Progress of genome editing technology and developmental biology useful for radiation research

Kento Miura\(^1,2\), Atsuo Ogura\(^2,3,4\), Kohei Kobatake\(^1,5\), Hiroaki Honda\(^6\) and Osamu Kaminuma\(^1,2,\)*

\(^1\)Department of Disease Model, Research Institute of Radiation Biology and Medicine, Hiroshima University, Hiroshima 734-8553, Japan
\(^2\)RIKEN BioResource Research Center, Tsukuba, Ibaraki 305-0074, Japan
\(^3\)RIKEN Cluster for Pioneering Research, Wako, Saitama 351-0198, Japan
\(^4\)Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan
\(^5\)Department of Urology, Hiroshima University, Hiroshima 734-8553, Japan
\(^6\)Field of Human Disease Models, Major in Advanced Life Sciences and Medicine, Institute of Laboratory Animals, Tokyo Women’s Medical University, Tokyo 162-8666, Japan

*Corresponding author. Department of Disease Model, Research Institute of Radiation Biology and Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan. Tel: +81-82-257-1556; Fax: +81-82-257-1556; Email: okami@hiroshima-u.ac.jp
(Received 18 October 2020; revised 26 November 2020; editorial decision 27 November 2020)

ABSTRACT

Following the development of genome editing technology, it has become more feasible to create genetically modified animals such as knockout (KO), knock-in, and point-mutated animals. The genome-edited animals are useful to investigate the roles of various functional genes in many fields of biological science including radiation research. Nevertheless, some researchers may experience difficulty in generating genome-edited animals, probably due to the requirement for equipment and techniques for embryo manipulation and handling. Furthermore, after obtaining F0 generation, genome-edited animals generally need to be expanded and maintained for analyzing the target gene function. To investigate genes essential for normal birth and growth, the generation of conditional KO (cKO) animals in which a tissue- or stage-specific gene mutation can be introduced is often required. Here, we describe the basic principle and application of genome editing technology including zinc-finger nuclease, transcription-activator-like effector nuclease, and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated protein (Cas) systems. Recently advanced developmental biology methods have enabled application of the technology, especially CRISPR/Cas, to zygotes, leading to the prompt production of genome-edited animals. For pre-implantation embryos, genome editing via oviductal nucleic acid delivery has been developed as an embryo manipulation- or handling-free method. Examining the gene function at F0 generation is becoming possible by employing triple-target CRISPR technology. This technology, in combination with a blastocyst complementation method enables investigation of even birth- and growth-responsible genes without establishing cKO strains. We hope that this review is helpful for understanding and expanding genome editing-related technology and for progressing radiation research.

Keywords: blastocyst complementation; chimeric mice; CRISPR/Cas; genome editing

INTRODUCTION

Since the appearance of zinc-finger nuclease (ZFN) in 1996 [1], progress and spread of the technology, genome editing, defined as ‘a targeted manipulation of genomes with site-specific artificial endonucleases’, have been remarkable. Notably, Charpentier and Doudna, who discovered one of the most useful genome editing tools, clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated protein (Cas), won the Nobel Prize in Chemistry 2020 [2, 3]. The CRISPR/Cas system has made it more feasible to create genome-edited animals such as knockout (KO), knock-in (KI) or point-mutated mouse, rat and other animals (Table 1) [4, 5], and therefore provided much benefit in analyzing functional
genes in almost all fields of biological science. Also in the field of radiation research, genome editing has been applied for investigating radiosensitive genes and creating animal models representing various diseases initiated by radiation effects [6, 7]. Recently, the participation of protein phosphatase magnesium-dependent 1 delta (Ppm1d) in stem cell response to ionizing radiation-induced genotoxic stress in colon was investigated by generating genome-edited mice in which a truncating mutation was introduced into the locus [8]. For monitoring ionizing radiation-mediated DNA damage, Sabol et al. generated a mouse line expressing fluorescence protein-tagged Fanconi anemia complementation group D2 by genome editing [9].

However, some researchers unfamiliar with this technology and developmental engineering techniques may not feel that genome-edited animals can be easily generated by their own hands. This is caused, at least in part, by the requirement for expensive equipment and training in techniques for embryo manipulation and handling [10]. Furthermore, particularly in radiation research, the investigation of radiosensitive genes by generating KO mice is often unsuccessful because of their embryonic lethality [7]. In such cases, conditional KO (cKO) animals that involve tissue- or stage-specific gene mutation are produced [11]. Even when genome-edited animals are successfully obtained at F0 generation, they usually need to be expanded by backcross or intercross for use as established strains, and the strains must be maintained properly. These time-consuming processes may also be a burden for researchers employing the genome editing technology in their studies.

Despite these concerns, the genome editing technology is promising to provide benefits in the fields of not only medical science but also the agroindustry. Therefore, in order to help researchers, especially those participating in radiation research, to understand and utilize the genome editing technology, we describe its basic principles and methods for producing genome-edited animals. In combination with new developmental biology methods, the application of this technology has been further expanded so that embryo manipulation or handling skills are dispensable. We also introduce new CRISPR/Cas-based gene analysis procedures in which the establishment of genome-edited animal strains would not be necessary.

### GENOME EDITING SYSTEMS

Artificial endonucleases used for genome editing are restriction enzymes that cleave phosphodiester bonds within a polynucleotide chain of targeted DNA sequences in living cells. In cells, nuclease-induced double strand breaks (DSBs) in DNA are repaired by two main pathways: non-homologous end-joining (NHEJ) and homology-directed repair (HDR) (Fig. 1). These pathways also work in the repair of DSB caused by ionizing radiation [12, 13]. In NHEJ, the broken ends are directly reconnected regardless of their sequence, frequently associated with the deletion or insertion of a random number of nucleotide bases [14]. Therefore, by introducing NHEJ in the translation site of target genes, we can destroy their functions by deleting functional sequences or causing frame shifts. In HDR, a homologous DNA sequence of undamaged chromatid is used for repairing DSB by annealing or recombination [15]. A donor single- or double-stranded DNA with the homology arm can be artificially provided and integrated into the broken locus. By utilizing these DSB-repairing pathways, we can introduce mutations and artificial sequences into the target site.

Various types of artificial endonucleases have been developed for genome editing so far [16, 17]. Among them, ZFN [18], transcription-activator-like effector nuclease (TALEN) [19, 20] and CRISPR/Cas [21–23] are mainly used. Both ZFN and TALEN have a Fok I nuclease domain which is responsible for DNA cleavage [1], though they have different DNA-binding domains for recognizing their target sites. ZFN contains a series of zinc-finger (ZF) motifs as DNA-binding domains. The single motif unit composed of about 30 amino acids recognizes three bases of target sequences (Fig. 1). In contrast, a single unit of transcription-activator-like effector (TALE) domain in TALEN, composed of 34 amino acids, recognizes a single base of target sequences. ZFN and TALEN recognize their targets based on a protein–DNA binding reaction and need to be used as fusion proteins of the nuclease domain and the DNA-binding domain. Therefore, the requirement for multi-step procedures to construct the corresponding vectors for targeting genome sequences is one of the weak points of ZFN and TALEN systems [4].

### Table 1. Representative methods to generate genome-edited animals by the CRISPR/Cas system

| Genome editing target | Microinjection | Electroporation | GONAD | SCNT |
|-----------------------|----------------|----------------|-------|------|
| Zygote/embryo         | Yes            | No             | Yes   | Yes  |
| Requirement of manipulation technique | Yes           | Yes            | No    | No   |
| Requirement of embryo handling | Yes           | Yes            | No    | No   |
| Mosaicism             | Yes            | Yes            | No    | Yes  |
| Rat (2013) [95]       | Mouse (2013) [40] | Mouse (2015) [42, 43] | Mice (2015) [48] | Pig (2015) [103] |
| Hamster (2014) [96]   | Rat (2014) [41] | Rat (2018) [102] | Rat (2020) [52] | Cattle (2017) [104] |
| Rabbit (2014) [97]    | Pig (2016) [45] | | | Sheep (2018) [105, 106] |
| Monkey (2014) [98]    | | | | Goat (2014) [107] |
| Pig (2014) [99]       | | | | |
| Sheep (2015) [100]    | | | | |
| Goat (2015) [101]     | | | | |
CRISPRs were identified as a region of functionally unknown repeated sequences in the *Escherichia coli* genome in 1987 [24]. It was later shown that CRISPRs were derived from foreign DNA sequences, and a complex of CRISPR RNA (crRNA) derived from CRISPR, trans-activating crRNA (tracrRNA), and Cas exhibited endonuclease activity against the foreign DNA in the presence of a complementary sequence, suggesting that CRISPR/Cas originally works as an adaptive defense system in bacteria [25, 26]. An artificial genome editing tool based on the CRISPR/Cas system was developed in 2012 [3] and applied to genome modification in cells of mammals including humans in 2013 [27–29]. In contrast to ZFN and TALEN, CRISPR/Cas recognizes its target DNA adjacent to a protospacer adjacent motif (PAM) [30] with the help of guide RNA, such as crRNA–tracrRNA duplex and its chimeric RNA (single guide RNA) (Fig. 1). Cas works as an endonuclease for initiating DSB repair responses. Among Cas enzymes, Cas9 derived from *Streptococcus pyogenes* is the most popular for genome editing, though other Cas9 variants have also been applied [31–34]. Since the recognition of target sequences by CRISPR/Cas is based on RNA–DNA interaction, we can easily choose the target genes/sites by designing the base-pairing part (≈20 bp) of the guide RNA. This simplicity and high efficiency give the CRISPR/Cas9 system a big advantage as the most useful genome editing tool. Although several disadvantages such as off-target effects have been argued, the effects occurring in genome-edited zygotes do not seriously affect their phenotypes in most cases [35–37] and can be removed by repeat backcrossing of the born animals with wild-type animals. Procedures to decrease the frequency of off-target effects have also been developed [38, 39].

**PRODUCTION OF GENOME-EDITED ANIMALS WITH A CRISPR/CAS SYSTEM**

In 2013, Wang et al. reported for the first time the generation of KO mice by co-injection of Cas9 mRNA and guide RNA into mouse eggs. The CRISPR/Cas9 system allows for efficient and precise genome editing, enabling the creation of knockout models with high efficiency and reduced off-target effects compared to other genome editing technologies. This has greatly advanced the field of radiation research and developmental biology, providing valuable tools for studying radiation-induced genetic changes and cellular responses. The ability to create targeted gene deletions in a controlled manner has led to a deeper understanding of radiation biology and the mechanisms of DNA damage repair.
Fig. 2. Methods to produce genome-edited animals with CRISPR/Cas system. (A) Microinjection of Cas9 and guide RNA (gRNA) into zygotes and transfer of the embryos into recipient females. (B) Introduction of Cas9 and gRNA into zygotes or embryos by electroporation. (C) In vivo genome editing to pre-implantation embryos in oviducts of pregnant female mice by GONAD. (D) Nuclear transfer of genome-edited somatic cells.

zygotes and transferring the resulting embryos into recipient females [40]. They also generated KI mice by injecting Cas9, guide RNA and donor DNA oligos into zygotes. Currently, the microinjection of Cas9 into embryos is one of the standard methods to generate genome-edited mice as well as other animals whose embryos can be handled and manipulated (Fig. 2A, Table 1). However, in addition to various pieces
Fig. 3. Schematic procedure of the triple-target CRISPR system. Three guide RNAs are designed to distinct protein coding regions of target gene exons. The sgRNAs and Cas9 mRNA/protein are introduced into wild-type zygotes by microinjection or electroporation. The individual Cas9/sgRNA complexes generate DSBs in the target regions in both alleles. The mice derived from the resulting zygotes contain biallelic mutations at nearly 100% efficiency.

The direct genome editing of zygotes described above is often accompanied by problems related to genome editing efficiency or mosaicism [53, 54]. Since successful genome editing in zygotes is usually confirmed after the birth of neonates, this procedure may not be suitable especially for several large animals with long sexual maturation and pregnancy periods. To apply genome editing to such animals, somatic cell nuclear transfer (SCNT) technology can be utilized (Fig. 2D) [55, 56]. In contrast to zygote-based genome editing, modified sequences in target genes can be confirmed in somatic cells in vitro within several days after introducing Cas9 and guide RNA. SCNT of successfully modified somatic cells can efficiently provide the desired genome-edited animals at F0 generation (Fig. 2D). In order to increase the application of this genome editing/SCNT-combined method, particularly for investigating large animal models, further improvement of SCNT efficiency may be desirable [57, 58].

TRIPLE-TARGET CRISPR METHOD

Although the CRISPR/Cas system is a powerful tool for genome editing, born animals derived from edited zygotes at F0 generation are often a mosaic of the edited and wild-type cells [53], therefore a backcross or intercross is necessary to obtain biallelic edited animals. A modified CRISPR/Cas9 system using three guide RNAs to each target gene (triple-target CRISPR) has been developed to enable the production of biallelic mutated mice at F0 generation [59, 60]. In the triple-target CRISPR system, three guide RNAs are designed to distinct protein coding regions of target gene exons (Fig. 3). These guide RNAs and Cas9 mRNA/protein are introduced into zygotes by microinjection or electroporation [61–63]. The pups born from the resulting zygotes contain biallelic mutations at nearly 100% efficiency.
Fig. 4. Triple-target CRISPR method with blastocyst complementation. (A) F0 generation male mice produced by triple-target CRISPR-induced Nanos3 KO showed a loss of spermatozoa. (B) Spermatozoa in fertile chimeric male mice generated by injecting Dnmt3b−/− ESCs into blastocyst containing biallelic Nanos3 mutations are fully derived from the ESCs. (C) Animals deficient for specific organs/tissues could be produced at F0 generation by subjection of genes responsible for the development of target organs/tissues (“gene X”) to the triple-target CRISPR method. (D) The target organs/tissues fully derived from ESCs, even if the lethal gene mutation is introduced in the ESCs, could be reproduced in F0 chimeric mice derived from blastocysts carrying biallelic ’gene X’ mutations.
Since screening and analysis of the gene function can be achieved without establishing or maintaining multi-generated KO animal strains, this method has already been applied to KO of various genes [59, 61, 62, 64, 65] and mouse strains [63].

Triple-target CRISPR is an outstanding system to induce whole-body biallelic KO in animals, though analyzing birth- and growth-responsible genes is still complicated, because KO of those genes usually causes embryonic or neonatal lethality. To circumvent the problem, organ- and tissue-specific cKO methods have been developed, especially using systems such as Cre-loxP-mediated genetic recombination [11]. In this system, cKO can be achieved in vivo by mating gene Ki mice carrying a target gene flanked by two loxP sites (flox mice) with a tissue/cell-specific promoter-driven Cre-transgenic mouse strain (Cre mice). In fact, in the 4th International Symposium of the Network-type Joint Usage/Research Center for Radiation Disaster Medical Science, we demonstrated our recent work regarding the contribution of an X chromosome gene, Kdm6a, to the pathogenesis of bladder cancer by generating its cKO mice [66]. That was because female homozygous systemic Kdm6a KO embryos die around embryonic day 12.5 to 13.5 [67–69], while the majority of systemic Kdm6a KO males (Kdm6a−/−) showed perinatal lethality [67, 68, 70].

However, there are several concerns in using the Cre-loxP system. Additional mating to insert the loxP sites in two alleles is required for generating homozygous cKO mice. The generation of flox mice was often technically troublesome, though it has been improved by the development of the CRISPR/Cas9 method [71, 72]. The Cre transgene sometimes causes non-negligible phenotypes [73, 74] and expresses in unexpected tissues [75]. Organ- and tissue-specific cKO still causes embryonic or neonatal lethality in some cases [76–78]. To circumvent the drawbacks of the Cre-loxP system, a tamoxifen-inducible Cre-ERT2 system that enables not only organ/tissue- but also time-specific KO of the targeted gene was developed [79]. Since Cre-ERT2 is translocated into the nucleus and works at the time of tamoxifen treatment, cKO of genes that result in lethality with the standard Cre-loxP system can be achieved in live animals by using the Cre-ERT2/tamoxifen system. We also used this system for investigating a role of Kdm6a in the regulation of aging-associated gene expression in a murine hematopoietic system by achieving tamoxifen-inducible KO of the Kdm6a gene [80].

Furthermore, a new system combining the triple-target CRISPR system with a blastocyst complementation method has recently been developed [63]. In the blastocyst complementation method, a target organ exclusively derived from embryonic stem cells (ESCs) or induced pluripotent cells (iPSCs) can be generated in chimeric animals by injecting these cells into blastocysts in which the development of the original target organ is prevented [81, 82]. The triple-target CRISPR system would be useful for inducing the organ deficiency by targeting genes essential for its development (e.g., Fgf2 or Fgf10 for the lung, Pdx1 for the pancreas, and Sall1 for the kidney) [83–86]. Since the deficient organs are expected to be complimented by pluripotent stem cells injected into the blastocysts, the production of chimeric mice containing the organ, even carrying a lethal gene mutation, could be achieved at F0 generation.

The concept of the blastocyst complementation/triple-target CRISPR-combined method was validated in germ cells, whose loss was caused by KO of the gene coding nanos C2HC-type zinc finger 3 (Nanos3) [87]. The germ loss phenotype was confirmed in several mouse strains by targeting Nanos3 in the triple-target CRISPR method [63]. Mutating the DNA methyltransferase 3B (Dnmt3b) coding gene was reported to cause embryonic death [88], though the dispensable role of Dnmt3b in germ cell development was demonstrated by a germ cell-specific cKO study [89]. From fertile chimeric male mice generated by injecting Dnmt3b−/− ESCs into blastocyst carrying biallelic mutations of Nanos3, only pups with ESC-derived coat color were born. Since the contribution of pluripotent stem cells to germ cells of F0 chimeric mice can be evaluated by the coat color of F1 offspring, it was suggested that sprematooza of the fertile chimeras were fully derived from the ESCs (Figs 4A and B) [63].

These technical improvements would make it more feasible to screen and analyze the gene function in a specific organ in adulthood (Figs 4C and D). Many researchers have studied the influence of ionizing radiation by focusing on various DNA repair genes, though mutating those genes in animals frequently results in lethality [7]. Therefore, new technologies combining the blastocyst complementation and triple-target CRISPR methods would also be useful for investigating those radiosensitive genes [90].

**CONCLUSION**

Genome editing technology has been increasingly applied in the field of agriculture. We may soon be able to eat tomato containing a large amount of gamma-aminobutyric acid, non-allergic eggs, muscular sea bream, etc. In addition, attempts to use genome editing in the medical field are currently being explored. As its originators have become Nobel Prize winners, the utilization of this technology is expected to be further accelerated. Although, several concerns regarding ethical problem as well as reputational damage may remain, particularly in these fields of study, genome editing is already an indispensable tool for progressing biological science. Coupled with evolving techniques in developmental biology, genome editing is fast becoming a powerful tool for analyzing gene function. The CRISPR/Cas9 system has recently been available not only for genome editing but also for RNA editing, epigenome manipulation, etc. [34, 91–94]. Furthermore, some of the methods are already available even for researchers who are unfamiliar with developmental biology, because expensive equipment and trained skills are no longer necessary. Genome editing technology, together with developmental biology, is promising to cause a revolution in various fields of biological science including radiation research.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**FUNDING**

This study was supported by grants from the Japan Society for the Promotion of Science KAKENHI Grant Numbers 19K16017 (K.M.), JP25112009 (A.O.), JP19H05758 (A.O.), and 19H03145 (O.K.), Epigenome Manipulation Project of the All-RIKEN Projects (A.O.), and the Program of the Network-type Joint Usage/Research Center for Radiation Disaster Medical Science of Hiroshima University, Nagasaki University, and Fukushima Medical University (O.K.).
SUPPLEMENT FUNDING

This supplement has been funded by the Program of the Network-type Joint Usage/Research Center for Radiation Disaster Medical Science of Hiroshima University, Nagasaki University, and Fukushima Medical University.

ACKNOWLEDGMENT

The authors thank the staff in the Bioresource Engineering Division, RIKEN Bioresource Research Center, and Norimasa Yamasaki and Sawako Ogata in the Department of Disease Model, Research Institute of Radiation Biology and Medicine, Hiroshima University for excellent technical assistance and advice. The part of this work was presented at the 4th International Symposium of the Network-type Joint Usage/Research Center for Radiation Disaster Medical Science.

REFERENCES

1. 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.
2. Deltcheva E, Chylinski K, Sharma CM et al. CRISPR RNA maturation by trans-encoded small RNA and host factor RNAse III. Nature 2011;471:602–7.
3. Jinek M, Chylinski K, Fonfara I et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 2012;337:816–21.
4. Chandrasegaran S, Carroll D. Origins of programmable nucleases for genome engineering. J Mol Biol 2016;428:963–89.
5. Meek S, Mashimo T, Burdon T et al. From engineering to editing the rat genome. Mamm Genome 2017;28:302–14.
6. Castle KD, Chen M, Wisdom AJ et al. Genetically engineered mouse models for studying radiation biology. Transl Cancer Res 2017;6:S900–S13.
7. Miyamoto T, Akutsu SN, Tauchi H et al. Exploration of genetic variants: Optimizing the repertoire, specificity and delivery of genome engineering tools. Mamm Genome 2017;33:1709–12.
8. Burocziova M, Burdova K, Martinikova AS et al. Truncated PPM1D impairs stem cell response to genotoxic stress and promotes growth of APC-deficient tumors in the mouse colon. Cell Death Dis 2019;10:818.
9. Sabol M, Akbudak MA, Fricova D et al. Novel TALEN-generated mCitrine-FANCD2 fusion reporter mouse model for in vivo research of DNA damage response. DNA Repair (Amst) 2020;94:102936.
10. Ogura A. Cloning mice. Cold Spring Harb Protoc 2017;2017:pdb prot094425.
11. Lobe CG, Nagy A. Conditional genome alteration in mice. Bioessays 1998;20:200–8.
12. Chapman JR, Taylor MR, Boulton SJ. Playing the end game: DNA double-strand break repair pathway choice. Mol Cell 2012;47:497–510.
13. Vignard J, Mirey G, Salles B. Ionizing-radiation induced DNA double-strand breaks: A direct and indirect lighting up. Radiat Oncol: J Eur Soc Therap Radiol Oncol 2013;108:362–9.
14. Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem 2010;79:181–211.
15. Morrical SW. DNA-pairing and annealing processes in homologous recombination and homology-directed repair. Cold Spring Harb Perspect Biol 2015;7:a016444.
16. Rouet P, Smith F, Jasin M. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Mol Cell Biol 1994;14:8096–106.
17. Porteus MH. A new class of medicines through DNA editing. N Engl J Med 2019;380:947–59.
18. Urnov FD, Redar EJ, Holmes MC et al. Genome editing with engineered zinc finger nucleases. Nat Rev Genet 2010;11:636–46.
19. Mashimo T, Kaneko T, Sakuma T et al. Efficient gene targeting by TAL effector nucleases coinfected with exonucleases in zygotes. Sci Rep 2013;3:1253.
20. Sommer D, Peters AE, Baumgart AK et al. TALEN-mediated genome engineering to generate targeted mice. Chromosome Res 2015;23:43–55.
21. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 2014;346:1258096.
22. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell 2014;157:1262–78.
23. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 2014;32:347–55.
24. Ishino Y, Shinagawa H, Makino K et al. Nucleotide sequence variants: Optimizing the repertoire, specificity and delivery of CRISPR-Cas9 nuclease with expanded targeting space. Sci Rep 2013;3:1253.
25. Sommer D, Peters AE, Baumgart AK et al. TALEN-mediated genome engineering to generate targeted mice. Chromosome Res 2015;23:43–55.
26. Brouns SJ, Jore MM, Lundgren M et al. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 2008;321:960–4.
27. Cong L, Ran FA, Cox D et al. Multiplex genome engineering using CRISPR-Cas9 systems. Science 2013;339:819–23.
28. Jinek M, East A, Cheng A et al. RNA-programmed genome editing in human cells. Elife 2013;2:e00471.
29. Mali P, Yang LH, Esvelt KM et al. RNA-guided human genome engineering via Cas9. Science 2013;339:823–6.
30. Mojica FJM, Diez-Villasenor C, Garcia-Martinez J et al. Short motif sequences determine the targets of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J Bacteriol 1987;169:5429–33.
31. Barrangou R, Fremaux C, Deveau H et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science 2007;315:1709–12.
32. Brouns SJ, Jore MM, Lundgren M et al. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 2008;321:960–4.
33. Cong L, Ran FA, Cox D et al. Multiplex genome engineering using CRISPR-Cas9 systems. Science 2013;339:819–23.
34. Jinek M, East A, Cheng A et al. RNA-programmed genome editing in human cells. Elife 2013;2:e00471.
35. Mali P, Yang LH, Esvelt KM et al. RNA-guided human genome engineering via Cas9. Science 2013;339:823–6.
36. Mojica FJM, Diez-Villasenor C, Garcia-Martinez J et al. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. Microbiology (Reading) 2009;155:733–40.
37. Celbrain-Serrano A, Davies B. CRISPR-Cas orthologues and variants: Optimizing the repertoire, specificity and delivery of genome engineering tools. Mamm Genome 2017;28:247–61.
38. Komor AC, Badran AH, Liu DR. CRISPR-based Technologies for the Manipulation of eukaryotic genomes. Cell 2017;168:20–36.
39. Nishimasu H, Shi X, Ishiguro S et al. Engineered CRISPR-Cas9 nuclease with expanded targeting space. Science 2018;361:1259–62.
34. Pickar-Oliver A, Gersbach CA. The next generation of CRISPR-Cas technologies and applications. Nat Rev Mol Cell Biol 2019;20:490–507.
35. Mashiko D, Young SA, Muto M et al. Feasibility for a large scale mouse mutagenesis by injecting CRISPR/Cas plasmid into zygotes. Dev Growth Differ 2014;56:122–9.
36. Iyer V, Boroviak K, Thomas M et al. No unexpected CRISPR-Cas9 off-target activity revealed by trio sequencing of gene-edited mice. PLoS Genet 2018;14:e1007503.
37. Willi M, Smith HE, Wang C et al. Mutation frequency is not increased in CRISPR-Cas9-edited mice. Nat Methods 2018;15:756–8.
38. Shen B, Zhang W, Zhang J et al. Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. Nat Methods 2018;15:512–4.
39. Anderson KR, Haeussler M, Watanabe C et al. CRISPR off-target analysis in genetically engineered rats and mice. Nat Methods 2018;15:512–4.
40. Wang H, Yang H, Shivalila CS et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genomic engineering. Cell 2013;153:910–8.
41. Kaneko T, Mashimo T. Simple genome editing of rodent embryos by electroporation of engineered endonucleases into intact rat embryos. Sci Rep 2014;4:6382.
42. Hashimoto M, Takemoto T. Electroporation enables the efficient mRNA delivery into the mouse zygotes and facilitates CRISPR/Cas9-based genome editing. Sci Rep 2015;5:11315.
43. Qin W, Dion SL, Kutny PM et al. Efficient CRISPR/Cas9-mediated genome editing in mice by zygote electroporation of nuclease. Genetics 2015;200:423–30.
44. Kaneko T, Mashimo T. Simple genome editing of rodent intact embryos by electroporation. PLoS One 2015;10:e0142755.
45. Tanihara F, Takemoto T, Kitagawa E et al. Somatic cell reprogramming-free generation of genetically modified pigs. Sci Adv 2016;2:e1600803.
46. Mizuno N, Mizutani E, Sato H et al. Intra-embryo gene cassette Knockin by CRISPR/Cas9-mediated genomic editing with adeno-associated viral vector. Sci Adv 2018;9:286–97.
47. Honda A, Tachibana R, Hamada K et al. Efficient derivation of knock-out and knock-in rats using embryos obtained by in vitro fertilization. Sci Rep 2019;9:11571.
48. Takahashi G, Gurumurthy CB, Wada K et al. GONAD: Genome-editing via Oviductal nucleic acids delivery system: A novel microinjection independent genome engineering method in mice. Sci Rep 2015;5:11406.
49. Takabayashi S, Aoshima T, Kabashima K et al. I-GONAD (improved genome-editing via oviductal nucleic acids delivery), a convenient in vivo tool to produce genome-edited rats. Sci Rep 2018;8:12059.
50. Gurumurthy CB, Sato M, Nakamura A et al. Creation of CRISPR-based germline-genome-engineered mice without ex vivo handling of zygotes by i-GONAD. Nat Protoc 2019;14:2452–82.
51. Hirose M, Ogura A. The golden (Syrian) hamster as a model for the study of reproductive biology: Past, present, and future. Reprod Med Biol 2019;18:34–9.
52. Hirose M, Honda A, Fulka H et al. Acrosin is essential for sperm penetration through the zona pellucida in hamsters. Proc Natl Acad Sci U S A 2020;117:2513–8.
53. Yen ST, Zhang M, Deng JM et al. Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. Dev Biol 2014;393:3–9.
54. Gurumurthy CB, O’Brien AR, Quadros RM et al. Reproducibility of CRISPR-Cas9 methods for generation of conditional mouse alleles: A multi-center evaluation. Genome Biol 2019;20:171.
55. Kals D, Zhou S, Cai B et al. Sheep and goat genome engineering: From random Transgenesis to the CRISPR era. Front Genet 2019;10:750.
56. Menchaca A, Dos Santos-Neto PC, Mulet AP et al. CRISPR in livestock: From editing to printing. Theriogenology 2020;150:247–54.
57. Matoba S, Wang H, Jiang L et al. Loss of H3K27me3 imprinting in somatic cell nuclear transfer embryos disrupts post-implantation development. Cell Stem Cell 2018;23:343–54 e5.
58. Matoba S, Zhang Y. Somatic cell nuclear transfer reprogramming: Mechanisms and applications. Cell Stem Cell 2018;23:471–85.
59. Sunagawa GA, Sumiyama K, Ukai-Tadenuma M et al. Mammalian reverse genetics without crossing reveals Nr3a as a short-sleeper gene. Cell Rep 2016;14:662–77.
60. Susaki EA, Ukai H, Ueda HR. Next-generation mammalian genetics toward organism-level systems biology. NPJ Syst Biol Appl 2017;3:15.
61. Matoba S, Nakamuta S, Miura K et al. Paternal knockout of Slc38a4/SNAT4 causes placental hypoplasia associated with intrauterine growth restriction in mice. Proc Natl Acad Sci U S A 2019;116:21047–53.
62. Inoue K, Ogonuki N, Kanimura S et al. Loss of H3K27me3 imprinting in the Sfmbt2 miRNA cluster causes enlargement of cloned mouse placenta. Nat Commun 2020;11:2150.
63. Miura K, Matoba S, Hirose M et al. Generation of chimeric mice with spermatozoa fully derived from embryonic stem cells using a triple-target CRISPR method for Nanos3. Biol Reprod 2020;ioaa176. (Online ahead of print.)
64. Tatsuki F, Sunagawa GA, Shi S et al. Involvement of Ca(2+) dependent hyperpolarization in sleep duration in mammals. Neuron 2016;90:70–85.
65. Niwa Y, Kanda GN, Yamada RG et al. Muscarinic acetylcholine receptors Chrm1 and Chrm3 are essential for REM sleep. Cell Rep 2018;24:2231–47 e7.
66. Kobatake K, Ikeda KI, Nakata Y et al. Kdm6a deficiency activates inflammatory pathways, promotes M2 macrophage polarization, and causes bladder cancer in cooperation with p53 dysfunction. Clin Cancer Res 2020;26:2065–79.
67. Wang C, Lee JE, Cho YW et al. UTX regulates mesoderm differentiation of embryonic stem cells independent
of H3K27 demethylase activity. Proc Natl Acad Sci U S A 2012;109:15324–9.
68. Welstead GG, Creyghton MP, Bilodeau S et al. X-linked H3K27me3 demethylase Utx is required for embryonic development in a sex-specific manner. Proc Natl Acad Sci U S A 2012;109:13004–9.
69. Thieml S, Gyarfas T, Richter C et al. The histone demethylase UTX regulates stem cell migration and hematopoiesis. Blood 2013;121:2462–73.
70. Shpargel KB, Sengoku T, Yokoyama S et al. UTX and UTY demonstrate histone demethylase-independent function in mouse embryonic development. PLoS Genet 2012;8:e1002964.
71. Abe T, Inoue KI, Furuta Y et al. Pronuclear microinjection during S-phase increases the efficiency of CRISPR-Cas9-assisted Knockin of large DNA donors in mouse zygotes. Cell Rep 2020;31:107653.
72. Horii T, Kobayashi R, Kimura M et al. Calcium-free and Cytochalasin B treatment inhibits Blastomere fusion in 2-cell stage embryos for the generation of Floxed mice via sequential electroperoration. Cell 2020;9:1088.
73. Forni PE, Scuoppo C, Imayoshi I et al. High levels of Cre expression in neuronal progenitors cause defects in brain development leading to microencephaly and hydrocephaly. J Neurosci 2006;26:9593–602.
74. Lewis AE, Vasudevan HN, O’Neill AK et al. The widely used Wnt1-Cre transgene causes developmental phenotypes by ectopic activation of Wnt signaling. Dev Biol 2013;379:229–34.
75. Payne S, De Val S, Neal A. Endothelial-specific Cre mouse models. Arterioscler Thromb Vasc Biol 2018;38:2550–61.
76. Shen TL, Park AY, Alcaraz A et al. Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. J Cell Biol 2005;169:941–52.
77. Jones RG, Li X, Gray PD et al. Conditional deletion of beta1 integrins in the intestinal epithelium causes a loss of hedgehog expression, intestinal hyperplasia, and early postnatal lethality. J Cell Biol 2006;175:505–14.
78. Govoni KE, Wergedal JE, Florin L et al. Conditional deletion of insulin-like growth factor-I in collagen type 1alpha2-expressing Cell Biol 2007;148:5706–15.
79. Indra AK, Warot X, Brocard J et al. Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: Comparison of the recombining activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. Nucleic Acids Res 1999;27:4324–7.
80. Sera, Y, Nakata, Y, Ueda, T, et al. UTX maintains functional integrity of murine hematopoietic system by globally regulating aging-associated genes. Blood 2020;blood.2019001044.(Online ahead of print.)
81. Chen J, Lansford R, Stewart V et al. RAG-2-deficient blastocyst complementation: An assay of gene function in lymphocyte development. Proc Natl Acad Sci U S A 1993;90:4528–32.
82. Kobayashi T, Yamaguchi T, Hamanaka S et al. Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. Cell 2010;142:787–99.
83. Yamaguchi T, Sato H, Kato-Itoh M et al. Interspecies organogenesis generates autologous functional islets. Nature 2017;542:191–6.
84. Goto T, Hara H, Sanbo M et al. Generation of pluripotent stem cell-derived mouse kidneys in Sall1-targeted anephric rats. Nat Commun 2019;10:451.
85. Mori M, Furushashi K, Danielsson JA et al. Generation of functional lungs via conditional blastocyst complementation using pluripotent stem cells. Nat Med 2019;25:1691–8.
86. Kitahara A, Ran Q, Oda K et al. Generation of lungs by blastocyst complementation in Apneumic Fgf10-deficient mice. Cell Rep 2020;31:107626.
87. Tsuda M, Sasaoka Y, Kiso M et al. Conserved role of nanos proteins in germ cell development. Science 2003;301:1239–41.
88. Okano M, Bell DW, Haber DA et al. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 1999;99:247–57.
89. Kaneda M, Okano M, Hata K et al. Essential role for de novo DNA methyltransferase Dnmt3a in maternal and paternal imprinting. Nature 2004;429:900–3.
90. Andreassen CN, Alsnør J, Overgaard M et al. Prediction of normal tissue radiosensitivity from polymorphisms in candidate genes. Radiotherapy and oncology: Journal of the European Society for Therapeutic Radiology and Oncology 2003;69:127–35.
91. Dominguez AA, Lim WA, Qi LS. Beyond editing: Repurposing CRISPR-Cas9 for precision genome regulation and interrogation. Nat Rev Mol Cell Biol 2016;17:5–15.
92. Fujita T, Kitaura F, Oji A et al. Transgenic mouse lines expressing the 3xFLAG-dCas9 protein for enChIP analysis. Genes Cells 2018;23:318–25.
93. Knott GJ, Doudna JA. CRISPR-Cas guides the future of genetic engineering. Science 2018;361:866–9.
94. Horii T, Morita S, Hino S et al. Successful generation of epigenetic disease model mice by targeted demethylation of the epigenome. Genome Biol 2020;21:77.
95. Li W, Teng F, Li T et al. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. Nat Biotechnol 2013;31:684–6.
96. Fan Z, Li W, Lee SR et al. Efficient gene targeting in golden Syrian hamsters by the CRISPR/Cas9 system. PLoS One 2014;9:e109755.
97. Yang D, Xu J, Zhu T et al. Effective gene targeting in rabbits using RNA-guided Cas9 nucleases. J Mol Cell Biol RNA-guided Cas9 nucleases. Nat Biotechnol 2013;31:684–6.
98. Niu Y, Shen B, Cui Y et al. Generation of gene-modified cystcomplementation in Apneumic Fgf10-deficient mice. Cell Rep 2020;31:107626.
99. Hai T, Teng F, Guo R et al. One-step generation of knock-out pigs by zygote injection of CRISPR/Cas9 system. Proc Natl Acad Sci U S A 2015;10:e0136690.
100. Crispo M, Mulet AP, Tesson L et al. Efficient generation of MSTN and FGF5 via zygote injection of CRISPR/Cas9 system. Cell Rep 2014;9:e109755.
102. Kobayashi T, Namba M, Koyano T et al. Successful production of genome-edited rats by the rGONAD method. *BMC Biotechnol* 2018;18:19.

103. Zhou X, Xin J, Fan N et al. Generation of CRISPR/Cas9-mediated gene-targeted pigs via somatic cell nuclear transfer. *Cell Mol Life Sci* 2015;72:1175–84.

104. Gao Y, Wu H, Wang Y et al. Single Cas9 nickase induced generation of NRAMP1 knockin cattle with reduced off-target effects. *Genome Biol* 2017;18:13.

105. Fan Z, Perisse IV, Cotton CU et al. A sheep model of cystic fibrosis generated by CRISPR/Cas9 disruption of the CFTR gene. *JCI Insight* 2018;3:e123529.

106. Zhang Y, Wang Y, Yulin B et al. CRISPR/Cas9-mediated sheep MSTN gene knockout and promote sSMSCs differentiation. *J Cell Biochem* 2019;120:1794–806.

107. Ni W, Qiao J, Hu S et al. Efficient gene knockout in goats using CRISPR/Cas9 system. *PLoS One* 2014;9:e106718.