Differential expression of FSHR and LHR genes and proteins during development of rabbit ovarian follicles

Author for correspondence:
Ahmed A. Kassab, Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Benha University, Egypt.
Tel: +20 1117313734.
E-mail: kassab_aa@yahoo.com

Eman Khalil, Mohamed Metwally, Hatem Bahgaat, Ahmed Kassab [ID] and Anwar El-Shafey

Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Benha University, Egypt

Summary

The development of an ovarian follicle is a complex process at the cellular and molecular level that is mainly regulated by follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR). To elucidate the contribution of these receptors to ovarian follicle development, it is necessary to determine their expression profiles during this biological process. Therefore, this study aimed to investigate the relationship between ovarian development pattern and the differential ovarian expression pattern of *FSHR* and *LHR* genes as well as proteins at different developmental stages. Ovaries were collected from 30 New Zealand rabbits at day 0 (birth), week 2 (neonate), week 4 (cub), week 16 (maturity), and day 18 pregnancy. Ovarian histology, and gene as well as protein expression were determined using light microscopy, real-time PCR and western blotting, respectively. The results showed that the expression levels of *FSHR* mRNA and protein increased coincidently with age and the growth of ovarian follicles. The levels of *LHR* mRNA and protein remained low from the day of birth until week 4 and became significantly higher by week 16 coinciding with appearance of growing and antral follicles, which have a defined thecal layer. FSHR gene and protein expression decreased with pregnancy, whereas LHR increased, reaching a peak level during pregnancy. It can be concluded that changes in FSHR and LHR gene and protein expression could be related to the growth and development of follicles, indicating the regulatory role for these receptors in rabbit folliculogenesis.

Introduction

In contrast with most other mammals, the formation, activation and development of ovarian follicles occurs entirely postnatally in rabbits (Hutt *et al.*, 2006). Germ cells first appear in the ovary as oogonium, arranged in cell nests that disappear late in development (Ricken and Viebahn, 2002; Takagi *et al.*, 2007). Folliculogenesis starts 2 weeks after birth. By that time, primordial follicles have already developed (Daniel-Carlier *et al.*, 2013). Primary follicles and growing primary follicles are visible at the fourth week of age (Hutt *et al.*, 2006). Secondary follicles with clearly distinct theca cell layers are first identified at 6 weeks of age, while growing and antral follicles are noticed in the ovaries of 8-week-old and 12-week-old rabbits (Hutt *et al.*, 2006). The interstitial glands are typical steroid-secreting cells that were first demonstrated in the rabbit ovary at 8 weeks of age (Abd-Elkareem, 2010). These glands are mostly derived from the theca interna cells of the atretic antral follicle (Abd-Elkareem, 2010).

Ovarian follicle development is a complex process that is controlled by the integration of endocrine, cellular and molecular mechanisms (Wear *et al.*, 2016) and its dynamic regulation is an integral part of the reproductive process (Wu *et al.*, 2015). This process is highly orchestrated by endocrine signalling from the hypothalamus and the pituitary gonadotropins (Jin *et al.*, 2017). However, the effects of gonadotropins on ovarian development depend not only on the level of circulation, but also on the expression of their respective receptors (FSHR and LHR) (Mehl *et al.*, 2017). These receptors belong to the superfamily of G-protein coupled receptors that are characterized by the presence of a large extracellular domain (Narayan *et al.*, 2018). Pituitary gonadotropins are necessary for promoting ovarian development, maturation of the follicles and development of granulose cells through interaction with their receptors (Dias *et al.*, 2002; Zachos *et al.*, 2003; Hunter *et al.*, 2004; Durlej *et al.*, 2011). Genetic mutation of these receptors can result in pathological conditions in which follicular growth is arrested and the female becomes infertile (Narayan *et al.*, 2018).

Regarding the onset of *FSHR* gene expression, some investigators have found earlier expression before the formation of histologically distinct primordial follicles in hamster ovaries (Chakraborty and Roy, 2015). In contrast, other studies have reported a later onset of expression at the beginning of mouse folliculogenesis (Lei *et al.*, 2010) or granulose cells proliferation

(Saraiva *et al.*, 2011). An *in situ* hybridization-based study showed restricted FSHR expression in granulose cells (Hillier, 2001). Whereas, LHR was mainly expressed in the thecal cells (Zhang *et al.*, 2001) and partially in corpus luteal cells (Ziecik *et al.*, 2007).

Ovarian expression of the *LHR* and *FSHR* genes has been detailed in the mouse, rat, hamster, horse, cat, and duck (Rannikki *et al.*, 1995; O'Shaughnessy *et al.*, 1996, 1997; Ni *et al.*, 2007; Chakraborty and Roy, 2015; Scarlet *et al.*, 2015; Mehl *et al.*, 2017). However, this expression at transcriptional and translational levels has not yet been elucidated in rabbit. Therefore, this study aimed to investigate the changes in FSHR and LHR gene and protein expression in rabbit ovary from birth to maturity, in addition to pregnant rabbits. In addition, we correlated these changes with granulosa and theca layer thickness to improve the understanding of the expression underlying ovarian follicle development, which is considered a fundamental step in reproduction success, as it can be utilized in rabbit farming as predictors of disrupted reproductive capabilities in rabbits.

Materials and methods

Tissue sample collection

Thirty healthy female New Zealand rabbits at birth (0 day), neonate (2 week), cub (4 week), maturity (16 week), and pregnancy (18 days post-coitum, 18dpc) ($n = 6$ animals/group) were obtained from the animal house, Faculty of Veterinary Medicine, Benha University. Their body weights were 43.19 ± 2.51, 225.00 ± 31.05, 404.75 ± 11.87, 2275 ± 515, and 2635 ± 523 g, respectively. The animals were housed in individual cages at a controlled temperature ($23 \pm 2°C$) under a 12 h:12 h, light:dark cycle and provided with free access to water and food. The animals were euthanized by intramuscular injection of sodium pentobarbitone (140 mg/kg) in compliance with recommendations of the animal care committee of the Faculty of Veterinary Medicine, Benha University (approval number: BUFVTM 07–3–21). Right ovaries were kept in Bouin's fluid for no longer than 24 h for the histological technique, while the left ovaries were stored at $-80°C$ until RNA and protein extraction.

Histological preparation

Fixed ovaries were dehydrated in a graded series of ethanol and embedded in paraffin wax. Ovaries were sectioned (5–7 μm) and stained with haematoxylin and eosin for general histological structure and Masson's trichrome stain for staining collagen fibres, as described by Feldman and Wolfe (2014).

Histomorphometric measurements

Morphometrical studies were applied to images of stained histological sections of ovaries at all developmental stages using ImageJ software for measuring granulosa and theca layer thickness as previously described (Lan *et al.*, 2014). Ovarian follicles were classified according to Hutt *et al.* (2006).

Quantitative real- time PCR

Total RNA from 15 rabbit ovarian samples ($n = 3$/group) was extracted using a Total RNA Purification Kit following the manufacturer's guidelines (iNtRON Biotechnology, easy-REDTM Total RNA Extraction Kit) as previously described (Abd-Allah *et al.*, 2015). cDNA was prepared from 5 μg of total RNA using the M-MuLV reverse transcriptase enzyme following the

protocol of the manufacturer (Thermo Scientific, Fermentas, #EP0451). Quantitative PCR was conducted using the 2X Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer's protocol (Thermo Scientific, USA, #K0221). PCR cycling conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s (denaturation step) and 60°C for 1 min (annealing step). The melting curve was produced by increasing the temperature from 60 to 95°C. RT-PCR was performed using three sets of primers, *FSHR* (forward, 5′-GAGGAATGCCATTGAA CTGAGG-3 and reverse, 5′-GTTGGAGAACACATCTG-3′); *LHR* (forward, 5′-CTGGAGAAGATGCACAATGG-3′ and reverse, 5′-AATTAGCCTCTGAATGGACTC-3′) and *GAPDH* (forward, 5′-TGTTTGTGATGGGCGTGAA-3′ and reverse, 5′-CCTCCACAATGCCGAAGT-3′).

The $\Delta\Delta C_T$ method was used to calculate the data with GAPDH as an internal control (Livak and Schmittgen, 2001). Three replicates of RT-PCR assays were performed for each sample

Western blot

In RIPA buffer, 15 ovarian samples ($n = 3$/group) were homogenized as previously described (Yang *et al.*, 2004). In total, 20 μg proteins were segregated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA). The membrane was blocked with 3% bovine serum albumin in Tween-20/Tris-buffered saline (TPST) at room temperature for 1–2 h. After blocking, the membranes were incubated with primary antibodies: anti-β-actin (1:1000, ab8226, Abcam), anti-FSHR (1:500, ab75200, Abcam), and anti-LHR (1:500, ab179780, Abcam). PVDF membranes were then incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody that was diluted to 1:5000 in blocking solution at room temperature. Densitometry analysis of protein bands was carried out using ImageJ software. The density of each band was normalized to β-actin. Three replicates were conducted for each protein sample.

Statistical analysis

Data were assessed using SPSS 16.0 software and one-way variance analysis (ANOVA). Duncan's multi-range test was used to assess differences in the mean values. Correlations between granulosa and theca layers thickness of different types of ovarian follicles and mRNA as well as protein expression of FSHR and LHR were calculated using Pearson's correlation coefficient (r). *P*-values < 0.05 or $P < 0.01$ were considered statistically significant. All data were presented as mean ± standard error of the mean (SEM).

Results

Ovarian follicle development

At birth, the ovarian cortex mainly contained germ cells, not yet assembled into primordial follicles and arranged in cell nests. Each cell nest was surrounded by a continuous basal lamina and elongated flattened stromal cells, and formed of germ cells of large size and spherical shape, and somatic cells, which by comparison were smaller and slightly oblong in shape (Figure 1A). At 2 weeks of age, the cell nests were completely resolved, as each oocyte individually acquired follicular cells and was organized into newly formed primordial follicles that appeared for the first time in the inner portion of the cortex. These follicles consisted of an oocyte surrounded by a one-cell layer of flattened follicular cells
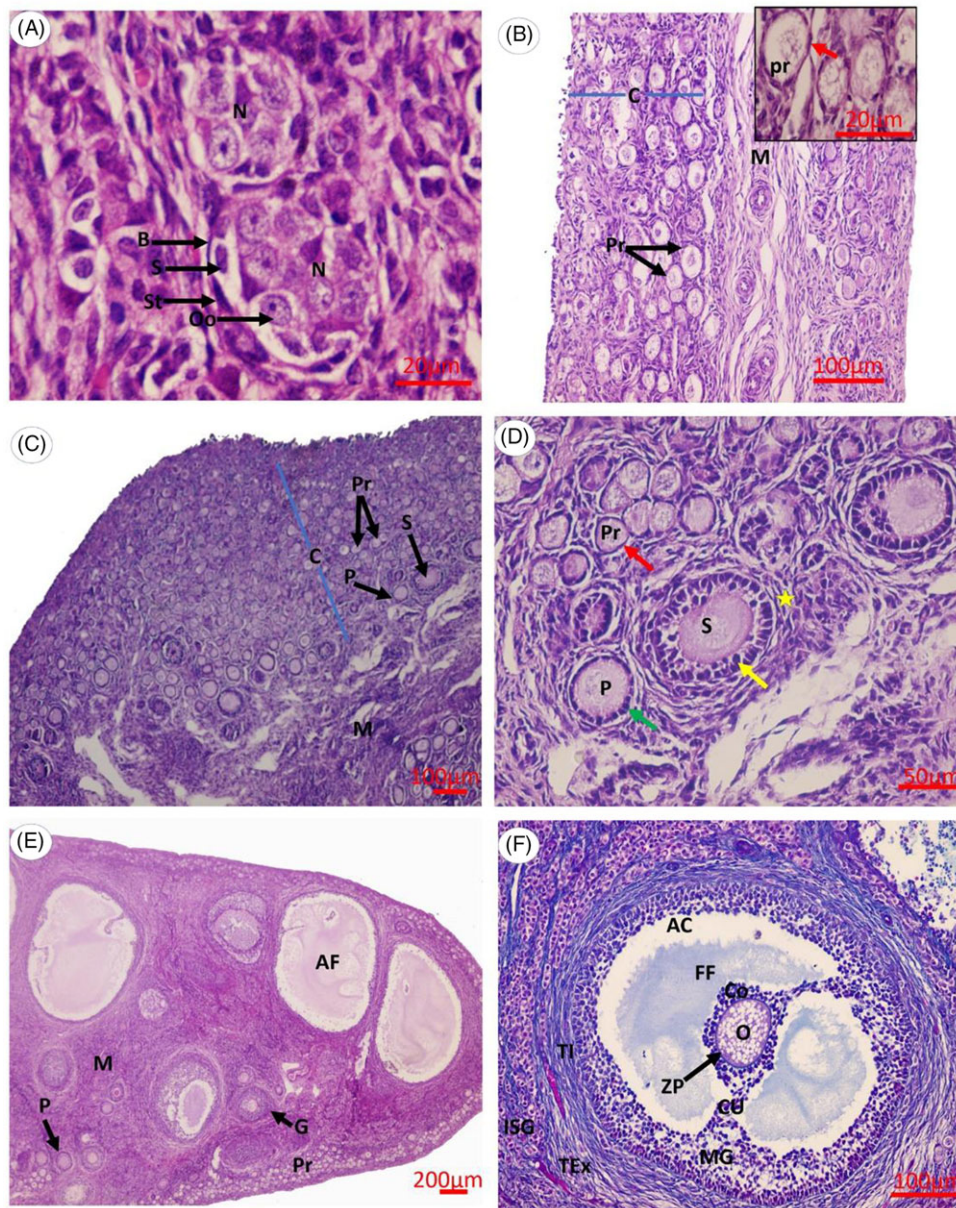
Figure 1. Photomicrograph of the rabbit ovary at different postnatal development stages. (A) 0 day, (B) 2 weeks, (C, D) 4 weeks, (E, F) 16 weeks. Abbreviations: AC, antrum cavity; AF, antral follicle; B, basal lamina; C, cortex; Co, corona radiata; CU, cumulus oophorous cells; FF, follicular fluid; G, growing follicle; ISG, interstitial gland; M, medulla; MG, mural granulosa cells; N, oogonial nest; O, primary oocyte; Oo, oogonia; P, primary follicle; Pr, primordial follicle; S, secondary follicle/somatic cells; St, stromal cells; TEx, theca externa; TI, theca interna. Red arrow, flattened follicular cells; green arrow, cuboidal follicular cells; yellow arrow, granulosa cells; yellow asterisk, theca cells. Scale bars: 20 μm (A), 100 μm (B, C, F), 50 μm (D), 200 μm (E). (A–E) Haematoxylin and eosin stain. (F) Masson's trichrome stain.

(Figure 1B). By the fourth week, a pool of primordial follicles occupied the majority of the ovarian cortex. In addition, primary and secondary follicles were often first observed near the interface of the cortex and medulla (Figure 1C). Each of these primary follicles consisted of an oocyte surrounded by a single cell layer of cuboidal follicular cells, while the secondary follicles were occasionally enclosed by a theca-cell-like layer (Figure 1D). At week 16 (age of maturity), all ovarian follicle types (primordial, primary, growing, and antral) were observed in the ovarian cortex. Primordial and primary follicles were located peripherally, whereas the growing and antral follicles were located centrally (Figure 1E). The antral follicle was distinguished by the formation of a fluid-filled cavity adjacent to the oocyte known as the antrum. This follicle was formed of an oocyte surrounded by acidophilic zona pellucida. A layer of columnar follicular cells was organized radially around the zona pellucida, forming the corona radiata. The follicular cavity was lined by stratified epithelium that constituted the mural granulosa layer. Cumulus oophorous cells connected the ovum and its surrounding cells with mural granulosa cells. Well organized theca folliculi surrounded the large antral follicles from outside. The theca folliculi were differentiated into theca interna that was more cellular and less fibrous, while the theca externa was more fibrous and less cellular. The cells of the theca interna were polyhedral or elongated in shape. (Figure 1F). A prominent and characteristic feature of the mature rabbit ovary was the presence of fully developed interstitial glands that were represented by groups of polyhedral or rounded cells separated from each other by connective tissue (Figure 1F). At the pregnancy stage (18 days post-coitum), the rabbit ovary was mostly occupied by corpora lutea with a low number of ovarian follicles at different developmental stages (Figure 2A,B).

Histomorphometry for different follicular structures

The thickness of the granulosa layer in primordial follicles increased significantly (three-fold) from 1.54 ± 0.16 μm to 4.37

Table 1. Micromorphometric measurements showed thickness of granulosa and theca layers of different types of ovarian follicles in the rabbit ovary

Follicle classes	Measurements	Different ages of female rabbits				
		0 D	2 weeks	4 weeks	16 weeks	18 dpc
Primordial follicle	Thickness of granulosa cells (µm)	–	1.54 ± 0.16[Ab]	1.58 ± 0.08[Ac]	1.84 ± 0.33[Ae]	1.64 ± 0.06[Ae]
	Thickness of theca cells (µm)	–	–	–	–	–
Primary follicle	Thickness of granulosa cells (µm)	–	4.37 ± 0.44[Aa]	4.72 ± 1.93[Ab]	5.98 ± 0.370[Ad]	5.05 ± 0.43[A]
	Thickness of theca cells (µm)	–	–	–	–	–
Secondary follicle	Thickness of granulosa cells (µm)	–	–	18.55 ± 1[Aa]	19.53 ± 0.27[Ac]	18.90 ± 0.47[Ac]
	Thickness of theca cells (µm)	–	–	12.66 ± 1.2[A]	13.35 ± 0.6[Ac]	13.21 ± 0.65[Ac]
Growing follicle	Thickness of granulosa cells (µm)	–	–	–	41.06 ± 0.87[Ab]	39.5 ± 0.54[Ab]
	Thickness of theca cells (µm)	–	–	–	44.97 ± 3.55 [Ab]	44.52 ± 0.95[Ab]
Antral follicle	Thickness of granulosa cells (µm)	–	–	–	65.19 ± 7.5 [Aa]	62.34 ± 6.4[A]
	Thickness of theca cells (µm)	–	–	–	85.44 ± 2.9[Aa]	85.39 ± 16.2[Aa]

Data presented in form of mean ± SEM. The same uppercase letters (A) in the same row mean that there was no significant difference ($P < 0.05$). Values within the same column for the thickness of granulosa and theca layers (µm) and carrying different lowercase letters (a–e) are significantly different at $P < 0.05$.
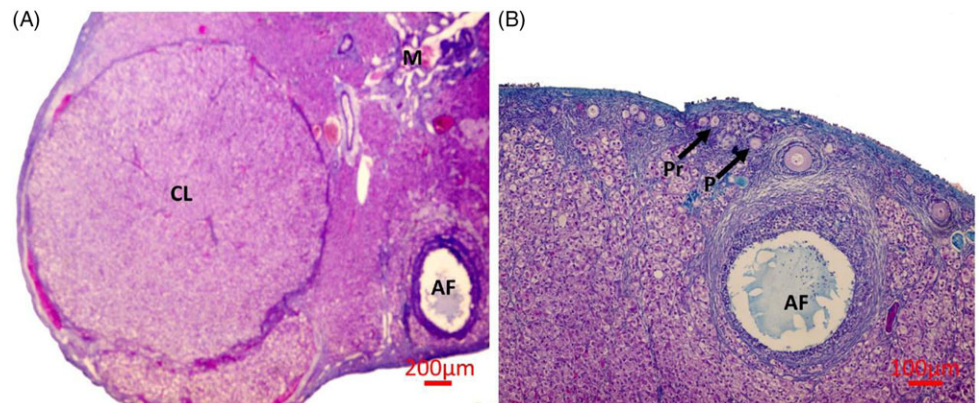

Figure 2. Photomicrograph of 18 day pregnant rabbit ovary. (A, B) Pregnant rabbit ovary containing AF, antral follicle; CL, corpus luteum; M, ovarian medulla; P, primary follicle; Pr, primordial follicle. Scale bars: 200 µm (A), 100 µm (B). Masson's trichrome stain.

± 0.44 µm in primary follicles at 2 weeks of age ($P < 0.05$) and increased significantly (12-fold) from 1.58 ± 0.08 µm in primordial follicles to 18.55 ± 1 µm in secondary follicles at 4 weeks of age ($P < 0.05$). This thickness increased significantly (35-fold) from the primordial stage (1.84 ± 0.33 µm) to the antral stage (65.19 ± 7.5 µm) at 16 weeks of age ($P < 0.05$) and ~38-fold from 1.64 ± 0.6 µm in primordial follicles to 62.34 ± 6.4 µm in antral follicles at pregnancy ($P < 0.05$). The theca cell layer appeared at the secondary stage in rabbit follicles. Theca cell layer thickness increased significantly (six-fold) from the secondary (13.35 ± 0.6 µm) to the antral stage (85.44 ± 2.9 µm) at 16 weeks of age and six-fold from 13.21 ± 0.65 µm in secondary follicles to 85.39 ± 16.2 µm in antral follicles at pregnancy ($P < 0.05$) (Table 1).

Differential expression of *FSHR* and *LHR* genes

The data obtained from RT-qPCR revealed that ovarian *FSHR* gene expression was detectable at a low level in the newly born rabbit around the time of birth (0D) followed by a significant increase by week 2 to week 4 after birth. Thereafter, *FSHR* gene expression significantly increased (~11-fold more than the 0D) by week 16, then decreased notably by pregnancy (18 dpc) (Figure 3A). The levels of *LHR* transcripts remained low from the day of birth

(0D) to week 4 and became significantly higher by week 16. At pregnancy, *LHR* expression increased and reached the maximum level (Figure 3B).

Differential expression of FSHR and LHR proteins

The expression levels of FSHR protein as detected by western blot exhibited a significant gradual increase with highest expression at week 16 (Figure 4A,B). The expression was decreased at pregnancy (18 dpc), but remained higher than at the earlier stages (from 0D to 4 weeks) (Figure 4A, B). In contrast, LHR protein did not show any significant difference from birth to week 4, but at further stages its expression gradually increased until reaching the highest expression at pregnancy (Figure 4A,C).

Correlation between genes and proteins expression and ovarian parameters

It was noted from our results that a significant increase in LHR and FSHR gene and protein expression was observed by the appearance of the advanced ovarian follicles that have many layers of granulosa cells and defined thecal layers. Therefore, we investigated the correlation between FSHR and LHR gene and protein expression and
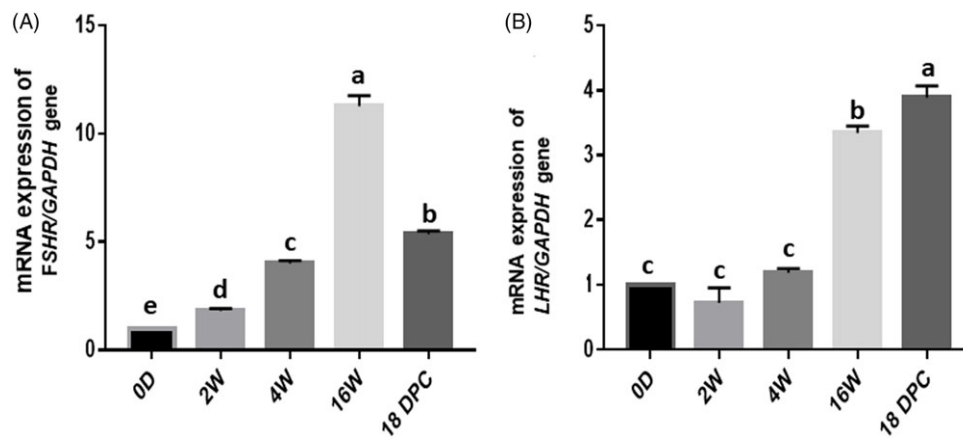
Figure 3. RT-PCR analysis of mRNA levels in 15 rabbit ovarian samples ($n = 3$ per each group). Three replicates of RT-PCR assays were performed for each sample. (A) Relative *FSHR* gene expression. (B) Relative *LHR* gene expression. Different letters above the bars indicate significant differences between different ovarian developmental stages ($P < 0.05$). Data presented as mean ± SEM. Abbreviations: FSHR, follicular stimulating hormone receptor; LHR, luteinizing hormone receptor. 0D, 0 day; 2W, 2 weeks; 4W, 4 weeks; 16W, 16 weeks; 18 DPC, 18 days post-coitum.
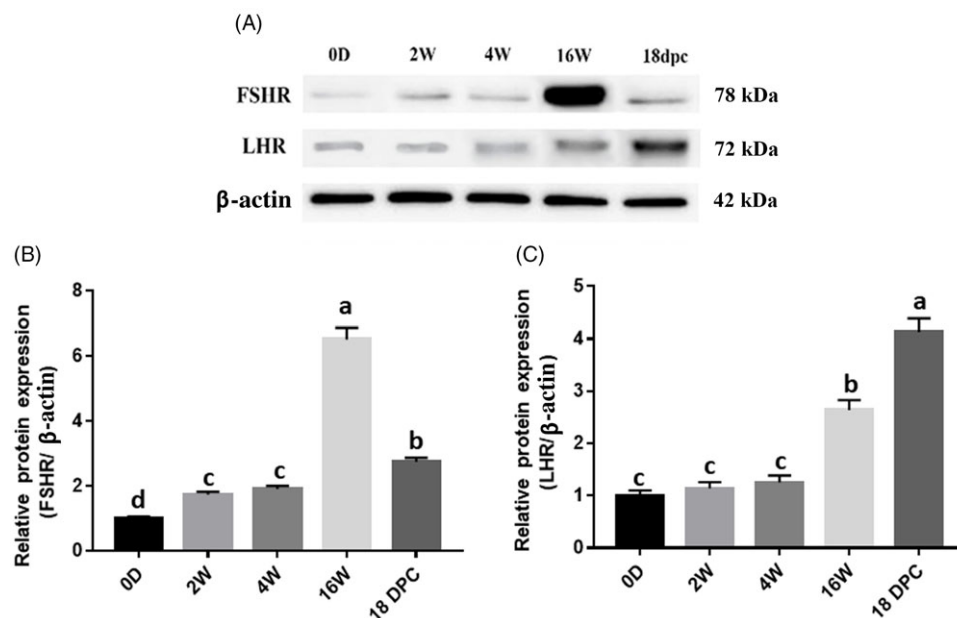
Figure 4. Western blot analysis of FSHR and LHR protein in 15 rabbit ovarian samples ($n = 3$ per each group). Three replicates were conducted for each protein sample. (A) Western blots of FSHR, LHR, and β-actin (internal control) at different ages. Densitometric analysis of (B) FSHR and (C) LHR. Different letters above bars indicate significant differences between the different ovarian developmental stages ($P < 0.05$). Data presented as mean ± SEM. Abbreviations: FSHR, follicular stimulating hormone receptor; LHR, luteinizing hormone receptor; 0D, 0 day; 2W, 2 weeks; 4W, 4 weeks; 16W, 16 weeks; 18 DPC, 18 days post-coitum.

granulosa and theca layer thickness. The obtained results showed that FSHR gene and protein expression had a significant positive correlation with increases in granulosa layer thickness (Table 2). LHR gene and protein expression revealed a significant positive correlation with increases in theca layer thickness (Table 3).

Discussion

Based on the role of FSHR and LHR in reproduction and the limited knowledge of transcriptional and translational expression of rabbit FSHR and LHR throughout folliculogenesis, we described the expression pattern of FSHR and LHR genes and proteins during the development of rabbit ovary, using folliculogenesis as a model system, and correlated their expression changes with the ovarian histomorphological changes, which would contribute to the creation of a theoretical basis for understanding the potential biological mechanisms of hormone receptors in ovarian development. The present study found that there was a correlation between linear morphological development of the ovarian follicles and the expression profiles of FSHR and LHR.

At birth, the ovarian cortex primarily consisted of germ cells arranged in cell nests. A similar result was obtained by Ricken and Viebahn (2002) and Picut *et al.* (2015). The data from RT-qPCR at this age exhibited low FSHR expression, indicating an earlier expression before the start of folliculogenesis. This result is similar to the data obtained by Chakraborty and Roy (2015) on hamster, but differs from that reported by Lei *et al.* (2010) and Hu *et al.* (2021) who found subsequent FSHR expression coinciding with the start of folliculogenesis.

By week 2 of age, the *FSHR* gene significantly increased compared with its level at birth. This is the period during which the newly formed primordial follicles first appeared in the inner part of the ovarian cortex that was a hallmark of initiating folliculogenesis. In line with these findings, Durlej *et al.* (2011) also reported a negligible expression of FSHR in porcine ovary at birth, with only an appreciable expression observed when primordial follicles appeared. The increase in FSHR expression at this age could be due to the appearance of follicular cells of the primordial follicles, as these cells showed higher FSHR expression (Rannikki *et al.*, 1995; Hillier, 2001; Durlej *et al.*, 2011).

At week 4, FSHR was significantly upregulated and this elevated expression coincided with the development of primary and

Table 2. Correlation between the thickness of the granulosa layer of different types of follicles and the expression of the FSHR gene and protein

Follicle classes	FSHR gene *r*	FSHR protein *r*
Primordial follicle	0.605*	0.565*
Primary follicle	0.695**	0.640*
Secondary follicle	0.756**	0.614*
Growing follicle	0.799**	0.759**
Antral follicle	0.820**	0.772**

r: Pearson's correlation coefficient.
Statistical significances were set at *$P < 0.05$ or **$P < 0.01$.

Table 3. Correlation between the thickness of the theca layer of different types of ovarian follicles and the expression of the LHR gene and protein

Follicle classes	LHR gene *r*	LHR protein *r*
Primordial follicle	–	–
Primary follicle	–	–
Secondary follicle	0.729**	0.607*
Growing follicle	0.965**	0.824**
Antral follicle	0.964**	0.876**

r: Pearson's correlation coefficient.
Statistical significances were set at *$P < 0.05$ or **$P < 0.01$.

secondary follicles (O'Shaughnessy *et al.*, 1997). This upregulation could be due to the transformation and proliferation of granulosa cells during the growth phase (Wu *et al.*, 2015). Similarly, one previous study showed that FSHR was higher in primary follicles than in primordial follicles (Zheng *et al.*, 1996).

By week 16 (age of maturity), a marked morphological growth of the rabbit ovary has occurred and has become fully developed containing ovarian follicles in all developmental stages including primordial, primary, growing and antral follicles (Al-Saffar and Almayahi, 2018). During this period, the level of the *FSHR* gene dramatically increased. Notably, this increase coincided with the appearance of growing follicles, and small and large antral follicles that contained numerous layers of granulosa cells (Kishi *et al.*, 2018).

In general, this study found a positive correlation between FSHR mRNA and protein expression and thickness of the granulosa layer, as the ovary grows during postnatal life. Therefore, the results of this study supported the hypothesis that FSHR is essential for the proliferation of granulosa cells (Johnson and Woods, 2009; Donaubauer and Hunzicker-Dunn, 2016).

Follicular development and steroidogenic activity are necessary to maintain pregnancy in the rabbit, as estradiol is the luteotropin principle in rabbits (Goodman *et al.*, 1998), and only follicles are the major source of oestrogen (Niswender *et al.*, 2000). The number and size of large follicles in pregnant rabbit ovaries decreased compared with the number and size of follicles in non-pregnant rabbit ovaries, as high progesterone during pregnancy appears to suppress follicular development (Saleh, 2013; Fawzy *et al.*, 2021). In agreement with previous studies, our results indeed found that the ovarian cortex of the pregnant rabbit ovary was mostly occupied by corpora lutea, with few different types of ovarian follicles. By analyzing the expression of the *FSHR* gene during this period, we found an expected decrease in the expression of this gene. Therefore, it was speculated that the decrease in the expression of the *FSHR* gene might be due to the decrease in the number of developing follicles in the pregnant ovary. This reflects that the number of follicles may affect the total level of *FSHR* expression (Quan *et al.*, 2019). Furthermore, this result demonstrated that *FSHR* expression is affected by reproductive status (pregnancy).

Data from RT-PCR showed that *LHR* transcripts remained low from the day of birth (0D) to week 4. This is the period in which no theca cells are morphologically distinguished, although at week 4 the theca cells surrounding the secondary follicles can be identified. Nonetheless, they were still not well differentiated, which was consistent with previous *in situ* studies showing that LHR is expressed by differentiating theca cells (Kishi *et al.*, 2018). Thereafter, at week 16, we found that LHR expression became significantly higher and coincided with the appearance of growing and antral follicles, which have a defined thecal layer. These results are compatible with *in situ* hybridization studies in which *LHR* mRNA was expressed exclusively in ovarian theca cells (Zhang, *et al.*, 2001). Also, this study found a significant positive correlation between *LHR* mRNA, protein expression and the thickness of theca cell layer, suggesting that LHR is essential for the development of the thecal compartment (Kishi *et al.*, 2018).

The histological sections of the pregnant rabbit ovary revealed that the rabbit ovary contained several well developed corpora lutea, occupying most of the ovarian cortex in addition to some different ovarian follicles as reported by Saleh (2013). By following the expression of LHR at this time, we found that the expression of *LHR* mRNA increased and reached the maximum level. It is expected that the appearance of several corpus lutea in addition to the presence of growing and antral follicles, which have a defined thecal layer leading to an increase in the total level of LHR expression as the earlier studies, showed that LHR was demonstrated to be an integral component of the luteal cells of corpus lutea, theca cells and large follicles in the ovary (Zhang *et al.*, 2001; Ziecik *et al.*, 2007).

Post-transcriptional, translational, and degradation regulation of proteins have recently been shown to be as significant as transcription itself (Vogel and Marcotte, 2012). Therefore, FSHR and LHR expression was analyzed at the protein level in ovarian tissue using western blot. Notably, the protein expression of FSHR and LHR had similar mRNA pattern changes. This compatibility between mRNA and protein profiles supports the hypothesis that both gonadotropin receptors are involved in the regulation of folliculogenesis in rabbits

In conclusion, this is the first study to reveal mRNA and protein expression of FSHR and LHR throughout rabbit folliculogenesis from birth to maturity, as well as in pregnant rabbit, and to correlate their expression changes with the thickness of granulosa and theca layers. These findings indicated that variations in the expression profiles of the FSHR gene and protein are associated with granulosa layer thickness, while LHR expression profiles are associated with theca layer thickness, suggesting that each gene/protein may have a different way to regulate ovary development in rabbits.

References

Abd-Allah SH, Shalaby SM, Abd-Elbary E, Saleh AA and El-Magd MA (2015). Human peripheral blood CD34^{+} cells attenuate oleic acid–induced acute lung injury in rats. *Cytotherapy* **17**, 443–53.

Abd-Elkareem M (2010). Histomorphological studies on the development of the ovary of the rabbits during the postnatal period. MSc thesis. Assiut Univ. Assiut.

Al-Saffar FJ and Almayahi MS (2018). Histomorphological postnatal developmental study of the ovaries of the local rabbits (*Oryctolagus cuniculus*). *Bas J Vet Res* **17**, 124–46.

Chakraborty P and Roy SK (2015). Expression of FSH receptor in the hamster ovary during perinatal development. *Mol Cell Endocrinol* **400**, 41–7.

Daniel-Carlier N, Harscoët E, Thépot D, Auguste A, Pailhoux E and Jolivet G (2013). Gonad differentiation in the rabbit: evidence of species-specific features. *PLoS ONE* **8**, e60451.

Dias JA, Cohen BD, Lindau-Shepard B, Nechamen CA, Peterson AJ and Schmidt A (2002). Molecular, structural, and cellular biology of follitropin and follitropin receptor. *Vitam Horm* **64**, 249–322.

Donaubauer EM and Hunzicker-Dunn ME (2016). Extracellular signal-regulated kinase (ERK)-dependent phosphorylation of Y-box-binding protein 1 (YB-1) enhances gene expression in granulosa cells in response to follicle-stimulating hormone (FSH). *J Biol Chem* **291**, 12145–60.

Durlej M, Knapczyk-Stwora K, Duda M, Galas J and Slomczynska M (2011). The expression of FSH receptor (FSHR) in the neonatal porcine ovary and its regulation by flutamide. *Reprod Domest Anim* **46**, 377–84.

Fawzy AM, Ibrahim S, Mahmoud K, Heleil BA, Ismail IE, Almadaly EA, El-Magd MA and Ramoun AA (2021). Differential molecular and hormonal changes in oocytes, granulosa cells and follicular fluid of pregnant and non-pregnant camels. *Zygote*, 1–8. doi: 10.1017/S096719942000091X.

Feldman AT and Wolfe D (2014). Tissue processing and hematoxylin and eosin staining. *Methods Mol Biol* **1180**, 31–43.

Goodman SB, Kugu K, Chen SH, Preutthipan S, Tilly KI, Tilly JL and Dharmarajan AM (1998). Estradiol-mediated suppression of apoptosis in the rabbit corpus luteum is associated with a shift in expression of bcl-2 family members favoring cellular survival. *Biol Reprod* **59**, 820–7.

Hillier SG (2001). Gonadotropic control of ovarian follicular growth and development. *Mol Cell Endocrinol* **179**, 39–46.

Hu S, Zhu M, Wang J, Li L, He H, Hu B, Hu J and Xia L (2021). Histomorphology and gene expression profiles during early ovarian folliculogenesis in duck and goose. *Poult Sci* **100**, 1098–108.

Hunter MG, Robinson RS, Mann GE and Webb R (2004). Endocrine and paracrine control of follicular development and ovulation rate in farm species. *Anim Reprod Sci* **82–83**, 461–77.

Hutt KJ, McLaughlin EA and Holland MK (2006). Primordial follicle activation and follicular development in the juvenile rabbit ovary. *Cell Tissue Res* **326**, 809–22.

Jin H, Won M, Shin E, Kim HM, Lee K and Bae J (2017). EGR2 is a gonadotropin-induced survival factor that controls the expression of IER3 in ovarian granulosa cells. *Biochem Biophys Res Commun* **482**, 877–82.

Johnson AL and Woods DC (2009). Dynamics of avian ovarian follicle development: cellular mechanisms of granulosa cell differentiation. *Gen Comp Endocrinol* **163**, 12–7.

Kishi H, Kitahara Y, Imai F, Nakao K and Suwa H (2018). Expression of the gonadotropin receptors during follicular development. *Reprod Med Biol* **17**, 11–9.

Lan RX, Liu F, He ZB, Chen C, Liu SJ, Shi Y, Liu YL, Yoshimura Y and Zhang M (2014). Immunolocalization of GnRHRI, gonadotropin receptors, PGR, and PGRMCI during follicular development in the rabbit ovary. *Theriogenology* **81**, 1139–47.

Lei L, Jin S, Mayo KE and Woodruff TK (2010). The interactions between the stimulatory effect of follicle-stimulating hormone and the inhibitory effect of estrogen on mouse primordial folliculogenesis. *Biol Reprod* **82**, 13–22.

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402–8.

Mehl NS, Khalid M, Srisuwatanasagul S, Swangchan-uthai T and Sirivaidyapong S (2017). Comparison of the ovarian and uterine reproductive parameters, and the ovarian mRNA and protein expression of LHR and FSHR between the prepubertal and adult female cats. *Reprod Domest Anim* **52** (Supplement 2), 41–4.

Narayan P, Ulloa-Aguirre A and Dias JA (2018). Gonadotropin hormones and their receptors. In: *Yen and Jaffe's Reproductive Endocrinology* (eds Strauss J, Barbieri A and Gargiulo A), pp. 25–57. Philadelphia: Elsevier.

Ni Y, Zhou Y, Lu L, Grossmann R and Zhao R (2007). Developmental changes of FSH-R, LH-R, ER-β and GnRH-I expression in the ovary of prepubertal ducks (*Anas platyrhynchos*). *Anim Reprod Sci* **100**, 318–28.

Niswender GD, Juengel JL, Silva PJ, Rollyson MK and McIntush EW (2000). Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev* **80**, 1–29.

O'Shaughnessy PJ, Dudley K and Rajapaksha WR (1996). Expression of follicle stimulating hormone-receptor mRNA during gonadal development. *Mol Cell Endocrinol* **125**, 169–75.

O'Shaughnessy PJ, McLelland D and McBride MW (1997). Regulation of luteinizing hormone-receptor and follicle-stimulating hormone-receptor messenger ribonucleic acid levels during development in the neonatal mouse ovary. *Biol Reprod* **57**, 602–8.

Picut CA, Dixon D, Simons ML, Stump DG, Parker GA and Remick AK (2015). Postnatal ovary development in the rat: morphologic study and correlation of morphology to neuroendocrine parameters. *Toxicol Pathol* **43**, 343–53.

Quan Q, Zheng Q, Ling Y, Fang F, Chu M, Zhang X, Liu Y and Li W (2019). Comparative analysis of differentially expressed genes between the ovaries from pregnant and nonpregnant goats using RNA-seq. *J Biol Res (Thessalonike, Greece)* **26**, 3.

Rannikki AS, Zhang FP and Huhtaniemi IT (1995). Ontogeny of follicle-stimulating hormone receptor gene expression in the rat testis and ovary. *Mol Cell Endocrinol* **107**, 199–208.

Ricken AM and Viebahn C (2002). Stage-specific expression of the mitochondrial germ cell epitope PG2 during postnatal differentiation of rabbit germ cells. *Biol Reprod* **67**, 196–203.

Saleh AM (2013). Histological study of ovary through last periods (*Oryctolagus cuniculus*) of pregnancy in domestic rabbit. *Kufa J Vetmed Sci* **4**, 11–9.

Saraiva MVA, Celestino JJH, Araújo VR, Chaves RN, Almeida AP, Lima-Verde IB, Duarte ABG, Silva GM, Martins FS, Bruno JB, Matos MH, Campello CC, Silva JR and Figueiredo JR (2011). Expression of follicle-stimulating hormone receptor (FSHR) in goat ovarian follicles and the impact of sequential culture medium on *in vitro* development of caprine preantral follicles. *Zygote* **19**, 205–14.

Scarlet D, Walter I, Hlavaty J and Aurich C (2015). Expression and immunolocalisation of follicle-stimulating hormone receptors in gonads of newborn and adult female horses. *Reprod Fertil Dev* **28**, 1340–8.

Takagi K, Yamada T, Miki Y, Umegaki T, Nishimura M and Sasaki J (2007). Histological observation of the development of follicles and follicular atresia in immature rat ovaries. *Acta Med Okayama* **61**, 283–98.

Vogel C and Marcotte EM (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* **13**, 227–32.

Wear HM, McPike MJ and Watanabe KH (2016). From primordial germ cells to primordial follicles: a review and visual representation of early ovarian development in mice. *J Ovarian Res* **9**, 36.

Wu W, Han J, Cao R, Zhang J, Li B, Liu Z, Liu K, Li Q, Pan Z, Chen J and Liu H (2015). Sequence and regulation of the porcine FSHR gene promoter. *Anim Reprod Sci* **154**, 95–104.

Yang P, Wang J, Shen Y and Roy SK (2004). Developmental expression of estrogen receptor (ER) α and ERβ in the hamster ovary: regulation by follicle-stimulating hormone. *Endocrinology* **145**, 5757–66.

Zachos NC, Billiar RB, Albrecht ED and Pepe GJ (2003). Developmental regulation of follicle-stimulating hormone receptor messenger RNA expression in the baboon fetal ovary. *Biol Reprod* **68**, 1911–7.

Zhang M, Shi H, Segaloff DL and Van Voorhis BJ (2001). Expression and localization of luteinizing hormone receptor in the female mouse reproductive tract. *Biol Reprod* **64**, 179–87.

Zheng W, Magid MS, Kramer EE and Chen YT (1996). Follicle-stimulating hormone receptor is expressed in human ovarian surface epithelium and fallopian tube. *Am J Pathol* **148**, 47–53.

Ziecik AJ, Kaczmarek MM, Blitek A, Kowalczyk AE, Li X and Rahman NA (2007). Novel biological and possible applicable roles of LH/hCG receptor. *Mol Cell Endocrinol* **269**, 51–60.