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Sex- and age-dependent expression of *Pax7*, *Myf 5*, *MyoG*, and *Myostatin* in yak skeletal muscles

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ABSTRACT. The aim of this study was to investigate the myogenic factor mRNA expression pattern of *Pax7*, *Myf5*, *MyoG*, and *Myostatin* mRNA at different ages, sexes, and muscle tissues of Datong yaks. The expression patterns in semimembranosus (SM), quadriceps femoris (QF), and triceps muscle of arm (TM) were detected by quantitative real-time polymerase chain reaction and compared using biostatistics. The results showed that the *Pax7* gene expression levels were higher in the hindquarters (SM and QF) than in the forequarters, and was higher in male compared to in female muscle ($P \leq 0.05$). The *Myf5* gene expression levels of male yaks were highest in QF ($P \leq 0.05$), whereas the expression levels of female yaks were highest in TM ($P \leq 0.05$). Female *MyoG* gene expression levels were higher in QF and TM compared to in male yaks. The *MyoG* expression was higher in all muscles at 6 months old compared to in 3-year-old muscle. The

highest *MSTN* gene expression was found in 6-month-old TM muscle and in QF muscle of 3 years ($P \geq 0.05$). In conclusion, yak muscles showed different growth patterns depending on position. At 6 months of age, the satellite cells in the male hindquarter muscles and the female forequarter muscle showed a strong proliferative ability, at the same time the satellite cells in all female muscles had a powerful differentiation ability. Hindquarter muscles appear to mainly grow at younger ages and forequarters mainly grow at older ages.

Key words: Myogenic factor; Sex; Age; Expression pattern

INTRODUCTION

Yak (*Bos grunniens*) is an important livestock in the Qinghai Tibet Plateau. It is the only species of cattle that is able to provide high quality meat and dairy products under the extremely harsh ecological conditions of the above region. However, the slow growth and development, a yak production trait, is difficult to adapt to the currently rapidly growing development of the national economy. Hence, growth rate and muscularity have become the most important economic traits in yak breeding. The number and size of muscle fibers determine postnatal muscle growth (Muráni et al., 2007) and the presence and activity of satellite cells play an important role in skeletal muscle hyperplastic and hypertrophic growth (Zammit et al., 2006). A few studies of changes in yak muscle histology at different ages (Yang et al., 2001; Liu et al., 2013) and in different breeds (Zhang et al., 2013) have been reported. However, a study of the expression levels of the myogenic regulatory factors *Pax7*, *Myf5*, *MyoG*, and *Myostatin* (*MSTN*) have thus far not been performed.

The fiber formation in cattle ceases and the total fibers number is established around day 210 of gestation (Du et al., 2010). The mechanisms of hyperplasia and hypertrophy in muscle growth are controlled by sequential expression of muscle regulatory factors (MRFs; including *MyoD*, *Myf6* (*Mrf4*), *Myf5*, and *myogenin*), which commit cells to the myogenic program and control differentiation and fusion (Yin et al., 2014). *Myf5* regulates myogenesis and homeostasis (Gayraud-Morel et al., 2007). *MyoG* and *Mrf4* play an important role in myoblast differentiation and hypertrophy, whereas *MyoG* controls myotube formation (Knapp et al., 2006). In adult myogenesis, *MyoD* and *Myf5* expression could indicate myoblast proliferation and hyperplasia. In pig, *Pax7* has been shown to induce satellite cell self-renewal, affecting the satellite cell physiology and the dynamic stage of postnatal growth (Patruno et al., 2008). *Pax7* also activates *Myf5* and *MyoD*, regulating the satellite cells' entry into the myogenic program. *MSTN* has been shown to maintain satellite cells in a state of reversible quiescence (Wagner et al., 2005; Amthor et al., 2006), as well as to inhibit the *MyoD* synthesis and activity through interactions with MRFs (Amthor et al., 2004; Guttridge, 2004).

The aim of this study was to assess and compare the expression levels of *Pax7*, *Myf5*, *MyoG*, and *MSTN* genes in three different skeletal muscles from different sex and age of postnatal yak. Our data provide a better understanding of the molecular mechanism of muscle growth regulation and for screening the candidate genes related to growth rate and muscularity in yak breeding.

MATERIAL AND METHODS

All experimental procedures were performed in accordance with the Guide for Animal Care and Use of Laboratory Animals in the Institutional Animal Care and Use Committee of QingHai University. The experimental protocol was approved by the Department Animal Ethics Committee of QingHai University.

Reagents

All reagents were of analytical grade of the highest purity that is commercially available. Prime Script RT-reagent Kit with gDNA Eraser (Perfect Real Time) and SYBR Premix Ex Taq were purchased from Takara Biotechnology (Dalian, China) (Asadpour et al., 2013). *Pax7*, *MyoG*, *Myf5*, and *MSTN* primers were synthesized by Sangon Biotech (Sangon Biotech, Shanghai, China) (Kang et al., 2013).

Animal treatment

Animal experiments were carried out according to the guidelines for animal experiments at the National Institute of Animal Health. The experimental group comprised 30 healthy yaks: 10 female and 10 male 6 month old yaks (weight: 70.00 ± 5.00 kg), and 10 three year old female yaks (280.00 ± 5.00 kg). The animals were provided by the Datong Cattle farm from the Qinghai province and kept under the same housing and feeding conditions. Muscle tissues were collected from three different skeletal muscles, semimembranosus (SM) and quadriceps femoris (QF) belong to hindquarter; and triceps muscle from arm (TM: the caput longum of triceps brachii muscle) belongs to forequarters, within 45 min after slaughter and immediately frozen in liquid nitrogen until further analyses.

Real-time polymerase chain reactions (RT-PCR) and analysis

The myogenic factors were determined by quantitative qRT-PCR. Muscle RNA was extracted using TRIzol reagent following the manufacturer protocols (TaKaRa, Dalian, China). Total RNA (1 μg) was reverse transcribed to synthesize cDNA using the Prime Script RT-reagent Kit for RT-PCR. RT-PCR amplification was carried out on a Bio-Rad iQ5 (Bio-Rad, USA) by SYBR Premix Ex TaqTM II (TaKaRa) chemistry detection under the followed amplification conditions (Each reaction contained 150 ng of template DNA, 12.5 mL of SYBRII green PCR master mix and 2 mM of each primer. The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The mRNA data was quantified using the comparative threshold cycle (40 CT) method. *β-actin* was used as a housekeeping gene with which the expression levels of all other genes were compared Subsequently, the measured expression levels and $2^{-\Delta\Delta Ct}$ were used to calculate the relative mRNA expression levels of the myogenic genes (Livak and Schmittgen, 2001). The primers used are presented in Table 1.

Statistical analyses

All data are reported as means ± SE. All statistical analyses were done with SPSS v. 21.0. Differences between experimental groups were evaluated by the Student *t*-test. The level of statistical significant difference was set at $P \leq 0.05$.

Table 1. Primers used for the RT-PCR of yak (*Bos taurus*).

Gene	Primer (5'-3')	Product size (bp)
Myf5	GGCATGCCTGAATGTAACAG	162
	TTGCTCTGAGTTGGTGATCC	
Pax7	GCTCAGACGTGGAGTCAGAA	352
	CCGTCTTGGGAGATGGTAGT	
MyoG	ACTACCTGCCTGTCCACCTC	229
	ACCTTCTTGAGTCTGCGCTT	
MSTN	CATCAAACCCATGAAAGACG	372
	GCAATAATCCAATCCCATCC	
β-actin	CCCTGGAGAAGAGCTACGAG	247
	TCTTTCTGCATCCTGTTTGC	

RESULTS

Sex-dependent changes of myogenic factors

The myogenic factor expression level in 6 month old yak indicated that the *Pax7* gene expression levels were SM > QF > TM, but with no significant difference between SM and QF ($P \geq 0.05$). *Pax7* expression was higher in males than in females ($P \leq 0.05$) (Figure 1A). The *Myf5* expression levels were the highest in male QF ($P \leq 0.05$) and were higher in female TM than in male TM ($P \geq 0.05$) (Figure 1B). The *MyoG* expression levels were higher in female QF and TM than in males ($P \leq 0.05$), but showed no sex differences in SM (Figure 1C). The highest *MSTN* expression levels were found in female SM and in male QF, but were not statistically significant among the sexes in TM ($P \geq 0.05$; Figure 1D).

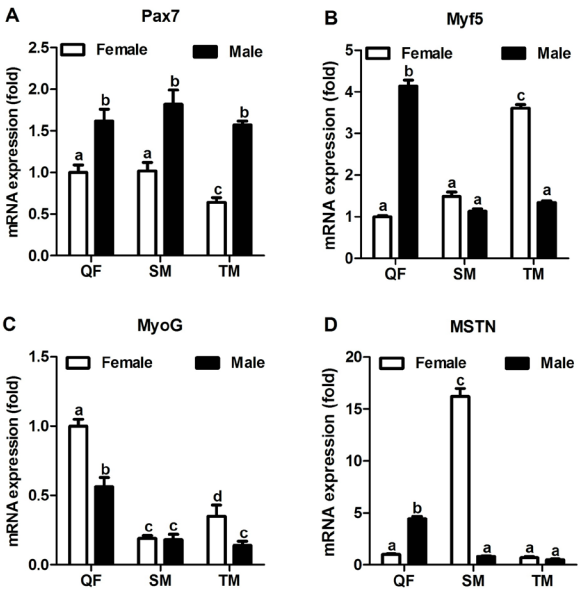

Figure 1. Relative mRNA expression of myogenic regulatory genes in muscle tissues of male and female yak. The mRNA expression levels were measured by qRT-PCR. **A.** *Pax7*; **B.** *Myf5*; **C.** *MyoG*; **D.** *MSTN*. The muscle tissues were QF: quadriceps femoris; SM: semimembranosus; and TM: Triceps muscle of arm. All muscle samples were collected at 6 months. *β-actin* was used as a housekeeping gene with which the expression levels of all other genes were compared. Data are reported as means ± SE. Letters a-d above the bars within each figure indicate significant differences at $P \leq 0.05$.

Age-dependent changes in myogenic factors

The myogenic factor expression levels in females at different ages are shown in Figure 2. Compared to in 3 year old muscle tissue, *Pax7* expression levels were higher in 6 month old QF and SM ($P \leq 0.05$), whereas the opposite was found in TM (Figure 2A). The expression levels of *Myf5* were the highest in QF at 3 years of age, and the lowest in SM at the same age (Figure 2B). The *MyoG* expression levels were higher at 6 months than at 3 years, across all muscle tissues ($P \leq 0.05$) (Figure 2C). *MSTN* expression levels were the highest in female TM of 6 months and the lowest in SM of the same age (Figure 2D).

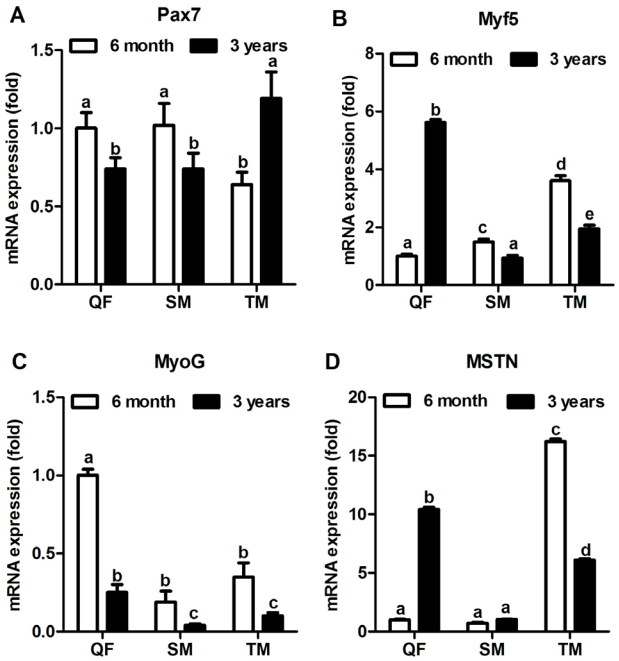

Figure 2. Relative mRNA expression of myogenic regulatory genes in muscle tissues collected from female yak of two different ages (six months and three years). The mRNA expression levels were measured by qRT-PCR. **A.** *Pax7*; **B.** *Myf5*; **C.** *MyoG*; **D.** *MSTN*. The muscle tissues were QF: quadriceps femoris; SM: semimembranosus; and TM: Triceps muscle of arm. *β-actin* was used as a housekeeping gene with which the expression levels of all other genes were compared. Data are reported as means ± SE. Letters a-d above the bars within each figure indicate significant differences at $P \leq 0.05$.

DISCUSSION

Many reports have indicated that satellite cells play an important role in muscle hypertrophy (Wigmore and Stickland, 1983; Mesires and Doumit, 2002; Ishido et al., 2004; Wang et al., 2015), which is involved in normal muscle growth and regeneration. Satellite cells are located in myofibers between the plasmalemma and the basal lamina, and can be activated by injury or other signals (Cooper et al., 1999; Ishido et al., 2004; Wilschut et al., 2008). Patruno et al. (2008) investigated the number of activated satellite cells in the porcine semitendinosus muscle after birth. They found that all myogenic cells had 10-15% $Pax7^{+}$ cells

at birth and noticed that *Pax7*$^{+}$/*MyoD*$^{+}$ cells increased at one month after birth (Patruno et al., 2008). Furthermore, Campion et al. (1981) observed that the absolute number of satellite cells of the peroneus longus and sartorius muscles in pigs increased over the first 32 weeks of postnatal growth, but that the number decreased again from 32 to 64 weeks. Ropka-Molik et al. (2011) showed that the *Pax7* mRNA level of young pietrain pigs (from 60 to 120 days) increased, and that a weak decline was observed at 210 days. The transcript levels of *Pax7* in the largest muscle (SM) were the lowest (Ropka-Molik et al., 2011). Our results showed that in the hindquarters (QF and SM), *Pax7* expression was higher at 6 months than at 3 years, meanwhile, in the forequarters (TM), *Pax7* expression was higher at 3 years than at 6 months; At 6 months, *Pax7* expression among all three muscle tissues was higher in males than in females. *MSTN* belongs to the transforming growth factor beta family, which negatively regulates skeletal muscle mass. Our results indicated that at 6 months, the *MSTN* expression was highest in the TM, whereas at 3 years it was the highest in QF. This suggests that the growth pattern of yak muscle differs depending on its position. The forequarters muscle mainly grew in younger muscles and the hindquarters mainly grew in older muscles, which was consistent with the *Pax7* results.

The *MyoG* gene is expressed after the *Myf5* and *MyoD* genes and is mainly engaged in the muscle differentiation process. *MyoG*$^{-/-}$ mice continue to specify the muscle lineage through the formation of myoblasts, but show perinatal lethality because of severe disruption of myoblast differentiation and muscle fiber formation, (Hasty et al., 1993; Moncaut et al., 2013). *MyoG* and *Myf5* genes regulate their own expression and could interact with other members of the MRF family. Genetic variation in *MyoG* and *Myf5* could affect muscle formation and ultimately lead to a change in meat production and quality (Singh and Dilworth, 2013). Previous studies have found that the generation of satellite cells is accompanied by low expression of *Myf5* in C2C12 cells, suggesting that *Myf5* plays a role in maintaining satellite cell populations (Bhuiyan et al., 2009; Hu et al., 2010; Biressi et al., 2013). Thus, *Myf5* down-regulation in skeletal muscle may reduce myoblast proliferation during proliferation phase I, maintain quiescent satellite cell pools, and reduce the number of satellite cells activated to proceed as myoblasts. Our results suggested that in the hindquarters of males and the forequarters of females, satellite cells have a strong proliferative ability, which was consistent with the observed *Pax7* and *MSTN* expression. In all muscle tissues, the *MyoG* expression was higher in females and at 6 months of age. This indicates that the satellite cells in all female muscles have powerful differentiation ability.

In summary, our results indicate that identifying the main myogenic gene mRNA expression levels in different muscles, different sexes, and at different ages may aid us in our understanding of the postnatal myogenesis process. However, the relationships between the main myogenic regulatory factors and the postnatal myogenesis processes need further studies to be completely elucidated.

Conflicts of interest

The authors declare no conflict of interest.

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