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Effect of MSTN propeptide protein on the growth and development of Altay lamb muscle

W. Du[1], Y. Zhang[1], J.Z. Yang[2], H.B. Li[1], J. Xia[1], N. Li[1], J.S. Zhang[1], X.M. Yan[1] and Z.Y. Zhou[1]

[1]Xinjiang Academy of Animal Science, Urumqi, China
[2]Agriculture Lab, University of Hawaii at Manoa, Honolulu, HI, USA

Corresponding author: Y. Zhang
E-mail: zhanyang2015@126.com

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ABSTRACT. Prokaryotic expression technology was used to express maltose-binding protein binding myostatin (MSTN) propeptide fusion protein. Six disease-free Altay lambs were used in this study. The right leg gastrocnemii were injected with MSTN recombinant propeptide protein. The left leg gastrocnemii (the control group) were injected with the same dose of phosphate based saline. The lambs were fed during four months under the same conditions and then slaughtered. Gastrocnemius samples were hematoxylin-eosin stained and the size of the muscle fibers was measured. A real-time polymerase chain reaction (RT-PCR) showed that single gastrocnemius cells in the experimental group had an average area of 1163.01 μm^2, while it was 845.09 μm^2 in the control group ($P < 0.05$). This indicates that the MSTN propeptide biological agents had an inhibitory effect on MSTN. In order to reveal its mechanism, RT-PCR was conducted to detect the expression of the differentiation-associated genes *MyoD*, *Myf5*, *Myogenin*, *p21*, and *Smad3*. The results showed that, in the MSTN propeptide biological agent injected group, expression levels of *MSTN*, *Smad3*, and *p21* were lower than the control group, while *Myf5*, *MyoD*, and *Myogenin* were higher compared to the control group. This indicates that, when

expression of the *MSTN* gene was inhibited, muscle cell differentiation and growth can be promoted by *Smad3* up-regulated expression of *Myf5*, *MyoD*, and *Myogenin*.

Key words: MSTN; Propeptide; Real-time-PCR

INTRODUCTION

In 1997, McPherron et al. reported a new transforming growth factor (TGF) beta, which was cloned from mouse skeletal muscle cDNA library. By comparisons of protein homology, it was shown to be a new member of the TGF-β superfamily, and was named growth/differentiation factor-8 (GDF-8) (McPherron et al., 1997). The gene mutational Pyrmont and Belgian Blue cattle show a significant double-muscled phenomenon (Kambadur et al., 1997). *Myostatin* (*MSTN*) gene silenced mice (Magee and Sayegh, 1997) and zebrafish, which were bred in the laboratory, also showed significant muscle hyperplasia. Skeletal muscle-specific over-expression of MSTN propeptide in transgenic mice resulted in a dramatic increase in skeletal muscle mass (Lee and McPherron, 2001; Yang et al., 2001). In mice, a two-fold increase in muscle growth following injection of the mutated propetide in neonatal stages lasted for about five weeks after injection, primarily as the result of fiber hypertrophy (Yang et al., 2001; Lee and McPherron, 2011). These results suggest that a reduction of myostatin activity by bone morphogenetic protein 1/ Tolloid proteinase-resistant myostatin propeptide can be an effective way to promote muscle growth. Li et al. (2010) compared eukaryotic expression with monoclonal antibody technology. Prokaryotic expression technology has a large expression quality and has the advantages of easy purification, low cost, and an easy industrialized production process. In view of this, prokaryotic expression technology was used in the present study, to express maltose binding protein (MBP) binding MSTN propeptide fusion protein. The protein was injected into Altay lambs with the aim to investigate whether, in the early days of muscle development, over-expression of MSTN propeptide in lamb skeletal muscle can speed up the growth rate of muscle, and thus, provide a scientific basis for increased muscle production.

MATERIAL AND METHODS

pMal-MProM recombinant bacteria were kept by laboratory. Protein Marker, IPTG, protein purification kit, embedding agent, alcohol, ammonia, hematoxylin dye, eosin dye, OTC viscose, restriction endonuclease *Xho*I, *Bam*HI, and T4 DNA ligase were purchased from Takara Bio Engineering Co., Ltd. in Dalian. Taq DNA polymerase was purchased from Promage Company.

Protein expression and purification

Using pMal-MProM recombinant bacteria (kept and maintained at Xinjiang Academy of Animal Science), induced temperature and time that may affect pMal-MProM expression were investigated. At 37°C, a final concentration of 0.5 mM IPTG (Isopropyl β -D-1-Thiogalactopyranoside) was added. The bacteria were cultured for 5 h, at which point the expression was optimal. Following this methodology, Rosetta protein was expressed. Following centrifugation (1300 g), the bacteria, in pellet form, were re-suspended in a cell lysis

buffer containing protein inhibitors. Following sonication on ice, the supernatant, containing an inducible protein, was retrieved and the protein was purified by affinity chromatography. At this time, 1 mL maltose resin gel was added to the chromatography column. The column was first washed with distilled water three times, and then washed three times in gel buffer, until the column was equilibrated. The supernatant containing the target protein was then slowly added, so that the protein could adsorb the gel. The gel buffer was used to wash the column approximately 10 times to re-balance, and then 0.5 mM maltose was used to wash the maltose resin gel five additional times to create the hybrid protein. Finally, the targeted protein was eluted by 10 mM maltose.

Injection of MSTN propeptide

Six healthy and disease-free Altay sheep were selected; ewes had sufficient milk to ensure normal growth and development of the lambs. One week (7-10 days) after birth, the selected lambs were injected with MSTN propeptide protein, MSTN propeptide protein was injected into the lambs. The gastrocnemius of each left leg was used as the control group by injecting 0.5 mL phosphate based saline (PBS). In contrast, the gastrocnemius of the right leg, the experimental group, was injected with 0.5 mL MSTN propeptide. During the second week, booster injections were administered in similar places. During the entire experiment, an adequate energy and protein feed supply was ensured for ewes. Likewise, sanitation control, adequate exercise and temperature was ensured. After feeding for four months, all six experimental lambs were slaughtered and measured. Part of the tissue was embedded for hematoxylin-eosin (H&E) staining to measure muscle fiber size, while the other part was flash frozen in liquid nitrogen and stored in a -80°C freezer until further analysis.

Muscle sectioning, immunofluorescence, and measurement

Each sample to be used for frozen sectioning was coated with Tissue-Tek OCT and cut at 8-μm thickness using a freezing microtome (CM1100, Leica, Berlin, Germany) at -20°C. The slices were stained with H&E. All sections were photographed using a digital microscope (Nikon, Berlin, Germany). The myofiber diameter and the area of cross-section (CSA) were measured using Image-Pro Plus v. 6.0 (Media Cybernetics, Bethesda, MD, USA).

RNA isolation and RT-PCR

Total RNA of all samples from the six Altay lambs was isolated using the TRIzol (Invitrogen, Chicago, IL, USA) method. Expression levels of *MSTN*, *Myf5*, and *MyoD* were detected after MSTN propeptide treatment, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as inner control. Reactions of quantitative real-time PCR (qRT-PCR) were performed using an iCycler IQ5 Multicolor detection system (Bio-Rad, Chicago, IL, USA). The qRT-PCR contained 1 μL cDNA template, 12.5 μL SYBR Premix Ex Taq, 10.5 μL sterile water, and 0.5 μL gene specific primers (Table 1). The thermal cycling parameters were one cycle at 95°C for 30 s, 40 cycles of 95°C for 10 s, and 60°C for 40 s. The qRT-PCR analysis of each sample was repeated three times. The relative mRNA expression levels of the genes of interest were calculated using the normalized relative quantification method followed by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Table 1. Primers for RT-PCR.

Gene	Primer sequence (5'→3')	Product length (bp)	Annealing temperature (°C)
GAPDH	F: CAAGTTCCACGGCACAGTCA	248	60
	R: GGTTCACGCCCATCACAAA		
Myogenin	F: AACCAGGAGGAGCGTGATCT	175	58
	R: GATTGTGGGCATCTGTAGGG		
p21	F: GGAACTTCGACTTTGTCACCG	321	59
	R: AGATCAGCCTGCGTTTGGA		
MyoD	F: GCTCCAGAACCGCAGTAAGT	194	57
	R: CGAAACACGGGTCATCATAGA		
Smad3	F: GCCGACAAAGACAAATAAAAGG	97	59
	R: TCAGGGGAAAGATGACAGACC		
MSTN	F: GATTTTGGGCTTGATTGTGATG	204	60
	R: ACCTTTGGGGTTTGCTTGG		
Myf5	F: CAGCAGAAACGCAGTAAAACC	120	58
	R: TTAGCATATCCCACCGCAAC		

Design and synthesis of primers

Referring to the mRNA sequences of sheep GAPDH, the *Myogenin*, *p21*, *MyoD*, *Smad3*, *MSTN*, and *Myf5* genes published in GenBank were selected. The primer design software PRIMER 5.0 was used to design primers. The primer sequences are shown in Table 1.

Data analysis

Differences between the groups were evaluated by the two-tailed Student *t*-tests and $P < 0.05$ was considered significant. All data are reported as mean ± SE.

RESULTS

Analysis of protein expression product

The recombinant bacterial lysates were subjected to SDS-PAGE electrophoresis. The protein expression analysis showed that the induced bacteria had a thick protein band at 75 kDa, close to the theoretically-speculated relative molecular mass of expression product. The results of this analysis are shown in Figure 1.

Analysis of tissue sections

There were no significant differences in weight between the right (MSTN-injected) and left (PBS-injected) leg of the lambs. The CSAs of a total of 1000 muscle fibers analyzed in 6 performed by Motic Images Advanced 3.2 image processing software, showed that the average single cell areas of gastrocnemius in the experimental and control groups were 1163.01 and 845.09 μm^2, respectively ($P < 0.05$) (Figures 2 and 3). This indicates that MSTN propeptide played a certain role in muscle production. Likely, the administered dose was not high enough to produce a significant difference in leg weight between the experimental and control groups.

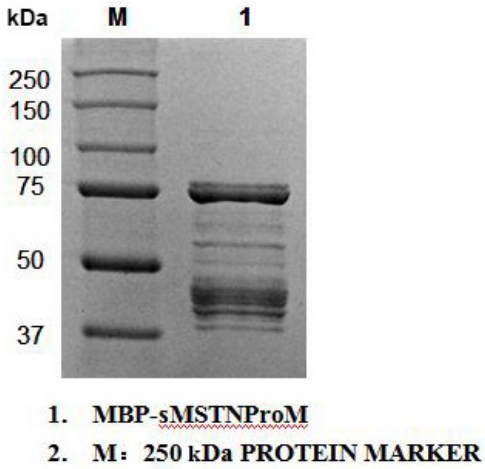

Figure 1. Protein expression and purification.

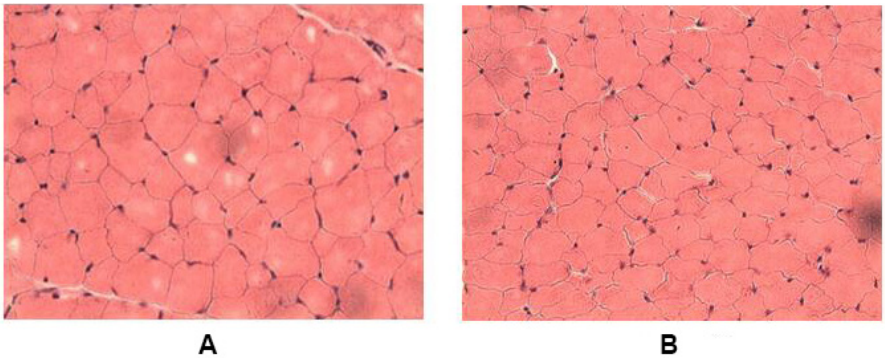

Figure 2. Effects of MSTN propeptide on lamb muscle fiber (N = 10). Shown are muscle sections stained by H&E in the MSTN propeptide treatment group **A.** and in the control group **B**. The magnification is 40X in both cases.

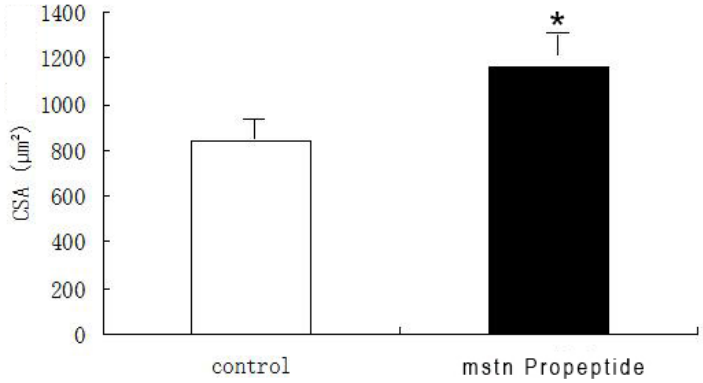

Figure 3. Effects of myostatin propeptide on the muscle fiber cross-sectional area (CSA). An asterisk indicates a significant difference ($P < 0.05$).

RT-PCR analysis

Through the injection of MSTN propeptide biological agent, we were able to inhibit *MSTN* expression to a certain extent. The expression of the differentiation-associated genes *MyoD*, *Myf5*, *Myogenin*, *p21*, and *Smad3* (Figure 4) showed that the expression levels of *MSTN*, *Smad3*, and *p21* were lower in the experimental group than in the control group. On the other hand, the expression levels of *Myf5*, *MyoD*, and *Myogenin* were higher in the experimental group than in the control group. This indicates that, when the *MSTN* gene expression was suppressed, expression levels of *Myf5*, *MyoD*, and *Myogenin* were up-regulated by *Smad3*, resulting in a promotion of muscle cell differentiation and growth.

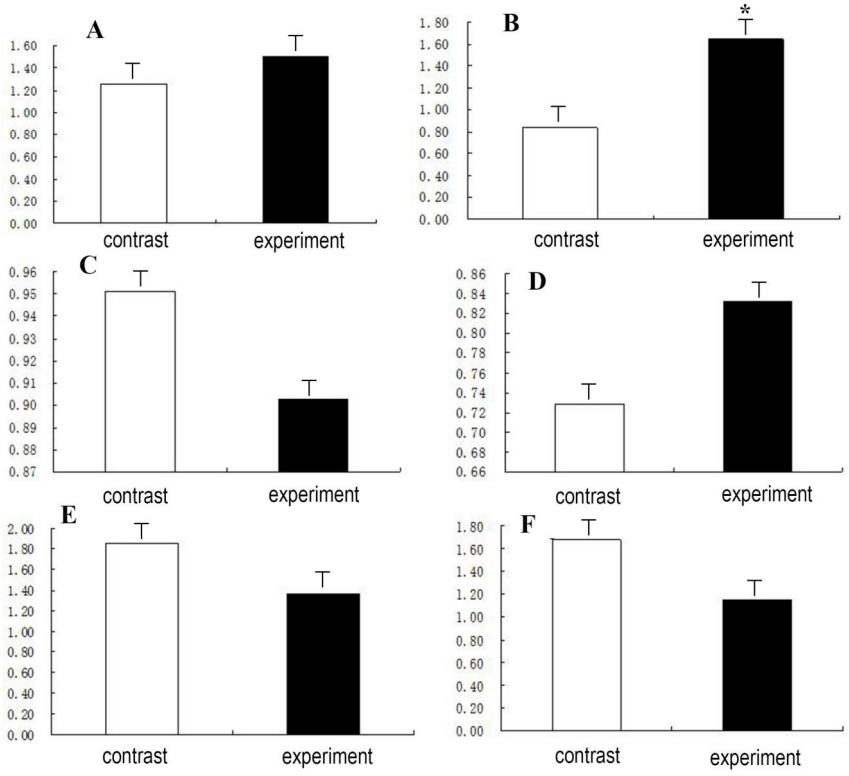

Figure 4. RT-PCR detecting the expression of differentiation-associated genes *Myf5* (**A**), *MyoD* (**B**), *MSTN* (**C**), *Myogenin* (**D**), *p21* (**E**), and *Smad3* (**F**). An asterisk indicates a significant difference ($P < 0.05$).

DISCUSSION

Muscle cell differentiation and growth are bidirectionally regulated by a number of positive and negative regulators. The insulin-like growth factor (IGF) axis is considered to play an important positive role in regulating the differentiation and growth of muscle cells. Expression of IGFs increased in the formation of secondary fibers and their role is to stimulate myoblast proliferation and differentiation, and gene family regulation of myogenic determination genes (such as *MyoD*). *MyoD* includes four genes; *MyoD1* (*Myf3*), *Myogenin*

(*MyoG*), *Myf5*, and *Myf6* (*herculinh* or *MRF4*). *MyoD* and *Myf5* share similar roles in the process of myogenesis, while *Myogenin* and *Myf6* control muscle differentiation (Tian et al., 2005).

In this experiment, MSTN propeptide biological agent was injected into lambs. We found that this agent inhibited MSTN to certain extent; the average CSAs of the gastrocnemius were significantly larger in the experimental group (1163.01 μm^2) than in the control group (845.09 μm^2). To further study the active mechanism, we used RT-PCR to detect the expression of genes associated with differentiation; *MyoD*, *Myf5*, *Myogenin*, *p21*, and *Smad3*. The results showed that *MSTN* gene expression was suppressed by *Smad3*-increased expression of *Myf5*, *MyoD*, and *Myogenin*.

These results could be brought about through a number of mechanisms: 1. The decreased *MSTN* expression resulted in a general reduction in cell inhibition. 2. MSTN transformed the signal into the nucleus through the mediation of the smad protein to act on the regulatory regions of target genes (Liu et al., 2001; Langley et al., 2002). *Smad3* is the downstream target gene of the MSTN receptor complexes, participating in MSTN-mediated regulation of myoblast differentiation (Ge et al., 2011). Therefore, it is possible that decreased *MSTN* expression levels resulted in a reduction in *Smad3* (MSTN has been shown to inhibit the differentiation of skeletal muscle satellite cells by *Smad3* down-regulated *MyoD* expression). Following the reduced expression of *Smad3*, cell proliferation was faster than normal in cells (Gulbis et al., 1996). 3. *MSTN* expression levels decreased and the negative feedback effects on *MyoD* decreased, resulting in increased expression levels of *MyoD*, *Myf5*, and *Myogenin*. The *MyoD* concentration during cell proliferation was greater than the normal cell concentration and may be accelerating cell proliferation. After *MyoD* gene interference, the *MSTN* gene expression was also reduced and the cell growth rate was the same as the normal growth rate. On the other hand, after *MSTN* interference, the expression of *MyoD* increased and skeletal muscle satellite cells grew rapidly, resulting in a growth rate that was higher than the normal cell growth rate. 4. *MSTN* expression levels decreased, resulting in reduced expression levels of *p21*. The *p21* gene regulates the cell cycle mainly through two aspects: p21 combines with cyclin, cyclin-dependent kinases (CDK), and proliferating cell nuclear antigen (PCNA) to form a tetramer complex. This complex can effectively inhibit the activity of various cyclin-CDK complexes causing the cell cycle to arrest in the G1-phase (Gulbis et al., 1996). In addition, p21 can combine with the PCNA subunit of DNA polymerase at CDK deficiency to block DNA replication. Once the *p21* gene fuction was removed, it allowed the cells to proliferate in the presence of negative growth signals. It is known that, as the downstream effector of p53, p21 is primarily related to radiation-induced Gl-phase arrest. It makes the cells exit the cell cycle to coordinate cell cycle progression, therefore, it is considered a cell proliferation-suppressing gene (El-Deiry et al., 1993).

In conclusion, our study confirmed that the MSTN propeptide played a certain role in the differentiation and proliferation of Altay lamb muscle. We detected the expression of differentiation-associated genes *MyoD*, *Myf5*, *Myogenin*, *p21* and *Smad3* by RT-PCR, to reveal their mechanism of action. Through this experiment we believe that using MSTN propeptide to inhibit *MSTN* gene expression might be a way to increase sheep muscle growth and improve meat production rate.

Conflicts of interest

The authors declare no conflict of interest.

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