Effect of HIF-1a/VEGF signaling pathway on plasma progesterone and ovarian prostaglandin F$_{2a}$ secretion during luteal development of pseudopregnant rats

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ABSTRACT. The corpus luteum is a temporary endocrine structure in mammals that plays an important role in the female reproductive cycle and is formed from a ruptured and ovulated follicle with rapid angiogenesis. Vascular endothelial growth factor (VEGF) is thought to be vital in normal and abnormal angiogenesis in the ovary, but the molecular regulation of luteal VEGF expression during corpus luteum development *in vivo* is still poorly understood at present. Therefore, we examined whether hypoxia-inducible factor-1a (HIF-1a) is induced and regulates VEGF expression and luteal function *in vivo* using a pseudopregnant rat model treated with a small-molecule inhibitor of HIF-1a, echinomycin. Corpus luteum development in the pseudopregnant rat ovary was determined after measuring plasma progesterone concentration and ovarian prostaglandin F$_{2a}$ content to reflect changes

in HIF-1a and VEGF on different days of this developmental process. At day 7, the corpus luteum was formed and the expression of HIF-1a/VEGF reached a maximum, while a significant decrease in HIF-1a/VEGF expression was observed when luteolysis occurred at day 13. Additionally, echinomycin blocked luteal development by inhibiting VEGF expression mediated by HIF-1a and following luteal function by detecting the progesterone changes at day 7. These results demonstrated that HIF-1a-mediated VEGF expression might be an important mechanism regulating ovarian luteal development in mammals *in vivo*, which may provide new strategies for fertility control and for treating some types of ovarian dysfunction, such as polycystic ovarian syndrome, ovarian hyperstimulation syndrome, and ovarian neoplasia.

Key words: Corpus luteum; Hypoxia-inducible factor-1a; Echinomycin; Progesterone; Vascular endothelial growth factor

INTRODUCTION

The corpus luteum is a temporary endocrine structure in mammals that plays an important role in the female reproductive cycle and is formed temporarily from a ruptured and ovulated follicle through rapid angiogenesis (Young et al., 2000; Wulff et al., 2001; Fraser et al., 2005; Fraser and Duncan, 2005; Nishimura and Okuda, 2010). Rupture of the follicle just after ovulation is thought to occur under hypoxia conditions because of bleeding and the presence of an immature vasculature (Amselgruber et al., 1999). Recent studies have indicated that hypoxia is important for establishing the vascular system during luteal development (Nishimura and Okuda, 2010), which induces hypoxia-inducible factor-1a (HIF-1a) expression in luteal cells (Molitoris et al., 2009; Miyazawa et al., 2010). In areas in which vascular endothelial growth factor (VEGF) is regulated by hypoxia, there is up-regulation of specific transcription factors, including HIF-1a (Semenza, 2000; Critchley et al., 2006). However, the physiological role of HIF-1a in luteal development remains poorly understood.

VEGF plays an important role in the regulation of normal and abnormal angiogenesis in the ovary (Neulen et al., 1995; Lee et al., 1997; Fraser et al., 2005; Fraser and Duncan, 2005; Shimizu et al., 2007; Shimizu and Miyamoto, 2007; van den Driesche et al., 2008; Khandrika et al., 2009), particularly in the newly formed corpus luteum (Redmer and Reynolds, 1996; Fraser and Wulff, 2003; Tamanini and De Ambrogi, 2004). Hypoxia is a potent stimulus of VEGF expression (Koos, 1995; Lee et al., 1997), as ovulation causes a decrease in local oxygen concentration to produce a hypoxic environment, and thus may be the main stimulator of VEGF production in the developing corpus luteum (Nishimura and Okuda, 2010). However, gonadotropins play a clear role in regulating follicular growth and angiogenesis, as gonadotropin-releasing hormone antagonist treatment in the luteal phase of marmoset results in luteolysis and associated vascular regression (Young et al., 2000). Therefore, it is likely that VEGF expression is also regulated by gonadotropins during mammalian luteal formation in the ovary. Indeed, human chorionic gonadotropin (HCG) stimulates VEGF synthesis in human luteinized granulosa cells (Koos, 1995; Neulen et al., 1995; Christenson and Stouffer, 1997; Lee et al., 1997; Fraser et al., 2005; Fraser and Duncan, 2005). In addition, luteal vascularization and the development of ovarian hyperstimulation syndrome are absolutely dependent on

luteinizing hormone/HCG stimulation (Neulen et al., 1995; Nastri et al., 2010). Furthermore, in a fully formed and highly vascularized corpus luteum, exogenous HCG also up-regulates VEGF expression (Wulff et al., 2001; Shimizu et al., 2007; Shimizu and Miyamoto, 2007). These results indicate that VEGF is regulated by HIF-1a under both hypoxic and normoxic conditions, as HIF-1a can regulate VEGF mRNA expression under gonadotropin-stimulated conditions in luteal cells *in vitro* (Zhang et al., 2011). However, there have been no reports regarding the contribution of HIF-1a signaling in VEGF-dependent angiogenesis during ovarian luteal formation *in vitro*.

Given the important role of HIF-1a in regulating VEGF expression *in vitro* (Zhang et al., 2011), we hypothesized that the HIF-1a signaling pathway contributes to VEGF-dependent angiogenesis and the subsequent functions of the corpus luteum in the ovary *in vivo*. Therefore, we examined the expression of HIF-1a and VEGF during luteal development in pseudopregnant rats by measuring plasma progesterone levels and ovarian prostaglandin F_{2a} contents on days 2, 7, 13, and 19 of pseudopregnancy. Further analysis showed that the HIF-1a small-molecule inhibitor echinomycin (Ech) not only inhibited luteal functions, but also decreased HIF-1a-mediated VEGF expression in the early corpus luteum of pseudopregnant rats. These results demonstrated that HIF-1a is an important mediator of luteal functions. This may be an important mechanism of regulating VEGF-dependent angiogenesis during luteal development in mammals.

MATERIAL AND METHODS

Animals

Sprague-Dawley rats were purchased from Wushi Experimental Animal Supply Co., Ltd. (Fuzhou, China). The animals were maintained under a 14-h light/10-h dark schedule with a continuous supply of chow and water. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee, Fujian Normal University.

Experimental design

The rats were allowed to acclimate for 1-2 weeks before mating with males was commenced, which occurred at 2-3 months of age (179-250 g body weight). Previously unmated female rats (3/cage) were mated with a vasectomized male (1/cage) and checked every morning for the presence of a vaginal plug. Day 1 of pseudopregnancy was defined as the day when a vaginal plug was recovered. Pseudopregnant females were removed and used for experiments. The duration of pseudopregnancy has been previously determined to be 13 ± 1 days based on plasma steroid levels and vaginal smears.

To further confirm this rat model, we measured plasma progesterone levels and ovarian prostaglandin F_{2a} contents in the animals on days 2, 7, 13, and 19 of pseudopregnancy. The rats were decapitated and trunk blood was collected for progesterone determination. The abdomen was opened and the ovaries were rapidly excised and chilled in ice-cold 0.154 M NaCl containing 14.0 µM indomethacin (Sigma-Aldrich, St. Louis, MO, USA) just after perfusion to measure prostaglandin F_{2a} and HIF-1a/VEGF expression.

To estimate the possible role of HIF-1a/VEGF signaling during corpora luteal formation in pseudopregnant rats, 1 mg/kg body weight Ech, a small-molecule inhibitor of HIF-1a, was intraperitoneally injected on day 2 of pseudopregnancy and then plasma progesterone levels were determined. We also measured prostaglandin F_{2a} content and HIF-1a/VEGF expression in the perfused ovaries on day 7.

One ovary from each rat was fixed in 4% paraformaldehyde for histological and immunohistochemical examination, while the other ovary was snap-frozen and used in other experiments.

Ovarian perfusion

To avoid the influence of vascular system, ovarian perfusion was performed before collecting the ovaries. Female rats were perfused *in vivo* through the abdominal aorta with 0.154 M NaCl containing 14.0 μM indomethacin. Animals were anesthetized with 0.05 mg atropine per kg body weight subcutaneously and 2.5 mg diazepam per kg body weight intraperitoneally (Sigma). The abdomen was opened by a mid-ventral incision and an intravenous cannula (1.4 mm in outer diameter) was inserted via the aortic bifurcation. The abdominal aorta was clamped caudal of the celiac artery, and the inferior vena cava was severed. Next, 40 μL indomethacin solution was perfused at ambient temperature through the lower abdominal vascular system for approximately 5 min at constant pressure using a hand-held syringe. Perfusion was discontinued when the ovaries, particularly the corpus luteum, were completely pale. The ovaries were then quickly removed for subsequent analysis of prostaglandin F_{2a} levels and gene expression.

Radioimmunoassay of progesterone and prostaglandin F_{2a}

The levels of plasma progesterone and ovarian prostaglandin F_{2a} were determined using specific radioimmunoassay kits according to manufacturer instructions. The progesterone radioimmunoassay kit [intra-assay coefficient of variation (CV) < 4.3% and inter-assay CV < 7.1%] and the prostaglandin F_{2a} radioimmunoassay kit (intra-assay CV < 10.0% and inter-assay CV < 15.0%) were from Atomic Gaoke Co., Ltd., Department of Isotope, China Institute of Atomic Energy (Beijing, China). Protein concentrations were determined using a Bio-Rad (Hercules, CA, USA) assay with bovine serum albumin standards.

RNA extraction and real-time polymerase chain reaction analysis of VEGF mRNA and HIF-1a mRNA

Total RNA was extracted from the ovaries or granulosa cells using TRIzol solution (Life Technologies, Carlsbad, CA, USA) and then reverse-transcribed (cDNA Synthesis Kit; Bio-Rad). The reverse-transcribed products were amplified using a TaqMan Gene Expression Assay kit (Applied Biosystems, Foster City, CA, USA), including TaqMan Universal PCR Master Mix, HIF-1a primer, and proliferating cell nuclear antigen primer. To detect the levels of 18S ribosomal RNA, a kit was used as an endogenous control. Relative gene expression was calculated in accordance with the ΔΔCt method. Relative mRNA levels are reported as $2^{-\Delta\Delta Ct}$ values.

Western blot analysis of VEGF and HIF-1a proteins

Protein samples were obtained using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology, Haimen, China). Protein concentrations were determined using a Bio-Rad assay with bovine serum albumin standards. Next, 20 μg protein samples were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrophoretically transferred onto a polyvinylidene fluoride membrane. The membrane was washed and probed with anti-HIF-1a antibody (1:500, Abcam, Cambridge, UK) overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-labeled goat anti-mouse IgG (1:5000, Novus Biologicals, Littleton, CO, USA) for 60 min at room temperature, and then the film was developed to obtain the images. After HIF-1a, the membrane was reprobed for VEGF expression with anti-VEGF antibody (1:1000, Abcam). To detect the immunoblotting signal, 2 mL enhanced chemiluminescence detection solution was added, and the membrane was wrapped and exposed to Kodak OMAT film.

Statistical analysis

Data are reported as means ± SE. The significance of differences in mean values within and between multiple groups was evaluated using one-way analysis of variance followed by the Tukey multiple range test. The Student t-test was used to evaluate statistical significance of differences between the 2 groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

Changes in plasma progesterone and ovarian prostaglandin F_{2a} of pseudopregnant rats on different days of the luteal lifespan

To further confirm the rat model of pseudopregnancy, the levels of plasma progesterone and ovarian prostaglandin F_{2a} of pseudopregnant rats on days 2, 7, 13, and 19 of the luteal lifespan were examined and the highest level of progesterone was found on day 7 ($P < 0.05$), indicating that the corpus luteum had formed (Figure 1A). The significantly decreased level of progesterone on day 13 implied that luteolysis had occurred ($P < 0.05$; Figure 1A). Changes in ovarian prostaglandin F_{2a} also confirmed this rat model of luteal development (Figure 1B).

Changes in VEGF and HIF-1a mRNA levels in the ovaries of pseudopregnant rats on different days of the luteal lifespan

To illustrate the possible role of HIF-1a/VEGF during corpus luteum development in pseudopregnant rats, we also detected the expression of VEGF and HIF-1a mRNA in the ovaries of pseudopregnant rats on different days of the luteal lifespan. A significant increase was observed in VEGF mRNA expression on day 7 (Figure 2A), while a significant decrease was observed in VEGF mRNA expression on day 13 (Figure 2A). These results were consistent with the development of pseudopregnant corpus luteum, implying that VEGF plays a vital role in the formation of the corpus luteum as reported previously (Neulen et al., 1995; Redmer and Reynolds, 1996; Fraser and Wulff, 2003; Tamanini and De Ambrogi, 2004; Fraser et al.,

2006). Interestingly, a significant correlation ($R^2 = 0.7867$) was also observed (Figure 2B), indicating that HIF-1a also participated in the development of the corpus luteum through the VEGF signaling pathway.
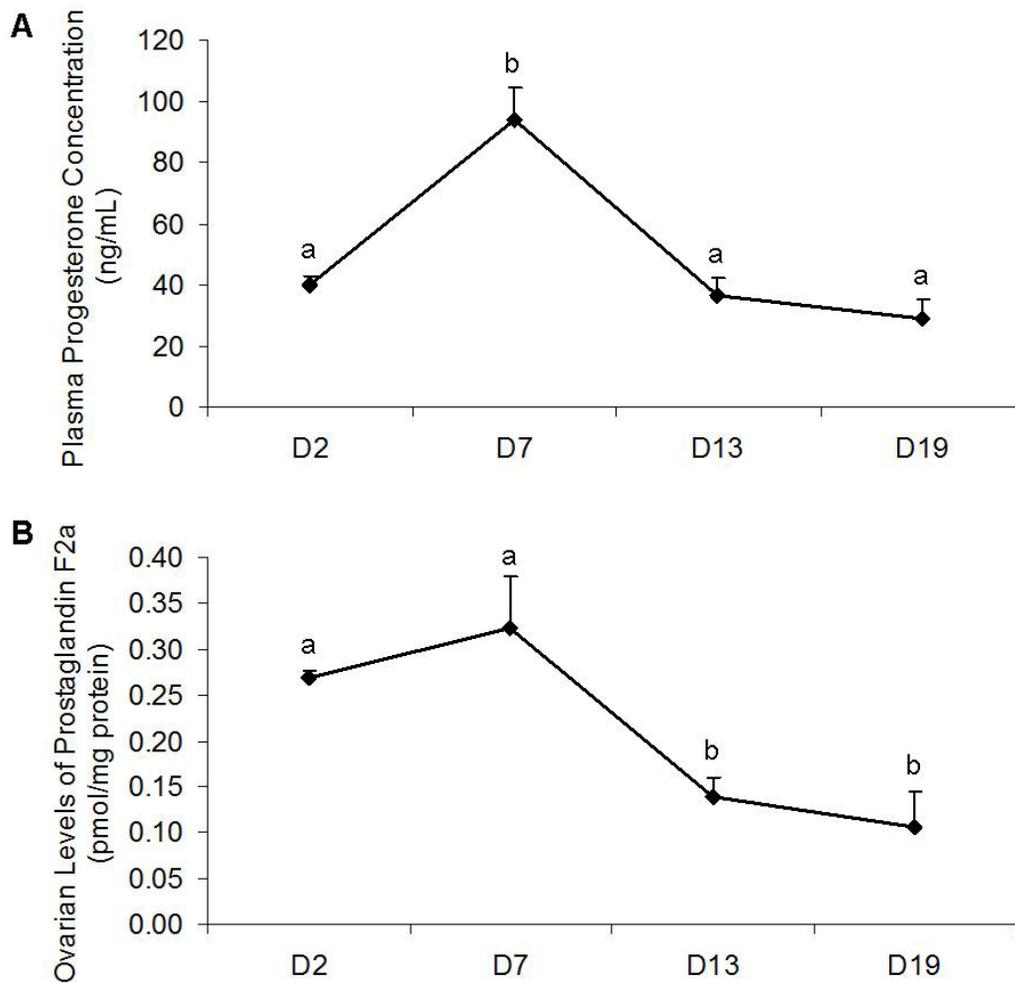

Figure 1. Levels of plasma progesterone (**A**) and ovarian prostaglandin F_{2a} (**B**) of pseudopregnant rats on different days of the luteal lifespan. Adult female rats were mated with vasectomized males to induce pseudopregnancy. Day 1 of pseudopregnancy was defined as the day when a vaginal plug was recovered. Animals were sacrificed by decapitation and trunk blood was collected for progesterone determination on days (D) 2, 7, 13, and 19. The abdomen was opened and the ovaries were rapidly excised and chilled in ice-cold 0.154 M NaCl containing 14.0 μM indomethacin just after perfusion to measure prostaglandin F_{2a} and HIF-1a/VEGF expression. Each value represents means ± SE. One-way analysis of variance was used to analyze the data. Different superscripts denote significant values ($P < 0.05$) by the Tukey multiple range test. N = 6 animals in each group.
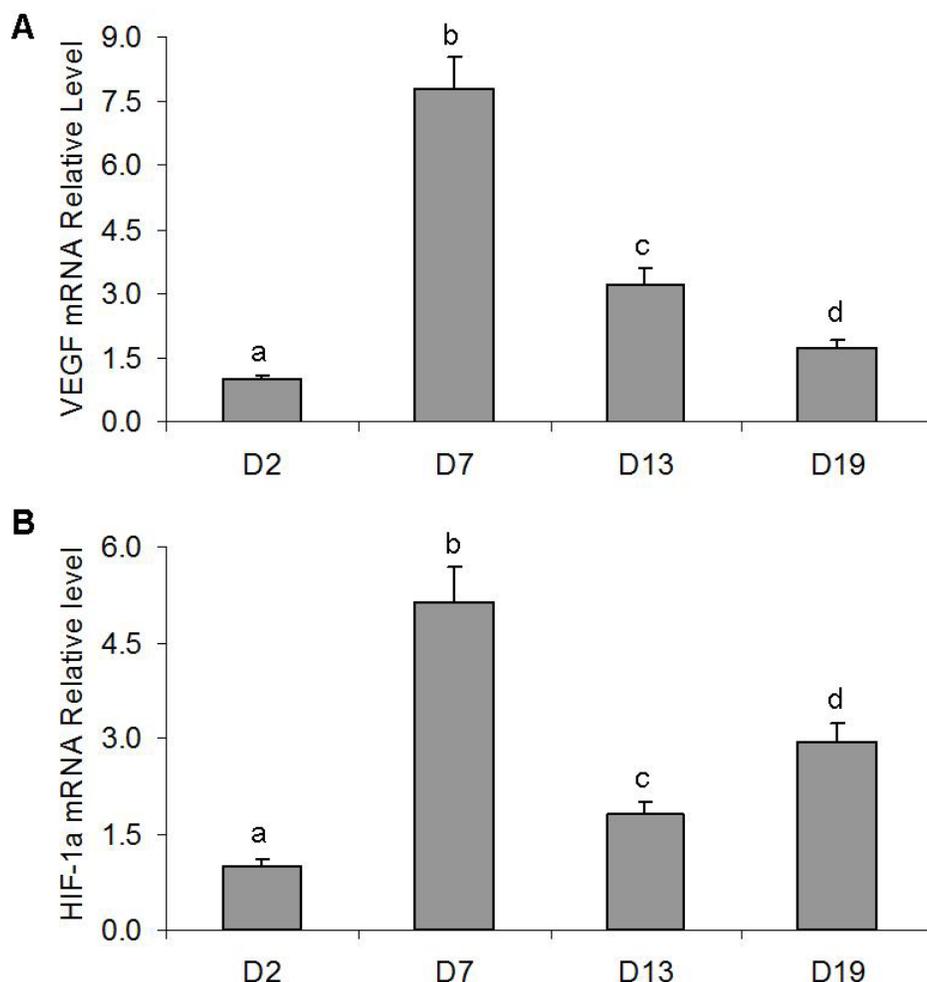
Figure 2. VEGF and HIF-1a mRNA levels in the ovaries of pseudopregnant rats on different days of the luteal lifespan. **A.** Relative mRNA levels of VEGF by real-time reverse transcription-polymerase chain reaction analysis. **B.** Relative mRNA levels of HIF-1a by real-time reverse transcription-polymerase chain reaction analysis. Each value represents means ± SE. One-way analysis of variance was used to analyze the data. Different superscripts denote significant values ($P < 0.05$) by the Tukey multiple range test. N = 6 animals in each group. D = day.

Effects of Ech on luteal development in pseudopregnant rats

To further clarify the participation of HIF-1a in luteal development, pseudopregnant rats were injected with Ech, a small-molecule inhibitor of HIF-1a, on day 2 of pseudopregnancy and then the levels of plasma progesterone and ovarian prostaglandin F_{2a} were examined in pseudopregnant rats on days 2 and 7 of the luteal lifespan with and without Ech treatment (Figure 3). The results showed that Ech significantly decreased the level of plasma progesterone (Figure 3A) and inhibited the production of ovarian prostaglandin F_{2a} (Figure 3B) in pseudopregnant rats, further demonstrating the important role of HIF-1a in corpus luteum formation *in vivo*.
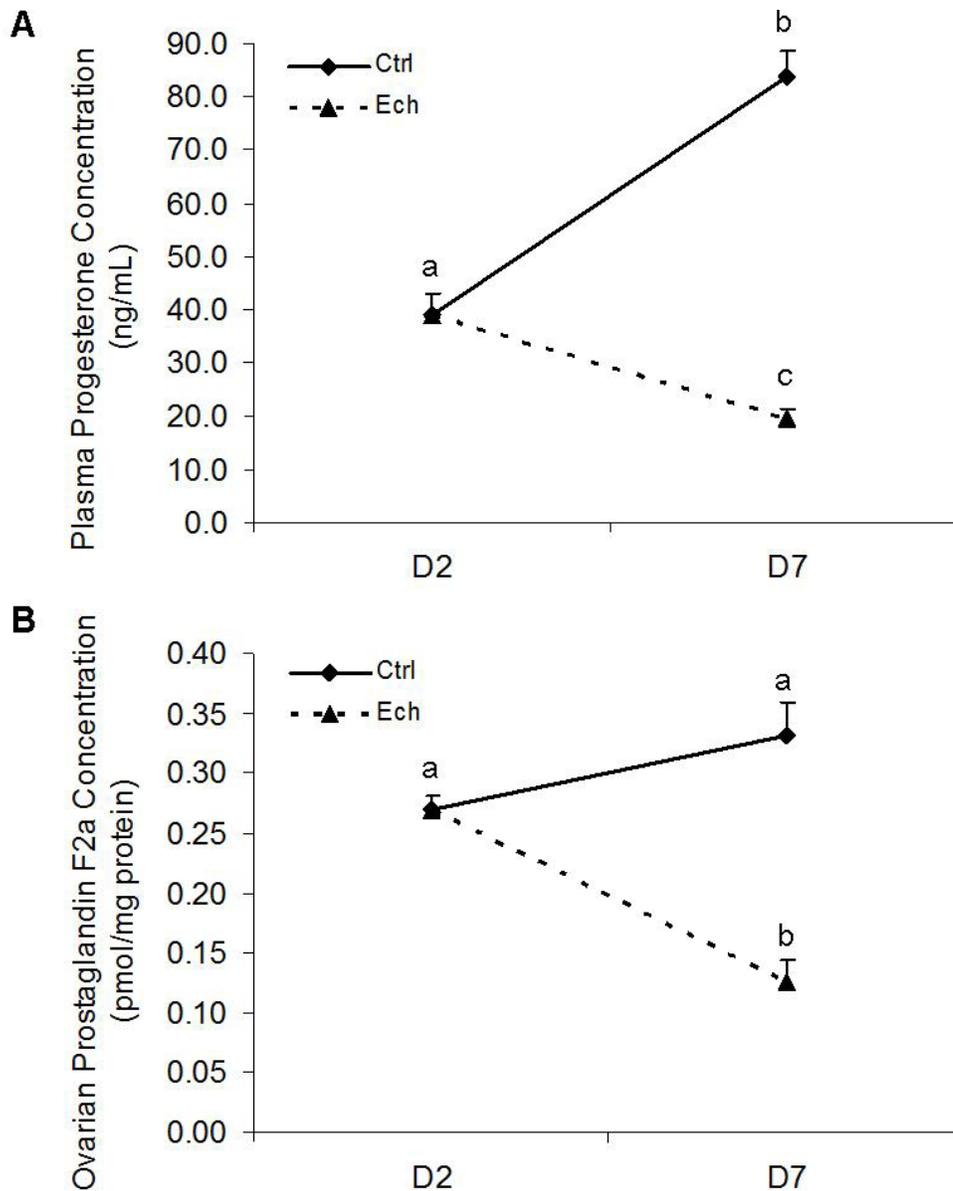
A

B

Figure 3. Levels of plasma progesterone (**A**) and ovarian prostaglandin F_{2a} (**B**) in pseudopregnant rats on days (D) 2 and 7 of luteal lifespan with and without Ech treatment. Adult female rats were mated with vasectomized males to induce pseudopregnancy. Day 1 of pseudopregnancy was defined as the day when a vaginal plug was recovered. Pseudopregnant rats were intraperitoneally injected with Ech (1 mg/kg body weight), a small-molecule inhibitor of HIF-1a, on day 2 of pseudopregnancy. Animals were sacrificed by decapitation and trunk blood was collected for progesterone determination on days 2 and 7. The abdomen was opened and the ovaries were rapidly excised and chilled in ice-cold 0.154 M NaCl containing 14 µM indomethacin just after perfusion for measuring prostaglandin F_{2a} and HIF-1a/VEGF expression. Each value represents means ± SE. One-way analysis of variance was used to analyze the data. Different superscripts denote significant values (P < 0.05) by the Tukey multiple range test. N = 6 animals of each group. Ech = echinomycin.

Effects of Ech on VEGF and HIF-1a mRNA expression during luteal development in pseudopregnant rats

To identify the possible role of HIF-1a/VEGF signaling during luteal development *in vivo*, the expression levels of VEGF and HIF-1a mRNA were tested in the ovaries of pseudopregnant rats with and without Ech treatment. The results indicated that Ech blocked VEGF mRNA expression in the ovary of pseudopregnant rats on day 7 (Figure 4A), implying that HIF-1a took part in VEGF mRNA expression during this developmental process. Interestingly, there was no obvious change in HIF-1a mRNA expression in the ovaries after Ech treatment on day 7 of pseudopregnancy (Figure 4B). Therefore, HIF-1a protein expression was determined.
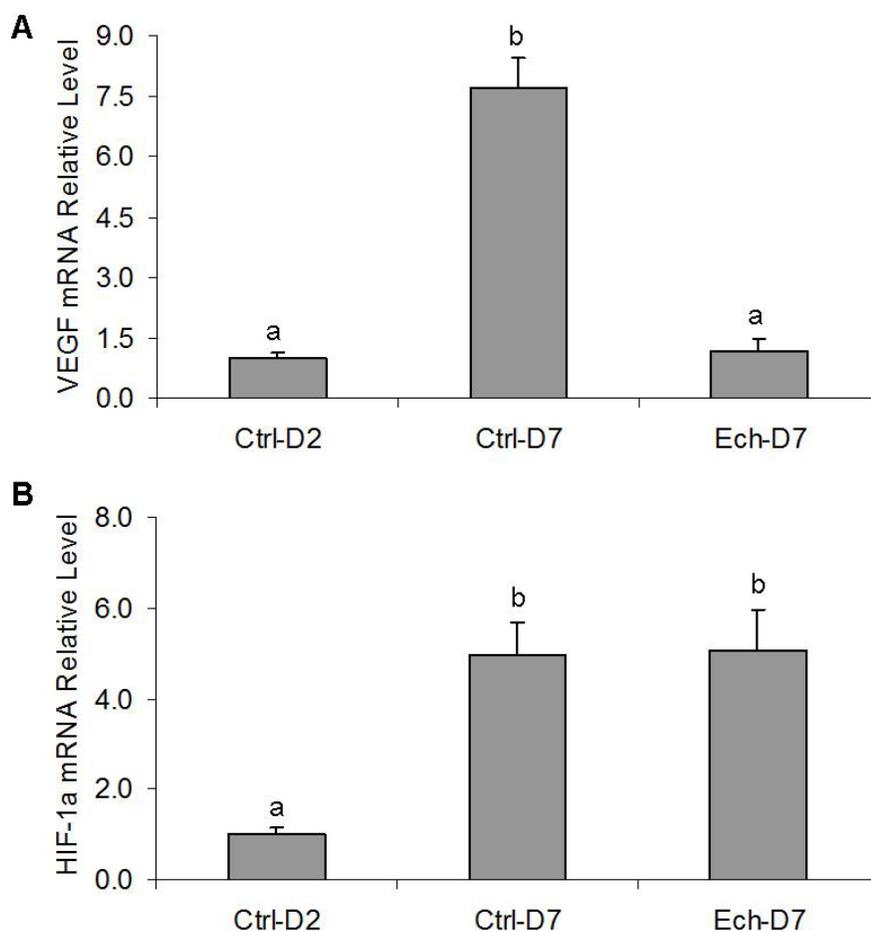
Figure 4. VEGF and HIF-1a mRNA levels in the ovaries of pseudopregnant rats on days (D) 2 and 7 of the luteal lifespan with and without Ech treatment. **A.** Relative mRNA levels of VEGF by real-time reverse transcription-polymerase chain reaction analysis. **B.** Relative mRNA levels of HIF-1a by real-time reverse transcription-polymerase chain reaction analysis. Each value represents means ± SE. One-way analysis of variance was used to analyze the data. Different superscripts denote significant values ($P < 0.05$) by the Tukey multiple range test. N = 6 animals in each group. Ech = echinomycin.

Effects of Ech on HIF-1a protein expression during luteal development in pseudopregnant rats

HIF-1a protein expression in pseudopregnant rat ovaries indicated that there was also no significant change after Ech treatment on day 7 of pseudopregnancy (Figure 5), which may be because Ech is an inhibitor of HIF-1a activity. Therefore, HIF-1 binding activity was further analyzed.
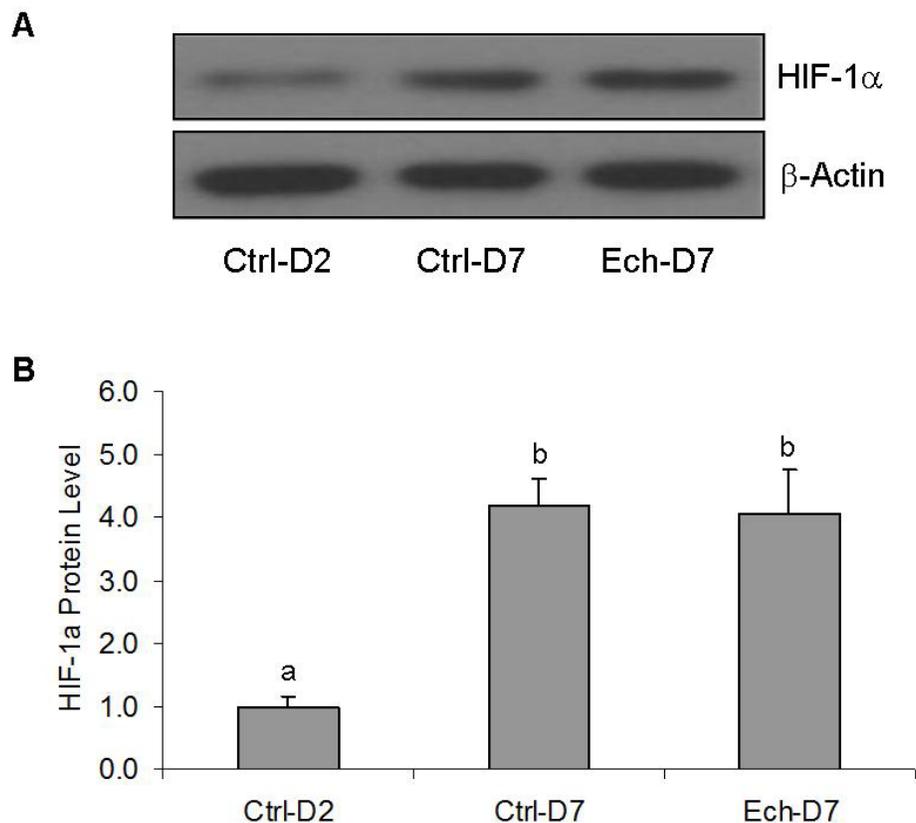

Figure 5. HIF-1a protein levels in the ovaries of pseudopregnant rats on days (D) 2 and 7 of the luteal lifespan with and without Ech treatment. **A.** Representative electrochemiluminescence gel documents of western blot analyses depicting the protein levels of HIF-1a. **B.** Summarized intensities of HIF-1a blots normalized to the control. Each value represents means ± SE. One-way analysis of variance was used to analyze the data. Different superscripts denote significant values ($P < 0.05$) by the Tukey multiple range test. N = 6 animals of each group. Ech = echinomycin.

Effects of Ech on HIF-1 binding activity during luteal development in pseudopregnant rats

To further identify the regulatory role of HIF-1a in VEGF mRNA expression, we also examined the effects of Ech on HIF-1 binding activity during luteal development in pseudopregnant rats. The results showed that Ech significantly inhibited HIF-1 binding activity

(Figure 6), which contributed to the decrease in VEGF mRNA expression in pseudopregnant ovaries treated with Ech and inhibition of subsequent luteal functions on day 7 of pseudopregnancy.
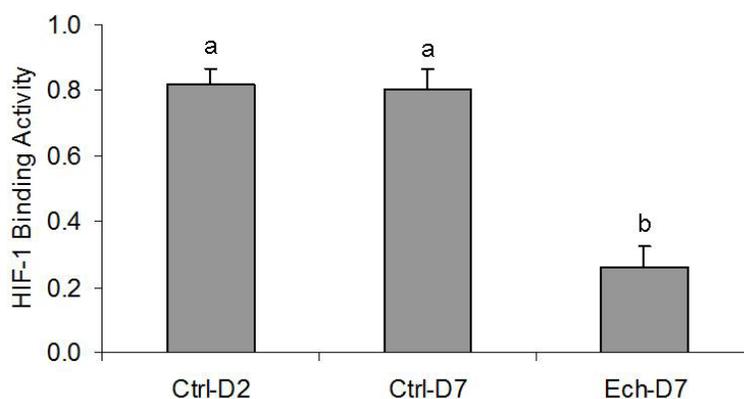

Figure 6. HIF-1 binding activity in the ovaries of pseudopregnant rats on days (D) 2 and 7 of the luteal lifespan with and without Ech treatment. Each value represents means ± SE. One-way analysis of variance was used to analyze the data. Different superscripts denote significant values (P < 0.05) by the Tukey multiple range test. N = 6 animals in each group. Ech = echinomycin.

DISCUSSION

The results of the present study clearly demonstrated that HIF-1a and VEGF were expressed in the ovaries and expression changed in a similar manner during corpus luteum development in pseudopregnant rats, suggesting that HIF-1a/VEGF signaling may play an important regulatory role in corpus luteum formation and luteal functions in mammals.

It is well-known that the corpus luteum is a temporary endocrine structure that plays an important role in the female reproductive cycle and is formed from a ruptured and ovulated follicle through rapid angiogenesis in mammals (Young et al., 2000; Wulff et al., 2001; Fraser et al., 2005; Nishimura and Okuda, 2010; Zhang et al., 2011). VEGF is thought to play a critical role in the regulation of normal and abnormal angiogenesis in the ovary (Neulen et al., 1995; Lee et al., 1997; Fraser et al., 2005; Kaczmarek et al., 2005; Shimizu and Miyamoto, 2007; Shimizu et al., 2007; van den Driesche et al., 2008; Zhang et al., 2011), particularly in the newly formed corpus luteum. In primates, the VEGF protein is localized to the hormone-producing cells of the corpus luteum, and is highest in the granulosa-derived cells (Ravindranath et al., 1992; Christenson and Stouffer, 1997; Lee et al., 1997; Kaczmarek et al., 2005; Shimizu et al., 2007; Shimizu and Miyamoto, 2007). VEGF plays a fundamental role in the physiological angiogenesis and vascularization of the follicular luteinizing granulosa layer during corpus luteum formation (Christenson and Stouffer, 1997; Kaczmarek et al., 2005; Shimizu et al., 2007; Shimizu and Miyamoto, 2007). Because inhibition of VEGF *in vivo* during the luteal phase will prevent luteal angiogenesis and subsequent progesterone secretion (Wulff et al., 2001; Fraser and Duncan, 2005; Fraser et al., 2005, 2006; Duncan et al., 2008), excess VEGF generation during the vascularization of multiple follicles is also thought to cause ovarian hyperstimulation syndrome (Neulen et al., 1995; Nastri et al., 2010).

Furthermore, if VEGF is inhibited, the corpus luteum will have a rudimentary vascular bed with poor functions (Duncan et al., 2008). VEGF is also required for the ongoing function and vasculature maintenance of the mature corpus luteum (Fraser and Duncan, 2005; Fraser et al., 2005, 2006). The results of previous studies are consistent with our results, revealing changes in VEGF mRNA expression in the corpus luteum of pseudopregnant rats.

Our previous experiments demonstrated that VEGF is transcriptionally activated by an HIF-1-mediated mechanism in luteal cells *in vitro* under hypoxia conditions (Zhang et al., 2011), which are caused by ovulation of a ruptured follicle with bleeding and an immature vasculature *in vivo* (Kaczmarek et al., 2005; Nishimura and Okuda, 2010). However, many reports have also shown that reproductive hormones such as HCG take part in the primary regulation of VEGF expression in the ovary. For example, VEGF mRNA expression in human luteinized granulosa cells has been shown to be dose- and time-dependently enhanced by HCG *in vitro* (Neulen et al., 1995; Nastri et al., 2010). Chronic or acute exposure to HCG directly stimulates VEGF production and secretion in monkeys (Christenson and Stouffer, 1997) and human luteinized granulosa cells (Neulen et al., 1995; Lee et al., 1997; Wulff et al., 2001; Nastri et al., 2010). Administration of a gonadotropin-releasing hormone antagonist decreased VEGF mRNA expression in the monkey corpus luteum (Ravindranath et al., 1992). In addition, luteal vascularization and the development of ovarian hyperstimulation syndrome were found to be dependent on luteinizing hormone/HCG stimulation (Neulen et al., 1995; Nastri et al., 2010). Furthermore, in a fully formed, highly vascular corpus luteum, HCG also up-regulates VEGF expression (Wulff et al., 2001). Therefore, the present study also examined changes in HIF-1a mRNA expression during corpus luteum development in pseudopregnant rats. The results showed that HIF-1a expression occurred in a stage-specific manner and was highly correlated with VEGF expression, suggesting that HIF-1a is vital to VEGF-dependent development of the corpus luteum and subsequent functions *in vivo*.

HIF-1, a helix-loop-helix transcriptional factor, which consists of HIF-1a and HIF-1b, has been cloned and characterized as a transcriptional activator of many oxygen-sensitive genes, including erythropoietin, heme oxygenases, and transferring and several glycolytic enzymes (Wang et al., 1995; Wang and Semenza, 1995), whose protein products play critical roles in developmental and physiological processes, including angiogenesis, erythropoiesis, glycolysis, iron transport, and cell proliferation/survival (Zhong et al., 2000; Wulff et al., 2001; Yaba et al., 2008; Miyazawa et al., 2009, 2010; Nishimura and Okuda, 2010). It has been indicated that HIF-1a is induced by a decrease in O_2 concentration in tissue or cells. HIF-1b is not inducible, but can bind to HIF-1a to form a dimer, which activates the transcription of many genes containing cis-hypoxia-response element in their promoter or enhancer regions (Kazi and Koos, 2007; Molitoris et al., 2009). Therefore, we used an HIF-1a-specific small-molecule inhibitor Ech to treat the pseudopregnant rats and then examined gene expression and hormone levels. A significant decrease in plasma progesterone and decreased production of ovarian prostaglandin F_{2a} were observed after Ech treatment. Further analysis found that the ovarian VEGF mRNA level was clearly inhibited, which may contribute to insufficient luteal functions. Interestingly, there was no obvious change in HIF-1a mRNA expression in the ovaries. Therefore, we also detected HIF-1a protein expression and found that Ech had no effect on HIF-1a protein expression. Analysis of HIF-1 binding activity showed that this decrease in VEGF mRNA expression might have been caused by the inhibition of Ech on HIF-1 binding activity during luteal development in pseudopregnant rats. HIF-1a participated in the develop-

ment of the corpus luteum and its functions through the VEGF signaling pathway in the ovary of pseudopregnant rats.

In summary, we demonstrated that VEGF expression changes in a stage-dependent manner during corpus luteum development in the ovary. Further investigation showed that this change in VEGF expression was regulated by HIF-1a signaling. This HIF-1a-mediated VEGF expression may be an important mechanism regulating luteal development and its functions in the mammalian ovary. Furthermore, HIF-1a antagonism may be useful for developing novel treatments for fertility control and for some types of ovarian dysfunction (Chan and Giaccia, 2010; Miyazawa et al., 2009, 2010), particularly conditions characterized by pathological angiogenesis and excessive vascular permeability, such as polycystic ovarian syndrome, ovarian hyperstimulation syndrome, and ovarian neoplasia.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

Amselgruber WM, Schäfer M and Sinowatz F (1999). Angiogenesis in the bovine corpus luteum: an immunocytochemical and ultrastructural study. *Anat. Histol. Embryol*. 28: 157-166.

Chan DA and Giaccia AJ (2010). PHD2 in tumour angiogenesis. *Br. J. Cancer* 103: 1-5.

Christenson LK and Stouffer RL (1997). Follicle-stimulating hormone and luteinizing hormone/chorionic gonadotropin stimulation of vascular endothelial growth factor production by macaque granulosa cells from pre- and periovulatory follicles. *J. Clin. Endocrinol. Metab*. 82: 2135-2142.

Critchley HO, Osei J, Henderson TA, Boswell L, et al. (2006). Hypoxia-inducible factor-1alpha expression in human endometrium and its regulation by prostaglandin E-series prostanoid receptor 2 (EP2). *Endocrinology* 147: 744-753.

Duncan WC, van den Driesche S and Fraser HM (2008). Inhibition of vascular endothelial growth factor in the primate ovary up-regulates hypoxia-inducible factor-1alpha in the follicle and corpus luteum. *Endocrinology* 149: 3313-3320.

Fraser HM and Wulff C (2003). Angiogenesis in the corpus luteum. *Reprod. Biol. Endocrinol*. 1: 88.

Fraser HM and Duncan WC (2005). Vascular morphogenesis in the primate ovary. *Angiogenesis* 8: 101-116.

Fraser HM, Bell J, Wilson H, Taylor PD, et al. (2005). Localization and quantification of cyclic changes in the expression of endocrine gland vascular endothelial growth factor in the human corpus luteum. *J. Clin. Endocrinol. Metab*. 90: 427-434.

Fraser HM, Wilson H, Wulff C, Rudge JS, et al. (2006). Administration of vascular endothelial growth factor Trap during the 'post-angiogenic' period of the luteal phase causes rapid functional luteolysis and selective endothelial cell death in the marmoset. *Reproduction* 132: 589-600.

Kaczmarek MM, Schams D and Ziecik AJ (2005). Role of vascular endothelial growth factor in ovarian physiology - an overview. *Reprod. Biol*. 5: 111-136.

Kazi AA and Koos RD (2007). Estrogen-induced activation of hypoxia-inducible factor-1alpha, vascular endothelial growth factor expression, and edema in the uterus are mediated by the phosphatidylinositol 3-kinase/Akt pathway. *Endocrinology* 148: 2363-2374.

Khandrika L, Lieberman R, Koul S, Kumar B, et al. (2009). Hypoxia-associated p38 mitogen-activated protein kinase-mediated androgen receptor activation and increased HIF-1alpha levels contribute to emergence of an aggressive phenotype in prostate cancer. *Oncogene* 28: 1248-1260.

Koos RD (1995). Increased expression of vascular endothelial growth/permeability factor in the rat ovary following an ovulatory gonadotropin stimulus: potential roles in follicle rupture. *Biol. Reprod*. 52: 1426-1435.

Lee A, Christenson LK, Patton PE, Burry KA, et al. (1997). Vascular endothelial growth factor production by human luteinized granulosa cells *in vitro*. *Hum. Reprod*. 12: 2756-2761.

Miyazawa M, Yasuda M, Fujita M, Kajiwara H, et al. (2009). Therapeutic strategy targeting the mTOR-HIF-1alpha-VEGF pathway in ovarian clear cell adenocarcinoma. *Pathol. Int*. 59: 19-27.

Miyazawa M, Yasuda M, Fujita M, Hirabayashi K, et al. (2010). Granulosa cell tumor with activated mTOR-HIF-1alpha-VEGF pathway. *J. Obstet. Gynaecol. Res*. 36: 448-453.

Molitoris KH, Kazi AA and Koos RD (2009). Inhibition of oxygen-induced hypoxia-inducible factor-1alpha degradation unmasks estradiol induction of vascular endothelial growth factor expression in ECC-1 cancer cells *in vitro*. *Endocrinology* 150: 5405-5414.

Nastri CO, Ferriani RA, Rocha IA and Martins WP (2010). Ovarian hyperstimulation syndrome: pathophysiology and prevention. *J. Assist. Reprod. Genet*. 27: 121-128.

Neulen J, Yan Z, Raczek S, Weindel K, et al. (1995). Human chorionic gonadotropin-dependent expression of vascular endothelial growth factor/vascular permeability factor in human granulosa cells: importance in ovarian hyperstimulation syndrome. *J. Clin. Endocrinol. Metab*. 80: 1967-1971.

Nishimura R and Okuda K (2010). Hypoxia is important for establishing vascularization during corpus luteum formation in cattle. *J. Reprod. Dev*. 56: 110-116.

Ravindranath N, Little-Ihrig L, Phillips HS, Ferrara N, et al. (1992). Vascular endothelial growth factor messenger ribonucleic acid expression in the primate ovary. *Endocrinology* 131: 254-260.

Redmer DA and Reynolds LP (1996). Angiogenesis in the ovary. *Rev. Reprod*. 1: 182-192.

Semenza GL (2000). Expression of hypoxia-inducible factor 1: mechanisms and consequences. *Biochem. Pharmacol*. 59: 47-53.

Shimizu T and Miyamoto A (2007). Progesterone induces the expression of vascular endothelial growth factor (VEGF) 120 and Flk-1, its receptor, in bovine granulosa cells. *Anim. Reprod. Sci*. 102: 228-237.

Shimizu T, Jayawardana BC, Tetsuka M and Miyamoto A (2007). Differential effect of follicle-stimulating hormone and estradiol on expressions of vascular endothelial growth factor (VEGF) 120, VEGF164 and their receptors in bovine granulosa cells. *J. Reprod. Dev*. 53: 105-112.

Tamanini C and De Ambrogi M (2004). Angiogenesis in developing follicle and corpus luteum. *Reprod. Domest. Anim*. 39: 206-216.

van den Driesche S, Myers M, Gay E, Thong KJ, et al. (2008). HCG up-regulates hypoxia inducible factor-1 alpha in luteinized granulosa cells: implications for the hormonal regulation of vascular endothelial growth factor A in the human corpus luteum. *Mol. Hum. Reprod*. 14: 455-464.

Wang GL and Semenza GL (1995). Purification and characterization of hypoxia-inducible factor 1. *J. Biol. Chem*. 270: 1230-1237.

Wang GL, Jiang BH, Rue EA and Semenza GL (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc. Natl. Acad. Sci. U. S. A*. 92: 5510-5514.

Wulff C, Dickson SE, Duncan WC and Fraser HM (2001). Angiogenesis in the human corpus luteum: simulated early pregnancy by HCG treatment is associated with both angiogenesis and vessel stabilization. *Hum. Reprod*. 16: 2515-2524.

Yaba A, Bianchi V, Borini A and Johnson J (2008). A putative mitotic checkpoint dependent on mTOR function controls cell proliferation and survival in ovarian granulosa cells. *Reprod. Sci*. 15: 128-138.

Young FM, Rodger FE, Illingworth PJ and Fraser HM (2000). Cell proliferation and vascular morphology in the marmoset corpus luteum. *Hum. Reprod*. 15: 557-566.

Zhang Z, Yin D and Wang Z (2011). Contribution of hypoxia-inducible factor-1alpha to transcriptional regulation of vascular endothelial growth factor in bovine developing luteal cells. *Anim. Sci. J*. 82: 244-250.

Zhong H, Chiles K, Feldser D, Laughner E, et al. (2000). Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res*. 60: 1541-1545.