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Transcriptome analysis of skeletal muscle at prenatal stages in Polled Dorset versus Small-tailed Han sheep

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ABSTRACT. The objectives of the present study were to identify additional genes that may play important roles in the regulation of skeletal muscle growth and development, and to provide fundamental information for understanding the underlying molecular mechanisms. Eighteen cDNA libraries were constructed from the longissimus muscle of Polled Dorset (PD) and Small-tailed Han (SH) fetuses. To reveal the differences between the two species, we analyzed the differences in gene expression in 60-, 90- and 120-day fetal skeletal muscle by applying Agilent ovine genome-wide microarray. In this study, we obtained 17,704 genes using a chip containing 39,242 probes. There were 88 differentially expressed genes in the 60-day group ($P < 0.05$), 128 genes in the 90-day group ($P < 0.05$), and 340 genes in the 120-day group ($P < 0.05$) between the two breeds. The differentially expressed genes were grouped in different GO categories and signaling pathways. These

results suggested that there are many genetic differences in the muscle growth and development transcriptomes between these two breeds. This study laid the foundation for future genomic research in sheep.

Key words: Skeletal muscle; Transcriptome; Gene expression; Sheep

INTRODUCTION

Prenatal skeletal muscle development is an important determinant factor for muscularity and meat quality (Rehfeldt et al., 2004). In large precocial species such as sheep (Ashmore et al., 1972; Maier et al., 1992) and cattle (Russell and Oteruelo, 1981), the maximum myofiber complement of a muscle is achieved prior to birth. More than three waves of myogenic cells appear in sheep, and most myofibers form after 60 days of gestation (Wilson et al., 1992; McCoard et al., 2000). Among many factors, genetics is the most fundamental. With the development of high-throughput analytical technologies, the relationships between various genetic factors from a global and systematic aspect become easy to understand. Polled Dorset sheep (PD) is a foreign breed with a white wool-free face. Its main distinguishing features are hornless appearance, long, lean square body set on short legs, pink skin and 'spongy' short-stapled wool. The characteristics of this sheep breed such as rapid growth rate, superior fleshing and muscular development as well make it ideally suited for the meat trade. Also, PD rams are the most commonly used as sires for the production of prime lambs, while the Small-tailed Han sheep (SH) is an excellent local breed in China because of its significant characteristic of hyperprolificacy, with a mean litter size of 2.61(National Commission for Livestock and Poultry Genetic Resources , 2011). Also, with long, strong limbs and an elliptical fan-shaped tail, it has a slower growth rate and is a dual-purpose breed, where it is used as a female parent in cross breeding programs. In the present study, we performed transcriptomic analysis of longissimus muscle, collected from PD and SH sheep by using the specialized transcriptome-wide sheep oligo DNA microarray. Our objectives were to identify some putative candidate genes that are valuable for meat yield and quantity and to provide a theoretical basis for understanding the molecular mechanisms underlying muscle growth and development.

MATERIAL AND METHODS

Animals and tissue preparation

All animal procedures were performed according to protocols approved by the Committee on Animal Care and Use for Biological Studies, Shandong Province, China. PD and SH ewes (nine for each breed) were selected on the basis of similar age, body weight and body size. After these animals were subjected to pre-feeding for 45 days, they were mated with the ram of corresponding breed, and the date of mating was recorded. The fetuses were selected at 60, 90 and 120 days of gestation, and the longissimus muscle tissue was rapidly and manually dissected from each fetus. These samples were snap-frozen in liquid nitrogen and stored at -80°C until further use. At least three fetuses from each stage were used for constructing the library. Subsequently, skeletal muscles from 18 fetuses were used for QPCR validation. Total RNA was extracted from the frozen longissimus muscle using TRIzol reagent (Invitrogen Inc., Carlsbad, CA, USA) according to manufacturer instructions. RNA integrity was evaluated by

gel electrophoresis and RNA purity was checked by the 28S/18S ratio and RIN value. RNA samples with RIN value greater than seven and 28S/18S ratio greater than 0.7 were selected for microarray analysis.

Microarray experiment

The new specialized and standardized transcriptome-wide sheep microarray (Agilent Technologies, Santa Clara, CA, USA) contained 39,242 sheep probes sourced from RefSeq and UniGene. The oligo DNA microarray was 44,000 nucleotides in size. Total RNA was amplified and labeled by Low Input Quick Amp Labeling kit, One-Color (Agilent Technologies), following manufacturer instructions. Labeled cRNA was purified using the RNeasy mini kit (QIAGEN, GmBH, Germany). Each slide was hybridized with 1.65 μg Cy3-labeled cRNA using the Gene Expression Hybridization kit (Agilent Technologies) in a Hybridization Oven (Agilent Technologies), according to manufacturer instructions. After 17-h hybridization, slides were washed in staining dishes (Thermo Shandon, Waltham, MA, USA) with Gene Expression Wash Buffer kit (Agilent Technologies), following manufacturer instructions. Slides were scanned with an Agilent Microarray Scanner (Agilent Technologies) with default settings, i.e., dye channel: green, scan resolution = 5 μm, PMT 100%, 10%, 16 bit.

Statistical analysis of microarray data

Data were extracted with Feature Extraction software 10.7 (Agilent Technologies). Raw data were normalized by the Quantile algorithm, Gene Spring Software 11.0 (Agilent Technologies). One-way ANOVA and the *t*-test were used for screening differentially expressed genes (DEGs), on the premise that no relationships existed between the data at different time points. The thresholds for significance were $P < 0.05$ and a 2-fold change.

Gene Ontology (GO) enrichment analysis

The GO project is a major bioinformatics initiative with the aim of standardizing the representation of gene and gene product attributes across species and databases. The project provides a controlled vocabulary of terms for describing gene product characteristics and gene product annotation data from GO Consortium members, as well as tools to access and process these data. In this study, the analysis was performed by DAVID database, taking $P < 0.05$ as the threshold. This kind of analysis is able to recognize the main biological functions that DEGs exercise.

Quantitative real-time PCR (qRT-PCR) for microarray data

cDNA was synthesized from 1 μg total RNA using the PrimeScript RT reagent kit (Takara Technologies, Code: DRR037A) in a 20-μL reaction mixture. qRT-PCR was performed and the amount of amplified DNA was monitored by fluorescence at the end of each cycle using CFX 96 (Bio-Rad Laboratories). Thermal cycling consisted of an initial step at 95°C for 110 s followed by 40 cycles at 95°C for 10 s and 61°C for 33 s. *RPLP0* was chosen as the reference gene for normalization of all data because it was expressed more stably (Laborda, 1991; Fleming-Waddell et al., 2007; Vuocolo et al., 2007; White et al., 2008). Each qRT-PCR

(in 20 µL) contained 10 µL 2X real-time mix, 0.8 µL of each primer, 2 µL normalized template cDNA, and 6.4 µL water. Each plate was repeated three times in independent runs for all reference and selected genes. Gene expression was evaluated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Gene expression ratios were normalized to the *RPLP0* gene in the same sample, and the primer information are shown in Table 1.

Table1. Primer sequences and PCR product sizes of genes selected for validation by qRT-PCR.

Gene		Primer sequence (5'-3')	Annealing temperature (°C)	Product size
CD19	F	CTGTGCTCCACCTAACAGCA	60	252
	R	TCGGGCAGTAACAGAACCAC	60	
MRCL3	F	TGCTGACCACAATGGGAGAC	60	293
	R	AGAGATGCATGTGCCCCAAA	58	
MSTN	F	TGAGAACAGCGAGCAGAAGG	60	183
	R	GGAGCCTTGGGCAAAAGTTG	60	
IFNAR1E	F	GAAAACAGCTGCACATGCCA	58	637
	R	AAATGCCACCCCTCAGGATG	60	
HOXA5	F	AGACCCTGGAGCTGGAGAAG	62	93
	R	TCTCCGAGAGGCAAAGAGCA	60	
CA4	F	CGTGCAAAACAACGGGCATA	58	241
	R	CTCATCTTCGGCGAACTGGT	60	
MYO9A	F	GGCTGCTGAGGTGATTGACT	60	168
	R	CGATTTTCCAGAGCCATGCG	60	
CLDN7	F	CCATGACGGGAGGCATCATT	60	225
	R	CAGCTTTGCTCTCACTCCCA	60	
TPM4	F	GCCGAGGTGTCCGAACTAAA	60	167
	R	CGGGTCTCAGCCTCTTTCAG	62	
GATAD2A	F	CTCTGCTACTGCTCATCCCG	62	343
	R	CCCAAACGCAAACCCTGAAG	60	
CAV1	F	AGGGCAACATCTACAAGCCC	60	121
	R	TCGTTGAGATGCTTGGGGTC	60	
MYOT	F	ACTCCATCAAAGCCAGGAGC	60	128
	R	GGGGTGGGGTCTTGAATCTG	62	
GAPDH	F	CCGCATCCCTGAGACAAGAT	60	209
	R	TTCCCGTTCTCTGCCTTGAC	60	
RPLPO	F	TCTCCTTCGGGCTGGTCATC	62	131
	R	GCACACGCTGGCAACATTG	60	

RESULTS

Microarray data summary

Eighteen cDNA libraries were constructed using longissimus muscles from PD and SH fetuses. We applied the first specialized and standardized transcriptome-wide sheep oligo DNA microarray to determine gene expression levels. The results showed that the average detectable rate of all probe sets reached 71.14% (Table 2), which enabled us to evaluate the changes in gene expression. The raw data were normalized and evaluated. We analyzed gene expression levels of fetuses at three different stages of individuals of two sheep breeds, using a cutoff of |fold change| < 2 with $P < 0.05$. The volcano plot is shown in Figures 1 and 2. A pool of differentially expressed probes from the two groups was used for a systematic hierarchical clustering to gain insight into the transcriptome-wide similarities between 18 individuals (Figure 3). We found that individuals at the same developmental stage were clustered together, indicating that differences derived during development were larger than those from the genetic backgrounds between the breeds.

Table 2. Results for quality control of oligo DNA microarray.

Name	CV value (%)	Detectable rate (%)
SH60-1	5.67	74.53
SH60-2	4.58	72.68
SH60-3	4.66	72.9
SH90-1	4.12	71.82
SH90-2	5.55	72.00
SH90-3	4.4	70.88
SH120-1	4.95	67.66
SH120-2	4.93	68.52
SH120-3	5.35	66.26
PD60-1	4.55	71.3
PD60-2	4.84	73.65
PD60-3	4.38	69.1
PD90-1	5.89	70.51
PD90-2	4.93	71.15
PD90-3	4.48	72.66
PD120-1	5.04	68.76
PD120d-2	5.07	68.84
PD120-3	4.68	70.82

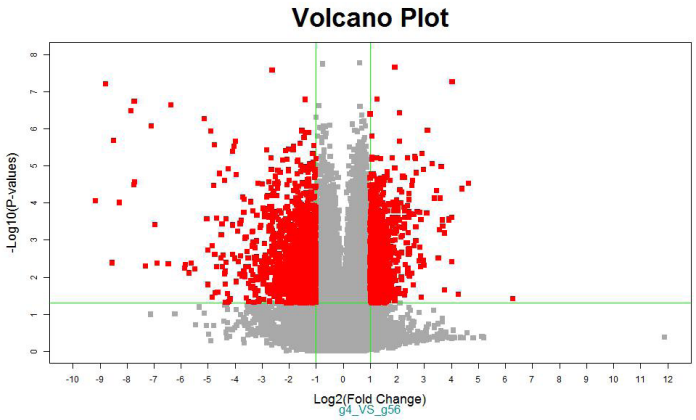

Figure 1. Volcano plot of PD at different stages.

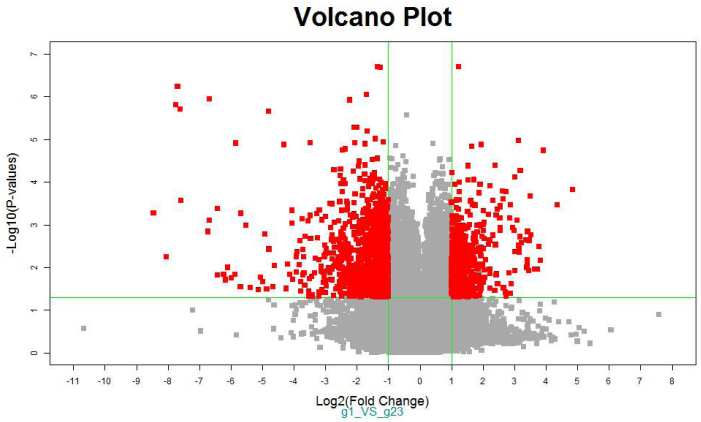

Figure 2. Volcano plot of SH at different stages.

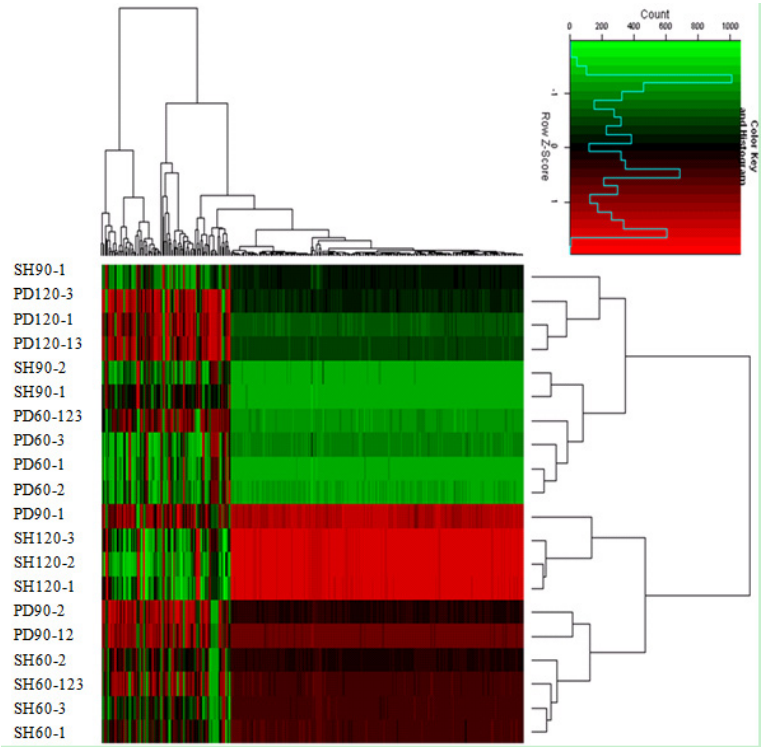

Figure 3. Hierachical clustering of differentially expressed genes.

Gene expression in skeletal muscle at various developmental stages between two sheep breeds

At 60-day stage, a total of 88 genes were differentially expressed between the two sheep breeds, including 65 known genes, in which 33 genes were upregulated and 32 downregulated in PD. The *PPP1R15A* and *MRPL1* genes were uniquely expressed in SH, and the *RARG*, *CA4* and *C1QTNF1* genes were uniquely expressed in PD. At 90-day stage, a total of 128 genes were shown to be significantly differentially expressed between the two breeds, including 97 known genes, in which 39 genes were upregulated and 58 downregulated in PD. The *ITGBL1*, *CPXM2*, *MRPL1* genes were uniquely expressed in SH, and *SIGLEC14* and *APOBEC3G* were uniquely expressed in PD. At 120 days, a total of 340 genes were differentially expressed between the two breeds, including 280 known genes. Compared with PD, 231 genes were upregulated and 49 downregulated in SH. The *HPSE*, *RAB33B*, *MRPL1*, and *PPP1R15A* genes were uniquely expressed in SH, and *F2*, *ADIPOQ*, *ASGR1*, *FABP4*, *MGAT1*, *HINT1*, and *HLA-DQB1* were uniquely expressed in PD. The numbers of known DEG at various developmental stages between the two breeds were 65, 97 and 280, respectively, and the upregulated genes were 33, 39 and 231 in PD sheep, as shown in Figure 4. With fetal growth and development, the number of DEGs increased, consistent with the rules of sheep skeletal muscle development.

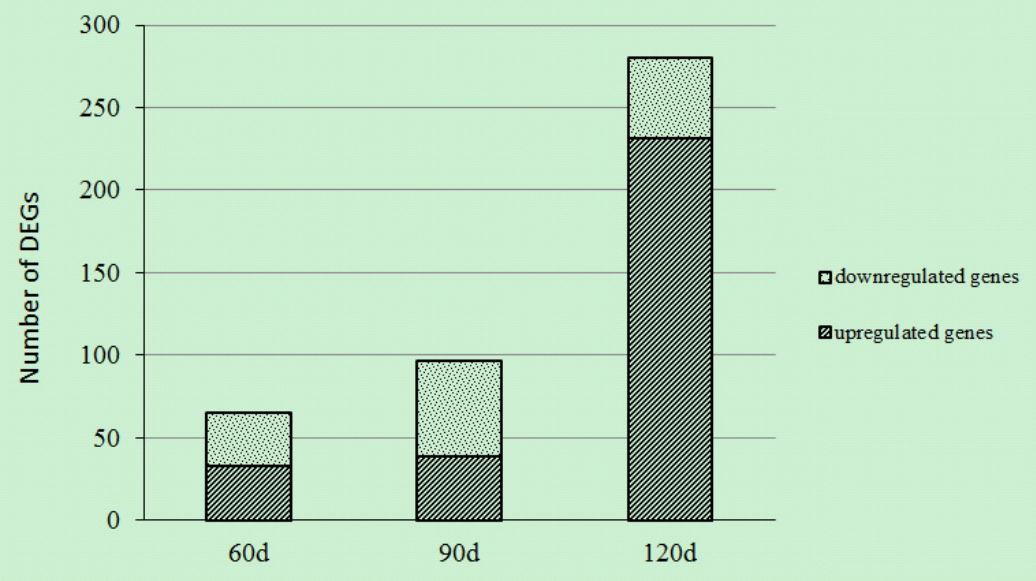

Figure 4. Number of differentially expressed genes (DEGs) between two breeds.

DEG GO analysis

To gain further insight into the biological importance of the DEGs, a GO analysis was performed by applying the DAVID database, and the results are shown in Figure 5A, B and C. The DEGs were grouped in the cellular component, molecular function and biological process categories. In general, the categories of molecular function involved in myogenesis were similar between PD and SH sheep. However, the numbers of DEGs involved in certain biological processes were quite different between PD and SH. For instance, more genes involved in cyclase activity and toxin binding were expressed in SH. In contrast, more DEGs involved in ectopic enzyme activity, demethylation activity of bone structure, muscle structure composed of prominent vitamin transport activity, lipid-binding aspects of metalloenzymes, and kinase activity were identified in PD. In the cellular component category, there were more genes involved in the intermediates and the outer edge of the structure of cells in SH, and more genes were grouped in the spindle fibers, perikaryon, killer cells, their positive or negative regulation of immune response, and immune system development in PD. For the biological process category, there were more genes involved in cell aging, control of virus propagation, fetal development and the developmental process of pigmentation, and organ growth in SH. Also, more genes were categorized in transposition, positive or negative regulation of virus propagation, embryo implantation, keratinization, positive and negative regulation of killer cells and apoptosis in PD.

DEG pathway analysis

A pathway analysis was performed by Biocarta and KEGG, to show the most repre-

sented pathways among the DEGs. Of the known genes, 582 DEGs in PD versus SH, were enriched in 327 pathways including 172 Biocarta and 155 KEGG pathways. A total of 934 DEGs in PD versus SH were enriched in 390 pathways including 220 Biocarta and 170 KEGG pathways.

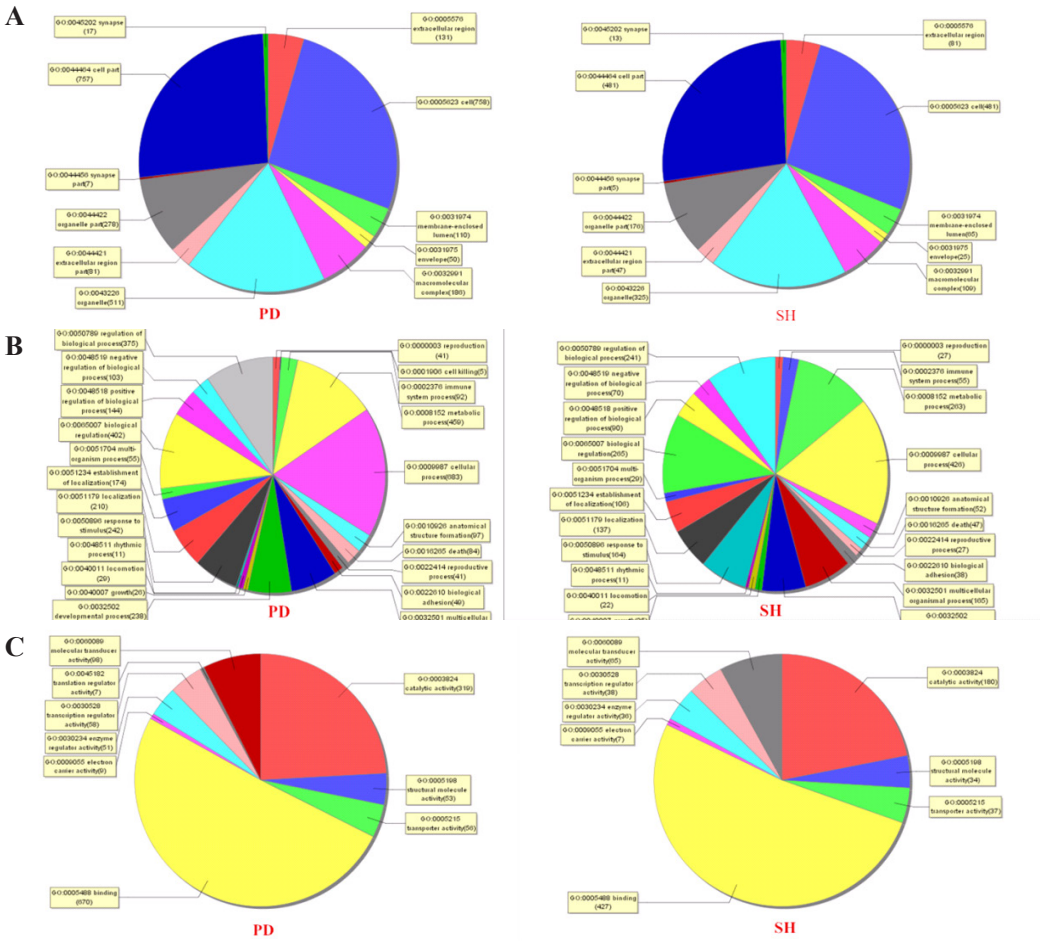

Figure 5. A. Results of GO analysis, which differentially expressed genes grouped in the cellular component categories; **B.** results of GO analysis, which differentially expressed genes grouped in the biological process categories; **C.** results of GO analysis, which differentially expressed genes grouped in the molecular function categories.

Validation of microarray data

To validate the microarray data，13 genes were randomly selected for qRT-PCR. Housekeeping genes such as those encoding β-actin (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), commonly used as internal controls for such analysis, were not suitable for normalization in these experiments, for their transcription was altered during myogenesis (Radonic et al., 2004; Pas et al., 2005). As in previous studies (Laborda, 1991;

Fleming-Waddell et al., 2007; Vuocolo et al., 2007; White et al., 2008), *RPLP0* was chosen as the ideal reference gene to normalize the data. The results for a panel of the 11 genes were in good agreement with the microarray data, indicating that our microarray data results reliably revealed the differences in gene expression profiles in skeletal muscle.

DISCUSSION

The sheep is an important domestic animal and model for many types of medically relevant research. An investigation of gene expression in ovine muscle would significantly advance our understanding of muscle growth. DNA microarray technology offers a novel approach to analyze transcriptome-wide expression profiling in cells or tissues under different stages or conditions. In this study the DEGs in sheep fetal skeletal muscle at different stages of gestation were analyzed, and the results that showed several genes were specifically expressed in PD and SH. *PPP1R15A* is a member of a group of genes whose transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents. It plays a general role in negative growth control, including growth suppression and apoptosis in many cell types. *MRPL1* may play a role in restricting the ability of bacteria to invade and grow *in vivo* (Rojo et al., 2007). However, its effects on muscle and adipose development is unknown. *RARG*, a member of the nuclear hormone receptor family, is also known as *NR1B3*, and is known to interact with *NCoR1* (Dowell et al., 1999). Loss of function of NCoR1 protein significantly increases the strength and power of mouse muscles (Yamamoto et al., 2011). *CA4*, a member of the *CA* family, belongs to the zinc metalloenzymes and has a role in inherited renal abnormalities of bicarbonate transport (Alvarez et al., 2007; Tian et al., 2010). *C1QTNF1*, a protein-coding gene, has a role in the regulation of glucose metabolic processes (Jeon et al., 2008; Chalupova et al., 2013). In our study, the *PPP1R15A* and *MRPL1* genes were uniquely expressed in SH, while the *RARG*, *CA4* and *C1QTNF1* genes were uniquely expressed in PD at the stage of 60 days, which suggests that these genes may play different roles in fetal growth and development in sheep. *ITGBL1*, a member of the EGF-like protein family, is associated with isolated growth hormone deficiency and abnormally short height. Too little growth hormone can cause short stature in children, and changes in muscle mass, cholesterol levels, and bone strength in adults (Eckman, 2010). *CPXM2*, a protein-coding gene, may be involved in cell-cell interactions. *SIGLEC14* is a putative adhesion molecule. Sialic acid-binding receptor may activate associated receptors (Angata et al., 2006). *APOBEC3G* is a member of the cytidine deaminase gene family, a family of proteins that has been suggested to play an important role in innate anti-viral immunity (Takaori, 2005). In the present study, the *ITGBL1*, *CPXM2*, *MRPL1* genes were only expressed in SH, and *SIGLEC14* and *APOBEC3G* only expressed in PD at 90 days. This suggested that they may play different roles in the two breeds. *HPSE* is involved in embryo development, heparan sulfate turnover, hair growth and wound healing (Baker et al., 2012). *RAB33B*, a member of RAS oncogene family, plays important roles in defined steps of vesicular transport in protein secretion and the endocytosis pathway (Zheng et al., 1998). *F2* plays a role in migration and proliferation of vascular smooth muscle cells, apoptosis and angiogenesis (Borissoff et al., 2010, 2011). *ADIPOQ* is expressed in adipose tissue exclusively and modulates a number of metabolic processes, including glucose regulation and fatty acid oxidation (Diez and Iglesias, 2003). *ASGR1*, encoding a subunit of the asialoglycoprotein receptor, plays a critical role in serum glycoprotein homeostasis (Benyair et al., 2011). *FABP4* encodes the fatty acid-binding pro-

tein found in adipocytes and delivers long-chain fatty acids and retinoic acid to their cognate receptors in the nucleus (Maeda et al., 2005). *MGAT1* encodes a protein that is considered to be essential for normal embryogenesis (Jacobsson et al., 2012). *HINT1* has been found to be a tumor-suppressing gene (Lee et al., 2004). *HLA-DQB1* belongs to the HLA class II beta chain paralogs, and it plays a central role in the immune system and is also expressed in skeletal muscle tissue (Murray et al., 2007). In our research, *HPSE*, *RAB33B*, *MRPL1*, and *PPP1R15A* were only expressed in SH, and *F2*, *ADIPOQ*, *ASGR1*, *FABP4*, *MGAT1*, *HINT1*, and *HLA-DQB1* only expressed in PD at the stage of 120 days. For the three prenatal stages studied, the *MRPL1* gene was especially expressed in SH in all stages. *PPP1R15A* was expressed in SH at 60 and 120 days. This suggested that these genes may be the genetic reasons for causing the skeletal muscle growth and development differences between PD and SH sheep in the embryonic stage. Additionally, the number of DEGs, i.e., 88, 128 and 340, was shown to be significantly differentially expressed between the two sheep breeds for the three stages. Also, the maximum number of DEGs was seen at 120 days, indicating that rapid myofiber proliferation included numerous genes during the myogenic process. With fetal growth and development, the number of DEGs increased, consistent with the rules of sheep skeletal muscle development. These results indicated that the difference in body weight between adult PD and SH sheep may depend on some DEGs at the embryonic stage.

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