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Research Article

Associations Between Forkhead Box L2 Expression and Ovary Development in Laying Hens

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Abstract

Make sure healthy ovary or follicle is critical for extending egg laying performance in poultry. Transcription factor forkhead box L2 (FOXL2) gene have key role in regulate development of ovary. In the present research, different aged Hy-line Brown hens were maintained to explore relationships between ovarian developing and FOXL2 expression. Through histological observation, different quantities of follicles from various phases of age were observed. It was displayed that FOXL2 expression and number mature follicle were increased as the days of age increased and then decreased. In comparison, the expression of FOXL2 in hypothalamus and eyelid were remained in a relative stable level. Taken together, these data in our research establish a framework for understanding the potential functions of FOXL2 in regulate chicken ovarian developing and may provide a new perspective on the theory and practice to increase egg production or others.

Keywords: *FOXL2, Follicle, Ovary, Laying, Poultry*

Yumurtacı Tavuklarda Ovaryum Gelişimi İle Forkhead Box L2 Ekspresyonu Arasındaki İlişki

Öz

Kanatlılarda yumurtlama performansını artırmada sağlıklı ovaryum veya foliküller kritik öneme sahiptir. Transkripsiyon faktörü forkhead box L2 (FOXL2) geni, ovaryum gelişimini düzenlemede anahtar rol oynamaktadır. Bu araştırmada, ovaryum gelişimi ile FOXL2 ekspresyonu arasındaki ilişkiyi araştırmak için farklı yaşlarda Hy-line Brown tavuklar kullanılmıştır. Histolojik olarak, değişik yaş evrelerindeki folikül miktarları incelendi. FOXL2 ekspresyonu ve olgunlaşmış folikül sayısının, yaşla birlikte arttığı ve sonrasında ise azaldığı gözlemlenmiştir. Hipotalamus ve gözkapağında FOXL2 ekspresyonu karşılaştırıldığında göreceli olarak sabit kaldıkları belirlenmiştir. Sonuçlar birlikte değerlendirildiğinde, çalışmada elde edilen veriler tavuklarda ovaryum gelişimini düzenlemede FOXL2'nin muhtemel fonksiyonunu anlamada bir çerçeve oluşturabilir, teorik ve uygulamada yumurta üretimini artırmada yeni bir bakış açısı geliştirmeye yardımcı olabilir.

Anahtar sözcükler: *FOXL2, Folikül, Ovaryum, Yumurtacı, Kanatlı*

INTRODUCTION

As egg laying poultry, the most important economic trait is egg production, which is influenced by a variety of factors, including genetics, nutrition, and environment conditions (such as light time and intensity). All of these factors influence egg production could be attributed to affecting ovarian development. The ovarian development of chickens starts from the early stage of embryos and continues until the end of the whole reproductive cycle. And the growth, maturation, and differentiation of follicles in ovary were regulated under the synergistic of local regulatory factors (growth factors and cytokines) and/or exogenous hormones (follicle stimulating hormone and luteinizing hormone) [1,2].

FOXL2 is the first discovered factors that participate in ovarian development in a variety of animals such as mammals, birds, reptiles and fish. And it's a highly conserved gene that continuously expressed in mammalian ovaries

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from sexual differentiation to adulthood especially in granulosa cells. In addition to causing Blepharophimosis-ptosis-epicanthus-inversus syndrome BPES, mutations in FOXL2 cause many ovarian diseases. Over 95% granulosa cell tumors (GCTs) are associated with abnormal expression of FOXL2 [3,4]. In many animals, FOXL2 directly activates the transcription of CYP19A1 to regulate estrogen synthesis [5,6]. FOXL2 is a molecular marker of the early mammalian ovary, which begins to express in the mouse embryo of 12.5 d old and has female specificity. Further studies identified FOXL2 mice cannot form primordial follicles that became sterile [7,8]. These data demonstrated that FOXL2 plays an important role in the process of sex differentiation and and ovarian granulosa cell differentiation.

Although the function of FOXL2 has been well studied in mammals, it remains unclear in chicken. Studies in chicken embryos have found that FOXL2 and CYP19A1 are both female-specific and the expression patterns are highly correlated. The expression of FOXL2 was decreased in chicken embryos supplemented with CYP19A1 inhibitors, but did not completely disappear or have obvious sexual reversal, while over expression of CYP19A1, the expression of FOXL2 was increased [9,10]. Therefore, it can be speculated that during embryonic period, FOXL2 has a certain interaction with aromatase and participates in the sex determination of chicken embryo gonads. On the other side, a non-synonymous replacement of FOXL2 SNP A238G causes isoleucine 77-proline mutations associated with egg production and egg weight in Chinese Big Bone Chicken, and FOXL2 can enhance the regulate role of GDF9 in pre-follicular cells proliferation [11,12]. However, there is no systematic study on the role of FOXL2 in the development of chicken embryo gonads or in the development of adult ovarian follicles.

The laying performance of hens is a compelling problem in poultry production, and egg production performance is closely related to the development of hen ovary. Ovarian development is a dynamic process that continues the entire process of female reproductive life. FOXL2 has been considered as a key factor in controlling normal reproductive physiology in mammals. Therefore, we identified FOXL2 as a candidate gene for controlling ovarian development in poultry, and systematically studied its role in the development of poultry ovary. In this research, we will compare the expression of FOXL2 and follicle developing at different age in egg-laying hens that further provide a suggestion for extending egg production stage.

MATERIAL and METHODS

Ethics Statement

Experimentation with animals was approved by the Experimental animal management methods of Xinxiang Medical University (Approval number 201206078) and followed the Regulations of Experimental Animals of Henan Authority.

Animals and Sample Collection

Several different-aged Hy-Line Brown hens were purchased from Siqing chicken farm (Xinxiang, China) and maintained on open floor space under free food and water intake. Chickens were divided into 8 groups according to their age and 3 chickens in each group (30 d, 60 d, 90 d, 120 d, 160 d, 220 d, 330 d, 480 d). At times, the hens was selected and euthanized by decapitation. Ovary was removed and weighted at different age to investigate the follicle development of hens. At the same time, different stage follicles in ovary were counted. While other organ such as hypothalamus and eyelid were excised at the indicated ages. Scissors cut appropriate size of organ (50-100 mg) and stored in liquid nitrogen until used for RNA extraction.

Histopathological Analysis

A part of ovary (1 cm^3) were removed and fixed with 4% neutral formalin at room temperature for 48 h. Serial tissue sections were cut to 5-μm thickness after embedding in paraffin. Each slide was stained with hematoxylin and eosin (H&E) and then examined by light microscopy (Olympus BX41, Olympus Optical Co., Tokyo, Japan).

Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was prepared from 10 mg of collected organ homogenized in Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The DNaseI-treated RNA (0.2 μg) was reverse-transcribed into cDNA via an EasyScript First-Strand cDNA Synthesis Super Mix (TransGen Biotech, Beijing, China). The following primers were used in the qPCR: Foxl2 forward primer, 5'-CTACT CCTACGTGGCCCTGA-3', and reverse primer, 5'-TGATGAAG CACTCGTTGAGG-3'; β-actin forward primer, 5'-AGTACCCC ATTGAACACGGT-3', and reverse primer, 5'-ATACATGGCT GGGGTGTTGA-3'. The reaction was run on a 7500 thermal cycler (Applied Biosystems) with an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 40 s. The expression of FOXL2 was determined using the relative quantification method and normalized to control using the $2^{-\Delta\Delta CT}$ method with β-actin as an internal standard.

Statistical Analysis

Data are expressed as means ± standard error (SE). Differences in variability among different groups were determined by one-way tests of variance using the GraphPad Prism (version 6.0; GraphPad Software, San Diego, CA, USA); statistical significance was set at $P<0.05$.

RESULTS

It was demonstrated that the weight of ovary was obviously

increased as the days of age increasing and reached a peak value until 220 d and then began a slow decline during aging (30, 60, 90, 120, 160, 220, 330, 480) *(Fig. 1)*. Before age of 160 d, we could hardly see any mature egg in ovary.

An obvious different morphology of follicle was observed in different age's hens by using HE staining. To further explore the relationships between laying and ovary weight, the histopathological features of the different aged ovary are shown in *Fig. 2*. Histologically, the normal ovarian cavities were infiltrated with small homogeneous follicles in low-aged chicken. While the higher aged hens contained with a comparatively high number of primordial and early follicles. To further deeply exploit follicle developing kinetics, the number of primary follicles and secondary follicles were detected according to the follicle diameter. The table exhibit secondary follicles number was obviously increased as the days of age increasing and reached a peak value until 160 d and then began a slow decline between different ages *(Table 1)*. Above these data, it was exhibited that the development of ovary has a relationship with hens' age.

Many researchers reported that FOXL2 is one of the most important sex determination genes. To examine the role of FOXL2 on development of follicle or egg production, analyses of its expression in ovary were performed by real-time PCR on different age's hens. As shown in *Fig.2a*, the expression of FOXL2 was up-regulated and reached a peak value at 120 d, thereafter with a continuous down-regulated expression. To further confirm its regulated role, the expression of FOXL2 in eyelid and hypothalamus were also detected *(Fig. 3)*. Different from in ovary, FOXL2 expression was maintained at a constant level. In hypothalamus, FOXL2 high expression kinetics started earlier than 60 d, and sustained a peak value at 60-120 d, and there after returning to near basal levels at high age. Based

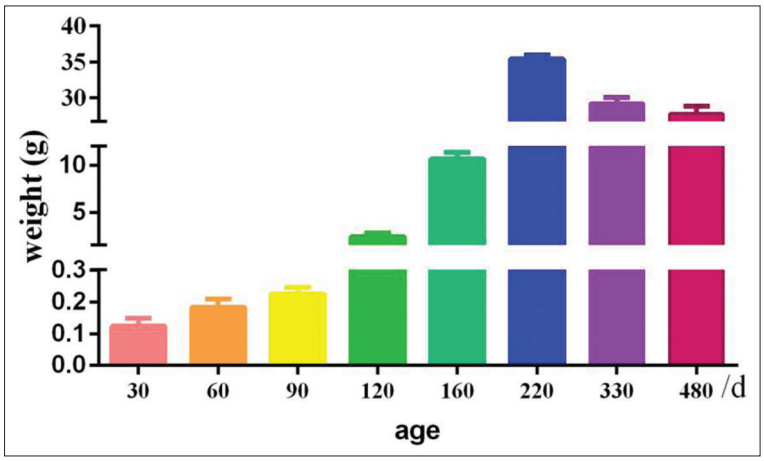

Fig 1. Weight of ovary. The weight of ovary were measured at indicated age. (N= 3)

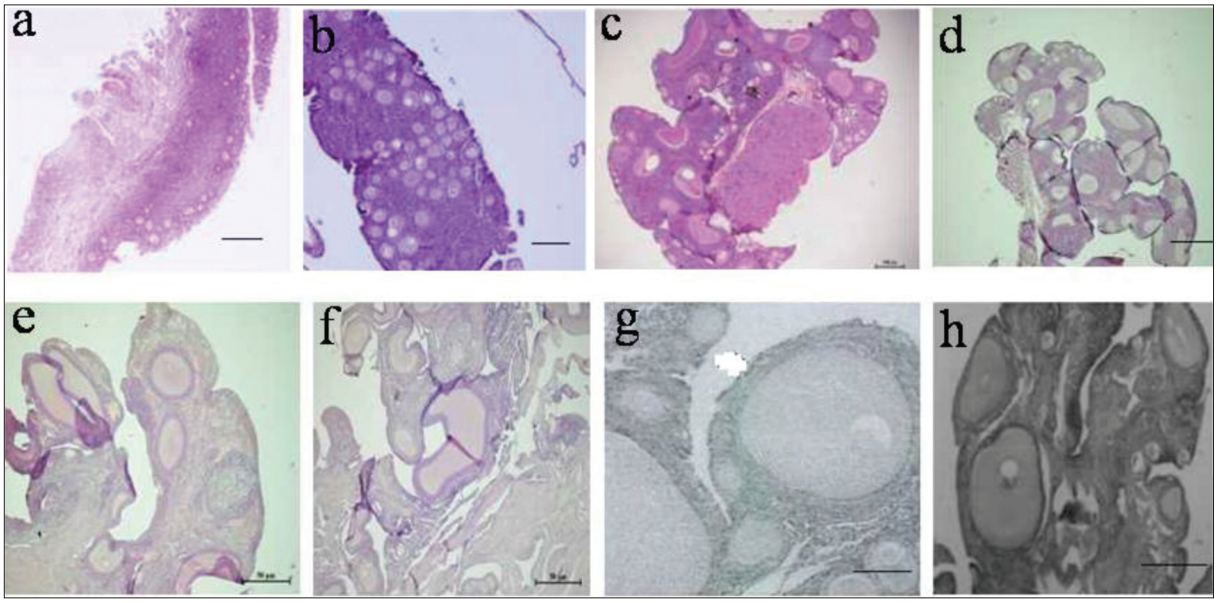

Fig 2. Ovary histopathology at day indicated age. Ovary were subjected to H&E staining. a:30d; b:60d; c:90d; d:120d; e:160d; f:220d; g:330d; h:480d

Table 1. *Diameter of follicle in ovary*

Age	Primary Follicle	Secondary Follicle	Shrinkage of Secondary Follicle
30	88165.27±4252.37	648.55±866.71	1.57±0.34
60	78231.87±6106.40	5595.47±886.64	2.07±0.64
90	62820.34±11434.69	7553.120±2302.44	2.53±0.65
120	63018.30±10068.41	8684.20±1875.39	2.48±0.53
160	61380.10±7743.69	9868.10±1206.37	2.60±0.50
220	61380.10±7743.69	8168.70±1306.33	3.30±0.49
330	56230.20±8206.77	7168.80±1236.19	3.60±0.59
480	40724.10±3240.64	5164.65±1024.26	5.12±0.84

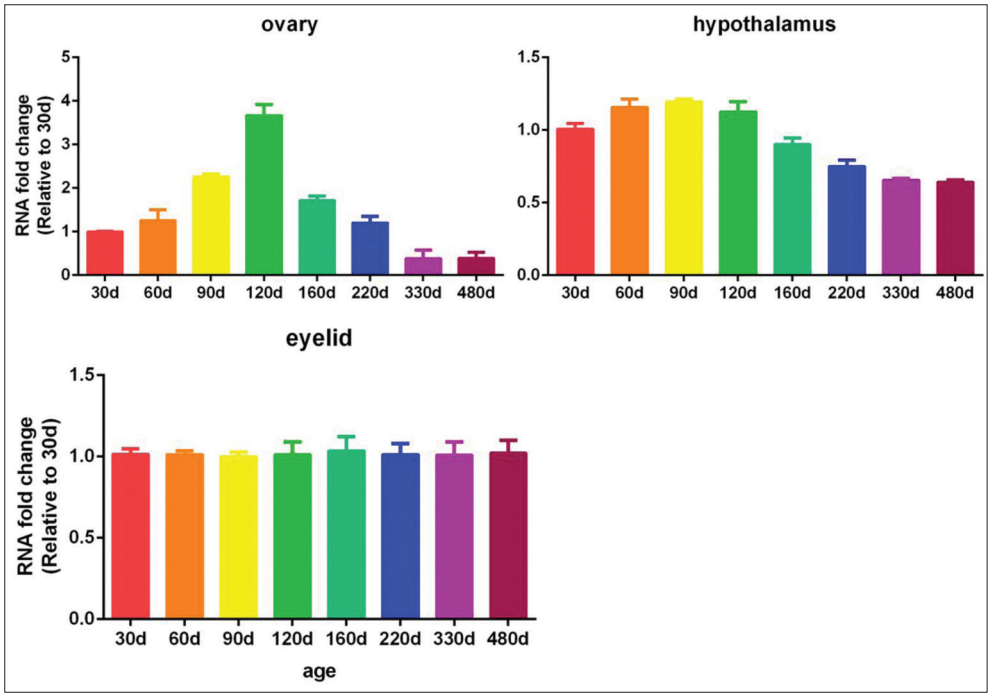

Fig 3. The expression of FOXL2 gene in different organ. The mRNA levels of FOXL2 in the ovary, eyelid, hypothalamus at indicated age were determined by real-time PCR (N=3)

on these data, it's suggested that a relationship between FOXL2 and ovarian developing.

DISCUSSION

Ovarian reserve is a critical factor affecting the function of ovary in human being. At present, the ovarian reserve function is usually evaluated based on the age, the number of follicles. Age was the only independent factor that affected the ovarian reserve, the number of follicle and high quality embryo could be reduced with advancing age [13]. Therefore, the physiological age of women is sometimes not exactly compatible with ovarian reserve function. FOXL2 is an important regulator in early stage of human ovarian differentiation and involved in the proliferation and differentiation of granulosa cells. Studies have shown that the expression level of FOXL2 in ovarian granulosa cells is negatively correlated with serum basal FSH, indicating that the expression level of FOXL2 in luteinized granulosa cells decreases with the increase of FSH, suggesting that the decrease of FOXL2 mRNA expression may reflect the decrease of ovarian reserve function [14]. Fuhrer et al.[15] reported that FOXL2 may have an anti-follicular apoptosis effect, and the reduced expression of FOXL2 may promote apoptosis of follicles and decrease the number of follicles in the ovary, leading to a decrease in ovarian reserve function. Therefore, FOXL2 can be used as a direct indicator of ovarian reserve function.

Similarly, there are currently no specific markers for independent evaluation of ovarian reserve function and ovarian response in laying hens. The follicular development process of mature ovary in poultry is different from that of mammals and has priority characteristics. The expression level of FOXL2 is up-regulated during this process, indicating that it has a certain effect on the differentiation of granulosa cells during chicken follicle selection [16]. The results were consistent with the results of Govoroun et al.[17] and Qin et al.[12]. Thus, similar to the role in mammals, FOXL2 may also affect follicular development

by participating in the regulation of the function of ovarian granulosa cells in sexually mature chicken ovaries. In our study, we aimed to explore the relationship between FOXL2 and ovarian developing in Hy-Line chicken. From our data, with growth of age, the expression of FOXL2 in ovarian was increased, and then slowly decreased. By comparison, the expression of FOXL2 in hypothalamus and eyelid was not change very much over time. Our data was identical with other research that expression of FOXL2 has critical roles in the regulation of hen ovarian development and may be used as a indicator.

In summary, this experiment demonstrated that FOXL2 plays an important role in the development of chicken ovary, but its specific regulatory mechanism needs further research. In depth study of the gene regulatory network of chicken ovary development and candidate key genes can provide ideas for understanding the regulation mechanism of chicken follicle development, and provide a theoretical basis for genetic improvement of chicken laying performance.

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