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Single-step generation of rabbits carrying a targeted allele of the tyrosinase gene using CRISPR/Cas9

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Abstract: Targeted genome editing of nonrodent mammalian species has provided the potential for highly accurate interventions into gene function in humans and the generation of useful animal models of human diseases. Here we show successful clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas)-mediated gene targeting via circular plasmid injection in rabbits. The rabbit tyrosinase gene (*TYR*) was effectively disrupted, and we confirmed germline transmission by pronuclear injection of a circular plasmid expressing humanized Cas9 (hCas9) and single-guide RNA. Direct injection into pronuclear stage zygotes was possible following an *in vitro* validation assay. Neither off-target mutagenesis nor hCas9 transgenesis was detected in any of the genetically targeted pups and embryos examined. Gene targeting with this rapid and simplified strategy will help accelerate the development of translational research using other nonrodent mammalian species.

Key words: CRISPR/Cas9, gene targeting, rabbit, tyrosinase

Introduction

Gene targeting and disruption in mice are valuable tools for understanding gene function in mammalian development and disease [2]. They generally involve the production of chimeric progeny using homologous recombined pluripotent stem cells (PSCs), which have the ability to transmit their genome into the germline (naïve PSCs). However, effective germline transmission of PSCs in chimeric animals has so far been achieved only in the mouse and rat (rodent species) [15]. Pluripotent

stem cells in nonrodent mammalian species have been generated as primed-state PSCs, but these have proved difficult to include in the embryo proper when introduced into host preimplantation embryos [8, 20].

Recently engineered endonucleases, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), are useful for gene targeting [7]. They allow efficient and specific cleavage of genomic sequences in animals by directly injecting mRNAs of site-specific nucleases into 1-cell embryos to generate a double-strand breakage (DSB) in the DNA

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[7]. The enzymes recognize target DNA by peptide-DNA affinity, and fused FokI nucleases generate a DSB; subsequently, error-prone nonhomologous end joining results in small insertions or deletions (indels). However, even though several methods such as Golden Gate assembly [4] or the Platinum Gate system [18] have been developed to decrease the steps, the complex and time-consuming design and generation of ZFNs or TALENs for each target gene limit the application of these methods.

The type II bacterial clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein (Cas) have been developed as an efficient gene targeting system [10]. In such type II systems, the complex of a CRISPR RNA (crRNA) annealed to a transactivating crRNA is sufficient to guide the Cas9 endonuclease to a specific genomic sequence to generate a DSB in the target DNA. Recent reports demonstrated gene targeting of several genes in mice and rats using the CRISPR/Cas system [11, 21]. The ease of design, construction, and delivery of multiple synthetic single-guide RNAs (sgRNAs) suggest the possibility of multiplexed genome editing in mammals [21]. Recent studies of Cas9 specificity have demonstrated that although each base within the 20 nt guide sequence contributes to its overall specificity, multiple mismatches between the sgRNA and its complementary target DNA sequence can be tolerated depending on the quantity, position, and base identity of the mismatches [11].

This leads to potential off-target DSBs and indel formation [6, 9]. To improve the specificity of Cas9-mediated genome editing, a combination of the D10A mutant nickase version of Cas9 with a pair of offset sgRNAs complementary to opposite strands of the target site has been suggested [17]. Generally, for targeted disruption of a candidate gene in mammalian embryos, the injected mRNA must be prepared using several steps. Target sequences need to be evaluated by *in vitro* assay using plasmid vectors, reconstruction of mRNA expression vectors, and then preparation of the desired mRNA by *in vitro* transcription [12, 17, 20, 22, 23, 24]. However, Mashiko *et al.* reported direct injection of a plasmid DNA, pX330 [5], containing a humanized Cas9 (hCas9) expression cassette with a gene-targeting sgRNA expression cassette. This enabled a rapid (within 1 month), simple, and reproducible method for targeted mutagenesis in mice [13]. For these reasons, the CRISPR/Cas9 system is now developing as a valuable strategy for

genome editing research and possibly for generating targeted gene modification in nonrodent mammalian species [16, 23].

Here we confirmed successful gene targeting in the rabbit via a simplified and rapid strategy using pX330, a circular plasmid DNA encoding hCas9, and sgRNA. Thus, we demonstrated efficient generation of a genetically engineered nonrodent mammalian species. Potentially, this approach could be used for the functional annotation of genes or for modeling human genetic diseases.

Materials and Methods

Animals

All rabbits were maintained and used for experiments in accordance with the guidelines for animal experimentation of the RIKEN Bioresource Center and Tsukuba Primate Research Center after approval by the responsible committees.

Plasmid preparation

To perform the single-strand annealing (SSA) assay, a pCAG-EGxxFP plasmid expressing a 5' and 3' enhanced green fluorescent protein (EGFP) sequence was used according to Mashiko *et al.* [13]. The 499-bp genomic fragment of the rabbit tyrosinase gene containing an sgRNA target sequence was amplified by polymerase chain reaction (PCR) with a cycling program of 94°C for 3 min and 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s and placed between the EGFP fragments at the multiple cloning site (*Bam*HI–*Eco*RI) of pCAG-EGxxFP. Plasmids coexpressing hCas9 and sgRNA were prepared by ligating oligonucleotides into the *Bbs*I site of pX330 (<http://www.addgene.org/42230/>). The primers and oligonucleotides used are listed in Table S1.

Cell transfection

Ten micrograms of pCAG-EGxxFP-target was mixed with 10 μ g of pX330 with or without the sgRNA sequence and then introduced with a NEPA21 electroporator (Nepa Gene Co., Ltd., Chiba, Japan) into 3×10^5 human embryonic kidney (HEK) 293 cells/well cultured in 6-well plates. The EGFP fluorescence was observed and assessed using a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan) and a BZ-II Analyzer image analysis system (Keyence).

Production of the rabbits carrying the targeted allele of the tyrosinase gene

Mature Dutch Belted rabbits were purchased from Kitayama Labes (Nagano, Japan). Fertilized embryos were obtained from mature female rabbits that had been treated with 75 IU of follicle stimulating hormone (Antrin R10; Kyoritsu Seiyaku, Tokyo, Japan) twice daily for 3 days, followed by 100 IU of human chorionic gonadotropin (hCG; Gonatropin; Teikoku Zoki, Tokyo, Japan) 12 h later. The does were mated with fertile males immediately after hCG treatment. Twenty hours after mating, fertilized embryos (zygotes) were flushed from the oviducts using warmed HEPES-buffered RD medium [3] containing 4 mg/ml of bovine serum albumin (ICN Biomedicals, Irvine, CA, USA). The pronuclei of zygotes were microinjected with 5 ng/ μ l of the pX330 plasmid. After pronuclear injection of the plasmid, the embryos were cultured in RD medium at 37°C in 6% CO₂ and 5% O₂ in air for 24 h and then transferred into the oviducts of pseudopregnant Japanese White (JW) rabbits (Kitayama Labes) that had been treated with 100 IU hCG and given manual vulval stimulation 24 h before transfer. To confirm germline transmission, a heterozygous mutant female rabbit that had a targeted allele of the tyrosinase gene, was mated with male JW rabbit. For genotyping of the offspring, skin samples were collected 2-days after birth, and genomic DNAs were extracted and sequenced.

DNA sequencing

Genomic DNA was extracted from small tissues of embryos or pups. Approximately 500–700 bp genomic fragments containing the target site were amplified by PCR and sequenced. After confirmation of an indel mutation at the target site, PCR fragments were subcloned into a pT7 Blue T-vector (Merck Millipore, Darmstadt, Germany) and sequenced for the determination of each allele.

Off-target analysis

According to Yang *et al.*, each sgRNA of the 12 nt target sequence immediately upstream of the Protospacer Adjacent Motif (PAM) and the 3 nt PAM sequence (NGG, with four variations, i.e., AGG, TGG, GGG, CGG) was searched against the rabbit genome in Ensembl (http://www.ensembl.org/Oryctolagus_cuniculus/Info/Index). The “E value” was set to 100000. The number of exactly matched sequences in the query results

was the total number of hits in the rabbit genome, including the designed target sequence. If the hit was within an exon region (defined as from 100 bp upstream to 100 bp downstream of any exon based on the Ensembl output), we considered it as a significant hit and considered a potential off target site. PCR and sequencing were conducted on potential off-target loci to identify if there were any mutations. Moreover, the other potential off-target sites were analyzed using BLAST searching of the *Oryctolagus cuniculus* (rabbit) nucleotide sequences (<http://www.ncbi.nlm.nih.gov/BLAST/>). Twenty bases preceding the PAM sequence were aligned with the rabbit genome. Approximately 500–700 bp genomic fragments containing a centrally situated off-target site were amplified by PCR and sequenced. The primers used for off-target PCR analysis are listed in Table S1.

Results

Evaluation of sgRNA Sequences to disrupt the rabbit Tyrosinase Gene Using CRISPR/Cas9

To establish a system that could produce genetically targeted rabbits effectively, we used the pX330 plasmid expressing hCas9 and sgRNA for serial gene targeting, after assessing the efficacy of producing appropriate DSBs by SSA assays *in vitro* [13]. The efficacy of DSB-mediated homology-dependent repair was validated by cotransfection with the plasmid pCAG-EGxxFP (Fig. 1A). This system does not need to construct additional plasmids or require the use of further sgRNA synthesis for the microinjection of CRISPR/Cas9 after evaluating nuclease activity. To disrupt the rabbit tyrosinase gene, four target sites in the first exon were evaluated by SSA assays (Fig. 1B). Each pX330 plasmid was co-transfected with pCAG-EGxxFP, which contains a 499-bp genomic fragment of tyrosinase containing sgRNA target sequences. EGFP fluorescence was evaluated to determine which sgRNA sequence in pX330 caused a DSB in the pCAG-EGxxFP sequence in HEK293T cells (Figs. 2A and B). A candidate sequence, tyrosinase CR2, showed the most effectiveness in causing a suitable DSB.

Single-step production of genetically targeted rabbits using CRISPR/Cas9

Following validation of the sgRNA sequence by SSA assay, the most effective plasmid—pX330 with the target sequence tyrosinase CR2—was microinjected directly into the pronuclei of rabbit zygotes. Of 77 injected eggs,

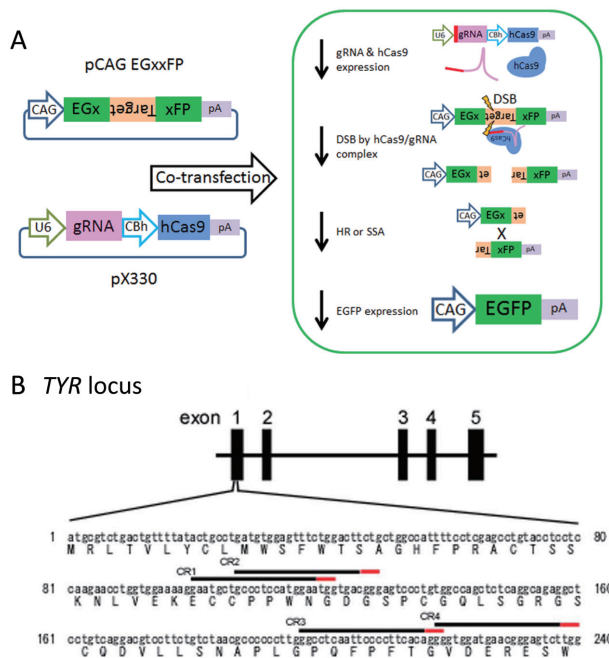


Fig. 1. Scheme for CRISPR/Cas9-mediated gene targeting. (A) Scheme for the SSA assay using pCAG EGxxFP and pX330 in HEK293 cells. The transduced hCas9 and sgRNA complex caused DSB in the target sequence of pCAG-EGxxFP by homology-dependent repair via homologous recombination (HR) or caused single-strand annealing (SSA). (B) Schematic representation of sgRNAs targeting tyrosinase (*TYR*). The sgRNA target sequence and PAM sequence are highlighted in black and red, respectively.

67 (87%) embryos were transplanted into the oviducts of pseudopregnant JW rabbits. Nine of these (13%) developed to term. Of them, two (3%) pups had the targeted allele at the tyrosinase locus (Table 1). One live pup with a normal coat color (Dutch Belted) had a heterozygous mutation (a 2-bp deletion) at the tyrosinase locus (Fig. 3A). Unfortunately, one pup with an albino coat-color phenotype over its entire body that carried a homozygous mutation (a 7-bp deletion) at the tyrosinase locus was stillborn (Fig. S1). Two of the wild-type pups also died prenatally. To detect off-target mutations in mutant pups, 20-base sequences of the sgRNA sequence of tyrosinase CR2 were searched in the rabbit genome using Ensemble and BLAST. Five potential off-target sites with two or three mismatch sequences were found (Supplementary Table S2). We amplified and sequenced the genomic DNAs around the regions that contained the potential off-target sites in these mutant rabbits. No off-target mutations were detected. Additionally, no hCas9 transgenes were detected in any of the pups examined (data not shown). To confirm germline transmission, a

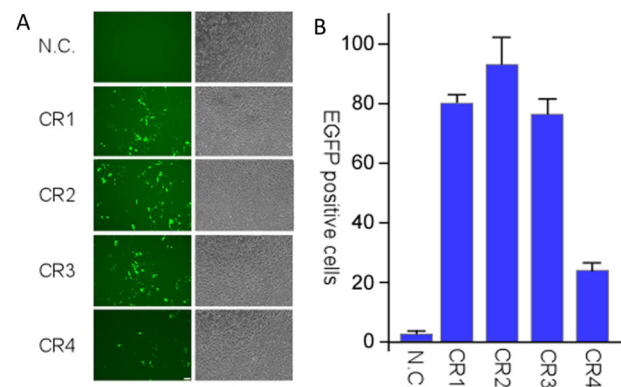


Fig. 2. Evaluation of sgRNA sequences for disrupting the rabbit tyrosinase gene. (A) The efficiency of DSB-mediated homology-dependent repair was validated by observing EGFP fluorescence at 24 h after transfection (left panels, EGFP signals; right panels, phase contrast microscopy images). (N.C., normal control pX330 without an sgRNA sequence; CR1-CR4, pX330 plasmid with tyrosinase CR1/CR2/CR3/CR4 sequences, respectively). (B) Quantitative representation of the DSB efficiency of each sgRNA sequence. Data are shown as the mean \pm standard deviation (SD). Scale bar=100 μ m.

Table 1. Generation of genetically targeted rabbits using CRISPR/Cas9

	Injected	Survived	Pups	GMO (hetero:homo)
Tyrosinase CR2	77	67 (87%)	9 (13%)	2 (3%) (1:1)

GMO, genetically modified organism.

heterozygous female mutant rabbit that showed a Dutch coat color was crossed with a male JW rabbit. We successfully obtained does that had the white coat color phenotype over their entire body and red eyes (Fig. 3B and C). In fact, sequencing analysis suggested that the white does carried the targeted alleles of the tyrosinase gene (Fig. 3B). Thus, pronuclear injection of this circular plasmid DNA proved to be a very effective strategy for the generation of genetically targeted rabbits using CRISPR/Cas9.

Discussion

In this report, we document simplified gene targeting via the CRISPR/Cas9 system in the rabbit. Using microinjection of pronuclei with our circular plasmid DNA—validated by *in vitro* SSA—it was not necessary to reconstruct mRNA expression vectors, to carry out sequence confirmation, or to prepare mRNAs by *in vitro* transcription as reported previously [12, 17, 20, 22, 23,

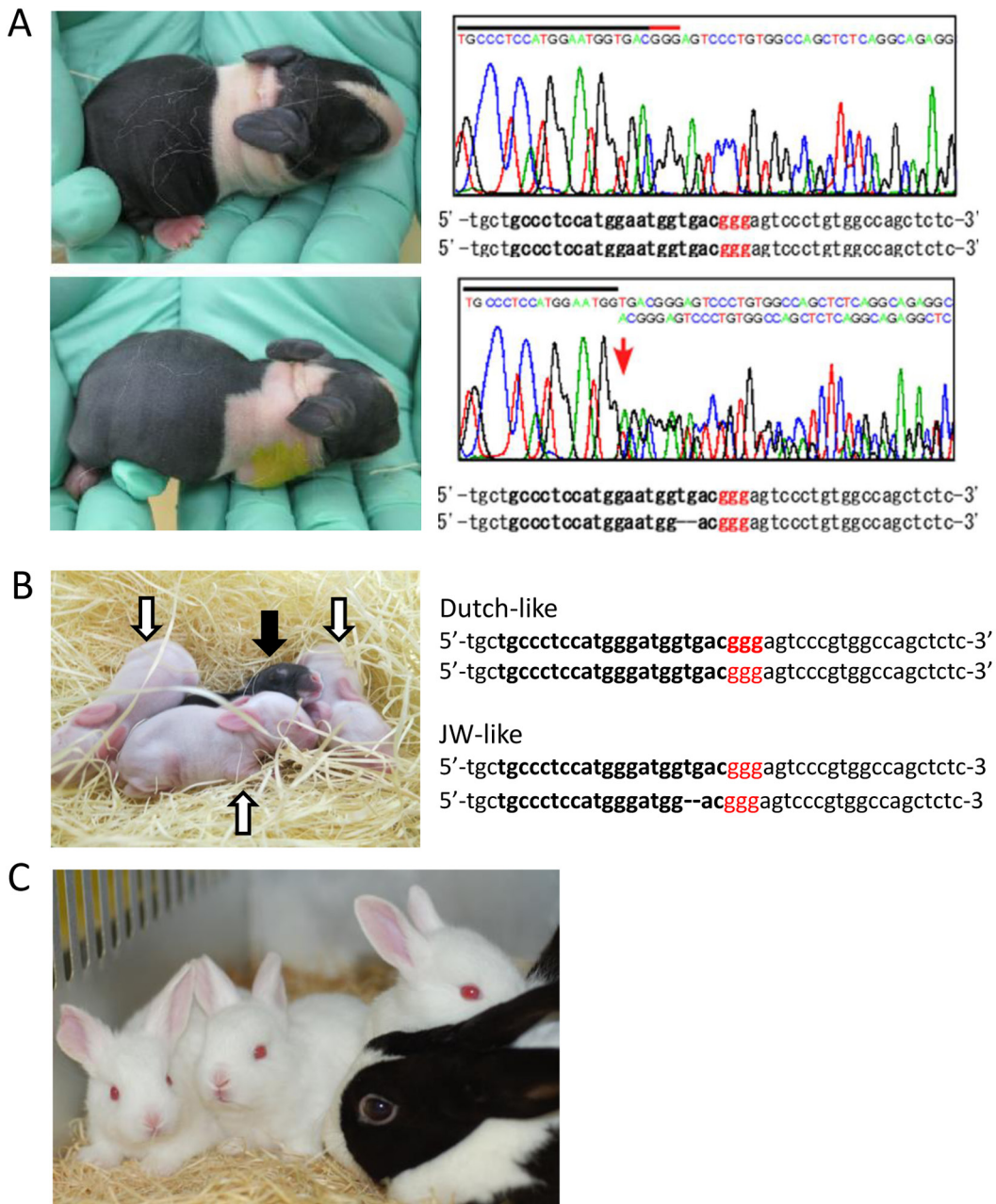


Fig. 3. CRISPR/Cas9-mediated modification of the tyrosinase gene in founder rabbits. (A) Five-day-old founder pups with representative tyrosinase genomic sequences (top panels, wild-type pup; bottom panels, heterozygous pup with a 2-bp deletion). The arrow indicates the indel mutation in this pup. Subcloning and sequencing its PCR fragment confirmed each allele's identity. (B) Photograph and representative tyrosinase genomic sequences of F1 pups that showed a Dutch-like coat color (closed arrow) and JW-like (white) coat color (open arrows). (C) Photograph of JW-like does that have a white coat color and red eyes.

24]. Because no off-target mutation or hCas9 transgenesis was detected in this experiment, this method of circular plasmid DNA injection into zygotes will be a valuable and rapid strategy for achieving gene targeting not only in rodents but also in other mammalian species.

In the mouse model, gene knockout and knockin via cytoplasmic coinjection of mRNAs encoding hCas9 and sgRNA have been used widely and successfully [17, 20, 24]. Mashiko *et al.* [13] have reported that the pronuclear injection of a circular plasmid expressing the hCas9/

sgRNA complex is a rapid, simple, and reproducible method for achieving targeted mutagenesis in mice. This method can skip the need for constructing mRNA expression vectors and sgRNA synthesis. Here we employed this strategy in the rabbit and showed its easy and rapid capacity to accomplish gene targeting with high accuracy. Although no transgene was detected in our experiments, fewer than 5% of the genetically targeted offspring expressed the hCas9 transgene in the mouse system [12]. Moreover, compared with mouse data in general (more than 10% of genetically modified offspring per injected embryos), the success rate of our experiment (3%) seems low. It is well known that the efficiency of gene transfer into rabbits by the microinjection of plasmid-based constructs into pronuclei varies between 0.5% and 3% [1]. Rabbit pronuclear-stage embryos seem to have much more chromosomal fragility than mouse embryos. Therefore, we need to assess the efficiency of gene modification in the rabbit using plasmid microinjection not into the pronuclei but also into the zygote cytoplasm to prevent hCas9 transgenesis and/or to prevent chromosomal damage. Unfortunately, one albino pup carrying a homozygous mutation (a 7-bp deletion) at the tyrosinase locus was stillborn (Fig. 3). A null mutation in tyrosinase was unlikely to be the cause of this death, as two wild-type pups also died prenatally in this experiment and healthy offspring carrying tyrosinase null mutations have been born in other laboratories [14, 19]. Some unidentified genetic aberrations might have occurred during micromanipulation of the oocytes and embryos. We successfully confirmed germline transmission of the targeted allele and the effect in the form of the white coat color phenotype by natural mating using the heterozygous mutant individual with a JW rabbit.

This report offers a valuable candidate strategy for appropriate gene targeting using the CRISPR/Cas9 system in the rabbit. This will facilitate investigations of gene function and the production of useful animal models of human diseases.

Abbreviations

Cas, CRISPR-associated; hCas9, humanized Cas9; CRISPR, clustered regularly interspaced short palindromic repeat; crRNA, CRISPR RNA; DSB, double-strand break; EGFP, enhanced green fluorescence protein; FSH, follicle stimulating hormone; GMO,

genetically modified organism; hCG, human chorionic gonadotropin; JW, Japanese White; NHEJ, nonhomologous end joining; PAM, Protospacer Adjacent Motif; PCR, polymerase chain reaction; PSC, pluripotent stem cell; sgRNA, single-guide RNA; SSA, single-strand annealing; TALEN, transcription activator-like effector nuclease; ZFN, zinc-finger nuclease.

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References

1. Bosze, Z., Hiripi, L., Carnwath, J.W., and Niemann, H. 2003. The transgenic rabbit as model for human diseases and as a source of biologically active recombinant proteins. *Transgenic. Res.* 12: 541–553. [Medline] [CrossRef]
2. Capecchi, M.R. 2005. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nat. Rev. Genet.* 6: 507–512. [Medline] [CrossRef]
3. Carney, E.W. and Foote, R.H. 1991. Improved development of rabbit one-cell embryos to the hatching blastocyst stage by culture in a defined, protein-free culture medium. *J. Reprod. Fertil.* 91: 113–123. [Medline] [CrossRef]
4. 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. [Medline] [Cross-Ref]
5. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., and Zhang, F. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339: 819–823. [Medline] [CrossRef]
6. 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. [Medline] [Cross-Ref]
7. Gaj, T., Gersbach, C.A., and Barbas, C.F. 3rd. 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* 31: 397–405. [Medline] [Cross-Ref]
8. Honda, A., Hatori, M., Hirose, M., Honda, C., Izu, H., Inoue, K., Hirasawa, R., Matoba, S., Togayachi, S., Miyoshi, H., and Ogura, A. 2013. Naive-like conversion overcomes the limited differentiation capacity of induced pluripotent stem cells. *J. Biol. Chem.* 288: 26157–26166. [Medline] [Cross-Ref]

9. 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., Cradick, T.J., Marraffini, L.A., Bao, G., and Zhang, F. 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* 31: 827–832. [[Medline](#)] [[CrossRef](#)]
10. 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. [[Medline](#)] [[CrossRef](#)]
11. Kuscu, C., Arslan, S., Singh, R., Thorpe, J., and Adli, M. 2014. Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease. *Nat. Biotechnol.* 32: 677–683. [[Medline](#)] [[CrossRef](#)]
12. Li, D., Qiu, Z., Shao, Y., Chen, Y., Guan, Y., Liu, M., Li, Y., Gao, N., Wang, L., Lu, X., Zhao, Y., and Liu, M. 2013. Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. *Nat. Biotechnol.* 31: 681–683. [[Medline](#)] [[CrossRef](#)]
13. Mashiko, D., Fujihara, Y., Satouh, Y., Miyata, H., Isotani, A., and Ikawa, M. 2013. Generation of mutant mice by pronuclear injection of circular plasmid expressing Cas9 and single guided RNA. *Sci. Rep.* 3: 3355. [[Medline](#)] [[CrossRef](#)]
14. Mashimo, T., Kaneko, T., Sakuma, T., Kobayashi, J., Kunihiro, Y., Voigt, B., Yamamoto, T., and Serikawa, T. 2013. Efficient gene targeting by TAL effector nucleases coinjected with exonucleases in zygotes. *Sci. Rep.* 3: 1253. [[Medline](#)] [[CrossRef](#)]
15. Nichols, J. and Smith, A. 2009. Naive and primed pluripotent states. *Cell Stem Cell* 4: 487–492. [[Medline](#)] [[CrossRef](#)]
16. Niu, Y., Shen, B., Cui, Y., Chen, Y., Wang, J., Wang, L., Kang, Y., Zhao, X., Si, W., Li, W., Xiang, A.P., Zhou, J., Guo, X., Bi, Y., Si, C., Hu, B., Dong, G., Wang, H., Zhou, Z., Li, T., Tan, T., Pu, X., Wang, F., Ji, S., Zhou, Q., Huang, X., Ji, W., and Sha, J. 2014. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell* 156: 836–843. [[Medline](#)] [[CrossRef](#)]
17. 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., and Zhang, F. 2013. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 154: 1380–1389. [[Medline](#)] [[CrossRef](#)]
18. Sakuma, T., Ochiai, H., Kaneko, T., Mashimo, T., Tokumasu, D., Sakane, Y., Suzuki, K., Miyamoto, T., Sakamoto, N., Matsuura, S., and Yamamoto, T. 2013. Repeating pattern of non-RVD variations in DNA-binding modules enhances TALEN activity. *Sci. Rep.* 3: 3379. [[Medline](#)] [[CrossRef](#)]
19. Suzuki, K.T., Isoyama, Y., Kashiwagi, K., Sakuma, T., Ochiai, H., Sakamoto, N., Furuno, N., Kashiwagi, A., and Yamamoto, T. 2013. High efficiency TALENs enable F0 functional analysis by targeted gene disruption in *Xenopus laevis* embryos. *Biol. Open* 2: 448–452. [[Medline](#)] [[CrossRef](#)]
20. Tachibana, M., Sparman, M., Ramsey, C., Ma, H., Lee, H.S., Penedo, M.C., and Mitalipov, S. 2012. Generation of chimeric rhesus monkeys. *Cell* 148: 285–295. [[Medline](#)] [[CrossRef](#)]
21. 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. [[Medline](#)] [[CrossRef](#)]
22. Xiao, A., Wang, Z., Hu, Y., Wu, Y., Luo, Z., Yang, Z., Zu, Y., Li, W., Huang, P., Tong, X., Zhu, Z., Lin, S., and Zhang, B. 2013. Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. *Nucleic Acids Res.* 41: e141. [[Medline](#)] [[CrossRef](#)]
23. Yang, D., Xu, J., Zhu, T., Fan, J., Lai, L., Zhang, J., and Chen, Y.E. 2014. Effective gene targeting in rabbits using RNA-guided Cas9 nucleases. *J. Mol. Cell Biol.* 6: 97–99. [[Medline](#)] [[CrossRef](#)]
24. 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. [[Medline](#)] [[CrossRef](#)]