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Double-Muscled Phenotype in Mutant Sheep Directed by the CRISPR-Cas9 System

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Abstract

Myostatin (MSTN) is a well-known negative regulator of muscle growth. The double-muscled sheep caused by natural loss-of-function mutations of MSTN have very strong skeletal muscle. In this study, our results demonstrate the successful generation of MSTN mutant sheep via specific targeting of an exon 1 site using Cas9 technology. The MSTN-knockout sheep in our study had increased muscle significantly just like double-muscled phenotype. Our study suggests that the direct injection of Cas9: sgRNA into zygotes could be widely used to create gene knockouts in large domestic animals. Notably, on the basis of our findings, sheep can be added to the growing list of species for which genome editing is now practical. The generation of MSTN mutant sheep has implications for the genetic improvement of local sheep varieties, and also for the usage of sheep as a model for large animal medical research.

Keywords: Genome editing; Knockout; Targeted mutagenesis; Sheep; Myostatin

Introduction

Myostatin (MSTN), a transforming growth factor-β family member, functions as a negative regulator of skeletal muscle development and growth. MSTN is also directly or indirectly involved in regulation of fat and glucose metabolism [1-5], Animals with mutated MSTN genes show an enhanced phenotype. Natural gene mutations of MSTN have been reported in humans [6], cattle [7], dogs [8] and sheep [9]. MSTN knockout mice display 2 to 3-fold increase in both myofiber size (hypertrophy) and myofiber number (hyperplasia) [10]. The double-muscled cattle caused by natural loss-of-function mutations of MSTN have very strong skeletal muscle and contain much less fat [11]. MSTN-knockout mice have a remarkable increase in muscle mass and significant decrease in fat compared to their corresponding wild-type littermates [12,13]. Therefore, MSTN disruption provides a potential agricultural strategy for promoting animal growth and performance [9]. Gene targeting is the most effective means of introducing mutations in animals and can be used for analyzing gene function, generating animal models for human genetic diseases and optimizing livestock production. CRISPR/Cas9 has been vigorously pursued as an efficient method for genetic modification in a wide variety of animals, including livestock species [14]. The components of the prokaryotic clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system is a recently developed technology for targeted genome modification in mammalian cells, bacteria, zebra fish, mice, monkey and pigs [15-18]. When these indels introduce a frame shift mutation or disrupt important functional domains, the functions of the target genes will be damaged [19-21]. Co-injection of zygotes with Cas9 mRNA and single guide (sg) RNA has proven to be an efficient gene-editing strategy. Redirecting Cas9 to a new target site requires only the alteration of a gene-specific 20-nt DNA sequence in sgRNAs, which can be synthesized at a large scale [22]. Recently, Zhou and colleagues reported the first gene-knockout pigs generated using one-step zygote injection of the CRISPR/Cas9 system, demonstrating a highly promising and rapid method to create large domestic gene-knockout animals [23]. Here, we succeeded in generating knock-out transgenic sheep with CRISPR/Cas9 system. The method is simple, and flexible in design. Our results show that the Cas9-based method induced MSTN disruptions with high efficiency, and the levels of MSTN mRNA and protein were significantly lower in the sheep with the double-muscled phenotype. Furthermore, the efficiency of obtaining transgenic founders is 15.6%. We propose that CRISPR/Cas9 mediated knock-out will become a standard method for the generation of transgenic sheep lines.

Materials and Methods

All 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 College of Biological Sciences, China Agricultural University.

Cell culture to determine gene expression

A sheep fetal fibroblast cell line was cultured in the medium containing 10% fetal bovine serum in 5% CO_2 at 37°C [11]. The cells were seeded in 6-well plates (Thermo Scientific, USA). After 24 h, cells were co-transfected with a mixture of plasmid, pcDNA 3.1(+)-Cas9

and pMD-19T-U6-sgRNA with the mass ratio of 2:1 (2500 ng in total per well), following the instruction of Lipofectamine 3000 (Life Technologies, USA). Cells were harvested 48 h after transfection.

Preparation of Cas9 mRNA and sgRNA

Cas9 and sgRNA coding regions containing T7 promoter were PCR amplified by TransStart FastPfu DNA Polymerase (TransGen Biotech, China) from each plasmid constructed above. The T7-Cas9 PCR products were gel purified and used as the template for *in vitro* transcription (IVT) using mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Life Technologies, USA). The poly (A) tailing reaction was performed after the completion of capping using Poly (A) Tailing Kit (Life Technologies, USA) according to the manufacturer's instruction. The T7-sgRNA PCR product was gel purified and used as the template for IVT using MEGA short script T7 Kit (Life Technologies, USA). The sgRNA was purified by ethanol precipitation and the Cas9-encoding mRNA was by Lithium Chloride (LiCl) precipitation. All the products were re-dissolved in RNase-free water and stored at -80°C.

Microinjection of zygotes and embryo transfer

The sheep zygotes were obtained by super-ovulation of females and artificial insemination. The zygotes were flushed using sterile filtered embryo flushing solution from the oviduct of the sheep. After that, 2-5 pl TE solution containing 40 ng/μl of sgRNA and 80 ng/μl of Cas9 mRNA were injected into the cytoplasm of pronucleus embryos using injection needles. Injections were performed by an Eppendorf transferMan NK2 micromanipulator.

Detection and analysis of indel mutation in lambs

Ear and muscle in back were collected and digested in lysis buffer (10 μM Tris-HCl, 0.4 M EDTA, 1% SDS, and 100 μg/ml Proteinase K). And incubated at 50 for 1 h, followed by extraction in 400 ml of phenolchloroform. The mixture was then centrifuged at 12000 rpm for 20 min at 4. The supernatant was transferred to a new tube. An equal volume of isopropanol was added, and the tube was vortexed thoroughly. The mixture was then kept at -20 for at least 1 h, followed by centrifugation at 12000 rpm for 15 min at 4. The supernatant was removed, and the DNA pellet was washed with 500 l of 75% ethanol. Followed by centrifugation at 12000 rpm for 5 min at 4. Finally, the pellet was dried for 10 min and resuspended in 50 μl of DNase-free water. For each samples, at least 50 clones were picked up randomly and sequenced by Sanger sequencing.

RT-PCR analysis of MSTN mRNA

Total RNA was extracted using the Trizol reagent (Invitrogen) and chloroform, and the RNA was quantified using a Nano drop spectrophotometer. First-strand cDNAs of MSTN and GAPDH (endogenous control) were generated by RT using 1 mg of total RNA and oligo-dT primers. Thermal cycling was performed using 35 cycles of denaturation at 94 for 30 sec, annealing at 60for 30 sec, and elongation at 72. The sizes of the RT-PCR products were estimated by electrophoresis of a 5 ml aliquot on a 2.0% agarose gel.

Western blotting of MSTN protein

For Western blot analysis, total proteins were isolated from the samples by homogenization in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, and complete protease inhibitor cocktail (Beyotime, Beijing, China). The concentration of proteins was measured by Bradford reagent (Sigma), separated on 10% SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore). After blocking in 5% low-fat milk in PBST (0.1% Tween 20 in PBS) for 1 h, the membranes were incubated with tGFP antibody (1:500, Santa Cruz Biotechnology), Firsts antibody (1:500, Santa Cruz Biotechnology) or mouse GAPDH antibody (1:2000, Santa Cruz Biotechnology) overnight at 4°C. After washing in PBST, the membranes were incubated in goat anti-rabbit antibody conjugated with horseradish peroxidase (1:5000) for 1381h, followed by three washes in PBST. The signals were detected by ECL Chemi-luminescent kit (Amersham Pharmacia Biotech, Arlington Heights).

Statistical Analysis

Statistical comparisons of body weight, body height, body length and myofibers among different genotypes of sheep were performed by the Student t-test and $p<0.05$ was considered as statistically significant. Statistical analyses were carried out using SAS release 8.1 (SAS Institute Inc., Cary, NC).

Results and Discussion

Design of sgRNAs

To test the feasibility of gene targeting in sheep using the CRISPR/Cas9 system, 4 guide sgRNAs that target the first exon of the sheep MSTN gene (Supplementary information, Tables S1A and S1B) were designed and assembled. To validate the targeting efficiency of these sgRNAs, T7E1 assays were performed. All sgRNAs efficiently guided Cas9 for genome editing *in vitro* (Figure 1A). To determine whether the sgRNAs also work *in vivo*, a mixture of Cas9 mRNA and the sgRNAs were co-transfected into sheep myoblasts. sgRNA #2 and #4 had the highest targeting efficiency (Figure 1B) and were consequently selected for subsequent experimentation (Figure 1C).

Gene	Number of embryos injected	Number of embryos transferred	Number of new borns	Number of mutations
MSTN	154	130	32	5
Ration	--	84.40%	24.60%	15.60%

Table 1: Numbers of injected and transferred embryos during the establishment of knock-out sheep.

Cas9 mRNA and sgRNA were mixed and injected into sheep zygotes. The injected eggs were transferred into pseudopregnant females. The mutations were identified by sequencing PCR amplified 0.5 kbp genomic fragment containing target in the center.

Generation of gene-modified lambs

In vitro synthesized Cas9 mRNA and the sgRNAs were co-injected into single-celled sheep embryos (Figure 2A). Out of 154 injected embryos, 130 healthy embryos were transferred into surrogate mothers, and 32 lambs were born. PCR was used to assess the presence of the transgene. Of the 32 lambs, 5 were confirmed to be positive for MSTN mutation (#003, #004, #008, #022 and #063; Table 1). Tissue

samples from the ears of the 32 lambs were dissected for MSTN genotyping (Figure 2B).

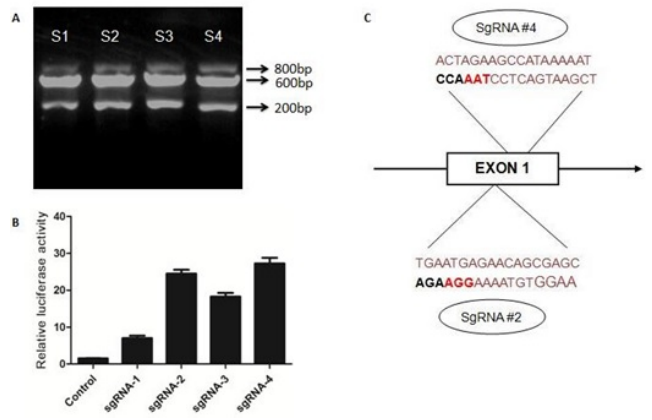

Figure 1: (A): Indel mutations were detected by T7E1. Three independent experiments were performed, and the cleavage efficiency was estimated. All sgRNAs efficiently guided Cas9 for genome editing. (B): Analysis of sgRNA activity. Four sgRNAs were designed to target the sequence of the MSTN gene. The targeting efficiency was obviously higher for sgRNA#2 and #4 than for sgRNA#1 and #3. (C): Schematic diagram of Cas9 binding to the sheep MSTN gene. The sheep MSTN exon1 is displayed as a box and its introns as solid lines. The sgRNA#2 and #4 targeting sites are shown with the PAM sequences marked in red.

Figure 2: (A): MSTN mutant sheep generation via zygote injection of Cas9 mRNA/sgRNA. The positive rate was 15.6%. (B): Sanger sequencing of the targeted site in mutant sheep. The sizes of insertions (+) or deletions (Δ) are indicated (right). (C) Photographs of 30-day-old gene-modified lambs.

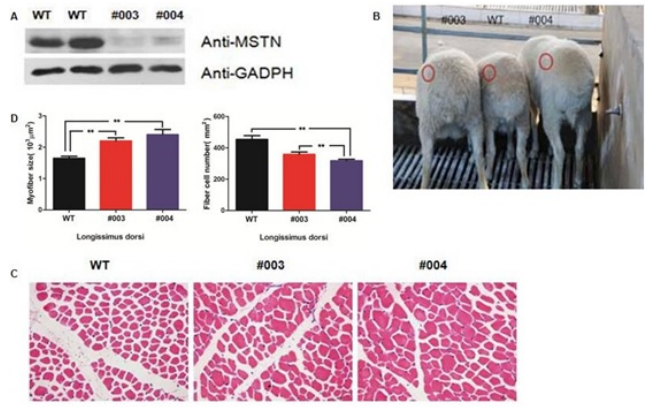

Figure 3: (A): Western blot analysis of MSTN mutant sheep. The MSTN expression for #003 and #004 was lower than for the control. Total protein from mutant sheep was subjected to SDS-PAGE on a 12% acrylamide gel, and MSTN was detected using an anti-myostatin antibody. GADPH was tested as a loading control. (B): Sheep with MSTN mutations displayed the doubled-muscled phenotype. The muscle mass (red arrow) was greater in MSTN mutants (left and right) than in wild-type lambs (middle). (C): Histological cross section of longissimus dorsi. H&E staining showed myofiber hypertrophy in muscles of mutant sheep compared with control sheep. Scale bar = 50 μm. (D): Changes in myofiber size and density in longissimus dorsi. The average size of myofibers in the longissimus dorsi from mutant lambs was 2407 ± 245.36 μm^2 and the control lambs was 1658.51 ± 181.14 μm^2. The average number of myofibers from the control lambs was 444.49 ± 27.59/mm^2 and the mutant lambs was 338.92 ± 17.10/mm^2

Sanger sequencing of the region surrounding the target site showed that each of the 5 positive lambs contained new base insertions or deletions. To confirm these mutations, we cloned the PCR products and randomly selected more than 50 clones from each lamb. Consistent with the cleavage assays, the 5 sheep had mutant alleles that were different from each other (Figures 2C and Supplementary information, Figure S1A).

Expressions of MSTN

QPCR and Western blotting were then performed to assess MSTN expression in the five mutant sheep. MSTN mRNA and protein expression for sheep #003 and #004 was lower than for other sheep (Supplementary information, Figure S2A and Figure 3A). At three month of age, the #003 and #004 MSTN mutant sheep exhibited the double-muscled phenotype without other apparent abnormality (Figure 3B). The muscle mass of the MSTN mutants (left and right) was greater than that of the wild-type lambs (middle). To determine if the increased muscle mass was due to hyperplasia and/or hypertrophy of muscle fibers, the longissimus dorsi from #003 and #004 mutant sheep were dissected for H&E staining. The myofibers in the mutant sheep were hypertrophic and there were more myofibers than in the control sheep (Figure 3C). The average size of myofibers in the longissimus dorsi from mutant lambs (2407 ± 245.36 μm^2) was substantially greater than that of control lambs (1658.51 ± 181.14 μm^2). Furthermore, the average number of myofibers from the control

lambs (444.49 ± 27.59/mm^2) was significantly higher than from mutant lambs (338.92 ± 17.10/mm^2) (Figure 3D).

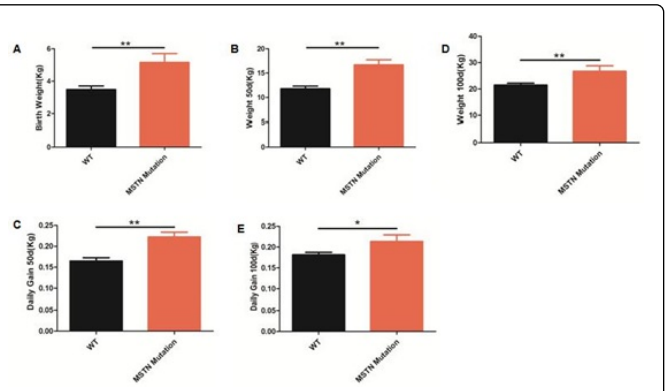

Figure 4: (A): The birth weight of mutant and control sheep. (B): The weight of mutant and control sheep at 50 days of age. (C): The daily gain of mutant sheep and control sheep in 50 days. (D): The weight of mutant and control sheep at 100 days of age. (E): The daily gain of mutant sheep and control sheep in 100 days.

Double-muscled phenotype of MSTN mutant sheep

When the mutant lambs were born, their body weights were obviously higher than that of the other lambs (Figure 4A), and at 50 days, their body weights, daily gain, body length were elevated (Figure 4B, Figure 4C, Supplementary information, Figure S-3A,), though the body heights were similar (Supplementary information, Figure S-3B). When the mutant lambs were 100 days old, their body weights (Figure 4D), daily gain (Figure 4E) body length (Figure S-3C) and body height (Figure S-3D) were obviously higher than the controls.

Discussion

MSTN dysfunction resulted in dramatic increase of animal muscle mass due to hypertrophy and hyperplasia of muscle fibers [24–27]. Inhibition of MSTN expression by gene knockout could promote the muscle growth and meat production of livestock animals. Due to the low efficiency of gene target in livestock animal somatic cells, Cas9/gRNA is an ideal alternative for production of MSTN-knockdown transgenic livestock. In recent years, other engineered endonucleases, such as TALENs and clustered regularly interspaced short palindromic repeats and their associated systems, have been used for genome alteration [27-31]. In comparison to ZFNs and TALENs, the CRISPR/Cas9 system is suitable for this method because of the ease of donor-vector construction and of the multiple gRNA design [32,33]. Unlike other ways of editing genes, which require construction of double-stranded DNA templates, this CRISPR/Cas9 system allows rapid and seamless editing of the genome at precise locations [34-36]. So the Cas9/gRNA-mediated gene editing was a more effective genome-editing tool. The use of gene editing in sheep species poses particular challenges based on the structure of the fertilized egg and early embryo. In our study we demonstrated the feasibility of Cas9/gRNA-mediated gene editing at the MSTN locus of sheep genome. Our results demonstrate the successful generation of MSTN mutant sheep via specific targeting of an exon 1 site using Cas9 technology and microinjection. The partial silencing of MSTN in livestock may weaken some negative effects of null mutations while increasing meat performance [26]. Some disadvantages of double-muscled cattle include the reduction in female fertility, lower viability of offspring, and delay in sexual maturation [11]. The MSTN-knockout sheep in our study had increased muscle phenotype, but no abnormal performance was noted (Figure 3B). Our study suggests that the direct injection of Cas9: sgRNA into zygotes could be widely used to create gene knockouts in large domestic animals. Notably, on the basis of our findings, sheep can be added to the growing list of species for which genome editing is now practical. In this study, we successfully generated several off-targeted MSTN mutant sheep with a high efficiency (15.6%). This high efficiency could be due to the fact that the sgRNA target site covered the site of mutation, as it has been shown that the closer the point of sgRNA digestion is to the desired mutation locus, the more efficiently the desired mutation occurs [37-39]. Together, these data indicated that injection of sgRNAs only into oocyte-specific Cas9transgenicsheep embryo is a convenient, efficient and reliable approach for sheep genome editing.

Conclusion

In summary, our study reports the production of healthy myostatin KO lambs using the CRISPR/Cas9 system in an efficient way to increase muscle growth and body weight. We have shown that zygote injection of the CRISPR/Cas system can efficiently generate genome-modified sheep in one step. It may become a powerful tool for assessing the functions of genes, altering critical residues in proteins to create desirable gain-of-function or loss-of-function mutations, and generating targeted mutagenesis in highly conserved proteins in sheep to facilitate the study of corresponding human diseases and agricultural production. The generation of MSTN mutant sheep has implications for the genetic improvement of local sheep varieties, and also for the usage of sheep as a model for large animal medical research.

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Author Contributions

W.C.H and W.X.G participated in study design, Z.L and Z.C.Y performed the experiments, L.R.Z and Z.F.P analyzed the data, W.M.M wrote the manuscript. W.Y.Y, F.H.Y and Y.Z.H conducted the animal experiments. L.J.F and Y.L feeding management, carcass dissection and sample collection, X.J.L, M.X.M and H.S.J performed the pathological analyses. D.L.X. and W.C.D. oversaw the study. All authors discussed the data. All authors reviewed the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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