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Association between *DLK1* and *IGF-I* gene expression and meat quality in sheep

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ABSTRACT. The aim of the present study was to detect delta-like 1 homolog (*DLK1*) and insulin-like growth factor-I (*IGF-I*) gene expression in the longissimus dorsi of Hu sheep at different growth stages and study the association between these genes and meat quality. The diameter and density of muscle fibers and tenderness of the longissimus dorsi were measured. Growth stage, but not sex, significantly affected *DLK1* and *IGF-I* expression. *DLK1* and *IGF-I* expression in the sheep longissimus dorsi gradually increased with growth, but also decreased during some periods. These results suggest that different growth stages significantly affect *DLK1* and *IGF-I* gene expression in sheep muscle tissue. The expression of *DLK1* and *IGF-I* genes were positively and significantly ($P < 0.01$) correlated with muscle fiber diameter and muscle fiber shear stress, and negatively and significantly ($P < 0.01$) correlated with muscle fiber density. Muscle fiber diameter was positively and significantly ($P < 0.01$) correlated with muscle fiber shear stress, and negatively and significantly ($P < 0.01$) correlated with muscle fiber density. In addition,

DLK-1 expression was significantly ($P < 0.01$) and positively correlated with *IGF-I* expression.

Key words: *DLK1*; Gene expression; Hu sheep; *IGF-I*; Muscle trait

INTRODUCTION

Most livestock production traits are complex, involving contributions from a large number of additive genetic variants each of small effect size, interactions between genes, and environment influences (Tellam et al., 2012). Sheep meat production has recently improved through selective breeding and improved animal husbandry (Fogarty, 2009; Gardner et al., 2010). DNA marker-assisted breeding strategies in sheep are now positioned to markedly accelerate the rates of genetic gain for desirable production traits, especially those that are difficult to measure, costly, and only expressed late in life (Tellam et al., 2012). According to McIlveen and Buchanan (2001) flavor, tenderness and juiciness appear to be the three most important determinants of sensory enjoyment for the UK consumer. Many factors affect the quality of meat, including diet and management, slaughter, and processing practices (Mullen et al., 2006).

Growth is a complex process that involves the regulated coordination of a wide array of neuroendocrine pathways. *DLK1* (delta-like homolog 1) is a cell surface transmembrane glycoprotein that belongs to the epidermal growth factor-like family of homeotic proteins and plays an important role in regulating fetal and postnatal development (Oczkowicz et al., 2010). Increased expression of *DLK1* is the primary cause of muscle hypertrophy in callipyge sheep exhibiting overgrowth of fast-twitch muscles and reduced adiposity (Cao et al., 2010). The role of *DLK1* in adipogenesis has been well-documented (Smas and Sul 1996; Nueda et al., 2007; Sul, 2009). Recently, *DLK1* was shown to regulate the fate of myogenic cells (Andersen et al., 2009) and human skeletal stem cells (Abdallah et al., 2004).

Insulin-like growth factor (IGF)-I and -II play important roles in the regulation of skeletal muscle growth and act as mediators of growth hormone during muscle development and differentiation (Zhang et al., 2008; Wang et al., 2011). During postnatal skeletal muscle growth *in vivo* or in fully differentiated muscle cells, IGFs increase the rate of protein synthesis and decreases the rate of protein degradation, thereby enhancing myofiber hypertrophy (Le Grand and Rudnicki, 2007; Rhoads et al., 2009). Genetic variation influences *IGF-I* expression; extensive progress has been made towards understanding the molecular biology of this hormone (Rotwein, 1999), and has made the *IGF-I* gene a suitable target for genetic manipulation (Pursel et al., 2004) and an attractive candidate gene for identifying DNA polymorphisms, both in humans (Rasmussen et al., 2000) and in domestic species (Ge et al. 2001). The genetic variation of plasma *IGF-I* in crossbred ewe lambs post-weaning was evaluated together with its potential use as a physiological marker for selection in meat sheep (Afolayan and Fogarty, 2008). Hu sheep are characterized by earlier sexual maturity, greater fecundity, low lean meat percentage, and low growth rate (Zhang, 2003). The aim of the present study was to investigate the association between *DLK1* and *IGF-I* gene expression and the meat quality of Hu Sheep.

MATERIAL AND METHODS

Experimental animals

Thirty-six Hu sheep were reared intensively under the same conditions; their diets con-

tained adequate minerals and vitamins. Animals were slaughtered at 6 different growth stages (2 days old and 1, 2, 3, 4, and 6 months old), including 6 animals per stage and an equal number of males and females. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Jiangsu Province and of the Animal Care and Use Committee of the Chinese Ministry of Agriculture. The protocol was approved by the government of Jiangsu Province (Permit Number: 45) and the Ministry of Agriculture of China (Permit Number: 39). All efforts were made to minimize the animals' suffering. Samples were collected from the longissimus dorsi muscle tissues within 10 min of slaughter, immediately frozen in liquid nitrogen after collection, and stored at -80°C before RNA isolation. In order to measure the diameter and density of muscle fiber and muscle tenderness, muscle tissues were fixed in 4% formaldehyde and measured using the paraffin sectioning method.

DLK1 and *IGF-I* gene expression

Total RNA was extracted from longissimus dorsi muscle tissues according to Trizol Regent Kit instructions (Takara Biotechnology Dalian, Co. Ltd., Dalian, China). RNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA with purity (A_{260}/A_{280}) >1.8 was used. Total RNA was detected by 1.2% agarose gel electrophoresis. Approximately 250 ng total RNA from each sample was transcribed into cDNA using a reverse transcription kit (Takara Biotechnology) according to manufacturer instructions. Real-time polymerase chain reaction (PCR) was performed using an FQ-PCR ABI 7900 for the *DLK1*, *IGF-I*, and *18S* genes according to standard protocols with the primers indicated in Table 1. The 18S ribosomal RNA gene (eukaryon) was used as a reference gene to analyze *DLK1* and *IGF-I* gene expression. The annealing temperature ranged from 53°-63°C and primer concentrations were optimized according to the SYBR Green I kit system (Takara Biotechnology). Optimum reaction conditions were a 10-μL reaction volume, which included 0.2 μL of each primer, 0.2 μL ROX Reference Dye, 3.4 μL H_2O, 5 μL SYBR Green Real-Time PCR Master Mix, and 1 μL cDNA template. PCR conditions were 40 cycles for 15 s at 95°C, 5 s at 95°C, and 30 s at 60°C. Additionally, 1 μL sterile water (instead of template) was used as a negative control and 3 parallel experiments were included each time PCR was conducted. Fluorescent signals were analyzed and transformed into copy number (Ct value) for the *DLK1* and *IGF-I* genes based on ΔCt as Ct (target gene) minus Ct (reference gene). For the comparison of Hu sheep of the same age but of different sex, ΔΔCt was determined as ΔCt (male) minus ΔCt (female). The ΔΔCt was ΔCt (other months old) minus ΔCt (2 days old) when individual Hu sheep were of the same sex but different ages. The $2^{-\Delta\Delta Ct}$ represented the differential expression of target genes between the experimental group and the control group.

Table 1. Primer sequence of *DLK1*, *IGF-I*, and *18S* genes for RT-PCR.

Gene	Reference sequence	Primers [forward (F) and reverse (R)]	Products (bp)
DLK1	NM-174037	F: CGTCTTCCTCAACAAGTGCGA R: TCCTCCCCGCTGTTGTAGTG	102
IGF-I	M30653	F: TCCAGTTCGTGTGCGGAGA R: TCCTCAGATCACAGCTCCGG	126
18S (eukaryon)	AY753190	F: CGGCTACCACATCCAAGGAA R: GCTGGAATTACCGCGGCT	187

Statistical analysis

SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used to calculate the Ct value and standard error among repeating samples. Difference in relative gene expression level was analyzed using the $2^{-\Delta\Delta Ct}$ method (Liu, 2007). Two-factor analysis of variance was used to measure the interaction between sex and various development stages after birth. Additionally, we examined whether *DLK1* and *IGF-I* expression were affected by sex and growth stages. The mRNA transcriptional quantity was indicated as the average ± standard error. A histogram of ΔCt was used to verify the conclusion that the value of ΔCt had a negative relationship with transcriptional quantity.

RESULTS

Determination of muscle fiber indicators

We measured muscle fiber diameter (Table 2), density (Table 3), and shear stress (Table 4) in different sex and growth stages to analyze the correlation between these factors and the relative expression of *DLK1* and *IGF-I*. Table 2 shows that Hu sheep muscle fiber diameters were extremely and significantly different ($P < 0.01$) among different growth stages in the same sex; however, in the same growth stage, the differences between rams and ewes were not significant ($P > 0.05$) before 2 months of age. After 2 months, the differences became significant. Table 3 shows that muscle fiber density in 2-day-old sheep was significantly greater than in other growth stages; with growth stage, muscle fiber density decreased, reaching a minimum at 6 months old. In the same growth stage, the muscle fiber density in ewes was greater than that in rams, and except for those 2 days old and 6 months old, the differences between rams and ewes were significant ($P < 0.05$) in other growth stages. Table 4 shows that for sheep of the same sex, muscle fiber tenderness was inversely proportional to muscle fiber shear stress decreased with growth stage; at the same growth stage, muscle fiber tenderness in ewes was higher than in rams. Except for 2-day-old and 1-month-old sheep, the differences between rams and ewes were significant between growth stages ($P < 0.05$).

Table 2. Comparison of muscle fiber diameter in different genders and at different growth stages.

Groups	Two days old	One month old	Two months old	Three months old	Four months old	Six months old
Male	$7.603 \pm 0.139^{m,F}$	$9.634 \pm 0.218^{m,E}$	$12.241 \pm 0.231^{m,D}$	$14.475 \pm 0.43^{m,C}$	$20.358 \pm 0.917^{m,B}$	$27.320 \pm 0.912^{m,A}$
Female	$7.191 \pm 0.228^{m,F}$	$9.356 \pm 0.225^{m,E}$	$11.022 \pm 3.228^{m,D}$	$13.943 \pm 0.186^{n,C}$	$19.978 \pm 0.255^{n,B}$	$26.230 \pm 0.738^{n,A}$

Serial (A), (B), and (C) show the results of multiple comparisons of the same sex at different stages. Means with different lowercase letters within the same row indicate a significant difference between different columns. Means with different capital superscript letters within the same row indicate extremely significant differences between different columns. Serial (m) and (n) indicate the results of multiple comparisons of the same stage for different sex. Means with different lowercase letters within the same column indicate significant differences between different rows. Means with different capital superscript letters within the same column indicate extremely significant differences between different rows.

Table 3. Comparison of muscle fiber density in different genders and growth.

Groups	Two days old	One month old	Two months old	Three months old	Four months old	Six months old
Male (root/mm^2)	4647.329 ± 132.594^{m,a}	4347.165 ± 47.566^{n,b}	3743.103 ± 758.438^{n,b}	3629.118 ± 92.445^{n,c}	3093.416 ± 22.155^{n,d}	1871.076 ± 130.173^{m,e}
Female (root/mm^2)	4780.541 ± 19.410^{m,a}	4524.395 ± 106.395^{m,b}	4349.370 ± 57.525^{m,b}	3874.513 ± 58.902^{m,c}	3286.724 ± 151.360^{m,d}	2067.813 ± 139.234^{m,e}

Serial (a), (b), and (c) show the results of multiple comparisons of in the same sex at different stages. Means with different lowercase letters within the same row indicate a significant difference between different columns. Means with the different capital superscript letters within the same row indicate extremely significant differences between different columns. Serial (m) and (n) show the results of multiple comparisons of the same stage for different sexes. Means with different lowercase letters within the same column indicate significant differences between different rows. Means with different capital superscript letters within the same column indicate extremely significant differences between different rows.

Table 4. Comparison of muscle fiber shear stress in different genders and growth stages.

Groups	Two days old	One month old	Two months old	Three months old	Four months old	Six months old
Male (root/mm^2)	2.470 ± 0.129^{m,eD}	2.764 ± 0.064^{m,dC}	2.847 ± 0.516^{m,bcB}	2.830 ± 0.332^{m,cC}	2.912 ± 0.050^{m,abA}	2.968 ± 0.065^{m,aA}
Female (root/mm^2)	2.295 ± 0.114^{m,dC}	2.642 ± 0.139^{m,cB}	2.741 ± 0.412^{n,bcB}	2.767 ± 0.542^{n,bB}	2.813 ± 0.106^{n,aB}	2.882 ± 0.232^{n,aA}

Serial A (a), B (b), and C (c) show the results of multiple comparisons for the same sex at different stages. Means with different lowercase letters within the same row indicate a significant difference between different columns. Means with different capital superscript letters within the same row indicate extremely significant differences between different columns. Serial (m) and (n) show the results of multiple comparison of the same stage for different sexes. Means with different lowercase letters within the same column indicate significant differences between different rows. Means with different capital superscript letters within the same column indicate extremely significant differences between different rows.

Specificity of the amplified fragment

The dissociation curves of *DLK1*, *IGF-I*, and *18S* PCR products showed sharp single peaks (Figure 1). This indicates that dimers of primers were not produced and that the primers were specific. The melting temperatures of *DLK1*, *IGF-I*, and *18S* genes were 86.7°, 87.2°, and 85.0°C, respectively, and no amplification product was generated in the negative control sample. The efficiencies of *DLK1*, *IGF-I*, and *18S* gene amplifications were 99.7, 99.8, and 99.8%, respectively.

DLK1 gene expression

Table 5 and Figure 2 show that there was an interaction between sex and various development stages after birth for *DLK1*, but the values were not significant (P = 0.143). Growth stage was significantly related to *DLK1* expression (P = 0.000), while sex was not (P = 0.481).

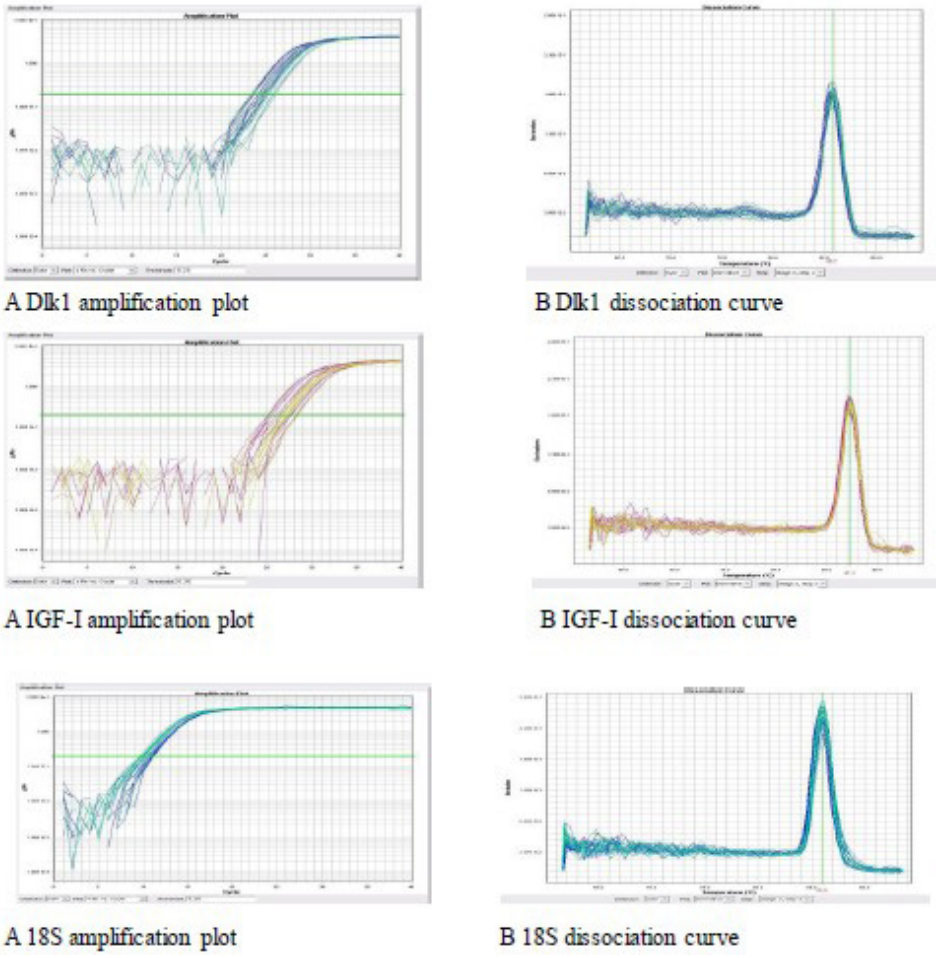

Figure 1. Amplification plot and dissociation curve of *DLK1*, IGF-1, and 18S genes.

Table 5. Interaction of gender and various development stages after birth in *DLK1*.

Source	Type III sum of squares	d.f.	Mean squares	F	P
Growth stage	59.098	5	11.820	20.907	0.000
Gender	0.283	1	0.283	0.500	0.481
Growth stage x gender	4.798	5	0.960	1.698	0.143

Expression of the *DLK1* gene in the sheep longissimus dorsi gradually increased with growth development stage (Figure 3). However, *DLK1* expression at 2 months of age was lower than that at 1 month, and then gradually increased in the following stages, peaking at 6 months of age. Table 6 shows that the expression of *DLK1* at 6 months of age was significant ($P < 0.01$) and greater than at 1, 2, 3, and 4 months old. The expression of *DLK1* at 1, 2, 3, and 4 months old was significant ($P < 0.01$) and greater than in 2-day-old sheep.

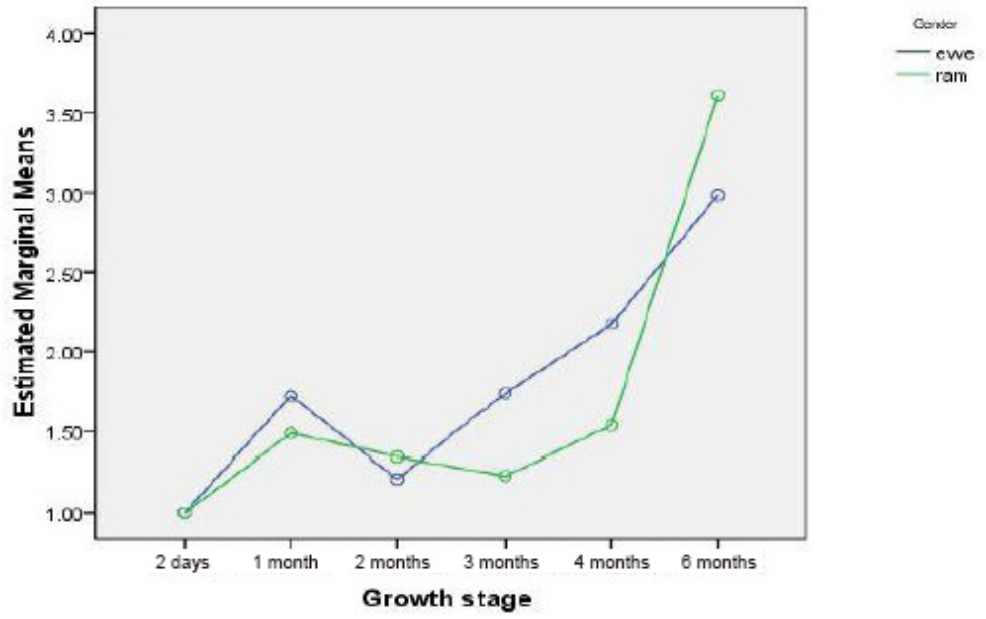

Figure 2. Interaction between gender and various development stages after birth for *DLK1*.

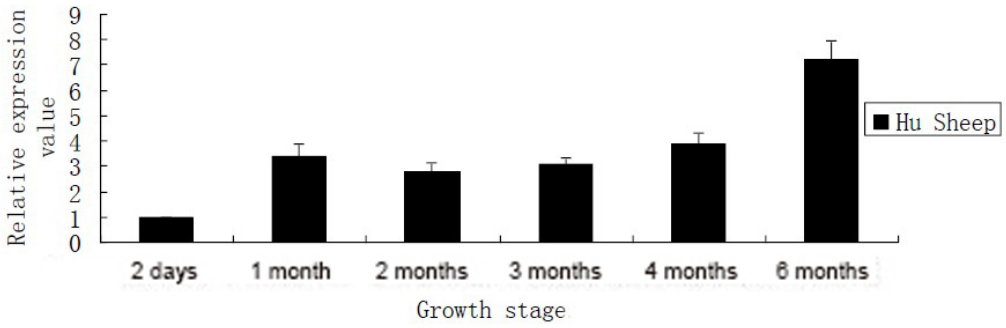

Figure 3. Changes in *DLK1* expression among Hu sheep at different development stages ($2^{-\Delta\Delta Ct}$ method, 2 days old for the control group).

Table 6. Interaction of gender and various development stages after birth in *IGF-1*.

Source	Type III Sum of squares	d.f.	Mean squares	F	P
Growth stage	60.508	5	12.102	9.514	0.000
Gender	0.155	1	0.155	0.122	0.728
Growth stage x gender	7.758	5	1.552	1.220	0.306

IGF-I gene expression

Table 7 and Figure 4 show that sex was correlated with various development stages after birth for IGF-I, but the value was not significant (P = 0.306). Growth stage had a significant effect on *IGF-I* expression (P = 0.000), while sex did not (P = 0.728).

Table 7. Variance analysis of *DLK1* and *IGF-I* expression among different development stages.

Groups	Two days old	One month old	Two months old	Three months old	Four months old	Six months old
DLK1	1^{C}	3.4194 ± 0.46945^{B}	2.7799 ± 0.36339^{B}	3.1012 ± 0.24157^{B}	3.8825 ± 0.40471^{B}	7.2251 ± 0.73703^{A}
IGF-I	1^{C}	3.9739 ± 0.71000^{B}	3.4708 ± 0.23705^{B}	3.1100 ± 0.38902^{B}	4.0652 ± 0.49917^{B}	7.2837 ± 1.05843^{A}

Serial of (A), (B), (C) show the results of multiple comparison of Hu sheep at different stages. Means with different capital superscript letters within the same row show that there are extreme significant differences between different columns.

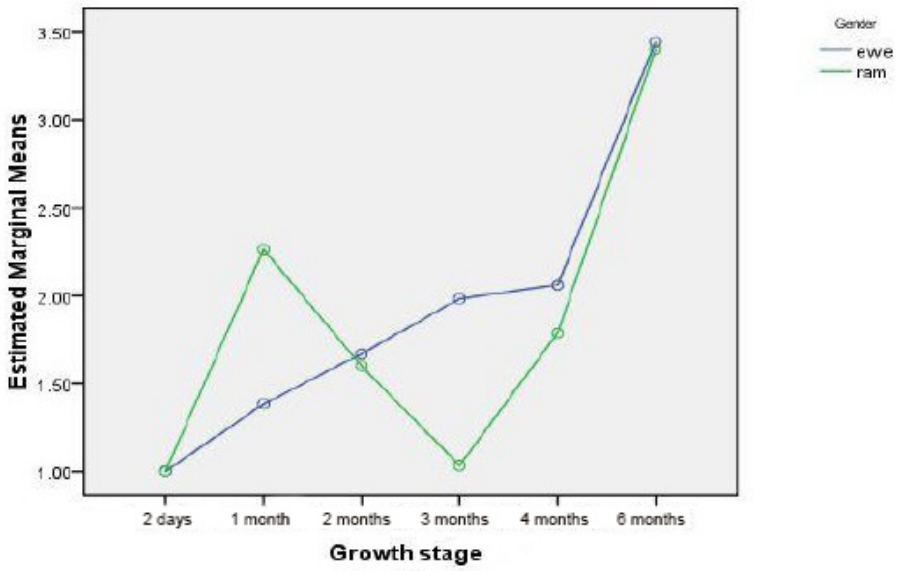

Figure 4. Interaction between gender and various development stages after birth for IGF-I.

IGF-I expression in the sheep longissimus dorsi increased during the first 2 stages and then continuously decreased until 3 months of age; thereafter, *IGF-I* expression gradually increased and reached the peak at 6 months of age (Figure 5). Table 6 shows that the expression of *IGF-I* in sheep that were 6 months of age was significant (P < 0.01) and greater than at 1, 2, 3, and 4 months of age. *IGF-I* expression at 1, 2, 3, and 4 months of age was significant (P < 0.01) and greater than that in 2-day-old sheep.

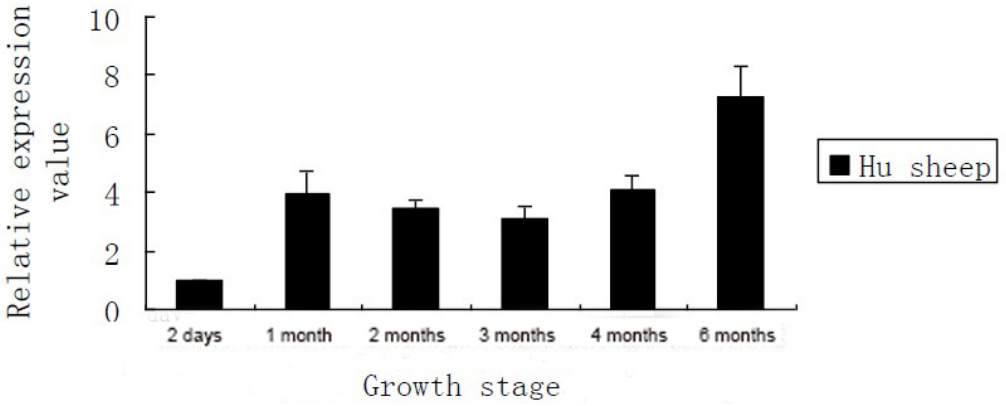

Figure 5. Changes in *IGF-I* expression among Hu sheep in different development stages ($2^{-\Delta\Delta Ct}$ method, 2 days old for the control group).

Association between *DLK1* and *IGF-I* gene expression and meat quality

DLK1 and *IGF-I* gene expression was positively and significantly ($P < 0.01$) correlated with muscle fiber diameter and muscle fiber shear stress, but negatively and significantly ($P < 0.01$) correlated with muscle fiber density. Muscle fiber diameter was positively and significantly ($P < 0.01$) correlated with muscle fiber shear stress, and negatively and significantly ($P < 0.01$) correlated with muscle fiber density. In addition, DLK-1 expression was significantly ($P < 0.01$) and positively correlated with *IGF-I* expression (Table 8).

Table 8. Association of *DLK1* and *IGF-I* gene expression with meat quality traits.

Index	*DLK1*	*IGF-I*	Muscle fiber diameter	Muscle fiber density	Muscle fiber shear stress
DLK1	1	0.576**	0.658**	-0.659**	0.453**
IGF-I	0.576**	1	0.509**	-0.515**	0.401*
Muscle fiber diameter	0.658**	0.509**	1	-0.987**	0.760**
Muscle fiber density	-0.659**	-0.515**	-0.987**	1	-0.776**
Muscle fiber shear stress	0.453**	0.401*	0.760**	-0.776**	1

*Shows that there are significant relationship between two different index ($0.01 < P < 0.05$); **shows that there are extreme significant relationship between two different index ($P < 0.01$).

DISCUSSION

Selective breeding programs with the goal of increasing the productivity and profitability of the sheep meat industry use elite, progeny-tested sires. The broad genetic traits of primary interest in the progeny of these sires include skeletal muscle yield, fat content, meat quality, and reproductive efficiency. Natural mutations in sheep that enhance muscling have been identified, while a number of genome scans have identified and confirmed quantitative trait loci for skeletal muscle traits (Tellam et al., 2012).

The *DLK1* gene is the ligand of the Notch signaling pathway, inhibits the differentiation of adipocytes, and regulates muscle development (Lee et al., 1995). In transgenic mice, skeletal muscle *DLK1* is overexpressed, caused hypertrophy traits and affected muscle growth; similarly, a double muscled trait was caused in sheep (Davis et al., 2004; Magee et al., 2011; Bi and Kuang, 2012). In the present study, *DLK1* expression was higher in the longissimus dorsi of males than in females at different growth stages after birth. It is well-documented that muscle growth is faster and muscle fiber is thicker in rams than in ewes; ewes accumulate fat more easily. In addition, sex hormones may also affect the growth of skeletal muscle, and thus may inhibit fat cell differentiation and regulate muscle development (Davis et al., 2004). The expression of *DLK1* gene beginning at birth through 12 weeks of age gradually increased for the NN and NCpat phenotypes in muscle, but NCpat phenotype individuals showed a more significant increase (White et al., 2008). In addition, Fleming-Waddell et al. (2009) showed that the weight of both NCpat and NN phenotype individuals increased linearly after birth, but the slope in NCpat growth weight was greater than that in NN, suggesting that the NNpat genotype grows faster. In the present study, expression of the *DLK1* gene generally increased gradually in the longissimus dorsi in both rams and ewes, reaching a maximum at 6 months of age. We found very significant or significant differences for the different stages. Therefore, *DLK1* may regulate the activity of muscle development and promote muscle growth (White et al., 2008; Fleming-Waddell et al., 2009).

Previous studies have suggested that fetal *IGF-I* and *IGF-II* are independently regulated in sheep fetal circulation and show a different expression pattern (Fowden and Silver, 1995; Oliver et al., 1996). Expression of the *IGF-I* and *IGF-II* genes depend on the origin of the cells and their development stage (Wang et al., 2011). *IGF-I* and *IGF-II* mRNA was up-regulated in rat and ovine muscle satellite cells from proliferation to differentiation (Wang et al., 2011). In contrast, *IGF-II* gene expression was decreased in turkey satellite cells from proliferation to differentiation (Ernst et al., 1996). In addition, *IGF-I* expression was not detected in humans (Crown et al., 2000) or avian (Kocamis et al., 2001) satellite cells. The expression patterns of IGF for *in vitro* muscle cells suggested autocrine regulation of myogenesis by IGFs (Wang et al., 2011). In the present study, the *IGF-I* gene showed significantly higher expression in the ram longissimus dorsi than in that of ewes at different growth stages after birth, suggesting that the difference between rams and ewes was associated with growth stage. In contrast, Xu (2002) indicated that *IGF-I* expression was not significantly different ($P < 0.05$) between the male and female in longissimus dorsi. The previous study suggested that the differences in *IGF-I* gene expression in muscle between rams and ewes were associated with the species type or variety. Consistent with the results of Gu et al. (2009) and Huang and Xie (2009), *IGF-I* expression in the ewes' longissimus dorsi was gradually increased with their growth and reached a peak at 6 months of age. Gerrard et al. (1998) found that *IGF-I* mRNA expression increased gradually from 44 days of pregnancy to the late pregnancy in the pig fetal semi-tendinosus and further increased after birth, peaking at 21 days old and declining during adulthood. Gotz et al. (2001) used immunohistochemistry methods to find that *IGF-I* expression in the pig skeletal muscle remained at the same level as that in the blood from 11-22 weeks old.

Nueda et al. (2008) found that it was beneficial to release *IGF-I* when the *DLK1* protein was bound to the membrane with the IGFbp1/*IGF-I* complex in cells, which increased the local *IGF-I* concentration to enhance receptor signaling and fat formation. The result of the study revealed a positive correlation between *DLK1* and *IGF-I* expression in muscle (Nueda et al., 2008). Similarly, in the present study, we identified significant ($P < 0.05$) and positive

correlation between the expression of *DLK1* and *IGF-I*. Abe et al. (2009) indicated that *IGF-I* expression showed the same trend as that of MyHC 2b from prenatal days 16-18. The increase in MyHC 2b expression enlarged the muscle fiber diameter; thus, IGF-I may be a regulatory factor associated with the growth of muscle and muscle fiber diameter enlargement. The Hu sheep meat quality traits are affected by muscle fiber diameter, density, and muscle tenderness. However, muscle growth is not included on this list because of the increase in muscle fiber number; however, this feature may be related to the thickness of the cross-sectional area of muscle fibers. With increased thickness of muscle fibers, intramuscular fat increased and muscle fiber density declined as muscle tenderness was reduced.

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