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Analysis of the spatiotemporal expression of major genes in the TGF-β/*Smad* signaling pathway and correlation analysis using Hu sheep muscle tissue

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ABSTRACT. The mRNA expression levels of key genes (*Smads*, *MSTN*, and *MyoG*) in the TGF-β/*Smad* signaling pathway in Hu sheep at different growth stages (2 days, 2 months, and 6 months of age) and in different skeletal muscles (longissimus dorsi muscle and soleus muscle) and different genders were detected; and correlation of the *Smad* family (*Smad*2, *Smad*3, *Smad*4, and *Smad*7), *MSTN*, *MyoG* expressions was analyzed in Hu sheep. The results showed that the expression of *Smads* was higher in the soleus muscle than in the longissimus dorsi muscle; the expressions of *Smad*2, *Smad*3, and *Smad*4 were significantly higher in 2-day-old sheep than in sheep belonging to the other age groups ($P < 0.05$); the expressions of *Smad*2, *Smad*4, and *Smad*7 were higher in rams than in 2-day-old ewes, but lower in rams than in 2-month-old and 6-month-old ewes; and the expression of *Smad*3 was higher in rams than

in 2-day-old and 2-month-old ewes, but lower in rams than in 6-month-old ewes. In the 2 different muscle tissues, expression of *Smad*2 was significantly positively correlated ($P < 0.01$) with that of *Smad*3. The expression of *Smad*3 was significantly positively correlated ($P < 0.01$) with that of *Smad*4, which showed that the *Smad* family genes could have an inhibitory effect on the TGF-β/*Smad* signaling pathway.

Key words: Hu sheep; *Smads*; *MSTN*; *MyoG*; Gene expression

INTRODUCTION

The TGF-β/*Smad* signaling pathway regulates skeletal muscle development, repair, and locomotory mechanism in all mammals, such as sheep and cattle (Kollias and McDermott, 2008) (Figure 1). The TGF-β/*Smad* signaling pathway is composed of the TGF-β receptor and *Smads* protein family. When TGF-β binds to TbRII, the TbRI receptor is activated to stimulate the phosphorylation of *Smad*2 and *Smad*3, resulting in heterogeneous composites with *Smad*4 to regulate target gene transcription by interacting with transcription factors, inhibitors, or activators in the cell nucleus. *Smad*7, the antagonistic protein of the type I receptor, can firmly bind to TbRI to inhibit the phosphorylation of *Smad*2 and *Smad*3; thus, TGF-β has a negative feedback effect on the regulation of target gene transcription (Li et al., 2008; Droguett et al., 2010), and it can be involved in the process of cell proliferation and differentiation. *MSTN*, a significant member of the TGF-β superfamily, has a negative effect on skeletal muscle growth and development. Blocking the expression of *MSTN* by using RNA interference or gene knockout technology leads to double muscular traits (Lee, 2007; Liu et al., 2008), and the number of muscle fibers and fiber diameter of transgenic mice increase significantly. Overexpression of *MSTN* in mice resulted in muscle atrophy and weight loss (Abe et al., 2009). *MyoG* belongs to MRFs (Myogenic regulatory factors), and it is the key gene in the regulation of cell differentiation and plays an important role in muscle cell growth; moreover, *MyoG* can induce myoblast proliferation and promote mononuclear myoblasts to fuse to multinucleated myotubes (Sonstegard et al., 1998). The *Smad* family is classified into three different types (Kaivo-oja et al., 2006): the first type is R-*Smad* composed of *Smad*2 and *Smad*3; the second type, called Co-*Smad* composed of *Smad*4; and the third type, I-*Smad* consists of *Smad*6 and *Smad*7. The *Smad* signal transduction pathway is composed of extracellular ligands, specific receptors on the cell surface, and *Smad* signal transduction molecules that cause a cascade reaction, transfer the extracellular signal into the nucleus, and regulate the transcription of target genes.

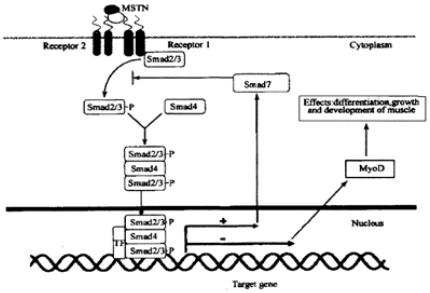

Figure 1. TGF-β/*Smad* signaling pathway.

To understand the expression and internal relationships between genes in the TGF-β/*Smad* signaling pathway in Hu sheep, we detected the spatiotemporal expression of genes in muscular tissues at the mRNA expression level. In this study, we evaluated the changes in gene expression in the TGF-β/*Smad* signaling pathway in Hu sheep muscular tissues and discussed the differences in gene expressions in different parts of the body and at different ages, to collect information for further studies on the functions of the pathway during muscle growth and development in Hu sheep.

MATERIAL AND METHODS

Experimental animals

Hu sheep were purchased from Suzhou Sheep Breeding Farm in Jiangsu Province. The sheep were divided into 3 growth stages (i.e., 2 days, 2 months, and 6 months of age), and 3 rams and 3 ewes were used for each stage. The animals were raised under the same conditions. All animals were slaughtered, and the longissimus dorsi and soleus muscles were rapidly collected and preserved in liquid nitrogen.

rTaq, dNTP, PrimerScript RT reagent Kit, SYBR® Premix Ex TaqTM II (Tli RNaseH Plus), and TRIzol were purchased from (TaKaRa Biotechnology Dalian, Co., Ltd., Dalian, China).

Total RNA isolation

Total RNA was isolated using the TRIzol method.

Primer design and synthesis for real-time PCR

Real-time PCR primers were designed and synthesized (Table 1).

Table 1. Primer sequences for *Smads*, *MSTN*, *MyoG*, and 18S genes for RT-PCR.

Genes	Reference sequences	Primer sequences	Products (bp)
Smad2	XM004020532	SF: CTTGAGAAAGCCATCACCAC	180
		SR: TCGATGGGACACCTGAAG	
Smad3	XM004010875	SF: ATTGAGCTGCACCTGAACGGAC	116
		SR: CTCCCTCTTCGCTCGCAGTGT	
Smad4	GAAI01002997	SF: GAATAGCCCCAGCCATCAGT	97
		SR: GCAACACAGCCTCTTGACTTCCG	
Smad7	GAAI01006724	SF: CCCTCCAACTACTCGCTCCC	90
		SR: GCAACACAGCCTCTTGACTTCCG	
MSTN	AF019622	SF: CGCCTGGAAACAGCTCCTAAC	119
		SR: CCGTCGCTGCTGTCATCTCT	
MyoG	AF433651	SF: AATGAAGCCTTCGAGGCCC	101
		SR: CGCTCTATGTACTGGATGGCG	
18S	AY753190	SF: CGGCTACCACATCCAAGGAA	187
		SR: GCTGGAATTACCGCGGCT	

Complementary DNA (cDNA) synthesis

Total RNA was transcribed into cDNA by using the TaKaRa reverse transcription kit (TaKaRa Biotechnology Dalian, Co., Ltd.), according to the manufacturer instructions.

RT-PCR was performed in 10-μL solution containing 0.5 μL 1000 ng/μL total RNA, 2 μL PrimerScript Buffer, 0.5 μL Oligo dT, 0.5 μL Random 6 mers, 0.5 μL PrimerScript RT Enzyme Mix I, and RNase-free water to a final volume of 10 μL. PCR conditions were as follows: 15 min at 37°C and 15 s at 85°C.

Quantitative PCR detection

The cDNA products were diluted and tested using fluorescent quantitative PCR (FQ-PCR) with ABI 7500 (ABI, USA); the 18s ribosomal RNA gene (eukaryon) was used as the reference gene to analyze the expression levels of *Smads*, *MSTN*, and *MyoG*. Under the same conditions, annealing temperature (53°-63°C) and primer concentrations were optimized for use in the experiment, according to the SYBR Green I Kit (TaKaRa Biotechnology Dalian, Co., Ltd.). The optimal reaction system was a 20-μL reaction volume containing 0.8 μL 10 μM PCR Forward Primer, 0.8 μL 10 μM PCR Reverse Primer, 0.4 μL ROX Reference Dye II, 7 μL H_2O, 10 μL SYBR Green real-time PCR master mix, and 1 μL template. The PCR conditions were as follows: 40 cycles of 30 s at 95°C, 5 s at 95°C, and 34 s at 60°C; 1 μL sterile water (instead of the template) was used as the negative control, and 3 parallel experiments were conducted for each sample. Fluorescence signals were converted into Ct values of *Smad*2, *Smad*3, Samd4, *Smad*7, *MSTN*, and *MyoG* and used to calculate the initial template copies.

Statistical analysis

SPSS 16.0 was used to calculate the Ct values and standard errors among repeat samples, and differences in relative gene expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). ΔCt was calculated as Ct of the target gene minus Ct of the reference gene. For Hu sheep of similar age and skeletal muscles but different gender, ΔΔCt was calculated as ΔCt for rams minus ΔCt for ewes. ΔΔCt was calculated as ΔCt for other age groups minus ΔCt for the 2-day-old group when Hu sheep were of the same gender and had similar skeletal muscles but belonged to different age groups. ΔΔCt was calculated as ΔCt for the soleus muscle minus ΔCt for the longissimus dorsi muscle when Hu sheep were of the same gender and age group but different skeletal muscles. The $2^{-\Delta\Delta Ct}$ represented differential expression of the target gene between the experimental and control groups. Data of Hu sheep in similar age groups and skeletal muscles but different genders were compared by the *t*-test, while data of Hu sheep of the same gender and skeletal muscles but different age groups and the same gender and age groups but different skeletal muscles were compared by ANOVA. Meanwhile, a variation trend histogram of ΔCt was used to verify the conclusion that the value of ΔCt showed a negative relationship with transcriptional quantity.

RESULTS

Total RNA analysis

RNA concentration was measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA with a purity (A_{260}/A_{280}) of >1.8 was used. Total RNA was detected by 1% agarose gel electrophoresis (Figure 2).

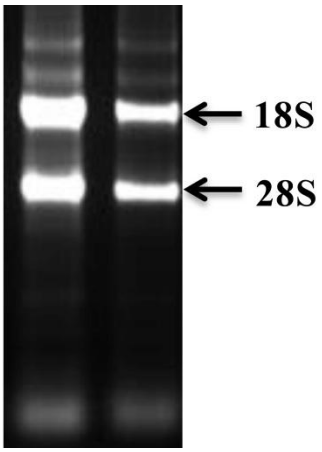

Figure 2. Agarose gel electrophoresis for total RNA.

Spatial and temporal expression analyses of *Smads* in sheep muscle

Spatial and temporal expression analyses of Smads in the same month, same gender, and different muscle tissues

Expression of *Smad*2 in the longissimus dorsi muscle was lower than that in the soleus muscle. In 2-day-old rams and ewes and 2-month-old ewes, expression of *Smad*2 showed significant differences ($0.01 < P < 0.05$); in 6-month-old rams and ewes, expression of *Smad*2 showed no significant differences between the longissimus dorsi and soleus muscles (Figure 3I). Expression of *Smad*3 in the longissimus dorsi muscle was lower than that in the soleus muscle. In 2-day-old rams and ewes, expression of *Smad*3 showed significant differences ($0.01 < P < 0.05$); in 2-month-old rams and ewes, expression of *Smad*3 showed no significant differences between the longissimus dorsi and soleus muscles (Figure 3II). Expression of *Smad*4 in the longissimus dorsi muscle was lower than that in the soleus muscle. In 2-day-old rams and 2-month-old ewes, expression of *Smad*4 showed significant differences between the longissimus dorsi and soleus muscles ($0.01 < P < 0.05$); in 6-month-old rams and ewes, there were no significant differences in the longissimus dorsi and soleus muscles (Figure 3III). Expression of *Smad*7 in the longissimus dorsi muscle was lower than that in the soleus muscle. In 2-day-old rams and 2-month-old ewes, expression of *Smad*7 showed significant differences between the longissimus dorsi and soleus muscles ($0.01 < P < 0.05$); in 2-day-old ewes and 2-month-old rams, there were no significant differences in the longissimus dorsi and soleus muscles (Figure 3IV).

Spatial and temporal expression analyses of Smads in the same muscle tissues, same gender, and different months

With the increase in age, expression of *Smad*2 decreased at first and then increased. There were no significant differences in different genders ($P > 0.05$, Figure 4I). Expression of *Smad*3 decreased at first and then increased, and the expression of *Smad*3 in male and female sheep showed a highly significant difference in 2-day-old and 2-month-old sheep (Figure 4II).

Expression of *Smad*4 showed a gradually decreasing trend in the extensor digitorum longus of male lambs and gastrocnemius muscle of female lambs, but it increased at first and then decreased. In different growth stages, significant or highly significant differences were observed between male and female lambs (Figure 4III). Expression of *Smad*7 decreased at first and then increased, and this expression was higher in 6-month-old sheep than in 2-month-old sheep. There were significant or highly significant differences between male and female lambs (Figure 4IV).

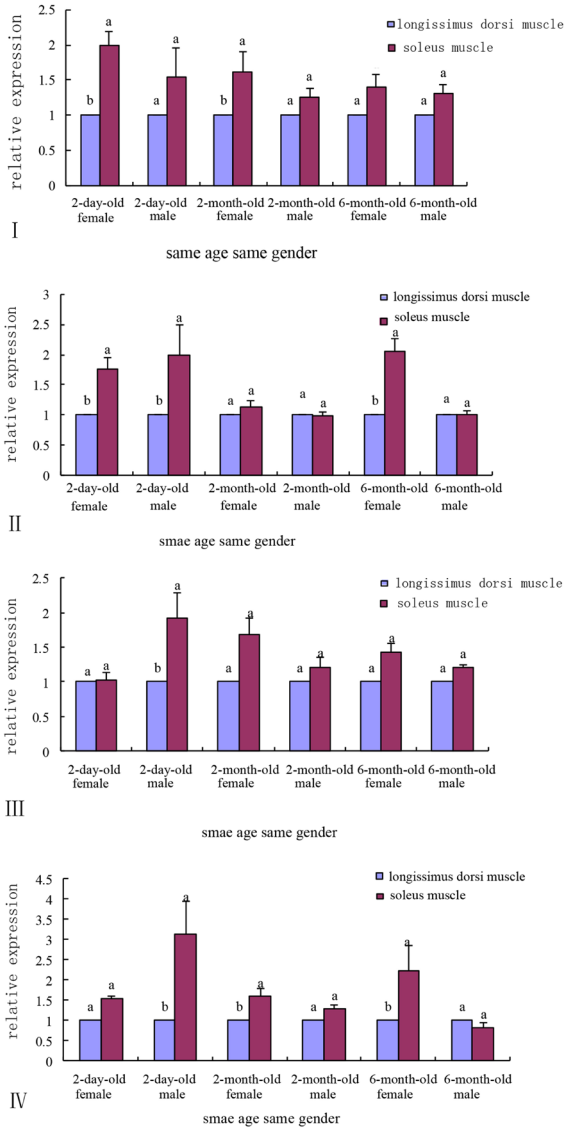

Figure 3. Comparison of different expressions of Smad in different muscles of the sheep. **I.** Smad2; **II.** Smad3; **III.** Smad4; **IV.** Smad7. A, B, C, a, b, and c show the results of multiple comparisons of same sex and same growth stages in different muscles of the sheep. Same letters are not significantly different ($P > 0.05$), values with different letters are significantly different ($0.01 < P < 0.05$), and values with different capitals are extremely different ($P < 0.01$).

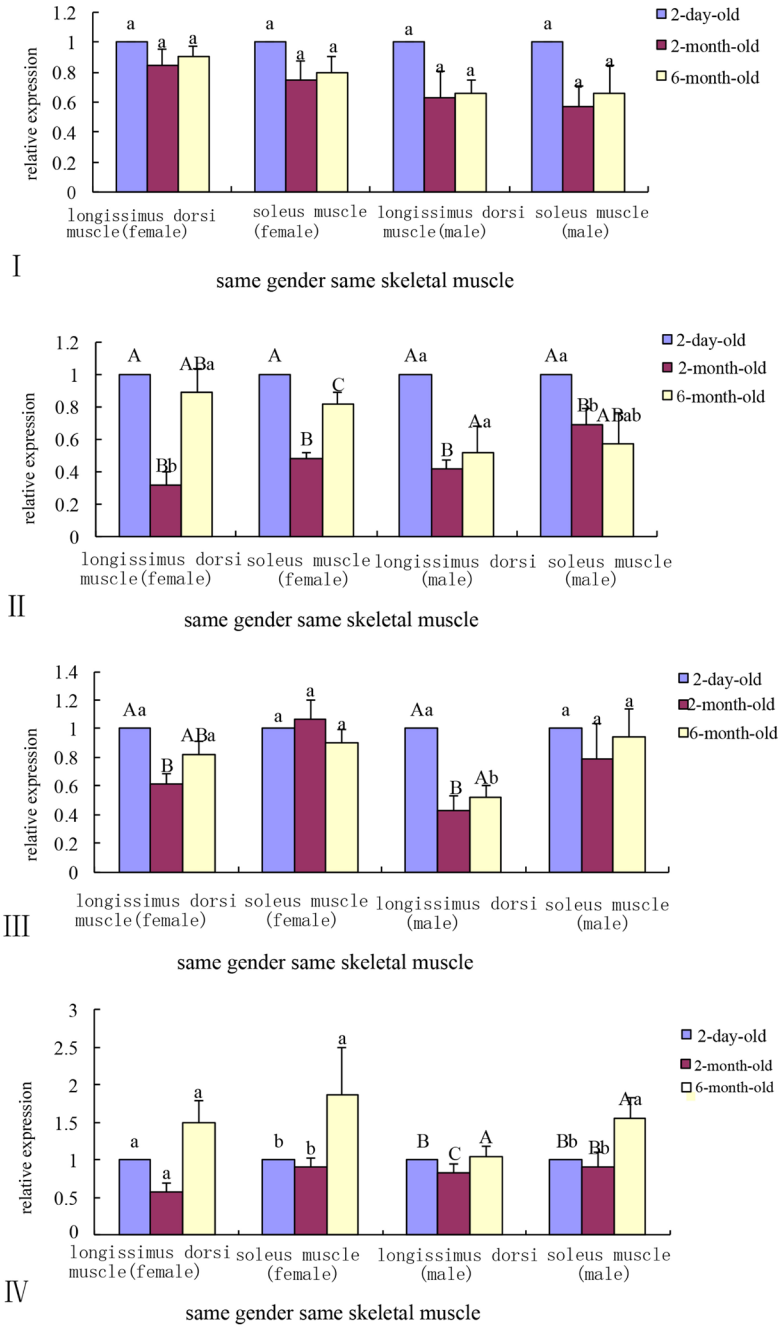

Figure 4. Comparison of different expressions of Smads at different growth stages of the sheep. **I.** Smad2; **II.** Smad3; **III.** Smad4; **IV.** Smad7. A, B, C, a, b, and c show the results of multiple comparisons of same sex and same muscles at different growth stages. Same letters are not significantly different ($P > 0.05$), values with different letters are significantly different ($P < 0.05$), and values with different capitals are extremely different ($P < 0.01$).

Spatial and temporal expression analyses of Smads in the same months, same muscle tissues, and different genders

In 2-day-old sheep, expression of *Smad*2 showed extremely significant differences in the longissimus dorsi muscle between rams and ewes; in 2-month-old and 6-month-old sheep, significant differences in the longissimus dorsi muscle were observed between rams and ewes ($0.01 < P < 0.05$, Figure 5I). In 2-day-old longissimus dorsi muscle and 6-month-old soleus muscle, expression of *Smad*3 showed significant differences between rams and ewes; in 6-month-old longissimus dorsi muscle, extremely significant differences were observed ($0.01 < P < 0.05$, Figure 5II). In 2-day-old soleus muscle and 2-month-old longissimus dorsi muscle, expression of *Smad*4 showed significant differences between rams and ewes ($0.01 < P < 0.05$); in the other age groups, there were no significant differences in different genders ($P > 0.05$, Figure 5III). In 2-day-old longissimus dorsi muscle, expression of *Smad*7 showed extremely significant differences between rams and ewes ($P < 0.01$); in the other age groups, there were no significant differences in different genders ($P > 0.05$, Figure 5IV).

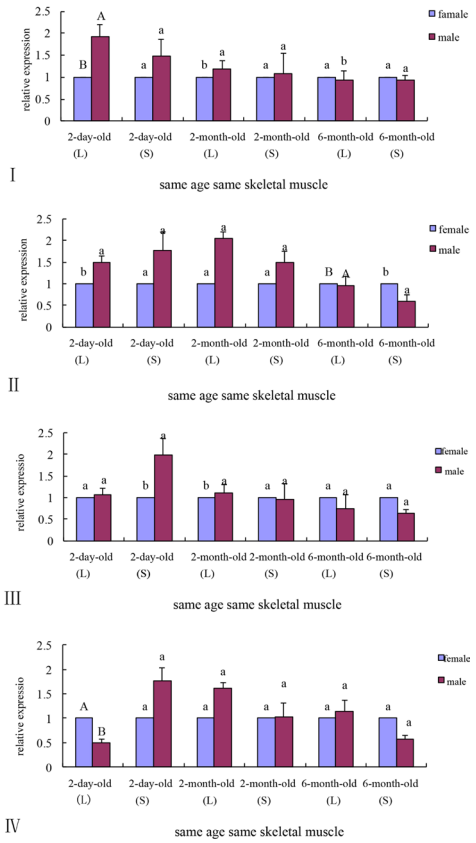

Figure 5. Comparison of different expressions of Smads in different genders of the sheep. **I.** Smad2; **II.** Smad3; **III.** Smad4; **IV.** Smad7. A, B, C, a, b, and c show the results of multiple comparisons of same growth stages and same muscles in different genders of the sheep. Same letters are not significantly different ($P > 0.05$), values with different letters are significantly different ($0.01 < P < 0.05$), and values with different capitals are extremely different ($P < 0.01$).

Correlation analysis between the expressions of *Smads*, *MSTN*, and *MyoG*

In 2-day-old longissimus dorsi muscle, expression of *Smad*2 was extremely significantly and negatively correlated with *MSTN* ($P < 0.01$, Table 2). In 2-month-old longissimus dorsi muscle, expression of *Smad*2 was significantly and positively correlated with *Smad*3 ($P < 0.05$); expression of *Smad*3 was significantly and positively correlated with *Smad*4 and *Smad7* ($P < 0.05$); expression of *Smad*3 was significantly and negative correlated with *MSTN* and *MyoG* ($P < 0.05$); expression of *Smad*4 was extremely significantly and positively correlated with *Smad*7 ($P < 0.01$); and expression of *MSTN* was extremely significantly and positively correlated with *MyoG* ($P < 0.01$, Table 3).

Table 2. Correlation of *Smads*, *MSTN*, and *MyoG* gene expressions in the longissimus dorsi muscles of 2-day-old Hu sheep.

Index	*Smad2*	*Smad3*	*Smad4*	*Smad7*	*MSTN*	*MyoG*
Smad2	1	0.332	-0.154	-0.084	-0.785**	-0.094
Smad3	0.332	1	-0.102	-0.099	-0.275	-0.008
Smad4	-0.154	-0.102	1	-0.339	-0.143	-0.311
Smad7	-0.084	-0.099	-0.339	1	0.246	0.183
MSTN	-0.785**	-0.275	-0.143	0.246	1	0.330
MyoG	-0.094	-0.008	-0.311	0.183	0.330	1

Table 3. Correlation of *Smads*, *MSTN*, and *MyoG* gene expressions in the longissimus dorsi muscles of 2-month-old Hu sheep.

Index	*Smad2*	*Smad3*	*Smad4*	*Smad7*	*MSTN*	*MyoG*
Smad2	1	0.476*	0.440	0.373	-0.184	-0.465
Smad3	0.476*	1	0.560*	0.543*	-0.558*	-0.564*
Smad4	0.440	0.560*	1	0.875**	-0.191	-0.426
Smad7	0.373	0.543*	0.875**	1	-0.080	-0.336
MSTN	-0.184	-0.558*	-0.191	-0.080	1	0.691**
MyoG	-0.465	-0.564*	-0.426	-0.336	0.691**	1

In 6-month-old longissimus dorsi muscle, expression of *Smad*3 was significantly or extremely significantly and positively correlated with *Smad*4, *Smad*7, and *MSTN* ($P < 0.05$, Table 4).

Table 4. Correlation of *Smads*, *MSTN*, and *MyoG* gene expressions in the longissimus dorsi muscles of 6-month-old Hu sheep.

Index	*Smad2*	*Smad3*	*Smad4*	*Smad7*	*MSTN*	*MyoG*
Smad2	1	0.218	0.218	0.436	-0.109	-0.038
Smad3	0.218	1	0.534*	0.643**	0.509*	0.271
Smad4	0.218	0.534*	1	0.511*	0.019	0.530*
Smad7	0.436	0.643**	0.511*	1	0.640**	0.196
MSTN	-0.109	0.509*	0.019	0.640**	1	0.000
MyoG	-0.038	0.271	0.530*	0.196	0.000	1

In the longissimus dorsi muscle, expression of *Smad*2 was extremely significantly and positively correlated with *Smad*3 ($P < 0.01$), was negatively correlated with *MSTN* ($P > 0.05$), and was positively correlated with *Smad*4, *Smad*7, and *MyoG* ($P > 0.05$); expression of *Smad*3 was extremely significantly and positively correlated with *Smad*4 ($P < 0.01$), was negatively

correlated with *MSTN* and *MyoG* (P > 0.05), and was positively correlated with *Smad*7 (P > 0.05); expression of *Smad*4 was extremely significantly and positively correlated with *MyoG* (P < 0.01) and was positively correlated with *Smad*7 and *MSTN* (P > 0.05); expression of *Smad*7 was positively correlated with *MyoG* (P > 0.05) and was negatively with *MSTN* (P > 0.05); and expression of *MSTN* was not significantly and positively correlated with *MyoG* (P > 0.05, Table 5).

Table 5. Correlation of *Smads*, *MSTN*, and *MyoG* gene expressions in the longissimus dorsi muscles.

Index	*Smad2*	*Smad3*	*Smad4*	*Smad7*	*MSTN*	*MyoG*
Smad2	1	0.368**	0.272	0.092	-0.229	0.086
Smad3	0.368**	1	0.496**	0.182	-0.091	0.181
Smad4	0.272	0.496**	1	0.118	0.182	0.419**
Smad7	0.092	0.182	0.118	1	-0.020	0.046
MSTN	-0.229	-0.091	0.182	-0.020	1	0.246
MyoG	0.086	0.181	0.419**	0.046	0.246	1

In 2-day-old soleus muscle, expression of *Smad*2 was significantly or extremely significantly and positively correlated with *Smad*3 and *Smad*7 (P < 0.05); expression of *Smad*3 was extremely significantly and positively correlated with *Smad*4 and *Smad*7 (P < 0.01); expression of *Smad*4 was extremely significantly and positively correlated with *Smad*7 (P < 0.01); and expression of *MSTN* was extremely significantly and positively correlated with *MyoG* (P < 0.01, Table 6).

Table 6. Correlation of *Smads*, *MSTN*, and *MyoG* gene expressions in the soleus muscles of 2-day-old Hu sheep.

Index	*Smad2*	*Smad3*	*Smad4*	*Smad7*	*MSTN*	*MyoG*
Smad2	1	0.920**	0.543*	0.873**	0.216	0.346
Smad3	0.920**	1	0.656**	0.891**	0.034	0.129
Smad4	0.543*	0.656**	1	0.830**	-0.671**	-0.545*
Smad7	0.873**	0.891**	0.830**	1	-0.219	-0.069
MSTN	0.216	0.034	-0.671**	-0.219	1	0.912**
MyoG	0.346	0.129	-0.545*	-0.069	0.912**	1

In 2-month-old soleus muscle, expression of *Smad*2 was extremely significantly and positively correlated with *Smad*3, *Smad*4, and *Smad*7 (P < 0.01); expression of *Smad*3 was extremely significantly and positively correlated with *Smad*4 and *Smad*7 (P < 0.01); and expression of *Smad*4 was significantly and positively correlated with *Smad*7 (P < 0.05, Table 7).

Table 7. Correlation of *Smads*, *MSTN*, and *MyoG* gene expressions in the soleus muscles of 2-month-old Hu sheep.

Index	*Smad2*	*Smad3*	*Smad4*	*Smad7*	*MSTN*	*MyoG*
Smad2	1	0.780**	0.822**	0.738**	0.130	-0.195
Smad3	0.780**	1	0.588*	0.578*	-0.100	-0.061
Smad4	0.822**	0.588*	1	0.520*	0.328	-0.114
Smad7	0.738**	0.578*	0.520*	1	-0.067	-0.137
MSTN	0.130	-0.100	0.328	-0.067	1	0.571*
MyoG	-0.195	-0.061	-0.114	-0.137	0.571*	1

In 6-month-old soleus muscle, expression of *Smad*2 was significantly or extremely significantly and positively correlated with *Smad*3, *Smad*4, and *Smad*7 (P < 0.05); expression of *Smad*3 was extremely significantly and positively correlated with *Smad*4 and *Smad*7 (P < 0.01); and expression of *Smad*4 was extremely significantly and positively correlated with *Smad*7 (P < 0.01, Table 8).

Table 8. Correlation of *Smads*, *MSTN*, and *MyoG* gene expressions in the soleus muscles of 6-month-old Hu sheep.

Index	*Smad2*	*Smad3*	*Smad4*	*Smad7*	*MSTN*	*MyoG*
Smad2	1	0.651**	0.541*	0.625**	0.032	0.236
Smad3	0.651**	1	0.805**	0.742**	-0.264	0.373
Smad4	0.541*	0.805**	1	0.763**	-0.170	0.357
Smad7	0.625**	0.742**	0.763**	1	0.199	0.461
MSTN	0.032	-0.264	-0.170	0.199	1	0.352
MyoG	0.236	0.373	0.357	0.461	0.352	1

In soleus muscle, expression of *Smad*2 was extremely significantly and positively correlated with *Smad*3, *Smad*4, *Smad*7, and *MyoG* ($P < 0.01$) and was not significantly and positively correlated with *MSTN* ($P > 0.05$); expression of *Smad*3 was extremely significantly and positively correlated with *Smad*4 and *Smad*7, was significantly and positively correlated with *MyoG* ($P < 0.05$), and was not significantly and negatively correlated with *MSTN* ($P > 0.05$); expression of *Smad*4 was extremely significantly and positively correlated with *Smad*7 ($P < 0.01$), was not significantly and negatively correlated with *MSTN*, and was not significantly and positively correlated with *MyoG*; expression of *Smad*7 was not significantly and negatively correlated with *MyoG* and *MSTN*; and expression of *MSTN* was extremely significantly and positively correlated with *MyoG* ($P < 0.01$, Table 9).

Table 9. Correlation of *Smads*, *MSTN*, and *MyoG* gene expressions in the soleus muscles.

Index	*Smad2*	*Smad3*	*Smad4*	*Smad7*	*MSTN*	*MyoG*
Smad2	1	0.793**	0.703**	0.615**	0.016	0.362**
Smad3	0.793**	1	0.687**	0.587**	-0.095	0.338*
Smad4	0.703**	0.687**	1	0.603**	-0.009	0.252
Smad7	0.615**	0.587**	0.603**	1	-0.210	-0.018
MSTN	0.016	-0.095	-0.009	-0.210	1	0.622**
MyoG	0.362**	0.338*	0.252	-0.018	0.622**	1

DISCUSSION

Growth and development of skeletal muscles are regulated by cell factors such as TGF-β and IGF-I, and functions of the TGF-β superfamily are the most significant. The TGF-β superfamily is composed of TGF-β, activins, inhibins, and BMPs, which have a wide range of biological activities and are involved in early embryonic development, formation of cartilage and bone, tumor formation and development, and other functions (Wan et al., 2012). TGF-β can activate several signal pathways, but the *Smad* pathway is considered the most significant. *Smad* is a general designation of Mad and Sma protein and its analogues, namely, *Smad* (Sma-Mad) proteins, which are directly involved with the TGF-β superfamily (Derynek and Zhang, 2003). To date, 9 different *Smad* proteins have been identified in mammals (Kohei, 1998). The *Smad* family of proteins participates in the regulation of cell activity, which can regulate cell proliferation, apoptosis, differentiation, and migration, as well as regulation of immune and endocrine functions.

The *Smad* family of proteins is the key component of TGF-β family signal transduction in the cell. We mainly studied four genes: *Smad*2, *Smad*3, *Smad*4, and *Smad*7. *Smad*2 and *Smad*3 belong to R-*Smad* and are highly homologous in structure, but their functions are not the same. *Smad*2 is crucial for embryo formation, and *Smad*2 or *Smad*2rexon3 can be detected in early embryos; however, *Smad*3 cannot be detected (Faure et al., 2000). *Smad*3

can be directly combined with DNA, but *Smad*2 cannot (Roberts, 1998). *Smad*4 is the only co-mediated *Smad* (Co-*Smad*) in mammals. *Smad*4 and other *Smad* proteins form complexes through the regulation of p21 and other downstream genes for cell cycle arrest in the G1 phase and inhibition of cell proliferation. *Smad*7 is a general inhibitory factor that can antagonize growth inhibition of the cell, matrix formation, apoptosis induction, embryonic lung formation by TGF-β, or activin signaling. In this study, we found that the expression of *Smads* in the soleus muscle was higher than that in the longissimus dorsi muscle, which may be related to the type of muscle fiber. Expressions of *Smad*2, *Smad*3, and *Smad*4 were significantly higher in 2-day-old sheep than in sheep of other age groups ($P < 0.05$), and expression of *Smad*7 was lower in 2-day-old sheep than in 6-month-old; 2-month-old sheep showed the lowest expression, which may be the reason for the different functions of *Smads*. Expressions of *Smad*2, *Smad*4, and *Smad*7 were higher in rams than in 2-day-old ewes, but lower in rams than in 2-month-old and 6-month-old ewes; expression of *Smad*3 was higher in rams than in 2-day-old and 2-month-old ewes, but lower in rams than in 6-month-old ewes.

MSTN through the regulation of *Smad*3 plays a negative regulatory role on skeletal muscles. *MSTN* with its receptor-binding activity induces phosphorylation of *Smad*3 and enhances the role of *Smad*3 with *MyoG*enic factors, inhibiting the expression of *MyoG*enic factors and formation of secondary structures. Myoblasts could not be differentiated into myotubes. *MyoG* is a stimulative muscle growth factor, which can regulate the differentiation of muscle cells and muscle fiber. At different ages (0-6 months), *Smad*2 was not significantly and positively correlated with *MSTN* in Altai sheep and Turpan black sheep muscles ($P > 0.05$), and *Smad*2 was significantly and positively correlated with *Smad*3 (Anwyl, 2014; Tuleliyan, 2014). However, in this experiment, *Smad*2 was extremely significantly or not significantly and negatively correlated with *MSTN* in the longissimus dorsi muscle, but *Smad*2 was not significantly and positively correlated with *MSTN* in the soleus muscle. There may be different relationships between *Smad*2 and *MSTN* in different muscle tissues. There is a positive correlation between *Smad*2 and *Smad*3, which is consistent with the results. We found that *MSTN* in mice transmits a signal through the *Smads* pathway, thereby upregulating the expression of the p21 gene, inhibiting the cell proliferation, and downregulating the expression of *MyoG* (Van Hoef et al., 2011). Similarly, through transfecting *MSTN* lentiviral vectors in Xinjiang Merino Sheep myoblasts, overexpression of *MSTN* can significantly downregulate the expression of *MyoG* and upregulate the expression of *Smad*3 (Liu et.al., 2012). This indicates that there is a negative correlation between *MSTN* and *MyoG*. However, in this study, the expression of *MSTN* was positively correlated with the expression of *MSTN*. This is contradictory with the abovementioned point of view. The reason may be that we selected only Hu sheep in the early growth stages as research subjects,; therefore, further experiments need to be performed.

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