Immunohistochemistry and Expression Profile of Estrogen Receptor 2 Gene in Different Grade Size Ovarian Follicles of Leizhou Black Ducks

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Immunohistochemistry and expression profile of estrogen receptor 2 gene in different grade size ovarian follicles of Leizhou black ducks

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Abstract

Background: Estrogen receptor 2 (ESR2) plays significant biological roles in the reproductive system and ovarian follicle development. This study, therefore, aimed to reveal the expression pattern and cell-specific localization of ESR2 in the ovarian follicles of Leizhou black ducks.

Method: Four laying Leizhou black ducks at 43 weeks old were annihilated and different grade-sized follicles were collected for immunohistochemistry and expression profile study. The follicles were grouped into seven (7) as small white follicles (SWF), large white follicles (LWF), small yellow follicles (SYF), large yellow follicles (LYF), follicle 5 (F5), follicle 2 (F2), and follicle 1 (F1).

Results: The qRT/PCR results displayed that *ESR2* mRNA was expressed in all follicles with the highest ($P < 0.05$) level of expression found in F1 compared to other follicles. Immunohistochemistry analysis of the cell-specific localization of *ESR2* protein revealed that *ESR2* was distributed in both granulosa and theca cells region in all the follicles examined. There was a significantly higher localization of *ESR2* protein in the granulosa cells than the theca cells of SWF, SYF, LYF, F2, and F1. Comparatively, *ESR2* was highly expressed in the granulosa cells of LYF than in all the other follicles.

Conclusion: These results provide theoretical knowledge for the in-depth study of the related biological functions of the *ESR2* gene and its application at the cellular level.

Key words: ovarian follicles, granulosa cells, theca cells, ESR2, Leizhou black ducks.

Background

Leizhou black duck is an egg-type duck breed known to have excellent egg production and quality traits enriched in nutrients and trace elements (1,2). As a high-quality local duck breed, much research has been done to improve its meat and egg performance and environmental adaptability (2–9).

Estrogens belong to the gonadal steroid hormone family synthesized from cholesterol mainly in the ovaries, granulosa cells, and corpora lutea (10,11). Estrogens regulate several biological and physiological functions in the reproductive system (10,12,13) by binding to its cognate receptors; estrogen receptor 1 (*ESR1/*ERα/*ER1*) and estrogen receptor 2 (*ESR2/ERβ*/*ER2*) which are found in the nuclear receptor superfamily (14–16). The estrogen receptors act as transcription factors to initiate gene transcription through estrogen response elements (EREs) in the target tissues and also interact with other transcription factors (17).

The female reproductive organ, the ovary produces and releases eggs and serves as an endocrine gland that produces and discharges hormones (18,19). The ovary also mediates ovulation and provides proteins and steroid hormones for estrous cycle maintenance, secondary sex characteristics, and prepare the uterus for implantation (20–22). The functional unit of the ovary is the ovarian follicles which contain three different types of cells; oocytes, granulosa cells, and theca cells. The oocytes (germ cells) which may form an embryo are surrounded by and form intracellular connections with granulosa cells (somatic cells) which are also surrounded by and form connections with the theca cells (somatic cells) (Figure 1) (20,23–25).

Ovarian follicles go through several stages of development throughout the stroma of the cortex from primordial, primary, secondary to preovulatory follicles (26,27). Primordial follicles are inactive with oocytes arrested in the first meiotic prophase in the early postnatal period with

flattened granulosa cells. When primordial follicles become activated, the oocyte grows and granulosa cells differentiate from a flattened appearance to a more cuboidal form and proliferate into primary follicles. As the primary follicle grows, they are surrounded by several granulosa cell layers and recruited theca cells from the interstitial stromal cells on the basement membrane to form Secondary or Pre-antral follicles (28–32). Finally, fluid-filled vesicles emerge in the granulosa cells to form early antral follicles which later amalgamate to form a single large fluid-filled antral follicle. The proliferation of granulosa cells slows down and the last stage of follicular growth is primarily due to the increase of antral follicles to form preovulatory or Graafian follicles (Figure 1). Granulosa cells differentiate into cumulus and mural cells (20,22,30). The hypothalamic, pituitary and gonadal (HPG) axis regulate follicle development and ovulation by synthesizing gonadotropin hormones (follicle-stimulating hormones, FSH and luteinizing hormone, LH) which coordinate to stimulate the synthesis and discharge of estrogen and progesterone in the somatic cells whose increase may have positive feedback on the HPG axis and gonadotropin hormones to coordinate the development, maturation, and oviposition of follicles (Figure 2) (24,33–38).

Generally, the follicles can be categorized into two based on their sizes during development as prehierarchical and hierarchical or preovulatory follicles. Prehierarchical follicles are divided into small white follicles (SWF), large white follicles (LWF), small yellow follicles (SYF), and large yellow follicles (LYF). The hierarchical follicles are also divided from large to small as F1, F2, F3, F4, and F5/6 (Figure 3). After F1 maturation and ovulation, F2 and F3 respectively become new F1 and F2 whereas LYF becomes preovulatory follicles (33,39,40). The ovarian follicle development in poultry is dependent on the efficient differentiation and proliferation of the

granulosa and theca cell layers to enhance prehierarchical follicles to mature into hierarchical follicles. (39,41,42).

Therefore, this study elucidated the expression profile of *ESR2* in the different grade-sized follicles and the localization of *ESR2* protein in the granulosa and theca cells of the follicles.

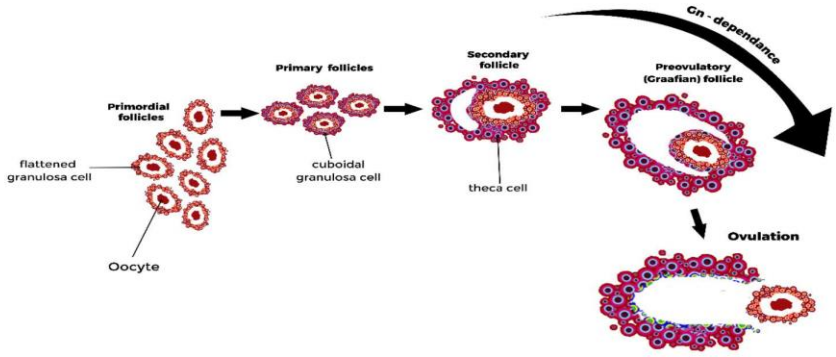


Figure 1. Ovarian follicle development in poultry. Primordial follicles become activated, the oocyte grows and granulosa cells differentiate from a flattened appearance to a more cuboidal form and proliferate to form primary follicles. Primary follicles grow and are surrounded by several granulosa cell layers and recruited theca cells into Secondary follicles. The proliferation of granulosa cells slows down and the last stage of follicular growth is primarily due to the increase of secondary follicles to form preovulatory or Graafian follicles. Gn, gonadotropin.

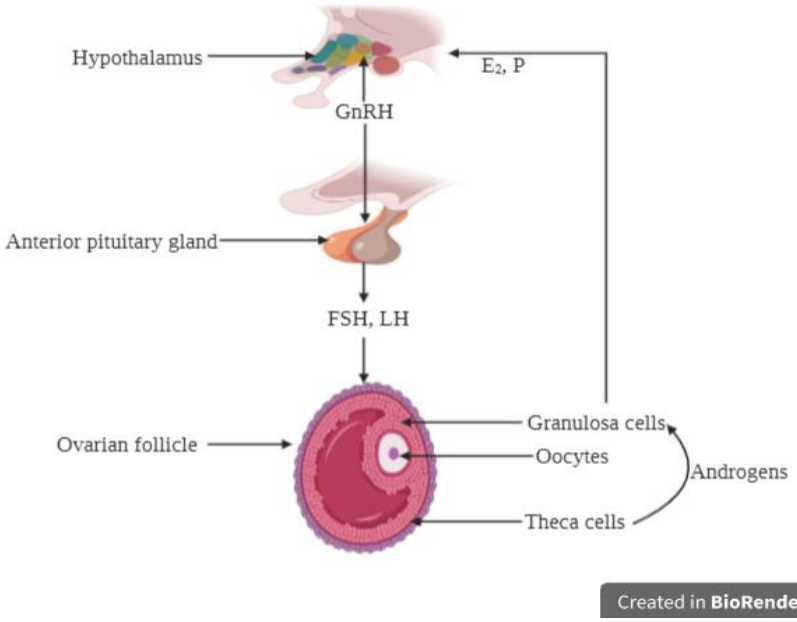


Figure 2. Hormonal regulation of ovarian follicle development. GnRH is discharged from the hypothalamus into the portal circulation to operate on the gonadotrope cells in the anterior pituitary gland to excite the synthesis and discharge of FSH and LH. FSH and LH enhance the synthesis and discharge of estrogen and progesterone in granulosa and theca cells whose increase may have positive feedback on the HPG axis and gonadotropin hormones to coordinate the development, maturation, and oviposition of follicles. Androgens from theca cells are aromatized to E_2 in the granulosa cells. GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E_2, estradiol (estrogen); P, progesterone. Arrows indicate positive stimulatory effects.

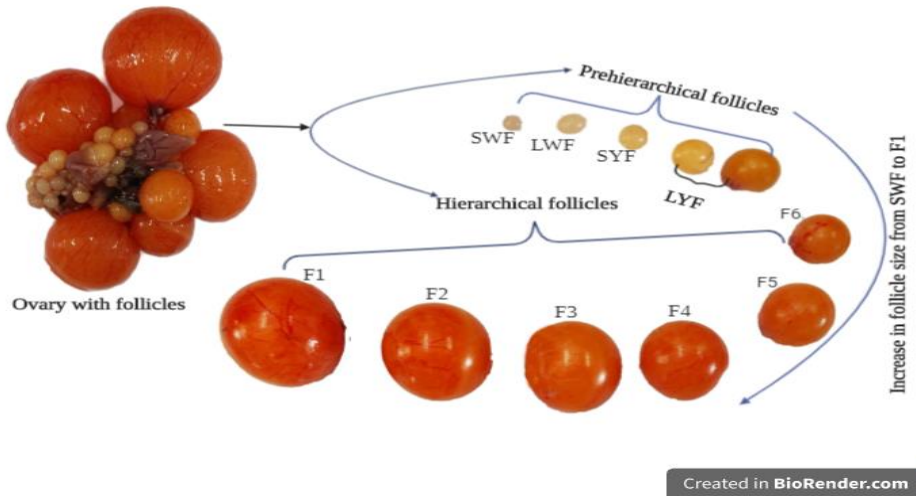


Figure 3. Diagram of Leizhou black duck ovary and ovarian follicles showing numerous rounded and different grade size follicles. The follicles are divided into two categories; prehierarchical and hierarchical follicles. F1-F6, follicles 1-6; LYF, large yellow follicles; SYF, small yellow follicles; LWF, large white follicles, and SWF, small white follicles.

Materials and methods

Animals and sample collection

All the animals were maintained and studied following the National Institute of Health (NIH) guidelines for care and use of laboratory animals, and all protocols were approved in advance by the Animal Care and Ethics Committee of Guangdong Ocean University of China (No. NXY20160172).

Four (4) female Leizhou black ducks at 43 weeks of laying were used in this study. Follicles at different growth stages were collected from laying Leizhou black ducks based on their diameter according to the "Poultry Entity Anatomy Atlas" (43), Leizhou black duck follicles were grouped as; SWF: <4 mm, LWF: 4.49±0.43 mm, SYF: 5.81±0.37 mm, LYF: 6.94±0.29 mm, F6: 10.63±0.88 mm, F5: 14.11±0.61 mm, F4: 17.33±1.13 mm, F3: 24.31±1.24 mm, F2: 30.70±1.42 mm, F1: 34.34±0.44 mm. In this study, however, we selected SWF, LWF, SYF, LYF, F5, F2, and

F1. All follicular tissues were quickly collected into tubes containing liquid nitrogen and stored in a refrigerator at -80°C for later use. Again, samples of all the follicle tissues were collected and quickly fixed into formalin and stored at room temperature for immunohistochemistry studies. Egg yolk in bigger grade-sized follicles (LYF, F5, F2, and F1) were rinsed and washed in PBS and quickly fixed in the formalin while smaller follicles (SWF, LWF, and SYF) were directly fixed into the formalin.

RNA extraction and reverse transcription

Total RNA was extracted from each tissue using Magzol reagent (Beijing, Quanshijin), following the manufacturer's protocol. The quality and concentrations of the RNA were detected respectively by 1% agarose gel electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA) at 260:280nmratio. Reverse transcription was performed to synthesize cDNA using PrimeScript RT Reagent kit with gDNA Eraser (Beijing, Quanjin) according to the manufacturer's protocol.

Expression profile of the *ESR2* gene in Leizhou black duck

According to the ChamQTM SYBR qPCR Master Mix 7750 (Trans, Guangzhou) fluorescence quantification kit, the fluorescence quantification of each sample tissue was performed on the Applied Biosystems StepOnePlus (USA) fluorescence quantitative PCR. All primers were designed using Primer Premier 6.0 (Palo Alto, USA) and synthesized by Sangon Biotechnology (Shanghai, China). The detailed information of all primers used in this study is provided in table 1. Three replicates for *ESR2* and β-actin were performed in every tissue. PCR reaction system: 10μL ChamQTM SYBR qPCR Master Mix, 0.5μL PCR Forward Primer (0.5μM), 0.5μL PCR Reverse Primer (0.5μM), 0.5μL cDNA, 8.5 μL ddH2O, a total volume of 20μL amplification reaction. Reaction procedure to amplify the template was 95°C, 30 s; 40 cycles (95°C, 10s; 56°C,

30s; lighting; 72°C, 25s); 95°C, 15s; 60°C, 1 min; 95°C, 15s. The relative expression levels of the genes test were calculated using the 2–ΔΔCt method (44).

Table 1. Primer pairs designed for quantitative real-time PCR

Gene	Sequence（5'-3'）	Product length（bp）	Annealing temperature (℃)	Login ID
ESR2	F: CAGTGCTACCTGTGACCAGA R: TGCAGCCTTCACATGACCAG	168	60.0	XM_021274553.2
B-actin	F: CGCAAATGCTTCTAAACC R: AGACTGCTGCTGATACCTT	167	55.9	NM_001310421.1

Immunohistochemistry of *ESR2* gene in ovarian follicles

Immunohistochemistry (IHC) for ESR2 was performed according to Wuhan servicebio technology CO., LTD standard operating procedures.

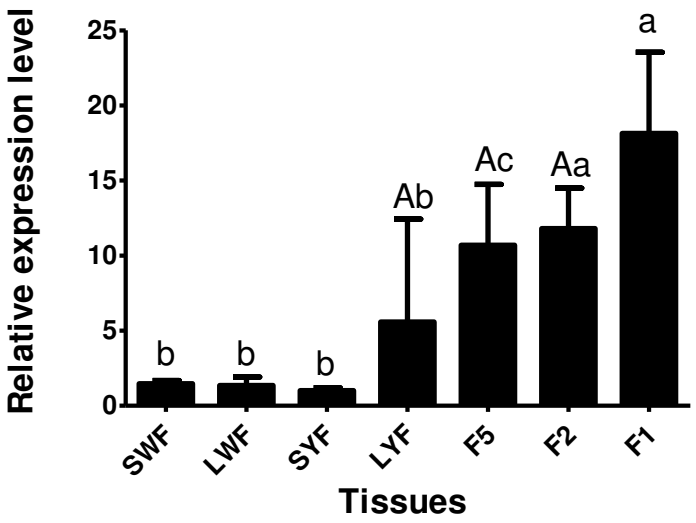
Freshly dissected follicular tissues (less than 3 mm thick) were fixed in 4% paraformaldehyde (Servicebio, G1101) for 24 hours at room temperature. The sections were then sequentially deparaffinized with xylene (Sinopharm Chemical Reagent Co., Ltd.) and rehydrated. Antigen retrieval was performed with EDTA (PH9.0) antigen retrieval solution (Servicebio, G1203) in a microwave oven at medium heat for 8 minutes and after cooling, the slides were washed 3 times, 5 minutes each in PBS (PH7.4). To block endogenous peroxidase, the slides were put in 3% hydrogen peroxide solution (Servicebio, G0115), incubated at room temperature for 25 min in the dark, and washed 3 times, 5 minutes each in PBS (PH7.4). The slides were incubated with 3% BSA solution (Servicebio, G5001) to block for 30 minutes at room temperature. Sections were

incubated overnight at 4°C with primary mouse *ESR2* ab288 antibodies diluted with PBS followed by washing with PBS (PH7.4) 3 times for 5 minutes each. After the sections were dried slightly, they were incubated in the HRP mouse secondary antibody at room temperature for 50 minutes. After 3 washes with PBS (PH7.4) for 5 minutes each, the sections were incubated for 30 min in diaminobenzidine (DAB) staining solution (Servicebio, G1211) with 30% H_2O_2 and then counterstained with hematoxylin for 3 minutes (Servicebio, G1340) and differentiated with hematoxylin differentiation solution (Servicebio, G1309) for a few seconds. The sections were dehydrated with graded series of alcohol, ethanol, and xylene and then dried slightly and mounted on a microscope (CIC, XSP-C204) with neutral gum (Servicebio, G1403).

Results

Expression profile of *ESR2* gene in different grade-sized follicles of laying Leizhou black ducks

To evaluate the expression pattern of *ESR2* in the follicles of laying Leizhou black ducks, seven (7) different grade-sized follicles were selected from the ducks and detected by RT-qPCR. The results showed that *ESR2* was expressed in all tissues (Figure 4). The highest expression level of *ESR2* was found in F1 compared to other tissues followed by F2, F5, and LYF. Obvious *ESR2* mRNA expression was discovered in SWF and LWF with lower expression levels in SYF. *ESR2* gene was significantly ($P < 0.01$) expressed in F1 compared to the other grade-sized follicles except for F2 (Figure 4). *ESR2* expression was not significantly ($P > 0.05$) different in F2, F5, and LYF. There was a significant ($P < 0.01$) difference in the expression of *ESR2* in F2 and F5 compared to SWF, LWF, and SYF. The expression of *ESR2* was not significantly ($P > 0.05$) different in SWF, LWF, SYF, and LYF.



186

187 **Figure 4. Expression pattern of *ESR2* in different grade-sized follicles of laying Leizhou**
188 **black ducks.**

189 **NB: SWF- small white follicles; LWF-large white follicles; SYF- small yellow follicles; LYF-**
190 **large yellow follicles; F5- follicle 5; F2- follicle 2; F1-follicle 1; GC- granulosa cells; TC- theca**
191 **cells. Different lower and upper cases show a significant difference (P < 0.01; 0.05).**

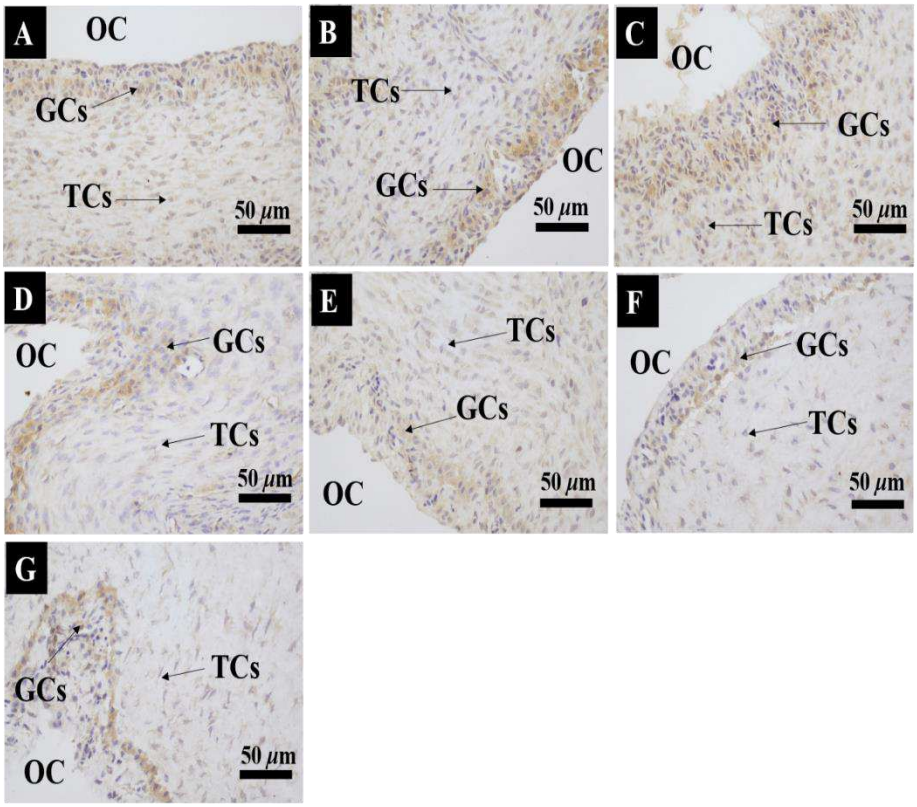
192 **Localization of *ESR2* in the ovarian follicular cells**

193 In situ localization results indicated the intense and specific signals in the granulosa and theca
194 cells. The staining and the proportion of positive cells varied between the different cell types
195 (Figure 5). The nucleus stained with hematoxylin is blue, and the positive expression of DAB is
196 brown. The presence of *ESR2* mRNA, identified by brown cytoplasmic staining was found in all
197 the follicular stages in both cells. Generally, the *ESR2* protein was highly localized in the granulosa
198 region than in the theca region (Figure 6C). In LYF and F1, the *ESR2* protein was extremely and
199 significantly ($P < 0.01$) localized in the granulosa region than the theca region. Also, the *ESR2*
200 protein was significantly ($P < 0.05$) localized in the granulosa region than the theca region in SWF,

SYF, and F5. Even though, the expression of *ESR2* was higher in the granulosa region than the theca region in LWF and F5, the difference was not significant ($P > 0.05$).

The localization of *ESR2* protein in granulosa cells was highest in LYF followed by F1, F2, SWF, F5, SYF, with the lowest expression in LWF (Figure 6A). Expression pattern of *ESR2* in the granulosa cells across the different grade-sized follicles revealed that *ESR2* protein expression was significantly ($P < 0.01$; 0.05 respectively) higher in LYF granulosa cells than LWF and SYF granulosa cells. There was no significant ($P > 0.05$) difference in the localization of *ESR2* protein in LYF, F1, F2, F5, and SYF granulosa cells (Figure 6A).

The localization of *ESR2* protein in theca cells was highest in F1 followed by F2, LYF, F5, SYF, SWF, with the lowest expression in LWF (Figure 6B). The expression pattern of *ESR2* in the theca cells across the different grade-sized follicles revealed that *ESR2* protein expression in F1, F2, and LYF was significantly ($P < 0.01$) higher than LWF. There was no significant ($P > 0.05$) difference in the localization of *ESR2* protein in F1, F2, LYF, F5, SYF, and SWF theca cells (Figure 6B).



215

216 **Figure 5. Representative immunolocalization of *ESR2* proteins (brown) in Leizhou black**
217 **duck ovarian follicles**. Paraformaldehyde-fixed tissue sections were immunostained using anti-
218 mouse *ESR2*. Panels A to G, showing strong immunostaining were observed in all granulosa cells
219 (GCs) within the various-sized ovarian follicles sampled. The scale bar is 50 μm.
220 NB: A- small white follicles; B-large white follicles; C- small yellow follicles; D- large yellow
221 follicles; E- follicle 5; F- follicle 2; G-follicle 1; GCs- granulosa cells; TCs- theca cells; OC- oocyte

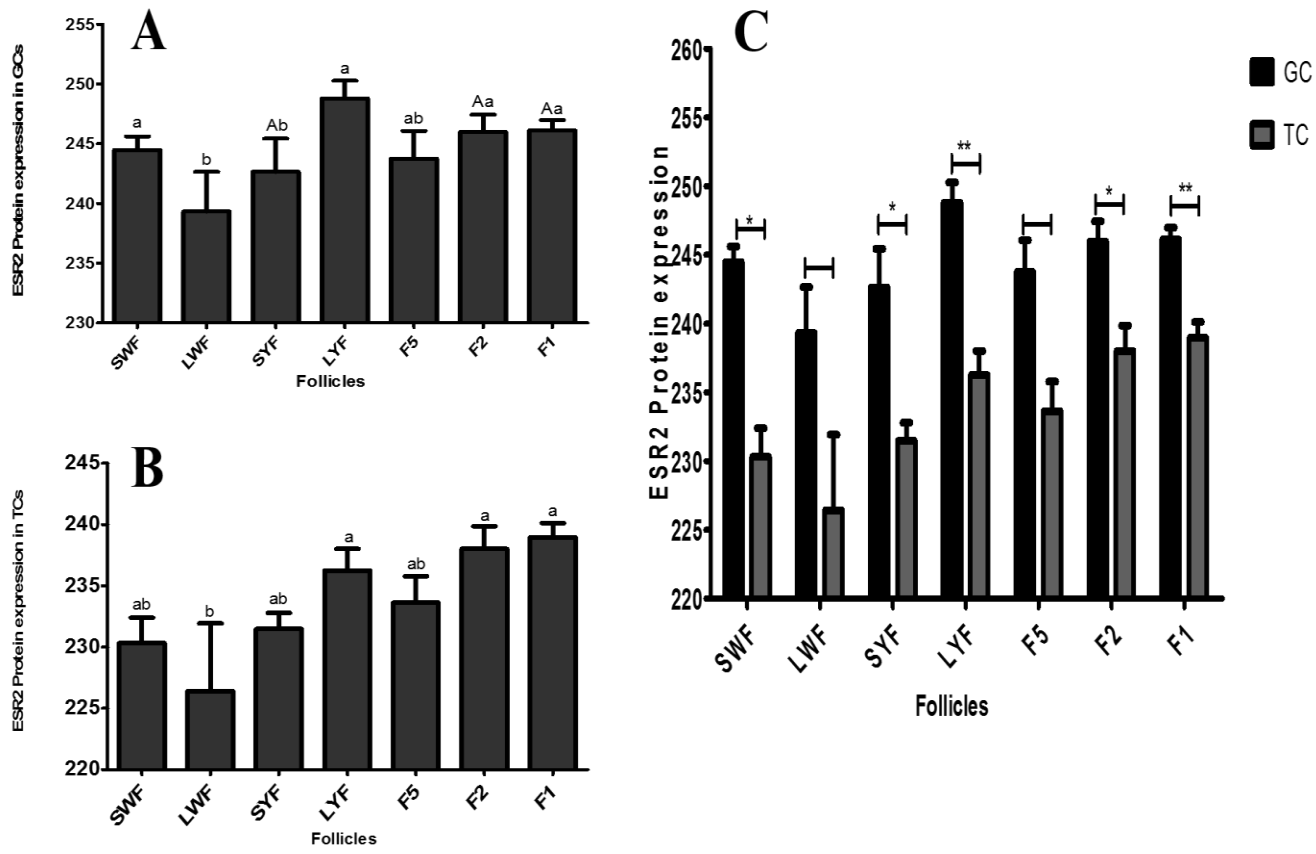


Figure 6. The localization of *ESR2* protein in GCs and TCs of different grade-sized follicles

NB: SWF- small white follicles; LWF-large white follicles; SYF- small yellow follicles; LYF-large yellow follicles; F5- follicle 5; F2- follicle 2; F1-follicle 1; GC- granulosa cells; TC- theca cells. * signifies a significant difference (P < 0.05), ** signifies a significant difference (P < 0.01). Different lower and upper cases show a significant difference (P < 0.01; 0.05).

Discussion

Expression profile of *ESR2* gene in different grade-sized follicles of laying Leizhou black ducks

The ovarian follicles are the functional unit of the ovary which contains three different types of cells; oocytes, granulosa cells, and theca cells. The poultry follicles can be categorized as prehierarchical and hierarchical follicles. Prehierarchical follicles are divided into SWF, LWF, SYF, and LYF whilst the hierarchical follicles are divided from large to small as F1, F2, F3, F4, F5/6. As the granulosa and theca layers proliferate and differentiate, the prehierarchical follicles mature into hierarchical follicles (39,41,42). FSH and LH promote the production of estrogen (estradiol) by the granulosa cells to enhance the development of the ovarian follicles (22).

Given this, we selected four prehierarchical (SWF, LWF, SYF, and LYF) and three hierarchical (F1, F3, and F5) follicles to detect the expression pattern of *ESR2* in the different grade sized follicles. After qPCR analysis, the results showed that *ESR2* was expressed in all the grade-sized follicles with the highest expression in F1, F3, F5, LYF, LWF, SWF, and SYF respectively. Generally, *ESR2* mRNA increased as the follicle size increased, thus, the higher the follicles, the higher the *ESR2* mRNA.

Preovulatory/Graafian follicles are a major source of estrogen because the dominant selected follicle has an aromatase activity that is more sensitive to FSH than the other smaller follicles. Thus the dominant follicle is capable of producing larger amounts of estrogen than the smaller follicles (45,46) which may be the cause of higher *ESR2* expression in hierarchical follicles than the prehierarchical follicles in this study. Similar to our findings, a study in the chicken ovary revealed that *ESR2* was highly expressed in white follicles followed by yellow follicles and small

yellow follicles (47). The expression of *ESR2* mRNA was examined in prehierarchical (white and yellow follicles) after treating Hy-Line hens with recombinant chicken GH. The study revealed that *ESR2* mRNA increased progressively in white follicles from 14 weeks to 17 weeks whereas, in yellow follicles, it decreased on the 17th week (48). A recent study that investigated the secretion of the reproductive hormone during follicle development in Yangzhou, Carlos, and Zhejiang geese revealed that estradiol (E_2) concentration at LWF to SYF stages was highest in Zhejiang than the other two breeds. However, there was no significant difference in E_2 concentration in hierarchical follicles in all three breeds. This signifies that E_2 plays a vital role in follicle development from the prehierarchichal form into hierarchical forms (39). There was a higher expression of ESR in all the follicles examined with increasing photoperiods which greatly improved the reproductive organ and follicle development in layer ducks at the pullet phase (33).

Localization of *ESR2* in the granulosa and theca cells of the ovarian follicles

Both granulosa and theca cells synthesize several hormones to promote and regulate follicle maturation and development (24,30). FSH and LH work in the plasma membranes of granulosa and theca cells respectively to promote the synthesis and discharge of estrogen and progesterone respectively (24,33,39,49,50). Androgens produced by the theca cells are taken and up by the granulosa cells and converted by P450 aromatase into estrogen (51). Thus, this study considered the localization of *ESR2* in the two cells due to their proximity in functions for follicle growth and development in seven different grade-sized follicles.

The findings of this study showed the cell-specific localization of *ESR2* in the follicles of Leizhou black ducks. *ESR2* protein was more pronounced and localized in the granulosa cells than theca cells of all the different grade-sized follicles in this study. Similarly, a study in chicken pre-ovulatory follicles (F3, F2, and F1) revealed that *ESR2* was significantly expressed in the granulosa

277 cells than theca cells of F3 and F2 follicles. However, the vice-versa was the case for F1 follicles
278 (47). Again, during the gonadal development of Tammar wallaby, the ontogeny of ERs revealed
279 that *ESR2*was found in both GC and TC of follicles at all stages of development where *ESR2* was
280 highly cytoplasmic in GC with weak staining in TC (52).

281 In this study, *ESR2* was expressed in the granulosa cells of all the different grade-sized follicles
282 which is consistent with an earlier study in the ovaries of humans and marmosets where *ESR2* was
283 expressed in the granulosa cells of all follicle stages examined (53). Another study in bovine
284 revealed a moderate to high *ESR2* expression in the follicle cells of primordial and primary follicles
285 which were higher than in secondary follicle cells. The granulosa cells showed higher expression
286 of *ESR2* in tertiary follicles than that in the secondary follicle cells (46). In humans (54), cattle
287 (55), and rats (56), there was a high expression of *ESR2* in the granulosa cells of pre-antral and
288 antral follicles which same was revealed in this study. Contrary to the findings of this study, there
289 was a highly specific expression of *ESR2* in the GC layer of small and developing follicles that
290 declined with increasing follicle size (57). Regardless of the follicle-specific expression
291 differences between different species, our findings and previous ones show that estrogens are
292 involved in folliculogenesis by interacting with *ESR2*.

293 Findings in this study revealed that the highest expression of *ESR2* was found in the GC of LYF
294 which is necessary for follicular development and selection into the hierarchical follicle. In poultry,
295 after the ovary has matured, the ovulation procedure is strictly based on hierarchical order (F6-
296 F1). After F1 ovulation, a follicle is selected from LYF to replace F6 and the yolk material in the
297 selected LYF follicle is quickly deposited for grade follicular development (58). LYF is an
298 important reserve follicle that develops into grade follicles and its development and recruitment
299 may affect the ovulation cycle of poultry (59). In that order, the recruited SWF, LWF, SYF, and

LYF have a high probability of entering the grade follicular stage (40,60,61). Therefore, *ESR2* was localized in SWF, LWF, SYF, and LYF to enhance their growth and development for recruitment and selection into the hierarchical order in Leizhou black duck.

Pre-ovulatory follicles are a major source of estrogen because the dominant selected follicle has an aromatase activity that is more sensitive to FSH than the other smaller follicles. Thus the dominant follicle is capable of producing larger amounts of estrogen than the smaller follicles (45,46). Affirmatively, in this study, *ESR2* was high in all the cells of the pre-ovulatory follicles (F5, F2, and F1) to bind to the large amounts of estrogen to enhance its activity in the follicles for growth, oocyte maturation, and ovulation in Leizhou black ducks.

Conclusion

ESR2 mRNA was expressed in all follicles with the highest expression level found in F1. Immunohistochemistry analysis of the cell-specific localization of *ESR2* protein revealed that *ESR2* was distributed in both granulosa and theca cells region in all the follicles examined. *ESR2* protein was highly localized in the granulosa cells than the theca cells in all the follicles. Comparatively, *ESR2* was highly expressed in the granulosa cells of LYF than in all the other follicles. These results provide theoretical knowledge for the in-depth study of the related biological functions of the *ESR2* gene and its application at the cellular level.

Abbreviations

ESR2: Estrogen receptor 2; GC: granulosa cell; TC: theca cell; OC: oocyte; LYF: large yellow follicles; SYF: small yellow follicles; LWF: large white follicles; and SWF: small white follicles.

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Authors' contribution

CAA: Conceptualization, design, experimentation, data curation and analysis, writing-original draft; writing-review & editing. **YL:** Experimentation, methodology, data curation and analysis, software. **RY:** Experimentation and data curation. **YP**: Experimentation and data curation**. LL:** Experimentation, methodology, data curation and analysis, software. **YS:** Conceptualization, funding acquisition, methodology, project administration; supervision; writing-review & editing. **ZZ:** Funding acquisition, project administration, supervision; Writing-review & editing. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

All the animals were maintained and studied following the National Institute of Health (NIH) guidelines for care and use of laboratory animals, and all protocols were approved in advance by

the Animal Care and Ethics Committee of Guangdong Ocean University of China (No. NXY20160172).

Consent for publication

Not applicable.

Competing Interest

The authors declare that they have no competing interests.

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