*Journal of Reproduction and Development*, Vol. 49, No. 3, 2003

**—Original—**

# Messenger Ribonucleic Acid Expressions of Hepatocyte Growth Factor, Angiopoietins and Their Receptors During Follicular Development in Gilts

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**Abstract.** Angiogenic factors are associated with angiogenesis during follicular development in the mammalian ovary. The aim of the present study was to determine the relationships between the vascular network and mRNA expressions of angiopoietins (Ang)-1, Ang-2 and hepatocyte growth factor (HGF), and their receptors in follicles at different developmental stages during follicular development. Ovaries in gilts were collected 72 h after equine chorionic gonadotropin (eCG, 1250 IU) treatment for histological observation of the capillary network. Granulosa cells and thecal tissues in small (<4 mm), medium (4–5 mm) or large (>5 mm) individual follicles were collected for detection of mRNA expression of HGF, Ang-1 and Ang-2 in granulosa cells, and HGF receptor (HGF-R) and Tie-2 in the theca cells by semi-quantitative RT-PCR. The number of capillaries in the thecal cell layer increased significantly in healthy follicles at all developmental stages in the eCG group compared with those in controls. The expression of Ang-1 mRNA declined in granulosa cells of medium and large follicles and the level of Ang-2 mRNA increased in granulosa cells of small follicles after eCG treatment. The ratio of Ang-2/Ang-1 increased in small, medium and large follicles from ovaries after eCG treatment, but Tie-2 mRNA expression in the theca cells did not change. The level of HGF mRNA increased in granulosa cells of small follicles after eCG treatment but HGF-R in theca cells was not increased by eCG. These data suggested that the angiopoietins might be associated with thecal angiogenesis during follicular development in eCG-treated gilts.

**Key words:** Follicular Development, Angiogenesis, angiopoietins, HGF, Pig

(**J. Reprod. Dev. 49:** 203–211, 2003)

The development of the vascular network in the ovary is closely associated with follicular development. In the mammalian ovarian follicle only the thecal cell layer is well vascularized, whereas the granulosa layer is avascular [1]. The development of vascular networks in the theca cell layer of the follicle is induced by angiogenic factors. In the ovary, the angiogenic factors produced by granulosa cells [2, 3] help to maintain the vasculature and health of the dominant follicles [4].

Recently, many angiogenic factors involved in the regulation of angiogenesis have been identified. There is increasing evidence that vascular endothelial growth factor (VEGF) is an essential factor for follicular development [2, 3, 5]. VEGF effects are complemented and coordinated by another class of angiogenic factors, the angiopoietins [6]. VEGF acts during the early stages of vessel development [7, 8], whereas angiopoietin-1 (Ang-1) acts later to promote angiogenic remodeling including vessel maturation and stabilization [9–12]. In addition, Ang-1 acts as an endothelial cell survival factor [9, 13, 14]. Ang-2 is a natural antagonist for Ang-1, opposing the

Accepted for publication: March 3, 2003
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effect of Ang-1-mediated stabilization by promoting a more plastic state in the capillary endothelium [6]. Previous studies have reported that Ang-1 and Ang-2 mRNA expression was detected in the ovary of the rat [6], human [15], monkey [16, 17] and cow [18].

Hepatocyte growth factor (HGF), also called scatter factor, is a powerful activator of growth and motility of mouse and human endothelial cells [19, 20]. The inhibitors of tyrosine kinases, effective on HGF receptors, inhibited HGF-induced activation of endothelial cells [19]. HGF is a potent angiogenic molecule and is overexpressed in invasive human cancers, including breast cancer, relative to non-invasive cancers [21]. In the ovary, HGF mRNA expression has been detected in the human [22, 23] and bovine [24] ovary.

The diverse biological activities of angiopoietins (Ang-1 and Ang-2) and HGF are mediated via their receptors in target cells. A receptor ligand of angiopoietins has been identified which activates endothelial cell-specific tyrosine kinase receptors known as Tie-2 receptors [6, 14]. The expression of Tie-2 mRNA is detected in the human corpus luteum [15]. The receptor of HGF (HGF-R) is the product of the c-met protooncogene that is primarily localized to epithelial cells [25–28].

The action of angiopoietins on angiogenesis in human corpus luteum is well documented [15] but virtually nothing is known about the role of it during thecal angiogenesis of porcine follicle development. Also the action of HGF during thecal angiogenesis is unknown. Therefore, to gather some fundamental information about follicular angiogenesis, we investigated thecal angiogenesis and mRNA expression of Ang-1, Ang-2 and HGF in granulosa cells and of Tie-2 and HGF-R in thecal tissues in individual follicles isolated from the ovaries of eCG-treated gilts.

## Materials and Methods

*Animals and hormone treatment*

Eight prepubertal gilts with an average weight of 65 kg were used and divided into two groups. One group (n=4) was injected i.m. with 1250 IU of equine chorionic gonadotropin (eCG, Teikoku Zouki Pharmaceutical Co., Tokyo, Japan) to induce follicular development, and the other group (n=4) was injected with saline as controls. After anesthetization by injection of ketamine hydrochloride (6 ml/gilt; Sankyo Co., LTD., Tokyo, Japan) and atropine sodium salt (0.5 mg/gilt; Tanabe Co., LTD., Tokyo, Japan), all eight animals were ovariectomized 72 h after the eCG or saline injections. The present study was approved by the Ethics Committee for Care and Use of Laboratory Animals for Biomedical Research of the Graduate School of Agricultural Science, Tohoku University.

*Histological examination of the follicular capillaries*

The right ovary from each animal was fixed in 4% paraformaldehyde solution. After fixing, the ovary was embedded in paraffin wax and sectioned serially at 8 $\mu$m in thickness. Every tenth section was mounted and stained with hematoxylin-eosin. To avoid counting individual follicles more than once, oocytes with nuclei were used as a mark, and the size of the follicle in which the oocyte was present was measured using an ocular micrometer. On the basis of their diameter, the follicles were separated into three classes: small, 3.0–3.9 mm; medium, 4.0–5.0 mm and large, >5.0 mm. All capillaries larger than 10 $\mu$m in diameter in the whole theca interna section of follicles larger than 3.0 mm in diameter were counted under 200× magnification (20× objective lens and 10× ocular lens). Sixteen, thirteen and forty-five follicles 3.0–3.9 mm; 4.0–5.0 mm and >5.0 mm in diameter, respectively, were counted (one section per follicle) to quantify the average numbers of capillaries surrounding follicles.

*Analysis of mRNA expression of angiopoietins, HGF and their receptors*

Follicles were isolated from the left ovaries following the method reported previously [2]. Briefly, single follicles were isolated in dissection medium (Dulbecco's phosphate buffer medium supplemented with 0.4% BSA). After measuring the diameter with a calibrated grid, each healthy follicle was punctured in a 1.5 ml eppen tube. The follicle wall obtained from each follicle was transferred to dissection medium for mechanical separation of the granulosa cells from thecal tissues by the gentle scraping of the follicles with a small spatula. The medium containing dispersed granulosa cells was collected and centrifuged, and the theca cells were then vigorously vortexed and carefully washed in order to remove any possible granulosa cell contamination. Thecal tissues and

granulosa cells were then stored in liquid nitrogen before analysis of mRNA expression.

Total cellular RNA was extracted from granulosa cell and thecal tissues with RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan). A semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using Ready To Go RT-PCR Beads (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) with 100 ng RNA following the method described by the manufacturer. The RT reaction was carried out at 42 C for 15 min and samples were incubated for reactions at 95 C for 5 min to inactivate the reverse transcriptase and to completely denature the template. The oligonucleotide primers for angiopoietins, HGF and their receptors, and the amplification profiles (dissection, annealing, extension and cycle) are shown in Tables 1 and 2, respectively. The RT-PCR products were electrophoresed on a 2% agarose gel. The bands were quantified by densitometry using the NIH Image 1.63 analysis program (NIH, Bethesda, Maryland). Beta-actin mRNA has been found in pig follicle cells with levels that are independent of follicle status and size [29], and its expression is not affected by growth factors and gonadotropins [30, 31]. Therefore, each gene mRNA level in the present study was normalized on the basis of beta-actin mRNA content. The results obtained were referred to as single follicles and are classified on the basis of diameters: small, <4 mm; medium, 4–5 mm; large, >5 mm.

*Statistical analysis*

All data are presented as mean ± SEM. Significant differences in the number of capillaries in theca interna between the control and eCG groups were analyzed by Student's *t*-test. The mRNA levels of angiopoietins, HGF and their receptors were normalized on the basis of $\beta$-actin mRNA content, and significant differences in each of the genes among three developmental stages between the control and eCG groups were analyzed by ANOVA, followed by Fisher's LSD as a multiple comparison test. Differences were considered significant at $P<0.05$ or less.

## Results

*Vascular population following eCG treatment*

eCG treatment significantly increased the number of capillaries in the theca interna of follicles at different developmental stages (Fig. 1). Moreover, in the eCG group, the number of capillaries in the theca interna increased as the follicles grew. The number of capillaries in the atretic follicles did not change in both groups (data not shown).

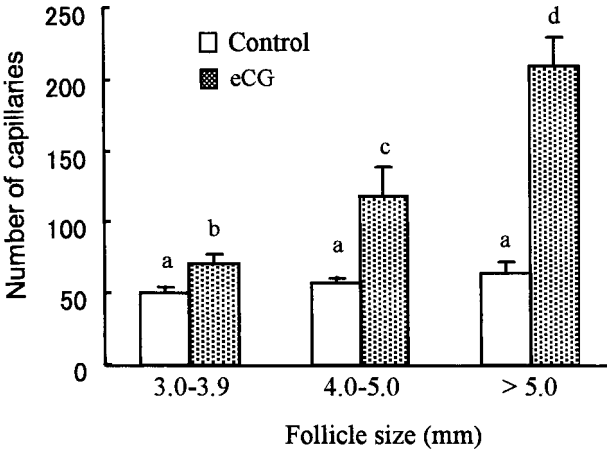


**Fig. 1.** The number of capillaries in the theca interna of small (3.0–3.9 mm), medium (4.0–5.0 mm) and large (>5.0 mm) follicles in ovaries of gilts with (n=4) or without (n=4) eCG treatment. Values with different superscripts are significantly different from each other at $P<0.05$.

*Expression of Ang-1 and -2 mRNA, and ratio of Ang-2/Ang-1 in granulosa cells and Tie-2 mRNA in thecal tissues*

The expressions of Ang-1 and Ang-2 mRNA in granulosa cells in both groups decreased as the follicles grew. The mRNA levels of Ang-1 decreased significantly in granulosa cells of medium and large follicles in the eCG group compared with those in the control group (Fig. 2, upper panel). The expression of Ang-2 mRNA in granulosa cells of medium and large follicles did not change between the two groups. However, the mRNA levels of Ang-2 in small follicles increased more significantly in the eCG group than in the control group (Fig. 2 middle panel). As Ang-1 and Ang-2 have opposing effects, the relative ratio of Ang-2/Ang-1 was calculated to demonstrate the net effect of their changes during follicular development. The Ang-2/Ang-1 ratio increased significantly in follicles at all developmental stages after eCG treatment (Fig. 2, lower panel). The mRNA level of Tie-2 in thecal tissues in both

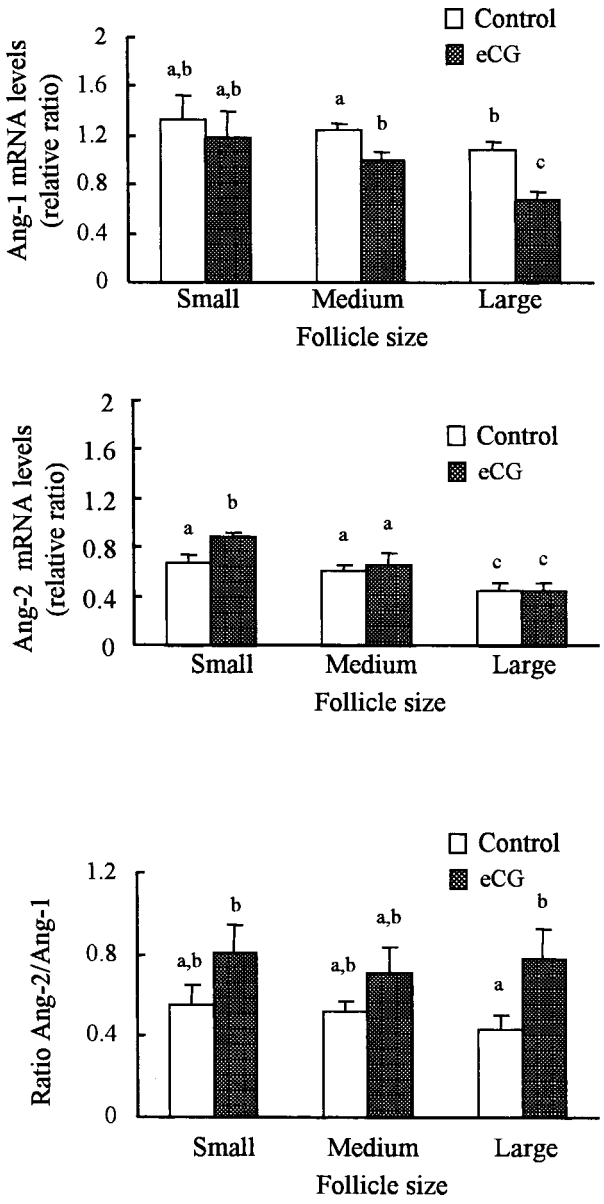


**Fig. 2.** Expression of Ang-1 (upper panel) and Ang-2 (middle panel) mRNAs and Ang-2/Ang-1 ratio (lower panel) in granulosa cells of small (diameter, <4 mm), medium (diameter, 4–5 mm) and large (diameter, >5 mm) follicles from prepubertal gilts with (closed bar) or without (open bar) eCG treatment. The expressions of each factor were normalized on the basis of $\beta$-actin mRNA content. The relative ratio of Ang-2/Ang-1 was calculated to demonstrate the net effect of their changes during follicular development. The data are indicated as the mean ± se (n=4). Different superscripts denote significantly different values ($P<0.05$).

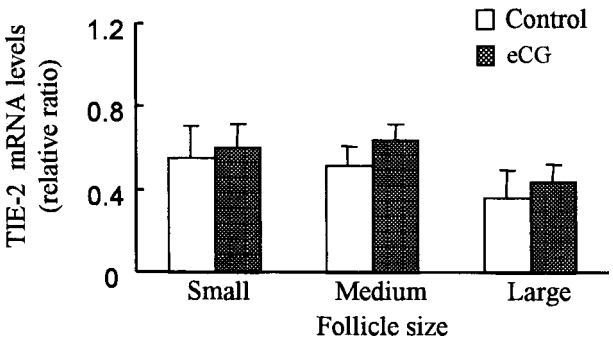


**Fig. 3.** Expression of Tie-2 mRNAs in thecal tissues of small (diameter, <4 mm), medium (diameter, 4–5 mm) and large (diameter, >5 mm) follicles from prepubertal gilts with (closed bar) or without (open bar) eCG treatment. The expressions of Tie-2 were normalized on the basis of $\beta$-actin mRNA content. The data are indicated as the mean ± se (n=4).

groups remained unchanged among the three follicle sizes (Fig. 3).

*Expression of HGF mRNA in granulosa cells and HGF-R mRNA in thecal tissues*

The level of HGF mRNA in granulosa cells of follicles at different developmental stages in the control group did not change, whereas those in the eCG group decreased as the follicles grew (Fig. 4, upper panel). The level of HGF mRNA in small follicles in the eCG group only increased significantly compared with those in the control group. The expression of HGF-R mRNA in thecal tissues of follicles at different developmental stages were not changed by eCG treatment (Fig. 4, lower panel).

## Discussion

The present study demonstrated that the number of capillaries in theca interna and the Ang-2/Ang-1 ratio increased in follicles at different developmental stages in eCG-treated gilts. Tie-2, Ang-1 and Ang-2 receptor, was expressed in sufficient quantity in thecal tissues before eCG treatment. The level of HGF mRNA increased in granulosa cells of small follicles after eCG treatment but HGF-R in theca cells was not increased by eCG. These findings are the first evidence that the angiopoietins-Tie-2 system may be involved in the formation of the capillary

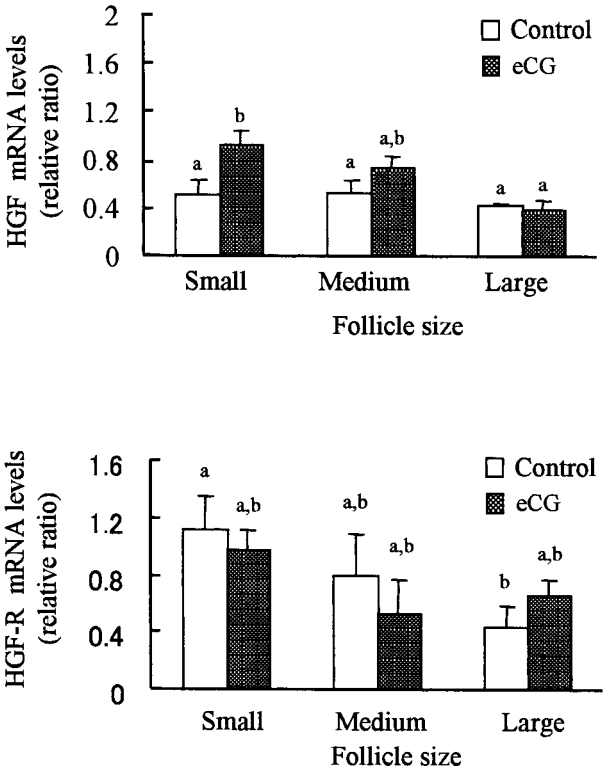


**Fig. 4.** Expression of HGF and HGF-R mRNAs in granulosa cells and thecal tissues, respectively, of small (diameter, <4 mm), medium (diameter, 4–5 mm) and large (diameter, >5 mm) follicles from prepubertal gilts with (closed bar) or without (open bar) eCG treatment. The expressions of mRNA were normalized on the basis of $\beta$-actin mRNA content. The data are indicated as the mean ± se (n=4). Different superscripts denote significantly different values ($P<0.05$).

network in the theca cell layer during eCG-induced porcine follicular development.

During folliculogenesis the development of the vascular network required to mature the antral follicles, and the increase in VEGF expression is compatible with its established role in stimulating angiogenesis [2]. Ang-1 has been shown to be required for stabilization of newly formed, leakage-resistant capillaries [11, 12]. Our results show that the expression of Ang-1 mRNA decreased in granulosa cells of medium and large follicles after eCG treatment. The effects of angiopoietins are complemented and coordinated by VEGF [6]. We previously have reported that expression of VEGF mRNA increased in medium and large follicles that have more capillaries in the thecal layer in eCG-treated gilts [2]. Thus, although expression of Ang-1 mRNA decreased, the effect (stabilization) of Ang-1 during follicular angiogenesis may be compensated by VEGF. Medium and large follicles have been known to possess high estrogenic activity after eCG treatment [13,33]. Daily administration of 17-$\beta$-estradiol for 8 days in ovariectomized rats resulted in a significant reduction in Ang-1 expression [34]. Thus, in the present study, the decreased Ang-1 mRNA expression in granulosa cells may have been caused by estradiol produced by eCG stimulation. On the other hand, Ang-2 mRNA expression differed markedly from Ang-1 expression in follicles at different developmental stages after eCG treatment. Whereas Ang-1 mRNA expression decreased in medium and large follicles after eCG treatment, Ang-2 expression increased in small

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

| Genes | PCR primers | Fragment size (bp) | EMBL/references* |
|---|---|---|---|
| Ang-1 | for: 5′-AGGAGCAAGTTTTGCGAGAG-3′<br>rev: 5′-CTGCAGAGCGTTTGTGTTGT-3′ | 251 | AF233227 |
| Ang-2 | for: 5′-AAAGTTGCTGCAGGGAAAGA-3′<br>rev: 5′-TCACAGCTCAGAGCGAAGAA-3′ | 191 | AF233228 |
| HGF | for: 5′-TGGCAATGAATTTGACCTCT-3′<br>rev: 5′-CTTCTTCCCCTCGAGGATTT-3′ | 218 | AF213397 |
| Tie-2 | for: 5′-TTGCATCACCGTGCTGTT-3′<br>rev: 5′-ATGCGTGCCTTCAGAACC-3′ | 255 | E08401 |
| HGF-R | for: 5′-GTAAGTGCCCGAAGTGTAAG-3′<br>rev: 5′-GCCCTCTTCCTATGACTTC-3′ | 313 | [24] |
| $\beta$-actin | for: 5′-ATCGTGCGGGACATCAAGGA-3′<br>rev: 5′-AGGAAGGAGGGCTGGAAGAG-3 | 169 | [3] |

for=forward; rev=reverse.
*: EMBL accession number of reference of published sequence.

**Table 2.** PCR conditionds used for detection of mRNA

| Gene | Dissociation | Annealing | Extension | Cycles |
|---|---|---|---|---|
| Ang-1 | 94 C for 0.5 min | 60 C for 1 min | 72 C for 1.5 min | 32 |
| Ang-2 | 94 C for 0.5 min | 63 C for 1 min | 72 C for 45 sec | 35 |
| HGF | 94 C for 0.5 min | 60 C for 1 min | 72 C for 1.5 min | 35 |
| Tie-2 | 94 C for 1 min | 58 C for 1 min | 72 C for 1 min | 38 |
| HGF-R | 95 C for 0.5 min | 60 C for 2 min | 72 C for 3 min | 35 |
| $\beta$-actin | 95 C for 1 min | 60 C for 1 min | 72 C for 1 min | 24 |

follicles. However, the effect of eCG on Ang-2 mRNA expression in granulosa cells during follicular development is still unknown.

The Ang-2/Ang-1 ratio increased in follicles at different developmental stages after eCG treatment. We previously reported that VEGF mRNA was expressed in granulosa cells of medium and large follicles, but not small ones, during follicular development in eCG-treated gilts [2]. As for relationships between angiopoietins and VEGF, there is one suggestion that, in the presence of VEGF, Ang-2 can promote vessel sprouting by blocking a constitutive (stabilizing) Ang-1 signal, whereas in the absence of VEGF, Ang-2 inhibition of a constitutive Ang-1 signal can contribute to vessel regression [14]. The results of the present and our previous studies [2] suggest that VEGF present in medium and large follicles may assist the action of Ang-2 causing capillary development and maturation in the theca cell layer during eCG-treated porcine follicular development. Additional studies are needed to define the expression and action of these local factors in thecal angiogenesis during follicular development.

A family of endothelium-specific receptor tyrosine kinases, the Tie family, has been identified, and consists of the receptors Tie-1 and Tie-2 [10, 35–40]. The transmembrane protein Tie-2 serves as a receptor for the polypeptide ligands Ang-1 and Ang-2 [6]. Immunolocalization of Tie-2 was observed in the newly vascularized theca interna, but no staining was observed in the avascular granulosa cells of the mature follicle in the rat ovary [41]. Our results indicate that Tie-2 mRNA was expressed in thecal tissues of follicles at different developmental stages in both groups, and that eCG treatment did not induce the expression of Tie-2 mRNA in thecal tissues, suggesting that Tie-2 mRNA may be sufficiently expressed in theca cells before eCG treatment.

HGF is produced by mesenchymal-derived theca cells, and stimulates epithelial granulosa cell proliferation during follicular development in the ovary [42]. It has been shown that HGF mRNA is expressed in bovine and human thecal cells [23, 42]. Other studies, however, showed the expression of HGF mRNA in rat granulosa cells [43]. Our study using eCG-treated porcine follicles showed that HGF mRNA was expressed in granulosa cells of follicles at all developmental stages in both groups. This result coincides with those of previous studies using rat ovaries [43], but not those in which bovine and human ovaries were used [23, 42]. In the present study, the expression of HGF mRNA increased in granulosa cells of small follicles after eCG treatment, whereas those of medium and large follicles remained unchanged. No information, however, is available on the molecular mechanisms that regulate the HGF mRNA level in the granulosa cells during follicular development. The activation of the HGF-HGFR signaling pathway has been reported to be associated with stimulation of angiogenesis by HGF itself and/or indirectly through VEGF [44]. We found that the levels of HGF-R mRNA in thecal tissues were unchanged after eCG treatment. HGF induced rat ovarian surface epithelial cell mitosis [45] and suppressed apoptotic susceptibility in human endometrial epithelial cells [46]. Therefore, we speculate that, in the small follicle at least, HGF stimulates granulosa cell proliferation and is indirectly associated with thecal angiogenesis during eCG-treated porcine follicular development.

The present study demonstrated that expression patterns of angiopoietins mRNA alter in the process of follicular development in eCG-treated gilts. In fact, the expression of Ang-1 mRNA decreased in granulosa cells of medium and large follicles and the expression of Ang-2 mRNA increased in small ones. The results of the present and our previous studies [2] suggest that angiogenic factors associated with the development

of the capillary network in the theca interna may alter in medium sized follicles (diameter, 4–5 mm), and that VEGF and angiopoietins may control the development and stabilization of blood vessels during follicular development in eCG-treated gilts.

In conclusion, the present study demonstrated that growing porcine follicles induced by eCG treatment have a greater number of blood vessels in the thecal cell layer and showed an increase in the Ang-2/Ang-1 ratio. Tie-2 was expressed in sufficient quantity in thecal tissues before eCG treatment. These findings suggest that the angiopoietins-Tie-2 system may be involved in perifollicular angiogenesis during follicular development in eCG-treated gilts.

## Acknowledgment

We thank the undergraduate and graduate students in our laboratory for their assistance. This study was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences.

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