Heritable Multiplex Genetic Engineering in Rats Using CRISPR/Cas9

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Abstract

The CRISPR/Cas9 system has been proven to be an efficient gene-editing tool for genome modification of cells and organisms. Multiplex genetic engineering in rat holds a bright future for the study of complex disease. Here, we show that this system enables the simultaneous disruption of four genes (ApoE, B2m, Prf1, and Prkdc) in rats in one-step, by co-injection of Cas9 mRNA and sgRNAs into fertilized eggs. We further observed the gene modifications are germline transmitted, and confirmed the off-target mutagenesis and mosaicism are rarely detected by comprehensive analysis. Thus, the CRISPR/Cas9 system makes it possible to efficiently and reliably generate gene knock-out rats.

Introduction

Rats have returned back to the laboratory as a “renaissance animal” and are becoming an indispensable experimental animal model for understanding the human genome through the establishment of biological links between disease phenotypes and genetic networks. Gene targeting via embryonic stem (ES) cells provides a powerful tool for the generation of precise genetic alterations, but genome manipulation in rat ES cells is inefficient and technically challenging [1–2]. Other genome-editing technologies, such as zinc-finger nucleases (ZFNs) [3–4] and transcription activator-like effector nucleases (TALENs) [5–7] have proven effective for genomic manipulation but are limited because of the need to engineer specific protein pairs for each target site. Recently, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9) system has been proven to a simpler way to edit the eukaryotic genome even in a multiplex manner [8–14]. A small single-guide RNA (sgRNA) is produced by fusion of the crRNA and tracrRNA sequences, as the template for in vitro transcription using the T7 Ultra Kit (Ambion, AM1908). Cas9 protein and sgRNA are the only components necessary for the induction of targeted DNA cleavage in zebrafish [13], mammal cells [8,14] and mice [12–14]. Furthermore, Cas9 and sgRNA can effectively disrupt multiple genes in cells and different organisms [8,14]. Here, we extend the application of the CRISPR/Cas9 system to multiplex genetic engineering in the rat.

Materials and Methods

Animals

Rats were bred in standard cages in an Assessment and Accreditation Of Laboratory Animal Care-accredited SPF animal facility. All animal protocols were approved by the Animal Care and Use Committees of the Institute of Laboratory Animal Science of Peking Union Medical College (ILAS-GC-2010-044).

DNA constructs

The paired synthesized oligonucleotides for sgRNAs were annealed and cloned into the pUC57-sgRNA expression vector (Table S1 and Fig. S1 in File S1). The oligonucleotide sequences are listed in Table S1 in File S1.

In vitro transcription

The Cas9 expression plasmid was linearized with Age I and used as the template for in vitro transcription using the T7 Ultra Kit (Ambion, AM1345) [12]. sgRNA expression plasmids were linearized with Dna I and used as templates for in vitro transcription using the MEGAplescript Kit (Ambion, AM1354). Transcribed Cas9 mRNA and sgRNA were both purified by using the MEGAclear Kit (Ambion, AM1908).

Cas9/sgRNA injection into fertilized rat eggs

Sprague Dawley (SD) rats purchased from Beijing Vital River Laboratories animal center are housed in standard cages and maintained on a 12-h light/dark cycle with food and water. The microinjection of fertilized rat eggs was described previously [3].
In brief, four-week-old donor rats were injected with 30 units of pregnant mare serum gonadotropin (PMSG, Sigma-Aldrich), followed by an injection of 30 units of human chorionic gonadotropin (hCG, Sigma-Aldrich) 48 h later, and immediately mated with SD males. Zygotes were obtained on the next day and cultured in KSOM (Millipore) at 37°C, 5% CO2 for 2 h and then prepared for microinjection. In the first experiment, zygotes were injected with a mixture of Cas9 mRNA (20 ng/µl) and sgRNAs containing 8 sgRNAs (5 ng/µl) targeting site A. The corresponding PCR products were sub-cloned in the T7EN1 cleavage assay indicated modification of the targeted loci were amplified as 500 bp fragments around each targeting site (Fig. S2 and Table S3 in File S1). The PCR products were digested by T7 endonuclease 1 (T7ER1) and analyzed on the FlowJo software.

T7EN1 cleavage assay

Genomic DNA was extracted from the tails of 7-day-old rats using phenol-chloroform and recovered by alcohol precipitation. The T7EN1 cleavage assay was performed as described by Shen et al. [12]. PCR was used to amplify the targeting loci using the following conditions: 95°C, 5 min; [95°C 30 s, 63°C 30 s, 72°C 40 s] ×35 cycles; 72°C 10 min; hold at 4°C. PCR products were purified using a PCR clean-up kit (Axygen, AP-PCR-50). Purified PCR products were denatured and re-annealed in NEB Buffer 2 (NEB) prior to digestion with T7EN1 (NEB, M0302L) for 40 min and separation by 2% agarose gel electrophoresis. Cleavage bands in the T7EN1 cleavage assay indicated modification of the targeting site. The corresponding PCR products were sub-cloned for sequencing analysis to detect mutations. The PCR primers used to amplify sgRNA target fragments of ApoE, B2m, Ptf1 and Prkdc are listed in Table S3 in File S1. If no wild type allele was observed by sequencing, we assumed the rat had bi-allelic mutation. If wild type allele was detected by sequencing, we assumed the rat harbored monoallelic mutation. If more than two genotypes were detected by sequencing, we assumed the rat had mosaic mutation.

Identification and analysis of off-target sites

The following potential off-target loci were searched by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using 20 bp sgRNA sequence closed to the PAM (N/G/A)G and PAM, as NN NNNNN NNNNN A/G/A/G; NN NNNNN NNNNN C/G/A/G; NN NNNNN NNNNN G/G/A/G; and NN NNNNN NNNNN T/G/A/G, where N is the seed base matching the target site exactly [10,16–17]. The ApoE, B2m, Ptf1, and Prkdc genes were applied to off-target assays and potential target sites (OTS) highly homologous to the target sites summarized in Table S5 in File S1. The selected OTS were amplified from tail genomic DNA and subjected to the T7EN1 cleavage assay and sequencing analysis.

Germline transmission assay

Germline transmission of the modified genes was determined in the F1 rats from crossing an F0 rat with a wild-type SD rat. To determine the genotypes of the F1 offspring, PCR products of tail genomic DNA from F1 rats were subjected to the T7EN1 cleavage assay and sequencing analysis. Detection of mutation from F1 rats identical to the mutation from F0 parents was considered as germline transmission.

Biochemical parameters

Fasting blood sample was collected from tail of the rat after hungry for the night. Whole blood was centrifuged at 3000 g for 15 min at room temperature to obtain the serum and prepared for serum total cholesterol (CHO), triglycerides (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) detection using HITACHI 7100 Automatic Analyzer.

Flow cytometry analysis

The peripheral blood cells were lysed with BD Pharm Lyse™ Lysing buffer and filtered with a sterile nylon mesh. After counted, cells were stained with B cell marker, Anti-Rat CD45RA APC (eBioscience) and T cell marker Anti-Rat CD3 PE (eBioscience). Data acquisition was performed on FACS Aria I (Becton Dickson) and analyzed on the FlowJo software.

Western blot analysis

Equal amounts of soluble protein were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Immobilon NC; Millipore, Molshiem, France). Immunoblotting was carried out with antibodies specific for B2M (1:1000, proteintech). Primary antibodies were visualized with anti-rabbit HRP-conjugated secondary antibodies (Santa Cruz) using a chemiluminescent detection system (Western blotting Luminal Reagent; Santa Cruz). Variations in sample loading were corrected by normalizing to β-actin levels.

Results

Generation of multiple gene modification using one sgRNA for each gene by zygote injection

Four genes, including apolipoprotein E (ApoE), beta-2 microglobulin (B2m), perforin 1 (Ptf1), and protein kinase DNA-activated catalytic polypeptide (Prkdc), were selected to determine whether the CRISPR/Cas9 system could be used to disrupt multiple genes in rats. The CRISPR/Cas9 targeting sites were designed using the rules described as before [10,16–17]. The Cas9 (Addgene No. 44758) and sgRNAs were transcribed by T7 RNA polymerase in vitro as described by Shen et al. [Fig. S1 in File S1]. Twenty nanogram Cas9 mRNA and sgRNA mixtures were pooled and microinjected into one-cell fertilized eggs of SD rats (Table S2 in File S1).

A mixture of 4 sgRNAs, each targeting one gene (targeting site A) (Fig. 1b), was used at 10 ng/µl/sgRNA. Total 125 injected zygotes were transferred to 5 pseudopregnant female SD rats, and 15 pups were born (Table S2 in File S1). To detect the gene modifications, the targeted loci were amplified as 500–700 bp fragments around each targeting site (Fig. S2 and Table S3 in File S1). The PCR products were digested by T7 endonuclease 1 (T7EN1), an enzyme that is capable of recognizing and cleaving mismatched DNA (Fig. 1a). The results showed, the cleavages were detected at all the 4 targeted sites with efficiency of 73.33% at ApoE (founders #1, #2, #4~6, #8~10, #13~15), 60% at B2m (founders #1~3, #6, #9~11, #13, #15), 26.67% at Ptf1 (founders #5, #8, #13, #14), and 66.67% at Prkdc (founders #2, #4, #7, #8, #10~15), respectively (Fig. 1a, Table 1). Further sequencing confirmed the loci were indeed mutagenized in these rats (Fig. 2). Our results indicated the CRISPR-Cas9 function efficiently at all targeted loci. Furthermore, the T7EN1 cleavage assay and sequencing results showed three rats (3/15) contained one mutant gene, six rats (6/15) contained two mutant genes, five rats (5/15) contained three mutant genes, and one rat (1/15) contained four mutant genes (Fig. 1b, Table S4 in File S1). It
suggests that Cas9:sgRNA system can disrupt multiple genes in one step.

Two sgRNAs were used for multiple genes modification in rats

Considering that two sgRNAs targeting adjacent sites efficiently deleted the intervening region in cells [8], we tested whether using dual sgRNAs targeting at one gene enable fragment deletion by CRISPR/Cas9 system in rats. Therefore, another distinct sgRNA of each gene, together with the tested 4 sgRNAs were co-microinjected into one-cell-stage SD rat embryo at 5 ng/μl/sgRNA (Table S2 in File S1). A total of 26 pups from 9 recipients were born from 276 transferred embryos (one died after birth) (Table S2 in File S1). The modifications of the different loci were also analyzed by PCR (Fig. 3a), T7EN1 cleavage assay (Fig. 3b), and sequencing (Fig. 2). The CRISPR/Cas9 mediated the cleavage of target loci with high efficiencies of 76%, 56%, 52%, and 52% at ApoE, B2m, Prf1, and Prkdc, respectively (Table 1). Compared with single sgRNA, dual sgRNAs targeting yielded more fragment deletion from PCR and sequencing results (Fig. S2 in File S1, Fig. 2, Fig. 3a). Notably, almost all the deleted fragments cover the dual sgRNAs targeting sites or locate between the two sites (Fig. 2). Interestingly, targeted ApoE locus (in potential founder #36 and #38) and B2m locus (in potential founder #38) can’t be PCR amplified using normal primers, suggesting a larger fragment was deleted. Indeed, using new primers we confirmed large fragment deletion up to 983 bp in length in the ApoE (potential founder #38, Fig. 2 & 3), 1922 bp in length in the B2m

Table 1. Summary of the mutation of the founder rats.

| Mutant rats/Total rats (%) | Bi-allelic mutant rats/Total rats (%) |
|---------------------------|-------------------------------------|
|                           | ApoE  | B2m  | Prf1 | Prkdc | ApoE  | B2m  | Prf1 | Prkdc |
| Single sgRNA/Gene         | 15/15 (100%) | 11/15 (73.3%) | 9/15 (60%) | 4/15 (26.7%) | 10/15 (66.7%) | 10/15 (66.7%) | 6/15 (40%) | 1/15 (6.7%) | 5/15 (33.3%) |
| Dual sgRNAs/Gene          | 24/25 (96%) | 19/25 (76%) | 14/25 (56%) | 13/25 (52%) | 13/25 (52%) | 15/25 (60%) | 11/25 (44%) | 10/25 (40%) | 9/25 (36%) |

Single sgRNA/Gene: A mixture of 4 sgRNAs, each targeting a single site in each of the 4 genes. Dual sgRNAs/Gene: Another distinct sgRNA of each gene, together with the tested 4 sgRNAs targeting double site in each of the 4 genes.

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Figure 1. Generation of multiplex genetic modified rats using CRISPR/Cas9 system. (a) Detection of Cas9:sgRNA-mediated site-specific cleavage of the endogenous ApoE, B2m, Prf1, and Prkdc by T7EN1 cleavage assay. PCR amplicon of the targeted fragment at the ApoE, B2m, Prf1, and Prkdc in 15 founder rats (#1–15) were subjected to T7EN1 cleavage assay. Founder #13, which is quadruple gene mutant, was marked with asterisks. (b) DNA sequences of four loci in founder #13. PCR amplicon with cleaved bands in T7EN1 cleavage assay were cloned and sequenced. The PAM sequence was underlined and highlighted in green; the targeting site are red; the mutations are blue, lower case; insertions (+) or deletions (−) are shown to the right of each allele.
More importantly, dual sgRNAs targeting yielded 6 rats (6/25) harboring all 4 mutant genes, which is higher than single sgRNA targeting (Table S4 in File S1). Taken together, dual sgRNAs targeting at one gene yielded more fragment deletion events.

We assumed a founder rat harbors bi-allelic mutations when the wild type allele was undetectable by PCR genotyping. And for

Figure 2. Schematic diagrams of sgRNAs and DNA sequences of targeting genomic loci. PCR amplicon of the targeted fragment in the ApoE, B2m, Prf1, and Prkdc in all founder rats (1–40) were sequenced. The PAM sequence is underlined and highlighted in green; the targeting site are red; the mutations are blue, lower case; insertions (+), deletions (−) or mutant (m) are shown to the right of each allele. N/N indicates positive colonies out of total sequenced. (a) ApoE locus. (b) B2m locus. (c) Prf1 locus. (d) Prkdc locus.

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simplicity, we regarded a founder rat carries a monoallelic mutation when the wild type allele was detected. In this study, efficient bi-allelic modifications by the Cas9:sgRNA were observed in 25 potential founders in the ApoE gene (Table 1, Fig. 2), 17 potential founders in the B2m gene (Table 1, Fig. 2), 11 potential founders in the Prf1 gene (Table 1, Fig. 2), and 14 potential founders in the Prkdc gene (Table 1, Fig. 2). Consequently, potential founder #38 with bi-allelic ApoE mutation was sacrificed and observed the level of serum low density lipoprotein (LDL) increased up to 275.5% compared with wild-type control rats (Fig. S3a in File S1); potential founder #36 with bi-allelic B2m mutation was sacrificed and showed no B2M expression in the lung (Fig. S3b in File S1); no mature T cell was detected in potential founders #31 and decreased mature B cells with bi-allelic Prkdc mutation (Fig. S3c in File S1).

Off-target analysis

Recent work suggested that CRISPR-Cas9 can tolerate 1~3 base pairs mismatch, and then induce off-target-mutation [19–21]. Next, we comprehensively investigated off-target damage in mutant rats. We examined 13 potential off-target sites (OTS) for ApoE-A sgRNA, 51 OTS for B2m-A sgRNA, 8 Prf1-A sgRNA and 47 OTS for Prkdc-A sgRNA in 6 selected founders by T7EN1 cleavage assay. Surprisingly, only one real off-target mutation (Prkdc-A OTS-4) was detected from total 119 OTS (Fig. S4 and Table S5 in File S1), demonstrating CRISPR-Cas9 is a reliable rat gene targeting tool. Then we checked Prkdc-A OTS-4 in the other F0 rats and found mutations indeed occurred in 23 F0 rats by T7EN1 cleavage assay and sequencing (Fig. 4a & 4b). Notably, 8 out of 25 founders from the 5 ng/μl Prkdc-A sgRNA group contained mutations, while all founders (15/15) from the 10 ng/μl Prkdc-A sgRNA group contained mutations, suggesting off-target effect can be minimized by decreasing the concentration of sgRNA.

Germline transmission analysis and mosaicism

In addition, we determined the transmission of gene modifications by crossing 3 F0 mutants (potential founder #3, #19, #26) with wild-type SD rats and determined the genotypes of the F1 offspring. The offspring yielded PCR product of same size as potential founders. The PCR products were further analyzed by sequencing. Sequencing results showed the same mutations appeared in offspring as their mutant parent rats (Fig. S5 in File S1), demonstrating that mutations induced by Cas9:sgRNA can be transmitted through germline. Off-target mutation was also determined in founder #3, and the result showed mutation in potential off-target site was transmitted through germline (Fig. 4c). Cas9:sgRNA may continue to induce DNA double strand break

Figure 3. Cas9:sgRNA-mediated modifications in 4 genes by a mixture of dual sgRNAs for each gene. (a) PCR identification of sgRNA:Cas9-mediated site-specific cleavage of the endogenous ApoE, B2m, Prf1, and Prkdc loci. The genetic modification analysis was performed by PCR amplification of the targeted fragment in the ApoE, B2m, Prf1, and Prkdc in 25 founder rats (#16~40) derived from co-microinjection of a mixture of dual sgRNAs for each genes as described in Table S2 in File S1. Primer used for PCR amplification was described in Table S3 in File S1. Additionally, founder #36, #38 had a larger deletion in the ApoE, the primer ApoE-NS2 and ApoE-NAS2 used for amplification. Founder #38 had a larger deletion in the B2m, the primer B2m-S2 and B2m-AS2 used for amplification. (b) Detection of Cas9:sgRNA-mediated site-specific cleavage of the endogenous ApoE, B2m, Prf1, and Prkdc by T7EN1 cleavage assay. PCR products from (a) were subjected to T7EN1 cleavage assay as described in material and methods. doi:10.1371/journal.pone.0089413.g003
Figure 4. Analysis of the transmission of the off-target mutation. (a) Detection of Cas9:sgRNA-mediated off-target cleavage of Prkdc OTS-4 in all founders (1–40) by T7EN1 cleavage assay. PCR amplicon of Prkdc OTS-4 in all 40 founder rats were subjected to T7EN1 cleavage assay as described in methods. Total 23 founders (*) displayed cleavage bands. (b) PCR products with cleavage bands were cloned and sequenced. Sequence result showed OTS-4 indeed mutagenized in the 23 founders. Indels were also detected around 270 bp downstream of the OTS-4 in most colonies, which may be introduced by PCR amplification when Taq encountering repeat sequence. (c) Detection of Cas9:sgRNA-mediated off-target cleavage of Prkdc OTS-4 in 8 F1 pups derived from founder #3 by T7EN1 cleavage assay. Mutations were detected in 2 F1 pups (4 and 8). (d) DNA sequences of...
was heritable.

transmit a mutation detected in the tail DNA to its F1 offspring.

genetic mosaicism. Our results showed that only one rat did not
a very low level. As described above, this system may induce
sites, demonstrating CRISPR-Cas9 induced off-target-mutation at

target mutation was detected from total 119 potential targeting
may induce off-target-mutation. From our result, only one off-

of rats with multiple mutations in tightly linked genes.

CRISPR/Cas9 system can tolerate sequence mismatch, which
may induce off-target-mutation. From our result, only one off-
target mutation was detected from total 119 potential targeting
sites (potential founder #3), two (founder #3) or three (founder #3)
mutant genes were selected to cross with wild-type SD rat. (a)
Detection of Cas9 sgRNA-mediated on-target cleavage of the
endogenous B2m in 8 F1 pups derived from potential founder #3
by T7EN1 cleavage assay. Mutations were detected in 3 F1 pups
(1, 4, and 5). (b) DNA sequences of genomic loci in F1 pups 1, 4
and 5. PCR amplicon of the targeted fragment at the B2m
in potential founder #3-derived F1 pups 1, 4, and 5 were cloned and
sequenced. Sequencing result showed one kind of mutation same
as the founder #3 was detected in the offspring, indicating that
off-target mutation induced by Cas9 sgRNA was heritable.

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(DSB) beyond one-cell stage embryos, resulting in genetic
mosaicism. If a mutation cannot be transmitted through germline,
or if more than two mutations were detected in the progeny,
we assumed the rat had genetic mosaicism. From the germline
transmission assay, only one mutation (Fig. S3 in File S1) was not
detected in the F1 offspring. More than two mutants were detected
at ApoE (potential founder #10), B2m (potential founder #15),
Prkdc (potential founder #29 and #31) (Figure 2). These results
indicate that Cas9 sgRNA also induces genetic mosaicism at low
frequency, similar to TALENs. This mosaicism may result from
persistent Cas9 sgRNA activity in later embryogenesis, or
recleavage of certain already alleles [5,6].

Discussion

Genetic manipulation of rats is a crucial technique for the study
of diseases, especially in the fields of neuroscience, physiology and
drug discovery. Generation of precise genetic alterations is limited
by rat embryonic stem (ES) cells culture [1–2]. In 2009, the gene
targeted rat became technically feasible using ZFNs, which can
bypass ES cells screening and chimeric rat germline generation
procedure [3]. However, the generation of specific mutant rat
is labor intensive by ZFNs or Talens for the need to engineer specific
protein pairs for each target site. Recently, another genome
editing tool CRISPR/Cas9 has proven to be a simpler way to edit
the eukaryotic genome.

Here, we successfully disrupt four genes (ApoE, B2m, Pyf1, and
Prkdc) in one rat in one-step at efficiencies of 24% by co-injection of
Cas9 mRNA and sgRNA into one-cell fertilized eggs. During
the revision process of this work, two independent studies reported
the success of rat genome modifications using CRISPR-Cas9
system [22,23]. This system eliminates the necessity of engineering
specific protein pairs to each target site [3–6,24] and makes it
possible to produce mutant rats in a few weeks, which suggests that
the CRISPR/Cas9 system is an efficient tool for accelerating the
propagation of mutant rats.

Furthermore, rats with mutations in multiple closely linked
genes are hard to produce by breeding rats with a single mutation.
In this study, we showed that co-injection of Cas9 with multiple
sgRNAs can generate rats with multiplex genetic mutations in one
step with low off-target effects and mosaicism. In theory, the
CRISPR/Cas9 system should enable straightforward generation
of rats with multiple mutations in tightly linked genes.

CRISPR/Cas9 system can tolerate sequence mismatch, which
may induce off-target-mutation. From our result, only one off-
target mutation was detected from total 119 potential targeting
sites, demonstrating CRISPR-Cas9 induced off-target-mutation at
a very low level. As described above, this system may induce
genetic mosaicism. Our results showed that only one rat did not
transmit a mutation detected in the tail DNA to its F1 offspring.
This may be caused by mosaicism or a small sample size. Somatic
genetic mosaicism in tail DNA was also at low levels.

Our results confirmed the versatility and reliability of CRISPR/
Cas9 system for rat genome editing. It will be interesting to expand
this targeting system to produce precise deletions, conditional
alleles and insertion of larger DNA fragments to generate knock-in
and conditional knock-out rats for the genes of interest.

Supporting Information

File S1 Figure S1. The pUC57-sgRNA expression vector. The
gRNA expression vector was constructed using the backbone of
the pUC57 vector with a Kanamycin resistance gene. The
annealed oligos were inserted between the two Bso I restriction
sites (blue) downstream of the T7 promoter (red). The construct
was linearized by Dra I (green) for in vitro transcription. Figure S2.
Cas9 sgRNA-mediated 4 gene modifications by a mixture of 4
single sgRNAs. (a) PCR identification of sgRNA:Cas9-mediated
site-specific cleavage of the endogenous ApoE, B2m, Pyf1, and
Prkdc loci. The genetic modification analysis was performed by PCR
amplification of the targeted fragment in the ApoE, B2m, Pyf1, and
Prkdc in 15 potential founder rats (#1–15) derived from co-
microinjection of a mixture of 4 single sgRNAs as described in
Table S2 in File S1. Primers used for PCR amplification were
described in Table S3 in File S1. Figure S3. Phenotypes of the
potential founder rats (#29 and #31) (Figure 2). These results
indicate that Cas9 sgRNA also induces genetic mosaicism at low
frequency, similar to TALENs. This mosaicism may result from
persistent Cas9 sgRNA activity in later embryogenesis, or
recleavage of certain already alleles [5,6].
the endogenous \textit{B2m} and \textit{Pkhdc} in 12 F1 pups derived from potential founder #19 by T7E1 cleavage assay. The mutations were detected in all 12 F1 pups. (d) DNA sequences of genomic loci in mutant pups. PCR amplicon of the targeted fragment at the \textit{B2m} and \textit{Pkhdc} in potential founder #19-derived F1 pups were cloned and sequenced. Sequencing result showed the mutations same as the founder #19 were detected in the offspring. Two kinds of mutation of \textit{Prkdc} were all transmittable, indicating mosaicism induced by Cas\textsubscript{9}\textsubscript{-}sgRNA. (e) Detection of Cas\textsubscript{9}\textsubscript{-}sgRNA-mediated on-target cleavage of the endogenous \textit{ApoE} by PCR, \textit{B2m} and \textit{Pkhdc} by T7E1 cleavage assay in 10 F1 pups derived from potential founder #26. The mutations were detected in F1 pups. (f) DNA sequences of genomic loci in mutant pups. Smaller band of PCR amplicon of \textit{ApoE} were gel extracted and sequenced. PCR amplicon of the targeted fragment at the \textit{B2m} and \textit{Pkhdc} in potential founder #26-derived F1 pups were cloned and sequenced. Sequencing result showed the mutations same as the founder #26 were detected in the offspring. Table S1. Oligonucleotides for generating sgRNA expression vectors. Table S2. Summary of embryo injections of sgRNA:Cas9. Table S3. Primers for amplifying sgRNA targeted loci. Table S4. Summary of mutations of multiple genes. Table S5. Summary of the alleles for putative off-target sites. Table S6. Primers for amplifying off-target sites.

\textbf{Author Contributions} 

Conceived and designed the experiments: LZ XH YM BS. Performed the experiments: YM BS XZ YL WC JM. Analyzed the data: LZ XH YM BS. Contributed reagents/materials/analysis tools: LZ XH YM BS XZ YL WC JM. Wrote the paper: LZ XH YM BS. N/A.

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