Temporal expression of myogenic regulatory genes in different chicken breeds during embryonic development

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Research Article

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Abstract

Background

The basic units of skeletal muscle in all vertebrates are multinucleate myofibers, which are formed from the fusion of mononuclear myoblasts during the embryonic period. Understanding the regulation of embryonic skeletal muscle development is important to improve animal production efficiency and meat quality. The processes of myofiber formation and maturation are controlled by multiple regulatory factors. Therefore, we selected four chicken breeds, namely, Cornish, White Plymouth Rock, White Leghorn and Beijing-You Chicken, for evaluation of their temporal expression patterns of known key regulatory genes (Myomaker, MYOD and MSTN) during pectoral and thigh muscle development to characterize the embryonic muscle development of different chicken breeds.

Results

The highest expression level of Myomaker, a vital gene for controlling myoblast fusion, was occurred from E13 to E15 for all breeds, while its expression level was close to zero at D1, indicating that E13 to E15 was the crucial stage of myoblast fusion in chickens. Interestingly, the gene expression level of Myomaker in both pectoral and thigh muscle of Cornish chickens, which are fast-growing, was the highest during the crucial stage. The expression of the MYOD gene in muscles at D1 was much higher than that in the embryonic development period, implying that MYOD might have an important role in chicken growth after hatching. In addition, a significant difference was found in the expression level of MYOD among the four chicken breeds at D1. Hematoxylin and eosin staining of both pectoral and thigh muscles suggested that the formation of myofibers was largely complete at E17 in chicken embryos, and myofiber hypertrophy subsequently began, which was speculated to have occurred because of the increase in MSTN gene expression and the decrease in Myomaker gene expression at E17.

Conclusion

These findings revealed the same temporal expression patterns of the key regulatory genes among different breeds, but they also showed some variation across different breeds within the long-term selection for growth. Our research contributes to lay a foundation for the study of myofiber development during the embryonic period in different chicken breeds.

Introduction

The major component of the vertebrate carcass is skeletal muscle, which accounts for ~ 40% of body mass [1]. The development and growth of skeletal muscle are complex, dynamic processes and begin with the proliferation and differentiation of progenitor cells that arise from the mesoderm [2]. Skeletal muscle is composed of bundles of myofibers. A critical event in myogenesis is the fusion of myoblasts
either with one another to generate new multinucleated myofibers (hyperplasia) or with an existing myofiber, thereby increasing the pool of myonuclear (hypertrophy) and allowing muscle growth [3]. These processes are controlled through a series of steps by the regulation of gene expression and posttranslational modification.

Millay et al. [4] identified the muscle-specific membrane protein Myomaker, which controls myoblast fusion in the early embryonic development of mice. Several recent studies detected the gene expression of Myomaker in the skeletal muscle of adult mice and showed that the gene expression level was low, but the gene was reactivated during myofiber repair after muscle injury, which indicated that Myomaker is necessary for myoblast fusion in skeletal muscle growth and development [5, 6]. The role of the Myomaker gene in muscle development has also been illustrated in zebrafish [7]. In contrast, the function of Myomaker in chicken is lagging behind that in other model species. Luo et al. [8] published one of the first studies and confirmed that Myomaker was a muscle-specific gene in chickens. The functional verification of Myomaker has also been studied in primary chicken myoblasts by overexpression and knockdown of Myomaker, and the results showed that the expression of Myomaker could promote the fusion of chicken myoblasts [8].

In addition, numerous genetic screens performed in mice, Drosophila and zebrafish have demonstrated that myogenic regulatory factors are involved in myoblast fusion. Members of this gene family, such as Myf5, MYOD, MYOG and MRF4, constitute an interactive regulatory transcriptional network that controls the determination and terminal differentiation of myoblasts [9]. Among these genes, MYOD is considered to act as one of the determinants [10]. MYOD is essential for both embryonic and adult skeletal muscle growth, and it both induces transcription and promotes myogenesis in embryonic skeletal muscle and is committed to determining muscle plasticity in adult skeletal muscle [11]. MYOD can specifically recognize DNA sequences and coordinate myogenic gene expression by binding to a palindromic E-box motif (5'-CANNTG-3') [12]. During the early differentiation of primary chicken myoblasts, MYOD binds to the E-box1 of Myomaker to promote the regulation of the promoter and induce the transcription and regional histone modification of Myomaker, thereby facilitating the formation of myofibers [8].

The gene function of MSTN is distinctly different from that of Myomaker and MYOD. Double muscling in animals refers to marked hypertrophy of muscle, which more often occurs in cattle, sheep and pigs. Kambadur et al. [13] conducted a sequence analysis of Belgian Blue, Piedmontese and normal cattle and found mutations in heavy-muscled cattle breeds that inhibited the expression of MSTN, which demonstrated the negative regulatory role of MSTN in muscle development. Further studies have found that MSTN can inhibit myoblast differentiation by blocking genes induced through the Akt/TORC1/p70S6K signaling pathway [14]. Recently, Kim et al. [15] utilized the D10A-Cas9 nickase technique to generate MSTN-knockout chickens by primordial germ cells. Compared with wild-type chickens, the MSTN-knockout chickens exhibited significantly larger muscle mass and less abdominal fat deposition in pectoral and thigh muscles. However, the degree of skeletal muscle hypertrophy and hyperplasia caused by MSTN loss varied with sex and muscle type.
With the increasing demand for animal meat, more studies on the growth and differentiation of skeletal muscle are needed to improve growth rates. The understanding of the regulation of embryonic and postnatal skeletal muscle growth and development is extremely important in this regard. Additionally, the development of poultry muscle and the amelioration of meat quality have been a major focus of breeders. Muscle growth rate differs among the various breeds of chicken; thus, investigating the expression of myogenesis-related genes in various types of chicken could be a breakthrough to regulate muscle development [16]. However, studies investigating the *Myomaker*, *MYOD*, and *MSTN* gene expression profiles during embryonic development among different chicken breeds are largely unclear. Here, we collected pectoral muscle and thigh muscle tissue of four chicken breeds at embryonic days 11, 13, 15, 17 (E11, E13, E15, E17) and day 1 (D1) for the study of gene expression patterns and to characterize the embryonic muscle development of different chicken breeds.

**Materials And Methods**

**Bird and sample collection**

White Leghorn (WL) is an egg-type chicken breed with a slow growth rate and excellent laying performance. Beijing-You Chicken (BYC) is a native Chinese breed with good meat quality. Cornish (CN) and White Plymouth Rock (WPR) are two popular breeds due to their rapid growth rate and have been extensively used worldwide as the paternal and maternal lines of commercial broilers, respectively. The fertile eggs of WL and BYC were obtained from the Poultry Genetic Resource and Breeding Experimental Unit of China Agricultural University. The fertile eggs of WPR and CN were provided by Beijing Huadu Yukou Poultry Industry Co., Ltd. (China). All fertile eggs were sterilized with Benzalkonium bromide solution before incubation. The temperature and humidity in the incubator were maintained at 37.8°C and 60%, respectively.

The overall flow of the present study is shown in Fig. 1A. More than 40 fertile eggs were randomly examined for the presence of viable embryos at each embryonic timepoint (E11, E13, E15 and E17). The viable embryos were sacrificed by decapitation for subsequent experiments. The remaining fertile eggs were left in the incubator until the chicks hatched. After hatching, more than 20 healthy chicks were killed by cervical dislocation. The right pectoral muscle and thigh muscle, which were trimmed free of fat, were collected from each chicken embryo. All muscle tissues were immediately saved in RNase-free tubes containing RNA preservation solution and stored at -20°C until RNA extraction. All experiments with chicken in this study were approved by the guidance of ethical regulations from the Animal Care and Use Committee of China Agriculture University, Beijing, China.

**Sex determination**

Sex was first judged by gonadal development after the abdomen was opened. In brief, both sides of the gonads being the same size indicated males, while a left side being larger than the right side indicated females (Fig. 1B). To reconfirm the sex of the chicken embryos, a PCR amplification reaction of *CHD1* genes located on the Z chromosome was conducted [17]. The males were identified by the presence of
one band (600 bp) after agarose gel electrophoresis, while the females were identified by the presence of two bands (600 bp and 450 bp) (Fig. 1C). After sex determination, six males and six females collected at each time point were used for the subsequent analysis.

**Total RNA extraction and cDNA synthesis**

Approximately 20 mg of muscle tissue was transferred to a 1.5 mL tube (Axygen, USA) with 300 µL of lysis reagent (Promega, Wisconsin, USA) and then homogenized three times. Then, 300 µL of RNA dilution liquid was added to the homogenate and incubated for 5 min until centrifugation at room temperature at 13,000 rpm, and total RNA was extracted according to the manufacturer's protocol (Promega, Wisconsin, USA). The RNA concentration was detected a NanoDrop spectrophotometer (Thermo, USA), and the quality was assessed by agarose gel electrophoresis. Total RNA was qualified and used for the next manipulation (Fig. 1D). cDNA was synthesized using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan) in a 10 µL volume according to the manufacturer's instructions.

**Quantitative real-time PCR**

Two microliters of cDNA were added to 18 µL of PCR master mix containing 10 µL TB Green Premix Ex Taq (Takara, Shiga, Japan), 2 µL of ROX Reference Dye II (Takara, Shiga, Japan), 0.8 µL of forward primer (10 µM), 0.8 µL of reverse primer (10 µM), and 6 µL of sterilized water. The primer sequences for target genes, which included *Myomaker*, *MYOD*, *MSTN*, and the internal reference gene (*β*-actin), are listed in Table 1. The reactions were run on an Applied Biosystems 7500 real-time PCR instrument using the following settings: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 34 s; and a dissociation step consisting of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. For all qPCR analyses, three technical replicates were included for each sample. Relative expression was calculated with *β*-actin as a housekeeping gene using the $2^{-\Delta\Delta Ct}$ method [18]. Changes in gene expression were calculated separately for pectoral muscle and thigh muscle using the average ΔCt value of *Myomaker* for pectoral muscle of WL males at E11 as the control.
Table 1
The RT-qPCR primer sequence of genes

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5'-3')</th>
<th>Annealing Temperature (℃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHD1</td>
<td>F-GTTACTGATTGGTCTACGAGA</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>R-ATTGAAATGATCCAGTGCTTG</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F-ATCTTTCTTGGGTATGGAGTC</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R-GCCAGGGGTACATTGTGG</td>
<td></td>
</tr>
<tr>
<td>Myomaker</td>
<td>F-TGGGTGTCCCTGATGGGC</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R-CCCGATGGGTCTCCTGAGTAG</td>
<td></td>
</tr>
<tr>
<td>MYOD</td>
<td>F-GCTACTACACGGAATCACCAAAAT</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R-CTGGGCTCCACTGTCACTCA</td>
<td></td>
</tr>
<tr>
<td>MSTN</td>
<td>F-ACAGTAGCGATGGCTCTTT</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R-CCGTTGTAGGTTTTTGGA</td>
<td></td>
</tr>
</tbody>
</table>

Hematoxylin-Eosin (HE) staining

The pectoral muscle and thigh muscle samples were carefully collected from E15 to D1 chicken embryos and fixed in 4% paraformaldehyde for more than 48 h. The samples were dehydrated in 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 95% ethanol solutions successively and then embedded in paraffin wax. The transverse sections of 10 µm were cut and stained with hematoxylin and eosin for morphology examination [19].

Statistical analysis

The results are expressed as the means ± SEs of six independent biological replicates. Statistical analyses were performed using one-way analysis of variance followed by least significant difference (LSD) tests via the R program (ver 3.6.1). *P* values of less than 0.05 and 0.01 were considered to be significant and extremely significant, respectively.

Results

Temporal expression of myogenic regulatory genes during embryonic development

During the embryonic development of both males and females, the expression levels of the Myomaker gene in pectoral and thigh muscles among the four chicken breeds showed a wave change trend of first increasing and then decreasing (Fig. 2A-D). The Myomaker mRNA abundance in both muscle tissues showed an overall increase from E11 to E15 and reached the highest level at E13 of BYC (Fig. 2B) and at
E15 (Fig. 2A) of the other three breeds (Fig. 2C-D), which means that the critical period of myogenic fusion in chicken embryos was from E13 to E15. Afterward, the expression of the Myomaker gene across muscle tissues dropped linearly, although an unusual increase was found in the pectoral muscle of females of BYC at D1 (Fig. 2B) and the thigh muscle of females of WPR at E17 (Fig. 2C). Notably, the expression level of the Myomaker gene was nearly zero at D1 (except for BYC in the pectoral muscle) due to the decrease in myoblast fusion and the basic formation of myofibers during the late embryonic stage.

The expression of the MYOD gene in the pectoral muscle and thigh muscle of the four chicken breeds was clearly lower during the embryonic stage compared with D1 (Fig. 2E-H). The overall expression trends of the MYOD gene in the males were similar to those in the females. In brief, the gene expression level increased from E11 to E15, decreased slightly from E15 to E17, and then increased sharply from E17 to D1. Unlike that of the previous two genes, the expression level of MSTN in all breeds increased greatly with embryonic development and peaked at E17. After the chickens hatched, the gene expression level of MSTN significantly declined (Fig. 2I-L), especially in CN, in which it decreased to nearly zero for both males and females.

**Meat-type chicken breed had the highest gene expression of Myomaker at the crucial period**

The fusion of myoblasts increased from E13 to E15 due to the highest gene expression level of Myomaker. This result was further corroborated by analyzing the myofiber morphology of chicken embryos. Histological sections of the myofibers at each time point were generated, and it could be seen that the myoblasts at E13 to E15 were in the process of rapid fusion, which was consistent with the gene expression characteristics of Myomaker. The contours of myofibers were not clear until E17 (Fig. 3), indicating that the formation of myofibers had been completed at E17, after which myofiber hypertrophy was observed.

Since the crucial period of myoblast fusion was E13 to E15, we further analyzed the differential gene expression of Myomaker among various chicken breeds. WL and BYC are the egg-type chicken and native breeds, respectively. CN and WPR are the paternal and maternal lines of modern commercial broilers, respectively. There was no obvious difference in Myomaker gene expression among the males of the four chicken breeds at E13 (Fig. 4A-B). However, the muscles of CN females had higher Myomaker gene expression than did those of the other three breeds at E13 (Fig. 4C-D). The relative expression of Myomaker in the pectoral muscle in CN males at E15 was 1.81 ± 0.24, which was extremely significantly higher than that in the WL, BYC and WPR males (P < 0.01, Fig. 4E). Similar results were found in the thigh muscle of males (Fig. 4F) and both muscle tissues of the females (Fig. 4G-H). These results indicate that the Myomaker gene expression in CN was significantly higher than that in the other three chicken breeds at the crucial period and that the data were robust.

**The various gene expression of MYOD among the four chicken breeds at D1**

As noted above, the expression of the MYOD gene in muscles at D1 was higher than that during the embryonic period, which showed little change in MYOD gene expression. This result suggested that the
MYOD gene may more strongly regulate myogenesis after hatching. Therefore, the important time point of D1 was selected to investigate the differential gene expression of MYOD among various chicken breeds. There was an extremely significant difference in MYOD gene expression in the pectoral muscle (72.68 ± 10.34) and thigh muscle (75.20 ± 10.04) of males between WPR and the other three chicken breeds at D1 (P < 0.01, Fig. 5A-B). In addition, MYOD gene expression in the muscles of males of CN was significantly lower than that in WPR at D1 (P < 0.01, Fig. 5A-B). The pectoral muscle and thigh muscle of females of WPR still had the highest MYOD gene expression among the four chicken breeds at D1 (Fig. 5C-D). The difference in gene expression between CN females and the other chicken breed was changed compared to that of CN males at D1. In particular, the MYOD gene expression in the thigh muscle of CN females was significantly higher than that in egg-type chickens and Chinese native breeds (P < 0.01, Fig. 5D), which implied that the gene expression patterns of MYOD were slightly different between males and females at D1.

The inhibition of MSTN for the myoblast fusion

The expression level of the MSTN gene was low during the critical period of myoblast fusion, while it reached the highest level at E17, at which time the myoblast development of chicken embryos was repressed. The gene expression of MSTN in pectoral muscle of BYC males (23.10 ± 0.95) was significantly higher than that of WL and WPR males at E17 (P < 0.01, Fig. 6A). Similar results were obtained in the thigh muscle of BYC males (Fig. 6B). Interestingly, there was no significant difference in MSTN in the muscles of females among various chicken breeds at E17 (Fig. 6C-D), which indicated that MSTN gene expression in muscles might be different between male and female chicken embryos.

Discussion

Our findings demonstrated that E13 to E15 was the crucial period of myoblast fusion in chicken embryos and that Myomaker gene expression in CN was the highest. The Myomaker gene is closely related to the fusion of myoblasts to myoblasts during the embryonic stage [20]. Myomaker gene expression decreased after the crucial period, which implied that the process of myoblast formation was essentially complete. This hypothesis was confirmed by the increase in MSTN gene expression at E17. Since the MYOD gene expression of D1 in muscles was significantly higher than that in the embryonic period, we thought MYOD was more important in myoblast hypertrophy after the chicken hatched.

The Myomaker gene is closely related to the fusion of myoblasts to myoblasts during the embryonic stage [20]. Like in a previous study [8], Myomaker gene expression was highest at E14 but sharply decreased after E16. We found that E13 to E15 was associated with the highest expression of Myomaker among the four different types of chicken breeds, suggesting that E13 to E15 was considered a critical stage in myoblast development. The increase in MSTN gene expression at E17, together with the decrease in Myomaker gene expression, implied that myoblast fusion and muscle development events were inhibited from E17 to D1, which in turn proved the occurrence of atrophy of skeletal muscle in late-term chicken embryos [21]. The atrophy of skeletal muscle was associated with the rapid growth of the
intestinal tract and the inhibition of protein synthesis in the liver, leading to the catabolism of protein in skeletal muscle to provide energy for chicken embryos [22, 23]. Our results showed that the Myomaker gene was barely expressed in muscles at D1, which verified previous the findings of studies showing that muscle morphology formation occurred during embryogenesis [24].

We found that MYOD gene expression in muscles at D1 was much higher than that during the embryonic period, which was consistent with the findings of a previous study [8]. Unlike the results in chickens, MYOD gene expression in mice increased until birth, at which point the expression decreased [25]. This finding indicated a difference in MYOD gene expression between vertebrates and mammals during the embryonic period. However, the distinction among various chicken breeds is not yet clear; thus, we carried out this study and found MYOD gene expression differences in males and females between broilers and laying hens and local chickens. The results suggested that there might be breed variations in the MYOD gene expression pattern in chickens.

Studies have found that the MSTN gene can inhibit the activity and expression of the MYOD gene, thereby controlling the differentiation of myoblasts into myotubes, and that this inhibition is mediated through the Smad3 pathway [26]. The 55% reduction in MSTN expression along with the upregulation of MYOD by 4.65 times in chicken embryonic myoblasts further demonstrated the negative correlation between the two gene [27]. Nonetheless, relatively little research has been conducted on the expression patterns of the MSTN gene during the embryonic period of chickens. In the present study, MSTN gene expression in both pectoral muscle and thigh muscle increased from E11 to E17 in all four chicken breeds, which was consistent with the results for other organs, such as the liver, heart, brain and intestine [28]. As the chicken embryo develops to E17, the nutrients in amniotic fluid are used for the functional development of the gastrointestinal tract, and the morphological changes of the intestinal mucosa are accelerated [22]. The gene expression of MSTN during E15 to E17 increased sharply, suggesting that chicken embryos prepare for feeding after hatching at this stage through rapid development of digestive and metabolic organs; therefore, muscle growth in the late embryonic period is temporarily inhibited.

E13 to E15 is considered a critical stage of myoblast fusion in embryonic development. The formation of myofibers in various chicken breeds at the possible critical stage is an important foundation for the growth rates of skeletal muscle in chickens after incubation. The differences between broilers and laying hens have increased due to the intensive genetic selection of modern breeding for important traits [29]. Selection effects are often detected in chicken embryos, which are excellent models for development mechanisms [30, 31]. Previous studies have shown that the body weight and pectoral muscle weight of broilers were larger than those of laying hens from E11 to E18 [32]. The increased proliferation and differentiation activity of myoblasts, increased number of nuclei in myofibers and increased diameter of myofibers at E15 also proved that broilers grow faster during the embryonic stage. We found that the expression of the Myomaker gene in the muscles of CN was the highest at the critical stage of myoblast fusion and was significantly higher than that of other chicken breeds at E15. These results implied that the myoblasts fused to myofibers more in CN, a cornerstone broiler breed that develops rapidly, than in
other chicken breeds. This might be the reason why fast-growing broilers such as CN chickens had greater muscle mass a few weeks after hatching.

**Conclusion**

Together, this study reported the dynamic expression of key genes that influence muscle development in the late stage of chicken embryos. The expression of genes in pectoral and thigh muscle could be controlled by a shared myogenic regulatory program. We found that E13 to E15 was the critical period of myoblast fusion and contributed significantly to the rapid growth of broilers. Since the increase in *MSTN* gene expression occurred together with the decrease in *Myomaker* gene expression at E17, we speculate that the formation of myofibers is nearly complete at E17 and that the process of myofiber hypertrophy begins after that. There might be breed variations in the *MYOD* gene expression pattern in chickens, since differences in gene expression were detected among meat-type, egg-type and native chickens between males and females at D1. Our research also lays a foundation for the study of myofiber development during the embryonic period in different chicken breeds.

**Abbreviations**

WL: White Leghorn;
BYC: Beijing-You Chicken;
WPR: White Plymouth Rock;
CN: Cornish;
PM: Pectoral muscle;
TM: Thigh muscle;
PMM: Pectoral muscle of males;
PMF: Pectoral muscle of females;
TMM: Thigh muscle of males;
TMF: Thigh muscle of females.

**Declarations**

**Ethics approval and consent to participate**

All experiments with chicken in this study were approved by the guidance of ethical regulations from the Animal Care and Use Committee of China Agriculture University, Beijing, China.
Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors’ contributions

The contributions of the authors were as follows: NY conceived the project; JL prepared the incubation of fertile eggs; SG and HL conducted the animal trial and collected muscle samples; SG and QH performed the experiment; SG prepared the original draft of the manuscript; CW and CS modified the manuscript. All authors read and approved the final manuscript.

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References


Figures
Figure 1

Experimental design and procedure

A. Experimental design. In this study, four chicken breeds and five important time points were selected to explore the expression levels of three muscle-related genes in pectoral muscle and thigh muscle. B-C. Graphical representation of different sexes. For males, both sides of the gonads were the same size, and one band (600 bp) was visible; for females, the left side of the gonads was larger than the right side, and two bands (600 bp and 450 bp) were visible.

D. RNA quality was assessed by agarose gel electrophoresis. The 2:1 ratio of 28S:18S rRNA was employed as a threshold for intact RNA.

Figure 2
Time series of the three genes in the late embryonic stage

**A-D.** Expression trends of the *Myomaker* gene in the pectoral and thigh muscles of male and female White Leghorn chicken (WL), Beijing-You Chicken (BYC), White Plymouth Rock (WPR) and Cornish (CN) chickens. **E-H.** Expression trends of the *MYOD* gene in the pectoral and thigh muscle of male and female WL, BYC, WPR and CN chickens. **I-L.** Expression trends of the *MSTN* gene in the pectoral and thigh muscle of male and female WL, BYC, WPR and CN chickens. For A-L, the red and cyan lines represent changes in the expression levels of the corresponding gene in pectoral muscle of males (PMM) and females (PMF), respectively. The green and purple lines represent changes in the expression levels of the corresponding genes in the thigh muscle of males (TMM) and females (TMF), respectively.

**Figure 3**

Hematoxylin and eosin staining of the pectoral and thigh muscles at different timepoints
The two columns represent the pectoral muscle (PM) and thigh muscle (TM) of WL chickens. The myoblasts were still fusing at E15, and the contours of myofibers were not clear until E17, indicating that the formation of myofibers had been completed at E17. The diameter of myofibers of TM was obviously larger at D1. Scale bars: 50 μm.

**Figure 4**

**Gene expression of Myomaker among the various chicken breeds**

**A-D.** *Myomaker* gene expression differences among the four chicken breeds in the pectoral muscle of males, the thigh muscle of males, the pectoral muscle of females and the thigh muscle of females at E13. **E-H.** *Myomaker* gene expression differences among the four chicken breeds in the pectoral muscle of males, the thigh muscle of males, the pectoral muscle of females and the thigh muscle of females at E15. **, * and ns represent adjusted $P$ values of < 0.01, < 0.05 and > 0.05, respectively.
Figure 5

Gene expression of *MYOD* among the various chicken breeds at D1

**A.** Gene expression differences of *MYOD* in the pectoral muscle of males among the four chicken breeds at D1. **B.** Gene expression differences of *MYOD* in the thigh muscle of males among the four chicken breeds at D1. **C.** Gene expression differences of *MYOD* in the pectoral muscle of females among the four chicken breeds at D1. **D.** Gene expression differences of *MYOD* in the thigh muscle of females among the four chicken breeds at D1. ** and * represent adjusted *P* values < 0.01 and < 0.05, respectively.
Figure 6

Gene expression of *MSTN* among various chicken breeds at E17

**A.** Gene expression differences of *MSTN* in the pectoral muscle of males among the four chicken breeds at E17. **B.** Gene expression differences of *MSTN* in the thigh muscle of males among the four chicken breeds at E17. **C.** Gene expression differences of *MSTN* in the pectoral muscle of females among the four chicken breeds at E17. **D.** Gene expression differences of *MSTN* in the thigh muscle of females among
the four chicken breeds at E17. **, * and ns represent adjusted $P$ values < 0.01, < 0.05 and > 0.05, respectively.