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Sheep *YAP1* temporal and spatial expression trend and its relation with *MyHCs* expression

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ABSTRACT. RT-PCR was used to study the temporal and spatial pattern of Yes-associated protein 1 (*YAP1*) and myosin heavy chain (*MyHC*) expression in four different skeletal muscles (i.e., longissimus dorsi muscle, soleus muscle, gastrocnemius muscle, and extensor digitorum longus) and three growth stages (i.e., 2 days old, 2 and 6 months old) of Hu Sheep. The results showed that *YAP1* was differentially expressed in skeletal muscles of sheep, that expression increased gradually with age, and that there were high levels of expression in the gastrocnemius muscle and lower levels in the longissimus dorsi muscle. *MyHCI* was expressed at high levels in the soleus muscle and at lower levels in the longissimus dorsi muscle. In contrast, *MyHCIIA* and *MyHCIIX* were expressed at high levels in the extensor digitorum longus and at lower levels in the soleus muscle. The expression of *MyHCI* and *MyHCIIA* decreased with increasing age while that of *MyHCIIX* increased. *YAP1* expression was negatively correlated with *MyHCII* ($P < 0.01$) and positively correlated with *MyHCIIX* ($P < 0.01$)

across all growth stages and skeletal muscle types studied. We speculate that after birth, the thicker muscle fiber diameter is associated with the high expression of *MyHCIIX*. Therefore, we conclude that *YAP1* expression affects sheep muscle fiber development after birth and provides important genetic information for the selection candidate genes for sheep muscle growth.

Key words: Hu sheep; *YAP1*; *MyHCs*; Gene expression; Muscle fiber development

INTRODUCTION

Yes-associated protein 1 (*YAP1*), known as YAP, YAP2, YAP65, and YKI, is a major downstream effector of the Hippo pathway, which acts as a co-activator or co-repressor. *YAP1* is directly phosphorylated by large tumor suppressor on five HXRXXS consensus motifs, which inhibits the ability of *YAP1* to enter the nucleus. *YAP1* plays important roles in cell proliferation and apoptosis, organ size control, cell contact inhibition, and tumorigenesis (Zhao et al., 2008; Li et al., 2011; Liu et al., 2011; Zhang and Zhu, 2011). In addition, it is a novel regulator of C2C12 myogenesis (Watt et al., 2010). Mature skeletal muscle fibers are composed of four main isoforms of myosin heavy chain (MyHCI, IIA, IIB, and IIIX) (Lefaucheur, 2010). Muscle fiber-type composition affects meat indicators such as muscle flesh, pH, marbling, intramuscular fat, tenderness, water loss rate, and muscle fiber diameter. Oxidative muscle fibers (*MyHCI* and *MyHCIIA*) have a high myoglobin and phospholipid content and low ATPase activity, and the muscle fiber diameter is smaller than that of other types of skeletal muscle fiber. In contrast, glycolytic muscle fibers (*MyHCIIB*) comprise skeletal muscle fibers that are thicker in diameter; metabolic activity, and the contractile properties of intermediate muscle fibers (*MyHCIIX*) are intermediate to those of the oxidative and glycolytic muscle fibers (D'Antona et al., 2006; Delbono, 2010). Muscle fiber types are regulated constantly during growth and development and during adaptation to environment changes. The proportion of *MyHCIIB* is too low to be detected in sheep and goat mature skeletal muscle fibers (Argüello et al., 2001; Hemmings et al., 2009). Therefore, we analyzed temporal changes in *MyHCI*, *MyHCIIA*, and *MyHCIIX* gene expression during changes in the development of sheep and goat mature skeletal muscle fibers. We speculated that after birth, thicker muscle fiber diameter is associated with higher expression of *MyHC*. In the present study, the temporal and spatial patterns of *YAP1* and *MyHC* expression and their correlations were analyzed in sheep skeletal muscle fibers; the result show the effect of *YAP1* gene expression on the development of sheep muscle fiber after birth and provide important genetic information for the selection of sheep muscle growth candidate genes.

MATERIAL AND METHODS

Experimental animals

Eighteen experimental Hu sheep were purchased from Suzhou Sheep Breeding Farm. Sheep were divided into three growth stages (i.e., 2 days old, 2 and 6 months old), including three rams and three ewes in each stage, and were raised under the same conditions. All animals were slaughtered at the end of the experiment and the longissimus dorsi muscle, soleus muscle, gastrocnemius muscle, and extensor digitorum longus were rapidly collected and conserved in liquid nitrogen.

Reagents and kits

rTaq, dNTP, Primer Script RT reagent Kit, SYBR® Premix Ex Taq™ II (Tli RNaseH Plus), and TRIzol were purchased from TaKaRa; primers were designed by Oligo 7.0 and synthesized by Shanghai Sangon Biological Engineering Company. DEPC was purchased from Beijing BioTeke Corporation, Goldview was purchased from SBS Genetech Company, and other reagents were purchased from China National Medicines Corporation Ltd.

MyHC gene expression

Total RNA was extracted in the presence of buffer containing β-mercaptoethanol and guanidine using an RNAiso plus kit (TaKaRa Biotechnology Dalian, Co. Ltd., China) following the manufacturer instructions. RNA was eluted in 40 μL RNase-free water. RNA concentration was measured using a Nano Drop ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA) and samples with purity (A_{260}/A_{280}) of >1.8 was used. Exactly 250 ng total RNA from each sample was transcribed into cDNA using the Takara reverse transcription kit (TaKaRa Biotechnology) according to manufacturer instructions. Primers listed in Table 1 were used for q-PCR.

Table 1. Primer sequences used to amplify *MyHCs*, *YAP1*, *MyoG*, and 18S rRNA genes by RT-PCR.

Gene	Reference sequence	Primer sequence	Product (bp)
MyHCI	AB058898	SF: TCGTCAAGGCCACAATTTG	101
		SR: CTGCTGCAACACCTGGTCCT	
MyHCIIA	AB058896	SF: AAGCCTTTTGATGCCAAGACT	115
		SR: TTCACCGTCACTTTCCCACC	
MyHCIIX	AB058897	SF: CTTCGTGGCGGACCCTAAG	101
		SR: CAGTTACTGTCGCCCCAGCT	
YAP1	JQ714252	SF: GACAGCGGACTGAGCATGAG	108
		SR: CAGGGTGCTTTGGTTGATAGG	
18S	AY753190	SF: CGGCTACCACATCCAAGGAA	187
		SR: GCTGGAATTACCGCGGCT	

Data processing and statistical analysis

SPSS 16.0 was used to calculate the Ct values and standard errors of replicate samples, and the difference in relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. To compare differences in Hu sheep of the same age, from equivalent skeletal muscle types taken from different genders, the following equation was used: ΔΔCt was ΔCt (male) - ΔCt (female). The ΔΔCt was calculated as follows: ΔCt (other ages, in months) - ΔCt (2 days old) when the sheep were of the same gender, same skeletal muscle, but were of different ages. The ΔΔCt was calculated using the following equation: ΔCt (other skeletal muscles) - ΔCt (longissimus dorsi muscle), when the sheep were of the same gender, age, but samples were of different skeletal muscles. The value of Hu sheep of the same age but different gender, using the same skeletal muscle, was compared using the *t*-test, whereas data from individuals of the same gender, using the same skeletal muscle, but of different ages, and individuals of the same gender, age, but using samples from different skeletal muscles were compared using one-way ANOVA. In this analysis, the longissimus muscle of 2-day-old female lambs was used as a reference.

RESULTS

YAP1 spatial and temporal expression analysis in sheep muscle

There was almost no difference in the level of *YAP1* expression in different skeletal muscles of ram and ewe taken from 2-day-old sheep. In 2-month-old sheep, *YAP1* was expressed higher in gastrocnemius muscle and lower in longissimus dorsi muscle; the expression was significant ($P < 0.05$) or highly significant ($P < 0.01$, $P < 0.05$) between rams and ewes. In 6-month-old sheep, *YAP1* was expressed higher in the gastrocnemius muscle and extensor digitorum longus, and lower in the longissimus dorsi muscle. The expression of *YAP1* between skeletal muscles differed at a high level of significance ($P > 0.01$, $P < 0.05$; Figure 1). *YAP1* expression increased gradually across the growth stages; however, in the soleus muscle and gastrocnemius of rams, no statistically significant difference ($P > 0.05$) was observed in the downward trend from 2 to 6 months (Figure 2). In 2-day-old sheep, *YAP1* was more highly expressed in ewes than in rams, especially in the gastrocnemius and extensor digitorum longus ($P < 0.01$). In 2-month-old sheep, *YAP1* was more highly expressed in rams than in ewes, but no significant difference ($P > 0.05$) was found. In 6-month-old sheep, *YAP1* was more highly expressed in rams than in ewes ($P < 0.05$) in the extensor digitorum longus muscle, while there was no significant difference in *YAP1* expression between other skeletal muscles from rams and ewes ($P > 0.05$; Figure 3).

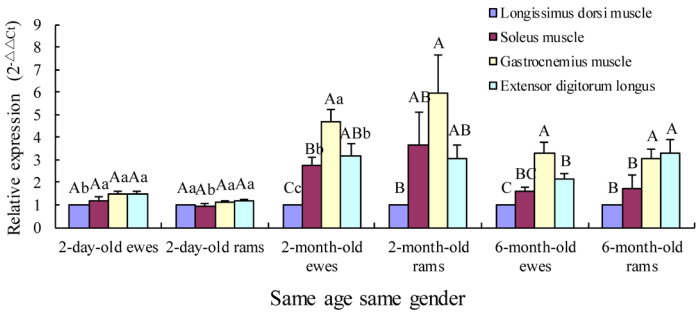

Figure 1. Expression of sheep *YAP1* in different muscles. A, B, C, a, b, and c show results from multiple comparisons between different muscles from animals of the same sex and growth stages. Values with the same letters are not significantly different ($P > 0.05$), those with different letters are significantly different ($P < 0.05$), and those with different capitals are highly significantly different ($P < 0.01$).

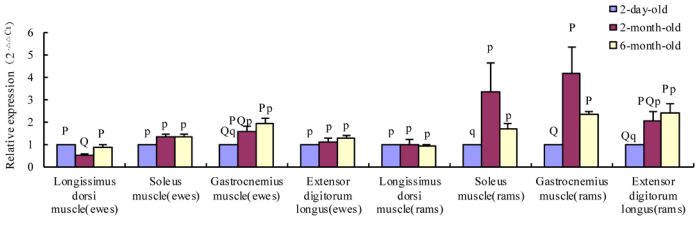

Figure 2. Expression of sheep *YAP1* at different growth stages. P, Q, p, and q show results from multiple comparisons between same muscles from animals of the same sex and different growth stages. Values with the same letters are not significantly different ($P > 0.05$), those with different letters are significantly different ($P < 0.05$), and those with different capitals are highly significantly different ($P < 0.01$).

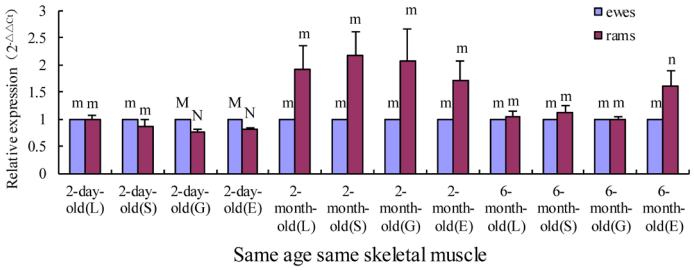

Figure 3. Effect of gender on the expression of sheep *YAP1*. M, N, m, and n show results of multiple comparisons of the same muscles from animals of different genders at the same growth stages. Values with the same letters are not significantly different (P > 0.05), those with different letters are significantly different (P < 0.05), and those with different capitals are highly significantly different (P < 0.01). L, longissimus dorsi muscle; S, soleus muscle; G, gastrocnemius muscle; E, extensor digitorum longus.

Spatial and temporal expression of MyHCs in sheep muscle

The three *MyHC* genes were expressed in a muscle tissue-specific manner (P < 0.05), wherein, *MyHCI* was expressed at high levels in the soleus muscle and at low levels in the longissimus dorsi muscle. In contrast, *MyHCIIA* and *MyHCIIX* were highly expressed in the extensor digitorum longus and showed lower expression in the soleus muscle. Low levels of *MyHCIIA* and *MyHCIIX* expression were observed in the four skeletal muscle types taken from 2-day-old animals. When age increased, levels of *MyHCIIA* and *MyHIIX* expression also increased in the gastrocnemius muscle compared to that in the three other skeletal muscle types (Figure 4).

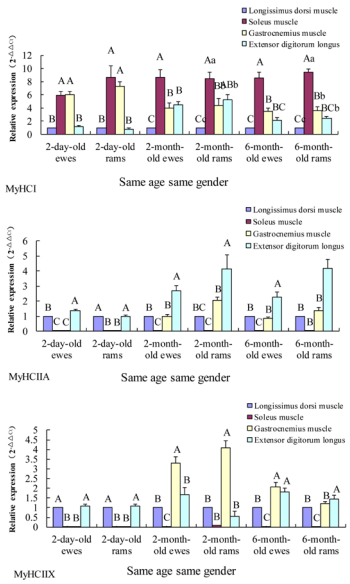

Figure 4. Expression of *MyHCs* genes in different muscles. A, B, C, a, b, and c show results from multiple comparisons between different muscles from animals of the same sex and growth stages. Values with the same letters are not significantly different (P > 0.05), those with different letters are significantly different (P < 0.05), and those with different capitals are highly significant different (P < 0.01).

Expression of *MyHCI* in the four skeletal muscle types decreased gradually in a significant manner ($P < 0.05$) with increasing age, from 2 days to 2 months. Conversely, there was very little increase in the expression of *MyHCI* from 2 to 6 months. There was no significant difference in *MyHCI* expression between the two growth stages in the four skeletal muscle types ($P > 0.05$), except in the extensor digitorum longus (ewes), the longissimus dorsi muscle (rams), and the extensor digitorum longus (rams) ($P < 0.01$). Overall, the expression of *MyHCIIA* in skeletal muscles decreased with increasing age, except in the gastrocnemius, in which the expression first increased and then decreased; the expression between different ages was significantly different ($P < 0.01$). Overall, the expression of *MyHCIIX* in skeletal muscles increased with age except in the gastrocnemius, in which the expression first increased and then decreased; the expression between different ages differed significantly ($P < 0.01$ or $P < 0.05$; Figure 5).

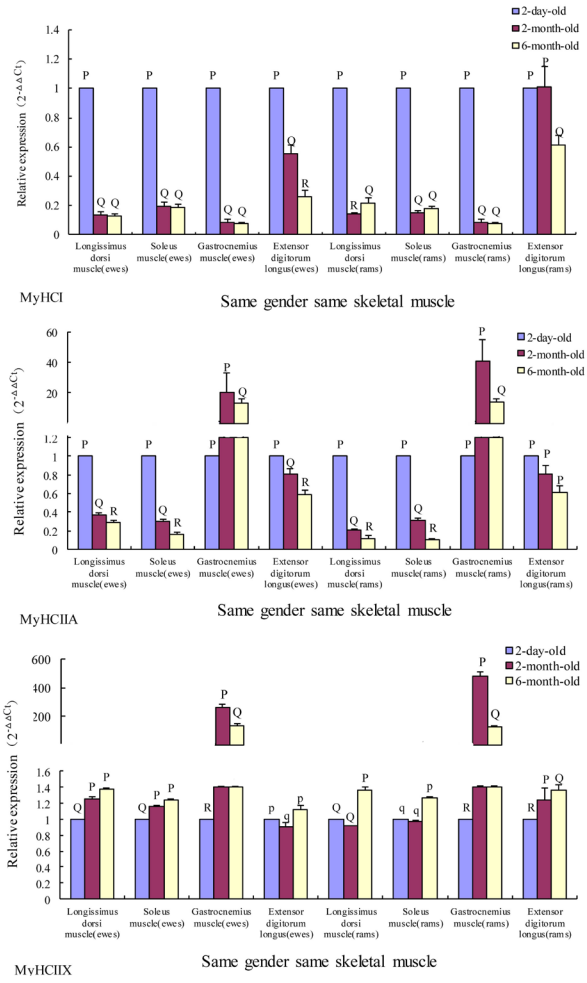

Figure 5. Differential expression of sheep MyHCs at different growth stages. P, Q, R, p, q, and r show results of multiple comparisons of the same muscles from animals of the same sex but different growth stages. Values with the same letters are not significantly different ($P > 0.05$), those with different letters are significantly different ($P < 0.05$), and those with different capital letters are highly significantly different ($P < 0.01$).

There was no significant difference in the expression of *MyHCs*, with the exception of *MyHCI*, which exhibited significant differences in expression ($P < 0.05$) in the extensor digitorum longus (2 days old), longissimus dorsi muscle, gastrocnemius, and extensor digitorum longus (6 months old). The expression of *MyHCIIA* was significantly different ($P < 0.01$) in the extensor digitorum longus (2 days old), longissimus dorsi muscle (2 months old), longissimus dorsi muscle, and extensor digitorum longus (6 months old); and *MyHCIIX* expression differed significantly ($P < 0.01$) between the soleus muscle and gastrocnemius muscle (6 months old) between ewes and rams in different skeletal muscles from different growth stages (Figure 6).

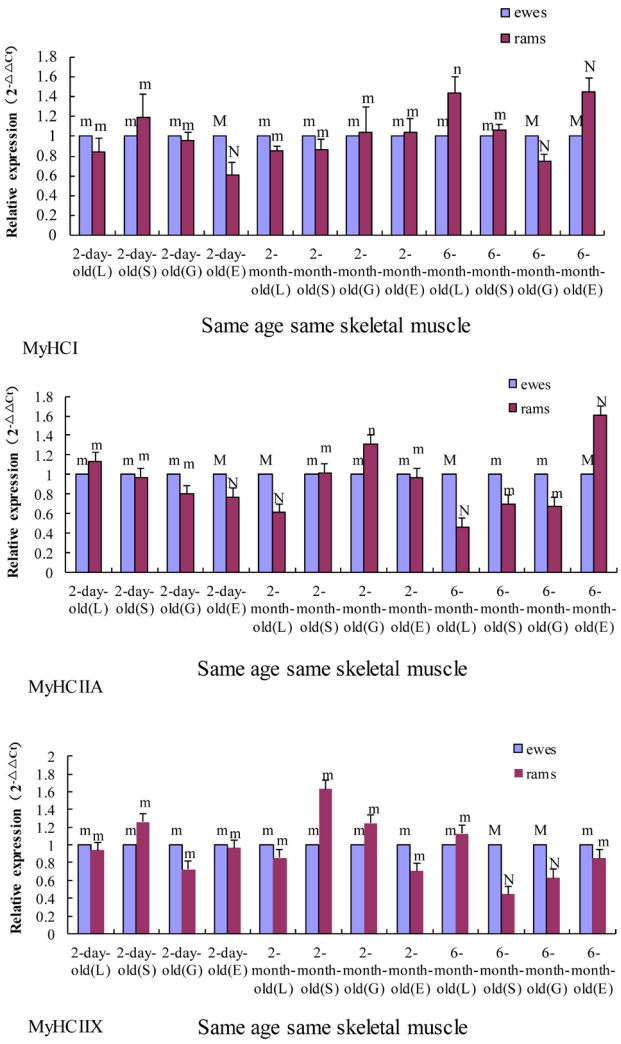

Figure 6. Differential expression of MyHCs in sheep of different genders. M, N, m, and n show the results of multiple comparisons of the same muscles taken from sheep of the same growth stage but different genders. Values with the same letters are not significantly different ($P > 0.05$), those with different letters are significantly different ($P < 0.05$), and those with different capital letters are highly significantly different ($P < 0.01$). L, longissimus dorsi muscle; S, soleus muscle; G, gastrocnemius muscle; E, extensor digitorum longus.

Association of *YAP1* with *MyHC* gene expression

YAP1 expression was not significantly correlated with *MyHCI*, *MyHCIIA*, or *MyHCIIX* (P > 0.05) in different skeletal muscle types from 2-day-old animals. (Table 2A). Whereas, *YAP1* expression was significantly negatively correlated with that of *MyHCI* (P < 0.05) and significantly positively correlated (P < 0.01) with *MyHCIIA* and *MyHCIIX* in different skeletal muscle types from 2-month-old animals (Table 2B). Similarly, *YAP1* expression was significantly negatively correlated (P < 0.01) with *MyHCI* and significantly positively correlated (P < 0.01) with *MyHCIIA*, *MyHCIIX,* in different skeletal muscle types from 6-month-old animals (Table 2C).

Table 2. Association of *YAP1* with *MyHC* gene expression in different growth stages.

Index	MyHCI	MyHCIIA	MyHCIIX	YAP1
A				
MyHCI	1	-0.864	-0.849**	0.149
MyHCIIA	-0.864**	1	0.965**	0.022
MyHCIIX	-0.849**	0.965**	1	0.002
YAP1	0.149	0.022	0.002	1
B				
MyHCI	1	-0.368**	-0.390**	-0.280*
MyHCIIA	-0.368**	1	0.882**	0.655**
MyHCIIX	-0.390**	0.882**	1	0.461*
YAP1	-0.280*	0.655**	0.461**	1
C				
MyHCI	1	-0.478**	-0.574**	-0.374*
MyHCIIA	-0.478**	1	0.923**	0.864**
MyHCIIX	-0.574**	0.923**	1	0.911**
YAP1	-0.374**	0.864**	0.911**	1

A. 2-day-old animals; **B.** 2-month-old animals; and **C.** 6-month-old animals.

YAP1 expression was significantly positively correlated with that of *MyHCI* and *MyHCIIX* in the longissimus dorsi muscle at different growth stages (Table 3A). There was no significant correlation between YAP1 expression and *MyHCI*, *MyHCIIA*, *MyHCIIX* expression, respectively (P > 0.05) in the soleus muscle at different growth stages (Table 3B). The expression of *YAP1* was significantly negatively correlated (P < 0.01) with that of *MyHCI* and was significantly positively correlated (P < 0.01) with *MyHCIIA* and *MyHCIIX* expression in the gastrocnemius muscle at different growth stages (Table 3C). The expression of *YAP1* was significantly negatively correlated with that of *MyHCIIA* and *MyHCIIX* (P > 0.05) in the extensor digitorum longus at different growth stages (Table 3D). In addition, *YAP1* expression exhibited a highly significant negative correlation (P < 0.01) with *MyHCI* expression and a highly significant positive correlation (P < 0.01) with *MyHCIIX* expression in all four skeletal muscle types across all three growth stages (Table 3E).

Table 3. Association of YAP1 with MyHCs genes expression in different muscles.

Index	MyHCI	MyHCIIA	MyHCIIX	YAP1
A				
MyHCI	1	0.806**	0.726**	0.466*
MyHCIIA	0.806**	1	0.685**	0.240
MyHCIIX	0.726**	0.685**	1	0.429*
YAP1	0.466**	0.240**	0.429**	1
B				
MyHCI	1	0.678**	0.393**	-0.172
MyHCIIA	0.678**	1	0.733**	-0.157
MyHCIIX	0.393**	0.733**	1	0.124
YAP1	-0.172	-0.157	0.124	1
C				
MyHCI	1	-0.836**	-0.888**	-0.496**
MyHCIIA	-0.836**	1	0.930**	0.636**
MyHCIIX	-0.888**	0.930**	1	0.683**
YAP1	-0.496**	0.664**	0.683**	1
D				
MyHCI	1	0.517**	0.306*	0.033
MyHCIIA	0.517**	1	0.619**	0.309*
MyHCIIX	0.306*	0.619**	1	0.394**
YAP1	0.033	0.309*	0.394**	1
E				
MyHCI	1	-0.488**	-0.643**	-0.288*
MyHCIIA	-0.294**	1	0.550**	0.040
MyHCIIX	-0.372**	0.550**	1	0.459**
YAP1	-0.288**	0.040	0.459**	1

A. Longissimus dorsi muscle; **B.** soleus muscle; **C.** gastrocnemius muscle; **D.** extensor digitorum longus; and **E.** muscles.

DISCUSSION

Type *MyHCI* fibers (red muscle fibers) and type *MyHCIIB* fibers (white muscle fibers) are present at different ratios in different skeletal muscle types. Soleus muscle is a slow-red muscle fiber and contains 60% red muscle fibers and 30% intermediate muscle fibers. The extensor digitorum longus is a fast-white muscle fiber and contains 45% white muscle fibers and 45% intermediate muscle fibers. Gastrocnemius and longissimus dorsi muscles contain roughly equivalent levels of red and white muscle fibers. In the present study, we explored the role of *YAP1* in regulating muscle development by studying the trend of *YAP1* expression in different skeletal muscles and across different growth stages, and by investigating the correlation between *YAP1* and *MyHC* expression.

Spatial and temporal expression pattern of *YAP1* and *MyHCs*

In the present study, *YAP1* expression was found to differ significantly among the four skeletal muscles studied in 2-month and 6-month-old animals. It was found to be expressed at high levels in the gastrocnemius muscle and at lower levels in the longissimus dorsi muscle. *YAP1* expression increased gradually with age in different skeletal muscles of sheep. Sun et al. (2011)

indicated that the diameter of muscle fibers in rams was thicker than that in ewes, while the muscle fibers in rams were less tender than those in ewes. This indicates that *YAP1* expression might be associated with the enlargement of muscle fiber diameter; however, there was no significant difference in *YAP1* expression between rams and ewes.

Currently, eight kinds of *MyHC* isoforms, which are encoded by separate genes, have been found in mammals, and are located on chromosomes 7 and 12 (Weiss et al., 1999; Shrager et al., 2000; Davoli et al., 2002). Only *MyHCI*, *MyHCIIA*, and *MyHCIIX* are expressed in sheep skeletal muscle. Since a high proportion of *MyHCI* muscle fibers can lead to better quality meat (Lefaucheur et al., 2004), many scholars have committed to improving the quality of livestock and poultry meat by improving the regulation of muscle fiber types or by increasing the proportion of type *MyHCI* muscle fibers. Of the three types of *MyHC* that are differentially expressed in different types of skeletal muscle (*MyHCI*, *MyHCIIA*, and *MyHCIIX*), *MyHCI* is expressed at higher levels in the supraspinatus, followed by semitendinosus, and is expressed at lower levels in the longissimus dorsi muscle. *MyHCIIX* is expressed at the highest level in the longissimus dorsi muscle, followed by the semitendinosus, and is expressed at the lowest level in the supraspinatus (D'Antona et al., 2006). *MyHCIIA* and *MyHCIIX* were most highly expressed in the longissimus dorsi muscle and the semimembranosus of Laiwu pigs compared to Duroc pigs (Hu et al., 2008). Zhang et al. (2010) studied the pattern of *MyHC* expression in pig skeletal muscle, and showed that *MyHCI* is highly expressed in the soleus muscle, and is expressed at lower levels in the longissimus dorsi muscle. *MyHCIIA* was highly expressed in soleus muscle, and at lower levels in the extensor digitorum longus. *MyHCIIX* was highly expressed in the longissimus dorsi muscle, and at lower levels in the soleus muscle. The expression of *MyHCI* decreased gradually with age, while that of *MyHCIIB* increased gradually with age. Sex had no significant effect on *MyHCI* expression (Yang et al., 2005). In the present study, *MyHCI* was found to be expressed at high levels in the soleus muscle and at lower levels in the longissimus dorsi muscle, whereas *MyHCIIX* was highly expressed in extensor digitorum longus and at lower levels in the soleus muscle. This is because the soleus muscle is a slow-red muscle fiber, whereas the extensor digitorum longus is a fast-white muscle fiber. In addition, *MyHCI* and *MyHCIIA* expression decreased with animal age while *MyHCIIX* expression increased with animal age. In contrast, Yang et al. (2005) found that the expression of *MyHCIIB* was higher in 2-month-old animals than in new-born pigs. Sun et al. (2011) and Chen et al. (2009) independently studied longissimus dorsi fiber diameter in sheep and pigs and showed that muscle fiber diameter in adult pigs was nearly twice the length of that in adult sheep because of the lack of *MyHCIIB*. The diameter of muscle fiber in sheep is smaller, and is more tender than that in pigs.

Correlation between YAP1 and MyHC

Pellegrino et al. (2003) and Lefaucheur et al. (2002) considered that under normal physiological conditions, the transformation of muscle fiber was based on a certain regularity, such that those from new-born pigs were of the oxidative type, and the glycolytic fibers did not differentiate. With increasing age, a decrease in the number of oxidative muscle fibers and an increase in the number of glycolytic muscle fibers was observed. *MyHCI* transformed to *MyHCIIA*, *MyHCX*, and *MyHCIIB* at a high rate and the *MyHC* isoform was expressed in the following order during development: I to IIA to IIX to IIB, in pig muscle. On the other hand, oxidative metabolism weakened and the muscle fiber diameter increased. *YAP1* expression was significantly and negatively correlated with *MyHCI* and significantly and positively correlated with *MyHCIIX* ($P < 0.01$). These results showed that *YAP1* may be associated with muscle fiber thickness and that

it may be involved in the transformation of myosin heavy chain. In the correlation analysis using 2 days old, *YAP1* exhibited no significant correlation with *MyHC* isoforms, while in 2-month and 6-month-old animals, *YAP1* expression was significantly and negatively correlated with *MyHCI*, and significantly positively correlated with *MyHCIIA* and *MyHCIIX*. This indicates that right after birth, *YAP1* is not involved in *MyHC* gene transformation, but as the age increases, *YAP1* is involved in *MyHC* gene transformation. Correlation analysis of the gastrocnemius muscle revealed that *YAP1* expression was highly negatively correlated with *MyHCI*, and was positively correlated with *MyHCIIA* and *MyHCIIX*. Correlation analysis of the soleus muscle revealed that there was no significant correlation between *YAP1* and *MyHCs*. Correlation analysis of the extensor digitorum longus revealed that there was no significant correlation between *YAP1* and *MyHCI*, but was significantly positively correlated with *MyHCIIA* and significantly positively correlated with *MyHCIIX*. Correlation analysis of the longissimus dorsi muscle revealed that YAP1 significantly positively correlated with *MyHCI* and *MyHCIIX*, and was not significantly correlated with *MyHCIIA*. These results indicated that in correlation analysis of different growth stages in the same skeletal muscle type, YAP1 did not always show a significant negative correlation. At the same time, YAP1 may exhibit positive correlation with MyHCII, even significant positive correlation in some other muscle tissue, and it showed that YAP1 did not play a catalytic role in thickening muscle fiber diameter in the skeletal muscle. Overall, *YAP1* was associated with muscle fiber thickening as evidenced through the correlation analysis between *YAP1* and *MyHCs*.

Conflicts of interest

The authors declare no conflict of interest.

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