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RESEARCH ARTICLE

Generation of CRISPR/Cas9-mediated lactoferrin-targeted mice by pronuclear injection of plasmid pX330

Mengxu GE[1*], Fei LIU[2*], Fei CHANG[1], Zhaolin SUN[1], Jing FEI[1], Ying GUO[1], Yunping DAI[1], Zhengquan YU[1], Yaofeng ZHAO[1], Ning LI (✉)[1,2], Qingyong MENG (✉)[1]

1 State Key Laboratory of Agrobiotechnology, College of Biological Science, China Agricultural University, Beijing 100193, China
2 College of Animal Science and Technology, Yunnan Agricultural University, Kunming 650201, China

Abstract Lactoferrin is a member of the transferrin family of multifunctional iron binding glycoproteins. While numerous physiological functions have been described for lactoferrin, the mechanisms underlying these functions are not clear. To further study the functions and mechanisms of lactoferrin, we modified the lactoferrin promoter of mice using the CRISPR/Cas9 system to reduce or eliminate lactoferrin expression. Seven mice with lactoferrin promoter mutations were obtained with an efficiency of 24% (7/29) by injecting the plasmid pX330, expressing a small guide RNA and human codon-optimized SpCas9, into fertilized eggs of mice. Plasmid integration and off-targeting of pX330 were not detected. These results confirmed that pronuclear injection of a circular plasmid is a feasible and efficient method for targeted mutagenesis in mice.

Keywords lactoferrin, promoter, CRISPR/Cas9, plasmid pX330

1 Introduction

Lactoferrin (LF) is a multifunctional iron binding glycoprotein belonging to the transferrin family, with a molecular weight of 80 kDa. In 1938, LF was first identified as a red protein that was present in bovine milk[1]. In 1960, it was isolated from human milk by Groves and was subsequently recognized as a major iron binding protein[2–4]. LF is not only present in milk and several other exocrine secretions, but it is also present in the secondary granules of neutrophils. Several biological functions have been described for LF, including iron homeostasis, immunomodulation, cellular growth and differentiation, antimicrobial, antioxidant, anti-inflammatory, antiviral and anticancer properties[5–14]. However, the mechanisms underlying these functions are not clear. In this study, we aimed to obtain mice in which LF expression was reduced or eliminated, thereby providing a model to study the functions and mechanisms of LF.

Of the next-generation genome editing technologies, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system has been demonstrated to be a robust tool for genome engineering. The CRISPR/Cas system is an RNA-based adaptive immune system in bacteria and archaea that can destroy invasive plasmids, phages and viruses[15–17]. Compared with the complex and time-consuming design and generation of zinc finger nucleases and transcription activator-like effector nucleases, the CRISPR/Cas gene-editing approach only requires Cas9 and small guide RNAs (sgRNAs). Because of its simplicity, low cost and high efficiency, the CRISPR/Cas system has become a widely used platform for genome editing in animals and plants[18]. Recently, the type II CRISPR/Cas system has been demonstrated to be an efficient gene-editing technology that has been used successfully to produce many gene-targeted cell lines and animals[19–24].

In this study, we injected plasmid pX330 expressing an sgRNA and human codon-optimized SpCas9 (hSpCas9) into mouse zygotes to modify the mouse *LF* promoter, and we obtained *LF* gene promoter mutation mice without detectable random plasmid integration and off-targeting. These mutated mice can provide a model for studying the functions and mechanisms of LF.

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Correspondences: ninglcau@cau.edu.cn, qymeng@cau.edu.cn
*These authors contribute equally to the work

2 Materials and methods

2.1 Animals

Kunming mice used in this project were bought from the Chinese Experimental Animal Resources Research Institute for Food and Drug Control. Animals were handled according to the Guidelines for the Care and Use of Laboratory Animals established by the Beijing Association for Laboratory Animal Science. Animal experiments were approved by the Animal Ethics Committee of the College of Biological Sciences, China Agricultural University.

2.2 pX330-sgRNA and 2Se-sgRNA plasmid construction

The targeting sites were designed in the basic *LF* promoter areas using the free website (http://crispr.mit.edu). We synthesized oligonucleotides that were complementary to the targeting sites with the three highest scores (Table 1). The polymerase chain reaction (PCR) primers used in this work are shown in Table S1 (Appendix A). Six pairs of oligonucleotides were designed for three target sites. Three pairs of oligonucleotides (C-mRJM-1-1-F/R, C-mRJM-1-2-F/R, C-mRJM-1-3-F/R) were annealed to double-stranded DNA and then individually ligated to the *Bbs* I sites of plasmid pX330 (authorized by Dr. Feng Zhang from the Massachusetts Institute of Technology and obtained from Dr. Bo Zhang at the College of Life Sciences, Peking University) to construct three pX330-sgRNA plasmids. The other three pairs of oligos (DC-mRJM-1-1-F/R, DC-mRJM-1-2-F/R, DC-mRJM-1-3-F/R) were individually ligated into the *Eco*RI and *Bam*HI (New England Biolabs, Ipswich, MA, USA) sites of the dual-color reporter vector 2Se (which was constructed in our laboratory). Then, the pX330-sgRNA and 2Se-sgRNA plasmids were confirmed by sequence analysis.

Table 1 Three target sites within the basic *LF* promoter sequences

Score	gRNA
92	CCCTACACAGGCGCTGGTACAGG
88	AGTACCCCCTACACAGGCGCTGG
82	AGCGCCTGTGTAGGGGGTACTGG

Note: The gRNAs are scored by inverse likelihood of offtarget binding.

2.3 Verifying the targeting efficiency in HEK293T cells

The human embryonic kidney HEK293T cell line was cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) under 5% CO_2 at 37°C. The cells were seeded into 24-well plates (Thermo Fisher Scientific, Waltham, MA, USA). After 24 h, cells were co-transfected with a 1∶1 mixture of plasmid pX330-sgRNA and plasmid 2Se-sgRNA (200 ng per well) using Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY, USA). Cell fluorescence was detected by fluorescence microscopy at 24 h post-transfection. Mutation frequencies were counted by flow cytometry 48 h after transfection.

2.4 Construction of pX330-EF1α-sgRNA

Plasmid pX330 was digested with *Kpn*I-HF and *Age*I-HF (New England Biolabs, Ipswich, MA, USA) to remove the CBh promoter, and then the linearized pX330 vector was gel purified. The EF1α promoter of plasmid pBudCE 4.1 (purchased from Invitrogen, Carlsbad, CA, USA) was PCR amplified using Q5 high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) with a pair of primers (*Kpn*I-EF1a-F/*Xma*I-EF1a-R). After electrophoresis, the PCR products were purified using the E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). The purified PCR products were digested by *Kpn*I-HF and *Xma*I (New England Biolabs, Ipswich, MA, USA), and then ligated to the linearized pX330 vector. Finally, the construction of pX330-EF1α-sgRNA was confirmed by sequence analysis.

2.5 Verifying the targeting efficiency of pX330-EF1α-sgRNA in C2C12 cells

The C2C12 cell line was cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) under 5% CO_2 at 37°C. Cells were seeded into six-well plates (Thermo Fisher Scientific, Waltham, MA, USA). After 24 h of culture, plasmids pX330-sgRNA, pX330-EF1α-sgRNA and pGFP (expressing the green fluorescent protein) (4 μg per well) in 150 μL of electroporation medium were electro-transfected into C2C12 cells using electroporation cuvettes. The cells were harvested at 48 h post-transfection, and genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). The genomic region flanking the CRISPR target site was PCR amplified using Q5 high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) with the primer pair LF-F1-1/LF-R1-1. After electrophoresis, PCR products were purified using the E.Z.N.A Gel Extraction Kit. A total of 400 ng of the purified PCR products were mixed with 1 μL 10 × LA Buffer, and ultrapure water was added to a final volume of 10 μL, and subjected to a re-annealing process to enable heteroduplex formation: 95°C for 10 min, 95 to 85°C with ramping at −2°C/s, 85°C for 1 min, 85 to 75°C with ramping at −0.3°C/s, 75°C for 1 min, 75 to 65°C with ramping at −0.3°C/s, 65°C for 1 min, 65 to 55°C with ramping at −0.3°C/s, 55°C for 1 min, 55 to 45°C with ramping at −0.3°C/s, 45°C for 1 min, 45 to 35°C with ramping at −0.3°C/s, 35°C for 1 min, 35 to 25°C with ramping at −0.3°C/s, 25°C for 1 min, and a final hold at

4°C. After annealing, 10 μL of the products were treated with 2 μL of NEB buffer 2 and 0.5 μL T7 endonuclease 1 (T7EN1) (New England Biolabs, Ipswich, MA, USA), and 7.5 μL of ultrapure water was added, resulting in a final volume of 20 μL. After incubation at 37°C for 1 h, the products were added to 4 μL of 6 × loading buffer and analyzed on 8% Tris-borate-EDTA (TBE) polyacrylamide gels. Gels were stained with ethidium bromide for 5 min and imaged with a Gel Logic 212 Imaging System (Eastman Kodak, Rochester, NY, USA). Quantification was based on relative band intensities. The indel percentage was determined by the formula $100 \times (1 - (1 - (b + c)/(a + b + c))^{1/2})$, wherein a is the integrated intensity of the undigested PCR product and b and c are the integrated intensities of each cleavage product.

2.6 Pronuclear microinjection

Kunming female mice were superovulated and mated with Kunming male mice, and fertilized eggs were collected from the oviduct. Pronuclear stage eggs were injected with pX330-EF1α-sgRNA at a concentration of 1 ng·μL^{-1}. The eggs were cultivated in M16 (Sigma–Aldrich, St. Louis, MO, USA) for a moment and then transferred into both sides of the fallopian tubes of pseudopregnant Kunming females.

2.7 Detection of mutations in mice

Twenty days after birth, genomic DNA was extracted from the tails of mice. The regions surrounding the target locus in the *LF* gene of the postnatal mice were genotyped by PCR (the primers for the 11 mice used in the first microinjection were LF-F1-1/LF-R1-1; the primers for the 18 mice used in the second microinjection were TB-LF-F/TB-LF-R). Then, genomic DNA was PCR amplified using Q5 high-fidelity DNA polymerase. After purifying the PCR products, a total of 400 ng of the purified PCR products were then denatured, annealed and treated with T7EN1 nuclease. Then, the products with mutations detected by the T7EN1 assay were subcloned into the pMD-19T vector (Takara, Dalian, China), and the clones for each sample were randomly picked and sequenced.

2.8 Plasmid integration analysis

Four pairs of primers were randomly designed for different locations in the plasmids (cas9-ZL-F1/R1, cas9-ZL-F2/R2, cas9-ZL-F3/R3, cas9-ZL-F4/R4). To determine whether the plasmid was integrated into the mouse genome, genomic DNA isolated from the mutants was subjected to PCR amplification with the four pairs of primers.

2.9 Off-target analysis

To test whether there were off-target mutations, 12 potential off-target candidates were selected using CRISPR Genome Engineering Resources. We designed 12 pairs of primers (OT-LF-F1/1-OT-LF-F12/12) that were complementary to approximately 600 bp regions of potential off-target sites. Then, the regions containing the potential off-target sites were amplified with KOD-plus high-fidelity DNA polymerase (TOYOBO, Osaka, Japan) and sequenced.

3 Results

3.1 Design of sgRNA and preparation of the CRISPR/Cas9 plasmids

The size of the mouse lactoferrin gene promoter is 7.5 kb. The transcription level directed by the LF minimal promoter is controlled by three regions, including both positive (between nucleotides −1739 and −922) and negative (between nucleotides −2644 and −1739, and between nucleotides −589 and −291) regulatory sequences. Moreover, the basic promoter of the mouse lactoferrin gene is located between nucleotides −234 and −21; deletion of this region completely abolishes its activity[25]. Thus, we designed three sgRNAs (sgRNA1-1, sgRNA1-2, sgRNA1-3) targeting this critical area (Fig. 1a), and completed the construction of three pX330-sgRNA and three 2Se-sgRNA plasmids. To validate which sgRNA sequence works, a dual-color reporter vector (2Se) was used to indicate the activity of RNA-guided hSpCas9. The reporter comprises a monomeric red fluorescent protein (*mRFP*) gene, an RNA-guided hSpCas9 target sequence, and one enhanced GFP (*eGFP*) gene. The reporter-transfected cells will express only mRFP in the absence of hSpCas9 nuclease activity; in the presence of nuclease activity, the transfected cells will express both mRFP and eGFP[26]. To select the best pX330-sgRNA, we co-transfected pX330-sgRNA and 2Se-sgRNA into HEK293T cells. Twenty-four hours after the transfection, most of the cells expressed mRFP. Only a small fraction of cells expressed both mRFP and eGFP, suggesting that RNA-guided hSpCas9 indeed worked (Fig. 1b). Forty-eight hours after the transfection, the mutation frequencies of the three targets (sgRNA1-1, sgRNA1-2, sgRNA1-3) were 28.6%, 26.0%, and 22.0%, respectively, as assessed by flow cytometry (Fig. 1c).

The promoter of hSpCas9 is CBh in the initial pX330 plasmid. We replaced the CBh promoter with the EF1α promoter, which is constitutively expressed in mouse cells, to improve the expression efficiency of hSpCas9[27]. This promoter replacement was applied to pX330-sgRNA1-1, which had the highest targeting efficiency. To verify that the new plasmid, pX330-EF1α-sgRNA1-1, could still play a role, we transfected pX330-sgRNA1-1, pX330-EF1α-sgRNA1-1, and pGFP into mouse C2C12 cells. Then, we isolated genomic DNA and performed a T7EN1 assay.

Apparent cleavages of bands were found in pX330-EF1α-sgRNA1-1-transfected cells (Fig. 1d), indicating that it cleaved the target locus efficiently.

3.2 One-step generation of *LF* gene promoter mutant mice by pronuclear injection of circular plasmid

Following validation of the pX330-EF1α-sgRNA1-1 plasmids in mouse C2C12 cells, we directly injected it into the pronucleus of fertilized eggs twice. The two microinjections are summarized in Table 2. The regions surrounding the target locus in the *LF* gene of postnatal mice were genotyped by PCR (Appendix B, Fig. S1). The PCR results demonstrated that the CRISPR/Cas9 system did not produce long insertions or deletions and the T7EN1 assay was performed to assess the targeted cleavage efficiency (Fig. 2a, Fig. 2b). Clear cleavage bands were detected in three mice born after the first microinjection and four mice born after the second microinjection. To confirm this gene mutation, subsequent sequence analysis (Fig. 2c) showed that the seven mice had *LF* gene promoter mutations at the target site (three mice from the second microinjection) or 40 bp upstream of the target locus (three mice from the first microinjection) or both (one mouse from the second microinjection).

3.3 Plasmid integration and off-target analysis

An injected plasmid may integrate into the gene target in the mouse genome. To determine whether the plasmid integrated, four pairs of primers were randomly designed

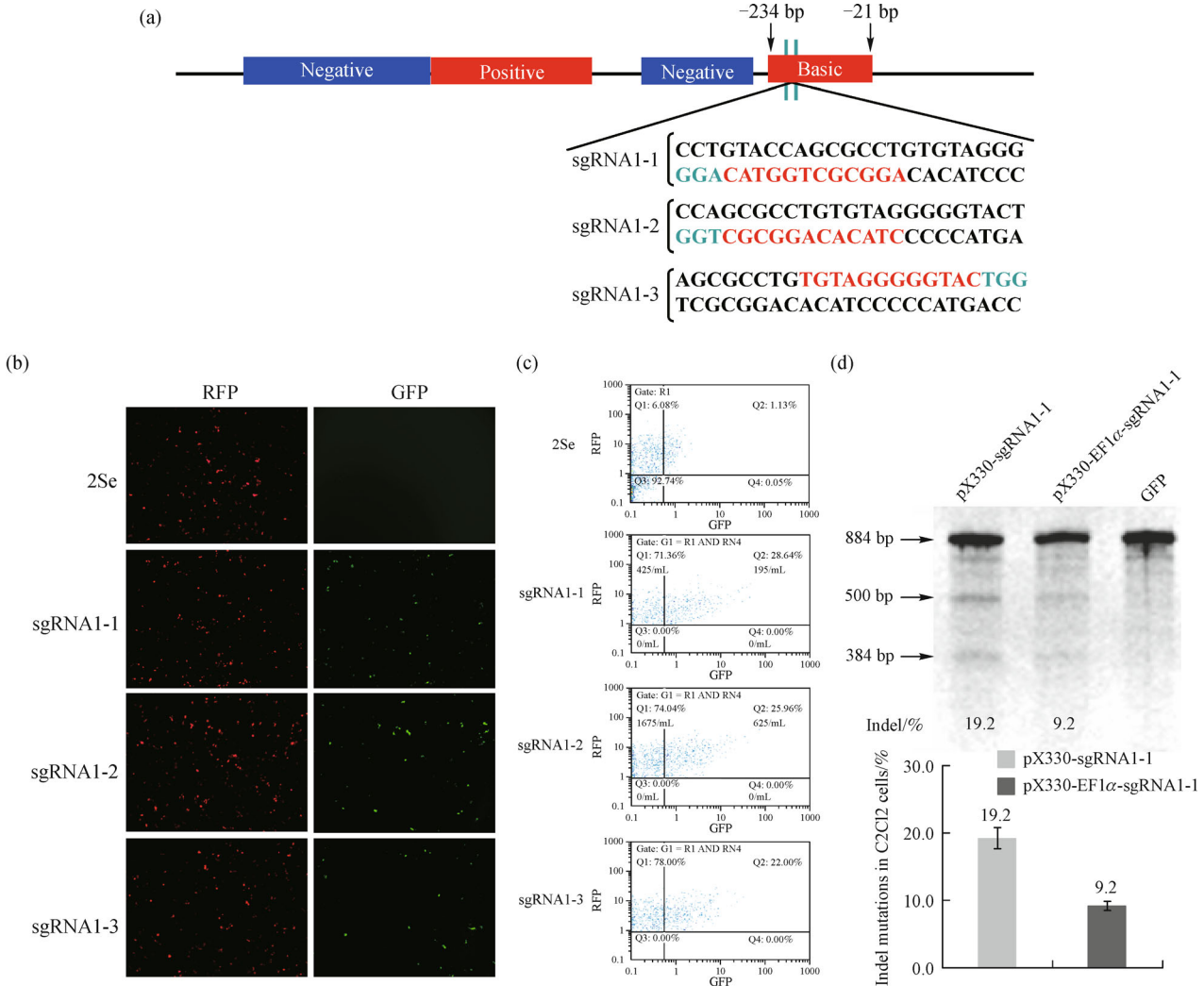

Fig. 1 Preparation of the CRISPR/Cas9 plasmids. (a) Schematic of sgRNAs targeting sites at critical regions of the *LF* promoter. The protospacer adjacent motif (PAM) sequence is labeled in blue and a 12 bp seed sequence is highlighted in red; (b) observed expression of red fluorescent protein (RFP) and green fluorescent protein (GFP), 24 h after co-transfection of pX330-sgRNA and 2Se-sgRNA, by fluorescence microscopy; (c) the percentage of $RFP^{+}GFP^{+}$ cells by flow cytometry 48 h after co-transfection is shown; (d) T7EN1 assay for Cas9-mediated cleavage in C2C12 cells and its densitometry analysis by Image J.

Table 2 Generation of mutant mice via pX330 plasmid injection

No.	Injected	Pregnancy	Newborn	Mutation	Indel mutation frequency	Mutation type
1	238	4	11	3	3/11 (27%)	Three mice were all mutated 40 bp upstream of the target locus
2	325	5	18	4	4/18 (22%)	Three of four were mutated at the target site, and the other one was mutated at both the target site and 40 bp upstream of the target locus

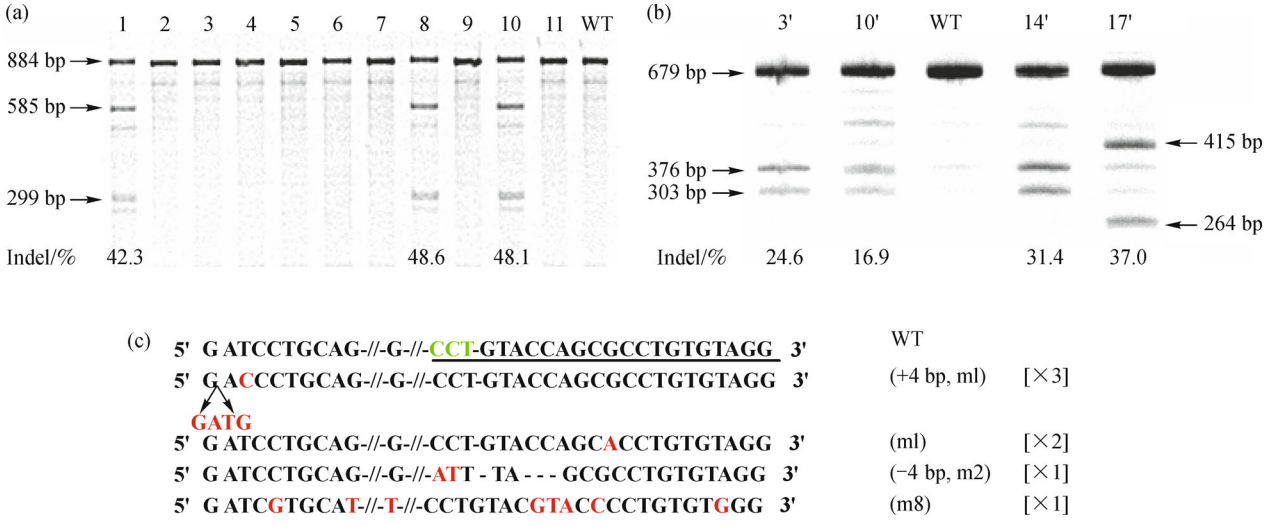

Fig. 2 Generation of *LF* gene promoter mutation mice via the CRISPR/Cas9 system. (a) T7EN1 assay for Cas9-mediated cleavage in newborn mice from the first microinjection. Four pregnant mice resulting from the first microinjection gave birth to 11 mice. WT is the genome of Kunming wild-type mice; (b) T7EN1 assay for Cas9-mediated cleavage in newborn mice from the second microinjection. 3′, 10′, 14′, and 17′ represent the genomes of the newborn mice from the second microinjection; (c) DNA sequences of the WT and four mutant alleles in seven mice. The target site is underlined, and the PAM sequence is labeled in green. -//- represents an omitted base, and 18 and 15 bp are omitted from left to right. The numbers of mutant mice are in brackets.

in different locations of the plasmid. Genomic DNA, isolated from the seven mice generated by CRISPR/Cas9, was subjected to PCR amplification with the four pairs of primers. The final PCR results showed that the plasmid did not integrate into the mouse genome at this level of detection (Appendix B, Fig. S2).

It is known that a small number of mismatches between sgRNAs and the target DNA can be tolerated, and one to four base-pair mismatches did not affect the ability of sgRNAs to identify their potential targets[28,29]. To test whether there were off-target mutations in these genetically modified mice, we searched for off-target sites in the mouse genome containing up to three or four base-pair mismatches compared with the 20 bp sgRNA coding sequence. We predicted 12 potential off target candidates, then the approximate 600 bp regions containing the potential off-target sites were amplified and sequenced. No mutations were observed at these 12 hypothetical off-target loci in any of the seven gene-targeted mice (Table 3).

4 Discussion

In this study, we used CRISPR/Cas-mediated genome editing and obtained mice genetically modified in the *LF* promoter by injecting plasmid pX330, which expresses sgRNA and hSpCas9, into fertilized eggs of mice. The mutation efficiency was 24% (7/29), which indicates that zygote injection of circular plasmid is a viable and efficient method for generating mouse models with gene modification. Previously, the generation of gene-targeted animals via the CRISPR/Cas9 system was realized through microinjection of a Cas9 RNA:sgRNA mixture into fertilized eggs. Instead, in this study, we avoided the complex preparation, careful handling and storage of RNA, and injected plasmid pX330 expressing sgRNA and hSpCas9 instead of RNA. Differences have been found when using mRNA and plasmids to generate mutant mice via pronuclear injection of circular plasmid[30] and injecting exogenous components may have an adverse effect on mice. To minimize such a risk, we used a circular plasmid to reduce plasmid integration into the mouse genome. Moreover, we also analyzed plasmid integration in the mouse genome, and the results showed that the injected plasmid did not integrate into the genome. In addition, although prolonged existence of plasmid may increase the chance of off-target mutations, transient expression by pronuclear injection could decrease this risk.

Table 3 Off-target analysis in *LF* mutant mice

Site name	Sequence	Indel mutation frequency (Mutant/Total)	Coordinate	Strand
Target site	CCCTACACAGGCGCTGGTACAGG	/	chr 9: 111019095–111019117	–
OT1	**a**C**a**TAC**t**CAGGC**t**CTGGTACAGG	0/7	chr 19: 48087277– 48087299	–
OT2	C**t**gTACACAGG**t**GCTGGTA**a**TGG	0/7	chr 18: 35579256–35579278	+
OT3	**t**CCT**g**CACAGGC**t**CTGGTA**t**AGG	0/7	chr 2: 121955005–121955027	–
OT4	**t**CCT**g**CACAGGC**t**CTGGTA**t**AGG	0/7	chr X: 91332097–91332119	+
OT5	**t**CCT**g**CACAGGC**t**CTGGTA**t**AGG	0/3	chr X: 92015969–92015991	–
OT6	CC**ac**ACACAGG**ga**CTGGTACAGG	0/3	chr 4: 135896156–135896178	+
OT7	C**a**CTACACAGG**a**GCT**t**GTACTGG	0/3	chr 7: 107035334–107035356	–
OT8	CCCT**g**C**g**C**t**GGC**c**CTGGTACAGG	0/3	chr 3: 27673749–27673771	–
OT9	CCC**c**ACACA**a**GC**t**CTG**c**TACTGG	0/3	chr 10: 111084383–111084405	+
OT10	CCC**c**A**a**ACAGG**a**GCTGGTA**g**GGG	0/3	chr X: 134240517–134240539	+
OT11	CCCT**tt**ACAG**t**C**c**CTGGTACAGG	0/3	chr 1: 166363559–166363581	–
OT12	CCCTACA**t**AG**at**GCTG**t**TACAGG	0/3	chr 19: 48586214–48586236	–

Note: +, sense strand; –, antisense strand. Mismatches from the on-target sequence are lower-case and boldfaced.

The CRISPR/Cas9 system makes rapid, simple and cost-effective gene mutations, but the high off-target mutation rate cannot be ignored. Previous reports have suggested that one to four base-pair mismatches will not affect the ability of sgRNAs to identify their potential targets. This degree of mismatch reduces, rather than completely preventing, Cas9 cleavage, which leads to a high frequency of off-target mutations[28,29]. We analyzed the off-target rate of seven genetically modified mice, and no mutations were detected in 12 potential off-target sites. However, with the optimization of the CRISPR/Cas9 system, there are also solutions to improve CRISPR-Cas nuclease specificity, for example, using truncated sgRNAs (17 or 18 bp)[31], double-nick[32] and dimeric CRISPR RNA-guided *Fok*I nucleases[33].

The CRISPR/Cas9 system mediated gene mutations at the target sites in previous reports[28,34], but it also mediated gene mutations at both the target site and 40 bp upstream of the target locus in our study. In the first microinjection, we obtained three mice that had a 4 bp insertion 40 bp upstream of the target locus, and in the second microinjection, we still generated one mouse with a mutation 40 bp upstream of the designated target locus. The reason for this is more than likely to be due to the structure of the *LF* gene promoter. Furthermore, double-stranded break and non-homologous end joining mediated by the CRISPR/Cas9 system may occur at non-target sites.

5 Conclusions

We have successfully obtained *LF* gene promoter mutation mice by injecting plasmid pX330 expressing Cas9/gRNA complexes into zygotes without detectable random plasmid integration and off-target mutations. This simple and quick gene editing system will have many potential applications for generating genetically modified small animal models.

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Compliance with ethics guidelines Mengxu Ge, Fei Liu, Fei Chang, Zhaolin Sun, Jing Fei, Ying Guo, Yunping Dai, Zhengquan Yu, Yaofeng Zhao, Ning Li and Qingyong Meng declare that they have no conflict of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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