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Transcript Characteristic of Myostatin in Sheep Fibroblasts

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ABSTRACT

Myostatin, a secreted growth factor highly expressed in skeletal muscle, negatively regulates skeletal muscle growth and differentiation. Recently, myostatin is emerged as a potential target for anti-atrophy and anti-fibrotic therapies. Therefore, to investigate the regulation of myostatin in sheep adult fibroblasts, we used the RNA interference mediated by lentiviral vector to gene silence myostatin. Simultaneously, we also had constructed the sheep myostatin overexpression vector to further explore the function of myostatin in fibroblasts. The results here demonstrated that the lentiviral vector could significantly reduce myostatin gene both at mRNA and protein level by 71% and 67%, respectively ($P < 0.01$). Inhibition of myostatin also resulted in a remarkable increase of activin receptor 2B (ACV2B), p21, PPARγ, leptin, C/EBPβ, and MEF2A expression, and a decrease of Akt1, CDK2, MEF2C, and Myf5 expression. Ectopic myostatin mRNA and protein were also present in the fibroblasts transfection. Furthermore, we observed that overexpression of myostatin contributed to an increase of Akt1, CDK2, Myf5 and PPARγ, and a decrease of p21, C/EBPα and leptin at the transcript level. These results suggested that myostatin positively regulated Akt1, CDK2, Myf5, leptin, and C/EBPα, but negatively regulated p21 mRNA expression in adult fibroblasts, and it also expanded our understanding of the regulation mechanism of myostatin. Moreover, the lentiviral system inactivated myostatin gene in fibroblasts would be used to generate transgenic sheep and to ameliorate muscle fibrosis and atrophy by gene therapy in the future. J. Cell. Biochem. 113: 2652–2660, 2012.

KEY WORDS: MYOSTATIN; GENE SILENCE; OVEREXPRESSION; FIBROBLASTS; REGULATORY MECHANISM

Myostatin, a member of the transforming growth factor β (TGFβ) superfamily of secreted growth factors, plays an important role in regulating of skeletal muscle development and adult homeostasis. Gene targeted mice show that myostatin is a muscle-specific negative regulator of skeletal muscle mass with hypermuscularity [McPherron et al., 1997]. Inactivating mutations of myostatin gene in a number of mammalian species are associated with increased muscle mass and decreased fat mass indicating conservation of function in mammals [Lee, 2007].

Myostatin regulates muscle mass in part by inhibiting the proliferation of myoblasts. Inhibited proliferation of myoblast, induced by myostatin, involves the activation of Smad, Akt, p38MAPK and p21 pathways [Langley et al., 2002; Philip et al., 2005; Trendelenburg et al., 2009; Han et al., 2010]. Overexpression

Abbreviations used: TGFβ, transforming growth factor β; MRF, myogenic regulatory factor; PPARγ, peroxisome proliferator-activated receptor γ; C/EBP, CCAAT/enhance-binding protein.

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of myostatin in transgenic mice results in induction atrophy of adult skeletal muscle by downregulating Akt/mammalian target of rapamycin signaling pathway and decreasing muscle gene expression, such as MyoD, Pax7, etc. [Durieux et al., 2007; Amirouche et al., 2009]. In addition to the regulation of muscle mass, myostatin has a profound impact on the fibrosis of skeletal muscle [Li et al., 2008]. Treatment of fibroblasts with myostatin results in the proliferation of muscle and tendon fibroblasts [Li et al., 2008; Mendias et al., 2008]. Continued proliferation of fibroblasts in muscle would contribute to the pathological process of muscle fibrosis. It also demonstrates that the signaling pathways in fibroblasts are similar to that in myoblasts [Li et al., 2008]. Therefore, myostatin inhibitors have generated great interest as candidates for treatment of muscle wasting diseases, as well as promoting livestock production.

To date, the effects of myostatin on adipogenic differentiation are poorly understood, and the underlying mechanisms are unknown. The regulation mechanisms between myostatin and adipocygenesis are complex. In vitro, overexpression of myostatin inhibits adipocyte differentiation, such as 3T3-L1, bovine preadipocyte and human bone marrow-derived mesenchymal stem cells [Hirai et al., 2007; Guo et al., 2008; Stolz et al., 2008]. However, in C3H10T(1/2) mesenchymal multipotent cells, myostatin promotes adipogenesis [Artaza et al., 2005]. Adipose-specific transgenic overexpression of myostatin in vivo also resulted in decreased adipocyte size [Feldman et al., 2006]. In addition, muscle-specific overexpression of myostatin caused a decrease in muscle mass and an increase in epididymal fat pad mass [Reisz-Porszasz et al., 2003]. Ignoring the endogenous myostatin, these evidences suggest that myostatin directly regulates adipogenesis.

However, some studies in vivo had inconsistent results. In adult mice administration of myostatin does not alter fat mass, but reduces muscle mass [Stolz et al., 2008]. Similarly, treatment with myostatin-specific antibody in mice had no effect on fat mass accretion in adipose tissue, but also increased muscle mass [Bernardo et al., 2010]. In the mice with diet-induced obesity, inhibition of myostatin by soluble activin receptor type IIB treatment does not cause fat loss [McPherron et al., 2011]. These evidences suggested that the effect of myostatin on adipose tissue was indirectly. Furthermore, in vivo, inhibition of myostatin signaling specifically in muscle causes the same metabolic phenotypes as global myostatin gene deletion. While inhibition of myostatin signaling specifically in fat does not cause any increase of lean or fat mass [Guo et al., 2009].

Inactivation of myostatin in transgenic mice, such as knockout and overexpression the propetide domain of myostatin, resulted in the increase of muscle mass and the decrease of fat mass [McPherron et al., 1997; Yang et al., 2001]. Myostatin was produced in skeletal muscle with high expression, while its expression in fat was very low [Allen et al., 2008]. Taken together, it is seem that the effect of myostatin on adipocytes is mediated by the action of myostatin on skeletal muscle rather than adipose tissue [Allen et al., 2011].

Adipocytes are found in stereotypical depots throughout the body, but can also be found mixed with other cell types in other locations especially in loose connective tissue [Rosen and MacDougald, 2006]. Most of our knowledge about the mechanisms of adipocyte differentiation has been established by studying immortalized fibroblast cell lines (e.g., 3T3-L1 and 3T3-F442A) that can be induced to differentiate into adipocytes in vitro by the administration of a hormonal cocktail [Green and Kehinde, 1975; Siersbaek et al., 2010]. Recent report also have shown that absent of myostatin resulted in enhanced peripheral tissue fatty acid oxidation and increased thermogenesis, culminating in increased fat utilization and reduced adipose tissue mass [Zhang et al., 2011]. Therefore, study of myostatin in fibroblasts also could help us to understand the regulation mechanisms between myostatin and adipogenesis related genes.

RNA interference (RNAi) is a powerful tool to manipulate gene expression in cells and even whole animals. Lentiviral vectors are particularly efficient tools for generating transgenic animals because they are able to infect non-dividing and dividing mammalian cells, stem cells, zygotes and their differentiated progeny. It is shown that lentivirus-delivered shRNA are capable of specific, highly stable and functional silencing of gene expression in a variety of cell types and also in transgenic mice [Rubinson et al., 2003]. Of course, overexpression of the targeted gene is another method to explore its function in vitro or in vivo.

In the previous work, we had generated the RNAi vector mediated by lentiviral to gene silencing goat myostatin in fetal fibroblasts. However, it is unknown whether this vector is efficient in sheep adult fibroblasts or the transcript characteristic of myostatin in sheep adult fibroblasts. In addition, we also had constructed sheep myostatin expression vector pAcGFP-myostatin to identify the signaling mechanisms of myostatin by overexpression in sheep fibroblasts.

MATERIALS AND METHODS

CULTURE OF SHEEP ADULT FIBROBLASTS

The sheep fibroblast cell lines were isolated from ear tissue of 4-year-old adult female Ujumuqin sheep and cut into 1-mm^3 pieces and allowed to adhere to 60-mm dishes (Costar) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) at 37°C and 5% carbon dioxide (CO_2). All experimental procedures were approved by the Biological Studies Animal Care and Use Committee, Beijing, Peoples Republic of China. Low passages (5–10 passages) of cultured fibroblasts were used in the present experiment.

DESIGN, CLONING AND TRANSDUCTION OF LENTIVIRAL VECTOR EXPRESSING SHEEP MYOSTATIN SHRNAS

In the present study, the target site 322 (GACGATGACTAC-CACGTTA) was used to knockdown myostatin in sheep fibroblasts, whereas the target site 219 (CAAAGATGCTATAAGACAA) of sheep myostatin for RNAi was used as the positive control. The irrelevant sequence (TTCTCCGAACGTGTCACGT) known not to block any mammalian mRNA was also selected as the negative control. The shRNA annealed oligonucleotides had been cloned into the pFU-GW-RNAi vector between the *Hpa*I and *Xho*I sites. To detect any

mutations (insertion/deletion), the region of the plasmid containing the insert was amplified by colony PCR and sequenced.

The lentiviral particles were produced by co-transfection of the transfer vector pFU-GW-RNAi (20 μg) together with two helper plasmids (15 μg pCMV8.9 packaging plasmid and 10 μg pVSV-G envelope plasmid) into HEK293T cells seeded in 15-cm dishes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The virus-containing cell culture supernatants were collected 48 h posttransfection, centrifuged at 4,000*g* for 10 min at 4°C to remove cell debris, passed through a 0.45-μm filter, and concentrated by centrifugation in a swing bucket at 4,000*g* for 15 min at 4°C using a Centricon Plus-20 centrifugal filter device (Millipore, Billerica, MA). The titer of the virus concentrate was determined by transducing 293T cells with serial dilutions. The virus preparation used for transgenesis had a final titer of greater than 10^8 transducting units/ml.

The sheep fibroblasts were seeded in 6-well plates and cultured until 50–60% confluent. The lentivirus with the optimal multiplicity of infection (MOI) of 50 had been added into the opti-MEM (Gibco) containing 8 μg/ml polybrene (H9268, Sigma, St. Louis, MO). The medium was changed to the fresh growth medium after 8 h. The green fluorescence excited by the lentiviral vector would be observed under a fluorescence microscope (ECLIPSE TE2000-U, Nikon, Tokyo, Japan) 48 h later. We also selected the non-transfected cells as a negative control in the present study.

CONSTRUCTION AND TRANSFECTION OF SHEEP MYOSTATIN EXPRESSION VECTOR PACGFP-MYOSTATIN IN SHEEP FIBROBLASTS

The sheep myostatin expression construct was generated by the RT-PCR amplification of the coding region of myostatin and its subsequent cloning into the pAcGFP-N1 vector (Clontech, Carlsbad, CA). The PCR primers used for this amplification were shown in Table I. Sheep muscle total RNA was used as the RT template. Plasmids were amplified in DH5α bacteria, purified (EndoFree Plasmid Mega Kit; Qiagen) and dissolved in nuclease-free water.

In the sheep myostatin overexpression transient transfection, fibroblasts were seeded in 6-well plates and cultured until 80–90% confluent. Lipofectamine 2000™ reagent (Invitrogen, Carlsbad, CA) was used as the transfection reagent. Four microgram of sheep myostatin expression plasmid pAcGFP-myostatin had been transfected into fibroblasts by lipofectamin 2000 (4 μl) according to the protocol recommended by the manufacturer. Six hours later, the transfected medium was replaced by fresh medium containing 10% FBS. Similarly, we also selected the non-transfected cells as the negative control for further study.

RNA EXTRACTION AND REAL-TIME PCR

Total RNA was isolated 96 h later of the lentiviral infection or 48 h after sheep myostatin expression plasmid transfection respectively, using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quality of the total RNA was

TABLE I. Primers Used for RT-PCR and Quantitative RT-PCR

Gene	GB accession #		Primer sequence 5' → 3'	Amplicon (bp)
Myostatin	NM_001009428	Forward primer	CAAAACTGGCTCAAACAACCTG	194
		Reverse primer	GTGGAGTGCTCATCACAATCAAG	
ACVR2B	NM_174495	Forward primer	TCCCTCACGGATTACCTCAAG	149
		Reverse primer	TCCCTGTGGGCAATAGATGG	
GAPDH	NM_001190390	Forward primer	CAAGTTCCACGGCACAGTCA	249
		Reverse primer	TGGTTCACGCCCATCACAA	
Myostatin	pAcGFP-myostatin	Forward primer	CGGCCCGCTCGAGATGCAAAACTGCAAATC	1148
		Reverse primer	ATCCCCGCGGTGAGCACCCACAGCGATCTA	
Smad3	NM_001205805.1	Forward primer	GACGAGAAGTGGTGCGAGAA	197
		Reverse primer	CACAGGCGGCAGTAGATGA	
Akt1	NM_001161857.1	Forward primer	CAGCATCGTGTGGCAGGAC	138
		Reverse primer	TCTTGGTCAGGTGGCGTAA	
P38MAPK	NM_001142894.1	Forward primer	CAGATGCCGAACATGAAC	145
		Reverse primer	GGTACTGGGCAAAGTAGG	
CDK2	NM_001142509.1	Forward primer	CTTGTTATCGCAAATGCTG	103
		Reverse primer	GTGAGGTACTGGCTTGGTC	
P21	NM_001098958	Forward primer	GCAGACCAGCATGACAGATTT	70
		Reverse primer	GGATTAGGGCTTCCTCTTGGA	
MyoD	NM_001040478.2	Forward primer	CGACTCGGACGCTTCCAGT	181
		Reverse primer	GATGCTGGACAGGCAGTCGA	
MyoG	NM_001174109.1	Forward primer	GTGCCCAGTGAATGCAGCTC	111
		Reverse primer	GTCTGTAGGGTCCGCTGGGA	
Myf5	NM_174116.1	Forward primer	ACCAGCCCCACCTCAAGTTG	150
		Reverse primer	GCAATCCAAGCTGGATAAGGAG	
MEF2C	NM_001159277.1	Forward primer	CAGTCATTGGCTACCCCAGT	152
		Reverse primer	GCGGTGTTAAAGCCAGAGAG	
MEF2A	NM_001083638.1	Forward primer	CAGGTGGTGGCAGTCTTG	169
		Reverse primer	GGCAGGCTTGGAGTTGTC	
Pax7	XM_616352.5	Forward primer	GAGAAGAAAGCCAAGCACAGC	106
		Reverse primer	TACGCTTCAGAGGGAGGTCG	
PPARγ	NM_001100921.1	Forward primer	GATAAAGCGTCAGGGTTCCA	183
		Reverse primer	AGACATCCCCACAGCAAGG	
Leptin	NM_173928.2	Forward primer	CTGTGCCCATCTGCAAGGT	132
		Reverse primer	CAGGGATGAAGTCCAAACCAG	
C/EBPα	NM_176784.2	Forward primer	GCGGCAAAGCCAAGAAGTCC	188
		Reverse primer	GCGGCTCAGTTGTTCCACCC	
C/EBPβ	NM_176788.1	Forward primer	ACAGCGACGAGTACAAGATCC	154
		Reverse primer	GACAGTTGCTCCACCTTCTTCT	

measured by spectrophotometer (AstraGene; AstraNet, Cambridge, UK). RNA samples (1 μg) with A260/280 ratio between 1.8 and 2.0 were reverse-transcribed by using M-MLV reverse transcriptase (Promega, Madison, WI) with 50 μM oligo (dt) 18 primers. Primers for PCRs (Table I) were designed to generate amplicons that span multiple exons. Real-time PCR was performed using Power SYBR-Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA) on the BIO-RAD iQ5 real-time PCR Detection System (Bio-Rad, Hercules, CA). The 20 μl PCR included 1 μl RT product, 10 μl 2× SYBR-Green PCR master mix, 2.5 μM forward and reverse primers and autoclaved nuclease free water. The reactions were started with 3 min of UNG incubation step at 55°C, 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. The melting curve was constructed for verification of specificity of PCR products by increasing the temperature from 60 to 95°C in sequential steps of 0.2°C for 15 s. All reactions were run in triplicate and included no template controls. Glyceraldhyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as housekeeping gene. Gene expression was normalized to GAPDH expression by using the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001].

WESTERN BLOTS

The protein was extracted from lentiviral vector transduction after 96 h or sheep myostatin expression plasmid pAcGFP-myostatin transfection 48 h later, respectively. The cells were resuspended in RPRA buffer containing a protease inhibitor and homogenized. Protein concentrations were determined in accordance with the manufacturer's instructions (BioRad).

Protein (20 μg) was loaded for electrophoresis on a 10% gel and transferred to nitrocellulose membrane (Millipore, Billerica, MA) by wetting transferring. The standard western analysis protocol was used thereafter. The following primary antibodies were used in this study: anti-myostatin antibody (AB3239, 1:1,000; Millipore), anti-GFP antibody (G1546, 1:5,000; Sigma) or anti-GAPDH antibody (G8795, 1:5,000; Sigma). HRP-conjugated anti-mouse IgG antibody (115-035-174, 1:10,000; Jackson ImmunoResearch, West Grove, PA) and anti-rabbit IgG (111-035-003, 1:10,000; Jackson ImmunoResearch) antibodies were used as secondary antibodies, and ECL reagent (WBKLS0500; Millipore) was used for developing. The protein expression was detected by chemiluminescence, quantified by densitometry by the GelQuantNET software, and normalized to GAPDH.

STATISTICS ANALYSIS

All data are presented as the mean ± SE. The experiments were repeated in triplicate. One-way ANOVA was performed to identify significant changes between different groups. The statistical significance was inferred from P values <0.05 or 0.01.

RESULTS

THE LENTIVIRAL VECTOR WAS SUFFICIENT TO GENE SILENCE SHEEP MYOSTATIN IN FIBROBLASTS

In the previous study, we had constructed the lentiviral system to gene silence goat myostatin in fetal fibroblasts. Therefore, it should be tested the actual knockdown efficiency of myostatin gene in sheep fibroblasts as application in sheep transgenic research. In the present experiment, quantitative RT-PCR indicated that the lentiviral vector Lv322 had significantly reduced sheep myostatin by 71% at the transcript level ($P < 0.01$), whereas the positive control Lv219 also could significantly decrease myostatin mRNA by 78% ($P < 0.01$), compared with the non-transfected control (Fig. 1A). Western blot showed that the expression of myostatin at the molecular weight of approximately 104 kDa had also significantly been decreased by 67% ($P < 0.05$). The positive control Lv219 had reduced myostatin expression by 35% ($P < 0.05$; Fig. 1B). To verify the accuracy of the result, therefore, we still had checked the specificity of the polyclonal antibody in fetal longissimus dorsi muscle between Texel (known as myostatin mutation) and Ujumqin (wild-type) sheep by Western blot analysis. Our results demonstrated that the mature form of myostatin was at the molecular weight of approximately 52 kDa, unlike the result of 12.5 kDa detected by the JA16 monoclonal antibody (data not shown). Similar results of the myostatin protein at 104 kDa were observed in the goat fetal fibroblasts (data not shown).

Activin receptor 2B (ACV2B), the putative receptor for myostatin, had been increased to 260% and 151% detected by real-time PCR in the Lv322 and Lv219 transduction, respectively ($P < 0.01$; Fig. 1A). Because the Lv322 and the positive control Lv219 both had the ability to silence myostatin in sheep adult fibroblasts, therefore, the lentiviral vector Lv322, not the positive control Lv219, was selected to study the transcript characteristic of myostatin in sheep adult fibroblasts. In this study, we also demonstrated that the negative control with no effect on myostatin and ACV2B gene expression could used in the further study.

OVEREXPRESSION OF MYOSTATIN IN SHEEP ADULT FIBROBLASTS

In this experiment, myostatin mRNA level was strongly increased in response to myostatin gene transfection (Fig. 2A). When primers were designed to specifically amplify myostatin mRNA encoded by the vector, RT-PCR analysis showed that ectopic myostatin mRNA was not detected in the non-transfected control and pAcGFP-N1 empty vector, whereas ectopic myostatin mRNA was abundantly present in fibroblasts transfected with the myostatin expression plasmid (Fig. 2A). The fusion protein was also detected at the molecular weight 78 kDa by the GFP antibody in Western blotting, whereas the GFP protein was detected at the molecular weight 26 kDa in the empty vector and was non-detectable in the non-transfected cells (Fig. 2B). It demonstrated that the myostatin protein produced by the overexpression vector was precursor form of myostatin (52 kDa), without the mature form (26 kDa).

DIFFERENTIAL EXPRESSION OF SMAD3, AKT1, P38MAPK, P21, AND CDK2 IN ADULT FIBROBLAST CELLS

In this study, we noted that Inhibition of myostatin had significantly reduced the expression of Akt1 and CDK2 mRNA by 26% and 33%, respectively ($P < 0.05$). The expression of p21 had been significantly increased to 141% ($P < 0.05$; Fig. 3). In contrast to the RNAi model, overexpression of myostatin had significantly increased the expression of Akt1 and CDK2 to 172% and 124%, respectively ($P < 0.05$). In addition, p21 mRNA expression had been reduced to 80% ($P > 0.05$; Fig. 4). No matter in the knockdown model or

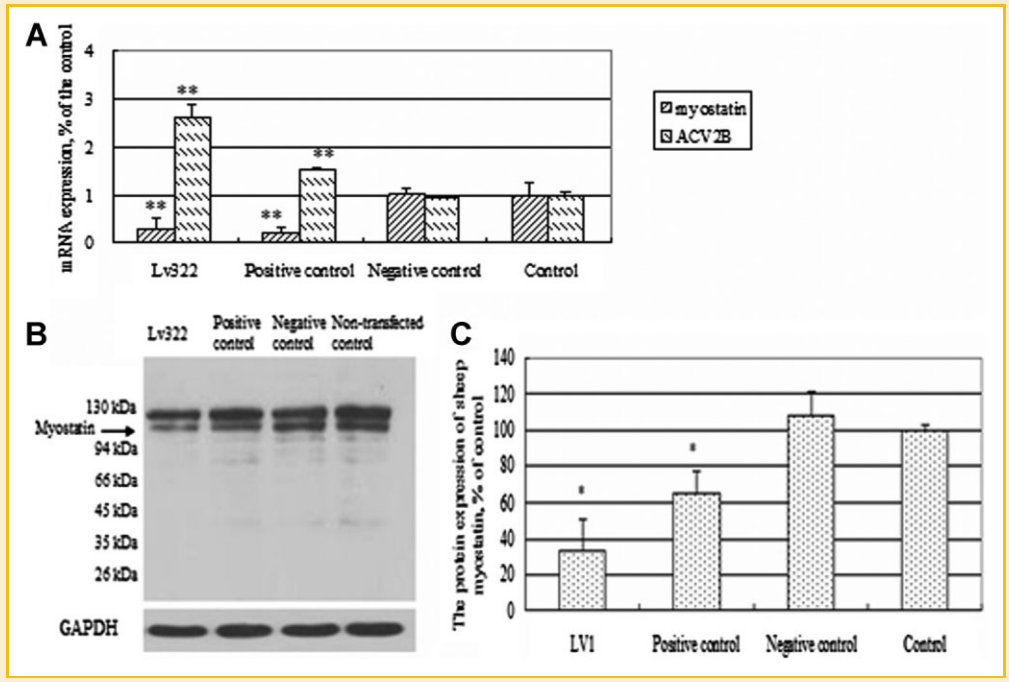

Fig. 1. Expression of myostatin and ACV2B genes in the transduction of lentiviral mediated RNA interference in sheep adult fibroblasts. A: Myostatin and ACV2B genes were determined using real-time (RT)-PCR and normalized to GAPDH mRNA. B: Western-blot analysis of myostatin protein expression. Non-transfected cells were chosen as the control. The irrelevant sequence known not to block any mammalian mRNA was also selected as the negative control. $^{**}P < 0.01$ or $^{*}P < 0.05$ with non-transfected control.

overexpression model, there were no changes of Smad3 and p38MAPK expression at the transcript level.

DIFFERENTIAL EXPRESSION OF MYOGENIC TRANSCRIPTION FACTORS (MYOD, MYOG, MYF5, MEF2A, AND MEF2C) AND PAX7 AS THE INACTIVATION AND OVEREXPRESSION OF MYOSTATIN IN FIBROBLASTS

Myostatin inhibits myoblast proliferation and differentiation by down-regulating MRFs expression. Similarly, myostatin signals through Pax7 to negatively regulate satellite cell (muscle stem cells) activation and self-renewal. In sheep adult fibroblasts, we did not detect the expression of MyoD, MyoG and Pax7, consistent with the previous result in goat fetal fibroblasts (data not shown). In Lv322 transfection, mRNA expression of MEF2C and Myf5 had been non-significantly decreased to 61% and 58%, respectively, however, the expression of MEF2A was strongly increased to 1,248% ($P < 0.01$), as the inactivation of myostatin in sheep fibroblasts compared with the controls (Fig. 3). Interestingly, overexpression of myostatin had little impact on the expression of MEF2A and MEF2C, however, the expression of Myf5 had significantly increased to 144% ($P < 0.05$), compared with the control (Fig. 4).

DIFFERENTIAL EXPRESSION OF ADIPOGENESIS MARKER GENES AS THE INACTIVATION AND OVEREXPRESSION OF MYOSTATIN IN FIBROBLASTS

Recently, some reports have indicated that regulation of myostatin in adipogenesis is dependent on the cell types. In the Lv322 transduction, we found that PPARγ, leptin and C/EBPβ were significantly increased to 708%, 332%, and 493% at the transcript level by real-time PCR ($P < 0.01$), respectively (Fig. 3). However, there were no significant changes of C/EBPα mRNA expression in the Lv322 transduction compared to the control.

In the sheep myostatin overexpression experiment, we observed that C/EBPα and leptin gene expression had been reduced by 32% and 37% ($P < 0.05$), respectively. Unexpectedly, the expression of PPARγ had been increased to 360% ($P < 0.01$), compared with the control. In addition, we noted that there were no significant changes of C/EBPβ mRNA expression (Fig. 4).

DISCUSSION

Although myostatin is highly conserved among species as the master regulator of muscle mass, it is also expressed in cardiac muscle and adipose tissue with lower level [Sharma et al., 1999; Allen et al., 2008]. Recently, it was demonstrated that myostatin was also expressed in fibroblasts with lower level [Li et al., 2008; Mendias et al., 2008; Stewart et al., 2008; Jain et al., 2009]. Fibroblasts play an important role in the repair response of tissues to injury by secreting extracellular matrix proteins including collagen and growth factors. In muscle regeneration, connective tissue fibroblasts coordinating interaction with satellite cells also are likely to play a role in regeneration [Murphy et al., 2011]. However, recent reports had shown that myostatin stimulated fibroblast proliferation in vitro and directly regulated skeletal muscle fibrosis [Zhu et al., 2007; Li et al., 2008]. To date, most studies of myostatin have focused on its role as an inhibitor of muscle growth that occurs via

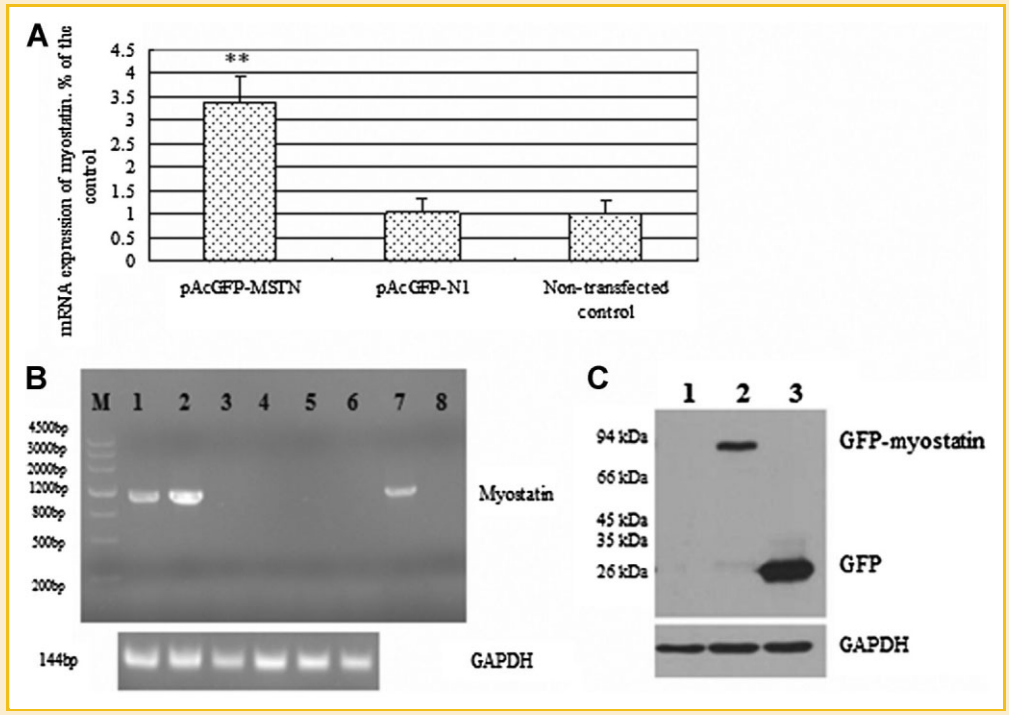

Fig. 2. Expression of myostatin in the transfected sheep adult fibroblasts. A: Expression of myostatin detected by real-time (RT)-PCR B: myostatin mRNA level. Reverse transcript PCR analysis was performed with primers specifically designed to amplify myostatin mRNA encoded by the expression vector. Lines 1–2, the myostatin expression vector pAcGFP-myostatin transfected cells; lines 3–4, the empty vector pAcGFP-N1 transfected cells; lines 5–6, the non-transfected cells; line 7, the pAcGFP-myostatin plasmid as the positive control; line 8, the pAcGFP-N1 as the negative control. The length of the myostatin product was 1,148 bp. C: Western blot analysis of the GFP-myostatin fusion protein by GFP antibody. Line 1, the non-transfected cells; line 2, the pAcGFP-myostatin transfected cells; line 3, pAcGFP-N1 transfected cells. The molecular weight of GFP-myostatin fusion protein was 78 kDa, whereas the molecular weight of GFP protein was 26 kDa.

its effects on the activation, proliferation, and differentiation of myoblasts. Therefore, myostatin also has provided a potential target for anti-fibrotic therapies.

In the present study, fibroblasts were used as the tool to identify the regulation mechanism through RNAi and overexpression model of myostatin. In the previous study, we had generated the lentiviral system to gene silence goat myostatin. Therefore, the result here indicated that the lentiviral system with the high efficiency also could gene silence myostatin both at the transcript and protein level in sheep adult fibroblasts and could be used to generate transgenic animal by zygote injection or oocyte injection in the future. With the higher affinity than ACV2A, ACV2B, the putative receptor of myostatin, had been increased caused by silencing of myostatin, indicating the effectiveness of the lentiviral system.

In the present experiment, we also had successfully constructed the sheep myostatin overexpression vector. The result here were shown that we could detect the GFP-myostatin fusion protein in sheep adult fibroblasts, indicating the pAcGFP-myostatin could be used to understand the mechanism of myostatin in overexpression model in vitro, even in vivo.

In myoblasts, myostatin regulated its proliferation and differentiation through Smad, Akt, p38MAPK and p21 pathways [Thomas et al., 2000; Philip et al., 2005; Amirouche et al., 2009]. Some reports also showed that myostatin regulated the proliferation of fibroblasts through the phosphorylation of Smad3, Akt and p38MAPK at the protein level [Li et al., 2008; Mendias et al., 2008]. However, in this study, we found that myostatin positively regulated Akt1 and CDK2 gene and negatively regulated p21 expression at the transcript level in adult fibroblasts. Similar to our result, it was reported that myostatin deletion regulated Akt expression at the transcript level in mice quadriceps [Morissette et al., 2009]. The cause of no changes of p38 and Smad3 at the transcript level might is that myostatin regulated p38 and Smad3 expression at the protein phosphorylation.

In addition to controlling muscle mass, myostatin appears to regulate muscle fiber-type composition postnatally. It has recently shown that in cattle lacking myostatin or myostatin knockout mice there is an increased number of fast glycolytic fibers [Stavaux et al., 1994; Girgenrath et al., 2005]. It was also suggested that myostatin could regulate fiber-type composition by regulating the expression of MEF2C and MyoD during myogenesis. While myostatin positively regulates MEF2C level, it negatively regulates MyoD expression in muscle [Hennebry et al., 2009]. However, the result here demonstrated that the adult fibroblasts did not express the myogenesis marker genes such as MyoD, MyoG and Pax7, consistent with the characteristics of fibroblasts. The result in the present study indicated that myostatin also positively regulated MEF2C expression, because the decreased MEF2C was caused by the inhibition of myostatin. However, in the goat fetal fibroblasts, we noted that inhibition of myostatin resulted in an increase of MEF2C, indicating that the relationship between myostatin and MEF2C was changed

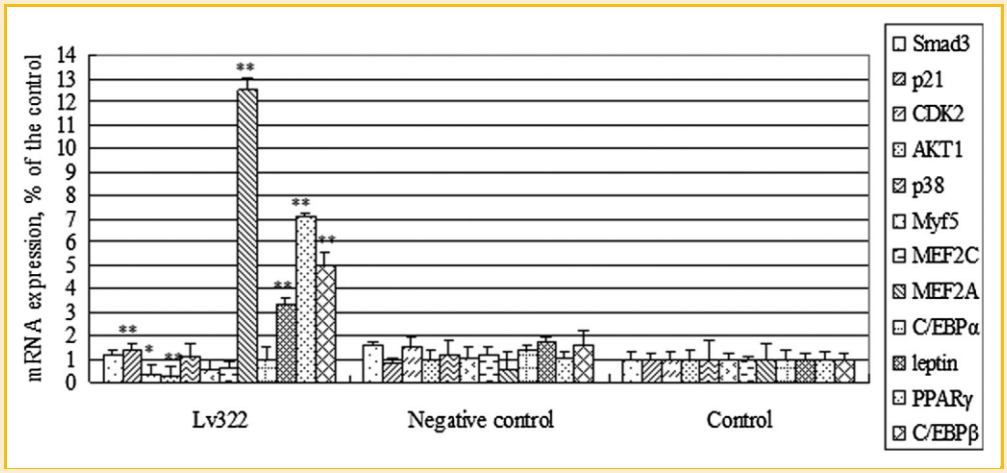

Fig. 3. Genes expression after myostatin knockdown in sheep adult fibroblasts. The non-transfected fibroblasts were selected as the control. The irrelevant sequence known not to block any mammalian mRNA was also selected as the negative control. $^{**}P < 0.01$ or $^{*}P < 0.05$ with non-transfected control.

during the development stage. It was also supported by the result that myostatin acts via by changing the specification of the fibers during development through altered MEF2C expression [Hennebry et al., 2009]. In the present study, we observed highly MEF2A expression levels as the absence of myostatin, compared to the control. Previous reported had shown that loss of functional myostatin increased both Myf5 and MEF2A gene expression in the pectoralis muscles of myostatin$^{-/-}$ mice. Consistent with the result of loss of functional myostatin in mice, the increased level of MEF2A in our results indicated that myostatin negatively regulated MEF2A expression both in muscle and fibroblasts. However, in myostatin overexpression model, we did not find gene expression changes of MEF2A and MEF2C, we reasoned that it could be due to the proliferation effect of myostatin in fibroblasts, whereas MEF2 had no effect on fibroblasts growth.

Myf5, another important regulator of skeletal myogenesis, is the earliest to be expressed among the MRFs. Besides in muscle, Myf5 expression is also found in non-muscle tissues, such as predipocytes and neurons [Francetic and Li, 2011]. It suggested that Myf5 induced laminin assembly. It also had shown that Myf-5^{m1} cells may fail to express extracellular matrix components [Braun et al., 1992]. Taken together, it is implied that Myf5 plays an important role in regulating the formation of extracelluar matrix. Non-consistent with the regulation mechanism in myoblast, our studies have addressed that myostatin might positively regulate Myf5 expression in fibroblasts.

In the present study, we observed that inactivation of myostatin in adult fibroblasts resulted in the increased of PPARγ, leptin and C/EBPβ mRNA expression, but not C/EBPα. Similar result was also found in the transgenic over-expression of myostatin propetide mice, in which myostatin function was blocked. The transgenic mice fed either a high or normal fat diet also displayed significantly high levels of PPARγ expression above the wild-type littermates in epididymal fat [Suzuki et al., 2008]. The results above together

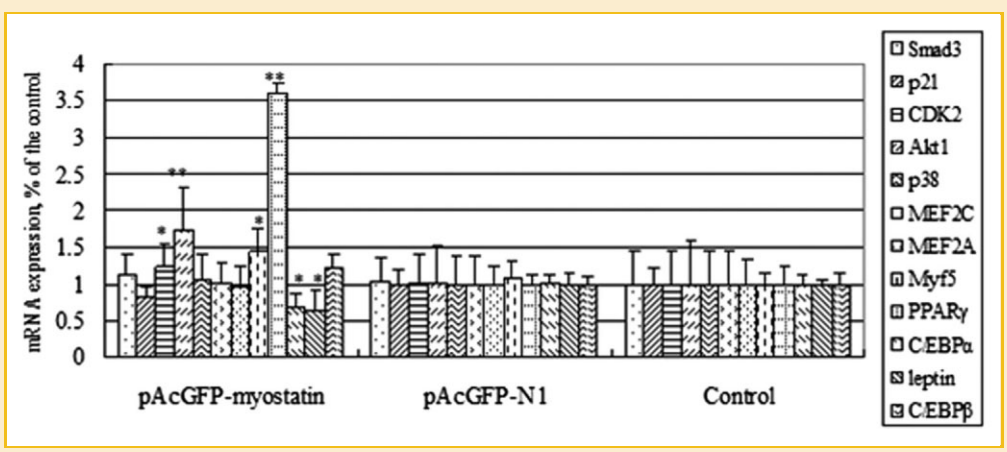

Fig. 4. Genes expression in the myostatin overexpression model. The non-transfected fibroblasts were selected as control. The transfection of empty vector pAcGFP-N1 was selected as another control. $^{**}P < 0.01$ or $^{*}P < 0.05$ with non-transfected control.

showed that myostatin might negatively regulated PPARγ and leptin expression in vivo and in vitro. However, we noted that over-expression of myostatin down-regulated C/EBPα and leptin mRNA expression in adult fibroblasts. The result here was consistent with the results that myostatin significantly reduced leptin and C/EBPα expression in hMSCs [Guo et al., 2008]. Surprisingly, in this study, PPARγ expression had been increased by ectopic expression of sheep myostatin in fibroblasts. Sequence analysis of human myostatin promoter showed a putative PPARγ binging site, suggesting the existence of regulation relationship between myostatin and PPARγ [Ma et al., 2001]. Recently, some reports demonstrated that the nuclear receptor PPARγ control the cell proliferation and apoptosis, depending on the presence of an RB-HDAC3 complex [Fajas et al., 2003]. It was also demonstrated that PPARγ agonists inhibited profibrotic phenotypes in human lung fibroblasts and bleomycin-induced pulmonary fibrosis [Milam et al., 2008]. In addition, PPARγ agonists also inhibited the ability of transforming growth factor β1 to induce myofibroblast differentiation and collagen secretion. In contrast, some reports demonstrated that myostatin stimulated fibroblast proliferation in vivo and also stimulated TGFβ1 secretion in C2C12 myoblasts. It also demonstrated that myostatin directly regulated skeletal muscle fibrosis. Therefore, unlike in adipocyte, the relationship between myostatin and PPARγ in fibroblasts was complex and the elucidation of this question required further investigation.

In conclusion, our study demonstrated that myostatin positively regulated Akt1, CDK2, Myf5, leptin, and C/EBPα, but negatively regulated p21 mRNA expression, suggesting that the signal pathways of myostatin in adult fibroblasts were similar to that of the muscle. Furthermore, it also expanded our understanding of the regulation mechanism of myostatin in adult fibroblasts. Our observations supported the negative role for myostatin in regulation of adipocygenesis, but still need to verify in adipocytes and in vivo. In addition, our results demonstrated that the lentiviral system could knockdown myostatin gene in sheep adult fibroblasts, which could be used to generate transgenic sheep for the promotion of meat production and to ameliorate muscle fibrosis by gene therapy in the future.

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