

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

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## 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

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

	Microinjection	Electroporation	GONAD	SCNT
Genome editing target	Zygote/embryo	Zygote/embryo	Zygote/embryo	Somatic cell
Requirement of manipulation technique	Yes	No	No	Yes
Requirement of embryo handling	Yes	Yes	No	Yes
Mosaicism	Yes	Yes	Yes	No
Applied animals (year of publication)	Mouse (2013) [40] Rat (2013) [95] Hamster (2014) [96] Rabbit (2014) [97] Monkey (2014) [98] Pig (2014) [99] Sheep (2015) [100] Goat (2015) [101]	Mouse (2015) [42, 43] Rat (2014) [41] Pig (2016) [45]	Mouse (2015) [48] Rat (2018) [102] Hamster (2020) [52]	Pig (2015) [103] Cattle (2017) [104] Sheep (2018) [105, 106] Goat (2014) [107]

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 *Ppm1d* 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].

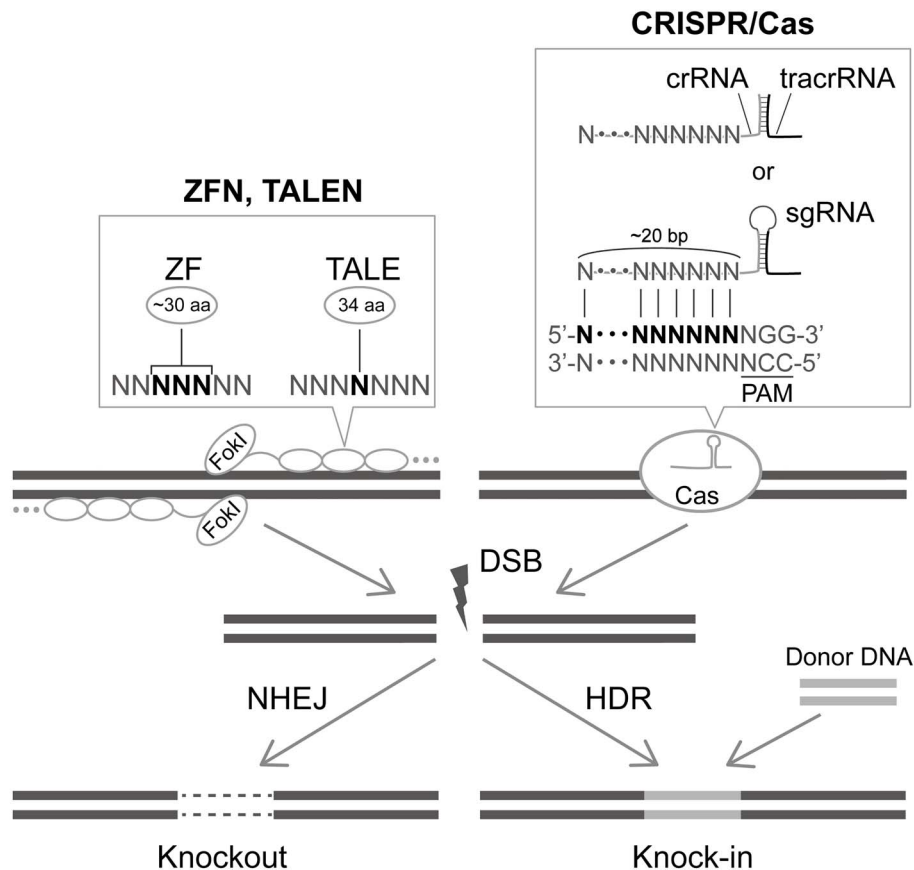
However, some researchers unfamiliar with this technology and developmental engineering techniques may not feel that genome-edited animals can be easily generated by their own hands. This is caused, at least in part, by the requirement for expensive equipment and training in techniques for embryo manipulation 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].



**Fig. 1.** Nuclease-induced DSB in DNA followed by repairing through NHEJ or HDR. ZFN and TALEN contain ZF and TALE motifs, respectively, as DNA-binding domains. A Fok I nuclease domain is involved in both ZFN and TALEN for DNA cleavage. The CRISPR/Cas system recognizes DNA via RNA–DNA interaction between guide RNA, such as crRNA–tracrRNA duplex and single guide RNA (sgRNA), and its target DNA sequence adjacent to PAM. Then, Cas complexed with guide RNA works as an endonuclease and cleaves the target DNA. The resulting DSB is repaired through the NHEJ or HDR pathway. In NHEJ, the broken ends are directly reconnected, frequently accompanied by random deletion or insertion. In HDR, homologous recombination with the corresponding sequence in undamaged chromatid occurs. When a donor DNA with a homology arm is provided artificially, it can be integrated into the broken locus.

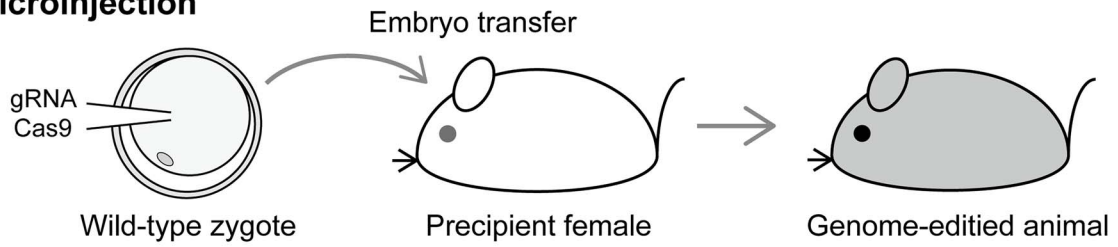
CRISPRs were identified as a region of functionally unknown repeated sequences in the *Escherichia coli* genome in 1987 [24]. It was later shown that CRISPRs were derived from foreign DNA sequences, and a complex of CRISPR RNA (crRNA) derived from CRISPR, trans-activating crRNA (tracrRNA), and Cas exhibited 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 a 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].

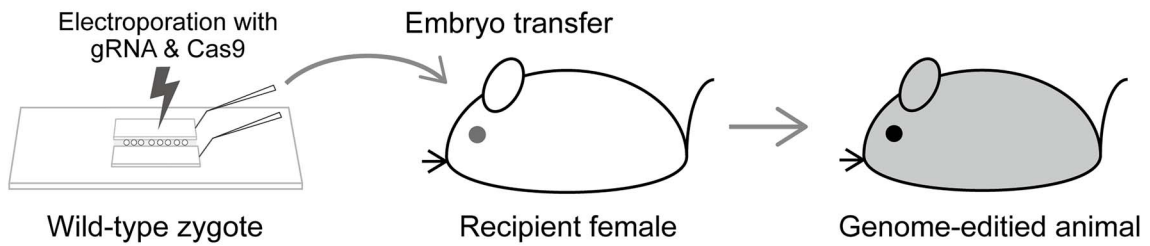
#### PRODUCTION OF GENOME-EDITED ANIMALS WITH A CRISPR/CAS SYSTEM

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

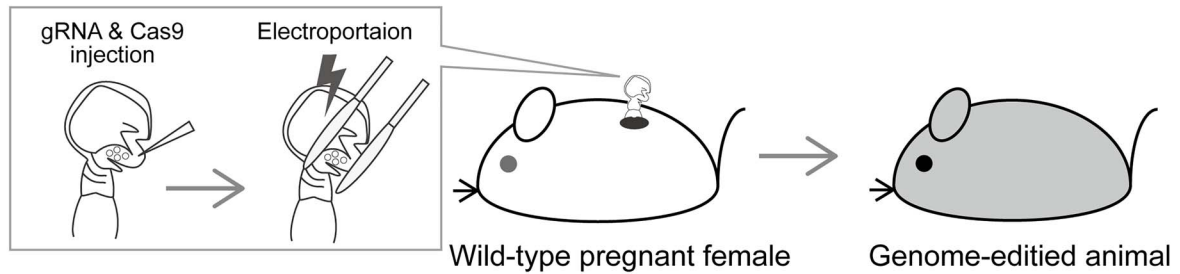
### A. Microinjection



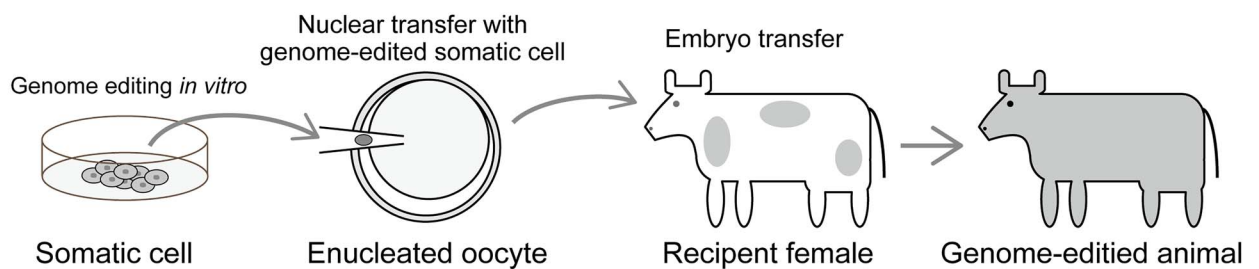
### B. Electroporation



### C. GONAD



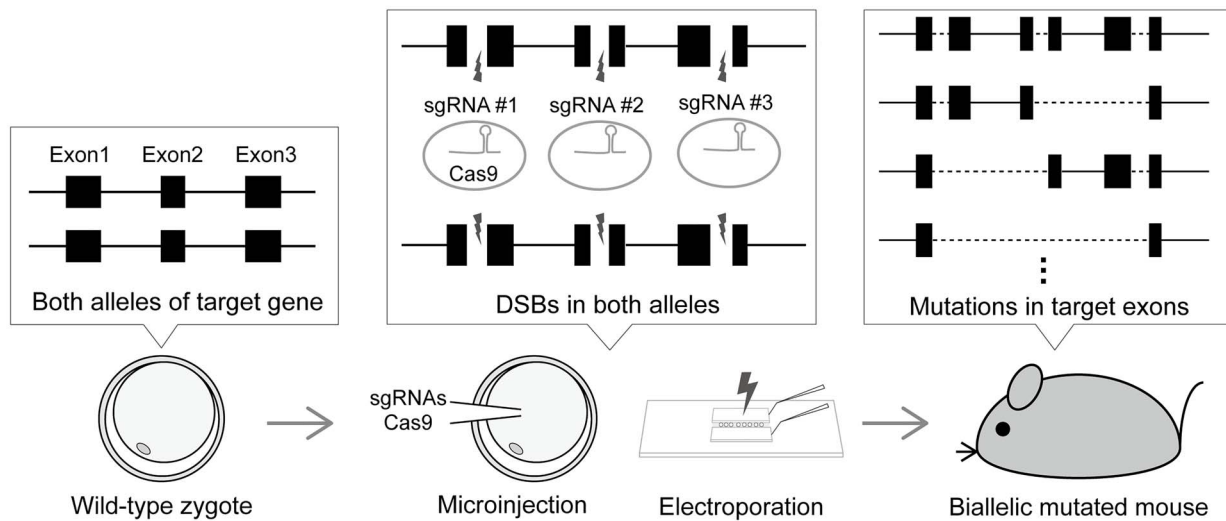
### D. Nuclear transfer



**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.**

of equipment such as an inverted microscope and micromanipulator, highly skilled techniques for their proper usage are needed to employ the basic CRISPR/Cas system [10].

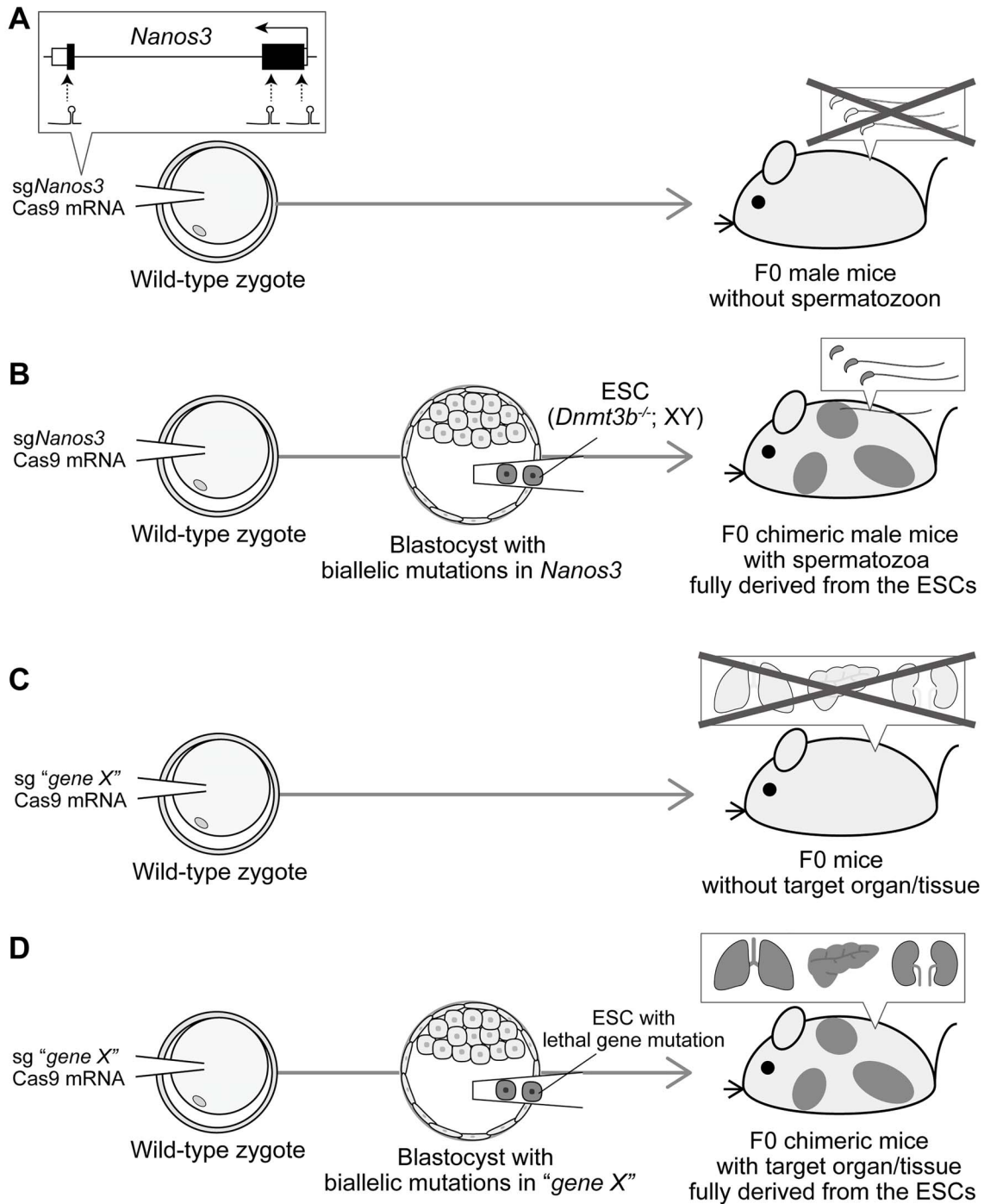
From 2014, several groups succeeded in producing genome-edited animals by direct introduction of Cas9 and guide RNA into embryos using electroporation [41–43]. In this method, embryos are placed between two metal plates suspended in Cas9- and guide RNA-containing solution (Fig. 2B). Since Cas9 and guide RNA are automatically introduced into embryos upon electroporation, genome-edited embryos are easily produced without using special equipment or trained skills for micromanipulation [44, 45]. Furthermore, an efficient KI system in which Cas9/guide RNAs and DNA fragments are introduced into zygotes by electroporation and adeno-associated virus vectors, respectively, has recently been reported [46, 47].

Takahashi *et al.* developed a further improved genome editing method, called genome editing via oviductal nucleic acids delivery (GONAD) [48]. GONAD enables the *in vivo* genome editing of pre-implantation embryos in oviducts of pregnant female mice (Fig. 2C). In this method, we simply prepare pregnant female mice, surgically expose their oviducts, and inject a genome editing solution containing Cas9 mRNA and guide RNA into the oviduct. Since this oviduct already contains pre-implantation embryos, we can introduce the Cas9 and guide RNA into the embryos by conducting *in vivo* electroporation through electrodes sandwiching the oviduct. Genome-edited pups can be obtained from this pregnant female at a certain rate. Because of the successful avoidance of *ex vivo* handling of embryos outside the oviduct, the utility of GONAD has been further expanded. The improved GONAD (*i*-GONAD), containing some modifications such as using Cas9 protein instead of its mRNA, can be utilized not only for mouse and rat [49, 50] but also hamster whose embryo is very sensitive to its surrounding environment *in vitro* (Table 1) [51, 52].

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 spermatozoon. (B) Spermatozoa in fertile chimeric male mice generated by injecting *Dnmt3b*<sup>-/-</sup> 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 subsection 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*<sup>+/Y</sup>) 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., *Fgfr2* or *Fgf10* for the lung, *Pdx1* for the pancreas, and *Sall1* for the kidney) [83–86]. Since the deficient organs are expected to be complemented 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*<sup>-/-</sup> male 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 spermatozoa 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/Cas 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.

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