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Importance of vascular endothelial growth factor (VEGF) in ovarian physiology of mammals

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Summary

Ovarian folliculogenesis in mammals is a complex process. Several compounds have been tested during *in vitro* culture of follicular cells for a better understanding of the mechanisms and factors related to ovarian folliculogenesis in mammals. From these compounds, vascular endothelial growth factor (VEGF) can be highlighted, as it is strongly associated with angiogenesis and, in recent years, its presence in ovarian cells has been investigated extensively. Previous studies have shown that the presence of VEGF protein, as well as mRNA expression of its receptor 2 (VEGFR-2) increases during follicular development. Therefore, it is likely that the interaction between VEGF and VEGFR-2 is crucial to promote follicular development. However, few studies on the influence of this factor on follicular development have been reported. This review addresses aspects related to the structural characterization and mechanism of action of VEGF and its receptors, and their biological importance in the ovary of mammals.

Keywords: Angiogenesis, Folliculogenesis, *In vitro* culture, Maturation, Tyrosine kinase

Introduction

Ovarian folliculogenesis in mammals is a complex process that is comprised of interactions between several autocrine, paracrine and endocrine factors. With respect to paracrine factors, the role of vascular endothelial growth factor (VEGF) is noteworthy. VEGF was initially identified and named vascular permeability factor (VPF). Subsequently, its angiogenic activity was described, and the renamed VEGF is now considered possibly the most potent angiogenic agent ever described. VEGF also stimulates the survival of endothelial cells in vessels through the inhibition of apoptosis, as well as promoting their proliferation, migration and differentiation, and causing changes in gene expression patterns and inhibition of senescence (Dvorak, 2000).

The VEGF family is comprised of several members: VEGF-A, placental growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. VEGF-A is the most studied subtype and has been detected in preantral follicles from several mammalian species such as humans (Otani *et al.*, 1999; Harata *et al.*, 2006), rats (Celik-Ozenci *et al.*, 2003), pigs (Barboni *et al.*, 2000), goats (Sharma and Sudan, 2010) and cows (Greenaway *et al.*, 2005). Moreover, regulatory effects of VEGF on mammalian folliculogenesis and luteogenesis have been observed (Quintana *et al.*, 2004; Roberts *et al.*, 2007; Yang *et al.*, 2008).

For preantral folliculogenesis, the importance of VEGF for the survival and growth of early (Bruno *et al.*, 2009) and advanced (Fisher *et al.*, 2009) follicles has been reported. Based on the observation of a positive correlation between follicle diameter and VEGF production, Fisher *et al.* (2009) demonstrated that this compound might play an important role during *in vitro* follicle development.

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The involvement of VEGF in the regulation of the various phases of follicle development has been shown, however more studies are necessary for a better understanding of the mechanisms by which this factor (ligand and receptors) acts in mammalian ovarian folliculogenesis.

Features of the ovarian vascular system and follicular angiogenesis

The ovary in mammalian species is comprised of two distinct portions: (1) the cortex, which is the outermost part with a stroma of conjunctive tissue, and follicles and corpora lutea at several developmental stages; and (2) the medulla, the inner region, which contains loose conjunctive tissue highly vascularized and originating from ovarian arteries. Histologically, the limits between these two regions are not well defined.

The folliculogenesis process takes place within the cortex, from the formation of the primordial follicle to the development to the preovulatory stage, which comprises the preantral (primordial, primary and secondary follicles) and antral (tertiary and preovulatory follicles) phases. Despite the fact that preantral follicles do not possess their own vascular supply, the formation of the capillary network that surrounds the follicle is critical for growth beyond this phase. Angiogenesis begins within the stroma during early follicular development (Suzuki *et al.*, 1998). Up to this point, nutrition and oxygenation of primordial and primary follicles rely on passive diffusion from stromal blood vessels, which are thin and single layered at this time. At the secondary stage or later, stromal cells that surround the follicles become organized in thecal layers, in which the innermost part (theca internal) contains many blood vessels, whilst the outer layer (theca external) is composed mainly of fibrous conjunctive tissue. Thereafter, during the appearance of the antral cavity full of follicular fluid, follicles become surrounded by a capillary network, which promotes the nutrition of both these cells and granulosa cells. This vascular system is divided into two distinct parts that enters either the external and internal thecal cells layers (Stouffer *et al.*, 2001), and both contribute to the production of follicular fluid (Van den Hurk & Zhao, 2005), which is rich in VEGF (Ferrari *et al.*, 2006). The number and diameter of blood vessels increase as the follicle develops, but these never penetrate the basement membrane that separates theca interna and granulosa cells layers.

Structural characterization of VEGF and its receptors

VEGF is a cytokine and is a homodimeric glycoprotein that is expressed in several tissues as various types, with a molecular weight of about 45 kD (Ferrara & Henzel, 1989). Its structure forms an antiparallel homodimer that is linked covalently by two disulphide bridges between cystine residues. The cystine knot motif consists of an eight-residue ring formed by the disulphide bridges and is conserved in the same position by a third disulphide bond (Muller *et al.*, 1997). VEGF-A is formed by two monomers that contain a cystine knot motif determined by three intrachain disulphide bridges, whilst the homodimer is assembled by two interchain disulphide bridges linking the monomers. Overall, the VEGF monomer resembles that of other cystine knot growth factors such as platelet-derived growth factor (PDGF), but its N-terminal segment is helical rather than extended. The dimerization mode of VEGF is similar to that of PDGF and is very different from that of transforming growth factor (TGF)-β. Mutational analysis of VEGF reveals that symmetrical binding sites for the receptor kinase domain receptor (KDR) are located at each pole of the VEGF homodimer (Muller *et al.*, 1997).

In humans, the gene that encodes VEGF is comprised of eight exons that are separated by seven introns, and the coding region is approximately 14 kb (Tischer *et al.*, 1991; Houck *et al.*, 1991). VEGF mRNA undergoes alternative splicing events that lead to the production of mature homodimeric proteins. Each monomer is designated in accordance with the number of amino acids along their chains ($VEGF_{110}$, $VEGF_{111}$, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{162}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$; Fig. 1). The isoforms $VEGF_{110}$ (Keyt *et al.*, 1996) and $VEGF_{121}$ (Park *et al.*, 1993) do not bind to heparin as the carboxy-terminal domain located between amino acids 111 and 165 is not present, which makes both molecules freely diffusible within cells. In contrast, $VEGF_{165}$ and $VEGF_{189}$ bind to heparin with greater affinity. The use of heparinase either *in vivo* (Sasisekharan *et al.*, 1994) or *in vitro* (Rathjen *et al.*, 1990) indicates the potential of heparin molecules to be an important element of the binding complex VEGF receptor. In both cases, cell proliferation and neovascularization were inhibited. The absence of binding may not be due to a loss of VEGF receptors (Gitay-Goren *et al.*, 1992), as this activity could be recovered by the use of exogenous heparin (Rathjen *et al.*, 1990). Therefore, it was observed that successful signal transduction depends on the formation of a complex of VEGF, its receptors and heparin (VEGF–heparin–receptor) (Gitay-Goren *et al.*, 1992). These data suggest that the stability of VEGF–heparin–receptor complexes probably contributes to effective signal transduction and stimulation of endothelial cell proliferation (Keyt *et al.*, 1996).

All transcripts contain exons one to five and exon eight, with diversity generated through the alternative

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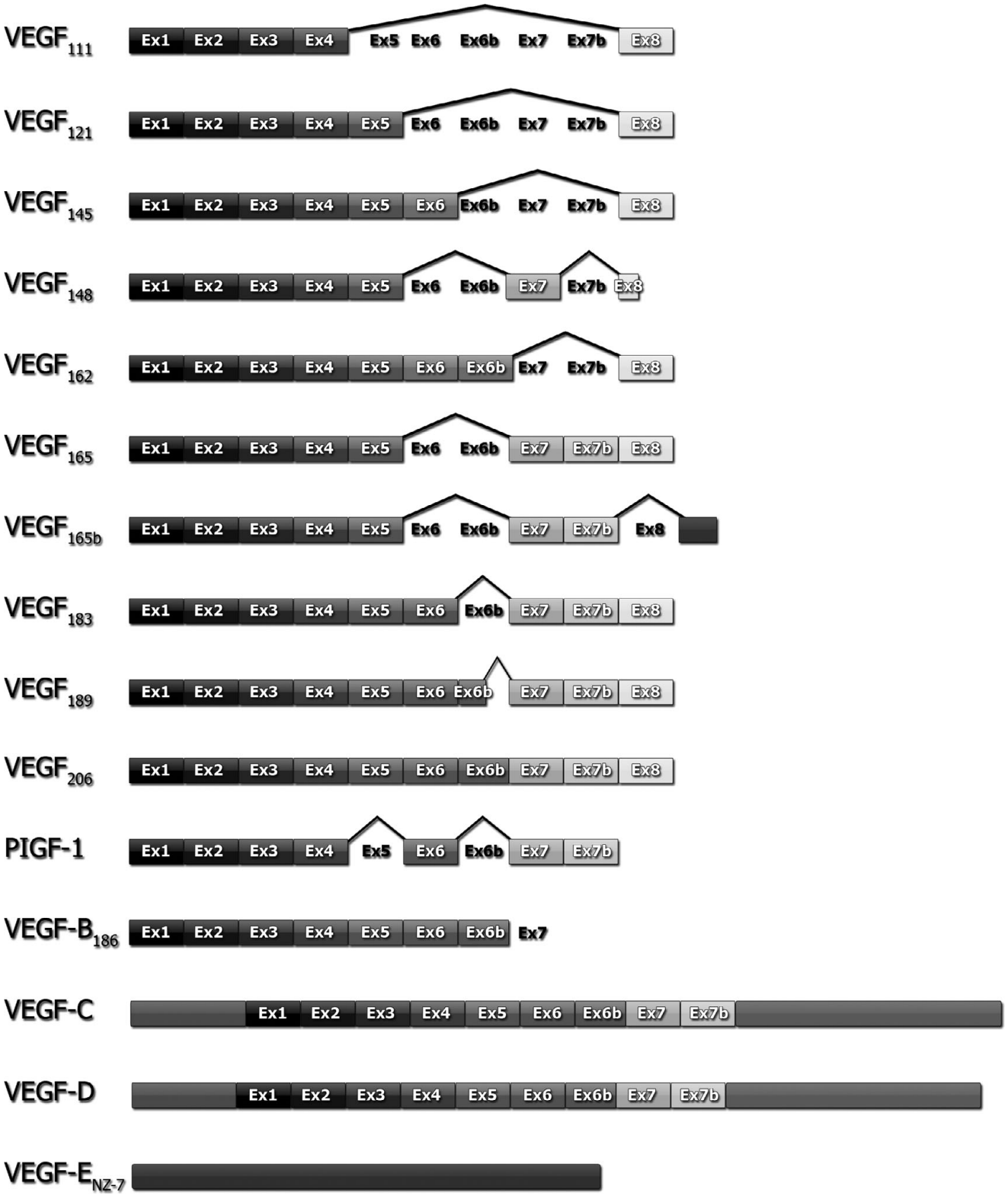

Figure 1 VEGF isoforms generated by alternative splicing. VEGF-A comprises monomers designated according to the number of amino acids in the polypeptide chain ($VEGF_{110}$, $VEGF_{111}$, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{162}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$). VEGF, vascular endothelial growth factor.

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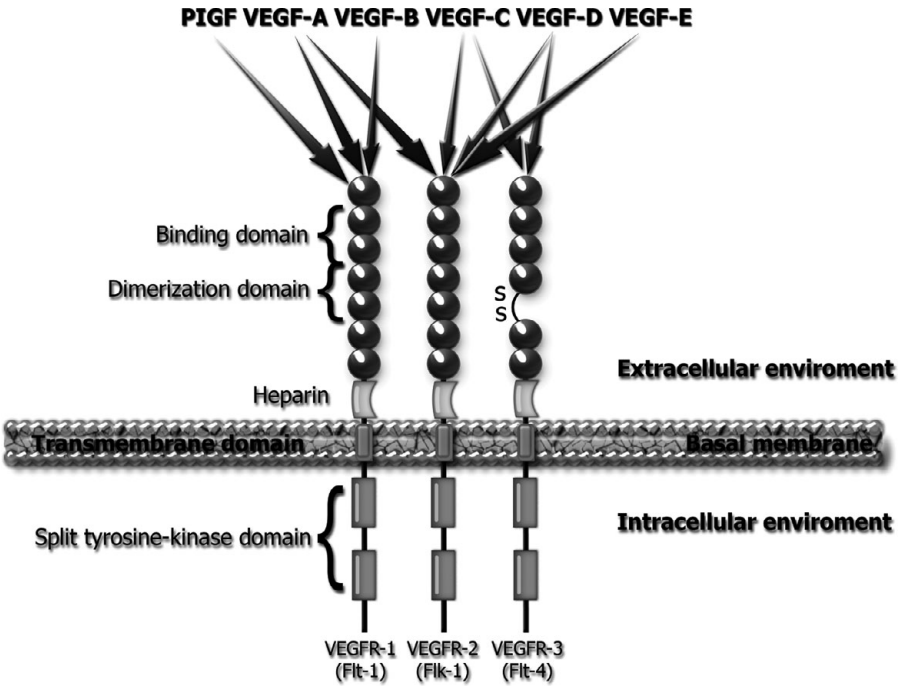

Figure 2 Binding complex VEGF-heparin-receptor involved in biological responses to VEGF in various cells and tissues. VEGF-A binds both to VEGFR-1 and VEGFR-2, whilst PIGF and VEGF-B interact only with VEGFR-1. VEGF-C and VEGF-D bind to receptors VEGFR-2 and VEGFR-3, and VEGF-E binds only to VEGFR-2. Flt, Fms-like tyrosine kinase-1; PIGF, placental growth factor; VEGF, vascular endothelial growth factor.

splicing of exons six and seven, except for VEGF-A_{165b}, which contains an alternative exon eight (Holmes & Zachary, 2005). This variant is an endogenous inhibitory VEGF molecule that does not contain exon six, but possesses an alternative exon (eight) that encodes a new carboxy terminus that increases the chances of the occurrence of a family of isoforms with this novel carboxy-terminal end (Bates *et al.*, 2002).

In relation to the necessity of the above complex for the activity of VEGF, it is known that this molecule binds directly to three receptor types: VEGFR-1/Flt-1 (Fms-like tyrosine kinase-1; De Vries *et al.*, 1992), VEGFR-2/KDR (kinase insert domain containing region; Terman *et al.*, 1992) and VEGFR-3/Flt-4 (Fms-like tyrosine kinase-4; Kaipainen *et al.*, 1995; Karkkainen *et al.*, 2002) (Fig. 2). These receptors are members of the tyrosine kinase family, and have as common features the presence of seven immunoglobulin-like domains in the extracellular portion, a single transmembrane region and a tyrosine kinase sequence interrupted by the kinase insertion domain in its intracellular portion (Shibuya *et al.*, 1990). Nevertheless, VEGF binds with high affinity to only two of these three receptors (VEGFR-1/Flt-1 and VEGFR-2/KDR), whilst VEGFR-3/Flt-4 is involved in interactions with other VEGF forms (VEGF-C and VEGF-D; Neufeld *et al.*, 1999). The cleavage of VEGF165 by plasmin, which is an important process in the angiogenesis cascade (Mignatti *et al.*, 1989), releases an N-terminal fragment that is comprised of amino acids 111–165. This polypeptide binds to two of the VEGF receptors, VEGFR-1/Flt-1 and VEGFR-2/KDR, in the absence of heparin (Keyt *et al.*, 1996). Once VEGF has bound, VEGFR-2 dimerizes and autophosphorylates, which in turn activates several signal transduction cascades (Byrne *et al.*, 2005).

Expression, immunolocalization and mechanism of action of VEGF and its receptors

VEGF and its receptors VEGFR-1/Flt-1 and VEGFR-2/KDR are expressed in the ovary of mammals, and have been identified in several reproductive tissues such as the ovarian follicle, corpus luteum, endometrial vessels and at embryo implantation sites (Jakeman *et al.*, 1993; Shweiki *et al.*, 1993; Gordon *et al.*, 1996; Neufeld *et al.*, 1999; Krussel *et al.*, 2001; Al-zi'abi *et al.*, 2003). Previous studies have demonstrated the presence of VEGF and its respective mRNA expression in the endometrium of fertile women with normal uteri (Shifren *et al.*, 1996). Both the protein and mRNA corresponding to VEGF and its receptors were also detected in the granulosa and in thecal cells of bovine secondary ovarian follicles (Yang & Fortune, 2007). In sows, expression of mRNA for VEGF was observed in granulosa cells of antral follicles (Shimizu *et al.*,

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2002, 2003) during the early and mid-luteal phases (Kaczmarek *et al.*, 2007), whilst mRNA for receptors VEGFR-1 and VEGFR-2 were expressed especially within the layers of thecal cells (Shimizu *et al.*, 2002, 2003).

VEGF expression was also reported in granulosa and thecal cells of secondary follicles in rats, and could be enhanced in response to the gonadotropins follicle stimulating hormone (FSH), luteinizing hormone (LH) and human chorionic gonadotrophin (hCG) (Koos, 1995; Yang *et al.*, 2008). Conversely, at the end of the growth phase in porcine folliculogenesis, a progressive decrease in the production of VEGF is observed in response to the LH surge or to the administration of hCG (Barboni *et al.*, 2000).

Immunolocalization of VEGF in caprine ovarian tissue reveals the presence of VEGF in follicles at all developmental stages, with a progressive increase from the primary to the preovulatory stage, as well as in surrounding stroma cells (Sharma & Sudan, 2010). With regard to the immunoreactivity of goat ovaries in relation to receptor 2 (VEGFR-2/KDR), Bruno *et al.* (2009) observed the expression of this receptor in all follicle categories, but antral follicles displayed weak positive reactions. Furthermore, their study demonstrated the presence of this receptor in oocytes of primordial follicles, which indicated the involvement of VEGF in the growth and development of these cells (Bruno *et al.*, 2009). Immunolocalization reactions for VEGF are stronger in cells of the theca interna in comparison with granulosa cells and stromal vascular tissue (Sharma & Sudan, 2010). As VEGF levels rise whilst the respective receptors decline throughout follicle development, the role of this factor may rely more probably on its function of promoting cellular permeability.

VEGF and its receptors VEGFR-1/Flt-1 and VEGFR-2/KDR are detected in cells from the endothelium and during pregnancy corpora lutea in sows (Kaczmarek *et al.*, 2007), and also in endothelial cells of bovine ovaries (Berisha *et al.*, 2000). In humans, mRNA and proteins corresponding to these receptors, as well as to VEGF-A (protein), were expressed in oocytes, granulosa and stroma cells (Abir *et al.*, 2010). In addition, VEGF was also immunolocalized in granulosa and theca interna cells of healthy follicles from rats (Koos, 1995) and cows (Berisha *et al.*, 2000), as well as in luteinized granulosa cells in buffalos (Papa *et al.*, 2007) and mares (Al-zi'abi *et al.*, 2003).

Biological activity and role of VEGF in mammalian folliculogenesis

The selective activation of each of the VEGF receptor types results in distinct biological responses. Binding to VEGFR-1/Flt-1 leads to organizational effects on vascular structures, which are important for the interaction of endothelial cells and for blood vessels formation. In contrast, activation of VEGFR-2/KDR induces the formation, migration and proliferation of vascular endothelial cells (Neufeld *et al.*, 1999; Ho & Kuo, 2007), as well as contributing to cellular survival. Binding to VEGFR-3/Flt-4, predominantly expressed in lymphatic vessels, resulted in lymphatic angiogenesis (Ho & Kuo, 2007). Some biological responses to VEGF binding to its receptors important for follicle development are described in more detail below (Fig. 3).

Angiogenic action of VEGF

VEGF was discovered originally as a compound that was capable of enhancing the permeability of vessels, thus enabling proteins and other molecules to exit blood vessels and enter perfused tissues (Senger *et al.*, 1983; Dvorak *et al.*, 1995). With regard to the mammalian ovary, VEGF properties were demonstrated first in the bovine corpus luteum (Tischer *et al.*, 1989), and later in the same tissue from ewes (Redmer *et al.*, 1996). The cyclic changes during formation and regression of the corpus luteum comprise the formation of new blood vessels (Redmer & Reynolds, 1996; Wulff *et al.*, 2001) from pre-existing vessels, and is named angiogenesis.

Later studies have revealed that this factor was also involved in other processes such as the promotion of growth of vascular cells derived from arteries, veins and lymphatic vessels (Ferrara & Davis-Smyth, 1997; Ferrara & Alitalo, 1999). Moreover, VEGF was found to induce a potent angiogenic response in a wide range of *in vivo* (Leung *et al.*, 1989) and *in vitro* (Pepper *et al.*, 1992; 1994) models.

In the ovary, angiogenesis facilitates oxygenation and nutrition of target cells, and secures an increasing supply of gonadotropins, growth factors, oxygen, steroid precursors, as well as other substances to the growing follicle (Kaczmarek *et al.*, 2005). Such rise in the delivery of nutrients can be a decisive factor for the selection of the dominant follicle (Zimmermann *et al.*, 2001). Therefore, there is evidence that thecal angiogenesis plays a pivotal role in follicle development (Tamanini & De Ambrogi, 2004). Furthermore, granulosa cells are important for the angiogenic process, as these cells secrete several angiogenic factors that act on thecal cells.

VEGF and cell permeability

VEGF can also act indirectly through reorganization or formation of a primitive capillary plexus for supply of tissue needs, increase in vascular permeability and enabling a higher availability of growth factors,

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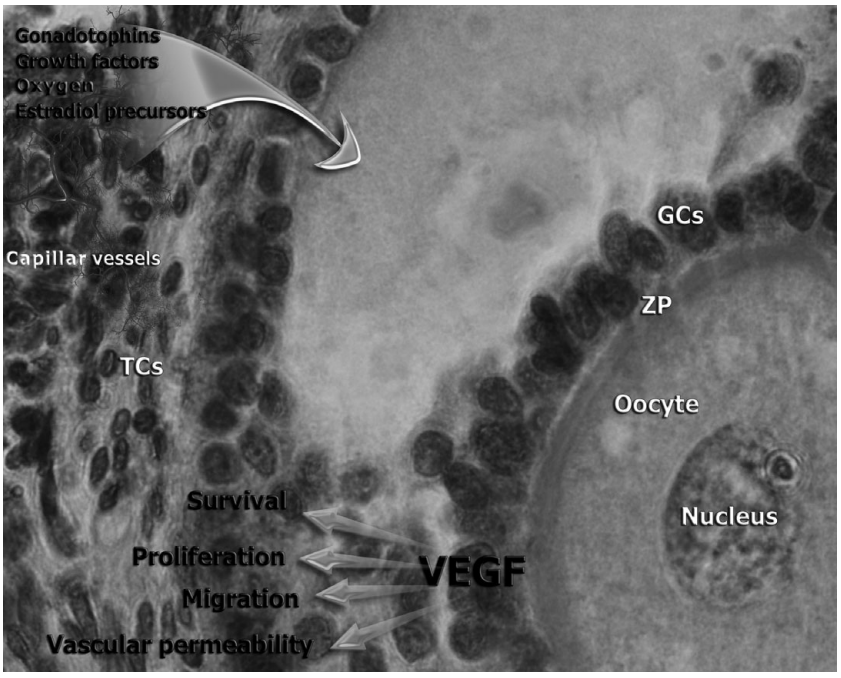

Figure 3 Biological activities of VEGF in the mammalian ovarian follicle. The expansion of the vascular network during follicle development enhances oxygenation and diffusion of several substances important for follicle cells, and leads to the discussed biological responses. GC, granulosa cell; TC, theca cell; VEGF, vascular endothelial growth factor; ZP, zona pellucida.

gonadotropins, steroids and oxygen, which are important for follicle growth. This fact was confirmed *in vivo* by Danforth *et al.* (2003) and Quintana *et al.* (2004) through direct injection of VEGF into the ovarian bursa in mice that enhanced neovascularization, increased the numbers of primary and secondary follicles and vascular permeability for developing follicles, and, as a consequence, reduced apoptosis. *In vitro,* Mattioli *et al.* (2001) observed that VEGF production raised blood supply and activated primordial follicles.

The cellular permeability induced by VEGF is attributed to the appearance of fenestrations that, through a not well defined mechanism, enables a rise in the efflux of small solutes (Roberts & Palade, 1995). Dvorak (2000) observed that the interaction between VEGF and its receptors VEGFR-1 and VEGFR-2 triggers a cascade of events that includes an increase in microvascular permeability, leading to deposition of pro-angiogenic fibrin in the extracellular matrix and formation of new vessels. Furthermore, VEGF induces an increase in calcium influx, as well as a rise in the concentration of this ion within endothelial cells (Bates & Curry, 1997).

In the ovarian follicle, the promotion of vascular permeability, vasodilation and development of endocrine function by theca cells resulted in a gradual rise in ovarian blood flux, and supported antrum formation and functional adaptation events for ovulation, which led to follicle rupture (Jiang *et al.*, 2003; Tamanini & De Ambrogi, 2004). Thus, the establishment of an adequate vascular supply is possibly a limiting step in the selection and maturation of the one dominant follicle that will ovulate (Stouffer *et al.*, 2001).

The formation of the antral cavity is a spontaneous event during the *in vitro* culture of advanced preantral follicles, however mitogenic factors such as VEGF may enhance rates of occurrence of this process (Araújo *et al.*, unpublished data). One study showed that VEGF secretion is stage dependent and increases as the follicle grows, which reflects in the amounts of VEGF in the follicular fluid (Barboni *et al.*, 2000). VEGF is also produced by cells of preovulatory follicles, as well as by luteinized cells (Taylor *et al.*, 2004).

VEGF and cell survival

The role of VEGF as a survival factor was observed either *in vitro* or *in vivo* with endothelial cells (Alon *et al.*, 1995; Yuan *et al.*, 1996), as well as with other cell types. VEGF inhibits apoptosis induced by absence of serum in culture medium (Gerber *et al.*, 1998a) or by injuries that result from cryopreservation (Shin *et al.*, 2006). This property may be mediated via PI3kinase/Akt (Gerber *et al.*, 1998a), which is a signalling pathway fundamental for regulation of cell proliferation, survival, migration and metabolism, and also plays an important role in the activation of primordial follicles (Cantley, 2002). Moreover, VEGF induces the expression of anti-apoptotic proteins such as Bcl-2 and A1 in endothelial cells (Gerber *et al.*,

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1998b). The addition of VEGF to *in vitro* culture supported the maintenance of viability and ultrastructure of goat early preantral follicles (Bruno *et al.*, 2009).

Mitogenic action of VEGF

In addition to its angiogenic properties, VEGF is also a potent mitogenic factor that is secreted by many differentiated cells in response to several stimuli such as, for instance, hypoxia. Nonetheless, the loss of its carboxy-terminal domain reduces significantly the potency for induction of proliferation in endothelial cells (Keyt *et al.*, 1996). VEGF exerts direct mitogenic effects on granulosa cells, and then acts on follicle growth in human ovaries (Otani *et al.*, 1999). The presence of VEGF-A receptors, especially in granulosa cells, suggests that this factor may be involved in proliferation events, as well as in the onset of development of primordial follicles in humans (Abir *et al.*, 2010). Furthermore, during the transition of these follicles to the primary stage, an increase in VEGF and its mRNA takes place in rats (Kezele *et al.* 2005). Yang & Fortune (2007) observed the transition of primary follicles to the secondary stage, and also the increase in follicle diameter, through the *in vitro* culture of ovarian tissue retrieved from bovine fetuses in medium supplemented with VEGF. Similarly, in addition to follicular growth, an increase in oocyte diameter could also be seen in early (Bruno *et al.*, 2009) and advanced (Araújo *et al.*, unpublished data) goat preantral follicles.

Role of VEGF on oocyte maturation

As VEGF expression increases progressively from the primary to the preovulatory stage, which is directly correlated to the expansion of vascularization and oxygenation of follicles (Sharma & Sudan, 2010), selection of the dominant follicle depends on the formation and the differentiation of a rich vascular supply with an increment in the permeability of the respective vessels (Kawano *et al.*, 2003). Such conditions are very important because hypoxia may reduce oocyte metabolism and cause changes in intracellular pH, which in turn affects organization and stability of the meiotic spindle (Gaulden, 1992). Such an effect can result in chromosomal disorders (non-disjunction of chromosomes) (Van Blerkom *et al.*, 1997). Moreover, deficiencies in blood supply impair the delivery of substances that are essential for the development of follicles to the preovulatory phase (Zimmermann *et al.*, 2003). Therefore, VEGF is an important factor for the development of mammalian oocytes, and contributes to making these gametes competent for fertilization, embryo development and pregnancy.

The incomplete cytoplasmic maturation commonly observed after *in vitro* maturation of oocytes (First & Barnes, 1989) may explain the low rates of fertilization and extrusion of the first polar body (Trounson *et al.*, 1977). The use of VEGF in culture of bovine cumulus–oocyte complexes promoted nuclear (Einspanier *et al.*, 2002; Luo *et al.*, 2002) and cytoplasmic (Luo *et al.*, 2002) maturation of the oocytes, and enhanced normal fertilization rates and the subsequent embryo development to the blastocyst stage. Moreover, Iijima *et al.* (2005) observed that treatment of rats with VEGF promoted ovarian follicular angiogenesis, stimulated follicle development and increased the number of ovulated oocytes, which showed normal fertilization and developmental competence to term.

Despite the evidence that VEGF can contribute to oocyte maturation, the mechanisms by which this factor acts in this process are still unclear. It has been postulated that VEGF may exert its main paracrine effects directly on oocytes or indirectly via cumulus cells that express VEGF receptors type 2 (VEGFR-2/KDR) (Bruno *et al.*, 2009) and are expanded in bovine (Einspanier *et al.*, 2002; Luo *et al.*, 2002) and caprine (Araújo *et al.*, unpublished data) cumulus–oocytes complexes cultured with VEGF.

Conclusions

A full understanding of the role of VEGF on the modulation of ovarian physiology is very important as this growth factor controls vascularization and therefore the availability of oxygen and nutrients for the follicles. Studies have demonstrated that VEGF influences cell survival, proliferation and thus follicular development positively, along with the stimulation of secretion of some steroid hormones such as, for instance, progesterone. In spite of the recognized potential of VEGF for enhancing follicle and oocyte developmental processes, studies on the functions of this factor in folliculogenesis are still scarce. Therefore, more investigation is necessary in order to explore the various biological properties of VEGF and its receptors.

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