МОЛЕКУЛЯРНАЯ БИОЛОГИЯ, 2012, том 46, № 6, с. 900–906

МОЛЕКУЛЯРНАЯ БИОЛОГИЯ КЛЕТКИ

UDC 577.21;577.17.171.52

EXPRESSION AND REGULATION OF Ang-2 IN MURINE OVARIES DURING SEXUAL MATURATION AND DEVELOPMENT OF CORPUS LUTEUM

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Received March 14, 2012
Accepted for publication April 03, 2012

The aim of this study was to examine the expression and regulation of angiopoietin-2 (Ang-2) in murine ovaries during sexual maturation, gonadotropin treatment and luteal development by *in situ* hybridization and RT-PCR. By *in situ* hybridization *Ang-2* mRNA was mainly localized in granulosa cells, thecal cells and corpus luteum, otherwise in oocytes. Moreover, *Ang-2* mRNA was highly expressed in corpus luteum and granulosa cells of atretic follicles. According to RT-PCR data, *Ang-2* mRNA was lowly expressed on day 10 after birth, then expression levels gradually increased and reached their highest values on day 25 after birth. In the superovulated model of immature mice, *Ang-2* expression was strongly induced by equine chorionic gonadotropin (eCG) 48 h post the eCG injection, and was high from 0.5 to 13 h after hCG treatment. *In situ* hybridization showed that *Ang-2* mRNA was highly expressed in corpus luteum from day 2 to 9 post the hCG injection, then the expression levels gradually declined on days 11 and 13 after hCG treatment. According to RT-PCR data, the levels of *Ang-2* mRNA expression showed a decline after the hCG injection, with a nadir on day 3, followed by an increase, reaching the highest level on day 9 post-hCG injection. Then again *Ang-2* expression gradually declined from day 11 to 15 after hCG injection. These results suggest that Ang-2 may be involved in follicular development, atresia, ovulation, and corpus luteum formation and regression.

Keywords: **Ang-2, mouse, ovary, follicle, corpus luteum.**

Angiogenesis, the development of new blood vessels from preexistent vasculature, in healthy adult animals is mainly limited to the reproductive system [1]. The development of new blood vessels in the ovaries is essential to guarantee the necessary supply of nutrients and hormones to promote follicular growth and corpus luteum formation. Accumulating data has shown that the vascular endothelial growth factor (VEGF) is present within both ovary follicles and corpora luteum and appears to play an integral role in endothelial cells and blood vessel regulation within the ovary [2, 3]. However, recent molecular analysis of the mechanism of angiogenesis has focused on the role of other growth factors, angiopoietins, which function in concert with VEGF on the formation, stabilization, and regression of blood vessels [4].

Angiopoietin-1 (Ang-1) affects vascular endothelial cells through an endothelial cell-specific tyrosine kinase receptor (Tie-2) and is involved in the maturation and stabilization of new blood vessels. On the other hand, Ang-2, an endogenous antagonist of Ang-1, plays an important role in loosening the supporting cell matrix and destabilization of existing blood vessels together with VEGF. In the presence of VEGF, Ang-2 could stimulate endothelial cell proliferation and migration and promote further angiogenesis, whereas in the absence of VEGF, Ang-2 could induce blood vessel destabilization and regression [4, 5]. At present, a number of studies found that Ang-2 is dynamically expressed in ovarian follicles and corpus luteum of different species, suggesting a regulating role of Ang-2 in ovarian angiogenesis. In rat ovaries, Ang-2 is strongly expressed in theca cells from early antral follicles and preovulatory follicles [6]. In ewe ovaries, Ang-2 was detected in granulosa cells and theca cells of follicles [7]. Intrafollicular injection of Ang-2 can prevent ovulation and development and function of the subsequent corpus luteum [8]. In addition, Ang-2 was also detected in corpus luteum of rats, cows, mares, monkeys and humans [9–13]. During prostaglandin $F_{2\alpha}$-induced luteolysis, Ang-2 expression increases [14–16]. These results indicate that Ang-2 is necessary for follicular growth and atresia, ovulation, and subsequent development and regression of corpus luteum. However, Ang-2 expression and regulation in murine ovaries during sexual maturation and luteal development are still not defined in detail. The aim of this study was to investigate *Ang-2* expression in mouse ovaries dur-

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ing sexual maturation, gonadotropin treatment, and luteal development by *in situ* hybridization and reverse transcription polymerase chain reaction (RT-PCR).

EXPERIMENTAL

Sexual maturation. Immature female mice (Kunming White outbred strain, 21 days old) were caged in a controlled environment with a cycle of 14 L : 10 D. All animal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University. Mouse ovaries were collected on days 10, 15, 20, 25, 30 and 40 after birth. There were at least three mice per group.

Gonadotropin treatment. The mice (21 days old) were superovulated with an i.p. injection of 5 IU equine chorionic gonadotropin (eCG). Ovaries were collected from the treated mice at 0.5, 1, 3, 6, 12, 24, 36 and 48 h after the eCG injection. Ovaries were collected directly from 21-day-old immature mice as controls. In addition, 21-day-old mice were superovulated with an i.p. injection of 5 IU eCG followed by administration of 5 IU of human chorionic gonadotropin (hCG) 48 h later. Ovaries were collected from these treated mice at 0.5, 1, 3, 5, 7, 9, 11 and 13 h post the hCG injection and on days 1, 2, 3, 4, 5, 7, 9, 11, 13 and 15 post the hCG injection respectively. Day 0 was designated as the day when hCG was injected. There were at least three mice per group

***In situ* hybridization.** Total RNA extracted from the mouse uteri was reverse-transcribed and amplified with *Ang-2* primers. *Ang-2* forward primer 5'-GCATCTACACACTGACCTTC and reverse primer 5'-CTGGTTGGCTGATGCTAC were designed according to *Mus musculus Ang-2* gene (Genbank accession number NM_007426). The amplification of *Ang-2* cDNA was performed with 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The amplified fragment (372 bp) of *Ang-2* was recovered from the agarose gel and cloned into a pGEM-T plasmid (pGEM-T Vector System 1, "Promega", Madison, WI). The orientation of the *Ang-2* fragment in the pGEM-T plasmid was determined by PCR using a combination of primers for T7, SP6 and mouse *Ang-2*. The cloned *Ang-2* fragment was further verified by sequencing. An *Ang-2*-containing plasmid was amplified with the primers for T7 and S6 to prepare templates for labeling. Digoxigenin (DIG)-labeled antisense and sense cRNA probes were transcribed *in vitro* using a DIG RNA labeling kit ("Roche Diagnostics GmbH", Mannheim, Germany).

Mouse ovaries were flash frozen in liquid nitrogen. Frozen sections (10 μm) were mounted onto 3-aminopropyltriethoxy-silane ("Sigma")-coated slides and fixed in 4% paraformaldehyde solution in PBS. The sections were washed in PBS twice, treated with 1% Triton-100 for 20 min and washed again in PBS 3 times. After prehybridization in a solution of 50% formamide and 5 × SSC (1 × SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) at room temperature for 15 min, the sections were hybridized in the hybridization buffer (5 × SSC, 50% formamide, 0.02% BSA, 250 mg/mL yeast tRNA, 10% dextran sulfate, 1 mg/mL denatured DIG-labeled antisense or sense RNA probe for mouse Ang-2) at 55°C for 16 h. After hybridization, the sections were washed sequentially in 50% formamide/5 × SSC at 55°C for 15 min, 50% formamide/2 × SSC at 55°C for 30 min, 50% formamide/0.2 × SSC at 55°C twice for 30 min each, and 0.2 × SSC at room temperature for 5 min. After nonspecific binding was blocked in a 1% blocking reagent ("Roche") for 1 h, the sections were incubated with sheep anti-DIG antibodies, conjugated with alkaline phosphatase (1 : 5000, "Roche") in a 1% block reagent overnight at 4°C. The signal was visualized with 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.4 mM nitrobluetetrazolium in a buffer containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$. Endogenous alkaline phosphatase activity was inhibited with 2 mM levamisole ("Sigma"). All of the sections were counterstained with 1% methyl green in 0.12 M glacial acetic acid and 0.08 M sodium acetate for 30 min. The positive signal was visualized by a dark brown color.

RT-PCR. Total RNA was extracted from mouse ovaries with the TRIZOL reagent, digested with RQ1 DNase I and reverse-transcribed into cDNA with M-MLV Reverse Transcriptase ("Promega", Madison, WI). The amplified PCR fragment for *Ang-2* was separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and quantitated by optical density using the UVP laboratory imaging and analysis system ("UVP, Inc.", Upland, CA). The band densities for *Ang-2* were normalized to *GAPDH* expression. To compare the intensities of RT-PCR products in a semi-quantitative way, we determined the exponential phase of amplification by performing 25–28–30–35 cycles for *Ang-2* and *GAPDH*. The final amplification cycles were 25 for *Ang-2* and *GAPDH*. As a negative control, each sample was run through PCR in the absence of reverse transcriptase or cDNA to rule out genomic DNA contamination. All the RT-PCR reactions were repeated at least three times with three batches of ovaries. Quantitative data is expressed as mean ± SEM and was analyzed by one-way ANOVA using the SPSS software program. The differences were considered significant at $P < 0.05$.

RESULTS

Ang-2 mRNA expression in mouse ovary during sexual maturation

By *in situ* hybridization *Ang-2* mRNA was mainly localized in granulosa cells and thecal cells of follicles, but could not be detected in oocytes and stroma of ovaries. In the granulosa cells of atretic follicles, *Ang-2*

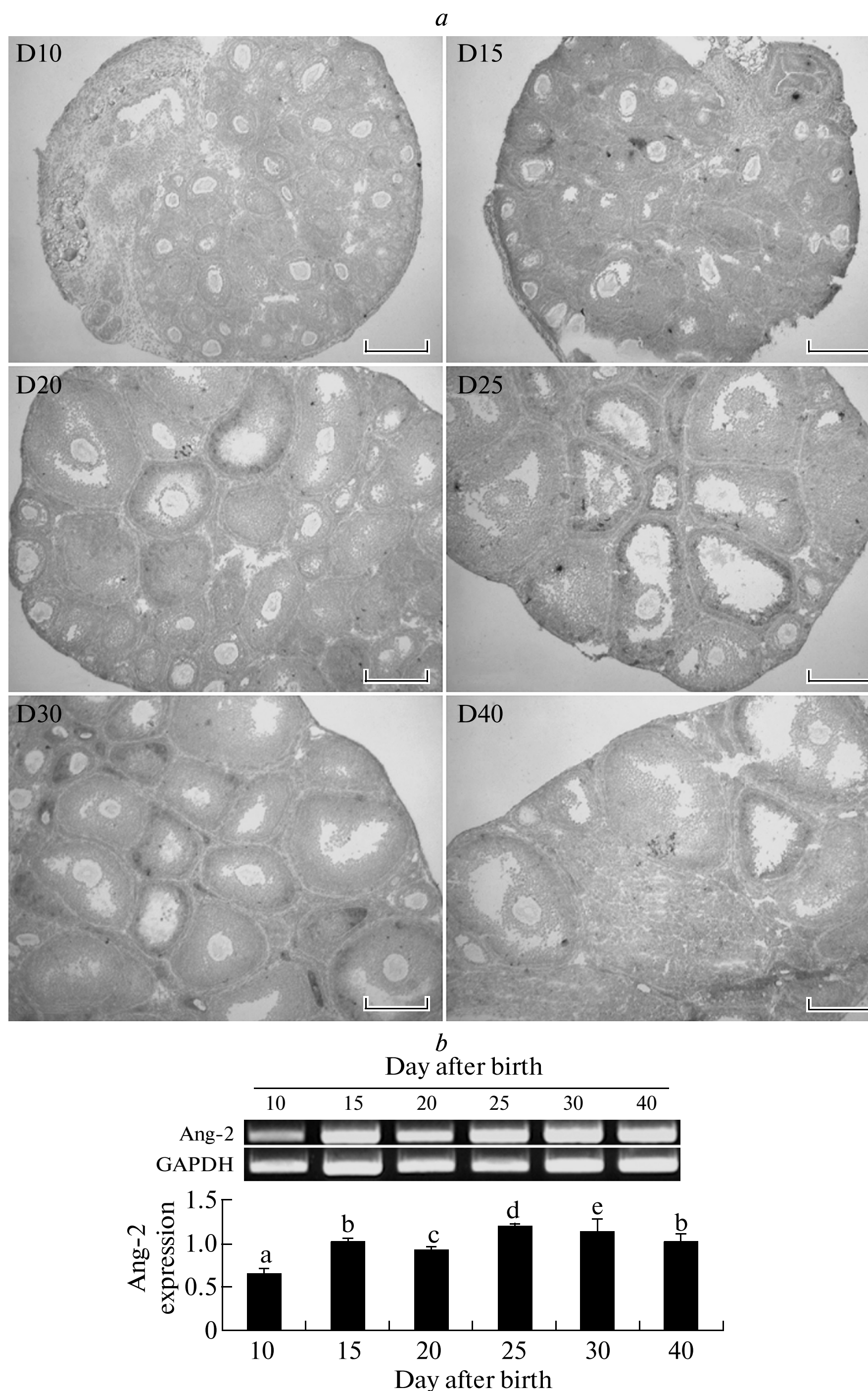

Fig. 1. *Ang-2* expression in mouse ovary during sexual maturation. *a* – *In situ* hybridization of *Ang-2* expression in mouse ovaries on days 10, 15, 20, 25, 30 and 40 after birth. Bar = 60 μm. *b* – Representative photograph of a reverse-transcription polymerase chain reaction of *Ang-2* expression in mouse ovaries on days 10, 15, 20, 25, 30 and 40 after birth. Ratio between Ang-2 and GAPDH. *Ang-2* expression was normalized to *GAPDH*. Each bar shows mean ± SEM of samples. Bars with different letters at the top differ significantly.

mRNA was highly expressed. On day 40 after birth, the *Ang-2* mRNA signal was also strongly detected in corpus luteum (fig. 1*a*). RT-PCR was also used to semi-quantitate the level of *Ang-2* mRNA expression in mouse ovaries during sexual maturation. *Ang-2* mRNA was lowly expressed on day 10 after birth, then the expression levels gradually increased and reached their highest values on day 25 after birth, and then expression gradually decreased on days 30 and 40 after birth (fig. 1*b*).

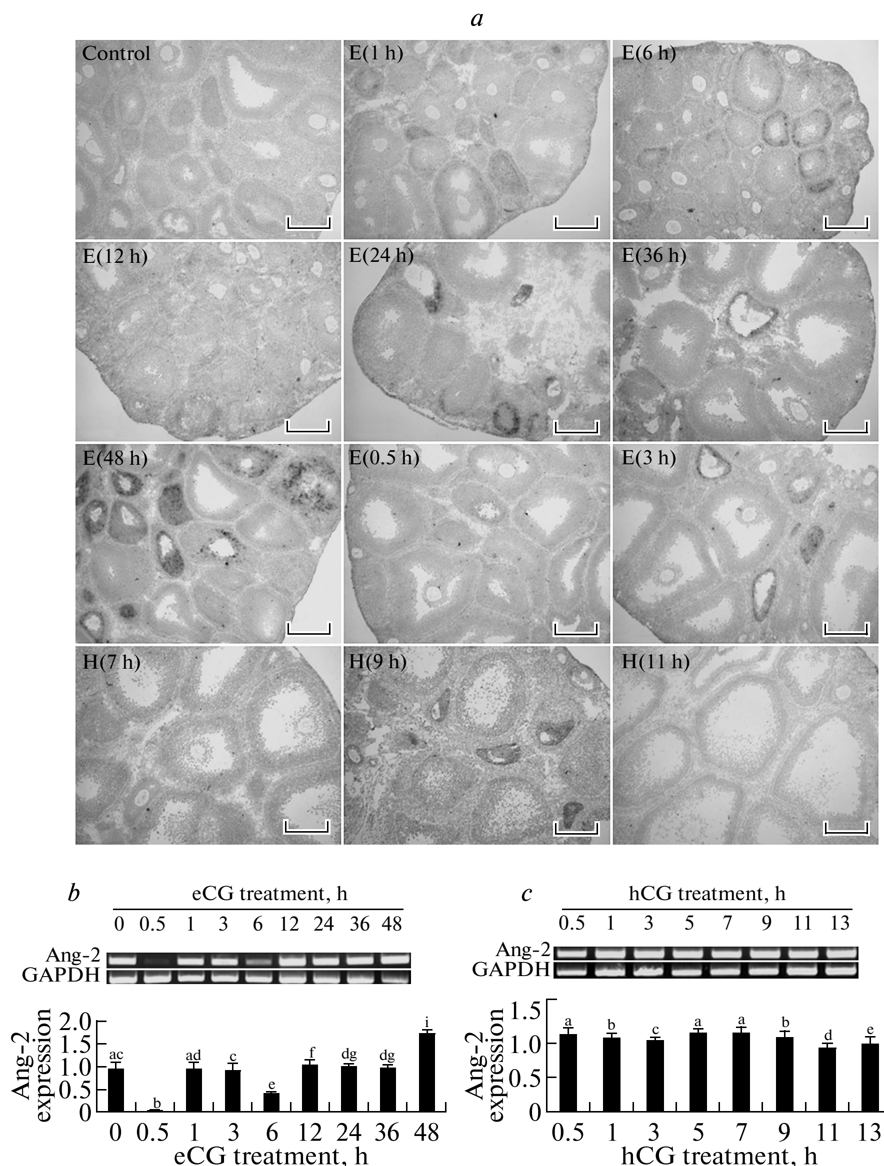

Fig. 2. *Ang-2* expression in mouse ovary after gonadotropin treatment. *a* – *In situ* hybridization of *Ang-2* in the immature mouse ovary after treatment with eCG for 1, 6, 12, 24, 36 and 48 h, followed by a treatment with hCG for 0.5, 3, 7, 9 and 11 h. No hybridization signals were observed in mouse ovaries 48 h post the eCG injection,when a DIG-labeled *Ang-2* sense probe was used for *in situ* hybridization. Bar= 60 μm. *b* – Representative photograph of a reverse-transcription polymerase chain reaction of *Ang-2* expression after eCG treatment. *c* – Representative photograph of a reverse-transcription polymerase chain reaction of *Ang-2* expression after hCG treatment.

Ang-2 mRNA expression after gonadotropin treatment

Ang-2 mRNA expression was low in thecal cells of follicles after the eCG injection, while it was high in follicular granulosa cells, which occur in atresia at different stages of follicular development. However, the *Ang-2* mRNA signal was not detected in oocytes and stroma of the ovaries post-eCG treatment (fig. 2*a*). After the hCG injection, *Ang-2* was also lowly expressed in thecal cells of follicles (fig. 2*a*). To further confirm *Ang-2* expression, RT-PCR was also performed. Although *Ang-2* expression was seen after the eCG treatment, a significantly higher level of *Ang-2* expression was detected 48 h post the eCG injection, (fig. 2*b*). After hCG treatment *Ang-2* mRNA was highly expressed at 0.5–13 h (fig. 2*c*).

Ang-2 mRNA expression during the formation and regression of corpus luteum

There was a low level of *Ang-2* mRNA expression in the newly formed corpus luteum on day 1 post the hCG injection. *Ang-2* mRNA was highly expressed in corpus luteum from day 2 to 9 after the hCG injection, then expression gradually declined on days 11 and 13

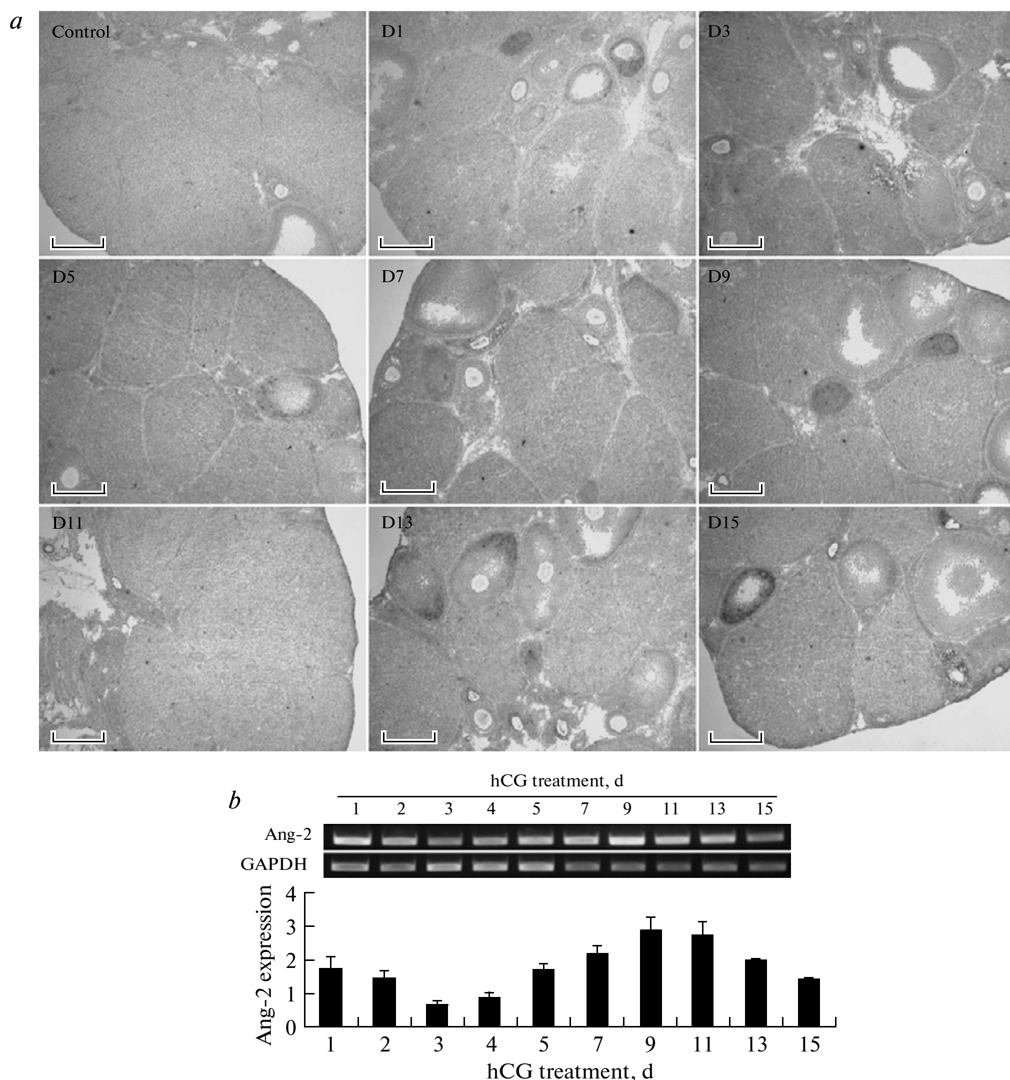

Fig. 3. *Ang-2* expression in mouse ovary during the formation and regression of corpus luteum. *a* – *In situ* hybridization of *Ang-2* in the corpus luteumof mouse ovaries on days 1, 3, 5, 7, 9, 11, 13 and 15 after treatment with hCG. No hybridization signals were observed in mouse ovaries on day 9 post the hCG injection,when a DIG-labeled *Ang-2* sense probe was used for *in situ* hybridization. Bar = 60 μm. *b* – Representative photograph of a reverse-transcription polymerase chain reaction of *Ang-2* expression in the corpus luteumof mouse ovary. A significant difference could be observed between each group ($P < 0.05$).

(fig. 3*a*). According to RT-PCR, the levels of *Ang-2* mRNA expression showed a decline after the hCG injection, with a nadir on day 3, followed by an increase, reaching the highest level on day 9 post the hCG injection. Then, *Ang-2* expression gradually declined from day 11 to 15 after the hCG injection (fig. 3*b*).

DISCUSSION

In this study expression and regulation of Ang-2 in mouse ovaries during sexual maturation and luteal development were examined by *in situ* hybridization and RT-PCR. Our results showed that *Ang-2* mRNA was expressed in granular cells and theca cells of the developing follicles. In mature follicles, *Ang-2* mRNA was only expressed in theca cells. These findings were consistent with a previous report [17]. However, in rat ovaries, Ang-2 was mainly localized in theca cells of follicles [6]. The inconsistency in data observed between mice and rats might be attributable to the differences in species or the techniques used.

Ang-2 might regulate vascular remodeling together with the vascular endothelial growth factor (VEGF). In the presence of VEGF, Ang-2 could enable endothelial cell migration and proliferation and promote angiogenesis; in the absence of VEGF, Ang-2 could result in blood vessel destabilization and regression [18]. Previous studies have found that VEGF was expressed in theca cells, indicating that Ang-2 could regulate follicular angiogenesis [17, 19]. However, Ang-2 was lowly expressed in theca cells of follicles. These results showed that VEGF might play a key role during

mouse follicular development. Further studies found that a direct injection of VEGF into the mouse ovary resulted in a significant increase in the number of preovulatory follicles, while injection of VEGF antibodies significantly reduced the number of primordial follicles within 1–3 days after administration without affecting the primary or secondary follicle numbers [20, 21]. In addition, Ang-2 was highly expressed in granular cells of atretic follicles, meanwhile VEGF was not detectable in these follicles, indicating that Ang-2 could contribute to vessel regression. Simultaneously, after an injection of Ang-1 antibodies, along with the increase of Ang-2/Ang-1 ratio, also increased the number of atretic follicles in gonadotropin-treated rat ovaries, while the number of antral follicles and preovulatory follicles decreased [22]. However, in marmoset ovaries, high expression of Ang-2 is not apparent in atretic follicles, so further work is required to ascertain whether the differences existedamong species [23].

In the present study Ang-2 was mainly detected in theca cells of mature follicles after the hCG injection. RT-PCR results showed that *Ang-2* mRNA was highly expressed in ovaries after the hCG treatment. These results imply that Ang-2 might play an important role during ovulation. The expression of Ang-2 inpreovulatory follicles has also been confirmed in rats and cows. In rat ovaries, the *Ang-2* mRNA level increased during the preovulatory period and ovulation, and the immunohistochemistry results demonstrated that Ang-2 expression was greater in early antral follicles and preovulatory follicles than in the preantral follicles [7, 24]. In gonadotrophin- releasing hormone (GnRH)-treated cow ovaries, Ang-2 expression was detected throughout the periovulatory phase and decreased at 10 and 25 h (ovulation period) after the GnRH treatment [25]. Moreover, using a microdialysis system *in vitro* infusion of Ang-2 could stimulate progesterone release from bovine preovulatory follicles [26]. Further studies have found that an intrafollicular injection of Ang-2 could disrupt the maturation of a preovulatory follicle and prevent ovulation [8, 27]. However, the concentration of Ang-2 in macaque and human serum remained constant throughout the natural menstrual cycle. After removal of the ovaries in macaques the serum concentration of Ang-2 also was not changed [28]. These results indicated that the ovaries are not major contributors to the circulation levels of Ang-2, and it is more likely that the Ang-2 of ovarian origin serves as a local factor.

The corpus luteum, a transient endocrine gland, is formed in the ovary after ovulation and secretes progesterone to establish and maintain pregnancy. The present study showed that Ang-2 was dynamically expressed in corpus luteum obtained from mice after hCGteatment, indicating that Ang-2 could be related to the formation, maintenance and regression of corpus luteum in mice. Likewise, the effects of Ang-2 in corpus luteum have also been demonstrated in rats, cows, ewes, monkeys and humans [7, 9–11, 13]. VEGF is the major factor regulating angiogenesis, and inhibition of VEGF can markedly decrease the corpus luteum volume and progesterone concentrations in plasma [29]. In mouse ovaries, VEGF is highly expressed in the developing corpus luteum [17, 19]. Therefore, VEGF might collaborate with Ang-2 to regulate angiogensis during corpus luteum formation in mouse ovaries. Further studies have found that inhibition of VEGF resulted in localized increased expression of Ang-2 [30]. In addition, Ang-2 was also detectable during luteolysis. During prostaglandin $F_{2\alpha}$-induced luteolysis Ang-2 expression acutely increased at 2 h in bovine corpus luteum, but only at 8 h in sheep corpus luteum[14–16]. The discrepancies might be attributable to the difference in species. Further studies using an *invitro* microdialysis system have found that an infusion of Ang-2 results in an acute decrease of progesterone release [14]. These results further demonstrated that Ang-2 might play an important role during corpus luteum regression.

In conclusion, our results suggest that Ang-2 may be involved in follicular development, atresia, ovulation, and formation and regression of corpus luteum.

ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (31072099 and 31101778), Research Fund for the Doctoral Program of Higher Education of China (20100061110078) and the Scientific Research Foundation of Jilin University (4305050102PO).

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