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IGF-1 and FoxO3 Expression Profiles and Developmental Differences of Breast and Leg Muscle in Pekin Ducks (*Anas platyrhynchos domestica*) During Postnatal Stages

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Abstract: Breast Muscle (BM) and Leg Muscle (LM) in Pekin duck belong to different types. However, myofiber differences of BM and LMcle and relationship between myofiber development with the expressions of IGF-1 and FoxO3 are not clear. The difference of BM and LM fibres was investigated and relationship between the two gene expressions and myofiber development was explored at D2 (the 2nd day), W2 (the 2nd week), W4, W6. Results showed the development of BM and LM fibres were different as the Relative Growth Areas (RGAs) of BM and LM fibres reached their maximum values at W4-W6 and W2-W4, respectively. The expression of IGF-1 peaked while that of FoxO3 bottomed, at W6 and W2 in BM and LM, respectively. The results of this study could provide basic insight into the skeletal muscle development, the expressions of IGF-1 and FoxO3 and the association between the two aspects.

Key words: Pekin ducks, skeletal muscle, IGF-1 and FoxO3, expression profiles, tissue section

INTRODUCTION

In developing avian muscles, it has been shown that embryonic muscle fibers, as well as adult muscle fibers are heterogeneous with regard to myosin isoform content (Crow *et al*., 1983; Crow and Stockdale, 1984, 1986). Generally, the leg muscle is mainly constituted of slow fibers while the breast muscle is mainly constituted of fast fibers (Li *et al*., 2010). These two muscle fibers are different from each other in many aspects such as protein constitution, physical properties and taste which are related to the meat production in an agricultural view (Li *et al*., 2009). Therefore, exploring the developmental characteristics of the two muscle fibres in poultry could be very helpful to provide some useful data for revealing the mechanisms of the skeletal muscle development.

The 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muscle development is a multistep process, in which mesodermal precursor cells are selected to form myoblasts that are withdrawn from the normal cell cycle and subsequently differentiate into myotubes (Merlie *et al*., 1977; Buckingham, 2006). In avian species, the skeletal muscle undergoes development and maturation in structure and function during the incubation period. Also, during this period, myoblasts are proliferating, differentiating into multinucleated myotubes and finally forming mature muscle fibers, the process is chiefly involved in myofiber hyperplasia (Picard *et al*., 2002). In postnatal stage, as opposed to the number increase during embryonic stage skeletal muscle hypertrophy is defined as an increase in muscle mass which comes as the result of an increase in the size of pre-existing skeletal muscle fibers (Glass, 2005). In this stage, it has been demonstrated that Insulin-like Growth Factor 1 (IGF-1) is sufficient to induce skeletal muscle hypertrophy and Forkhead box O (FoxO) can cause dramatic atrophy of muscle fibres (Sandri *et al*., 2004).

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The IGF-1/PI3K/Akt signalling pathway plays a key role in the regulation of muscle mass and promotes fiber hypertrophy by stimulating overall protein synthesis and suppressing proteolysis (Bodine *et al*., 2001; Rommel *et al*., 2001; Frost and Lang, 2007). In skeletal muscle, Akt is activated by IGF-1 stimulating protein translation through induction of mammalian Target of Rapamycin (mTOR) which activates p70S6K and inactivates the inhibitor of translational initiation 4EBP1 and GSK-3β which stimulates the initiation factor eIF2B (Bodine *et al*., 2001; Rommel *et al*., 2001). In contrast, during atrophy process, the activity of the IGF-1/PI3K/Akt pathway decreases, leading to the activation of FoxO3 which leads to a stimulation of protein breakdown not only by the ubiquitin-proteasome pathway (Sandri *et al*., 2004; Stitt *et al*., 2004) but also, by the autophagic/lysosomal system (Mammucari *et al*., 2007; Zhao *et al*., 2007). Therefore, IGF-1 and PoxO3 could be used as the positive and negative indicators of skeletal development.

A number of studies have investigated the characteristics of skeletal muscle development during the embryonic stage (Callis *et al*., 2007; Li *et al*., 2010; Liu *et al*., 2012; Chen *et al*., 2012). However, there is seldom comparison about the developmental morphologic difference between the breast muscle and leg muscle tissues of duck in postnatal stages. Furthermore, evidences have shown that IGF-1 promotes the muscle mass growth (Liu *et al*., 2011, 2012) and FoxO3 plays crucial role in skeletal muscle autophagy (Sanchez *et al*., 2012) but their roles in skeletal development of Pekin duck and the association of their expression profiles with these two muscle fibers in duck are still unclear.

Here, researchers compared the morphologic differences of breast muscle and leg muscle fibers of Pekin duck in postnatal stages using tissue section technology, performed the expression patterns of IGF-1 and FoxO3 using Real-Time quantitative PCR technology (qRT-PCR) and tried to explored the relationship between the expression patterns of IGF-1 and FoxO3 and the skeletal muscle development of Pekin duck. The results of this study would provide preliminary understanding of the developmental characteristics of postnatal skeletal muscle in Pekin duck and the association of the expression patterns of IGF-1 and FoxO3 with the skeletal muscle development of Pekin duck.

MATERIALS AND METHODS

Birds and sample collection: Pekin ducks were obtained from Pekin Ducks Breading Farms of Institute of Animal Science, Chinese Academy of Agricultural Sciences. A total of 40 eggs with approximately 98±5 g per egg were selected to incubate in an incubator with a temperature of 37±0.5°C and humidity of 86-87%. After birth, the ducklings were raised in four pens with ten ducklings per pen. Four individuals with approximately average body weight were slaughtered to collect breast and leg samples at each stage of D2 (the 2nd day after birth), W2 (the 2nd week after birth), W4 and W6. For each individual at each stage, two pieces of Breast Muscle (BM) samples from pectoralis major and two pieces of Leg Muscle (LM) samples from the center of biceps femoris were collected, respectively. One of the two samples from the same tissue was frozen immediately in liquid nitrogen for RNA extraction and the other was immersed in 10% neutral formalin for morphologic research.

Morphologic analysis of skeletal muscle fibres: Four fixed samples washed by running water were dehydrated in series diluted ethanol, i.e., 75% for 4 h, 85% for 4 h, 95% for overnight and then 100% ethanol for 2 h, two changes. Dehydrated tissues were treated with xylene for three times and then embedded into paraffin blocks, trimmed and cut at 4 μm by using Leica RM2235 (German). Paraffin ribbon was placed in water bath at about 37°C. Sections were mounted onto slides, dried in air for 30 min and then baked in 45°C oven overnight. Sections were dewaxed 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 Haematoxylin-Eosin (H&E). Digital microscope (Nikon 90i) was used to observe and analyse sections. The average area of 200 muscle fibres at each stage was measured with Axioversion 40 Software. Unpaired t-test was used to compare the independent means from the diameters of breast muscle fibre and leg muscle fibre (SAS Package 9.0). The equation of relative growth areas is:

$$RGA = (Af-Al)$$

Where:

RGA = Relative Growth Area
Af = The mean area of former stage
Al = The mean area of later stage

Total RNA extraction and first-strand cDNA synthesis: Total RNAs were isolated from all the samples using the RNAiso plus kit (Takara, Dalian, China) and the integrity of RNAs was tested by 1.2% denaturing agarose gel electrophoresis. The quantity of RNAs was estimated by measuring the spectrophotometric absorption at 260 nm in a Nanodrop ND-1000® Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand

Table 1: The primer pairs used in this study

Primer name	GenBank access No.	Primer sequence (5'-3')	Tm (°C)	Length of product (bp)
FoxO3	XM_001234495.2	F CAAGGGCGACAACAACAG	54.9	106
		R GCTCTTCCCAGTGCCTTC	54.9	
IGF-1	EU049611.1	F CCTTTGGGATGGTGTATGAG	55.1	176
		R ACCAAGCAACCGAACCAC	55.9	
β-actin	EF667345.1	F ATGTCGCCCTGGATTTCG	55.5	165
		R CACAGGACTCCATACCCAAG	57.3	

cDNAs were obtained from 10 μg of total RNA using cDNA synthesis kit (TaKaRa, Japan) following the manufacturer's instruction.

Real-time quantitative RT-PCR: The SYBR PrimeScript RT-PCR kit (TaKaRa, Dalian, China) was used for the quantification of IGF-1, FoxO3 and reference gene (β-actin). The qRT-PCR reactions were carried out with an iCycler IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA). The qRT-PCR reaction contained 1 μL of cDNA template, 12.5 μL of SYBR Premix ExTaq, 9.5 μL of sterile water and 1 μL of each gene-specific primer (Table 1). Thermal cycling parameters were 1 cycle at 95°C for 2 min, 40 cycles of 95°C for 15 sec, 60°C for 34 sec. Dissociation curve analysis was carried out after each real time experiment to ensure that there was only one product. The qRT-PCR analysis of each sample was repeated three times.

Statistical analysis: The relative gene expression levels were determined by the comparative cycle threshold (C_T) method. The AC_T value was calculated by subtracting the target C_T of each sample from its β-actin C_T value. The gene expression at different stages was analysed by one-way ANOVA followed by the Bonferroni test for pairwise comparison. Unpaired t-test was used to compare the average area and RGAs of the muscle fiber between BM and LM (SPSS for Windows, Version 13.0, Chicago, IL, USA).

RESULTS AND DISCUSSION

Morphologic analysis of BM and LM fibres in Pekin duck: Figure 1 and 2 showed the cross-sections of LM and BM fibres from D2 to W6, respectively. The Figure 1 and 2 showed that LM and BM fibres continuously developed from D2 to W6. However, the development of BM fibres lagged behind of that of LM fibres in all the postnatal stages.

The average areas of BM and LM fibres at each stage were showed in Fig. 3. It could be clearly seen that the average area of LM and BM fibres both increased continuously from D2 to W6 while the average area of LM fibres increased more rapidly than that of BM fibres. At each stage, the average area of LM fibres was

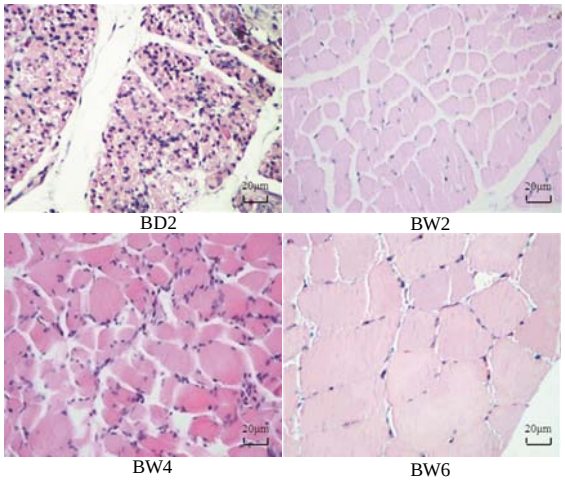

Fig. 1: Cross-sections of leg muscle fibers at various stages of Pekin duck. LD2, LW2, LW4 and LW6 represent the cross-sections of leg muscle fibers at the 2nd day, the 2nd, 4th and 6th weeks, respectively

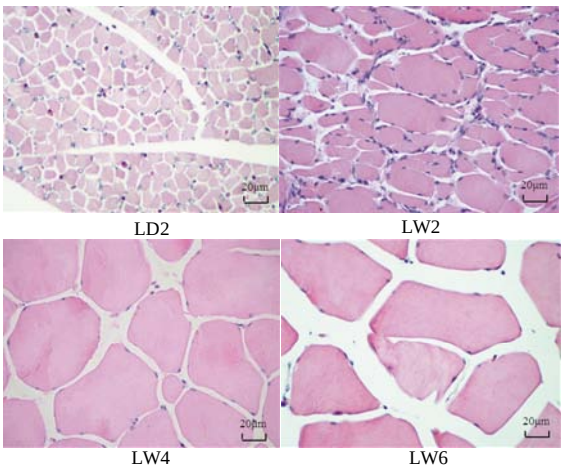

Fig. 2: Cross-sections of breast muscle at various stages of Pekin duck. BD2, BW2, BW4 and BW6 represent the cross-sections of breast muscle fibers at the 2nd day, the 2nd, 4th and 6th weeks, respectively

significantly larger than that of BM fibres ($p<0.01$ at all stages). The RGAs of LM and BM fibres at different

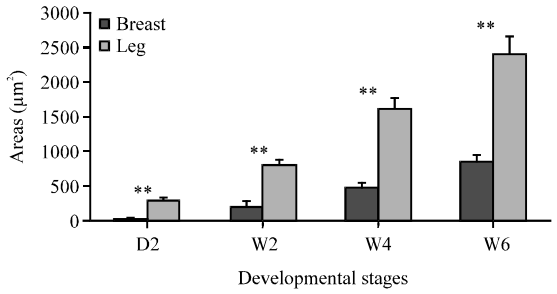

Fig. 3: The myofiber areas of Pekin duck breast and leg muscle at various stages. **indicates there is extremely significant difference between the area of breast muscle fibers and that of leg muscle fibers; D2, W2, W4 and W6 represent the areas of breast and leg muscle fibers at the 2nd day, the 2nd, 4th, and 6th weeks, respectively. Breast: 21.47, 198.45, 497.69, 857.41; Leg: 289.87, 805.73, 1602.38, 2389.47

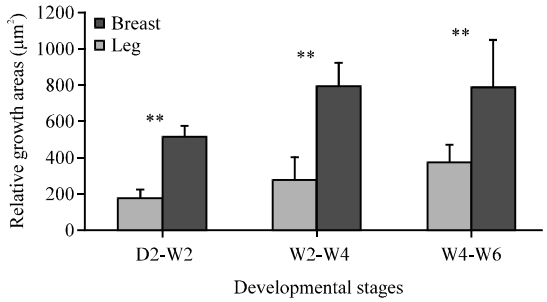

Fig. 4: The relative growth areas of myofiber of Pekin duck breast and leg muscle at various stages. **indicates there is extremely significant difference and *indicates there is significant difference between the areas of breast muscle fibers and that of leg muscle fibers; D2-W2, W2-W4 and W4-W6 represent the relative growth areas of breast and leg muscle fibers during the 2nd day to the 2nd weeks, the 2nd to the 4th weeks and the 4th to the 6th weeks, respectively. Breast: 176.98, 281.24, 377.72; Leg: 515.86, 796.65, 787.09

stages were showed in Fig. 4. It demonstrated that the RGAs of BM fibres and LM fibres shared a similar trend (increased first and then decreased) but the RGAs of LM fibres. For BM fibres, the RGAs continuously increased from D2-W2 to W4-W6 (from 176.98-377.72 μm^2) but the RGAs of LM fibres reached its peak at W2-W4 (796.65 μm^2).

Expression patterns of *IGF-1* and *FoxO3* gene in Pekin duck skeletal muscle: The mRNA expression patterns of

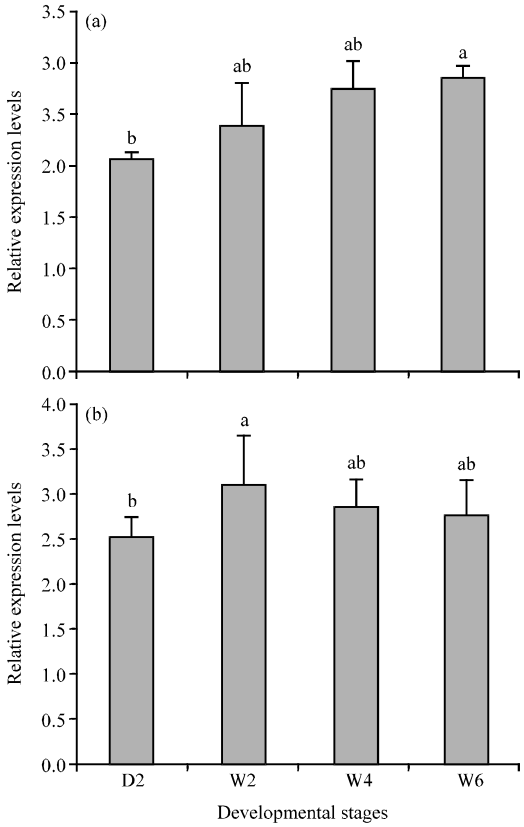

Fig. 5: Relative expression levels (means) of Pekin duck *IGF-1* gene. The relative expression levels headed by the same letter denote no significant difference ($p>0.05$) and headed different letter denote significant difference ($p<0.05$); D2, W2, W4 and W6 represent the relative expression levels of; a) IGF-1 in breast and b) leg muscle at the 2nd day, the 2nd, 4th and 6th weeks, respectively

IGF-1 and *FoxO3* genes in LM and BM at different stages were detected by qRT-PCR and the results were shown in Fig. 5, 6 and Table 2. In BM, IGF-1 expression level increased from D2 to W6 (from 2.07-2.87). In LM, similarly, IGF-1 expression increased from D2 to W2 (from 2.48-3.07). Comparing with IGF-1, FoxO3 expression level showed a converse trend. The expression level of FoxO3 in BM declined from D2 (3.25) slowly and reached its minimum value at W6 (2.42). In LM, FoxO3 expression level showed a similar trend with that in BM and the minimum expression level was at W4 (2.54).

Morphologic analysis such as tissue section is effective because of its intuitionistic and quick evaluation without much data. White *et al.* (2013) studied the myofibre cross sectional area of the mice muscle stained by H&E after nandrolone decanoate administration. He *et al.* (2012) elucidated the mechanism of

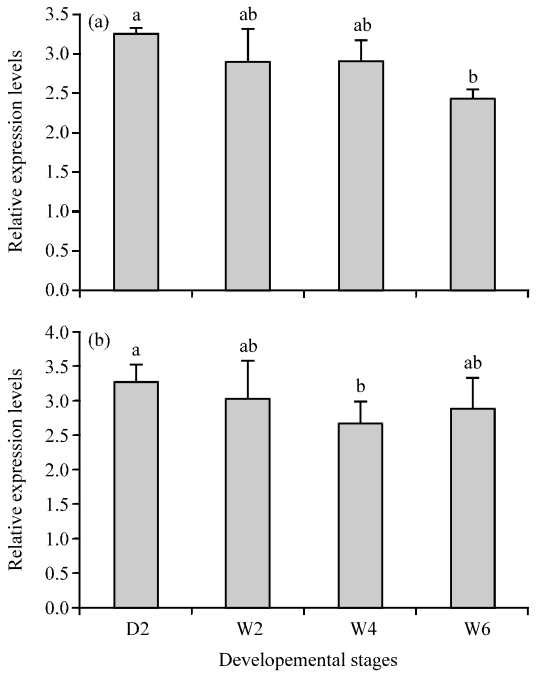

Fig. 6: Relative expression level (means) of Pekin duck *FoxO3* gene. The relative expression levels headed by the same letter denote no significant difference ($p>0.05$) and headed different letter denote significant difference ($p<0.05$); D2, W2, W4 and W6 represent the relative expression levels of; a) *FoxO3* gene in breast fibers and b) leg muscle fibers at the 2nd day, the 2nd, 4th and 6th weeks, respectively

Table 2: The expression levels of *IGF-1* and *FoxO3* genes in breast muscle and leg muscle at various stages[a]

Gene name	Organs	D2	W2	W4	W6
IGF-1	Leg	2.48±0.23	3.07±0.52	2.81±0.30	2.72±0.41
	Breast	2.07±0.05	2.40±0.40	2.76±0.26	2.87±0.11
FoxO3	Leg	3.13±0.21	2.90±0.48	2.54±0.27	2.76±0.38
	Breast	3.25±0.08	2.91±0.43	2.90±0.19	2.42±0.14

[a]Data were shown as mean±SD. There were four stages at each organ

exercise-induced BCL2-regulated autophagy for muscle glucose homeostasis using multiple methods including representative images. In this study, researchers performed the analysis of myofibre cross sectiona areas stained by H&E and found that both BM and LM fibres of duck underwent a series of developmental process in postnatal stages. Meanwhile the development of BM fibres always lagged behinds that of LM fibres. This suggests that the fibre development of BM and LM of duck is different in postnatal stages.

Muscle mass increase by hypertrophy (increased cellular protein content) is regulated by numerous factors including IGF-1, one of the most prominent known muscle growth factors (Braun and Gautel, 2011). IGF-1 is known to activate the PI3K/Akt pathways and hereby represses FoxO3 protein activity and promotes muscle growth. Muscle-specific over-expression of IGF-1 causes muscle hypertrophy (Musaro *et al*., 2001) suggesting the expression level of IGF-1 is closely related to the development of skeletal muscle. In the present research, the expression level of IGF-1 in BM reached the maximum values at W6 while that in LM peaked at W2 indicating W6 is the fastest developmental stage for BM and W2 is the fastest growth stage for LM.

Generally, the FoxO3 transcription factor is believed to be crucial in muscle wasting and for the induction of atrogin-1 and MuRF-1 whose induction is essential for rapid atrophy (Sandri *et al*., 2004; Stitt *et al*., 2004; Hasselgren, 2007). At the same time, FoxO3 reduces total protein synthesis in adult muscle in mammals (Reed *et al*., 2012) and thus reduces muscle hypertrophy. Therefore, FoxO3 is regarded as a negative regulator of skeletal muscle growth. In the current study, the expression level of FoxO3 peaked at W6 in BM and that at W2 in LM indicating BM grows fastest at W6 and LM develops most slowly at W2.

CONCLUSION

Taking the results of the morphologic analysis of BM and LM fibres together with the results of the expression patterns of *IGF-1* and *FoxO3* genes, researchers could find that the expression level of IGF-1 is positively correlated with skeletal development while that of FoxO3 is negatively associated with skeletal muscle development of Pekin duck. The RGAs of BM fibres peaked at W4-W6 and that of LM fibres reached the maximum value at W2-W4 indicating W4-W6 is the crucial stage for BM development and W2-W4 is the key stage for leg muscle development of Pekin duck. Interestingly, the expression levels of IGF-1 peaked while that of FoxO3 bottomed, at W6 and W2 in BM and LM, respectively. All the results above suggest that IGF-1 and FoxO3, like in the skeletal muscle of other animals are the potential positive and negative regulators in the skeletal muscle development of Pekin duck, respectively.

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