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# Regulation by FSH of the dynamic expression of *retinol-binding protein 4* in the mouse ovary

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## Abstract

**Background:** Ovarian retinoid homeostasis plays an important role in the physiological function of the ovary. Retinol-binding protein 4 (RBP4) acts as the mediator for the systemic and intercellular transport of retinol and is heavily involved in cellular retinol influx, efflux, and exchange. However, the expression patterns and regulatory mechanisms of *Rbp4* in the ovary remain unclear.

**Methods:** The expression pattern of ovarian *Rbp4* was examined in immature mice during different developmental stages and in adult mice during different stages of the estrous cycle. The potential regulation and mechanisms of ovarian *Rbp4* expression by estrogen and related gonadotropins in mouse ovaries were also investigated.

**Results:** The present study demonstrated that the ovarian expression of *Rbp4* remained constant before puberty and increased significantly in the peripubertal period. In adult female mice, the expression of *Rbp4* increased at proestrus and peaked at estrus at both the mRNA and protein levels. The protein distribution of RBP4 was mainly localized in the granulosa cell and theca cell layer in follicles. In addition, the expression of *Rbp4* was significantly induced by follicle-stimulating hormone (FSH) or FSH + luteinizing hormone (LH) in combination in immature mouse (3 weeks old) ovaries *in vivo* and in granulosa cells cultured *in vitro*, both at the mRNA and protein levels. In contrast, treatment with LH or 17 $\beta$ -estradiol did not exhibit any observable effects on ovarian *Rbp4* expression. Transcription factors high-mobility group AT-hook 1 (HMGA1), steroidogenic factor 1 (SF-1), and liver receptor homolog 1 (LRH-1) (which have been previously shown to be involved in activation of *Rbp4* transcription), also responded to FSH stimulation. In addition, H-89, an inhibitor of protein kinase A (PKA), and the depletion of HMGA1, SF-1, and LRH-1 by small interfering RNAs (siRNAs), resulted in a dramatic loss of the induction of *Rbp4* expression by FSH at both the mRNA and protein levels.

**Conclusions:** These data indicate that the dynamic expression of *Rbp4* is mainly regulated by FSH through the cAMP-PKA pathway, involving transcriptional factors HMGA1, SF-1, and LRH-1, in the mouse ovary during different stages of development and the estrous cycle.

**Keywords:** Follicle-stimulating hormone, Retinol, Ovary, Granulosa cells

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### Summary sentence

Ovarian *Rbp4* expression remains constant before puberty, increases significantly around puberty in immature mice, and peaks at estrus in adult mice, which is mainly regulated by FSH through the cAMP-PKA pathway partly and involves transcriptional factors HMGA1, SF-1, and LRH-1.

### Background

Retinol (vitamin A) and its derivatives, collectively known as retinoids, play crucial roles in ovarian development and normal physiological function [1]. Retinol is not biologically active per se, and within cells can be oxidized to retinal and retinoic acid (RA) by dehydrogenases. Most of the cellular actions of retinoids can be accounted for by the transcriptional regulatory activity of RA through their nuclear receptors, known as RA receptors (RARs) and retinoid X receptors (RXRs), which associate with RA response elements (RAREs) within the promoters of retinoid-responsive genes [1]. RA in ovarian antral follicles enhanced FSH-mediated ovarian follicular cell differentiation and female fertility, and vitamin A deficiency inhibited oocyte development and decreased ovulated oocytes in mice [2, 3]. RA also plays a crucial role in both nuclear and cytoplasmic maturation of mouse and bovine oocytes [4, 5] and can also stimulate steroidogenesis, such as testosterone production in human theca cells and estradiol production in mouse granulosa cells [1, 6]. In addition, ovarian retinoid levels vary with the estrous cycle [7], and the concentration of retinol is greater in the follicular fluids of the dominant follicles than that of small follicles [8, 9]. However, the regulatory mechanisms of ovarian retinoid homeostasis have not yet been fully understood.

The data from our laboratory suggest that FSH enhances retinol uptake, accumulation, and metabolism in the mouse ovary (unpublished data), but the mechanisms remain unclear. Retinol-binding protein 4 (RBP4), which acts as the mediator for the systemic and intercellular transport of retinol, plays an important role in cellular retinol influx, efflux, and exchange [10]; and seems to play an important role in retinol intercellular transport and accumulation in follicular fluids of the dominant follicles. Evidence shows that the RBP4 immunostaining was observed in the layers of theca and granulosa cells of antral follicles with the most intense staining noted in the cells of large and healthy follicles. Furthermore, the levels of RBP4 and retinol in the fluids of large follicles were higher than those in the fluids of medium or small follicles [8]. High RBP4 levels are also observed in the serum of women with polycystic ovary syndrome (PCOS) and in the fluids from swine follicular cysts [11, 12]. Based on these data [8–12], the regulation of *Rbp4* expression during follicular development

remains an interesting and important point of study and would provide an explanation for the possible mechanisms involved in changing ovarian retinoid levels during follicular development.

The regulatory mechanisms of follicular development and ovarian function are primarily realized through neuroendocrine activities in the hypothalamus–pituitary–ovary (HPO) axial, although early stage occurs independently of the HPO axis. Follicle-stimulating hormone (FSH) or FSH+ luteinizing hormone (LH), which are released by the pituitary gland, principally control follicular development and ovulation by regulating estradiol ( $E_2$ ) production and the functions of granulosa and theca cells. FSH and LH exert their actions by activating their membrane receptors (namely FSHR and LHR, G-protein coupled receptors) thereby resulting in an increase in intracellular cyclic AMP (cAMP), a second messenger involved in the transduction of hormonal or growth signals that regulate cell functions such as proliferation, differentiation, and metabolism [13, 14]. Elevated cAMP activates protein kinase A (PKA), which phosphorylates certain transcription factors and regulates downstream gene transcription. However, it is not until puberty (at approximately 4 weeks of age in mouse) that gonadotropin pulsatility is sufficient to stimulate fully-grown mature follicles and induce ovulation.

In the adult female mouse (after 6–8 weeks old), under the influence of reproductive hormones (FSH, LH and  $E_2$ ), ovarian activities (follicular growth, ovulation, and corpus luteum formation and regression) alternate periodically during the estrous cycle (averages 4–5 days: proestrus for 9–18 h, estrus for about 12 h, metestrus for 24–48 h, and diestrus for 48–72 h) [15, 16]. During proestrus, some recruited follicles in the ovary start to grow rapidly under the stimulation of FSH [17]. During the estrus, the dominant follicles mature and ovulate under the influence of FSH, LH, and estradiol [17–19]. After ovulation, the corpus luteum starts to form under the influence of LH and mice enter metestrus [20]; and during diestrus, the mature corpus luteum releases progesterone which inhibits LH and FSH release by the anterior pituitary and inhibits follicular development [17]. Metestrus terminates with the regression of the corpus luteum under the influence of the pulses of prostaglandin ( $PG_{F2\alpha}$ ) released from the uterus [20, 21].

The structural protein high mobility group AT-hook 1 (HMGA1) is important in promoter regulation by uncovering chromatin and facilitating the recruitment of multiple transcription factors [22]. The *cis*-regulatory sequences of the mouse *Rbp4* gene contain several copies of an AT-rich motif, known to be the binding site for HMGA1 proteins. It has also been reported that HMGA1 can bind the *Rbp4* promoter and recruit the transcription factors steroidogenic factor 1 (SF-1) and

liver receptor homolog 1 (LRH-1) to form an activation complex and induce *Rbp4* transcription in Hepa1 mouse hepatoma cells [22].

The present study investigated the expression patterns of *Rbp4* during different stages of development and the estrous cycle. We also explored the potential regulatory mechanisms governing ovarian *Rbp4* expression by estrogen and related gonadotropins in mouse ovaries and examined the involvement of HMGA1, LRH-1, and SF-1. The results of this study are expected to provide mechanistic evidence for the fluctuations in ovarian retinoid levels during follicular development and estrous cycle.

## Methods

### Animals

Immature (3 weeks old, the same below) and adult (8 weeks old, the same below) BALB/c mice were obtained from the Medical Department of Jilin University (Changchun, China). The mice were raised in an environment with controlled temperature (22–24 °C) and humidity (60–70%) in a 12 h light/dark cycle and provided with food and water ad libitum. Vaginal smear tests were performed on adult female mice (8 weeks old), and ovaries were collected from the mice at various stages of the estrous cycle. Some of the adult female mice (8 weeks old) were mated with male mice, and the ovaries of newborn female mice were collected at different developmental stages (postnatal 1, 2, 3, 4 and 5 weeks). To avoid the effect of endogenous gonadotrophins and  $E_2$ , we choose immature female mice (3 weeks old) to investigate hormonal effects on ovarian *Rbp4* expression. They were injected intraperitoneally with a single dose of FSH (10 IU/mouse; Ningbo Second Hormone Factory, Ningbo, China), LH (10 IU/mouse; Ningbo Second Hormone Factory, Ningbo, China), or 17 $\beta$ -estradiol (1  $\mu$ g/mouse/day in corn oil; Sigma, USA) [23]. Mice were anesthetized with isoflurane and sacrificed via cervical dislocation at 24 or 48 h after injection, and ovarian samples used for RNA isolation were rapidly placed in EZNATM RNA safe stabilizer reagent (Omega, USA) and then stored at –80 °C. All animal studies were conducted in accordance with the protocol approved by the Animal Care and Use Committee of Jilin University.

### Vaginal smear tests

To retrieve ovarian samples from adult mice (8 weeks old) during each of the four stages of the estrous cycle, we performed the vaginal smear tests to identify the specific estrous cycle stages. Briefly, we placed the tip of a plastic pipette filled with 40  $\mu$ l of phosphate buffered saline after (PBS; Hyclone, USA) into the mouse vagina, and gently flushed the vagina 3–5 times. Vaginal contents (cells and vaginal fluids) from each mouse were collected into a PCR tube and then smeared onto a glass

slide. The evaluation of the vaginal smear images was performed under a microscope IX71 system (Olympus, Japan). Proestrus was characterized by the mostly nucleated and some cornified epithelial cells in the smear; estrus by cornified squamous epithelial cells without visible nuclei; metestrus by the predominance of leukocytes and a few nucleated epithelial and/or cornified squamous epithelial cells; and diestrus was characterized by a predominance of leukocytes [24, 25]. The reader is directed to further citations for a full description [24, 25]. Vaginal smear tests were performed at 08:00 and 20:00 each day. When the stages were identified, the mice were sacrificed via cervical dislocation and their ovaries were collected.

### Immunohistochemistry

Intact ovaries collected from immature mice (3 weeks old) that were that were untreated controls or treated with FSH for 48 h were fixed in 4% paraformaldehyde (Boster, Wuhan, China) for 12 h at 4 °C. After washed with PBS and dehydrated, these tissues then were embedded in paraffin wax (Thermo, USA); and paraffin-embedded tissue sections (5  $\mu$ m) were deparaffinized. After washing with PBS, the sections were treated with 10 mM citric acid buffer (pH 6.0) (Boster, Wuhan, China) for antigen activation for 15 min in boiling water. The sections were washed with PBS and blocked by 5% (v/v) BSA in PBS (Boster, Wuhan, China). Sections were incubated overnight at 4 °C with mouse monoclonal antibody specific to RBP4 at a 1:200 dilution in PBS (Proteintech, USA). In order to validate the specificity of RBP4 antibody, 5% nonimmune goat serum [26–29] or PBS [30] was used as negative controls instead of RBP4 antibody. After washed by 0.3% (v/v) Triton X-100 in PBS, sections were incubated with goat-anti-mouse second antibody (Boster, Wuhan, China) for 1 h at room temperature, stained using ABC kits, and counterstained with hematoxylin.

### Follicular granulosa cell isolation and culture in vitro

Primary granulosa cells were isolated from immature female mouse (3 weeks old) ovaries, as described previously [31, 32]. The mice were sacrificed via cervical dislocation after being anesthetized, and the follicles were isolated with no. 5 fine needles. Follicles were then treated with trypsin (Hyclone, USA) for 1 h and filtered using a 100- $\mu$ m filter (Life Technologies, USA). The isolated granulosa cells were cultured in Dulbecco's Modified Eagle Medium/F12 1:1 (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA), 1% insulin–transferrin–selenium (Sigma, USA), and 1% antibiotics (100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin; Hyclone, USA) at 37 °C in an atmosphere of 5% CO<sub>2</sub> in air. Twenty-four hours later, non-adherent cells

were removed and adherent cells were treated with FSH (100 IU/L), LH (100 IU/L), 8-Br-cAMP (500 μM; Sigma, USA), or H-89 (10 μM; PKA inhibitor; Beyotime, Beijing, China).

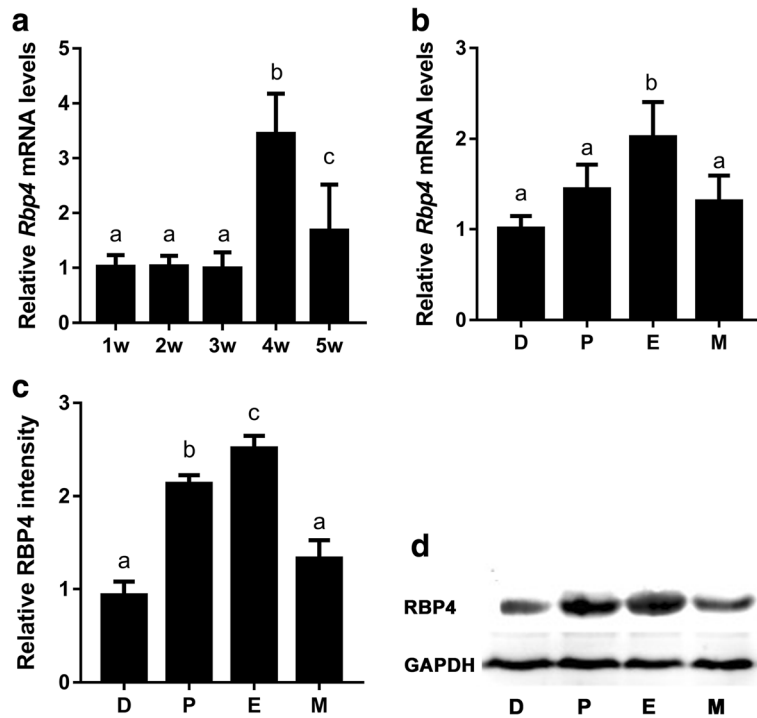
**RNA interference**

siRNA oligonucleotides specific for mouse *Hmga1*, *Sf-1*, and *Lrh-1* were designed and synthesized by GenePharma (Shanghai, China). The sequences that provided successful knockdown were *Hmga1* siRNA, 5'-GAGT CAGAAAGAGCCCAGT-3'; *Sf-1* siRNA, 5'- GCCTCG ATGTGAAATTCCT-3'; and *Lrh-1* siRNA, 5'-GCAG AAGAAAGCCCTCATT-3'. The negative control (NC) siRNA was 5'-TTCTCCGAACGTGTCACGT-3'. Cells were transfected at 50–70% confluency with 50 nM of siRNA duplexes using the FuGENE HD transfection reagent (Roche Applied Science, USA) in accordance with the manufacturer's instructions. FSH was added to the culture medium 24 h after siRNA transfection and cells were treated for an additional 24 h.

**Total RNA extraction and real-time quantitative PCR assay**

Total RNA from ovarian tissue and granulosa cells cultured in vitro were extracted using an RNAprep pure

Micro Kit (Tiangen, Beijing, China) and reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara, Japan) according to the manufacturer's instructions. Real-time PCR was performed on a sequence detection system (Agilent Technologies, USA) using a SYBR Premix Ex TaqII kit (Takara, Japan). The gene-specific primers (forward and reverse, respectively) that we used for real-time quantitative PCR amplification were as follows: *Rbp4* (NM\_011255.3), 5'-AGTCAAG GAGAACTTCGACAAGG-3', 5'-CAGAAAACCTCAGC GATGATGTTG-3'; *Hmga1* (NM\_016660.2), 5'-GCAG GAAAAGGATGGGACTG-3', 5'-AGCAGGGCCTTCCA GTCCAG-3'; *Sf-1* (NM\_139051.3), 5'-CCAGACCTT TATCTCCATTGTCG-3', 5'-AGTGCATCTGGTCAG CCACCT-3'; *Lrh-1* (NM\_030676.3), 5'-TCATGCTG CCCAAAGTGGAGA-3', 5'-TGGTTTTGGACAGTTC GCTT-3'. The expression level of mouse  $\beta$ -actin (NM\_007393.3), 5'-TCTGGCACCACACCTTCTA-3', 5'-AGGCATACAGGGACAGCAC-3', was used as an internal reference. The relative gene expression levels were calculated using the  $2^{-\Delta\Delta C_t}$  method. All primers were obtained from Sangon Biotech (Shanghai, China), all experiments were repeated at least three times.



**Fig. 1** Dynamic expression of *Rbp4* in mouse ovaries during the different stages of development and the estrous cycle. (A) Real-time qPCR analyses of *Rbp4* mRNA levels in mouse ovaries during different developmental stages. Data are presented as means ± SEM, n = 8. (B) Relative *Rbp4* mRNA levels in mouse ovaries during diestrus (D), proestrus (P), estrus (E), and metestrus (M). Data are presented as mean ± SEM, n = 8. RBP4 protein levels in the ovaries of mice during the estrous cycle were determined via Western blot analyses (D), and blots were quantified via densitometry (C). The protein levels were normalized to GAPDH. Data are presented as means ± SEM, n = 3. Means with different letters represent a significant difference at the 0.05 level

**Western blot analyses**

Protein samples were obtained by homogenizing whole ovaries and by lysing granulosa cells in lysis buffer (Beyotime, Beijing, China) with a 10 µg/ml protease and phosphatase inhibitor cocktail (Thermo, USA). Samples were then centrifuged at 13,000 g at 4 °C. Tissue and cell extracts were normalized to the sample protein concentration, as determined by a protein assay kit (Beyotime, Beijing, China). Proteins (40 µg) were separated via sodium dodecyl sulfate polyacrylamide gel (12%) electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were then blocked in Tris-buffered saline with Tween 20 (TBST) containing 5% non-fat instant milk and incubated overnight at 4 °C with mouse monoclonal antibodies specific to RBP4 or GAPDH (Proteintech, USA); and rabbit monoclonal antibodies specific to HMGA1, SF-1, or LRH-1 (Abcam, USA) each at 1:5000 dilution. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Proteintech, USA) for 1 h. Enhanced chemiluminescence (ECL) detection was

performed using an ECL system according to the specifications of the manufacturer (Beyotime, Beijing, China). Protein levels were normalized to GAPDH and quantified via densitometry using a Tanon gel imaging system (Tanon, Shanghai, China).

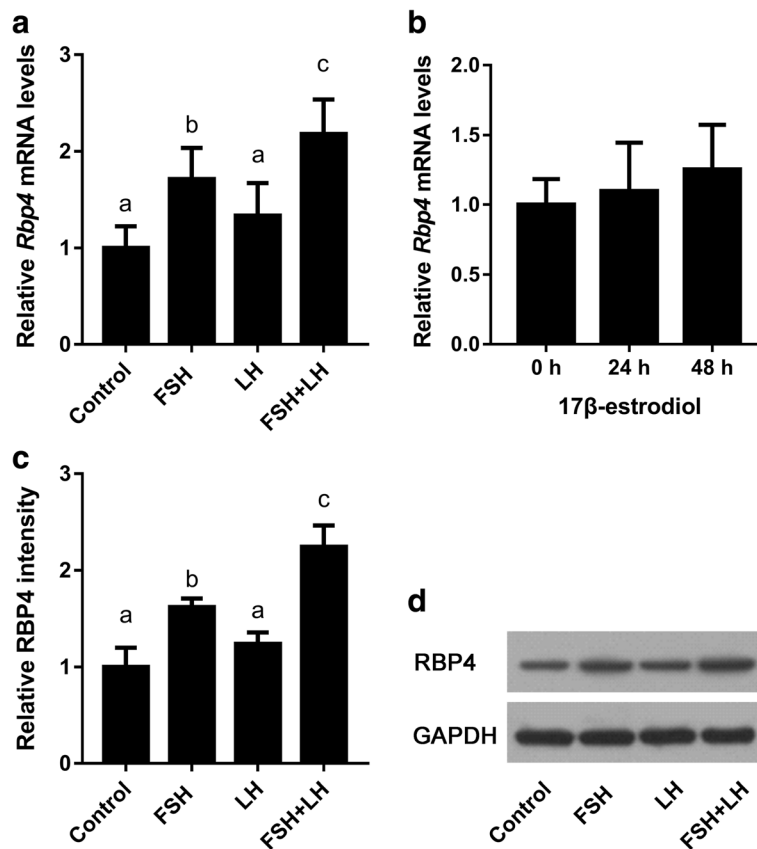
**Statistical analyses**

The statistical analyses of the data were conducted via one-way ANOVA followed by Tukey’s multiple-range test. Differences were considered significant at  $P < 0.05$ . All the statistical analyses were performed using SPSS 22.0 for Windows (StatSoft, USA).

**Results**

**Variable expression of *Rbp4* in mouse ovaries during the different stages of development and the estrous cycle**

We first performed experiments to investigate the expression pattern of *Rbp4* in whole mouse ovaries during different developmental stages. *Rbp4* mRNA expression in mouse ovaries remained constant at 1 to 3 weeks postnatally and notably increased at 4 weeks (i.e., peripubertally),



**Fig. 2** Effects of gonadotropins and ovarian steroids on the expression of *Rbp4* in the 3-week-old mouse ovary. (A) *Rbp4* mRNA expression levels in the ovaries of mice treated with FSH, LH, or FSH + LH for 48 h. Data are presented as means ± SEM, n = 8. (B) *Rbp4* mRNA expression levels in the ovaries of mice treated with 17β-estradiol for 24 or 48 h. Data are presented as mean ± SEM, n = 8. RBP4 protein levels in the ovaries of mice treated with FSH or LH were determined using Western blot analyses (D), and blots were quantified via densitometry (C). The protein levels were normalized to GAPDH. Data are presented as means ± SEM, n = 3. Means with different letters represent a significant difference at the 0.05 level

and dropped at 5 weeks (Fig. 1A). We also investigated the expression patterns of *Rbp4* in whole mouse ovaries during the estrous cycle. The expression of *Rbp4* mRNA in the ovaries of adult mice (8 weeks old) having normal cycles increased at estrus (Fig. 1B), and the levels of RBP4 protein in the ovaries of normally cycling adult mice increased at proestrus and peaked at estrus (Fig. 1C and D). These data imply a potential relationship between *Rbp4* expression and follicular growth [8].

**Effects of gonadotropins and ovarian steroids on *Rbp4* expression in mouse ovaries**

Follicular growth is mainly controlled by gonadotropins and estrogen. Therefore, we determined the effects of these hormones on *Rbp4* expression in mouse ovaries. First, immature mice (3 weeks old) were treated with FSH, LH, or FSH + LH. The results showed that the expression levels of *Rbp4* in whole ovarian samples increased significantly after treatment with FSH (Fig. 2A) or FSH + LH (Fig. 2A), but not with LH alone (Fig. 2A), and the induction with combined FSH + LH was greater than that with FSH alone (Fig. 2A). The levels of RBP4 protein in the ovaries of mice treated with FSH and/or LH in proportion to the mRNA levels (Fig. 2C and D). 17β-Estradiol showed no evident effect on ovarian *Rbp4* expression (Fig. 2B). Thus, *Rbp4* expression in mouse ovaries appeared to be predominantly regulated by FSH.

**Localization of RBP4 protein in mouse ovary**

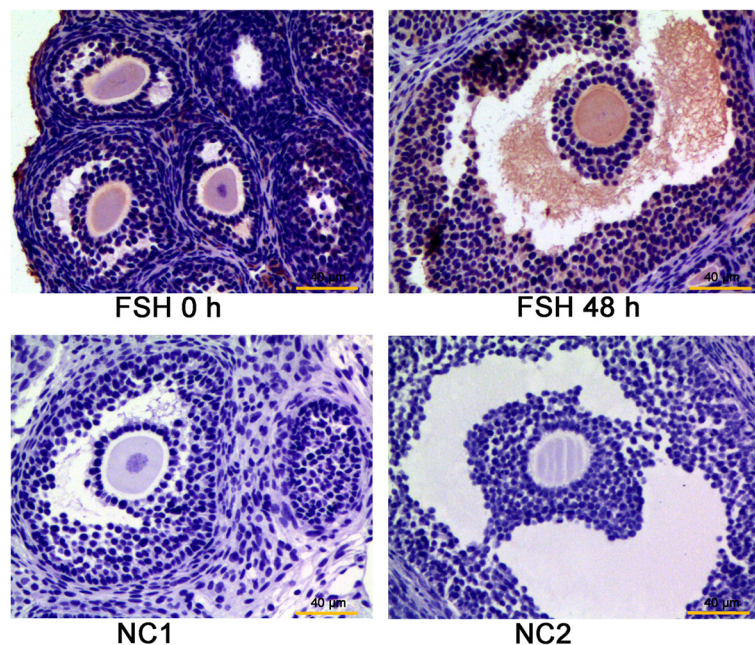
The localization of RBP4 protein in mouse ovaries was analyzed using immunohistochemical staining with specific antibody (Fig. 3). Positive signals for RBP4 were detected in the layers of granulosa and theca cells, and marked staining was also observed in the follicular antrum of large antral follicles in the ovaries of mice treated with FSH for 48 h (Fig. 3).

**FSH stimulates the expression of *Rbp4* in mouse granulosa cells**

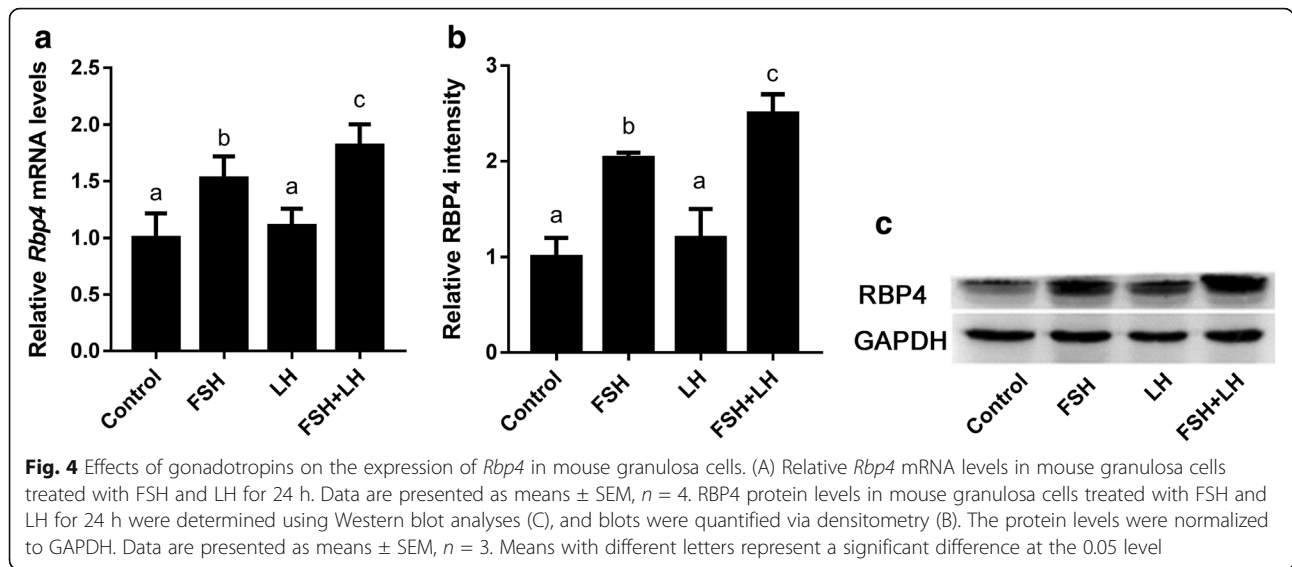
Granulosa cells are one of the two most important types of gonadotropin-sensitive somatic cells in the mammalian ovary. We examined the effect of FSH and FSH + LH on the expression of *Rbp4* using an in vitro culture model of granulosa cells, and our results showed that the expression of *Rbp4* was induced by both FSH and FSH + LH treatments, and that the induction by combined FSH + LH treatment was greater than that by FSH alone (Fig. 4A, B and C).

**cAMP-PKA pathway mediates FSH-induced *Rbp4* expression**

As previously reported, FSH and LH perform their actions by activating their membrane receptors, the FSH receptor (FSHR) and the LH receptor (LHR), both of which belong to the G-protein coupled receptors [13, 14]. Stimulation of these receptors results in an increase in intracellular cAMP [13, 14], which promotes the rapid activation of

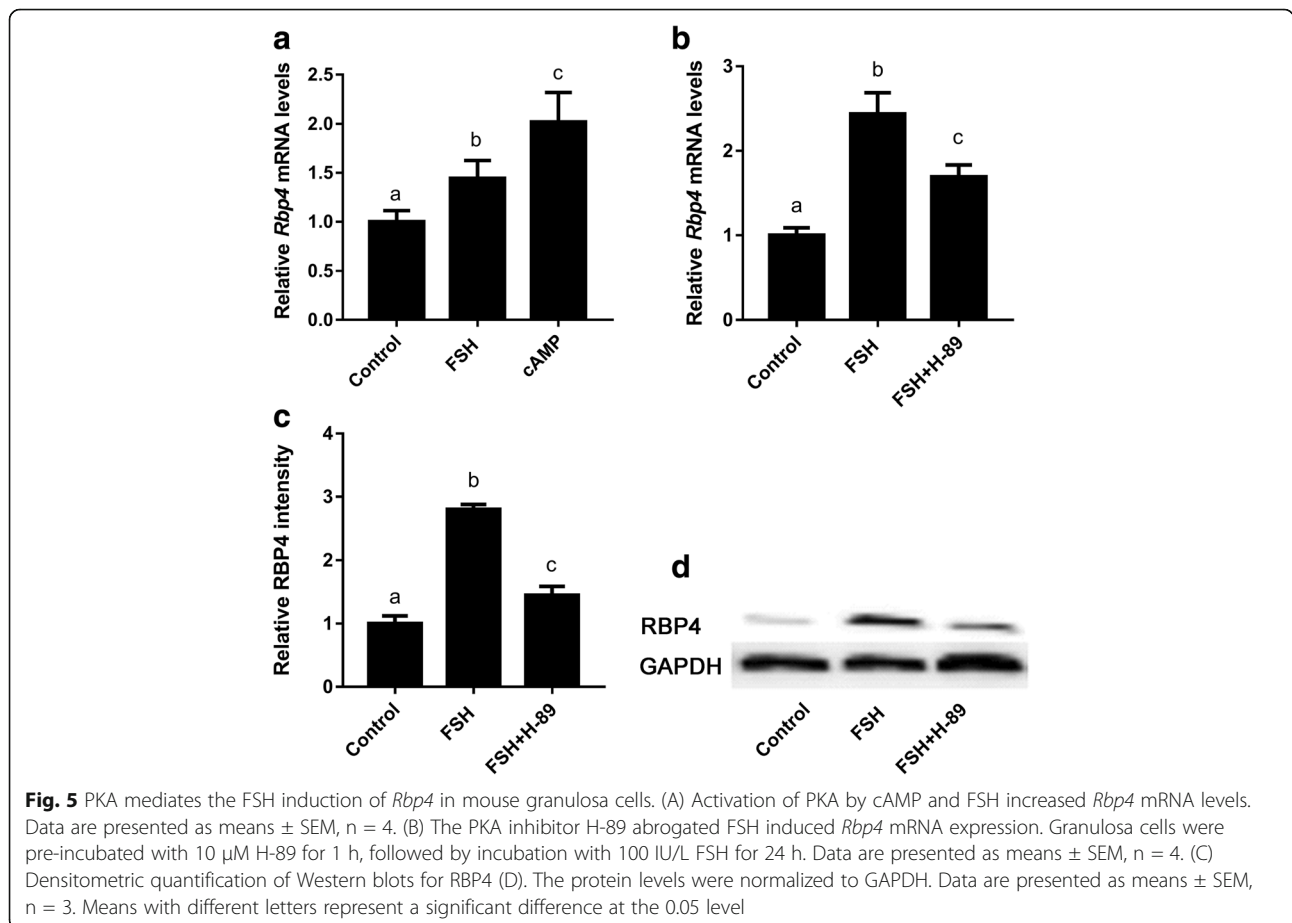


**Fig. 3** Localization of RBP4 protein in the ovaries of the immature mice (3 weeks of age) treated with or without FSH for 48 h. Negative controls (NC), which were incubated with 5% goat serum (NC1) or PBS (NC2) instead of the primary antibody, were used to validate the specificity of RBP4 antibody. Positive signals for RBP4 were detected in granulosa and theca cell layers. Bar = 40 μm



protein kinase A (PKA) [33]. To determine whether the cAMP-PKA pathway was involved in FSH-induced *Rbp4* expression in mouse granulosa cells, we first treated the cells in vitro with 500  $\mu$ M 8-Br-cAMP (a cAMP agonist) or 10  $\mu$ M H-89 (a PKA inhibitor). If the cAMP-PKA

pathway was involved in FSH induction, 8-Br-cAMP would show similar induction effects and H-89 would prevent FSH induction. As expected, the treatment of mouse granulosa cells with 500  $\mu$ M 8-Br-cAMP for 24 h resulted in a dramatic increase in *Rbp4* expression (Fig. 5A), and



the induction of *Rbp4* by FSH was prevented with 10  $\mu$ M H-89 (Fig. 5B, C and D). Thus, the cAMP-PKA pathway likely mediates FSH-induced *Rbp4* expression.

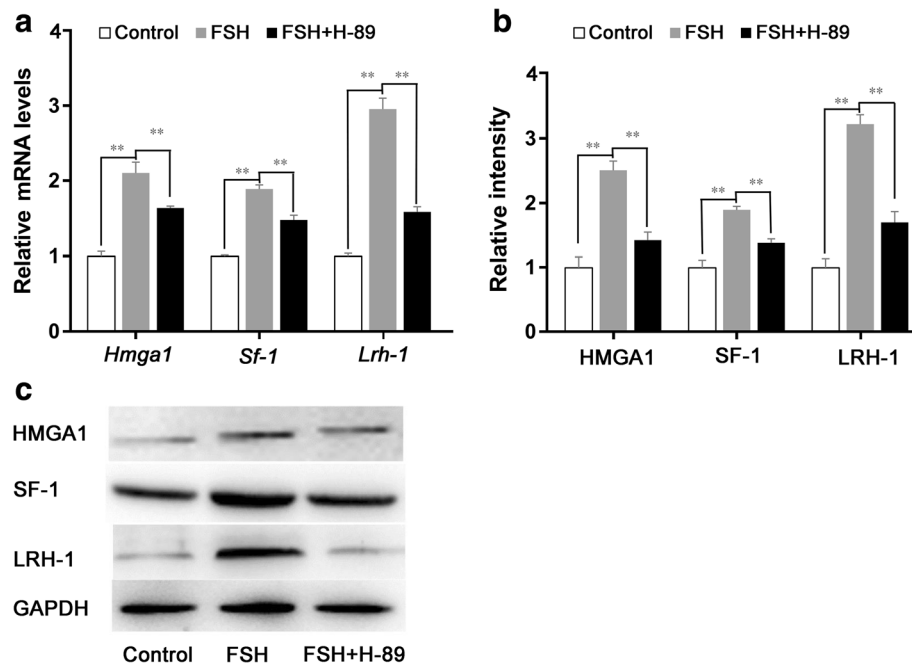
**HMGA1, SF-1, and LRH-1 mediate FSH stimulation of *Rbp4***

HMGA1 can bind to the promoter of *Rbp4* and recruit SF-1 and LRH-1 to regulate the transcriptional activity of *Rbp4* [22]. The present study examined the effects of FSH on the expression of these transcription factors, and the results showed that FSH significantly stimulated the expression of these genes in granulosa cells. Moreover, the PKA inhibitor H-89 significantly inhibited FSH induction of these genes (Fig. 6A, B, and C). These data suggest that FSH stimulated the expressions of HMGA1, SF-1, and LRH-1 via PKA. To determine whether HMGA1, SFI, and LRH-1 mediated the FSH induction of *Rbp4*, we sought to knock down the corresponding genes using specific siRNAs. As expected, the expressions of these genes at the mRNA and protein levels were diminished to approximately 50% in cells transfected with 50 nM siRNA compared with controls (Fig. 7A, B, C, D, E, and F). After 24 h of transfection, the cells were treated with FSH for an additional 24 h. The cells were harvested and *Rbp4* mRNA and protein levels were examined. *Rbp4* expression was significantly reduced in cells transfected with *Hmga1* siRNA (Fig. 7G and H); while, knockdown of *Lrh-1* or *Sf-1* also prevented FSH induction of *Rbp4* (Fig. 7G, I, and J). Thus,

the transcription factors HMGA1, SF-1, and LRH-1 are likely to be involved in the induction of *Rbp4* expression by FSH.

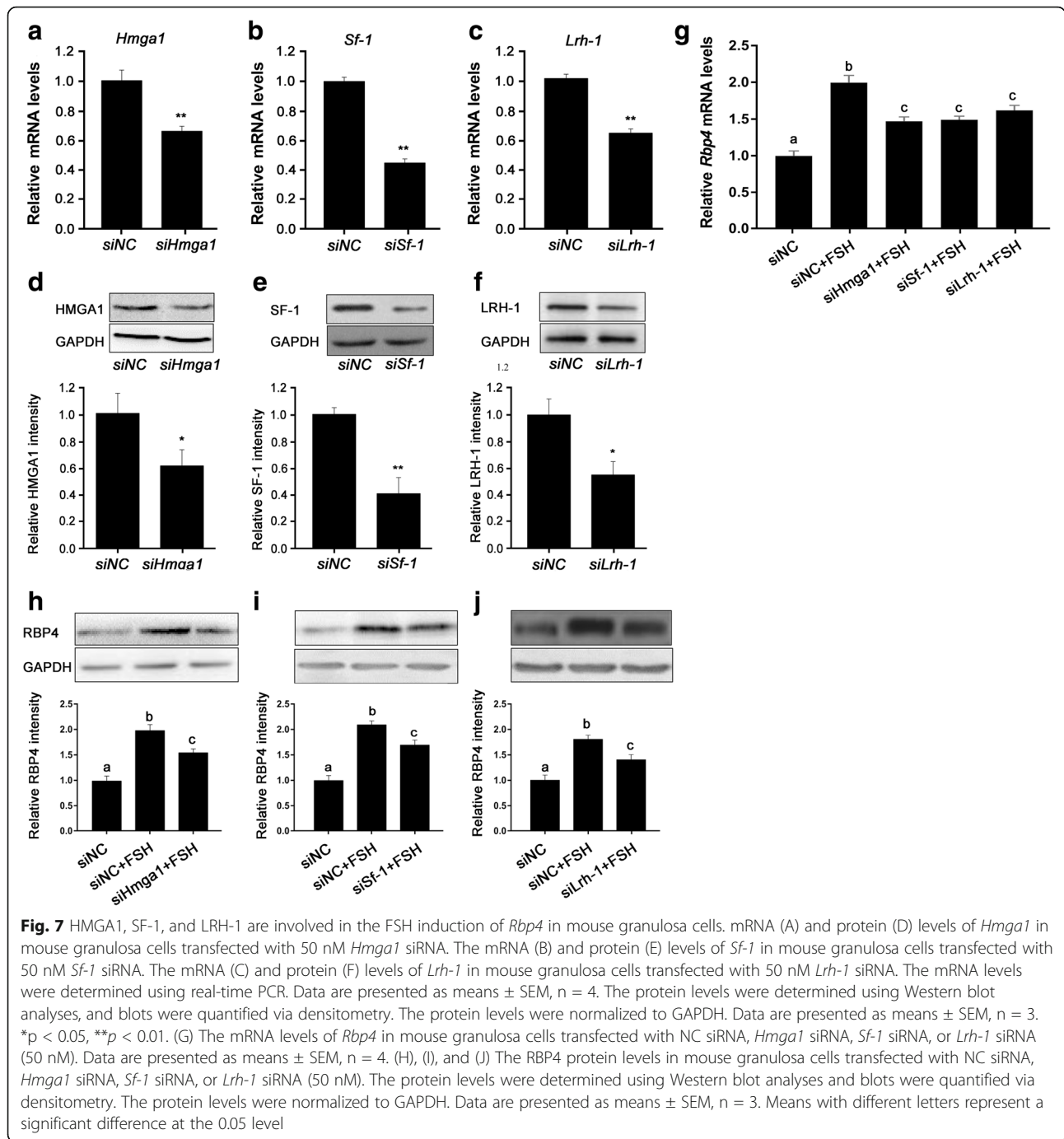
**Discussion**

Firstly, the present study presented the dynamic expression patterns for *Rbp4* in mouse ovaries during different stages of development and the estrous cycle. The expression of ovarian *Rbp4* remained constant at 1 to 3 weeks postnatally, notably increased at 4 weeks (i.e., peripubertally), and dropped at 5 weeks. Numerous studies have supported the concept that the gonadotropin-releasing hormone (GnRH) neuronal network generates pulse and surge modes of gonadotropin secretion that are critical for puberty [34], and stimulate the secretion of a mass of local ovarian factors and follicular growth in females. This modulation in *Rbp4* expression may be related to the pituitary gonadotropin wave at the onset of puberty. After puberty, some follicles in ovary start to grow and ovulate irregularly under the influence of serum gonadotropins, and at 5 weeks of age, some mice may be in the follicular-growth phase and some in the post ovulatory period. The different levels of gonadotrophins in these mice may constitute a possible explanation for the drop in *Rbp4* expression at 5 weeks. Furthermore, the expression of *Rbp4* in the ovaries of adult mice (8 weeks old) having normal cycles was found to increase at proestrus and peaked at estrus, the phase with higher serum levels



**Fig. 6** FSH upregulates HMGA1, SF-1, and LRH-1 via PKA. (A) Relative mRNA levels of *Hmga1*, *Sf-1*, and *Lrh-1* in mouse granulosa cells incubated with FSH and H-89 for 24 h. Data are presented as means  $\pm$  SEM, n = 4. SF-1 and LRH-1 protein levels were determined using Western blot analyses (C), and blots were quantified via densitometry (B). The protein levels were normalized to GAPDH. Data are presented as means  $\pm$  SEM, n = 3. \* $p$  < 0.05, \*\* $p$  < 0.01





of FSH and LH. In a previous study, higher RBP4 protein concentrations were observed in the follicular fluids of dominant follicles relative to small follicles [8, 9]. The dynamic expression patterns of *Rbp4* in mouse ovaries during different stages of development and the estrous cycle motivated us to pose an alternative hypothesis: that the expression of ovarian RBP4 is affected by gonadotropins and gonadal steroids and is related to follicular growth.

We then investigated the potential regulatory effects of gonadotropins and estrogen on the dynamic expression of *Rbp4* in the mouse ovary. The expression of *Rbp4* was significantly stimulated by administration of FSH and combined FSH + LH, but unaffected by administration of LH or 17 $\beta$ -estradiol. In addition, intense immunostaining was observed in granulosa cell layers of large antral follicles in the ovaries of mice treated with FSH. To determine whether the induction of *Rbp4* by FSH

and FSH + LH occurred due to increased expression of *Rbp4* in granulosa cells, we examined primary granulosa cells cultured in vitro. Our results showed that the expression of *Rbp4* was induced by FSH and combined FSH + LH treatments, and that the induction by combined FSH + LH treatment was stronger than that by FSH alone. The administration of LH alone did not exert an effect. Because the primary granulosa cells were isolated from the immature female mouse (3 weeks old) ovaries without FSH or equine chorionic gonadotropin (eCG) pretreatment and with no or low LHR expression, the cells were mainly immature granulosa cells without LHR expression [35]. This may be the reason as to why LH alone manifested no obvious effects on granulosa cells and immature ovaries in the present study. However, LHR in granulosa cells can be induced by FSH [35], and during late maturation of granulosa cells in antral follicles, LH exerts a relatively more notable effect than FSH on cAMP formation [14, 36, 37], suggesting that LHR density was relatively greater than FSHR density or that LHR was more effectively coupled to cAMP generation. These findings may explain the stronger induction of *Rbp4* expression by FSH + LH than by FSH alone, and the higher levels of RBP4 in the follicular fluids of the large antral follicles [8].

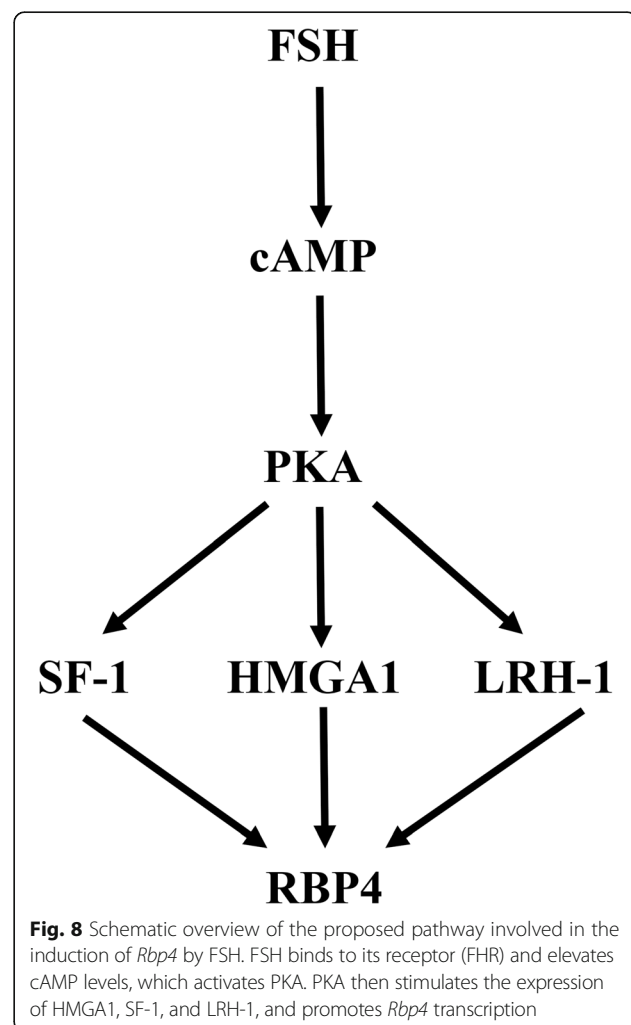
To determine whether the induction of *Rbp4* by FSH was mediated by the cAMP-PKA pathway, we examined the effects of the cAMP analog 8-Br-cAMP and the PKA inhibitor H-89 on the induction of *Rbp4* in granulosa cells. The treatment of cells with 500  $\mu$ M 8-Br-cAMP showed a dramatic increase in *Rbp4* expression, whereas 10  $\mu$ M H-89 significantly prevented the induction of *Rbp4* by FSH. Thus, the cAMP-PKA pathway likely participates in the induction of *Rbp4* by FSH.

The cis-regulatory sequences of the mouse *Rbp4* gene contain a bipartite promoter, i.e., a proximal region necessary for basal expression and a distal segment that contains several binding sites for the structural HMGA1 proteins that are structural components of chromatin [22, 38]. HMGA1 binds to the adenine thymine (AT)-rich regions of DNA, changes the structure of DNA and recruit transcription factors to the promoter, and facilitates gene transcription [39]. There are several AT-rich motifs homologous to the binding sites of HMGA1 in the promoter region of mouse *Rbp4*, and HMGA1 proteins can bind upstream sequences of the *Rbp4* promoter. In the present study, *Hmga1* was sensitive to FSH and this response was prevented by the PKA inhibitor H-89; whereas the knockdown of *Hmga1* with siRNA resulted in a dramatic loss of FSH-induced *Rbp4* expression. In addition, HMGA1 can interact with SF-1 and LRH-1 and recruit them to the complex [22]. In the present study, the depletion of SF-1 and LRH-1 resulted in a dramatic loss of FSH-induced *Rbp4* expression. SF-1

was shown to be expressed at higher levels in theca cells, and also found in granulosa cells; whereas LRH-1 expression was found to be abundant and highly restricted to the granulosa cells of developing follicles [40, 41]. Consistent with our data, the two transcription factors were shown to be induced in granulosa cells by FSH/cAMP through the activation of PKA [40, 41], as cAMP signals are mediated largely via PKA [33] and can be elevated in granulosa cells by FSH [42, 43] or LH [44]. Thus, the expression of ovarian *Rbp4* was likely regulated by FSH via the cAMP-PKA pathway, involving HMGA1, SF-1, and LRH-1.

### Conclusion

In conclusion, the present study showed a dynamic expression pattern of *Rbp4* in the mouse ovary during different stages of development and the estrous cycle, which was predominantly regulated by FSH. Furthermore, the cAMP-PKA pathway and transcription factors HMGA1, SF-1, and LRH-1 were likely involved in the induction of *Rbp4* by FSH (Fig. 8). This work expands



our understanding of the mechanisms underlying the regulation of *Rbp4* expression patterns in the ovary during different stages of development and the estrous cycle. Although further research needs to be conducted in this area, our data are important for the understanding of the molecular regulation of retinol transport and accumulation in the follicular fluids during ovarian follicular growth.

#### Abbreviations

8-Br-cAMP: 8-Bromoadenosine 3':5'-cyclic monophosphate; FSH: Follicle-stimulating hormone; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HMGA1: High-mobility group AT-hook 1; LH: Luteinizing hormone; LRH-1: Liver receptor homolog 1; PKA: Protein kinase A; RBP4: Retinol-binding protein 4; SF-1: Steroidogenic factor 1

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#### Availability of data and materials

Data sharing was not applicable to this article as no datasets were generated or analyzed during the current study.

#### Authors' contributions

YJ, CL, and XZ conceived and designed the study; YJ performed the experiments; YJ and YZ analyzed the data; YJ, SC, and LC wrote the manuscript. All authors read and approved the final manuscript.

#### Ethics approval

The animal protocols used herein were conducted according to the Guide for the Administration of Laboratory Animals (Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes, 1986), and were approved by the Institutional Animal Care and Use Committee (IACUC) of Jilin University, China.

#### Consent for publication

Not applicable

#### Competing interests

The authors declare that they have no competing interests.

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