THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR ISOFORMS IN EARLY FOLLICLE DEVELOPMENT

by

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Abstract

Since vascularization of the theca layer increases as follicles progress in size through preantral and antral stages, the principal angiogenic factor, vascular endothelial growth factor A (VEGFA), may influence follicle growth via regulation of angiogenesis. However, VEGFA may also influence follicular development through nonangiogenic mechanisms since its expression has been localized to nonvascular follicles and cells. Alternative mRNA splicing of 8 exons from the *VEGFA* gene results in the formation of different VEGFA isoforms. Each isoform has unique properties and is identified by the number of amino acids within the mature protein. Proangiogenic isoforms are encoded by exon 8a while a sister set of isoforms with antiangiogenic properties are encoded by exon 8b. The antiangiogenic isoforms comprise the majority of VEGFA expressed in most tissues while expression of the proangiogenic VEGFA isoforms is upregulated in tissues undergoing active angiogenesis. The *Vegfa* angiogenic isoforms (*Vegfa_120*, *Vegfa_164*, and *Vegfa_188*) were detected in developing rat ovaries, and quantitative RT-PCR determined that *Vegfa_120* and *Vegfa_164* mRNA was more abundant after birth, while *Vegfa_188* mRNA was highest at embryonic day 16. The antiangiogenic isoforms, *Vegfa_165b* and *Vegfa_189b*, were amplified and sequenced from rat ovaries and quantitative RT-PCR determined that *Vegfa_165b* mRNA was more abundant around embryonic day 18, but *Vegfa_189b* lacked a distinct pattern of abundance. VEGFA and its receptors were localized to pregranulosa and granulosa cells of all follicle stages and to theca cells of advanced-stage follicles. Antiangiogenic VEGFA isoforms were localized to pregranulosa and granulosa cells of all follicle stages and to theca cells of advanced-stage follicles. To determine the role of VEGFA in developing ovaries, postnatal day 3/4 rat ovaries were cultured with VEGFR-TKI, a

tyrosine kinase inhibitor that blocks signaling through the VEGFA receptors, FLT1 and KDR. Ovaries treated with VEGFR-TKI had vascular development reduced by 94%. In addition, treated ovaries had more primordial follicles, fewer early primary, transitional, and secondary follicles, and greater total follicle numbers compared with control ovaries. This suggests that VEGFA promotes follicle recruitment and early follicular development. These effects may be dependent upon increased ovarian vascularization or they may be mediated by nonvascular mechanisms.

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Chapter 1 - Literature Review

Introduction

Angiogenesis is the term used to describe the formation of new vessels from the remodeling and expansion of the existing vascular network. This process involves both proliferation and migration of endothelial cells and can lead to vascularization of previously avascular tissues (Patan 2000, Shimizu *et al.* 2003b). Ovarian and follicular vasculature allows for delivery of nutrients, oxygen, and systemic hormones, as well as release of ovarian hormones (Shimizu *et al.* 2003b, Robinson *et al.* 2009). Due to recurring cyclical changes and development of follicles, continued angiogenesis is essential for these ovarian functions.

Follicle assembly and initial recruitment of primordial follicles begins near the corticomedullary border and progresses outward to the periphery (Rajah *et al.* 1992, van Wezel & Rodgers 1996). At this stage of development, the major ovarian vessels are only located within the medulla (Brennan *et al.* 2002). Primordial, primary, and early secondary follicles are not directly supplied with vasculature but they are able to receive nutrients and oxygen by passive diffusion from vessels in the surrounding stroma (Suzuki *et al.* 1998, Shimizu *et al.* 2003b, Robinson *et al.* 2009). Therefore, follicle assembly and growth at these stages may be influenced by close association with vasculature and the associated delivery of mediating factors.

In order for follicles to progress past these early developmental stages, an individual capillary network needs to form around each follicle (Suzuki *et al.* 1998). Vascularization is first visible in follicles that contain four layers of granulosa cells (Wulff *et al.* 2001). All capillaries are located outside of the basement membrane of the follicle and granulosa cells will remain avascular throughout follicle development (Suzuki *et al.* 1998, Tamanini & De Ambrogi 2004).

Proliferation of the theca layer significantly increases from the early to late secondary follicle stage and approximately ¼ of these proliferating cells are endothelial cells (Wulff *et al.* 2001). A significant increase in vasculature has been demonstrated during preantral follicle development in pigs (Martelli *et al.* 2006) and follicular blood flow has been shown to be necessary for continued growth of small antral follicles in cows (Acosta *et al.* 2005). In rabbits, both vasodilation and extension of thecal capillaries support the increase of blood flow during follicle growth (Macchiarelli *et al.* 1993).

Vascular changes continue through preovulatory development. It has been demonstrated in women that there is a decrease in blood flow to the apical aspect of preovulatory follicles while flow to the basal and lateral follicle walls remains unchanged. Presumably, this change in blood flow is necessary for eventual follicle rupture (Brännström *et al.* 1998). Furthermore, subcutaneous injection of adult rats with TNP-470, an angiogenic inhibitor previously used to reduce tumor growth, not only causes a reduction in follicular angiogenesis but also prevents ovulation (Iijima *et al.* 2005).

In addition to regulating follicle development, alterations in the follicular vasculature may also be involved in follicular degeneration. Early indicators of follicular atresia include a reduction in follicle vascularity and decreased DNA synthesis in endothelial cells within the theca layer (Greenwald 1989). In sheep, the capillary network in the theca layer has been shown to undergo a significant reduction as atresia progresses (Hay *et al.* 1976). In humans, the capillaries within atretic follicles are thin, have reduced branching, and are not uniformly distributed (Macchiarelli *et al.* 1993).

Angiogenesis is a highly regulated process that involves control from both proangiogenic and antiangiogenic factors. Principal proangiogenic factors include, vascular endothelial growth

factor A (VEGFA), fibroblast growth factor 2, members of the platelet-derived growth factor family, and angiopoietins (Carmeliet 2000, Robinson *et al.* 2009). Of these factors, VEGFA has been extensively studied in regards to its role in angiogenic regulation (Ferrara & Davis-Smyth 1997, Ferrara 2004) and has also been investigated as a factor involved in follicular development. For example, intraperitoneal injection of mature rats with VEGFA results in increased numbers of preovulatory follicles, decreased numbers of atretic follicles, and increased numbers of ovulated oocytes compared to control rats (Iijima *et al.* 2005). In contrast, subcutaneous administration of truncated versions of the VEGFA receptors, KDR (kinase insert domain protein receptor) and FLT1 (FMS-like tyrosine kinase 1), fused to IgG (Trap compounds that inhibit VEGFA) to adult marmoset monkeys during the follicular phase inhibits ovulation and results in twice as many atretic follicles compared to control monkeys (Wulff *et al.* 2002). VEGFA may also be necessary for increased oocyte competence since the concentration of VEGFA protein in follicular fluid from antral follicles was significantly greater in women that became pregnant after in vitro fertilization compared to those that did not (Zhao *et al.* 2010).

VEGFA and Its Receptors

VEGFA (also known as VEGF) has been shown to promote migration, proliferation, and tube formation in endothelial cells (Patan 2000) and is a member of the platelet-derived growth factor and vascular endothelial growth factor family. This family also includes placenta growth factor, VEGFB, VEGFC, and VEGFD (Ferrara 2004). VEGFA is essential for both vasculogenesis and angiogenesis. Loss of VEGFA in mouse models leads to severe vascular abnormalities and is embryonic lethal between 11-12 dpc (Carmeliet *et al.* 1996, Ferrara *et al.* 1996). Messenger RNA expression for VEGFA is prominently stimulated by hypoxia but

VEGFA expression is also upregulated by several other factors, such as platelet derived growth factor, insulin-like growth factor-1, tumor necrosis factor-alpha, fibroblast growth factor, transforming growth factor alpha and beta, and epidermal growth factor (Robinson & Stringer 2001, Ferrara 2004).

There are 2 tyrosine kinase receptors that bind VEGFA with high affinity, FLT1 (also referred to as VEGFR1) and KDR (also referred to as VEGFR2). Both of these receptors have 7 extracellular immunoglobulin-like domains, a single transmembrane region, and an intracellular tyrosine kinase sequence with a kinase insert domain (Robinson & Stringer 2001, Ferrara 2004). FLT1 was the first VEGFA receptor identified and has high affinity for VEGFA; however, VEGFA binding results in only weak tyrosine phosphorylation and does not appear to induce a proliferative response (de Vries *et al.* 1992, Park *et al.* 1994, Waltenberger *et al.* 1994, Seetharam *et al.* 1995). Thus, it has been proposed that FLT1 negatively regulates VEGFA activity by sequestering it and limiting its availability to bind KDR (Park *et al.* 1994). Mutant mice that lack FLT1 die between 8.5-9.5 dpc and display severe vascular disorganization and increased number of endothelial progenitor cells (Fong *et al.* 1995, Fong *et al.* 1999). However, mutant mice that possess an intact VEGFA binding region on FLT1 but lack a tyrosine kinase domain are able to fully develop and display normal vascular development (Hiratsuka *et al.* 1998).

Although FLT1 has a higher affinity for VEGFA than KDR, VEGFA binding to KDR induces stronger tyrosine phosphorylation (Waltenberger *et al.* 1994). It is believed that KDR mediates most, if not all, of VEGFA's regulation of endothelial cell proliferation and migration. Mutant mice that lack KDR die between 8.5-9.5 dpc and fail to develop organized blood vessels (Shalaby *et al.* 1995). Mutated VEGFA proteins that lack affinity for the KDR receptor fail to

stimulate proliferation and migration of bovine endothelial cell cultures. In contrast, treatment of these cell cultures with VEGFA protein mutants that lack affinity for the FLT1 receptor stimulates endothelial cell proliferation similar to that induced by treatment with wild-type VEGFA protein (Keyt *et al.* 1996).

In addition to FLT1 and KDR, VEGFA has also been shown to bind to neuropilins. Mutant mice that lack neuropilin-1 (NRP1) die between 10.5-12.5 dpc with multiple vascular defects and mutant mice that overexpress NRP1 die at 17.5 dpc with excessive and dilated vasculature (Kitsukawa *et al.* 1995, Kawasaki *et al.* 1999). NRP1 appears to function as a coreceptor by presenting VEGFA to KDR. NRP1 has a very short intracellular domain and is unable to stimulate cellular responses in the absence of KDR (Soker *et al.* 1998). NRP1 has also been shown to directly bind FLT1. Therefore, one of the mechanisms by which FLT1 negatively regulates VEGFA activity may be through competing for NRP1 binding (Fuh *et al.* 2000).

VEGFA Isoforms

VEGFA is encoded by a single gene but different isoforms exist due to alternative mRNA splicing of 8 exons (Figure 1-1). The different isoforms are identified by number of amino acids and each isoform has unique properties (Houck *et al.* 1991, Tischer *et al.* 1991). Of the predominant isoforms, VEGFA_121 (exons 1-5 and 8a; Figure 1-1) is the shortest protein and is unable to bind heparin. VEGFA_165 (exons 1-5, 7 and 8a; Figure 1-1) and VEGFA_189 (exons 1-5, 6a, 7 and 8a; Figure 1-1) contain additional amino acid sequences encoded by exons 6 and 7. VEGFA_165 has moderate affinity for heparin due to the amino acid residues encoded by exon 7. VEGFA_189 has additional residues encoded by exon 6 and thus, has a high affinity for heparin binding. (Robinson & Stringer 2001). VEGFA_189 is almost entirely bound to either

cell surfaces or the extracellular matrix (ECM), presumably due to interactions with heparin-containing proteoglycans. Approximately 50-70% of VEGFA_165 is bound to cells or the ECM while VEGFA_121 is freely diffusible (Houck *et al.* 1992). NRP1 is able to bind to VEGFA_165 but not VEGFA_121. NRP1 enhances the binding of VEGFA_165 to KDR and thus, its regulation of proliferation and migration of endothelial cells. (Soker *et al.* 1998). Several other isoforms have been isolated in different cells and species, including VEGFA_115, VEGFA_145, VEGFA_162, and VEGFA_183, and VEGFA_206 (Anthony *et al.* 1994, Cheung *et al.* 1995, Lei *et al.* 1998, Sugihara *et al.* 1998, Jingjing *et al.* 1999, Lange *et al.* 2003).

In addition to differences in amino acid length, VEGFA isoforms have been identified that originate from alternative splicing of exon 8. The previously described VEGFA isoforms are encoded by exon 8a (Figure 1-1). Replacement of exon 8a with exon 8b generates a novel, sister set of isoforms (VEGFA_XXXB) referred to as the "B" isoforms (Figure 1-1). The VEGFA_XXXB isoforms only differ from the other VEGFA isoforms by the 6 amino acids located at the C´-terminal end and instead of being proangiogenic, the presence of residues encoded by exon 8b confers antiangiogenic properties (Bates *et al.* 2002, Cui *et al.* 2004, Woolard *et al.* 2004b, Konopatskaya *et al.* 2006, Harper & Bates 2008).

In most normal, adult tissues, the VEGFA_XXXB isoforms comprise at least half of the total VEGFA expressed. In normal tissues undergoing active angiogenesis, such as the placenta or in pathologic states, such as neoplasia, VEGFA_XXXB isoform expression is downregulated (Woolard *et al.* 2004b, Harper & Bates 2008). For example, mRNA for the *VEGFA_165B* isoform was detected in 94.4% of normal kidney samples but only 22.2% of renal cell carcinoma samples from the same human patients (Bates *et al.* 2002). Similarly, *VEGFA_XXXB* isoforms comprised approximately 91% of the total amount of the *VEGFA* mRNA amplified from normal

colorectal tissue but less than 55% of the total mRNA from colorectal tumor tissue from the same human patients (Varey *et al.* 2008). Differential expression of the proangiogenic versus antiangiogenic VEGFA isoforms is also evident in proliferative diabetic retinopathy. This condition develops from hypoxia-mediated blood vessel growth which extends from the retina into the vitreous chamber in human diabetic patients. The VEGFA_XXXB isoforms represented approximately 64% percent of the VEGFA protein isolated from the vitreous of non-diabetic human patients but only 12.5% of the VEGFA protein isolated from the vitreous in diabetic patients (Bevan *et al.* 2008). Therefore, it appears that alternate splicing of the *VEGFA* gene and the resulting ratio of increased proangiogenic versus antiangiogenic isoforms is an important regulator of angiogenesis.

The VEGFA_XXXB isoforms were considered antiangiogenic shortly after identification because treatment with VEGFA_165B inhibited VEGFA_165-mediated proliferation and migration of cultured endothelial cells (Bates *et al.* 2002). One study demonstrated that while VEGFA_165 can stimulate angiogenesis in rabbit corneas, VEGFA_165B does not stimulate angiogenesis and can even inhibit VEGFA_165-mediated corneal angiogenesis (Woolard *et al.* 2004b). In another study, intraocular injections of VEGFA_165B resulted in a nearly 50% reduction in the area of hypoxia-induced retinal neovascularization in mice (Konopatskaya *et al.* 2006). In addition, melanoma cells expressing VEGFA_165B injected into nude mice produce significantly smaller tumors than melanoma cells expressing VEGFA_165 (Woolard *et al.* 2004b).

The antiangiogenic properties of the VEGFA_XXXB isoforms are believed to be related to inefficient stimulation of downstream signaling. VEGFA_165B has been shown to bind to KDR with similar affinity as VEGFA_165 but does not activate downstream signaling via KDR.

Not only does treatment of human endothelial cells with VEGFA_165B result in less KDR phosphorylation than treatment with VEGFA_165, but VEGFA_165B-treated cells demonstrated similar phosphorylation as untreated cells. Treatment of these cells with both VEGFA_165 and VEGFA_165B also resulted in less phosphorylation than treatment with VEGFA_165 alone (Woolard *et al.* 2004b). Another study demonstrated that VEGFA_165B was able to induce phosphorylation by binding KDR but caused significantly less phosphorylation in certain KDR sites, including one of the positive mouse regulatory sites, Y1052. Furthermore, VEGFA_165B is not able to bind NRP1 and this might explain the ineffectiveness of these antiangiogenic VEGFA at signaling upon binding to KDR (Kawamura *et al.* 2008).

The different VEGFA isoforms are named for the number of amino acids that comprise each protein; however, the number of amino acids for the similar isoforms can vary between species. For example, the predominant proangiogenic isoform in humans has 165 amino acids (VEGFA_165) but the corresponding isoform in mice, rats, and cattle consists of only 164 amino acids (VEGFA_164) (Breier *et al.* 1992, Bacic *et al.* 1995, Robinson & Stringer 2001, Shimizu & Miyamoto 2007). The antiangiogenic isoform that has been most extensively studied is human VEGFA_165B which has the same number of amino acids as its proangiogenic counterpart, VEGFA_165 (Bates *et al.* 2002, Harper & Bates 2008). We have sequenced the mRNA for *VEGFA_164B* (GenBank accession number EU017524.1) from bovine granulosa cells. Based on the predicted amino acid sequence, the antiangiogenic isoforms appear to have the same number of amino acids as the proangiogenic isoforms in the bovine. However, we have also sequenced the mRNA for *Vegfa_165b* from rat ovaries and based on the predicted amino acid sequence, the antiangiogenic isoforms appear to have an additional amino acid compared to their respective proangiogenic isoforms in rats (Artac *et al.* 2009). To avoid confusion, all

VEGFA isoforms will be referred to in this chapter using the number of amino acids found in humans (VEGFA_121, VEGFA_165, VEGFA_189, VEGFA_165B, etc.) regardless of the species being discussed.

Establishment of the Primordial Follicle Pool

In mammals, it is primarily believed that the oocyte population is nonrenewable and the number of primordial follicles formed during fetal or early perinatal life is what limits the reproductive life span (Perez *et al.* 1999, Hansen *et al.* 2008, McLaughlin & McIver 2009). A decrease in the number of primordial follicles can result in reduced fertility, premature ovarian failure, or sterility. Exposing fetal rats to gamma-irradiation severely depletes the number of oocytes and premature ovarian failure occurrs at approximately 6 months-of-age despite normal onset of puberty and initial fertility (Mazaud *et al.* 2002). In addition, treating fetal rats with busulphan results in a dose-dependent reduction in the number of primordial follicles that develop in treated animals compared to controls and severely depleted rats will exhaust their supply of follicles early in adulthood (approximately 60 days-of-age) (Hirshfield 1994).

During follicle assembly, approximately 1/3 of the oocytes are arrested at the diplotene stage of the first meiotic division and are incorporated into primordial follicles while the remaining 2/3 of germ cells are lost through apoptosis (Pepling & Spradling 2001). Overexpression of the anti-apoptotic factor, BCL2 (B-cell leukemia/lymphoma 2), in mouse ovaries leads to an increase in the initial primordial follicle pool but this difference is lost by 2 months-of-age (Flaws *et al.* 2001). Interestingly, loss of BCL2 function in mice results in a dramatic reduction in the number of normal primordial follicles and in the development of numerous primordial follicle-like structures that contain granulosa cells but lack an oocyte (Ratts

et al. 1995). Furthermore, deletion of the pro-apoptotic factor, BAX (BCL2-associated X protein), in mice leads also to an increase in the initial primordial follicle pool and this surplus is maintained into late adult life, resulting in sustained ovarian function to nearly 2 years-of-age (Perez *et al.* 1999). It is possible that proangiogenic VEGFA isoforms may be involved in stimulating an increase in BCL2 which promotes germ cell survival. Although studies in females are lacking, experiments utilizing 4-week-old, bovine testes have demonstrated a role for VEGFA in regulating germ cell death. Increased numbers of germ cells along with increased mRNA levels of *BCL2* relative to *BAX* is seen after VEGFA_165 treatment (Caires *et al.* 2009).

Previous studies in our laboratory have demonstrated immunohistochemical staining for VEGFA protein and VEGFA_XXXB isoforms in oocyte cysts and pregranulosa cells of primordial follicles in embryonic and postnatal rat ovaries. In addition, VEGFA_XXXB isoforms are also localized to the oocytes of primordial follicles (Artac *et al.* 2009, McFee *et al.* 2009). The VEGFA receptors, FLT1 and KDR, are localized to oocyte cysts and both pregranulosa cells and oocytes of primordial follicles (McFee *et al.* 2009). Other studies have demonstrated weak VEGFA immunostaining of ooctyes in primordial follicles from human ovaries (Otani *et al.* 1999) and KDR expression has been localized to oocytes of primordial follicles from goat ovaries (Bruno *et al.* 2009).

Our laboratory has also demonstrated mRNA expression for the *Vegfa_121*, *Vegfa_165*, *Vegfa_189*, *Vegfa_165b*, and *Vegfa_189b* isoforms in rat ovaries during late embryonic and early postnatal development (Artac *et al.* 2009, McFee *et al.* 2009). In contrast to male gonadal development, distinct morphologic structures do not appear in the developing rat ovary until ovigerous cords form at the end of gestation (~E16-18). Oocytes are contained within these cord-like structures until P1 when primordial follicles begin to assemble (Rajah *et al.* 1992,

Fröjdman *et al.* 1993, Hirshfield & DeSanti 1995). Expression of mRNA for the predominant proangiogenic isoform, *Vegfa_165*, and its comparable antiangiogenic isoform, *Vegfa_165b* (Artac *et al.* 2009, McFee *et al.* 2009) significantly increases between E13 and E18 in the rat ovary (Figure 1-2a) and the timing of this upregulation coincides with ovigerous cord formation. Therefore, upregulation of angiogenic *Vegfa* and downregulation of antiangiogenic *Vegfa_xxxb* isoforms may help promote follicle assembly.

Systemic (IP) administration of adult mice with an antibody designed to neutralize VEGFA reduces the number of primordial follicles by approximately 50% without having an effect on primary or secondary follicles. Similar results are seen with intrabursal administration of the same antibody to prepubertal (6 to 8-weeks-old) mice. Intrabursal administration of a KDR antibody also reduced primordial follicle numbers in prepubertal mice but injection with a FLT1 antibody had no effect. Although these differences in primordial follicles numbers were lost between 30 days (immature mice) or 6 months (adults) after treatment, (Roberts *et al.* 2007) these data suggest that VEGFA and KDR play a role in the maintenance of the primordial follicle pool.

Recruitment of Primordial Follicles

The duration of a female's reproductive lifespan is determined not only by the number of primordial follicles that are formed but also by the rate at which this pool of quiescent follicles is depleted. Primordial follicles can remain quiescent or arrested in development for months to years to decades, depending on the female's normal reproductive lifespan. A primordial follicle is defined as an individual oocyte surrounded by a single layer of pregranulosa cells. A primary follicle is characterized by a single layer of granulosa cells. (Parrott & Skinner 1999, Kezele *et*

al. 2002a, Smitz & Cortvrindt 2002). The transformation of flattened pregranulosa to cuboidal granulosa cells is a relatively slow process (greater than 4 months in humans) and follicles are not considered to be actively growing until they have reached the primary stage (Gougeon & Chainy 1987, Smitz & Cortvrindt 2002). This transition is also an irreversible process and a follicle will continue to grow until its eventual demise, either through atresia or ovulation.

Initial follicle recruitment begins near the corticomedullary border (Hirshfield 1992, van Wezel & Rodgers 1996). This is similar to the pattern that occurs during follicle formation. Close association of recruited follicles to the medulla may be related to these follicles being exposed to factors diffusing from the medullary vasculature. It has also been proposed that the total number of follicles within the pool influences the rate in which primordial follicles are recruited. Administration of busulphan to pregnant rats to destroy primordial germ cells in developing fetuses revealed an inverse correlation between the number of primordial follicles in the initial pool and the rate in which these follicles were recruited to the growing pool (Hirshfield 1994). Morphometric studies have also demonstrated an accelerated rate of follicle recruitment in women as they approach menopause and their supply of primordial follicles dwindles (Richardson *et al.* 1987, Faddy *et al.* 1992, Gougeon *et al.* 1994, Hansen *et al.* 2008).

Several studies have investigated possible factors that are involved in the regulation of primordial follicle recruitment. Ovarian microarray analysis revealed upregulation of 148 genes and downregulation of 50 genes in PO rat ovaries that were cultured for 1 week and contained predominately primary follicles compared to freshly isolated P4 ovaries that contain predominately primordial follicles. A high proportion of primary follicles are found in cultured ovaries because the primordial to primary transition occurs at a faster rate in culture (Kezele &

Skinner 2003). One of the 148 genes upregulated in this study was *Vegfa*. QPCR analysis has also identified the same increase in *Vegfa* gene expression (Kezele *et al.* 2005b).

One might assume that this upregulation of *Vegfa* has a role in promoting the recruitment of primordial follicles into the growing follicle pool. However, a similar study produced conflicting results. Culture of P4 rat ovaries with AMH reduced the number of primordial follicles that transitioned to the primary stage; however, microarray analysis of these cultured ovaries revealed an upregulation of *Vegfa* (Nilsson *et al.* 2007). The results from this study might suggest that upregulation of *Vegfa* has a role in suppressing the recruitment of primordial follicles. One reason for the conflicting results is that these studies did not distinguish which *Vegfa* isoforms are upregulated. It is possible that proangiogenic *Vegfa* isoforms are upregulated during the primordial to primary follicle transition and that the antiangiogenic isoforms are upregulated when this process is suppressed. In fact, studies by our laboratory using developing rat ovaries showed that mRNA expression for both *Vegfa_165* and *Vegfa_165b* increases dramatically from E13 to E18. After birth, mRNA expression of both of these isoforms declines (Artac *et al.* 2009, McFee *et al.* 2009). However, *Vegfa_165b* expression dramatically declines to levels less than those measured at E13 while *Vegfa_165* expression rebounds from P0 to P5 (Figure 1-2a). No trends were apparent for the mRNA expression for either *Vegfa_189* or *Vegfa_189b* during these developmental time points (Figure 1-2b). Therefore, these data suggest that upregulation of angiogenic *Vegfa* and downregulation of antiangiogenic *Vegfa_xxxb* isoforms may help promote follicle assembly and early follicular recruitment.

Expression of VEGFA isoforms may be related to angiogenesis of the ovary and/or follicles; however, both primordial and primary follicles are avascular. Despite being avascular, we have demonstrated localization of VEGFA, VEGFA_XXXB isoforms, FLT1, and KDR to

pregranulosa/granulosa cells of both of these follicle types using immunohistochemical staining of postnatal rat ovaries. Protein expression for FLT1 and KDR has also been localized to oocytes within these follicles (Artac *et al.* 2009, McFee *et al.* 2009). Other studies have demonstrated weak VEGFA immunostaining of oocytes in primary follicles from adult rat ovaries (Celik-Ozenci *et al.* 2003). In goat ovaries, KDR expression has been localized to oocytes of primordial and primary follicles and to granulosa cells of primary follicles (Bruno *et al.* 2009). These data provide further support that VEGFA signaling plays a role in maintenance and/or activation of primordial follicles.

Experiments in our laboratory utilizing P3/4 rat ovary cultures also support a role for VEGFA isoforms in initial follicle recruitment. Treatment with a VEGFA receptor tyrosine kinase inhibitor (blocks signal transduction through both FLT1 and KDR) significantly reduced vascular density, increased the percentage of primordial follicles, and decreased the percentage of developing follicles compared to control ovaries (Figure 1-3) (McFee *et al.* 2009). In contrast, treatment with a NRP1 inhibitor (V1) (Starzec *et al.* 2006) only minimally reduced vascular density and did not alter the percent of primordial vs. growing follicles in treated ovaries compared to controls (McFee *et al.* 2009). However, the percentage of early primary follicles was reduced and the percentage of primary follicles was increased (Figure 1-3) (McFee *et al.* 2009). This suggests that VEGFA helps to promote the activation of primordial follicles from the resting state but this regulation does not appear to require NRP1 binding. Although, VEGFA binding to NRP1 may help stimulate the development of follicles to the primary state. Furthermore, localization of VEGFA and its receptors to non-vascular cells, along with alterations of early follicular development with and without reduced vascularization indicates

that VEGFA regulation of follicle development does not necessarily depend on vascular mechanisms.

Further experiments using the same P3/4 rat ovary culture system demonstrated that treatment with recombinant VEGFA_165 or a VEGFA_XXXB antibody (neutralizes all antiangiogenic VEGFA isoforms) results in an increase in vascular density. In addition, treated ovaries had a decreased percentage of primordial follicles and an increased percentage of developing follicles compared to controls (Figure 1-3) (Artac *et al.* 2009). Both experiments lend further support for VEGFA's role in promoting follicle activation and early development. However, neutralization of the antiangiogenic VEGFA_XXXB isoforms produced more pronounced changes than administration of excess proangiogenic isoforms. This suggests that the antiangiogenic isoforms have a prominent role in regulating follicle progression.

Other studies utilizing postnatal rat ovary cultures have demonstrated a role for E_2 and P_4 in initial follicle recruitment. Approximately ½ the number of primordial follicles transitioned to primary follicles in PO rat ovary cultures that were treated with E_2 or P_4 compared with control ovary cultures (Kezele & Skinner 2003). Circulating levels of E_2 and P_4 are high in the developing embryonic and neonatal rat but drop substantially within 2 days after birth (Weisz & Ward 1980, Montano *et al.* 1995, Kezele & Skinner 2003). In larger mammals, steroid concentrations decrease around the same time follicles begin to develop (Thau *et al.* 1976). Taken together, this suggests that E_2 and P_4 can help prevent premature primordial follicle recruitment in embryonic and postnatal development (Kezele & Skinner 2003).

Another study supports this role of E_2 and also suggests that E_2 regulates ovarian expression of VEGFA, which promotes follicle recruitment. Intrabursal injection of diethylstilbestrol (DES) in prepubertal (P21) rat pups significantly increases the number of both

primary and small secondary follicles in the treated ovary compared to the contralateral ovary. Intrabursal injection with recombinant VEGFA had similar effects on follicle dynamics. In the same study, both systemic and intrabursal administration of E_2 resulted in an increase in ovarian VEGFA protein expression (Danforth *et al.* 2003).

Preantral and Antral Follicle Development

Initiation of follicle growth is characterized by 2 distinct phases. The first is the transition from primordial to primary follicle where squamous pregranulosa cells transform into cuboidal granulosa cells. The next phase includes an increase in the number of granulosa cells along with an increase in oocyte size (Braw-Tal & Yossefi 1997). Secondary, or preantral, follicles are classified as an oocyte surrounded by more than 2 granulosa cell layers (Parrott & Skinner 1999). Complete zonae pellucidae develop in large preantral follicles (Braw-Tal & Yossefi 1997). Early antral follicles have patchy spaces between granulosa cells that contain fluid and follicles are classified as antral, or tertiary, when the fluid spaces develop into a larger cavity (Smitz & Cortvrindt 2002). In cattle, antral formation first occurs in follicles with at least 250 granulosa cells in the largest histologic cross section (Braw-Tal & Yossefi 1997). Theca cells first begin to associate with small preantral follicles but a distinct theca layer is only apparent with late preantral or early antral follicles (Braw-Tal & Yossefi 1997). The first theca cells form the theca interna layer which is located closest to the granulosa cells and as development progresses, a theca externa layer forms which is highly vascularized. It takes a few weeks for an antral cavity to develop after initial follicle recruitment in mice and rats. The same process takes approximately 2 months in humans and several months in large domestic animals (Smitz & Cortvrindt 2002).

Granulosa cells begin to develop FSH receptors during the preantral stage and theca cells express LH receptors as soon as they form (Roy *et al.* 1987, Sokka *et al.* 1996, Smitz & Cortvrindt 2002). The follicles that are initially recruited and begin development in the prepubertal period never ovulate and are lost via atresia. Cyclic follicle recruitment begins after puberty due to the increase in circulating FSH levels and ovulation of the first follicle coincides with the first LH surge (Mazaud *et al.* 2002). During cyclic recruitment, a cohort of antral follicles is able to escape atresia due to the survival action of FSH (Chun *et al.* 1996, Scheele & Schoemaker 1996, McGee & Hsueh 2000). Loss of gonadotropins via hypophysectomy or follicle culture without hormonal treatments eventually leads to atresia and apoptosis of developing rat follicles (Chun *et al.* 1996, Nahum *et al.* 1996). FSH treatment but not LH or hCG treatment can significantly prevent follicular atresia in cultured rat follicles (Chun *et al.* 1996). Each growing follicle has a threshold requirement for stimulation by FSH and this threshold needs to be surpassed to achieve continued development. The recruited cohort of follicles represents a group of follicles that is at a comparable stage of development and thus, has similar developmental requirements (Fauser & Van Heusden 1997).

In postnatal rat ovaries, VEGFA, FLT1, and KDR has been localized to granulosa cells, theca cells, and the cytoplasm of oocytes of preantral follicles (McFee *et al.* 2009). In postnatal mice with KDR-LacZ-expressing cells, KDR expression is also seen in granulosa cells, theca cells, and the cytoplasm of oocytes within secondary follicles (Bott *et al.* 2010). Other studies have also demonstrated strong VEGFA immunostaining in secondary and antral follicles from rat ovaries, especially in the theca cells (Celik-Ozenci *et al.* 2003). In one study analyzing human ovaries, VEGFA was localized to theca interna cells throughout follicle development and to granulosa cells after the primary stage (Yamamoto *et al.* 1997). Another study identified

immunostaining for VEGFA and FLT1 in granulosa cells of all developing follicles and in theca cells of medium and large antral follicles (Otani *et al.* 1999). VEGFA protein is also expressed in bovine fetal ovaries and is predominately localized to blood vessels and secondary follicles. In adult bovine ovaries, immunohistochemistry has localized VEGFA expression to both theca and granulosa cells from preovulatory follicles. KDR expression was predominately localized to granulosa cells but some expression was present in theca cells (Berisha *et al.* 2000, Greenaway *et al.* 2004).

Expression of mRNA for *VEGFA_121*, *VEGFA_165*, *VEGFA_189*, *FLT1*, and *KDR* has also been demonstrated in fetal bovine ovaries and levels of *VEGFA_121* and *VEGFA_189* increase as development proceeds. Messenger RNA levels for *VEGFA* were consistent across follicle development in theca cells while expression for *KDR* and *FLT1* was weak in granulosa cells but strong in theca cells (Yang & Fortune 2007). Both granulosa and theca cells in developing follicles from adult bovine and porcine ovaries express mRNA for the *VEGFA_165* and *VEGFA_121* isoforms and expression of *VEGFA* increases as antral follicles increase in size (Berisha *et al.* 2000, Mattioli *et al.* 2001). Cultured bovine granulosa cells predominately express the *VEGFA_120* and *VEGFA_164* isoforms but also weakly express mRNA for *VEGFA_189*. These cultured cells also express mRNA for *KDR* (Greenaway *et al.* 2004). In marmoset monkeys, mRNA for *VEGFA* is expressed in both granulosa and theca cells of secondary and tertiary follicles while *FLT1* and *KDR* is expressed by the endothelial cells within the theca layer in marmoset monkeys. The mRNA levels for *VEGFA* in granulosa cells increase from the secondary follicle stage to the tertiary stage (Wulff *et al.* 2001, Wulff *et al.* 2002).

VEGFA protein in follicular fluid and granulosa cells have been demonstrated to increase in both bovine and porcine follicles as they increase in size from small to large antral follicles

(Berisha *et al.* 2000, Mattioli *et al.* 2001, Greenaway *et al.* 2004). Protein levels of KDR also increase significantly between small and large bovine antral follicles and protein levels in theca cells are similar to those in granulosa cells from large antral follicles (Greenaway *et al.* 2004). Medium antral follicles from gilts had distinct differences in VEGFA protein expression. Those follicles with high follicular fluid concentrations of VEGFA also had high follicular fluid levels of E_2 and had wider vascular networks within the follicular wall than medium follicles with low VEGFA levels (Mattioli *et al.* 2001).

Based upon mRNA and protein expression alone, VEGFA appears to be involved in the growth of follicles from the preantral and early antral stages to later antral stages. Numerous other studies have added support for the role of VEGFA in follicle development. Culturing pieces of bovine fetal ovarian cortex in the presence of VEGFA has no effect on the number of primordial or primary follicles but does increase the number of secondary follicles (Yang & Fortune 2007). Injection of *VEGFA* gene fragments into the ovaries of miniature gilts results in increased numbers of large antral follicles, increased mRNA expression of the *VEGFA_165* and *VEGFA_121* isoforms in granulosa cells, and increased VEGFA protein levels in follicular fluid. In addition, the capillary density within the theca interna is increased in follicles from VEGFA-injected gilts compared to control animals (Shimizu *et al.* 2003a, Shimizu 2006). Intramuscular injection of prepubertal gilts with eCG induces an increase in VEGFA protein levels in follicular fluid and *VEGFA* mRNA levels in granulosa cells of follicles larger than 4 mm in diameter (Barboni *et al.* 2000). VEGFA treatment also results in an increased number of preovulatory follicles, a decreased number of atretic follicles, and an increase number of ovulated oocytes (Iijima *et al.* 2005).

VEGFA regulation of preantral and antral follicle growth appears to be mediated through FLT1 and KDR signaling. Inhibition of VEGFA with intrabursal injection of a soluble FLT1/Fc chimera Trap does not alter the number of preantral or early antral follicles in prepubertal rats treated with eCG. However, there is an increased number of atretic follicles compared to control rats along with increased BAX and decreased BCL2 protein levels in follicular cells (Abramovich *et al.* 2006). Neutralization of VEGFA by administration of a VEGF Trap (truncated versions of FLT1 or FLT1 and KDR fused to IgG) significantly reduces granulosa cell proliferation, theca proliferation, and thecal vascularization in secondary and tertiary follicles in marmoset monkeys (Wulff *et al.* 2001, Wulff *et al.* 2002).

Conclusion

There is a strong body of evidence that supports a role for VEGFA in initial follicle recruitment and development. Even though VEGFA is considered a prominent proangiogenic factor, VEGFA has been localized to nonvascular follicles and cells and may influence follicular development via nonvascular mechanisms. In addition, VEGFA isoforms have now been identified that have antiangiogenic properties (Harper & Bates 2008). Specific agents have been developed that differentiate between the antiangiogenic and proangiogenic VEGFA isoforms; however, many of the studies evaluating VEGFA in regards to ovarian and follicular development up until this point have not differentiated proangiogenic VEGFA from antiangiogenic VEGFA. One must take this into consideration when interpreting the findings from these studies. In vitro experiments from our laboratory indicate that proangiogenic VEGFA promotes while antiangiogenic VEGFA suppresses follicle recruitment and early follicular development (Figure 1-4). Further studies are warranted to help elucidate how the different

VEGFA isoforms regulate follicular formation and progression. In vivo experiments utilizing cell-specific mutant models that lack or overexpress proangiogenic and antiangiogenic isoforms would further define the role of VEGFA in these processes and aid in our understanding of the balance of proangiogenic versus antiangiogenic VEGFA isoforms in follicular development.

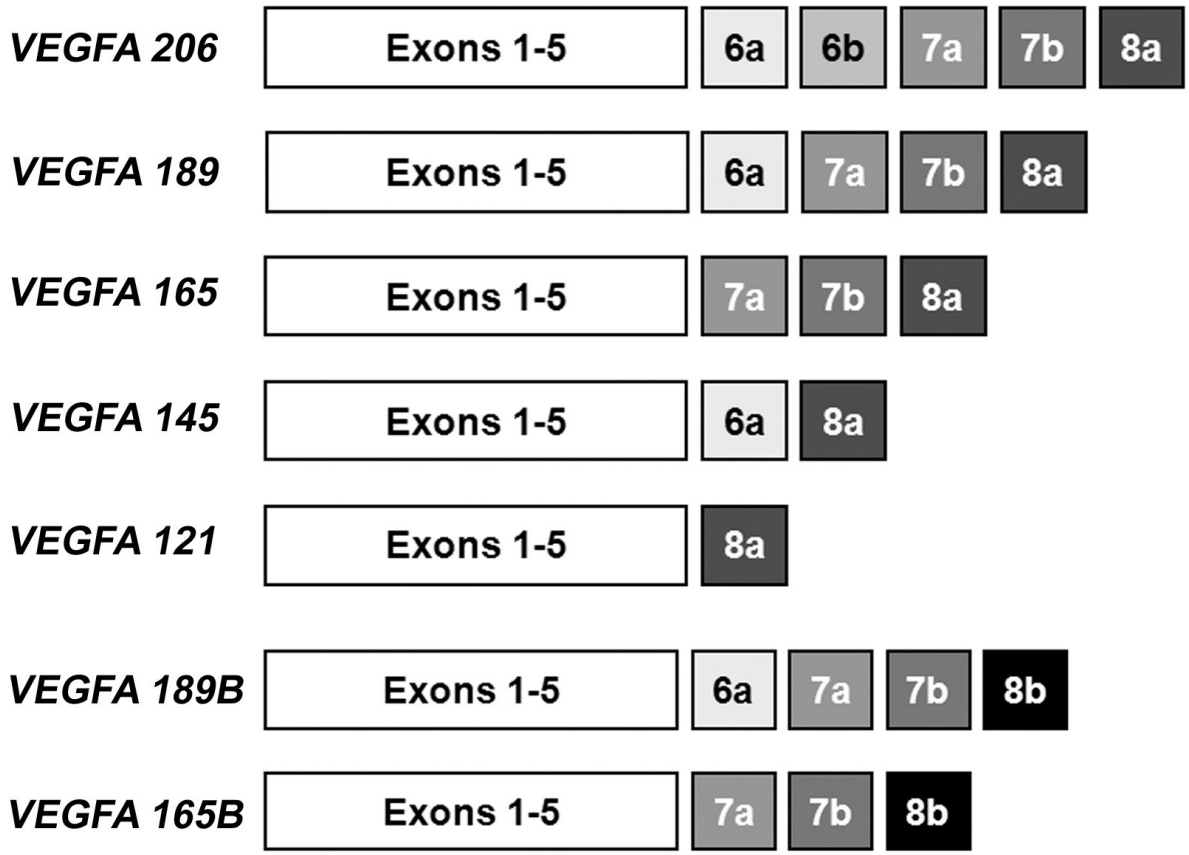

Figure 1-1

Alternate splicing of the human *VEGFA* gene results in different VEGFA isoforms. Each isoform is encoded by a specific set of exons and the resulting proteins are named by their unique number of amino acids. Exon 8a encodes the proangiogenic isoforms while exon 8b encodes the antiangiogenic "B" isoforms.

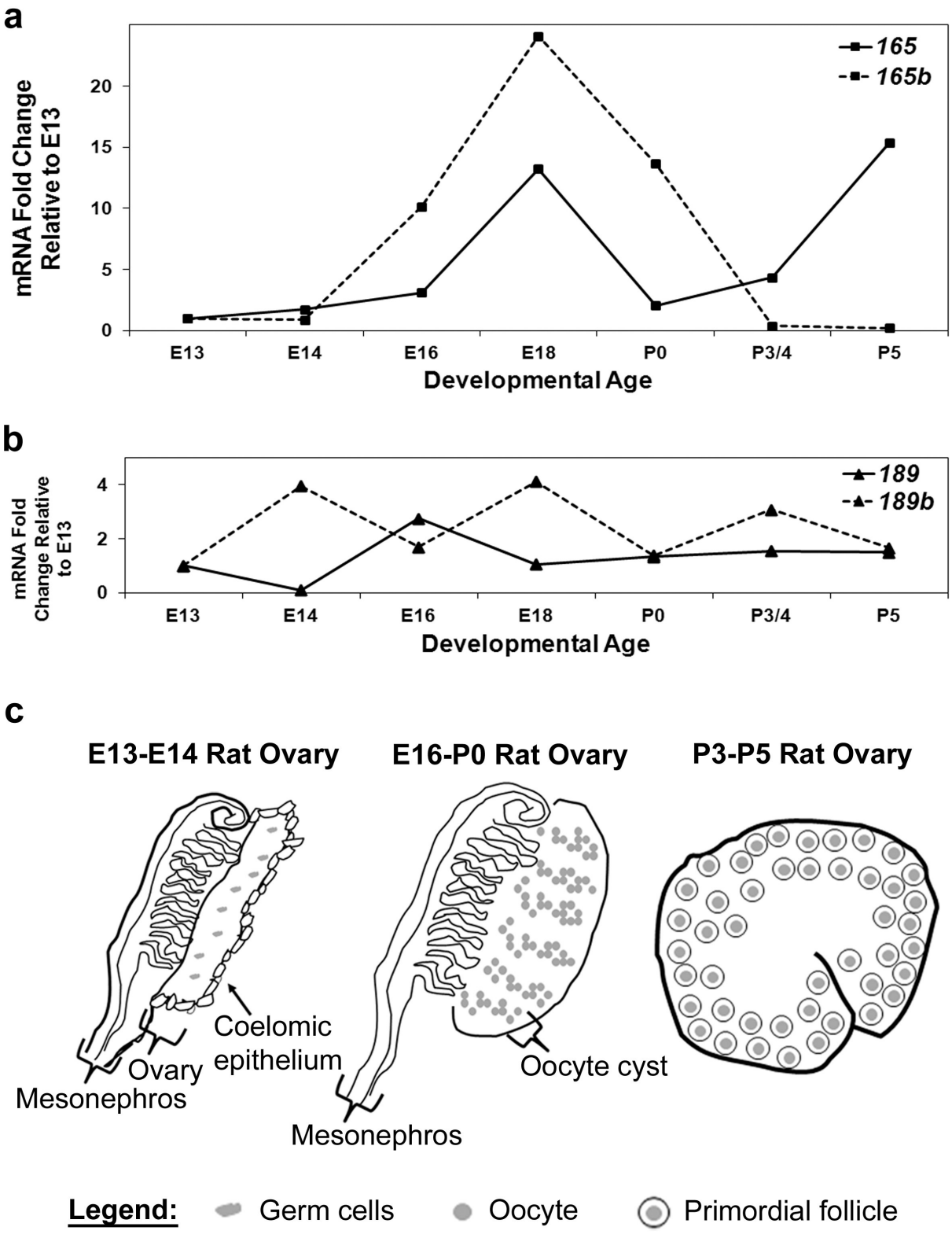
a
mRNA Fold Change Relative to E13
20
15
10
5
0
E13
E14
E16
E18
P0
P3/4
P5
Developmental Age
165
165b
b
mRNA Fold Change Relative to E13
4
2
0
E13
E14
E16
E18
P0
P3/4
P5
Developmental Age
189
189b
c
E13-E14 Rat Ovary
E16-P0 Rat Ovary
P3-P5 Rat Ovary
Coelomic epithelium
Ovary
Mesonephros
Oocyte cyst
Mesonephros
Legend:
Germ cells
Oocyte
Primordial follicle

Figure 1-2

Quantitative RT-PCR was conducted to detect mRNA levels for *Vegfa_165*, *Vegfa_165b*, *Vegfa_189*, and *Vegfa_189b* in rat ovaries from E13 through P5 of ovarian development. *Gapdh* was used as an endogenous control to account for differences in starting material. These data are the result of at least three different pools of tissue from each age group. The mean normalized values obtained for E13 have been set at 1 and the values for the other developmental ages are presented as a fold change from E13. Therefore, values greater than 1 indicate increased mRNA levels and values less than 1 indicate reduced mRNA levels in comparison to E13 (**a**,**b**). The primary morphologic stages that occur during development of the rat ovary from E13-P5 are represented (**c**).

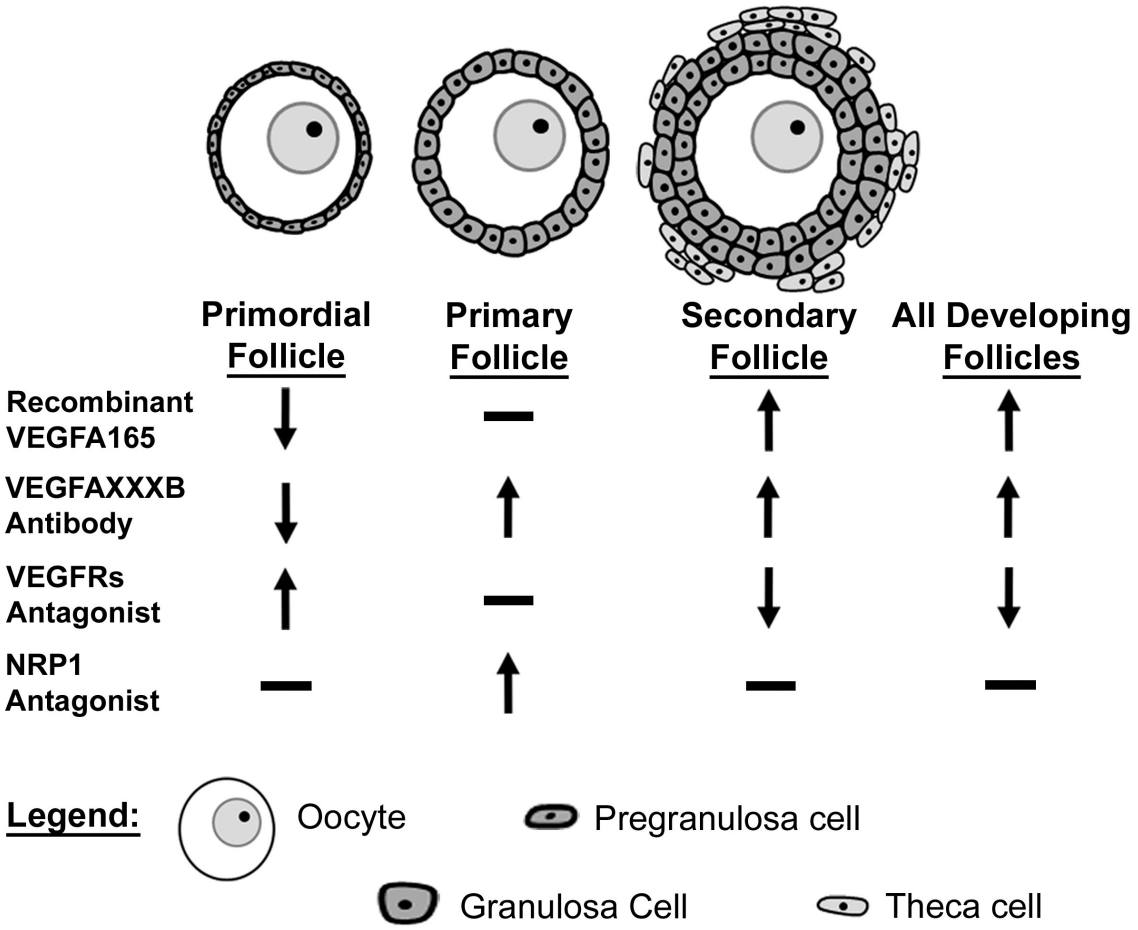

Figure 1-3

One ovary from postnatal day 3/4 rat was cultured for 2 weeks with either recombinant VEGFA_165, antibodies to VEGFA_XXXB isoforms, an inhibitor of KDR and FLT1 (VEGFA receptors), or an inhibitor to NRP1. The paired ovary from each rat was cultured without treatment to serve as a control. The mean number of follicles at each stage of development was calculated as a percent of total follicles and these percentages were compared between treated and control ovaries. Arrows pointing up indicate a significant increase, arrows pointing down indicate a significant decrease, and dashes indicate no difference in the percent of follicles at each stage in treated ovaries in comparison to controls. All developing follicles include early primary, primary, transitional, and secondary follicle stages.

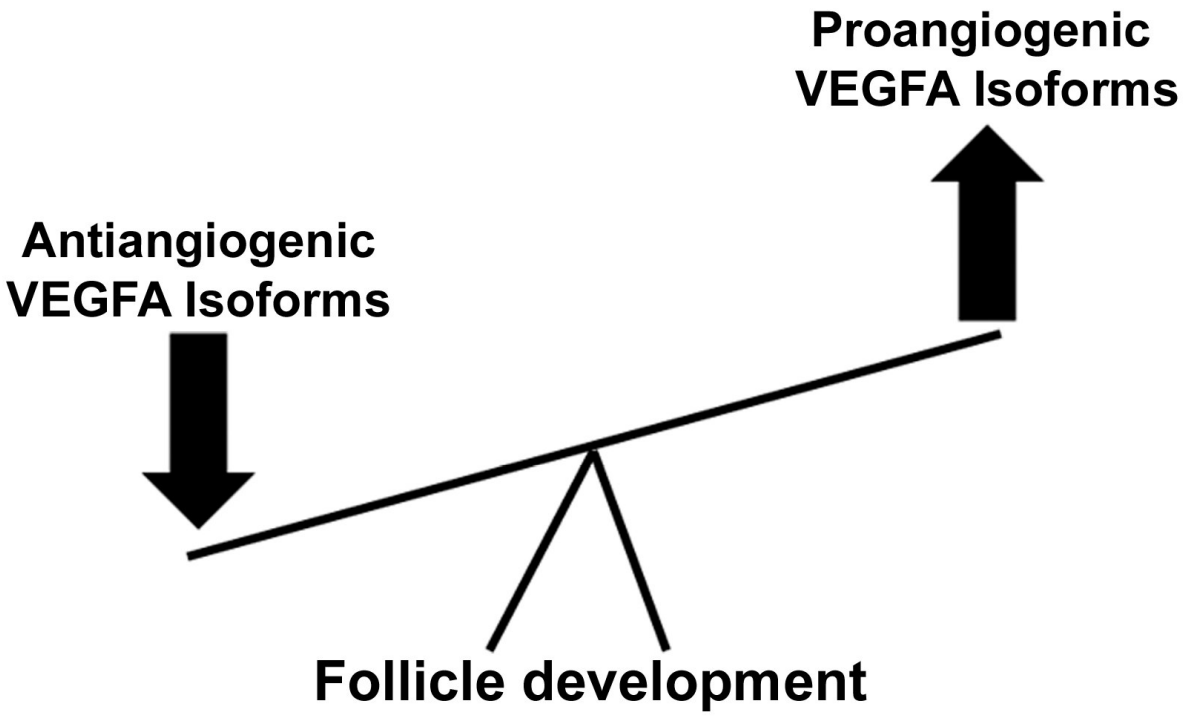

Figure 1-4

Representation of the proposed role for VEGFA isoforms in follicle development. Proangiogenic isoforms appear to promote initial recruitment and development of ovarian follicles while antiangiogenic isoforms appear to suppress these processes.

Chapter 2 - Inhibition of Vascular Endothelial Growth Factor Receptor Signal Transduction Blocks Follicle Progression in Perinatal Rat Ovaries

Introduction

The primordial follicle pool plays a vital role in female reproduction. Abnormal development or regulation of the primordial follicle pool can lead to ovarian dysfunction, including an impairment of reproductive capacity or premature ovarian failure (Hirshfield 1994, Mazaud *et al.* 2002, Guigon *et al.* 2003). Furthermore, manipulation of the primordial follicle pool may provide a means to increase reproductive efficiency and/or increase our understanding of follicular development.

During the late gestational period in rodents, oocytes are located within cord-like structures. These ovigerous cords consist of clusters of oocytes (oocyte cysts) surrounded by mesenchymal-like somatic cells (Konishi *et al.* 1986). Primordial follicles are formed when a single layer of squamous pre-granulosa cells envelop individual oocytes, disrupting the oocyte cysts (Hirshfield 1992, Rajah *et al.* 1992, Pepling & Spradling 2001). Folliculogenesis is initiated during the first few days of life in the rodent and by postnatal days 3 and 4 (P3/4), the rat ovary consists predominately of primordial follicles (Rajah *et al.* 1992). As soon as the primordial follicle pool is formed, subsets of follicles are recruited into the growing follicle pool with small antral follicles first appearing during the second and third week of life (Gelety & Magoffin 1997). Non-recruited primordial follicles can remain quiescent for months or years (Peters *et al.* 1962).

Although the process of primordial follicle recruitment is incompletely understood, specific growth factors must either stimulate primordial follicles to leave the dormant state or inhibit primordial follicles from entering the growing pool. Microarray analysis has identified that *Vegf* mRNA expression is significantly upregulated during the primordial to primary follicle transition in postnatal rat ovaries (Kezele *et al.* 2005b) and in vivo injections of a VEGF antibody have demonstrated that primordial follicles may be affected (Roberts *et al.* 2007). These findings are interesting since there is relatively no vasculature surrounding primordial or primary follicles. Neither of the previous studies addressed whether VEGF's actions were indirectly regulated through vascular development or directly at the level of the somatic cells or oocytes.

The principal angiogenic gene, VEGF, consists of 5 family members: *Vegfa*, *Vegfb*, *Vegfc*, *Vegfd*, (officially called *Figf,* c-fos induced growth factor) and placenta growth factor (*Pgf*). The best characterized family member of these is *Vegfa*. The *Vegfa* gene consists of 8 exons which undergo alternative splicing to form different mRNA splice variants and are translated into VEGFA protein isoforms with different numbers of amino acids. The predominant isoforms expressed in most tissues throughout the body are VEGFA_188, 164, and 120 (Carmeliet & Collen 1999). VEGFA isoforms are structurally different based upon the exons they are developed from and these differences make them unique in their function. The larger isoforms containing exons 6 and 7 have heparin binding domains, making them less diffusible. The smaller isoforms lack these exons and are freely diffusible. This difference in diffusion allows VEGFA isoforms to form a chemoattractant gradient to induce endothelial cell migration and the formation of vascular networks within developing organs or tumors (Grunstein *et al.* 2000, Springer *et al.* 2000).

Two tyrosine kinase receptors, FMS-like tyrosine kinase 1 (FLT1, also known as VEGFR1) and kinase insert domain protein receptor (KDR, also known as VEGFR2 and FLK1), bind to VEGFA. The primary receptor involved in VEGFA-induced angiogenesis is KDR. Binding of VEGFA to KDR promotes endothelial cell survival, differentiation, and migration (Claesson-Welsh 2003) and mice missing KDR lack endothelial cells and do not survive past mid-gestation (Shalaby *et al.* 1995). Although *Flt1* knockout mice have abundant numbers of endothelial cells, they also die during embryonic development because endothelial cells are unable to assemble a functional vascular network (Fong *et al.* 1995). It has been proposed that FLT1 regulates vascular development by trapping VEGFA and suppressing VEGFA levels within an appropriate range (Shibuya 2001).

Previous work in our laboratory has demonstrated that VEGFA is necessary for development of both seminiferous cords and sex-specific vasculature during testis morphogenesis in the rat (Bott *et al.* 2006). In this study, we hypothesized that VEGFA is involved in early follicle development which may be independent of vasculature development. The objectives of the present study were to determine if inhibition of both VEGFA receptors (FLT1 and KDR) or KDR alone affected vascular development and follicle progression in perinatal rat ovarian cultures.

Materials and Methods

Embryonic and postnatal ovaries were obtained from our own Sprague-Dawley rat colony at the University of Nebraska-Lincoln Animal Science Department, with founders purchased from Charles River (Wilmington, MA). Ovaries were dissected from embryonic day 13 (E13) to P10 rats in order to evaluate ovaries across important developmental stages: the formation of oocyte cysts, the formation of primordial follicles, and the initiation of follicular

activation and development. Embryonic age was calculated from days post coitus. Postnatal age was determined using day of birth as postnatal day zero. All animal procedures were approved by the University of Nebraska Animal Care and Use Committee.

Isolation of RNA for RT-PCR

Total RNA from ovaries at different ages was extracted using Tri Reagent (Sigma-Aldrich, St. Louis, MO) per manufacturer's protocol. After isolation, total RNA was resuspended in 20 µl DEPC water and reverse transcription (RT) was performed on 5 µg of template using SuperScript II (Invitrogen, Carlsbad, CA) according to manufacturer's recommended protocol (Itoh *et al.* 1998). The resulting cDNA was then stored at -20°C for subsequent RT-PCR.

Vegfa, Flt1, *and* Kdr *RT- PCR*

Primers for rat *Vegfa* (Table 2.1) were used with an annealing temperature of 58°C for 35 cycles to generate different products, depending on the *Vegfa* mRNA isoform expressed (Dulak *et al.* 2000). These primers generate products of 99 bp for *Vegfa_120*, 171 bp for *Vegfa_144*, 231 bp for *Vegfa_164*, 303 bp for *Vegfa_188*, and 354 bp for *Vegfa_205*. For *Flt1*, a nested PCR approach with an annealing temperature of 54ºC for 35 cycles was utilized to produce a 202 bp product. Outer and inner primer sets (Table 2.1) were designed with the PrimerQuest primer design program (Integrated DNA Technologies, Coralville, IA). A nested approach was also used with human specific *Kdr* primers (Table 2.1) at an annealing temperature of 52ºC for 30 cycles to amplify a 213 bp product (Ergün *et al.* 1998). Primers for glyceraldehyde-3-phosphate dehydrogenase (*Gapdh;* Table 2.1) were used at an annealing temperature of 60ºC for 40 cycles to produce a 460 bp product. *Gapdh* was used as an endogenous control for RNA isolation and amplification (Wesolowski SR 2003). All PCR products were subcloned and confirmed using

restriction digest analysis. PCR products were subcloned into pCRII (Invitrogen) using the TOPO TA Cloning kit (Invitrogen) and sequenced with primers for the T7 promoter region (data not shown). RT-PCR was conducted on 3-5 different samples for each developmental time point.

Vegfa, Flt1, *and* Kdr *QRT- PCR*

Primers were designed using Primer Express 1.5 (software that accompanied the 7700 Prism sequence detector; Applied Biosystems, Foster City, CA) for rat *Vegfa_120, 164, and 188* (Table 2.1). Fluorescent probes were obtained from Applied Biosystems (Table 2.1). Quantitative RT-PCR (QRT-PCR) for the *Vegfa* isoforms was performed with TaqMan Universal Master Mix (Applied Biosystems), 900 nM of both forward and reverse primers, and 200 nM of probe. *Gapdh* was amplified for all samples to serve as a basis for calculating relative expression. *Gapdh* primers and probes were obtained from Applied Biosystems. Experimental and *Gapdh* PCRs were carried out in separate wells in triplicate. An arbitrary value of template was assigned to the highest standard and corresponding values to the subsequent dilutions. These relative values are plotted against the threshold value for each dilution to generate a standard curve. The relative amount for each experimental and *Gapdh* triplicate was assigned an arbitrary value based on the slope and y-intercept of the standard curve. The average of the experimental triplicate is divided by the average of the *Gapdh* triplicate and the resulting normalized values are used for statistical analysis (Wood *et al.* 2003). At least three different pools of each age tissue were utilized to obtain these data.

Embedding, Histology and Immunohistochemistry

Ovaries were fixed in Bouins solution and paraffin embedded according to standard procedures (Itoh *et al.* 1998). Tissues were sectioned (5 μm), deparaffinized, rehydrated, and

microwaved in 0.01M sodium citrate to boil for 5 minutes. After boiling, tissues were cooled for 1-2 hours and sections were blocked with 10% normal goat serum in PBS for 30 minutes at room temperature. Immunohistochemistry was performed as previously described (Cupp *et al.* 1999a). The VEGFA antibody was a rabbit polyclonal IgG raised against a peptide corresponding to amino acids 1-140 of human VEGFA (Santa Cruz Biotechnology, Santa Cruz, CA). The FLT1 antibody was a rabbit polyclonal IgG raised against a peptide at the C-terminus of human FLT1 (Santa Cruz Biotechnology). The KDR (FLK1) antibody was a mouse monoclonal IgG_1 antibody raised against a peptide corresponding to amino acids 1158-1345 of mouse KDR (Santa Cruz Biotechnology). All antibodies were diluted 1:50-1:100 in 10% normal goat serum. As a negative control, serial sections were processed without primary antibody. Biotinylated goat anti-rabbit and goat anti-mouse secondary antibodies were diluted 1:300. Secondary antibody was detected with aminoethyl carbazole (AEC) chromagen substrate solution (ZYMED Laboratories, San Francisco, CA). The same immunohistochemical procedures localized VEGFA and KDR staining to Sertoli cells, germ cell cytoplasm, and certain interstitial cells within E14 to P5 rat testes in previous studies from our laboratory (Bott *et al.* 2006). Specificity of antibodies was determined through blocking proteins for each protein (data not shown). Immunohistochemistry for each protein was performed on at least three different sections of tissue from each age group.

Organ Cultures

Ovaries were dissected from P3/4 rats. One ovary from each animal was designated as a control while its pair was subjected to VEGFA receptor signal transduction inhibitor (VEGFR-TKI; Calbiochem, La Jolla, CA) treatment. Tyrosine kinase activity through FLT1 and KDR is inhibited by VEGFR-TKI, blocking signal transduction of VEGFA (Hennequin *et al.* 1999).

Organs were cultured for 14 days as previously described (Parrott & Skinner 1999). Ovaries were cultured in drops of medium on 0.4 μm Millicell-CM filters (Millipore, Bedford, MA) floating on the surface of 0.4 ml of medium in 4-well plates. The medium consisted of DMEM/Hams F-12 Medium (1:1, vol:vol; Gibco, Grand Island, NY) supplemented with 0.1% BSA, 0.1% Albumax (Gibco), 5X ITS-X (insulin, sodium transferrin, sodium selenite, ethanolamine; Gibco), 0.05 mg/ml L-ascorbic acid (Sigma-Aldrich), penicillin, and streptomycin. Doses of VEGFR-TKI (diluted in DMSO) were added directly to the culture medium of the treated wells at the start of culture and treatment was repeated daily. Similar doses of DMSO were added to the paired control wells. The medium was changed after every two days of culture.

We cultured P3/4 ovaries because ovaries from rats of this age consist predominately of primordial follicles (Rajah *et al.* 1992) and similar ovarian organ culture procedures have demonstrated that primordial follicles can spontaneously initiate development to early primary follicles (Parrott & Skinner 1999). We utilized a dose of 8 μM VEGFR-TKI in our organ culture system because we have demonstrated this dose affects testis morphogenesis and vascular development in rat testis organ cultures while not affecting germ cell mitosis (Bott *et al.* 2006). The IC50 (concentration required for 50% inhibition) for single layer cells treated with VEGFR-TKI is 2 μM for FLT1 and 0.1 μM for KDR (Hennequin *et al.* 1999). We are well within the IC50 for both VEGFA receptors.

Imaging and Area Analysis of Organ Cultures

After culture treatment, ovaries were imaged with a Spot camera imaging capture system (Diagnostic Instruments, Spot Advance, Sterling Heights, MI). The NIH Scion image program (Scion Image, Frederick, MD) was utilized to obtain individual ovary areas (total number of

pixels) at 40X magnification**.** Each ovary was outlined twice and these two areas were averaged to obtain an accurate area measurement for each ovary (Cupp *et al.* 1999b). The mean area for each control ovary was set to 100% and the mean area of each treated ovary was calculated as a percentage of its paired control. A total of 45 ovary pairs were imaged for area measurements.

Whole-mount Immunohistochemistry of Organ Cultures

After imaging, ovaries were either fixed in Bouins solution and paraffin embedded for histology (see below) or fixed in 4% paraformaldehyde for whole mount immunohistochemistry. VEGFR-TKI organ culture ovaries were fixed overnight at 4°C. Ovaries were then washed in PBS for two hours at room temperature and incubated overnight at 4°C in blocking buffer (PBS; 5% BSA; 0.1% Triton X-100, Sigma-Aldrich). After one hour at room temperature, ovaries were incubated overnight at 4°C with primary antibody, washed with washing buffer (PBS; 1% BSA; 0.1% Tween 20, Sigma-Aldrich) for 4-5 hours at 4°C (changing the buffer once), followed with two, one hour rinses of washing buffer at room temperature. The first primary antibody utilized was a mouse monoclonal IgG_1 raised against rat platelet endothelial cell adhesion molecule (PECAM1 or CD31; BD Pharmingen, San Jose, CA; 1:50 dilution in blocking buffer). Ovaries were then incubated overnight at 4°C with Cy5-conjugated secondary antibody (Jackson Laboratories, West Grove, PA; 1:500 dilution in blocking buffer) and washed with washing buffer, as with the primary antibody. These blocking, incubation, and washing steps were repeated using an anti-laminin primary antibody and a Cy2-conjugated secondary antibody. The second primary antibody was a rabbit polyclonal immunoglubin raised against rat laminin (DAKO, Carpinteria, CA; 1:100). Ovaries were mounted in Gel/Mount (Biomeda, Foster City, CA) for subsequent confocal microscopy.

Confocal Microscopy of Organ Cultures

VEGFR-TKI organ culture images were collected using a Bio-Rad MRC1024ES confocal laser scanning microscope (Bio-Rad Laboratories, Hercules, CA). Whole ovaries were scanned thru a series of Z-sections at 100X, 200X, and 600X magnifications (thickness of sections being 10 μm, 5 μm, and 3 μm respectively) with green, red, and merged channels to determine positive staining for PECAM1 and laminin. Organ depths were estimated at the 100X magnification by the number of 10 μm Z-steps required to scan through the entire organ.

Vascular Density Quantification

Red channel confocal images at 600X magnification were used to analyze vascular density of control and treated organ culture ovaries. Vascular density or staining index (SI) was quantified with the use of Scion image. Densitometry was performed on 3 fields for each organ. Within each field, the SI was defined as the number of pixels exceeding an arbitrary grayscale value. Mean SI for each organ was defined as the average of the SI's from all 3 fields. The mean vascular density for each control ovary was set to 100% and the mean of each treated ovary was calculated as a percentage of its paired control. A total of 17 ovary pairs were analyzed for vascular density.

Follicle Staging and Quantification of Organ Cultures

Organ culture ovaries not used for whole mount immunohistochemistry were fixed and embedded as described above. Embedded ovaries were sectioned (5μm), deparaffinized, stained with hemotoxylin and eosin, and rehydrated. With brightfield microscopy, follicles from each ovary were staged into 0 to 4 classifications (Parrott & Skinner 1999). A stage 0 primordial follicle consists of an oocyte surrounded by a single layer of squamous pre-granulosa cells. A stage 1 early primary follicle consists of an oocyte surrounded by a single layer of cells

composed of a combination of pre-granulosa and granulosa cells. A stage 2 primary follicle consists of an oocyte surrounded by a single layer of cuboidal granulosa cells. A stage 3 transitional follicle is developing a second layer of granulosa cells while a stage 4 follicle has theca cells beginning to organize around the granulosa cell layers (Parrott & Skinner 1999). For the VEGFR-TKI organ cultures, 3 ovary pairs were imaged at 400X magnification for follicle quantification. The middle 12 histology sections were evaluated for each ovary and follicles were staged and counted from 3 non-overlapping cortical areas. Middle sections were utilized in order to count follicles over a full cross-section of ovarian tissue and to evaluate differences in follicle numbers over comparable regions between treated and control ovaries. Follicle staging and counting was performed independently by two individuals and discrepancies in counts, if any, were averaged. The mean number of follicles in each stage of development per area counted was statistically analyzed between control and treated ovaries. Additionally, the mean number of follicles from each treated ovary was expressed as a percentage of its paired control and the percentages from all ovary pairs were analyzed. The mean number of each follicle stage was also expressed as a percentage of the total number of follicles from each area. These percentages were statistically analyzed between control and treated ovaries.

Statistical Analysis

All data were analyzed by one-way ANOVA using JMP software (SAS Institute, Cary, NC). A Student t-test was used to compare mean normalized QRT-PCR values between different developmental ages. A Student t-test and a Dunnett test were used to compare ovarian area, vascular density, and follicle counts between control and treated organs. Differences in data were considered to be statistically significant at a P value of < 0.05, unless otherwise stated.

Results

Vegfa, Flt1, *and* Kdr *mRNA Expression During Ovarian Development*

Conventional RT-PCR was used to evaluate mRNA expression during early ovarian development. Nine developmental time points were evaluated (E13, E14, E16, E18, P0, P3, P4, P5, P10). Both *Vegfa_120* and *164* mRNA was detected at all time points evaluated (Figure 2-1). Messenger RNA from *Vegfa_188* was detected at all developmental time points except E14 (Figure 2-1). The *Vegfa_205* mRNA isoform was distinctly present at P10 with a faint band detected at E18 (Figure 2-1). The upper bands from E16 and P0 were cut out of the gel and determined to be *Vegfa_188* and not *Vegfa_205* through subcloning and sequencing. Messenger RNA for Flt1 and Kdr was also detected throughout all time points evaluated (Figure 2-1). Quantitative real-time PCR (QRT-PCR) demonstrated differential abundance of *Vegfa_120*, *164*, and *188* during pre- and perinatal development of the ovary (Figure 2-2). Specifically, *Vegfa_120* levels increased from embryonic ages to P0; however, after birth, levels declined from P0 to P3 (Figure 2-2A; $P < 0.05$). Messenger RNA levels for *Vegfa_164* increased from E13 to E18, declined after birth, and then increased again at P5 (Figure 2-2B; $P < 0.05$). Levels for *Vegfa_188* mRNA peaked at E16 (Figure 2-2C; $P < 0.05$).

Localization of VEGFA, FLT1, and KDR Within Developing Ovaries

After confirmation of *Vegfa*, *Flt1*, and *Kdr* mRNA expression during early ovarian development, immunohistochemistry was utilized to localize expression to specific cell types. Immunohistochemistry was performed on ovaries from P0, 3, 4, 6 and 10 rats. Staining for VEGFA protein was localized to oocyte cysts and to the pre-granulosa or granulosa cells of follicles from primordial through antral stages (Figure 2-3A,D,G). In pre-antral and antral follicles, VEGFA staining was also detected in the cytoplasm of oocytes and in theca cells

(Figure 2-3G). No staining was identified in ovaries processed without primary antibody (Figure 2-3J). Expression of FLT1 (Figure 2-3B,E,H) and KDR (Figure 2-3C,F,I) protein was localized to oocyte cysts and to the oocytes and pre-granulosa or granulosa cells of primordial through antral stage follicles. Staining for FLT1 and KDR was also identified in theca cells of advanced stage follicles (Figure 2-3H,I) but was not detected in negative control ovaries (Figure 2-3K,L).

Effects of VEGFA Signal Transduction Inhibitor, VEGFR-TKI, on Rat Ovarian Organ Cultures

To determine the function of VEGFA in follicle progression, P3/4 rat ovarian organ cultures were treated with VEGFR-TKI, a VEGFA tyrosine kinase signal transduction antagonist which inhibits both KDR and FLT1. Ovaries treated with VEGR-TKI (Figure 2-4D) had a 25% reduction in ovarian area when compared to their paired controls (Figure 2-4A,G; $P < 0.0001$). Depth of cultured ovaries, determined by the total number of 10 μm Z-series confocal microscopy images taken of each organ, was not statistically different between treated and control ovaries.

Whole mount immunohistochemistry and confocal microscopy was utilized to further evaluate the role of VEGFR-TKI treatment on ovarian cultures. Laminin staining was used to localize basement membranes (Aumailley & Krieg 1996) and thus outline individual follicles (Figure 2-4B,E). VEGFA signal transduction inhibition did not appear to alter follicle formation or organization (Figure 2-4E). PECAM1 staining was used to localize endothelial cells (Vecchi *et al.* 1994) and thus identify the vasculature within ovaries (Figure 2-4C,F). Ovarian vascular density was diminished by 94% in treated ovaries (Figure 2-4F,H; $P < 0.0001$) compared to controls (Figure 2-4C).

Effects of VEGFA Signal Transduction Inhibitor, VEGFR-TKI, on Follicle Development

The number of follicles in control ovaries was compared to VEGFR-TKI treated ovaries to determine differences due to treatment. Ovaries treated with VEGFR-TKI (Figure 2-5B) had 119% more primordial (stage 0) follicles and 43% more total follicles per area evaluated than control ovaries (Figure 2-5A,C; $P < 0.0001$). VEGFR-TKI treatment also resulted in 40% less transitional (stage 3) and secondary (stage 4) follicles (Figure 2-5C; $P < 0.005$). Furthermore, treated ovaries consisted of 51% primordial follicles and 49% developing follicles (stages 1-4) compared to control ovaries with 32% primordial and 68% developing follicles (Figure 2-5F; $P < 0.0001$). Specifically, VEGFR-TKI treated ovaries consisted of 19% more primordial follicles, 8% less early primary follicles (stage 1), and 7% less transitional and secondary follicles (Figure 2-5E; $P < 0.003$). Evaluation of total follicle counts from treated ovaries as a percentage of control ovaries did not reveal any significant differences (Figure 2-5D).

Discussion

The current study demonstrates a role for VEGFA and its receptors in follicle progression in the perinatal rat ovary. Inhibition of VEGFA signal transduction through a tyrosine kinase inhibitor affecting both KDR and FLT1 dramatically arrested vascular development and increased primordial follicle numbers, suggesting that VEGFA may be a regulator of initial follicle activation.

The *Vegfa* gene is complex and produces multiple isoforms through alternative splicing. The different isoforms have unique functions during vascular development in many organs (Carmeliet & Collen 1999). In the current study, we detected expression of smaller *Vegfa* isoforms early, with larger isoforms appearing later during ovarian development. Further analysis with QRT-PCR demonstrated that *Vegfa_188* mRNA was most abundant at E16 with

elevated amounts of *Vegfa_164* and *120* after E18 and P0, respectively. Around E18 in the rat, primordial germ cells have formed germline cysts that are ceasing to proliferate and are undergoing meiosis. Germline cysts are composed of clusters of oogonia connected by intracellular bridges formed through incomplete cytokinesis. These cell clusters synchronously proliferate and waves of oogonia enter meiosis in a non-synchronous manner until they are arrested around birth (Pepling & Spradling 1998, Bristol-Gould *et al.* 2006). Thus, during embryonic development, VEGFA_188 may aid in mitosis, transition into meiosis, or allow for survival of oogonia directly or indirectly through reorganization of vasculature.

There is no information to suggest ovarian vasculature undergoes reorganization during the time oocyte cysts are present. The VEGFA_188 isoform is the least diffusible isoform and it increases branching of vasculature and development of capillaries from larger vasculature. The VEGFA_164 isoform recruits endothelial cells, aiding in the formation of major blood vessels (Grunstein *et al.* 2000, Ferrara *et al.* 2003). Thus, greater expression of *Vegfa_188* and *Vegfa_164* may be necessary to increase branching of vasculature as large vessels form between nests to aid in maintenance and survival of oogonia prior to cyst breakdown.

After birth and meiotic arrest, oocyte cysts undergo a programmed breakdown where pre-granulosa cells invade, divide cytoplasm, and surround oocytes to form primordial follicles (Pepling & Spradling 1998, Pepling & Spradling 2001). During this time, there is a tremendous amount of oocyte loss (Pepling & Spradling 2001). In our study, greater concentrations of *Vegfa_120* were detected after P0. Since the highly diffusible VEGFA_120 isoform is primarily thought to be involved in endothelial cell recruitment (Grunstein *et al.* 2000), it is possible that VEGFA_120 may be involved in the oocyte cyst to primordial follicle transition, if vascular reorganization is necessary. To date, there have been no studies to indicate that vascular

development is involved in the breakdown of oocyte cysts. However, estrogen, estrogen-like compounds, and progesterone affect oocyte cyst breakdown, increase the number of multi-oocytic follicles (MOFs), and interfere with assembly of primordial follicles (Iguchi & Takasugi 1986, Iguchi *et al.* 1986, Jefferson *et al.* 2002, Kezele & Skinner 2003, Jefferson *et al.* 2006, Jefferson *et al.* 2007). Both estrogen and progesterone have been demonstrated to regulate the *Vegfa* gene to cause differential expression of *Vegfa* isoforms in many other tissues (Hyder *et al.* 2000, Mueller *et al.* 2000, Hyder *et al.* 2001, Shi *et al.* 2001, Hyder & Stancel 2002, Mueller *et al.* 2003, Schafer *et al.* 2003, Wu *et al.* 2004, Koos *et al.* 2005). Thus, VEGFA could be a candidate gene involved directly in the process of oocyte cyst breakdown or in vascular reorganization that results from oocyte cyst breakdown and primordial follicle formation.

Non-angiogenic survival roles have been attributed to VEGFA in several different systems (Jin *et al.* 2000, Nishijima *et al.* 2007) and survival genes such as *Bcl2* (Abramovich *et al.* 2006) have been demonstrated to be upregulated by VEGFA. Therefore, it is possible that VEGFA may act to reduce oogonial loss during the oocyte cyst to primordial follicle transition. In the current study, we localized VEGFA, FLT1, and KDR, to oocyte cysts at P0. Previous studies have also reported that *Bcl2* was localized to oocyte cysts in porcine ovaries at similar stages of development (Garrett & Guthrie 1999). We speculate that VEGFA is acting in a novel non-angiogenic manner to upregulate survival factors during the oocyte cyst to primordial follicle transition to allow for increased numbers of primordial follicles.

Previous immunohistochemical studies have determined VEGFA, KDR, and FLT1 were present in oocytes of primordial and primary follicles (Otani *et al.* 1999, Celik-Ozenci *et al.* 2003, Harata *et al.* 2006) and in granulosa and theca cells (Berisha *et al.* 2000, Garrido *et al.* 2001, Celik-Ozenci *et al.* 2003, Greenaway *et al.* 2004, Greenaway *et al.* 2005) from adult rats,

humans, cattle, and primates. In our current study in postnatal rat ovaries, oocyte cysts, pre-granulosa cells, and granulosa cells expressed VEGFA. Furthermore, VEGFA was present in theca cells and the cytoplasm of oocytes of pre-antral and antral follicles. FLT1 and KDR were localized to oocyte cysts, pre-granulosa cells, granulosa cells, and oocytes of all follicle stages, as well as theca cells of more advanced stage follicles. Localization of VEGFA and its receptors to non-vascular cells (granulosa, oocyte) and vascular cells (theca) within the follicle implies both angiogenic and non-angiogenic roles for VEGFA in the regulation of follicle development.

Postnatal ovarian organ culture data from the current study supports a role for VEGFA in initial primordial follicle activation. The VEGFR-TKI inhibitor, which blocked both KDR and FLT1, resulted in increased numbers of primordial follicles compared to the cultured controls, suggesting that primordial follicle activation was impaired without VEGFA. Treatment with VEGFR-TKI also resulted in an increase in the total number of follicles. Based on the follicle counts for each stage of development, this increase appears to be the result of less follicles being stimulated to leave the primordial follicle pool and thus, less growing follicles being lost through atresia. Vasculature may be necessary to transport growth factors and nutrients to primordial follicles to help transition them to later stages of development. Even if there is no blood flow through the vasculature in organ cultures, the development of vasculature may provide an indirect method for angiogenic factors to affect follicular development.

In support of the data in the current study, microarray analysis of perinatal rat ovaries revealed the *Vegfa* gene is up-regulated during the primordial to primary follicle transition (Kezele *et al.* 2005b). In addition to VEGFA, a number of growth factors have already been identified through in vitro culture systems or knockout mice models to be important in primordial follicle progression (Parrott & Skinner 1999, Dissen *et al.* 2001, Nilsson *et al.* 2001,

Durlinger *et al.* 2002, Kezele *et al.* 2002b, Nilsson *et al.* 2002, Kezele & Skinner 2003, Nilsson & Skinner 2003, Kezele *et al.* 2005a). For example, knocking out PTEN, a suppressor of PIK3, within the oocytes of mice results in activation of all primordial follicles during early adulthood and premature ovarian failure (Reddy *et al.* 2008). Since VEGFA has been shown to activate the PIK3 signaling pathway (Guo *et al.* 1995, Fujio & Walsh 1999, Abid *et al.* 2004) and the current study localized VEGFA and its receptors to primordial follicles, VEGFA signaling through the PIK3 pathway may be involved in regulating primordial follicle activation.

Treatment with VEGFR-TKI also affected the number of follicles that progressed to the secondary stage. Later stage follicles require vascularization of theca cells to provide increased nutrients to the growing follicle. Inhibition of VEGFA through neutralization of VEGFA or VEGFA receptors has widely been studied in many different species. In most cases, development of later stage follicles is disrupted and ovulation is impaired (Fraser & Wulff 2001, Zimmermann *et al.* 2001, Zimmermann *et al.* 2002). Therefore, these previous data, in combination with our current study, indicate that VEGFA may be a potential target gene involved in regulation of early stages of follicle initiation as well as later stages of follicle development.

Furthermore, VEGFR-TKI treatment resulted in an overall reduction in ovarian area. We speculate that this change in area is the result of a greater composition of primordial follicles, which are smaller in size than developing follicles, in treated ovaries compared to controls. It is not clear why an increase in area (horizontal growth along the filter) was seen in some ovaries without an increase in depth (vertical growth above the filter); however, it is likely the result of our organ culture system. Ovaries were cultured in a drop of media on top of floating filters.

The filter may have provided more structural support for horizontal growth and the limited amount of media within the drop may have restricted vertical growth.

In conclusion, the current study supports a role for VEGFA in early follicle development. Expression patterns of VEGFA, FLT1, and KDR suggest that VEGFA could potentially be involved in non-angiogenic or angiogenic events regulating oocyte cyst breakdown to development of later stage follicles. Ovarian organ culture data with a VEGFA signal transduction inhibitor suggests that VEGFA affects primordial follicle activation. A reduction in the number of early stage follicles would reduce the number of follicles developing to the pre-antral stage, affect the pool of follicles available for ovulation, and impair the reproductive capacity of females. Our study demonstrates a novel role for VEGFA in the recruitment of primordial follicles into the growing follicle pool, as well as a potential survival factor for primary and later stage follicles.

Conventional RT-PCR Primer Sequences	
Gene	**Primers**
Vegfa	5'-GCACATAGGAGAGATGAGCTTCC-3' 5'- CACCGCCTTGGCTTGTCACAT-3'
Flt1	outer: 5'-ACACTTGTCGTGTGAAGAGTGGGT-3' outer: 5'-ACGATGAGAGTGGCTGTGAGGTTT-3' inner: 5'-TATCAGCGTGAAGCATCGGAAGCA-3' inner: 5'-TGCGTCCTCGGCAGTTACATCTTT-3'
Kdr	5'-CAGCTTCCAAGCGGCTAAGG-3' 5'-TCAAAAATTGTTTCTGGGGC-3'
Gapdh	5'-ACCACAGTCCATGCCATCAC-3' 5'-TCCACCACCCTGTTGCTGTA-3'
Quantitative RT-PCR Primer Sequences	
Gene	**Primers**
Vegfa120	5'-GCACATAGGAGAGATGAGCTTCC-3' 5'-CGCCTTGGCTTGTCACATTT-3'
Vegfa164	5'-GAAAAATTCACTGTGAGCCTTGTTC-3' 5'-CTTGGCTTGTCACATCTGCAA-3'
Vegfa188	5'-GCGTTCACTGTGAGCCTTGTT-3' 5'-CTTGGCTTGTCACATCTGCAA-3'
Quantitative RT-PCR Probe Sequences	
Gene	**Probes**
Vegfa120	5'-ATAGCAGATGTGAATGCAGACCAAAGAAAG-3' FAM-TAMRA
Vegfa164 & Vegfa188	5'-CAGCTTGAGTTAAACGAA-3' FAM-MGB

Table 2.1

Conventional and quantitative RT-PCR primers and probes.

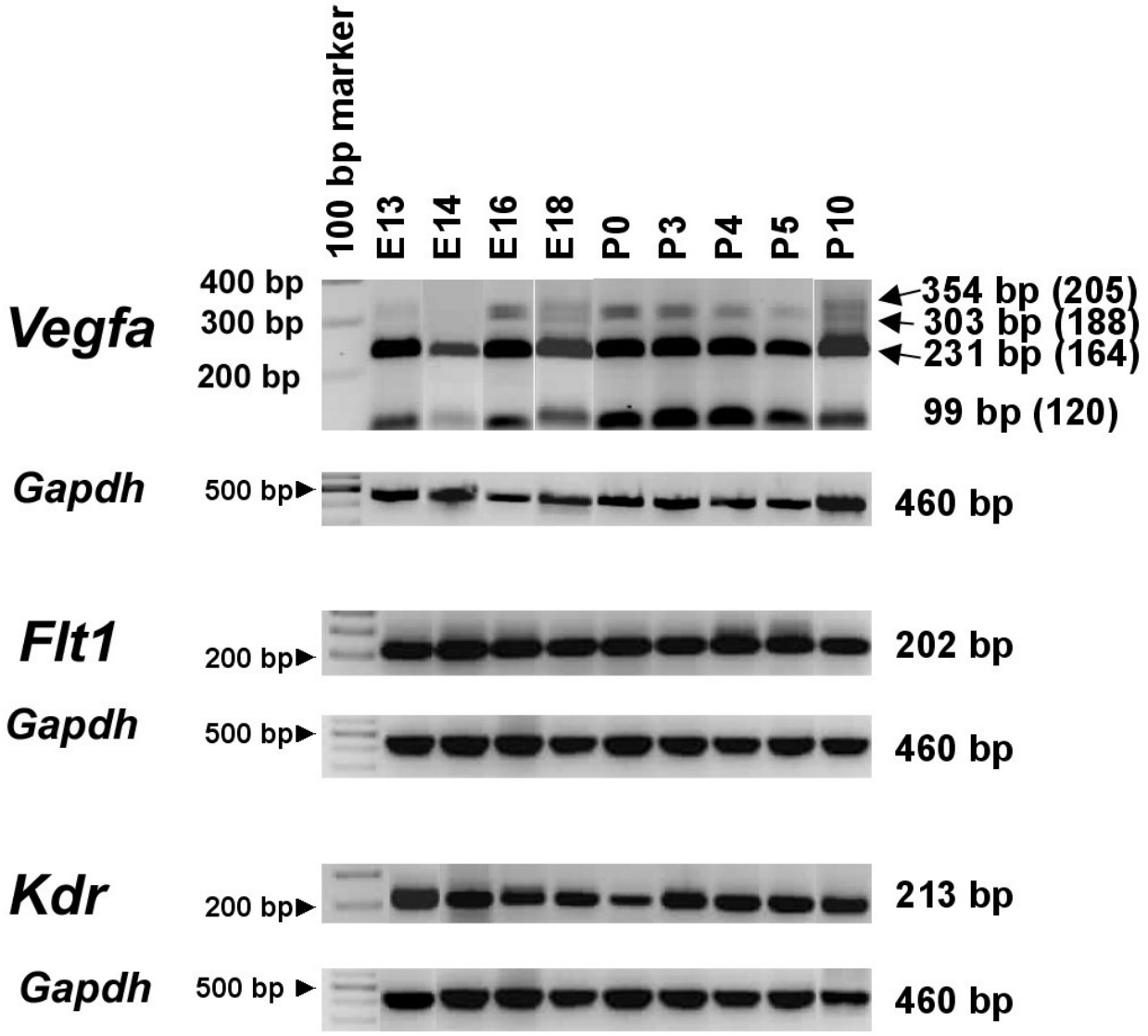

Figure 2-1

Conventional RT-PCR for Vegfa isoforms, Flt1, and Kdr from embryonic day (E) 13 to postnatal day (P) 10 of ovarian development. Gapdh served as a control for RNA isolation and amplification. Negative control samples (without template) did not produce a band (data not shown). These data are the result of at least 3 different pools of each age tissue.

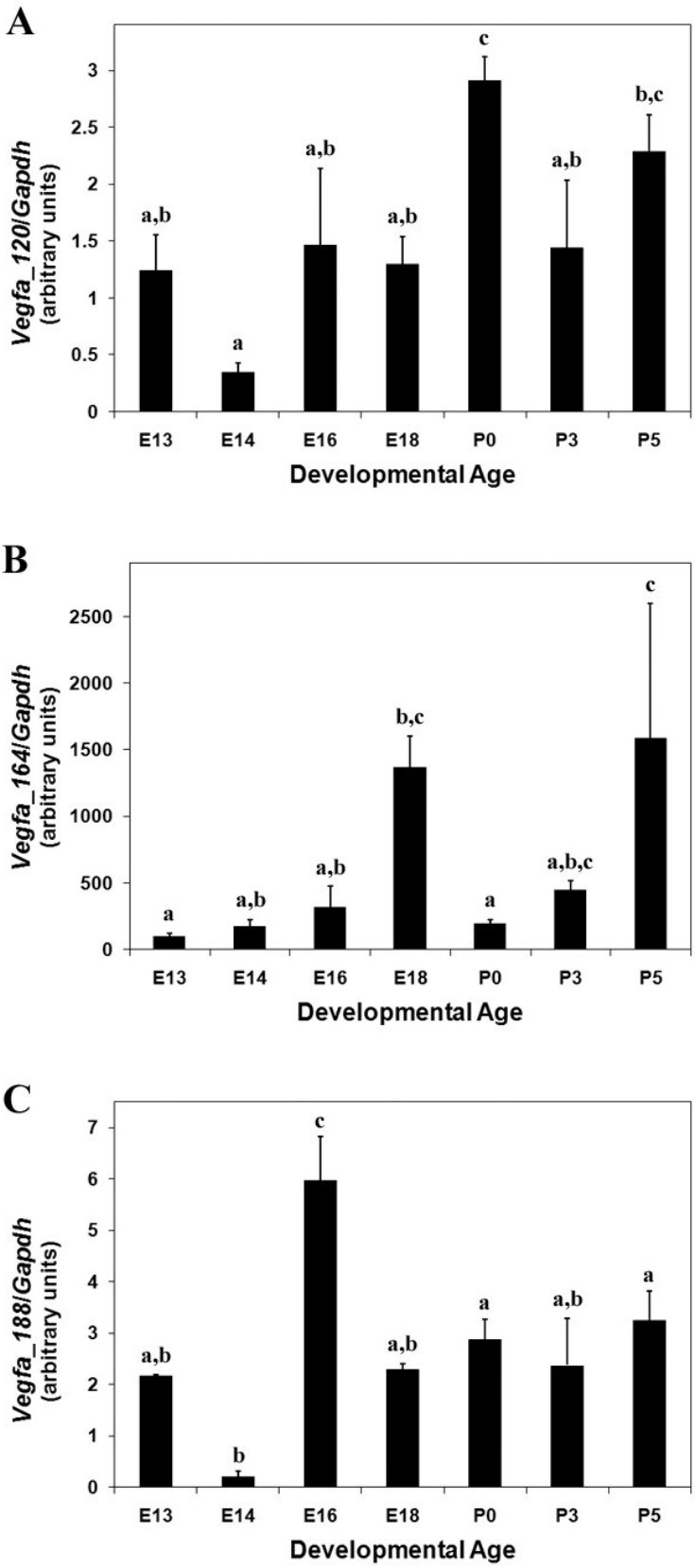
A
Vegfa_120/Gapdh
(arbitrary units)
0
0.5
1
1.5
2
2.5
3
a,b
a
a,b
a,b
c
a,b
b,c
E13
E14
E16
E18
P0
P3
P5
Developmental Age
B
Vegfa_164/Gapdh
(arbitrary units)
0
500
1000
1500
2000
2500
a
a,b
a,b
b,c
a
a,b,c
c
E13
E14
E16
E18
P0
P3
P5
Developmental Age
C
Vegfa_188/Gapdh
(arbitrary units)
0
1
2
3
4
5
6
7
a,b
b
c
a,b
a
a,b
a
E13
E14
E16
E18
P0
P3
P5
Developmental Age

Figure 2-2

Quantitative Real Time PCR was conducted to detect Vegfa isoforms 120 (A), 164 (B), and 188 (C) from embryonic day (E) 13 through postnatal day (P) 10 of ovarian development. Gapdh was used as an endogenous control to account for differences in starting material. These data are the result of at least 3 different pools of each age tissue. The mean normalized values ± SEM are presented and different letters represent a statistically significant difference in means @ $P < 0.05$.

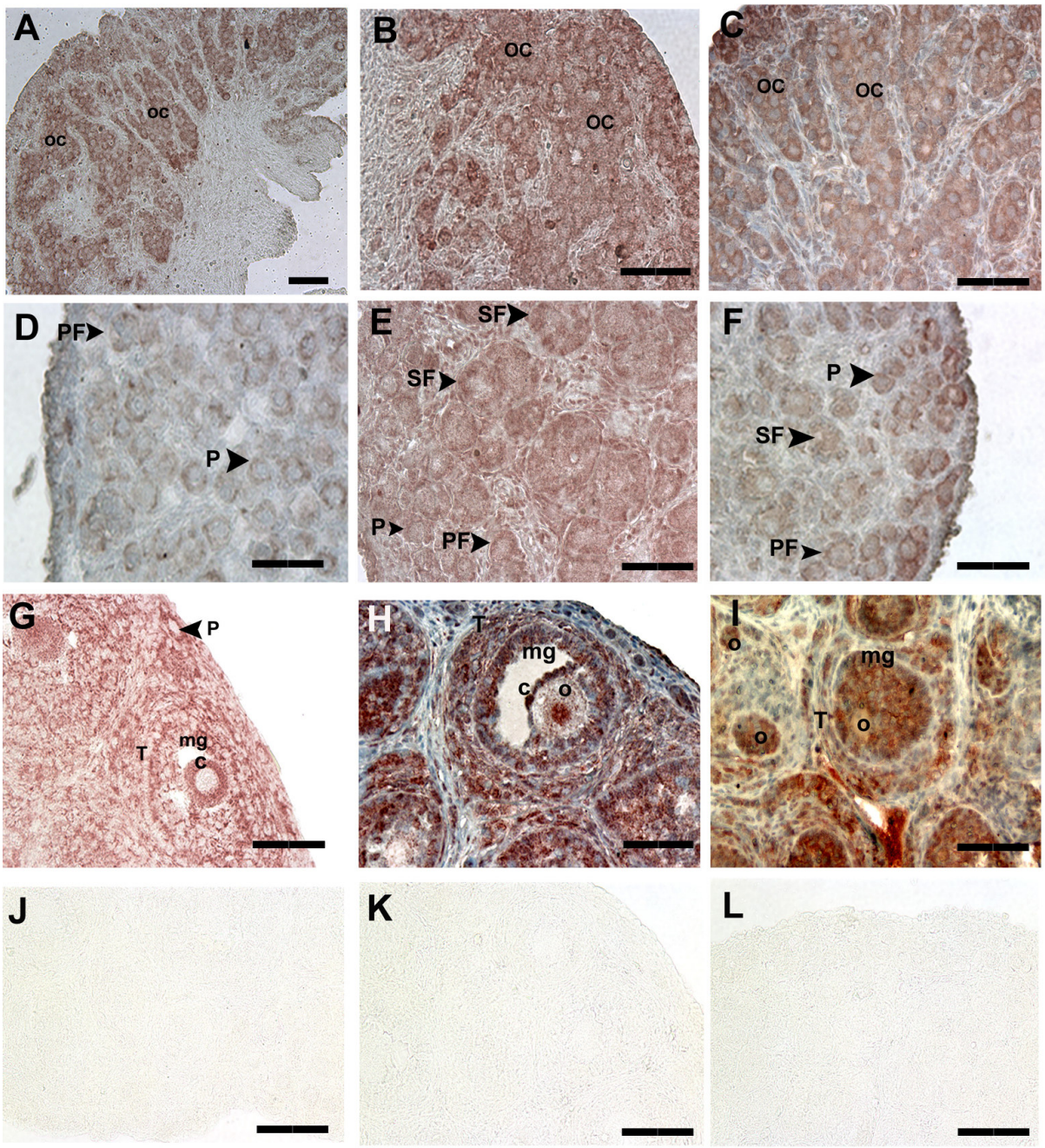

Figure 2-3

Immunohistochemistry for VEGFA in postnatal day 0 (P0; A), P4 (D), and P10 (G) ovaries; FLT1 in P0 (B), P3 (E), and P10 (H) ovaries; and KDR in P0 (C), P6 (F), and P10 (I) ovaries. All figures except G, J, K, and L were lightly counterstained with hemotoxylin. P3 ovarian sections with no primary antibody (J,K,L) served as negative controls. These data are

the result of at least 3 different ovaries from each developmental time point. Scale bars = 50 μm. oc = oocyte cyst, P = primordial follicle, PF = primary follicle, SF = secondary follicle, o = oocyte, c = cumulus granulosa, mg = mural granulosa, T = theca.

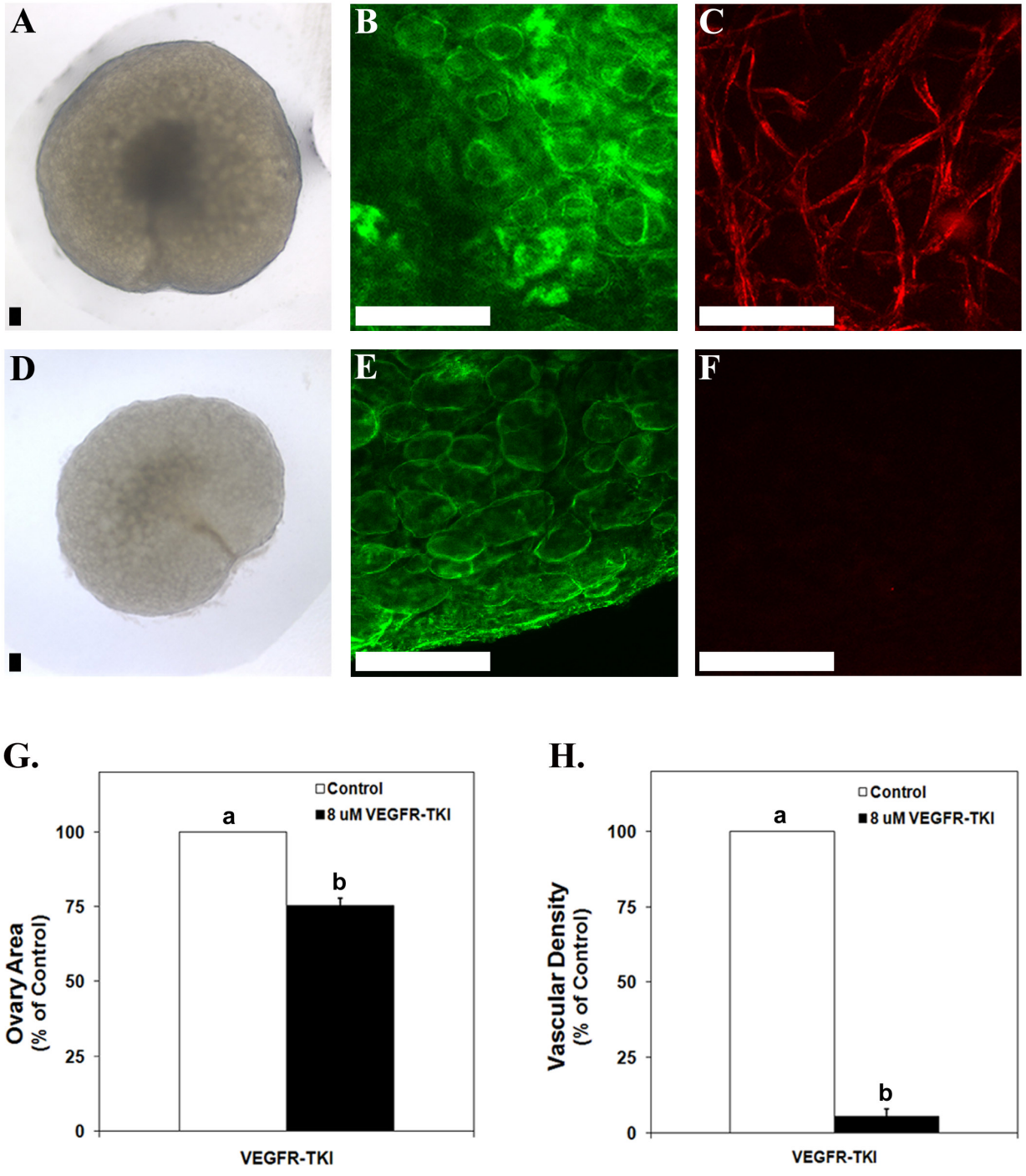

Figure 2-4

Postnatal day 3/4 ovarian organ cultures either without (A,B,C) or with (D,E,F) 8 µM of VEGFR-TKI. Brightfield images (A,D). Confocal images of whole mount immunohistochemistry staining for laminin (basement membrane marker - green; B,E) to

localize individual follicles or PECAM1 (endothelial cell marker - red; C,F) to localize vasculature. Effect of VEGFR-TKI on ovarian area (G) and vascular density (H) expressed as a percentage of control organs. Scale bars = 50 μm. Data are the result of 45 (G) and 17 (H) ovary pairs. The mean areas ± SEM are presented and different letters represent a statistically significant difference between treated and control groups (G,H; $P < 0.0001$).

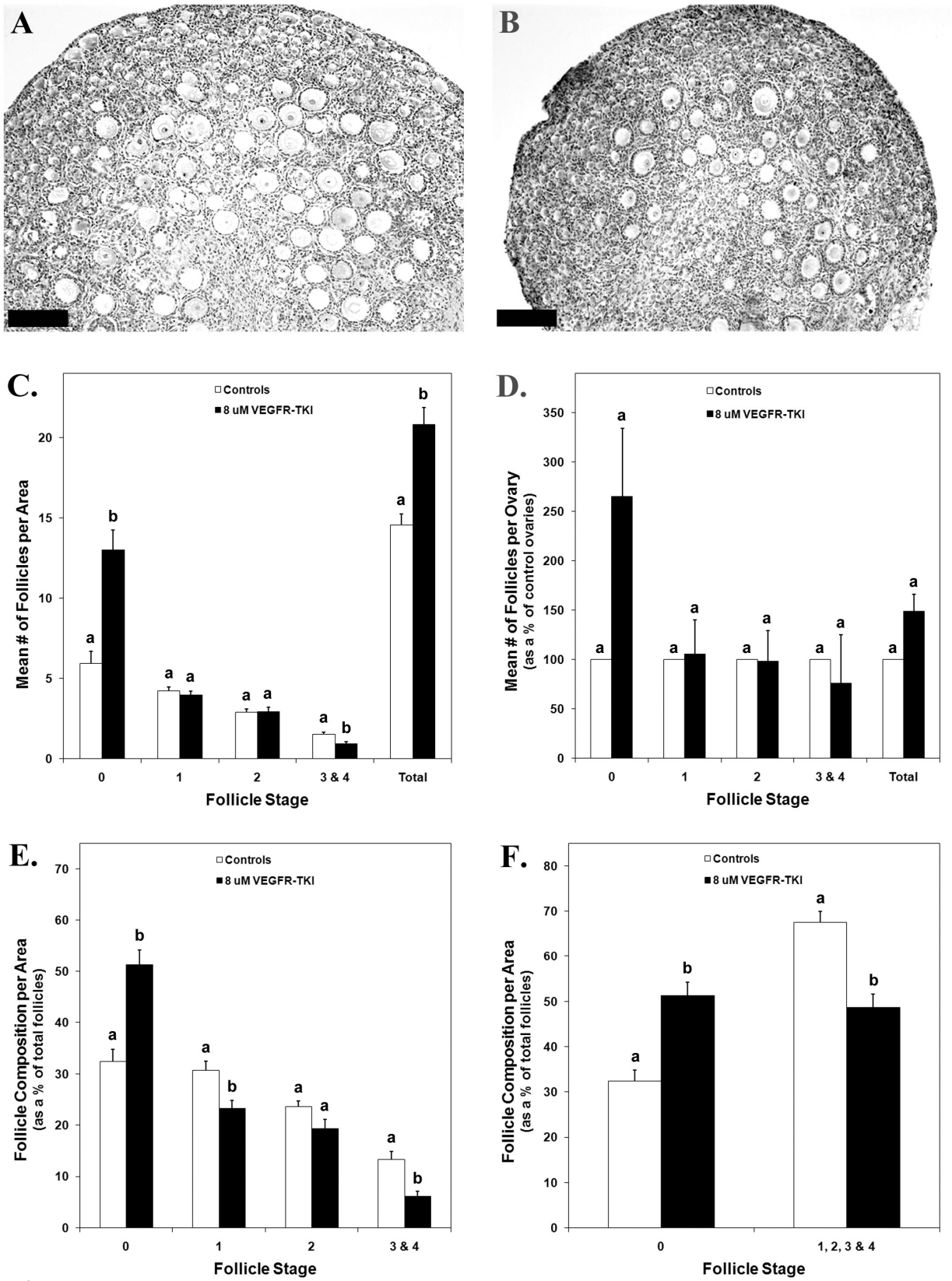
A
B
C.
Controls
8 uM VEGFR-TKI
Mean # of Follicles per Area
Follicle Stage
0
1
2
3 & 4
Total
D.
Mean # of Follicles per Ovary
(as a % of control ovaries)
E.
Follicle Composition per Area
(as a % of total follicles)
F.
1, 2, 3 & 4

Figure 2-5

Postnatal day 3/4 cultured control ovaries (A) or ovaries treated with 8 μM VEGFR-TKI (B) stained with hemotoxylin and eosin. Mean number of follicles per area from control and VEGFR-TKI ovary pairs (C). Mean number of follicles for VEGFR-TKI treated ovaries expressed as a percentage of their paired controls (D). Mean number of follicles per area for control and treated ovaries expressed as a percentage of the total number of follicles per area (E,F). Scale bars = 150 μm. Data are the result of 3 ovary pairs and 12 histological sections evaluated at 400X magnification per ovary (C,D,E,F). The mean numbers of follicles ± SEM are presented and different letters within each follicle stage represent a statistically significant difference of $P < 0.005$ (C,D,E,F).

Chapter 3 - Expression of Antiangiogenic VEGFA Isoforms in Developing Rat Ovaries

Introduction

During late embryonic development, oocytes are organized in large clusters called oocyte cysts. After birth, oocyte cysts begin to develop into individual follicles known as primordial follicles. A primordial follicle contains an oocyte surrounded by a single layer of squamous pre-granulosa cells and is considered to be the first stage of development in follicle progression (Hirshfield 1992, Rajah et al. 1992, Nilsson & Skinner 2001, Pepling & Spradling 2001). By postnatal day 3 to 4 (P3-4) in the rat, 90% of the ovary consists of primordial follicles (Rajah et al. 1992). Only a small portion of primordial follicles will develop into a pre-ovulatory follicle, while the majority of follicles will undergo atresia (Eppig et al. 1996, Parrott & Skinner 1999). Although much has been elucidated about factors involved in folliculogenesis, there is still a great deal to be determined. Vascular endothelial growth factor A (VEGFA) has been shown to be up-regulated during the primordial to primary follicle transition (Kezele et al. 2005b). VEGFA is a potent mitogen that is highly involved in angiogenesis, the formation and differentiation of blood vessels. It has also been shown that follicular development is accelerated in cycling female rats after administration of VEGFA and injection of a VEGFA antibody into the ovary decreases follicular angiogenesis and completely inhibits ovulation (Iijima et al. 2005).

The *Vegfa* gene is composed of eight exons and produces different mRNA splice variants. These splice variants are translated into VEGFA protein isoforms with different numbers of amino acids. The predominant isoforms expressed in most tissues throughout the body are VEGFA_188, 164, and 120 (Carmeliet & Collen 1999) and VEGFA_164 is the

predominant isoform involved in the recruitment of endothelial cells and the formation of major blood vessels (Grunstein *et al.* 2000, Ferrara *et al.* 2003). The human VEGFA protein contains one more amino acid residue on each isoform compared to the rodent, thus, the human VEGFA_165 isoform is homologous to the rodent VEGFA_164 isoform.

Until recently, all VEGFA isoforms were thought to be pro-angiogenic; however, anti-angiogenic splice variants to VEGFA have now been identified (Bates et al. 2002). VEGFA_165B, the newly identified human anti-angiogenic isoform, is formed by differential splicing from the end of exon 7 into what was thought to be the 3' untranslated region of the gene. This region has now been identified as exon 8b. Isoforms that contain exon 8b instead of exon 8a are anti-angiogenic isoforms. It has been proposed that there is a proximal splicing site that allows for production of pro-angiogenic isoforms and a distal splicing site that results in anti-angiogenic isoforms (Harper & Bates 2008). In addition to the VEGFA_165B isoform, it has been proposed that every pro-angiogenic isoform has a corresponding anti-angiogenic isoform where exon 8a has been substituted by 8b (Woolard et al. 2004a).

Previous studies have shown that VEGFA_165B can bind kinase insert domain protein receptor (KDR, also known as VEGFR2 and FLK1) with the same affinity as VEGFA_165; however, it is incapable of activating or stimulating downstream signaling pathways. Furthermore, anti-angiogenic VEGFA isoforms are down-regulated in renal and prostate tumors, potentially allowing for enhanced tumor metastasis (Woolard et al. 2004b). A possible mechanism for the anti-angiogenic effects of the anti-angiogenic isoforms is blocking the pro-angiogenic isoforms from binding to their receptors, FMS-like tyrosine kinase 1 (FLT1, also known as VEGFR1) and KDR.

Previous experiments in our laboratory demonstrated a novel role for VEGFA in the recruitment of primordial follicles into the growing follicle pool, as well as, a potential survival factor for primary and later stage follicles through both vascular dependent and independent mechanisms (McFee et al. 2009) . In the current study, we investigated the expression of antiangiogenic VEGFA isoforms in developing rat ovaries.

Materials and Methods

Animals

Embryonic, postnatal, and adult ovaries were obtained from our own Sprague-Dawley rat colony at the University of Nebraska-Lincoln Animal Science Department with founders purchased from Charles River (Wilmington, MA). Ovaries were dissected from embryonic day 13 (E13) to P10 rats in order to evaluate ovaries across important developmental stages: the formation of oocyte cysts, the formation of primordial follicles, and the initiation of follicular activation and development. Embryonic age was calculated from days post coitus. Postnatal age was determined using day of birth as postnatal day zero. All animal procedures were approved by the University of Nebraska Animal Care and Use Committee.

Vegfa *Anti-angiogenic Isoforms RT- PCR and QRT-PCR*

Total RNA from ovaries at different ages was isolated and converted to cDNA for subsequent RT-PCR according to previously reported methods (McFee *et al.* 2009). The forward primer utilized for *Vegfa* anti-angiogenic isoform conventional RT-PCR was previously used for *Vegfa* pro-angiogenic isoform RT-PCR in our laboratory (McFee *et al.* 2009). The reverse primer (Table 3.1) was designed with the PrimerQuest primer design program (Integrated DNA Technologies, Coralville, IA). These primers were used with an annealing temperature of 54.5°C for 35 cycles to generate products of 220 bp for *Vegfa_165b* and 292 bp for *Vegfa_189b*.

Gapdh is a constitutively expressed gene and was used as a control for RNA isolation and amplification (Wesolowski *et al.* 2003) and previously reported protocols (McFee *et al.* 2009) were utilized to produce a 460 bp product. All PCR products were subcloned and confirmed using restriction digest analysis. PCR products were subcloned into pCRII (Invitrogen) using the TOPO TA Cloning kit (Invitrogen) and sequenced with primers for the T7 promoter region (data not shown). RT-PCR was conducted on 3-5 different samples for each time point. Quantitative RT-PCR (QRT-PCR) primers were designed using Primer Express 1.5 (software that accompanied the 7700 Prism sequence detector; Applied Biosystems, Foster City, CA) for rat *Vegfa_164b* and *Vegfa_189b* (Table 3.1). Fluorescent probe (Table 3.1) was obtained from Integrated DNA Technologies. Procedures and analyses for the anti-angiogenic *Vegfa* QRT-PCR were performed as previously described (McFee *et al.* 2009). At least three different pools of each age tissue were utilized to obtain these data.

Embedding, Histology and Immunohistochemistry

Ovaries were histologically prepared and immunhistochemistry was performed according to standard protocols in our laboratory (McFee *et al.* 2009). The primary antibody was a mouse polyclonal IgG_1 raised against a peptide corresponding to the 9 amino acid C-terminus of human VEGFA_165B (Abcam, Cambridge, MA). This antibody has been characterized previously to bind VEGFA_189B, VEGFA_183B, VEGFA_165B, VEGFA_145B and VEGFA_121B (Woolard *et al.* 2004b, Perrin *et al.* 2005, Bates *et al.* 2006). Therefore, this antibody is collectively termed VEGFA_XXXB but is sold commercially (R&D Systems and Abcam) as a VEGFA_165B antibody. In other experiments in our laboratory, this VEGFA_XXXB antibody has been shown to bind rat VEGFA_165B, 189B, and 121B via western blot analysis in gonadal tissue (unpublished data). As a negative control, serial sections were processed without primary

antibody. The biotinylated goat anti-mouse secondary antibodies were diluted 1:300. The secondary antibody was detected with aminoethyl carbazole (AEC) chromagen substrate solution (ZYMED Laboratories, San Francisco, CA). Immunohistochemistry was performed on at least three different sections of tissue from each age group.

Statistical Analysis

All data were analyzed by one-way ANOVA using JMP software (SAS Institute, Cary, NC). A Student t-test was used to compare mean normalized QRT-PCR values between different developmental ages. Differences in data were considered to be statistically significant at a P value of < 0.05, unless otherwise stated.

Results

Vegfa *Anti-angiogenic Isoforms mRNA Expression During Ovarian Development*

Through conventional RT-PCR and subcloning, our laboratory confirmed the presence of messenger RNA from two *Vegfa* anti-angiogenic isoforms (*165b* – GenBank: EU040284.1 and Figure 3-1A; *189b* – GenBank: EU040285.1 and Figure 3-1B. Partial 3' UTR determined from *Rattus norvegicus* vascular endothelial growth factor gene, 3' UTR region, GenBank: U22372.1.) in developing and adult rat ovaries. We utilized these mRNA sequences to predict the protein sequence for rat VEGFA_165B (Figure 3-2A) and VEGFA_189B (Figure 3-2B). Interestingly, these protein sequences are one amino acid longer than rat VEGFA_164 (GenBank: AAI68708.1; Figure 3-2A) and 188 (GenBank: AAF19211.1; Figure 3-2B), respectively. The rat *Vegfa_165b* mRNA sequence is 90% homologous to human *VEGFA_165B* (GenBank: AAL27435.1; Figure 3-3A) and the predicted rat VEGFA_165B protein sequence is 88% homologous to human VEGFA_165B (GenBank: AAL27435.1; Figure 3-3B).

Conventional RT-PCR was used to evaluate *Vegfa* anti-angiogenic isoform mRNA expression in developing and adult rat ovaries. Eight developmental time points (E13, E14, E16, E18, P0, P4, P5, P10) and one adult time point (P200) were evaluated. Messenger RNA from both *Vegfa_165b* and *Vegfa_189* was detected at all time points evaluated (Figure 3-4A).

Quantitative real-time PCR (QRT-PCR) demonstrated differential abundance of *Vegfa_165b* and *189b* during pre- and perinatal development of the ovary (Figure 3-4B,C). Specifically, *Vegfa_165b* levels increased from E13/14 to E18 but levels decreased again by P3/5 (Figure 3-4B; $P < 0.05$). Although a distinct *Vegfa_189b* mRNA pattern was not present, increased levels were detected at E14, E18, and P3 compared to E13 (Figure 3-4C; $P < 0.05$).

Localization of VEGFA Anti-angiogenic Isoforms Within Developing Ovaries

After confirmation of *Vegfa_165b and 189b* mRNA expression during early ovarian development, immunohistochemistry was utilized to localize expression to specific cell types. Immunohistochemistry was performed on P0, P3, and P10 rat ovaries (Figure 3-5). Staining for anti-angiogenic isoforms was localized to the pre-granulosa and granulosa cells of follicles from primordial through antral stages and in theca cells of pre-antral and antral follicles (Figure 3-5A,B,C). Positive staining was also localized to oocyte cysts and to oocytes within primordial follicles (Figure 3-5A,B,C).

Discussion

The current study was the first to identify mRNA expression of anti-angiogenic Vegfa isoforms in rat ovarian tissue. By utilizing the mRNA sequences to predict the anti-angiogenic VEGFA protein sequences, we also determined that the rat anti-angiogenic isoforms, VEGFA_165B and 189B, are one amino acid longer than their comparable pro-angiogenic isoforms, VEGFA_164 and 188.

In previous experiments in our laboratory, we detected mRNA expression for three pro-angiogenic *Vegfa* isoforms (120, 164, and 188) and the *Vegfa* receptors, *Kdr* and *Flt1*, in developing rat ovaries (McFee *et al.* 2009). In the current study, messenger RNA expression was also detected for *Vegfa_165b* and *189b* in developing and adult rat ovaries. Analysis with QRT-PCR has demonstrated that *Vegfa_188* mRNA is most abundant at E16 and there are elevated amounts of *Vegfa_164* and *120* after E18 and P0, respectively (McFee *et al.* 2009). For the anti-angiogenic isoforms, QRT-PCR revealed that *Vegfa_165b* mRNA is most abundant around E18 but there is not a distinct pattern of abundance for *Vegfa_189b*.

Around E18 in the rat, primordial germ cells have formed germline cysts that are ceasing to proliferate and are undergoing meiosis. Germline cysts are composed of clusters of oogonia connected by intracellular bridges formed through incomplete cytokinesis. These cell clusters synchronously proliferate and waves of oogonia enter meiosis in a non-synchronous manner until they are arrested around birth (Pepling & Spradling 1998, Bristol-Gould *et al.* 2006). Thus, during embryonic development, the actions of VEGFA_188 and the anti-angiogenic actions of VEGFA_165B may be important in regulating VEGF-mediated vascular development and reorganization or other non-vascular functions of VEGF that may affect oocyte maintenance or meiosis.

Between E18 and P3/4, the developing rat ovary transitions from being composed predominately of oocyte cysts to being composed predominately of primordial follicles (Rajah *et al.* 1992, Kezele & Skinner 2003). Oocyte cysts undergo a programmed breakdown where pre-granulosa cells invade, divide cytoplasm, and surround oocytes to form primordial follicles (Pepling & Spradling 1998, Pepling & Spradling 2001). During late embryonic and early

postnatal development, VEGFA_120 and 164 may act via vascular or non-vascular mechanisms to regulate the oocyte cyst to primordial follicle transition.

Previous immunohistochemical studies in developing rat ovaries have localized VEGFA to oocyte cysts, pre-granulosa cells, granulosa cells, theca cells, and the cytoplasm of oocytes of pre-antral and antral follicles (McFee *et al.* 2009). FLT1 and KDR have also been localized to oocyte cysts, pre-granulosa cells, granulosa cells, theca cells, and oocytes of all follicle stages (McFee *et al.* 2009). In the current study, anti-angiogenic VEGFA isoforms were localized to oocyte cysts, pre-granulosa cells, granulosa cells, theca cells, and oocytes within primordial follicles. Anti-angiogenic VEGFA isoforms were localized to similar non-vascular (granulosa, oocyte) and vascular (theca) follicular cells as pro-angiogenic VEGFA isoforms and receptors; however, pro-angiogenic isoforms were localized to oocytes of advanced follicle stages (pre-antral and antral follicles) while anti-angiogenic isoforms were localized to oocytes of non-developing follicles (primordial follicles).

Previous experiments in our laboratory demonstrated a dramatic role for VEGFA in the formation and maintenance of vasculature, as well as the regulation of follicle progression in perinatal rat ovaries (McFee *et al.* 2009). Taken together with the results from the current study, this suggests that the antiangiogenic VEGFA isoforms may be important in dampening the proangiogenic and nonangiogenic functions of VEGFA in the regulation of follicle development.

RT-PCR Primer & Probe Sequences			
PCR	**Product**	**Gene**	**Sequence**
conventional	reverse primer	Antiangiogenic *Vegfa* isoforms	5'-GGTGAGAGGTCTGCAAGTACGTTCG-3'
quantitative	forward primer	*Vegfa_165b*	5'-CAGAAAATCACTGTGAGCCTTGTT-3'
quantitative	forward primer	*Vegfa_189b*	5'-TCCTGGAGCGTTCACTGTGAG-3'
quantitative	reverse primer	*Vegfa_165b* & *Vegfa_189b*	5'-GGTGAGAGGTCTGCAAGTACGTT-3'
quantitative	probe	*Vegfa_165b* & *Vegfa_189b*	5'-AGCGGAGAAAGCATTTGTTTGTCCAAG-3' FAM-TAMRA

Table 3.1

Conventional and quantitative RT-PCR primers and probes.

A. Rat *Vegfa_165b* mRNA Sequence

```
  1 ATGAACTTTC TGCTCTCTTG GGTGCACTGG ACCCTGGCTT TACTGCTGTA CCTCCACCAT
 61 GCCAAGTGGT CCCAGGCTGC ACCCACGACA GAAGGGGAGC AGAAAGCCCA TGAAGTGGTG
121 AAGTTCATGG ACGTCTACCA GCGCAGCTAT TGCCGTCCAA TTGAGACCCT GGTGGACATC
181 TTCCAGGAGT ACCCCGATGA GATAGAGTAT ATCTTCAAGC CGTCCTGTGT GCCCCTAATG
241 CGGTGTGCGG GCTGCTGCAA TGATGAAGCC CTGGAGTGCG TGCCCACGTC GGAGAGCAAC
301 GTCACTATGC AGATCATGCG GATCAAACCT CACCAAAGCC AGCACATAGG AGAGATGAGC
361 TTCCTGCAGC ATAGCAGATG TGAATGCAGA CCAAAGAAAG ATAGAACAAA GCCAGAAAAT
421 CACTGTGAGC CTTGTTCAGA GCGGAGAAAG CATTTGTTTG TCCAAGATCC GCAGACGTGT
481 AAATGTTCCT GCAAAAACAC AGACTCGCGT TGCAAGGCGA GGCAGCTTGA GTTAAACGAA
541 CGTACTTGCA GACCTCTCAC CGGAAAGACC GATTAACCAT GTCACCACCA CACCACCATC
```

Exon 8b — 3' UTR

B. Rat *Vegfa_189b* mRNA Sequence

```
  1 ATGAACTTTC TGCTCTCTTG GGTGCACTGG ACCCTGGCTT TACTGCTGTA CCTCCACCAT
 61 GCCAAGTGGT CCCAGGCTGC ACCCACGACA GAAGGGGAGC AGAAAGCCCA TGAAGTGGTG
121 AAGTTCATGG ACGTCTACCA GCGCAGCTAT TGCCGTCCAA TTGAGACCCT GGTGGACATC
181 TTCCAGGAGT ACCCCGATGA GATAGAGTAT ATCTTCAAGC CGTCCTGTGT GCCCCTAATG
241 CGGTGTGCGG GCTGCTGCAA TGATGAAGCC CTGGAGTGCG TGCCCACGTC GGAGAGCAAC
301 GTCACTATGC AGATCATGCG GATCAAACCT CACCAAAGCC AGCACATAGG AGAGATGAGC
361 TTCCTGCAGC ATAGCAGATG TGAATGCAGA CCAAAGAAAG ATAGAACAAA GCCAGAAAAA
421 AAATCAGTTC GAGGAAAGGG AAAGGGTCAA AAACGAAAGC GCAAGAAATC CCGGTTTAAA
481 TCCTGGAGCG TTCACTGTGA GCCTTGTTCA GAGCGGAGAA AGCATTTGTT TGTCCAAGAT
541 CCGCAGACGT GTAAATGTTC CTGCAAAAAC ACAGACTCGC GTTGCAAGGC GAGGCAGCTT
601 GAGTTAAACG AACGTACTTG CAGACCTCTC ACCGGAAAGA CCGATTAACC ATGTCACCAC
```

Exon 8b — 3' UTR

Figure 3-1

Messenger RNA sequences for rat *Vegfa_165b* (GenBank: EU040284.1; A) and *189b* (GenBank: EU040285.1; B) with exon 8b mapped. The sequence regions which were subcloned in our laboratory are italicized and the primers utilized are shaded in gray.

A. Alignment of Rat VEGFA_164 and 165B Proteins

```
  1 MNFLLSWVHWTLALLLYLHHAKWSQAAPTTEGEQKAHEVVKFMDVYQRSYCRPIETLVDI  Rat 164
  1 MNFLLSWVHWTLALLLYLHHAKWSQAAPTTEGEQKAHEVVKFMDVYQRSYCRPIETLVDI  Rat 165b

 61 FQEYPDEIEYIFKPSCVPLMRCAGCCNDEALECVPTSESNVTMQIMRIKPHQSQHIGEMS  Rat 164
 61 FQEYPDEIEYIFKPSCVPLMRCAGCCNDEALECVPTSESNVTMQIMRIKPHQSQHIGEMS  Rat 165b

121 FLQHSRCECRPKKDRTKPENHCEPCSERRKHLFVQDPQTCKCSCKNTDSRCKARQLELNE  Rat 164
121 FLQHSRCECRPKKDRTKPENHCEPCSERRKHLFVQDPQTCKCSCKNTDSRCKARQLELNE  Rat 165b

181 RTCRCDKPRR                                                    Rat 164
181 RTCRPLTGKTD                                                    Rat 165b
```

B. Alignment of Rat VEGFA_188 and 189B Proteins

```
  1 MNFLLSWVHWTLALLLYLHHAKWSQAAPTTEGEQKAHEVVKFMDVYQRSYCRPIETLVDI  Rat 188
  1 MNFLLSWVHWTLALLLYLHHAKWSQAAPTTEGEQKAHEVVKFMDVYQRSYCRPIETLVDI  Rat 189b

 61 FQEYPDEIEYIFKPSCVPLMRCAGCCNDEALECVPTSESNVTMQIMRIKPHQSQHIGEMS  Rat 188
 61 FQEYPDEIEYIFKPSCVPLMRCAGCCNDEALECVPTSESNVTMQIMRIKPHQSQHIGEMS  Rat 189b

121 FLQHSRCECRPKKDRTKPEKKSVRGKGKGQKRKRKKSRFKSWSVHCEPCSERRKHLFVQD  Rat 188
121 FLQHSRCECRPKKDRTKPEKKSVRGKGKGQKRKRKKSRFKSWSVHCEPCSERRKHLFVQD  Rat 189b

181 PQTCKCSCKNTDSRCKARQLELNERTCRCDKPRR                            Rat 188
181 PQTCKCSCKNTDSRCKARQLELNERTCRPLTGKTD                            Rat 189b
```

Figure 3-2

Predicted protein sequences for rat VEGFA165B (A) and 189B (B) aligned with rat VEGFA164 (GenBank: AAI68708.1) and 188 (GenBank: AAF19211.1), respectively. Homologous amino acids are shaded in gray.

A. Alignment of Human *VEGFA_165B* and Rat *Vegfa_165b* mRNA

```
  1 ATGAACTTTCTGCTGTCTTGGGTGCATTGGAGCCTTGCCTTGCTGCTCTACCTCCACCAT  Human
  1 ATGAACTTTCTGCTCTCTTGGGTGCACTGGACCCTGGCTTTACTGCTGTACCTCCACCAT  Rat

 61 GCCAAGTGGTCCCAGGCTGCACCCATGGCAGAAGGAGGAGGGCAG-AATCATCACGAAGT  Human
 61 GCCAAGTGGTCCCAGGCTGCACCCACGACAGAAGG-GGAG--CAGAAAGC-CCATGAAGT  Rat

120 GGTGAAGTTCATGGATGTCTATCAGCGCAGCTACTGCCATCCAATCGAGACCCTGGTGGA  Human
117 GGTGAAGTTCATGGACGTCTACCAGCGCAGCTATTGCCGTCCAATTGAGACCCTGGTGGA  Rat

180 CATCTTCCAGGAGTACCCTGATGAGATCGAGTACATCTTCAAGCCATCCTGTGTGCCCCT  Human
177 CATCTTCCAGGAGTACCCCGATGAGATAGAGTATATCTTCAAGCCGTCCTGTGTGCCCCT  Rat

240 GATGCGATGCGGGGGCTGCTGCAATGACGAGGGCCTGGAGTGTGTGCCCACTGA-GGAGT  Human
237 AATGCGGTGTGCGGGCTGCTGCAATGATGAAGCCCTGGAGTGCGTGCCCAC-GTCGGAGA  Rat

299 CCAACATCACCATGCAGATTATGCGGATCAAACCTCACCAAGGCCAGCACATAGGAGAGA  Human
296 GCAACGTCACTATGCAGATCATGCGGATCAAACCTCACCAAAGCCAGCACATAGGAGAGA  Rat

359 TGAGCTTCCTACAGCACAACAAATGTGAATGCAGACCAAAGAAAGATAGAGCAA-GACAA  Human
356 TGAGCTTCCTGCAGCATAGCAGATGTGAATGCAGACCAAAGAAAGATAGAACAAAGCCA-  Rat

418 GAAAATCCCTGTGGGCCTTGCTCAGAGCGGAGAAAGCATTTGTTTGTACAAGATCCGCAG  Human
415 GAAAATCACTGTGAGCCTTGTTCAGAGCGGAGAAAGCATTTGTTTGTCCAAGATCCGCAG  Rat

478 ACGTGTAAATGTTCCTGCAAAAACACAGACTCGCGTTGCAAGGCGAGGCAGCTTGAGTTA  Human
475 ACGTGTAAATGTTCCTGCAAAAACACAGACTCGCGTTGCAAGGCGAGGCAGCTTGAGTTA  Rat

538 AACGAACGTACTTGCAGATCTCTCACCAGGAAAGACTGA                        Human
535 AACGAACGTACTTGCAGACCTCTCACC-GGAAAGACCGATTAA                  Rat
```

B. Alignment of Human and Rat VEGFA165_B Proteins

```
  1 MNFLLSWVHWSLALLLYLHHAKWSQAAPMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVD  Human
  1 MNFLLSWVHWTLALLLYLHHAKWSQAAPTTEGE-QKAHEVVKFMDVYQRSYCRPIETLVD  Rat

 61 IFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLECVPTEESNITMQIMRIKPHQGQHIGEM  Human
 60 IFQEYPDEIEYIFKPSCVPLMRCAGCCNDEALECVPTSESNVTMQIMRIKPHQSQHIGEM  Rat

121 SFLQHNKCECRPKKDRARQENPCGPCSERRKHLFVQDPQTCKCSCKNTDSRCKARQLELN  Human
120 SFLQHSRCECRPKKDRTKPENHCEPCSERRKHLFVQDPQTCKCSCKNTDSRCKARQLELN  Rat

181 ERTCRSLTRK-D                                                  Human
180 ERTCRPLTGKTD                                                  Rat
```

Figure 3-3

Alignment of the human *VEGFA_165B* (GenBank: AAL27435.1) and rat *Vegfa_165b* mRNA sequences (GenBank: EU040284.1; A). Alignment of human VEGFA165B (GenBank: AAL27435.1) with the predicted protein sequence for rat VEGFA165B (B). Homologous nucleotides and amino acids are shaded in gray.

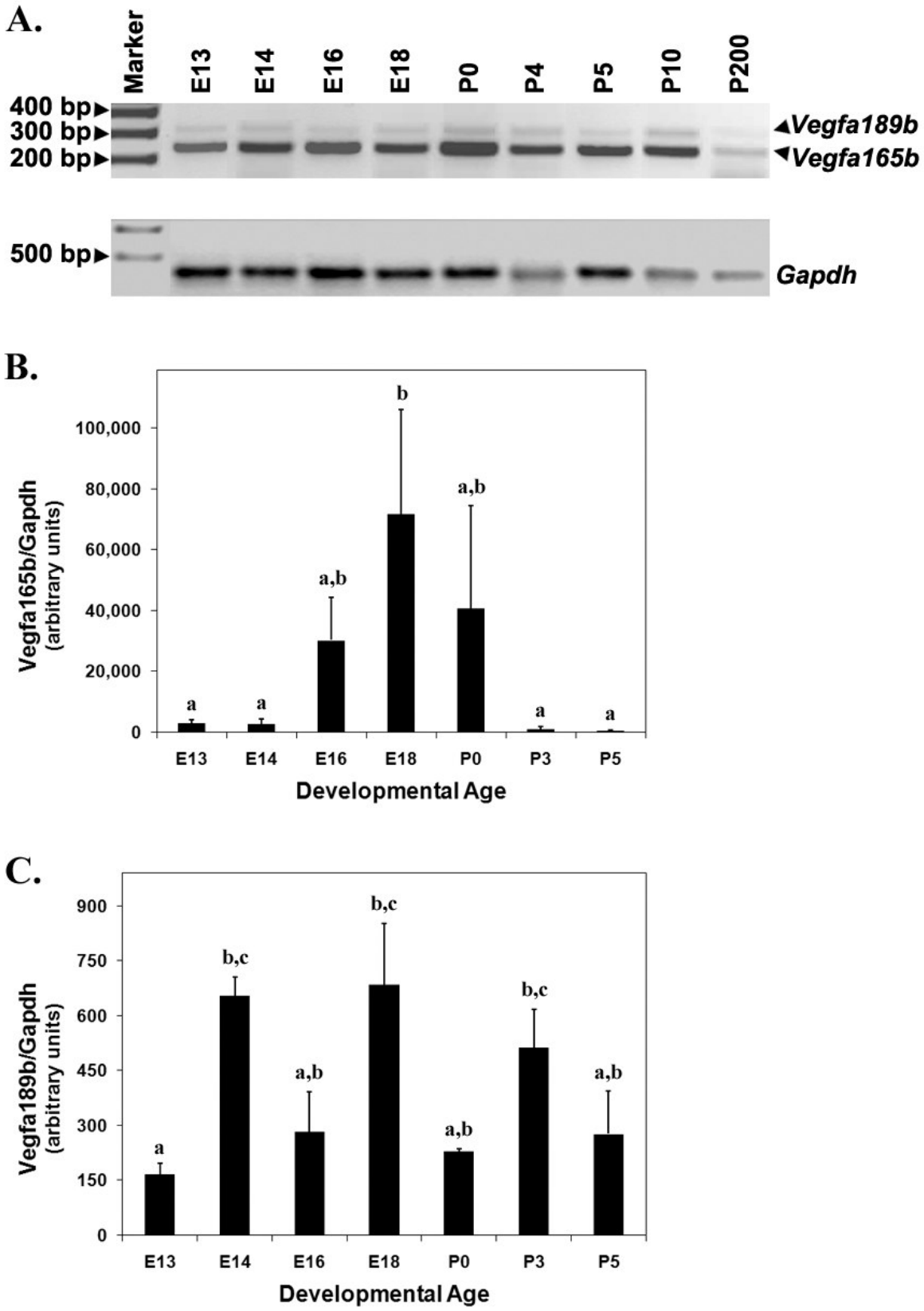
A.
Marker
E13
E14
E16
E18
P0
P4
P5
P10
P200
400 bp
300 bp
200 bp
Vegfa189b
Vegfa165b
500 bp
Gapdh
B.
Vegfa165b/Gapdh (arbitrary units)
100,000
80,000
60,000
40,000
20,000
0
a
a
a,b
b
a,b
a
a
E13
E14
E16
E18
P0
P3
P5
Developmental Age
C.
Vegfa189b/Gapdh (arbitrary units)
900
750
600
450
300
150
0
a
b,c
a,b
b,c
a,b
b,c
a,b
E13
E14
E16
E18
P0
P3
P5
Developmental Age

Figure 3-4

Conventional RT-PCR for *Vegfa* anti-angiogenic isoforms from embryonic day 13 (E13) to postnatal day 10 (P10) developing ovaries and P200 adult ovaries. *Gapdh* served as a control for RNA isolation and amplification (A). Negative control samples (without template) did not produce a band (data not shown). Quantitative Real Time PCR to detect *Vegfa_165b* and *Vegfa_189b* from E13 through P5 of ovarian development. *Gapdh* was used as an endogenous control to account for differences in starting material (B,C). These data are the result of at least 3 different pools of each age tissue (B,C). The mean normalized values ± SEM are presented and different letters represent a statistically significant difference in means at $P < 0.05$ (B,C).

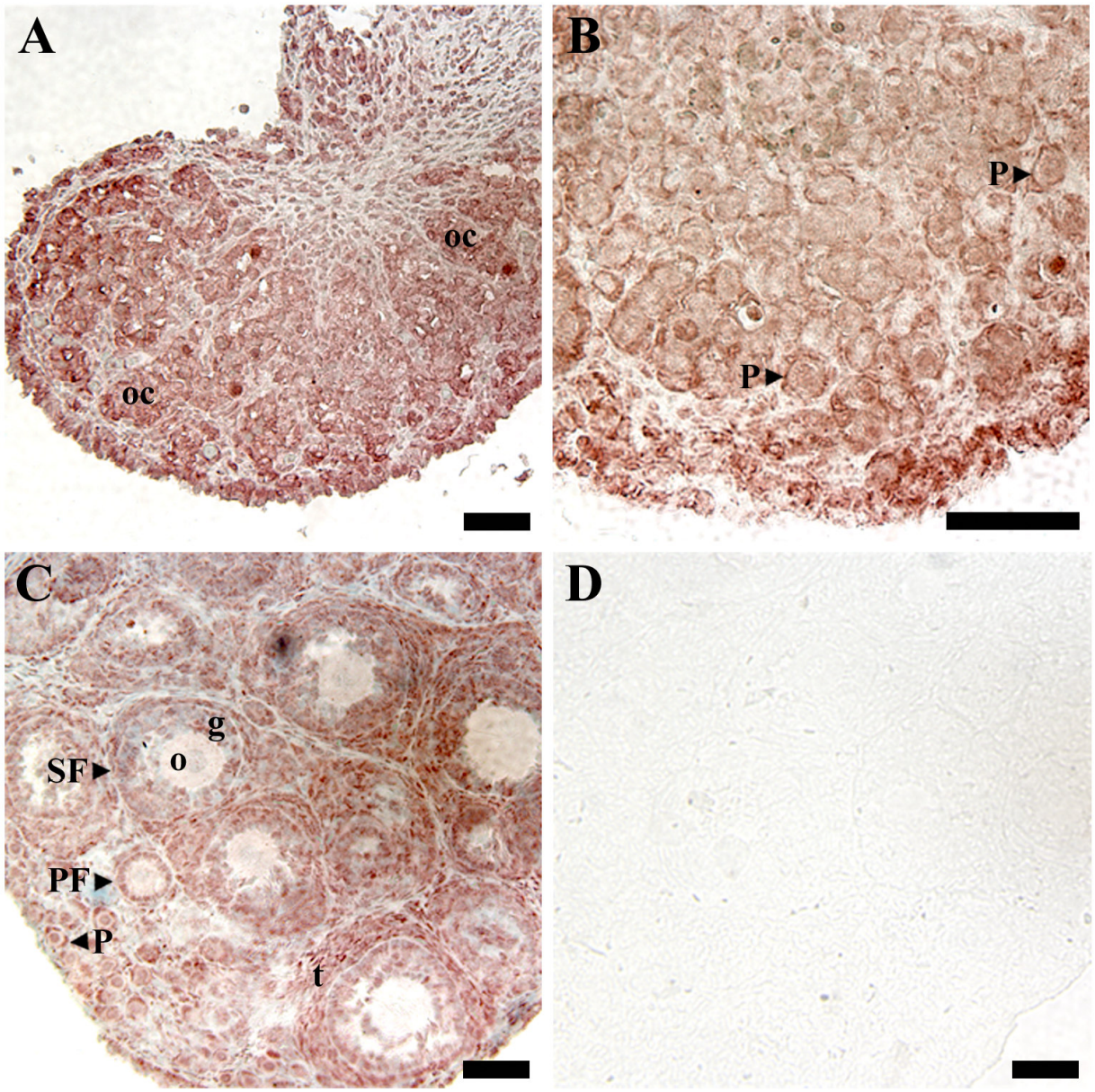

Figure 3-5

Immunohistochemistry for VEGFA anti-angiogenic (xxxB) isoforms in postnatal day 0 (P0; A), P3 (B), and P10 (C,D) ovaries. Figures A and C were lightly counterstained with hemotoxylin. P10 ovarian sections with no primary antibody (D) served as negative controls. These data are the result of at least 3 different ovaries from each developmental time point. Scale bars = 50 µm. oc = oocyte cyst, P = primordial follicle, PF = primary follicle, SF = secondary follicle, o = oocyte, g = granulosa cells, t = theca.

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