CRISPR/Cas9-mediated loss of FGF5 function increases wool staple length in sheep

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Keywords
FGF5; loss-of-function; Merino sheep; staple length; wool

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(Received 17 April 2017, accepted 15 June 2017)
doi:10.1111/febs.14144

Fibroblast growth factor 5 (FGF5) regulates hair length in humans and a variety of other animals. To investigate whether FGF5 has similar effects in sheep, we used clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) to generate loss-of-function mutations with the FGF5 gene in Chinese Merino sheep. A total of 16 lambs were identified with genetic mutations within the targeting locus: 13 lambs had biallelic modifications and three lambs had monoallelic modifications. Characterization of the modifications revealed that 13 were frameshift mutations that led to premature termination, whereas the other three were in-frame deletions. Thus, CRISPR/Cas9 efficiently generated loss-of-function mutations in the sheep FGF5 gene. We then investigated the effect of loss of FGF5 function on wool traits in 12 lambs and found that wool staple length and stretched length of genetically modified (GM) yearling sheep were significantly longer compared with that of wild-type (WT) control animals. The greasy fleece weight of GM yearling sheep was also significantly greater compared with that of WT sheep. Moreover, the mean fiber diameter in GM sheep showed no significant difference compared with WT sheep, suggesting that the increase in greasy fleece weight was likely attributed to the increase in wool length. The results of this study suggest that CRISPR/Cas9-mediated loss of FGF5 activity could promote wool growth and, consequently, increase wool length and yield.

Introduction

Wool length is a critical economic trait in wool-producing sheep because it is closely associated with wool productivity and quality. Under conditions of similar wool density and fineness, wool length has a positive correlation with wool yield. In addition, wool length is also an important quality trait in wool textile industry that influences textile product quality and also determines the system of manufacture. Therefore, considerable effort has been made to increase wool length.

Unexpectedly, improvement of wool length by conventional genetic means has been slow and not obvious. Therefore, substantial efforts have been made to establish novel effective techniques that increase wool length. With the utility of concurrent genomic approaches, several candidate genes or loci have been found that are related to hair length. Interestingly, all of the proposed or candidate genes responsible for hair length are attributed to fibroblast growth factor 5

Abbreviations
CRISPR/Cas9, clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9; FGF5, fibroblast growth factor 5; FGF, fibroblast growth factor; FGFRI, fibroblast growth factor receptor 1; FGFRII, fibroblast growth factor receptor 2; GM, genetically modified; InDel, insertion–deletion; PAM, protospacer adjacent motif; sgRNA, single-guide RNA.
(FGF5). So far, a number of spontaneous mutations of FGF5 associated with hair length have been identified in humans [1], mice [2,3], dogs [4,5], cats [6], donkeys [7], goats [8] and hamsters [9]. It is indicative that the FGF5 gene is the leading candidate for hair length variation. However, to date, no causal mutations have been identified that are associated with wool length in fine wool sheep.

The cycle of hair growth in most fur mammals consists of three phases: growth (anagen), regression (catagen) and rest (telogen). FGF5 is a member of FGF family with 23 related genes, and the role of FGF5 in hair growth is recognized with respect to inhibition of the anagen phase [10–12]. FGF5 carries out its biological activity by binding and activating the FGF receptor 1 (FGFR1) or 2 (FGFR2). Its function can be regulated by alternative splicing of truncated protein FGF5s that would cancel the inhibition of hair growth by FGF5 [13–16]. By contrast to other animals, wool growth in fine wool sheep has a distinct by life-long growth, without seasonal spontaneous hair removal, and the function of FGF5 in fine wool sheep is still far from understood. However, high similarities between the gene sequence for FGF5 in Merino sheep and other mammalian orthologs have been observed in a previous study [17], which indicated that the role of FGF5 in fine wool sheep could be similar to that in other animals. Knockout of the FGF5 gene in mice led to a long hair phenotype similar to the spontaneous mutation designated as angora (go) in mice [2]. Disruption of FGF5 in cashmere goats by clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) led to a significant increase in cashmere fiber length, as well as cashmere yield [18,19]. However, no studies so far have investigated the function of FGF5 in wool-producing sheep by gene targeting.

With the fast development of genome modification approaches, investigators have applied state-of-the-art gene manipulation approaches to modify genes associated with specific traits. Knockout or disruption of FGF5 by gene targeting in mice or cashmere goats resulted in an increase in hair or cashmere length. In this study, we targeted the FGF5 gene in Chinese merino fine wool sheep with CRISPR Cas9. A total of 16 targeted lambs were generated and 12 founders lived for more than 1 year. In these 12 targeted animals, staple length and wool yield were significantly greater compared with those of controls. The results of this study show that blockade of FGF5 in fine wool Merino sheep could increase both wool length and yield, and such findings also contribute toward future molecular breeding studies in the fine wool sheep.

Results

Targeting of FGF5 gene in in vitro sheep embryos

A single-guide RNA (sgRNA) targeting exon1 of FGF5 gene was designed and evaluated by an in vitro essay and used for in vitro embryo targeting. A total of 199 one-cell-stage in-vitro-fertilization (IVF) embryos were subjected to injection of sgRNA/Cas9 into the cytoplasm. By screening the mutants in differential developmental embryos with T7EN1 digestion along with PCR sequencing, we detected high efficient gene targeting events. In total, 58 of 155 embryos were identified with modifications introduced by CRISPR/Cas9, with an efficiency of up to 37.4% (Fig. 1A,B). The modifications could be screened out from the two-cell stage to blastocyst. The outcomes validated the efficacy of the sgRNA/Cas9 system in sheep FGF5 gene editing.

Generation of FGF5 gene targeting sheep

Based on the results from in vitro embryos, we carried out FGF5 gene targeting in sheep. A total of 303 embryos were harvested from 35 super-ovulation donor ewes, and 170 cleaved embryos were transferred to 101 surrogate ewes. Finally, 20 surrogates completed full-term concept and produced 20 offsprings. By T7EN1 digestion followed by PCR sequencing, 16 lambs were identified with insertion–deletion (InDel) modifications, of which 13 were biallelic and three were monoallelic (Fig. 1C,D). The targeting efficiency was as high as 80% and the biallelic rate was up to 81%. The results showed that the sgRNA/Cas9 system functions with high efficiency to target FGF5 gene.

Characterization of disruptive modifications in the FGF5 gene

According to PCR sequencing data, half of the genetically modified (GM) lambs exhibited two kinds of InDels in the targeting region of FGF5. Further analysis of each GM individual by T-A cloning sequencing precisely characterized the variety of modifications (Fig. 2). The 28-bp deletion (c.84_111del,D28) was the predominant modification in all GM lambs, accounting for 50% of GM lambs. The other two major modifications were a 13-bp deletion (c.84_96del,D13) and a 17-bp deletion (c.85_101del,D17), which were detected in three GM lambs, respectively. In all GM lambs, 13 frameshift mutations and three in-frame deletions were identified. All of the frameshift deletions resulted in premature termination and led to truncated proteins.

The FEBS Journal 284 (2017) 2764–2773 © 2017 The Authors. The FEBS Journal published by John Wiley & Sons Ltd on behalf of Federation of European Biochemical Societies.
that could completely or partially abolish FGF5 function (Fig. 3). This indicated that targeting FGF5 by CRISPR/Cas9 could efficiently bring out loss-of-function modifications.

To precisely assess the putative functional impact of the targeted FGF5 modification, we performed prediction of protein secondary structure and construction of 3D models for the three major targeting deletions noted above. The predicted protein structure showed that the truncated proteins resulted from premature termination and lost the critical β-strands domain, which is necessary for FGF5 function (Fig. 4). This inferred that major targeting events led to disruption of FGF5 function.

**Off-target analysis**

To confirm the off-target modifications in the targeted lambs, we determined the putative off-target sites predicted by the software. A total of three potential off-target sites across the entire sheep genome were sequenced. No off-target mutations were detected in these loci in all founder animals. This demonstrated the high specificity of the sgRNA used in this study.

**Phenotype analysis of gene-targeted lambs**

Except for four lambs that died, the other 12 yearling alive GM founders and 11 wild-type (WT) control animals were subjected to phenotype evaluation. The wool staple length and stretched length of targeted sheep were 10.73 and 12.29 cm versus 9.23 and 10.86 cm of control animals, respectively. Both the staple length and stretched length of FGF5-targeted lambs were significantly longer compared with those of nontargeted controls ($P < 0.01$; Fig. 5A,B and Tables 1 and 2). This demonstrated that disruption of FGF5 stimulated wool growth and increased wool
length. Concordantly, the greasy fleece weight of targeted lambs was significantly heavier compared with that of nontargeted controls: 3.74 kg for targeted lambs versus 2.96 kg for controls \( (P < 0.01; \text{Fig. 5D and Tables 1 and 2}) \). Notably, the fineness of the two groups showed no significant difference: 19.83 \( \mu \text{m} \) for targeted lambs versus 19.60 \( \mu \text{m} \) for controls \( (P < 0.01; \text{Fig. 5C and Tables 1 and 2}) \). This suggested that the increase in wool weight is most possibly attributed to the increase in wool length.

**Discussion**

In sheep, wool growth predominantly refers to the production of large quantities of wool fibers, which are destined for the use at the top end of the market. Once formed in wool-bearing areas of the sheep skin, wool follicles grow down into the dermis through a maturation phase that culminates in fiber production [20]. As follicle maturation proceeds, the dermal papilla that resides within the base of the follicle acts as a control center for follicle activity. Continuous cell division in the follicle bulb region facilitates the movement of concentrically arranged inner root sheath, cuticles, and cortical cells upwards toward the skin surface, accompanied by terminal differentiation of these cells [21]. Thus, the production of hardened wool fiber requires efficient activation of dermal papilla to satisfy constant cell division in the follicle bulb region.

As a regulator of hair growth, FGF5 has been shown to be responsible for the long hair phenotype by targeted and spontaneous loss-of-function mutations. It inhibits the activation of dermal papilla cells proliferation and the synthesis of hair fiber [22]. The WT FGF5 protein has a critical β-stand motif involved in the interaction between FGF5 and its receptor to trigger receptor activation and biological

![Fig. 2. InDel forms and loci around PAM for all targeted lambs. (A) Number of nontargeted control (NTC) and targeted lambs. (B) WT and targeted sequences. 'ʌ' in conjunction with lowercase red italics represents the insertion of base pair; small italic letters indicate the substitution of a base pair. (C) InDel forms and their ratio of correspondent colonies of clone sequencing.](image-url)
Table: Loss of FGF5 function in sheep

| Lambs | Sequence | Indel type | Frame shift/in frame | Allelic modification |
|-------|----------|------------|----------------------|----------------------|
| WT    | GAGAAGCGCTCGACCACCAAA | 3N+0 | N = 0 | In frame | FGF5+/FGF5+ |
| GM003 | GAGAAGCGCTCGACCACCAAA | 3N+1 | N = 4 | Premature termination | FGF5–/FGF5– |
| GM007 | GAGAAGCGCTCGACCACCAAA | 3N+1 | N = 9 | Premature termination | FGF5–/FGF5– |
| GM009 | GAGAAGCGCTCGACCACCAAA | 3N+1 | N = 9 | Premature termination | FGF5–/FGF5– |
| GM010 | GAGAAGCGCTCGACCACCAAA | 3N+0 | N = 0 | Premature termination | FGF5–/FGF5– |
| GM011 | GAGAAGCGCTCGACCACCAAA | 3N+0 | N = 0 | Premature termination | FGF5–/FGF5– |
| GM012 | GAGAAGCGCTCGACCACCAAA | 3N+1 | N = 4 | Premature termination | FGF5–/FGF5– |
| GM013 | GAGAAGCGCTCGACCACCAAA | 3N+1 | N = 4 | Premature termination | FGF5–/FGF5– |
| GM021 | GAGAAGCGCTCGACCACCAAA | 3N+0 | N = 6 | In frame (six aa del) | FGF5–/FGF5– |
| GM022 | GAGAAGCGCTCGACCACCAAA | 3N+2 | N = 8 | Premature termination | FGF5–/FGF5– |
| GM026 | GAGAAGCGCTCGACCACCAAA | 3N+0 | N = 4 | In frame (four aa del) | FGF5–/FGF5– |
| GM039 | GAGAAGCGCTCGACCACCAAA | 3N+2 | N = 5 | Premature termination | FGF5–/FGF5– |
| GM041 | GAGAAGCGCTCGACCACCAAA | 3N+0 | N = 6 | In frame (six aa del) | FGF5–/FGF5– |
| GM046 | GAGAAGCGCTCGACCACCAAA | 3N+1 | N = 4 | Premature termination | FGF5–/FGF5– |
| GM050 | GAGAAGCGCTCGACCACCAAA | 3N+1 | N = 1 | Premature termination | FGF5–/FGF5– |
| GM056 | GAGAAGCGCTCGACCACCAAA | 3N+2 | N = 2 | Premature termination | FGF5–/FGF5– |
| GM066 | GAGAAGCGCTCGACCACCAAA | 3N+1 | N = 9 | Premature termination | FGF5–/FGF5– |
| GM1135 | GAGAAGCGCTCGACCACCAAA | 3N+1 | N = 9 | Premature termination | FGF5–/FGF5– |

Fig. 3. Modifications and editing events of FGF5 targeted sheep. Lambs, FGF5 targeted sheep numbered as GM003–GM1135; nontargeted lambs as WT. Sequence: targeted sequence near PAM region and Indel modifications defined by sequencing of PCR products and further T-A clone sequencing; small letters in blue indicate a base pair substitution, letters in red refer to insertion. The PAM sequence is underlined and shown in bold; all modifications were determined by alignment with the WT sequence. Frame Shift/in Frame: predictive translation events (frameshift or in-frame) resulting from targeting events based on exact mutations. Allelic modification: characterization of targeting by biallelic modification or monoallelic modification; +/+, WT; –/–, biallelic modification; +/–, monoallelic modification.

It is reasonable to hypothesize that, by targeting the deficiency of FGF5, efficient activation of dermal papilla could be induced. Therefore, more cells would be committed for fiber generation and follicle maturation would eventually be promoted. The targeted and spontaneous FGF5 mutations that resulted in abnormally long hair were evidently attributed to the disruption of FGF5 protein; therefore, loss-of-function mutations as a result of targeting sheep FGF5 would act to cancel its inhibition upon activation of dermal papilla and increase the wool length.

Accordingly, we targeted the FGF5 gene of Chinese merino sheep by CRISPR/Cas9 and created 16 edited founders with disruption of FGF5. Subsequent investigation of the wool traits of 12 lambs showed that wool staple length, stretched length and yearling grease fleece weight were significantly longer and heavier compared with those of WT control animals. As a result of sgRNA targeting FGF5 sequence of exon 1, the truncated FGF5 proteins resulting from editing abolished the most parts of the WT FGF5 protein. Among the edited modifications, the predominantly modified allele D28 (c.84_111del, 28 base deletion) induced a frameshift that led to a premature stop codon at position 110; the other two major modifications, D13 (c.84_96del, 13 base deletion) and D17 (c.85_101del, 17 base deletion), also created a premature stop codon and led to the generation of 115 and 45 amino acid truncated proteins, respectively. Based on the predicted secondary structure and their 3D models of three major truncated proteins, D28, D13 and D17 FGF5 truncated proteins totally lacked all β-strands compared with the WT FGF5 protein that had 14 β-strands. The β-strands within the canonical FGF5 trefoil determine its unique binding profiles to FGFRs, which is a mandatory step for FGF5 function. Thus, even when expressed and secreted correctly, the modified FGF5 proteins without β-strands were unlikely to
medicate the FGF5 signal cascade. It was strongly postulated that D28, D13 and D17 were loss-of-function mutants of FGF5.

Further investigation of the wool traits of the targeted founders indicated that GM sheep with loss-of-function modifications exhibited an increase in wool staple length, wool stretched length and grease fleece yield. The improvement of wool traits is in agreement with long-haired phenotypes that were previously observed in spontaneous loss-of-function mutations of FGF5 in mice [2,3] and donkeys [7], as well as in targeted dysfunctional modification in FGG5 knockout Dorper sheep [24]. Overall, we deduced that the targeted effect most possibly resulted from loss-of-function modifications of the FGF5 gene because of a lack of the β-strand domain of mutant FGF5.

Taken together, a set of loss-of-function modifications of FGF5 were successfully generated by CRISPR-Cas9 in fine wool sheep. An elongated wool staple length, stretched length and greater grease fleece weight were observed as expected in yearling edited founders. The results of this study highlight that blockade of FGF5 activity by gene targeting could result in the promotion of wool growth and lead to an increase in wool staple length and grease fleece yield.

Materials and methods

Animals

The animals used in this study were regularly maintained in the Research Base of Sheep Breeding of Xinjiang Academy.
of Animal Science, located at Urumqi city in Xinjiang province of China. Surgeries were performed under strict aseptic conditions, and efforts were made to minimize animal suffering. All animal handling procedures were carried out in strict accordance with the approved guidelines of the Institutional Animal Care of Xinjiang Academy of Animal Science.

sgRNA design

The sgRNA targeting sheep FGF5 gene was designed using online software (http://www.e-crisp.org/E-CRISP/reannotate_crispr.html) based on the 361-bp exon 1 sequence (GenBank: NM_001246263). The schematic structure of the sheep FGF5 gene and the sgRNA design are shown in Fig. 1A.

Preparation of Cas9 mRNA and sgRNA

In vitro transcription of Cas9 mRNA and sgRNA was performed as described previously [25]. Briefly, paired synthesized oligonucleotides (FGF5-sgRNA-CF/CR; Table S1) for sgRNA preparation were annealed and then subcloned into the linearized pX330 vector. With respect to the production of sgRNA by in vitro transcription, the primers (FGF5-sgRNA-TF/TR) used in the preparation of the template for in vitro transcription are listed in Table S1. The template of the T7-sgRNA structure was amplified by PCR using PrimeSTAR Max Premix (Takara Bio Inc., Otsu, Japan). The PCR products were subjected to gel purification (Qiagen, Hilden, Germany) and used as the template for in vitro transcription with the MEGAscriptTMT7 transcription kit (Ambion, Austin, TX, USA). A three-nucleotide spacer (AGA) and T7 promoter were added to the 5’ flanking of the Cas9 coding region by PCR with the primers of Cas9 F/R (Table S1) and with px330 as the template. The amplified T7-Cas9 products were purified and used as the template for in vitro transcription with the mMESSAGEmMACHINE® T7 ULTRA transcription kit (Ambion) in accordance with the manufacturer’s instructions. Both the Cas9 mRNA and the sgRNA were purified using the MEGAclear™ transcription clean-up kit (Ambion).

Verification of FGF5 targeting by CRISPR Cas9 in in vitro ovine embryos

Collection, maturation, and culture of sheep oocytes in vitro were carried out as described by Wang et al. [26]. Briefly, ovaries were collected from a local abattoir.
Immature oocytes were aspirated from medium-sized (3–6 mm) antral follicles. After 22–24 h of maturation, the surrounding cumulus cells were removed from the oocytes by blowing repeatedly with a needle in the presence of 0.1% hyaluronidase. A frozen semen pellet was thawed and added into 1 mL of IVF medium, and the mixture was co-incubated for 30 min. Some 14–15 h after fertilization, the mixture of 100 ng·µL⁻¹ Cas9 mRNA and 50 ng·µL⁻¹ FGF5-sgRNA was injected into the cytoplasm of one-cell fertilized eggs using a micro-injection system (Nikon, Tokyo, Japan). Injected embryos were cultured in M16 medium (Sigma, St Louis, MO, USA) for 7 days. The cleavage rate of embryos was calculated at 48 h post fertilization, and embryos were harvested from the two-cell stage to blastocyst for modification screening. For genotyping of the targeted mutation, embryos were treated by lysis buffer, and lysate was used as a primary template for PCR amplification by primers (FGF5-F/R1; Table S1). The PCR products were immediately used as a secondary template for further nested PCR by primers (FGF5-F/R2; Table S1). The final PCR products were used in a T7EI assay [27] and sequencing.

Generation of gene-modified sheep by injection of Cas9/sgRNA into zygote cytoplasm

Gene-modified sheep were generated via microinjecting the mixture of Cas9/sgRNA into the cytoplasm of zygotes. Sheep zygotes were collected via surgical oviduct flushing from the donors using estrus synchronization and a super-ovulation treatment as described previously [28].

Then, the zygotes were subjected to cytoplasmic microinjection with 100 ng·µL⁻¹ Cas9 mRNA and 50 ng·µL⁻¹ sgRNA. After injection, zygotes were cultured in in vitro culture medium (SOF supplemented with 3 mg·mL⁻¹ BSA) for 24 h at 38.6 °C, 5% CO₂, until they divided into two to four cells. A total of 101 ewes aged 2–4 years old regular estrus cycles were selected as recipients. For embryo transfer, all recipients were synchronized by the same treatment as donor ewes. The divisive embryos were transferred into the ampullary-isthmic junction of the oviduct of recipients. After 60 days of transplantation, pregnancy was determined by an ultrasound scan.

PCR-based assay for screening of genetic modified animals

For editing event analysis, tissues from lamb tail were sampled and the genomic DNA was extracted from ground tissue lysate by phenol–chloroform, and recovered by alcohol precipitation. DNA with an equal amount (300 ng) was used as a template for PCR amplification by primers (FGF5-F/R2; Table S1) according to the program: 95 °C for 5 min followed by 95 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s, 72 °C 45 s, ending with 72 °C for 7 min after 35 cycles. All PCR products were subjected to a T7EI assay [27] and further Sanger sequencing.

T7EI cleavage assay

The T7EI cleavage assay was performed as described previously [29]. In brief, targeted fragments were amplified from extracted DNA by TaqDNA polymerase (BBI, Toronto, ON, Canada). Approximately 200 ng of PCR product for each sample was denatured and annealed to generate...
heteroduplexes in NEB Buffer 2 (NEB, Beverly, MA, USA) using the program settings: 95 °C for 10 min, 95 °C to 85 °C at −2 °C·s−1, 85 °C to 25 °C at −0.3 °C·s−1, holding at 25 °C for 1 min, and finally at 10 °C. After reannealing, the products were treated with 0.2 μL of T7EI (NEB) and incubated at 37 °C for 30 min. Then, the digested PCR products were subjected to 2% agarose gel electrophoresis.

**Prediction of the modified FGF5 protein structure of targeted lambs**

To evaluate the effects of targeted deletion, three main targeted modifications were chosen. The secondary structures of three truncated proteins were predicted by Phyre2 software, and their 3D models were constituted using the Phyre server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The WT of the FGF5 protein was set as the control.

**Off-target prediction and analysis**

All potential off-target sites with homology to the 23-bp sequence [sgRNA plus protospacer adjacent motif (PAM)] were retrieved by a base-by-base scan of the whole Ovis aries genome (3.1) using an online tool (http://www.e-crisp.org/E-CRISP/reannotate_crispr.html). Three potential off-target sites were predicted in the sheep genome. These potential off-target sites were amplified from all of the targeted sheep and embryos. The primers used for amplifying the off-target sites are listed in Table S1. The PCR products were first subjected to a T7E1 cleavage assay, and then the off-target sites are listed in Table S1. The PCR products were subjected to TA cloning and sequencing.

**Sampling wool and measurement of wool phenotype**

Wool samples for determining staple length and fiber mean diameter were removed from scapular region of side of the body (around the first rib area) when the GM sheep were growing up to age 1 year. Next, 10 g for each sample was required for subsequent measurement of staple length and fiber mean diameter. The staple length, stretched length and wool greasy weight were measured manually in accordance with the National Sheep Wool Quality Inspection Standards of China (GB1523-2013). Fiber mean diameter was determined by OFDA 2000, according to the GB/T21030-2007 instruction.

**Statistical analysis**

Student's t-test was used for the statistical analysis (spss, version 15.0; SPSS Inc., Chicago, IL, USA). P < 0.05 was considered statistically significant.

**Acknowledgements**

This study was funded by grant 2016ZX08008001-002-001 from the China Agriculture Ministry genetically modified organisms project. It was also partly supported by grant U1303284 from the China National Natural Science Foundation, a subcontract of grant 2013AA102506 from the China National High Technology Research Development Program (863 Program) and the Key Laboratory Research Grant of Xinjiang Province (2014KL007).

**Author contributions**

All authors contributed directly to the planning, execution, and analysis of this work. M-JL, W-RL, C-XL, and S-GH conceived and designed the experiments. C-XL and X-MZ prepared the Cas9 and SgRNA and identified the targeted lambs. LC performed phenotype measurement and analysis of targeted sheep. X-RP, J-PL, BH, L-QW, and J-CH contributed to generating the targeted sheep. This article was written by M-JL, W-RL, C-XL, and X-MZ.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Primers used in this study.