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ORIGINAL ARTICLE

Relationships among muscle fiber type composition, fiber diameter and *MRF* gene expression in different skeletal muscles of naturally grazing Wuzhumuqin sheep during postnatal development

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ABSTRACT

The aim of this study was to determine the relationships among muscle fiber-type composition, fiber diameter, and myogenic regulatory factor (*MRF*) gene expression in different skeletal muscles during development in naturally grazing Wuzhumuqin sheep. Three major muscles (i.e. the Longissimus dorsi (LD), Biceps femoris (BF) and Triceps brachii (TB)) were obtained from 20 Wuzhumuqin sheep and 20 castrated rams at each of the following ages: 1, 3, 6, 9, 12 and 18 months. Muscle fiber-type composition and fiber diameter were measured using histochemistry and morphological analysis, and *MRF* gene expression levels were determined using real-time PCR. In the LD muscle, changes in the proportion of each of different types of fiber (I, IIA and IIB) were relatively small. In the BF muscle, a higher proportion of type I and a 6.19-fold lower proportion of type IIA fibers were observed ($P < 0.05$). In addition, the compositions of type I and IIA fibers continuously changed in the TB muscle ($P < 0.05$). Moreover, muscle diameter gradually increased throughout development ($P < 0.05$). Almost no significant difference was found in *MRF* gene expression patterns, which appeared to be relatively stable. These results suggest that changes in fiber-type composition and increases in fiber size may be mutually interacting processes during muscle development.

Key words: *gene expression, MRFs, muscle fiber-type composition, postnatal development, Wuzhumuqin sheep.*

INTRODUCTION

It is widely accepted that muscle development consists of both hyperplasia and hypertrophy. Feeding behavior (Vestergaard *et al.* 2000; Redgate *et al.* 2014), exercise intensity (Bond *et al.* 2004) and activity patterns (Reimers *et al.* 2014) may influence the regulation of muscle development or meat quality.

In general, skeletal muscles are classified as three or four major fiber types (i.e. I, IIA, IIB and IIX) according to differences in contractility, structure and metabolic properties. Myosin adenosine triphosphatase (mATPase) histochemistry was used for fiber typing. This technique reports differences in the sensitivity of mATPase activity in tissues pre-incubated at specific pH levels (Brooke & Kaiser 1970; Lind & Kernell 1991). The three major fiber types (I, IIA and IIB) co-exist at birth (Choi & Kim 2009), and their proportions (Wojtysiak & Połtowicz 2014) in different muscles can influence the metabolic properties of muscles (Choe & Kim 2014), muscle fiber characteristics (Kim *et al.* 2014), meat quality traits (Lim *et al.* 2015) and muscle tenderness (Sazili *et al.* 2005). Moreover, the relative frequency of the IIB fiber type is strongly correlated with postmortem

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glycolysis, postmortem muscle pH, meat lightness, drip loss, juiciness and flavor (Lee *et al.* 2012; Kim *et al.* 2013). The number of muscle fibers is thought to be determined by the embryonic stage, after which sequential fiber-type conversion occurs during muscle development (Ashmore *et al.* 1972; Pette & Staron 1997; Ciciliot *et al.* 2013). However, the precise mechanism underlying fiber-type transformation remained unclear until now. In addition, voluntary wheel exercises (Talmadge *et al.* 2014; Soffe *et al.* 2015), resistance-type exercise training (Aguiar *et al.* 2013; Kim *et al.* 2015), other physical exercises (Zampieri *et al.* 2015) and repeated high-intensity exercises (Kassar-Duchossoy *et al.* 2004; Gejl *et al.* 2015) have been shown to exert special effects on fiber-type composition in both men and rats of different ages.

Growth rates and muscularity are economically important traits in sheep breeding. Muscle growth is regulated by several factors, including non-coding RNAs (Horak *et al.* 2016), myogenic regulatory factors (*MRFs*) (Zhong *et al.* 2013), satellite cells (Bunprajun *et al.* 2012) and *myostatin* (*MSTN*) (Shibata *et al.* 2006) and so on. *MRFs* are a vital class of basic helix-loop-helix (bHLH) transcription factors that consist of four specific genes, including *MyoD*, *Myf5*, *MRF4* and *myogenin*. In the *MRF* family, *MyoD* and *Myf5* participate in early myogenesis (Rudnicki *et al.* 1993; Wood *et al.* 2013), while *MRF4* and *myogenin* may be highly associated with the later stages of differentiation (Watabe 1999; Averous *et al.* 2012). However, recent studies have shown that *MyoD* controls myogenesis, while *Myf5* and *myogenin* mRNA expression do not, during the terminal differentiation phase of bovine myoblasts (Muroya *et al.* 2005). Our review of previous studies of *MRFs* revealed that they have focused primarily on gene expression (Ropka-Molik *et al.* 2011), cloning (Zhang *et al.* 2014) and functional experiments (Kablar *et al.* 2003) in rats and humans, while few have explored the mRNA expression patterns of muscle-specific genes during external stimulation (Aguiar *et al.* 2013; Kamikawa *et al.* 2013) in the postnatal development of ruminant animals. The transcript profiles of *MyoD* and *Myf5* have been shown to be related to myosin heavy chain isoform types in bovine adult muscles (Muroya *et al.* 2002). Additionally, single nucleotide polymorphisms (SNPs) in the *MRF4* (Wang *et al.* 2011), *Myf5* (Li *et al.* 2004) and *MyoG* (Sun *et al.* 2014) genes were found to be significantly and positively correlated with growth/muscle traits in cattle and sheep.

Wuzhumuqin sheep (Fig. 1), a type of Mongolian sheep that possesses 14 pairs of ribs, were used in the study. In general, Wuzhumuqin sheep are accustomed to voluntary movements and a high amount of free-feeding (also called natural grazing) for their entire lifespan. Traditional feeding methods remain in common use for raising livestock in a large number of places, including Inner Mongolia, Qinghai, Xinjiang and Tibet. Most Chinese consumers prefer Wuzhumuqin sheep that are reared under naturally grazing conditions to other commercial breeds because they are labeled as an 'organic meat product'. Their slaughter rate out of the total number of slaughtered sheep in Inner Mongolia is 50.6% (the original data regarding Wuzhumuqin sheep were provided by Agricultural Technology Demonstration Bases in East Wuzhumuqin, Inner Mongolia, China). To our knowledge, few domestic reports have been published regarding the characteristics of muscle fibers in commercial animals except for reports

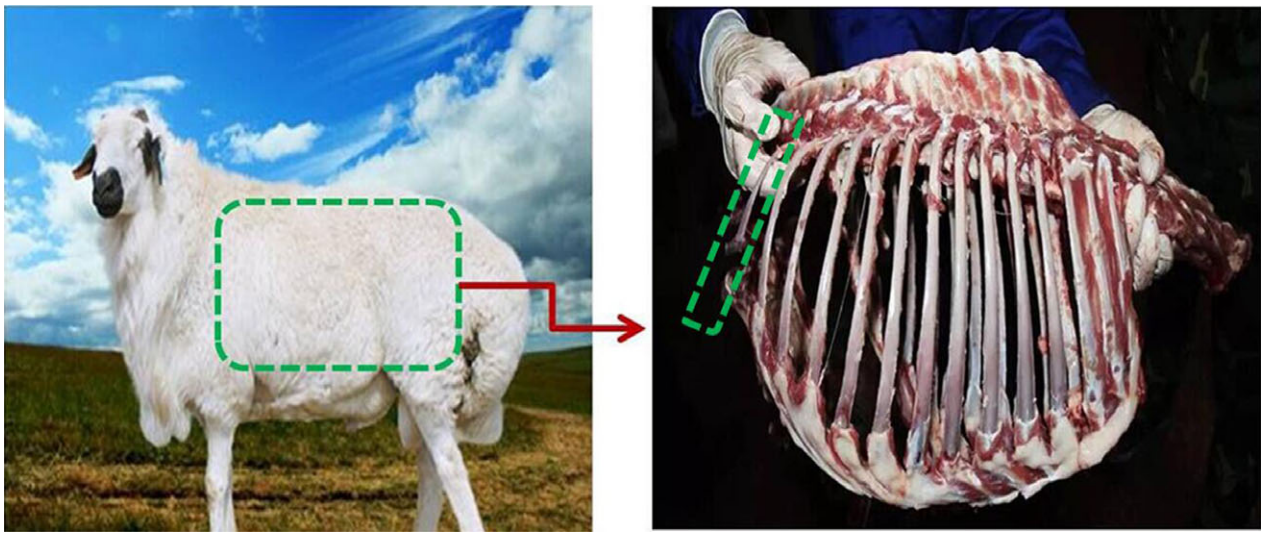

Figure 1 Anatomy of Wuzhumuqin sheep ribs.

describing Wuzhumuqin sheep of different ages grown under natural feeding conditions. Therefore, the objective of this study was to investigate the relationships among muscle fiber-type composition, muscle diameter and *MRF* gene expression (i.e. the three major *MRFs*: *MRF4*, *Myf5* and *myogenin*) in skeletal muscles obtained from naturally grazing Wuzhumuqin sheep during postnatal development.

MATERIALS AND METHODS

Animals and muscle samples

The selected study animals were Wuzhumuqin sheep that were born at the Agricultural Technology Demonstration Bases (The Original Breeding Farm of Wuzhumuqin Sheep) in East Wuzhumuqin, Xilingol, Inner Mongolia, China. The Original Breeding Farm (E115°10′-120°07′, N44°40′-46°) where the Wuzhumuqin sheep used in this study were raised is at a relatively high altitude (range, 800-1500 m) and consists of a broad range of natural grassland (37 million hm^2 available). The extreme maximum summer temperature (39.7°C) and extreme minimum winter temperature (−40.7°C) were recorded, and 1027 species of wild vascular plants were collected in 2011 in this region. These wild vascular plants represented 455 different feeding values. The Wuzhumuqin sheep were reared under natural feeding conditions during the day for their whole lifespan. Approximately 1000–1500 sheep form the group that is relatively extensively managed at this location.

All procedures performed in this study were conducted using a protocol approved by The Institutional Animal Care and Use Committee at the College of Animal Science and Technology, Inner Mongolia Agricultural University, China. Three major muscles (i.e. the Longissimus dorsi (LD), Biceps femoris (BF) and Triceps brachii (TB)) were collected from Wuzhumuqin sheep (n = 120, male) aged at 1, 3, 6, 9, 12 and 18 months (20 sheep at each stage). Muscle samples were collected from the following tissues: for LD, adjacent to the 13th thoracic vertebra; and for BF and TB, from the central portions of the BF and TB muscles. Ten grams of each muscle were collected immediately after slaughter, the visible fat and connective tissues were removed, and the samples were snap-frozen in liquid nitrogen and then stored at −80°C until further analysis.

Histochemical analysis

Transverse serial sections (10 μm thickness) were cut from whole blocks (1.0 × 1.0 × 1.5 cm) using a cryostat (HM525 NX UV; Microm GmbH, Waldorf, Germany) at −20°C and subsequently incubated to histochemically detect mATPase using a conventional method (Schiaffino *et al.* 1989) with some modifications. Unfixed muscle sections were pre-incubated at room temperature for 5 min in a buffer consisting of 200 mmol/L sodium acetate that was adjusted to pH 4.3 using acetic acid. The sections were then washed for 15 min in a buffer containing 180 mmol/L potassium chloride, 100 mmol/L pentobarbital sodium and distilled water at a ratio of 1 : 1 : 3 (pH 10.4). Subsequently, the sections were incubated at 37°C for 45 min in adenosine triphosphatase (ATPase) pre-incubation buffer (pH 9.4) containing 180 mmol/L $CaCl_2$, 100 mmol/L pentobarbital sodium and distilled water at a ratio of 2 : 1 : 7 and an additional 25 mg of ATP disodium salt. Next, the muscle sections were washed three times within 10 min in a 1% $CaCl_2$ buffer. The sections were placed in 2% $CoCl_2$ for 3 min and then washed three times for 30 s per wash in flowing distilled water to remove the overflowing buffer. Finally, the muscle sections were immersed in a 1% solution of yellow ammonium sulfide for 30 s, washed in distilled water for 10 min, and then dehydrated through a series of alcohol gradient solutions (70%, 80%, 90% and then 95% anhydrous alcohol for 2 min at each gradient). The stained sections were examined using an image analysis system (Image-Pro® plus 5.1; Media Cybernetics Inc., Rockville, MD, USA). The muscle fibers were divided into fiber types I, IIA and IIB according to current definitions (Brooke & Kaiser 1970; Schiaffino *et al.* 1989). Approximately 600 fibers per sample were counted to estimate the distributions of muscle fibers. The percentage of each fiber type refers to the ratio of the number of fibers counted for each fiber type to the total number of counted fibers.

Morphometric analysis

The diameters of the myofibers in the LD, BF and TB muscles were measured using traditional hematoxylin and eosin (HE) staining. Briefly, the muscle samples were excised perpendicular to the direction of the myofibers, and serial tissue sections (10 μm thickness) were cut using a cryostat (Microm, HM525) at −20°C. The muscle sections were air-dried at room temperature for 18 min. Then, the cells were fixed for 5 min in 4% paraformaldehyde adjusted to pH 7.4 using phosphoric acid. Subsequently, the sections were washed in distilled water, and the nuclei were counterstained using Harry's hematoxylin for 15 min. Afterwards, the staining solution was washed away using distilled water, and the color reaction was performed by incubating the sections in 1% acid alcohol for 30 s and then immediately washing the sections with distilled water. Next, the muscle sections were

stained with 0.5% eosin for 3 min and then dehydrated in an alcohol gradient (70%, 80%, 90% and then 95% anhydrous alcohol for 2 min at each gradient). Finally, the muscle sections were mounted, dehydrated in xylene for 2 min and covered with a cover slip. In the stained sections, photographic documentation was obtained of three random histological fields (100× lens) in each sample using a microscope connected to a computer. Muscle hypertrophy was determined by measuring muscle fiber diameter (Fiber Dia.) in approximately 500 muscle fibers in each sample using an image analysis system (Image-Pro® plus 5.1; Media Cybernetics Inc.).

Primer design and quantitative real-time PCR

Based on the regions in *MRF* sequences that were identified as conserved in the National Center for Biotechnology Information (NCBI), we designed three pairs of primers to amplify the *Myf5* (NM_001287037), *MRF4* (NM_001134782) and *myogenin* (NM_001174109) genes in sheep (Table 1). First, total RNA was extracted using the RNAiso Plus reagent (Takara, Tokyo, Japan) according to the manufacturer's protocol, and RNA integrity was verified using a 0.8% agarose gel. RNA quality and quantity were assessed using optical density $(OD)_{260/280\ nm}$ and $OD_{230/260\ nm}$ ratios in a Nano Drop ND-1000 UV Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). After they were extracted, the RNA samples were used to synthesize first-strand complementary DNA (cDNA) using an Ex Script™ RT reagent Kit (Takara), in which genomic DNA was removed using a standard procedure at 42°C for 2 min before the transcription reaction was performed. Finally, quantitative real-time PCR was performed using a Cycler I Q Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the SYBR green method. *β-actin* (NM_001009784) was used as a housekeeping gene control. All PCRs were conducted in a total volume of 25 μL that consisted of 2 μL of first-strand cDNA (500 ng/μL), 12.5 μL of SYBR® Premix Ex Taq TM II (Takara), 1 μL of each primer (10 μmol/L), and 8.5 μL of ddH_2O (Takara). The amplification program included an initial denaturation step at 95°C for 30 s, 30 cycles of denaturation at 95°C for 5 s and annealing at 55.7–63°C (Table 1) for 30 s, and a final extension at 72°C for 1 min. A melting program was performed in a range of 55–95°C at a heating rate of 0.5°C/10 s to generate melting curves. The reactions were performed at least in triplicate, and a negative control was also run in parallel. The $2^{-\Delta\Delta Ct}$ method was used to analyze the real-time PCR data (Livak & Schmittgen 2001).

Statistical analysis

The fiber-type proportion, fiber diameter, and real-time PCR data are presented as the mean ± SD, and each sample was measured at least in triplicate. The experimental data were analyzed using the variance analysis procedure in Statistical Analysis Systems (SAS Institute, Cary, NC, USA). Duncan's multiple-range tests were used to identify significant differences among the means at a 5% level of significance (SAS Institute). Pearson's correlation coefficients were evaluated to determine the relationships between fiber-type composition, fiber diameter, and the expression levels of myogenic regulatory genes (SAS Institute). Differences were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Muscle fiber-type composition

The muscle fibers in all of the muscle samples obtained from the Wuzhumuqin sheep (1–18 months of age) were categorized as one of the following types: I, IIA or IIB (Fig. 2). A clear difference in fiber-type composition was observed during growth among the LD, BF and TB muscles (Fig. 3). Throughout the experimental period, the proportion of and changes in the proportion of fiber-type I muscles were relatively small in the LD muscle, excluding the value observed at 9 months (Fig. 3A). In the

Table 1 Primer sequences and PCR conditions for real-time PCR

Gene	Sequence 5′ to 3′	Product (bp)	Annealing temperature (°C)
Myf5	F: CACGACCAACCCTAACCAGA R: TGGTGATCCGATCCACTATGCT	263	58.6
MRF4	F: ATGCAGGAGTTAGGGGTGGAC R: TGTTCCTCCGAGGAAATGCTGT	224	57.2
Myogenin	F: CACTCTGAGGGAGAAGCGCAG R: TGTGGACTGCAGGAGGCACTAT	258	55.7
β-actin	F: AGAGCAAGAGAGGCATCC R: TCGTTGTAGAAGGTGTGGT	103	57

β-actin was used as a housekeeping gene control for real-time PCR analysis.

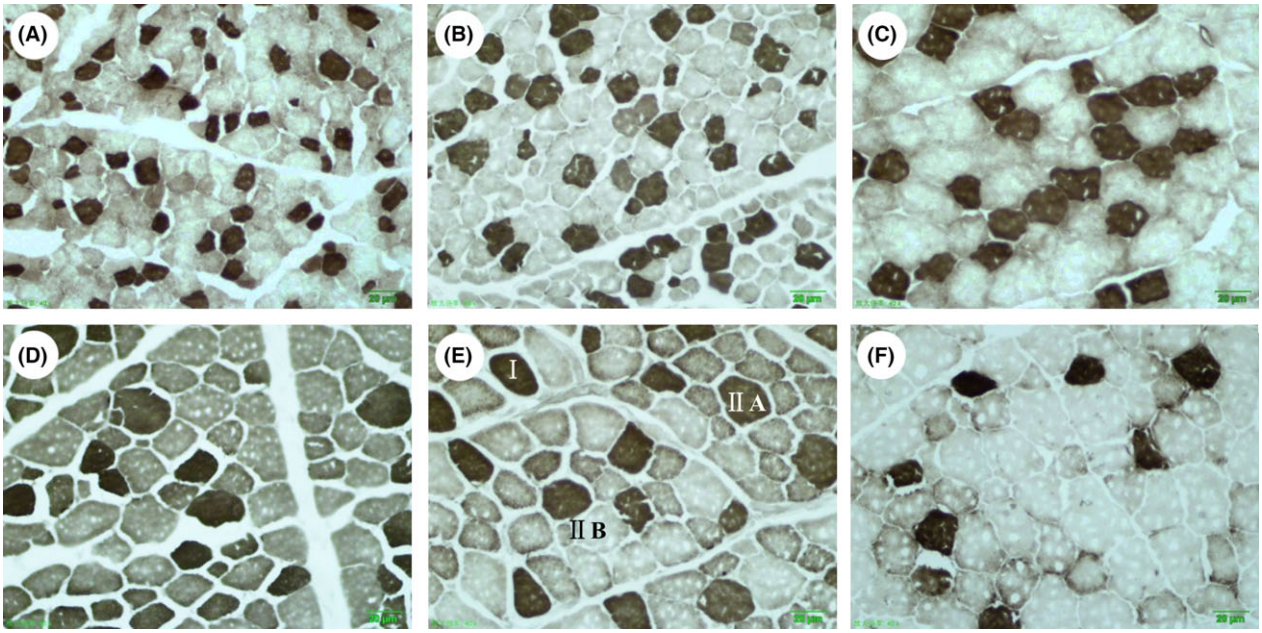

Figure 2 Serial muscle sections were made from tissues obtained from Wuzhumuqin sheep during aging. Abbreviations: A, 1 month; B, 3 months; C, 6 months; D, 9 months; E, 12 months; and F, 18 months. I, IIA and IIB represent muscle types. The serial muscle sections were stained for myosinadenosine triphosphatase (ATPase) reactivity after they were pre-incubated at pH 4.30. Magnification, 40× (bar = 20 μm).

same muscle, the frequency of type IIA fibers declined in the early stages (from 1 to 6 months), and this change was accompanied by small changes in the ratio of type IIB fibers ($P < 0.05$), which changed only weakly throughout the experimental period (Fig. 3A). In other words, the proportions of each of the three types of muscle fibers appeared to be relatively stable during growth in the LD muscle. Based on the accumulated evidence, changes in the proportions of each fiber type may be induced by fiber transformation during postnatal development (Yan *et al.* 2011; Wilson *et al.* 2012; Hopker *et al.* 2013; Mosole *et al.* 2014). A previous study found that a shift from type IIA fibers to type IIB fibers occurred during the early months after birth, but the proportion of type I fibers was unaffected by aging (Wegner *et al.* 2000). This finding was partially confirmed by our results in the LD muscle during the early experimental period. The proportion of type I fibers in the LD muscle was different during later stages (from 9 to 12 months). Normally, fiber type conversion occurs in a graded and sequential manner (Ashmore *et al.* 1972). Because they are allowed autonomous activity and completely voluntary feeding, the dietary structure of Wuzhumuqin sheep might be affected by a large number of potential factors (including feeding behavior, aging processes, pasture changes, seasonal alterations and climate impact). Throughout the study, the proportion of type IIA fibers rapidly declined at certain points, and this might have been because of the mixed effects of the previously mentioned potentially impacting factors or perhaps because of normally occurring transformations in fiber types (e.g. from type IIA to I and IIB) in autonomously feeding sheep during postnatal development. According to the results of previous studies, a transformation from type II to type I fibers occurs during longer-duration, higher-volume, endurance-type events (Wilson *et al.* 2012).

Similarly, the fiber population of the BF muscle might be continuously adjusted by fiber-type variations during postnatal development (Fig. 3B). A decrease in the percentage of type IIA units was observed until the animals were 6 months old ($P < 0.05$), and the changes in the proportion of type I and type IIB fibers were significantly different in the BF muscle (Fig. 3B). During late aging, an increase was observed in the frequency of type I fibers ($P < 0.05$), but no further enhancement was observed at 18 months. A decrease in the number of type IIB fibers was observed until the animals were 12 months old. This was followed by a rapid increase in this type of fiber in the BF muscle at 18 months ($P < 0.05$) (Fig. 3B). Furthermore, the results of a previous study showed that the percentage of type I muscle fibers was higher in mice in a trained group but that there were no differences between age groups (Hopker *et al.* 2013). However, other researchers have suggested that muscle fiber transformations depend more strongly on activity

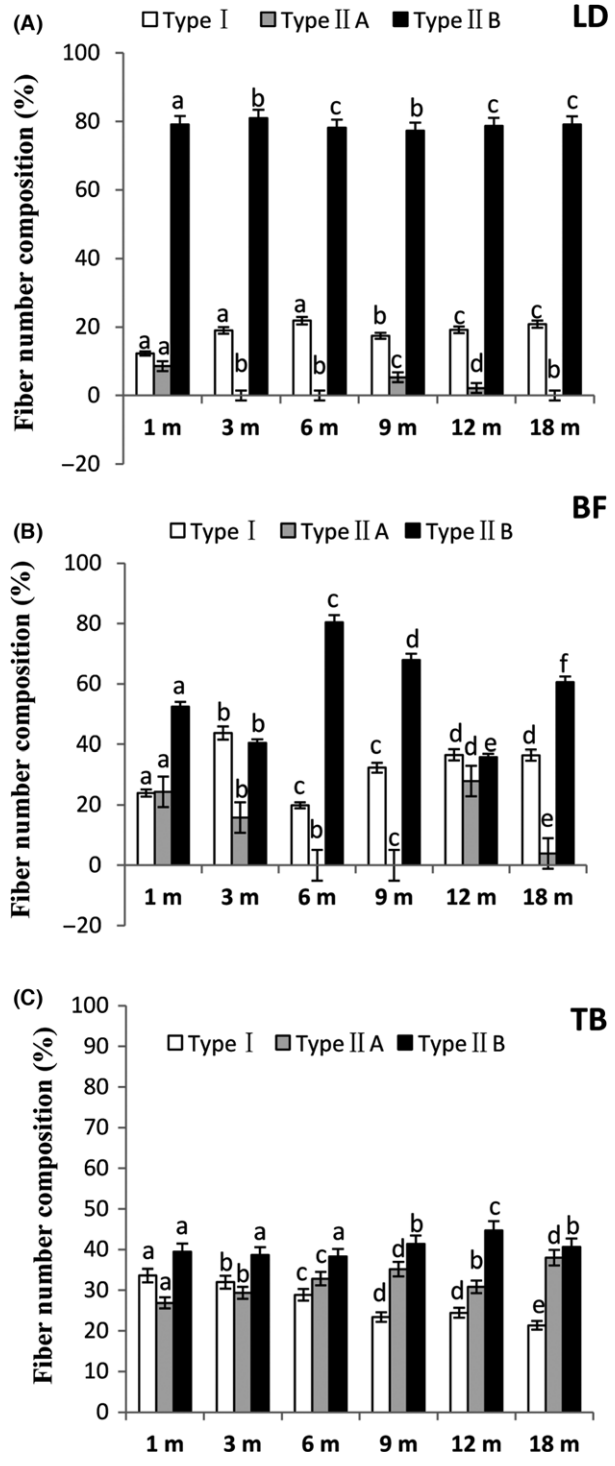

Figure 3 Changes in skeletal muscle fiber type composition during postnatal development in Wuzhumuqin sheep. The bars represent the mean ± SD. Different letters on the bars indicate significant differences between the age groups ($P < 0.05$). Abbreviations: LD, Longissimus dorsi; BF, Biceps femoris and TB, Triceps brachii.

than on the pattern of stimulation (Sreter *et al.* 1982). During postnatal life, a higher proportion of type I fibers (except for the values found at 1 month and 6 months) was observed in BF muscles than in the other muscles examined in the current study. However, it is difficult to determine the reason for this difference, which likely results from the mixed effects of life-long voluntary movement (Hopker *et al.* 2013) and aging processes in these animals. Specifically, there was 6.19-fold lower type IIA ratio in the BF muscle during growth, suggesting that a higher proportion of type IIA fibers may be required for fiber-type transformations over long-term periods in naturally grazing sheep.

Interestingly, there was a continuous decrease in the proportion of type I fibers in the TB muscle during the first few stages ($P < 0.05$) and a significant increase in the percentage of type IIA fibers during the same time ($P < 0.05$) (Fig. 3C). Then, the proportion of type IIA fibers slowly decreased up to 12 months ($P < 0.05$), and this change was accompanied by a nearly complementary change in the number of type IIB fibers ($P < 0.05$). The direction of fiber conversion (i.e. a shift from type I to II or type II to I fibers) has been shown to be affected by differences in exercise patterns during aging (Wilson *et al.* 2012). In the current study, we observed that a shift from type I to IIA fibers in the TB muscle occurred during the first few months, and we suggest that this might have resulted in a shift from type IIA to I fibers in the BF muscle. However, these findings were not consistent in corresponding later ages, and further research is therefore required to determine the reason underlying these changes and associations.

Muscle fiber diameter

Representative images of hematoxylin and eosin (HE)-stained sections were used to examine changes in morphology, as shown in Figure 4, and the corresponding fiber diameter data are presented in Table 2. There was a progressive increase in fiber diameter until the animals were 9 months old, when significant differences were observed between the age groups in the LD and BF muscles ($P < 0.05$). In both muscles, the highest values were recorded at 9 months, and no further increases were observed in later stages (Table 2). In addition, a continuous increase in fiber diameter was observed in the TB muscle during development, and there was a significant difference between the age groups ($P < 0.05$, Table 2). Our results suggest that the proportion of muscle fibers became stabilized in one or both fiber types (I and/or IIB fiber-type), and that this process was accompanied by an increase in stabilization in fiber diameters during muscle development. In other words, the successive increases in fiber diameter are potentially associated with the continuous changes that were observed in fiber-type composition during the aging process.

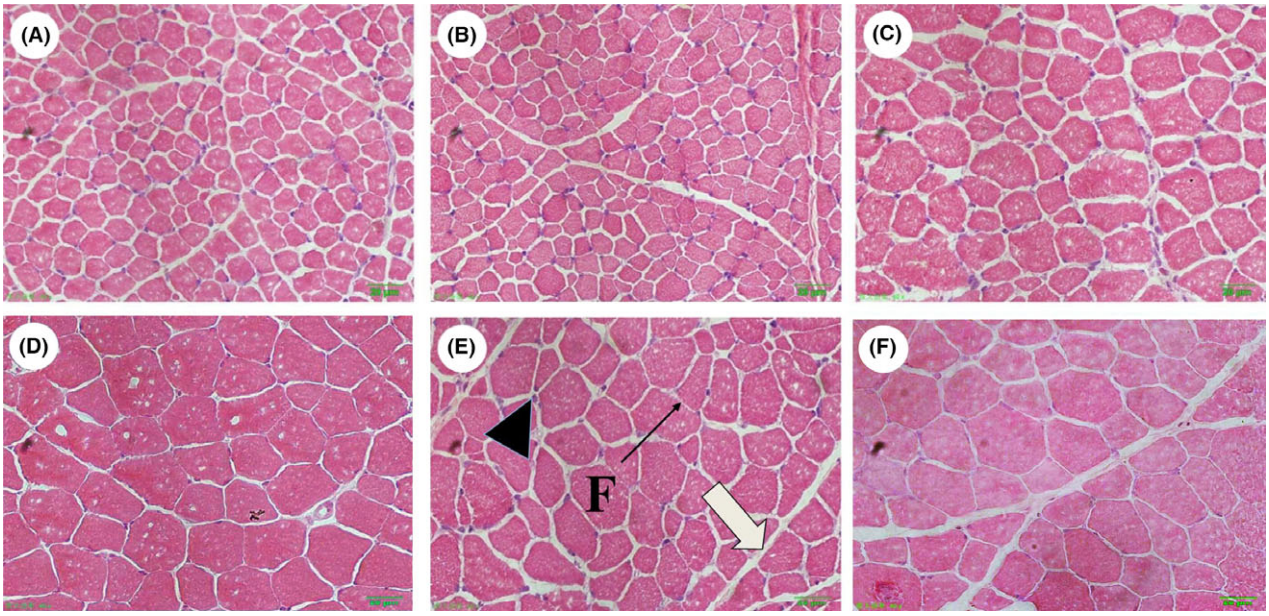

Figure 4 Muscle cross-sections of tissues obtained from Wuzhumuqin sheep were stained with hematoxylin and eosin (HE). Abbreviations: A, 1 month; B, 3 months; C, 6 months; D, 9 months; E, 12 months; and F, 18 months. Muscle fibers (F); endomysium (black arrow); perimysium (white arrow); and myonucleus (arrowhead). Magnification, 40× (bar = 20 μm).

Table 2 Changes in muscle fiber diameter in different skeletal muscles of Wuzhumuqin sheep during postnatal development

Fiber Diameter (μm)	LD	BF	TB
1 month	$9.1411^{A} \pm 0.1293$	$15.7769^{A} \pm 3.7395$	$17.0218^{A} \pm 0.2076$
3 months	$14.7489^{B} \pm 0.3799$	$17.9721^{B} \pm 3.2115$	$24.6895^{B} \pm 0.5989$
6 months	$25.8697^{C} \pm 0.6088$	$26.5905^{C} \pm 8.8395$	$31.4059^{C} \pm 1.4166$
9 months	$28.9007^{D} \pm 1.4242$	$28.2256^{D} \pm 5.8509$	$32.2096^{C} \pm 1.0709$
12 months	$27.8258^{E} \pm 0.8467$	$27.6503^{E} \pm 3.9714$	$34.1798^{D} \pm 1.3776$
18 months	$27.8641^{E} \pm 0.2997$	$27.4456^{E} \pm 4.3213$	$41.5717^{E} \pm 0.7721$

The data represent the mean ± SD. Different letters on the mean value denote significant differences between the age groups in each muscle ($P < 0.05$). BF, biceps femoris; LD, longissimus dorsi; TB, triceps brachii.

Profiles of *MRF4*, *Myf5* and *myogenin* gene expression

The gene expression profiles of *MRF4*, *Myf5* and *myogenin* in the three major muscles that were obtained from Wuzhumuqin sheep are shown in Figure 5. The results revealed that each *MRF* member exhibited a unique and stable gene expression pattern and that there were no significant differences between age groups during development ($P > 0.05$). Data from previous studies have indicated that *MRFs* have different gene expression patterns and perform specific functions during embryonic stages and postnatal development (Zhong *et al.* 2013). *MRF* gene expression patterns were distinct in different muscles, potentially because of differences in fiber types (Muroya *et al.* 2002) and exercise activity (Kim *et al.* 2015) in the animals. Some authors have suggested that *MRF* gene expression levels are increased by short-term passive repetitive stretching (Kamikawa *et al.* 2013). However, other researchers have reported that *MRFs* regulate muscle mass but not fiber-type conversion during long-term resistance training (Aguiar *et al.* 2013). In addition, in red muscle, *myogenin* transcript levels were significantly higher than the levels of *MyoD*, implying that each *MRF* mRNA is expressed at a different level in each fiber type (Alves-Costa *et al.* 2014). Our experimental results indicate that *MRF* expression patterns are relatively stable and that they are not significantly influenced by long-term free movement or aging processes in these animals. However, the observed expression levels may be correlated with changes in fiber type proportions and muscle hypertrophy in naturally grazing sheep.

According to the results of earlier studies, the *MyoD* and *Myf5* genes primarily control muscle growth in early stages (e.g. in proliferating satellite cells), whereas *MRF4* and *myogenin* primarily regulate muscle mass in later stages (e.g. myoblast

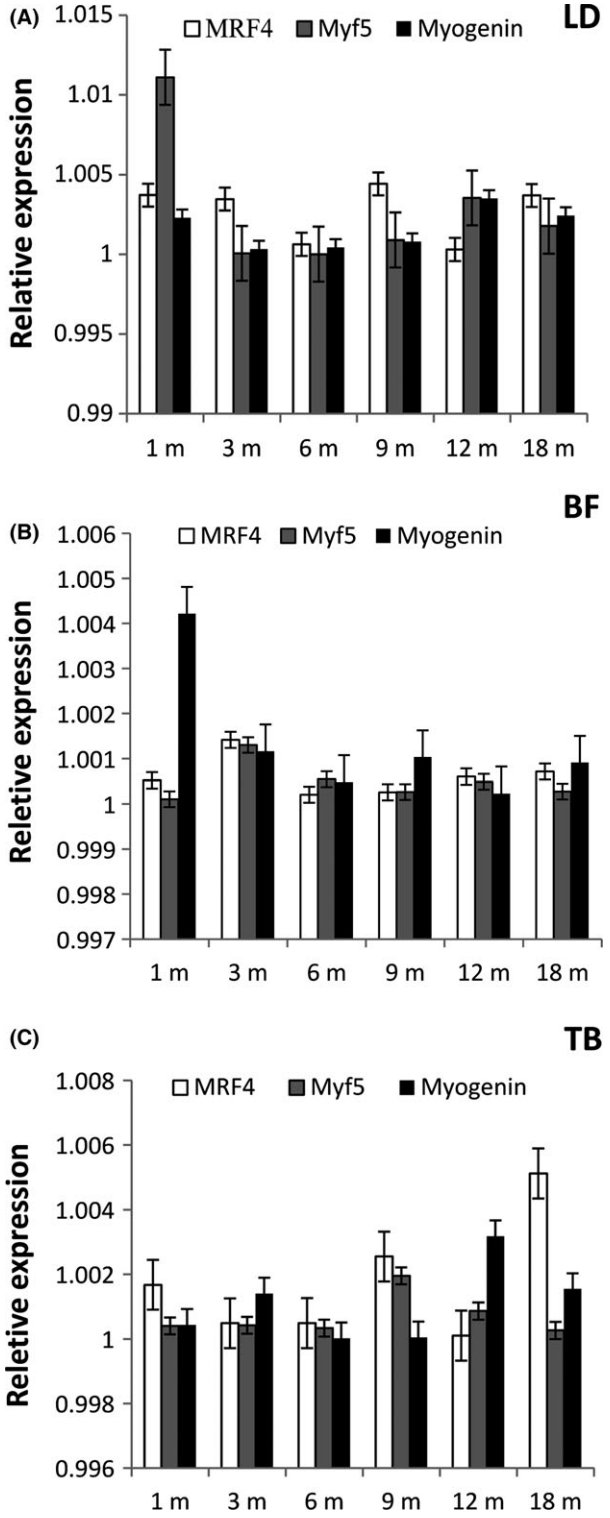

Figure 5 Myogenic regulatory factor (*MRF*) messenger RNA (mRNA) expression profiles in skeletal muscles obtained from Wuzhumuqin sheep during postnatal development. The bars represent the mean ± SD. No significant differences were observed between the age groups ($P > 0.05$). Abbreviations: LD, Longissimus dorsi; BF, Biceps femoris and TB, Triceps brachii.

differentiation and fusion during myogenesis). Moreover, *MRFs* are closely associated with myogenesis (i.e. with self-adjustment and mutual activation) (Le Grand & Rudnicki 2007; Schnapp *et al.* 2009). However, the specific mechanisms that are involved in *MRF* activities during muscle development remain unclear.

Correlation coefficients between muscle fiber-type composition, *MRF* gene expression and muscle fiber diameter

The correlation coefficients between muscle fiber-type composition, *MRF* gene expression, and muscle fiber diameter in the three major muscles are shown in Table 3. Skeletal muscles consist of different types of fibers. Thus, in our data, we evaluated the relative ratios of fiber types, and the following results had higher r values ($r \geq 0.61$). In LD muscles, the expression of the *Myf5* gene was negatively correlated with the fiber type I content and the ratio of type I to type IIB fibers ($P < 0.05$) and positively correlated with the percentage of type IIA fibers and the ratio of type IIA to type IIB fibers as well as the ratio of type IIA to type I fibers ($P < 0.05$). Similarly, in the BF muscle, *myogenin* gene expression was associated with changes in the percentages of fiber types in the BF muscle, specifically with the ratio of type I to type IIB fibers ($P < 0.05$). Moreover, in the TB muscle, a series of significant correlations were observed between fiber diameter and type I fibers ($r = -0.93$) ($P < 0.01$) and between fiber diameter and the ratio of type IIA to type I fibers ($r = 0.92$) ($P < 0.01$). Other significant correlations were also found in the TB muscle between fiber diameter and fiber type IIA content and between fiber diameter and the ratio of type I to type IIB fibers ($P < 0.05$). Additionally, in the TB muscle, *myogenin* gene expression was correlated with the percentage of type I fibers and fiber diameter.

Although a considerable number of reports have addressed how *MRFs* and muscle fiber conversion are affected by different types of exercises or stimuli, the specific molecular mechanisms that govern muscle development remain unclear. To our knowledge, the current study is the first to show that an increase in fiber diameter (resulting from aging processes or long-term voluntary movement) is associated with the *MRF* gene expression patterns and changes in fiber types in different skeletal muscles in naturally grazing Wuzhumuqin sheep during postnatal development (Fig. 6). Some authors have previously reported that fiber type-specific differences are associated with *MRFs*. Other researchers have suggested that there is no significant change in *MRF* transcript levels during changes in fiber types in animals exposed to low-frequency electrical stimulation

Table 3 Correlation coefficients (*r*) between the muscle fiber-type composition, *MRF* gene expression and fiber diameter in Wuzhumuqin sheep during growth

Measurements	Gene expression (LD)				Gene expression (BF)				Gene expression (TB)			
	MRF4	*Myf5*	*MyoG*	Fiber Dia.	*MRF4*	*Myf5*	*MyoG*	Fiber Dia.	*MRF4*	*Myf5*	*MyoG*	Fiber dia.
Type I	−0.47	−0.87*	−0.19	0.70	0.11	0.09	0.62	−0.01	0.31	0.34	0.63	−0.93**
Type IIA	0.40	0.81*	0.18	−0.45	−0.46	−0.23	0.64	−0.51	−0.29	−0.25	−0.59	0.90*
Type IIB	0.13	0.05	0.01	−0.63	0.30	0.11	−0.80	0.38	−0.15	−0.29	−0.33	0.41
Type I/IIB	−0.49	−0.86*	−0.25	0.68	−0.30	−0.06	0.81*	−0.18	0.29	0.37	0.59	−0.88*
Type IIA/IIB	0.39	0.81*	0.24	−0.43	−0.53	−0.15	−0.25	−0.34	−0.21	−0.11	−0.46	0.72
Type IIA/I	0.39	0.89*	0.23	−0.58	−0.34	−0.25	0.41	−0.61	−0.27	−0.35	−0.55	0.92**
Fiber diameter	−0.31	−0.64	0.06	1	−0.52	−0.28	−0.79	1	−0.18	−0.12	−0.78	1

*$P < 0.05$, **$P < 0.01$. BF, biceps femoris; Fiber Dia., fiber diameter; LD, longissimus dorsi; *MRF*, myogenic regulatory factor; TB, triceps brachii.

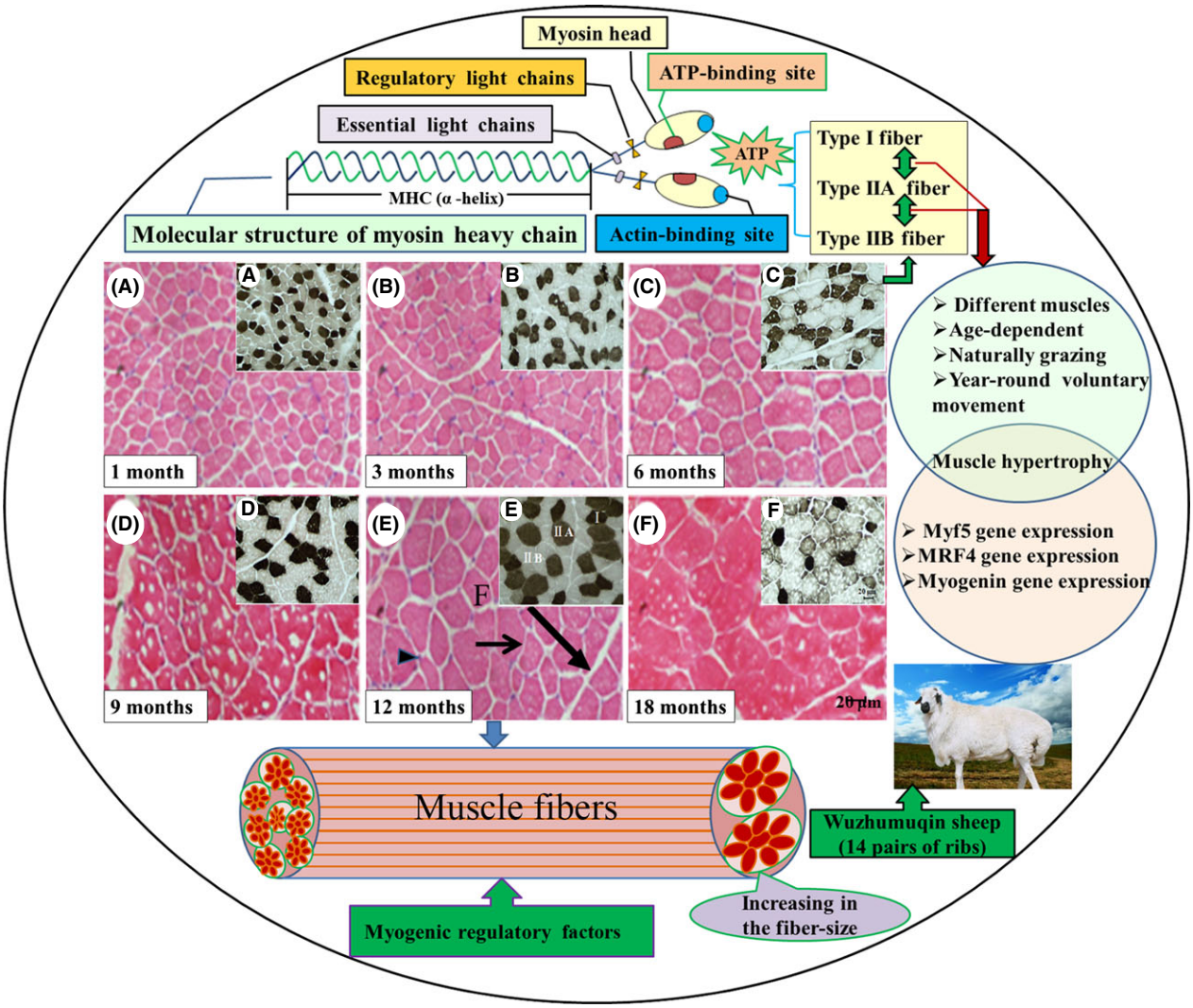

Figure 6 Graphic abstract – graph demonstrating muscle fiber diameter, fiber type composition, and myogenic regulatory factor (*MRF*) gene expression in Wuzhumuqin sheep of different ages that were grown under natural feeding conditions.

(Kraus & Pette 1997). However, our data indicate that changes in fiber type composition and *MRF* expression levels might have mixed effects on muscle hypertrophy in naturally grazing Wuzhumuqin sheep during postnatal development. Further investigations are therefore required to determine the

precise mechanisms involved in muscle development in animals grown under natural feeding conditions.

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