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Expression dynamics of gonadotropin-releasing hormone-I and its mutual regulation with luteinizing hormone in chicken ovary and follicles

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ABSTRACT

Gonadotropin-releasing hormone-I (*GnRH-I*) has been identified in the ovaries of vertebrate species, and this decapeptide is a key regulator of reproductive functions. However, its biological action and regulatory mechanism in the chicken ovary remain to be characterized. In this study, the expression of *GnRH-I* gene in chicken hypothalamus and ovaries at different developmental stages and different sizes of follicles was investigated, and the effect of *GnRH-I* mRNA on chicken follicular cells was analyzed in vitro. The results showed that the expression of *GnRH-I* was dramatically decreased in the hen ovary compared to that in the hypothalamus after sexual maturation. In the mature ovarian follicles, *GnRH-I* mRNA levels were significantly higher in theca cells than that in granulosa cells. Overexpression of *GnRH-I* decreased the expression of luteinizing hormone receptor (*LHR*) mRNA in theca cells from preovulatory follicles but had no effect on granulosa cells. Treatment of theca cells with different concentrations of luteinizing hormone (LH) significantly increased *GnRH-I* mRNA expression at low doses (50 ng/ml) but significantly decreased it at higher doses (200 ng/ml). Furthermore, *GnRH-I* inhibited LH-induced *LHR* expression at the lower dose of LH (50 ng/ml). These findings provide strong evidence indicating that *GnRH-I* is an important regulator in the chicken ovary.

1. Introduction

Gonadotropin-releasing hormone I (*GnRH-I*) is a hypothalamic neuronal decapeptide that plays an important role in the reproductive system. This decapeptide is secreted and released by hypothalamic neurosecretory cells and stimulates the synthesis and secretion of gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary, which in turn modulate the gonadal functions (Péczely, 1989; Marshall et al., 1991; Kaiser, 1998). Although the principal source and target sites for GnRH are in the hypothalamus and pituitary, in vivo and in vitro studies have shown that *GnRH* and its receptor also have functional expression in some non-hypothalamic reproductive tissues, including the ovaries, oviducts, endometrium, mammary glands and testes, indicating that this hormone may have extra-pituitary functions in addition to its role in the pituitary (Sakamoto et al., 1993; Raga et al., 1998; Ortmann and Diedrich, 1999; Walters et al., 2008; Li et al., 2015).

There is now abundant evidence that GnRH is present in the ovaries (Knecht et al., 1985; Aten et al., 1986; Leung et al., 2003) and exerts direct effects on ovarian functions, such as oocyte maturation, follicular development and steroidogenesis (Hsueh and Erickson, 1979; Sharp et al., 1990; Kang et al., 2003, p. 16. Metallinou et al., 2007). By in situ hybridization, it has been shown that *GnRH-I* and its receptor mRNA are expressed in human granulosa-luteal cells (hGLCs) and normal ovarian surface epithelial (OSE) cells (Peng et al., 1994; Kang et al., 2000) as well as in rat granulosa cells of primary, secondary and tertiary follicles (Dekel et al., 1985; Clayton et al., 1992). In the ovaries of non-mammalian vertebrates, including birds, fish, frogs, and reptiles, *GnRH-I* mRNA and protein are mainly found in the oocytes of early-growing follicles and in theca cells and granulosa cells of previtellogenic follicles (Yu et al., 1998; Svetlana et al., 2002; Singh et al., 2007). Functionally, it has been shown that *GnRH-I* and its agonist exert both inhibitory and stimulatory effects on ovarian cellular functions by autocrine or paracrine action (Ranta et al., 1984). Studies have demonstrated that *GnRH-I* stimulated progesterone secretion in hGLCs (Olsson et al., 1990) and induced transcription of the progesterone receptor (Natraj and Richards, 1993). However, other groups have reported an inhibitory or no effect of *GnRH-I* on progesterone production

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in porcine and human ovaries (Ledwitz-Rigby, 1990; Török et al., 1992). In the bovine ovary, GnRH agonists had dose-dependent stimulatory effects on steroid hormone secretion in both granulosa cells and luteal tissues (Ramakrishnappa et al., 2005). GnRH agonists also exerted action on granulosa cells at varying follicular stages in the porcine ovary (Takekida et al., 2003). However, the actual biological action and regulatory mechanism of *GnRH-I* in the ovary remain to be further elucidated.

Granulosa cells (GCs) and theca cells (TCs) are the main components of follicles, and the functional interactions between them play a crucial role in steroidogenesis and follicular development (Qiu et al., 2013; Liu et al., 2015). The chicken ovary contains five to seven large, yolk-filled preovulatory follicles (F1-F7) arranged in a hierarchy. In avian species, androgens and estrogens are primarily produced by theca cells of the preovulatory follicle (Nitta et al., 1991; Johnson and Bridgham, 2001; Rangel et al., 2009). Previously, many functional studies of GnRH in the ovary have mainly focused on GCs, GLCs and the corpus luteum tissue of mammals (Parinaud et al., 1988; Minaretzis et al., 1995; Ortmann, 2001); however, the regulation of GnRH in the chicken ovarian follicle, especially in theca cells, is poorly understood. Therefore, in this study, we investigated the expression of *GnRH-I* in the chicken ovary and hypothalamus at different developmental stages. We also examined the effects of over expression of *GnRH-I* mRNA on the expression levels of *FSHR*, *PGR*, *LHR* and *ERα* in chicken preovulatory follicular cells.

2. Materials and methods

2.1. Birds and tissue collection

Jining Bairi hens at the ages of 30-, 60-, 90-, 110-day-old, 140-day-old and not laying eggs (sexually immature individuals with several 4–8 mm follicles in the ovary, here named 140a) and 140-day-old egg-laying hens (sexually mature individuals, here named 140b) were used for analyzing the expression pattern of the chicken *GnRH-I* gene. All hens were housed in batteries under the same environmental conditions with *ad libitum* access to feed and water, and were exposed to a 13L: 11D photoperiod when sample collection. The hens were killed by cervical dislocation and the tissues of the hypothalamus, whole ovary and ovarian follicles at different developmental stages were collected and immediately stored in liquid nitrogen. The hypothalamus and ovary were harvested for RNA extraction to investigate the expression characteristics of *GnRH-I* in chickens at different developmental stages. Follicles with varied sizes, including SW (small white follicles, < 3 mm), SY (small yellow follicles, 4–8 mm), F5 (9–12 mm), F3 (21–25 mm), F1 (31–35 mm) and POF1 (the newly post-ovulatory follicle tissue) follicles, were manually dissected from 140b chicken ovaries for RNA extraction to compare the levels of *GnRH-I* expression among different sized follicles. The yolk in the follicles was carefully removed with a syringe. The birds were handled and treated according to the Animal Care and Use Committee of Shandong Agricultural University.

2.2. RNA extraction and real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the frozen tissues using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA quality and quantity were then assessed using a spectrophotometer (Eppendorf, Hamburg, Germany), and minimum OD 260/280 ratios of 1.8 were obtained. The cDNA was synthesized using a PrimeScript RT reagent kit with a gDNA Eraser (TaKaRa, Dalian, China) in a 20 μl total reaction volume: 1 μg of total RNA mixed with 1 μl of oligo (dT) 18 primer, 1 μl of PrimerScript RT Enzyme Mix I, 4 μl of 5 × PrimeScript Buffer and RNase-free ddH_2O up to a total volume of 20 μl. The reaction mixture was incubated at 37 °C for 15 min and then at 85 °C for 5 s. The resulting cDNA was stored at −20 °C for mRNA expression analysis.

Table 1
Primers used for real-time quantitative RT-PCR and plasmid construction of chicken GnRH-I gene.

Primer Name	Sequence (5′–3′)	Annealing Temperature (°C)	Size (bp)
GnRH-I-F	GGCTCAACACTGGTCTTATGG	56	101
GnRH-I-R	TCTTCTGGCTTCTCCTTCG		
β-actin-F	TGGATGATGATATTGCCTGC	56	253
β-actin -R	ATCTTCCTCCATATCATCC		
pcDNA3.1-GnRH-I-F	CCCTCGAGCAGCCTCTCTCAGCAAAC	55	297
pcDNA3.1-GnRH-I-R	CCAAGCTTAATCTCCTTTCTTCTGGC		

qPCR was conducted on an MX3000P (Stratagene, La Jolla, CA) in a 15 μl volume containing 7.5 μl of 2 × SYBR Premix Ex Taq II (TaKaRa, Dalian, China), 0.2 μl of each forward and reverse primer (10 μM, β-actin-F/R and GnRH-I-F/R, Table 1), 0.3 μl of 50 × Rox Reference Dy II, and 1.5 μl of cDNA sample at a dilution of 1:4 according to the following program: 95 °C for 5 s, 56 °C for 20 s, and 72 °C for 15 s for 40 cycles. Melting curves were used to confirm the specificity of each product, and the PCR efficiencies were determined by analysis of twofold serial dilutions of cDNA and were designed to detect all of the signals in the spanning region. The PCR efficiency was close to 100%, allowing the use of the $2^{-\Delta\Delta Ct}$ method for the calculation of relative gene expression levels (Livak and Schmittgen, 2001). The β-actin gene was used as the internal control. All samples were amplified in triplicate, and the data were normalized to β-actin expression.

2.3. Immunohistochemistry

Chicken ovaries were collected from 45-day-old (sexually immature) and 160-day-old (sexually mature) hens. Tissues were fixed in 10% buffered formalin, paraffin embedded, and then cut into 5 μm tissue sections. All the sections were deparaffinized, rehydrated through a graded ethanol series, boiled in 10 mM sodium citrate buffer, quenched in 3% hydrogen peroxide, and blocked with 10% goat serum for 20 min. Next, the slides were incubated with rat anti-chicken GnRH-I antibodies (GHPRLSDLKETMAS of chicken GnRH-I was as the immunogens and synthesized by Abmart, 1:100) at 37 °C for 2 h. Then, the sections were incubated with the biotinylated secondary antibody and avidin–biotin-peroxidase complex for 30 min according to the Histostain-Plus Kit instructions (Zhongshan Golden Bridge Biotechnology, China). Finally, immunoprecipitates were visualized by incubation using a diaminobenzidine kit (Zhongshan Golden Bridge Biotechnology, China). After immunostaining, the sections were counterstained with hematoxylin, dehydrated, and covered. Negative control staining was performed by using normal rat serum and PBS instead of primary antibody. No specific staining was found on the control slides (Fig. 3A and B).

2.4. Plasmid construction

The complete open reading frame (ORF) of the chicken *GnRH-I* (NM_001080877.1) gene was inserted into the pcDNA3.1(+) plasmid vector (Invitrogen, Carlsbad, CA, USA) using the primer pair pcDNA3.1-GnRH-1-F/R (Table 1). *Hind*III and *Xho*I restriction enzyme sites were added at the 5′-end of the forward and reverse primers. PCR amplification was performed in 20 μl volumes containing 2 μl 10 × Ex-Taq-Buffer, 1.6 μl (2.5 mM) of dNTPs (TaKaRa, Dalian, China), 0.1 μl (5 U/μl) of Ex-Taq DNA polymerase (TaKaRa, Dalian, China), 0.4 μl each of

forward and reverse primers, 1 μl chicken genomic DNA (50–100 ng), and 14.5 μl of nuclease ddH_2O and run on a Mastercycler gradient (Eppendorf, Germany) according to the following program: 94 °C for 4 min; 35 cycles of 94 °C for 30 s, annealing at 58 °C for 30 s and 72 °C for 20 s; and final extension at 72 °C for 5 min. The construct was sequenced in both directions to confirm the authenticity of the sequences. The plasmid was reproduced in *E. coli* DH5α-competent cells and purified using an Endo-Free Plasmid Purification Kit (Tiangen).

2.5. Cell separation and culture

The five largest follicles (F5-F1) from the ovaries of egg-laying hens were separated and placed in cold phosphate-buffered saline (PBS, HyClone). The theca and granulosa layers were isolated according to the protocol described in Gilbert et al. (1977). The theca and granulosa cells were dispersed by treatment with 0.1% (w/v) collagenase II at 38.5 °C for 35 or 6 min with gentle agitation in a flask. After centrifugation, the cells were suspended in culture medium containing M199 (HyClone) with 10% (v/v) fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Gibco) and subsequently seeded in 24-well culture plates at a density of 1×10^6/well. The number of viable cells (90%) was estimated using Trypan blue. Cells were cultured at 38.5 °C in a water-saturated atmosphere of 95% air and 5% CO_2. The cells were used for qPCR or transfection.

2.6. Theca cell treatment and transient transfection

To examine overexpression of the chicken *GnRH-1* gene, theca cells were grown to 70% confluency and then transfected with plasmids using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) following the supplier's protocol. Briefly, the theca cells were grouped randomly and transfected by the 800 ng reconstructed plasmid pcDNA3.1-GnRH-1 or a pcDNA3.1(+) empty vector as a control. For the LH treatment assay, the freshly isolated chicken theca cells were seeded into 24-well plates; after 24 h, the cultured cells were subsequently treated with different concentrations (0, 5, 10, 25, 50, 100, 200, 250 ng/ml) of equine LH (Sigma, St. Louis, MO) according to the description of Wei et al. (Wei et al., 2013) or co-transfected with the 800 ng pcDNA3.1-GnRH-1 plasmid and 50 ng/ml equine LH. All the treated cells were collected after another 24 h for RNA extraction and qPCR analysis.

2.7. Protein extraction and Western blot analysis

The theca cells with different treatment were lysed in cold lysis buffer with protease inhibitors (10 mM Tris–HCl, 30% glycerol, 1% Triton X-100, 1 mM EDTA, 4 μg/ml leupeptin, 22 μg/ml aprotinin, 1 mM PMSF). Proteins were obtained by centrifugation at 15,000 rpm, 4 °C for 15 min, and quantified by the method of Bradford (Bio-Rad, CA, USA). Protein concentration was determined by the bicinchoninic acid assay (BCA Protein Array kit, TIANGEN Biotech). An equal amount of protein was separated by running on 12% SDS gel electrophoresis under denaturing and nonreducing conditions and then transferred to nitrocellulose filter membrane. The membranes were blocked with a solution of 5% bovine serum albumin (BSA) in PBS for 1 h at 37 °C and then incubated with rat anti-chicken GnRH-1 primary antibody (1:500) in a 5% bovine serum albumin/PBS solution for 2 h at 37 °C. After washing in PBST (G-Biosciences), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rat secondary antibodies (1:1000; Beyotime) in a 5% bovine serum albumin/PBS solution for 1 h at 37 °C and washed with PBST. The immunoreactive proteins were detected using an ECL™ Western blotting detection system. Total protein was normalized with the anti-β-actin antibody (1:1000; Beyotime) on another gel. A parallel negative control experiment omitting the primary antibody was conducted to observe any cross-reactivity of the secondary antibody.

2.8. Statistical analysis

Differences between the experimental groups were evaluated by one-way ANOVA followed by Duncan's multiple range test ($P < 0.05$) using the General Linear Model procedure of SAS (version 9.2). The same-sized follicles from three to four individuals' ovaries were collected and mixed as one experimental repeat in cell culture and total RNA isolation. In one experiment each treatment was repeated at least four times, and all data were presented as the mean ± SEM (n = 4).

3. Results

3.1. The expression of GnRH-I in chicken ovary and hypothalamus at different developmental stages

By using qPCR, the mRNA expression of *GnRH-I* in the ovary and hypothalamus was analyzed in the hens of 30 d, 60 d, 110 d and 140 d. For the hens at 140 d, *GnRH-I* mRNA expression was compared between not laying (140 a) and laying (140 b) individuals. The results showed that ovarian *GnRH-I* mRNA expression increased significantly between 30 and 60 d and then decreased from 90 to 110 d. In the nearly sexually mature ovaries of group 140a, the *GnRH-I* mRNA was dramatically increased, but it was significantly decreased in sexually mature ovaries of 140b chickens (Fig. 1A). A similar expression pattern for the ages 30–140a of *GnRH-I* mRNA in chicken hypothalamus was observed. At the ages of 60 and 140a, the *GnRH-I* mRNA expression was significantly higher than at other stages of development. However, in 140b chickens, *GnRH-I* mRNA expression was significantly decreased in the ovaries but not in the hypothalamus (Fig. 1B).

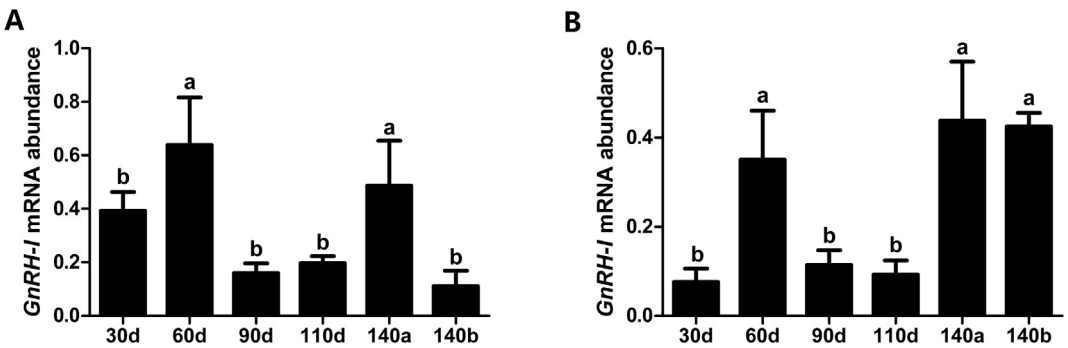

Fig. 1. The expression of *GnRH-I* mRNA in the ovary and hypothalamus from 30-, 60-, 90-, 110- and 140-day-old (140a and 140b) Jining Bairi hens was analyzed by qPCR. (A) The expression of *GnRH-I* in the ovary. (B) The expression of *GnRH-I* in hypothalamus. All data are presented as the mean ± SEM. Bars with different lowercase letters are significantly different ($P < 0.05$, n = 4).

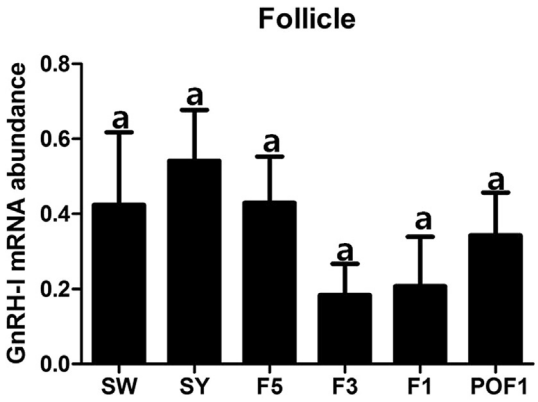

Fig. 2. The expression of *GnRH-I* mRNA in different sized follicles of 140-day-old Jining Bairi hens. Relative expression levels of *GnRH-I* mRNA in small white follicles (SW), small yellow follicles (SY), the fifth largest follicles (F5), the third largest follicles (F3), the first largest follicles (F1) and the newly post-ovulatory follicles (POF1). All data are presented as the mean ± SEM. Bars with different lowercase letters indicate statistically significant differences ($P < 0.05$, n = 4).

3.2. The expression of chicken GnRH-I mRNA in different sized ovarian follicles

The highest abundance of *GnRH-I* mRNA was found in the small follicles (SW, SY and F5), while the abundance of *GnRH-I* mRNA remained low in large follicles (F3 and F1) and then slightly increased in POF1 follicles (Fig. 2). However, no statistically significant differences were observed in mRNA expression of *GnRH-1* among all the follicles studied ($P > 0.05$).

3.3. The localization and expression of the chicken GnRH-I gene in follicular cells

The cellular localization of the GnRH-I protein was examined in sexually immature (45 d) and laying (160 d) hen ovaries by immunohistochemistry. In 45-day-old ovaries, a weak signal of GnRH-I protein was detected in the ovarian interstitial and granulosa cells of growing follicles (Fig. 3C). In 160-day-old hen ovaries, GnRH-I protein was strongly stained in theca cell layers but weak in granulosa cells of follicles (Fig. 3D). To corroborate the protein expression patterns, the mRNA expression of *GnRH-1* in theca cells was validated by qPCR. In the five largest preovulatory follicles, *GnRH-I* mRNA levels were significantly higher in theca cells than that in granulosa cells (Fig. 4).

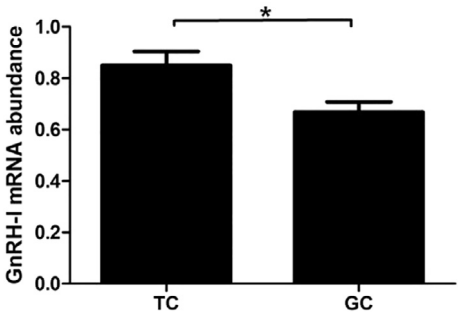

Fig. 4. The mRNA expression of chicken *GnRH-I* in theca cells (TCs) and granulosa cells (GCs) by qPCR. All data are presented as the mean ± SEM. * represents $P < 0.05$ (n = 4).

3.4. Effects of GnRH-I mRNA overexpression on the expression of LHR, FSHR, PGR and ERα genes in preovulatory follicle granulosa and theca cells

Reproductive hormones play a key role in the control of follicular development and ovulation via their receptors. Therefore, we investigated the influence of over expression chicken *GnRH-I* mRNA on the expression of *LHR*, *FSHR*, *PGR* and *ERα* mRNA in the theca and granulosa cells of the preovulatory follicles. Theca cells and granulosa cells were transfected with either *GnRH-I* overexpressing plasmids (pcDNA3.1-GnRH-I) or empty plasmids of pcDNA3.1 (+) as a control. The results showed that *GnRH-I* mRNA was significantly up-regulated in transfected theca and granulosa cells compared to the empty vector

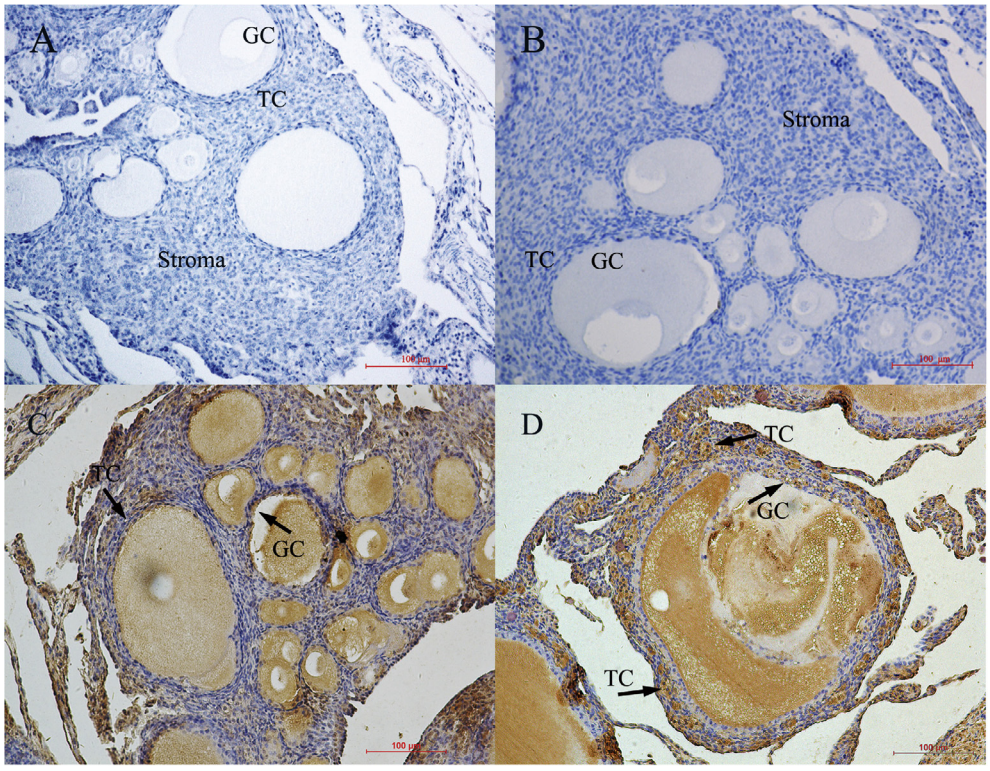

Fig. 3. Immunohistochemical staining for GnRH-I in ovarian follicles from 45- and 160-day-old chickens. (A) Negative control with PBS used in place of the primary antibody. (B) Negative control with rat serum used in place of the primary antibody. (C) Follicles in 45-day-old chicken ovaries. (D) Follicles in 160-day-old chicken ovaries; arrowheads in C and D indicate the position of strongly stained protein. GC, granulosa cells; TC, theca cells. Bar = 100 μm.

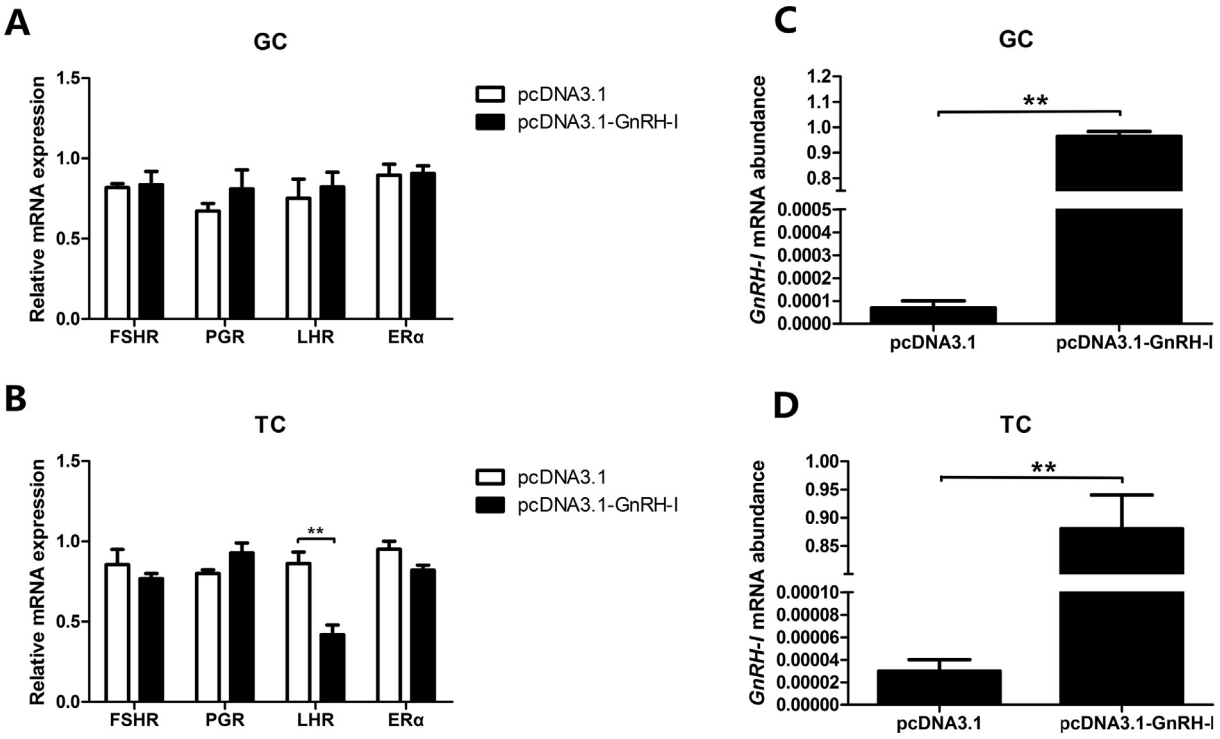

Fig. 5. The effect of overexpression *GnRH-I* on *FSHR*, *PGR*, *LHR* and *ERα* mRNA levels in chicken theca and granulosa cells. (A and B) The expression of *FSHR*, *PGR*, *LHR* and *ERα* mRNA levels in GCs and TCs. (C and D) Overexpression of *GnRH-I* in granulosa cells (GCs) and theca cells (TCs). All data are presented as the mean ± SEM. ** represents $P < 0.01$ (n = 4).

control ($P < 0.01$) (Fig. 5C and D). In granulosa cells, the expression of *LHR*, *FSHR*, *PGR* and *ERα* mRNA was not influenced by *GnRH-I* (Fig. 5A). However, in theca cells, the mRNA expression of *LHR* was significantly decreased by *GnRH-I* overexpression ($P < 0.01$) while the other three genes was not significantly affected (Fig. 5B).

3.5. The effects of LH hormone on GnRH-I and LHR mRNA expression in preovulatory follicle theca cells

To examine the effects of LH on the expression of *GnRH-I*, we isolated theca cells from the five largest preovulatory follicles (F5-F1) and treated them with different concentrations of LH for 48 h. The results indicated that treatment with 50 ng/ml LH resulted in a significant increase in *GnRH-I* mRNA, but higher doses of LH (200 ng/ml) significantly inhibited the expression of *GnRH-I* mRNA (Fig. 6A). In the same experiment, LH gradually increased *LHR* gene expression in a dose-dependent manner; the mRNA level of *LHR* was significantly up-regulated with increasing LH concentrations (Fig. 6B).

3.6. The effects of GnRH-I mRNA overexpression on LH-induced LHR mRNA expression in preovulatory follicle theca cells

To further investigate the interaction relationship between *GnRH-I* and LH, theca cells were treated with pcDNA3.1-GnRH-I plasmids in the presence of LH or were left untreated. Because the 50 ng/ml LH treatment significantly facilitated *GnRH-I* mRNA expression and because 800 ng *GnRH-I* overexpression plasmids significantly decreased *LHR* mRNA expression in theca cells, we detected LHR expression by *GnRH-I* overexpression and LH treatments. After overexpression, the protein levels of GnRH-I were also detected by using Western blot. The results showed that the level of GnRH-I protein in the overexpression group was significantly higher than that in the empty vector control group ($P < 0.01$) (Fig. 7). The concomitant treatment of *GnRH-I* overexpression plasmids and 50 ng/ml LH significantly inhibited LH-induced expression of *LHR* mRNA (Fig. 8).

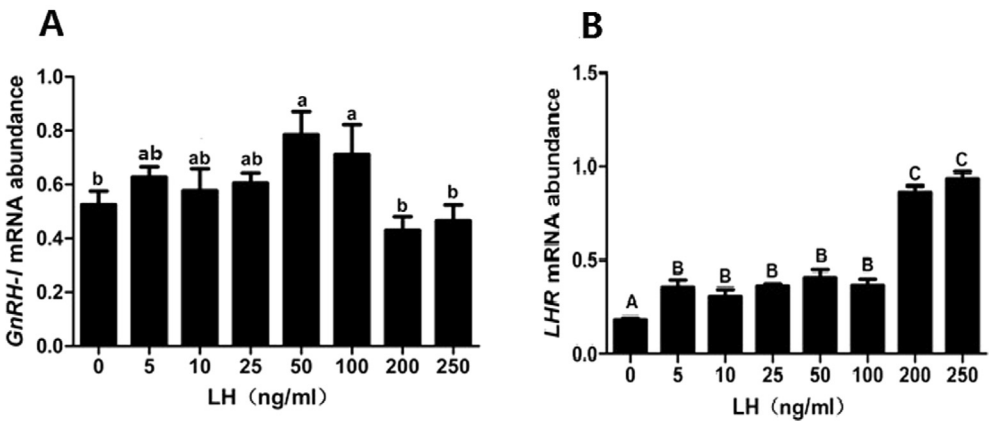

Fig. 6. Gonadotropins regulate *GnRH-I* mRNA expression in theca cells. Primary theca cells were treated with different doses of FSH and LH. Expression of *GnRH-I* mRNA was analyzed by qPCR. All data are presented as the mean ± SEM. Bars with different lowercase letters are significantly different at $P < 0.05$ and different uppercase letters are significantly different at $P < 0.01$ (n = 4).

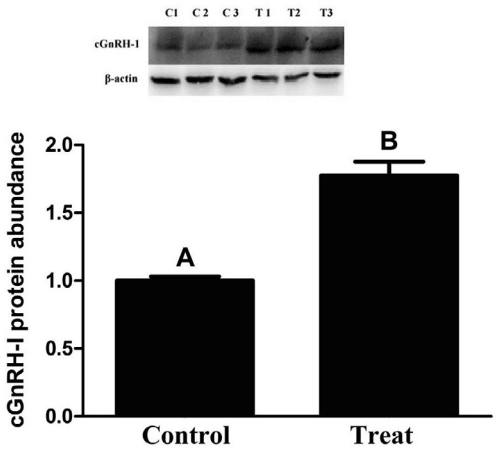

Fig. 7. The effect of overexpression of *GnRH-1* mRNA on its protein expression. C1-C3 was the control (pcDNA3.1 empty vector plasmid), T1-T3 was the overexpression group. Data are presented as the mean ± SEM. Bars with different uppercase letters are significantly different at $P < 0.01$ (n = 3).

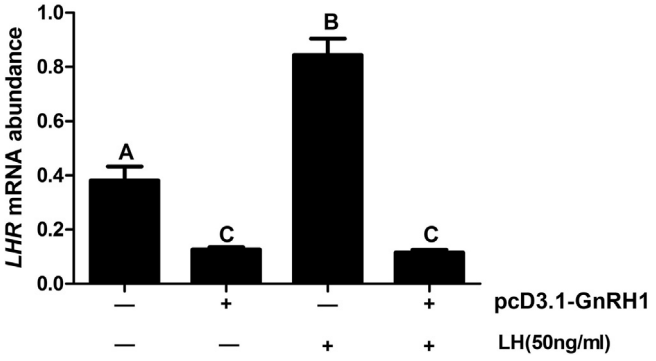

Fig. 8. *GnRH-I* inhibited mRNA expression of LH-induced *LHR*. Overexpression of *GnRH-I* and treatment with LH in chicken preovulatory follicle theca cells decreased *LHR* mRNA expression. All data are presented as the mean ± SEM. Bars with different uppercase letters are significantly different at $P < 0.01$ (n = 4).

4. Discussion

In recent years, the extra-hypothalamic roles of GnRH have attracted special interest in the field of reproductive biology. In this study, we found that from 30 d to approaching sexual maturation (140a d), the expression of *GnRH-I* mRNA in the chicken ovary had the same trend as that in the hypothalamus. However, a different expression pattern existed in the chicken hypothalamus and ovary after sexual maturation (140b d). *GnRH-I* mRNA expression was significantly decreased in the ovary after sexual maturation, yet in the hypothalamus, *GnRH-I* mRNA expression maintained a high level. The high expression of *GnRH-I* in the immature ovary suggests that it may be responsible for the delay in ovarian function until the initiation of sexual maturation. *GnRH-1* mRNA expression also increased in the ovary of prepubertal ducks (Ni et al., 2007). Studies of immature hypophysectomized diethylstilbestrol-implanted rats revealed that whether GnRH exerts a stimulatory or inhibitory ovarian function is largely determined by the maturational status of the ovary and the stage of follicular development and that the major effects of GnRH in immature follicles are inhibitory (Ranta et al., 1984). In porcine species, GnRH agonist treatment significantly decreased the proliferative activity of small and medium follicle granulosa cells but showed a negligible response in mature granulosa cells from large follicles (Knecht et al., 1985). Furthermore, a GnRH agonist significantly decreased P4 and E2 secretion in granulosa cells from large follicles but not from small or medium follicles (Takekida et al., 2003). This observation is consistent with *GnRH-R* expression. In the rat ovary, in situ hybridization analysis showed that *GnRH-R* expression was dependent on the degree of follicular development and the stage of the estrous cycle (Bauer-Dantoin and Jameson 1995; Schirman-Hildesheim et al., 2005). Higher levels of the *GnRH-R* transcripts were detected in granulosa cells from small follicles, whereas the transcript levels were slightly lower in both medium and large follicles in the rat ovary (Bauer-Dantoin and Jameson 1995). In the present study, by using qPCR, we also found that the expression of *GnRH-I* mRNA was higher in small follicles than in large follicles, and by immunohistochemical methods, the immunoreactivity was detected mainly in the granulosa layer of the immature ovaries, whereas immunoreactivity was detected mainly in the theca layer of the mature ovaries.

The in vitro effects of *GnRH-I* on chicken ovarian function were also examined in preovulatory follicular cells. Overexpression of the chicken *GnRH-I* gene in granulosa and theca cells from preovulatory follicles revealed that *LHR* gene expression in theca cells was significantly inhibited by *GnRH-I*, while *FSHR*, progesterone receptor (*PGR*) and estrogen receptor (*ERα*) were not affected in both granulosa cells and theca cells (Fig. 5). These results suggest that *GnRH-I* was mainly interaction with LH/LHR in chicken preovulatory follicular theca cells. A previous study of human GLCs showed that GnRH agonist treatment resulted in the down-regulation of FSH receptor and LH receptor (Kang et al., 2001). GnRH and its agonistic analogs inhibited the FSH-induced increase in estrogen and progesterone production in cultured rat ovarian granulosa cells (Otani et al., 1982). Another study also showed that GnRH analogs suppressed FSH-induced steroidogenesis in rat granulosa cells (Wickings et al., 1990). In prepubertal female cats, it was also found that a GnRH agonist (deslorelin) suppressed reproductive function through a change in ovarian *LHR* mRNA expression (Mehl et al, 2017). These findings suggested that GnRH-I may exert its anti-gonadotrophic effect by down-regulating gonadotrophin receptors.

One of the novel findings of the present study was the demonstration that LH regulated *GnRH-I* mRNA expression in a dose-dependent manner. The results from this study indicate that treatment with LH at low doses (50 ng/ml) increased *GnRH-I* gene expression, whereas there was an inhibitory effect on *GnRH-I* mRNA levels at higher doses (200 ng/ml). Furthermore, *GnRH-I* inhibited LH-induced *LHR* mRNA expression in chicken theca cells from preovulatory follicles. Concomitant treatment with a GnRH-I overexpression plasmid and LH (50 ng/ml) resulted in a significant decrease in *LHR* mRNA expression in comparison to the treatment with LH alone. It has been reported that LH/hCG down-regulated the expression of *GnRHR* mRNA levels in the rat and human ovary (Harwood et al., 1980; Peng et al., 1994; Choi et al., 2006). Treatment with FSH or hCG resulted in down-regulation of *GnRH-I* mRNA expression in hGLCs (Kang et al., 2001). In addition, treatment with estrogen induced a dose-dependent decrease in *GnRH-I* mRNA expression in hGLCs (Khosravi and Leung, 2003). The modulation of GnRH-I/GnRHR via LH/FSH suggests a regulatory feedback loop between the two hormones in the ovary, although the precise physiological mechanism remains unknown.

In conclusion, we have demonstrated that 1) *GnRH-I* mRNA is expressed in both the immature chicken ovary and the hypothalamus, but its expression significantly decreases in the ovary compared to the hypothalamus when chickens become sexually mature; 2) *GnRH-I* gene expression is up-regulated by LH at low doses but down-regulated at high doses in chicken theca cells; and 3) *GnRH-I* gene inhibits LH-induced *LHR* mRNA expression when the LH dose is low in chicken preovulatory follicle theca cells. These results suggest that the presence of *GnRH-I* in the chicken ovary may be involved in the processes of follicle development and sexual maturation.

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