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Increasing the efficiency of CRISPR/Cas9-mediated precise genome editing in rats by inhibiting NHEJ and using Cas9 protein

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ABSTRACT
Precise modifications such as site mutation, codon replacement, insertion or precise targeted deletion are needed for studies of accurate gene function. The CRISPR/Cas9 system has been proved as a powerful tool to generate gene knockout and knockin animals. But the homologous recombination (HR)-directed precise genetic modification mediated by CRISPR/Cas9 is relatively lower compared with nonhomologous end-joining (NHEJ) pathway and extremely expected to be improved. Here, in this study 2 strategies were used to increase the precise genetic modification in rats. Scr7, a DNA ligase IV inhibitor, first identified as an anti-cancer compound, and considered as a potential NHEJ inhibitor, was used to increase the HR-mediated precise genetic modification. Meanwhile, the Cas9 protein instead of mRNA was used to save the mRNA to protein translation step to improve the precise modification efficiency. The Fabp2 and Dbnd1 loci were selected to knockin Cre and CreERT2, respectively. Our result showed that both Scr7 and Cas9 protein can increase the precise modification.

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated; sgRNA, single guide RNA; NHEJ, nonhomologous end joining; HR, homologous recombination; Fabp2, fatty acid binding protein 2; Dbnd1, dysbindin (dystrobrevin binding protein 1) domain containing 1

Introduction
Genome editing tools such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) have proved as effective tools for generation of genetically modified cells and animals. This strategy to generate genetically modified animals by injection of engineered nucleases into zygotes bypasses the embryonic stem cell (ESCs) and chimera animal stage. This is particularly suited for rat, an important laboratory animal model especially for the toxicology and pharmacology studies, for which the ES cells is inefficient and technically challenging.

Those engineered nucleases can cleave the defined DNA sequence to generate DNA double-strand breaks (DSBs) and stimulate DNA repair through nonhomologous end-joining (NHEJ) and/or homologous recombination (HR)-directed repair mechanisms. Precise genomic modifications such as site mutation, codon replacement, reporter insertion and conditional gene knockout are usually mediated by HR supplying with a DNA template. But NHEJ induced mutation occurs more frequently than HR-mediated precise genetic modification because NHEJ happens throughout cell cycle, while HR only in S and G2 phase. Precise genetically modified animals are expected especially for precise gene function analysis. In previous studies, we successfully applied the CRISPR/Cas9 to generate gene floxed allele and Cre knockin rats. But the efficiency of HR-mediated precise genetic modification by CRISPR/Cas9 is relatively low compared with NHEJ induced mutations. Therefore, the efficiency of HR-mediated precise genetic modification is still to be improved. In this study, the Scr7, a DNA ligase IV inhibitor was added to the microinjection mixture to inhibit NHEJ. Meanwhile the Cas9 protein was used to replace the Cas9 mRNA to enhance the precise genome modification.

Results and discussion
CRISPR/Cas9-mediated Cre insertion at endogenous Fabp2 locus
In our previous work, we have succeeded in generating conditional gene knockout and fragment knockin rats using 2-cut strategy. But the NHEJ induced mutation at a much higher efficiency than HR-mediated precise genetic modification. Therefore, to improve the HR-mediated precise genetic modification by CRISPR/Cas9 is extremely expected, especially for large fragments manipulation. In this study we employed the 2-cut strategy used as before. The sgRNA targeting sites were selected and assembled as previously described (Table S1). The circular donor plasmid was used as template to induce HR-mediated precise genetic modification (Sequence S1).
Microinjection was performed in one-cell embryos of Sprague Dawley (SD) rats (Table 1). We first tried to knock in the Cre coding sequence to the N terminus of an interesting gene, fatty acid binding protein 2 (Fabp2). The Fabp2 functions in fatty acid absorption, and intracellular transport of dietary long chain fatty acids. To achieve Fabp2 targeting, 2 sgRNA targeting sites were selected, one located upstream of exon 1, the other one located on the exon 1 (Fig. 1A). The circular donor plasmid was designed to fuse a Cre-p2a sequence with the start codon of Fabp2 gene. This circular donor plasmid donor-Cre-p2a-Fabp2, which contains 2 ~ 500 bp homology arms flanking the Cre coding sequence, was severed as template for HR-mediated modification (Supplementary sequence). Schematic overview of the strategy to generate Fabp2-Cre knockin rats was shown in Fig. 1A. A total of 81 injected zygotes were transferred to 3 pseudopregnant female SD rats and 11 pups were born (Table 1). The primers (Del-F/Del-R) flanking the 2 sgRNA target sites were used to detect the mutations from NHEJ (Fig. 1A, Table S2). To detect the HR-mediated modification, we designed 2 pairs of primers (upstream and downstream). For the upstream primer pair (Up-F/Up-R), one locates out of the left homologous arm and the other localizes inside the Cre coding sequence. For the downstream primer pair (Dw-F/Dw-R), one locates out of the right homologous arm and the other localizes upstream of the Up-R primer in the Cre coding sequence. An overlap region was included in the upstream amplicon and downstream amplicon (Fig. 1A, Table S3). All PCR products were sequenced to characterize the mutations (Fig. 1B). Five rats with the efficiency up to 45% (5/11) (potential founders #1, #3, #4, #10, #11) had NHEJ-induced mutations (Fig. 1B, Table 1, Fig. S1 and Table S3). Two rats, with efficiency of 18% (potential founders #6, #7) had Cre insertion at Fabp2 locus. The Cre insertion was further confirmed by sequencing (Fig. 1D and Table 1). These results suggested that selected sgRNA targeting sites accompanied with donor can be applied to insert the Cre coding sequence at Fabp2 locus. Our results also showed that the length of homologous arm can be as short as ~500 bp.

**Scr7 enhances the efficiency of Cre insertion at Fabp2 locus**

Enhancement of the HR-mediated precise modification could be achieved by inhibiting NHEJ or improving HR. Scr7, first identified as a potential drug for cancer treatment, inhibits DNA ligase IV which is critical for patching DSBs in NHEJ pathway. Recently, the Scr7 has been reported to increase the rates of HR-mediated modification in mammalian cells. To assess the effects of Scr7 on the insertion rate of large DNA fragment at endogenous locus, the Scr7 (at final concentration of 0.5 μM, 1 μM, 2 μM) was added to the CRISPR/Cas9 mixture (Cas9 mRNA, sgRNAs and circular donor plasmid) for microinjection to produce Fabp2-Cre knockin rats.

The results showed, with 0.5 μM Scr7, a total of 152 injected zygotes were transferred to 5 pseudopregnant female SD rats and 23 pups were born (Table 1 and Fig S2). The above described primer pairs (Del-F/Del-R, Up-F/Up-R and Dw-F/Dw-R) were used to detect the CRISPR/Cas9-mediated mutations. All PCR products were sequenced to characterize the mutations (Fig. S2). Ten rats with the efficiency up to 43% (10/23) (potential founders #3, #6, #8, #11, #13, #14, #15, #18, #21, and #22) had NHEJ-induced mutations (Table 1 and Table S3). Five rats, with efficiency of 21% (potential founders #10, #13, #16, #17, and #19) had Cre insertion at Fabp2 locus (Fig. 1C, Table 1, and Fig. S2). With 1 μM Scr7, a total of 130 injected zygotes were transferred to 5 pseudopregnant female SD rats and 28 pups were born (Table 1 and S3). The PCR and sequencing results showed that 14 rats with the efficiency up to 50% (14/28) (potential founders #1, #2, #5, #6, #7, #9, #11, #16, #18, #19, #22, #23, #24, and #25) had NHEJ-induced mutations (Fig. S3, Table 1 and Table S3). Eleven rats, with efficiency of 39% (11/28) (potential founders #1, #5, #9, #10, #11, #12, #13, #18, #20, #26, and #28) had Cre insertion at Fabp2 locus (Fig. 1C, Fig. S3, and Table 1). With 2 μM Scr7, a total of 115 injected zygotes were transferred to 4 pseudopregnant female rats. Nine pups were born (Table 1). The results showed that 3 rats with the efficiency up to 33% (3/9) (potential founders #1, #3, and #4) had NHEJ-induced mutations (Fig. S4, Table 1 and Table S3). Three rats, with efficiency of 33% (3/9) (potential founders #2, #6, and #7) had correct Cre insertion at Fabp2 locus (Fig. 1C, Fig. S4, and Table 1). These results showed that, with Scr7, the precise insertion efficiency was much higher. Furthermore, the result suggested the optimal concentration of Scr7 is 1 μM (Fig. 1C and Table S3).

**Table 1. Summary of generation of Fabp2-Cre and Dbndd1-CreERT2 knockin rats.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Injection mix</th>
<th>Scrm (μM)</th>
<th>Blastocyst/Injected zygotes</th>
<th>Transferred embryos (Recipients)</th>
<th>Newborns (Dead)</th>
<th>Corrected Knockin Rats</th>
<th>Indels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fabp2</td>
<td>Cre insertion</td>
<td>Cas9 mRNA (25ng/μl) + sgRNA (10 ng/μl/each)</td>
<td>0.5</td>
<td>210/127</td>
<td>81 (3)</td>
<td>11 (0)</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>280/175</td>
<td>130 (5)</td>
<td>28 (0)</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>300/150</td>
<td>115 (4)</td>
<td>9 (0)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Dbndd1</td>
<td>CreERT2 insertion</td>
<td>Cas9 mRNA (25ng/μl) + sgRNA (10 ng/μl/each)</td>
<td>0</td>
<td>270/176</td>
<td>98 (4)</td>
<td>13 (0)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>390/190</td>
<td>140 (5)</td>
<td>17 (0)</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>320/171</td>
<td>135 (5)</td>
<td>29 (0)</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>265/135</td>
<td>100 (4)</td>
<td>13 (0)</td>
<td>10</td>
<td>2</td>
</tr>
</tbody>
</table>

Cas9 mRNA (25 ng/μl) or protein (30 ng/μl), sgRNA (10 ng/μl/each) targeting Fabp2, Dbndd1 together with a plasmid template (4 ng/μl) for each gene were injected into fertilized eggs. The oligos for sgRNAs plasmid preparation were shown in Table S1. The sequences of plasmid template were shown in supplementary sequence. Primers used for detection and sequencing were shown in Table S2.
Scr7 enhances the efficiency of precise modification at Dbndd1 locus

To further confirm the effect of Scr7 in CRISPR/Cas9-mediated precise modification in rats, we knockin the CreER<sup>72</sup> cassette to C terminus of Dbndd1 gene. The Dbndd1-CreER<sup>72</sup> rats were produced by pronuclear co-injection of Cas9 mRNA, sgRNAs, and donor plasmid together with or without Scr7. The donor plasmid was designed to fuse a p2a-CreER<sup>72</sup> sequence with the last codon of the Dbndd1 gene (Fig. 2A). The circular donor plasmid contains ~0.8 kb left homology arm and ~1.2 kb right homology arm flanking the p2A-CreER<sup>72</sup> cassette (Fig. 2A, Supplementary sequence). A similar strategy was used to detect the modifications in Dbndd1 locus (Fig. 2B, Table 1 and Table S2). At the absence of the Scr7, 98 injected zygotes were transferred to 4 recipients and 13 pups were obtained (Table 1). The results showed that 6 (potential founders #4, #6, #7, #8, #9, #13) out of 13 rats with the efficiency up to 46% contained the CreER<sup>72</sup> insertion (Fig. 2F, Table 1, and Table S4). Six rats (potential founders #1, #2, #3, #6, #9, #11) had the NHEJ-induced indels.
Figure 2. Generation of rats carrying CreERT2 coding sequence at endogenous Dbndd1 locus. (A) Strategy overview for the insertion of a CreERT2 coding sequence at endogenous Dbndd1. The sgRNA targeting sites are shown as black arrows and the sgRNA sequences are showed underlined in blue and the PAM in red. The template donor plasmid was designed to fuse p2a-CreERT2 to the last codon of Dbndd1 gene. Targeting sites in donor plasmid were mutated to avoid the cleavage recognized by sgRNAs. (B) The PCR amplification of Cas9/sgRNA-mediated Cre insertion at the endogenous Dbndd1 locus. Up: PCR amplification of upstream of the insertion part including the left homologous arm and part of CreERT2 coding sequence using Up-F/Up-R primers. The Up-R primer should be located out of the left homologous arm. Down: PCR amplification of the downstream of the insertion part including the right homologous arm and part of Cre coding sequence using Dw-F/Dw-R primers. Dw-R primer should be located out of the right homologous arm. Indel: PCR amplification of Cas9/gRNA induced NHEJ modification at the endogenous Dbndd1 locus. The primers used for PCR amplification and sequencing were indicated in A and shown in Table S2. (C) Purification of recombinant Cas9 protein. The Cas9 was cloned to pET28a vector and expressed in E. coli strain BL21 (DE3). Lane 1 was loaded with pET28a contained control E. coli. Lane 2 was loaded with pET28a-Cas9 expression E. coli. Lane 3 and Lane 4 are the purified Cas9 protein. (D) In vitro DNA cleavage assay to detect the purified Cas9 protein activity. Different concentration of purified Cas9 protein mixed with gRNA (100 nM) were used to digest 100 ng plasmid DNA at 37 °C for 1 h. Lane P: control plasmid with Nestin sgRNA targeting site and no Cas9 protein was added. Lane 1:1 μl of Cas9 protein ordered from company (M0306S, NEB). Lane 2-6: DNA was digested with purified Cas9 protein (1 μl, 1/10 μl, 1/20 μl, 1/30 μl, 1/40 μl, respectively). (E) Increased frequency of Cas9/sgRNA-mediated CreERT2 insertion at Dbndd1 locus. The Cas9 mRNA was replaced with Cas9 protein in the Cas9/sgRNA components for zygotes microinjection. The Scr7 at the concentrations 1 μM was added to the Cas9/sgRNA mixture (Cas9 mRNA/protein, sgRNAs and circular donor plasmid) for microinjection. The pups with specific band were counted as CreERT2 insertion. (F) The chromatographs of CreERT2 integration at the Dbndd1 locus.
(Fig. S5, Table 1, and Table S4). At the presence of Scr7 (1 μM), 140 injected zygotes were transferred to 5 recipients and 17 pups were born (Table 1). Our results showed that 11 rats (potential founders #1, #6, #7, #8, #9, #10, #11, #13, #14, #15, #16) with the efficiency up to 64% contained the CreER2 insertion (Fig. S6, Table 1, and Table S4). The rats carry NHEJ induced indels at an efficiency of 41% (7/17) (potential founders #1, #4, #7, #8, #13, #14, #15) (Fig. S6, Table 1, and Table S4). Our results further confirmed that the Scr7 can increase the efficiency of CRISPR/Cas9-mediated precise modification in rats (Fig. 2B and E, Fig. S6, Table 1 and Table S4).

Cas9 protein instead of mRNA can increase the efficiency of HR-mediated gene insertion

The Cas9 protein instead of mRNA for pronuclear microinjection can avoid the translation steps may thereby increase the HR-mediated modification in rats. To test the possibility, we use the Cas9 protein instead of the Cas9 mRNA to generate Dbdndd1-CreERT2 rats. The Cas9 proteins with expected size of 160 kDa were purified as described and showed only one major band (Fig. 2C). The Cas9 protein activity was tested by in vitro cleavage assay (Fig. 2D). For microinjection, 30 ng/μl Cas9 protein was used. A total of 135 injected zygotes were transferred to 5 recipients and 29 pups were delivered (Table 1). The results showed that 15 rats (potential founders #3-8, #12, #14, #16, #17, #21, #22, #24, #26, #27) with the efficiency up to 51% (15/29) contained the CreER2 insertion (Fig. S7, Table 1, and Table S4). Five rats (potential founders #1, #3, #11, #20, #28) with efficiency of 17% (5/29) had indel mutations in the target sites (Fig. S7, Table 1, and Table S4). The results showed that Cas9 protein significantly improves the CRISPR/Cas9-mediated precise modification in rats (Fig 2B and 2E, Fig. S7, Table 1, and Table S4).

Then, we ask whether the insertion efficiency could be improved further by combined using Cas9 protein and Scr7. To this end, we added the Scr7 (at final concentration of 1 μM) to the CRISPR/Cas9 mixture (Cas9 protein, sgRNAs and circular donor plasmid) for microinjection. A total of 100 injected zygotes were transferred to 4 recipients and 13 pups were born (Table 1). Our results showed that 10 rats (potential founders #1-3, #5, #6, #8-12) with the efficiency up to 76% (10/13) contained the CreER2 insertion (Fig. S8, Table 1, and Table S4). Two rats (potential founders #8, #9) with efficiency of 15% (2/13) had indel mutations (Fig. S8, Table 1, and Table S4). The results showed that Cas9 protein instead of Cas9 mRNA at the presence of Scr7 further improve CRISPR/Cas9-mediated precise modification in rats (Fig. 2E).

Off-target and transmission analysis

Although recent work suggested that CRISPR/Cas9 induced off-target mutations are rare in mice, our previous work showed that CRISPR-Cas9 induces off-target effects at an even lower level. Therefore, using the online off-target analysis tool, we examined 33 potential off-target sites (OTS) for Fabp2-A sgRNA, Fabp2-B sgRNA, Dbdndd1-A sgRNA and Dbdndd1-A sgRNA (Table S5). Primers designed for PCR amplification of potential off-target sites were shown in Fig. S6. The PCR products are subject to the T7EN1 cleavage assay, but no off-target site was found (data not shown). Our work further confirmed that the CRISPR/Cas9 induced off-target effect is not a major concern for application in rats.

In addition, we tested the transmission of gene modifications by crossing genetically modified F1 rats (potential founders #12 and #2 from Fabp2-Cre with Scr7 at 1 μM; #8 from Dbdndd1-CreERT2 with Cas9 protein and Scr7 at 1 μM) with wild-type SD rats. The F1 offspring showed the similar genotyping PCR bands with their parents, suggesting that mutations induced by Cas9:sgRNA have been successfully transmitted through germline (Fig. 3).

In summary, our studies demonstrated that using Cas9 protein and the addition of Scr7 to the microinjection mixture to inhibit NHEJ can increase the efficiency of the CRISPR/Cas9-mediated precise modification in rats significantly.

Materials and methods

Animals

Rats were housed in a standard facility in an Assessment and Accreditation Of Laboratory Animal Care-accredited animal facility and fed a standard diet. All the rat experiments were approved by the Animal Care and Use Committee of the Institute of Laboratory Animal Science of Peking Union Medical College (ILAS-GC-2015-001).

DNA constructs

The sgRNA constructs were prepared by cloning the ordered oligonucleotides into the Bsa I site of pUC57-sgRNA (Addgene, 51132). The oligonucleotides for the gRNAs construction are listed in Table S1. All donor plasmids were constructed on the basis of the Brown Norway rat genomic sequence (assembly Rnor_6.0) and performed by Taihe biotech (Beijing, China). The donor template sequences were cloned into pGSI plasmid (Supplementary sequence).

In vitro transcription

Both Cas 9 mRNA and sgRNA were prepared as before, and purified using the MEGAClear Kit (Ambion, AM1908). All solutions used for Cas9 mRNA and sgRNA preparation were RNase-free.

S. pyogenes Cas9 purification

The plasmid pMJ825 (addgene, 39315) was mutated the Alanine at 10 of Cas9 back to Aspartate (A10D) and cloned into pET28a vector named pET28a-Cas9. The pET28a-Cas9 plasmid with a histag at the N terminal of Cas9 and expressed in E. coli BL21 (DE3) to collect the Cas9 protein (Supplementary sequence). The E. coli BL21 with pET28a-Cas9 plasmid was cultured in Luria-Bertani (LB) medium with 50 µg/mL kanamycin at 37°C at 200 rpm. The Cas9 protein purification procedure was performed according to the protocol described by Jinek et al. In brief, after the overnight culture, 1% inoculum was transferred to a fresh LB broth (1 L) and incubated at 37°C with shaking.
(220 rpm) till OD600 = 0.4~0.8 (about 2.5 ~ 3 h), IPTG was added to a final concentration of 1 mM for 20 h at 20°C with shaking (220 rpm). Cells were harvested and washed with PBS and resuspended in 20 ml lysis Buffer (20 mM Tris pH 8.0, 0.5 M NaCl, 1 mM PMSF). Cells were sonicated at the condition of 20 kHz, 30 cycles of 10 s shock followed by 10 s of break. After sonication, the cellular debris and total proteins were separated by centrifugation at 27,000 × g for 45 min at 4°C. After removing impurities by 0.4 μM filter, the supernatant was loaded on 10 ml Ni-NTA agarose (Qiagen). The agarose was washed extensively with 20 mM Tris pH 8.0, 500 mM NaCl and 10 mM - 350 mM imidazole by using FPLC (Bio-Rad). Dialysis was performed to remove imidazole. After affinity chromatography, the Cas9 protein purified with SP sepharose HiTrap column (GE Life Sciences), eluted with a linear gradient of KCl (100 mM ~ 1M). After dialysis, Cas9 was further purified by size chromatography on a superdex 200 16/60 column in PB buffer pH 7.5 prepared by DEPC treated water. After concentration determination, the purified Cas9 protein was subpackaged into 0.6 ml tubes. After flash-frozen in liquid nitrogen, the Cas9 protein was store at −80°C and prepared for use.

**In vitro cleavage assay using Cas9 proteins**

The Nestin target site (gggccagaccagcagacgag) was used to detect the Cas9 activity. The targeting site was amplified using primer: Cas9 dect-F1 (5'-GCACCTTTGTTTCTTCTT

![Figure 3. Genotyping of the F1 rats by crossing the potential founders with wildtype SD rats. (A) PCR amplification of Fabp2 locus in 9 F1 pups derived from founder #12. The potential founder #12 (Fig. S3) was crossed with wildtype SD rats and determined the genotypes of the F1 to confirm the transmission of Cre insertion at Fabp2 locus. The Cre insertions were detected in 5 F1 pups (#2, #3, #5, #7, and #8). (B) PCR amplification of Fabp2 locus in 7 F1 pups derived from founder #2. The potential founder #2 (Fig. S3) was crossed with wildtype SD rats and determined the genotypes of the F1 to confirm the transmission of NHEJ induced mutation. Mutations were detected in all F1 pups for their parents are modified bi-allelic. (C) PCR amplification of Dendd1 locus in 9 F1 pups derived from founder #8. The potential founder #8 (Fig. S8) was crossed with wildtype SD rats and determined the genotypes of the F1 to confirm the transmission of modifications at Fabp2 locus. The CreERT2 insertions were detected in 5 F1 pups (#2, #4, #5, #8, and #9) and NHEJ induced indels were detected in 4 F1 pups (#1, #3, #6, and #7).]
CTGC-3′) and Cas9 dect-R1 (5′-GTCTCTTGCCATCTAAAG-TACTTCCCT-3′) from the rat tail genomic DNA. The expected amplicon was 1148 bp. The PCR product was TA cloned to pMD18T vector and sequenced. The target construct (3.8 kb) contains the target site was digested in the presence of Cas9 protein/sgRNA complexes at 37°C for 1 h. An ordered Cas9 protein (M0386S, NEB) was used as the positive control. The digested samples were analyzed on the 1% agarose gel (Fig. 2D).

**Cas9/sgRNA injection into fertilized rat eggs**

Sprague Dawley rats purchased from Beijing Vital River Laboratories animal center are housed with a standard condition. In brief, Zygotes were obtained from female donor rats mated with SD males after treatment with 30 units of pregnant mare serum gonadotropin (PMSG, Sigma-Aldrich) and 30 units of human chorionic gonadotropin (hCG, Sigma-Aldrich). Microinjections were performed in fertilized eggs using a Nikon Microinjection system under standard conditions. Microinjections were performed both in the cytoplasm and male pronucleus as described. After microinjection, the zygotes were transferred to pseudopregnant SD rats (20–30 zygotes per pseudopregnant SD rat). Cas9 mRNA (20 ng/μl) /Cas9 protein (30 ng/μl) and sgRNAs (10 ng/μl/each) for targeting genes were mixed together with Scr7 (0.5 μM, 1 μM or 2 μM) for microinjection. For microinjection with Cas9 protein, the injection mixture was pre-warmed at 37°C for 20 min before microinjection.

**Genomic DNA preparation and genotyping**

Genomic DNA was prepared according to the methods described before and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). The PCR primers were designed according to the targeting sequence and insertion sequence. The PCR primers used for genotyping of the genetically modified rats were shown in Fig. 1A, Fig. 2A and the sequences were listed in the Table S2. The PCR products were sub-cloned for sequencing analysis by using a TA clone kit (Takara).

**Germline transmission**

Germline transmission of the gene modified rats was confirmed by offspring genotyping. The selected potential founder rats are crossed with wild-type SD rats. The offspring with identical genotype to their parents was considered as successful germline transmission.

**Methods**

Methods and any associated references are available in the Supplementary Information.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Author contributions**

L. Z., X. H., and Y. M. designed experiments. Y. M., W. C., X. Z., Y. L., S. P., and W. D. performed the experiments. Y. M., X. H., and L. Z. analyzed the data and wrote the manuscript.

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