Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9

The use of ZFNs, TALENs, and CRISPR-Cas9 for precision genome editing in mammals

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The past decade has been one of rapid innovation in genome-editing technology. The opportunity now exists for investigators to manipulate virtually any gene in a diverse range of cell types and organisms with targeted nucleases designed with sequence-specific DNA-binding domains. The rapid development of the field has allowed for highly efficient, precise, and now cost-effective means by which to generate human and animal models of disease using these technologies.

This review will outline the history and recent development of genome-editing technology, culminating with use of CRISPR-Cas9 to generate novel mammalian models of disease. While the road to using this same technology for treatment of human disease is long, the pace of innovation over the past 5 years and early successes in model systems builds anticipation for this prospect.

The emergence of genome-editing technology

The classical method for gene modification is homologous recombination. This approach has been widely used in mouse embryonic stem cells to generate germline knockout or knockin mice(1, 2). A disadvantage is that it typically takes more than a year to generate a genetically modified mouse using the standard approach. Furthermore, similar attempts at using homologous recombination in human cells have been proven to be far more challenging, and alternative approaches to knock down gene expression, such as antisense oligonucleotides and short interfering RNAs, have instead become standard. However, these approaches only transiently reduce gene expression, and the effect is usually incomplete and can often affect off-target

genes(3) . These shortcomings have fueled the demand for more effective methods of gene modification.

A new wave of technology that is variously termed “gene editing,” “genome editing,” or “genome engineering” has emerged to address this demand by giving investigators the ability to precisely introduce a variety of genetic alterations, ranging from knockin of single nucleotide variants to insertion of genes to deletion of chromosomal regions, into mammalian cells far more efficiently than traditional homologous recombination. We describe the key advantages and disadvantages of the three most popular genome-editing tools (summarized in Table 1). This description is not meant to be a comprehensive review of the work leading to the development of the tools, but rather to give readers a working knowledge of the tools and be able to select among the tools for desired tasks.

Zinc finger nucleases

Zinc finger nucleases (ZFNs) are increasingly being used in academic and industry research for a variety of purposes ranging from the generation of animal models to human therapies(4). ZFNs are fusion proteins comprising an array of site-specific DNA-binding domains—adapted from zinc finger-containing transcription factors—attached to the endonuclease domain of the bacterial FokI restriction enzyme. Each zinc finger domain recognizes a 3- to 4-basepair (bp) DNA sequence, and tandem domains can potentially bind to an extended nucleotide sequence (typically with a length that is a multiple of 3, usually 9 bp or 12 bp) that is unique in a cell’s genome.

To cleave a specific site in the genome, ZFNs are designed as a pair that recognizes two sequences flanking the site, one on the forward strand, the other on the reverse strand. Upon binding of the ZFNs on either side of the site, the pair of FokI domains dimerize and cleave the DNA at the site, generating a double-strand break (DSB) with 5' overhangs(4). Cells repair DSBs using either (1) non-homologous end-joining (NHEJ), which is more straightforward and can occur during any phase of the cell cycle, but occasionally results in erroneous repair, or (2) homology-directed repair (HDR), which typically occurs during late S phase or G2 phase when a sister chromatid is available to serve as a repair template (Fig. 1).

The error-prone nature of NHEJ can be exploited to introduce frameshifts into the coding sequence of a gene, potentially knocking out the gene by either of two mechanisms: premature truncation of the protein and nonsense-mediated decay of the mRNA transcript (Fig. 2). Alternatively, HDR can be utilized in a fashion similar to homologous recombination, with the introduction of a repair template with a desired mutation flanked by homology arms (Fig. 2). Though mechanistically similar, the efficiency of genome editing with HDR is significantly improved over traditional homologous recombination, because the first step of the process (generation of a DSB) is induced rather than occurring spontaneously. The exogenous repair template can be either a double-strand DNA vector or a single-stranded DNA oligonucleotide (ssODN). For ssODNs, homology arms of as little as 20-nucleotide length can enable introduction of mutations into the genome(5-7). In many cases, the efficiency is sufficiently improved that antibiotic selection to identify correctly targeted clones is unnecessary. If antibiotic selection is not used, then extra steps to remove the cassette from the genome using

systems like Cre-lox and FLP-FRT are unnecessary, in contrast to traditional homologous recombination.

Despite the advantages of genome editing with ZFNs, there are several potential disadvantages. It has not proven to be straightforward to assemble zinc finger domains to bind an extended stretch of nucleotides with high affinity(8). This has made it difficult for non-specialists to routinely engineer ZFNs. To surmount this difficulty, an academic consortium has developed an "open-source" library of zinc finger components and protocols to perform screens to identify ZFNs that bind with high affinity to a desired sequence(9, 10); nonetheless, it can still take months for non-specialists to obtain optimized ZFNs. A commercial option to obtain optimized ZFNs is available, but the expense may be prohibitive for some investigators.

Another potential disadvantage is that target site selection is limited—the "open-source" ZFN components can only be used to target binding sites every few hundred bp throughout the genome. While this may be a non-issue if an investigator seeks to knock out a gene, since a frameshift introduced anywhere in the early coding sequence of the gene can produce the desired result, it may present challenges if a particular site is required, e.g., to knock in a specific mutation into a gene. Since the introduction of the "open-source" platform, alternative platforms to engineer optimized ZFNs have since emerged, with varying degrees of speed, flexibility in site selection, and success rates(11-14).

Finally, a significant concern about the use of proteins designed to introduce DSBs into the genome is that they will do so not only at the desired site but also at off-target sites. In one study

in which ZFNs were used for genome editing in human pluripotent stem cells, the investigators identified 10 possible off-target genomic sites based on high sequence similarity to the on-target and found a single off-target mutation in 184 clones assessed(15). Two subsequent studies of ZFNs using unbiased genome-wide methods to identify potential off-target sites for several ZFN pairs revealed infrequent off-target events at numerous loci in a cultured human tumor cell line(16, 17). Thus, investigators should be cognizant of the possibility that ZFNs designed for a particular purpose may incur undesired off-target events at a low rate. One strategy to reduce off-target events is to use a pair of ZFNs that have distinct FokI domains that are obligate heterodimers(18, 19). This prevents a single ZFN from binding to two adjacent off-target sites and generating a DSB; rather, the only way an off-target event could occur is if both ZFNs in a pair bind adjacently and thus allow the FokI dimer to form.

Transcription activator-like effector nucleases

The recent discovery of a class of proteins called transcription activator-like effectors (TALEs), exclusive to a group of plant pathogens, has led to the characterization of a novel DNA-binding domain, termed TAL repeats. The naturally occurring TAL repeats comprise tandem arrays with 10 to 30 repeats that bind and recognize extended DNA sequences(20). Each repeat is 33 to 35 amino acids in length, with two adjacent amino acids (termed the repeat-variable di-residue, or RVD) conferring specificity for one of the four DNA basepairs(21-25). Thus, there is a one-to-one correspondence between the repeats and the basepairs in the target DNA sequences. Understanding the RVD code has made it possible to create a new type of engineered site-specific nuclease that fuses a domain of TAL repeats to the Fok1 endonuclease domain, termed

TAL effector nucleases (TALENs)(26, 27)(Fig. 1). TALENs are similar to ZFNs in that they can generate DSBs at a desired target site in the genome and so can be used to knock out genes or knock in mutations in the same way (Fig. 2).

In comparison to ZFNs, TALENs have turned out to be much easier to design. The RVD code has been employed to engineer many TAL repeat arrays that bind with high affinity to desired genomic DNA sequences; it appears that more often than not a *de novo* engineered TAL repeat array will bind to a desired DNA sequence with high affinity(27, 28). TALENs can be designed and constructed in as short a time as two days and in as large a number as hundreds at a time(29, 30); indeed, a library with TALENs targeting all of the genes in the genome has been constructed(31).

One potential advantage over ZFNs is that the TAL repeat array can be easily extended to whatever length desired. Whereas engineered ZFNs typically bind 9- or 12-bp sequences, TALENs are often built to bind 18-bp sequences or even longer, with the theoretical possibility of achieving greater affinity and specificity with TALENs. Another possible advantage of TALENs over ZFNs is that there appear to be fewer constraints on site selection, with at least a few potential sites available in each 100 bp of genomic DNA. However, it also appears that methylation of the target site attenuates the binding affinity(32), although it is possible to tweak the RVD code to accommodate methylated DNA bases(33, 34).

As with ZFNs, off-target effects are a significant concern with TALENs. A study in which TALENs were used for genome editing in human pluripotent stem cells found low but

measurable rates of mutagenesis at some of 19 possible off-target sites based on sequence similarity to the on-target site(28). Although comparative data is scarce, one study found that for TALENs and ZFNs targeting the same site in the *CCR5* gene, the TALENs produce fewer off-target mutations than the ZFNs at a highly similar site in the *CCR2* gene(35). Furthermore, the ZFNs produced greater cell toxicity (i.e., inhibited their growth) when introduced into cells compared to the TALENs. Thus, there is a general perception in the field that TALENs are "cleaner" than ZFNs. As with ZFNs, TALENs with obligate heterodimer FokI domains are routinely used to minimize the possibility of off-target events.

A clear disadvantage of TALENs is their significantly larger size compared to ZFNs. The typical size for a cDNA encoding a TALEN is ~3 kb, whereas a cDNA encoding a ZFN is only ~1 kb. In principle, this makes it harder to deliver and express a pair of TALENs into cells compared to ZFNs, and the size of the TALENs make them less attractive for therapeutic applications in which they must be delivered in viral vectors with limited cargo size or as RNA molecules. Furthermore, the highly repetitive nature of the TALENs may impair their ability to be packaged and delivered by some viral vectors(36), though this can apparently be overcome by diversifying the coding sequences of the TAL repeats(37).

CRISPR-Cas9

The recent discovery of bacterial adaptive immune systems known as clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems have led to the newest set of genome-editing tools. CRISPR-Cas systems use a combination of proteins

and short RNAs to target specific DNA sequences for cleavage. The bacteria collect "protospacers" from foreign DNA sequences (e.g., from bacteriophages), incorporate them into their genomes, and use them to express short guide RNAs, which can then be used by a CRISPR-Cas system to destroy any DNA sequences matching the protospacers.

In early 2013, four groups demonstrated that heterologous expression of a CRISPR-Cas system from *Streptococcus pyogenes*, comprising the Cas9 protein along with guide RNA(s) (either two separate RNAs, as found in bacteria, or a single chimeric RNA), in mammalian cells results in DSBs at target sites with (1) a 20-bp sequence matching the protospacer of the guide RNA and (2) an adjacent downstream NGG nucleotide sequence (termed the protospacer-adjacent motif, or PAM)(38-41) . This occurs via the formation of a ternary complex: Cas9 binds the non-protospacer portion of the guide RNA, the protospacer of the guide RNA hybridizes with one strand of the genomic DNA, and Cas9 binds to the PAM in the DNA. Cas9 then catalyzes the DSB in the DNA at a position three basepairs upstream of the PAM(40)(Fig. 1).

In contrast to ZFNs and TALENs, which must be built from scratch for each new target site, CRISPR-Cas9 can be easily adapted to target any genomic sequence by changing the 20-bp protospacer of the guide RNA, which can be done with simple molecular biology. The Cas9 protein component remains unchanged. This ease of use for CRISPR-Cas9 is a significant advantage over ZFNs and TALENs, especially in generating a large set of vectors to target numerous sites(39) or even genome-wide libraries(42-44). Another potential advantage of CRISPR-Cas9 is the ability to multiplex, i.e., to use multiple guide RNAs in parallel to target multiple sites simultaneously in the same cell(38, 39). This makes it straightforward to mutate

multiple genes at once or to engineer precise deletions in a genomic region, although it should be noted that simultaneous use of multiple ZFN or TALEN pairs can achieve the same outcomes.

One potential disadvantage of CRISPR-Cas9 is site selection, though in this regard it compares favorably with ZFNs and TALENs. Even with the most flexible version of the *S. pyogenes* CRISPR/Cas system, site selection is limited to 23-bp sequences on either strand that end in an NGG motif (the PAM for *S. pyogenes* Cas9), which occur on average once every 8 bp(38). However, CRISPR-Cas systems from other species are starting to be employed in mammalian cells(38, 45, 46), and their versions of Cas9 have different PAM requirements, which allows for targeting of sites in the genome for which the *S. pyogenes* system is not optimal. For example, the canonical *Neisseria meningitides* Cas9 PAM has been reported to be NNNNGATT, although it appears to be more tolerant of variation in the PAM compared to *S. pyogenes* Cas9(45, 46).

Another disadvantage of CRISPR-Cas9 is the size of the Cas9 protein. The cDNA encoding *S. pyogenes* Cas9 is ~4 kb in size, making it somewhat larger than a TALEN and much larger than a ZFN. This size makes it challenging to deliver via viral vectors or as an RNA molecule. The chimeric version of the guide RNA is only ~100 nucleotides in size, but it needs to be delivered in parallel with Cas9, either as a separate RNA molecule or via a DNA cassette with a separate promoter (typically a RNA polymerase III promoter such as U6). Here again, the emerging availability of CRISPR-Cas systems from other species may prove helpful. The cDNA encoding *N. meningitidis* Cas9 is ~3.2 kb in size and so should allow for easier delivery, which may be important for therapeutic applications.

Perhaps the biggest concern regarding CRISPR-Cas9 is the issue of off-target effects. It has recently been demonstrated that although that each nucleotide within the 20-nt protospacer contributes to overall *S. pyogenes* Cas9 binding and specificity, single mismatches are often well tolerated, and multiple mismatches can sometimes be tolerated depending on their locations in the protospacer(47-50). Systematic analysis of the effect of alterations in the protospacer reveals an increasing tolerance for mismatches with increasing distance from the PAM. A number of studies in mammalian cells have documented off-target mutations occurring at significant rates at sites with sequence similarity to the on-target sites, occasionally rivaling or even surpassing mutagenesis at the on-target sites(47-52). It has been posited that alternative CRISPR-Cas systems such as that from *N. meningitides* may offer better targeting specificity by virtue of their longer protospacers (24 nt for *N. meningitides*) and longer PAMs. Experimental confirmation of improved specificity in mammalian cells remains to be shown. Early results with the *N. meningitides* CRISPR-Cas9 system suggest that it may be less tolerant of mismatches in the protospacer compared to the *S. pyogenes* system(45).

Efforts to improve the specificity of CRISPR-Cas9 in mammalian cells are in progress. One strategy has been to use a mutant version of Cas9 that can only introduce a single-strand nick into the target DNA, rather than a DSB. Use of a pair of "nickase" CRISPR-Cas9 complexes with binding sites on opposite strands flanking the target site can produce the equivalent of a DSB with 5' overhangs (Fig. 1), which is then repaired by NHEJ or HDR and can result in an on-target alteration. At an off-target site, a single-strand nick would be fixed by a different mechanism (base excision repair pathway) that is much less likely to result in a mutation. Because the likelihood of two nickases binding near each other elsewhere in the genome is very

low, the off-target mutation rate should be dramatically reduced. Indeed, testing of this strategy in mammalian cells has demonstrated a reduction in off-target activity by up to three orders of magnitude with at most a modest reduction in on-target efficacy(49, 52, 53). Another strategy to reduce off-target effects is to reduce the length of the protospacer portion of the guide RNA, which makes it less tolerant of mismatches and thus can preserve the on-target efficacy while reducing off-target mutagenesis(54).

Genome editing in mammalian models

Although the creation of mouse lines with genetic alterations such as gene knockouts or conditional alleles has long been feasible with traditional homologous recombination employed in mouse embryonic stem cells, the last few years have seen the application of novel genome-editing tools for the generation of genetically modified mice with unprecendented ease and efficiency. Furthermore, these tools have made it possible to genetically modify animals for which embryonic stem cell lines are not widely available.

Initial studies of the efficacy of genome-editing tools in the mutagenesis of mammalian embryos were performed with rats. Inspired by studies in which injection of RNAs encoding ZFNs directly into the embryos of fruit flies and zebrafish yielded stable, heritable genomic alterations, injection of ZFN-encoding RNAs into one-cell rat embryos successfully generated monoallelic and biallelic frameshift mutations resulting in gene knockout(55, 56). Numerous knockout rats have since been generated using this ZFN strategy. Subsequently, both TALENs and CRISPR-Cas9 have been used in similar fashion to generate knockout rats(57-59).

A particular advantage is that it is possible to obtain knockout animals in the first generation (assuming the targeted gene is not embryonic lethal), dramatically speeding up the time needed to do genetic studies in animals. Another advantage of this approach is that embryos from any of a variety of animal strains can be used; in the case of mice, there is no longer a restriction to a limited number of embryonic stem cell lines that necessitate backcrossing to an inbred strain of choice. Embryos from that inbred strain can be used to directly generate the knockout mice. Similarly, embryos from a strain that already carries genetic alterations can be used, relieving the need for many generations of interbreeding to obtain mice with multiple genetic alterations. The ability to perform multiplex gene targeting with CRISPR-Cas9 is also helpful in this regard.

All three engineered nucleases outlined above have proven effective at producing targeted mutations in mouse embryos(60-68). The efficiencies vary wildly depending on the nuclease, target site in the genome, and amount of RNAs injected. The most striking demonstration of efficiency has been with CRISPR-Cas9, with simultaneous targeting of both alleles of two genes in 80% of mice(66). CRISPR-Cas9 has also been used along with ssODNs or double-strand DNA donor vectors in mouse embryos to knock in tags and fluorescent markers into endogenous gene loci and, most impressively, to generate conditional knockout mice in one step by simultaneously knocking in two loxP sites flanking an exon of a gene(67).

Finally, the high efficiencies of the genome-editing tools, particularly CRISPR-Cas9, has made it possible to generate targeted mutations in animals far beyond the reach of the traditional homologous recombination/embryonic stem cell approach. Both TALENs and CRISPR-Cas9

have now been used to generate genetically modified monkeys (69, 70), in each case targeting genes involved in human diseases. This is a remarkable accomplishment that suggests that there is no technical barrier to using genome-editing tools to modify human embryos, notwithstanding the profound social and ethical repercussions that would result if such attempts were to be made.

Genome editing in human cells

To date, there have been a number of reports demonstrating the feasibility of performing genome editing in human pluripotent stem cells (hPSCs) with ZFNs, TALENs, and CRISPRs(15, 28, 39, 53, 71-75). Genetically altered hPSCs offer the possibility of differentiating wild-type and mutant cell lines into whatever somatic cell type desired, potentially giving new insights into disease pathophysiology. In one such study, the investigators generated induced pluripotent stem cells (iPSCs) from patients with Parkinson disease caused by the G2019S mutation of the *LRRK2* gene as well as control individuals(75). Upon differentiation into midbrain dopaminergic neurons, the cell lines displayed striking differences in whole-genome gene expression patterns, with clustering analysis showing that in some cases a patient line and a control line were more closely matched than lines generated from two different patients. Indeed, even iPSC lines generated from the same patient failed to cluster together, demonstrating the high degree of heterogeneity among iPSC lines. As an alternative approach, the investigators used ZFNs to correct the G2019S mutation in three of the patient-derived iPSC lines and to insert the mutation into a control iPSC line. They found that the matched sets of wild-type/mutant cell lines clustered together very closely, confirming the superiority of the genome-editing strategy for disease modeling studies. The investigators consistently found that mutant neurons displayed less

neurite outgrowth and more apoptosis in response to oxidative stress than matched wild-type neurons.

Other human cell types have proven to be amenable to genome editing. In one study, the investigators isolated intestinal stem cells from cystic fibrosis patients homozygous for the common delta508 mutation in the *CFTR* gene(76). They used CRISPR-Cas9 targeting the site of the mutation, along with a double-strand DNA donor vector, to correct one mutant allele (sufficient to "cure" the disease in this recessive disorder). They then used the mutant and corrected stem cells to create intestinal organoids in culture. Whereas the mutant organoids failed to respond to forskolin treatment by swelling, consistent with a lack of functional CFTR protein, the corrected organoids did respond by swelling, demonstrating a functional rescue.

The remarkable efficiency and ease of use of CRISPR-Cas9, where only 20 nucleotides in the guide RNA need be changed to retarget the nuclease, has led to the development of genome-wide "CRISPR interference" or "CRISPRi" libraries with the potential to knock out each of the genes in the genome. Three groups have performed proof-of-principle, genome-wide knockout screens in cells, two in human cells(42, 43) and one in mouse cells(44). The results of the screens compared favorably with traditional genome-wide RNA interference screens, establishing a powerful new complementary approach to RNA interference to probe gene function in an unbiased fashion.

Conclusion

The rapid development and improvement of genome editing tools provides investigators with three well-characterized options for experiments as diverse as forward genetic screens to correction of pathogenic mutations in iPSC-derived human cells. ZFNs, TALENs, and CRISPRs can all generate site-specific double stranded breaks with varying degrees of specificity and efficiency. The early uses of these systems have demonstrated remarkable new possibilities and allowed for the creation of model systems in a wide variety of organisms. With each iteration the technology has improved, and the prospects for the study of human disease with genome editing has never been better.

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Given the explosive nature of the genome-editing field in recent years, especially in the past year, we regret that due to space limitations we were unable to cite and describe many worthy studies, and we apologize for their omission.

Competing Interests

The authors declare that they do not have any competing or financial interests.

References

1. **Smithies, O., Gregg, R.G., Boggs, S.S., Koralewski, M.A., and Kucherlapati, R.S. 1985. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination.** ***Nature*** **317:230-234.**
2. **Thomas, K.R., and Capecchi, M.R. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells.** ***Cell*** **51:503-512.**

3. Qiu, S., Adema, C.M., and Lane, T. 2005. A computational study of off-target effects of RNA interference. *Nucleic Acids Res* 33:1834-1847.
4. Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S., and Gregory, P.D. 2010. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* 11:636-646.
5. Radecke, S., Radecke, F., Cathomen, T., and Schwarz, K. 2010. Zinc-finger nuclease-induced gene repair with oligodeoxynucleotides: wanted and unwanted target locus modifications. *Mol Ther* 18:743-753.
6. Soldner, F., Laganiere, J., Cheng, A.W., Hockemeyer, D., Gao, Q., Alagappan, R., Khurana, V., Golbe, L.I., Myers, R.H., Lindquist, S., et al. 2011. Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. *Cell* 146:318-331.
7. Chen, F., Pruett-Miller, S.M., Huang, Y., Gjoka, M., Duda, K., Taunton, J., Collingwood, T.N., Frodin, M., and Davis, G.D. 2011. High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. *Nat Methods* 8:753-755.
8. Ramirez, C.L., Foley, J.E., Wright, D.A., Muller-Lerch, F., Rahman, S.H., Cornu, T.I., Winfrey, R.J., Sander, J.D., Fu, F., Townsend, J.A., et al. 2008. Unexpected failure rates for modular assembly of engineered zinc fingers. *Nat Methods* 5:374-375.
9. Maeder, M.L., Thibodeau-Beganny, S., Osiak, A., Wright, D.A., Anthony, R.M., Eichtinger, M., Jiang, T., Foley, J.E., Winfrey, R.J., Townsend, J.A., et al. 2008. Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. *Mol Cell* 31:294-301.
10. Maeder, M.L., Thibodeau-Beganny, S., Sander, J.D., Voytas, D.F., and Joung, J.K. 2009. Oligomerized pool engineering (OPEN): an 'open-source' protocol for making customized zinc-finger arrays. *Nat Protoc* 4:1471-1501.
11. Sander, J.D., Dahlborg, E.J., Goodwin, M.J., Cade, L., Zhang, F., Cifuentes, D., Curtin, S.J., Blackburn, J.S., Thibodeau-Beganny, S., Qi, Y., et al. 2011. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat Methods* 8:67-69.
12. Kim, H., Um, E., Cho, S.R., Jung, C., Kim, H., and Kim, J.S. 2011. Surrogate reporters for enrichment of cells with nuclease-induced mutations. *Nat Methods* 8:941-943.
13. Gupta, A., Christensen, R.G., Rayla, A.L., Lakshmanan, A., Stormo, G.D., and Wolfe, S.A. 2012. An optimized two-finger archive for ZFN-mediated gene targeting. *Nat Methods* 9:588-590.
14. Bhakta, M.S., Henry, I.M., Ousterout, D.G., Das, K.T., Lockwood, S.H., Meckler, J.F., Wallen, M.C., Zykovich, A., Yu, Y., Leo, H., et al. 2013. Highly active zinc-finger nucleases by extended modular assembly. *Genome Res* 23:530-538.
15. Hockemeyer, D., Soldner, F., Beard, C., Gao, Q., Mitalipova, M., DeKelver, R.C., Katibah, G.E., Amora, R., Boydston, E.A., Zeitler, B., et al. 2009. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat Biotechnol* 27:851-857.
16. Gabriel, R., Lombardo, A., Arens, A., Miller, J.C., Genovese, P., Kaeppel, C., Nowrouzi, A., Bartholomae, C.C., Wang, J., Friedman, G., et al. 2011. An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat Biotechnol* 29:816-823.

17. **Pattanayak, V., Ramirez, C.L., Joung, J.K., and Liu, D.R. 2011. Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. *Nat Methods* 8:765-770.**
18. **Guo, J., Gaj, T., and Barbas, C.F., 3rd. 2010. Directed evolution of an enhanced and highly efficient FokI cleavage domain for zinc finger nucleases. *J Mol Biol* 400:96-107.**
19. **Doyon, Y., Vo, T.D., Mendel, M.C., Greenberg, S.G., Wang, J., Xia, D.F., Miller, J.C., Urnov, F.D., Gregory, P.D., and Holmes, M.C. 2011. Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. *Nat Methods* 8:74-79.**
20. **Bogdanove, A.J., and Voytas, D.F. 2011. TAL effectors: customizable proteins for DNA targeting. *Science* 333:1843-1846.**
21. **Moscou, M.J., and Bogdanove, A.J. 2009. A simple cipher governs DNA recognition by TAL effectors. *Science* 326:1501.**
22. **Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A., and Bonas, U. 2009. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326:1509-1512.**
23. **Morbitzer, R., Romer, P., Boch, J., and Lahaye, T. 2010. Regulation of selected genome loci using de novo-engineered transcription activator-like effector (TALE)-type transcription factors. *Proc Natl Acad Sci U S A* 107:21617-21622.**
24. **Streubel, J., Blucher, C., Landgraf, A., and Boch, J. 2012. TAL effector RVD specificities and efficiencies. *Nat Biotechnol* 30:593-595.**
25. **Cong, L., Zhou, R., Kuo, Y.C., Cunniff, M., and Zhang, F. 2012. Comprehensive interrogation of natural TALE DNA-binding modules and transcriptional repressor domains. *Nat Commun* 3:968.**
26. **Christian, M., Cermak, T., Doyle, E.L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A.J., and Voytas, D.F. 2010. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 186:757-761.**
27. **Miller, J.C., Tan, S., Qiao, G., Barlow, K.A., Wang, J., Xia, D.F., Meng, X., Paschon, D.E., Leung, E., Hinkley, S.J., et al. 2011. A TALE nuclease architecture for efficient genome editing. *Nat Biotechnol* 29:143-148.**
28. **Hockemeyer, D., Wang, H., Kiani, S., Lai, C.S., Gao, Q., Cassady, J.P., Cost, G.J., Zhang, L., Santiago, Y., Miller, J.C., et al. 2011. Genetic engineering of human pluripotent cells using TALE nucleases. *Nat Biotechnol* 29:731-734.**
29. **Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.V., Bogdanove, A.J., and Voytas, D.F. 2011. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res* 39:e82.**
30. **Reyon, D., Tsai, S.Q., Khayter, C., Foden, J.A., Sander, J.D., and Joung, J.K. 2012. FLASH assembly of TALENs for high-throughput genome editing. *Nat Biotechnol* 30:460-465.**
31. **Kim, Y., Kweon, J., Kim, A., Chon, J.K., Yoo, J.Y., Kim, H.J., Kim, S., Lee, C., Jeong, E., Chung, E., et al. 2013. A library of TAL effector nucleases spanning the human genome. *Nat Biotechnol* 31:251-258.**
32. **Bultmann, S., Morbitzer, R., Schmidt, C.S., Thanisch, K., Spada, F., Elsaesser, J., Lahaye, T., and Leonhardt, H. 2012. Targeted transcriptional activation of silent**

oct4 pluripotency gene by combining designer TALEs and inhibition of epigenetic modifiers. *Nucleic Acids Res* 40:5368-5377.

33. Deng, D., Yin, P., Yan, C., Pan, X., Gong, X., Qi, S., Xie, T., Mahfouz, M., Zhu, J.K., Yan, N., et al. 2012. Recognition of methylated DNA by TAL effectors. *Cell Res* 22:1502-1504.

34. Valton, J., Dupuy, A., Daboussi, F., Thomas, S., Marechal, A., Macmaster, R., Melliand, K., Juillerat, A., and Duchateau, P. 2012. Overcoming transcription activator-like effector (TALE) DNA binding domain sensitivity to cytosine methylation. *J Biol Chem* 287:38427-38432.

35. Mussolino, C., Morbitzer, R., Lutge, F., Dannemann, N., Lahaye, T., and Cathomen, T. 2011. A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res* 39:9283-9293.

36. Holkers, M., Maggio, I., Liu, J., Janssen, J.M., Miselli, F., Mussolino, C., Recchia, A., Cathomen, T., and Goncalves, M.A. 2013. Differential integrity of TALE nuclease genes following adenoviral and lentiviral vector gene transfer into human cells. *Nucleic Acids Res* 41:e63.

37. Yang, L., Guell, M., Byrne, S., Yang, J.L., De Los Angeles, A., Mali, P., Aach, J., Kim-Kiselak, C., Briggs, A.W., Rios, X., et al. 2013. Optimization of scarless human stem cell genome editing. *Nucleic Acids Res* 41:9049-9061.

38. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., et al. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339:819-823.

39. Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. 2013. RNA-guided human genome engineering via Cas9. *Science* 339:823-826.

40. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337:816-821.

41. Cho, S.W., Kim, S., Kim, J.M., and Kim, J.S. 2013. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat Biotechnol* 31:230-232.

42. Wang, T., Wei, J.J., Sabatini, D.M., and Lander, E.S. 2014. Genetic screens in human cells using the CRISPR-Cas9 system. *Science* 343:80-84.

43. Shalem, O., Sanjana, N.E., Hartenian, E., Shi, X., Scott, D.A., Mikkelsen, T.S., Heckl, D., Ebert, B.L., Root, D.E., Doench, J.G., et al. 2014. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 343:84-87.

44. Koike-Yusa, H., Li, Y., Tan, E.P., Velasco-Herrera, M.D., and Yusa, K. 2013. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotechnol*.

45. Hou, Z., Zhang, Y., Propson, N.E., Howden, S.E., Chu, L.F., Sontheimer, E.J., and Thomson, J.A. 2013. Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. *Proc Natl Acad Sci U S A* 110:15644-15649.

46. Esvelt, K.M., Mali, P., Braff, J.L., Moosburner, M., Yaung, S.J., and Church, G.M. 2013. Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nat Methods* 10:1116-1121.

47. Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., and Sander, J.D. 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* 31:822-826.

48. Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O., et al. 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* 31:827-832.

49. Mali, P., Aach, J., Stranges, P.B., Esvelt, K.M., Moosburner, M., Kosuri, S., Yang, L., and Church, G.M. 2013. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat Biotechnol* 31:833-838.

50. Pattanayak, V., Lin, S., Guilinger, J.P., Ma, E., Doudna, J.A., and Liu, D.R. 2013. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat Biotechnol* 31:839-843.

51. Cradick, T.J., Fine, E.J., Antico, C.J., and Bao, G. 2013. CRISPR/Cas9 systems targeting beta-globin and CCR5 genes have substantial off-target activity. *Nucleic Acids Res* 41:9584-9592.

52. Cho, S.W., Kim, S., Kim, Y., Kweon, J., Kim, H.S., Bae, S., and Kim, J.S. 2014. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res* 24:132-141.

53. Ran, F.A., Hsu, P.D., Lin, C.Y., Gootenberg, J.S., Konermann, S., Trevino, A.E., Scott, D.A., 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.

54. Fu, Y., Sander, J.D., Reyon, D., Cascio, V.M., and Joung, J.K. 2014. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol*.

55. Geurts, A.M., Cost, G.J., Freyvert, Y., Zeitler, B., Miller, J.C., Choi, V.M., Jenkins, S.S., Wood, A., Cui, X., Meng, X., et al. 2009. Knockout rats via embryo microinjection of zinc-finger nucleases. *Science* 325:433.

56. Mashimo, T., Takizawa, A., Voigt, B., Yoshimi, K., Hiai, H., Kuramoto, T., and Serikawa, T. 2010. Generation of knockout rats with X-linked severe combined immunodeficiency (X-SCID) using zinc-finger nucleases. *PLoS One* 5:e8870.

57. Tesson, L., Usal, C., Menoret, S., Leung, E., Niles, B.J., Remy, S., Santiago, Y., Vincent, A.I., Meng, X., Zhang, L., et al. 2011. Knockout rats generated by embryo microinjection of TALENs. *Nat Biotechnol* 29:695-696.

58. 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.

59. Li, W., Teng, F., Li, T., and Zhou, Q. 2013. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. *Nat Biotechnol* 31:684-686.

60. Carbery, I.D., Ji, D., Harrington, A., Brown, V., Weinstein, E.J., Liaw, L., and Cui, X. 2010. Targeted genome modification in mice using zinc-finger nucleases. *Genetics* 186:451-459.

61. Cui, X., Ji, D., Fisher, D.A., Wu, Y., Briner, D.M., and Weinstein, E.J. 2011. Targeted integration in rat and mouse embryos with zinc-finger nucleases. *Nat Biotechnol* 29:64-67.

62. Meyer, M., de Angelis, M.H., Wurst, W., and Kuhn, R. 2010. Gene targeting by homologous recombination in mouse zygotes mediated by zinc-finger nucleases. *Proc Natl Acad Sci U S A* 107:15022-15026.

63. Sung, Y.H., Baek, I.J., Kim, D.H., Jeon, J., Lee, J., Lee, K., Jeong, D., Kim, J.S., and Lee, H.W. 2013. Knockout mice created by TALEN-mediated gene targeting. *Nat Biotechnol* 31:23-24.

64. Wefers, B., Meyer, M., Ortiz, O., Hrabe de Angelis, M., Hansen, J., Wurst, W., and Kuhn, R. 2013. Direct production of mouse disease models by embryo microinjection of TALENs and oligodeoxynucleotides. *Proc Natl Acad Sci U S A* 110:3782-3787.

65. Shen, B., Zhang, J., Wu, H., Wang, J., Ma, K., Li, Z., Zhang, X., Zhang, P., and Huang, X. 2013. Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell Res* 23:720-723.

66. Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., and Jaenisch, R. 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153:910-918.

67. Yang, H., Wang, H., Shivalila, C.S., Cheng, A.W., Shi, L., and Jaenisch, R. 2013. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* 154:1370-1379.

68. Wu, Y., Liang, D., Wang, Y., Bai, M., Tang, W., Bao, S., Yan, Z., Li, D., and Li, J. 2013. Correction of a genetic disease in mouse via use of CRISPR-Cas9. *Cell Stem Cell* 13:659-662.

69. 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.

70. Liu, H., Chen, Y., Niu, Y., Zhang, K., Kang, Y., Ge, W., Liu, X., Zhao, E., Wang, C., Lin, S., et al. 2014. TALEN-Mediated Gene Mutagenesis in Rhesus and Cynomolgus Monkeys. *Cell Stem Cell* 14:323-328.

71. Lombardo, A., Genovese, P., Beausejour, C.M., Colleoni, S., Lee, Y.L., Kim, K.A., Ando, D., Urnov, F.D., Galli, C., Gregory, P.D., et al. 2007. Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat Biotechnol* 25:1298-1306.

72. Zou, J., Maeder, M.L., Mali, P., Pruett-Miller, S.M., Thibodeau-Beganny, S., Chou, B.K., Chen, G., Ye, Z., Park, I.H., Daley, G.Q., et al. 2009. Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. *Cell Stem Cell* 5:97-110.

73. Yusa, K., Rad, R., Takeda, J., and Bradley, A. 2009. Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nat Methods* 6:363-369.

74. Sebastiano, V., Maeder, M.L., Angstman, J.F., Haddad, B., Khayter, C., Yeo, D.T., Goodwin, M.J., Hawkins, J.S., Ramirez, C.L., Batista, L.F., et al. 2011. In situ genetic correction of the sickle cell anemia mutation in human induced pluripotent stem cells using engineered zinc finger nucleases. *Stem Cells* 29:1717-1726.

75. Reinhardt, P., Schmid, B., Burbulla, L.F., Schondorf, D.C., Wagner, L., Glatza, M., Hoing, S., Hargus, G., Heck, S.A., Dhingra, A., et al. 2013. Genetic correction of a

LRRK2 mutation in human iPSCs links parkinsonian neurodegeneration to ERK-dependent changes in gene expression. *Cell Stem Cell* 12:354-367.

76. Schwank, G., Koo, B.K., Sasselli, V., Dekkers, J.F., Heo, I., Demircan, T., Sasaki, N., Boymans, S., Cuppen, E., van der Ent, C.K., 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.

Table 1. Relative characteristics of genome-editing tools in mammalian systems

	Origin	Typical genomic target site	Flexibility in site selection	Ease of use/ affordability	Size (ability to be packaged in viruses)	Efficacy	Specificity/ lack of off-target effects	References
ZFNs	Zinc finger proteins (widely found in nature)	Pair of 9- or 12-bp sequences	+	+	+++	++	++	(6, 9, 11-17, 72)
TALENs	TAL effector proteins in plant pathogens	Pair of 13-bp or longer sequences (no length limitation)	++	++	++	++	+++	(27-30)
CRISPR-Cas9	Bacterial immune system (*S. pyogenes*, other species)	20-nt protospacer + 3-nt PAM (*S. pyogenes*)	++	+++	+	+++	+ to +++ (depending on strategy used)	(37-41, 48-52, 54, 66)

+ indicates least favorable, +++ indicates most favorable