Identification of the crucial molecular events during the large-scale myoblast fusion in sheep

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UNLIKE THE SMALL MAMMALS with two myogenic waves during development, there are at least three generations of myotubes in sheep (63). Myoblast formation in the sheep fetus begins at ~32 days of gestation, and the full complement of myofibers is achieved by days 80–120 of gestation (3, 39). Previous studies provide the valuable information on association between gene expression and prenatal skeletal muscle development in sheep at transcriptome-wide level (10, 50, 62). However, to our best knowledge, we still do not know the specific developmental stage at which large-scale fetal myoblasts undergo differentiation and fusion in sheep, or the ongoing molecular mechanisms underpinning the phenotypic variations in muscle during the fetal period.

Myogenesis is a multiple-step process, including cell determination, proliferation, differentiation, and fusion, that is orchestrated by the expression of a cascade of transcriptional regulators such as Pax3/Pax7, members of the MyoD, MEF2, and E2F family, and so on (9, 44, 59). Myoblast fusion is a critical process that contributes to the growth of muscle during development and to the regeneration of myofibers upon injury. Myoblasts fuse with each other as well as with multinucleated myotubes to enlarge the myofiber. At the cellular level, the fusion process is characterized by the alignment of myoblast and myotube membranes and rearrangement of actin cytoskeleton at contact sites followed by membrane fusion. Most of the findings in understanding the process of myoblast fusion during development come from studies of the fruit fly Drosophila melanogaster (1, 51). The current understanding of myoblast fusion in mammals comes largely from experiments with a myoblast cell culture model in which the fusion steps can be recapitulated in vitro. Studies have suggested that myoblast fusion in mammals is regulated by various cell adhesion proteins, including the α3-, α9-, and β-integrin subunits, neogenin, M- and N-cadherin, CD36, and a disintegrin and metalloprotease 12; transmembrane lipids, including cholesterol and phosphatidylserine; and intracellular domain-associated signaling or adaptor proteins, including β-catenin, end binding 3, kindlin-2, myoferlin, creatine kinase B, diacylglycerol kinase-ξ, Rac1, focal adhesion kinase (FAK), and syntrophin, which accumulate at sites of contact between two myogenic cells either in a symmetrical or an asymmetrical manner (1, 47). Recently, Hindi et al. (26) reviewed the roles of the major signaling pathways involved in mammalian myoblast fusion, including integrins and FAK, Rho guanosine triphosphatases, ERK5, Calcineurin-NFATc2, NF-κB, Wnt signaling, transforming growth factor (TGF)-β, and nitric oxide signaling (26). Unfortunately, few in vivo studies have been reported on this field, let alone in large mammals.

In addition, among the myogenic regulatory factors, E2F and MEF2 families respectively play important roles in myogenic differentiation. E2Fs function to regulate cell cycle. Lazaro et al. (33) demonstrated MyoD, Myf-5, and MEF2D failed to engage the differentiation program unless the cells were exposed to low levels of mitogens and withdrew from the cell cycle. This highlights the significance of regulation of cell cycle in myogenesis. In skeletal myogenesis, myocyte proliferation, differentiation, or apoptosis are coordinated at the level of E2F (25, 59), and downregulation of E2F1 mRNA is
required for myogenic differentiation in C2 and C2C12 myoblasts (56, 61). PIK3R1 (phosphoinositide-3-kinase, regulatory subunit 1) and FST ( follistatin) are not only target genes of E2F1 (8), but also important members of signal pathways that regulate myoblast proliferation, differentiation, and fusion (4, 21, 23, 29, 36, 52, 53). During differentiation, MEF2A, a member of MEF2 family, plays a determining role in regulating muscle cell differentiation and inducing muscle specific gene transcription in conjunction with bHLH (basic helix-loop-helix) transcription factors (5, 6, 42, 44, 60). MEF2 families also participate in transcriptional activation of slow-fiber-specific transcription by a combinatorial mechanism involving proteins of the NFAT (14). Moreover, TPM2, TNC, and CASQ1 (MEF2A targets) are exclusively expressed in slow or fast myofibers. Interestingly, most investigations involved in E2Fs, MEF2, and their targets are performed with the myoblast lines in mice. There are few studies for them in skeletal muscle during the prenatal stage in large mammal. Here, using the sheep as a large animal model, we identified the crucial myogenic stage and the molecular events involved in developing skeletal muscle during five fetal stages (70, 85, 100, 120, and 135 days of gestation) by histological and microarray analyses. Our investigations update the regulatory network of myogenesis as well as contribute to promote the efficiency of management and production in sheep.

MATERIALS AND METHODS

Materials

Animals and sample collection were described in our recent study (50). As indicated in our recent study (49), the animal experiment protocols were approved by the Biological Studies Animal Care and Use Committee, Shanxi Province, Peoples Republic of China. In the present study, we used an indigenous Chinese breed, Ujumqin sheep, for investigation of muscle development at six developmental stages, including 70, 85, 100, 120, 135 days of gestation and birth. Three fetuses were sampled at each stage of development, and the longissimus dorsi muscles (LM) were analyzed.

Histological Analysis

Serial cross sections 10 μm thick were cut at −20°C with a cryostat, and these slides of six developmental stages (70, 85, 100, 120, 135 days of gestation and birth) were stained by hematoxylin and eosin (HE). Photomicrographs of five to eight randomly selected areas were used to estimate the diameter, and the myofiber density (number of myofibers per mm²) in the sections of muscle was examined. Muscle fiber measurements were conducted with DT2000 (V2.0) image analysis software (Nanjing East-image, Nanjing, China). To examine the myogenic potential for muscle cells, we identified Pax7-positive cells (undifferentiated myoblasts) in LM using immunofluorescence staining (IFS). The primary IFS antibodies were rabbit laminin antibody (Sigma, St. Louis, MO) and mouse monoclonal anti-pax7 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). The corresponding anti-rabbit (ALEXA594) and anti-mouse (ALEXA488) (Invitrogen, Carlsbad, CA) secondary antibodies were used. Staining was performed according to the manufacturer’s instructions. The IFS slides were analyzed using a system of epi-fluorescence microscopes (TE2000U; Nikon, Tokyo, Japan). The fetal myoblast nuclei were stained green and the basal lamina red. Images were subsequently analyzed with Image Pro Plus (v5.1) image analysis software (Media Cybernetics, Bethesda, MD). The numbers of fetal myoblast and myofiber in each section were counted, and the fetal myoblast concentration within a given area of view was also calculated for each slide. Photomicrographs of five to eight randomly selected areas were used to estimate the total number of fetal myoblast in muscle cross sections. One-way ANOVA unequal variance, in which only one effect of age included in this statistical model, were used for multiple comparisons of data in SAS 8.0, and the corrected P < 0.05 (post hoc) was taken to indicate statistical significance.

Microarray Experiment and Statistical Analysis

Here, we must make a statement that the original microarray data used in this study, which is deposited in the National Center for Biotechnology Information database (GSE23563), have previously already been published as a breed comparison (50). The previous analysis (50) is a static comparison of differential expression. But in the present study, we focus on one breed (Ujumqin) and investigate the dynamic characteristics of gene expression and muscle development across the different gestational time points. The LM from sheep fetuses were investigated for gene expression at five prenatal developmental stages (70, 85, 100, 120, and 135 days of gestation). The microarray of each developmental stage contained three biological replicates (n = 3). The procedure of the microarray experiment is described in Ref. 50. Statistical methods involved are derived in GeneSpring10.0 (Agilent Technologies). One-way ANOVA unequal variance (Welch) was applied to contrast the five developmental stages, and the unequal unpaired variance t-test (Welch’s t-test) method was used for a comparative analysis between two stages (85 days vs. 100 days). The threshold for significance was the corrected P < 0.05 (Benjamini Hochberg) for one-way ANOVA. In Welch’s t-test, a fold change of twofold or more and P < 0.05 with false discovery rate (FDR) < 0.4 were taken to indicate statistical significance.

Clustering, Gene Ontology, and Functional Annotation Analysis

Only 1,508 of the 15,008 probe sets were definitive in the Agilent Sheep Gene Expression Microarray. To further clarify the function of the differentially expressed genes in this study, we conducted a BLASTN search for highly homologous human sequences (search setting, query coverage not less than 50%; E value less than 1.00E-100). Hierarchical clustering (distance metric, Pearson’s centered: linkage rule, average linkage) were performed using GeneSpring GX 10.0 (Agilent). DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov) were used for further functional analysis to identify the molecular events or cascades involved in skeletal muscle development. Significance was expressed as a P value, which was calculated using the EASE score (P value < 0.05 was considered significant).

Identification of Conserved cis-Regulatory Motifs

The Molecular Signatures Database (MSigDB) (58) was used to identify cis-regulatory motifs that were enriched in the gene expression clusters. The database and methods used were as described previously (66). Number of enriched gene sets that are significant, as indicated by an FDR of < 25%. In general, given the lack of coherence in most expression datasets and the relatively small number of gene sets being analyzed, an FDR cutoff of 25% is appropriate. In the present study, only motifs with FDR < 10% are reported. It is assumed that the identified motifs are also conserved in the ovine genome.

Quantitative Real-time PCR Analysis

Total RNA was used to make cDNA using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). Quantitative Real-time PCR (qPCR) was performed on an ABI 7500 instrument (Applied Biosystems, Foster City, CA) using the Fast EvaGreen Master Mix (Biotium, Hayward, CA). Thermal cycling consisted of an initial step at 95°C for 10 min followed by 40 cycles at 95°C for 30 s and 62°C for 30 s. The gene primers are shown in Table 1. The
Table 1. Primers of genes examined by quantitative RT-PCR

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Tm, °C</th>
<th>Length, bp</th>
<th>Gene Symbol</th>
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<tr>
<td>FM: GGGTCGCTCAGTTTATTGG</td>
<td>56.8</td>
<td>131</td>
<td>RpLP0</td>
</tr>
<tr>
<td>RM: CTTGGCTGATGGAGTGGTCG</td>
<td>54.6</td>
<td>88</td>
<td>TPM2</td>
</tr>
<tr>
<td>FM: GCTGGAGGTCCTGGGTCAA</td>
<td>54.2</td>
<td>141</td>
<td>PIK3R1</td>
</tr>
<tr>
<td>RM: TGGATACAGCAGAGGGATT</td>
<td>57.8</td>
<td>84</td>
<td>CASQ1</td>
</tr>
<tr>
<td>FM: GGAGAGCAGCGAGAAGATG</td>
<td>56.2</td>
<td>112</td>
<td>ACTN3</td>
</tr>
<tr>
<td>RM: GCGGCTGATCAAGGTCCAG</td>
<td>56.2</td>
<td>92</td>
<td>INSIG1</td>
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</tr>
<tr>
<td>RM: GACCATCCCACCTCTCACAG</td>
<td>56.3</td>
<td>102</td>
<td>ALDOA</td>
</tr>
<tr>
<td>FM: GCCCTGAGAGTAAGTCTGAGGAGCC</td>
<td>54.8</td>
<td>200</td>
<td>MYL6B</td>
</tr>
<tr>
<td>RM: GGCAGGCTTGGAGTTGTC</td>
<td>59.1</td>
<td>152</td>
<td>E2F1</td>
</tr>
<tr>
<td>FM: CTGGGCCTGATGGAGTGGTCG</td>
<td>53.6</td>
<td>200</td>
<td>TRRAP</td>
</tr>
<tr>
<td>RM: GACGGACTGAACCTCTGTAG</td>
<td>56.8</td>
<td>174</td>
<td>MYO9A</td>
</tr>
<tr>
<td>FM: ATCGGCAATGAGCGGTTC</td>
<td>55.3</td>
<td>144</td>
<td>ACTB</td>
</tr>
<tr>
<td>RM: CGCAGTCCCTCCCATTATTC</td>
<td>55.8</td>
<td>144</td>
<td>ACTB</td>
</tr>
<tr>
<td>FM: AGACCGTATGCCAAGATGTG</td>
<td>56.2</td>
<td>174</td>
<td>MYO9A</td>
</tr>
<tr>
<td>RM: AGACCGTATGCCAAGATGTG</td>
<td>55.5</td>
<td>117</td>
<td>EEF1A2</td>
</tr>
</tbody>
</table>

F, forward; R, reverse; Tm, annealing temperature.

qPCR measurements were performed in triplicate for each cDNA sample (n = 19), and gene expression was quantified relative to RpLP0 expression by the 2−ΔΔCt method. Data were analyzed with SAS 8.0, in which a t-test or ANOVA procedure for the data from two groups or five groups to examine the significance of difference in gene expression. The threshold for significance in gene expression: *P < 0.05, **P < 0.01.

Cell Culture, Differentiation Analysis

The sheep myoblast cell lines were isolated from the longissimus muscle of adult female Ujumqin sheep in our lab. For myoblast differentiation assay, the sheep myoblasts were seeded in six-well plates at a concentration of 8,000–10,000 cells/cm². After adhesion, the basic medium (DME containing 10% FBS) was replaced with differentiation medium (DM) (DME containing 2% horse serum). The sheep myoblasts were continuously cultured at 37°C and 5% carbon dioxide for 7 days, and the cells were collected every 24 h for examination of differentiation by myosin heavy chain (MyHC) immunostaining. Expression of E2F1 and MEF2A in cell culture at 1 and 5 day in DM were examined by Western blot.

Western Blot

Equal amounts of proteins were used for Western blot. Briefly, 20 μg aliquots of protein were subjected to electrophoresis on 12% SDS-PAGE gels. The separated proteins were transferred onto nitrocellulose membranes, which were incubated overnight with primary antibodies [rabbit-polyclonal anti-E2F1 (Avitaveshibo), 1:1,000; rabbit-polyclonal anti-MEF2A (Baioworld), 1:1,000] at 4°C. The membranes were then incubated with secondary antibodies [goat anti-mouse IgG (H+L), HRP (Jackson)] at room temperature.

RESULTS

Period of 85–100 Days is Crucial for Large-scale Myoblast Fusion

A total of 3,521 differentially expressed probes were identified across five developmental stages, which matched 2,251 genes for further analysis. We created hierarchical clusters using the 3,521 differential probes. As shown in Fig. 1, patterns of gene expression in fetal LM altered significantly after 100 days; namely samples at 70, 85, and 100 days share one pattern, whereas samples at 120 and 135 days share another pattern. This suggests that day 100 is an important timing threshold for skeletal muscle development in the ovine fetus. To ascertain whether there are significant changes in histological morphology in fetal LM during the mid-late gestation in sheep, we examined morphologic variations in myofiber by HE staining and examined the myofiber density and diameter across the six developmental stages (70 days, 85 days,
100 days, 120 days, 135 days, birth). Results show that there indeed were marked differences of histological morphology in myofiber between earlier (70 days, 85 days) and later stages (100 days, 120 days, 135 days, and birth) (Fig. 2A). In particular, the characteristic “rosettes” (the early-formed larger myotubes, acting as scaffold for the formation of a later generation of myotubes, are surrounded by those late-formed smaller ones) in muscle sections at both 70 and 85 days typify the developing muscle at earlier stages. Moreover, there was a significant increment of both myofiber density ($P < 0.05$) and diameter ($P < 0.01$) between 85 and 100 days (Fig. 2, B and C). To explore the cell properties and myogenic potential at the cellular level, we identified Pax7-positive cells (undifferentiated myoblasts) in LM with IFS (Fig. 3A), as well as calculated the ratio of Pax7-positive cells to myofibers at each developmental stage (Fig. 3B). Results show that the ratio of Pax7-positive cells to myofibers significantly differed between 70, 85, 100, and 120 days ($P < 0.05$) and remained unchanged at later stages (120 days, 135 days, and birth) ($P > 0.05$).

Changes of Transcriptome in Fetal Skeletal Muscle Between 85 and 100 Days

To ascertain the intrinsic characteristics of myogenic differentiation, we performed a comparison of gene expression in LM between 85 and 100 days at the transcriptome-wide level. A total of 739 differentially expressed probes were identified, 387 of which were upregulated and 352 were downregulated with development (Supplemental Table S1). To gain a global view of the enriched functions of these differential genes, we used DAVID (http://david.abcc.ncifcrf.gov) to perform functional clustering.

For the increasingly expressed genes from 85 to 100 days, we identified 16 significant biological process terms, among which immunity, carbohydrate metabolism, calcium-mediated signaling, lipid, fatty acid, and steroid metabolism were the top four enriched terms (Table 2). For the clustering of differential...
genes by cellular component category, 38 terms were significantly overrepresented, most of which were associated with functions of immunity and cell junction (Table 3). In particular, more than one-third of the terms were related to plasma membrane and cell junction, which is an indispensable process in myoblast fusion, indicating that myoblast fusion is very intense at this stage. As for pathways, immune system signaling and carbohydrate metabolism were the two most enriched REACTOME pathways (Table 4). The above findings suggest that the immune system is probably involved in myogenic differentiation during the period of 85–100 days in sheep.

For the decreasingly expressed genes from 85 to 100 days, >68% of genes encode the components of cytoplasm in cells (Table 5), suggesting cell mitosis is declining. In addition, “cell cycle” was the most significantly enriched KEGG and REACTOME pathway at this developmental stage (Table 6). The transcriptome analysis explicitly indicates that myoblast proliferation was greatly attenuated during this period.

Conserved cis-Regulatory Motifs Overrepresented at the Transcriptome-wide Level During Large-scale Myoblast Fusion

To investigate the mechanisms orchestrating the concordant changes of gene expression in skeletal muscle during the intensive proliferation of myofiber (85–100 days) at the transcriptional level, we examined the enrichment of conserved cis-acting regulatory motifs of the differential genes using the MSigDB (58). For the increasingly expressed genes, five conserved motifs for MEF2 were most significantly enriched in the gene sets (Table 7). Alternative analysis under DAVID platform also demonstrates that transcription factor binding site (TFBS) for MEF2 were significantly overrepresented (Supplemental Table S2). For the decreasingly expressed genes from 85 to 100 days, two conserved motifs for E2F1 were most significantly enriched in the gene sets (Table 8). Meanwhile, we also found significantly overrepresented TFBS for E2F under DAVID platform (Supplemental Table S3).

Profiles of Gene Expression in LM by qPCR

E2F1 and MEF2A differentially regulate their targets during large-scale myoblast fusion. We used qPCR to examine the expression of E2F1, MEF2A, and their target genes in LM at 85 and 100 days, respectively (Fig. 4, A and B). Results demonstrate that both E2F1 and MEF2A mRNA were significantly decreased during this period (P < 0.01). Furthermore, we also found that the phosphorylated E2F1 and MEF2A were both downregulated significantly with progress of myogenic differentiation in vitro (P < 0.01) (Fig. 5B). This indicates that downregulation of E2F1 and MEF2A is required for the myoblast differentiation and fusion. To investigate expression of the target genes in LM, here we selected PIK3R1 and FST for analysis, which are not only target genes for E2F1 (8) but also important molecules in signal pathways associated with muscle development. Results demonstrate that transcription of PIK3R1 and FST were significantly upregulated (P < 0.01), whereas another target EEF1A2 (eukaryotic translation elongation factor 1 alpha 2) mRNA was not significantly changed at the 85–100 day stage (Fig. 4A). This suggests that E2F1 most likely regulates the myogenic events through PIK3R1 and FST, but not EEF1A2 at this stage. Among three MEF2A target genes examined, MYH1 (myosin, heavy chain 1, skeletal muscle, adult) encodes muscle structural protein, and CASQ1 (calsequestrin 1, fast-twitch, skeletal muscle) is expressed...
exclusively in fast (type II) myofiber. *ALDOA* (aldolase A, fructose-bisphosphate) functions as an important metabolic enzyme in muscle. qPCR analysis revealed that three *MEF2A* targets were significantly upregulated between 85 and 100 days (*P*/H11021 0.01).

### Developmental expression of muscle structural genes and regulatory genes.

To characterize gene expression during fetal muscle development, we examined six muscle structural genes including *MYL6B* (myosin, light chain 6B), *ACTN3* (actinin, alpha 3), *MYO9A* (myosin IXA), *TPM2* (tropomyosin 2), *TNC* (telopeptide C-terminal), and *TNNI3* (troponin I). The expression of these genes was monitored from 35 to 100 days of gestation.

### Table 2. Biological process for increasingly expressed genes from 85 to 100 days

<table>
<thead>
<tr>
<th>Term</th>
<th>P Value</th>
<th>Count</th>
<th>%</th>
<th>Fold Enrichment</th>
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</thead>
<tbody>
<tr>
<td>BP00150:MHCI-mediated immunity</td>
<td>0.0001</td>
<td>5</td>
<td>1.93</td>
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<tr>
<td>BP00149:T-cell mediated immunity</td>
<td>0.0001</td>
<td>12</td>
<td>4.63</td>
<td>4.16</td>
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<td>BP00001:Carbohydrate metabolism</td>
<td>0.0064</td>
<td>18</td>
<td>6.95</td>
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<tr>
<td>BP00148:Immunity and defense</td>
<td>0.0072</td>
<td>32</td>
<td>12.36</td>
<td>1.62</td>
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<tr>
<td>BP00112:Calcium mediated signaling</td>
<td>0.0087</td>
<td>7</td>
<td>2.70</td>
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<tr>
<td>BP00109:Lipid, fatty acid and steroid metabolism</td>
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<td>BP00180:Detoxification</td>
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<td>1.93</td>
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<td>BP00005:Glycolysis</td>
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<td>BP00298:Glycogen metabolism</td>
<td>0.0357</td>
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<td>BP00276:General vesicle transport</td>
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<td>BP00151:MHCI-mediated immunity</td>
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<td>BP00196:Oogenesis</td>
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<td>1.54</td>
<td>4.05</td>
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*Count* refers to the number of genes enriched in corresponding biological process. The percentage column shows the genes from our data set that overlap with the biological process in the total number of genes represented in that biological process.

### Table 3. Cellular component for increasingly expressed genes from 85 to 100 days

<table>
<thead>
<tr>
<th>Term</th>
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<th>Fold Enrichment</th>
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<td>GO:0044459—plasma membrane part</td>
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<td>GO:0042611—MHC complex protein</td>
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<td>GO:0005887—integral to plasma membrane</td>
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<td>GO:0031226—intrinsic to plasma membrane</td>
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<td>GO:0005886—plasma membrane</td>
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<td>GO:0042825—TAP complex</td>
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<td>GO:0042824—MHC class I peptide loading complex</td>
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<td>GO:0005743—mitochondrial inner membrane</td>
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<td>GO:0005625—soluble fraction</td>
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<td>GO:0044421—extracellular region part</td>
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<td>GO:0070161—anchoring junction</td>
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<td>6</td>
<td>2.32</td>
<td>0.023165</td>
<td>3.69</td>
</tr>
<tr>
<td>GO:0031225—anchored to membrane</td>
<td>9</td>
<td>3.47</td>
<td>0.024085</td>
<td>2.56</td>
</tr>
<tr>
<td>GO:0045177—apical part of cell</td>
<td>8</td>
<td>3.09</td>
<td>0.024202</td>
<td>2.80</td>
</tr>
<tr>
<td>GO:0005626—in soluble fraction</td>
<td>22</td>
<td>8.49</td>
<td>0.025687</td>
<td>1.64</td>
</tr>
<tr>
<td>GO:0005924—cell-substrate adherens junction</td>
<td>6</td>
<td>2.32</td>
<td>0.026843</td>
<td>3.55</td>
</tr>
<tr>
<td>GO:0005584—collagen type I</td>
<td>2</td>
<td>0.77</td>
<td>0.031512</td>
<td>62.66</td>
</tr>
<tr>
<td>GO:0005624—membrane fraction</td>
<td>21</td>
<td>8.11</td>
<td>0.032596</td>
<td>1.63</td>
</tr>
<tr>
<td>GO:0029885—cell-substrate junction</td>
<td>6</td>
<td>2.32</td>
<td>0.032966</td>
<td>3.36</td>
</tr>
<tr>
<td>GO:0044449—contractile fiber part</td>
<td>6</td>
<td>2.32</td>
<td>0.034065</td>
<td>3.33</td>
</tr>
<tr>
<td>GO:0005912—adherens junction</td>
<td>7</td>
<td>2.70</td>
<td>0.037231</td>
<td>2.83</td>
</tr>
<tr>
<td>GO:0044420—extracellular matrix part</td>
<td>6</td>
<td>2.32</td>
<td>0.038685</td>
<td>3.21</td>
</tr>
<tr>
<td>GO:0048471—perinuclear region of cytoplasm</td>
<td>10</td>
<td>3.86</td>
<td>0.040528</td>
<td>2.18</td>
</tr>
<tr>
<td>GO:0016323—basolateral plasma membrane</td>
<td>8</td>
<td>3.09</td>
<td>0.043433</td>
<td>2.47</td>
</tr>
<tr>
<td>GO:0043292—contractile fiber</td>
<td>6</td>
<td>2.32</td>
<td>0.043673</td>
<td>3.11</td>
</tr>
<tr>
<td>GO:0044431—Golgi apparatus</td>
<td>10</td>
<td>3.86</td>
<td>0.045159</td>
<td>2.13</td>
</tr>
<tr>
<td>GO:0005769—early endosome</td>
<td>5</td>
<td>1.93</td>
<td>0.046257</td>
<td>3.69</td>
</tr>
<tr>
<td>GO:0005829—cytosol</td>
<td>30</td>
<td>11.58</td>
<td>0.048281</td>
<td>1.41</td>
</tr>
<tr>
<td>GO:0031012—extracellular matrix</td>
<td>11</td>
<td>4.25</td>
<td>0.048809</td>
<td>2.00</td>
</tr>
<tr>
<td>GO:0044455—mitochondrial membrane part</td>
<td>6</td>
<td>2.32</td>
<td>0.049024</td>
<td>3.01</td>
</tr>
<tr>
<td>GO:0005901—caveola</td>
<td>4</td>
<td>1.54</td>
<td>0.049445</td>
<td>4.82</td>
</tr>
<tr>
<td>GO:0031966—mitochondrial membrane</td>
<td>12</td>
<td>4.63</td>
<td>0.049869</td>
<td>1.91</td>
</tr>
</tbody>
</table>
differentiation and fusion.

There were marked differences in mRNA levels of the four regulatory genes, \textit{INSIG1}, \textit{TXNRD1}, \textit{USP9Y}, and \textit{TRRAP}, between 70 and 85 days, the ratio of Pax7-positive cells increasing with development, while \textit{TPM2}, \textit{TNC}, and \textit{ACTN3} were inexpressed at 47 days. The switch of myofiber types occurred during the stage of 70–85 days. This may underpin changes of cellular properties in muscle progenitors and how to manipulate the feeding during pregnancy for better postnatal growth. It has been shown that any stress or stimulus to the fetus, such as maternal nutrient constriction, would markedly affect birth weight and postnatal muscle development before 100 days (16, 17, 68). Around day 85, myoblast differentiation and fusion begins to accelerate, and thus more new myofibers are formed until 100 days; meanwhile the resident primary myofibers increase their size (Fig. 2, A and B). Although there was no significant proliferation of myofibers between 70 and 85 days, the ratio of Pax7-positive cells relative to myofibers declined at this stage (Fig. 3B), which suggest changes of cellular properties in muscle progenitors occurred during the stage of 70–85 days. This may underpin the intense proliferation of myofibers at later stages, since decline of Pax7 expression is followed by myoblast proliferation and differentiation (12, 19, 45).

### DISCUSSION

Myofiber formation in the sheep fetus begins at ~32 days of gestation and the full complement of myofibers is achieved by days 80–120 of gestation (3, 39). In the present study, our findings strongly indicate that large-scale myoblast fusion in muscle occurs at the stage of 85–100 days and that myoblast proliferation is almost completed at 100 days, while increase of myofiber size continues thereafter in sheep. These results will provide the managers of farm animals information on when and how to manipulate the feeding during pregnancy for better postnatal growth. It has been shown that any stress or stimulus to the fetus, such as maternal nutrient constriction, would markedly affect birth weight and postnatal muscle development before 100 days (16, 17, 68). Around day 85, myoblast differentiation and fusion begins to accelerate, and thus more new myofibers are formed until 100 days; meanwhile the resident primary myofibers increase their size (Fig. 2, A and B). Although there was no significant proliferation of myofibers between 70 and 85 days, the ratio of Pax7-positive cells relative to myofibers declined at this stage (Fig. 3B), which suggest changes of cellular properties in muscle progenitors occurred during the stage of 70–85 days. This may underpin the intense proliferation of myofibers at later stages, since decline of Pax7 expression is followed by myoblast proliferation and differentiation (12, 19, 45).

### Table 4. KEGG pathways for increasingly expressed genes between 85 and 100 days

<table>
<thead>
<tr>
<th>Term</th>
<th>P Value</th>
<th>Genes</th>
<th>Fold Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa00150:Autoimmune thyroid disease</td>
<td>0.0001</td>
<td>HLA-DQB1, HLA-A, HLA-DRB4, HLA-C, HLA-B, HLA-E, TSHR, LOC652614, HLA-DRA</td>
<td>6.82</td>
</tr>
<tr>
<td>hsa05330:Allograft rejection</td>
<td>0.0001</td>
<td>HLA-DQB1, HLA-A, HLA-DRB4, HLA-C, HLA-B, HLA-E, LOC652614, HLA-DRA</td>
<td>8.45</td>
</tr>
<tr>
<td>hsa05416:Viral myocarditis</td>
<td>0.0002</td>
<td>HLA-DQB1, CAV1, MYH1, HLA-A, HLA-DRB4, HLA-C, HLA-B, HLA-E, LOC652614, HLA-DRA</td>
<td>5.51</td>
</tr>
<tr>
<td>hsa05332:Graft-versus-host disease</td>
<td>0.0002</td>
<td>HLA-DQB1, HLA-A, HLA-DRB4, HLA-C, HLA-B, HLA-E, LOC652614, HLA-DRA</td>
<td>7.80</td>
</tr>
<tr>
<td>hsa04940:type I diabetes mellitus</td>
<td>0.0003</td>
<td>HLA-DQB1, HLA-A, HLA-DRB4, HLA-C, HLA-B, HLA-E, LOC652614, HLA-DRA</td>
<td>7.24</td>
</tr>
<tr>
<td>hsa04612:Antigen processing and presentation</td>
<td>0.0005</td>
<td>HLA-DQB1, TAP1, HLA-A, HLA-DRB4, HLA-C, HLA-B, HLA-E, LOC652614, HLA-DRA, B2M</td>
<td>4.71</td>
</tr>
<tr>
<td>hsa00010:Glycolysis / Gluconeogenesis</td>
<td>0.0023</td>
<td>ALDOA, LDHA, ALDH2, ENO3, PGAM2, FBP2, GAPDH</td>
<td>5.07</td>
</tr>
<tr>
<td>hsa04910:Insulin signaling pathway</td>
<td>0.0035</td>
<td>PRKHz, PP1R3C, SLC2A4, PYGL, FLOT2, ACACA, PP1R3A, FB2, INP5D, CALM1</td>
<td>3.22</td>
</tr>
<tr>
<td>hsa04514:Cell adhesion molecules (CAMs)</td>
<td>0.0101</td>
<td>HLA-DQB1, F11R, PECAM1, HLA-A, HLA-DRB4, HLA-C, HLA-B, HLA-E, LOC652614, HLA-DRA</td>
<td>2.96</td>
</tr>
<tr>
<td>hsa04200:Calcium signaling pathway</td>
<td>0.0185</td>
<td>ADCY4, ENDR8, SLC25A4, ATP2A1, CHP2, OXTR, PLCD1, NOS2, PPP3CA, CALM1</td>
<td>2.47</td>
</tr>
<tr>
<td>hsa05310:Asthma</td>
<td>0.0275</td>
<td>FCER1A, HLA-DQB1, HLA-DRB4, HLA-DRA</td>
<td>6.00</td>
</tr>
<tr>
<td>hsa00330:Arginine and proline metabolism</td>
<td>0.0319</td>
<td>CKM, GATM, ALDH2, NOS2, ASL</td>
<td>4.10</td>
</tr>
<tr>
<td>hsa04640:Hematopoietic cell lineage</td>
<td>0.0458</td>
<td>CD3G, CD3D, IL4R, HLA-DRB4, CD1B, HLA-DRA</td>
<td>3.03</td>
</tr>
</tbody>
</table>

The “Genes” column shows differentially expressed genes enriched in corresponding KEGG pathway. Italics represent biased annotation or term not related to the aim of the study.

### Table 5. Cellular component for decreasingly expressed genes from 85 to 100 days

<table>
<thead>
<tr>
<th>Term</th>
<th>Count</th>
<th>%</th>
<th>P Value</th>
<th>Fold Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:00045177—apical part of cell</td>
<td>10</td>
<td>4.42</td>
<td>4.05E-04</td>
<td>4.47</td>
</tr>
<tr>
<td>GO:00005737—cytoplasm</td>
<td>115</td>
<td>50.88</td>
<td>4.95E-04</td>
<td>1.26</td>
</tr>
<tr>
<td>GO:00005622—intracellular</td>
<td>154</td>
<td>68.14</td>
<td>0.004055</td>
<td>1.12</td>
</tr>
<tr>
<td>GO:00044444—intracellular plasma</td>
<td>79</td>
<td>34.96</td>
<td>0.005229</td>
<td>1.29</td>
</tr>
<tr>
<td>GO:00044424—intracellular nonmembrane-bounded organelle</td>
<td>47</td>
<td>20.80</td>
<td>0.006686</td>
<td>1.45</td>
</tr>
<tr>
<td>GO:00044429—intracellular organelle</td>
<td>128</td>
<td>56.64</td>
<td>0.012881</td>
<td>1.14</td>
</tr>
<tr>
<td>GO:00031253—cell projection membrane</td>
<td>4</td>
<td>1.77</td>
<td>0.047135</td>
<td>4.92</td>
</tr>
<tr>
<td>GO:0005853—eukaryotic translation elongation factor 1 complex</td>
<td>2</td>
<td>0.88</td>
<td>0.006042</td>
<td>32.01</td>
</tr>
<tr>
<td>GO:00044446—intracellular organelle</td>
<td>63</td>
<td>27.88</td>
<td>0.073143</td>
<td>1.19</td>
</tr>
<tr>
<td>GO:0016324—apical plasma membrane</td>
<td>5</td>
<td>2.21</td>
<td>0.084282</td>
<td>3.01</td>
</tr>
</tbody>
</table>
A recent study in myostatin-null mice reveals the PI3K pathway to be identified between E2F1 and TGF-β. The present evidence (Fig. 4) implies that there may be a positive regulator of muscle development and growth by way acts as an MSTN target (11), which suggests that the PI3K pathway is a downstream target of FST-MSTN signal under control of E2F1. Interestingly, in human umbilical vein endothelial cells, AP-1- and E2F1-dependent expression of G1 cyclins were regulated by PI3K/Akt, JNK, and ERK signaling pathways regulated by E2F1 in myoblast differentiation and inducing muscle specific gene transcription (31), implying that there may be a feedback regulatory relationship between PI3K and E2F1 in muscle as well. Another intriguing question to be tackled concerns the mechanism of TGF-β pathways regulated by E2F1 in myoblast differentiation and fusion.

During differentiation, MEF2A, a member of the MEF2 family, plays a determining role in regulating muscle cell differentiation and inducing muscle specific gene transcription in conjunction with bHLH transcription factors (5, 6, 42, 44, 60). In the present study, the downregulation of MEF2A triggered significant higher expression of muscle structural genes (MYH1, CASQ1, and ALDOA) in LM at 85 days than at 100 days (P < 0.01) (Fig. 4B), which strongly suggests that the myofiber proliferation is increasing by myoblast fusion at this stage in sheep. Transition of myofiber type parallels muscle

Table 6. Pathways for decreasingly expressed genes from 85 to 100 days

<table>
<thead>
<tr>
<th>Term</th>
<th>P Value</th>
<th>Genes</th>
<th>Fold Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa04110:Cell cycle</td>
<td>0.0329</td>
<td>SKP2, BUB1, CDK6, MCM2, ZBTB17, MCM3</td>
<td>3.30</td>
</tr>
<tr>
<td>hsa05215:Prostate cancer</td>
<td>0.0382</td>
<td>HSP90AB1, HSP90B1, PGDC, TC7FL1, PIK3R1</td>
<td>3.86</td>
</tr>
<tr>
<td>hsa05218:Melanoma</td>
<td>0.0812</td>
<td>CDK6, PGDC, CDH1, PIK3R1</td>
<td>3.87</td>
</tr>
<tr>
<td>hsa05200:Pathways in cancer</td>
<td>0.0962</td>
<td>HSP90AB1, SM2, HSP90B1, SKP2, CDK6, CDH1, TC7FL1, TRAF4, PIK3R1</td>
<td>1.89</td>
</tr>
<tr>
<td>REACT_152:Cell cycle, mitotic</td>
<td>0.0379</td>
<td>XPO1, TUBB, TUBB3, CENPQ, KNTC1, SKP2, BUB1, CDK6, MCM2, MCM3, TUBA1A</td>
<td>2.02</td>
</tr>
<tr>
<td>REACT_71:Gene expression</td>
<td>0.0397</td>
<td>EEF1A1, YARS, HNRNPK, MED15, UBC, RPL27, RPS15A, RPS4X, VARS, EEF2B3, YBX1, PRPF6</td>
<td>1.91</td>
</tr>
<tr>
<td>REACT_6167:Influenza infection</td>
<td>0.0566</td>
<td>XPO1, HNRNPK, UBC, RPL27, RPS15A, RPS4X, YBX1</td>
<td>2.48</td>
</tr>
<tr>
<td>REACT_17015:Metabolism of proteins</td>
<td>0.0852</td>
<td>EEF1A1, UBC, RPL27, RPS15A, RPS4X, TUBA1A, EEF2B3, XRN2</td>
<td>2.05</td>
</tr>
</tbody>
</table>

In the “Term” column pathway titles labeled as “hsa” indicate they belong to the KEGG pathways, while those titled with “REACT” are derived from the REACTOME pathways in DAVID. Italicics represent biased annotation or term not related to the aim of the study.

Pik3r1 and FST are not only target genes of E2f1 (8), but also important members of signal pathways that regulate myogenesis. It has been shown, using C2C12 myoblasts, that the PI3K pathway regulates the myoblast migration (20), proliferation (21, 36), differentiation (21, 40, 53, 55, 67), and fusion (52), as well as increase of myotube size (28, 48) during myogenesis. Pik3r1 is a subunit of PI3K pathway, affecting muscle development and growth in zebrafish and mouse (37, 41). In the present study, significant upregulation of Pik3r1 in LM between 85 and 100 days suggests that the E2f1-regulated PI3K pathway is involved in large-scale myoblast differentiation and fusion in sheep. In mouse and chicks, FST functions as a positive regulator of muscle development and growth by inhibting TGF-β family members including myostatin (2, 23, 34, 43, 64). As to cellular behavior, FST regulates the proliferation (4, 7, 23, 35), differentiation (4, 32), and fusion (29) of muscle cells in myogenesis in vivo and in vitro of mouse and duck. The present evidence (Fig. 4A) implies that there may be a novel pathway to be identified between E2f1 and TGF-β. We suppose that E2f1 may regulate myostatin signal to modulate fetal myoblast differentiation and fusion by upregulating FST. A recent study in myostatin-null mice reveals the PI3K pathway acts as an MSTN target (11), which suggests that the PI3K pathway is a downstream target of FST-MSTN signal under control of E2F1. Interestingly, in human umbilical vein endothelial cells, AP-1- and E2F1-dependent expression of G1 cyclins were regulated by PI3K/Akt, JNK, and ERK signaling pathways (31), implying that there may be a feedback regulatory relationship between PI3K and E2F1 in muscle as well. Identification of the feedback loop between PI3K and E2F1 in myogenesis would be an interesting issue. Another intriguing question to be tackled concerns the mechanism of TGF-β pathways regulated by E2F1 in myoblast differentiation and fusion.

Table 7. Enrichment of conserved cis-regulatory motifs for the increasingly expressed genes from 85 to 100 days

<table>
<thead>
<tr>
<th>Gene Motif (number of genes)</th>
<th>Description</th>
<th>Genes in Overlap</th>
<th>FDR q Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSRSRFC4_Q2 (219)</td>
<td>Genes with promoter regions (~2 kb, 2 kb) around transcription start site containing the motif ANKCTAWAAATAGMNW, which matches annotation for MEF2A-MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)</td>
<td>44</td>
<td>0.0008</td>
</tr>
<tr>
<td>VSRSRFC4_01 (251)</td>
<td>Genes with promoter regions (~2 kb, 2 kb) around transcription start site containing the motif RNKCTATTWTTAGMWN, which matches annotation for MEF2A: MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)</td>
<td>48</td>
<td>0.029</td>
</tr>
<tr>
<td>CTAWWWATA_VSR_SRCF4_Q2 (370)</td>
<td>Genes with promoter regions (~2 kb, 2 kb) around transcription start site containing the motif CTAWWWATA, which matches annotation for MEF2A: MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)</td>
<td>82</td>
<td>0.035</td>
</tr>
<tr>
<td>VSRMEF2_Q6_01(251)</td>
<td>Genes with promoter regions (~2 kb, 2 kb) around transcription start site containing motif RGCTATWTTTAR; motif does not match any known transcription factor</td>
<td>58</td>
<td>0.0787</td>
</tr>
<tr>
<td>VSHMFEF2_Q6(142)</td>
<td>Genes with promoter regions (~2 kb, 2 kb) around transcription start site containing motif SKYTTAAAATACYCH; motif does not match any known transcription factor</td>
<td>33</td>
<td>0.0922</td>
</tr>
</tbody>
</table>

The increasingly expressed genes from 85 to 100 days of fetal stages were examined for enrichment of conserved cis-acting regulatory motifs by using the Molecular Signatures Database (MSigDB) and its associated analysis procedures (Subramanian et al., Ref. 58). The database contained motifs conserved in the human, mouse, rat, and dog genomes. The conserved transcription factor binding sites and anonymous conserved motifs were restricted to a sequence window corresponding to ±2 kb of the transcription start site, while the conserved miRNA recognition sites were restricted to the 3′-untranslated region of the genes in this cluster. FDR q value: number of enriched gene sets that are significant, as indicated by a false discovery rate (FDR) < 0.1.
development in animals. Almost all primary myofibers are slow, type I muscle fibers, while most of the secondary myofibers are fast (type II) muscle fibers during muscle development (15, 57). A previous study shows that MEF2 is involved in regulation of muscle fiber type, and several slow-fiber-specific skeletal muscle genes contain NFAT-binding sites adjacent to MEF2-binding sites in their control regions (14). In this study, the alternately higher expression of TPM2, TNC, and CASQ1 in LM at different developmental stages (Fig. 4, B and C) explicitly demonstrates the switch of myofiber types in parallel with development. However, this transition of myofiber type is mainly due to the complement of newly formed fast myofibers by myoblast fusion but is different from substitution of slow MyHC by fast MyHC isoforms in the postnatal

**Table 8. Enrichment of conserved cis-regulatory motifs for the decreasingly expressed genes from 85 to 100 days**

<table>
<thead>
<tr>
<th>Gene Motif (number of genes)</th>
<th>Description</th>
<th>Genes in Overlap</th>
<th>FDR q Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGCGSSAAA_VSE2F_1DP_01(171)</td>
<td>Genes with promoter regions (−2 kb, 2 kb) around transcription start site containing the motif SGCGSSAAA, which matches annotation for E2F1; E2F transcription factor 1 TFDP1: transcription factor Dp-1</td>
<td>44</td>
<td>0.035</td>
</tr>
<tr>
<td>VSE2F1DP1_01(239)</td>
<td>Genes with promoter regions (−2 kb, 2 kb) around transcription start site containing the motif TTTCSCGC, which matches annotation for E2F1; E2F transcription factor 1 TFDP1: transcription factor Dp-1</td>
<td>63</td>
<td>0.0945</td>
</tr>
</tbody>
</table>

The decreasingly expressed genes from 85 to 100 days of fetal stages were examined for enrichment of conserved cis-acting regulatory motifs as described in Table 7.

**Fig. 4. Developmental expression of genes involved in muscle development and function by quantitative RT-PCR (qPCR).** Gene expression was quantified relative to RpLP0 expression by the 2−ΔCt method. Fetal LM samples at 70, 85, 100, 120, and 135 days of development are shown. The results for each gene are shown as means ± SD (n = 3), *P < 0.05, ***P < 0.01. A: expression of E2F1 and its target genes (PI3K, FST, and EEF1A2). B: expression of MEF2A and its target genes (CASQ1, ACTN3, and ALDOA). C: developmental expression of muscle structural genes (MYL6B, ACTN3, MYO9A, TPM2, TNC, and ACTB). D: developmental expression of regulatory genes associated with muscle development and differentiation (TRRAP, TXNRD1, INSIG1, and USP9Y).
A recent study demonstrates that myosin-IXA (MYO9A) is required to sustain the collective migration of epithelial cells by targeting RhoGAP activity (46). But it is not clear whether MYO9A is involved in the myogenic process during the previous stage. The higher expression of MYO9A in fetal muscle at 70 days than at later stages (Fig. 4C) suggests this gene may be associated with muscle development at an early stage. However, the role of MYO9A in the myogenic process at an early fetal stage remains to be ascertained further. The downregulation of TRRAP is essential for the differentiation of embryonic stem cells, brain tumor-initiating cells, and HL60 cells (30, 54, 65). In this research, marked downregulation of TRRAP mRNA in muscle between 85 and 100 days implies that it functions similarly in muscle cells as does in other cell lines.

Our investigations clarify the threshold for increase in myofiber size and number during fetal sheep development, which enhances the efficiency of sheep management, especially for mutton production. Although previous studies in small animals or cell models have established the significance of cell cycle regulation in myogenesis for E2F (25, 33, 56, 59, 61) and MEF2’s determining role in regulating muscle cell differentiation and inducing muscle-specific gene transcription in conjunction with bHLH transcription factors (5, 6, 14, 42, 44, 60), the present study reveals for the first time that a large number of genes spanning the cis-regulatory motifs for E2F1 or MEF2A are simultaneously less expressed or more expressed in skeletal muscle during large-scale myoblast fusion, respectively. Furthermore, it has been demonstrated that E2F1 is involved in the chromatin assembly to modulate cell cycle by regulating its target genes such as chromatin assembly factor-1 large subunit, Hmgn1, Hmgn2, Uhrf1, and Bnip3 in Arabidopsis, mouse, and rat (22, 27, 38, 49), as well as MEF2A; it also regulates expression of muscle-specific genes by affecting the chromatin state in human COS cells and B-lymphocytes (18, 24). These strongly confirm that E2F1 and MEF2A work as the center of networks orchestrating the large-scale gene expression through modifying the chromatin status during fetal myogenic differentiation. It updates previous thinking on the roles and rank of E2F1 or MEF2A in the regulatory network of myogenesis. This study also presents informative clues about the mechanism of myogenic differentiation in vivo.

Conclusions

Day 100 is an important checkpoint for gene expression and fetal muscle development in sheep. During the 85–100 day period, the switch from a network centered on E2F1 to a network centered on MEF2A at the transcriptome-wide level is coincident with large-scale myoblast fusion. The mRNA and phosphorylated protein levels of E2F1 and MEF2A significantly decline with myogenic progression in vivo and in vitro. PI3K and FST, as targets of E2F1, may be involved in myoblast differentiation and fusion. Downregulation of MEF2A contributes to the transition of myofiber types by differential regulation of the target genes involved at 85–100 days. This study presents a repertoire of gene expression in muscle during large-scale myoblast fusion at the transcriptome-wide level, which contributes to elucidate the regulatory network of myogenic differentiation.

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AUTHOR CONTRIBUTIONS

Author contributions: C.W., L.L., H.S., J.L., and L.Z. performed experiments; C.W., L.X., and W.L. analyzed data; C.W., and H.R. drafted manuscript; L.L., H.R., and L.D. conception and design of research; L.L. and H.R. interpreted results of experiments; H.S. prepared figures; W.L. and L.D. edited and revised manuscript; H.R. and L.D. approved final version of manuscript.

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