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Full Length Research Paper

Postnatal expression of myostain (*MSTN*) and myogenin (*MYoG*) genes in Hu sheep of China

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The study of candidate genes is an important tool to identify genes associated with economic traits. Skeletal muscle development is an important physiological process in meat animals, and it directly affects meat production. The expression of myostain (*MSTN*) and myogenin (*MYoG*) genes in longissimus dorsi, during the early growth stage of Hu sheep, was studied by semi-quantitative Reverse transcription polymerase chain reaction (RT-PCR). The results demonstrate that age and gender were playing a very important role in the expression of sheep muscle. *MSTN* and *MYOG* genes showed similar variation pattern for the male and female. The expression level of the *MSTN* and *MYoG* genes all showed a positive correlation with live weight, carcass weight and meat percentage, but only showed a significant relationship with meat percentage. *MSTN* gene showed an extreme significant positive relationship with *MYoG*.

Key words: Sheep, myostain (*MSTN*), myogenin (*MYoG*), gene expression, muscle trait.

INTRODUCTION

Muscle growth and development is a very complicated process. Except for the impact of the nutrition and breed, it is also regulated by age, sex, and a number of growth genes. Investigation of genes expressed during skeletal muscle development is elementary in understanding molecular mechanism of muscle growth, and can contribute to the discovery of candidate genes associated with meat production and quality traits (Chen et al., 2008). The transforming growth factor β (TGF-β) super family encompasses an immense group of secreted growth and differentiation factors, which play significant roles in regulating development and tissue homeostasis (Kingsley, 1994; Griffith et al., 1996; McPherron and Lee, 1996). Myostain (*MSTN*), also known as growth and differentiation factor-8 (GDF-8), is a 25 kDa homodimeric TGF-β family peptide and a negative regulator of hyperplasia, as well as hypertrophy of the muscle (Langley et al., 2002; Lee, 2004; Bellinge et al., 2005; Yang et al., 2005). The loss-of-function mutation of this regulatory peptide results in an increase in muscle mass, the most well known is double-muscling in the Belgian blue and Piedmont breeds of cattle (McPherron and Lee, 1997).

The *MYoG* gene is a member of the *MYoD* gene family (MYoG, MYoD1, MYF-5, and MYF-6), which acts on myogenesis (Olson, 1990; Weintraub et al., 1991), synthesizes myofibrillar proteins in the skeletal muscles and regulates the number of their myofibers. It is also expressed in all the phases of myoblast differentiation to

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Abbreviations: *MSTN*, Myostain; ***MYoG*,** myogenin.

Table 1. Primer sequence of the target genes.

cDNA sequence	Target genes	Prime sequence	Products
AF019623	*MSTN*	5′-gtcccgtggatctgaatg-3′ 5′-ttccgtcgtagcgtgata-3′	265 bp
U14331	*MYoG*	5′-aggctacgagcggactga-3′ 5′-gcagggtgctcctcttca-3′	198 bp
Internal reference	GAPDH	5′-aaggtcatccacgaccactt-3′ 5′-aggccatgccagtgagtt-3′	429 bp

proliferation (Tepas et al., 1999). *MYoG* is closely associated with the number of muscle fibers at birth, which is most important in determination of maximal lean meat growth capacity in pigs (Handel and Stickland, 1988). Hu sheep is a local sheep breed of China, mainly distributed along the Taihu Lake valley in Jiangsu and Zhejiang province. Though the meat is tender and juicy, Hu sheep has a lower relatively growth speed, compared with other foreign sheep breeds. Up to now, no data about the mechanism of the muscle development of Hu sheep was reported. In the present study, semi quantitative conventional reverse transcriptase-polymerase chain reaction (RT-PCR) technique was performed to investigate the differences in *MSTN* and *MYoG* genes expression of longissimus dorsi tissues during Hu sheep growth stages.

MATERIALS AND METHODS

Animals and tissue collection

48 Hu sheep with similar reared conditions (all diets contained adequate minerals and vitamins) were collected and slaughtered at six different growth stages (two day, one month, two months, three months, four months and six months), eight animals per stage, with equal number of males and females were used for analysis on postnatal expression of *MSTN* and *MyoG* mRNA. Samples were collected from longissimus dorsi muscle tissues. All tissue samples were immediately frozen in liquid nitrogen after collection and stored at -80°C until RNA isolation. All experimental procedures were performed according to authorization granted by the Chinese Ministry of Agriculture.

RNA extraction and first-strand cDNA synthesis

The RNA was isolated by Trizol, based on the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomezynski and Sacchi, 1987). The concentration of RNA was measured using a Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, USA) and purity (A260/A280) of > 1.8 was used. Total RNA from each sample was transcribed into first-strand cDNA synthesis using the Takara reverse transcription kit (Takara Biotechnology Dalian, Company Limited, China) according to manufacturer's instructions. Briefly, oligo dT primer (50 μM) was used to reverse transcribe 250 ng/μg of respective RNA in the presence of dNTPs mixture (10 mM each), 5 × primer ScriptTM buffer, RNase Inhibitor (40 U/μl) and prime ScriptTM RTase (200 U/μl) at 42°C for 60 min, following inactivation at 95°C for 5 min.

PCR primers designation

The primers of target genes were designed based on *MSTN* (GenBank accession number AF019623) and *MYoG* (GenBank accession number U14331) gene sequen ce of pig. The primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services Company Limited (Table 1).

Reverse transcription polymerase chain reaction (RT-PCR)

Polymerase chain reactions (PCR) were performed according to the standard protocols, with the primers indicated in Table 1. Briefly, the total volume of PCR reaction system was 20 μl for *MSTN*, *MYoG* and *GAPDH* genes, including 2.5 μl cDNA, 3 μl 10× buffer, 1.25 μmol dNTP, 15 μmol upstream primer, 15 μmol downstream primer, 1.5 units rTaq polymerase and 13.7 μl dH2O. PCR conditions for *MSTN* and *MYoG* genes were as follows, 1 cycle of 94°C for 5 min, followed by 35 cycles of 35 s (*MSTN*) and 30 s (*MYoG*) at 94°C, 56.7°C (*MSTN*) for 45 s and 59.8°C for 35 s (*MYoG*), 72°C for 35 s (*MSTN*) and 30 s (*MYoG*) followed by 1 cycle at 72°C for 10 min. PCR conditions for *GAPDH* gene were as follows, 1 cycle of 94°C for 5 min, followed by 35 cycles of 45 s at 94°C, 59.8°C for 45 s, 72°C for 45 s, followed by 1 cycle at 72°C for 10 min. After amplification, 5 μl of each PCR products were analyzed by agarose gel electrophoresis (1.2%) (Gene Technology, Shanghai Company Limited) in 1× Tris-acetate-EDTA (TAE) buffer, and visualized with ethidium bromide under UV light (BIORAD). Three times experiments was performed to verify the expression data.

Statistical analysis

Gel image scan was performed to find the bands and to measure the gray values of bands by using BandScan software version 4.50 (http://www.Glyco.com). The expression of target gene was calculated as the ratio of the amplification product of the target genes and the internal reference GAPDH. The differences of gene expression in the same gender at various growth stages, the differences of gene expression in different genders at the same growth stage, and the interaction between growth stages and genders were analyzed using multivariate variance analysis, we placed more importance in the comparison of different growth stages and different genders, and their multiple comparison were analyzed using least significant difference (LSD) method. Pearson coefficient of correlation among live weight, carcass weight, meat

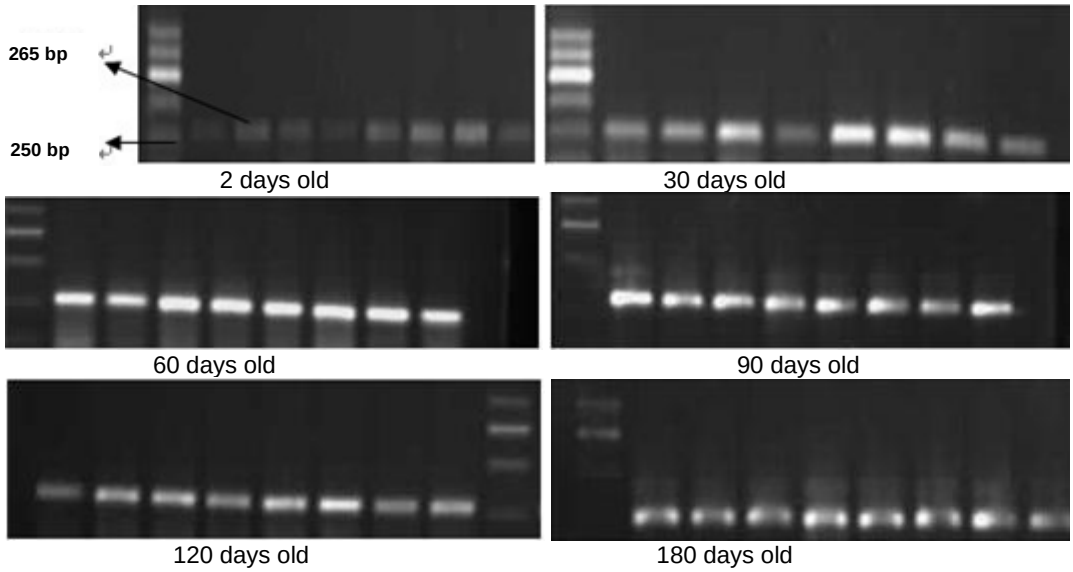

Figure 1. Agarose gel electrophoresis of *MSTN* gene.

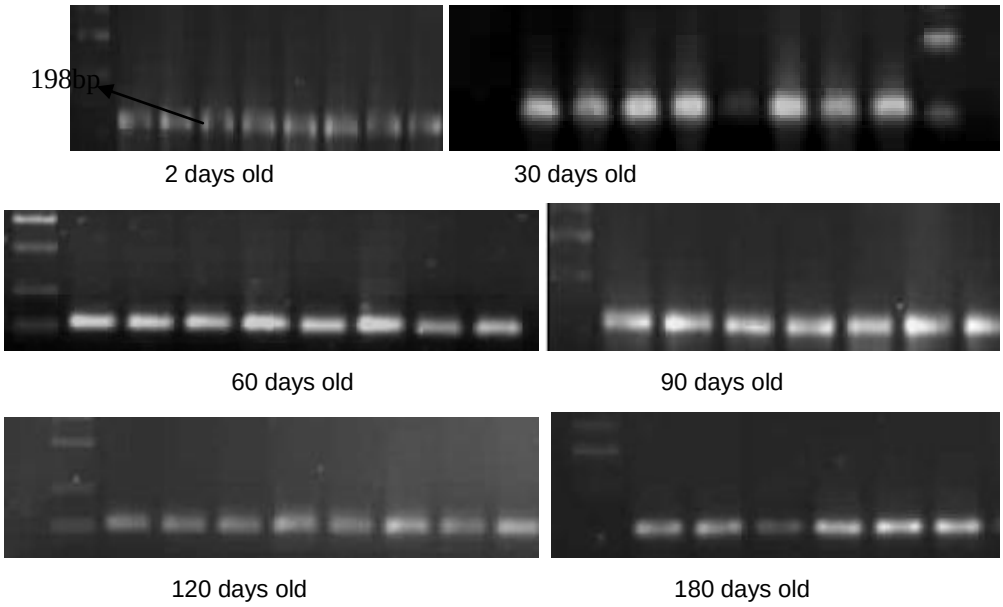

Figure 2. Agarose gel electrophoresis of *MYoG* gene.

percentage, expression of *MSTN* and *MYoG* were computed by the SAS software version 8.1 (SAS Institute Inc., NC. USA).

RESULTS

MSTN and *MYOG* genes RT-PCR product detection

The total RNA was amplified according to the above conditions by RT-PCR. After being detected by 1.2% agarose gel electrophoresis, *MSTN* had a specific band at 265 bp (Figure 1), and *MYoG* at 198 bp (Figure 2), and they were all consistent with the expected amplification result by primer designed.

MSTN and *MYOG* genes expression

MSTN and *MYoG* gene expression at different growth stages of different gender of Hu sheep were presented

Table 2. *MSTN* and *MYoG* gene expression at different growth stages of different gender of Hu sheep.

Gene	Sex	2 days	30 days	60 days	90 days	120 days	180 days
MSTN gene gray scale ratios	Male	$0.520\pm0.007^{Ef,m}$	$0.610\pm0.003^{De,N}$	$1.071\pm0.038^{Aa,n}$	$0.880\pm0.014^{Bb,M}$	$0.640\pm0.010^{Dd,N}$	$0.710\pm0.008^{Cc,n}$
	Female	$0.510\pm0.010^{Dd,m}$	$0.780\pm0.018^{Bb,M}$	$1.130\pm0.026^{Aa,m}$	$0.780\pm0.008^{Bb,N}$	$0.740\pm0.011^{Cc,M}$	$0.741\pm0.022^{Cc,m}$
MYoG gene gray scale ratios	Male	$0.570\pm0.009^{Ee,N}$	$0.970\pm0.006^{Aa,m}$	$0.921\pm0.031^{Bb,M}$	$0.820\pm0.016^{Cc,M}$	$0.720\pm0.017^{Dd,M}$	$0.740\pm0.002^{Dd,m}$
	Female	$0.640\pm0.013^{Ed,M}$	$0.940\pm0.020^{Aa,n}$	$0.850\pm0.020^{Bb,N}$	$0.590\pm0.006^{Fe,N}$	$0.680\pm0.006^{Dd,N}$	$0.730\pm0.021^{Cc,m}$

Values are mean expression levels of *MSTN* and *MYoG* genes and internal reference of male and female sheep with various ages, and were expressed by total gray scale ratios. The gray scale ratios between *MSTN* and *MYoG* genes were the ratios between the mean expression levels at various ages and the expression level of the internal reference. Means with the different superscripts within the same column (rows) shows that there are significant difference between different rows (columns) ($0.01 < P < 0.05$); means with the different capital superscripts within the same column (rows) shows that there were extreme significant difference between different rows (columns) ($P < 0.01$). The superscripts before comma showed the comparisons of different ages, and the superscripts after comma showed the comparisons of different sex.

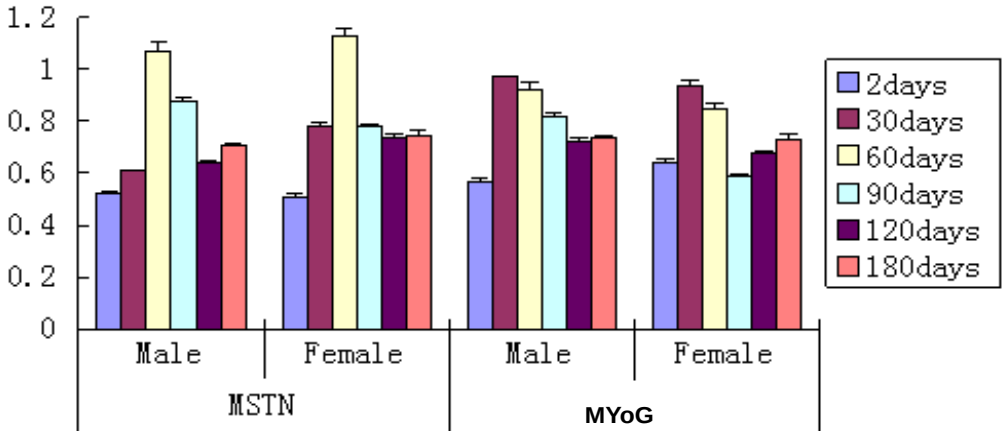

Figure 3. The effect of sex and growth in the expression of *MSTN* and *MYoG* genes.

in Table 2. The effects of sex and different growth stages on *MSTN* and *MYOG* genes were shown in Figure 3. *MSTN* and *MYoG* genes showed a similar variation pattern for the male and female. *MSTN* gene expression in muscle of Hu sheep were lowest at two days old, and tended to increase with the increasing age before 60 days, but after 60 days, tended to decrease with increasing age. In male, except that there was significant difference between 30 and 120 days old stages, there were identified extreme significant differences among the remaining different stages. In females, there was no significant difference between 30 and 90 days old, and 120 and 180 days old stages, there were identified extreme significant differences among the remaining different stages. Except that there was no significant difference between male and female in two days old stage, there were identified extreme significant or significant differences between the male and female sheep in the same stages. The *MYoG* gene expression in muscle of male sheep was lowest at two days old, and tended to increase with the increasing age before

Table 3. Correlation between *MSTN* and *MYoG* genes expression and the slaughter traits.

Index	Live weight	Carcass weight	Meat percentage	*MSTN*	*MYoG*
Live weight	1				
Carcass weight	0.992**	1			
Meat percentage	0.788**	0.828**	1		
MSTN	0.224	0.264	0.428*		
MYoG	0.057	0.116	0.302	0.484**	1

ns, No significant relationship between two different index (0.01<P<0.05); *significant relationship between two different index (0.01 < P < 0.05); **extreme significant relationship between two different index (P < 0.01).

30 days, and after 30 days the expression first tended to decrease before 120 days, and then tended to increase, while in female sheep, the gene expression level tended to increase with the increasing age before 30 days, and after 30 days the expression tended first to decrease before 90 days, and then tended to increase with the increasing age. In males, there was no significant difference between 120 and 180 days old stages; there were identified extreme significant differences among the remaining different stages; in female, except there was significant difference between two and 120 days old stages, there were identified extreme significant differences among the remaining different stages. In addition, there was no significant difference between male and female in 180 days old, but there were extreme significant differences or significant differences between the male and female sheep in other same stage.

The effect of *MSTN* and *MYoG* genes expression on the slaughter traits

The expression level of *MSTN* gene showed a positive correlation with live weight, carcass weight and meat percentage, but only showed significant relationship with meat percentage (Table 3). The expression level of *MYoG* gene showed non significant positive relationship with live weight, carcass weight and meat percentage. Similarly, there was an extreme significant positive correlationship among live weight, carcass weight, and meat percentage. An extreme significant positive relationship between *MSTN* and *MYoG* genes expression is shown in Table 3.

DISCUSSION

The muscle development was significantly correlated with animal ages (Souza et al., 2004) and body weight (Schwartz et al., 2004). In the present study, live weight, carcass weight and meat percentage were increased with age, from two days to six-months old (data not shown), sex had effect on muscle development. *MSTN* and *MYoG* genes showed a similar variation pattern for the male and female. *MSTN* (GDF-8) is expressed predominantly in skeletal muscle (Kambadur et al., 1997; Ji et al., 2002), and as a negative regulator of skeletal muscle development and growth in mice was suggested by McPherron and Lee (1997). The GDF-8 null mice revealed a dramatic increase in skeletal muscle mass. Notably, individual muscles of GDF-8 null mice weighed two to three-folds more than those of wild mice. GDF-8 coding sequence mutations compromise the biological activity of the protein, which leads to results in increased muscle mass hyperplasia and hypertrophy. Exploring increased muscle mass production in livestock and poultry, employing GDF-8 genotype selection or GDF-8 activity inhibition is meritorious. The physiological role of *MSTN* is largely associated with the prenatal muscle growth (Ji et al., 2002). However, the *MSTN* role in postnatal muscle growth of sheep remains unclear. Therefore, we determined that postnatal muscle growth occurs with detectable changes in GDF-8 expression. In Hu sheep, *MSTN* gene expression in muscle first tended to increase with the increasing age before 60 days, but after 60 days tended to decrease with increasing age, there were identified extreme significant differences among the remaining different stages, here we could find that *MSTN* gene also played a negative regulator of skeletal muscle development and growth in sheep. With the decrease of expression of *MSTN* gene, the weight of body and slaughter trait increased. In our study, we proposed a significant correlation between development and the postnatal change of *MSTN* gene expression in Hu muscle, and this was consistent with the precious study in pig (Lin et al., 2002).

The induction of the *MYoG* expression is associated with a rapid set-out of the myoblast differentiation (Buckingham, 1992; Montarras et al., 1991). The knock-out experiments on murine embryos revealed a crucial role of *MYoG* in myogenesis. The null *MYoG*-mutants in the homozygous state were stillborn and showed several muscle and skeletal defects (Hasty et al., 1993; Nabeshima et al., 1993). The *MYoG* mRNA expression in longissimus dorsi muscle (LDM) of Jinhua pig and Landrace pig (35, 80, 125 days of age) were determined.

The result show that the expression of *MYoG* gene increased with the age and muscle percentage of carcass in LDM of Jinhua pigs, the expression levels of *MYoG* gene and muscle percentage of carcass were directly correlated; in contrast, the expression of *MYoG* gene

decreased with the age and muscle percentage of carcass in Landrace pigs, and the expression levels of *MYoG* gene and muscle percentage of carcass were negatively correlated but did not display breed differences (Shan et al., 2009). Yang et al. (2006) determined the developmental changes of *MSTN* and *MYoG* genes expression in longissimus dorsi muscle of Erhualian and Large white pigs. The results indicate that, (1) at birth, the expression of the two breeds is lowest, and the *MSTN* mRNA expression in Large white pigs reached a peak at 20 days old; (2) the expression of *MSTN* increased in both Erhualian gilts and boars after birth, then reached a peak at 45 days old, and the sexual difference was observed at 120 days old ($P < 0.05$); (3) the expression of *MYoG* mRNA of the two breeds was high at 20 days old, then declined gradually; the results suggest that the high level of *MSTN* and *MYoG* genes expression might play a role in regulating the metabolic and contractile maturation of myofibers during the early postnatal growth; but did not appear in increasing or decreasing trends with increasing age. There is strain difference at 20 days old; around the sex maturation, the sexual difference was found in two genes expression in Erhualian pigs (Yang et al., 2006). In the present study, the trend of *MYoG* gene expression is different from Shan et al. (2009), and similar to Yang et al. (2006), maybe because of the different molecular mechanism of muscle development between sheep and pig.

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