

Temporal and Spatial Expression of the *Pax-7* Gene During Chicken Embryo and Postnatal Development

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Abstract: This study was conducted to investigate the different temporal and spatial expression pattern of *pax-7* gene in embryo stage and growth stage of chicken, aiming to explore associations between muscle development and molecular markers of *pax-7* gene. Breast and leg muscle tissue were selected from 8, 16, 20 and 21 days chick embryo and 2, 4, 6, 8, 10, 12 weeks rugao chickens. This study performed SYBR Green real-time fluorescence quantitative PCR to detect the expression pattern of *pax-7* gene. The results showed that the expression pattern of *pax-7* gene was similar between breast and leg muscles which it is lower during chicken embryo and reached to the highest point at 8-10 weeks of age. *Pax-7* gene was continual expressed during chicken embryo and postnatal development, not only expressed in the embryo stage. Finally, we reassess current models of early patterning based on the analysis of these dynamic spatiotemporal expression patterns.

Key words: Chicken, *pax-7* gene, RT-PCR, spatiotemporal expression, tissue, China

INTRODUCTION

Growth and development of chicken muscle fiber closely related to the muscle quality traits. The particular muscle fiber type, density and diameter have significant effects on meat quality. Chicken muscle fiber number has been determined in the embryonic period, muscle fiber size increases after incubation. Pax family plays a key role on proliferation and differentiation of muscle cell in the process of muscle fibers growth and development, it is considered to candidate genes affecting characteristic of the muscle fiber. At present, function of genes affecting muscle fiber almost has not been studied. There are pax gene family, *pax-3* and *7* gene which are related to growth and development of muscle fiber (Haunerland and Spener, 2004; Walther *et al.*, 1991; Stapleton *et al.*, 1993). Currently, there is no data on the growth and development law of chicken *pax-7* gene in different growth stages. *Pax-7* gene expression rule in different tissues of rugao chicken were studied in this study. The aim is to explore gene expression law of *pax-7* gene, reveal the developmental change the *pax-7* gene expression and provide preliminary information mechanism about the candidate genes of chicken muscle fiber growth.

MATERIALS AND METHODS

Six chickens were selected from the same batch of chicken hatching (8, 16, 20, 21 days after incubation) in

Yangzhou Xianglong Poultry Co., Ltd. while 2, 4, 6, 8, 10, 12 weeks rugao chicken were selected and slaughtered. Half male and half were female. Take chest, leg muscles sample and immediately frozen in liquid nitrogen, frozen samples at -70°C refrigerator for use.

Primer design and synthesis: Select the *β-actin* gene as reference gene, primers were designed according to the chicken *pax-7* gene mRNA sequence published in GenBank. Primers were synthesized by Shanghai Bioengineering Co., Ltd. The primer information and the reaction conditions are shown in Table 1.

Total RNA extraction and RT-PCR reaction: Chicken RNA was extracted using Trizol methods and the extraction step operating strictly according to kit instructions. RNA purity and concentration were detected with 1% agarose gel electrophoresis ultraviolet spectrophotometry. Chicken RNA were saved at -70°C refrigerator for use. cDNA synthesis contains 10 μL reaction mixture containing 5× PrimerScript buffer reaction mixture 2 μL, PrimerScript RT enzyme mix I 0.5 μL, Oligo dT 0.5 μL, Random 6 mers 0.5 μL, total RNA 500 ng, Rnase free H₂O to 10 μL. Reaction condition was 37°C 15 min, 85°C 5 sec. PCR amplified DNA fragment reaction is 20 μL. 10×PCR buffer 2.0 μL, 2.5 mmol L⁻¹ dNTP 1.5 μL, 10 μmol L⁻¹ primers (upstream) 1 μL, 10 μmol L⁻¹ primer (downstream) 1 μL, Taq enzyme 1.0 U, 100 ng μL⁻¹ DNA

Table 1: Primers used for RQ-PCR

Number	Sequences	Annealing	
		Size	Temp.(°C)
β-actin	F: 5' GAGAAATTGTGCGTGACATCA 3'	152	60
	R: 5' CCTGAACCTCTCATTGCCA 3'		
Pax-7	F: 5' CTGTCACCACTCTTTGC 3'	142	60
	R: 5' TGAAGCGGGTCACATA3'		

template 1 μL, ddH₂O 13.3 μL. Reaction conditions are that the samples were denaturing at 95°C 15 min, amplified by the following procedure; 95°C 20 min, 62°C 20 min, 72°C 20 sec, 33 cycles, 72°C for 7 min, 4°C preservation.

Construction of standard curve: Take a certain amount of cDNA template and dilute as 10-fold concentration. The dilute products were used to real time PCR while input concentration gradient values to quantitative PCR instrument. Based on the fluorescence response to real-time monitoring data, β-actin and pax-7 mRNA standard curve were constructed.

Quantitative PCR reaction system and reaction conditions: Reaction system 20 μL contains cDNA 2 μL, upstream and downstream of each primer 0.8 μL (10 μmol L⁻¹), ROX Reference Dye II (50 ×) 0.4 μL, SYBR Green real-time PCR master mix (2×) 10 μL, RNase free H₂O 6 μL. PCR reaction conditions are 95°C, 1 min; 95°C 15 sec; 60°C 34 sec, 40 cycles, analysis melting curve after amplification. PCR amplification owns unity if T_m peak is (85±0.8)°C in the melting curve each real-time PCR samples were detected 3 times, take average.

Data analysis: The quantitative PCR of all RNA samples were analysed by -ΔΔC_t method (Livak and Schmittgen, 2001). The fold changes in mRNA levels were determined as follows: ΔC(T) non-stimulated = C(T) target gene non-stimulated - C(T) 28 sec non-stimulated. ΔC(T) stimulated = C(T) target gene stimulated - C(T) 28 sec stimulated. The fold change in mRNA = 2(ΔC(T) non-stimulated - ΔC(T) stimulated).

RESULTS AND DISCUSSION

Purity and integrity of total RNA: The total RNA extracted from various tissues sampled in 1% agarose gel electrophoresis (Fig. 1). There are 28, 18 and 5 sec three bands and there are not significant DNA contaminated and degraded band. RNA purity were detected with UV spectrophotometer, OD₂₆₀/OD₂₈₀ is 1.8-1.9. It indicated that the high quality RNA extraction can be used for sub-sequent tests.

Specification of pax-7 gene primers: Figure 2 shows that the specification of pax-7 gene primers are very well.

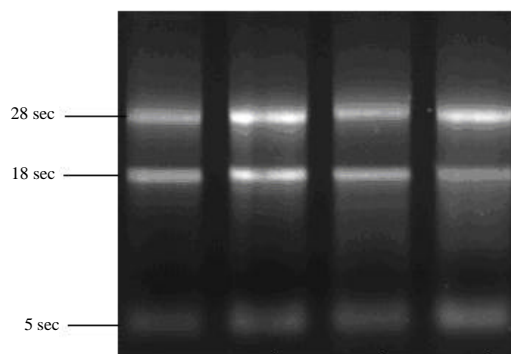


Fig. 1: 1% agarose gel electrophoresis of extracted total RNA

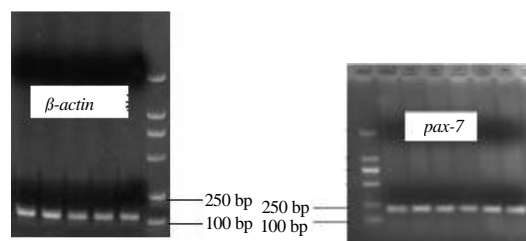


Fig. 2: Agarose electrophoresis results of PCR for RQ primers

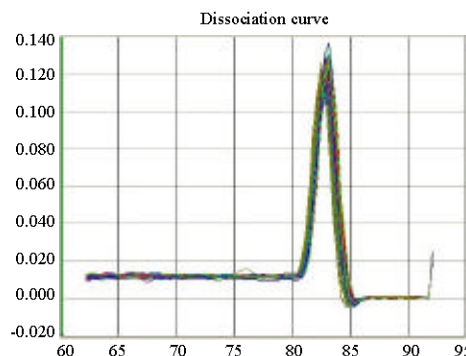


Fig. 3: RT-PCR dissociation curve for the pax-7 gene

Melting curve of fluorescence quantitative PCR: According to changes law of fluorescence after the reaction, amplification kinetics curve with cycle number and fluorescence changes detection was automatically generated, pax-7 gene mRNA RT-PCR product melting curve were constructed (Fig. 3). RT-PCR gene products had only one specific peak and there were not primer dimer and non-specific product.

Expression pattern of pax-7 gene

Pax-7 gene expression value in rugao chicken breast muscle: Pax-7 gene expression value in rugao chicken breast muscle in different developmental stages were showed in Table 2 and Fig. 4. Pax-7 gene expression of

Table 2: Relative expression value of *pax-7* gene in breast muscle of rugao chicken

Gender	Days				Weeks					
	8	16	20	21	2	4	6	8	10	12
Male	23.067 ±0.240	39.242 ±0.361	33.131 ±0.236	34.770 ±0.350	20.724 ±0.189	78.046 ±0.922	172.40 ±1.320	119.975 ±1.018	18.010 ±0.190	28.456 ±0.261
Female	18.959 ±0.170	20.111 ±0.212	23.012 ±0.218	21.478 ±0.210	35.166 ±0.272	35.832 ±0.382	23.372 ±0.214	78.046 ±0.837	13.917 ±0.150	20.482 ±0.186

Table 3: The RQ value of *pax-7* gene in leg muscle of rugao chicken

Gender	Days				Weeks					
	8	16	20	21	2	4	6	8	10	12
Male	26.73 ±0.175	32.942 ±0.277	30.244 ±0.180	37.040 ±0.201	19.770 ±0.163	64.920 ±0.535	124.842 ±1.1130	118.725 ±0.960	22.907 ±0.240	18.959 ±0.136
Female	32.954 ±0.321	20.128 ±0.129	26.102 ±0.250	32.396 ±0.236	25.531 ±0.164	34.984 ±0.377	68.0830 ±0.6440	100.45 ±0.839	32.082 ±0.243	29.254 ±0.301

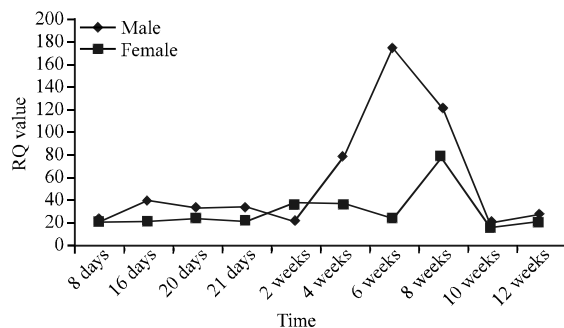


Fig. 4: *Pax-7* gene expression in different periods in breast muscle tissue

rugao chicken breast muscle showed in Fig. 4. It can be seen from Fig. 4 that gene expression of breast muscle of male chicken has been stable from incubation to born, the expression level increased rapidly after 2 weeks of age, gradually increased to the highest level of 6 weeks and began to decline at 8 weeks of age, down to low level at 10 weeks of age. The gene expression of breast muscle of female chicken keep stable from incubation to 6 weeks of age, rapidly rose to the highest point at 8 weeks of age. *Pax-7* gene expression was also maintained a high level then decreased rapidly and maintained the birth expression level after 10 weeks of age.

***Pax-7* gene expression value in rugao chicken leg muscle:** *Pax-7* gene expression value in rugao chicken leg muscle in different developmental stages were showed in Table 3 and Fig. 5. It can be seen from Fig. 5 that gene expression of leg muscle of male chicken are the same in the embryonic period and at different growth stages and gene expression of leg muscle of female chicken increased rapidly at 2 weeks age, reached a peak at 8 weeks of age then fall, kept the birth expression level at 12 weeks age. The expression level of female chicken is less than that of male chicken.

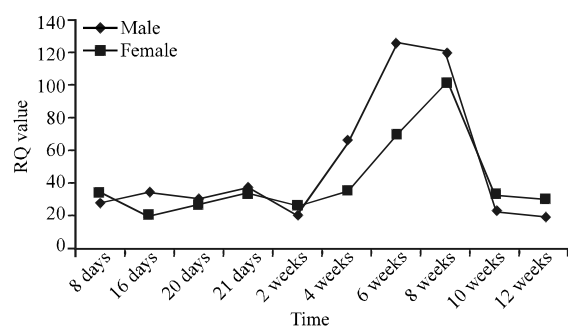


Fig. 5: *Pax-7* gene expression in different periods in leg tissue of rugao chicken

Relative quantification method: Relative quantification is used to quantification of gene expression is to calculate the relative gene expression level compared to the reference gene. Compared with the absolute quantification, the value calculated with this method is a relative value, not the copy number of gene transcription. But at the same time, this method eliminate the need for steps such as building a standard curve, the operating method is relatively simple and time-saving. Statistical results showed that the results of this method is very reliable in gene expression variation analysis. During the relative quantitative analysis, the following areas are optimized in order to ensure reliable results.

Choice of reference gene: GAPDH, β -actin, 18sRNA are generally selected as reference gene. Taking into account in animal muscle constant expression, the gene in humans, pigs, chickens, ducks and geese and other species is conservative so, β -actin gene was chosen in this study.

The choice of primers: In this study, we have chosen the SYBR Green method, the dye can not only combine with the target sequence but also combine primer dimers and nonspecific amplification products in PCR amplification process. In the primers design process, first consider the

intron design and reduce DNA contamination caused by non-specific amplification. To ensure the amplification efficiency between 90-110%, fragment length of primers is within 300 bp and primers were screened.

Construction of standard curve: The curve was constructed with recombinant plasmid as a template in this study. To solve different primer amplification efficiency problem, the ABI 7500 SDS V2.01 Version of the software were used to data analysis, the software can calculate the relative expression value when amplification efficiency is inconsistent.

CONCLUSION

Research has shown that *pax-7* gene interacts with *pax-3* in embryonic periods and control embryonic development of muscle fiber. *Pax-7* gene is related to muscle repair and regeneration of muscle cells in animals after birth. The number of muscle fibers have been identified before birth and *pax-7* can repair muscle and make muscle cell regeneration (Seale *et al.*, 2000; Oustanina *et al.*, 2004). Expression study of the *pax-7* gene in embryonic and postnatal expression showed that *pax-7* gene expression levels were the highest in embryonic periods, decreasing after birth. *Pax-7* gene expression of female chicken peaked at 6th week age and at 8th week age in male. It proved that *pax-7* gene had the repair function of animal muscle. This need to be further explored. The inconsistent early peak times of expression may be related to different physiological states during early growth and development process.

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