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Genome Editing in Mice Using CRISPR/Cas9: Achievements and Prospects

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Abstract

Animal models are a powerful tool to understand the mechanisms underlying physiological and pathological processes *in vivo*. To date, mice remain the species most commonly used for genetic manipulation. The recent development of engineered endonucleases such as Zinc Finger Nucleases (ZFN), Transcription activator-like effector nucleases (TALEN), and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) offered easy, flexible, and fast alternative to ES-Cell based gene targeting. Thanks to multiple advantages, the CRISPR system superseded its predecessors and became a popular method for genome editing. Here, we review the latest techniques to apply CRISPR editing to the mouse genome, and emphasize on the current methods used in transgenic laboratories and subsequent achievements in mice.

History

Animal models of diseases

Animal models, both vertebrates and invertebrates have been instrumental for dissecting the pathophysiology of human diseases. Despite divergent opinions over their use, animal models remain the most powerful tools to understand the mechanisms underlying physiological processes, and their pathological counterparts. They are also invaluable tools to search for disease modifiers and to develop and test novel treatment strategies. Although each model has intrinsic limitations, the use of animals as an entire systemic model is vital to biomedical research because they address metabolic and physiologic processes, which cannot be studied in isolated tissue culture.

The establishment of Mendelian genetics in the middle of the 19th century, and the later discovery of the structure of the DNA by Watson and Crick [1] were the basis of modern genetics that utilize the sequencing of the entire human genome to identify genes implicated in different diseases. A further application of modern genetics is recombinant DNA technology, resulting in the generation of the first transgenic animal (mouse) over three decades ago [2]. Since then, transgenesis, the artificial modification of an organism's genome, has been extensively used to identify the role of genes in the occurrence of diseases. One of the key achievements in manipulating the genome was reached when Mario Capecchi successfully disrupted a single gene in the mouse, opening the era of gene targeting [3].

To date, the mouse remains the species most commonly used for genetic manipulation. Nonetheless, the recent advances of new technologies such as endonucleases designed to target and cleave specific DNA sequences have emerged as alternative methods to accelerate the process of genome editing, and apply it to virtually any mammalian species.

Engineered endonucleases

During the last decade, the development of ES-Cell free methods for genomic modifications simplified and accelerated drastically the process of gene manipulation. It also revived microinjection as the favorite method for producing precise (targeted) manipulations in the mammalian genome. The first generation of these engineered endonucleases consisted of three types of protein-based molecular scissors: Zinc Finger Nucleases (ZFN), Transcription activator-like effector nucleases (TALEN), and Meganucleases (MN). Although these types of nucleases can be discriminated by their recognition sequences or their modular assembly (Meganucleases having the longest recognition sequence [4], they all rely on the interaction of a defined sequence of the genomic DNA with protein recognition elements. All three classes of nucleases have been successfully applied to mouse transgenesis by direct oocyte microinjection [4-8]. However, these proteins remain quite complex to design and assemble [9,10], and the overall process can be cumbersome and time consuming.

Recently, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system associated to the Cas9 endonuclease (CRISPR/Cas9) superseded its predecessors [11]. Contrarily to the previous systems, the CRISPR system relies on the hybridization of the genomic DNA with a short complimentary RNA sequence. In particular, CRISPR being a RNA-guided endonuclease (RGEN) system, it guaranteed an unprecedented ease of design and seamless synthesis (discussed herein), and contributed to the recent advent of the CRISPR/Cas9 for genome editing [12].

Identification and description of a bacterial immune system

In 1987, an odd sequence repeat has been identified in the *iap* gene of the bacterium *Escherichia coli* [13]. Subsequently, it took researchers over a decade to realize that these bacterial sequence repeats were indeed part of the bacterial immunity mechanism [14]. Specifically, the type II CRISPR/Cas9 constitutes a defense mechanism of the bacterium *Streptococcus pyogenes* to detect and destroy invading bacteriophages. This system relies on complexes made of three components: the Cas9 nuclease, guided by the CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA). In 2012, the mechanisms underlying the efficient targeting and cleavage of a specific DNA sequence were identified, and it has been shown that the crRNA and tracrRNA could be fused to a chimeric "single-guide" RNA (sgRNA), making up a two-component system sufficient for genome editing *in vitro* [15]. The short genomic

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target sequence consists of twenty base pairs (bp) complimentary to the sgRNA, and immediately upstream of a protospacer adjacent motif (PAM). The PAM is a three-nucleotide sequence of the form NGG (where N represents any nucleotide followed by two Guanines). The PAM is the only limiting factor when choosing a targeted sequence in a given genome. However, such target sites can be found on average every 8–12 bp in the human genome [16]. The second targeting limitations of the CRISPR system are the intrinsic restrictions of the U6 or T7 promoters used in CRISPR tools that require a G or GG (respectively) at the 5' end of the sgRNA for efficient transcription [17]. Nonetheless, these restrictions now tend to be completely ignored without many problems when researchers perform CRISPR-based gene editing. Consequently, the CRISPR system is far less restrictive than the repeat variable diresidues (RVD) cipher of the TALENs [18].

Harnessing CRISPR for genome editing

A milestone has been achieved when simultaneous reports showed that the CRISPR system could be harnessed *in vivo* [19,20]. In mice, the CRISPR technology has effectively targeted several genes at the same time (multiplex) and can induce either gene Knock-Out (KO) via the Non Homologous End Joining (NHEJ) pathway, or induce gene Knock-In (KI) upon Homologous Recombination (HR) of a "donor" template (plasmid or single strand oligonucleotides). Since then, the CRISPR system has rushed in all types of genetic manipulations, and genome editing has been achieved in an unprecedented number of species (animals and plants) including rats, rabbits, pigs, zebrafish, and even axolotls [21] or Rhesus monkeys [22]. To the best of our knowledge, there is no report to date of any species resistant to CRISPR editing. Ultimately, research on human cell lines [23], human stem cells [24], or human iPS cells [25] using CRISPR will tremendously speed up direct applications such as regenerative medicine. Nonetheless, such ubiquitous and unrivaled efficiency recently raised alarm over ethical consequences underlying genetic engineering in humans [26].

Genome editing in mice using CRISPR

For more than twenty years, ES-cell injection into blastocysts was the predominant way of editing the mouse genome. The main drawbacks of this method are the variable availability and potency of ES-cell lines, the time to obtain chimeras, and the inefficiency of transmission upon breeding of the chimeras. Despite sensible improvements over the years [27,28] this lengthy process may become obsolete. As previously mentioned, the direct injection of nucleases into the one-cell embryos has repositioned microinjection at the forefront of genome editing in mice.

Speed, precision, efficiency

Although the production of CRISPR edited mice via direct injection of CRISPR components is a very recent technique (the first report was published less than two years from the date of the present review), the plethora of publications [29-36] argue in favor of an incredibly fast pace. In average, from the design of the sgRNAs to the screening of genetically modified mice, the process takes about eight weeks, and most facilities and transgenic cores now produce modified mice within two to four months.

In the early days of CRISPR, potential off-target effects of the CRISPR system (Cas9 tolerates mismatches, especially in the 5' upstream region of the target site) have been documented [16,37]. The system has subsequently been fine tuned for increased specificity. The first modification consisted in engineering a "nickase" form of the Cas9 (referred to as Cas9-D10A). This mutated version of the nuclease cuts only one strand of DNA, and the double nicking approach greatly improved specificity both in human and mouse cells [38,39]. Furthermore, is has been shown that truncated guides could also increase specificity [40]. Finally, an attractive approach consisted in coupling sgRNAs to the *Fok1* endonuclease, which induces DNA cleavage only upon dimerization, thus doubling the length of the recognition sequence [41,42]. It is important to note here that off-target effects in mice are not as critical as in human since they can easily be outcrossed by way of breeding scheme.

The efficacy of the nucleases is generally dependent on chromatin accessibility and epigenetic mechanisms such as DNA methylation or histone modifications [43]. Nonetheless, several reports tend to prove that CRISPR is a much more efficient system than any other programmable endonuclease [44]. Several genes that failed to be edited using other nucleases were successfully targeted using CRISPR, and successful targeting of both alleles is much more efficient using CRISPR [34].

Seamless synthesis

There are many protocols to generate the two necessary components (Cas9 and sgRNA) for genome editing in mice [45]. Some of these protocols detail the complete procedure, from design of the target sequence to the identification of founders [46]. In short, there are few different ways of producing the readily available reagents for microinjection. One such fast method consists in cloning the desired 20bp sequence into a dual expression plasmid (e.g px330, Addgene #42230) expressing both sgRNA and Cas9. The direct injection of this circular plasmid into the pronucleus of fertilized eggs is a fast method to obtain KO mice [47]. However, this method is limited by the time required for cloning (sequencing is necessary for quality control) and the relative inefficiency of the expression vector to edit both alleles.

Traditionally, the injection mixture contains both sgRNA and Cas9 mRNA, rather than DNA. The *in vitro* transcription (IVT) of these two RNAs is then necessary, and they can both be generated using the same expression plasmid (e.g. px330). However, the Cas9 mRNA is slightly more difficult to synthesize because of its size and the polyA capping. An easy way to get fully validated Cas9 mRNA is to buy it from a commercial provider, as microinjection requires very low concentration of Cas9 reagent. Consequently, transgenic cores may obtain few micrograms of Cas9 mRNA, a very cheap investment that can last several months or up to a year.

This leaves the transgenic facilities with only one reagent to produce: the guide RNA. This is classically achieved by cloning the 20bp sequence into an expression vector, and then using this plasmid as template for IVT. Yet, the simplest and fastest way of producing several sgRNAs is the PCR-based "non-cloning" method. This method becomes very popular to create transgenic mice [48], as it takes a technician only one day to synthesize multiple sgRNAs (Figure 1).

The entire procedure can be divided into three steps.

It starts with the identification of guide sequences. This step is computerized and it takes only few minutes to get multiple guides (assessed against off-target likelihood) using one of several freely accessible computational tools (e.g. http://crispr.mit.edu). Some of these tools have been optimized and take genomic context (such as CpG islands) into account [49].

The second step is the synthesis of a linearized DNA template generated by PCR using a High Fidelity enzyme (e.g. Phusion polymerase or equivalent). The Forward primer is of the form 5'TTA

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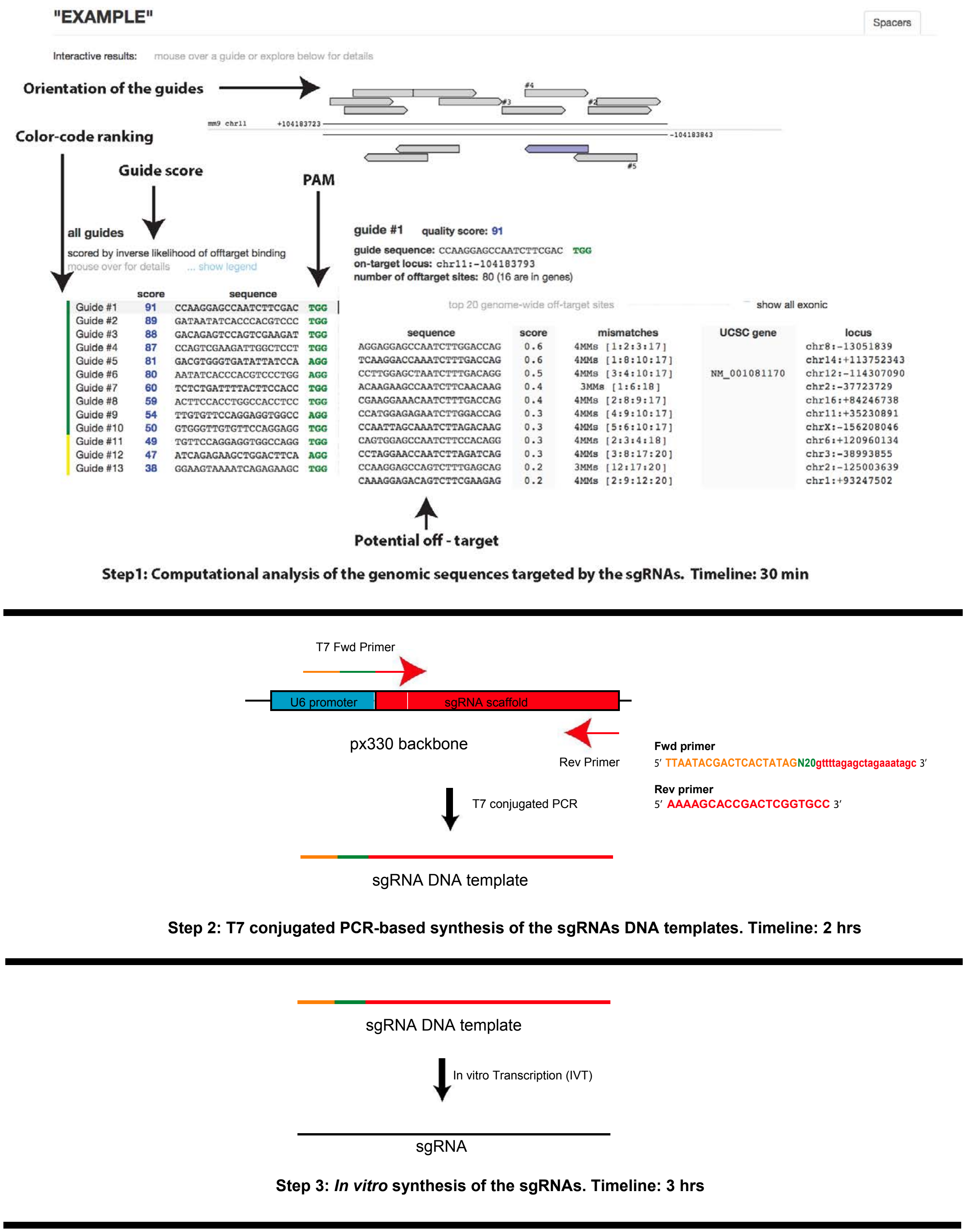

Figure 1: Flowchart of the "non-cloning" strategy to synthesize small guide RNAs for CRISPR genome editing in mice.
Design and selection of suitable guides are performed using freely accessible computer tools (step 1). The use of a Forward primer containing the T7 minimal sequence (orange), the guide sequence selected in step 1 (green), and a sequence complimentary to the sgRNA sca old (red) allows the synthesis of a suitable DNA template without any cloning step (step 2). Finally, *in vitro* transcription (IVT) is performed using a commercially available kit following manufacturer's recommendations (step 3).

ATACGACTCACTATAGN^{20}gttttagagctagaaatagc3'. It contains the T7 promoter minimal sequence (for subsequent IVT), upstream of the 20bp sequence identified in step 1, and a sequence complimentary to the expression vector. This strategy offers the advantage that cloning of the target sequence into the expression vector is no longer required.

For the last step, the mRNA is easily generated using an IVT kit (MEGAshortscript or equivalent). Because the sgRNA doesn't require capping, the process takes only few hours. The final purification is critical and can easily be done using *ad hoc* spin columns (Nucaway or the like).

Should a "donor" piece of DNA be required for homologous recombination, *de novo* synthesis of small oligonucleotides (for insertion of a LoxP site for instance) or larger plasmids containing homology arms is rapidly completed by specialized companies, and costs involved become relatively low.

Unprecedented achievements

In mice, the use of CRISPR generated tremendous excitement in the community, as unprecedented results are regularly published. Beside multiplexing, the creation of conditional KO mice in one step [20], single nucleotide variants (SNV) and point mutations [30], gene tags [20], or large targeted insertions [32] have all been reported in record time. Corrections of disease phenotypes have also been communicated [50,51]. Constitutive and Cre-dependent mouse lines expressing Cas9 into the Rosa26 safe harbor have been created and are readily available through the Jackson laboratory (Stock number 024857 and 024858).

Furthermore, the CRISPR system is broadly applicable and often coupled to other systems to create new hybrid technologies [52]. Therefore, there is no doubt the recent targeting of the RNA (rather than DNA) with the CRISPR system [53], which allows fine control (activation or repression) of the genes [54] will soon be applied to mice.

Outstanding Challenges

Perfecting the system

The most critical criterion that needs thorough evaluation when performing genome editing is the off-target effect. Besides computational analysis, several studies showed that off-target effects of CRISPR could be reduced below the detection limits of deep sequencing using previously described methods [55]. Although new approaches may well be developed in a close future, it is worth noting that only whole genome sequencing could guarantee the absence of off-target effects, although its price remains prohibitive. Few studies using whole genome analysis already showed that the mutation rates induced with endonucleases are not systematically higher than spontaneous (non-induced) natural mutations [56,57]. As previously mentioned, off-target remains ultimately much less critical for mice as for human genome engineering, as it is easily diluted out amongst generations.

The second perfectible criterion is the targeting restriction dictated by the NGG PAM. Other CRISPR systems than the type II system exist, and Cas9 orthologs [58] may have different PAM requirements, such as those of *Streptococcus thermophilus* and *Neisseria meningiditis*. These may represent attractive alternatives [45].

The mechanisms underlying the targeting and cleavage activities of the CRISPR system are not completely understood. Several studies of the mode of action provided valuable information that might help improving the system [59-62].

Finally, the physical form of the CRISPR reagents (DNA, RNA, or protein) used for microinjection in mice might also be critical. Since mouse genome editing is more powerful using the mRNA form than the DNA one, it is possible that direct injection of the Cas9 ribonucleoprotein might also improve the efficiency, as it has been successfully applied to human cell lines [63,64].

Mode of delivery

CRISPR is considered a "disruptive" technology, which is a technological innovation that creates a paradigm shift. In the mouse community, it indeed created several shifts on the way scientists create genetically modified mice.

Interestingly, early reports of factors influencing the outcome of microinjection in mice showed that cytoplasmic transgenesis, although quite inefficient, could still be successful [65].

Because the CRISPR components are made of mRNA, it makes sense to target the cytoplasm when microinjecting the mouse oocytes (even when containing a DNA donor, successful cytoplasmic injections have been reported [20]). The first reports of CRISPR edited mice used a piezo-assisted method for injecting into the cytoplasm of the oocytes [66]. However, non-assisted injection is also possible and the efficiency seems overall higher using cytoplasmic injection over pronuclear injection [67]. There are an increasing number of publications using cytoplasmic injection in mice, where the oocytes can accommodate very high concentration of reagents with no obvious toxicity. Another advantage of cytoplasmic injections is that the fertilized eggs do not need to have apparent pronuclei to be injected, thus allowing injection in a wider range of oocytes, eventually reducing the number of mice superovulated, and offering more flexibility in the timing of hormonal stimulation. In our hands, switching from pronuclear to cytoplasmic did not result in any pregnancy. However, this problem (probably due to the physical characteristics of the micropipettes once pulled) has easily been overcome in our laboratory by a short pre-incubation (five minutes) of the eggs with cytoskeletal inhibitors (e.g. Cytochalasin B - Sigma C6762 – 5 ug/ml), known for increasing the survival rate [68].

Another interesting change in the way investigators perform CRISPR genome editing in mice is the fact that the efficiency of the sgRNA tends not to be pre-assessed anymore. In the first publications, the investigators used to test their guides *in vitro*, using mouse cell lines (e.g. N2a or NIH3T3). There might be several reasons explaining why this step became less popular. First, this procedure is time-consuming and requires a lot of manpower. Secondly, high throughput experiments showed that only a very small proportion of the guides are inactive [69,70]. Additionally, the degree of activity of a given guide *in vitro* does not always correlate completely with the degree of activity *in vivo*. For that reason, the activity of the sgRNA is sometimes assessed directly by *in vivo* assay [71].

Since the mode of delivery of the CRISPR reagents to the oocytes is critical, we anticipate that alternative methods to microinjection [72,73] might ultimately change completely the way genetically modified mice are produced.

Concluding remarks

The flexibility and adaptability of the CRISPR–Cas9 system offer vast potential for genome manipulations. Despite controversy over the discovery of CRISPR genome engineering and patent disputes

[74], transgenic facilities around the world now offer this service. In mice, work is ongoing to elucidate discrete mechanisms inherent to the editing capacities of CRISPR elements. For instance, mosaicism following CRISPR injection has not been explored thoroughly [75].

New strategies for controlling the outcome of DNA cleavage are on their way. For example, when creating KO mice, the repair mechanisms of the oocyte following NHEJ generally create small deletions (indels) of random sizes. Conversely, the coinjection of two guides targeting two close sequences in opposite orientations allows efficient excision of a piece of DNA of a predefined size [76], which is convenient for genotyping of the progeny. Likewise, the efficiency of homologous recombination and the characteristics of the donor DNA for successful gene repair are being assessed [76,77]. Besides, the two main assays used to detect induced genomic modifications (e.g. Surveyor and T7E1 assays) lack sensitivity, and other methods are currently developed [78-81].

Collectively, nuclease-based technologies are revolutionizing contemporary molecular genetics, and are particularly applicable to the mouse genome. We anticipate that each of these systems will be thoroughly studied and enhanced [82], allowing researchers to take advantage of each type of nucleases according to their specificity and mechanisms of action. Other technologies based on nucleases [83,84]or not [85] may also appear as fast as the CRISPR/Cas9 system did.

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Competing interests statement

The authors declare no competing financial interest.

References

1. Watson JD, Crick FH (1953) Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature 171: 737-738.
2. Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci U S A 77: 7380-7384.
3. Capecchi MR (2005) Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat Rev Genet 6: 507-512.
4. Ménoret S, Fontanière S, Jantz D, Tesson L, Thinard R, et al. (2013) Generation of Rag1-knockout immunodeficient rats and mice using engineered meganucleases. FASEB J 27: 703-711.
5. Cui X, Ji D, Fisher DA, Wu Y, Briner DM, et al. (2011) Targeted integration in rat and mouse embryos with zinc-finger nucleases. Nat Biotechnol 29: 64-67.
6. Hermann M, Maeder ML, Rector K, Ruiz J, Becher B, et al. (2012) Evaluation of OPEN zinc finger nucleases for direct gene targeting of the ROSA26 locus in mouse embryos. PLoS One 7: e41796.
7. Sung YH, Baek IJ, Kim DH, Jeon J, Lee J, et al. (2013) Knockout mice created by TALEN-mediated gene targeting. Nat Biotechnol 31: 23-24.
8. Wang H, Hu YC, Markoulaki S, Welstead GG, Cheng AW, et al. (2013) TALEN-mediated editing of the mouse Y chromosome. Nat Biotechnol 31: 530-532.
9. Sanjana NE, Cong L, Zhou Y, Cunniff MM, Feng G, et al. (2012) A transcription activator-like effector toolbox for genome engineering. Nat Protoc 7: 171-192.
10. Carroll D, Morton JJ, Beumer KJ, Segal DJ (2006) Design, construction and in vitro testing of zinc finger nucleases. Nat Protoc 1: 1329-1341.
11. Pennisi E (2013) The CRISPR craze. Science 341: 833-836.
12. Baker M (2014) Gene editing at CRISPR speed. Nat Biotechnol 32: 309-312.
13. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J Bacteriol 169: 5429-5433.
14. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, et al. (2007) CRISPR provides acquired resistance against viruses in prokaryotes. Science 315: 1709-1712.
15. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, et al. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337: 816-821.
16. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, et al. (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 31: 827-832.
17. Sander JD, Joung JK (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 32: 347-355.
18. Moscou MJ, Bogdanove AJ (2009) A simple cipher governs DNA recognition by TAL effectors. Science 326: 1501.
19. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, et al. (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153: 910-918.
20. Yang H, Wang H, Shivalila CS, Cheng AW, Shi L, et al. (2013) One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 154: 1370-1379.
21. Fei JF, Schuez M, Tazaki A, Taniguchi Y, Roensch K, et al. (2014) CRISPR-mediated genomic deletion of Sox2 in the axolotl shows a requirement in spinal cord neural stem cell amplification during tail regeneration. Stem Cell Reports 3: 444-459.
22. Wan H, Feng C, Teng F, Yang S, Hu B, et al. (2015) One-step generation of p53 gene biallelic mutant Cynomolgus monkey via the CRISPR/Cas system. Cell Res 25: 258-261.
23. Hu W, Kaminski R, Yang F, Zhang Y, Cosentino L, et al. (2014) RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. Proc Natl Acad Sci U S A 111: 11461-11466.
24. Matsunaga T, Yamashita JK (2014) Single-step generation of gene knockout-rescue system in pluripotent stem cells by promoter insertion with CRISPR/Cas9. Biochem Biophys Res Commun 444: 158-163.
25. Horii T, Tamura D, Morita S, Kimura M, Hatada I (2013) Generation of an ICF syndrome model by efficient genome editing of human induced pluripotent stem cells using the CRISPR system. Int J Mol Sci 14: 19774-19781.
26. Lanphier E, Urnov F, Haecker SE, Werner M, Smolenski J (2015) Don't edit the human germ line. Nature 519: 410-411.
27. Dechiara TM, Poueymirou WT, Auerbach W, Frendewey D, Yancopoulos GD, et al. (2009) VelociMouse: fully ES cell-derived F0-generation mice obtained from the injection of ES cells into eight-cell-stage embryos. Methods Mol Biol 530: 311-324.
28. Poueymirou WT, Auerbach W, Frendewey D, Hickey JF, Escaravage JM, et al. (2007) F0 generation mice fully derived from gene-targeted embryonic stem cells allowing immediate phenotypic analyses. Nat Biotechnol 25: 91-99.
29. Gennequin B, Otte DM, Zimmer A (2013) CRISPR/Cas-induced double-strand breaks boost the frequency of gene replacements for humanizing the mouse Cnr2 gene. Biochem Biophys Res Commun 441: 815-819.
30. Inui M, Miyado M, Igarashi M, Tamano M, Kubo A, et al. (2014) Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system. Sci Rep 4: 5396.
31. Li D, Qiu Z, Shao Y, Chen Y, Guan Y, et al. (2013) Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. Nat Biotechnol 31: 681-683.
32. Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, et al. (2014) CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell 159: 440-455.
33. Shen B, Zhang J, Wu H, Wang J, Ma K, et al. (2013) Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. Cell Res 23: 720-723.
34. Yasue A, Mitsui SN, Watanabe T, Sakuma T, Oyadomari S, et al. (2014) Highly efficient targeted mutagenesis in one-cell mouse embryos mediated by the TALEN and CRISPR/Cas systems. Sci Rep 4: 5705.
35. Yin H, Xue W, Chen S, Bogorad RL, Benedetti E, et al. (2014) Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. Nat Biotechnol 32: 551-553.
36. Zhou J, Shen B, Zhang W, Wang J, Yang J, 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.

37. Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, et al. (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol 31: 822-826.
38. Fujii W, Onuma A, Sugiura K, Naito K (2014) Efficient generation of genome-modified mice via offset-nicking by CRISPR/Cas system. Biochem Biophys Res Commun 445: 791-794.
39. Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, et al. (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154: 1380-1389.
40. 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.
41. 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.
42. Tsai SQ, Wyvekens N, Khayter C, Foden JA, Thapar V, et al. (2014) Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. Nat Biotechnol 32: 569-576.
43. Daboussi F, Zaslavskiy M, Poirot L, Loperfido M, Gouble A, et al. (2012) Chromosomal context and epigenetic mechanisms control the efficacy of genome editing by rare-cutting designer endonucleases. Nucleic Acids Res 40: 6367-6379.
44. Mali P, Yang L, Esvelt KM, Aach J, Guell M, et al. (2013) RNA-guided human genome engineering via Cas9. Science 339: 823-826.
45. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, et al. (2013) Genome engineering using the CRISPR-Cas9 system. Nat Protoc 8: 2281-2308.
46. Harms DW, Quadros RM, Seruggia D, Ohtsuka M, Takahashi G, et al. (2014) Mouse Genome Editing Using the CRISPR/Cas System. Curr Protoc Hum Genet 83: 15 17 11-15 17 27.
47. Mashiko D, Fujihara Y, Satouh Y, Miyata H, Isotani A, et al. (2013) Generation of mutant mice by pronuclear injection of circular plasmid expressing Cas9 and single guided RNA. Sci Rep 3: 3355.
48. Dannappel M, Vlantis K, Kumari S, Polykratis A, Kim C, et al. (2014) RIPK1 maintains epithelial homeostasis by inhibiting apoptosis and necroptosis. Nature 513: 90-94.
49. Heigwer F, Kerr G, Boutros M (2014) E-CRISP: fast CRISPR target site identification. Nat Methods 11: 122-123.
50. Wu Y, Liang D, Wang Y, Bai M, Tang W, et al. (2013) Correction of a genetic disease in mouse via use of CRISPR-Cas9. Cell Stem Cell 13: 659-662.
51. Xue W, Chen S, Yin H, Tammela T, Papagiannakopoulos T, et al. (2014) CRISPR-mediated direct mutation of cancer genes in the mouse liver. Nature 514: 380-384.
52. Polstein LR, Gersbach CA (2015) A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. Nat Chem Biol 11: 198-200.
53. O'Connell MR, Oakes BL, Sternberg SH, East-Seletsky A, Kaplan M, et al. (2014) Programmable RNA recognition and cleavage by CRISPR/Cas9. Nature 516: 263-266.
54. Wu J, Wang Z, Shi Z, Zhang S, Ming R, et al. (2013) The genome of the pear (Pyrus bretschneideri Rehd.). Genome Res 23: 396-408.
55. Cho SW, Kim S, Kim Y, Kweon J, Kim HS, et al. (2014) Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. Genome Res 24: 132-141.
56. Smith C, Gore A, Yan W, Abalde-Atristain L, Li Z, et al. (2014) Whole-genome sequencing analysis reveals high specificity of CRISPR/Cas9 and TALEN-based genome editing in human iPSCs. Cell Stem Cell 15: 12-13.
57. Yang L, Grishin D, Wang G, Aach J, Zhang CZ, et al. (2014) Targeted and genome-wide sequencing reveal single nucleotide variations impacting specificity of Cas9 in human stem cells. Nat Commun 5: 5507.
58. Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, et al. (2011) Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol 9: 467-477.
59. Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, et al. (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science 343: 1247997.
60. Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, et al. (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell 156: 935-949.
61. Szczelkun MD, Tikhomirova MS, Sinkunas T, Gasiunas G, Karvelis T, et al. (2014) Direct observation of R-loop formation by single RNA-guided Cas9 and Cascade effector complexes. Proc Natl Acad Sci U S A 111: 9798-9803.
62. Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA (2014) DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. Nature 507: 62-67.
63. Kim S, Kim D, Cho SW, Kim J, Kim JS (2014) Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res 24: 1012-1019.
64. Ramakrishna S, Kwaku Dad AB, Beloor J, Gopalappa R, Lee SK, et al. (2014) Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. Genome Res 24: 1020-1027.
65. Brinster RL, Chen HY, Trumbauer ME, Yagle MK, Palmiter RD (1985) Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc Natl Acad Sci U S A 82: 4438-4442.
66. Yang H, Wang H, Jaenisch R (2014) Generating genetically modified mice using CRISPR/Cas-mediated genome engineering. Nat Protoc 9: 1956-1968.
67. Horii T, Arai Y, Yamazaki M, Morita S, Kimura M, et al. (2014) Validation of microinjection methods for generating knockout mice by CRISPR/Cas-mediated genome engineering. Sci Rep 4: 4513.
68. Brinster RL, Chen HY, Trumbauer ME, Avarbock MR (1980) Translation of globin messenger RNA by the mouse ovum. Nature 283: 499-501.
69. Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, et al. (2014) Genome-scale CRISPR-Cas9 knockout screening in human cells. Science 343: 84-87.
70. Zhou Y, Zhu S, Cai C, Yuan P, Li C, et al. (2014) High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. Nature 509: 487-491.
71. Sakurai T, Watanabe S, Kamiyoshi A, Sato M, Shindo T (2014) A single blastocyst assay optimized for detecting CRISPR/Cas9 system-induced indel mutations in mice. BMC Biotechnol 14: 69.
72. Aten QT, Jensen BD, Tamowski S, Wilson AM, Howell LL, et al. (2012) Nanoinjection: pronuclear DNA delivery using a charged lance. Transgenic Res 21: 1279-1290.
73. Lo CW (1983) Transformation by iontophoretic microinjection of DNA: multiple integrations without tandem insertions. Mol Cell Biol 3: 1803-1814.
74. Sheridan C (2014) First CRISPR-Cas patent opens race to stake out intellectual property. Nat Biotechnol 32: 599-601.
75. Yen ST, Zhang M, Deng JM, Usman SJ, Smith CN, et al. (2014) Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. Dev Biol 393: 3-9.
76. Parikh BA, Beckman DL, Patel SJ, White JM, Yokoyama WM (2015) Detailed phenotypic and molecular analyses of genetically modified mice generated by CRISPR-Cas9-mediated editing. PLoS One 10: e0116484.
77. Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR, et al. (2015) Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. Nat Biotechnol .
78. Certo MT, Ryu BY, Annis JE, Garibov M, Jarjour J, et al. (2011) Tracking genome engineering outcome at individual DNA breakpoints. Nat Methods 8: 671-676.
79. Nakagawa Y, Yamamoto T, Suzuki K, Araki K, Takeda N, et al. (2014) Screening methods to identify TALEN-mediated knockout mice. Exp Anim 63: 79-84.
80. Ota S, Hisano Y, Muraki M, Hoshijima K, Dahlem TJ, et al. (2013) Efficient identification of TALEN-mediated genome modifications using heteroduplex mobility assays. Genes Cells 18: 450-458.
81. Zhu X, Xu Y, Yu S, Lu L, Ding M, et al. (2014) An efficient genotyping method for genome-modified animals and human cells generated with CRISPR/Cas9 system. Sci Rep 4: 6420.
82. Boissel S, Jarjour J, Astrakhan A, Adey A, Gouble A, et al. (2014) megaTALs: a rare-cleaving nuclease architecture for therapeutic genome engineering. Nucleic Acids Res 42: 2591-2601.

83. Juillerat A, Bertonati C, Dubois G, Guyot V, Thomas S, et al. (2014) BurrH: a new modular DNA binding protein for genome engineering. Sci Rep 4: 3831.

84. Stella S, Molina R, López-Méndez B, Juillerat A, Bertonati C, et al. (2014) BuD, a helix-loop-helix DNA-binding domain for genome modification. Acta Crystallogr D Biol Crystallogr 70: 2042-2052.

85. Barzel A, Paulk NK, Shi Y, Huang Y, Chu K, et al. (2015) Promoterless gene targeting without nucleases ameliorates haemophilia B in mice. Nature 517: 360-364.