng A, Lin S, Ma E & Doudna J (2013) RNA-programmed genome editing in human cells. *Elife* **2**, e00471.
7 Shen B, Zhang J, Wu H, Wang J, Ma K, Li Z, Zhang X, Zhang P & Huang X (2013) Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell Res* **23**, 720–723.
8 Li D, Qiu Z, Shao Y, Chen Y, Guan Y, Liu M, Li Y, Gao N, Wang L, Lu X *et al.* (2013) Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. *Nat Biotechnol* **31**, 681–683.
9 Jiang W, Bikard D, Cox D, Zhang F & Marraffini LA (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat Biotechnol* **31**, 233–239.
10 DiCarlo JE, Norville JE, Mali P, Rios X, Aach J & Church GM (2013) Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. *Nucleic Acids Res* **41**, 4336–4343.
11 Li JF, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM & Sheen J (2013) Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nat Biotechnol* **31**, 688–691.
12 Nekrasov V, Staskawicz B, Weigel D, Jones JD & Kamoun S (2013) Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nat Biotechnol* **31**, 691–693.
13 Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL *et al.* (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat Biotechnol* **31**, 686–688.
14 Friedland AE, Tzur YB, Esvelt KM, Colaiacovo MP, Church GM & Calarco JA (2013) Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system. *Nat Methods* **10**, 741–743.
15 Wang Y, Li Z, Xu J, Zeng B, Ling L, You L, Chen Y, Huang Y & Tan A (2013) The CRISPR/Cas System mediates efficient genome engineering in Bombyx mori. *Cell Res* **23**, 1414–1416.
16 Bassett AR, Tibbit C, Ponting CP & Liu JL (2013) Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Rep* **4**, 220–228.
17 Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR & Joung JK (2013)

Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* **31**, 227–229.
18 Nakayama T, Fish MB, Fisher M, Oomen-Hajagos J, Thomsen GH & Grainger RM (2013) Simple and efficient CRISPR/Cas9-mediated targeted mutagenesis in Xenopus tropicalis. *Genesis* **51**, 835–843.
19 Yang D, Xu J, Zhu T, Fan J, Lai L, Zhang J & Chen YE (2014) Effective gene targeting in rabbits using RNA-guided Cas9 nucleases. *J Mol Cell Biol* **6**, 97–99.
20 Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F & Jaenisch R (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* **153**, 910–918.
21 Li W, Teng F, Li T & Zhou Q (2013) Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. *Nat Biotechnol* **31**, 684–686.
22 Hai T, Teng F, Guo R, Li W & Zhou Q (2014) One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. *Cell Res* **24**, 372–375.
23 Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L, Kang Y, Zhao X, Si W, Li W *et al.* (2014) Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell* **156**, 836–843.
24 Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE & Church GM (2013) RNA-guided human genome engineering via Cas9. *Science* **339**, 823–826.
25 Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823.
26 Cho SW, Kim S, Kim JM & Kim JS (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat Biotechnol* **31**, 230–232.
27 Ma Y, Shen B, Zhang X, Lu Y, Chen W, Ma J, Huang X & Zhang L (2014) Heritable multiplex genetic engineering in rats using CRISPR/Cas9. *PLoS One* **9**, e89413.
28 Zhou J, Shen B, Zhang W, Wang J, Yang J, Chen L, Zhang N, Zhu K, Xu J, Hu B *et al.* (2014) One-step generation of different immunodeficient mice with multiple gene modifications by CRISPR/Cas9 mediated genome engineering. *Int J Biochem Cell Biol* **46**, 49–55.
29 Jao LE, Wente SR & Chen W (2013) Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc Natl Acad Sci U S A* **110**, 13904–13909.
30 Xiao A, Wang Z, Hu Y, Wu Y, Luo Z, Yang Z, Zu Y, Li W, Huang P, Tong X *et al.* (2013) Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. *Nucleic Acids Res* **41**, e141.
31 Yang H, Wang H, Shivalila CS, Cheng AW, Shi L & Jaenisch R (2013) One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* **154**, 1370–1379.
32 Ma Y, Zhang X, Shen B, Lu Y, Chen W, Ma J, Bai L, Huang X & Zhang L (2014) Generating rats with conditional alleles using CRISPR/Cas9. *Cell Res* **24**, 122–125.
33 Ma Y, Ma J, Zhang X, Chen W, Yu L, Lu Y, Bai L, Shen B, Huang X & Zhang L (2014) Generation of eGFP and Cre knockin rats by CRISPR/Cas9. *FEBS J* **281**, 3779–3790.
34 Chang N, Sun C, Gao L, Zhu D, Xu X, Zhu X, Xiong JW & Xi JJ (2013) Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. *Cell Res* **23**, 465–472.
35 Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelsen TS, Heckl D, Ebert BL, Root DE, Doench JG *et al.* (2014) Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* **343**, 84–87.
36 Koike-Yusa H, Li Y, Tan EP, Velasco-Herrera Mdel C & Yusa K (2014) Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotechnol* **32**, 267–273.
37 Wang T, Wei JJ, Sabatini DM & Lander ES (2014) Genetic screens in human cells using the CRISPR-Cas9 system. *Science* **343**, 80–84.
38 Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA *et al.* (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **154**, 442–451.
39 Konermann S, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Platt RJ, Scott DA, Church GM & Zhang F (2013) Optical control of mammalian endogenous transcription and epigenetic states. *Nature* **500**, 472–476.
40 Wu Y, Liang D, Wang Y, Bai M, Tang W, Bao S, Yan Z, Li D & Li J (2013) Correction of a genetic disease in mouse via use of CRISPR-Cas9. *Cell Stem Cell* **13**, 659–662.
41 Schwank G, Koo BK, Sasselli V, Dekkers JF, Heo I, Demircan T, Sasaki N, Boymans S, Cuppen E, van der Ent CK *et al.* (2013) Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* **13**, 653–658.
42 Yin H, Xue W, Chen S, Bogorad RL, Benedetti E, Grompe M, Koteliansky V, Sharp PA, Jacks T & Anderson DG (2014) Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nat Biotechnol* **32**, 551–553.
43 Sung YH, Kim JM, Kim HT, Lee J, Jeon J, Jin Y, Choi JH, Ban YH, Ha SJ, Kim CH *et al.* (2014) Highly efficient gene knockout in mice and zebrafish with RNA-guided endonucleases. *Genome Res* **24**, 125–131.

44 Jiang W, Zhou H, Bi H, Fromm M, Yang B & Weeks DP (2013) Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic Acids Res* **41**, e188.
45 Tsai SQ, Wyvekens N, Khayter C, Foden JA, Thapar V, Reyon D, Goodwin MJ, Aryee MJ & Joung JK (2014) Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. *Nat Biotechnol* **32**, 569–576.
46 Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK & Sander JD (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* **31**, 822–826.
47 Fu Y, Sander JD, Reyon D, Cascio VM & Joung JK (2014) Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol* **32**, 279–284.
48 Guilinger JP, Thompson DB & Liu DR (2014) Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. *Nat Biotechnol* **32**, 577–582.
49 Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S & Kim JS (2013) Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res* **24**, 132–141.
50 Shen B, Zhang W, Zhang J, Zhou J, Wang J, Chen L, Wang L, Hodgkins A, Iyer V, Huang X *et al.* (2014) Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nat Methods* **11**, 399–402.
51 Montague TG, Cruz JM, Gagnon JA, Church GM & Valen E (2014) CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. *Nucleic Acids Res* **42**, W401–W407.
52 O'Brien A & Bailey TL (2014) GT-Scan: identifying unique genomic targets. *Bioinformatics* **30**, 2673–2675.
53 Lin Y, Cradick TJ, Brown MT, Deshmukh H, Ranjan P, Sarode N, Wile BM, Vertino PM, Stewart FJ & Bao G (2014) CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. *Nucleic Acids Res* **42**, 7473–7485.
54 Pattanayak V, Lin S, Guilinger JP, Ma E, Doudna JA & Liu DR (2013) High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat Biotechnol* **31**, 839–843.
55 Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O *et al.* (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* **31**, 827–832.
56 Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S & Kim JS (2014) Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res* **24**, 132–141.
57 Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y *et al.* (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* **154**, 1380–1389.
58 Sander JD & Joung JK (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol* **32**, 347–355.
59 Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH & Joung JK (2013) CRISPR RNA-guided activation of endogenous human genes. *Nat Methods* **10**, 977–979.
60 Perez-Pinera P, Kocak DD, Vockley CM, Adler AF, Kabadi AM, Polstein LR, Thakore PI, Glass KA, Ousterout DG, Leong KW *et al.* (2013) RNA-guided gene activation by CRISPR-Cas9-based transcription factors. *Nat Methods* **10**, 973–976.
61 Cheng AW, Wang H, Yang H, Shi L, Katz Y, Theunissen TW, Rangarajan S, Shivalila CS, Dadon DB & Jaenisch R (2013) Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res* **23**, 1163–1171.
62 Mendenhall EM, Williamson KE, Reyon D, Zou JY, Ram O, Joung JK & Bernstein BE (2013) Locus-specific editing of histone modifications at endogenous enhancers. *Nat Biotechnol* **31**, 1133–1136.
63 Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li GW, Park J, Blackburn EH, Weissman JS, Qi LS *et al.* (2013) Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* **155**, 1479–1491.