Developmental characteristics of pectoralis muscle in Pekin duck embryos

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ABSTRACT. To confirm the entire developmental process and transition point of embryonic Pekin duck pectoral muscle, and to investigate the association between pectoral muscle development and their regulating genes, anatomical and morphological analyses of embryonic Pekin duck skeletal muscles were performed, and the expression patterns of its regulating genes were investigated. The anatomical analysis revealed that body weight increased with age, while increases in pectoral muscle weight nearly ceased after the embryo was 20 days of hatching (E20). The developmental morphological characteristics of Pekin duck pectoral muscle at the embryonic stage showed that E20 was the transition point (from proliferation to fusion) of Pekin duck pectoral muscle. The expression patterns of MRF4, MyoG, and MSTN indicated that E19 or E20 was the fastest point of pectoral muscle development and the crucial transition for Pekin duck pectoral muscle development during the embryonic stage. Together, these findings imply that E20 is the crucial transition point (from proliferation to fusion) of Pekin duck pectoral muscle and that there is no

muscle fiber hypertrophy after E20. Results of this study provide further understanding of the developmental process and transition point of Pekin duck pectoral muscle during the embryo stage.

Key words: Pectoral muscle; Pekin duck; Embryos; Tissue sections; Expression pattern

INTRODUCTION

Skeletal muscle development is a multistep process, in which mesodermal precursor cells are selected to form myoblasts that are withdrawn from the normal cell cycle and subsequently differentiate into myotubes (Patel et al., 2002; Velleman et al., 2010). These myotubes further differentiate into muscle fibers (Buckingham, 2006; Robert et al., 2010). As the myofiber number does not increase during the postnatal period, and is determined during the embryonic stage in most animals, muscle production of adult livestock is determined during embryogenesis.

Hypertrophy of muscle fibers with the lengthening and thickening in the postnatal stage is the major event (Xu et al., 2011). Therefore, the study of muscle development during the embryonic stage is a popular research focus. A number of studies have investigated muscle development during the embryonic stage in different species (Sabourin and Rudnicki, 2000; Callis et al., 2007; Williams et al., 2009; Zhang et al., 2012). In chicken, Hartley et al. (1992) explored whether skeletal muscle satellite cells appeared during the late chicken embryo stage. In turkey, Moore et al. (2005) investigated the cross-section area of myofiber and satellite cell mitotic activity in pectoralis muscles of late-term turkey embryos. In duck, Chen et al. (2012) studied the developmental transition of pectoral muscles from late-term duck embryos to neonates. Liu et al. (2012) compared muscle weight, muscle fiber diameter, cross-sectional area, the number of myofibers per unit area (MFN), and the frequency of satellite cell activation and mitosis at the embryo stage of 27 days and at the postnatal stage of 2 days after hatching between ducks that were and were not fed insulin growth factor-1 *in vivo*.

However, no study has yet addressed the entire developmental process and transition points of Pekin duck pectoral muscles during the embryo stage. Improved knowledge of the developmental process and transition point of Pekin duck embryonic pectoral muscles will enhance our understanding of the mechanism of pectoral muscle development in the Pekin duck embryo. In this study, to confirm the entire developmental process and transition point of Pekin duck pectoral muscle during the embryo stage, and to investigate the association between pectoral muscle development and their regulated genes, anatomical and the morphological analyses of Pekin duck embryonic skeletal muscles were performed, and the expression patterns of their regulating genes were investigated. The results of this paper should provide further insight into the developmental process and transition points of Pekin duck pectoral muscle during the embryo stage.

MATERIAL AND METHODS

Anatomy experiment

The embryos of Pekin duck (*Anas platyrhynchos domestica*) were obtained from the

breeding farm of Z-type Pekin ducks at the Institute of Animal Science (IAS), Chinese Academy of Agricultural Science (CAAS). A total of 180 eggs with 98 ± 5 g weight were selected. All eggs were incubated under the same conditions at a temperature of $37° \pm 0.5°C$ and humidity of 86-87%. Ten eggs were picked out every day from 10 days after hatching (E10) to 27 days after hatching (E27). The embryos were removed from the eggs, and then the whole embryos and the pectoral muscle were weighed. The pectoral muscle rate of each embryo was calculated using the following formula: $PMR = PMW_n/BW_n$, where PMR is the pectoral muscle rate, PMW_n is the pectoral muscle weight at day n after hatching, and BW_n is the body weight at day n after hatching.

Morphological analysis of pectoral muscle

The pectoral muscle samples from six embryo stages (E11, E14, E17, E20, E23, and E26) were washed with running water and then dehydrated in a series of ethanol dilutions, i.e., 75% for 4 h, 85% for 4 h, 95% overnight, and 100% ethanol for 2 h with two changes. Dehydrated tissues were treated with xylene three times and then embedded into paraffin blocks, trimmed, and cut into 4 μm sections. A paraffin ribbon was placed in a water bath at approximately 40°C. Sections were mounted onto slides, air-dried for 30 min, and then incubated at 45°C overnight. Sections were de-waxed with two changes of xylene for 10 min each and then hydrated in two changes of 100% ethanol for 3 min each, 95 and 80% ethanol for 1 min each, and finally, rinsed in distilled water for 5 min. Slices were stained with hematoxylin and eosin. All sections were photographed using a digital microscope (Nikon). Five individuals were analyzed per group; five sections were studied per individual by monitoring five random fields per section. The average value of 125 myofibers was examined per bird. The myofiber diameter (MFD) and the mean number of myofibers per unit area (MFN) were measured in Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

Expression pattern of MRF4, MyoG, and MSTN

Total RNA of each of the samples described above (the pectoral muscle tissues of five embryos were pooled to form a sample) was isolated with Trizol (Invitrogen, USA) following manufacturer instructions. The SYBR PrimeScript RT-PCR Kit (TaKaRa, Japan) was used for reverse transcription-polymerase chain reaction (RT-PCR), quantification of MRF4, MyoG, and MSTN cDNA, and for the detection of the two reference genes, β-actin and NADPH. Quantitative RT-PCR (qRT-PCR) was carried out with an iCycler IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA). The qRT-PCR reaction contained 1 μL cDNA template, 12.5 μL SYBR Premix Ex-Taq, 10.5 μL sterile water, and 0.5 μL each gene-specific primer (Table 1). The thermal cycling parameters were 1 cycle at 95°C for 30 s, 40 cycles of 95°C for 10 s, and 60°C for 40 s. An 80-cycle melting curve analysis was performed after each PCR run to confirm product specificity, with 1 cycle at 95°C for 1 min, 1 cycle at 55°C, and then the temperature was increased by 0.5°C every 10 s to 95°C while continuously monitoring fluorescence. qRT-PCR analysis of each sample was repeated three times. Moreover, to generate gene-specific standard curves, plasmids containing each gene's cDNA were serially diluted from 10^{-1} to 10^{-8}, and were used as the PCR templates to detect the amplification efficiency of each primer set. Standard curve data (R^2, slope, and efficiency) are provided in Table 1.

Table 1. Primer sequences and standard curve data for real-time quantitative PCR analysis.

Target gene	Primer sequence (5'-3')	Product size (bp)	R²[a]	Slope[b]	Efficiency[c]
β-Actin	Forward: GCTATGTCGCCCTGGATTTC	168	0.998	-3.369	0.981
	Reverse: CACAGGACTCCATACCCAAGAA				
NADPH	Forward: AAGGCTGAGAATGGGAAAC	254	0.999	-3.458	0.946
	Reverse: TTCAGGGACTTGTCATACTTC				
MyoG	Forward: CGGATCACCTCCTGCCTGA	87	0.998	-3.354	0.951
	Reverse: CGTCCTCTAACGGCGATGCT				
MRF4	Forward: GGAGCGCCATCAGCTACATC	132	0.996	-3.231	0.976
	Reverse: CGAGGAAGTCCGAGCCATT				
MSTN	Forward: GCCGACGGAGTCTGATTTTC	196	0.997	-3.437	0.979
	Reverse: ACTCTGCCAAATACCAGTGCC				

[a]Regression coefficient of standard curve. [b]Slope of standard curve. [c]Amplification efficiency of PCR.

Data analysis

The relative mRNA expression levels of MRF4, MyoG, and MSTN genes were calculated using the normalized relative quantification method in BIO-RAD IQ5 software. Briefly, for each sample (three replicates per sample), mRNA expression of five genes (MRF4, MyoG, MSTN, β-actin, and NADPH) was quantified. The average threshold cycle (C_T) value of each gene in each sample was calculated. The expression level of each gene at the E10 stage was used as the control (= 1) to calculate the relative quantity (RQ, ΔC_T method) at other stages using the formula:

$$RQ_{sample} = E^{C_T (control) - C_{T(sample)}},$$

where E = efficiency + 1. Then, expressions of MRF4, MyoG, and MSTN genes in each stage were normalized using the relative quantity of both reference genes. The calculation formula was as follows:

$$NE_{sample(geneX)} = \frac{RQ_{sample(geneX)}}{(RQ_{sample(\beta-actin)} \times RQ_{sample(NAPDH)})^{1/2}},$$

where *NE* is the normalized expression of the gene.

RESULTS

Anatomy experiment

Figures 1 to 3 show the rough results of the anatomical analysis. Figure 1 shows that body weight increased with increasing age of Pekin duck embryos. Figure 2 shows that pectoral muscle weight development was proportional to body weight changes before E20. However, growth of the breast muscle nearly ceased after E20 (approximately 1.0 g). Figure 3 shows that the pectoral muscle rate increased with increasing age before E20, and then gradually declined after that.
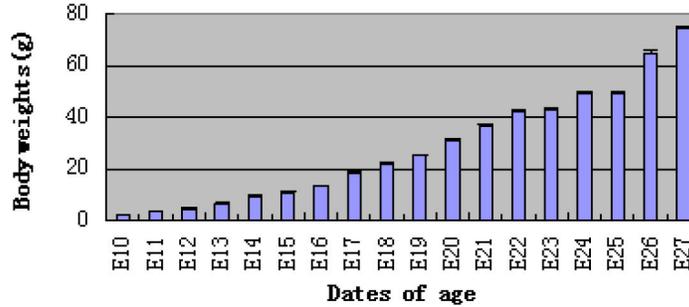
Figure 1. Growth of body weight of Pekin duck during embryos. E10 to E27 indicated embryos after 10 day hatching to embryos after 27 day hatching respectively.
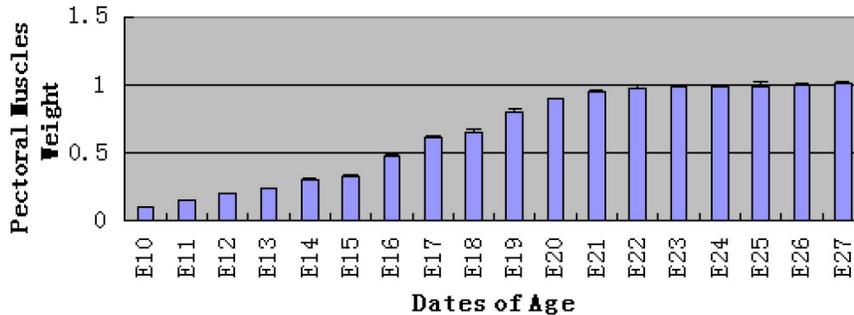
Figure 2. Growth of pectoral muscle weight of Pekin duck during embryos. E10 to E27 indicated embryos after 10 day hatching to embryos after 27 day hatching respectively.
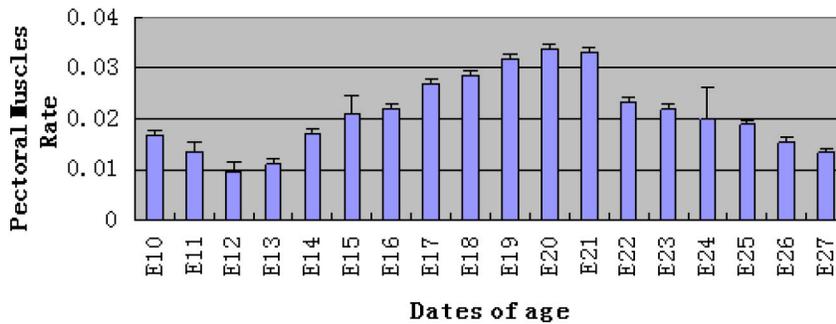
Figure 3. Pectoral muscle rate of Pekin duck during embryos. E10 to E27 indicated embryos after 10 day hatching to embryos after 27 day hatching respectively.

Morphological analysis of pectoral muscle

The process of muscle development at the embryonic stage is roughly demonstrated in Figure 4. Specifically, E11 to E17 represented the stage of proliferation, to form more mono-nucleated fibers, and fusing, to form multi-nucleated myotubes. At the E20 stage, myotubes

bound to the perimysium to form myolin. With myotubes developing continuously, muscle fibers became obvious at stage E23 in pectoral muscles of Pekin duck. Subsequently, muscle fibers continued to grow in circumference, and by stage E26, they already had the same shape as muscle fibers in adult animals.
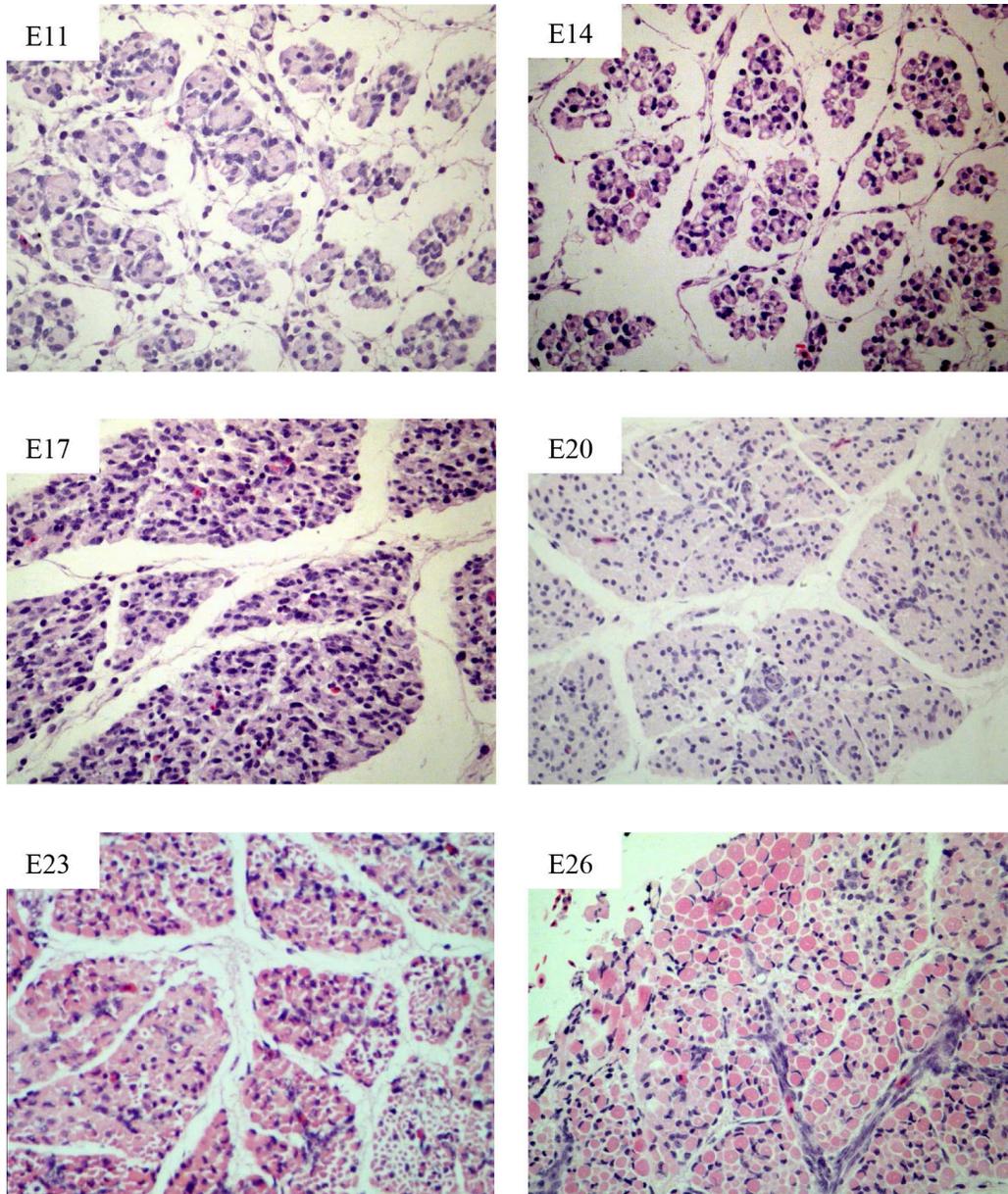
Figure 4. Cross-section of pectoral muscle in embryonic stage of Pekin duck. All the above images are amplified 400 times.

Figure 5 and 6 show the change in pectoral muscle fiber diameter and pectoral muscle fiber number in embryonic Pekin duck pectoral muscle tissues. Muscle fiber diameters increased with increasing age of the embryos, and two significant turning points (at E16 and E21, respectively) were observed (Figure 5). Conversely, the muscle fiber number decreased during development of the embryo. Interestingly, the significant turning points of muscle fiber number also occurred at E16 and E21 (Figure 6).
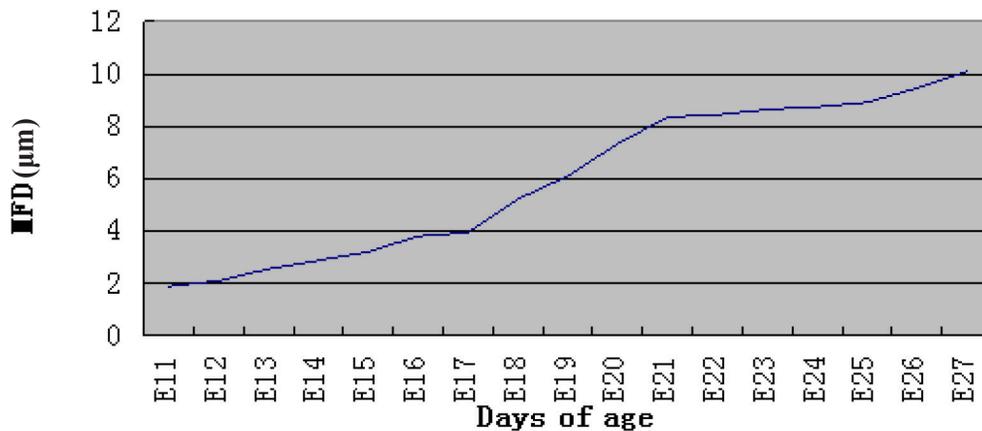
Figure 5. Myofiber diameter of pectoral muscle in embryonic stage of Pekin duck.
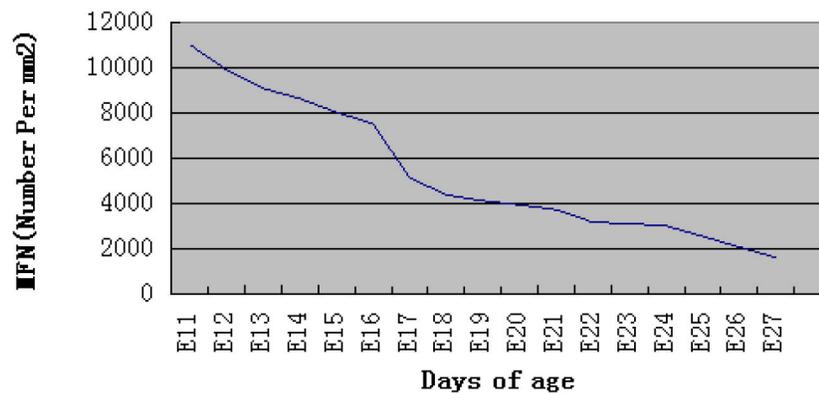
Figure 6. Myofiber number per mm^2 of pectoral muscle in embryonic stage of Pekin duck.

Expression profiling of MRF4, MyoG, and MSTN

The expression levels of MRF4, MyoG, and MSTN genes were detected using qRT-PCR. Figure 7 shows that the expression of MRF4 increased slowly, reached its peak value at E19, and then declined gradually. The expression of MyoG showed a similar trend as that of MRF4, and it reached its peak at E20. Conversely, the expression of MSTN declined slowly, reached its minimum value at E19, and then increased gradually (Figure 8).
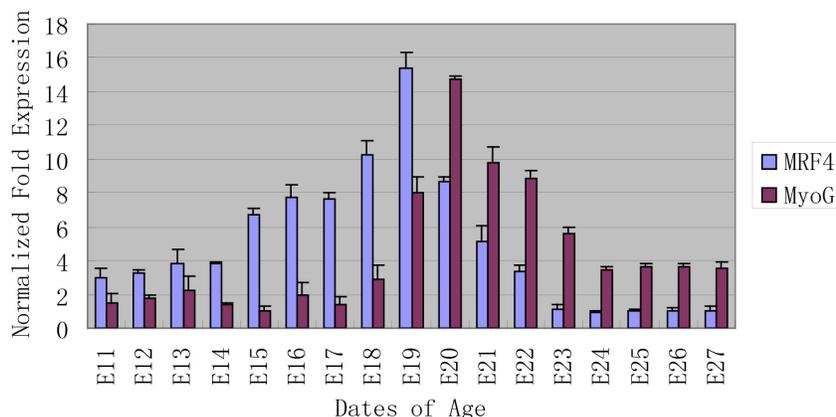
Figure 7. Relative expression level of MRF4 and MyoG genes in the pectoral muscle of embryonic stage Pekin duck.
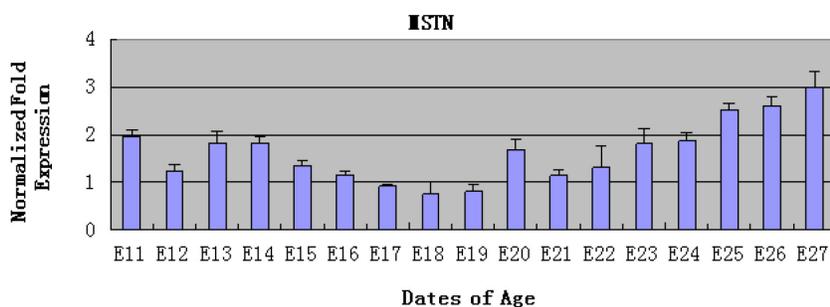
Figure 8. Relative expression level of MSTN gene in the pectoral muscle of embryonic stage Pekin duck.

DISCUSSION

In avian species, the skeletal muscle undergoes development and maturation in structure and function during the incubation period. During this time, myoblasts are proliferating, differentiating into multinucleated myotubes, and finally forming mature muscle fibers (Picard et al., 2002). In an exploration of the regulation and mechanism of skeletal muscle development, Moore et al. (2005) reported that the cross-section area of myofiber and satellite cell mitotic activity in the pectoralis muscle decreased with age in late-term turkey embryos, which indicated that the skeletal muscle might undergo atrophy during the final days of incubation. Chen et al. (2012) also found that the pectoralis muscle mass, muscle fiber bundles, and myofibers cross-section area showed marked reductions from E22 to hatching in duck. In the present study, body weight increased with age, while the pectoral muscle nearly ceased growth after E20. This suggested that the fusion of muscle fibers was the predominant event in the pectoral muscle of Pekin duck after E20, and that there was no hypertrophy during this stage. Therefore, E20 might be the crucial point at which muscle fibers of Pekin duck transition from proliferation to fusion.

Studying the developmental morphological characteristics of various tissues could provide intuitive evidence for the development and pathological changes of these tissues. Al-Ali et al. (2009) investigated the correlation between gross anatomical topography, sectional

sheet plastination, microscopic anatomy, and endoanal sonography of the anal sphincter complex in human males. Chen et al. (2006) found morphological changes of the skeletal muscle, tendon, and periosteum in senescence-accelerated mice (SAMP6) compared to age-matched normal SAMR1 mice. McClearn et al. (1995) explored the pervasiveness of myofiber degeneration and spatiotemporal patterns of degeneration during the development of head and neck muscles in chick embryos by evaluating morphological characteristics of muscle cells. In duck, Li et al. (2010) compared morphological characteristics of pectoral and leg muscle tissues during duck embryo stages to the neonatal stage through paraffin sections. Chen et al. (2012) studied the developmental transition of the pectoralis muscle from late-term duck embryos to neonates. In the current study, developmental morphological characteristics of Pekin duck pectoral muscles were investigated in the embryonic stage, and E20 was identified as the transition point (from proliferation to fusion) of the Pekin duck pectoral muscle. This suggested that the number of mono-nucleated myoblasts might be fixed before E20.

The myogenic regulatory factor (MRF) family (Tripathi et al., 2012), which includes MyoD, Myf5, MRF4, and MyoG, is important for muscle development. Two members of the family, MRF4 and MyoG, act as differentiation factors during skeletal muscle development, and MyoG is involved in muscle injury and repair after animal birth (Megeney and Rudnicki, 1995; Zhou and Bornemann, 2001; Wyszyńska-Koko et al., 2006). Myostatin (*MSTN*), also known as growth differentiation factor 8 (*GDF-8*), is an important negative regulator in skeletal muscle development. Inactivation of the *MSTN* gene in mice (Mendias et al., 2008) or mutations in bovine (McPherron and Lee, 1997), sheep (Boman et al., 2010), and humans (Schuelke et al., 2004) result in a similar phenotype of increased muscle growth. Therefore, these three genes, MRF4, MSTN, and MyoG, are always used as indicators of skeletal development. In this study, the expression patterns of MRF4, MyoG, and MSTN indicated that E19 or E20 is the fastest point of pectoral muscle development, and that E19 or E20 is the crucial transition for Pekin duck pectoral muscle development during the embryonic stage.

In conclusion, analytical analysis demonstrated that body weight increased with age, while the pectoral muscle nearly ceased growth after embryonic day 20 (E20). Further morphological analysis of the Pekin duck pectoral muscle in the embryonic stage revealed that E20 was the transition point (from proliferation to fusion) of Pekin duck pectoral muscles. The expression patterns of MRF4, MyoG, and MSTN indicated that E19 or E20 is the fastest point of pectoral muscle development, and that E19 or E20 is the crucial transition for Pekin duck pectoral muscle development during the embryonic stage.

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