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Efficient generation of genome-modified mice via offset-nicking by CRISPR/Cas system

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ABSTRACT

The mammalian zygote-mediated CRISPR/Cas system can efficiently generate targeted genome-modified animals. However, this system is limited by the risk of off-target mutations. Here we show that offset-nicking by Cas9 nickase and paired gRNAs allows us to generate region deleted mice and targeted knock-in mice without off-target mutations.

1. Introduction

The use of the CRISPR/Cas system, a recently developed genome engineering technology, on mammalian zygotes provides an easy, inexpensive and fast method for genome modification. The broad applicability of the CRISPR/Cas system has been demonstrated in recent reports showing the highly efficient generation of multiple knockout and knock-in animals [1,2]. On the other hand, high off-target effects of the CRISPR/Cas system have been suggested in culture cells [3–5], and we also reported the presence of off-target mutations in mouse zygotes treated with the CRISPR/Cas system [6]. Mali et al. reported that the off-target effect in culture cells could be avoided without affecting on-target mutation efficiencies by the use of two gRNAs engineered for sense and antisense strands within 100 base pairs of a target site and Cas9 nickase, a mutated Cas9 that makes single-strand breaks, and referred to this system as offset-nicking [7]. In addition, Ran et al. have reported that offset-nicking was utilizable for genome modification of mouse zygotes and the generation of 6 kbp genome deletions and precise point insertion of restriction enzyme sites into cultured cells [8].

However, no genome-modified organisms have yet been generated by offset-nicking, and thus it has not been demonstrated whether this method can actually be used to generate genome-modified mice, including mice with large-scale deletions and targeted knock-ins, and whether the off-target effects can actually be avoided by this method in mouse zygotes. In the present study, we investigated the potential use of offset-nicking by generating region-deleted mice and knock-in mice using this method, and examined the generation efficiency and the possibility of avoiding off-target effects by this method.

2. Materials and methods

2.1. Ethics statement

All animal care and experiments conformed to the Guidelines for Animal Experiments of The University of Tokyo, and were approved by the Animal Research Committee of The University of Tokyo.

2.2. Construction of Cas9 nickase coding vector

According to the sequence of Cas9^{D10A} [9], Cas9 nickase was constructed by mutation PCR of pCAG-T3-hCAS9-pA plasmid vector (Addgene plasmid 48625) [6] using the primers (Forward primer, 5′-AGTACTCCATTGGGCTCGccATCGGCACAAAC, and Reverse primer, 5′-CGAGCCCAATGGAGTACTTCTTGTCGGCTGC). The vector was sequenced using a commercial sequencing kit (Applied Biosystems, Foster City, CA, USA) and a DNA sequencer (Applied Biosystems) according to the manufacturer's instructions.

2.3. Construction of gRNA coding vectors

The gRNAs for target loci 1, 2 and 4 of *Rosa26* locus and target 5 and 6 of *Hprt* were designed (Supplementary Table S1), and plasmid vectors coding gRNAs with T3 promoter for each targets were

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synthesized according to previous work [6]. We also used a gRNA, which has been used in previous study [6], as a gRNA for target 3. These vectors were sequenced as described above.

2.4. In vitro transcription of Cas9^{D10A} and gRNAs

For the in vitro synthesis of Cas9^{D10A} mRNAs, Cas9^{D10A}-coding vector was linearized by SphI and transcribed in vitro with T3-RNA-polymerase (Promega) in the presence of m7G(5′)ppp(5′)G to synthesize capped RNA. In the case of gRNAs, each gRNA vector was linearized by DraI and transcribed by the same procedure but without m7G(5′)ppp(5′)G to avoid the formation of cap structure. The RNA transcripts were precipitated with absolute ethanol, washed and resuspended in RNase-free water. The RNA solutions were stored at −80 °C until use.

2.5. Microinjection

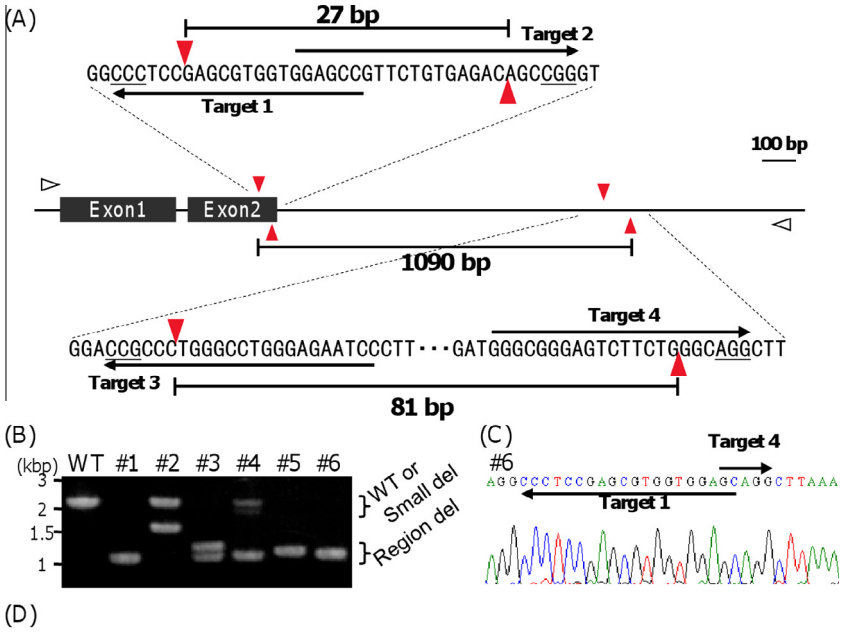
Sexually immature female C57BL/6NCr mice (4 weeks old) were superovulated by intraperitoneal injection of 7.5 IU eCG followed by 7.5 IU hCG at an interval of 48 h and mated overnight with C57BL/6NCr male mice that were >12 weeks old. Zygotes were collected after 20 h of hCG injection by oviductal flashing, and pronuclei-formed zygotes were put into the M2 medium. Microinjection was performed using a microinjector (Narishige) equipped microscope. Approximately 4 pl of RNA solution that contain 100 ng/μl of Cas9^{D10A} mRNA and 10 ng/μl of each gRNA were injected into the cytoplasm of each zygote using continuous pneumatic pressure. After injection, all zygotes were cultured in M16 medium and subjected for following experiments.

2.6. Embryo transfer and mutation analysis of obtained pups

Two-cell embryos injected with Cas9^{D10A} mRNA and gRNAs were transferred into the oviductal ampullas (10 embryos per oviduct) of 7 to 8-week-old female ICR mice mated with vasectomized ICR males at the previous night. After birth, genome DNA was extracted from the tail tips and subjected to PCR for on-target (Supplementary Table S1) and off-target loci (Supplementary Table S2) using the primers shown in Supplementary Table S3. PCR products were purified by agarose gel electrophoresis, and the extracted fragments were directly sequenced as described above. Predictive mutation pattern of each allele was analyzed by sequence data according to previous protocol [6].

2.7. Immunoblotting

Western blot analysis was performed according to standard protocol. One to 2 mm of tale tips captured from obtained and wild type pups were homogenized by potter homogenizer and were suspended in Laemmli buffer. The antibodies used were anti-Flag M2 monoclonal antibody (F1804, Sigma–Aldrich) and anti-β-actin polyclonal antibody (GTX109639, GeneTex, Inc., CA, USA). To visualize the protein-bound antibodies, horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (Jackson Immuno-Research Laboratories, Inc., West Grove, PA, USA) were used for



(D)

Transferred embryo	Total pups (%)	Mutated pups (%)	Region deletion (%)	Small deletion (%)
40	6 (15.0)	6 (100)	5*(83.3)	2**(33.3)

Fig. 1. Generation of region-deleted mice by the offset-nicking method. (A) Schematic illustration of the *Rosa26* gene structure and sequences around the target loci. The target sequences, PAM domain and PCR primer loci are indicated by arrows, underlining and white arrowheads, respectively. Red arrowheads show the break sites by Cas9 nickase and the distances between two break sites are indicated. (B) PCR analysis using tail tip DNAs of pups obtained from zygotes injected with Cas9^{D10A} mRNA and 4 kinds of gRNAs with a wild-type pup. PCR products of the wild type or small-deletion and region-deletion are indicated. (C) An example of a genome sequence in a region-deleted pup. The waveform data of a DNA sequencer obtained from a PCR fragment of tail tip DNA and the predicted sequence are shown with target positions. (D) Mutation efficiency and states of target sites in the obtained pups. *4 pups might have biallelic region deletion and 1 pup is monoallelic region deletion. **1 pup has a region deletion allele and double deletion allele with mosaicism, and the other pup has a double deletion allele and single deletion allele.

the second layer, respectively, followed by detection procedure using an ECL detection kit (Amersham-Pharmacia) according to the manufacturer's protocol.

3. Results and discussion

3.1. Generation of region deletion mice by offset-nicking method

First, we prepared 4 gRNAs, including a gRNA that showed a high off-target effect in a previous study [6], at two target sites of the Rosa26 locus (Fig. 1A and Supplementary Table 1), and tried to generate region-deleted mice, which deleted about 1000 base pairs between the two target sites using mouse zygotes. Forty mouse zygotes, in which Cas9D10A mRNA and 4 kinds of gRNAs were injected simultaneously, were embryo-transferred, and 6 pups were obtained. PCR analyses using tail tip DNA revealed the presence of mutations in all 6 pups (Fig. 1B and C). Direct sequencing analyses of the PCR products demonstrated that five of the pups (#1. #3–6) had region deletions between the two target sites, and four of these five pups (#1, #3, #5 and #6) might have had biallelic region deletions, while 1 pup (#4) had monoallelic region deletion and monoallelic double deletion with mosaicism (Fig. 1B, D and Supplementary Fig. 1). The remaining pup without region deletions had a double deletion allele and single deletion allele (#2 in Supplementary Fig. 1). These results show that the offset-nicking method could generate region-deletion mice with high efficiency.

In the present study, three pups (#1, #3 and #4) had a completely identical 1102-bp deletion. As there were homologous sequences around both ends of the deleted region, the deletion of this region might be promoted by a homologous recombination between the homologous sequences. In addition, only one kind of sequence could be detected in each of the two region-deletion pups (#5 and #6), indicating that the deletions in both alleles were completely the same in each of these two pups. This suggests that the deletion in one allele has some effects on the modification of another allele.

3.2. Offset-nicking-derived pups showed no off-target mutations

Of the four gRNAs used in the present study, the gRNA for target-3 showed high off-target effects in a previous study in which mutant mice were generated by the use of Cas9 nuclease on mouse zygotes [6]. In contrast, all six pups obtained in the present study by injection of the same concentration of Cas9 nickase mRNA had no off-target effects in any of the off-target sites (Supplementary Table 2), indicating that the off-target effects were also avoided in mouse zygotes by using the offset-nicking method.

3.3. Generation of knock-in mice by offset-nicking method

Second, we prepared paired gRNAs and single-stranded oligodeoxynucleotide (ssODN) template coding FLAG-tag, and tried to generate targeted knock-in mice by the offset-nicking method.

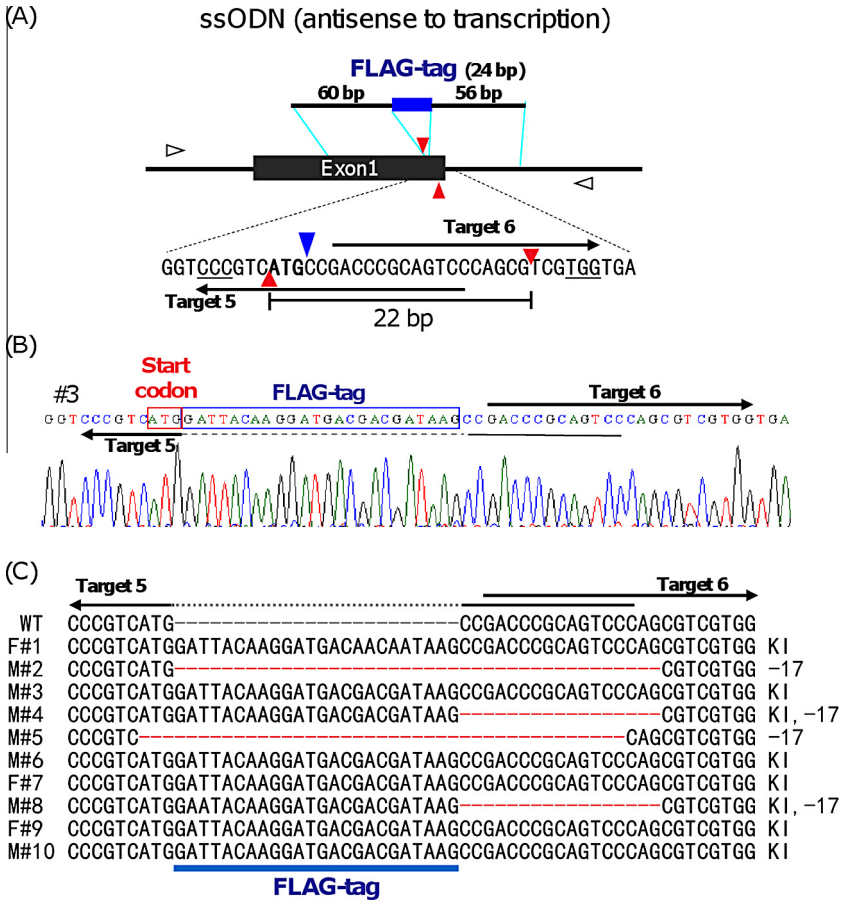


Fig. 2. Generation of knock-in mice by the offset-nicking method. (A) Schematic illustration of the *Hprt* gene structures, sequences around the target loci and ssODN template structure. The Target sequences, PAM domain and PCR primer loci are indicated by arrows, underlining and white arrowheads, respectively. The red arrowheads, blue arrowhead and blue box show break sites by Cas9 nickase, the FLAG-sequence insertion site and the FLAG-sequence in the ssODN template, respectively. The distances between two break sites are indicated. (B) An example of a genome sequence of a knock-in pup. A waveform data of a DNA sequencer obtained from PCR fragment of tail tip DNA and predicted sequence are shown with target positions, FLAG sequence and start codon. (C) Genome sequences around the target sites of pups obtained from the second experiment, in which the offset-nicking method was used to generate knock-in mice. The hyphens denote deleted nucleotides. F: female; M: male; KI: Knock-in.

Forty zygotes, which were injected with Cas9D10A mRNA, two gRNA targeted around the start-codon site of the Hprt gene and an ssODN template coding a FLAG-tag sequence with 60-bp and 56-bp homologous arms (Fig. 2A), were embryo-transferred, and 10 pups were obtained. PCR of the tale tip DNAs and direct sequencing identified that 8 of 10 pups were carrying an insertion of FLAG sequences with or without deletions, and the other two pups were carrying only deletion mutations (Fig. 2B and C). Only one kind of sequence could be detected in each of the 3 female pups (#1, #7 and #9), as shown in Fig. 2C, indicating the biallelic knock-in in these three pups. Western blot analysis of tail tip-derived proteins revealed specific bands at the predicted molecular weight of FLAG-tagged HPRT in 6 knock-in pups using FLAG-tag antibody (Supplementary Fig. 2). These results show that the offset-nicking method could efficiently generate targeted knock-in mice.

No off-target sites homologous with the 34-bp target sequence used in the present experiment could be found in the mouse genome sequences. Of the eight knock-in pups, two showed the same deletion at the target site of gRNA-6 (Fig. 2C). As the ssODN template used in the present experiment was the antisense strand of the Hprt gene, which included the target sequence and could be bound by gRNA-6, the template ssODN might be targeted by gRNA-6 and truncated by Cas9 nickase before contributing to the knock-in mouse generation. In order to avoid this risk, the ssODN template should be designed to be free of the target sequence or PAM domain. Alternatively, the use of a double-stranded oligodeoxynucleotide (dsODN) template should be effective not only to avoid the truncation by Cas9 nickase but also to allow for large-scale insertions longer than peptide-tags or loxP sequences, such as coding-gene insertions. The possibility of applying the offset-nicking method for the generation of knock-in animals using dsDNA templates should be investigated.

In summary, the present study revealed the availability of offset-nicking for the efficient generation of region-deleted and targeted knock-in mice without off-target risk. Hence, this system is expected to be useful as a standard genome modification technique in various organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.141.

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