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—Review—

Follicular Microvasculature and Angiogenic Factors in the Ovaries of Domestic Animals

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Abstract. The genetic and molecular mechanisms that control the development of capillary blood vessels during follicular development are beginning to be elucidated. Ovarian follicles contain and produce angiogenic factors that may act alone or in concert to regulate the process of thecal angiogenesis. These factors are ultimately controlled by endocrine, paracrine and autocrine regulation. A recent study indicated that vascular endothelial growth factor (VEGF) plays an important role in the process of thecal angiogenesis during follicular development. We are developing a novel technology for the induction of follicular development using the technique of *in vivo* gene administration. Here, we summarize the recent progress of our research.
Key words: Follicular development, Microvasculature, Angiogenic factor

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Specific Structure of The Microvasculature in the Porcine Ovary

During the reproductive period in female rabbits, the whole ovarian vascular bed undergoes specific and complex alterations that have an intimate relationship with the endocrine and ovulatory activities of the ovary [1]. In particular, these vascular modifications mainly involve the ovarian microvasculature and are related to development of the luteofollicular complex [2, 3]. The method of vascular corrosion casting associated with scanning electron microscopy (SEM) is especially well suited to the detailed morphological analysis of microvascular systems, as it offers quasi three-dimensional images of relatively high resolution [4]. Several SEM studies of vascular corrosion casts have been performed on the microvasculature of the ovary in rodents, rabbits, horses, cows, and sheep [5–9].

A recent study by Jiang *et al.* using corrosion casts for SEM revealed the structure of the microvasculature in the porcine ovary [10]. Porcine ovaries have coiled arteries and spiraling branches in the hilus and in the cortex, respectively (Fig. 1). In addition, small arterioles originating from the cortical coiled arteries straighten before entering the vascular complexes of the follicles. Follicles of 150–300 μm in diameter were surrounded by a polygonal meshwork of a few large capillary meshes, but no basket-like structure was visible (Fig. 2). Some follicles of 500–700 μm in diameter had a spherical meshwork of a few or several capillaries, and the capillary network was arranged as a thin single layer of capillaries (Fig. 3). The microvascular architecture of follicles of 1,000–

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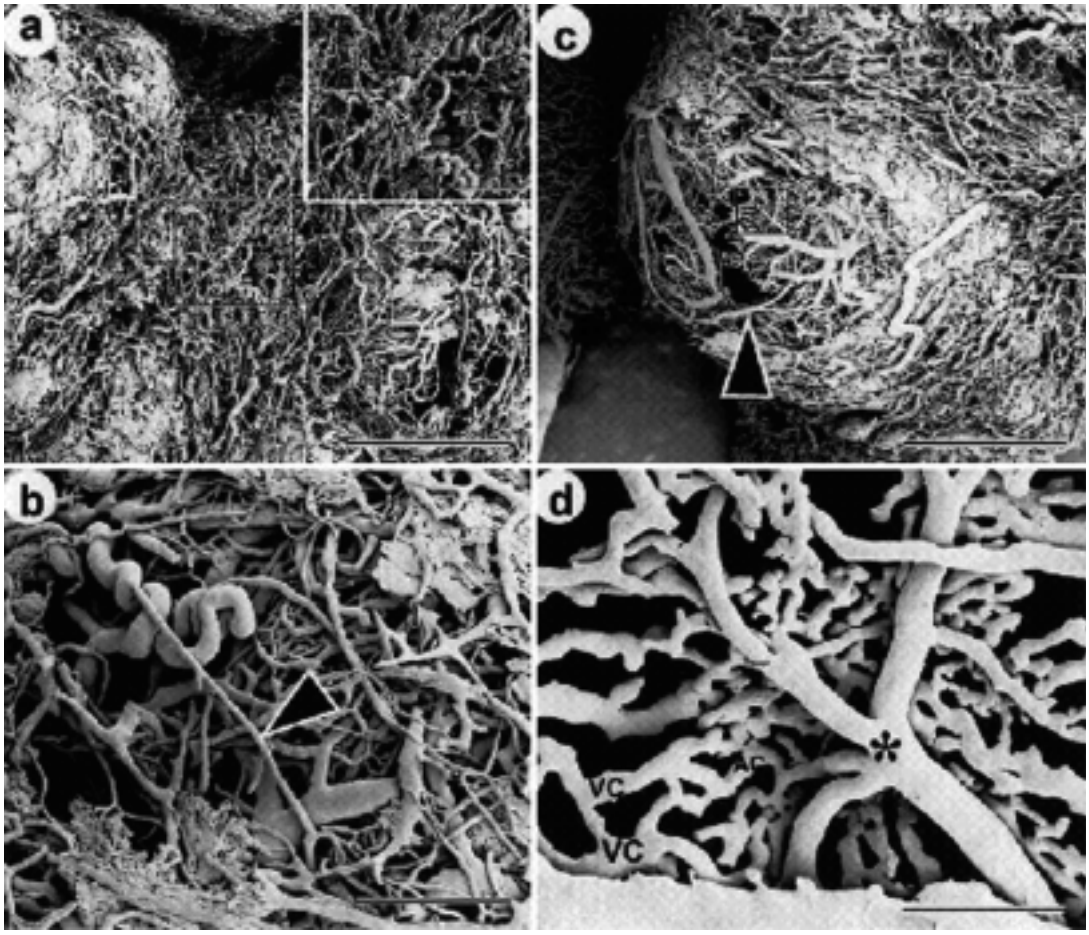

Fig. 1. Artery configurations in the cortex of the porcine ovary (a, b) and follicles (c, d). *Insert*: Higher magnification view of the selected area in a (*small triangle* coiled artery). b, c *Large arrowheads* Straight arterioles from coiled arteries before entering follicles. d Higher magnification view of the selected area in c (*asterisk* dicotomy of an arteriole in follicles). Connections between arteriole-derived capillaries (AC) and vein-derived capillaries (VC) were also observed (d). Bars=1.38 mm (a, c), 300 μm (insert in a), 400 μm (b), 120 μm (d).

2,000 μm in diameter was composed of three layers of vascular plexus (Fig. 3). The inner plexus consisted of a small, spherical, basket-like capillary network that was not well developed. The middle layer consisted of small arterioles and venules, and the outer layer was a coarse capillary plexus. In antral follicles with a diameter of more than 2,000 μm, the microvasculature was arranged as an inner vascular plexus of about 25 μm with dense capillaries, a middle layer of about 100 μm and an outer capillary plexus of about 30 μm in thickness (Fig. 3). The microvasculature of large follicles had sparse capillary networks or avascular areas in the apical region (Fig. 4).

The microvascular architecture and its changes in the follicles of pigs are different from those in rats, but similar to those in rabbits and cows, especially those of the large antral follicles. In the rat, the vascular bed of developing and mature follicles remains in a single-layered wreath configuration during development [11]. However, in ovaries of the rabbit [5, 6, 11, 12] and cow [9], the single-layered capillary wreath in the small-sized follicles becomes a multilayered structure in the thickened theca interna as the follicles develop into larger and more mature Graafian follicles. These morphological changes in follicular microvasculature support the need for an increase in blood supply as follicles develop.

The results of study by Jiang *et al.* indicate that the distribution of capillaries in the inner plexus varies greatly from region to region in the

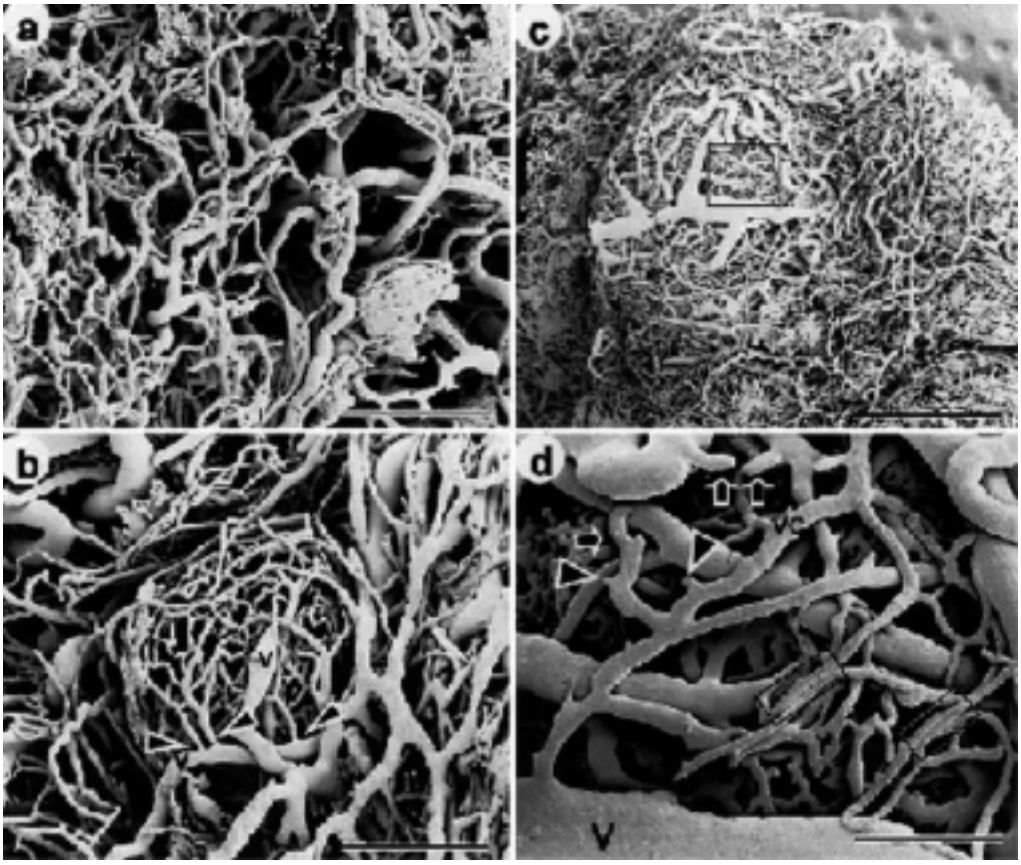

Fig. 2. Microvasculature of primordial or primary follicles (*stars* in a) and small antral (b) and medium antral (c, d) follicles in the porcine ovary. b *Small arrowheads* necks of drainage capillaries that connect to venular vessels (v). d Higher magnification view of the selected area in c. Budding (*arrowheads*) and sprouting (*arrows*) images indicate angiogenesis of venular capillaries (vc). Capillaries that contact each other in parallel are indicated by *open rectangles*. Bars 300= μm (a, b), 1.38 mm (c), 200 μm (d).

microvasculature of antral follicles in the pig. These findings are similar to those in unstimulated rabbits [11, 12] and support those reported previously in other animals. Studies on the non-invasive evaluation of ovarian and follicular blood flow in women by transvaginal color Doppler ultrasonography [13–17] have revealed marked regional differences in follicular blood flow with sustained increases in the basal and lateral walls of preovulatory follicles and concomitant decreases in the flow to their apical regions [18]. These vascular changes are probably necessary for follicle rupture as has been suggested on the basis of *in vivo* [19] and *in vitro* [20, 21] studies in the rat. A recent study on the microvascular network of bovine ovarian follicles indicates that von Willebrand factor (vWF, a specific marker of endothelial damage) positive areas are first observed in the outer layer of the theca interna in healthy follicles and vary from region to region in the same follicles and among follicles according to health or atresia. In the theca interna, the vWF-positive area is significantly greater in advanced and late atretic follicles compared with healthy and early atretic follicles in all regions, except for the apical region of advanced atretic follicles [22]. It has also been suggested that differences in vascular development are important in follicle selection and the selective maturation of preovulatory follicles [23]. In the atretic follicles of sheep, a progressive decrease in the number of capillaries lying close to the basal lamina occurs as atresia advances [24]. In contrast to those in developing follicles, a study of rabbit follicles has indicated that the capillaries in atretic follicles are usually thin and have few or no sprouts, are not uniformly distributed, and vary in

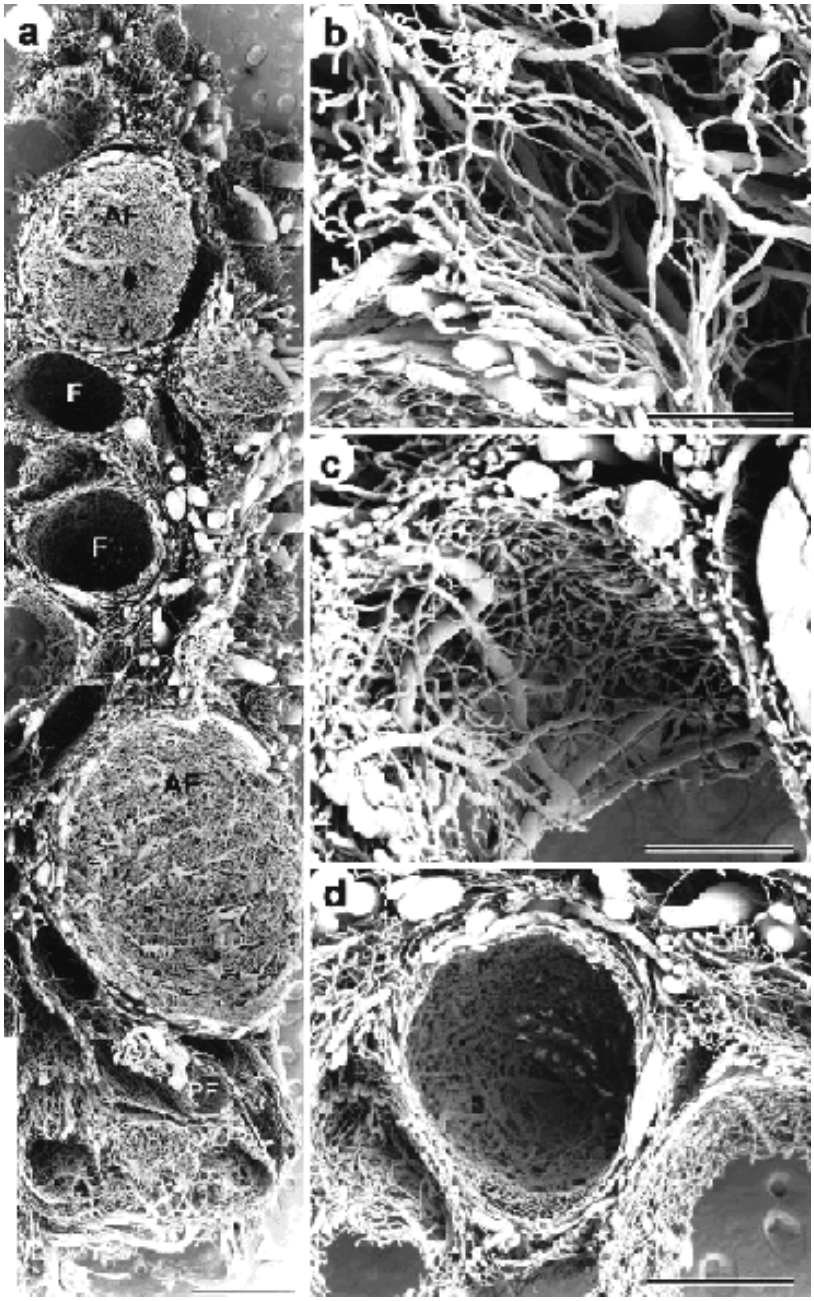

Fig. 3. View of a freeze-fractured vascular cast of the porcine ovary. a The vascular plexuses of pre-antral (PF) and antral (AF) follicles are exposed. Higher magnification views of the vascular plexuses of pre-antral (b) and small antral follicles (c, d) are shown (F follicles). Bars=1.38 mm (a), 300 μm (b), 400 μm (c), 900 μm (d).

size and arrangement especially in the inner layer of antral follicles [7].

Artifacts such as resin leakage, filling defects, and blunt capillary ends associated with vascular corrosion casts have been reported previously [8]. In a recent study by Jiang *et al.*, the vessels were well filled with the casting medium. Artifacts, if they occurred, were limited and did not affect the observations of the natural follicular microvasculature. Numerous round resin leakages

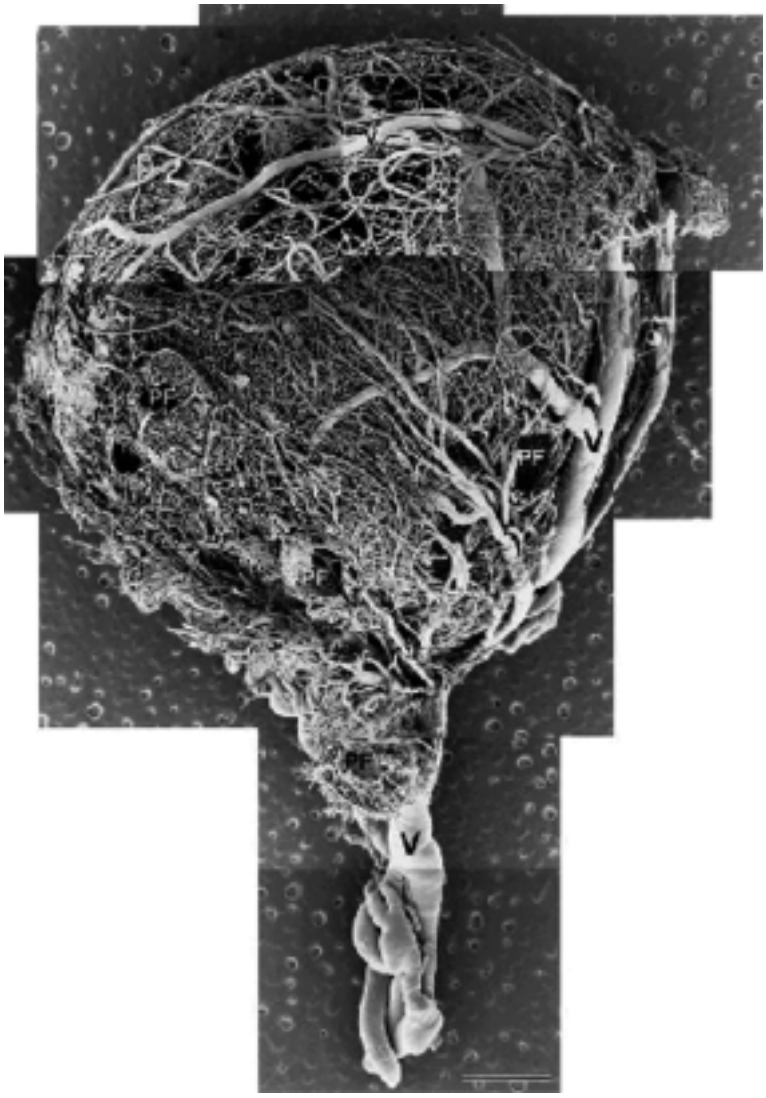

Fig. 4. Vascular architecture of large (9.9 mm) porcine follicles. Several vascular plexuses of pre-antral follicles (PF) around those of large follicles were observed. Arterial (a) and venous (v, V) vessels are indicated. Bar=1.38 mm.

projecting into the inner cavity were observed only in the capillaries of large porcine follicles (Fig. 6a). This finding was in agreement with those in man, rabbits, and primates but different from that in the rat [5, 12]. These marked leakages may be attributable to the extraordinary weakness of the vascular walls and/or the markedly increased permeability of the capillaries in preovulatory follicles [2, 3, 25, 26].

In conclusion, the structure and changes of the follicular microvasculature in pigs are different from those in rats, but similar to those in larger animals such as rabbits and cows.

Angiogenesis in the Mammalian Ovary

Vascular development occurs in two stages: an early stage of vasculogenesis, and a later stage that has been termed angiogenesis. Vasculogenesis is the mechanism by which the primary capillary network is formed from mesoderm-derived precursors (hemangioblasts), through a process of differentiation, proliferation, and coalescence to

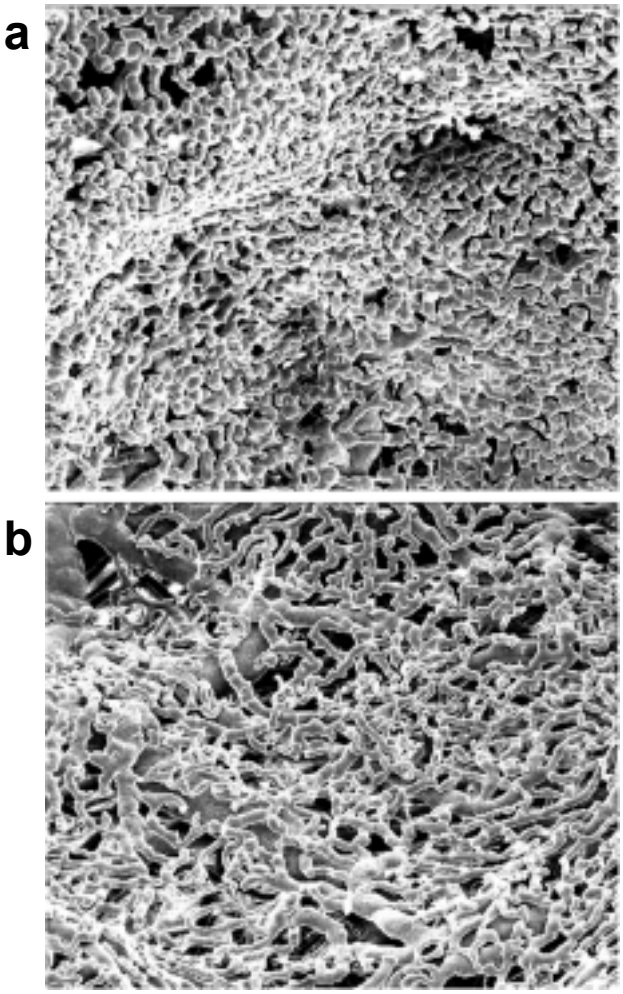

Fig. 5. Capillaries in the inner plexus of large follicles (7 mm in diameter) from gilts treated with eCG (a) and those (5 mm in diameter) from controls (b) are shown. Note well-developed capillaries with higher density in (a) compared with those in (b). Bar=200 μm.

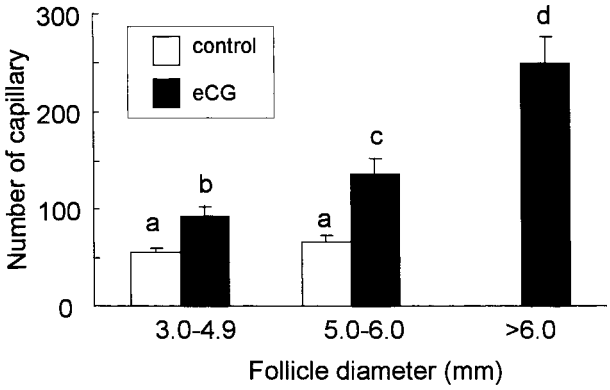

Fig. 6. The number of capillaries in theca interna of antral follicles larger than 3 mm in diameter in ovaries of prepubertal gilts with (n=4) or without (n=4) eCG injection. Values with different superscripts are significantly different from each other at P<0.05.

form the primitive vascular network [27, 28]. Vasculogenesis is mainly found during the prenatal period. Angiogenesis refers to the formation of new capillary blood vessels from preexisting microvessels by remodeling of the primary plexus. The angiogenic process begins with endothelial cell proliferation and migration into the primary vascular network, which lead to vascularization of previously avascular tissues and organs as well as to growth and remodeling of the initially homogeneous capillary plexus to form a new microcirculation.

Angiogenesis in the adult is related particularly to pathological situations such as tumor growth and metastasis. Physiologic angiogenesis in the adult is prominent only in the female reproductive system. It takes place in the uterus, placenta, mammary glands and ovaries [28]. In the ovaries, each specific phase of the hormonal cycle is accompanied by radical changes of the vascular bed. The ovarian vasculature is not distributed equally among the plethora of non-growing and growing follicles [29]; primordial and preantral follicles do not have any vascular supply of their own, but instead are supported by vessels in the surrounding stroma. However, as the antrum develops in the follicle, thecal angiogenesis involving the differentiation of two capillary networks located in the theca interna and externa occurs. Entering the theca interna, the arterioles break up into a rich network of capillaries that builds a basket-like network around the avascular stratum granulosum. The capillary networks are connected, and all capillary blood exits from the theca interna into small vessels, which become continuous with ovarian stromal veins. Since all capillaries remain outside the basement membrane of the follicle, the granulosa cell layer remains avascular until about the time of ovulation.

A previous study using immunohistochemical markers for vascular endothelial cells indicated that vascular density increases during follicle development from the preantral to antral stage [30]. By contrast, one of the earliest signs of follicular atresia is the association of reduced DNA synthesis in endothelial cells with reduced vascularity of the

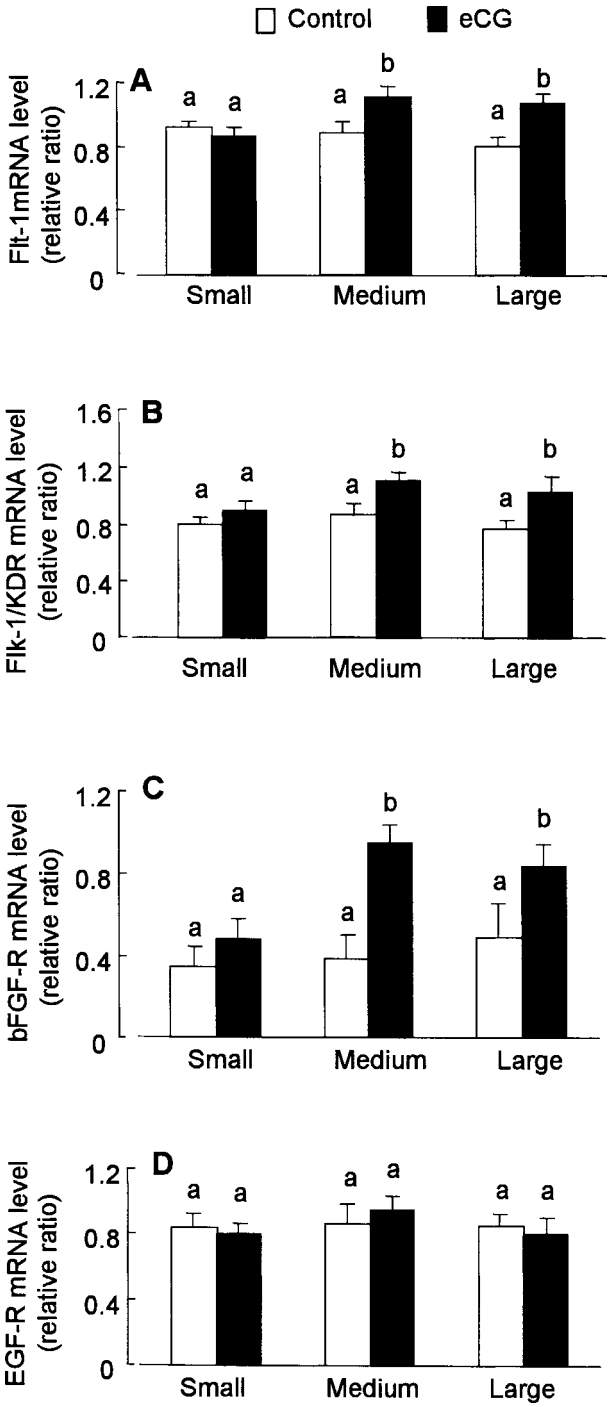

Fig. 7. Expression of VEGF 120, VEGF 164, bFGF and EGF mRNAs in granulosa cells of small (<4 mm), medium (4–5 mm) and large (>5 mm) follicles from prepubertal gilts with (closed bar) or without (open bar) eCG injection. The expressions of each factor were normalized on the basis of beta-actin mRNA content. The data indicate the mean ± se (n=6). Different superscripts denote significantly different values (P<0.05).

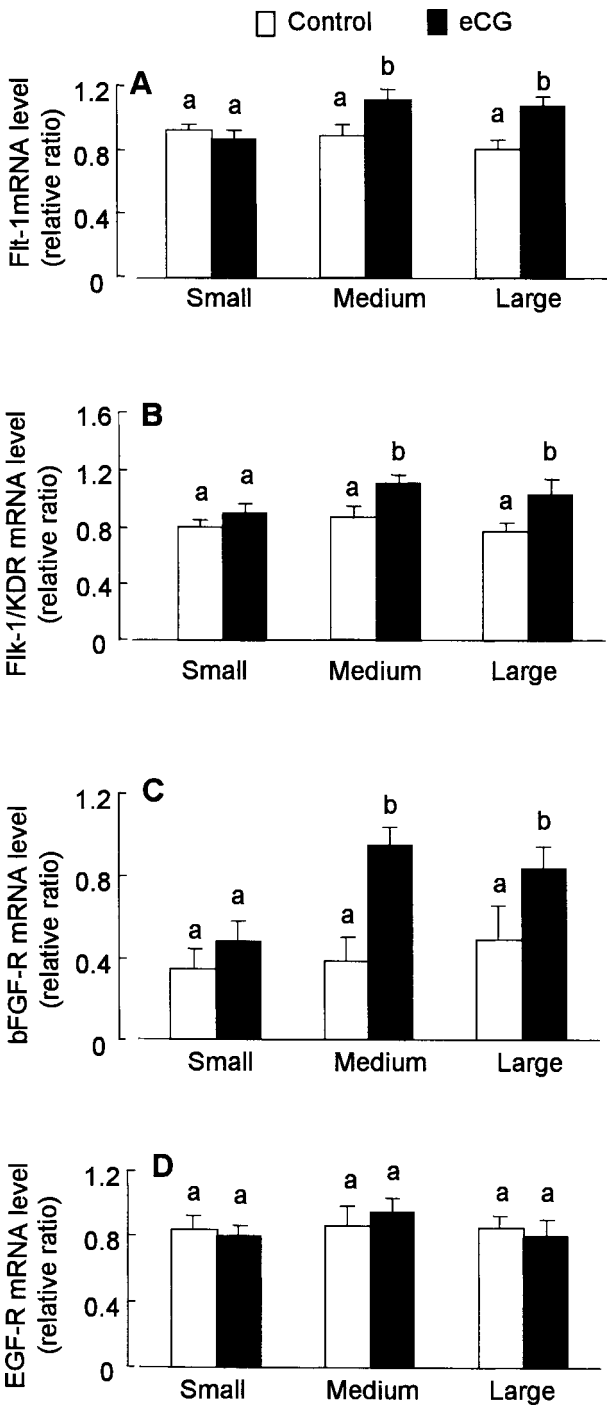

Fig. 8. Expression of Flt-1, Flk-1/KDR, bFGF-R and EGF-R mRNAs in theca shells of small (<4 mm), medium (4–5 mm) and large (>5 mm) follicles from prepubertal gilts with (closed bar) or without (open bar) eCG injection. The expressions of each factor were normalized on the basis of beta-actin mRNA content. The data are indicated as the mean ± se (n=6). Different superscripts denote significantly different values (P<0.05).

follicle [31]. The early atretic follicles regenerated when placed in an *in vitro* culture, suggesting that the follicle remains in its atretic state due to a decrease in vascularity, which limits access to nutrients, substrates and tropic hormones [32]. All preovulatory follicles of monkeys had similar concentrations of gonadotropin binding sites; however, only the follicle destined to ovulate (the dominant follicle) became heavily labeled after intravenous injection of radiolabeled gonadotropin [23]. This selective *in vivo* uptake of gonadotropin was associated with increased vascularity of the dominant follicles. Classic studies of ovarian morphology showed that the capillary network of preovulatory follicles was more extensive than that of other follicles [33, 34]. Taken together, follicular development as well as atresia is associated with, and dependent on, the degree of vascular development and support.

Angiogenic Factors and Their Roles in the Ovary

The extract of the thecal layer, but not the granulosa cell layer, of pig follicles stimulated migration and proliferation of endothelial cells *in vitro* [35]. This observation was potentially significant, since only the thecal layer of follicles is well vascularized, whereas the granulosa cell layer is avascular [32]. The media conditioned by granulosa cells stimulated the migration [36] and proliferation [37] of endothelial cells.

A recent study reported that vascular endothelial growth factor (VEGF), known as a potent mitogen for endothelial cells [38] and as a stimulator of vascular permeability [39, 40], was expressed in granulosa cells isolated from pig follicles [41, 42]. Moreover, it has been demonstrated that the short-term inhibition of angiogenesis after anti-VEGF antibody administration during the later growth phase of the dominant follicle interferes with its normal development [43], and the blocking of VEGF action by treatment with a soluble truncated form of the fms-like tyrosine kinase (Flt-1) receptor resulted in an 87% decrease of proliferation in the theca of secondary and tertiary follicles, a reduction in endothelial cell area and a marked decline in Flt-1 mRNA expression [44]. These studies suggest that VEGF is associated with follicular development in the mammalian ovary. Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) also have angiogenic action in the ovary [45–48]. bFGF mRNA was predominantly localized in the granulosa cells of the dominant follicles of the rat ovary [49]. In the bovine ovary, bFGF mRNA expression in the theca interna increased significantly during the final growth of follicles, whereas its expression in granulosa cells was very weak [50]. Previous studies indicated that EGF is an angiogenic factor [51, 52], and EGF was shown to enhance the proliferation of vascular endothelial cells *in vitro* [53] and affect neovascularization *in vivo* [48]. EGF is soluble in tissue fluid, can be translocated in tissues and induces endothelial cells to proliferate and form capillaries [53]. Immunocytochemical studies showed that EGF peptide was localized in the cumulus cells and granulosa cells [54], and in thecal and interstitial cells around growing follicles [46, 55]. Angiogenic factors such as VEGF, bFGF and EGF act via their receptors in target cells. There are two phosphotyrosine kinase receptors for VEGF, namely Flt-1 and fetal liver kinase (Flk-1), or the murine homolog of the kinase domain region (KDR), sharing 85% sequence identity with human KDR [56–60], bFGF receptor (bFGF-R) and EGF receptor (EGF-R). Flt-1, Flk-1/KDR and bFGF-R mRNAs are expressed in the theca interna of bovine ovarian follicles [65]. In the porcine ovary, a very strong EGF-R mRNA signal was observed in the cumulus, granulosa, and theca cells [54].

Initial reports on angiogenic activity of ovarian follicles were inconclusive, and the relationships among the stage of follicular development, gonadotropin treatment and follicular angiogenesis were unclear. To evaluate these relationships, we stimulated follicular development in gilts by equine chorionic gonadotropin (eCG) treatment [42]. 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 VEGF120, VEGF 164, bFGF and EGF in granulosa cells, and Flt-1, Flk-1 or KDR, bFGF receptor (bFGF-R) and EGF receptor (EGF-R) in thecal tissue. Although the number of capillaries in the theca interna in healthy follicles larger than 3 mm in diameter did not change in the controls, eCG treatment significantly increased the capillary population as the follicles grew (Figs. 5 and 6). The expression of VEGF 120, VEGF 164 and bFGF mRNAs increased in granulosa cells of medium

and large follicles from ovaries of prepubertal gilts after eCG treatment (Fig. 7). Gonadotropins have been shown to stimulate the production and expression of VEGF *in vitro* [61, 62]. We found that eCG induced the expression of VEGF 120 and VEGF 164 mRNAs in granulosa cells *in vivo*. In particular, the differences between the control and eCG groups in VEGF 120 mRNA expression in medium and large follicles were to a larger degree than those of VEGF 164, suggesting that VEGF 120 may be a major mediator in angiogenesis during porcine follicular development (Fig. 7A,B,C). VEGF mRNA expression is regulated by a variety of factors. Insulin-like growth factor I (IGF-I) has been shown to induce VEGF mRNA in cultured colorectal carcinoma cells [63]. In the ovary, follicle-stimulating hormone (FSH) stimulates the production of IGF-I in porcine granulosa cells *in vitro* [64]. Thus, in this study, the increase in VEGF mRNA expression in the granulosa cells may be caused by IGF-I produced by eCG.

Flt-1, Flk-1/KDR and bFGF-R mRNA expression increased in thecal cells of medium and large follicles after eCG treatment. Our results [42] indicated that the expression of Flt-1 and Flk-1/KDR mRNA increased in thecal tissue of medium and large follicles after eCG treatment and paralleled the expression of VEGF 120 and VEGF 164 mRNA, suggesting that the expression of Flt-1 and Flk-1/KDR might be activated by VEGF isoforms (Fig. 8A, B). The expression of Flt-1 and Flk-1/KDR was reported to be affected by hypoxia, although to a lesser extent than that of VEGF [65]. However, the effect of hypoxia on angiogenic receptors in thecal tissue during follicular development is still unknown. We observed that bFGF-R mRNA was expressed in thecal tissue, and paralleled the expression of bFGF mRNA in granulosa cells (Figs. 7 C and 8 C). Therefore, the results of that study supported the previous hypothesis that this factor may be involved in the vascularization of the theca interna [66].

The expression of EGF mRNA increased in granulosa cells of small, medium and large follicles from ovaries after eCG treatment, but the mRNA expression of EGF-R in thecal tissue did not change (Figs. 7D and 8D). The expression of EGF mRNA in granulosa cells increased with eCG treatment at all developmental stages, while the expression of EGF-R mRNA in thecal cells was unchanged. EGF causes the suppression of granulosa cell apoptosis [67] and granulosa cell proliferation [68]. The exposure of quiescent human keratinocytes to EGF resulted in a marked induction of VEGF mRNA expression [69]. In addition, EGF stimulated VEGF release by cultured glioblastoma cells [70]. Therefore, our results [42] suggest that EGF stimulates the granulosa cell proliferation and is indirectly associated with the formation of the capillary network during porcine follicular development.

In conclusion, our study [42] provides evidence that VEGF may be a major inducer of vascular development during follicular development in eCG-primed gilts and may contribute to ovarian disorders, including polycystic ovarian syndrome (PCOS), follicular cyst formation, luteal phase defects (LPD) and benign and malignant neoplasms [71]. Moreover, understanding the interaction between the ovarian vasculature and angiogenic factors during infertility therapy, such as controlled ovarian stimulation (COS) protocols, and associated side-effects, i.e. ovarian hyperstimulation syndrome (OHSS), is important.

PCOS is an aberrant condition of the ovary originally described by Stein and Leventhal [72]. While PCOS has been associated with healthy fertile women of reproductive age [73], it represents an astonishing 75% of cases involving anovulatory infertility [74]. Morphologically, PCOS is characterized by the presence of multiple small antral follicles localized around the periphery of the ovary. Although the aetiology of this multi-faceted disease remains subject to debate, non-surgical approaches to treating PCOS-related infertility include controlling gonadotropin secretion [74] and insulin sensitivity [75]. Therefore, we suggest that a technique to induce follicular development by gene injection may resolve the problems of infertility or ovarian dysfunction.

Prospects

We are developing a new technology for repeated collection of meiotically competent oocytes from antral follicles in live animals that represents an important tool for linking *in vitro* embryo production to animal breeding, and for treating ovarian diseases such as PCOS and OHSS. As angiogenic and anti-angiogenic agents are being

developed for clinical use, their potential application to the manipulation of the reproductive function and for the treatment of pathological conditions is being carefully monitored. Overexpression of VEGF in the ovary by gene injection, in contrast, may contribute to the collection of many high quality oocytes from animals. We believe that future study of thecal angiogenesis could offer an innovative protocol for the development of novel therapies for the prevention and treatment of infertility associated with ovarian dysfunction.

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