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Association of *Six1* and *Six4* gene expression with meat tenderness in Tan sheep

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ABSTRACT. Sine oculis-related homeobox 1 (*Six1*) and 4 (*Six4*) transcription factors are expressed in developing and adult muscle. These Six homeoproteins are required to activate the fast-type muscle program in the mouse primary myotome. Whether *Six1* and *Six4* genes have a role in meat tenderness in Tan sheep has not yet been established. To study the characteristics and mechanisms of meat tenderness in Tan sheep, we sampled the musculus triceps muscle, the longest neck muscles, abdominal muscles, gluteus and biceps femoris muscle and measured Warner-Bratzler shear force and texture profile analysis using a texture analyzer (TMS-Pro), and mRNA expression of *Six1* and *Six4*by real-time PCR, and we performed a correlation analysis. The Warner-Bratzler shear force was significantly different among the five different muscles, but there was no difference in this measure between the triceps and biceps femoris muscles. Hardness, gumminess and chewiness were significantly different among the different muscles. *Six1* and *Six4* mRNA expression was significantly higher in the longest neck muscles than in the other muscles. There was a significant negative correlation between *Six1* mRNA expression and the texture parameters gumminess and chewiness, and a significant positive correlation between *Six4* mRNA expression and gumminess and chewiness. Based on these results, we suggest that *Six1* and *Six4* not only regulate muscle fiber typing, but also meat tenderness in Tan sheep. They could thus be candidate genes for selecting for meat tenderness.

Key words: *Six1*; *Six4*; meat tenderness; Tan sheep

INTRODUCTION

Tan sheep are a short-tailed indigenous ovine breed distributed in the Ningxia Hui Autonomous Region in China that is well-known for its high-quality meat (Kang et al., 2013; Chen et al., 2013). Meat quality relates to human health as well as food safety; additionally, environmental protection, sustainable animal husbandry, human and animal well-being, customer satisfaction and producer income are all important considerations (Costa et al., 2002). Meat quality is usually evaluated by perception characteristics, such as tenderness, texture, color, palatability, fat content and fatty acid composition. Tenderness and texture are important components of meat quality, affecting consumer acceptability (Hopkins et al., 2013). Tenderness is often reported as the most important quality attribute of meat; however, relatively few studies have been done to investigate the tenderness of Tan sheep meat (Miller et al., 2001). Tenderness is affected by the structure of muscle and connective tissue. Although there is no standard method for measuring meat tenderness, some studies have assessed beef tenderness by the Warner-Bratzler shear force (WBS) method (Destefanis et al., 2008);others have used sensory evaluation, citing this as a convenient and efficient method fortesting meat tenderness (Miller et al., 2001; Elzo et al., 2012; Gonzalez et al., 2014; Holman et al., 2016). Genes that regulate muscle development and fiber type transformation can affect meat quality. Sine oculis-related homeobox 1 (*Six1*), a member of the Six homeobox gene family (Kawakami et al., 2000), has recently been shown to be a key regulatory factor in skeletal muscle development and muscle fiber type transformation (Wong et al., 2013). *Six1* regulates skeletal muscle development, muscle injury repair and transformation of muscle fiber type throughout the embryonic period. Moreover, *Six1* and *Six4* are important for the genes upstream of paired box 3 (*Pax3*) (Grifone et al., 2005). In the main muscle segment of mixed slow/fast myocytes, *Six1* and *Six4* are required to activate the fast muscle gene network. In a chromatin immunological precipitation experiment, *Six1* and *Six4* interacted with the regulatory region of the identified muscle genes, demonstrating direct control of the expression of these genes (Niro et al., 2010). We measured*Six1* and *Six4* mRNA expression and muscle tenderness in various muscles of Tan sheep and analyzed the correlations between these measures. We looked for evidence as to whether *Six1* and *Six4* are candidate genes influencing meat quality, especially tenderness, in Tan sheep, for application in the breeding of this type of sheep.

MATERIAL AND METHODS

Reagents

All reagents were of analytical grade and of the highest purity commercially available. The AxyPrep total RNA isolation kit was purchased from Corning Life Sciences Corporation (Wujiang, China), and TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix, TransStart Top Green qPCR SuperMix and 2×EcoTaq PCR SuperMix (with dye) were purchased from Transgene Biotech (Beijing, China).

Animal treatment

Twelve Tan sheep from Ningxia yanchi county Chunhao Grass industry specialized cooperatives with a pre-slaughter weight of 27.7 ± 2.8 kg aged 9 months were used in this study. The animals were slaughtered according to the National Standard of China (GB 13078-2001 and GB/T 17237-1998) and Agriculture Standards of China (NY 5148-2002-NY 5151-2002). As soon as possible after slaughter, samples of approximately 300 mg were taken from triceps muscles, the longest neck muscles, abdominal muscles, gluteus and biceps femoris muscle, packaged in foil, placed in liquid nitrogen and stored at -80°C until further use. Next, samples of approximately 200 g were taken from the same muscles for analysis of tenderness and texture at 4°C.

Tenderness Assay

Tenderness was measured using a texture analyzer (TMS-Pro, Food Technology Corporation) with samples at room temperature. Twelve muscle slices of 20×20×20 mm were placed on the platform of the analyzer, and tenderness was measured by piercing each sample to a depth of 10 mm using a penetration test fixture of diameter 5 mm at a speed of 100 mm/min. The thrust of the material was assessed using Warner-Bratzler shear force in Newton (N) units.

Texture profile analysis

A texture profile analysis (TPA) of 12 muscle slices 12.7 mm in diameter was conducted by orienting the samples according to muscle fiber orientation and shearing once using a texture analyzer (Ensoul Technology Ltd.). The detector moved at a crosshead speed of 200 mm/min, and the peak load (kg) of each core was recorded by a TMS-Pro using software provided by the FTC Corporation. Hardness, adhesiveness, cohesiveness, springiness, gumminess and chewiness were analyzed for each sample.

Design and synthesis of primers

Primers for real-time PCR were designed based on the mRNA sequences of the target genes using Primer Premier 5.0 software. *Six1* and *Six4* sequences were published in NCBI (GenBank), and the *β-actin* gene was used as an internal reference. Primers were synthesized at Sangong Biotech (Shanghai, China). The sequences of the primers, annealing temperatures and the lengths of the PCR products are shown in Table 1.

Table 1. Primer sequences used for RT-PCR

Gene	Oligos sequences	Product size (bp)	Tm (°C)
Six 1	F: 5'-CCACTCATGTCCAGCTCAGA-3' R: 5'-ACCCGGGAGAGAATAGTTGG-3'	120	60
Six 4	F: 5'-CAGGTCAGCAACTGGTTCAA-3' R: 5'-CTTCATGACCCTTGCTGGAT-3'	121	60
β-Actin	F: 5'-TGAACCCCAAAGCCAACC-3' R: 5'-AGAGGCGTACAGGGACAGCA-3'	107	61

Extraction and reverse transcription of RNA

Total RNA was extracted from muscle samples using an AxyPrep total RNA isolation kit, and the concentration and purity of the extracted total RNA were determined with a Maestro Nanomicro-spectrophotometer (MaestroGEN, Las Vegas, NV, USA). Reverse transcription of the total RNA was carried out using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix and the products were stored at −80°C until further analysis.

Quantitative PCR

Real-time PCR was performed with 25 mL reactions using SYBR Premix Ex Taq™ II. The PCR cycling conditions were 95°C for 30 s, followed by 50 cycles of 95°C for 5 s, 60°C for 34 s and 72°C for 30 s. A melting curve analysis was performed at 95°C for 10 s and 60°C for 1 min, followed by an increase in the temperature from 60 to 95°C at a rate of 0.5°C/10 s.

Statistical analyses

Data were analyzed by one-way analysis of variance followed by a Duncan test to compare the different muscles of Tan sheep, using SPSS 16.0 software (SPSS Inc., Michigan Avenue, Chicago, IL, USA). The relative expression amounts of the target genes were determined by the $2^{-\Delta\Delta CT}$ method. Correlation analyses were performed using Pearson's test. Statistical significance was set at $P< 0.05$ and $P< 0.01$. All pictures were analyzed using GraphPad Prism 5.0a (GraphPad Software, Inc.).

RESULTS

Comparison of the mRNA transcription levels (MRL) of *Six1* and *Six4* from various muscles of Tan sheep

The mRNA expression levels of *Six1* and *Six4* were significantly different among the different muscles of Tan sheep (Fig. 1). *Six1* mRNA expression was highest in the longest neck muscles and lowest in the gluteus. *Six1* mRNA expression levels in the longest neck muscles were 60.6% higher than those in triceps muscles ($P < 0.05$), 67.3% higher than those in biceps femoris muscles ($P < 0.05$), 75.3% higher than those in abdominal muscles ($P < 0.05$) and 79.3% higher than those in the gluteus ($P < 0.05$). *Six 1* mRNA expression levels in triceps muscles were 17.2% higher than those in biceps femoris muscles ($P < 0.05$), 37.4% higher than those in abdominal muscles ($P < 0.05$) and 47.5% higher than those in gluteus ($P < 0.05$). *Six1* mRNA expression levels in biceps femoris muscles were 24.4% higher than those in abdominal muscles ($P < 0.05$) and 36.6 higher than those in gluteus ($P < 0.05$). *Six1* mRNA expression levels in abdominal muscles were 16.2% higher than those in the gluteus ($P < 0.05$).

Six4 mRNA expression was highest in the longest neck muscles and lowest in the gluteus. *Six4* mRNA expression levels in the longest neck muscles were 49.2% higher than those in musculus triceps ($P < 0.05$), 65.5% higher than those in biceps femoris muscles (*P*

< 0.05), 79.4% higher than those in abdominal muscles ($P < 0.05$) and 82.2% higher than those in the gluteus ($P < 0.05$).*Six 4* mRNA expression levels in musculus triceps were 32.2% higher than those in biceps femoris muscles ($P < 0.05$), 59.6% higher than those in abdominal muscles ($P < 0.05$) and 65.1% higher than those in the gluteus ($P < 0.05$). *Six4* mRNA expression levels in biceps femoris muscles were 40.4% higher than those in abdominal muscles ($P < 0.05$) and 48.5% higher than those in the gluteus ($P < 0.05$). *Six4* mRNA expression levels in abdominal muscles were 13.6% higher than those in the gluteus ($P > 0.05$).

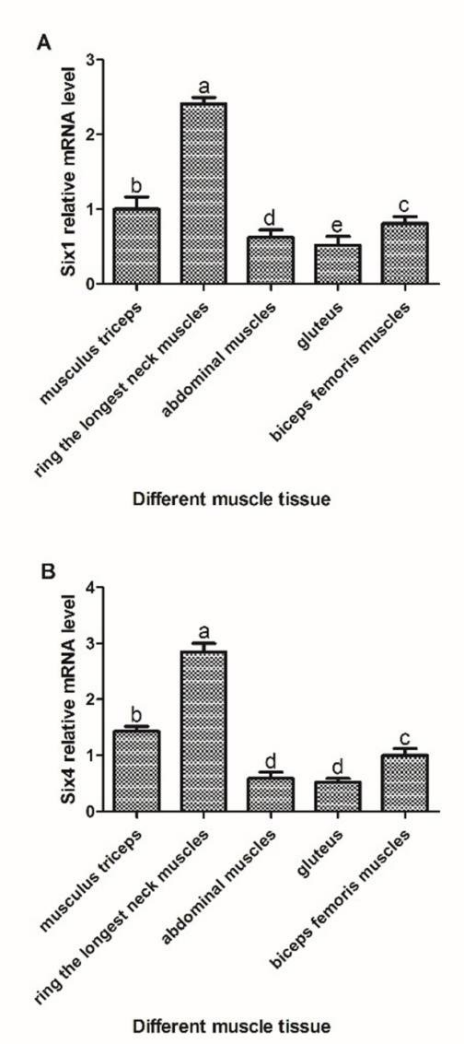

Figure 1. Six1 (A) and Six (B) relative mRNA expression level in triceps muscles, the longest neck muscles, abdominal muscles, gluteus and biceps femoris muscles of Tan sheep. Data are ratios of Six1 and Six4 genes' relative mRNA levels normalized to β-actin (housekeeping gene) mRNA levels. Each bar represents means ± SEM. Lowercases $P<0.05$.

Comparison of the Warner-Bratzler shear force (WBS) from various muscles of Tan sheep

WBS was determined in five different muscles in the Tan sheep (Fig. 2). There were significant differences in WBS among the five muscles ($P < 0.05$), but there was no significant difference between triceps and biceps femoris muscles ($P > 0.05$). WBS was highest in the longest neck muscles and lowest in the gluteus. WBS in the longest neck muscles was 29.5% higher than that in abdominal muscles ($P < 0.05$), 39.5% higher than that in biceps femoris muscles ($P < 0.05$), 39.8% higher than that in triceps muscles ($P < 0.05$) and 47.9% higher than that in gluteus ($P < 0.05$). WBS in abdominal muscles was 14.2% higher than that in biceps femoris muscles ($P < 0.05$), 14.6% higher than that in triceps muscles ($P < 0.05$) and 26.1% higher than that in the gluteus ($P < 0.05$). WBS in biceps femoris muscles was 0.5% higher than that in musculus triceps ($P > 0.05$) and 13.9% higher than that in the gluteus ($P < 0.05$). WBS in triceps muscles was 13.4% higher than that in the gluteus ($P < 0.05$).

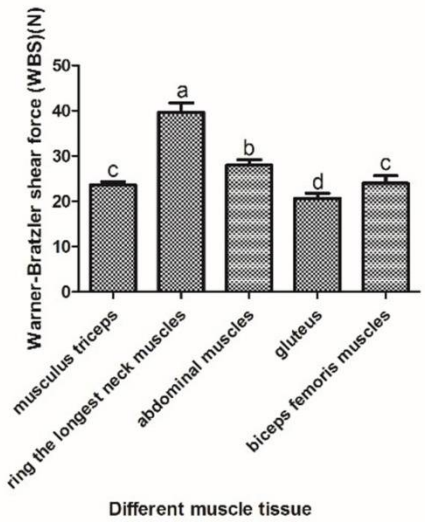

Figure 2. Warner-Bratzler shear force (WBS) in triceps muscles, the longest neck muscles, abdominal muscles, gluteus and biceps femoris muscles of Tan sheep. Each bar represents means ± SEM. Lowercases $P< 0.05$.
Note: In the same row, values with different small letter superscripts mean a significant difference ($P<0.05$), while those with the same superscripts mean no significant difference ($P>0.05$).

Comparison of the texture profile analysis (TPA) from different muscles of Tan sheep

We measured hardness (Fig. 3A), adhesiveness (Fig. 3B), cohesiveness (Fig. 3C), springiness (Fig. 3D), gumminess (Fig. 3E) and chewiness (Fig. 3F) in different muscles of Tan sheep for the TPA. Hardness in biceps femoris muscles was significantly different ($P < 0.05$) from that of triceps muscles, the longest neck muscles, abdominal muscles and the gluteus in Tan sheep, and the differences between different muscles were significant ($P < 0.05$). Adhesiveness, cohesiveness and springiness were not significantly different among

the different muscles ($P > 0.05$). Gumminess in biceps femoris muscles was significantly different ($P < 0.05$) among triceps muscles, the longest neck muscles, abdominal muscles and the gluteus in Tan sheep, and there was a significant difference in gumminess between gluteus and biceps femoris muscles ($P < 0.05$). Chewiness was significantly different between the longest neck muscles and biceps femoris muscles ($P < 0.05$), and there was a significant difference in chewiness between gluteus, abdominal muscles and biceps femoris muscles ($P < 0.05$).

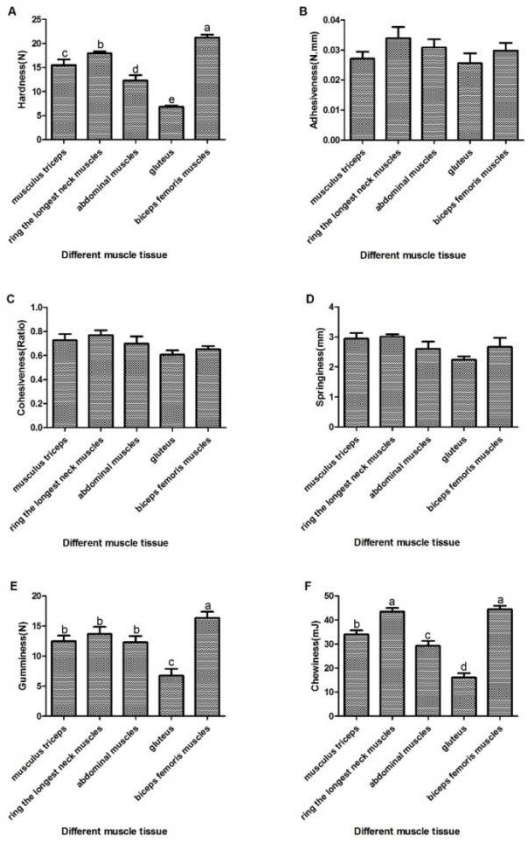

Figure 3. Texture profile analysis (TPA) in triceps muscles, the longest neck muscles, abdominal muscles, gluteus and biceps femoris muscles of Tan sheep. Each bar represents means ± SEM. In the same row, values with different small letter superscripts mean significant differences ($P< 0.05$),while those with the same superscripts or no superscripts mean no significant difference ($P> 0.05$).

Association of *Six1* and *Six4* Gene with Warner-Bratzler shear force and texture profile analysis from different muscles of Tan sheep

In an overall analysis, a significant relationship between *Six1* and *Six4* mRNA expression levels and WBS and TPA was found (Table 2). For musculus triceps, there was a significant negative correlation between *Six1* mRNA expression and the TPA parameters

gumminess and chewiness ($P < 0.01$), and no significant correlation was found between *Six4* mRNA expression and WBS, hardness, adhesiveness, cohesiveness, springiness, gumminess or chewiness ($P > 0.05$).For ring the longest neck muscles, there was no correlation between *Six1* mRNA expression and WBS, hardness, adhesiveness, cohesiveness, springiness, gumminess or chewiness ($P > 0.05$), and a positive correlation between *Six4* mRNA expression and chewiness ($0.01 < P < 0.05$). For abdominal muscles, there was a significant negative correlation between *Six1* mRNA expression and WBS ($0.01 < P < 0.05$) and gumminess ($P< 0.01$), and a significant positive correlation between *Six4* mRNA expression and WBS ($P< 0.01$) and gumminess ($0.01 < P < 0.05$). For the gluteus, there was a significant negative correlation between *Six1* mRNA expression and gumminess ($P < 0.01$), and a significant negative correlation between *Six4* mRNA expression and cohesiveness ($P < 0.01$). For biceps femoris muscles, there was a significant negative correlation between *Six1* mRNA expression and WBS ($P < 0.01$) and gumminess ($P < 0.01$), a significant positive correlation between *Six1* mRNA expression and chewiness ($P < 0.01$), and a significant positive correlation between *Six4* mRNA expression and WBS ($0.01 < P < 0.05$).

Table 2. Association of Six1 and Six4 Gene with Warner-Bratzler shear force and texture profile analysis from musculus triceps, ring the longest neck muscles, abdominal muscles, gluteus and biceps femoris muscles of Tan sheep.

	WBS	Hardness	Adhesiveness	Cohesiveness	Springiness	Gumminess	Chewiness
musculus triceps							
Six 1	0.208	0.413	0.194	0.042	0.030	-0.816**	-0.776**
Six 4	-0.260	-0.396	-0.278	0.214	-0.200	0.150	0.298
ring the longest neck muscles							
Six 1	-0.291	0.329	0.180	0.207	0.225	-0.421	0.290
Six 4	-0.232	-0.268	-0.173	0.422	0.128	0.141	0.621*
abdominal muscles							
Six 1	-0.594*	-0.513	0.491	0.362	0.176	-0.633**	-0.131
Six 4	0.735**	0.550	-0.546	-0.162	-0.032	0.669*	0.375
gluteus							
Six 1	-0.136	0.003	0.295	0.365	0.048	-0.775**	0.234
Six 4	0.217	-0.535	0.354	-0.802**	0.093	0.244	0.185
biceps femoris muscles							
Six 1	-0.798**	0.314	0.231	0.188	-0.109	-0.732**	0.622*
Six 4	0.591*	-0.067	0.279	-0.126	-0.217	0.542	-0.435

DISCUSSION

While tenderness is usually indicated by maximum shearing force in meat, the effect of the mouth on food is more than just cutting. The mouth wall, upper forehead, tongue and teeth will feel changes in food mechanical consistency and will affect tenderness evaluations. The concept of tenderness thus extends to hardness, adhesiveness, cohesiveness, springiness, gumminess and chewiness (Russo et al., 2002).

A strong correlation between WBS and the sensory evaluation of tenderness has resulted in the WBS becoming a common method for the analysis of meat tenderness. To address the parameters of tenderness not accounted for by WBS, the TPA was introduced by qualitative analysis of physical analysis in meat research; it has the advantages of good stability and high repeatability (Lalioti et al., 1997). The factors that affect meat tenderness are many and complex and include genes, nutrition, age and environment. Genes have an integral influence on meat quality, and meat quality traits are determined by complicated multigenetic networks and signaling pathways.

The primary aim of our study was to investigate the relationship between the gene expression levels of *Six1* and *Six4* and meat tenderness in Tan sheep muscles. We found a significant negative correlation between *Six1* mRNA expression and meat tenderness, and a positive correlation between *Six4* mRNA expression and WBS. Research suggests that Six4 protein expression is weak in satellite cells of resting adult muscle; but it increases substantially with satellite cell activation postinjury (Yajima et al., 2010). To determine whether *Six4* plays an active role in the regeneration process, siRNA duplexes were used in muscle to knock out *Six4*, leading to the loss of the *Six4* function in the activated satellite cells (Diao et al., 2012; Lu et al., 2013; Mueller et al., 2015). Under these conditions of intramuscular *Six4* knockout, several genes involved in myogenic differentiation or muscle function are down regulated, and repair after injury is significantly impaired, as assessed by fiber size measurement and marker gene expression analysis (Chakroun et al., 2015). Our experiments have revealed that appropriate levels of *Six4* are necessary for the process of muscle growth. In addition, some genes that are directly or indirectly controlled by *Six1* or *Six4* could amplify the "fast-type specialization" induced by Six homologous proteins by cellular autonomy or by directing adjacent cells. Myogenin (*MyoG*) interacts with *Six1* via myocyte enhancer factor 3 (*MEF3*) motifs during embryonic development. The results indicated that *MyoG* plays a central regulatory role in the formation of muscle cells. In piglets, the genetic equivalent of *MyoG* is closely related to body weight and muscle fiber number. As the thickness and density of the muscle fibers is highly correlated with meat tenderness, this suggests that *MyoG* affects meat tenderness. In mice, *MyoG* and *Six1* mRNA expression decreased as the differentiation time of C2C12 cells increased (Wu et al., 2012). *Six1* over expression inhibited *MyoG* expression in myoblasts in mice, and delayed myoblast differentiation (Wu et al., 2013; Wu et al., 2013). Six1 and Six4 proteins are known to absorb various cofactors, including Eya, Groucho and UTX proteins; thus, we can assume that chemical measurements in a given cell at the Six binding site (MEF3 site) will differ depending on the surrounding transcription factors (Grifone et al., 2007; Ikeda et al., 2002; Kobayashi et al., 2001; Seenundun et al., 2010). Many of the genes have MEF3 binding sites in their regulatory sequences, while Six proteins bind to the growth of C2C12 muscle cells (Liu et al., 2010). There are MEF3 sites in the core area of the MyoG enhancer, and when Six1 combines with MEF3, *MyoG* is expressed (Zhang et al., 2009). In zebrafish, more than half of the embryos were MyoG negative cells when *Six1a* expression was blocked, and the process of rapid muscle differentiation was inhibited (O'Brien et al., 2014). Previous studies have suggested that over expression of *Six1* up-regulates *MyoG* expression and over expression of *Six4* down-regulates *MyoG* expression (Yajima et al., 2010). *Six1* and *Six4* are co-expressed in muscles and are known to directly control *Pax3* expression and the genesis of hypaxial myogenic progenitors during embryogenesis. These hypaxial myogenic progenitor cells play a role in myogenicity through direct control of myogenic factor 5 (*Myf5*) and thus myogenin (Grifone et al., 2005; Giordani et al., 2007). Therefore, *Six1* and *Six4* play a key role in controlling the direction of muscle cell differentiation (by regulating the myogenic regulatory factors) and muscle fiber type decisions (by regulating Myh2/4/7 and fast and slow muscle genes), affecting livestock and poultry meat quality traits directly and indirectly, respectively. Further study is needed to understand the exact effects of Six homeobox gene families and these signaling pathways on the myogenic system. Research has shown that wingless-type MMTV integration site family members (Wnt) signaling affects differentiation of the phenotype in embryonic muscle fibers. Six

homeoproteins directly control Wnt4 expression in embryogenesis and may induce fast-type adjacent myogenic progenitor cells and/or muscle fiber myogenic commitment. The exact role of all these molecules in muscle development is not clear in the fetal period, though the characteristics of and number of cells expressing them will provide information (Anakwe et al., 2003; Hutcheson et al., 2009).

CONCLUSIONS

There have been few studies on correlation between muscle *Six1* and *Six4* expression and animal meat quality and production performance. *Six1* and *Six4* have been shown to regulate skeletal muscle cell differentiation and muscle fiber type transformation in mice. Therefore, *Six1* and *Six4* may play an important role in regulating livestock and poultry traits. However, the use of *Six1* and *Six4* as candidate genes for animal and poultry meat traits, and their specific mechanism of action, needs to be further studied.

CONFLICTS OF INTEREST

All authors declare the absence of any conflict of interests including any financial, personal, or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, this work.

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