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**—Full Paper—**

# 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

**Takashi SHIMIZU[1)], Barana C. JAYAWARDANA[1)], Masafumi TETSUKA[2)] and Akio MIYAMOTO[1)]**

*[1)]Graduate School of Animal and Food Hygiene, [2)]Department of Agriculture and Life Science, Obihiro University of Agriculture and Veterinary Medicine, Inada-machi, Obihiro, Hokkaido 080-8555, Japan*

**Abstract.** Vascular endothelial growth factor (VEGF) isoforms (VEGF 120 and VEGF 164) secreted by granulosa cells are involved in thecal angiogenesis during follicular development in the bovine ovary. However, whether the transcript of the VEGF120 and VEGF164 isoforms differs during follicular development in the ovary is still unknown. We first examined the gene expression of VEGF120, VEGF164, fms-like tyrosine kinase (Flt-1), and fetal liver kinase (Flk-1) in the granulosa cells (GCs) and theca cells (TCs) of pre-selection and post-selection follicles (PRF and POF respectively) from the bovine ovary. Then we examined the effects of FSH and estradiol (E2) on these factors in cultured bovine GCs. Messenger RNA (mRNA) expression was quantified using real-time PCR methods. The concentrations of E2 and P4 in the follicular fluid (FF) of the PRF and POF were estimated using an enzyme immunoassay (EIA). The concentrations of E2 and P4 in the FF were significantly higher in the POF than in the PRF. The ratio of E2/P4 in PRF and POF was 0.37 and 3.8, respectively. The expression levels of the VEGF120, VEGF164, and Flk-1 mRNAs in the GCs of POF with high E2 concentration were higher than those of PRF. The levels of the Flt-1 and Flk-1 mRNAs in the TCs were not different between PRF and POF. Since E2 in the FF of the POF used in the present study was high compared with the PRF, we examined the effects of E2 and FSH on the expression of the above genes using cultured GCs. Expression of VEGF120 mRNA was induced by a low concentration (1 ng/ml) of E2, whereas the levels of VEGF164 and Flk-1 mRNAs were not affected by E2. FSH stimulated the expression of the VEGF isoforms and Flk-1 genes. Moreover, the expression of those genes was enhanced when low E2 (1 ng/ml) was added to FSH. In conclusion, our data indicates that the VEGF isoforms have a follicle stage-dependent expression pattern. Thus, our results suggest that the expression of VEGF isoforms may be associated with characterization of the preovulatory phenotype during follicle development in the bovine ovary.

**Key words:** Estradiol, Fetal liver kinase (Flk-1), Fms-like tyrosine kinase (Flt-1), Follicle-stimulating hormone (FSH), Granulosa cell, Vascular Endothelial Growth factor (VEGF)

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Correspondence: T. Shimizu (e-mail: shimizut@obihiro.ac.jp)

Vascular endothelial growth factor (VEGF) was originally isolated based on its ability to specifically stimulate microvascular endothelial cells to proliferate [1–4]. VEGF mRNA was first

detected in the thecal and granulosa cell layers of late secondary follicles [5]. An increase in VEGF mRNA expression is observed within the granulosa cells of follicles in the tertiary stage, whereas in atretic follicles, VEGF expression is reduced in the granulosa cells and is no longer detectable in the theca cells [5]. Five molecular forms of VEGF are produced as a result of alternative splicing; three of them (VEGF120, VEGF 145, and VEGF 165) are soluble isforms, and the other two larger forms (VEGF 188 and VEGF 205) are generally membrane bound [6]. VEGF 120 and VEGF 164 are expressed in the mammalian ovary [7, 8] and are associated with follicular angiogenesis during follicular development [9, 10]. *In vivo* injection of VEGF gene or protein induces the emergence of a large number of preovulatory and antral follicles [11–13]. These reports indicate that VEGF is a crucial factor during follicular development in the ovary.

VEGF acts via two tyrosine kinase-family receptors, namely fms-like tyrosine kinase (Flt-1) and fetal liver kinase (Flk-1) [14]. The mRNA for the Flt-1 and Flk-1 receptors, which is localized in the endothelial cells of the theca of late secondary follicles, increases in the theca of tertiary follicles and decrease in atretic follicles [5]. Our previous study showed that the expression of the Flt-1 gene increased more than the expression of the Flk-1 gene in the theca cells when VEGF was overexpressed in the granulosa cells by direct ovarian injection of its gene fragment [11]. In addition, a study using the equine chorionic gonadotropin (eCG)-treated pigs indicated that the differences between the control and eCG groups in VEGF 120 gene expression in medium and large follicles were larger than those for VEGF 164 [9]. These results led us to hypothesize that the actions of the VEGF120 and VEGF164 isoforms may differ depending on the stage of follicular development in the ovary.

To test this hypothesis, we first investigated the expression of VEGF120 and VEGF 164 mRNAs in granulosa cells (GCs) and the mRNA levels for their receptors in the GCs and theca cells (TCs) of pre-selection and post-selection follicles collected from the bovine ovary. Then, we further examined the hormonal effects of FSH and estradiol-17$\beta$ (E2) on the levels of mRNA expression of VEGF120, VEGF164, and their receptors in cultured bovine granulosa cells.

## Materials and Methods

*Sample collection*

Paired ovaries were obtained from 21–26-month-old non-parous Holstein x Japanese Black F1 heifers at a local slaughterhouse. Only ovarian pairs with a corpus luteum (CL) and apparently normal follicles were used in the present study. Follicular fluid (FF) was aspirated from selected follicles using a syringe fitted with a 20G needle and kept at –20 C. The theca cell layer and granulosa cells were harvested from the aspirated follicles. The tissue samples were placed in RNA*later* (Ambion, Austin, TX, USA) and frozen at –30 C. Follicles were classified into two groups based on diameter [post-selection follicles (POF) were 11.0–19.0 mm in diameter; pre-selection follicles (PRF) were 7.0–8.5 mm in diameter].

*Bovine granulosa cell culture and hormone treatment*

Ovaries were obtained at a slaughterhouse from cows and heifers just after slaughter. After transport to the laboratory at 30 C, the ovaries were washed three times with prewarmed McCoy's 5A medium. Granulosa cells were collected from small size follicles (4–7 mm) by aspiration using a needle (18 gauge) and syringe (plastic, 10 ml) and were washed in Dulbecco's Modified Eagle's Medium/F12 medium (DMEM/F12). The number of viable cells was determined using trypan blue exclusion. Then, the cell suspension was centrifuged, resuspended, and seeded at a density of $5 \times 10^5$ viable cells per well (24-well culture plate) in 1 ml of DMEM/F12 containing 10% fetal calf serum (FCS) and antibiotics. The cells were cultured for 24 h at 37 C in a 5% $CO_2$ atmosphere, and then the wells were washed with DMEM/F12 to remove unattached cells and any remaining tissue debris. The medium was replaced with serum-free medium supplemented with E2 (1–100 ng/ml) and FSH (1–10 ng/ml) at several concentrations, and culture was continued for 6 h. Treatments were terminated by aspirating the medium and rinsing the cells twice with phosphate-buffered saline, and then the cells were used for RNA extraction. All experiments were repeated three times in triplicate.

*RNA extraction and reverse transcription (RT)*

Tissue samples were homogenized in denaturing solution containing 4 M guanidium thiocyanate (Wako Pure Chemical, Osaka, Japan), 25 mM

**Table 1.** Primer pairs used for detection of mRNAs

| Target gene | Sequence | Size (bp) | [a]EMBL/Reference |
|---|---|---|---|
| VEGF120 | Forward: 5′-GCC TCG GCT TGT CAC ATT TT-3′<br>Reverse: 5′-ACC GCC TCG GCT TGT CAC-3′ | 254 | [26] |
| VEGF164 | Forward: 5′- AGC AAG GCC CAC AGG GAT TT-3′<br>Reverse: 5′- ACC GCC TCG GCT TGT CAC-3′ | 310 | [26] |
| Flt-1 | Forward: 5′- GAA GGA CGG GAT GAG GAT GC-3′<br>Reverse: 5′-ATG GCG TTG AGC GGA ATG TA-3′ | 186 | X94263 |
| Flk-1 | Forward: 5′-TGG CCC AAC AAT CAG AGC AG-3′<br>Reverse: 5′-GAA CGG AGC CCA TGT CAG TG-3′ | 154 | X94298 |
| $\beta$-actin | Forward: 5′-CCA AGG CCA ACC GTG AGA AGA T -3′<br>Reverse: 5′-CCA CGT TCC GTG AGG ATC TTC A-3′ | 256 | K00622 |

a: EMBL accession number or reference for the published sequence.

sodium citrate, 0.5% sarkosyl (Sigma Chemical, St. Louis, MO, USA), and 0.1M $\beta$-mercaptoethanol (Kanto Chemical, Tokyo, Japan). Total RNA was extracted with phenol chloroform, further purified and treated with DNase using a commercial kit (SV total RNA Isolation System; Promega, Madison, WI, USA), and frozen at –20 C in THE RNA Storage Solution (Ambion). Single-stranded cDNA was reverse transcribed from total RNA (0.5–1.0 $\mu$g) using a 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Indianapolis, IN, USA) and a random primer. Each RT cycle consisted of 10 min of annealing at 25 C, 60 min of cDNA synthesis at 42 C, and 5 min of inactivation at 99 C.

*Quantitative real-time PCR*

Transcripts for VEGF120, VEGF164, Flt-1, Flk-1, and $\beta$-actin were quantified by real-time PCR with a LightCycler (Roche Diagnostics) using a commercial kit (QuantiTect™ SYBR® Green PCR; Qiagen, Hilden, Germany). The primers were designed using Primer-3 based on the bovine sequences (Table 1). The Primers for VEGF 120 and VEGF 164 were designed by Wellmann *et al*. [26]. The amplification program consisted of an initial 15-min activation at 95 C followed by 40 cycles of PCR (each cycle consisting of 15 sec of denaturation at 94 C, 30 sec of annealing at 58 C and 20 sec extension at 72 C). For quantification of the target genes, a series of standards were constructed by amplifying a fragment of DNA (to 700 bp) that contained the target sequence for real-time PCR (100 – 150 bp). The PCR products were subjected to electrophoresis, and the target band was cut out and purified using a DNA purification kit (SUPREC™-01, TaKaRa Bio, Ohtsu, Japan). Three to five serially diluted DNA standards were included in every PCR run. The values were normalized using $\beta$-actin as the internal standard.

*Steroid hormone assay*

Concentrations of E2 and progesterone (P4) in the FF samples were estimated by an enzyme immunoassay (EIA) as described previously [15, 16]. Steroid hormones were extracted with diethyl ether. The extraction efficiency was 85%. The ranges of the standard curves were 2–2,000 pg/ml for E2 and 0.05–50 ng/ml for P4. The intra- and interassay coefficients of variation were 6.2 and 8.5% for E2 and 4.5 and 7.4% for P4.

*Data analysis*

All data are presented as means ± SEM. The differences in expression of VEGF120, VEGF164, Flk-1, and Flt-1 and in the concentrations of estradiol and progesterone in follicular fluid between POF and PRF were analyzed by Student's *t*-test. Data for several factors in relation to the treated bovine granulosa cells were tested for the significance of differences using ANOVA followed by the Fisher's LSD test as a multiple comparison test. Differences were considered significant at $P<0.05$ or less.

## Results

*Characteristics of follicles*

Table 2 shows the conditions of the ovarian follicles used in this study. The concentrations of estradiol and progesterone in the follicular fluid increased significantly in the post-selection follicles

**Table 2.** Mean follicle diameter, concentrations of estradiol (E) and progesterone (P) in the follicular fluid, and E/P ratio in the cattle

| Follicle | Mean follicle diameter (mm) | Follicular fluid | | E/P ratio |
|---|---|---|---|---|
| | | Estradiol (ng/ml) | Progesterone (ng/ml) | |
| Pre-selection follicles (n=7) | 7.8 ± 0.2 | 3.14 ± 1.0* | 13.2 ± 3.0* | 0.37 ± 0.1* |
| Post-selection follicles (n=7) | 15.3 ± 1.2 | 81.8 ± 14.5 | 21.8 ± 2.1 | 3.8 ± 0.6 |

Values are means ± SEM. *: P< 0.05 versus post-selection follicles.

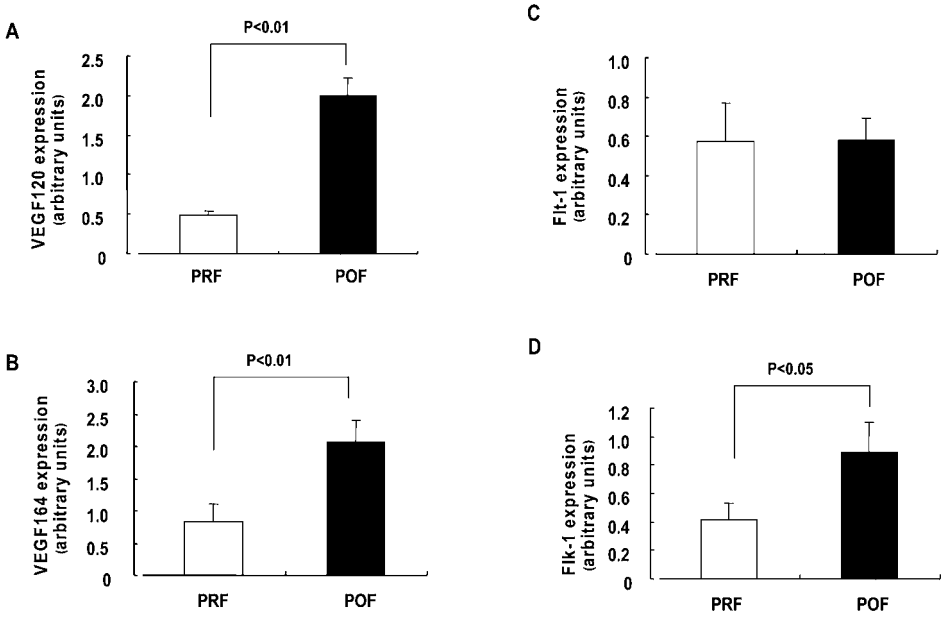


**Fig. 1.** Expression of VEGF120 (A), VEGF164 (B), Flt-1 (C) and Flk-1 (D) mRNAs in the granulosa cells of pre-selection (PRF, n=7) and post-selection (POF, n=7) follicles. The data are expressed as means ± SEM. The expression of each factor was normalized on the basis of $\beta$-actin expression.

(POF) compared to the pre-selection follicles (PRF). The ratio of E2/P4 in the POF and PRF were 3.8 ± 0.6 and 0.37 ± 0.1, respectively.

*Messenger RNA expression of VEGF120, VEGF164, and related receptors in the granulosa and theca cells of the POF and PRF*

The expression of VEGF120 and VEGF164 mRNAs in the GCs of the POF with a high E2 concentration was higher than in those of the PRF (Figs. 1A and B). On the other hand, for receptors, the levels of Flk-1 mRNA increased significantly in the GCs of the POF compared to those of the PRF, while the level of Flt-1 mRNA was the same in the POF and PRF (Figs. 1C and D). The levels of Flt-1 and Flk-1 mRNAs in the TCs did not differ between the POF and PRF (Figs. 2A and B).

*Effect of estradiol on the expression of VEGF120, VEGF164, and Flk-1 mRNAs in cultured granulosa cells*

Expression of VEGF120 mRNA was induced in the cultured GCs with a low concentration (1 ng/ml) of E2 (Fig. 3A). In contrast, the levels of VEGF164 and Flk-1 mRNA were not affected by E2 (Figs. 3B and C).

*Effect of FSH on the expression of VEGF120, VEGF164, and Flk-1 mRNAs in cultured granulosa cells*

Expression of VEGF120 mRNA in the cultured GCs increased when 10 ng/ml FSH was added to

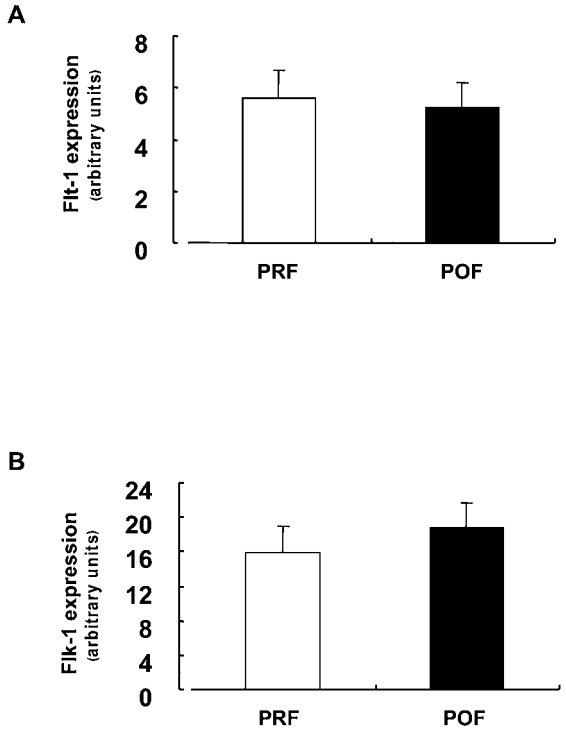


**Fig. 2.** Expression of Flt-1 (A) and Flk-1 (B) in the theca cells of pre-selection (PRF, n=7) and post-selection (POF, n=7) follicles. The data are expressed as means ± SEM. The expression of each factor was normalized on the basis of beta-actin expression.

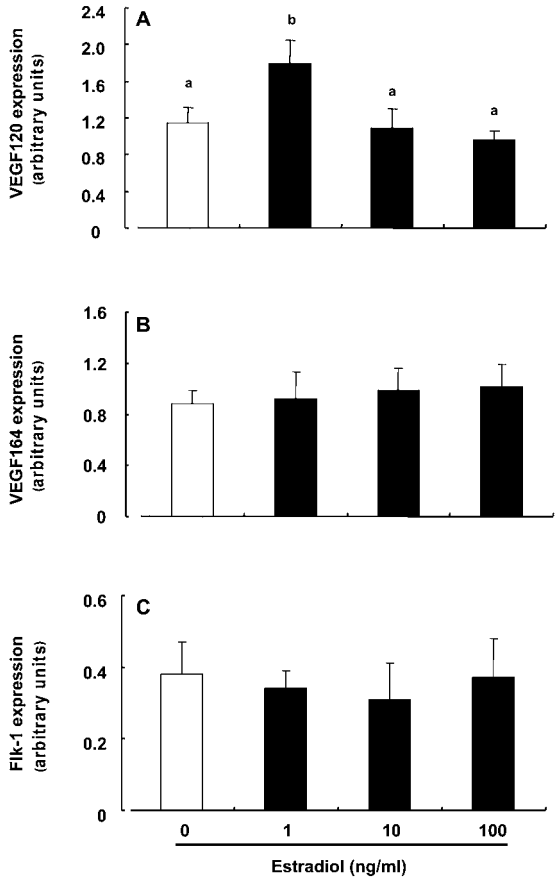


**Fig. 3.** Effect of estradiol on the expression of VEGF120 (A), VEGF164 (B), and Flk-1 (C) mRNAs in cultured bovine granulosa cells. E2 was added to the culture medium at each indicated concentration. The data are expressed as means ± SEM of three separate experiments with triplicate determinations in each. Different superscripts denote significantly different values ($P<0.05$).

the culture medium (Fig. 4A). Addition of 1 ng/ml FSH significantly increased VEGF164 mRNA expression compared with the other concentrations tested (Fig. 4B). Expression of the Flk-1 gene was significantly increased by 1 to 10 ng/ml FSH (Fig. 4C).

*Effect of E2 plus FSH on the expression of VEGF120, VEGF164, and Flk-1 mRNAs in cultured granulosa cells*

The combination of 1 ng/ml E2 and 1 ng/ml FSH stimulated expression of the VEGF120, VEGF164, and Flk-1 genes (Fig. 5 A-C). At a constant concentration of 1 ng/ml E2, the expression of the VEGF120 and Flk-1 genes was more markedly stimulated by 5 ng/ml FSH than 1 ng/ml FSH (Fig. 5A and C). In contrast, the expression of the VEGF164 gene did not change as the FSH concentration was increased (Fig. 5B).

## Discussion

The results of the present study indicate that expression of the VEGF120, VEGF 164, and Flk-1 genes increased in the GCs of the post-selection follicles, which possess high E2 levels compared with those of the pre-selection follicles, and that these factors were upregulated by E2 and FSH in the cultured GCs. We found that the expression level of Flt-1 gene in the GCs remained constant across the periods of follicle selection, whereas Flk-1 expression in the GCs increased from the pre-selection to post-selection period. A recent study

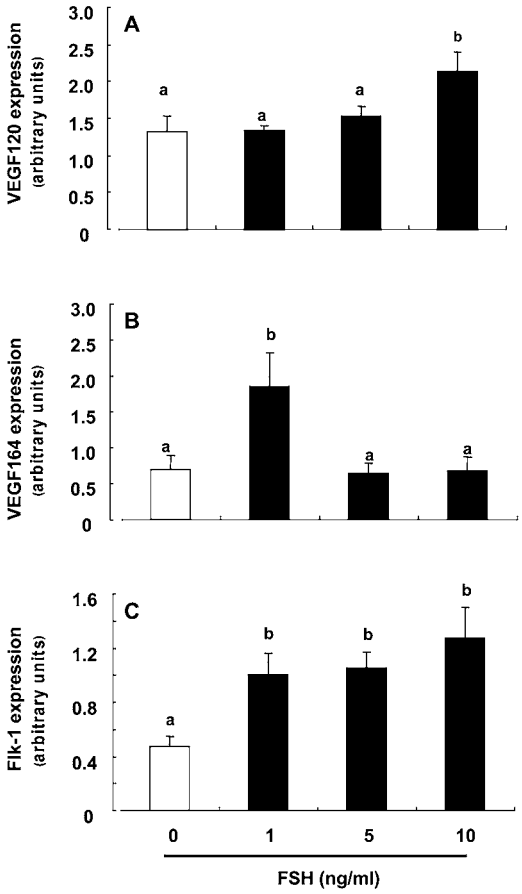


**Fig. 4.** Effect of FSH on the expression of VEGF120 (A), VEGF164 (B) and Flk-1 (C) mRNAs in cultured bovine granulosa cells. Each indicated concentration of E2 was added to the culture medium. The data are expressed as means ± SEM of three separate experiments with triplicate determinations in each experiment. Different superscripts denote significantly different values (P<0.05).

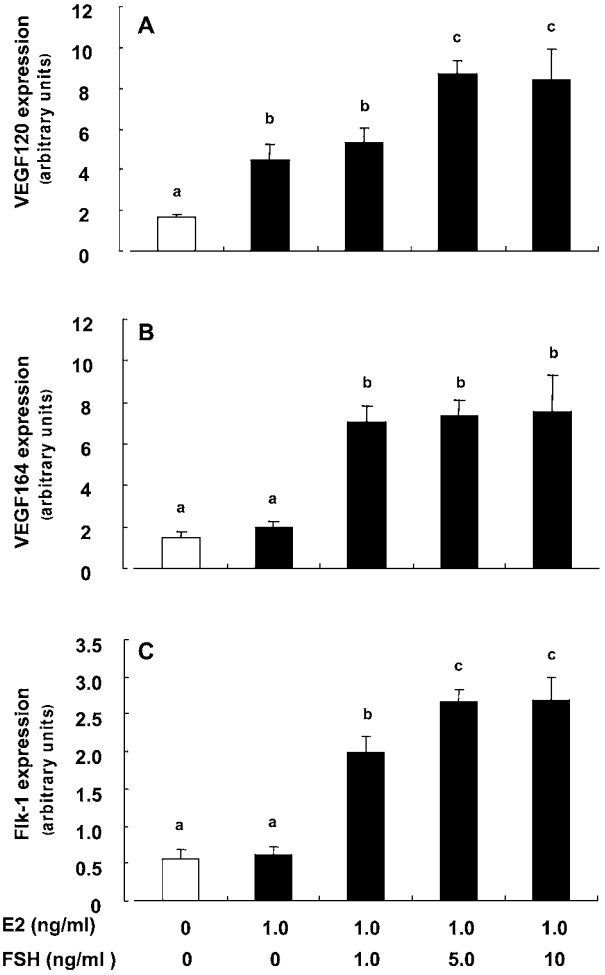


**Fig. 5.** Effect of E2 and FSH on the expression of VEGF120 (A), VEGF164 (B), and Flk-1 (C) mRNAs in cultured bovine granulosa cells. Each indicated concentration of estradiol was added to the culture medium. The data are expressed as means ± SEM of three separate experiments with triplicate determinations in each. Different superscripts denote significantly different values (P<0.05).

demonstrated that VEGF inhibited apoptosis in GCs via Flk-1 [17]. Therefore, we speculate that Flk-1 is essential for transducing the biological actions of VEGF in bovine GCs. On the other hand, expression of the Flt-1 and Flk-1 genes in TCs did not change developing on the period of follicle selection in the present study, which is similar to the findings of a previous study [8]. These results show that pre-selection follicles already sufficiently express the genes for both receptors in the TCs. In contrast, in the porcine ovary, the expression of Flk-1/KDR mRNA increases in the thecal tissue of medium and large follicles after eCG treatment in parallel with the expression of VEGF 120 and VEGF 164 mRNAs [9]. This difference may be related to the difference in the form of follicular growth (mono- versus multiovular).

The follicular fluid of dominant follicles contains higher amount of E2 than that of subordinate follicles in the bovine ovary [18, 19]. In fact, our results indicate that the concentrations of E2 and P4 in the follicular fluid of the post-selection follicles (E2/P4>1) were higher than those of the pre-selection follicles (E2/P4<1). Our data also indicated that E2 stimulates the expression of the

VEGF120 but not the VEGF164 gene in bovine GCs. These results suggest that the expression of VEGF genes induced by E2 may differ among isoforms in the GCs during follicle development in the bovine ovary. Estrogens increase the expression of VEGF mRNA by binding to the specific estrogen response elements in the VEGF gene [20] and have been shown to rapidly upregulate VEGF mRNA expression in the mouse [21], rat [22, 23], and sheep [24] uterus and in cultured bovine GCs [25]. Therefore, our data suggests that the transcription of VEGF 120 but not VEGF 164 genes may be induced via estrogen receptors.

VEGF 120 and VEGF 164 expression increases in the GCs of follicles from the eCG-treated porcine ovary [9]. Our results demonstrate that FSH stimulates the expression of the VEGF120, VEGF164, and Flk-1 genes in bovine GCs. Interestingly, expression of the VEGF164 gene was stimulated at low concentration of FSH (1 ng/ml), whereas VEGF 120 expression was induced by a high FSH concentration (10 ng/ml). This result suggests that FSH may influence the abundance of VEGF isoform transcript in bovine GCs. However, it is still unknown how FSH induces the different expression patterns of VEGF isoforms.

In the present study, the expression levels of the VEGF120, VEGF164, and Flk-1 genes in the GCs increased at low estradiol concentration (1 ng/ml) when FSH was also added. These results suggest that a basal level of estradiol enhances the effect of FSH on the expression of these genes. Further studies in vivo are necessary to confirm whether the preovulatory follicle in the cow shows enhanced expression of VEGF isoforms and their receptors.

In conclusion, our data demonstrates that estradiol and FSH stimulated the expression of the VEGF120 and VEGF 164 genes, and that Flk-1 expression is FSH-dependent, suggesting a follicle stage-dependent expression pattern for VEGF isoforms. Thus, our study suggests that the expression of VEGF isoforms may be associated with the characterization of the dominant follicle (preovulatory phenotype) during follicle development in the bovine ovary. Furthermore, our results strongly suggest that the transcription system for VEGF isoform genes may have different pathways by hormonal stimulation in bovine granulosa cells.

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