VEGF IN PRE-ANTRAL FOLLICULAR DEVELOPMENT

A Thesis

Presented in Partial Fulfillment of the Requirements for

the Degree Master of Science

in the Graduate School of The Ohio State University

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2006

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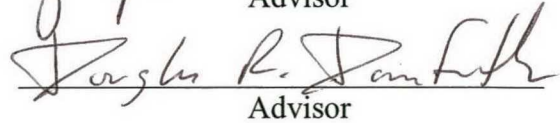

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ABSTRACT

The formation, survival, and growth of the primordial follicle pool in the mammalian ovary is a dynamic process. The complex interaction of these factors orchestrates the movement of primordial follicles into the growing pool or destines them for atresia. Central to the function of endocrine and paracrine signals is their ability to reach their target cell, via vascular delivery and/or diffusion.

One of the most important local regulators of ovarian vascular physiology is Vascular Endothelial Growth Factor (VEGF). Along with stimulating neovascularization, VEGF also regulates vascular permeability. VEGF is present in high amounts within the ovary and its role in the later stages of follicle growth and corpus luteum function is becoming well established. Neutralization of VEGF activity with antibodies or a soluble form of the VEGF receptor disrupts follicular development and corpus luteum function.

VEGF may also play an important role in the activation and development of primordial follicles. Data in the literature are mounting and suggest the presence and importance of VEGF in pre-antral follicles. As such, we hypothesized that neutralization of endogenous VEGF would inhibit initial follicle growth and perhaps disrupt maintenance of the primordial follicle pool. Our data indicate that systemic

administration of VEGF peptide antibodies significantly decreased the number of primordial follicles in treated mice. This decrease was also seen in ovaries that received intrabursal injections of either peptide VEGF antibodies or commercially available VEGF antibodies. VEGF may have exerted its effects on primordial follicles through KDR, the receptor that is recognized for the biological activity of VEGF. These data suggest that VEGF plays a vital role in the maintenance of the primordial follicle pool.

In addition to the aforementioned hypothesis, we were also interested in understanding VEGF mRNA expression at the level of the follicle. VEGF expression has been well characterized in latter stages of follicular development and in the corpus luteum. Data concerning the presence of VEGF in pre-antral follicles is controversial. To clarify the cellular sources of VEGF, we investigated objective quantification of mRNA expression. We performed laser capture microdissection (LCM) followed downstream by real-time PCR. This body of work establishes a reliable way to process and stain tissue without compromising mRNA integrity. These intact samples provide specific populations of cells through the use of LCM, and VEGF mRNA expression can be quantified in these specific populations using real-time PCR.

Dedicated to my parents Dr and Mrs. Gregory M. Roberts
And my soon-to-be husband Nicholas W Kin

ACKNOWLEDGMENTS

I wish to thank my advisers, Joseph Ottobre and Douglas Danforth. Thank you for your support, encouragement, and knowledge. My graduate career has been quite convoluted and I thank you most for your patience as I find my way.

Thank you to my committee members, Dr. Joy Pate for contributing the use of your lab and cryostat as well as your contributions to my graduate development. Dr. MacDonald Wick thank you for the many hours of listening to me vent my frustrations and for your support.

I am grateful to Laura Arbogast who showed me everything I know in the laboratory. You are a wonderful teacher and a great friend.

I thank Ann Ottobre who also supported me in not only the lab but also in life.

I am grateful to Nicholas Kin whose never-ending support and love has kept me going through graduate school. You and your own graduate work has been an inspiration to me.

Finally, I thank my parents and family who have given me the support and inspiration since I was a child to be successful in the scientific and academic environment.

Thank you for encouraging my love of science. I wouldn't be anywhere without you.

This work was supported by a grant from the Ohio Agriculture Research and Development Center.

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1. Roberts, AR, Arbogast, LK, Friedman, CI, Cohn, DE, Kaumaya, PT, Danforth, DR. *Neutralization of Endogenous VEGF Depletes Primordial Follicles in the Mouse Ovary.*

FIELD OF STUDY

Major Field: Animal Sciences
Area of Specialization: Reproductive Physiology

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α	alpha
β	beta
°C	degrees Celsius
CL	Corpus Luteum
FDA	Food and Drug Administration
flk-1	fetal liver kinase-1
flt-1	fms-like tyrosine kinase-1
flt-4	fms-like tyrosine kinase-4
g	gram(s)
h	hour(s)
HIF-1	Hypoxic-Inducible Factor 1
k	kilo

KDR	Kinase Domain Receptor
L	liter(s)
LCM	Laser Capture Microdissection
m	milli
mRNA	messenger RiboNucleic Acid
μ	micro
M	moles per liter
min	minute(s)
mol	mole(s)
rt	room temperature
sflt-1	soluble fms-like tyrosine kinase-1
VEGF	Vascular Endothelial Growth Factor
VEGFR1	Vascular Endothelial Growth Factor Receptor 1
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
VEGFR3	Vascular Endothelial Growth Factor Receptor 3
VPF	Vascular Permeability Factor

CHAPTER 1

LITERATURE REVIEW: VASCULAR ENDOTHELIAL GROWTH FACTOR AND THE MAMMALIAN OVARY

Introduction:

Follicular growth and development in the mammalian ovary is a dynamic evolution. A complex interaction of autocrine and paracrine factors is likely to coordinate the growth of a primordial follicle into the growing pool or destine it for atresia [1]. Some of the factors implicated in promoting the transition of primordial follicles to primary follicles are Kit Ligand (KL) [2], Bone Morphogenic Protein-4 (BMP-4) [3], Growth Differentiation Factor – 9 (GDF-9) [4] and VEGF. KL, and BMP-4 increase the number of primordial follicles in 4-day old rat ovaries in culture when compared to control [2]. This is a model system developed by M.K. Skinner. Kit Ligand is also referred to as stem cell factor due to its role in stem cell growth and development [5]. Mutations in the KL gene can lead to a variety of problems, including deficient gametogenesis [6] and arrest of follicular development at the primary stage [7, 8]. BMP-4 not only to increases the number of primordial follicles in culture (see above) but, the neutralization of BMP-4 results in the apoptotic loss of oocytes within 14 days [3]. BMP-15 [9] and BMP-7 [10] have been implicated in primordial follicle development but their roles are not yet clearly defined. GDF-9 also plays a role in early follicular development. Injecting cloned cDNA into the ovaries of gilts increases primary, secondary, and tertiary follicle populations [11]. Conflicting data exist suggesting that culturing ovaries with GDF-9 does not have an effect on primordial follicles [12]. GDF-9 knock-out mice have their follicular development arrested in the primary stage [13][14] Other factors have been implicated in primordial follicle development but may require further investigation. A variable that may be just as important as the factors themselves is the route of delivery these complex factors use to reach the "resting" follicular pool.

The most likely route of delivery is via the complex vasculature of the ovary. One of the key factors that has been implicated in angiogenesis of the ovary is Vascular Endothelial Growth Factor (VEGF). VEGF has an important role in the later stages of follicle growth and corpus luteum development, but its role in pre-antral follicular development is yet to be elucidated.

Since its discovery, VEGF has been the target of intense investigation. It has been investigated for not only its physiologic actions on endothelial and non-endothelial targets but also its pharmacological effects on various cancers. In addition to its well known effects on later stages of follicular development, VEGF may also play a key role in the growth and survival of pre-antral follicles.

Angiogenesis:

It is important to distinguish the terminology that refers to the growth and development of new blood vessels. Angiogenesis refers to the formation and differentiation of blood vessels [15]. Neovascularization is the formation and differentiation of blood vessels in abnormal quantity such as in pathologies of the retina and solid tumors [16]. Vasculogenesis is the formation and differentiation of the embryonic vascular system [17]. The diffusion of oxygen and nutrients and the removal of waste products is a necessary process for almost all cells and tissues. This process is accomplished via the vasculature in the body. Blood vessels are constantly being formed, redirected, and reorganized to meet the needs of the body. This process of formation and reorganization is called angiogenesis. There are 4 major steps that must be accomplished to complete both angiogenesis and neovascularization [18]. 1. Breaking through the basal lamina that envelopes existing blood vessels. 2. Migration of endothelial cells

toward a source signal. 3. Proliferation of endothelial cells. 4. Formation of tubes. VEGF has been identified as a primary growth factor that can induce all four steps. In the murine embryo, VEGF plays a vital role, the removal of even a single allele of the VEGF gene results in mortality between embryonic days 11 and 12 [19, 20]. Also the removal of the alleles coding for the VEGF receptors results in mortality between embryonic days 8.5 and 9.5 [21-23]. VEGF is also vital to solid tumor growth. Tumors that grow beyond $2mm^3$ need an adequate vascular supply to deliver oxygen and nutrients and remove waste products [24]. VEGF is a primary factor that tumors use to hijack existing vasculature and use it to further their own growth and development.

VEGF Discovery and History:

The discovery of VEGF centered around the vasculature associated with solid tumor growth. In 1939 Ide et al. observed the vasculature growth associated with a solid tumor transplanted into the ear of a rabbit [25]. This paper contained the first documented proposal of a factor responsible for neovascularization. Almost ten years later "factor X" was implicated in the neovascularization of retinal pathologies [26]. It was not until almost 1970 that the first experiments were conducted to check for the presence of a diffusible factor from solid tumors that could affect neovascualarization [27, 28]. These experiments suggested the existence of a diffusible factor that promoted blood vessel proliferation. Even before the discovery of VEGF, Judah Folkman [29] proposed that anti-angiogenics may be effective at treating cancer. This proposal began the chase to firmly identify a diffusible angiogenic factor. On the way to identifying VEGF, several groups were responsible for the discovery of related growth factors including Transforming Growth Factor - alpha(TGF-α), Transforming Growth Factor -

Beta(TGF-β), acidic Fibroblast Growth Factor(aFGF), and basic Fibroblast Growth Factor(bFGF) [30-32]. None of these growth factors had all of the characteristics that made it likely for them to be the main stimulator of tumor angiogenesis. For example, some of these growth factors are sequestered in the cell membrane and are not adequately secreted [33]. Also, there was little or no change in tumor neovascularization when some of these factors were neutralized [34, 35].

In the 1980's two independent lines of research led to the same discovery. Senger et al [36] isolated a factor from rodent and human tumor cell lines that induced vascular leakage without doing endothelial cell damage and aptly named it Vascular Permiablilty Factor (VPF). It wasn't until 1990 that Senger purified and sequenced VPF [37]. Around the same time Ferrara and Henzel identified a diffusible factor from bovine pituitary cells that was a potent mitogen. [38]. This "second" factor was named Vascular Endothelial Growth Factor for its confined effects to endothelial cells. The cloning and sequencing of both these factors revealed that they were the same protein [39, 40]. At this point VEGF moved to being recognized as the main growth factor that is responsible for both physiological and pathological angiogenesis.

VEGF General Information

Since its discovery VEGF has become a well-documented angiogenic factor. VEGF has many central roles including: endothelial cell proliferation, migration, and survival; vascular permeability; angiogenesis in the lymphatic system; and embryonic angiogenesis. For a complete review see refs [41, 42].

Several VEGF gene families have been discovered and more are being discovered. Currently, VEGF consists of the following families; VEGF-A, VEGF-B, VEGF-C,

VEGF-D, VEGF-E, VEGF-F. Predominant among them in the human is the VEGF-A family. Herein VEGF will indicate VEGF-A.

The human VEGF gene is organized into eight exons and seven introns located on chromosome 6p21.3 [43]. Alternative splicing of the VEGF gene results in a variety of isoforms including 121,145,165,189,206, each number indicating the number of amino acids in the isoform. Predominant among the isoforms is VEGF165. It has been well established that VEGF165 and VEGF 121 are the soluble isoforms most responsible for the biological activities of VEGF outside of the lymphatic system. VEGF 165 is a 45kDa heparin binding homo-dimer [38]. VEGF 121 lacks the heparin affinity of 165, which makes it a freely soluble protein, whereas 165 can be both soluble and sequestered in the extracellular matrix [44].

Hypoxia is the most potent upregulator of VEGF. Upstream of the code for the VEGF protein is a 28-base sequence which enhances transcription under hypoxic conditions. This transcription is mediated through Hypoxia-Inducible Factor 1 (HIF-1)[45, 46]. HIF-1 has long been identified as a key mediator in the cellular hypoxic response. Hypoxia has also been suggested to stabilize the mRNA of VEGF in posttranscriptional modifications. Other growth factors also upregulate VEGF including but not limited to TGF-α, TGF-β, IGF-I, FGF, PDGF, and IL-6 [47-49].

VEGF isoforms bind with varying affinity to three different receptor tyrosine kinases aptly named VEGFR1 (flt-1), VEGFR2 (KDR, flk-1), and VEGFR3 (flt-4). All three receptors contain 7 Ig-like domains in the extracellular domain and a single transmembrane region [50-52]. VEGFR3 binds VEGFC and VEGFD . VEGFR1 and VEGFR2 can be found on endothelial cells [53, 54] and was recently noted to be on

bone-marrow derived mononuclear phagocytes [55] . VEGFR1, named for being the first VEGF receptor to be discovered, has still evaded scientists as to what its specific function is. Conflicting research exists concerning this receptor. This conflict can be attributed to the cell type and stage of development in which the receptor is being studied. VEGFR1 has been suggested to be a "decoy" receptor because of its lack of signaling strength [56]. This view is strengthened by the presence of a soluble form of the receptor sVEGFR-1. VEGFR1 does play a pivotal role in development, as a knockout of this receptor results in embryonic lethality [21, 22]. VEGFR2 is the biologically active receptor for VEGF. See Figure 1.1 for a schematic representation of the VEGF families and receptors.

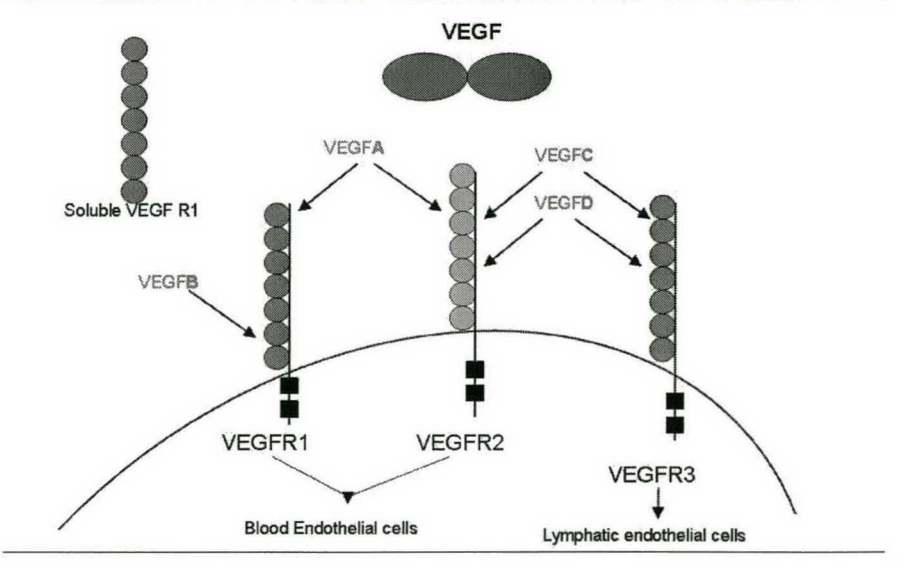


Figure 1.1 – Schematic of VEGF families and VEGF receptors. Circles represent Ig-like domains. Adjacent ovals represent the VEGF homo-dimer. Rectangles are a representation of the tyrosine-kinase intracellular domain. Adapted from Ferrara N. End Rev 2004, 25 (4):581-611

Upon binding the homo-dimer of VEGF to the second and third Ig-like domains [57] of VEGFR2, the receptor undergoes dimerization and phyosphorylation of several tyrosine residues which results in proliferation, migration, permiability and increased survival of endothelial cells [41]. VEGF accomplishes the aforementioned through a variety of signaling pathways including phosphorylation of phospholipases, PLCγ, PI3-kinase, src family and several other signaling proteins [58-60]. The PLCγ/PI3 kinase/Akt signaling pathway mediates both the anti-apoptotic effects of VEGF [61] and endothelial chemotaxis [62, 63]. The mitogenic properties of VEGF have been suggested to signal through the PLCγ/PKC/Raf/MAP pathway [64]. See figure 1.2 (below) for a schematic representation of VEGFR2 binding. Figure 1.3 represents some of the signaling pathways of VEGFR2.

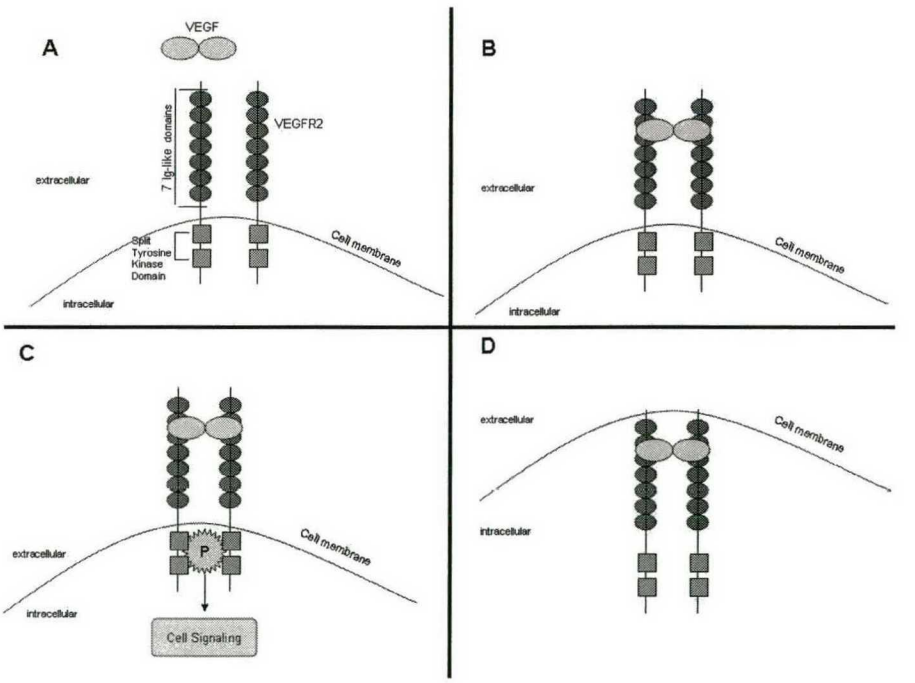


Figure 1.2 – Schematic of VEGF binding its receptor. **A.** VEGF and receptor labeled diagram. **B.** VEGF binds to its receptor causing receptor dimerization. **C.** Receptor Internalization. **D.** Phosphorylation of intracellular tyrosine kinase domains begins cell signaling cascades.

The actions of VEGF are numerous. VEGF is heavily involved in the angiogenic organization occurring during embryonic development, and a knockout of the VEGF gene or either one of the receptors results in embryonic lethality [19-23] . VEGF also plays a role in skeletal growth and in the transformation from cartilage to bone [65, 66]. In the adult, VEGF plays a smaller role in wound healing, adipose tissue formation [67] and the female reproductive tract. Most of the physiological effects of VEGF are mediated through endothelial targets. For example, VEGF induces endothelial proliferation, angiogenesis, vascular permeability, and increased survival.

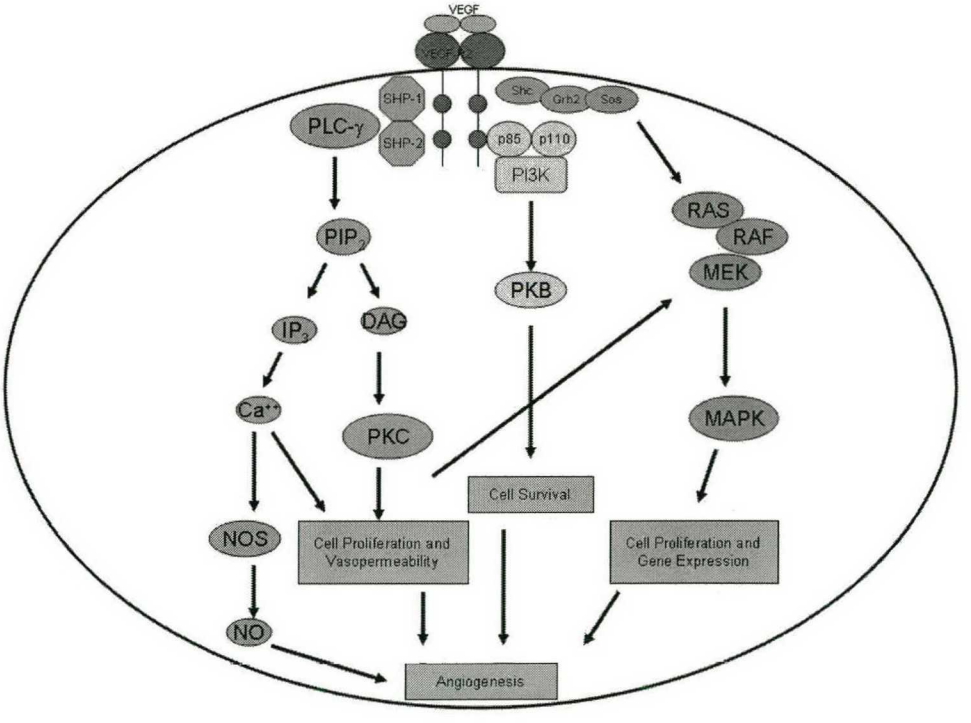


Figure 1.3 – Signaling pathways of VEGFR2 in an endothelial cell. Adapted from Sigma-Aldrich VEGF receptor signaling. Abbreviations can be found in table 1.1

Symbol	Full Name
Ca^{++}	Calcium
DAG	Diacyl Glycerol
Grb2	Growth factor receptor bound protein-2
IP3	Inositol Tri-Phosphate
MAPK	Mitogen Activated Protein Kinase
MEK	Map Kinase/Erk Kinase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
p85	Protein 85
p110	Protein 110
PI3K	Phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol 4,5 – bisphosphate
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC-γ	Phospholipase C-gamma
Raf	MAPKKK
Ras	Rat Sarcoma
Shp-1	Src homology 2 (SH2) domain containing protein tyrosine phosphotase -1
Shp-2	Src homology 2 (SH2) domain containing protein tyrosine phosphotase -2
Sos	Son of Sevenless
VEGF	Vascular Enothelial Growth Factor Receptor 2
VEGFR2	Vascular Endothelial Growth Factor

Table 1.1 – Symbols and Full Names of Cell Signaling Molecules seen in Figure 1.3

VEGF also plays a role in non-endothelial cells. VEGF has been shown to promote monocyte chemotaxis [55], induce colony formation in granulocyte-macrophage progenitor cells [68], enhance B cell generation [69], and may also play a role in the repopulation of hematopoetic stem cell populations after ablation [70].

VEGF in the Ovary

One of the most important local regulators of ovarian vascular physiology is VEGF [41, 71, 72]. As mentioned, along with stimulating neovascularization, VEGF also regulates vascular permeability. VEGF is present in high amounts within the ovary and its role in the later stages of follicle growth and corpus luteum function is becoming well established. VEGF production and increased blood vessel extension is associated with follicle activation [73]. VEGF is produced by the thecal and granulosa cells in the ovary [74-77] and granulosa cells secrete VEGF in response to stimulation by gonadotropins [74, 78-80]. After ovulation, luteal cells continue expression of VEGF [81, 82]. Neutralization of VEGF activity with antibodies [83] or a soluble form of the VEGF receptor [84]disrupts follicular/corpus luteum development and function [85-87]. In addition to its role in the later stages of follicle growth, VEGF may also play an important role in the activation and development of primordial follicles. Danforth et. al. [88], previously that VEGF administration increases the number of primary and small secondary follicles in the rodent ovary. This effect of VEGF is dose and time dependent. In addition, estrogen up-regulates VEGF expression in the rodent ovary. This up-regulation may be part of the mechanism by which estrogen supports follicular growth [88]. These data suggest a potential role for VEGF in primordial follicle activation.

Neutralization of VEGF

Before the discovery of VEGF the idea of neutralizing an angiogeneic factor had been heavily investigated. The neutralization of VEGF is becoming a popular

therapy when combating the vasculature of solid tumors and retinal neoangiogenesis. VEGF is upregulated in many human tumors [89]. In the early 90's several groups found that antibodies to VEGF inhibited the growth of tumors in-vivo [90]. These progressed to in-vivo studies and targeted both VEGF and its receptors. Most noteable of the anti-VEGF antibodies is bevacizumab which has recently been approved by the FDA for the treatment of metastatic colorectal cancer [91].

VEGF antibodies quickly became a way to help evaluate the role of VEGF in the ovary. The temporal expression of VEGF in the ovary increases with follicle growth. There is evidence in the literature that shows immunohistochemical staining of VEGF in the primordial follicle population [92] [93]. As the follicle begins to develop its own vascular network in the secondary stage, both thecal and granulosa cells begin to express VEGF [94]. Expression of VEGF remains constant as the follicle goes through antral development [76, 95]. The amount of VEGF also increases markedly in the follicular fluid nearing ovulation [79]. Once a follicle enters the later stages of development, VEGF mRNA has been shown to be stimulated by gondadotropins in-vitro [78][80]. Neutralizing VEGF and or VEGFR2 at any stage of follicular disrupts follicle growth. Early follicular phase neutralization of VEGFR2 results in delayed follicular selection [83]. Mid-follicular phase neutralization also delayed follicular selection and delayed ovulation [96]. Late follicular phase neutralization not only delayed ovulation but also blocked the characteristic rise in estradiol [83]. This implies that VEGF has a crucial role in the late development of ovarian follicles.

The changes to the ovarian vasculature during the formation of the corpus luteum are well documented [72, 81, 97] and it has long been accepted that these changes

are essential in order to maintain the corpus luteum for the normal ovarian cycle and pregnancy [76, 85, 86]. VEGF mRNA expression steadily increases from early to mid-late development in the corpus luteum and then slowly declines towards the end of the life-span of the CL [98]. Furthermore when soluble VEGFR-1 (sFlt-1) receptor was administered to rats that underwent gonadotropin induced ovulation they experienced a dramatic decrease in ovarian weight [86]. VEGFR-2 also plays a critical role in the blood vessel survival in the corpus lutuem. Pauli et al [99] demonstrated a regression of luteal vessels followed by a drop in progesterone within 24hrs of an injection of anti-VEGFR2 antibodies into pregnant mice. This resulted in the arrest of embryonic development.

Summary

In summary, since its discovery VEGF has been heavily pursued as the main protein responsible for angiogenesis and neovascularization. Although there are many factors that may play a role in neovascularization and angiogenesis, VEGF seems to play a central irreplaceable role, making it an attractive candidate to focus on when considering angiogenic pathologies. VEGF activity also exists outside of angiogenesis and VEGF is being implicated in several new roles in the body. VEGF is considered to be the primary mediator of angiogenesis during the late stages of folliculogenesis and corpus luteum development. Only recently has it been targeted as a potential candidate for mediating growth and development in the early stages of follicular development [88, 92, 99]. The data following herein will address the role of that VEGF in pre-antral follicular development, and examine the cellular sources of VEGF within the ovary.

REFERENCES

1. Danforth DR. Endocrine and paracrine control of oocyte development. Am J Obstet Gynecol 1995; 172:747-752.

2. Parrott JA, Skinner MK. Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. Endocrinology 1999; 140:4262-4271.

3. Nilsson EE, Skinner MK. Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. Biol Reprod 2003; 69:1265-1272.

4. Wang C, Roy SK. Expression of growth differentiation factor 9 in the oocytes is essential for the development of primordial follicles in the hamster ovary. Endocrinology 2006; 147:1725-1734.

5. Besmer P. The kit ligand encoded at the murine Steel locus: a pleiotropic growth and differentiation factor. Curr Opin Cell Biol 1991; 3:939-946.

6. Russell ES. Hereditary anemias of the mouse: a review for geneticists. Adv Genet 1979; 20:357-459.

7. Huang EJ, Manova K, Packer AI, Sanchez S, Bachvarova RF, Besmer P. The murine steel panda mutation affects kit ligand expression and growth of early ovarian follicles. Dev Biol 1993; 157:100-109.

8. Bedell MA, Brannan CI, Evans EP, Copeland NG, Jenkins NA, Donovan PJ. DNA rearrangements located over 100 kb 5' of the Steel (Sl)-coding region in Steel-panda and Steel-contrasted mice deregulate Sl expression and cause female sterility by disrupting ovarian follicle development. Genes Dev 1995; 9:455-470.

9. Dean J. Oocyte-specific genes regulate follicle formation, fertility and early mouse development. J Reprod Immunol 2002; 53:171-180.

10. Lee WS, Yoon SJ, Yoon TK, Cha KY, Lee SH, Shimasaki S, Lee S, Lee KA. Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. Mol Reprod Dev 2004; 69:159-163.

11. Shimizu T. Promotion of ovarian follicular development by injecting vascular endothelial growth factor (VEGF) and growth differentiation factor 9 (GDF-9) genes. J Reprod Dev 2006; 52:23-32.

12. Nilsson EE, Skinner MK. Growth and differentiation factor-9 stimulates progression of early primary but not primordial rat ovarian follicle development. Biol Reprod 2002; 67:1018-1024.

13. Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF. Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. Dev Biol 1998; 204:373-384.

14. Elvin JA, Yan C, Wang P, Nishimori K, Matzuk MM. Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. Mol Endocrinol 1999; 13:1018-1034.

15. Merriam-Webster Medical Dictionary [Internet]. Angiogenesis. 2005; 2006:1.

16. Merriam-Webster Medical Dictionary [Internet]. Neovascularization. 2005; 2006:1.

17. Merriam-Webster Medical Dictionary [Internet]. Vasculogenesis. 2005; 2006:1.

18. Tannock I, Hill R, Bristow R, Harrington L. The Basic Science of Oncology. United States: McGraw-Hill Professional; 2004: 558.

19. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996; 380:435-439.

20. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996; 380:439-442.

21. Fong GH, Zhang L, Bryce DM, Peng J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. Development 1999; 126:3015-3025.

22. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 1995; 376:66-70.

23. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995; 376:62-66.

24. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 2005; 23:1011-1027.

25. Ide AG, Baker NH, Warren SL Vascularization of the Brown Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. Am J Roentgenol 1939; 42: 891-899.

26. Michaelson IC. The mode of development of the vascular system of the retina with some observations on its significance for certain retinal disorders. Trans Ophthalmol Soc UK 1949; 68: 137-180.

27. Greenblatt M, Shubi P. Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique. J Natl Cancer Inst 1968; 41:111-124.

28. Ehrmann RL, Knoth M. Choriocarcinoma. Transfilter stimulation of vasoproliferation in the hamster cheek pouch. Studied by light and electron microscopy. J Natl Cancer Inst 1968; 41:1329-1341.

29. Folkman J, Merler E, Abernathy C, Williams G. Isolation of a tumor factor responsible for angiogenesis. J Exp Med 1971; 133:275-288.

30. Thomas KA, Rios-Candelore M, Gimenez-Gallego G, DiSalvo J, Bennett C, Rodkey J, Fitzpatrick S. Pure brain-derived acidic fibroblast growth factor is a potent angiogenic vascular endothelial cell mitogen with sequence homology to interleukin 1. Proc Natl Acad Sci U S A 1985; 82:6409-6413.

31. Esch F, Ueno N, Baird A, Hill F, Denoroy L, Ling N, Gospodarowicz D, Guillemin R. Primary structure of bovine brain acidic fibroblast growth factor (FGF). Biochem Biophys Res Commun 1985; 133:554-562.

32. Todaro GJ, De Larco JE, Fryling C, Johnson PA, Sporn MB. Transforming growth factors (TGFs): properties and possible mechanisms of action. J Supramol Struct Cell Biochem 1981; 15:287-301.

33. Vlodavsky I, Folkman J, Sullivan R, Fridman R, Ishai-Michaeli R, Sasse J, Klagsbrun M. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. Proc Natl Acad Sci U S A 1987; 84:2292-2296.

34. Matsuzaki K, Yoshitake Y, Matuo Y, Sasaki H, Nishikawa K. Monoclonal antibodies against heparin-binding growth factor II/basic fibroblast growth factor that block its biological activity: invalidity of the antibodies for tumor angiogenesis. Proc Natl Acad Sci U S A 1989; 86:9911-9915.

35. Dennis PA, Rifkin DB. Studies on the role of basic fibroblast growth factor in vivo: inability of neutralizing antibodies to block tumor growth. J Cell Physiol 1990; 144:84-98.

36. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983; 219:983-985.

37. Senger DR, Connolly DT, Van de Water L, Feder J, Dvorak HF. Purification and NH2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability factor. Cancer Res 1990; 50:1774-1778.

38. Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 1989; 161:851-858.

39. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989; 246:1306-1309.

40. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989; 246:1309-1312.

41. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004; 25:581-611.

42. Roy H, Bhardwaj S, Yla-Herttuala S. Biology of vascular endothelial growth factors. FEBS Lett 2006; 580:2879-2887.

43. Vincenti V, Cassano C, Rocchi M, Persico G. Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. Circulation 1996; 93:1493-1495.

44. Park JE, Keller GA, Ferrara N. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol Biol Cell 1993; 4:1317-1326.

45. Liu Y, Cox SR, Morita T, Kourembanas S. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. Circ Res 1995; 77:638-643.

46. Semenza G. Signal transduction to hypoxia-inducible factor 1. Biochem Pharmacol 2002; 64:993-998.

47. Frank S, Hubner G, Breier G, Longaker MT, Greenhalgh DG, Werner S. Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. J Biol Chem 1995; 270:12607-12613.

48. Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, Alitalo K. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. J Biol Chem 1994; 269:6271-6274.

49. Warren RS, Yuan H, Matli MR, Ferrara N, Donner DB. Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma. J Biol Chem 1996; 271:29483-29488.

50. Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene 1990; 5:519-524.

51. Matthews W, Jordan CT, Gavin M, Jenkins NA, Copeland NG, Lemischka IR. A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit. Proc Natl Acad Sci U S A 1991; 88:9026-9030.

52. Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB. Identification of a new endothelial cell growth factor receptor tyrosine kinase. Oncogene 1991; 6:1677-1683.

53. Vaisman N, Gospodarowicz D, Neufeld G. Characterization of the receptors for vascular endothelial growth factor. J Biol Chem 1990; 265:19461-19466.

54. Jakeman LB, Winer J, Bennett GL, Altar CA, Ferrara N. Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. J Clin Invest 1992; 89:244-253.

55. Shen H, Clauss M, Ryan J, Schmidt AM, Tijburg P, Borden L, Connolly D, Stern D, Kao J. Characterization of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes. Blood 1993; 81:2767-2773.

56. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem 1994; 269:26988-26995.

57. Fuh G, Li B, Crowley C, Cunningham B, Wells JA. Requirements for binding and signaling of the kinase domain receptor for vascular endothelial growth factor. J Biol Chem 1998; 273:11197-11204.

58. Eliceiri BP, Paul R, Schwartzberg PL, Hood JD, Leng J, Cheresh DA. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Mol Cell 1999; 4:915-924.

59. Matsumoto T, Claesson-Welsh L. VEGF receptor signal transduction. Sci STKE 2001; 2001:RE21.

60. Guo D, Jia Q, Song HY, Warren RS, Donner DB. Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with endothelial cell proliferation. J Biol Chem 1995; 270:6729-6733.

61. Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem 1998; 273:13313-13316.

62. Morales-Ruiz M, Fulton D, Sowa G, Languino LR, Fujio Y, Walsh K, Sessa WC. Vascular endothelial growth factor-stimulated actin reorganization and migration of endothelial cells is regulated via the serine/threonine kinase Akt. Circ Res 2000; 86:892-896.

63. Gille H, Kowalski J, Yu L, Chen H, Pisabarro MT, Davis-Smyth T, Ferrara N. A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR-1) constitutively inhibits vascular endothelial growth factor-dependent phosphatidylinositol 3'-kinase activation and endothelial cell migration. EMBO J 2000; 19:4064-4073.

64. Takahashi T, Ueno H, Shibuya M. VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. Oncogene 1999; 18:2221-2230.

65. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 1999; 5:623-628.

66. Carlevaro MF, Cermelli S, Cancedda R, Descalzi Cancedda F. Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. J Cell Sci 2000; 113 (Pt 1):59-69.

67. Fukumura D, Ushiyama A, Duda DG, Xu L, Tam J, Krishna V, Chatterjee K, Garkavtsev I, Jain RK. Paracrine regulation of angiogenesis and adipocyte differentiation during in vivo adipogenesis. Circ Res 2003; 93:e88-97.

68. Bautz F, Rafii S, Kanz L, Mohle R. Expression and secretion of vascular endothelial growth factor-A by cytokine-stimulated hematopoietic progenitor cells. Possible role in the hematopoietic microenvironment. Exp Hematol 2000; 28:700-706.

69. Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, Hicklin DJ, Zhu Z, Witte L, Crystal RG, Moore MA, Rafii S. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med 2001; 193:1005-1014.

70. Gerber HP, Malik AK, Solar GP, Sherman D, Liang XH, Meng G, Hong K, Marsters JC, Ferrara N. VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature 2002; 417:954-958.

71. Fraser HM, Wulff C. Angiogenesis in the primate ovary. Reprod Fertil Dev 2001; 13:557-566.

72. Hazzard TM, Stouffer RL. Angiogenesis in ovarian follicular and luteal development. Baillieres Best Pract Res Clin Obstet Gynaecol 2000; 14:883-900.

73. Mattioli M, Barboni B, Turriani M, Galeati G, Zannoni A, Castellani G, Berardinelli P, Scapolo PA. Follicle activation involves vascular endothelial growth factor production and increased blood vessel extension. Biol Reprod 2001; 65:1014-1019.

74. Lee A, Christenson LK, Patton PE, Burry KA, Stouffer RL. Vascular endothelial growth factor production by human luteinized granulosa cells in vitro. Hum Reprod 1997; 12:2756-2761.

75. Kamat BR, Brown LF, Manseau EJ, Senger DR, Dvorak HF. Expression of vascular permeability factor/vascular endothelial growth factor by human granulosa and theca lutein cells. Role in corpus luteum development. Am J Pathol 1995; 146:157-165.

76. Barboni B, Turriani M, Galeati G, Spinaci M, Bacci ML, Forni M, Mattioli M. Vascular endothelial growth factor production in growing pig antral follicles. Biol Reprod 2000; 63:858-864.

77. Friedman CI, Danforth DR, Herbosa-Encarnacion C, Arbogast L, Alak BM, Seifer DB. Follicular fluid vascular endothelial growth factor concentrations are elevated in women of advanced reproductive age undergoing ovulation induction. Fertil Steril 1997; 68:607-612.

78. Duffy DM, Stouffer RL. Luteinizing hormone acts directly at granulosa cells to stimulate periovulatory processes: modulation of luteinizing hormone effects by prostaglandins. Endocrine 2003; 22:249-256.

79. Koos RD. Increased expression of vascular endothelial growth/permeability factor in the rat ovary following an ovulatory gonadotropin stimulus: potential roles in follicle rupture. Biol Reprod 1995; 52:1426-1435.

80. Hazzard TM, Molskness TA, Chaffin CL, Stouffer RL. Vascular endothelial growth factor (VEGF) and angiopoietin regulation by gonadotrophin and steroids in macaque granulosa cells during the peri-ovulatory interval. Mol Hum Reprod 1999; 5:1115-1121.

81. Dickson SE, Bicknell R, Fraser HM. Mid-luteal angiogenesis and function in the primate is dependent on vascular endothelial growth factor. J Endocrinol 2001; 168:409-416.

82. Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG. Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest 1998; 78:1385-1394.

83. Zimmermann RC, Xiao E, Husami N, Sauer MV, Lobo R, Kitajewski J, Ferin M. Short-term administration of antivascular endothelial growth factor antibody in the late follicular phase delays follicular development in the rhesus monkey. J Clin Endocrinol Metab 2001; 86:768-772.

84. Wulff C, Wiegand SJ, Saunders PT, Scobie GA, Fraser HM. Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc (vascular endothelial growth factor Trap(A40)). Endocrinology 2001; 142:3244-3254.

85. Wulff C, Wilson H, Rudge JS, Wiegand SJ, Lunn SF, Fraser HM. Luteal angiogenesis: prevention and intervention by treatment with vascular endothelial growth factor trap(A40). J Clin Endocrinol Metab 2001; 86:3377-3386.

86. Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, Chisholm V, Hillan KJ, Schwall RH. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat Med 1998; 4:336-340.

87. Fraser HM, Dickson SE, Lunn SF, Wulff C, Morris KD, Carroll VA, Bicknell R. Suppression of luteal angiogenesis in the primate after neutralization of vascular endothelial growth factor. Endocrinology 2000; 141:995-1000.

88. Danforth DR, Arbogast LK, Ghosh S, Dickerman A, Rofagha R, Friedman CI. Vascular endothelial growth factor stimulates preantral follicle growth in the rat ovary. Biol Reprod 2003; 68:1736-1741.

89. Ferrara N. VEGF as a therapeutic target in cancer. Oncology 2005; 69 Suppl 3:11-16.

90. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature 1993; 362:841-844.

91. Zakarija A, Soff G. Update on angiogenesis inhibitors. Curr Opin Oncol 2005; 17:578-583.

92. Otani N, Minami S, Yamoto M, Shikone T, Otani H, Nishiyama R, Otani T, Nakano R. The vascular endothelial growth factor/fms-like tyrosine kinase system in human ovary during the menstrual cycle and early pregnancy. J Clin Endocrinol Metab 1999; 84:3845-3851.

93. Harata T, Ando H, Iwase A, Nagasaka T, Mizutani S, Kikkawa F. Localization of angiotensin II, the AT1 receptor, angiotensin-converting enzyme, aminopeptidase A,

adipocyte-derived leucine aminopeptidase, and vascular endothelial growth factor in the human ovary throughout the menstrual cycle. Fertil Steril 2006; 86:433-439.

94. Taylor PD, Hillier SG, Fraser HM. Effects of GnRH antagonist treatment on follicular development and angiogenesis in the primate ovary. J Endocrinol 2004; 183:1-17.

95. Greenaway J, Gentry PA, Feige JJ, LaMarre J, Petrik JJ. Thrombospondin and vascular endothelial growth factor are cyclically expressed in an inverse pattern during bovine ovarian follicle development. Biol Reprod 2005; 72:1071-1078.

96. Fraser HM, Wilson H, Rudge JS, Wiegand SJ. Single injections of vascular endothelial growth factor trap block ovulation in the macaque and produce a prolonged, dose-related suppression of ovarian function. J Clin Endocrinol Metab 2005; 90:1114-1122.

97. Fraser HM, Wulff C. Angiogenesis in the corpus luteum. Reprod Biol Endocrinol 2003; 1:88.

98. Tesone M, Stouffer RL, Borman SM, Hennebold JD, Molskness TA. Vascular endothelial growth factor (VEGF) production by the monkey corpus luteum during the menstrual cycle: isoform-selective messenger RNA expression in vivo and hypoxia-regulated protein secretion in vitro. Biol Reprod 2005; 73:927-934.

99. Pauli SA, Tang H, Wang J, Bohlen P, Posser R, Hartman T, Sauer MV, Kitajewski J, Zimmermann RC. The vascular endothelial growth factor (VEGF)/VEGF receptor 2 pathway is critical for blood vessel survival in corpora lutea of pregnancy in the rodent. Endocrinology 2005; 146:1301-1311.

CHAPTER 2

NEUTRALIZATION OF ENDOGENOUS VEGF DEPLETES PRIMORDIAL FOLLICLES IN THE MOUSE OVARY

Abstract:

The regulation of early follicular growth and development involves a complex interaction of autocrine, paracrine, and endocrine signals. The ability of these factors to regulate follicle growth may depend, in part, on the extent of vascular delivery to and perfusion of the ovary. Vascular endothelial growth factor A (VEGFA) is a major regulator of vascular physiology in the ovary. VEGFA is produced in numerous ovarian compartments, and likely plays a role in the regulation of all phases of follicular growth from preantral through preovulatory. The aim of the present study was to further evaluate the role of VEGF in early follicle growth by neutralization of endogenous VEGF or VEGF receptors. Adult mice were injected systemically or prepubertal mice were injected directly under the ovarian bursa with antibodies designed to neutralize VEGF or block interaction with its receptors in the ovary. Both systemic and intrabursal injections of VEGF antibody significantly reduced the number of primordial follicles within 1-3 days after administration without affecting primary or secondary follicle numbers. Primordial follicle numbers were not different from control levels by 30 days after VEGFA antibody administration. Administration of antibodies to the kinase domain receptor (KDR) for VEGF but not the FMS-like tyrosine receptor (FLT1) for VEGF also results in a significant decrease in primordial follicles. These data suggest that VEGF plays a vital role in the maintenance and growth of the primordial follicle pool.

Introduction:

The formation, survival, and growth of the preantral follicle pool in the mammalian ovary is a dynamic process regulated by a variety of autocrine, paracrine and endocrine signals [1]. The complex interaction of these factors may orchestrate the movement of primordial follicles into the growing pool or destine them for atresia. Central to the function of endocrine and paracrine signals is their ability to reach their target cell, via vascular delivery and/or diffusion.

One of the most important local regulators of ovarian vascular physiology is Vascular Endothelial Growth Factor A (VEGFA) [2-4]. Along with stimulating neovascularization, VEGFA also regulates vascular permeability. VEGFA production and increased blood vessel extension is associated with follicle activation [5]. VEGFA is produced by the thecal and/or granulosa cells in the ovary [6-10] and granulosa cells secrete VEGFA in response to stimulation by gonadotropins [6, 11-13]. After ovulation, luteal cells continue expression of VEGFA [14,15]. Neutralization of VEGFA activity with antibodies [16] or a soluble form of the VEGF receptor [17] disrupts follicular development and corpus luteum function [18-20].

In addition to its role in the later stages of follicle growth, VEGFA may also play an important role in the activation and development of pre-antral follicles. Although immunohistochemical studies generally confirm that follicular VEGFA expression increases as follicles mature [21-23], several reports reveal expression of VEGF in preantral follicular compartments. VEGFA protein has been identified in the oocytes of human primordial [24,25] and human and rat primary [25,26] follicles. Kezele et al [27]

identified VEGFA as one of the important genes upregulated during primordial follicle development in the mouse. We have shown previously that VEGFA administration increases the number of primary and small secondary follicles in the rodent ovary [28]. Administration of VEGFA directly to the ovary results in an increase in preantral follicle numbers in a dose and time dependent manner. In addition, estrogen up-regulates VEGFA expression in the rodent ovary similar to its effects on early follicle growth [28]. These data suggest a potential role for VEGFA in primordial follicle activation. As such, we hypothesized that neutralization of endogenous VEGFA might alter initial follicle growth and perhaps disrupt maintenance of the primordial follicle pool.

Materials and Methods:

Production of VEGF Antibodies

Antigen selection and generation was based on previous work in our laboratory with antibodies against HER2 [29]. In this approach an antigenic B-cell epitope of VEGFA (residues 127-144, common to VEGF A-G) was selected using Peptide Companion software (CSPS Pharmaceuticals Inc., San Diego, Ca). This peptide sequence is located in the carboxy terminal domain which is important for the mitogenic actions of VEGFA [30]. The B-cell epitope was co-linearly synthesized with a promiscuous T_H epitope (Measles Virus Fusion Protein (MVF) 288-302), using a 4-residue amino acid linker GPSL. All peptides were synthesized by solid phase peptide synthesis and were purified by semi preparative reversed phase HPLC as described previously [31]. The identity of the peptide MVF-VEGFA was confirmed by matrix-assisted laser desorption/ionization time of flight mass spectroscopy. Schematic representation of peptide construct is provided in Figure 2.1. Throughout this manuscript,

we have utilized VEGFA to describe that specific isoform and elsewhere utilized VEGF when describing our data since our antibody will recognize various forms of VEGF (A, B, C, D, etc).

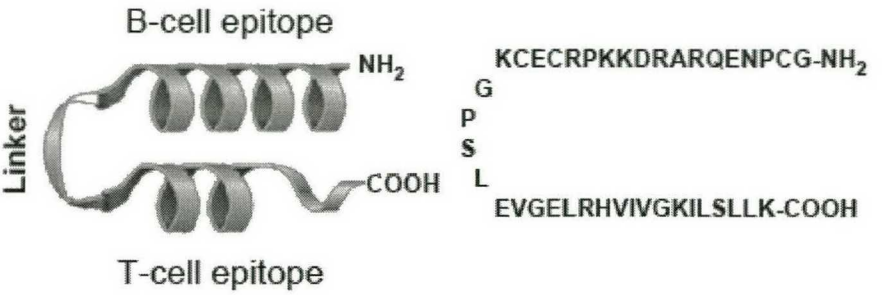


Figure 2.1 - Schematic representation of VEGF Chimeric Constructs. An antigenic B-cell epitope of VEGF (residues 127-144) was co-linearly synthesized with a promiscuous T-Cell epitope (Measles Virus Fusion Protein (MVF) residues 288-302) using a 4 residue (GPSL) amino acid linker. This construct was injected into adult New Zealand rabbits to generate polyclonal antisera as described in Methods.

Adult Female New Zealand White rabbits (Harlan, Indianapolis IN) received an initial injection of 1mg/ml of MVF-VEGFA, dissolved in H_2O with 100µg of nor-MDP (*N*-acetylglucosamine-3 yl-acetyl- L-alanyl-D-isoglutamine). Montanide ISA-720 was used to emulsify the peptide solution. Booster injections (0.5mg/ml) were given twice, three weeks apart. Blood samples were collected prior to the first injection and once a week thereafter. VEGF antibodies were purified from heat inactivated serum using a protein A column (Pierce Rockford, Il). Antibody titers were determined using an Enzyme-Linked Immunosorbent Assay as previously described [29]. Since MVF by itself is not immunogenic [29], we chose to utilize pre-immune serum from the same rabbits used for VEGF antibody generation as controls for our experiments.

All studies were approved by the Institutional Laboratory Animal care and Use Committee at The Ohio State University and were in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

VEGF Antibody Treatments

Twenty-four adult (6-8 week old) female C57Bl/6 mice (Harlan, Indianapolis IN) were injected IP with 55μg VEGF antibody in 100μl PBS. Control mice received a corresponding amount of pre-immune serum IgG. Injections were given every three days for two weeks. Mice were euthanized at various time points and both ovaries were removed and fixed for tissue processing. Vaginal smears were obtained via vaginal lavage every day for 30 days, and evaluated for estrous cycle status [32].

For some experiments, VEGF antibody was administered under the bursa of the ovary as a previously described [28]. Pre-pubertal female C57Bl/6 mice were anesthetized, and each ovary was injected under the bursa with 1μl VEGF antibody or a corresponding amount of pre-immune rabbit IgG. Mice were euthanized at various time points (8h, 24h, 48h, 72h, 30d) and both ovaries were removed and prepared for histological analysis. We used adult mice for the systemic (longer-term) study to avoid the potential confounding effects of VEGFA neutralization on pubertal development. Prepubertal mice were utilized for the intrabursal experiments to provide a more homogeneous ovarian physiology and histological architecture.

Tissue Processing

Tissues were processed as previously described [28]. Briefly, ovaries were removed from fixative, dehydrated, and embedded in Paraplast before being sectioned (thickness 7μm) and stained with Lillies allochrome. To avoid counting a follicle more

than once, follicles were counted on every fifth section throughout the ovary (approximately 20-30 sections/ovary were counted) and only follicles containing an oocyte were counted. Primordial follicles were described as those having a small oocyte with a single layer of squamous granulosa cells. Primary follicles had an intact enlarged oocyte with a visible nucleus and a single layer of cuboidal granulosa cells. Secondary follicles had two or more layers of cuboidal granulosa cells. Secondary follicles were further classified as small if they contained < 4 layers of granulosa cells and large if they contained 4 or more layers of granulosa cells.

Western Blot Analysis

Western blots characterizing VEGF antibodies were performed as described in [28]. Briefly, 10ng of recombinant human (h) VEGFA, 50ng of recombinant mouse (m) VEGFA (R&D Systems Minneapolis, MN), and 5 μg pancreatic tumor lysate were electrophoresed on a 12% polyacrylamide gel and transferred to nitrocellulose. The pancreatic tumor was obtained from the Rip1-Tag2 transgenic mouse which spontaneously develops pancreatic tumors expressing VEGFA [33]. Nitrocellulose membranes were probed with commercially available rabbit polyclonal VEGFA antibody (A-20, Santa Cruz Biotechnologies), VEGF peptide antibody, or purified IgG from preimmune serum. Primary antibodies were diluted 1:500 and detected with goat anti rabbit IgG horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnologies).

Statistical Analysis

Results are depicted as the mean + SEM. Potential differences in estrous cycle lengths and follicle numbers were analyzed by analysis of variance followed by a least-

significant-difference test or by a paired Student's *t*-test when appropriate. A *P* value of less than 0.05 was considered to be significant for all analyses

Results:

Western blotting experiments confirmed that our VEGF peptide antibodies recognized both mouse and human recombinant VEGFA proteins (Figure 2.2).

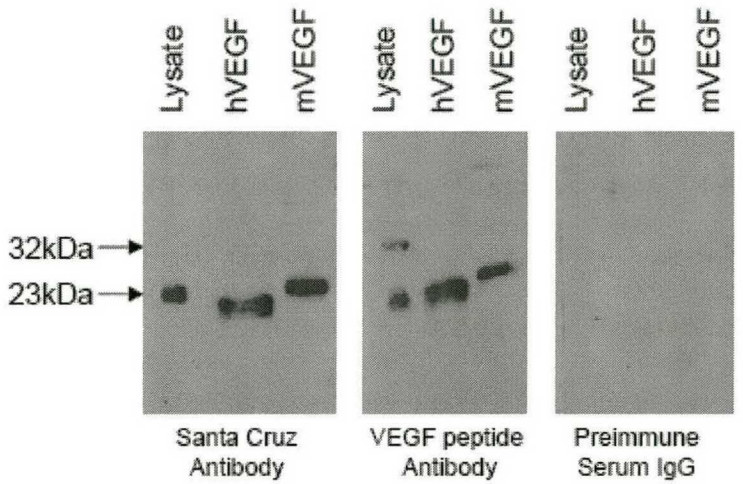


Figure 2.2 - Western Blot of pancreatic tumor cell lysate, human (h)VEGF and mouse(m)VEGF using commercially available (Santa Cruz) and VEGF peptide antibodies. Purified IgG from preimmune serum was included as a negative control.

It is unclear why the recombinant human VEGFA protein migrates slightly faster in our system than recombinant mouse VEGFA. In addition, these antibodies recognized a predominant 23 kDa band (presumably $VEGFA_{165}$) in pancreatic tumor lysate along with a slightly higher molecular weight protein (~ 32kDa) in this biological sample.

These data are similar to the results obtained with a commercially available antibody specific for VEGFA. Immunoblotting with purified IgG from preimmune serum revealed no specific binding to pure VEGFA proteins or tumor lysate.

Figure 2.3 reveals the effects of VEGF neutralization following intraperitoneal antibody administration on preantral follicles in adult mice.

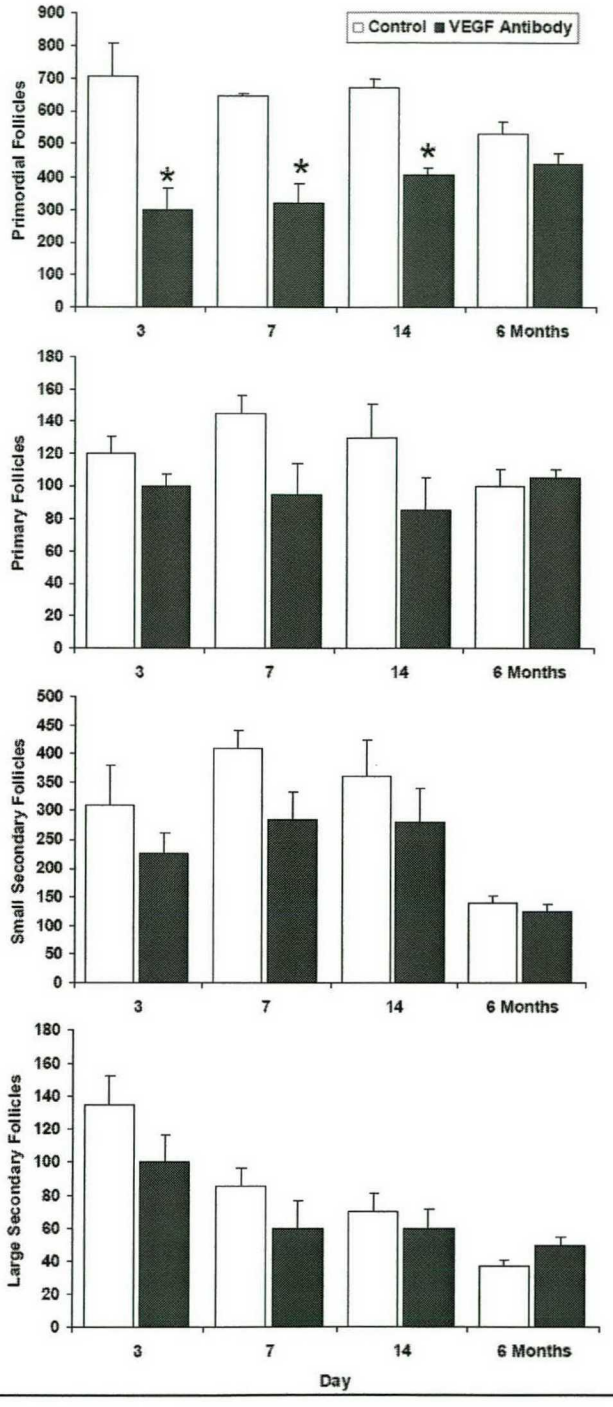


Figure 2.3 - Effect of systemic VEGF peptide antibody injection on preantral follicle survival. Adult mice (n=25) were injected twice/week for 2 weeks with 55 μg VEGF peptide antibody or purified IgG from preimmune serum and the ovaries were removed at various time points for quantitation of preantral follicle numbers as indicated in methods. Values are mean + SEM. * $P<0.05$ vs control, n=3 animals per time point except for 6 months in which 4 animals/group were utilized.

Within three days after a single VEGF peptide antibody injection, the number of primordial follicles was decreased by greater than 50%. Additional VEGF peptide antibody injections maintained but did not increase the destruction of primordial follicles seen after the first injection. In contrast, VEGFA antibodies had no effect on primary or secondary follicle numbers throughout the study. Six months after the initial injection (and 5.5 months after the last injection) primordial follicle numbers were not different from those observed in control mice. There were no significant changes in primordial follicles within either the control or VEGF antibody treatment groups in this experiment.

Systemic VEGF neutralization also disrupted estrous cyclicity in these mice. Within three days following the initial antibody injection, mice began to display persistent vaginal cornification; the interval between estrous vaginal smears was significantly reduced from 5.9 ± 0.2 days before treatment to 1.7 ± 0.2 days after VEGF antibody injection ($p < 0.05$, n=3/group).

It is possible that systemic administration of VEGF antibody could have effects outside the ovary which could impact primordial follicle growth and survival. Therefore we investigated whether direct ovarian administration of VEGF peptide antibodies would affect primordial follicle numbers. VEGF peptide antibody was injected under the bursa of one ovary and a corresponding amount of pre-immune IgG was injected under the bursa of the contralateral ovary. Thus, the effects of neutralization of "ovarian" VEGF could be determined with each animal serving as its own control. Direct ovarian administration of VEGF peptide antibody resulted in a dose-dependent decrease in the

number of primordial follicle numbers compared to control (pre-immune IgG) ovaries, with a maximal effect at 5-25 μg VEGF antibody (Figure 2.4, $p<0.05$, n=4-5/group).

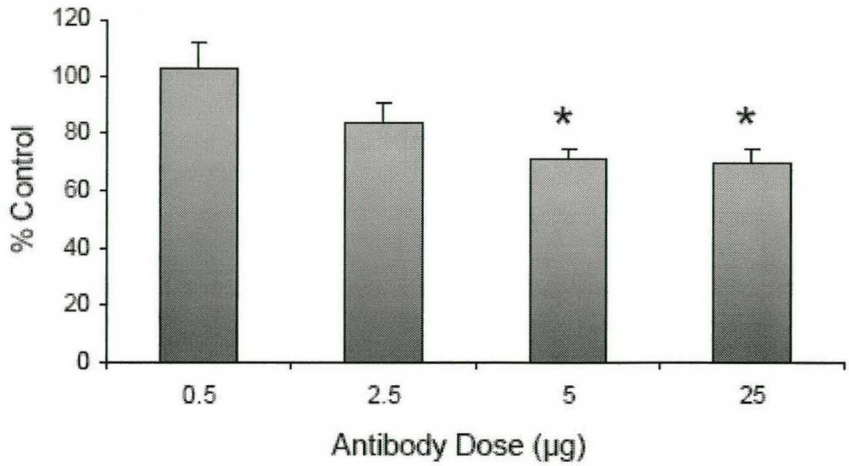


Figure 2.4 - Dose-response effect of intrabursal VEGF antibodies on primordial follicle survival. Prepubertal mice were injected with 0.5-25 μg VEGF antibodies under the bursa of one ovary. The contralateral ovary served as a control and was injected with purified IgG from pre-immune serum. The ovaries were removed 72 hours after injection and prepared for histology as described in methods. Values are Mean + SEM. *P<0.05 vs control, n=4 animals per dose except for the 2.5μg dose in which 3 animals/group were used.

We compared our VEGF peptide antibodies against a commercially available VEGF antibody preparation (AF-493-NA) from R&D Systems Inc (Figure 2.5). Both antibody preparations significantly depleted primordial follicles in the mouse ovary ($p<0.05$, n=4-6/group).

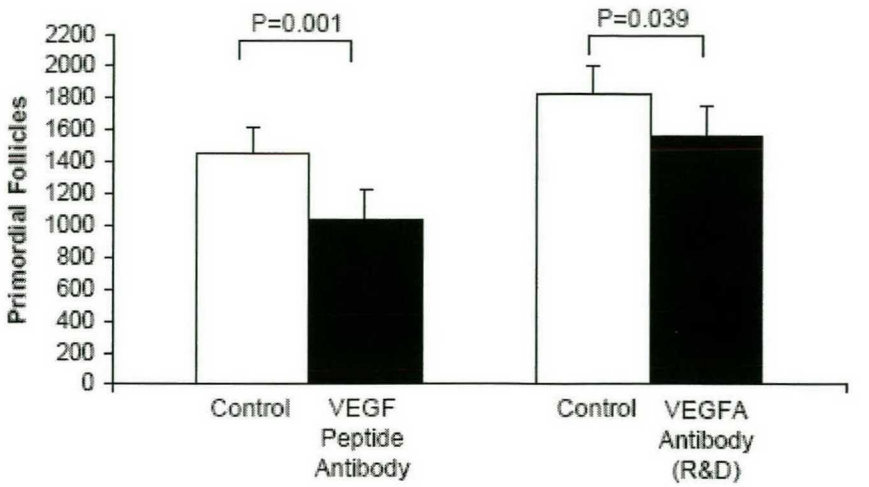


Figure 2.5 - Effect of intrabursal injection of VEGF peptide antibodies (left panel, n=4) and commercially available VEGFA antibodies (right panel, n=6). Prepubertal mice were injected with 5 μg anti-VEGF antibody in 1 μl saline under the bursa of one ovary. The contralateral ovary received 5 μg pre-immune IgG in 1 μl saline as control. Ovaries were removed 72 hours later and analyzed for primordial follicle numbers as described in methods. Values are mean + SEM.

Similar to the results obtained with systemic antibody administration, intrabursal administration of VEGF antibodies had no effect on primary or secondary follicle numbers (data not shown).

We also investigated the time course of primordial follicle destruction by VEGF antibody treatment (Figure 2.6). Intrabursal administration of VEGF peptide antibody resulted in a relatively rapid destruction of primordial follicles within 24hrs after injection. Follicle depletion was still evident at 3 days; however primordial follicle numbers were not different from control by 30 days after injection.

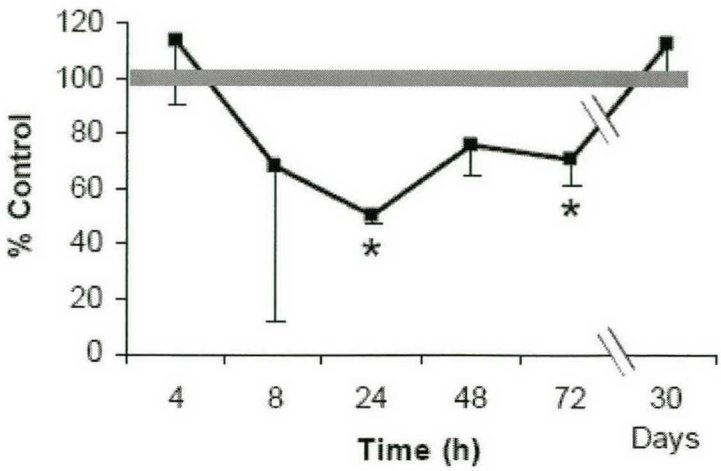


Figure 2.6 - Time course of primordial follicle destruction by VEGF peptide antibodies. Prepubertal mice were injected with 5 μg VEGF peptide antibody in 1 μl saline under the bursa of one ovary. The contralateral ovary received 5 μg pre-immune IgG in 1 μl saline as control. Ovaries were removed at specific time points and analyzed for primordial follicle numbers as described in methods. Values are the mean + SD of n=2 at 8h, n=3 at 24h, n=2 at 48h, n=4 at 72h and n=4 at 30 days. Note: the error bar for the 24h time point in contained within the symbol.

Since our antibody preparation binds VEGF directly it is not useful in determining which VEGF receptor (KDR or FLT1) might be important for primordial follicle growth and survival. Therefore we performed an additional experiment examining the effects of direct ovarian administration of commercially available antibodies that bind KDR (VEGFR2) or FLT1 (VEGFR1; R&D Systems Inc. Minneapolis, MN). Figure 2.7 reveals that treatment with antibodies that bind KDR but not FLT1 results in a statistically significant depletion of primordial follicles similar to that observed with anti-VEGF antibodies

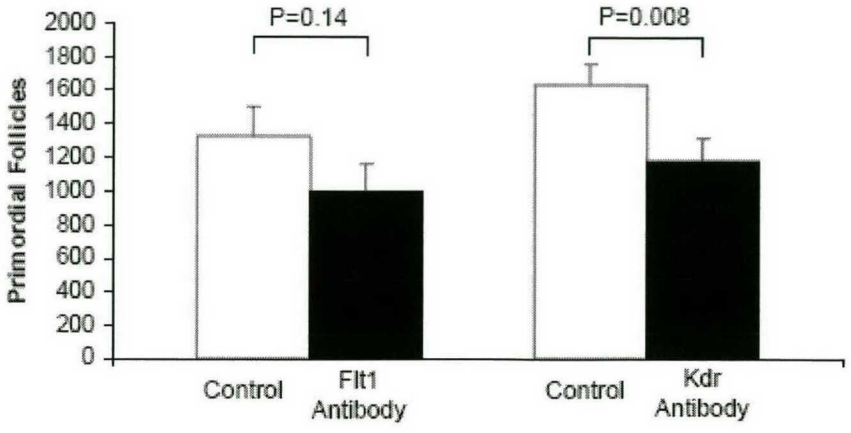


Figure 2.7 - Effect of antibodies against FLT1 and KDR on primordial follicle survival. Prepubertal mice (n=5/group) were injected with 5 μg anti-FLT1 or KDR antibody in 1 μl saline under the bursa of one ovary. The contralateral ovary received 5 μg pre-immune IgG in 1 μl saline as control. Ovaries were removed 72 hours later and analyzed for primordial follicle numbers as described in methods. Values are mean + SEM.

Discussion:

The data presented herein provide the first evidence that endogenous VEGF is important for primordial follicle survival in the rodent ovary. Administration of antibodies directed against an antigenic peptide region of the VEGF molecule result in a rapid and profound loss of primordial follicles in the ovary. Similarly, antibodies which specifically block the KDR receptor also result in primordial follicle depletion. Direct ovarian administration of antibodies also depletes primordial follicles indicating that neutralization of ovarian VEGF, as opposed to peripheral neutralization, is key to primordial follicle destruction.

The factors responsible for the formation, growth and survival of the primordial follicle pool are largely unknown. Although primordial follicle activation can occur in the absence of LH, FSH, or estrogen, it is clear that under normal physiological conditions,

primordial follicle growth occurs amid a rich milieu of endocrine, paracrine, and autocrine factors [reviewed in 34,35]. It is unknown if or how these factors might interact to influence the primordial follicle population. We and others have hypothesized that that the availability of an adequate vascular supply to provide endocrine and paracrine signals may play a key role in the regulation of early follicle growth [1, 36]. The data presented herein on the importance of endogenous VEGF for primordial follicle survival are consistent with this hypothesis.

Although the majority of research on the ovarian VEGF system has focused on the latter (antral) stages of follicle growth and corpus luteum function, previous data from our laboratory have suggested an important role for VEGFA in early follicle growth as well [28]. Administration of VEGFA directly to the rat ovary results in an increase in preantral follicle numbers in a dose and time dependent manner. In addition, estrogen up-regulates VEGFA expression in the rodent ovary similar to its effects on early follicle growth [28]. VEGFA has also been shown to be localized in the granulosa and thecal cells of pre-antral follicles [21] as well as the oocytes of primordial and primary follicles [24,25] . These, along with our present data provide important evidence that the ovarian VEGF system may be a critical component of early follicle physiology in the rodent ovary. Interestingly however, in the present study VEGF neutralization only depleted primordial follicles; there were no differences in primary or secondary follicle numbers either after systemic or intrabursal antibody administration. This may reflect the especially labile nature of the primordial follicle pool; this population is exquisitely sensitive to radiation- or chemotherapy-induced follicle destruction. Alternatively higher

doses or more prolonged exposure to neutralizing antibodies might be required for effects on primary and secondary follicles to be manifest.

In addition to depleting primordial follicles in the ovary, systemic administration of VEGF peptide antibodies also disrupts estrous cyclicity in the mouse. Shortly after VEGF antibody administration, mice displayed persistent vaginal cornification, similar to that observed in aging mice just prior to ovarian senescence [37]. Neutralization of endogenous VEGF has been demonstrated to inhibit ovulation [38] and it is possible that the presence of cornified cells in the vaginal smears is due to continued estrogen production by unovulated antral follicles persisting in the ovary for a few days after antibody administration. Indeed, 4 of 6 VEGF antibody treated mice had not ovulated by seven days after systemic antibody administration whereas 6 of 6 control mice had ovulated. Alternatively, systemic administration of VEGF antibodies might also alter uterine and vaginal function which might manifest in altered vaginal cytology. Other investigators have demonstrated effects of VEGF neutralization on uterine function in rodents [39,40].

The depletion of primordial follicles by VEGF neutralization is rapid, however over time this depletion of the follicular pool appears to abate. Primordial follicle numbers were decreased within 24 hours following intrabursal administration of VEGF peptide antibodies and remained below control levels for at least 3 days. Within thirty days the effect of VEGF neutralization was no longer apparent and primordial follicle numbers were not different from control. It is important to note however that the number of primordial follicles did not increase over time, either after systemic or intrabursal antibody administration.

It is unknown whether primordial follicle depletion following VEGF neutralization is due to a direct effect of VEGF on follicular units or is mediated via changes in the vascular compartment of the ovary. Certainly neutralization of endogenous VEGF would likely affect angiogenesis and vascular permeability as these are two of the primary effects of VEGFA in most tissues studied to date. It is perhaps less likely that changes in new vessel formation are involved since the effects of VEGF antibody treatment are observed within 24 hours, although neutralization of VEGF could result in endothelial cell apoptosis with resultant vascular compromise. We did not quantitate potential changes in angiogenesis in the present study. Changes in vascular permeability and, as such, delivery of important endocrine and paracrine modulators of early follicle growth may be a more important component of the effects observed.

Alternatively, increasing evidence suggests that VEGFA may have direct effects on non-vascular elements in a variety of tissues, including the ovary although many of these studies are somewhat limited. VEGFA and its receptors have tentatively been identified in granulosa cells, thecal cells, oocytes, and zona pellucida [21, 24-26, 41]. VEGFA has also been shown to directly stimulate granulosa cell function in vitro [22]. As such, it is possible that the effects of VEGFA on the preantral follicle might be mediated by direct actions on follicular components rather than indirectly via alterations in vascular physiology.

Our data indicate that inhibition of VEGF binding to the KDR but not FLT1 receptor is important for primordial follicle survival in the mouse ovary. Ovarian administration of antibodies to KDR resulted in significant depletion of primordial follicle numbers, whereas anti-FLT1 antibodies did not. This is consistent with previous

data in the literature indicating that the KDR receptor is the primary mediator of VEGF action and that the FLT1 receptor might serve as a decoy for VEGF [3]. Indeed, Zimmerman et al, [42] demonstrated that antibodies against KDR delayed follicular selection and development in the Rhesus monkey. However, it should be noted that we only utilized one dose of VEGF receptor antibodies and it is possible that higher doses, longer treatment, or different FLT1 antibody preparations might also result in primordial follicle depletion similar to our results with KDR antibodies.

A recent report by Johnson et al [43] proposed that the follicular pool is a dynamic rather than stagnant population. These authors suggest that a constant vascular influx of bone marrow-derived stem cells is required to maintain the primordial follicle population. VEGF neutralization might interfere with the delivery of stem cells to the ovary or disrupt new primordial follicle formation. Kezele et al [27] has shown that VEGF expression is up regulated during primordial follicle development. While the importance of bone marrow stem cell migration remains controversial, its potential role in the loss of primordial follicles following neutralization of endogenous VEGF warrants further investigation.

We chose to develop our own antibodies against VEGF in order to have an ample supply to conduct long term in vivo studies, and to investigate potential applications of active immunotherapy for other (cancer) studies. In order to develop a pan-specific antibody capable of neutralizing the majority of VEGF isoforms, we chose a conserved peptide sequence found in the majority of VEGF species (VEGF A – G). Coupling antigenic regions of the VEGF molecule with a promiscuous T-cell epitope is a useful strategy for development of novel antigens for generation of polyclonal antibodies, and

for exploring active immunization for a variety of therapies. This approach resulted in specific VEGF antisera, comparable to commercially available sources. Although we have not determined the affinity of this particular antibody, other antibodies developed using this technology display affinities similar to traditional polyclonal antibodies generated against intact proteins [31, 44-45]. Our VEGF peptide antibody does detect an additional higher molecular weight protein in the pancreatic tumor cell lysate and the identity of this protein is unknown at present although its molecular weight is roughly consistent with that of $VEGFA_{189}$. It is noteworthy that our protein-A purified antibody preparation is much less pure than the affinity purified antibody preparation we used for comparison from Santa Cruz Biotechnologies, yet the specificities are remarkably similar. Indeed, the data obtained with commercially available antibodies to VEGFA and KDR were nearly identical to those obtained with our VEGF peptide antibodies suggesting that the effects on primordial follicles we observed were due to specific inhibition of VEGF interaction with its receptor in the ovary, and not due to non-specific toxic effects of the antibody preparation. Moreover the similar response to acute versus "long-term" antibody treatment argues against non-specific toxic effects on the ovary. Finally, the lack of effect on primary and secondary follicles suggests that specific neutralization of VEGF is responsible for primordial follicle destruction in the ovary. In summary, these studies provide compelling evidence that endogenous VEGF is essential for primordial follicle survival in the rodent. Disruption of VEGF interaction with its receptors results in a rapid and pronounced loss of primordial but not other preantral follicles in the ovary.

Along with previous studies supporting the role of VEGF in early follicle growth we conclude that the ovarian VEGF system is an integral component of early follicle growth and survival in the rodent ovary.

REFERENCES

1. Danforth DR. Endocrine and paracrine control of oocyte development. Am J Obstet Gynecol 1995; 172:747-752.

2. Hazzard TM, Stouffer RL. Angiogenesis in ovarian follicular and luteal development. Baillieres Best Pract. Res. Clin. Obstet. Gynaecol. 2000; 14:883–900.

3. Fraser HM, Wulff C. Angiogenesis in the primate ovary, Reprod. Fertil. Dev. 2001; 13:557–566.

4. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat. Med. 2003; 9:669–676.

5. Mattioli M, Barboni B, Turriani M, Galeati G, Zannoni A, Castellani G, Berardinelli P, Scapolo PA. Follicle activation involves vascular endothelial growth factor production and increased blood vessel extension. Biol Reprod 2001; 65:1014-1019.

6. Lee A, Christenson LK, Patton PE, Burry KA, Stouffer RL. Vascular endothelial growth factor production by human luteinized granulosa cells in vitro. Hum Reprod 1997; 12:2756-2761.

7. Kamat BR, Brown LF, Manseau EJ, Senger DR, Dvorak HF. Expression of vascular permeability factor/vascular endothelial growth factor by human granulosa and theca lutein cells. Role in corpus luteum development. Am J Pathol 1995; 146:157-165.

8. Barboni B, Turriani M, Galeati G, Spinaci M, Bacci ML, Forni M, Mattioli M. Vascular endothelial growth factor production in growing pig antral follicles. Biol Reprod 2000; 63:858-864.

9. Friedman CI, Danforth DR, Herbosa-Encarnacion C, Arbogast L, Alak BM, Seifer DB. Follicular fluid vascular endothelial growth factor concentrations are elevated in women of advanced reproductive age undergoing ovulation induction. Fertil Steril 1997; 68:607-612.

10. Pietrowski D, Keck C. Differential regulation of ANG2 and VEGF-A in human granulosa lutein cells by choriogonadotropin. Exp Clin Endocrinol Diabetes. 2004; 112:208-214.

11. Duffy DM, Stouffer RL. Luteinizing hormone acts directly at granulosa cells to stimulate periovulatory processes: modulation of luteinizing hormone effects by prostaglandins. Endocrine 2003; 22:249-256.

12. Koos RD. Increased expression of vascular endothelial growth/permeability factor in the rat ovary following an ovulatory gonadotropin stimulus: potential roles in follicle rupture. Biol Reprod 1995; 52:1426-1435.

13. Hazzard TM, Molskness TA, Chaffin CL, Stouffer RL. Vascular endothelial growth factor (VEGF) and angiopoietin regulation by gonadotrophin and steroids in macaque granulosa cells during the peri-ovulatory interval. Mol Hum Reprod 1999; 5:1115-1121.

14. Dickson SE, Bicknell R, Fraser HM. Mid-luteal angiogenesis and function in the primate is dependent on vascular endothelial growth factor. J Endocrinol 2001; 168:409-416.

15. Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG. Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest 1998; 78:1385-1394.

16. Zimmermann RC, Xiao E, Husami N, Sauer MV, Lobo R, Kitajewski J, Ferin M. Short-term administration of antivascular endothelial growth factor antibody in the late follicular phase delays follicular development in the rhesus monkey. J Clin Endocrinol Metab 2001; 86:768-772.

17. Wulff C, Wiegand SJ, Saunders PT, Scobie GA, Fraser HM. Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc (vascular endothelial growth factor Trap(A40)). Endocrinology 2001; 142:3244-3254.

18. Wulff C, Wilson H, Rudge JS, Wiegand SJ, Lunn SF, Fraser HM. Luteal angiogenesis: prevention and intervention by treatment with vascular endothelial growth factor trap (A40). J Clin Endocrinol Metab 2001; 86:3377-3386.

19. Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, Chisholm V, Hillan KJ, Schwall RH. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat Med 1998; 4:336-340.

20. Fraser HM, Dickson SE, Lunn SF, Wulff C, Morris KD, Carroll VA, Bicknell R. Suppression of luteal angiogenesis in the primate after neutralization of vascular endothelial growth factor. Endocrinology 2000; 141:995-1000.

21. Yamamoto S, Konishi I, Tsuruta Y, Nanbu K, Mandai M, Kuroda H, Matsushita K, Hamid AA, Yura Y, Mori T. Expression of vascular endothelial growth factor (VEGF) during folliculogenesis and corpus luteum formation in the human ovary. Gynecol Endocrinol 1997; 11:371-381.

22. Greenway J, Connor K, Pedersen HG, Coomber BL, LaMarre J, Petrik J. Vascular Endothelial Growth Factor and Its Receptor, Flk-1/KDR, Are Cytoprotective in the Extravascular Compartment of the Ovarian Follicle. Endocrinology 2004; 145:2896-2905.

23. Taylor PD, Hillier SG, Fraser HM. Effects of GnRH antagonist treatment on follicular development and angiogenesis in the primate ovary. J Endocrinol. 2004; 183:1-17.

24. Otani N, Minami S, Yamoto M, Shikone T, Otani H, Nishiyama R, Otani T, Nakano R. The vascular endothelial growth factor/fms-like tyrosine kinase system in human ovary during the menstrual cycle and early pregnancy. J Clin Endocrinol Metab. 1999; 84:3845-3851.

25. Harata T, Ando H, Iwase A, Nagasaka T, Mizutani S, Kikkawa F. Localization of angiotensin II, the AT1 receptor, angiotensin-converting enzyme, aminopeptidase A, adipocyte-derived leucine aminopeptidase, and vascular endothelial growth factor in the human ovary throughout the menstrual cycle. Fertil Steril. 2006; 86:433-439.

26. Celik-Ozenci C, Akkoyunlu G, Kayisli UA, Arici A, Demir R. Localization of vascular endothelial growth factor in the zona pellucida of developing ovarian follicles in the rat: a possible role in destiny of follicles. Histochem Cell Biol 2003; 120:383-390.

27. Kezele PR, Ague JM, Nilsson E, Skinner MK. Alterations in the ovarian transcriptome during primordial follicle assembly and development. Biol Reprod. 2005; 72(1):241-255.

28. Danforth DR, Arbogast LK, Ghosh S, Dickerman A, Rofagha R, Friedman CI. Vascular endothelial growth factor stimulates preantral follicle growth in the rat ovary. Biol Reprod 2003; 68:1736-1741.

29. Dakappagari NK, Pyles J, Parihar R, Carson WE, Young DC, Kaumaya PT. A chimeric multi-human epidermal growth factor receptor-2 B cell epitope peptide vaccine mediates superior antitumor responses. J Immunol. 2003; 170:4242-4253.

30. Keyt BA, Berleau LT, Nguyen HV, Chen H, Heinsohn H, Vandlen R, Ferrara N. The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. J Biol Chem. 1996; 271:7788-7795.

31. Frangione-Beebe M, Albrecht B, Dakappagari N, Rose RT, Brooks CL, Schwendeman SP, Lairmore MD, Kaumaya PT. Enhanced immunogenicity of a conformational epitope of human T-lymphotropic virus type 1 using a novel chimeric peptide. Vaccine 2000; 19:1068-1081.

32. Nelson JF, Felicio LS, Randall PK, Sims C, Finch CE. A longitudinal study of estrous cyclicity in aging C57BL/6J mice: I. Cycle frequency, length and vaginal cytology. Biol Reprod 1982; 27:327-339.

33. Christofori G, Naik P, Hanahan D. Vascular Endothelial Growth Factor and Its Receptors, flt-1 and flk-1, are Expressed in Normal Pancreatic Islets and throughout Islet Cell Tumorigenesis. Molecular Endocrinology 1995; 9:1760-1770.

34. McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. Endocr Rev. 2000; 21:200-214.

35. Skinner MK. Regulation of primordial follicle assembly and development.Hum Reprod Update. 2005;11:461-471.

36. Hull ML, Charnock-Jones DS, Chan CL, Bruner-Tran KL, Osteen KG, Tom BD, Fan TP, Smith SK. Antiangiogenic agents are effective inhibitors of endometriosis. J Clin Endocrinol Metab 2003; 88:2889-2899.

37. vom Saal FS, Finch CE, Nelson JF Natural history and mechanisms of reproductive aging in humans, laboratory rodents, and other selected vertebrates. In: E. Knobil, JD Neill (eds), The Physiology of Reproduction, Vol 2, 2nd ed. New York, Raven Press 1994:1213-1314.

38. Xu F, Hazzard T, Evans A, Charnock-Jones S, Smith S, Stouffer R. Intraovarian actions of anti-angiogenic agents disrupt periovulatory events during the menstrual cycle in monkeys. Contraception 2005; 71:239-248

39. Heryanto B, Lipson KE, Rogers PA. Effect of angiogenesis inhibitors on estrogen-mediated endometrial endothelial cell proliferation in the ovariectomized mouse. Reproduction 2003; 125:337-346.

40. Rockwell LC, Pillai S, Olson CE, Koos RD. Inhibition of vascular endothelial growth factor/vascular permeability factor action blocks estrogen-induced uterine edema and implantation in rodents. Biol Reprod. 2002; 67:1804-1810.

41. Einspanier R, Schonfelder M, Muller K, Stojkovic M, Kosmann M, Wolf E, Schams D. Expression of the vascular endothelial growth factor and its receptors and effects of VEGF during in vitro maturation of bovine cumulus-oocyte complexes (COC). Mol Reprod Dev 2002; 62:29-36.

42. Zimmermann RC, Xiao E, Bohlen P, Ferin M. Administration of antivascular endothelial growth factor receptor 2 antibody in the early follicular phase delays follicular selection and development in the rhesus monkey. Endocrinology 2002 143:2496-2502.

43. Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT, Tilly JL. Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. Cell. 2005; 122(2):303-315.

44. Lairmore M D, DiGeorge AM, Conrad SF, Trevino AV, Lal RB, Kaumaya PT Human T-lymphotropic virus type 1 peptides in chimeric and multivalent constructs with promiscuous T-cell epitopes enhance immunogenicity and overcome genetic restriction J. Virol. 1995; 69:6077–6089.

45. Sundaram R, Lynch MP, Rawale SV, Sun Y, Kazanji M, Kaumaya PT De novo design of peptide immunogens that mimic the coiled coil region of human T-cell leukemia virus type-1 glycoprotein 21 transmembrane subunit for induction of native protein reactive neutralizing antibodies. J Biol Chem. 2004; 279:24141-51.

CHAPTER 3

USING LASER CAPTURE MICRODISSECTION AND REAL-TIME PCR TO EVALUATE VEGF mRNA EXPRESSION IN THE OVARY

Introduction:

Evaluating the levels of VEGF in specific follicle populations in the ovary has proven to be difficult. The current literature has conflicting results on the VEGF levels of primordial, primary, and small secondary follicles. While it is the general consensus that these pre-antral populations do not express VEGF [1], there are data that support the viewpoint that these populations do express VEGF [2, 3]. One of the most common methods for evaluating VEGF expression is immunohistochemistry (IHC). IHC is useful in detecting proteins but can by highly variable across laboratories. IHC can also has limitations with sensitivity. These caveats are the rationale for our group to find a more reliable way to evaluate VEGF levels in the ovary. To clarify the cellular sources of VEGF, we investigated objective quantification of mRNA expression instead of subjective assessment of the presence of VEGF protein. Real-time PCR allowed us to quantify mRNA levels using flouresence resonance energy transfer (FRET) probes. The only hurdle to overcome would be isolating a pure cell population for evaluation. Laser Capture Microdissection is a recently developed technique that allows the isolation of specific cell populations for use in other down stream applications such as real time-PCR.

Laser Capture Microdissection was developed in conjunction with the National Institutes of Health in the late 1990's. With the advancement of molecular analysis, specifically the evaluation of the mRNA, there was a call for more sophisticated ways of providing specific cellular samples. Molecular analysis can be limited by the quality of the sample to be tested when a specific, perhaps diseased, specimen is encased within healthy tissue. Several methods have attempted to isolate more specific populations of tissue such as, gross dissection of frozen blocks [4, 5], microdissection with manual tools

[6-9], touch preparation of frozen sections [10], and irradiation to get rid of unwanted genetic material [11]. Laser Capture Microdissection surpasses all these methods. LCM is done on a microscope stage, visualizing the tissue to be isolated through the microscope itself. LCM employs a transparent thermoplastic film that is stationed over the surface of a processed, sectioned specimen on a slide. A carbon dioxide laser is then pulsed through the thermoplastic film, adhering the film to the cells of interest. When the film is lifted the cells of interest are removed, while the extraneous parts of the specimen are left behind on the slide. The specimen on the film can then be processed for downstream applications. LCM can be used for both frozen and paraffin embedded specimens. A variety of staining protocols are also compatible with laser capture. The transparent films that adhere to the specimen are sterile and disposable, decreasing the likelihood of contamination of downstream applications. Currently there are several commercially available kits for use in laser capture with real time-PCR downstream. These kits contain a lysis buffer and columns for isolation and purification of genetic material. The objective of our work, done with laser capture microdissection, was to determine the expression of VEGF in specific cell populations of the ovary.

Materials and Methods:

Sample Preparation:

Due to the large amount of archived tissue available in our lab, it was important to evaluate the suitability of using frozen and fixed tissues for LCM followed by real-time PCR. Sheep were selected as a model because an abundant supply of tissue was available. Also, identifying histological structures, especially primordial follicles, is easier in sheep versus rodent ovaries, due to the larger size of the sheep ovary. Ovaries

from both lambs and ewes were either dehydrated in alcohol and xylene and then embedded in paraffin (i.e., fixed) for sectioning or embedded in OCT compound and slowly frozen in a beaker of isopentane submerged in liquid nitrogen (i.e., frozen). Both fixed and frozen samples were sectioned and mounted on non-charged slides for laser capture microdissection and RNA analysis.

Laser Capture Microdissection:

Slides were visualized on the Arcturus PixCell II. Adjustments were made for laser beam focus, power, and pulse. When the software was available pictures were taken before, after, and of the cap for each specimen. Specific cell populations were captured on Arcturus CapSure© Caps. These caps were placed directly onto a microcentrifuge tube for RNA isolation. Several experiments were conducted to validate the system and determine the number of cells needed to get reliable real-time PCR results.

RNA Isolation:

RNA was isolated through column purification with the Qiagen RNeasy© Mini kit and initial samples were analyzed for RNA integrity. The RNA underwent reverse transcription and was subsequently evaluated using real-time PCR.

RNA Integrity Testing:

RNA Integrity was evaluated at many steps in the process. Initial testing included scraping several sections into lysis buffer and isolating the RNA. Sample groups included, frozen, fixed, frozen stained, fixed stained, and frozen sections with prolonged exposure to room temperature. The integrity of the RNA in the samples was analyzed with the Agilent Bioanalyzer 2100. This machine employs capillary electrophoresis to

evaluate the 18S/28S ratio. As RNA degrades the 18S and 28S peaks decrease and additional, smaller, peaks begin to appear [12].

Selection of Normalizing Gene:

Due to the variability in the number of cells captured during LCM per sample it is necessary to have a reference with which to normalize the gene of interest. Normalization eliminates confounding variables when looking at the expression of mRNA. 18S is a popular gene for normalization because it is both abundant and consistent across cell types.

Plasmid Preparation for Standard Curve in RT-PCR.

Plasmids for 18S and VEGF were generated using Invitrogen's TOPO TA cloning system. After incorporating our insert (VEGF 127-144), colonies were selected based on growth. Plasmids containing 18S inserts were donated by the Callugri lab and were incorporated into E. coli. Colonies with plasmids that contained VEGF or 18S inserts were grown up in LB broth and the plasmids were purified using Qiagen's Midi Kit©. Correct insertion was verified through sequencing. These plasmids were used to generate standard curves for use in quantifying the initial concentration of VEGF mRNA and 18S rRNA in LCM samples. The plasmids were again validated on initial samples by visualizing the final products on a gel.

Primer Selection:

VEGF primers and probe were selected to bind to all isoforms of VEGF in the VEGFA family. When working with a small sample size, it is advantageous to include as many isoforms of VEGF as possible. All isoforms of VEGFA share the first and second exon. Our VEGF primers and probe were designed to target the junction between exons 1

and 2. Since DNA contains an intron between these two exons, this eliminates the possibility of picking up genomic DNA contamination in our purified RNA samples. The VEGF primer and probe sequences are as follows: Forward – GCCTTGCTGCTCTACCTTCA; Reverse – GGGTTTCTGCCCTCCTTCTG; Probe – CATGCCAAGTGGTCCC. The forward VEGF primer targets exon 1, the reverse primer targets exon 2 and the probe spans the junction between these two exons. 18S primers were designed by Applied Biosciences and sold in a kit containing the forward and reverse primers as well as the probe. Sequences of these primers and probe are regarded as proprietary information.

Real-Time PCR:

FRET probes were employed for two reasons; increased sensitivity when compared to available Sybr-Green options at the time, and also ability to multiplex. FRET probes were used for both VEGF and 18S. Standard curves were generated using the plasmid preparations described above. The range of detection was determined in several experiments investigating the maximum dilution of plasmid that still produced a reliable standard in triplicate. After determining the minimum range of detection for the standard curve, the sample size was determined. Several experiments were conducted to determine the number of cells per sample needed for that sample to appear on a reliable part of our standard curve.

Validation of Real-Time PCR

Following the beginning runs of real-time PCR, amplified products were run on a TBE gel and checked for accurate weights. A no-RT control was run to ensure that there

was no contamination with genomic DNA. Both amplicons (18S and VEGF) were of a similar size <100bp.

Results:

Comparisons were made between fixed and frozen tissues. There is evidence in the literature that supports frozen samples have higher RNA yields than fixed samples [13]. Several staining procedures were evaluated including traditional H&E staining, modified Lillies Allochrome staining, and a commercial stain marketed for laser capture microdissection, Histogene©. All staining protocols were evaluated for their ease in identifying ovarian cell types including primordial follicles and their effect on mRNA integrity. Although the histogene provided the fewest amount of steps and the quickest time from thawing to capturing, it did not allow for a clear identification of follicular structures. The modified H&E staining was problematic when identifying specific ovarian structures. The granulosa and thecal cells of large antral follicles were easily identified with bright stain, but the small primordial follicles were obscured in the stroma. The modified Lillies allochrome staining provided the clearest ovarian histology and was used throughout subsequent experiments.

RNA integrity was measured on both fixed and frozen specimens under several conditions. Figure 3.1 represents data from the Agilent Bioanalyzer 2100 indicating that frozen tissue maintains clear 28S and 18S peaks (A). Fixed tissue looses its clear 28S and 18S peaks due to degradation and this appears on the histogram as the combination of many smaller peaks (B). After determining that sample preparation would only include freezing the following conditions were examined; sheep frozen (C), frozen and stained(D), frozen, stained, and left at room temperature (25°C) for 10hrs (E),

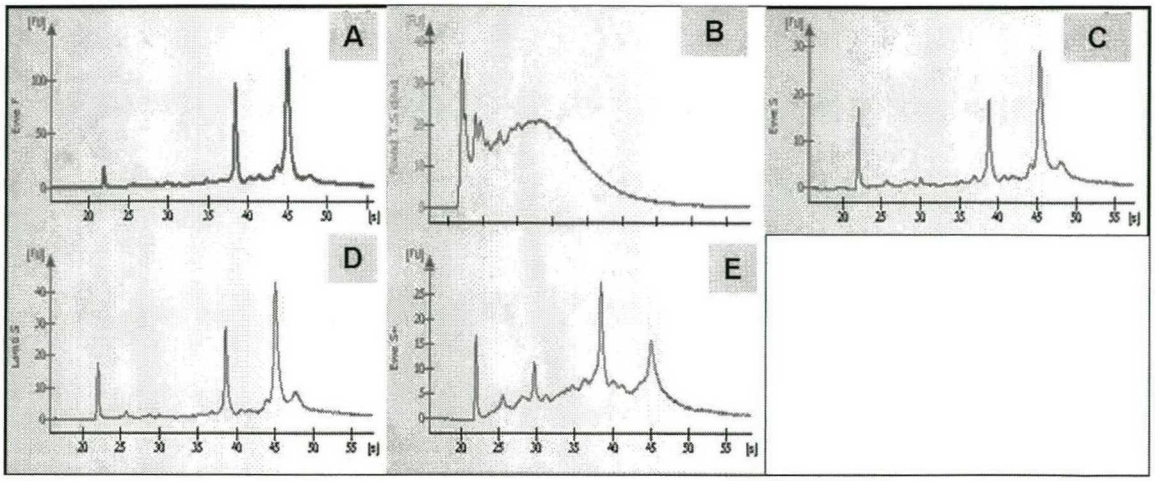


Figure 3.1 – Agilent Bioanalyzer 2100 data. Panel A is RNA from frozen sheep tissue. Panel B is RNA from fixed sheep tissue. Panel C is RNA from frozen, unstained, sheep tissue. Panel D is RNA from frozen, stained, sheep tissue. Panel E is RNA from frozen, stained, sheep tissue left at room temperature (25°C) for 10hrs. The staining tested was a modified Lillie's Allochrome.

Staining with the modified Lillie's Allochrome did not affect the RNA integrity of the sheep samples. Extended exposure to ambient temperatures caused moderate degradation of the RNA evident by the decrease in the 28S peak as well as the rise in baseline between the 18S and 28S peaks. Degradation was also characterized by an increase in the baseline to the left of the 18S peak.

Figure 3.2 is a representation of the process of laser capture microdissection. A large antral follicle was identified under the microscope (A). The thecal cells were removed while leaving the granulosa cells behind (B). Finally the granulosa cells were removed from the same follicle (C). Each of these specific cell populations was isolated on a separate cap.

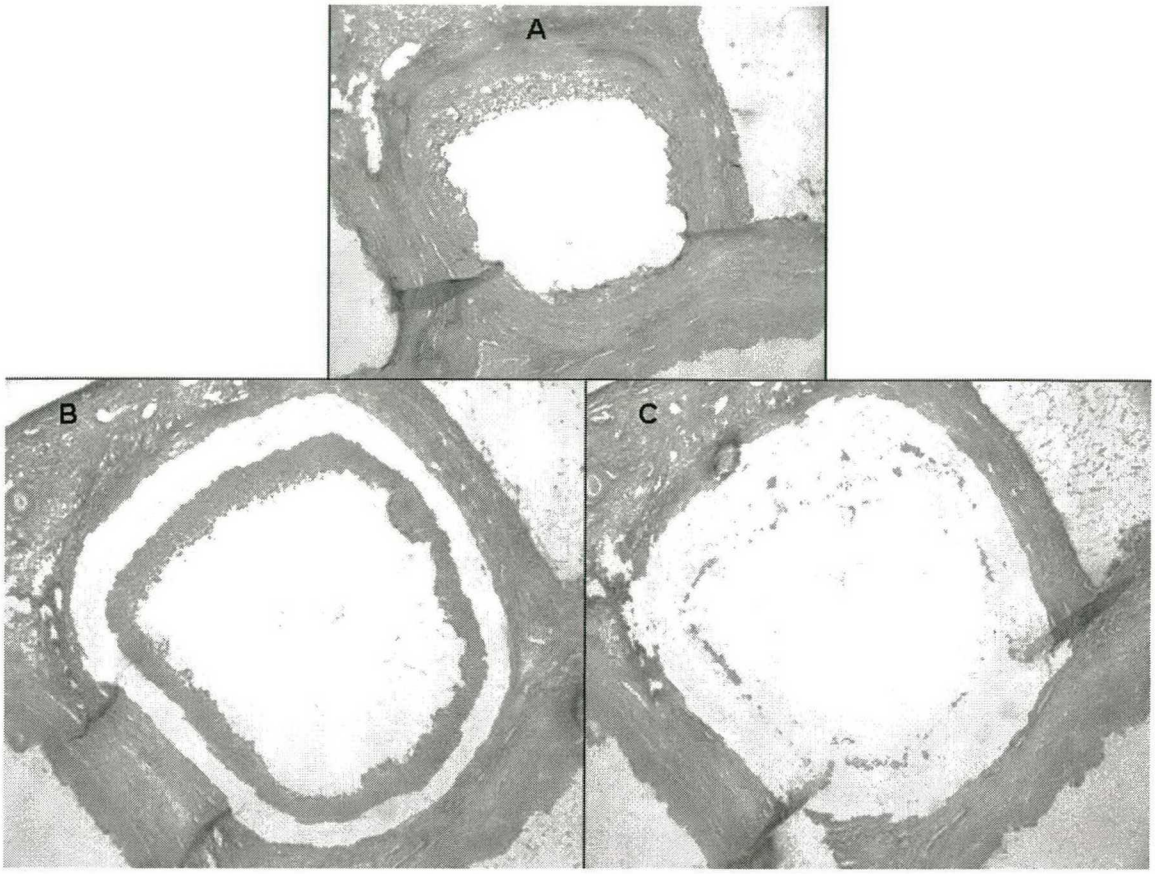
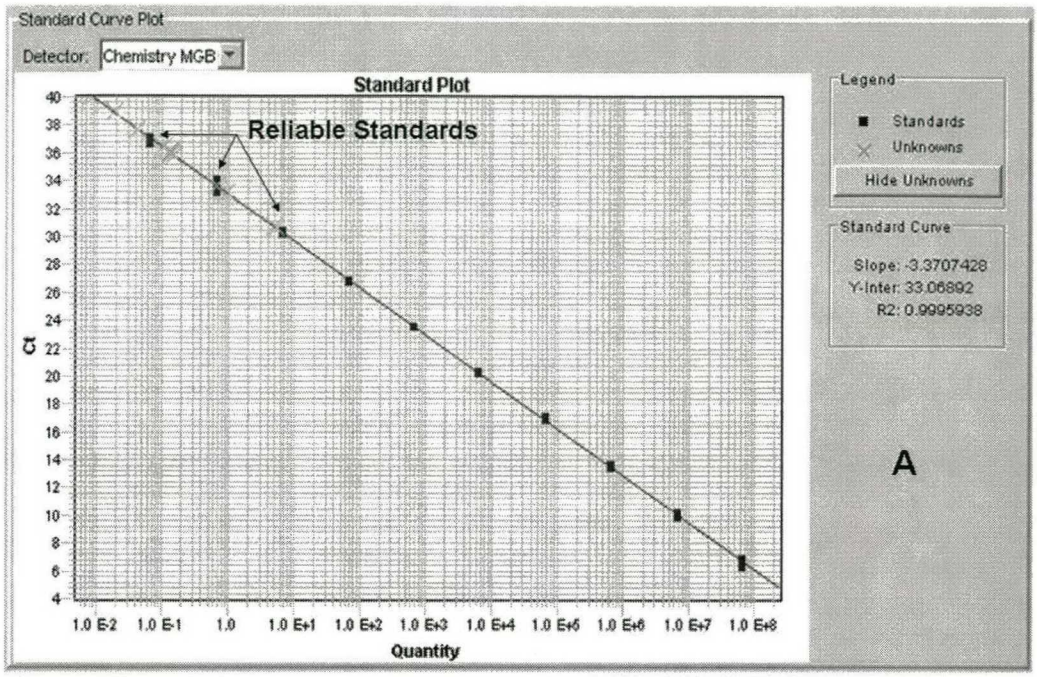


Figure 3.2 – Photographs of the process of Laser Capture Microdissection. Panel A is an intact large antral follicle. Panel B is the same follicle with the thecal cells removed. Panel C, again the same follicle with both the thecal and granulosa cells removed.

After isolating RNA from the caps containing specific cell populations the genetic material is put into the downstream application real-time PCR. In order to quantify RNA from LCM samples standard curves had to be constructed and the minimum limits of detection tested. Figures 3.3 and 3.4 illustrate the use of 18S and VEGF plasmids to establish reliable standard curves as well as the limits of our standard curve detection.



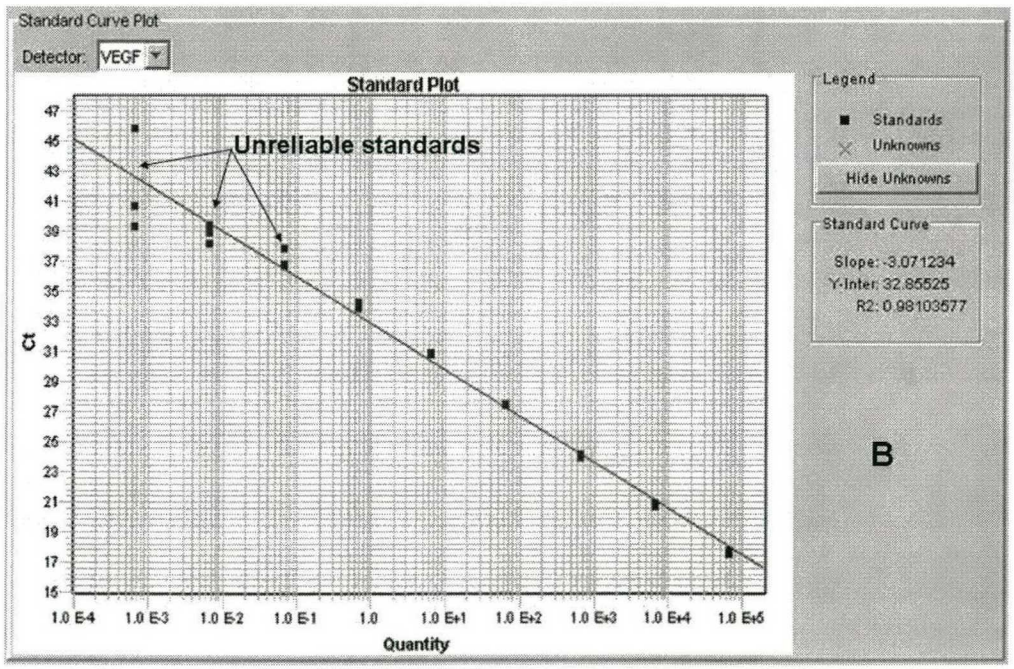


Figure 3.3 – Limits of VEGF standard curve. Panel A illustrates a minimum reliable level of detection using our VEGF plasmid to set up a standard curve. Panel B represents the same VEGF plasmid standards diluted out until the triplicate standard becomes unreliable.

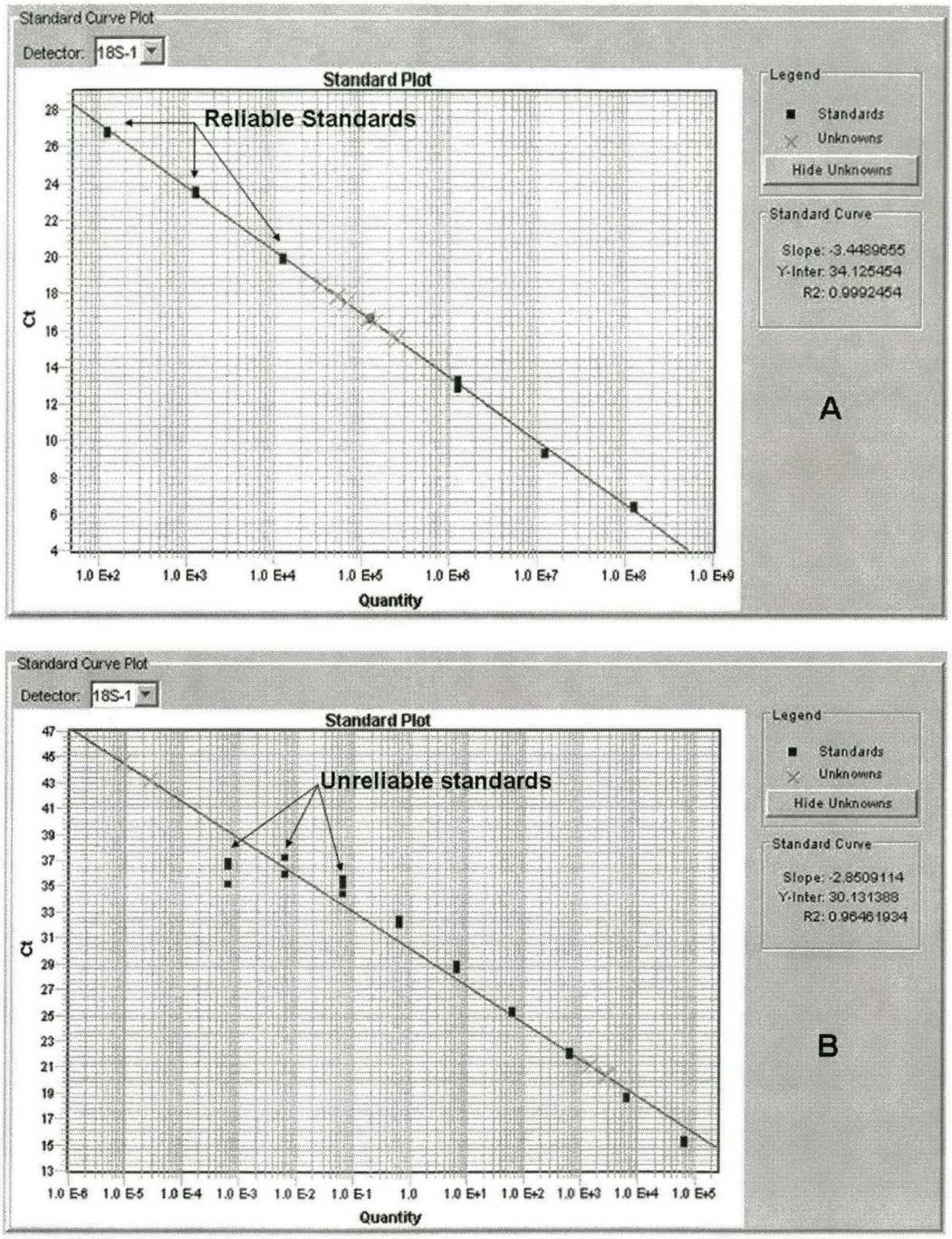


Figure 3.4 – Limits of 18S standard curve. Panel A illustrates a minimum reliable level of detection using our 18S plasmid to set up a standard curve. Panel B represents the same 18S plasmid standards diluted out until the triplicate standard becomes unreliable.

The minimum reliable standard we used to quantify the amount of VEGF in each LCM sample is 100 attograms for both 18S and VEGF. Any dilution beyond 100 attograms becomes unreliable as evident by the splaying of the triplicate standard points. This was also true of both 18S and VEGF. Figure 3.5 is a comparison between the different amounts of VEGF mRNA we captured or had available, and where they appeared in relation to the reliable points on our standard curve. RNA from 30-500um sections appeared on an unreliable section of the standard curve. When RNA was isolated from at least 4 consecutive sections of granulosa or thecal cells from large antral follicles the sample appeared at a reliable point on our standard curve. As a positive control we used RNA isolated from a 7mg piece of C.L. tissue. All 18S samples appeared on a reliable part of our standard curve (data not shown).

Finally a small collection of data served as both a validation of our system as well as a preliminary look at VEGF mRNA in the ovary. Table 3.1 provides the results of an experiment in which the VEGF mRNA was assessed in the granulosa and thecal cells of large antral follicles. All values have been normalized to 18S. Preliminary results found ranges of VEGF mRNA expression in specific ovarian compartments consistent with the literature [14].

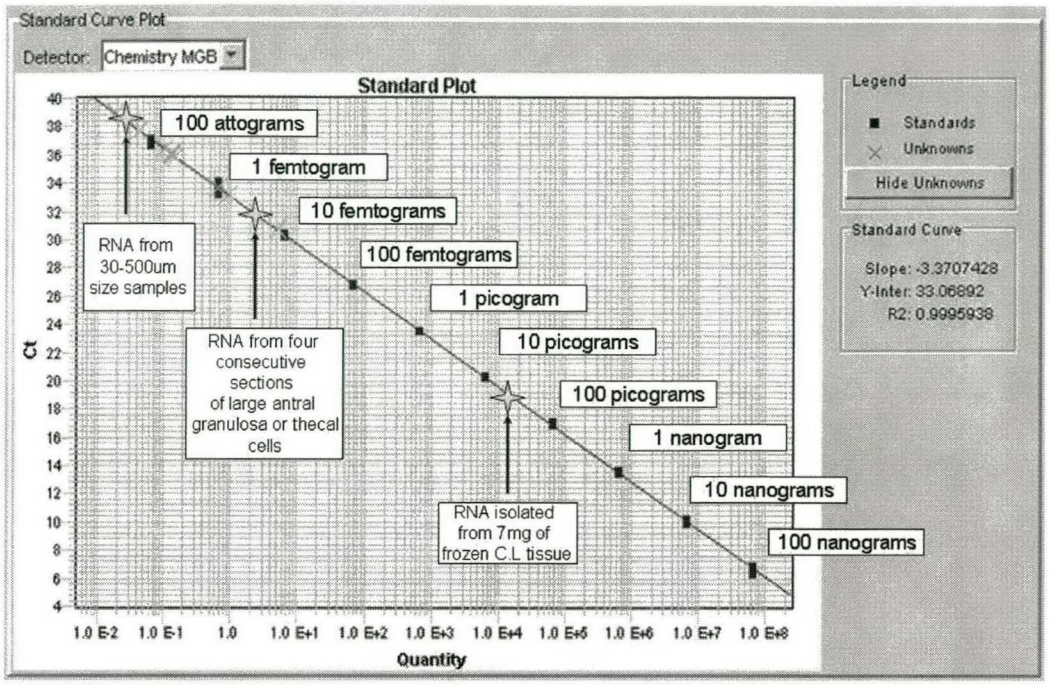


Figure 3.5 – Concentration comparisons between three different RNA samples. The RNA captured from a corpus luteum section ranging in diameter from 30-500um, RNA from four consecutive sections of large antral granulosa or thecal cells, and the RNA isolated from a 7mg piece of frozen corpus luteum tissue.

Sample	VEGF [attog] mRNA/18S [picog]
C.L. (30-500um sections)	Not detectable
Thecal Cells	352.9
Granulosa Cells	92.7± 24.5* (Range of 4 samples 44.4-152.9)
Positive Control (7mg frozen C.L. tissue)	6.25×10^4

Table 3.1 – Relative amounts of VEGF mRNA collected from the thecal and granulosa cells of large antral follicles in sheep ovaries. * Mean ± Standard Error

Discussion:

The work herein was done, largely to establish a set of new techniques. Initially, it was necessary to determine the amount of RNA needed from any one sample, so that the data point would appear on a reliable portion of the standard curve. The first experiments included taking a specimen rich in VEGF, the corpus luteum, and capturing different sizes of tissues ranging from 30um-500um. None of these samples provided enough VEGF RNA to appear on a reliable part of the standard curve. We gradually increased the amount of tissue we captured until it was determined that we had to take the same cells from at least four consecutive sections when isolating granulosa or thecal cells from large antral follicles. This number will have to increase greatly as smaller size follicles are investigated. Another variable that needs to be evaluated is the health of the follicles. LCM followed by real-time PCR would be a powerful tool for evaluating healthy and atretic follicles. Some guidelines need to be developed for identifying healthy, large antral follicles which could include a crisp border between the thecal and granulosa cells. Healthy granulosa cells should appear plump and round, not crenated or asymmetric, which would indicate atresia. The RNA from the healthy granulosa or thecal cells of four consecutive sections proved to be both intact and appearing at a reliable point on our standard curve. Preliminary data indicates that there is no significant difference between the VEGF mRNA expression in thecal and granulosa cells of large antral follicles. These results are similar to those in the literature [14]. Although LCM is one of the most accurate ways to collect specific cell types, the possibility of contamination still exists. The employment of cell specific markers for granulosa, thecal, and oocytes would be an objective way to ensure the purity of the cell population.

There is also some question as to the choice of normalizing gene. We have selected 18S which is a ribosomal RNA. The literature suggests that some commonly used normalizing genes such as GAPDH may have differing levels of expression throughout the estrous cycle [15]. Although we did not investigate the expression levels of 18S throughout the estrous cycle, 18S is often used to normalize the gene of interest, because it is abundant and consistent across cell types.

As science moves in a molecular direction it is important to validate techniques that allow for a molecular view of specific, homogeneous, cell populations. The employment of laser capture microdissection and real-time PCR will allow this project to move forward confidently when looking at the effects of VEGF in specific cell populations in the ovary.

REFERENCES

1. Fraser HM. Regulation of the ovarian follicular vasculature. Reprod Biol Endocrinol 2006; 4:18.

2. Otani N, Minami S, Yamoto M, Shikone T, Otani H, Nishiyama R, Otani T, Nakano R. The vascular endothelial growth factor/fms-like tyrosine kinase system in human ovary during the menstrual cycle and early pregnancy. J Clin Endocrinol Metab 1999; 84:3845-3851.

3. Harata T, Ando H, Iwase A, Nagasaka T, Mizutani S, Kikkawa F. Localization of angiotensin II, the AT1 receptor, angiotensin-converting enzyme, aminopeptidase A, adipocyte-derived leucine aminopeptidase, and vascular endothelial growth factor in the human ovary throughout the menstrual cycle. Fertil Steril 2006; 86:433-439.

4. Fearon ER, Hamilton SR, Vogelstein B. Clonal analysis of human colorectal tumors. Science 1987; 238:193-197.

5. Radford DM, Fair K, Thompson AM, Ritter JH, Holt M, Steinbrueck T, Wallace M, Wells SA,Jr, Donis-Keller HR. Allelic loss on a chromosome 17 in ductal carcinoma in situ of the breast. Cancer Res 1993; 53:2947-2949.

6. Emmert-Buck MR, Roth MJ, Zhuang Z, Campo E, Rozhin J, Sloane BF, Liotta LA, Stetler-Stevenson WG. Increased gelatinase A (MMP-2) and cathepsin B activity in invasive tumor regions of human colon cancer samples. Am J Pathol 1994; 145:1285-1290.

7. Zhuang Z, Bertheau P, Emmert-Buck MR, Liotta LA, Gnarra J, Linehan WM, Lubensky IA. A microdissection technique for archival DNA analysis of specific cell populations in lesions < 1 mm in size. Am J Pathol 1995; 146:620-625.

8. Noguchi S, Motomura K, Inaji H, Imaoka S, Koyama H. Clonal analysis of predominantly intraductal carcinoma and precancerous lesions of the breast by means of polymerase chain reaction. Cancer Res 1994; 54:1849-1853.

9. Park TW, Felix JC, Wright TC,Jr. X chromosome inactivation and microsatellite instability in early and advanced bilateral ovarian carcinomas. Cancer Res 1995; 55:4793-4796.

10. Kovach JS, McGovern RM, Cassady JD, Swanson SK, Wold LE, Vogelstein B, Sommer SS. Direct sequencing from touch preparations of human carcinomas: analysis of p53 mutations in breast carcinomas. J Natl Cancer Inst 1991; 83:1004-1009.

11. Shibata D, Hawes D, Li ZH, Hernandez AM, Spruck CH, Nichols PW. Specific genetic analysis of microscopic tissue after selective ultraviolet radiation fractionation and the polymerase chain reaction. Am J Pathol 1992; 141:539-543.

12. Auer H, Lyianarachchi S, Newsom D, Klisovic MI, Marcucci G, Kornacker K. Chipping away at the chip bias: RNA degradation in microarray analysis. Nat Genet 2003; 35:292-293.

13. Goldsworthy SM, Stockton PS, Trempus CS, Foley JF, Maronpot RR. Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. Mol Carcinog 1999; 25:86-91.

14. Shimizu T, Jiang JY, Sasada H, Sato E. Changes of messenger RNA expression of angiogenic factors and related receptors during follicular development in gilts. Biol Reprod 2002; 6: 1846-52.

15. Cardenas H, Burke KA, Bigsby RM, Pope WF, Nephew KP. Estrogen receptor beta in the sheep ovary during the estrous cycle and early pregnancy. Biol Reprod 2001; 65:128-134.

CHAPTER 4

DISCUSSION

The previous work has demonstrated that VEGF plays an important role in primordial follicle growth and survival. The specific role of VEGF and the mechanism by which it affects primordial follicles remains to be elucidated. The implications of the role of VEGF in the mammalian ovary are discussed here.

Role of VEGF in the mammalian ovary.

Although it is widely accepted that VEGF is an important regulator of the later stages of follicle development and corpus luteum function, the data presented in this thesis and other data in the literature provide convincing evidence that VEGF is an important growth factor for preantral follicle development. It has been shown previously that VEGF administration increases the number of primary and small secondary follicles in the rodent ovary [1]. Administration of VEGF directly to the ovary results in an increase in preantral follicle numbers in a dose and time dependent manner. In addition, estrogen up-regulates VEGF expression in the rodent ovary similar to its effects on early follicle growth [1].

In addition to stimulating preantral follicle growth, VEGF is also an important survival factor for the primordial follicle pool. Administration of antibodies directed against an antigenic peptide region of the VEGF molecule resulted in a rapid and profound loss of primordial follicles in the ovary. Similarly, antibodies which specifically block the KDR receptor also resulted in primordial follicle depletion. Direct ovarian administration of antibodies also depleted primordial follicles indicating that neutralization of ovarian VEGF, as opposed to peripheral neutralization, is key to primordial follicle destruction.

In addition to the destruction of primordial follicles, neutralization of endogenous VEGF also results in a rapid and pronounced increase in apoptosis in the ovary. Data from our laboratory and others [2] indicate that the pro-apoptotic protein BAX is rapidly up-regulated in the ovary following neutralization of endogenous VEGF. Similarly the anti-apoptotic protein BCL2 is rapidly decreased following neutralization of ovarian VEGF [2].

Localization of VEGF in the mammalian ovary

Although immunohistochemical studies generally confirm that follicular VEGFA expression increases as follicles mature [3-5], several reports reveal expression of VEGF in preantral follicular compartments. VEGF has been shown to be localized in the granulosa and thecal cells of pre-antral follicles [3] as well as the oocytes of primordial and primary follicles [6,7] .In the rat ovary, VEGF is highly expressed in the zona pellucida of selected secondary follicles [8]. Kezele et al [9] identified VEGFA as one of the important genes upregulated during primordial follicle development in the mouse. However, despite these recent studies suggesting preantral expression of VEGF, the majority of published studies indicate weak to absent VEGF expression in this follicular pool. The discrepancy likely resides in the techniques utilized to identify VEGF expression in the ovary. Almost all published studies to date have relied on immunohistochemisrty to localize VEGF in the ovary. This technique is exquisitely susceptible to subtle differences in antibody preparation and laboratory technique. As such, we chose to utilize a more objective and quantitative approach to identifying VEGF expression in the ovary.

Laser capture microdissection is proving to be a very useful way to identify and isolate specific cell populations. LCM coupled with real-time PCR provides a very powerful combination of specificity and sensitivity for evaluating the expression of VEGF in specific ovarian populations. The techniques developed herein are very useful for evaluating specific cell population within the heterogeneous ovarian architecture. We began our studies with larger antral follicles, because the structures of large antral follicles are easily identified and provided the optimal structures with which to establish and validate our laser capture and PCR protocols. With respect to LCM, we have optimized the system with respect to tissue preparation, staining protocols, RNA stability, and laser parameters including laser pulse duration, and strength. As such we have been able to successfully isolate specific cell types (granulosa, theca, and luteal cells) as well as individual oocytes. Furthermore we have identified minimal cellular requirements for subsequent real time PCR analysis. Similarly, optimal conditions for real time PCR of VEGF in these samples have been identified and the assay has been extensively validated with respect to sensitivity, specificity, and efficiency. With the techniques and validation in hand, future experiments will be developed towards evaluating the expression of VEGF (and its receptors) in pre-antral follicle populations which are perhaps the most relevant structures with respect to identifying the role of VEGF on preantral follicle growth and survival.

Mechanisms of action of VEGF in the mammalian ovary.

VEGF could increase the number of preantral follicles in the rat ovary by a variety of mechanisms. Enhanced vascularity or vascular permeability near developing follicles could increase the delivery of endocrine or paracrine factors, such as growth

factors, steroids, gonadotropins, or more generally oxygen and nutrients to the developing follicles. Increased delivery of folliculotrophic substances could result in an increase in the rate of follicular recruitment from the primordial pool (increased follicular growth), or an inhibition of follicular atresia.

In addition to its role as a potent inducer of angiogenesis, VEGF is also a direct mitogen for endothelial and tumor cells [10]. VEGF may be acting directly to provide a stimulus to primordial follicles that would allow them to progress into subsequent stages of follicular growth. Alternatively, VEGF could stimulate the production of factors that promote follicle survival. As previously discussed, VEGF down regulates BAX an apoptotic protein and up-regulates the production of Bcl-2 [11], a pro-survival factor that blocks the effects of BAX. Neutralization of VEGF could therefore not only remove a proliferation signal but also prevent the production of pro-survival factors. This combination could contribute to the destruction of the primordial follicle population.

Another possibility to explain the loss of primordial follicles is the effect of VEGF neutralization on the vasculature of the ovary. Although the primordial follicle population is generally sequestered away from the larger vessels in the ovary, primordial follicles must receive nutrients and oxygen and eliminate waste in association with the vasculature. Neutralizing VEGF would likely cause a disruption of the blood vessels within the ovarian stroma. This disruption could lead to a decrease in vasculature associated with the primordial follicles, in turn decreasing the amount of nutrients, oxygen, and growth factors the primordial follicles receive. However, alterations in vascularity would likely take longer than 24hrs, which is the time frame in which a rapid decline in the number of primordial follicles occurs. Alternatively, neutralization of

endogenous VEGF could result in a disruption of vascular permeability which is a much more rapid response to VEGF inhibition. Decreasing the permeability of existing vasculature would result in a loss of nutrients and growth factors to the primordial follicles which could result in primordial follicle apoptosis and atresia.

In contrast to acting via alterations in the ovarian vasculature, VEGF could have direct effects on non-vascular elements in the mammalian ovary. Recent evidence suggests that VEGF may have direct mitogenic effects on granulosa cells in vitro and could directly stimulate follicular growth in the rat ovary [12]. VEGFR1 mRNA and protein is expressed in granulosa cells in cows and VEGF is cytoprotective in the extravascular compartment of the bovine follicle [13].

Finally, recent evidence from Johnson et al. [14, 15] suggests that there is a renewable population of stem cells perhaps originating in the bone marrow. This population migrates to the ovary where it continually renews the primordial follicle population. Although these new data are controversial, one could speculate that VEGF could modulate these "stem cells" either in the bone marrow, ovary, or both and that neutralization of VEGF may cause either a direct or indirect depletion in this sensitive population.

In summary, the work presented herein provides compelling evidence that VEGF is an important regulator of preantral follicle growth and survival. Not only does VEGF stimulate preantral follicle growth [1] but neutralization of endogenous VEGF results in a rapid and profound destruction of the primordial follicle pool. In addition, to explore the ovarian physiology of VEGF we have developed techniques that will be important in evaluating the expression of VEGF and it's receptors in ovarian cell populations. Laser

capture Microdissection and real time-PCR are uniquely suited to identify the specific ovarian cells expressing VEGF and its cognate receptors in the heterogeneous mammalian ovarian architecture. These studies will provide real insight into the physiology and molecular biology of the ovarian VEGF system.

REFERENCES

1. Danforth DR, Arbogast LK, Ghosh S, Dickerman A, Rofagha R, Friedman CI. Vascular endothelial growth factor stimulates preantral follicle growth in the rat ovary. Biol Reprod 2003; 68:1736-1741.

2. Abramovich D, Parborell F, Tesone M. Effect of a vascular endothelial growth factor (VEGF) inhibitory treatment on the folliculogenesis and ovarian apoptosis in gonadotropin-treated prepubertal rats. Bio Reprod. 2006; 75: 434-41.

3. Yamamoto S, Konishi I, Tsuruta Y, Nanbu K, Mandai M, Kuroda H, Matsushita K, Hamid AA, Yura Y, Mori T. Expression of vascular endothelial growth factor (VEGF) during folliculogenesis and corpus luteum formation in the human ovary. Gynecol Endocrinol 1997; 11:371-381.

4. Greenway J, Connor K, Pedersen HG, Coomber BL, LaMarre J, Petrik J. Vascular Endothelial Growth Factor and Its Receptor, Flk-1/KDR, Are Cytoprotective in the Extravascular Compartment of the Ovarian Follicle. Endocrinology 2004; 145:2896-2905.

5. Taylor PD, Hillier SG, Fraser HM. Effects of GnRH antagonist treatment on follicular development and angiogenesis in the primate ovary. J Endocrinol. 2004; 183:1-17.

6. Otani N, Minami S, Yamoto M, Shikone T, Otani H, Nishiyama R, Otani T, Nakano R. The vascular endothelial growth factor/fms-like tyrosine kinase system in human ovary during the menstrual cycle and early pregnancy. J Clin Endocrinol Metab. 1999; 84:3845-3851.

7. Harata T, Ando H, Iwase A, Nagasaka T, Mizutani S, Kikkawa F. Localization of angiotensin II, the AT1 receptor, angiotensin-converting enzyme, aminopeptidase A, adipocyte-derived leucine aminopeptidase, and vascular endothelial growth factor in the human ovary throughout the menstrual cycle. Fertil Steril. 2006; 86:433-439.

8. Celik-Ozenci C, Akkoyunlu G, Kayisli UA, Arici A, Demir R. Localization of vascular endothelial growth factor in the zona pellucida of developing ovarian follicles in the rat: a possible role in destiny of follicles. Histochem Cell Biol 2003; 120:383-390.

9. Kezele PR, Ague JM, Nilsson E, Skinner MK. Alterations in the ovarian transcriptome during primordial follicle assembly and development. Biol Reprod. 2005; 72(1):241-255.

10. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004; 25:581-611.

11. Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 1998; 273:30336-30343.

12. Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, Chisholm V, Hillan KJ, Schwall RH. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat Med 1998; 4:336-340.

13. Greenway J, Gentry PA, Feige JJ, LaMarre J, Petrik JJ. Thrombospondin and vascular endothelial growth factor are cyclically expressed in an inverse pattern during bovine ovarian follicle development. Biol Reprod 2005; 1071-78.

14. Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 2004; 428:145-150.

15. Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT, Tilly JL. Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. Cell 2005; 122:303-315.

LIST OF REFERENCES

(Alphabetical)

Abramovich D, Parborell F, Tesone M. Effect of a vascular endothelial growth factor (VEGF) inhibitory treatment on the folliculogenesis and ovarian apoptosis in gonadotropin-treated prepubertal rats. Bio Reprod. 2006; 75: 434-41.

Auer H, Lyianarachchi S, Newsom D, Klisovic MI, Marcucci G, Kornacker K. Chipping away at the chip bias: RNA degradation in microarray analysis. Nat Genet 2003; 35:292-293.

Barboni B, Turriani M, Galeati G, Spinaci M, Bacci ML, Forni M, Mattioli M. Vascular endothelial growth factor production in growing pig antral follicles. Biol Reprod 2000; 63:858-864.

Bautz F, Rafii S, Kanz L, Mohle R. Expression and secretion of vascular endothelial growth factor-A by cytokine-stimulated hematopoietic progenitor cells. Possible role in the hematopoietic microenvironment. Exp Hematol 2000; 28:700-706.

Bedell MA, Brannan CI, Evans EP, Copeland NG, Jenkins NA, Donovan PJ. DNA rearrangements located over 100 kb 5' of the Steel (Sl)-coding region in Steel-panda and Steel-contrasted mice deregulate Sl expression and cause female sterility by disrupting ovarian follicle development. Genes Dev 1995; 9:455-470.

Besmer P. The kit ligand encoded at the murine Steel locus: a pleiotropic growth and differentiation factor. Curr Opin Cell Biol 1991; 3:939-946.

Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF. Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. Dev Biol 1998; 204:373-384.

Cardenas H, Burke KA, Bigsby RM, Pope WF, Nephew KP. Estrogen receptor beta in the sheep ovary during the estrous cycle and early pregnancy. Biol Reprod 2001; 65:128-134.

Carlevaro MF, Cermelli S, Cancedda R, Descalzi Cancedda F. Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. J Cell Sci 2000; 113 (Pt 1):59-69.

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996; 380:435-439.

Celik-Ozenci C, Akkoyunlu G, Kayisli UA, Arici A, Demir R. Localization of vascular endothelial growth factor in the zona pellucida of developing ovarian follicles in the rat: a possible role in destiny of follicles. Histochem Cell Biol 2003; 120:383-390.

Christofori G, Naik P, Hanahan D. Vascular Endothelial Growth Factor and Its Receptors, flt-1 and flk-1, are Expressed in Normal Pancreatic Islets and throughout Islet Cell Tumorigenesis. Molecular Endocrinology 1995; 9:1760-1770.

Dakappagari NK, Pyles J, Parihar R, Carson WE, Young DC, Kaumaya PT. A chimeric multi-human epidermal growth factor receptor-2 B cell epitope peptide vaccine mediates superior antitumor responses. J Immunol. 2003; 170:4242-4253.

Danforth DR, Arbogast LK, Ghosh S, Dickerman A, Rofagha R, Friedman CI. Vascular endothelial growth factor stimulates preantral follicle growth in the rat ovary. Biol Reprod 2003; 68:1736-1741.

Danforth DR. Endocrine and paracrine control of oocyte development. Am J Obstet Gynecol 1995; 172:747-752.

Dean J. Oocyte-specific genes regulate follicle formation, fertility and early mouse development. J Reprod Immunol 2002; 53:171-180.

Dennis PA, Rifkin DB. Studies on the role of basic fibroblast growth factor in vivo: inability of neutralizing antibodies to block tumor growth. J Cell Physiol 1990; 144:84-98.

Dickson SE, Bicknell R, Fraser HM. Mid-luteal angiogenesis and function in the primate is dependent on vascular endothelial growth factor. J Endocrinol 2001; 168:409-416.

Duffy DM, Stouffer RL. Luteinizing hormone acts directly at granulosa cells to stimulate periovulatory processes: modulation of luteinizing hormone effects by prostaglandins. Endocrine 2003; 22:249-256.

Ehrmann RL, Knoth M. Choriocarcinoma. Transfilter stimulation of vasoproliferation in the hamster cheek pouch. Studied by light and electron microscopy. J Natl Cancer Inst 1968; 41:1329-1341.

Einspanier R, Schonfelder M, Muller K, Stojkovic M, Kosmann M, Wolf E, Schams D. Expression of the vascular endothelial growth factor and its receptors and effects of VEGF during in vitro maturation of bovine cumulus-oocyte complexes (COC). Mol Reprod Dev 2002; 62:29-36.

Eliceiri BP, Paul R, Schwartzberg PL, Hood JD, Leng J, Cheresh DA. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Mol Cell 1999; 4:915-924.

Elvin JA, Yan C, Wang P, Nishimori K, Matzuk MM. Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. Mol Endocrinol 1999; 13:1018-1034.

Emmert-Buck MR, Roth MJ, Zhuang Z, Campo E, Rozhin J, Sloane BF, Liotta LA, Stetler-Stevenson WG. Increased gelatinase A (MMP-2) and cathepsin B activity in invasive tumor regions of human colon cancer samples. Am J Pathol 1994; 145:1285-1290.

Esch F, Ueno N, Baird A, Hill F, Denoroy L, Ling N, Gospodarowicz D, Guillemin R. Primary structure of bovine brain acidic fibroblast growth factor (FGF). Biochem Biophys Res Commun 1985; 133:554-562.

Fearon ER, Hamilton SR, Vogelstein B. Clonal analysis of human colorectal tumors. Science 1987; 238:193-197.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996; 380:439-442.

Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, Chisholm V, Hillan KJ, Schwall RH. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat Med 1998; 4:336-340.

Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat. Med. 2003; 9:669–676.

Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 1989; 161:851-858.

Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004; 25:581-611.

Ferrara N. VEGF as a therapeutic target in cancer. Oncology 2005; 69 Suppl 3:11-16.

Folkman J, Merler E, Abernathy C, Williams G. Isolation of a tumor factor responsible for angiogenesis. J Exp Med 1971; 133:275-288.

Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 1995; 376:66-70.

Fong GH, Zhang L, Bryce DM, Peng J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. Development 1999; 126:3015-3025.

Frangione-Beebe M, Albrecht B, Dakappagari N, Rose RT, Brooks CL, Schwendeman SP, Lairmore MD, Kaumaya PT. Enhanced immunogenicity of a conformational epitope of human T-lymphotropic virus type 1 using a novel chimeric peptide. Vaccine 2000; 19:1068-1081.

Frank S, Hubner G, Breier G, Longaker MT, Greenhalgh DG, Werner S. Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. J Biol Chem 1995; 270:12607-12613.

Fraser HM, Dickson SE, Lunn SF, Wulff C, Morris KD, Carroll VA, Bicknell R. Suppression of luteal angiogenesis in the primate after neutralization of vascular endothelial growth factor. Endocrinology 2000; 141:995-1000.

Fraser HM, Wilson H, Rudge JS, Wiegand SJ. Single injections of vascular endothelial growth factor trap block ovulation in the macaque and produce a prolonged, dose-related suppression of ovarian function. J Clin Endocrinol Metab 2005; 90:1114-1122.

Fraser HM, Wulff C. Angiogenesis in the corpus luteum. Reprod Biol Endocrinol 2003; 1:88.

Fraser HM, Wulff C. Angiogenesis in the primate ovary. Reprod Fertil Dev 2001; 13:557-566.

Fraser HM. Regulation of the ovarian follicular vasculature. Reprod Biol Endocrinol 2006; 4:18.

Friedman CI, Danforth DR, Herbosa-Encarnacion C, Arbogast L, Alak BM, Seifer DB. Follicular fluid vascular endothelial growth factor concentrations are elevated in women of advanced reproductive age undergoing ovulation induction. Fertil Steril 1997; 68:607-612.

Fuh G, Li B, Crowley C, Cunningham B, Wells JA. Requirements for binding and signaling of the kinase domain receptor for vascular endothelial growth factor. J Biol Chem 1998; 273:11197-11204.

Fukumura D, Ushiyama A, Duda DG, Xu L, Tam J, Krishna V, Chatterjee K, Garkavtsev I, Jain RK. Paracrine regulation of angiogenesis and adipocyte differentiation during in vivo adipogenesis. Circ Res 2003; 93:e88-97.

Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem 1998; 273:13313-13316.

Gerber HP, Malik AK, Solar GP, Sherman D, Liang XH, Meng G, Hong K, Marsters JC, Ferrara N. VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature 2002; 417:954-958.

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 1998; 273:30336-30343.

Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 1999; 5:623-628.

Gille H, Kowalski J, Yu L, Chen H, Pisabarro MT, Davis-Smyth T, Ferrara N. A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR-1) constitutively inhibits vascular endothelial growth factor-dependent phosphatidylinositol 3'-kinase activation and endothelial cell migration. EMBO J 2000; 19:4064-4073.

Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG. Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest 1998; 78:1385-1394.

Goldsworthy SM, Stockton PS, Trempus CS, Foley JF, Maronpot RR. Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. Mol Carcinog 1999; 25:86-91.

Greenaway J, Connor K, Pedersen HG, Coomber BL, LaMarre J, Petrik J. Vascular Endothelial Growth Factor and Its Receptor, Flk-1/KDR, Are Cytoprotective in the Extravascular Compartment of the Ovarian Follicle. Endocrinology 2004; 145:2896-2905.

Greenaway J, Gentry PA, Feige JJ, LaMarre J, Petrik JJ. Thrombospondin and vascular endothelial growth factor are cyclically expressed in an inverse pattern during bovine ovarian follicle development. Biol Reprod 2005; 1071-78.

Greenaway J, Gentry PA, Feige JJ, LaMarre J, Petrik JJ. Thrombospondin and vascular endothelial growth factor are cyclically expressed in an inverse pattern during bovine ovarian follicle development. Biol Reprod 2005; 72:1071-1078.

Greenblatt M, Shubi P. Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique. J Natl Cancer Inst 1968; 41:111-124.

Guo D, Jia Q, Song HY, Warren RS, Donner DB. Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with endothelial cell proliferation. J Biol Chem 1995; 270:6729-6733.

Harata T, Ando H, Iwase A, Nagasaka T, Mizutani S, Kikkawa F. Localization of angiotensin II, the AT1 receptor, angiotensin-converting enzyme, aminopeptidase A, adipocyte-derived leucine aminopeptidase, and vascular endothelial growth factor in the human ovary throughout the menstrual cycle. Fertil Steril. 2006; 86:433-439.

Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, Hicklin DJ, Zhu Z, Witte L, Crystal RG, Moore MA, Rafii S. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med 2001; 193:1005-1014.

Hazzard TM, Molskness TA, Chaffin CL, Stouffer RL. Vascular endothelial growth factor (VEGF) and angiopoietin regulation by gonadotrophin and steroids in macaque granulosa cells during the peri-ovulatory interval. Mol Hum Reprod 1999; 5:1115-1121.

Hazzard TM, Stouffer RL. Angiogenesis in ovarian follicular and luteal development. Baillieres Best Pract Res Clin Obstet Gynaecol 2000; 14:883-900.

Heryanto B, Lipson KE, Rogers PA. Effect of angiogenesis inhibitors on estrogen-mediated endometrial endothelial cell proliferation in the ovariectomized mouse. Reproduction 2003; 125:337-346.

Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 2005; 23:1011-1027.

Huang EJ, Manova K, Packer AI, Sanchez S, Bachvarova RF, Besmer P. The murine steel panda mutation affects kit ligand expression and growth of early ovarian follicles. Dev Biol 1993; 157:100-109.

Hull ML, Charnock-Jones DS, Chan CL, Bruner-Tran KL, Osteen KG, Tom BD, Fan TP, Smith SK. Antiangiogenic agents are effective inhibitors of endometriosis. J Clin Endocrinol Metab 2003; 88:2889-2899.

Ide AG, Baker NH, Warren SL. Vascularization of the Brown Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. Am J Roentgenol 1939; 42:891-899.

Jakeman LB, Winer J, Bennett GL, Altar CA, Ferrara N. Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. J Clin Invest 1992; 89:244-253.

Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT, Tilly JL. Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. Cell. 2005; 122(2):303-315.

Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 2004; 428:145-150.

Kamat BR, Brown LF, Manseau EJ, Senger DR, Dvorak HF. Expression of vascular permeability factor/vascular endothelial growth factor by human granulosa and theca lutein cells. Role in corpus luteum development. Am J Pathol 1995; 146:157-165.

Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989; 246:1309-1312.

Keyt BA, Berleau LT, Nguyen HV, Chen H, Heinsohn H, Vandlen R, Ferrara N. The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. J Biol Chem. 1996; 271:7788-7795.

Kezele PR, Ague JM, Nilsson E, Skinner MK. Alterations in the ovarian transcriptome during primordial follicle assembly and development. Biol Reprod. 2005; 72(1):241-255.

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature 1993; 362:841-844.

Koos RD. Increased expression of vascular endothelial growth/permeability factor in the rat ovary following an ovulatory gonadotropin stimulus: potential roles in follicle rupture. Biol Reprod 1995; 52:1426-1435.

Kovach JS, McGovern RM, Cassady JD, Swanson SK, Wold LE, Vogelstein B, Sommer SS. Direct sequencing from touch preparations of human carcinomas: analysis of p53 mutations in breast carcinomas. J Natl Cancer Inst 1991; 83:1004-1009.

Lairmore M D, DiGeorge AM, Conrad SF, Trevino AV, Lal RB, Kaumaya PT Human T-lymphotropic virus type 1 peptides in chimeric and multivalent constructs with promiscuous T-cell epitopes enhance immunogenicity and overcome genetic restriction J. Virol. 1995; 69:6077–6089.

Lee A, Christenson LK, Patton PE, Burry KA, Stouffer RL. Vascular endothelial growth factor production by human luteinized granulosa cells in vitro. Hum Reprod 1997; 12:2756-2761.

Lee WS, Yoon SJ, Yoon TK, Cha KY, Lee SH, Shimasaki S, Lee S, Lee KA. Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. Mol Reprod Dev 2004; 69:159-163.

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989; 246:1306-1309.

Liu Y, Cox SR, Morita T, Kourembanas S. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. Circ Res 1995; 77:638-643.

Matsumoto T, Claesson-Welsh L. VEGF receptor signal transduction. Sci STKE 2001; 2001:RE21.

Matsuzaki K, Yoshitake Y, Matuo Y, Sasaki H, Nishikawa K. Monoclonal antibodies against heparin-binding growth factor II/basic fibroblast growth factor that block its biological activity: invalidity of the antibodies for tumor angiogenesis. Proc Natl Acad Sci U S A 1989; 86:9911-9915.

Matthews W, Jordan CT, Gavin M, Jenkins NA, Copeland NG, Lemischka IR. A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit. Proc Natl Acad Sci U S A 1991; 88:9026-9030.

Mattioli M, Barboni B, Turriani M, Galeati G, Zannoni A, Castellani G, Berardinelli P, Scapolo PA. Follicle activation involves vascular endothelial growth factor production and increased blood vessel extension. Biol Reprod 2001; 65:1014-1019.

McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. Endocr Rev. 2000; 21:200-214.

Merriam-Webster Medical Dictionary [Internet]. Angiogenesis. 2005; 2006:1.

Merriam-Webster Medical Dictionary [Internet]. Neovascularization. 2005; 2006:1.

Merriam-Webster Medical Dictionary [Internet]. Vasculogenesis. 2005; 2006:1.

Michaelson IC. The mode of development of the vascular system of the retina with some observations on its significance for certain retinal disorders. Trans Ophthalmol Soc UK 1949; 68: 137-180.

Morales-Ruiz M, Fulton D, Sowa G, Languino LR, Fujio Y, Walsh K, Sessa WC. Vascular endothelial growth factor-stimulated actin reorganization and migration of endothelial cells is regulated via the serine/threonine kinase Akt. Circ Res 2000; 86:892-896.

Nelson JF, Felicio LS, Randall PK, Sims C, Finch CE. A longitudinal study of estrous cyclicity in aging C57BL/6J mice: I. Cycle frequency, length and vaginal cytology. Biol Reprod 1982; 27:327-339.

Nilsson EE, Skinner MK. Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. Biol Reprod 2003; 69:1265-1272.

Nilsson EE, Skinner MK. Growth and differentiation factor-9 stimulates progression of early primary but not primordial rat ovarian follicle development. Biol Reprod 2002; 67:1018-1024.

Noguchi S, Motomura K, Inaji H, Imaoka S, Koyama H. Clonal analysis of predominantly intraductal carcinoma and precancerous lesions of the breast by means of polymerase chain reaction. Cancer Res 1994; 54:1849-1853.

Otani N, Minami S, Yamoto M, Shikone T, Otani H, Nishiyama R, Otani T, Nakano R. The vascular endothelial growth factor/fms-like tyrosine kinase system in human ovary during the menstrual cycle and early pregnancy. J Clin Endocrinol Metab. 1999; 84:3845-3851.

Park JE, Keller GA, Ferrara N. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol Biol Cell 1993; 4:1317-1326.

Park TW, Felix JC, Wright TC,Jr. X chromosome inactivation and microsatellite instability in early and advanced bilateral ovarian carcinomas. Cancer Res 1995; 55:4793-4796.

Parrott JA, Skinner MK. Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. Endocrinology 1999; 140:4262-4271.

Pauli SA, Tang H, Wang J, Bohlen P, Posser R, Hartman T, Sauer MV, Kitajewski J, Zimmermann RC. The vascular endothelial growth factor (VEGF)/VEGF receptor 2 pathway is critical for blood vessel survival in corpora lutea of pregnancy in the rodent. Endocrinology 2005; 146:1301-1311.

Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, Alitalo K. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. J Biol Chem 1994; 269:6271-6274.

Pietrowski D, Keck C. Differential regulation of ANG2 and VEGF-A in human granulosa lutein cells by choriogonadotropin. Exp Clin Endocrinol Diabetes. 2004; 112:208-214.

Radford DM, Fair K, Thompson AM, Ritter JH, Holt M, Steinbrueck T, Wallace M, Wells SA,Jr, Donis-Keller HR. Allelic loss on a chromosome 17 in ductal carcinoma in situ of the breast. Cancer Res 1993; 53:2947-2949.

Rockwell LC, Pillai S, Olson CE, Koos RD. Inhibition of vascular endothelial growth factor/vascular permeability factor action blocks estrogen-induced uterine edema and implantation in rodents. Biol Reprod. 2002; 67:1804-1810.

Roy H, Bhardwaj S, Yla-Herttuala S. Biology of vascular endothelial growth factors. FEBS Lett 2006; 580:2879-2887.

Russell ES. Hereditary anemias of the mouse: a review for geneticists. Adv Genet 1979; 20:357-459.

Semenza G. Signal transduction to hypoxia-inducible factor 1. Biochem Pharmacol 2002; 64:993-998.

Senger DR, Connolly DT, Van de Water L, Feder J, Dvorak HF. Purification and NH2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability factor. Cancer Res 1990; 50:1774-1778.

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983; 219:983-985.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995; 376:62-66.

Shen H, Clauss M, Ryan J, Schmidt AM, Tijburg P, Borden L, Connolly D, Stern D, Kao J. Characterization of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes. Blood 1993; 81:2767-2773.

Shibata D, Hawes D, Li ZH, Hernandez AM, Spruck CH, Nichols PW. Specific genetic analysis of microscopic tissue after selective ultraviolet radiation fractionation and the polymerase chain reaction. Am J Pathol 1992; 141:539-543.

Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene 1990; 5:519-524.

Shimizu T. Promotion of ovarian follicular development by injecting vascular endothelial growth factor (VEGF) and growth differentiation factor 9 (GDF-9) genes. J Reprod Dev 2006; 52:23-32.

Skinner MK. Regulation of primordial follicle assembly and development.Hum Reprod Update. 2005;11:461-471.

Sundaram R, Lynch MP, Rawale SV, Sun Y, Kazanji M, Kaumaya PT De novo design of peptide immunogens that mimic the coiled coil region of human T-cell leukemia virus type-1 glycoprotein 21 transmembrane subunit for induction of native protein reactive neutralizing antibodies. J Biol Chem. 2004; 279:24141-51.

Takahashi T, Ueno H, Shibuya M. VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. Oncogene 1999; 18:2221-2230.

Tannock I, Hill R, Bristow R, Harrington L. The Basic Science of Oncology. United States: McGraw-Hill Professional; 2004: 558.

Taylor PD, Hillier SG, Fraser HM. Effects of GnRH antagonist treatment on follicular development and angiogenesis in the primate ovary. J Endocrinol. 2004; 183:1-17.

Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB. Identification of a new endothelial cell growth factor receptor tyrosine kinase. Oncogene 1991; 6:1677-1683.

Tesone M, Stouffer RL, Borman SM, Hennebold JD, Molskness TA. Vascular endothelial growth factor (VEGF) production by the monkey corpus luteum during the menstrual cycle: isoform-selective messenger RNA expression in vivo and hypoxia-regulated protein secretion in vitro. Biol Reprod 2005; 73:927-934.

Thomas KA, Rios-Candelore M, Gimenez-Gallego G, DiSalvo J, Bennett C, Rodkey J, Fitzpatrick S. Pure brain-derived acidic fibroblast growth factor is a potent angiogenic vascular endothelial cell mitogen with sequence homology to interleukin 1. Proc Natl Acad Sci U S A 1985; 82:6409-6413.

Todaro GJ, De Larco JE, Fryling C, Johnson PA, Sporn MB. Transforming growth factors (TGFs): properties and possible mechanisms of action. J Supramol Struct Cell Biochem 1981; 15:287-301.

Vaisman N, Gospodarowicz D, Neufeld G. Characterization of the receptors for vascular endothelial growth factor. J Biol Chem 1990; 265:19461-19466.

Vincenti V, Cassano C, Rocchi M, Persico G. Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. Circulation 1996; 93:1493-1495.

Vlodavsky I, Folkman J, Sullivan R, Fridman R, Ishai-Michaeli R, Sasse J, Klagsbrun M. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. Proc Natl Acad Sci U S A 1987; 84:2292-2296.

Vom Saal FS, Finch CE, Nelson JF Natural history and mechanisms of reproductive aging in humans, laboratory rodents, and other selected vertebrates. In: E. Knobil, JD Neill (eds), The Physiology of Reproduction, Vol 2, 2nd ed. New York, Raven Press 1994:1213-1314.

Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem 1994; 269:26988-26995.

Wang C, Roy SK. Expression of growth differentiation factor 9 in the oocytes is essential for the development of primordial follicles in the hamster ovary. Endocrinology 2006; 147:1725-1734.

Warren RS, Yuan H, Matli MR, Ferrara N, Donner DB. Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma. J Biol Chem 1996; 271:29483-29488.

Wulff C, Wiegand SJ, Saunders PT, Scobie GA, Fraser HM. Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc (vascular endothelial growth factor Trap(A40)). Endocrinology 2001; 142:3244-3254.

Wulff C, Wilson H, Rudge JS, Wiegand SJ, Lunn SF, Fraser HM. Luteal angiogenesis: prevention and intervention by treatment with vascular endothelial growth factor trap(A40). J Clin Endocrinol Metab 2001; 86:3377-3386.

Xu F, Hazzard T, Evans A, Charnock-Jones S, Smith S, Stouffer R. Intraovarian actions of anti-angiogenic agents disrupt periovulatory events during the menstrual cycle in monkeys. Contraception 2005; 71:239-248

Yamamoto S, Konishi I, Tsuruta Y, Nanbu K, Mandai M, Kuroda H, Matsushita K, Hamid AA, Yura Y, Mori T. Expression of vascular endothelial growth factor (VEGF) during folliculogenesis and corpus luteum formation in the human ovary. Gynecol Endocrinol 1997; 11:371-381.

Zakarija A, Soff G. Update on angiogenesis inhibitors. Curr Opin Oncol 2005; 17:578-583.

Zhuang Z, Bertheau P, Emmert-Buck MR, Liotta LA, Gnarra J, Linehan WM, Lubensky IA. A microdissection technique for archival DNA analysis of specific cell populations in lesions < 1 mm in size. Am J Pathol 1995; 146:620-625.

Zimmermann RC, Xiao E, Bohlen P, Ferin M. Administration of antivascular endothelial growth factor receptor 2 antibody in the early follicular phase delays follicular selection and development in the rhesus monkey. Endocrinology 2002 143:2496-2502.

Zimmermann RC, Xiao E, Husami N, Sauer MV, Lobo R, Kitajewski J, Ferin M. Short-term administration of antivascular endothelial growth factor antibody in the late follicular phase delays follicular development in the rhesus monkey. J Clin Endocrinol Metab 2001; 86:768-772.