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LETTER TO THE EDITOR

Generating rats with conditional alleles using CRISPR/Cas9

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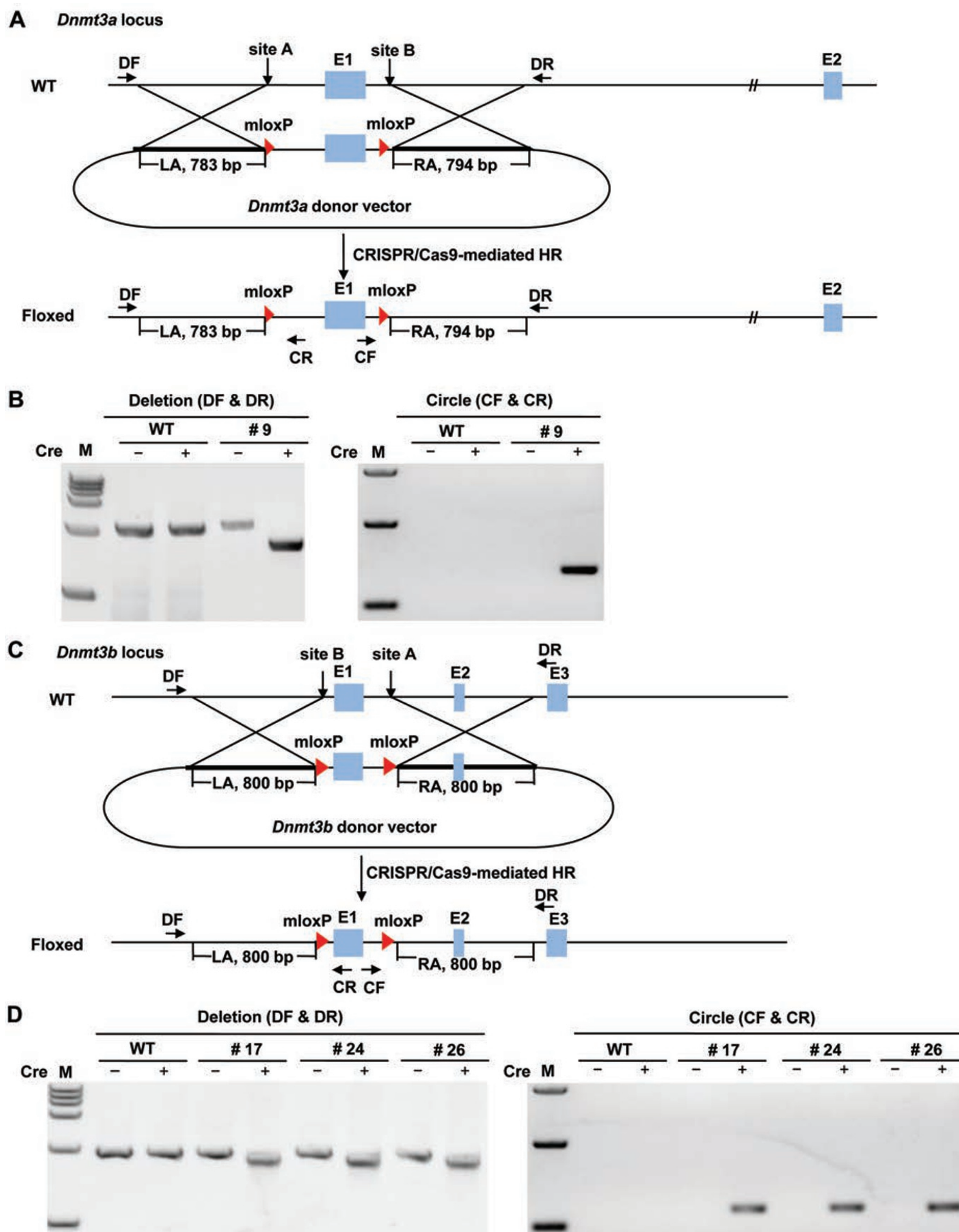
Dear Editor,

The rat is an important laboratory model and has many advantages over mouse models especially in toxicology and pharmacology studies. Several genome-editing technologies, such as zinc-finger nucleases (ZFNs) [1-2], transcription activator-like effector nucleases (TALENs) [3] and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9) system [4-5], have been used to produce knockout rat models by generating DNA double-strand breaks (DSBs) followed by non-homologous end joining (NHEJ)-mediated repair. However, the simple knockout strategies have limits for studying genes that are critical for embryogenesis. Conditional gene inactivation can circumvent this limitation and offers potentials to dissect the roles of such genes in specific tissues or developmental processes. Conditional gene modification is usually achieved by using the Cre/*loxP* system to inactivate a *loxP*-flanked (floxed) allele via Cre/*loxP*-mediated recombination. A recent report demonstrated that rats carrying a floxed gene can be successfully produced via microinjection of 2 pairs of ZFNs and 2 plasmid donors into fertilized eggs [2]. In comparison with ZFNs or TALENs, CRISPR/Cas9 provides a simpler way to edit the eukaryotic genome even in a multiplex manner [4-5]. Recently, mice carrying conditional alleles have been generated by using CRISPR/Cas9 system [7]. Here, we extend the application of the CRISPR/Cas9 system and report an effective strategy to generate rats with conditional alleles.

Three genes including *DNA (cytosine-5-)-methyltransferase 1* (*Dnmt1*), *DNA (cytosine-5-)-methyltransferase 3 alpha* (*Dnmt3a*), and *DNA (cytosine-5-)-methyltransferase 3 beta* (*Dnmt3b*) were selected to determine whether the CRISPR/Cas9 system could be used for the generation of gene-floxed rats. *Dnmt3a* and *Dnmt3b* are important for the establishment of *de novo* methylation in early development, while *Dnmt1* functions in the maintenance of methylation patterns [8]. For each targeting site, a single-guide (sgRNA) was designed using the rules described by Sapranauskas *et al*. [9] (Supplementary information, Figure S1 and Table S1). The Cas9 mRNA and sgRNA were transcribed by T7 RNA polymerase *in vitro* as described by Shen *et al*. [10]. A mixture of Cas9 mRNA (25 ng/µl) and sgRNA (10 ng/µl) was pooled with circular donor vectors (4 ng/µl), and microinjected into one-cell stage fertilized eggs of Sprague Dawley (SD) rat (Supplementary information, Table S2). The circular donor vector was used to minimize random integrations [2, 7].

For *Dnmt1* targeting, one sgRNA was designed to target a region downstream of the 3′ end of exon 1 (Supplementary information, Figures S2A and S4A). A circular donor vector containing exon 1 flanked by 2 *mloxP* sites and 2 homology arms of ~800 bp each was used as a template to repair the DSB by homologous recombination. In the donor vector, one *mloxP* site was located 3 bp upstream of the protospacer adjacent motif (PAM) of the sgRNA-targeting site, and the other was located upstream of exon 1. 131 injected zygotes were transferred to 4 pseudopregnant female SD rats and 12 pups were born (Supplementary information, Table S2). To detect the gene modifications, a pair of primers as indicated in Supplementary information, Figure S2A were used. The amplified fragment contains the whole right arm, part of the left arm, and the floxed exon 1 (Supplementary information, Figure S3A and Table S3). All PCR products were sub-cloned, and 20 clones for each rat were randomly selected for sequencing to detect the modifications (Supplementary information, Figure S4A). The results showed that 2 founder rats (#5 and #11) contained floxed exon 1 on the same allele. Interestingly, all the sequenced clones of founder #11 represented floxed alleles, suggesting a potential biallelic modification (Supplementary information, Figures S4A and S5A). Four rats (#2, #4, #8 and #10) only carried NHEJ-mediated mutations (Supplementary information, Figures S3A, S4A and Table S2). The genomic DNA of founder #11 was further analyzed by PCR using primers DF and DR to amplify the entire region covering the floxed exon1 and 2 homology arms. DNA sequencing of the PCR products confirmed the correct targeting (data not shown). More-



over, accurate excision of the floxed exon1 was further demonstrated by *in vitro* Cre/*loxP*-mediated recombination. The genomic DNA from the tail of founder #11 was incubated with Cre recombinase *in vitro*. Both truncated and circular products derived from Cre/*loxP*-mediated recombination can be detected by PCR amplifications (Supplementary information, Figure S2B). The PCR products were further sequenced, and the results con-

Figure 1 Generation of rats carrying floxed *Dnmt3a* or *Dnmt3b* by a two-cut strategy using the CRISPR/Cas9 system. **(A)** A schematic overview of the strategy to generate a *Dnmt3a* conditional allele. In the donor vector, *mloxP* sites are indicated as red triangles. The vector contains 2 homology arms of ~800 bp each flanking the *mloxP*-floxed exon 1. **(B)** *In vitro* Cre/*loxP*-mediated recombination of the floxed *Dnmt3a* allele. The genomic DNA of founder #9 was incubated with Cre recombinase. PCR analyses of the Cre-treated samples using primers DF and DR flanking the floxed allele produced shorter products. The circular product of Cre-mediated pop-out was also detected in founder #9 by CF and CR primers. The position of each primer is shown in **A**. The truncated fragment and circular PCR products were sequenced and the results are shown in Supplementary information, Figure S6A. **(C)** A schematic overview of the strategy to generate a *Dnmt3b* conditional allele. **(D)** *In vitro* Cre/*loxP*-mediated recombination of the floxed *Dnmt3b* allele. Primers DF/DR and CF/CR were used to amplify the truncated and circular products, respectively. The PCR products were sequenced and the results are shown in Supplementary information, Figure S6B. HR, homologous recombination; LA, left homology arm; RA, right homology arm.

firmed the accurate Cre/*loxP*-mediated recombination (Supplementary information, Figure S2B and S2C).

Considering that 2 sgRNAs can efficiently delete the intervening region [7] and thus might improve the targeting efficiency, we employed a two-cut strategy for *Dnmt3a* and *Dnmt3b* targeting. Two sgRNAs targeting exon 1 at 2 distinct locations were designed for each gene (Figure 1A, 1C, Supplementary information, Figure S4B and S4C). The circular donor vector for each gene contains 2 *mloxP* sites (each site locates 3 bp away from the corresponding PAM), the floxed exon1 and 2 homology arms (Figure 1A and 1C). For *Dnmt3a*, 178 injected zygotes were transferred into 6 recipients and 20 pups were born. Genotyping by PCR and sequencing showed that 6 rats (#6, #7, #8, #9, #11 and #18) contained floxed alleles. Among them, founder #9 likely contained floxed *Dnmt3a* on both alleles (Supplementary information, Figures S4B, S5B and Table S2), while for the other 5 founders, about half of the sequenced clones for each founder represented floxed alleles. For *Dnmt3b*, 149 injected zygotes were transferred into 5 recipients and 30 pups were born. Genotyping by PCR and sequencing showed that 9 rats (#6, #9, #13, #17, #19, #21, #24, #26 and #28) carried floxed alleles, and 3 of them (#17, #24 and #26) likely harbored biallelic mutations (Supplementary information, Figures S4C, S5C and Table S2). Seven *Dnmt3a* rats (#2, #4, #5, #10, #14, #16 and #20) and 6 *Dnmt3b* rats (#2, #11, #15, #16, #25 and #29) carried NHEJ-mediated indels (Supplementary information, Figure S4B, S4C and Table S2). PCR analysis using primers DF and DR, which yielded PCR products spanning the left and right arms, were performed to further examine the genomic DNA of founders that likely carried 2 floxed alleles. DNA sequencing of the PCR products further confirmed the correct targeting (data not shown). Similarly, *in vitro* Cre/*loxP*-mediated recombination was performed for all founders carrying floxed genes on both alleles (#9 for *Dnmt3a*, #17, #24 and #26 for *Dnmt3b*) (Figure 1B, 1D and Supplementary information, Figure S6A and S6B), and revealed an efficient Cre/*loxP*-mediated excision. Interestingly, the efficiency of generating rats carrying the conditional allele modification was ~16% (2/12) using one sgRNA, but increased to 30% (*Dnmt3a*, 6/20; *Dnmt3b*, 9/30) using 2 sgRNAs (Supplementary information, Table S2), indicating that the two-cut strategy significantly increases homologous recombination efficiency.

Next, primary fibroblast cells were isolated from the ears of biallele-floxed founder rats (*Dnmt1* #11, *Dnmt3a* #9, *Dnmt3b* #17) for *in vivo* Cre/*loxP*-mediated recombination assays. CMV-Cre was transfected into cultured rat fibroblasts, and the genomic DNA was isolated and analyzed by PCR. Consistently, truncated fragments derived from Cre/*loxP*-mediated recombination was detected by PCR amplification (Supplementary information, Figure S7B), confirming the correct Cre/*loxP*-mediated recombination. Then the expression of the target genes was assessed by reverse-transcription (RT)-PCR analysis using primers RT-F and RT-R (Supplementary information, Table S5). The results showed that no significant changes were detected in the mRNA levels of *Dnmt1*, *Dnmt 3a*, and *Dnmt3b* in conditional allele-carrying founders compared with those in wild-type rats (Supplementary information, Figure S7C), suggesting that the *mloxP* insertion did not affect the expression of target genes. In contrast, upon Cre transfection, the mRNA levels of *Dnmt1*, *Dnmt3a* and *Dnmt3b* decreased significantly, suggesting that the target genes were successfully disrupted by Cre/*loxP*-mediated recombination. It is worth noting that the incomplete *in vivo* recombination and gene disruption were likely due to the low transfection efficiency in primary rat fibroblasts.

Recent reports have suggested that the CRISPR/Cas9 system may tolerate sequence mismatches, and thereby generate off-target mutations [4-5, 7]. Therefore, we comprehensively investigated the potential off-target effects in mutant founders. We examined 9 potential off-target sites (OTS) for *Dnmt1*-A sgRNA, 7 OTS for *Dnmt3a*-A sgRNA, 45 OTS for *Dnmt3a*-B sgRNA, 4 OTS for *Dnmt3b*-A sgRNA and 9 OTS for *Dnmt3b*-B sgRNA. For each gene, 4 founders that contained CRISPR/Cas9-induced mutations were selected for off-target examina-

tion by the T7EN1 cleavage assay. Surprisingly, only 2 off-target mutations (*Dnmt1*-A OTS-3, *Dnmt3b*-B OTS-9) were detected from the total 74 OTS (Supplementary information, Figure S8, Tables S6 and S7), demonstrating that the CRISPR/Cas9 system is a reliable gene-targeting tool for rats. We also examined *Dnmt1*-A OTS-3 and *Dnmt3b*-B OTS-9 in the other corresponding founders by the T7EN1 cleavage assay and sequencing, and found that mutations at these 2 sites indeed occurred in 7 *Dnmt1* (7/12) and 9 *Dnmt3b* (9/30) founders, respectively (Supplementary information, Figure S9). Recent reports have also indicated that CRISPR/Cas9 induced off-target effects at a very low level in mouse and rat, suggesting that the potential off-target effect may not be a major concern for the application of the CRISPR/Cas9 system in genome modification [4-5, 7].

In summary, we described here for the first time the generation of rats carrying conditional alleles using the CRISPR/Cas9 system combined with a single circular donor vector. Our study provides a simple and flexible engineering strategy for the establishment of conditional knockout rats, which would facilitate the study of gene functions in a specific cell lineage or tissue in this model organism.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)