Journal of Human Genetics (2018) 63:107–113
https://doi.org/10.1038/s10038-017-0360-4

REVIEW ARTICLE

Genome editing for the reproduction and remedy of human diseases in mice

Satoshi Hara[1] · Shuji Takada[1]

Received: 25 June 2017 / Revised: 7 August 2017 / Accepted: 7 August 2017 / Published online: 27 November 2017

Abstract
With the recent progress in genome-editing technologies, such as the CRISPR/Cas9 system, genetically modified animals carrying nucleotide substitutions or large chromosomal rearrangements can be produced rapidly and at low cost. Such genome-editing techniques have been applied in the generation of animal models, especially mice, for reproducing human disease mutations, such as single-nucleotide polymorphisms (SNPs) or large chromosomal rearrangements identified by genome-wide screening analyses. While application methods are under development for various complex mutations involving genome editing for mimicking human disease-causing mutations in mice, functional studies of mouse models carrying replicated human mutations are gradually being published. In this review, we discuss the recent progress in application methods of the CRISPR/Cas9 system, focusing on the production of mouse models of diseases.

Introduction

Recent genome-wide screening by next-generation sequencing (NGS) techniques, such as exome sequencing, facilitates analysis of human disease-causing mutations at single-nucleotide resolution. Because more than one candidate disease-causing mutation is often indicated by in silico analysis of genome-wide screening, it is necessary to validate functions of the candidate mutations in vitro and/or in vivo by reproducing such mutations in cultured cells or introducing corresponding candidate mutations in animals. Of mammalian animal models, mice have been the first choice for reproducing human disease-causing mutations since the murine genome can be artificially manipulated by conventional gene targeting technology using embryonic stem (ES) cells [1]. However, there are some issues with the technology used for screening candidates to identify causative mutations. It is costly and time-consuming to produce mice carrying a mutation, inserts unnecessary exogenous sequences such as selection marker cassettes or loxP signals at a manipulated locus, and cannot be applied to repeat-rich regions. These issues have been resolved with the development of genome editing technology. In addition, this enables precise genome modification at the single-nucleotide level.

So far, several genome-editing tools have been developed such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and RNA-guided clustered regularly interspaced short palindrome repeat-associated Cas9 nuclease (CRISPR/Cas9) systems [2–5]. ZFNs and TALENs consist of a pair of DNA-binding domains fused with a FokI DNA nuclease domain. In contrast, the CRISPR/Cas9 system consists of a single guide RNA (sgRNA) for recognition of a target genomic locus and Cas9 for recognition of the sgRNA and digestion of DNA. sgRNA is a fusion of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) derived from *Streptococcus pyogenes* [6]. DNA nucleases of genome-editing tools introduce double-strand breaks (DSBs) into specific target genomic sequences that induce non-homologous end-joining (NHEJ)-mediated insertions and deletions (indels) or homology-directed repair (HDR)-mediated knock-in with an oligonucleotide or targeting vector containing exogenous DNA sequence(s) and homologous arms. Of the three genome-editing tools, the CRISPR/Cas9 system is the easiest to establish and has the highest efficiency of genome editing, and its use is now more widespread than that of the others.

In 2013, the first examples of CRISPR/Cas9-mediated genome editing of mammalian genomes were reported by

✉ Shuji Takada
takada-s@ncchd.go.jp

[1] Department of Systems BioMedicine, National Research Institute for Child Health and Development, Tokyo 158-8535, Japan

Table1 Production of mice carrying nucleotide substitutions using the CRIPSR/Cas9 system

Gene	Nucleotide substitution	Missense mutation	HDR frequency (mutated/total genotyped (%))	Microinjection	Reference
Tet1	GC>AT	N/A	6/9 (67%)	a sgRNA/Cas9^{WT} mRNA	Wang et al. [8]
Tet2	TA>AT	N/A	9/15 (60%)		
Sf1	C>T	p.R92W	26/181[a] (14%)	a sgRNA/Cas9^{WT} mRNA	Inui et al. [9]
Sox9	A>G[b]	p.K396R	5/24 (21%)	two sgRNAs/Cas9^{D10A} mRNA	
			9/26 (35%)	a sgRNA/Cas9^{WT} mRNA	
Cdk2	A>C[b]	p.Y15S	10/27 (37%)	a sgRNA/Cas9^{WT} mRNA	Singh et al. [18]
Mlh1	TC>AT	p.V384D	7/24 (29%)		
Smc1b	T>G	p.F1055L	3/14 (21%)		
Tex15	CG>TA	p.T218I	9/16 (56%)		
Sin1	GA>CG[b]	p.R84T	1/11 (9%)	a sgRNA/Cas9^{WT} mRNA	Wang et al. [31]
Sirt3	Several nucleotides[b]	N/A	2/21 (10%)	a sgRNA/Cas9^{WT} mRNA	
			2/9 (22%)	a sgRNA/Cas9^{WT} protein	
Lox	T>G	p.M292R	Not shown	a sgRNA/Cas9^{WT} mRNA	Lee et al. [19]
Prakg2	A>G	p.H527R	Not shown	a sgRNA/Cas9^{WT} mRNA	Xie et al. [28]

N/A not applicable

[a]Average of several experiments

[b]With silent mutations in ssODN to avoid re-cutting the target locus

Mali et al. [5] and Cong et al. [4]. Using mammalian cell lines, they demonstrated that indels and knock-ins can be introduced by co-transfection of sgRNA and Cas9 expression vectors with higher efficiency than that of TALENs. The first paper describing CRISPR/Cas9-mediated in vivo genome editing in mouse zygotes was also published in the same year [7, 8]. Shen et al. [7] showed that the *EGFP* gene in *Pou5f1*-IRES-*EGFP* knock-in mice can be mutated by microinjection of in vitro transcribed sgRNA and Cas9 mRNA into fertilized eggs. Using this strategy, Wang et al. [8] demonstrated that NHEJ-mediated indel mutations can be introduced at multiple endogenous genomic loci in vivo. Moreover, they have shown that mice with artificially defined nucleotide substitutions can be efficiently produced by microinjection of a single-stranded oligodeoxynucleotide (ssODN) with sgRNA/Cas9 via the HDR-mediated pathway. These studies clearly show that genetically modified mice, not only carrying simple indels but also nucleotide substitutions, can be obtained within 1 month.

At present, more than 3000 papers on the CRISPR/Cas9 system have been published, including not only methodologies but also functional studies using genome-edited cells or animals. Recently, there has been a gradual increase in the number of functional studies that analyzed phenotypes of mice carrying mutations corresponding to candidate human disease-causing mutations identified by exome sequencing. In this review, we discuss the recent progress of the CRISPR/Cas9 system, focusing on the production of mouse models of diseases.

HDR-mediated production of nucleotide substitution in mice

The ssODN-mediated nucleotide substitution in genomes is one of the essential methods for understanding the function of specific amino acids in proteins or for reproducing human mutations in mice for disease modeling. Wang et al. [8] reported the efficient production of mice with nucleotide substitutions, in which two nucleotides were converted at *Tet1* (GC>AT substitution) and *Tet2* (TA>AT substitution) loci by microinjection of ssODN, sgRNAs, and Cas9 mRNA in fertilized eggs. Inui et al. [9] also showed induction of point mutations in the *Sf1* (C>T substitution) gene by this strategy. Reports showing the production of animals with nucleotide substitutions are summarized in Table 1. These reports showed that the efficiencies of creation of substituted alleles using the above strategies are not always high, ranging from 3 to 67% (calculated from the ratio of the number of pups harboring mutations to that of total pups). These low efficiencies could be due to the low frequency of HDR-mediated processes.

Improvements in HDR-mediated substitution were reported in a knock-in experiment involving EGFP in a genomic locus of interest. Aida et al. [10] have demonstrated that the efficiency was improved by microinjection of recombinant Cas9 protein and crRNA/ tracrRNA, instead of Cas9 mRNA and sgRNA targeting *Actb*, with a targeting vector that contained an EGFP cassette with homologous arms at the *Actb* locus, as a donor for HDR-mediated editing, in fertilized eggs. They

reported that the microinjection of RNA–protein complexes into pronuclei enables delivery of the complex at earlier stages than that of Cas9 mRNA/sgRNA in the cytoplasm of zygotes. This would be one of the reasons for improvement in the knock-in efficiency.

Other approaches for enhancement of knock-in efficiencies have been reported based on the inhibition of NHEJ using cell lines: Chu et al. [11] demonstrated that inhibition of the NHEJ pathway improved the efficiencies of HDR-mediated knock-in by knock-down of KU70/80 or DNA ligase IV, which are key factors for NHEJ, or by overexpression of E1B55K/E4orf6, which is derived from Adenovirus and known as a suppressor of the Mre11/Rad50/NBS1 complex involved in the NHEJ pathway [12, 13]. In mice, Maruyama et al. [14] have shown that the frequency of HDR is improved by microinjection of a DNA ligase IV inhibitor, SCR7, into mouse zygotes with sgRNA/Cas9/ssODN [15]. Another way to improve the efficiency is enhancement of the HDR pathway. Song et al. [16] showed that overexpression of RAD51 in zygotes, an essential player in the HDR pathway, or treatment with a RAD51 enhancer, RS-1, also improved HDR-mediated in vivo knock-in efficiencies in rabbits [17].

The progress achieved with these methodologies for HDR-mediated nucleotide substitution enables us to generate mice harboring mutations at the single-nucleotide level without inserting any unnecessary sequence. In addition, the required time and cost to obtain model mice reproducing candidates of human disease-causing mutations are less, compared to that for conventional gene targeting technology. These facts enable us to plan functional screening for identification of the responsible gene from a list of candidates by producing mouse models.

Reproduction of diseases in mice carrying nucleotide substitutions

In this section, we introduce several examples of functional studies of murine models carrying mutations recapitulating human disease mutations generated by HDR-mediated genome editing using the above described methods.

The first example shows that generation of mice harboring mutations using genome editing is useful as a screening tool for functional analysis of single nucleotide changes. Singh et al. [18] tried to identify infertility-causing single-nucleotide polymorphisms (SNPs) involving meiosis-associated genes in humans through generation of mice with single-nucleotide substitutions corresponding to the human SNPs by microinjection of sgRNA, Cas9 mRNA, and ssODN into fertilized eggs. The candidate human SNPs, rs3087335 (*CDK2*), rs63750447 (*MLH1*), rs61735519 (*SMC1B*), and rs147871035 (*TEX15*), were selected based on the criteria of minor alleles in humans, altering evolutionarily conserved amino acids and changes being predicted as functionally deleterious by multiple algorithms. Phenotype analysis of the resulting mutation-bearing mice ($Cdk2^{p.Y15S}$, $MLH1^{p.V384D}$, $Smc1b^{p.F1055L}$, and $Tex15^{p.T218I}$) showed that $Cdk2^{p.Y15S/p.Y15S}$ mice exhibited severe spermatogenetic defects due to meiotic arrest at the pachytene stage, while spermatogenesis in homozygous mutants for the other SNPs was indistinguishable from wild type. This paper shows that generation of mice harboring mutations by the CRISPR/Cas9 system can be a screening tool.

The second example involves mice carrying CRISPR/Cas9-mediated single-nucleotide substitution used for validation of a disease-causing mutation for a candidate identified from human exome sequencing. Lee et al. [19] carried out exome-sequencing analysis of patients with familial thoracic aortic aneurysms and dissections (FTAAD) to identify a causal mutation of the disease. They finally identified a missense mutation at the *LOX* gene (p.M298R) as a strong candidate for FTAAD. To reveal the functional relevance of the mutation to the disease, they generated mice harboring a mutation (c.857 T > G encoding p.M292R) at the homologous position to the human mutation by microinjection of sgRNA, Cas9 mRNA and ssODN into fertilized eggs. $Lox^{p.M292R/+}$ animals had disorganized ultrastructural properties of the aortic wall and $Lox^{p.M292R/p.M292R}$ died from ascending aortic aneurysm and spontaneous hemorrhage shortly after parturition. They concluded that a missense mutation in *LOX* is associated with aortic disease in humans. It seems quite likely that the amount of literature dealing with the combination of NGS analysis, including exome sequencing and screening or validation of results by generation of CRISPR/Cas9-mediated single nucleotide substituted mice, is increasing enormously.

It should be noted that modeling of human mutations in mice has limitations in that phenotypes of a mutation, which corresponds to the same amino-acid position in orthologous human and mouse genes, are not always similar. Such a case was reported by Miyado et al. [20]. We found two unrelated patients with 46,XX testicular/ovotesticular disorders of sex development (DSD) and a p.R92W variant involving *NR5A1/SF1* [21]. We next generated mice carrying a homologous mutation of human p.R92W ($Nr5a1^{p.R92W}$) by microinjection of sgRNA, Cas9 mRNA, and ssODN into fertilized murine eggs to reproduce the patients' phenotype in the mouse; however $Nr5a1^{p.R92W/p.R92W}$ showed no signs of DSD.

Remedy of diseases in mice harboring mutations by nucleotide substitutions

As mentioned above, the CRISPR/Cas9 system can be used to substitute a wild-type nucleotide with a mutant one. This

also suggests that it is applicable to replace a mutation with a wild-type sequence.

Such a case was reported for remedy of a *mdx* (X chromosome-linked muscular dystrophy) mutant-carrying mouse strain, which is a model of human Duchene muscle dystrophy (DMD) [22]. The responsible *mdx* mutation is a C>T missense substitution involving exon 23 of the dystrophin (*Dmd*) gene [23]. Long et al. [24] carried out microinjection of sgRNA, Cas9 mRNA, and ssODN, which was designed to correct the mutated nucleotide of the *mdx* allele (T) to the wild-type (C), in zygotes collected from *mdx* mice. The resulting "mutation-corrected" mice showed no signs of muscular dystrophy. Interestingly, a mosaic individual, in which the contribution of the "corrected" allele was only 17%, was also obtained and muscular dystrophy manifestations were only partial [24]. This report implies that the CRISPR/Cas9 system could have potential for radical treatment of genetic diseases.

It could be possible to remedy a genetic disease that develops after birth by in vivo genome editing of somatic cells if a mutation in a causative gene is substituted with wild-type sequence in cells that express the gene. Such a case was reported in a mouse model for the remedy for human hereditary tyrosinemia type I (HTI), which is a fatal recessive genetic disease caused by mutation of an essential gene in the tyrosine catabolic pathway, fumarylacetoacetate hydrolase (*FAH*) [25]. The mouse model for human HTI, called *Fah5981SB* mouse strain, carries a G>A point mutation at the boundary of exon 8 and intron 8 of *Fah* locus [26], which leads to aberrant splicing of mRNA. *Fah* deficiency causes accumulation of toxic metabolites in hepatocytes and leads to liver damage and body weight loss. It is reasonable that this phenotype might be rescued by correction of the mutation in the liver. Yin et al. [27] introduced a plasmid coding sgRNA and Cas9 together with ssODN carrying wild-type sequence around the mutated "A" as a donor via hydrodynamic tail vein injection, which is a method of in vivo transfection of naked nucleic acids, primarily in hepatocytes of living adult mice through rapid injection of nucleic acids into the tail vein. The results showed that a small population of hepatocytes, but not all hepatic cells, in the mice thus treated carried the corrected allele, and liver damage and body weight loss were substantially rescued. This research demonstrates that the CRISPR/Cas9 system could have potential for in vivo treatment of recessive genetic diseases in individuals after birth.

Additionally, recently, CRISPR/Cas9-based in vivo treatment of dominant genetic diseases has been reported. Xie et al. [28] identified a c.1589 A>G missense mutation (H530R) of the *PRKAG2* gene encoding the γ^2 subunit of AMP-activated protein kinase in a familial case of Wolff–Parkinson–White syndrome, which is a dominant genetic disease and which usually exhibits cardiomyopathy characterized by fatal ventricular tachyarrhythmia and progressive heart failure. The association of the mutation and the phenotype was examined by generation of mice with mutations through microinjection of sgRNA, Cas9 mRNA, and ssODN carrying mouse sequence around the corresponding human mutation with A>G missense mutation (H527R) into fertilized eggs. The resulting mutant (*Prkag2* $^{+/p.H527R}$), which recapitulated human symptoms, indicated that the mutation is responsible, and is useful as a model for the disease. To treat the mutants, they intraventricularly injected *Prkag2*$^{+/p.H527R}$ mice with adeno-associated virus-9 (AAV9) carrying expression cassettes for Cas9 and sgRNA, which recognized only the mutant allele. Expression levels of *Prkag2* were reduced in the injected mutant hearts, suggesting that the mutant allele was cleaved by Cas9/sgRNA and transcribed mutant *Prkag2* mRNA was degraded by nonsense-mediated mRNA decay. The morphology and function of the heart were substantially restored after the effects of the gene product derived from mutant alleles were reduced.

The great potential of the CRISPR/Cas9 system for the remedy of recessive and dominant genetic diseases is evident, but efficient delivery of Cas9/sgRNA to causative cells remains challenging for most tissue types. One such tissue is the skin. Wu et al. [29] constructed a method of delivery and treated model mice for postnatal recessive dystrophic epidermolysis bullosa (RDEB), which causes skin fragility and blistering. They generated a model by microinjection of Cas9/sgRNA and a 1.5 kb DNA fragment carrying a part of the *Col7a1* gene containing c.6485 G>A involving exon 80, a homologous mutation of which is a causative mutation found in human RDEB patients, and homozygous mice with mutations exhibited typical pathologic features. As a therapy model, they intradermally injected Cas9/sgRNA ribonucleoproteins targeting exon 80 of the *Col7a1* locus into postnatal homozygous mutants. Genome editing of the target locus caused skipping of exon 80, resulting in a slightly shortened form of collagen VII protein, which retained its normal function, and the skin blistering phenotype of treated mutants was significantly diminished. It may be that development of various delivery systems for Cas9/gRNA will expand the CRISPR/Cas9 system-based therapy for genetic diseases.

Reproduction of diseases in mice carrying chromosome rearrangements

Reproducing human chromosomal rearrangements such as deletions, inversions, and duplications in mice is also an important approach to examine relationships between mutations and disease symptoms, and to establish animal

disease models. To date, CRISPR/Cas9 system-based methods for producing mice with chromosomal rearrangements have been developed. In principle, chromosomal rearrangements can be induced by introduction of Cas9 and a pair of sgRNAs targeting chromosomal breakpoints. Using this strategy, deletions can be generated using sgRNAs directed towards sequences flanking the deletion target fragment, with lesions flanking the deletion target being connected intra- or inter-chromosomally. Where the same reaction occurs inter-chromosomally, duplications can be generated. Additionally, inversions can be introduced when lesions generated with different sgRNAs are connected between the inside and outside of a target fragment. Translocations can be generated when lesions induced on different chromosomes are connected; however, the efficiency of this reaction is empirically low in mouse zygotes.

Successful generations of chromosomally rearranged mice have been reported using the above strategy. Wang et al. [30] reported a 95 kb deletion in mice. Recently, we demonstrated generation of mice carrying deletions of 0.5, 2, and 5 Mb and an inversion of 5 Mb [31, 32]. To induce the 2 and the 5 Mb deletions, we microinjected Cas9/two sgRNAs and a donor plasmid carrying the breakpoint sequences so that the breakpoint sequence could be artificially defined. Over the course of this experiment, we also obtained mice carrying a 5 Mb inversion but no duplications were obtained [32]. Boroviak et al. [33] also reported that they microinjected Cas9/two sgRNAs and ssODN as a donor to guide deletion in fertilized eggs and detected 1.1 Mb deletions and inversions in 30 and 21% of embryos genotyped, respectively, although no duplications were observed. When they tried to generate 155 and 545 kb rearrangements using the same strategy, only 1–2% of mice analyzed had the duplication, while 18–24 and 18–30% of mice contained deletions and inversions, respectively. It seems likely that the frequency of generation of duplications of the target locus using this strategy is much lower than that of deletions and inversions. However, recently, Birling et al. [34] reported efficient generation of large duplications in mice and rats by microinjection of Cas9 and two pairs of sgRNAs: one pair targeted upstream and the other pair downstream of the region of interest and where the distance between sgRNAs of a pair was less than 150 bp.

Generation of large duplications is useful for modeling human mutations and for understanding functions of largely "scattered" regulatory regions; however, while it is not impossible, it is not very efficient. Another method to generate large duplications may be introduction of Cas9/a pair of sgRNAs into ES cells instead of fertilized eggs, since the number of clones to be screened can be easily increased when ES cells are used [35]. A disadvantage of this approach is that it takes a longer time for the generation of mice with mutations from ES cells compared to that of zygote injection. Another method could be insertion of bacterial artificial chromosomes (BACs) containing a fragment of the duplication of interest at the target genomic locus by a method named the "two-hit two-oligo with plasmid" (2H2OP), in which Cas9, two sgRNAs, BAC DNA and two ssODNs are microinjected into fertilized eggs [36]. One of the pairs of sgRNAs recognizes a target in the genome and in the BAC DNA. One of two ssODNs contains terminal sequences generated by the Cas9/sgRNA of one end of the genomic target and one end of the BAC, and the other contains those of the other ends of the genome. The BAC is integrated in the genome at the target locus. With this method, the authors confirmed that 18% of offspring contained BAC DNA at the locus of interest. The method was originally developed to insert human BAC sequences into rat genomic loci of interest; however, it should also be applicable for the generation of duplications, at least in principle. So far, this is the most efficient means to insert large fragments into a target locus, but there are some limitations: the size to be inserted can be up to several hundred kb, available only when BAC clones containing entire target regions can be obtained and the backbone BAC vector can be introduced along with the insert. Further studies are needed to develop efficient methods for generation of disease model mice with large duplications.

Closing remarks

In this review, we discussed the current status of methods for creation of murine disease models and some examples of phenotypic analyses and remedies of genetic defects in such mice using the CRISPR/Cas9 system.

In the field of medical genomics, NGS is a powerful tool for identification of disease-causing mutations, especially small indels, and minor nucleotide substitutions and deletions. These mutations can be easily mimicked in living mouse genomes by the CRISPR/Cas9 system; therefore, the combination of NGS and the CRISPR/Cas9 system has been inextricably linked for recent medical genetic studies. Further advances in NGS and genome editing should strongly promote medical genomics.

As for other mutations, such as inversions and duplications, NGS is not very suitable, if not impossible. Instead, array comparative genomic hybridization (aCGH) is useful for detection of duplications, although resolution is not at the single nucleotide level. Identification of inversions and duplications at high resolution from patients' genomic DNA is not facile. Currently, requirements for modeling inversions and duplications do not seem very high compared to that of indels and local nucleotide substitutions. However, this requirement will be increased when such mutations can be easily identified at high resolution in the near future. It will be

necessary to construct efficient methods to reproduce inversions and duplications of various sizes in mice using genome editing technology as soon as a method for identification of inversions or duplications at high resolution is made available.

Before the emergence of genome editing technology, reproduction of human mutations in mice was carried out by ES cell-based technology, which required excessive cost, time and labor. This limited the number of mutant strains that could be generated. Genome editing requires much fewer of these, so it can be used even for screening purposes, whereas generation of mutant lines (up to dozens) is practical for screening. NGS analysis of patient sequences often resulted in listing of a large number of candidate mutations (typically more than one hundred). Further acceleration of the generation of mice harboring mutations by genome editing would be required.

Lastly, as we introduced in this review, it should be noted that reproduction of candidates of disease-causing mutations in mice is becoming more important to establish, since this enables validation of the functional importance of the candidates in vivo. However, it should be kept in mind that, when one analyzes model animals that carry the corresponding mutations as human disease-causing ones, the phenotype in animal models and human symptoms are sometimes different.

Acknowledgements This work was supported by JSPS KAKENHI Grant Number 17K07429 (to S.T.). We thank Editage (www.editage.jp) for English-language editing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

1. Capecchi MR. Altering the genome by homologous recombination. Science. 1989;244:1288–92.
2. Kim YG, Cha J, Chandrasegaran S. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proc Natl Acad Sci USA. 1996;93:1156–60.
3. Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, et al. Targeting DNA double-strand breaks with TAL effector nucleases. Genetics. 2010;186:757–61.
4. Cong L, Ran FA, Cox D, Lin S, Barretto R. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339:819–23.
5. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, et al. RNA-guided human genome engineering via Cas9. Science. 2013;339:823–6.
6. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337:816–21.
7. Shen B, Zhang J, Wu H, Wang J, Ma K, Li Z, et al. Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. Cell Res. 2013;23:720–3.
8. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell. 2013;153:910–8.
9. Inui M, Miyado M, Igarashi M, Tamano M, Kubo A, Yamashita S, et al. Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system. Sci Rep. 2014;4:823.
10. Aida T, Chiyo K, Usami T, Ishikubo H, Imahashi R, Wada Y, et al. Cloning-free CRISPR/Cas system facilitates functional cassette knock-in in mice. Genome Biol. 2015;16:507.
11. Chu VT, Weber T, Wefers B, Wurst W, Sander S. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. Nature. 2015;33:543–8.
12. Stracker TH, Carson CT, Weitzman MD. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature. 2002;418:348–52.
13. Xie A, Kwok A, Scully R. Role of mammalian Mre11 in classical and alternative nonhomologous end joining. Nat Struct Mol Biol. 2009;16:814–8.
14. Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR, Ploegh HL. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. Nat Biotechnol. 2015;33:538–42.
15. Srivastava M, Nambiar M, Sharma S, Karki SS, Goldsmith G, Hegde M, et al. An inhibitor of nonhomologous end-joining abrogates double-strand break repair and impedes cancer progression. Cell. 2012;151:1474–87.
16. Song J, Yang D, Xu J, Zhu T, Chen YE, Zhang J. RS-1 enhances CRISPR/Cas9- and TALEN-mediated knock-in efficiency. Nat Commun. 2016;7:10548.
17. Jayathilaka K, Sheridan SD, Bold TD, Bochenska K, Logan HL, Weichselbaum RR, et al. A chemical compound that stimulates the human homologous recombination protein RAD51. Proc Natl Acad Sci USA. 2008;105:15848–53.
18. Singh P, Schimenti JC. The genetics of human infertility by functional interrogation of SNPs in mice. Proc Natl Acad Sci USA. 2015;112:10431–6.
19. Lee VS, Halabi CM, Hoffman EP, Carmichael N, Leshchiner I, Lian CG, et al. Loss of function mutation in LOX causes thoracic aortic aneurysm and dissection in humans. Proc Natl Acad Sci USA. 2016;113:8759–64.
20. Miyado M, Inui M, Igarashi M, Katoh-Fukui Y, Takasawa K, Hakoda A, et al. The p.R92W variant of NR5A1/Nr5a1 induces testicular development of 46,XX gonads in humans, but not in mice: phenotypic comparison of human patients and mutation-induced mice. Biol Sex Differ. 2016;7:56.
21. Igarashi M, Takasawa K, Hakoda A, Kanno J, Takada S, Miyado M, et al. Identical NR5A1 missense mutations in two unrelated 46, XX individuals with testicular tissues. Hum Mutat. 2016;38:39–42.
22. Bulfield G, Siller WG, Wight PA, Moore KJ. X chromosome-linked muscular dystrophy (mdx) in the mouse. Proc Natl Acad Sci USA. 1984;81:1189–92.
23. Sicinski P, Geng Y, Ryder-Cook A, Barnard E, Darlison M, Barnard P. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science. 1989;244:1578–80.
24. Long C, McAnally JR, Shelton JM, Mireault AA, Bassel-Duby R, Olson EN. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. Science. 2014;345:1184–8.
25. Grompe M, al-Dhalimy M, Finegold M, Ou CN, Burlingame T, Kennaway NG, Soriano P. Loss of fumarylacetoacetate hydrolase is responsible for the neonatal hepatic dysfunction phenotype of lethal albino mice. Genes Dev. 1993;7:2298–307.
26. Aponte JL, Sega GA, Hauser LJ, Dhar MS, Withrow CM, Carpenter DA, et al. Point mutations in the murine

fumarylacetoacetate hydrolase gene: Animal models for the human genetic disorder hereditary tyrosinemia type 1. Proc Natl Acad Sci USA. 2001;98:641–5.
27. Yin H, Song C-Q, Dorkin JR, Zhu LJ, Li Y, Wu Q, et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. Nat Biotechnol. 2016;34:328–33.
28. Xie C, Zhang Y-P, Song L, Luo J, Qi W, Hu J, et al. Genome editing with CRISPR/Cas9 in postnatal mice corrects PRKAG2 cardiac syndrome. Cell Res. 2016;26:1099–111.
29. Wu W, Lu Z, Li F, Wang W, Qian N, Duan J, et al. Efficient in vivo gene editing using ribonucleoproteins in skin stem cells of recessive dystrophic epidermolysis bullosa mouse model. Proc Natl Acad Sci USA. 2017;114:1660–5.
30. Wang L, Shao Y, Guan Y, Li L, Wu L, Chen F, et al. Large genomic fragment deletion and functional gene cassette knock-in via Cas9 protein mediated genome editing in one-cell rodent embryos. Sci Rep. 2015;5:17517.
31. Hara S, Kato T, Goto Y, Kubota S, Tamano M, Terao M, Takada S. Microinjection-based generation of mutant mice with a double mutation and a 0.5 Mb deletion in their genome by the CRISPR/Cas9 system. J Reprod Dev. 2016;62:531–6.
32. Kato T, Hara S, Goto Y, Ogawa Y, Okayasu H, Kubota S, et al. Creation of mutant mice with megabase-sized deletions containing custom-designed breakpoints by means of the CRISPR/Cas9 system. Sci Rep. 2017;7:1156.
33. Boroviak K, Doe B, Banerjee R, Yang F, Bradley A. Chromosome engineering in zygotes with CRISPR/Cas9. Genesis. 2016;54:78–85.
34. Birling M-C, Schaeffer L, Andr P, Lindner L, Mar chal D, Ayadi A, et al. Efficient and rapid generation of large genomic variants in rats and mice using CRISMERE. Sci Rep. 2017;7:43331.
35. Kraft K, Geuer S, Will AJ, Chan WL, Paliou C, Borschiwer M, et al. Deletions, inversions, duplications: engineering of structural variants using CRISPR/Cas in mice. Cell Rep. 2015;10:833–9.
36. Yoshimi K, Kunihiro Y, Kaneko T, Nagahora H, Voigt B, Mashimo T. ssODN-mediated knock-in with CRISPR-Cas for large genomic regions in zygotes. Nat Commun. 2016;7:10431.