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RAPID COMMUNICATION: Generation of *FGF5* knockout sheep via the CRISPR/Cas9 system[1,2]

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ABSTRACT: Sheep are an important source of fiber production. Fibroblast growth factor 5 (FGF5) is a dominant inhibitor of length of the anagen phase of the hair cycle. Knockout or silencing of the *FGF5* gene results in a wooly coat in mice, donkeys, dogs, and rabbits. In sheep breeding, wool length is one of the most important wool quality traits. However, traditional breeding cannot accurately and efficiently mediate an advanced genotype into the sheep genome. In this study, we generated 3 *FGF5* knockout sheep via the 1-step clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system. Sequencing analysis confirmed that mutations in the *FGF5* gene existed in all germ lines of 3 founders: besides the intact sequence, 3 kinds of deletions in the *FGF5* gene (including 5, 13, and 33 bp) were detected. The changes in the primary and senior structure of the FGF5 protein due to the 3 deletions in founders suggested that the FGF5 protein was dysfunctional. In addition, the expression level of intact *FGF5* mRNA in heterozygous individuals decreased compared with the wild types ($P < 0.01$). Functionally, we discovered that wool length in founders was significantly longer than in wild types ($P < 0.05$). Collectively, the *FGF5* knockout sheep with the longer wool length phenotype will provide an efficient way for fast genetic improvement of sheep breeding and promote the development of wool industry.

Key words: *Cas9*, *fibroblast growth factor 5*, knockout, sheep, wool

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INTRODUCTION

The wool industry occupies an important share of the global agricultural economy. Wool length is one of the most important wool quality traits in sheep breeding. A cycle of hair follicles in mammals undergoes 3 phases, including the anagen (growth), catagen (involution), and telogen (rest) phases (Ryan, 2003). It has been verified that *FGF5* (*fibroblast growth factor 5*) is an inhibitor of hair elongation, as *FGF5*-null mouse exhibit an extremely long hair phenotype. The underlying mechanism was clarified by Hébert et al. (1994), who reported that silencing the expression of the *FGF5* gene prolonged the anagen VI phase of the hair cycle, resulting in a phenotype of extremely long hair. In addition, mutations in *FGF5* have been found in other species, including humans (Higgins et al., 2014), cats (Drögemüller et al., 2007; Kehler et al., 2007), dogs (Housley and Venta, 2006; Cadieu et al., 2009), rabbits (Li et al., 2008; Allain and Renieri, 2010), donkeys (Legrand et al., 2014), and mammoths (Roca et al., 2009). However, its low gene frequency in the natural genetic resources of sheep limits the genetic improvement of breeding in the short term.

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Recently, a novel and efficient genome editing tool based on the clustered regularly interspaced short palindromic repeat (**CRISPR**)/CRISPR-associated (**Cas**) system has been booming. The Cas9 endonuclease from the *Streptococcus pyogenes* type II CRISPR/Cas system has been used to modify the eukaryotic genome under the guidance of a synthetic single guide RNA (**sgRNA**) with simple base pair complementarities with a target genomic DNA sequence (Jiang et al., 2013; Hsu et al., 2014). The CRISPR/Cas system injected into a 1-cell embryo was used to efficiently generate genetically engineered model animals and livestock (Shen et al., 2013; Wang et al., 2013, 2015a,b; Ma et al., 2014; Niu et al., 2014). In the present study, we successfully generated *FGF5* knockout sheep using the CRISPR/Cas system by direct 1-step cytoplasmic injection of Cas9 mRNA and sgRNA into zygotes. These *FGF5* knockout sheep exhibited longer wool length compared with wild-type sheep, which would provide an efficient way for the genetic improvement of sheep breeding and promote the development of wool industry.

MATERIALS AND METHODS

Ethics Statement

All experimental animal protocols were approved and performed in accordance with the requirements of the Animal Care and Use Committee at China Agricultural University (approval number CAU20140910-2). To minimize the pain suffered by experimental animals, all surgical operations were performed under anesthesia.

Construction of a Template for Cas9 *mRNA and Single Guide RNA Transcription*

The strategy for the construction of the template for *Cas9* mRNA and sgRNA transcription is shown in Fig. 1A. Briefly, the *Cas9* sequence from *Streptococcus pyogenes* SF370 was codon optimized for sheep by an online platform (http://www.kazusa.or.jp/codon/) and designed, including 3X FLAG tags, nuclear localization signals, and a stop codon. Then, the sheep-optimized *Cas9* (*ShCas9*) was synthesized. The sgRNA scaffolds were designed online (http://www.genscript.com/gRNA-design-tool.html). The sequence of *FGF5* sgRNA was GAGGTTCCCCTTTCCGCACCT. The target site in the genome for *FGF5* sgRNA is shown in Fig. 1B. To avoid the off-target effect, the last 10 to 12 nucleotides of the 3′ end of the sgRNA targeting region of candidate sgRNA sequences were screened by basic local alignment search tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) in the sheep genome. A T7 promoter was linked to the 5′ ends of both *ShCas9* templates and the coding region of *FGF5* sgRNA by PCR. Details of the PCR primers were as follows: T7-*Cas9*F: TAATACGACTCACTATAGGGAGAATGGACTATAAGGACCATGAC; T7-*Cas9*R: TTATTTCTTTTTCTTAGCTTGACC; T7-*FGF5*F: TAATACGACTCACTATAGGAGGTTCCCCTTTCCGCACCT; and T7-*FGF5*R: CGACGCACTCGGTGCCACTT. Then, these PCR products, as transcription templates, were purified by E.Z.N.A. Cycle-Pure Kit (Omega Bio-tek Inc., Norcross, GA).

In Vitro Transcription

Cas9 mRNA and *FGF5* sgRNA were in vitro transcripted using a MEGA short script T7 kit (Thermo Fisher Scientific Inc., Carlsbad, CA) and purified using a MEGAclear kit (Thermo Fisher Scientific Inc.). *Cas9* mRNA was linked with the m7G(5′)ppp(5′)G cap on its 5′ terminal and poly A(3′) tail on its 3′ terminal using a mMACHINE T7 ULTRA kit (Thermo Fisher Scientific Inc.).

Pronuclear Microinjection and Transgenic Sheep Generation

Healthy Dorper ewes, ranging in age from 1 to 3 yr, were selected for use in this study. Embryo collection and transfer were performed as previously described (Deng et al., 2012). In brief, healthy Dorper ewes 1 to 3 yr old with regular estrous cycles were selected as embryo donors, and their estrous cycles were regulated by an intramuscular injection of a controlled internal drug release (**CIDR**) device (Pharmacia and Upjohn Co., Kalamazoo, MI). For superovulation, ewes were treated with a CIDR device + FSH (Merck Serono Co., Geneva, Switzerland). Fertilized 1-cell embryos were collected and microinjected with a mixture of *Cas9* mRNA (20 ng/L) and 5 sgRNA (5 ng/L each). Well-fertilized embryos were transplanted into the recipient oviducts within 1 h, and each recipient was transplanted with 2 to 5 zygotes.

SURVEYOR Assay and DNA Sequencing

Genomic DNA of lambs was extracted using E.Z.N.A. Tissue DNA Kit (Omega Bio-tek Inc., Norcross, GA). First, the sequence of the *FGF5* gene from microinjected individuals was amplified by PCR (PCR primers were as follows: forward: CTCCGCCATACACAGAACTG and reverse: CCAAGAAGTTGCCTTCAGAG), and the PCR products were slowly reannealed to generate heteroduplexes. The reannealed heteroduplexes were cleaved by SURVEYOR nuclease (Transgenomic, Inc.,

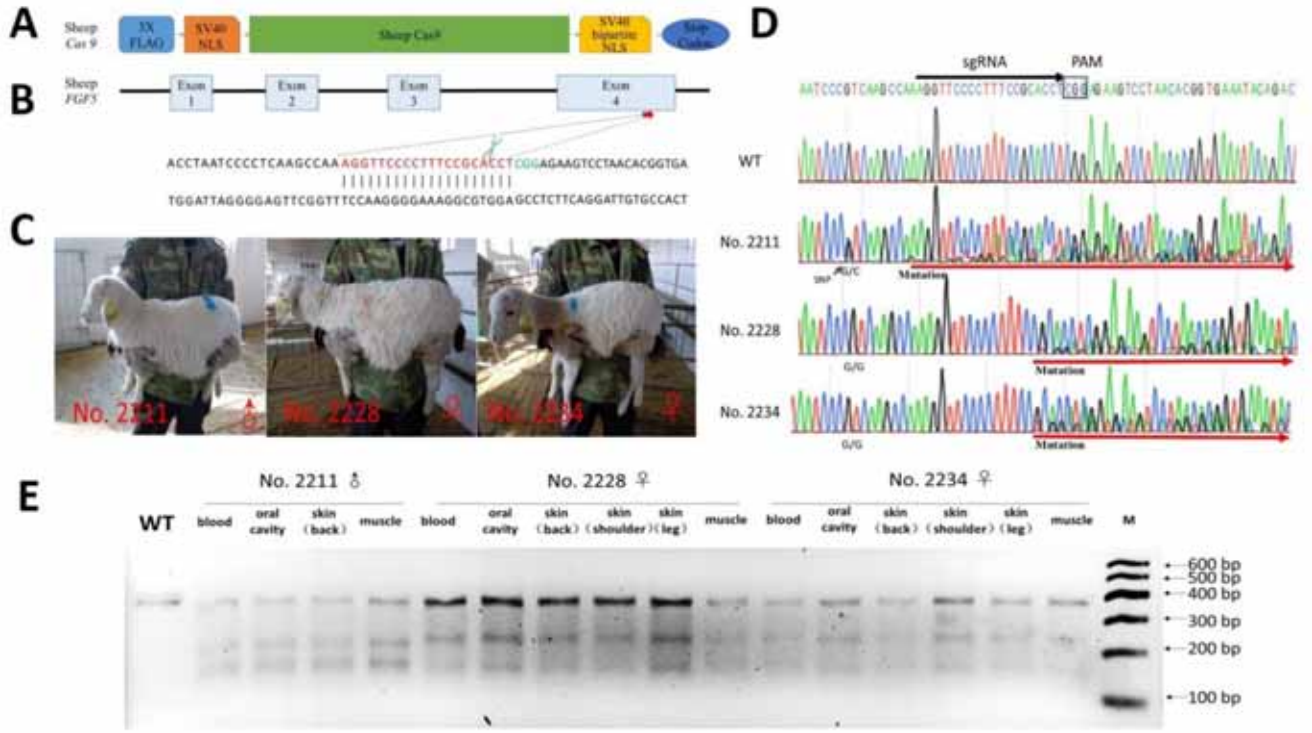

Figure 1. Generation of *FGF5* knockout sheep by *Cas9*-mediated modifications. (A) Schematic diagram of *Cas9* mRNA structure for 1-step microinjection into 1-cell embryos. The *Cas9* sequence was codon optimized for sheep using an online platform (http://www.kazusa.or.jp/codon/) and linked with 3X FLAG tags, nuclear localization signals (NLS), and a stop codon. (B) Schematic diagram of interaction between FGF5 single guide RNA (sgRNA) and the *FGF5* gene in sheep genome. The partial protein coding region sequences of FGF5 are presented in black, and sgRNA targeting sites are presented in red. (C) Photographs of 30-d-old genetically modified sheep. (D) Sequencing results of modified FGF5 loci detected in lambs. The 5-bp deletion (from 775 to 759 bp) was detected in 2 of the 3 founders (number 2228 and number 2234), and 2 deletions including 13 bp (from 770 to 762 bp) and 33 bp (from 739 to 771 bp) were confirmed in number 2211. (E) SURVEYOR mutation detection in different tissues from 3 genetically modified lambs. The SURVEYOR cleavage assay was used for extensive analysis of the target mutagenesis in 3 different somatic tissues including the oral cavity, muscle, and skin epidermis. SURVEYOR nuclease recognizes and cleaves mismatches due to the presence of SNP or small insertions or deletions. PAM = protospacer adjacent motifs; WT = wild type; ♂ = male; ♀ = Female.

Omaha, NE), whereas homoduplexes were left intact. The DNA concentration of each band was measured on an ethidium bromide–stained 2% Tris-boric acid-EDTA gel. Polymerase chain reaction products from founders confirmed by SURVEYOR nuclease were subcloned into a *pMD-18* vector (Takara Biotechnology (Dalian) Co., Dalian, China) for sequencing. Mutations were identified by alignment of sequenced alleles to the wild-type allele. MegAlign software was used to perform multiple alignments (Corpet, 1988).

Comparison of Protein Sequences and Structure Prediction

The Ensembl (http://asia.ensembl.org/index.html) sequence of *Ovis aries FGF5* was used (ENSOART00000020685.1). The resolved 3-dimensional structure file of the FGF5 protein form has not been published in the database, according to structure of other fibroblast growth factor (**FGF**) family members. The Phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id = index) was used for homology modeling and fold recognition (Kelley and Sternberg, 2009).

Real-Time PCR

Total RNA was extracted from the skin of founders and wild types using TRIzol reagent (Thermo Fisher Scientific Inc.). A RNA reverse kit (Takara Biotechnology (Dalian) Co., Dalian, China) was used to obtain the cDNA. Real-time PCR was performed on a Roche LightCycler 480 instrument, using a SYBR Green I Master kit (Roche Molecular Systems, Inc., Basel, Switzerland). The primer sequences were as follows: FT1F: TGTGTCTCAGGGGATTGTAGG; FT1R: GGCGGAGGCATAGGTATTATAG; FT2F: CACGTCTCTACCCACTTTCTG; and FT2R: TTAGGACTTCTCCGAGGTGC. The expression levels were analyzed using the ΔΔCt method and normalized against β-*actin* (Li et al., 2012). Each sample was run in triplicate.

Statistical Analysis

Statistical comparison of wool length was performed by the Student's *t* test. Statistical analyses were performed using SAS release 8.1 (SAS Inst. Inc., Cary, NC).

RESULTS AND DISCUSSIONS

Generation of FGF5 *Knockout Sheep*

FGF5 is expressed in the hair follicle and plays a critical role in inhibiting the length of the anagen phase of the hair cycle. Many researches have provided evidence that a dysfunctional *FGF5* gene leads to the phenotype of long hair. To obtain a breed with a long wool trait, CRISPR/Cas9 pronuclear microinjection was used for sheep *FGF5* gene modification. In total, 155 1-cell embryos were collected. *Cas9* mRNA and sgRNA were pooled and microinjected into the cytoplasm of embryos. One hundred injected 1-cell embryos were transferred into 53 surrogate females. Using B-mode ultrasound, it was observed that 14 recipients were pregnant on d 30 to 35 after embryo transfer. The pregnancy rate of recipients was 26.4%. In total, 18 lambs were born, including 5 twins. These data demonstrated that optimized *Cas9* mRNA and *FGF5* sgRNA were not obviously harmful to the embryos. To identify the *FGF5* gene modified rate of newborn sheep and the efficiency of Cas9 targeting in the genome, the blood DNA template was screened for positive individuals using PCR. Then, PCR products were subjected to DNA sequencing. The results demonstrated that the genomic modification occurred in 3 founder animals, including 2 females (number 2228 and number 2234) and a male (number 2211; Fig. 1C and 1D). The *FGF5*-mutated individuals presented, in their genomes, various amounts of integration; the integration efficiency was 15.8%. These results further substantiate the high efficiency of the CRISPR/Cas9 system in sheep. Compared with the classical embryonic nuclear transfer, although the success rate of positive founders is not high, this 1-step procedure leads to a high rate of healthy embryo development and a low rate of deformity. According to previous research results, chimerism existed in genetically modified animals obtained by the pronuclear microinjection Cas9 system (Yen et al., 2014). The SURVEYOR cleavage assay was used for extensive analysis of the target mutagenesis in 3 different somatic tissues differentiated from germ layers, including an oral cavity swab (entoderm), muscle (mesoderm), and skin epidermis (ectoderm), from 3 genetically modified lambs. The cleavage bands observed in every reaction indicated that the Cas9/sgRNA-mediated mutations existed in all germ layers (Fig. 1E).

Changes in Structure of FGF5 Protein Resulted from Gene Editing

To clarify details of the deletion in *FGF5* from founders, TA-clone and Sanger sequencing were used. Sequencing results demonstrated that mosaicism existed in all founders: besides intact *FGF5*, only a 5-bp deletion (from 775 to 759 bp) was detected in 2 of the 3 founders (number 2228 and number 2234), and 2 deletions including 13 bp (from 770 to 762 bp) and 33 bp (from 739 to 771 bp) were confirmed in number 2211 (Fig. 2A). It is interesting to note that more than 2 of the same genotypes were detected in female individuals. Further studies are required to investigate this phenomenon in sheep. It was highly conserved between the sheep FGF5 protein sequence and several other mammals. It is well known that FGF5 contains a conserved receptor binding domain, which interacts with FGFR1 (Beenken and Mohammadi, 2009). Fibroblast growth factors have a homologous core region ordered into 12 conserved antiparallel β strands (β1–β12) flanked by divergent amino and carboxyl termini, forming a contiguous, positively charged surface for binding FGF5 receptors (Mohammadi et al., 2005; Goetz and Mohammadi, 2013). In silico, the *FGF5* gene with deletions (5, 13, and 33 bp) results in a FGF5 protein frameshift or deletion of AA: the 5-bp deletion induces a frameshift that leads to a missense mutation (4 AA) from position 252 (Ser) to 256 (stop codon), the 13-bp deletion induces a frameshift form that leads to a missense mutation (4 AA) from position 249 (Leu) to 256 (stop codon), and the 33-bp deletion induces a frameshift that leads to a deletion (11 AA) from the position 247 (Lys) to 259 (Asn; Fig. 2A). Compared with the sequence of intact FGF5, we demonstrated that the β-12 motif was destroyed. Furthermore, to evaluate the putative functional impact of the FGF5 mutation, based on the structure of 5 FGF proteins, 3-dimensional models were built using the Phyre server. The models predicted that the mutated FGF5 lacked the 12 β strands that define the canonical trefoil of the FGF family (Fig. 2B). Therefore, we concluded that these 3 deletions of FGF5 affect the interaction between FGF5 and its receptors.

Expressions of Intact FGF5 *mRNA is Decreased in the Skin of* FGF5 *Mutated Heterozygous Individuals*

To test the impact of the *FGF5* gene mutations on *FGF5* mRNA expression, we extracted total RNA from skin of 1-mo-old founders and wild types. The transcription level of *FGF5* was measured by real-time PCR. To evaluate the intact *FGF5* mRNA levels, we designed 2 pairs of primers (primers FT1F and FT1R for total *FGF5* and primers FT2F and FT2R for intact *FGF5*) for amplification of *FGF5* mRNA. The primer pairs used for detecting both the mutated and the wild-type *FGF5* sequence are located in the region of 654 to 674 bp and the region of exon 2 (789–810 bp) of *FGF5* mRNA, and the primer pairs used for detecting intact *FGF5* are located in the region of 907 to 927 bp and 1,030 to 1,049 bp of *FGF5* mRNA (Fig. 2C). Because the segment with the 5-,

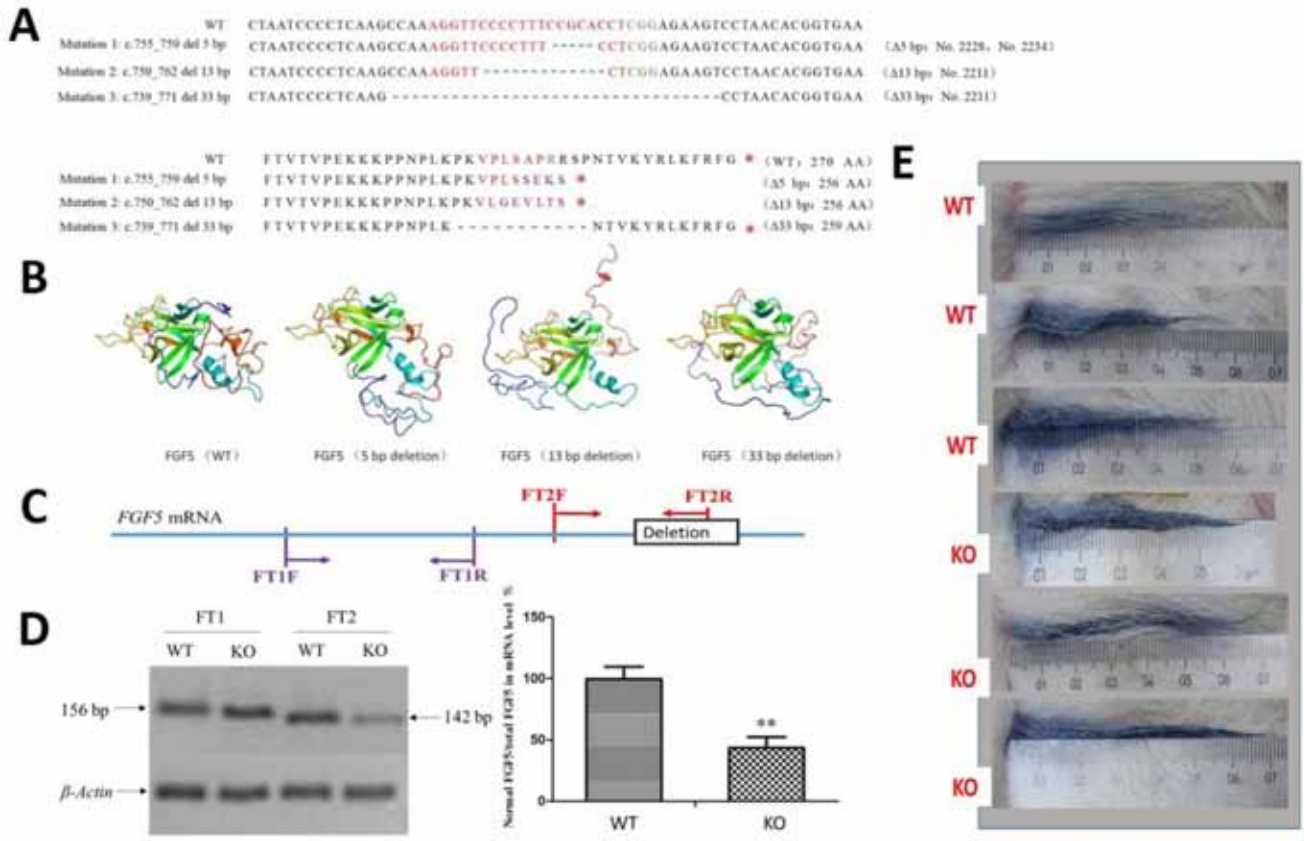

Figure 2. Changes in biological function of FGF5 in 1-mo-old founders. (A) Changes in protein AA sequence of FGF5 in founders. (B) Three-dimensional models built using the Phyre server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id = index) represent wild-type (WT) FGF5 (left) and FGF5 proteins with the 5-bp deletion, 13-bp deletion, and 33-bp deletion (right); the α-helix is highlighted in blue and the β strands are depicted by arrows. (C) Schematic diagram of primers designed for *FGF5*. Two pairs of primers (primers FT1F and FT1R for total *FGF5* and primers FT2F and FT2R for intact *FGF5*) were designed for amplification of FGF5 mRNA. The primer pairs used for detecting both the mutated and wild-type *FGF5* sequences are located in the region of 654 to 674 bp and the region of exon 2 (789–810 bp) of *FGF5* mRNA, and the primer pairs used for detecting intact *FGF5* are located in the region of 907 to 927 bp and 1,030 to 1,049 bp of *FGF5* mRNA. (D) Reverse transcription PCR and real-time PCR results of *FGF5*. Total RNA were isolated from skin of 1-mo-old founders and wild types. The expression levels were analyzed using the ΔΔCt method and normalized against β-*actin*. Each sample was run in triplicate. (E) Change of the loss-of-function mutation in FGF5 in wool length. Wool was collected from both transgenic and wild-type sheep at 1 mo of age. $^{**}P < 0.01$. Wool length was measured after being dyed with ink. KO = *FGF5* knockout.

13-, and 33-bp deletions in mRNA is located from 1,030 to 1,049 bp, the primer set of intact *FGF5* would not be able to amplify any fragment with mRNA samples from the mutant *FGF5* region. The results demonstrated that the mRNA from all lambs can be successfully amplified by the primer sets. Real-time PCR results showed that intact *FGF5* mRNA was significantly downregulated ($P < 0.01$) in the skin of *FGF5*-mutated sheep compared with the wild types (Fig. 2D).

Changes in Wool Phenotype of One-Month-Old FGF5 *Heterozygous Individuals*

To investigate the changes in wool length caused by the loss-of-function mutation in FGF5, the length of the wool was measured from 5 parts of both transgenic and wild-type sheep that were 1 mo old. Results showed that the wool length in all parts of *FGF5* knockout sheep was significantly longer than in wild-type sheep ($P < 0.05$; Fig. 2E). These results further confirmed that the loss-of-function mutation in FGF5 has positive effects on wool length and follicle activation. This is in agreement with long-haired phenotypes previously observed in FGF5-deficient mice, humans, dogs, and cats (Hébert et al., 1994; Housley and Venta, 2006; Drögemüller et al., 2007; Cadieu et al., 2009; Higgins et al., 2014).

In conclusion, we successfully generated genome-edited Dorper sheep with a dysfunctional *FGF5* gene through microinjecting Cas9 mRNA and sgRNA into 1-cell embryos. The *FGF5* knockout sheep with the longer wool phenotype will provide an efficient way for fast genetic improvement of sheep breeding and promote the development of the wool industry.

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