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Histological and Developmental Study of Prehierarchical Follicles in Geese*

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The development of the follicular wall and apoptosis of corresponding cells are dependent upon the stage of follicle growth and levels of endogenous hormones. However,the development and apoptosis of prehierarchical follicles in geese is insufficiently known.
In order to obtain an understanding about the microstructure, development and apoptosis of prehierarchical follicles in geese, firstly, a histological method was used to investigate the morphological structure of prehierarchical follicles. Results showed that the thickness of granulosa cell layers of the follicular wall increased first, then decreased to the lowest when follicles grew to 9~10 mm in diameter, and the theca layers also thinned to the lowest thickness at the same stage. Moreover, the expression of follicle-stimulating hormone receptor (FSHR) mRNA and the enzyme activity of caspase-3 were analyzed and the results showed that the expression of FSHR was highest when follicles grew to 8~9 mm in diameter ($p<0.05$); the enzyme activity of caspae-3 was the highest when follicles grew to 6~8 mm in diameter ($p<0.05$). These collective findings suggested that follicles 6~10 mm in diameter were especially significant, and perhaps represent a turning point from growing follicles to dominant follicles to be selected into a hierarchical sequence or to other follicles to be degenerated during prehierarchical follicle development

Key words: Goose, ovary, pre-hierarchical follicles, microstructure, FSHR, caspase-3.

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The avian ovary provides a unique model for the study of follicular development because it contains thousands of developing follicles and their developmental stages can be distinguished easily through observation. Follicles are arranged at the surface of the ovary and maintain a special hierarchy in their development, which is why some birds, e.g. chicken, can produce an egg each day or every two days, and many seasonal breeding birds, e.g. goose, need more days for producing an egg (CHEN *et al*. 2012). In birds, as in mammals, a large number of ovarian follicles cannot reach maturity and ovulate successfully but undergo degeneration at various stages of development. This is an important phenomenon for the regulation of clutch size, involving atresia (ONAGBESAN *et al*. 2009). Atresia is a frequently occurring phenomenon which can be found possibly in follicles of all sizes. However, the pre-hierarchal follicles, which are considered relatively undifferentiated, are susceptible to atresia, whereas pre-ovulatory follicles are resistant to atresia under normal physiological conditions (JOHNSON 2000; JOHNSON 2012; MCDERMENT *et al*. 2012). Therefore, the development of follicles is a homeostasis of selection and atresia, and the cells around the oocyte play an important role in this process.

The avian ovarian follicle is composed of an oocyte in the centre, surrounded successively by oocyte plasma membrane, the granulosa cell layer, the theca cell layer and surrounding connective tissues (KOVASC *et al*. 1992; WOJTYSIAK & KAPKOWSKA 2005; MADEKUROZWA & KIMARO 2006). All these circumambient structures constitute the follicle

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wall. It can provide mechanical support and transport nutrients for the growing oocytes and play a key role in the synthesis of steroid hormones (WOJTYSIAK & KAPKOWSKA 2005; HERNANDEZ & BAHR 2003; WOJTYSIAK *et al*. 2011), and it can also play important roles for the development of the oocyte and ovulation. All elements of the follicular wall undergo obvious structural changes during the phase of growth when observed under an electron microscope (KOVASC *et al*. 1992; MADEKUROZWA & KIMARO 2006). The normal maturation process of the oocyte within the ovarian follicle depends on the coordinated development of the follicular wall inducing granulosa and theca cells under the influences of various hormones and growth factors (WOJTYSIAK *et al*. 2011; LIN *et al*. 2011; HRABIA *et al*. 2008; ONAGBESAN *et al*. 2009; SECHMAN 2013). The reproductive axis hormone, follicle-stimulating hormone (FSH), plays a pivotal role in the process of follicular development. It is responsible for follicular recruitment and growth of the smaller follicles (HERNANDEZ & BAHR 2003). Circulating change of the expression levels of FSH and its receptor (FSHR) in granulosa cells play a decisive role in avian follicular selection (JOHNSON 2012; HERNANDEZ & BAHR 2003), and FSHR is considered as a gene marker for follicle selection (JOHNSON 2012; MCDERMENT *et al*. 2012; WOODS & JOHNSON 2005).

It is now well-established that ovarian follicular atresia occurs via apoptosis originating within the granulosa cell layer (JOHNSON & WOODS 2009). As in mammals, apoptosis within the pre-hierarchical follicle granulosa cell layer in avian species has been proposed to represent a primary cause of atresia during follicle development, because granulosa cells of these follicles are considered relatively undifferentiated, and are susceptible to apoptosis. Apoptosis, just as development, is a gonadotropin-dependent process (HSUEH *et al*. 1994), and caspase-3 could be looked upon as a marker for apoptosis. Based upon the stage-dependent state of sensitivity to apoptosis, the pre-hierarchal follicles which are apt to atresia have high enzyme activity of caspase-3 in theory (JOHNSON 2000). Terminal deoxytranceferase-mediated dUTP nick end labeling (TUNEL) could work as an effective method to test apoptosis, and has been used extensively (PACZOSKA-ELIASIEWICZ *et al*. 2003). Healthy cells should be TUNEL-negative, and the frequency of positive cells would significantly increase when the progress of follicular atresia occurs (KITAMURA *et al*. 2002).

Geese have some specific reproductive and physiological characteristics compared to chickens or ducks, such as low fecundity and seasonal breeding. Despite persistent interest in the bird reproductive system, there is less information on the ovarian system of the goose (KOVASC *et al*. 1992; WOJTYSIAK & KAPKOWSKA 2005), especially the morphological characteristics and development of prehierarchical follicles. It is valuable to investigate the development of goose prehierarchical follicles, and conjecture the turning points of apoptosis. In this study, the microstructure of the prehierarchical follicle wall was investigated by a histological method, and both indicators, including the FSHR expression level and the activity of caspase-3, were detected to obtain a preliminary understanding on the turning points of prehierarchical follicle development and apoptosis.

Material and Methods

Experimental animals

Eight Tianfu meat geese (*Anser cygnoides*), bred by Sichuan agricultural university, were selected as experimental animals. All geese were approximately 300 days old, laying five or more eggs in a sequence, kept in individual cages under natural light, provided with free access to feed and water. These geese were sacrificed by cervical bleeding after anesthesia with pentobarbital sodium. The breeding and slaughtering of geese conforms to the Experimental Animal Administration of Sichuan Agricultural University.

Classification of follicles, tissue collection, and H&E Staining

The ovaries were separated and the follicles were quickly dissected free and placed on filter paper (moistened with physiological saline). The follicles were distinguished according to their exterior and diameter (measured between the basement membrane with a vernier caliper). All prehierarchical follicles were placed into nine groups according to their diameter, i.e. < 2 mm, $<2\sim3$ mm, 3~4 mm, 4~5 mm, 5~6 mm, 6~7 mm, 7~8 mm, 8~9 mm, and 9~10 mm, meanwhile, the hierarchical follicle F5 and atretic follicle were also collected.

The ovarian tissues used for RNA isolation were immediately frozen in liquid nitrogen. The follicular walls of prehierarchical follicles in each group were sampled from three geese for the qRT-PCR of FSHR. The extracted follicles of another three geese were fixed as a whole in 4% (v/v) buffered paraformaldehyde in separate bottles, routinely processed and embedded in paraffin wax. Some sections (5 μm) were stained with haematoxylin and eosin and examined histologically; others were air-dried on slides, coated with

3-aminopropyltrieth oxylane (PROOIJEN-KNEGT *et al*. 1982), dried at 42°C for 45 min and left at 37°C overnight for the TUNEL. All the slides were observed under a Nicon-300 light microscope (Tokyo).

Variation in the thickness of granulosa layers and theca layers of prehierarchical follicles were measured by using the measuring software Image-Pro PLUS 6.0 .

RNA isolation and cDNA synthesis

Total RNA was extracted using the TRIzol method (TRIzol reagent, Invitrogen, USA). The procedure for RNA isolation and purification, as well as the on-column deoxyribonuclease treatment (Qiagen), were subsequently performed as detailed in the manufacturer's instructions. The quality and concentration of RNA were determined by a photometer (Eppendorf Biophotometer, Germany). The RNA integrity was assessed by RNA electrophoresis. First strand cDNA was synthesized from 10 μg of total RNA using a cDNA synthesis kit following the manufacturer's instructions (Takara, Japan). The newly synthesized cDNA product was immediately stored at -80°C for further study.

qRT-PCR of FSHR

The primer for the Quantitative RT-PCR (qRT-PCR) of FSHR was designed according to the sequence obtained by our laboratory. qRT-PCR was used to detect the expression level of the FSHR gene using the SYBR PrimerScript RT-PCR kit (Takara, Japan) in a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The whole PCR procedure was performed on the Cycler system with a reaction volume of 25 μL, which included 2.0 μL of cDNA, 12.5 μL of SYBR Premix EX Taq, 10.5 μL of sterile water, and 0.5 μL of each gene-specific primer. The relative expression ratio of target gene was calculated using the Multicolor Real-Time PCR Detection System CFX software (Bio-Rad). The calibrator-normalized relative quantification method $2^{-\Delta\Delta CT}$ method was employed. To normalize the target genes in similar cDNA samples, β-actin and ribosomal 18S rRNA were selected as the reference genes. The specific primer for the FSHR gene and reference genes are listed in Table 1. All reactions were completed in triplicate, and the data represent the mean of three independent experiments.

ELISA of Casepase-3 and TUNEL staining

The caspase-3 activity assay kit, using DEVD-pNA as substrate, was used to detect the activity of caspase-3 in the granulosa layers of prehierarchical follicles. A different classification of follicles was taken this time, i.e. follicles were distinguished every two millimeters in diameter compared with the above tests and according to the preceding results and improved detachment technology (GILBERT *et al*. 1977).

Granulosa layers were peeled off, the smashed tissues were cracked immediately according to manufacturer's instructions (BestBio, Product No. 4106, China) in order to obtain the protein. Definite protein quantitative determination (BestBio, Product No. 301003, China) detected the enzyme activity of caspase-3 (405 nm wavelengths) using a microplate reader (Thermo varioskan, USA).

Apoptotic cells were detected by terminal-deoxy-transferase-mediated dUTP nick end labeling (TUNEL). After deparaffinization, the sections were washed with distilled water and incubated with 20 μg/mL proteinase K at 37°C for 20 min. Then the apoptotic cells were detected with an in situ cell death detection kit according to manufacturer's instructions (Beyotime, Product No. C1088, China). For negative control, sections were stained by the same procedure except that the terminal deoxytransferase in the TUNEL reaction was replaced by PBS.

Table 1

Primers and annealing temperature for qRT-PCR

Genes	Primer Sequence (5'-3')	Annealing (°C)	Length (bp)	GenBankID
FSHR	F:GGACAACGATGTTCCCAGTGATAG R: ATGTGCCTTGCTCACCTAAACCT	58.5	123	KC477215
18S rRNA[a]	F:TTGGTGGAGCGATTTGTC R: ATCTCGGGTGGCTGAACG	60	129	AF173614
β-Actin[a]	F: CAACGAGCGGTTCAG GTGT R: TGGAGTTGAAGGTGGTCTCG	60	92	EF667345

[a] Reference gene for data normalization; FSHR, follicle-stimulating hormone receptor ; F: Forward primer; R: Reverse primer.

Statistical analysis

The statistical analysis was performed using one-way ANOVA and the independent sample test with SAS. The differences were considered to be significant when $P<0.05$.

Results

Morphological characteristic of prehierarchical follicles

The follicular wall consists of a granulosa cell layer, basal lamina, thecal layer and blood capillary, all these structures could be observed under a light microscope in this study (Figure 1. A, B, C, D). We observed variation in the thickness of granulosa layers and theca layers of prehierarchical follicles by using the measuring software Image-Pro PLUS 6.0. The granulosa layers and theca layers changed with the development of prehierarchical follicles, especially the thickness and the number of cell layers. The thickness of the granulosa layer changed obviously. Follicles 9~10 mm in diameter had the lowest thickness of both granulosa layer and theca layer (Figure 1. E, F).

Developmental expression of FSHR in prehierarchical follicles

Melting curve analyses showed the presence of a single PCR product for FSHR, β-actin and 18 s product, confirming the specificity of the reaction

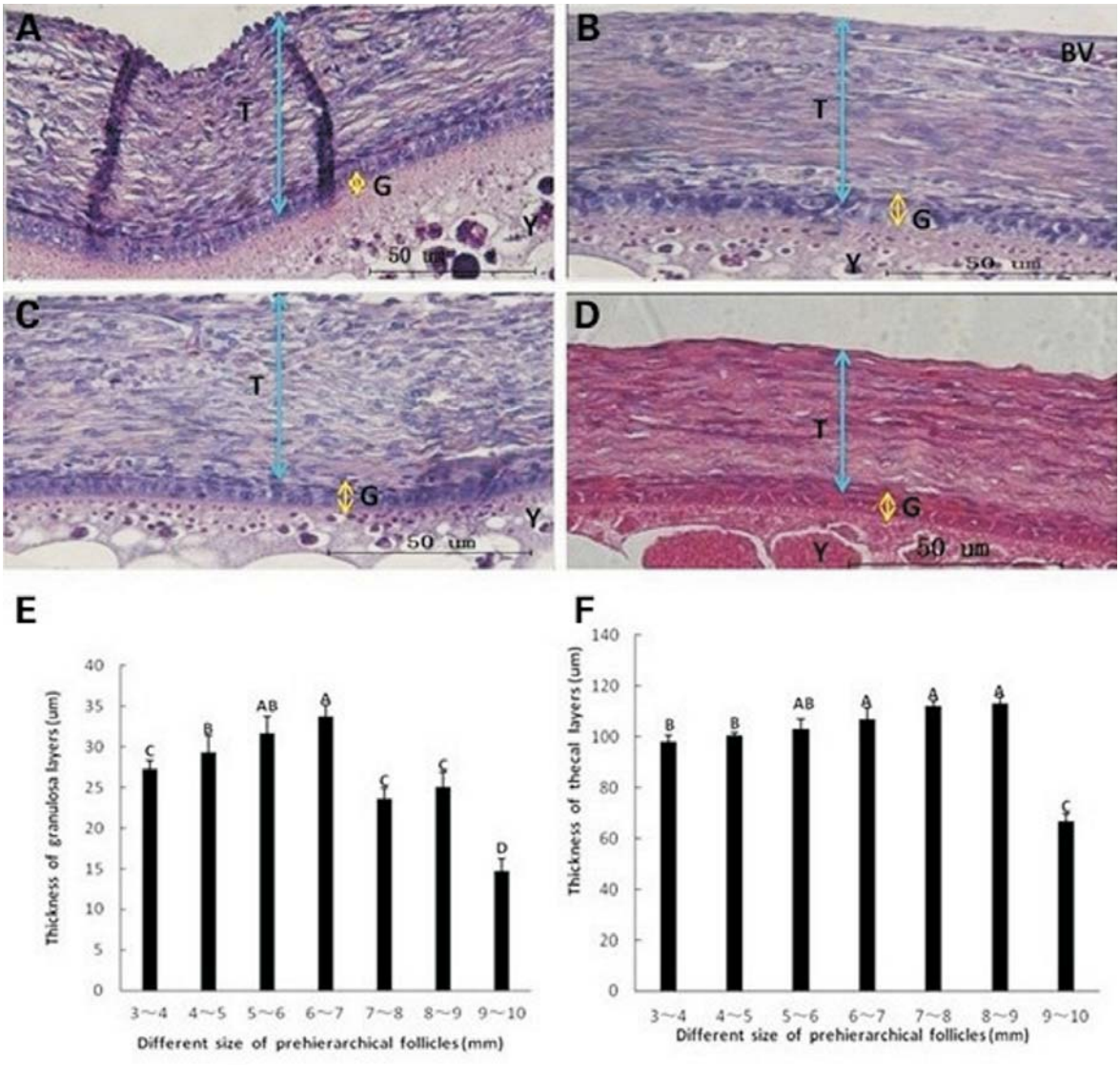

Fig. 1. Sections of the follicular wall of some prehierarchical follicles and the diversification of the thickness of granulosa layers and theca layers. T: theca layer; G: granulosa layer; BV: blood vessel; Y: yolk. Scale bar: 50 um. (A): 3~4 mm; (B): 6~7 mm; (C): 7~8 mm; (D): 9~10 mm. (E): Thickness of granulosa layers in goose prehierarchical follicles of different diameter; (F): Thickness of theca layers in goose prehierarchical follicles of different diameter. Values marked with different letters differ significantly ($P<0.05$).

(data not shown). FSHR cDNA sequences amplified in the qRT- PCR showed 100% homology to goose FSHR (KC477215). Total RNA from each sample reverse transcribed in the absence of reverse transcriptase resulted in C_T values not different from blanks (30 cycles), indicating that genomic DNA was not contributing to the FSHR mRNA quantity. The relative levels of the FSHR mRNAs in the isolated follicular wall of the developing prehierarchical follicles of goose are shown in Figure 2.

The qRT-PCR result revealed that FSHR expression was the greatest in follicles 8~9 mm in diameter. In addition, we found that the level of FSHR mRNA was significantly increased in follicles 3~4 mm in diameter that thereafter progressively declined in follicles 4~5 mm to 7~8 mm in diameter. The total fluctuations in the relative expression levels of FSHR were found to first rise, and then to drop significantly with follicular size increase, except for 8~9mm follicles.

The status of apoptosis in granulosa layers of prehierarchical follicles and other follicles

Caspase-3 activity of extracted protein samples from prehierarchical follicles was low in general, but follicles at 6~8 mm in diameter had relatively high enzyme activity, other follicles were almost at the same level (Figure 3A). The immunostaining method-TUNEL was used to test apoptosis for some follicles; these follicles were chosen according to the preceding results (8~9 mm, 9~10 mm, F5, atresia follicle). A relatively large number of TUNEL-positive cells was revealed in the atresia follicle, whereas a low number of these cells was found in the prehierarchical follicles (9~10 and 8~9 mm), yet they were negligible in preovulatory follicles during maturation (F5).

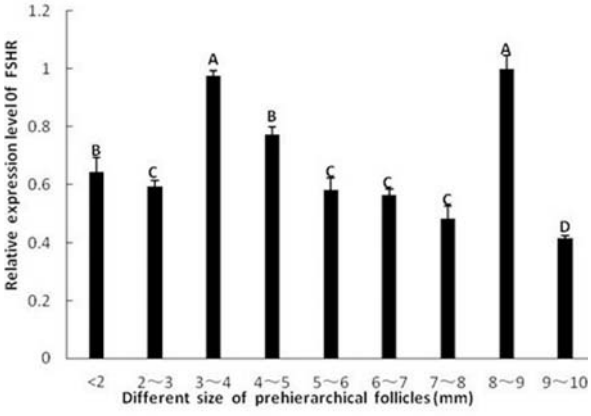

Fig. 2. Relative expression of FSHR in prehierarchical follicles of geese. Data are presented as means ± SEM; n = 3 geese/size. The mRNA expression level at 8~9mm was assigned as control. The different capital letters indicate significant differences between different size of prehierarchical follicles($P<0.05$).

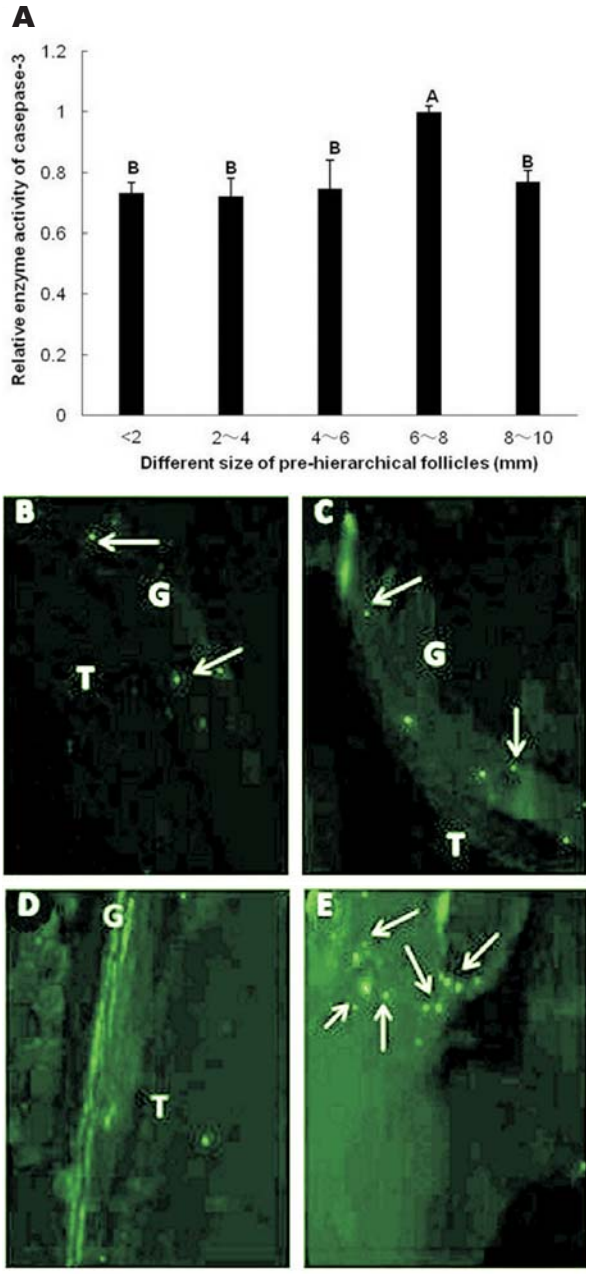

Fig. 3. The vitality of caspase-3 in the isolated granulosa layers of prehierarchical follicles and results of TUNEL. Data are presented as means ± SEM; n = 3 geese/size. The enzymatic activity level at 6~8mm was assigned as control. G: granulosa layer; T: thecal layer. The different capital letters indicate significant differences between different size of prehierarchical follicles ($P<0.05$). Straight arrows: TUNEL-positive groups of cells. (A).The vitality of caspase-3 in the isolated granulosa layers of prehierarchical follicles (B): 8~9 mm; (C): 9~10 mm; (D) : F5; (E): atresia follicle.

Discussion

We obtained a primary understanding of the morphological characteristics of the follicular wall of prehierarchical follicles in geese. From morphometric analysis of all the paraffin sections, it was found that the granulosa and theca layers changed with the development of prehierarchical follicles, especially their thickness. The thickness

of the granulosa layer increased to the highest values in follicles 6~7 mm in diameter, then decreased to the lowest in follicles 9~10 mm in diameter. The thickness of theca layers increased abidingly to a maximum in 8~9 mm follicles, then decreased abruptly to the lowest values in follicles of 9~10 mm. The diversification of the theca layer and granulosa layer and their interactions are associated with the maturation and function of the follicles (MADEKUROZWA & KIMARO 2006; SUBEDI *et al*. 2008). It was speculated that rapid proliferation of granulosa cells was associated with selection of the dominant follicles, and the thickness of theca layers increased to protect the granulosa cells and oocytes. Afterwards they began to decrease in thickness in follicles of 9~10 mm in diameter resulting in thinning of the wall, which is beneficial for impending ovulation after entering a hierarchic arrangement, i.e. the same stage of F6 as mentioned in chicken (MCDERMENT *et al*. 2012). The follicular wall consisted of perivitelline membrane, granulosa cell layer, theca cell layer and surrounding connective tissue (WANG *et al*. 2008), and almost all these structures could be observed under a light microscope. Based on the results it appeared that the histological morphology of healthy follicles in sexually mature geese, was similar to that of growing follicles in other avian species (SHEN *et al*. 1993). In summary, understanding the histological features of the prehierarchical follicles in geese can provide a morphological basis for studying their development, regulation, selection and atresia in future experiments.

The process of ovarian follicle development in birds and mammals is tightly coupled with the functional differentiation of the granulosa cell layer (JOHNSON & WOODS 2009). The transition of granulosa cells from an undifferentiated to a differentiated state is directly associated with follicle selection (JOHNSON 2012), FSHR is a marker for follicle selection and is expressed mainly in the granulosa layer of developing follicles (JOHNSON 2012; MCDERMENT *et al*. 2012; WEBB & CAMPBELL 2007), but there was no report about its dynamic expression in geese prehierarchical follicles. In this study, we found that the total fluctuation of FSHR expression increased at first in follicles of 3~4 mm, then dropped except for 8~9 mm follicles, which had greatest expression, whereas follicles 9~10 mm had the lowest expression. This indicated that 8~9 mm follicles are at a specific point for follicle selection in Tianfu meat geese, and follicles at this stage were more likely to be selected and become the dominant follicles at 9~10 mm in diameter. However, it could not be a decisive judgment because the follicles in this study were distinguished every one millimeter, and experimental error was unavoidable although the experiment was carried out extremely carefully. So, further experiments still need to be designed for verifying the inference at granulosa cells of these special stages.

The development of prehierarchical follicles is a homeostasis of selection and atresia, and thus can maintain the normal sequence of egg production in poultry. The follicles which cannot pass selection will diminish via apoptosis originating within the granulosa cell layer (JOHNSON 2000). According to our results, the enzyme activity of caspase-3, a marker for apoptosis, was detected in protein samples extracted from the granulosa layer of prehierarchical follicles. Cells that have higher caspase-3 activity will possibly undergo apoptosis (HURST *et al*. 2006; GLAMOCLIJA *et al*. 2005). So it can be speculated from our results that follicles of 6~8 mm diameter had high probability of atresia. The apoptotic cells at some satges were tested by the approach of TUNEL, and a relative large number of TUNEL-positive cells were revealed in the atretic follicles, whereas only a few were found in prehierarchical follicles. This was almost accordant with the results reported in chicken (KITAMURA *et al*. 2002; HRABIA *et al*. 2011), and suggested that apoptosis takes place mainly in prehierarchical follicles besides atretic follicles. These results confirmed the previous observations that hierarchical follicles in birds are resistant to atresia under normal physiological conditions (JOHNSON 2000; JOHNSON 2012; MCDERMENT *et al*. 2012). Atresia commonly occurs at the time of breeding, increasing during the advance of the breeding season to reach a peak at the time of nesting in seasonally breeding birds (GILBERT *et al*. 1983), so it was valuable to study the atresia of prehierarchical follicles to get more dominant follicles and then to prolong the ovulatory cycle in seasonally breeding birds, e.g. goose.

In conclusion, goose follicles of 6~8 mm undergo tough competition and selection, with high probability of apoptosis. The victorious will develop and achieve higher expression of FSHR. When they develop to 9~10 mm in diameter, the follicular wall becomes thinner to prepare for entering into a hierarchic arrangement. So, we deduced that the follicle stage of 6~10 mm in diameter is very important for development in geese. In order to study the mechanisms of apoptosis of granulosa cells of follicles at this special stage and to get more dominant follicles for improving the egg production of geese, more additional observations and studies should be done.

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