VEGF in the Placenta, and Maternal Circulation and Organs During Pregnancy in Mice

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Abstract

Whether vascular endothelial growth factor A (VEGF) plays an augmented role during pregnancy is unknown. In this thesis expression of VEGF in the placenta, maternal circulation and organs in mice was examined using qRT-PCR, LacZ expression, and/or ELISAs. Normal pregnancies and pregnancies with transgenic conceptuses that over-express VEGF in the placenta were examined. In normal pregnancies, VEGF$_{120/164}$ levels in the ovary increased in parallel with that of the maternal circulation. In pregnancies where the placenta over-expressed VEGF, maternal circulating VEGF$_{120/164}$ levels decreased and so did levels in the maternal ovary. Surprisingly, VEGF protein levels (per mg of total protein) decreased in the growing, highly vascular placenta during pregnancy. In conclusion this thesis provides evidence for an important ovarian source of maternal circulating VEGF$_{120/164}$ during pregnancy.
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## Abbreviations

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<th>Full Form</th>
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<tbody>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CL</td>
<td>corpus luteum</td>
</tr>
<tr>
<td>ECM</td>
<td>extra cellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>Flt1</td>
<td>VEGFR-1</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia inducible factor 1</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish-peroxidase</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<td>ISH</td>
<td>in situ hybridization</td>
</tr>
<tr>
<td>KDR</td>
<td>VEGFR-2</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PI GF</td>
<td>placental growth factor</td>
</tr>
<tr>
<td>RIP A</td>
<td>radiolymphoprecipitation assay buffer</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>sFlt1</td>
<td>soluble VEGFR-1</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
<tr>
<td>Vegf</td>
<td>vascular endothelial growth factor mRNA</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor protein</td>
</tr>
<tr>
<td>Vegf&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>Vegf&lt;sup&gt;hi&lt;/sup&gt;-LacZ</td>
</tr>
<tr>
<td>VEGF R1</td>
<td>vascular endothelial growth factor receptor 1</td>
</tr>
<tr>
<td>VEGF R2</td>
<td>vascular endothelial growth factor receptor 2</td>
</tr>
<tr>
<td>VPF</td>
<td>vascular permeability factor</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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Chapter 1

1 Introduction

Vascular endothelial growth factor (VEGF) is a potent stimulator of vascular development, as it induces differentiation, proliferation and migration of endothelial cells that contribute to vessel formation through both vasculogenesis and angiogenesis. Multiple isoforms of VEGF are produced through alternative splicing, and there are two membrane-bound receptors for the ligand, as well as soluble receptors. All organs express at least some VEGF, but the exact function of this protein during pregnancy and in organs such as the placenta is unknown. The placenta is a temporary organ present only during pregnancy, and is among the most vascular organs in the body. VEGF is thought to play a critical role in the development of the placental vasculature, but again, its exact function is unknown. Understanding the placental function of VEGF is important because excess placental sFlt1, a soluble receptor of VEGF, is thought to be a potential contributor to pre-eclampsia, a life-threatening complication of human pregnancy.

With VEGF signaling considered an important contributor to the pathology of pre-eclampsia, current studies are manipulating VEGF signaling to treat pre-eclampsia, but it should be noted that we don’t know what the normal function of VEGF in pregnancy is. Therefore, the goal of this thesis was to determine the distribution and expression of VEGF in the maternal circulation, placenta, and maternal organs during pregnancy in mice. Mice were used as a model for multiple reasons. First, the Vegf gene in the placenta can be manipulated, such that we can over-express VEGF in the placenta. We can also tag LacZ expression to Vegf mRNA expression to determine which cells specifically express Vegf mRNA. Mice are also used as an animal model in this thesis due to their placental similarities with the human placenta. By characterizing the changes in expression of the different isoforms of VEGF, it will further our understanding of the role VEGF plays during placentation, as well as in the maternal organs during pregnancy.
1.1 Pregnancy

Human pregnancy is a temporary state in which the formation of one or more embryos occurs. During pregnancy, many maternal changes occur, such as the development of a temporary organ called the placenta. The placenta is a transient and highly vascular organ with many purposes. It facilitates the transport of oxygen and nutrients to the fetus while removing fetal metabolic waste, acts as an immune barrier, is involved in hematopoiesis, and it synthesizes a variety of peptide and steroid hormones, such as progesterone, estrogen, human chorionic gonadotropin (hCG), placental lactogens, and relaxin (Myatt, 2002). Other changes that occur in the mother during normal human pregnancy include decreased peripheral vascular resistance, increased cardiac output, increased plasma volume, and increased renal plasma flow (Chapman et al., 1998, Clapp and Capeless, 1997). Most of these changes begin in early pregnancy. A significant increase in uterine blood flow also occurs, supplying the growing placenta and fetus with adequate oxygen and nutrients (Thaler et al., 1990). The site of gas and nutrient exchange in the human placenta is known as the intervillous space, and this region of the placenta is highly vascular. The exact mechanism by which this vascularity develops is not known, but VEGF is thought to play a role, as it is a potent angiogenic factor.

1.2 Vascular Endothelial Growth Factor (VEGF) and VEGF Receptors

1.2.1 History of VEGF

The identification of VEGF, a homodimeric glycoprotein of approximately 45kDa (Ferrara and Hanzel, 1989), took place over many years and through many independent and unrelated lines of research (Ferrara, 2002). Originally known as Vascular Permeability Factor (VPF) when first described in guinea pigs by Senger et al. in 1983, the protein was found to induce vascular leakage in the skin. The purification of the amino acid sequence by Senger et al. however did not occur until 1990. In 1989, Ferrara and Hanzel isolated a diffusable endothelial cell-specific mitogen from bovine pituitary follicular cells, which they named ‘vascular endothelial growth factor.’ As well in 1989, Connolly et al., Leung et al., and Keck et al., furthered the research into VEGF and found
through cDNA cloning that VEGF and VPF were the same molecule. Due to the many lines of research leading to the discovery of the same molecule, the protein is known as VEGF and VPF, but is more commonly recognized as VEGF because it acts primarily on vascular endothelial cells. It was later found that VEGF belonged to a family of secreted glycoproteins, including VEGF-B, -C, -D, and placenta growth factor (PIGF) (reviewed in Ferrara, 2003). Prokineticin 1 (PK1), also known as EG-VEGF, is considered a potent angiogenic factor (Hoffman et al., 2007). This gene is not related to the VEGF family however, therefore will not be discussed in this thesis. VEGF is also known as VEGFA, and is considered to be the most common and potent isoform in angiogenesis. This isoform will be the focus of interest in this thesis and I will refer to it as VEGF in my thesis.

Many biological roles have been shown for VEGF, some of which include: promoting the growth of vascular endothelial cells derived from arteries, veins, and lymphatics (reviewed by Ferrara and Davis-Smyth, 1997); survival factor for endothelial cells by preventing endothelial apoptosis induced by serum starvation (Alon et al., 1995) and also by inducing expression of anti-apoptotic proteins such as Bcl-2 and A1 (Gerber et al., 1998); as a chemoattractant for monocytes and endothelial cells (Ferrara et al., 2003); and on the enhancement of vascular permeability as originally described.

VEGF expression is regulated by many factors, such as oxygen tension, growth factors, and hormones. Vegf mRNA expression is induced by exposure to low pO₂ in a variety of pathophysiological circumstances (Dor et al., 2001; Semenza, 2003). In response to hypoxia, hypoxia inducible factor -1 (HIF-1) binds to specific oxygen response elements on the Vegf gene, resulting in increased gene transcription. Several growth factors, such as transforming growth factor (TGF) -α, -β, fibroblast growth factor (FGF), and platelet derived growth factor (PDGF), can also up-regulate Vegf expression (Frank et al., 1995; Pertovaara et al., 1994). This suggests that paracrine or autocrine release of such factors cooperates with local hypoxia in regulating VEGF release in the microenvironment. Hormones are also important regulators of Vegf gene expression. Thyroid stimulating hormone (TSH) has been shown to induce Vegf expression in several thyroid carcinoma cell lines (Soh et al., 1996) and estrogen has also been shown to be a potent inducer of Vegf transcription in the ovary, both in vivo (Shweiki et al., 1993; Ferrara et al., 1998) and in vitro (Christenson and Stouffer, 1997).
1.2.2 Isoforms of VEGF

The human *Vegf* gene is located on chromosome 6p21.3 (Vincenti et al. 1996) and is organized into eight exons, separated by seven introns (Houck et al. 1991). Alternative exon splicing results in different isoforms of VEGF, each with a distinct number of amino acids (e.g. 121, 165, 189, creating VEGF_{121}, VEGF_{165}, and VEGF_{189} respectively) (Figure 1.1). VEGF_{165} is the predominant isoform and lacks exon 6, whereas VEGF_{121} lacks exons 6 and 7, and VEGF_{189} contains all 8 exons. Since VEGF_{121} lacks exons 6 and 7, the regions containing the heparan binding residues, it fails to bind to heparan and is a freely diffusible protein (Park et al., 1993). Because VEGF_{165} lacks exon 6, it can be secreted, but a significant fraction remains bound to the cell surface and extracellular matrix (ECM) since it still contains exon 7. VEGF_{189} contains all eight exons thus is almost completely sequestered in the extracellular matrix (ECM). It should be noted that the ECM-bound isoforms may be released in a diffusible form by heparin or heparinase, which displaces them from their binding to heparan-like moieties (Houck et al., 1992), thus increasing the bioavailability of VEGF.

In mice, the animal model used in this thesis, the single *Vegf* gene also gives rise to multiple protein isoforms of VEGF by differential mRNA splicing: VEGF_{120}, VEGF_{164}, and VEGF_{188} (Figure 1.1) (Ferrera et al., 1992, Haigh, 2008). The mouse isoforms contain one less amino acid relative to their human counterpart, and are expressed differentially among organs (Table 1.1) (Ng et al., 2001). Similar to the human isoforms, VEGF_{120} does not bind heparan sulfate, thus is freely diffusible, whereas VEGF_{188} is heparan bound and is primarily associated with the cell surface and extracellular matrix. VEGF_{164} is the predominant isoform in mice and has intermediate properties like VEGF_{165} (Park et al. 1993, Ng et al., 2001). To exert their effects, the different isoforms of VEGF bind to two receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2.
Figure 1.1 - Exon structure of the Vegf gene. The Vegf gene consists of eight exons. All currently described isoforms contain exons 1–5 and exon 8. VEGF$_{189}$ contains all 8 exons, and is predominantly membrane bound. VEGF$_{165}$, the most common isoform, and lacks exon 6, whereas VEGF$_{121}$, the completely soluble isoform, lacks exons 6 and 7. Exons 6 and 7 contain the heparan binding residues that are responsible for making VEGF membrane bound rather than soluble. The number value in each isoform represents the number of amino acids in each isoform. In mice, the VEGF protein contains one less amino acid, therefore VEGF$_{189}$ is VEGF$_{188}$, VEGF$_{165}$ is VEGF$_{164}$, and VEGF$_{121}$ is VEGF$_{120}$.
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1.2.3 VEGF Receptors

There are two main cell surface receptors for VEGF, each with seven Ig-like domains in the extracellular domain, a single transmembrane region, and a consensus tyrosine kinase sequence that is interrupted by a kinase insert domain (Shibuya et al., 1990, and Terman et al., 1991).

VEGFR1 is also known as Flt1 (Figure 1.2). VEGF binds primarily to the second Ig-like domain in this receptor (Davis-Smyth et al., 1996) and, although it was the first receptor to be identified, the exact function of this receptor is yet to be determined. Studies have shown that when VEGF binds to Flt1, a weak tyrosine autophosphorylation response occurs. The weak response suggests that Flt1 may not be the primary receptor transmitting VEGF’s mitogenic signal (Park et al., 1994). Some studies have also shown that Flt1 inhibits endothelial cell migration (Gille et al., 2000) while others have indicated that it can mediate a weak mitogenic signal (Maru et al., 1998), therefore it may have other roles. An alternatively spliced soluble form of Flt1, known as sFlt1, binds to VEGF in the circulation. It inhibits the activity of VEGF (Kendall and Thomas, 1993) by preventing VEGF from binding to VEGFR2, the second cell-surface VEGF receptor. PlGF can also bind to Flt1, but is not considered as potent an angiogenic factor compared to VEGF because it does not bind to VEGFR2 (Ferrara, 2003), therefore will not be studied in detail in this thesis.

VEGFR2 is known as KDR and is the second major receptor for VEGF (Figure 1.2). VEGF binds primarily to the second and third Ig-like domain (Fuh et al., 1998) of KDR. KDR can mediate the mitogenic, angiogenic, and permeability-enhancing effects of VEGF (Ferrara, 2004) even though VEGF binds to KDR with a lower affinity compared to Flt1 (Terman et al., 1992).

The importance of the two VEGF receptors is shown by knock-out studies in mice. Homozygous mutation of either receptor results in embryonic lethality between embryonic days 8.5 and 9.5 due to abnormal vasculogenesis and development of blood islands (Fong et al., 1995; Shalaby et al., 1995; de Vries et al., 1992).

VEGF can also bind to Neuropilin 1 and Neuropilin 2 (NRP1 and NRP2), cell-surface receptors adjacent to KDR. VEGF121 does not bind to these binding sites,
Figure 1.2 - Interaction of VEGF and its receptors, Flt1, KDR, and sFlt1. VEGF binds can bind to two cell surface receptors Flt1 and KDR. VEGF binds to Flt1 with a higher affinity than KDR, but Flt1 is considered to be a ‘decoy’ receptor, as it does not mediate as strong a mitogenic signal. An alternatively spliced soluble form of Flt1, sFlt1, is thought to play a similar role, as it sequesters VEGF in the circulation and prevents it from binding to KDR, which the primary receptor for mediating the angiogenic and permeability effects of VEGF. Figure modified from Ferrara, 2003.
indicating that exon 7-encoded basic sequences are required for binding to this receptor (Soker et al., 1996). When coexpressed in cells with KDR, NRP1 enhanced the binding of VEGF_{165} to KDR and VEGF_{165}-mediated chemotaxis (Soker et al., 1998). The role of NRP1 in the development of the vascular system has also been shown by gene targeting studies, where Nrp1-null mice were embryonic lethal (Kawasaki et al., 1999).

In addition to the three major isoforms of VEGF that bind to Flt1 and KDR, another family of VEGF isoforms that can bind to Flt1 and KDR has been discovered in the past decade. This family is collectively known as VEGF

1.2.4 VEGF\_xxx\_b

The VEGF\_xxx\_b family of proteins is generated by differential splicing of exon 8 (Bates et al., 2002) (Figure 1.3), and they are believed to be anti-angiogenic because VEGF_{165}b inhibits VEGF_{165}-mediated endothelial cell proliferation and migration \textit{in vitro} (Bates et al, 2002; Rennel et al, 2008a,b) and vasodilation \textit{ex vivo} (Bates et al, 2002). For example, Qiu et al. (2008) showed that female transgenic mice which over-expressed VEGF_{165}b in their mammary tissue had fewer blood vessels, less blood in the mammary tissue, impaired alveolar coverage of the fat pad, and did not produce sufficient milk for nourishment of their pups, resulting in the death of their offspring shortly after birth. The over-expression of VEGF_{165}b in the ovaries of pregnant mice also resulted in reduced litter size, as the number of oocytes released into oviducts by the transgenic females was reduced compared to the control mice (Qiu et al., 2010).

VEGF_{165}b differs from VEGF_{165} only in the carboxy-terminal six amino acids (66 bp downstream from the original stop codon), where a change from CDKPRR to SLTRKD occurs (Bates et al., 2002). The replacement of highly positively charged arginine residues with neutral lysine–aspartic acid residues is what causes a functional
Figure 1.3 – Alternative splicing of *Vegf* gene. (A) Differential splicing of exon 8 in humans, 66 base pairs downstream of the proximal splice site, results in an inhibitory isoform of VEGF, denoted as VEGF\(_{xxx}\)b, with \(xxx\) representing the number of amino acids present in the isoform. The replacement of the last six amino acids, CDKPRR to SLTRKD, is thought to create the inhibitory effects. Figure modified from Ladomery et al., 2007. (B) A comparison of exon 8a and 8b in human and mouse VEGF\(_{xxx}\) and VEGF\(_{xxx}\)b is done. The VEGF\(_{xxx}\) sequence is identical between humans and mice. In VEGF\(_{xxx}\)b, an extra amino acid is added to the mouse sequence, creating VEGF\(_{165}\)b, and not VEGF\(_{164}\)b, as assumed. Figure A modified from Ladomery et al., 2007.
difference between the two proteins. When I compared the mouse and human *Vegf* gene sequences, I found the predicted exon 8b sequence located 66 base pairs downstream of the original exon 8a stop codon in the mouse *Vegf* gene. I noticed that an extra 3 base pairs (one amino acid) was present in the mouse *Vegf* exon 8b sequence. In rats, VEGF164 also becomes VEGF165b by differential splicing (Artac et al., 2009), and the presence of endogenous *Vegf*165b mRNA has been confirmed in rats using conventional RT-PCR and subcloning (Artact et al., 2009). Given that the sequence of mouse and rat *Vegf* mRNA is identical, we predict that VEGF165b is also present endogenously in mice.

A proposed mechanism for how VEGFxxxb causes anti-angiogenesis is reviewed by Harper and Bates (2008), and is briefly described here. When VEGF165 binds to KDR and neuropilin 1 (NRP1), a conformational change in KDR occurs (Ruch et al., 2007), resulting in the internal rotation of the intracellular domain. This however is not thought to occur with VEGF165b, which binds to KDR with the same affinity as VEGF165. Studies have shown that VEGF165b only partially activates KDR. A partial intracellular rotation causes the kinase domain to be activated, but tyrosine 1054, which is in the kinase regulatory site, is not phosphorylated. This is thought to be due to insufficient torsional rotation (Figure 1.4) (reviewed in Harper and Bates, 2008). This results in rapid closure of the ATP binding site of the kinase and rapid inactivation (Kawamura et al., 2008), leading to a poorly activated kinase and a weak, transient phosphorylation of extracellular-signal-regulated kinase 1 (ERK1) and ERK2 (Cebe-Suarez et al., 2006). Additionally, Cebe-Suarez et al. have shown in 2008 that VEGF165b does not bind NRP1, because the basic carboxy-terminal amino acids essential for NRP1 binding are absent. As mentioned above, the binding of VEGF to NRP1 is important in activating the receptor and causing downstream pathways such as cell proliferation and migration to occur.

When measuring total *Vegf* mRNA, it was interestingly found that greater than 50% of the total *Vegf* can consist of anti-angiogenic isoforms in certain human tissues (Qiu et al., 2009). An exception to this finding was the human placenta, where extensive angiogenesis is known to occur. In the human placenta VEGFxxxb makes up less than half of the total VEGF mRNA and protein (Bates et al., 2006 and Bevan et al., 2007).

1.3 Pregnancy and VEGF

The source and function of increased VEGF activity in pregnancy is unknown, but
evidence suggests that it plays a functional role. For example, when sFlt1 was infused into the circulation of rats, therefore reducing circulating free VEGF, hypertension and proteinuria were observed (Maynard et al., 2003). Infusion of VEGF however into the maternal circulation attenuated these symptoms (Maynard et al., 2003; Siddiqui et al., 2011), supporting that circulating VEGF during pregnancy plays a functional role. Possible sources of increased VEGF would be organs that express higher levels during pregnancy, and these organs are most likely those that are growing and hence require an increase in pro-angiogenic signals during pregnancy.

In pregnancy, Vegf mRNA is upregulated in the ovary, where it sustains the highly vascularized corpus luteum (Kaczmarek et al., 2005). The ovary is a prominent endocrine organ necessary for pregnancy to occur. The mature corpus luteum, where hormones such as estrogen and progesterone are produced and secreted into the bloodstream, receives one of the greatest rates of blood flow per gram of any tissue in the body (Niswender and Nett, 1998). Intensive proliferation of endothelial cells is also observed in the rodent corpus luteum during early pregnancy (Tamura and Grenwald, 1987). In humans, Vegf mRNA is upregulated in the corpus luteum of women in early pregnancy when compared to non-pregnant women (Sugino et al., 2000), but no cross-gestational studies have been conducted. No cross-gestational studies in mice have been conducted either, but this deficiency will be addressed in this thesis.

Expression of VEGF has also been studied in the decidua and mammary gland during pregnancy. Decidual Vegf mRNA increases at E8.0, where blood vessel formation is occurring (Chakraborty et al., 1995), and VEGF in the mammary gland increases during pregnancy (5 fold) and lactation (5-19 fold) (Pepper et al., 2000). Other maternal organs have not been looked at during pregnancy and this is important because there may be multiple sources for the increase in maternal circulating VEGF witnessed during pregnancy. Other likely sites for upregulation of VEGF and angiogenesis during pregnancy include maternal organs that grow during pregnancy, such as the spleen and liver (Davis et al., 1961; Milona et al., 2009). Blood flow into many maternal organs such as the ovary, lungs, heart, liver, and kidney also increases during pregnancy (Ahokas et. 1984 and Conrad, 2004), but since maternal arterial blood pressure does not rise during
Figure 1.4 – Interaction of VEGF$_{165}$ and VEGF$_{165b}$ with KDR. (A) Binding of VEGF$_{165}$ with KDR results in the activation of the split kinase domains (green lines) and the phosphorylation of tyrosine residues. The charged residues are required for VEGF receptor activation and, in receptor tyrosine kinases, this is thought to occur through torsional rotation of the intracellular domain. Tyrosine 1054 is located at the ATP binding pocket of the tyrosine kinase, and once phosphorylated, prevents the binding pocket from closing, thus resulting in a stable open structure. Tyrosine phosphorylation also results in the activation of angiogenic signalling pathways (Figure 1.2). (B) VEGF$_{165b}$ binds to KDR with equal affinity as VEGF$_{165}$, but does not bind neuropilin 1 (NRP1). The last six amino acids of VEGF$_{165b}$ are neutral in charge, and therefore it is not able to create sufficient torsional rotation for tyrosine 1054 to be phosphorylated (purple phosphates), although weak phosphorylation of other tyrosines can occur. As a result, the ATP binding pocket closes and the phosphorylated tyrosines are rapidly dephosphorylated by phosphatases, and the angiogenic signalling pathways are not activated. Figure modified from Harper and Bates, 2008.
pregnancy, this means that increased blood flow to these tissues must be a consequence of reduced vascular resistance due to vasodilation and/or angiogenesis; both of which could be mediated by increased VEGF activity (Maynard et al., 2003 and Szukiewicz et al., 2005).

Organs that release VEGF into the circulation likely express relatively high levels of the diffusible form of VEGF, VEGF<sub>120</sub>. The study by Ng et al., 2001 (Table 1.1) showed the mRNA distribution of Vegf isoforms across a variety of organs in non-pregnant adult mice. The three major VEGF isoforms were examined, VEGF<sub>120</sub>, VEGF<sub>164</sub>, and VEGF<sub>188</sub>. The ovary, eye, and small intestine expressed the greatest amount of Vegf<sub>120</sub> and Vegf<sub>164</sub>, both of which have soluble properties. Taking this information with the fact that the ovary receives a significant amount of blood flow during pregnancy, it seems plausible that the ovary may be a source for the increase in maternal circulating VEGF during pregnancy. In addition to the many maternal organs that may contribute to increases in maternal circulating VEGF, the placenta should also be considered. The placenta is a highly vascular organ present only during pregnancy, and placental blood flow progressively increases in gestation, thus making it a top candidate for increased VEGF secretion in the maternal blood.

1.4 Placenta in Mice

The placenta is a transient organ that appears following the implantation of the blastocyst into the endometrial stroma of the uterus. The mouse placenta, although not identical to that of the human, shares considerable structural and functional similarities with that of the human (Figure 1.5) (Adamson et al., 2002; Georgiades et al., 2002). For example, the epithelial component in both species originates from the trophoectoderm of the blastocyst. The trophoectoderm in mice is composed of two main layers, the mural, and polar trophoectoderm. Primary trophoblast giant cells (TGC) derive from the mural trophoectoderm and they invade the endometrial stroma where they contact maternal blood sinuses (Benirschke et al., 2005; Bevilacqua and Abrahamsohn, 1998). As a result of this contact, the endometrium undergoes stromal decidualization (Cross et al., 1994; Simmons and Cross, 2005). The polar trophoectoderm proliferates and forms the ectoplacental cone, and the extraembryonic ectoderm (chorion). The polar trophoectoderm gives rise to the variety of trophoblast cell subtypes that will later make
Figure 1.5 – Similarities between the human and mouse placenta. Analogous structures are labeled. The spongiotrophoblast layer in mouse is analogous to the trophoblast cell columns in humans. Direction of blood flow is the same in both species. Figure modified from Moore 1988, and Adamson et al., 2002.
Figure 1.6 – Schematic diagrams of the mouse placenta. (A) Schematic diagram of the conceptus at E9.5. Umbilical flow to the labyrinth (in the ectoplacental cone) begins at E9.5. (B) Schematic diagram of the mature mouse placenta at E13.5/E17.5. The maternal decidua, junctional zone, labyrinth, and chorionic plate are well defined by this time point and the basic structure of the placenta does not change hereafter. Figure 1.6B modified from Proctor 2008.
up the mature mouse placenta (Cross et al., 1994; Simmons and Cross, 2005; Simmons et al., 2007).

In the early mouse placenta (Figure 1.6a), between E8.5 and E9.5, the fusion of chorionic trophoblast cells with the allantoic mesoderm leads to the formation of primary villi (Cross et al., 2006). These villi become vascularized by fetal blood vessels in the labyrinth region of the placenta, where nutrient and gas exchange occurs. Proliferation and differentiation of cells in the ectoplacental cone gives rise to the junctional zone, which contains spongiotrophoblast cells, glycogen trophoblast cells, and secondary trophoblast giant cells (TGCs) (Simmons and Cross, 2005). The trophoblast giant cells are invasive, hormone-secreting cells that line the interface between the decidua and the conceptus. The glycogen trophoblast cells can migrate interstitially into the decidua, as do some of the secondary TGCs, which can line the spiral arteries (Shweiki et al., 1993).

The basic structure of the mature mouse placenta is formed by embryonic day 10.5 (Mu and Adamson, 2006) at which point the chorionic plate, labyrinth, junctional zone, and maternal decidua are well defined. By E13.5 (Figure 1.6b), the maternal decidua contains spiral arteries, maternal immune cells, and migratory glycogen trophoblast cells from the junctional zone. The function of the junctional zone is yet to be fully understood (Georgiades et al. 2002), but it is essential for fetal survival as knocking out Ascl2, a junctional zone specific marker in mice is lethal around E10.5 (Guillemot et al. 1994). Bordering the junctional zone and the maternal decidua is a unicellular discontinuous layer of trophoblast giant cells (Pijnenborg et al. 1981). These large mononuclear, polyploid (Zybina & Zybina, 1996) trophoblasts produce a variety of hormones and chemokines (Malassiné et al. 2003), which may alter maternal physiology and immunology. The labyrinth region is the zone closest to the fetus, and it is the site of nutrient and gas exchange. Maternal blood from the spiral arteries is transported to the labyrinth where it bathes the villi of the fetal labyrinth, allowing for the exchange of gas and nutrients, where the enriched fetal blood then travels through the umbilical vein to the fetus. From E14.5 to term (~E19), the mouse placenta does not change in size significantly, but vascularity in the labyrinth increases (Coan et al., 2004).

In the mouse placenta, Vegf mRNA is highly expressed at E9.5 by TGCs, which are thought to establish a VEGF gradient to attract maternal endothelial cells towards the
conceptus (Shweiki et al., 1993). By E10.5, Vegf mRNA expression decreases in primary TGCs, and punctate mRNA expression is observed in the labyrinth. This punctate expression is still observed in the labyrinth at E17.0 (Breier et al., 1992) where it may promote the large increase in capillary density that occurs (Coan et al., 2004).

1.5 Pre-eclampsia and Pre-eclampsia Mouse Models

Abnormal levels of VEGF and its receptor, sFlt1, in the placenta have been implicated in defective placentation as well as the pathogenesis of pre-eclampsia, a pregnancy related disorder (Maynard et al., 2003). Pre-eclampsia is a pregnancy-specific disorder characterized clinically by sudden onset or worsening maternal hypertension and proteinuria after 20 weeks of gestation (Wang et al., 2009). It is one of the most frequently encountered medical complications during pregnancy, as it affects ~5% of pregnant women worldwide (Myatt, 2002, World Health Organization, 2005). While the exact cause of the disorder is unknown, acute symptoms can be alleviated with the delivery of the placenta, thus pointing to the placenta as the prime suspect in the etiology of the disease (Myatt, 2002). Although pre-eclampsia appears to begin with the abnormal formation of trophoblast cells in the placenta, the target organ responsible for maternal clinical symptoms is believed to be the maternal endothelium (Roberts, 1999, reviewed in Wang et al., 2009). Thus, there has been an extensive search for factors released by abnormal trophoblast cells that can circulate and cause maternal endothelial dysfunction.

Abnormal placentation is thought to underlie pre-eclampsia, and much research has focused on abnormalities in the maternal vascular remodeling in this disease (Wang et al., 2009). In normal human placental development, the invasion of extravillous cytotrophoblasts of fetal origin to the maternal spiral arteries of the decidua causes the endothelial layer to be replaced and this transformation results in the replacement of small, high-resistance vessels with high-caliber capacitance vessels (Wang et al., 2009). In PE, this transformation to high-caliber capacitance vessels is incomplete, as the extravillous cytotrophoblasts are unable to adequately invade the myometrial spiral arteries (Zhou et al., 1997), and as a result, the myometrial segments remain narrow (Meekins et al., 1994).

sFlt1, the soluble receptor for VEGF has been shown to decrease cytotrophoblast invasiveness in vitro (Zhou et al., 1997), and sFlt1 in the maternal circulation is elevated
above normal levels during the third trimester of pregnancy in pre-eclampsia (Wang et al., 2002). Because sFlt1 levels are elevated, free VEGF levels are decreased in the maternal circulation during PE. Following the first days after delivery, free VEGF can be detected again in the maternal circulation (Vuorela-Vepsalainen et al., 1999; Wikstrom et al., 2007), suggesting that its main binding protein, sFlt1, is secreted into the maternal circulation by the placenta. Despite considerable research, the cause(s) of pre-eclampsia remain(s) unclear, and there is no effective treatment (reviewed by Ahmed et al., 2010). Animal models that recapitulate this complex pregnancy-related disorder can be used in many ways to help our understanding of the mechanism for this disease as well as to help devise effective treatment.

Many pre-eclampsia mouse models have been created through either genetic means or by the administration of compounds that cause changes that mimic the effects of pre-eclampsia (Maynard et al., 2003; Kanaski et al., 2008; Falcao et al., 2009; Granger et al., 2006; Zhou et al., 2008). In many models, sFlt1 was experimentally elevated and/or VEGF was reduced to induce signs of pre-eclampsia, suggesting a causal role. For example, Ahmed et al. (2010) reported a new mouse model where mating CBA/J females with DBA/J males spontaneously produced the relevant key features of pre-eclampsia without any of the above manipulations. Their studies have shown that the administration of statins, such as pravastatin, may possibly restore the angiogenic balance in the placenta and therefore alleviating the preeclamptic features by increasing VEGF release from trophoblasts (Ahmed et al., 2010). Pravastatins are also thought to restore angiogenic balance in the placenta by inhibiting the release of sFlt1 from macrophages (Redecha et al. 2009) as well as endothelial cells and normal-term placental villous explants (Cudmore et al., 2007). As a result, treatments targeting VEGF activity may be considered, but such treatments should be approached with caution, given our lack of basic understanding of the roles of VEGF in pregnancy.

1.6 Hypotheses and Objectives

VEGF is considered an important contributor to the pathology of pre-eclampsia but little is known about its functions during pregnancy. Thus the goal of my thesis was to determine the distribution and expression of total Vegf mRNA, as well as VEGF120/164 protein in the placenta, decidua, and in selected maternal organs throughout pregnancy.
<table>
<thead>
<tr>
<th>Organ</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>One of the hallmark signs of PE is proteinuria, a symptom caused by improper filtration by the glomeruli in the kidney. Glomerular filtration also increases during pregnancy.</td>
</tr>
<tr>
<td>Brain</td>
<td>The brain is one of the maternal organs affected by PE. Edema in the brain can occur as well as seizures.</td>
</tr>
<tr>
<td>Ovary</td>
<td>The ovaries will be used as a positive control. Vegfa expression is known to increase during early pregnancy in the ovary.</td>
</tr>
<tr>
<td>Decidua</td>
<td>The decidua is a maternal organ unique to pregnancy. Vegf mRNA expression increases in the early decidua therefore can be used as a positive control. The decidua is also connected to the placenta, therefore it could affect VEGF expression in the placenta.</td>
</tr>
<tr>
<td>Liver</td>
<td>The liver increases in weight in pregnancy, thus we expect that VEGF expression increases in pregnancy to allow for increased growth and blood flow in the liver.</td>
</tr>
<tr>
<td>Spleen</td>
<td>The spleen increases in weight in pregnancy, thus we expect that VEGF expression increases in pregnancy to allow for increased growth and blood flow in the spleen.</td>
</tr>
<tr>
<td>Heart</td>
<td>Increase in cardiac output occurs during pregnancy in humans and mice and this results in increased cardiac work load. Thus we expect that VEGF expression increases in pregnancy to allow for increased blood flow in the heart to meet its increased metabolic requirements.</td>
</tr>
<tr>
<td>Lung</td>
<td>Increases in cardiac output are associated with pregnancy, therefore the lung is an organ that might grow during pregnancy. Organ growth is associated with an increase in vascularity, therefore VEGF expression may increase.</td>
</tr>
</tbody>
</table>

Table 1.2 – Maternal organs to study for VEGF expression during pregnancy. The kidney and brain are important to look at because these are the two maternal organs affected the most during pre-eclampsia. The ovary and decidua are important organs for maintaining pregnancy, and both receive significant amounts of maternal blood during pregnancy. The liver, spleen, heart, and lung increase in organ weight during pregnancy, therefore they may show increased VEGF expression to aid in the growth of the organ.
VEGF<sub>165</sub>b protein was also determined for those organs, as studies have shown the importance of this protein in inhibiting angiogenesis. The priority maternal organs to be examined were those that are most affected by pregnancy and/or are abnormal in pre-eclampsia (Table 1.2).

The second objective of my thesis was to determine if higher than normal VEGF protein is expressed in the placenta of Vegf<sup>hi</sup> mutant mice. If so, my goal was to then determine the effect of this over-expression on VEGF<sub>120/164</sub> and VEGF<sub>165</sub>b protein levels in the placenta, and whether placental over-expression affected VEGF levels in maternal organs as well as levels in the maternal and fetal circulation at E17.5.

It was expected that as pregnancy progressed, circulating VEGF levels in the mother would increase and would become much higher than in the fetus based on prior work in humans and mice (Koyama et al., 2006; Pinto et al., 2007). As gestation advanced, it was hypothesized that the expression of Vegf mRNA and protein would increase in the placenta. This would tend to aid in the formation of the placental vasculature by increasing angiogenesis. Specifically, it was expected that the labyrinth region would show the greatest expression of Vegf mRNA and protein because it is the most vascularized region of the placenta. In the labyrinth, the sinusoidal trophoblast giant cells were expected to express Vegf, as they may attract the feto-placental vasculature into the labyrinth and towards maternal blood in the labyrinthine sinusoids. VEGF<sub>165</sub>b protein expression was hypothesized to decrease in the placenta during pregnancy. This would tend to aid in the formation of the vasculature by decreasing its inhibitory influence on angiogenesis.

For the second aim of my thesis, if the placenta is an organ that over-expresses VEGF when CD1 females are bred with Vegf<sup>hi</sup> males, then it was hypothesized that we would see an increase in VEGF<sub>120/164</sub> protein expression in the placenta. This increase in the placenta would be expected to increase maternal circulating VEGF levels compared to control mice of the same gestational age. No change in expression of VEGF protein in the maternal organs would be expected because the mothers will be wild type (WT). If changes in VEGF protein do occur in any maternal organ, then this would suggest communication of that organ with the placenta. VEGF<sub>165</sub>b protein was hypothesized to decrease in the placenta of Vegf<sup>hi</sup> mice because insertion of the IRES-LacZ transgene
after exon 8 would be expected to disrupt the translation of the anti-angiogenic isoform.

Overall, the results of this thesis will give us a better understanding of the changes in VEGF that occur in the placenta and maternal organs during pregnancy. Through our VEGF over-expression studies, we will get a better understanding of the interactions between VEGF, the placenta, and the maternal circulation/organs.
Chapter 2

2 Material and Methods

The following is a brief outline of the methods used to characterize the expression of VEGF during pregnancy in mice. Detailed protocols can be found in Appendix 2. Mice were used due to their similarities to humans and their ability to have genes altered. Breeding schemes used can be found in Table 2.1. All samples were collected from female mice between 8-12 weeks of age and in their first pregnancy. Non-pregnant female mice were used as a control, and three time-points during pregnancy were studied. The first stage focused on was E9.5, when maternal and fetal vascular flows to the placenta begin. The second is at E13.5, which is the end of placental growth, and finally at E17.5, near term and when the exchange barrier in the placenta matures and capillaries proliferate. The following histological and molecular methods were used to study the expression of VEGF and/or sFlt1.

2.1 Animals

In these studies, mice were used as model for various reasons. For instance, mice have a quicker pregnancy period (~19 days in mice compared to ~9 months in humans). There are also similarities between humans and mice during pregnancy, such as placental morphology, hemodynamic changes during pregnancy, and increases in maternal plasma VEGF during pregnancy (Koyama et al., 2006). Additionally, studying the maternal effects of VEGF over-expression in the placenta is not feasible in humans, but is possible in mice.

CD1/ICR (WT) mice were used to study Vegf mRNA and protein expression in normal pregnancies. ICR mice are descendents of CD1, and the name was changed to ICR when Charles River, a commercial supplier, brought the strain to North America from Switzerland. I will refer to them as CD1 mice in this thesis. These mice were purchased from the Toronto Center for Phenogenomics (TCP), housed conventionally, and handled according to the guidelines established by the Canadian Council on Animal Care. Animals were mated overnight and embryonic day 0.5 (E0.5) was designated as noon of the day a vaginal plug was found. This was the first pregnancy for all mice used.
<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Fetal genotype</th>
<th>Collection</th>
<th>analysis</th>
<th>Variables</th>
<th>NP</th>
<th>E9.5</th>
<th>E13.5</th>
<th>E17.5</th>
</tr>
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<tbody>
<tr>
<td>Vegf&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>+/-</td>
<td>Adult tissues listed in table 4</td>
<td>Histology for LacZ</td>
<td>Tissue localization</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>x</td>
</tr>
<tr>
<td>CD1</td>
<td>Vegf&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>Placenta, ovary, kidney</td>
<td>Histology for LacZ ELISA</td>
<td>Tissue localization VEGF&lt;sub&gt;120/164&lt;/sub&gt; and VEGF&lt;sub&gt;165b&lt;/sub&gt;</td>
<td>N/A</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>CD1</td>
<td>CD1</td>
<td>Placental tissue</td>
<td>qRT-PCR ELISA</td>
<td>Total Vegf mRNA VEGF&lt;sub&gt;120/164&lt;/sub&gt; and VEGF&lt;sub&gt;165b&lt;/sub&gt;</td>
<td>N/A</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>CD1</td>
<td>CD1</td>
<td>Maternal blood for plasma and E17.5 fetal blood for plasma</td>
<td>ELISA</td>
<td>VEGF&lt;sub&gt;120/164&lt;/sub&gt; and sFlt1</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult and placental tissue</td>
<td>ISH</td>
<td>Vegf mRNA</td>
<td>x</td>
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</tbody>
</table>

**Table 2.1 – Breeding scheme and mouse lines used in this thesis.** CD1 (Charles River) are outbred, not genetically identical mice. They are a commonly used strain in reproductive research. Vegf<sup>hi</sup> (Dr. Andras Nagy) are transgenic mice expressing a hypermorphic Vegf allele and Vegf-LacZ reporter. The allele expresses a bicistronic message, which allows for the independent production of two separate proteins, VEGF and β-galactosidase, from the same mRNA sequence. The line was maintained in a heterozygous state bred into a CD1 background.
Vegf<sup>hi</sup> mice were used to study cellular expression of Vegf mRNA and to over-express VEGF protein in the placenta. The heterozygous Vegf<sup>hi</sup> mouse line was obtained from Dr. Andras Nagy and was created by placing an IRES (internal ribosome entry site)-lacZ cassette in the last exon of the gene after the stop codon. A SV40 polyadenylation signal was placed after the lacZ coding sequence. The IRES sequence allowed for independent production of VEGF and β-galactosidase (β-gal), from the same bicistronic mRNA. Previous studies of this mutant have shown that disruption of the 3’UTR increased the stability of the VEGF transcripts, which resulted in increased Vegf mRNA levels and increased VEGF protein production in select organs relative to WT mice (Miquerol et al., 1999).

Maternal effects of VEGF over-expression in certain concepti were studied by mating Vegf<sup>hi</sup> males with CD1 females. Cellular location of Vegf mRNA expression in maternal organs was studied in Vegf<sup>hi</sup> females as in prior work (Maharaj et al., 2006). Vegf<sup>hi</sup> males were mated with CD1 females to study cellular expression of Vegf mRNA in the placenta. Cells that express Vegf mRNA are marked by nuclear LacZ protein.

2.2 Tissue/Blood Collection

2.2.1 Tissue Collection for LacZ Histology/Immunohistochemistry

Cellular location of Vegf mRNA was determined using Vegf<sup>hi</sup> mice (see section 2.1 for breeding scheme). Once the mouse was sacrificed, organs of interest were dissected and placed into 4% PFA for 1 hour. After fixation, a midline cut was made to ensure penetration of the LacZ fix. Tissue was then placed into LacZ fixative for 30 min on a rocker inside a cold room. Thin midline sections were then created in the organs/embryo’s using a scalpel to allow for the penetration of the X-gal stain. Samples were then washed three times using the wash buffer and then stained overnight in the dark with X-gal. The following morning, the tissue was washed three times using PBS, and the samples were then stored in 10% formalin in the fridge until they were embedded.
2.2.2 Tissue Collection for In Situ Hybridization (ISH)

Tissue was dissected in 1xDEPC PBS and fixed overnight in 4% PFA/1xDEPC PBS. The following day, the tissue was washed in PBS and immersed in 15% sucrose overnight. The tissue was then immersed in 30% sucrose and left for overnight again. On the third day, the tissue was embedded in OCT and stored at -80 °C.

2.2.3 Maternal Blood Collection

Once weighed, the mouse was anesthetized [using isoflurane (set at 5%) in conjunction with oxygen (at 1000 ml/min)] and maternal blood was collected from the left ventricle (representing systemic circulation). To do this, a heparin-coated needle was inserted into the left ventricle via ultrasound guided cardiac puncture. The blood from the left ventricle was placed in a 1.5 ml microcentrifuge tube and spun at room temperature for 20 minutes. Once spun, the plasma was collected and aliquoted into 1.5 ml microcentrifuge tubes and stored at -20 °C.

For maternal blood collection through the uterine vein, the mouse was anesthetized [using isoflurane (set at 5%) in conjunction with oxygen (at 1000 ml/min)] and the abdominal cavity was opened. The uterine veins were exposed and punctured with a needle. Blood was then collected through heparin coated capillary tubes (70 µl capacity). The capillary tubes were centrifuged at room temperature for 5 minutes and the plasma was isolated and placed into 1.5 ml microcentrifuge tubes. The plasma was stored at -20 °C.

2.2.4 E17.5 Fetal Blood Collection

Fetal blood was collected via decapitation of fetal heads. Heparin coated capillary tubes (70 µl) were used and blood was pooled from multiple embryos before being centrifuged to isolate the plasma. The blood was centrifuged at room temperature for 20 minutes at 2000g. The plasma was then collected and aliquoted into 1.5 ml microcentrifuge tubes and stored at -20 °C.
2.2.5 Tissue Collection for mRNA and Protein Analysis

An incision was made on the mouse at the abdominal cavity. The uterine horns were removed and placed into ice cold PBS. The maternal organs (brain, heart, lungs, kidney, spleen, ovary, and liver) were then removed, placed into a 2 ml cryotubes and flash frozen using liquid nitrogen. Placental collection varied depending on age. At E9.5, the decidua and primitive placenta were enriched for via microdissection. Samples from the same mother were pooled to create a single ‘N’ due to the small tissue size. For E13.5 and E17.5, whole placentas without decidua and microdissected regions (Chorionic plate, labyrinth, junctional zone) were collected. Samples were pooled and flash frozen in liquid nitrogen. All samples were then stored at -80 °C.

2.3 Histology

2.3.1 Paraffin Embedding and Sectioning

Tissue samples were placed in embedding cassettes and dehydrated through a series of ethanol washes, from 70% to 100% ethanol. The tissue was then placed into xylene for 30 minutes, and then through three paraffin wax stages, each for 20 minutes. Once embedded, 5 µm sections were made using a microtome. Paraffin sections were placed on glass slides and allowed to dry overnight before staining.

2.3.2 Neutral Red background Staining

Tissue sections were deparaffinized in xylene and then re-hydrated down an ethanol gradient. Slides were then placed in deionzied water for five minutes and stained with 0.1% Neutral red for 45 seconds. The slides were then rinsed with deionized water, dehydrated up the ethanol gradient, immersed in xylene, then coverslipped with mounting medium and left to set in a biosafety cabinet.

2.3.3 Hematoxylin & Eosin and Tri-chrome Staining

Tissue sections were deparaffinized in xylene and then re-hydrated down an ethanol gradient. The slides were then placed in deionzied water for five minutes and stained with hematoxylin for 10 seconds. The slides were then rinsed with deionized water, placed in tap water for five minutes, dipped in acid ethanol 8-12 times, placed in
deionized water again and stained with Eosin for 30 seconds. Tissue sections were then dehydrated up the ethanol gradient, immersed in xylene, then coverslipped with mounting medium and left to set in a biosafety cabinet. Hematoxylin is used to stain nuclei blue and eosinophilic structures pink. The trichrome stain differentially colours erythrocytes orange, muscles red, and collagen blue.

2.3.4 Immunohistochemistry (IHC)

All IHC was conducted by the Pathology Lab of the Centre for Modeling Human Disease at the Toronto Center for Phenogenomics (TCP). Five different antibodies were used in this thesis: CD34 (Abcam #ab8158), Pancytokeratin (Dako #Z0622), CD45 (Abcam #ab10558), F4/80 (Abcam #ab6640), and VEGF (NeoMarkers #RB-222-P1). CD34 is found on capillary endothelial cells and is also associated with hematopoietic progenitor cells and is a differentiation stage-specific leucocyte antigen. Pancytokeratin is a stain that picks up most cytokeratins. Cytokeratins are proteins of keratin-containing intermediate filaments found in the intracytoplasmic cytoskeleton of epithelial tissue. In the mouse placenta, trophoblast and trophoblast-derived cells stain positive for cytokeratin. The CD45 antibody stains all cells of hematopoietic origin, except erythrocytes, and the F4/80 antibody reacts with the F4/80 antigen, which is a macrophage-restricted cell surface glycoprotein.

2.3.5 In situ hybridization (ISH)

*Vegf* ISH was conducted by Dr. Vera Eremina in Dr. Sue Quaggin’s lab. Dr. Quaggin’s lab has created an in-house probe for detecting *Vegf* mRNA therefore their expertise was used to help us confirm that our transgenic mouse model was expressing *Vegf* in the same cells as a normal WT mouse.

2.4 Real Time RT-PCR (qRT-PCR)

2.4.1 RNA Extraction

On day one, tissue was crushed into a fine powder in liquid nitrogen using a mortar and pestle. Once in powder form, the samples were placed into a new tube and kept on dry ice. Samples were homogenized in 1ml of TRIzol and placed on ice for 15 minutes. 200 µl of chloroform was then added to the sample. Samples were spun for 15
minutes and the top aqueous phase was isolated into a new eppendorf. 500 µl of isopropanol was then added and the sample was placed into the freezer overnight. On the following day, the samples were spun and the RNA pellet was isolated from the isopropanol solution. The pellet was washed with 70% ethanol and spun again for 10 minutes to pellet the RNA. The ethanol was then vacuumed out and the pellet was suspended in 5 µl of RNase-free water. An RNase-Free DNase kit from Qiagen was then used to remove DNA from the sample. Following the removal of DNA, RNA cleanup was conducted using the RNeasy MinElute cleanup kit from Qiagen. Once RNA was isolated, the quality of the sample was measured via nanodrop.

2.4.2 Reverse Transcription (RNA → cDNA)

1 µg of RNA was diluted in 10 µl of deionized water. 9.7 µl of a master mix from TaqMan Reverse Transcription Reagents was then added to the mix and the reverse transcription reaction (25° for 5min, 42° for 30min, 95° for 5 min, and hold at 4°) was run on a Mastercycler from Eppendorf.

2.4.3 Quantitative Real Time-PCR (qRT-PCR)

The cDNA was diluted with deionized water and a master mix was then made up containing SYBER Green, Vegf primers (Table 2.2), and water. To each PCR plate well, 20 µl of the master mix and 5ul of the cDNA was added. The PCR transcription reactions were then run.

2.5 ELISA

2.5.1 Protein Extraction

Tissue was crushed into a fine powder in liquid nitrogen using a mortar and pestle. Once in powder form, the samples were placed into a new tube and kept on dry ice. RIPA+++ (as in Appendix 2) was added to each sample (200 µl per 0.1 g, with a minimum of 200 µl added) and homogenized for 20 seconds on regular ice. The samples were kept in ice for 45 minutes to allow the bubbles to dissipate. The samples were then spun in for 15 minutes at 4 °C. Aliquots were made and they were stored at -80 °C.
<table>
<thead>
<tr>
<th>Total Vegf Forward</th>
<th>Total Vegf Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ GAG CAG AAG TCC CAT GAA GTG 3’</td>
<td>5’ TGT CCA CCA GGG TCT CAA TC 3’</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>β-actin Forward</th>
<th>β-actin Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ TCG TGC GTG ACA TCA AAG AGA 3’</td>
<td>5’ GAA CCG CTC GTT GCC AAT A 3’</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gapdh Forward</th>
<th>Gapdh Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ AGG AGT AAG AAA CCC TGG ACC 3’</td>
<td>5’ AGG CCC CTC CTG TTA TTA TGG 3’</td>
</tr>
</tbody>
</table>

**Table 2.2** – Vegf, β-actin, and Gapdh primers used for qRT-PCR.
2.5.2 Protein Quantification – Bradford Assay

A BSA standard curve in deionized water was created. In a 96-well microplate, 10 µl of the standard or sample was pipetted into each well. Samples and standards were done in duplicates, and 10 µl of deionized water was used as a blank. After all the samples and standards were loaded, 200 µl of the Bradford reagent (Diluted 1:5 with deionized water) was added to each well. The plate was incubated for 5 minutes at room temperature and absorbance was then measured at 595 nm.

2.5.3 ELISA for VEGF\textsubscript{120/164}

Mouse VEGF Quantikine ELISA kits from R&D systems (Cat# MMV00) were used to quantify free VEGF\textsubscript{120/164} protein. The assay was carried out according to the procedures manual. Values given in the results represent protein concentration and are expressed in pg/ml in plasma and pg/mg of total protein in tissue.

2.5.4 ELISA for VEGF\textsubscript{165b}

Human VEGF\textsubscript{165b} DuoSet ELISA assays from R&D systems (Cat# DY3045) were used to quantify VEGF\textsubscript{165b} protein. The assay was carried out according to the procedures manual. Values given in the results represent protein concentration and are expressed in pg/mg of total protein in tissue.

2.5.5 ELISA for sVEGFR1 (sFlt1)

The mouse sVEGFR1 Quantikine ELISA kit from R&D systems (Cat# MVR100) was used to quantify sFlt1 protein in plasma in the maternal circulation. The assay was carried out according to the procedures manual. Values given in the results represent protein concentration and are expressed in pg/ml in plasma.

2.6 Statistical Analysis

Statistical tests were carried out using GraphPad Prism 5.0 software (San Diego, CA, USA). Analysis tests performed for experiments include an unpaired t-test, and One-way ANOVA for multiple groups with a Tukey post-hoc test. Statistical significance was defined as p<0.05.
Chapter 3

3  Results - VEGF Expression during Normal pregnancy

3.1  Overview

To determine changes in VEGF expression during pregnancy in mice, maternal and fetal circulating levels of VEGF$_{120/164}$ and its soluble receptor, sFlt1 were measured. Maternal organs and placental tissue were then examined for VEGF expression to determine the possible sources and/or functions of increases observed. Maternal organs were collected from non-pregnant (NP) and pregnant female CD1 mice aged 8-12 weeks to profile gestational changes in VEGF expression. Non-pregnant mice were used without controlling for the estrous cycle. Placental tissue was also collected from pregnant female CD1 mice at three time points: E9.5, when umbilical blood flow begins to the placenta; E13.5, mature placental regions are formed and labyrinth growth slows; and E17.5, near term. Tissue was used for Vegf mRNA and VEGF protein expression.

The overall goal of the following experiments was to profile changes in expression of VEGF in select maternal organs and the placenta during normal pregnancies in mice.

3.2  Methods

Non-pregnant CD1 females and pregnant CD1 females bred with CD1 males were used for blood collection. Females were aged 8-12 weeks and were in their first pregnancy. Once plasma was obtained (see sections 2.2.3 and 2.2.4), free VEGF$_{120/164}$ and sFlt1 protein were quantified using ELISAs (see sections 2.5.3 and 2.5.5). Maternal E13.5 and E17.5 plasma samples were diluted 2x to obtain concentrations that were on the standard curve for the VEGF assay. It was not necessary to dilute maternal non-pregnant, E9.5, and fetal plasma samples. For sFlt1 analysis, maternal non-pregnant, E9.5, and fetal plasma samples were diluted 10x, and E13.5 and E17.5 samples were diluted 40x to get a reading on the curve for the assay.
After blood collection, maternal organs and placental samples were collected from the same non-pregnant and pregnant CD1 female mice. Tissue was collected, weighed, then snap-frozen for later mRNA and protein analysis. It should be noted that whole placentas collected for organ weight had some maternal decidua attached, whereas whole placenta samples for mRNA and protein quantification had the decidua carefully removed as much as possible. Total Vegf mRNA was measured and quantified by Dr. Shannon Bainbridge using qRT-PCR (see sections 2.4). VEGF_{120/164} and VEGF_{165} protein was quantified using VEGF ELISAs (see section 2.5).

Cellular location of Vegf mRNA expression was determined using Vegf^{hi} mice (see section 2.1 and 2.2.1) at non-pregnant and pregnant (E9.5, E13.5, and E17.5) states. Placental expression of Vegf was observed by mating CD1 females with Vegf^{hi} males. Cellular location of Vegf mRNA in maternal organs was observed using Vegf^{hi} females (for non-pregnant state) and during pregnancy by mating CD1 males with Vegf^{hi} females.

3.3 VEGF_{120/164} and sFlt1 Expression in Maternal and Fetal Plasma

3.3.1 Rationale

Measureable amounts of free, biologically active VEGF are not consistently reported in maternal plasma in human pregnancy. Some studies report a decline in early pregnancy (Vuorela-Vepsalainen et al., 1999; Levine et al., 2004; Molskness et al., 2004), and others report undetectable amounts when assessed near term (Maynard et al., 2003; Tsatsaris et al., 2003; Levine et al., 2004; Wikstrom et al., 2007). However, total VEGF concentrations in plasma increase during pregnancy (McKeeman et al., 2004) as do sFlt1 concentrations (Levine et al., 2004); but the source and function of these increases is relatively unknown. The first part of my study was conducted to determine whether free VEGF and free sFlt1 increase in maternal plasma during pregnancy in CD1 mice as in prior reports on CD1 and C57BL/6 mice (Koyama et al., 2006; Zhou et al., 2007; Lu et al., 2007). Fetal blood was also collected near term to determine if VEGF and/or sFlt1 protein was differentially regulated in the maternal or fetal circulation during pregnancy in mice.
3.3.2 Results

Maternal free VEGF₁₂₀/₁₆₄ protein in plasma increased significantly at E13.5 compared to non-pregnant and E9.5 levels. Levels then increased again at E17.5 (Figure 3.1). Free VEGF₁₂₀/₁₆₄ in fetal plasma at E17.5 was similar to that of non-pregnant female adults. Free sFlt1 protein in the maternal circulation (Figure 3.1) also increased significantly at E17.5 relative to non-pregnant and E9.5 levels; and sFlt1 expression at E17.5 in the fetal circulation was also similar to that of the non-pregnant female adults. Significance (p<0.05) from one-way ANOVA is indicated by different letters above the data.

3.4 Tissue VEGF Expression during Pregnancy

3.4.1 Rationale

Increases in free VEGF₁₂₀/₁₆₄ were witnessed in the maternal circulation with advancing gestation, but the source(s) or function(s) of this elevated VEGF₁₂₀/₁₆₄ are relatively unknown. It is believed to be of placental origin since circulating levels decrease post-delivery (McKeeman et al., 2004), but this is yet to be confirmed. Therefore, the aim of this study was to determine which organs displayed the greatest increases in VEGF₁₂₀/₁₆₄ expression during pregnancy, as a cross-gestational profile has not been done before. Our conjecture was that the organs with the greatest increases in VEGF protein levels would be most likely to contribute to the increase in VEGF seen in the maternal circulation. Vegf mRNA quantification was used to indicate which organs specifically may be responsible for the increased VEGF protein production during pregnancy. Vegf<sup>hi</sup> mice were used to determine cellular location of Vegf mRNA expression as in prior work (Maharaj et al., 2006).

To determine which of the many maternal organs to focus on, we considered which organs are believed to play a role in pregnancy and/or pre-eclampsia (Table 1.2). Organ weights were also recorded during pregnancy to help determine which organs were most affected by pregnancy. We speculated that increases in organ weight would occur in those organs playing an increased functional role during pregnancy, and that growing organs would require a growth in their vasculature and an increase in blood flow.
Figure 3.1 – VEGF$_{120/164}$ and sFlt1 Protein in the maternal and fetal circulations. (A) Significant increases in maternal free VEGF$_{120/164}$ are seen at E13.5 and again at E17.5 compared to NP and E9.5 levels. Fetal VEGF$_{120/164}$ expression is similar to that of the NP mother. (B) Significant increases in maternal sFlt1 are seen at E17.5 relative to NP and E9.5 levels. Fetal sFlt1 expression is similar to that of the NP mother. Significance (p<0.05) from one-way ANOVA is indicated by different letters above the data. N’s are indicated by the numbers below the time points.
3.4.2 Results

3.4.2.1 Maternal Organ and Placental Weights during Pregnancy

Mouse body weight increased during pregnancy. Similarly, significant increases in lung, heart, and liver weights were observed in the pregnant dam. Placenta weight was also observed to increase between E13.5 and E17.5. Although spleen weight significantly increased at E9.5 and E13.5 relative to non-pregnant values, weight then decreased to non-pregnant values at E17.5. Skeletal muscle (gastrocnemius muscle) from the lower hind leg was decreased in weight during pregnancy. There were no significant changes in organ weights for the brain, kidney, ovaries (left or right), or small intestine during pregnancy (Figure 3.2).

3.4.2.2 Vegf mRNA in Maternal Organs

Increases in Vegf mRNA

qRT-PCR showed that in the kidney (Figure 3.3), a significant increase in Vegf mRNA expression occurred at E9.5 relative to non-pregnant levels. Intermediate levels, not significantly different than non-pregnant or E9.5, were observed later in gestation. Results suggested that podocytes of the glomerulus as well as the epithelial cells of the proximal and distal convoluted tubules expressed Vegf mRNA in the kidney. This was based on cell morphology, and on results of a prior report examining Vegf expression in non-pregnant mice (Maharaj et al., 2006).

The spleen (Figure 3.4) also showed increased Vegf mRNA but at a different time point during pregnancy. Expression was significantly increased at E17.5 relative to non-pregnant levels. Histology showed that, based on morphology, the cells around the central artery expressed Vegf mRNA, as well as the endothelial cells of the splenic artery.

No Changes in Vegf mRNA

The ovary (Figure 3.5) had no significant change in Vegf mRNA expression. LacZ histology showed that there were multiple cell types expressing Vegf mRNA in the ovary. The corpus luteum was the primary site of expression, but the granulosa cells of the antral follicle, theca cells, and stromal cells also expressed Vegf mRNA. This was based on cell
Figure 3.2 – Maternal and placental organ weights during pregnancy. (A) Maternal organ weights during pregnancy. As mouse body weight increased, significant increases in liver, heart, and lung weights were seen at E17.5 compared to their respective non-pregnant values. Spleen weight increased at E9.5 and remained elevated at E13.5, but weight decreased to non-pregnant values by E17.5. The skeletal muscle, brain, kidney, ovaries (left and right) and small intestine showed no changes in weight during pregnancy. (B) The mature placenta with decidua increased in weight between E13.5 and E17.5. Significance (p<0.05) is indicated by different letters above the error bars (one-way ANOVA in A and t-test in B). All organs and time points have an N=5.
Figure 3.3 – VEGF in the maternal kidney. (A) Vegf mRNA expression significantly increased at E9.5 relative to NP levels. Expression did not change after that until term. (B) VEGF<sub>120/164</sub> protein did not significantly change throughout pregnancy. (C) VEGF<sub>165b</sub> protein expression did not change significantly throughout pregnancy. Significance in A is indicated by different letters above the error bars (one-way ANOVA, p<0.05). N’s are indicated by the numbers below the time points. (D) LacZ (blue) stain indicates Vegf mRNA expression. Expression appeared to be localized to endothelial cells of podocytes (arrow) and epithelial cells of proximal and distal convoluted tubules (star) based on cell morphology and prior work (refer to Marahaj et al., 2006). Scale bar in D represents 50 μm. Background stain was Neutral Red. LacZ histology was obtained from Vegf<sup>hi</sup> females mated with CD1 males.
Figure 3.4 – VEGF in the maternal spleen. (A) A significant increase in *Vegf* mRNA was seen at E17.5 compared to NP expression. (B) VEGF$_{120/164}$ protein expression paralleled that of mRNA, as a significant increase at E17.5 was also seen relative to NP levels. (C) No significant change in VEGF$_{165b}$ protein expression was observed during pregnancy. Significance in A and B is indicated by different letters above the error bars (one-way ANOVA, p<0.05). N’s are indicated by the numbers below the time points. (D,E) LacZ (blue) stain indicates *Vegf* mRNA expression. *Vegf* mRNA expression in the endothelial cells of the splenic artery (arrow) and central artery (arrow head) are observed consistently in the spleen throughout pregnancy. Scale bars shown in D and E represent 50 µm. Background stain was Neutral Red. LacZ histology was obtained from Vegf$^{hi}$ females mated with CD1 males.
Figure 3.5 – VEGF in the maternal ovary. (A) No significant changes in Vegf mRNA were observed in the ovary during pregnancy. (B) A significant increase in VEGF$_{120/164}$ protein was observed from E9.5 to E13.5, and levels remained elevated near term. (C) Significant decreases in VEGF$_{165}$b were witnessed between NP and E13.5 and levels remained low near term. Significance is indicated by different letters above the error bars (one-way ANOVA, p<0.05). N’s are indicated by the letters below the time points. (D,E) The distribution of LacZ (blue) stain indicated that Vegf mRNA was expressed in the corpus luteum (arrow in D) and granulosa cells (arrow head in E) based on cell morphology. This distribution was observed consistently throughout pregnancy. Background stain was Neutral Red. LacZ histology was obtained from Vegf$^{hi}$ females mated with CD1 males. Scale bars represent 300 µm in D and 100 µm in E.
morphology, and on results of a prior report examining Vegf<sup>hi</sup> expression in non-pregnant mice (Maharaj et al., 2006).

Liver (Figure 3.6) Vegf mRNA did not show any changes with gestation. Morphology as well as previous LacZ results in non-pregnant mice (Maharaj et al., 2006) suggested that hepatocytes were the primary cell type expressing Vegf mRNA in the liver.

The decidua (Figure 3.7) also did not change in Vegf mRNA expression throughout pregnancy when compared to the non-pregnant endometrium. Morphology from LacZ histology showed that Vegf was expressed by endothelial cells and decidual cells at E9.5. Morphology indicated that decidual and endothelial cells lining maternal blood vessels expressed Vegf mRNA at E13.5 (Figure 3.7).

**Vegf-LacZ Histology Only**

Based on morphology and previous studies in non-pregnant mice (Maharaj et al., 2006), type two alveolar epithelial cells appear to express Vegf mRNA in the lung (Figure 3.8), and cardiomyocytes expressed Vegf in the heart (Figure 3.9). Vegf mRNA was not quantified in these organs.

In the maternal organs examined, with the exception of the developing decidua, the same cells consistently expressed Vegf mRNA throughout pregnancy (e.g. corpus luteal cells were consistently primary expressers of Vegf mRNA in the ovary).

### 3.4.2.3 Vegf mRNA in the Placenta

In the whole placenta (Figure 3.10), Vegf mRNA was highest at E9.5. Expression decreased at E13.5 relative to E9.5. Vegf<sup>hi</sup> histology at E9.5 showed the allantois and chorion expressing Vegf mRNA. Primary trophoblast giant cells in the anti-mesometrial side were also positive for Vegf mRNA expression (Figure 3.11). The mature placenta (E13.5 and E17.5) (Figure 3.12) was looked at as a whole and microdissected to enrich for the junctional zone, labyrinth, and chorionic plate regions. When compared to the whole placenta at E9.5, the junctional zone (Figure 3.13) showed increased Vegf mRNA expression by E17.5, whereas the labyrinth (Figure 3.14) and chorionic plate (Figure 3.15) regions however showed no significant changes in Vegf expression.
Figure 3.6 - VEGF in the maternal liver. (A,B,C) No significant changes in Vegf mRNA or VEGF_{120/164} protein were observed during pregnancy. VEGF_{165b} protein expression decreased at E17.5 relative to E13.5 levels. Significance was tested for with one-way ANOVA. N’s are indicated by the numbers below the time points. (D) LacZ (blue) stain indicates Vegf mRNA expression in the non-pregnant liver of a Vegf^{hi} female mouse. Expression was primarily in hepatocytes (arrow), which are surrounding the central vein (star). Scale bar shown represents 100 μm. Background stain was Neutral Red.
Figure 3.7 – VEGF in the decidua. (A) No significant changes in Vegf mRNA were observed in the decidua during pregnancy relative to the NP endometrium. (B) A significant increase in VEGF_{120/164} protein was observed in the decidua at E9.5 relative to NP levels. At E13.5 and E17.5, VEGF_{120/164} protein expression was significantly lower than at E9.5 and the NP endometrium. (C) VEGF_{165b} protein expression was highest in the NP endometrium. Levels then decreased significantly at E9.5 and remained low until term. Significance is indicated by different letters above the error bars (one-way ANOVA, p<0.05). N’s are indicated by the numbers below the time points. (D,E,F) E9.5 conceptus with LacZ (blue) stain indicating Vegf mRNA expression. (D) The decidua expressed Vegf mRNA around the ectoplacental cone/chorion. The anti-mesometrial side also heavily expressed Vegf mRNA. (E) A clear boundary between decidual and placental cells expressing Vegf mRNA was observed. Morphology suggested that endothelial cells (star in E) and decidual cells (blue stain in F) expressed Vegf mRNA. (G) Wholemount LacZ staining of the placenta and decidua at E13.5. (H) Vegf mRNA is expressed by endothelial cells around maternal blood vessels at E13.5 (arrow in H) and by decidual cells (arrow in I). Scale bars shown represent 200 µm (E) 100 µm (F), 100 µm (H) and 50 µm (I). The background stain was Neutral Red. Vegf-LacZ histology was obtained from Vegf^{hi} females mated with CD1 males.
Figure 3.8 - VEGF in the maternal lung. No significant changes in (A) VEGF<sub>120/164</sub> or (B) VEGF<sub>165b</sub> protein expression were observed during pregnancy. Significance was tested for by one-way ANOVA. N’s are indicated by the numbers below the time points. (C) LacZ (blue) stain indicates Vegf mRNA expression. Previous studies (Maharaj et al., 2006) suggested that Vegf mRNA was expressed by type 2 alveolar epithelial cells (arrow). Scale bar shown in C represents 100 µm. Background stain was Neutral Red. Vegf-LacZ histology was obtained from Vegf<sup>hi</sup> females mated with CD1 males.
Figure 3.9 - VEGF in the maternal heart. No significant changes in (A) VEGF{sub}120/164 or (B) VEGF{sub}165b protein expression were observed during pregnancy. Significance was tested for with one-way ANOVA. N’s are indicated by the numbers below the time points. (C) LacZ (blue) stain indicates Vegf mRNA expression at E9.5. Morphology and previous studies suggested that Vegf mRNA expression was found in cardiomyocytes (arrow). Scale bar shown in C represents 50 μm. Background stain was Neutral Red. Vegf-LacZ histology was obtained from Vegf{sup}hi females mated with CD1 males.
Figure 3.10 – VEGF in the whole placenta. (A) A significant decrease in Vegf mRNA was observed in the placenta at E13.5 when compared to the median Vegf mRNA expression at E9.5. Intermediate expression was observed near term. (B,C) Significant decreases in VEGF_{120/164} and VEGF_{165} protein were also observed from E9.5 to E13.5, where protein levels remained low until near term. Significance is indicated by different letters above the error bars (one-way ANOVA, p<0.05). N’s are indicated by the numbers below the time points.
Figure 3.11 - VEGF-LacZ histology in the placenta at E9.5 established by mating CD1 females with Vegfhi males. (A) Wholemount LacZ picture of E9.5 conceptus. Embryo was removed to get a better view of the cells expressing Vegf mRNA (blue stain). (B) H&E-LacZ of E9.5 conceptus. The decidua and ectoplacental/chorion region are visible above the embryo. (C) Primary trophoblast giant cells (identified by brown staining from cytokeratin IHC) in the anti-mesometrial side expressed Vegf mRNA (blue colour in nuclei). (D) Ectoplacental/chorion region of the conceptus at E9.5. LacZ expression was observed in the chorion (E) as well as the yolk sac membrane (F). Background was stained with Hematoxylin and Eosin. (G) Negative control (WT littermate) placenta at E9.5 had no visible LacZ expression. Scale bars shown represent 1 mm (B), 50 µm (C), 200 µm (D), and 20 µm (E,F). EC = ectoplacental cone, E = embryo.
Whole Placenta at E13.5

A – E13.5

B – E13.5

C – E13.5

D – E13.5
Figure 3.12 - VEGF-LacZ histology in the mature placenta established by mating CD1 females with Vegf<sup>hi</sup> males. (A) Wholemount LacZ stain of the whole placenta at E13.5. Vegf mRNA (blue stain) was expressed in the junctional zone (*) and labyrinth (**). Staining in the decidua (arrow) is artifactual (see E). (B) Negative control (WT littermate) placenta at E13.5. No visible LacZ staining was observed in the chorionic plate, labyrinth (**), or junctional zone (*). Non-specific staining was observed in the decidua (arrow), which was also observed in wholemount Vegf<sup>hi</sup> placenta (arrow in A). (C) Histology of negative control (WT littermate) placenta at E13.5. No staining was observed in the chorionic plate, labyrinth or junctional zone. Background stain was Neutral Red. (D) VEGF immunohistochemistry showing VEGF protein expression (pink) in the junctional zone (*) and labyrinth (**).
Junctional Zone

A  Total Vegf mRNA

B  VEGF_{120/164} Protein

C  VEGF_{163b} Protein

D  – E17.5 VEGF^{hi}

E  – E17.5 VEGF^{hi} WT
Figure 3.13 – VEGF in the junctional zone. (A) A significant increase in Vegf mRNA was observed at E17.5 relative to the whole placenta at E9.5. (B,C) Significant decreases in VEGF$_{120/164}$ and VEGF$_{165b}$ protein were observed at E13.5 relative to the whole placenta at E9.5. Levels remained low near term as well. Significance is indicated by different letters above the error bars (one-way ANOVA, p<0.05). N’s are indicated by the numbers below the time points. (D) LacZ histology of the junctional zone at E17.5. Vegf mRNA (blue stain and arrow) was expressed by spongiotrophoblast and glycogen cells. (E) Negative control (WT littermate) placenta at E17.5 stained with beta-galactosidase. No LacZ expression was observed in the junctional zone. Scale bars represent 50 µm in D and E. Backgrounds stain was Tri-chrome. Vegf-LacZ histology was obtained from CD1 females mated with Vegf$^{hi}$ males.
A  Total *Vegf* mRNA

B  VEGF<sub>120/164</sub> Protein

C  VEGF<sub>165b</sub> Protein

D – E17.5 VEGF<sup>hi</sup>

E – E17.5 VEGF<sup>hi</sup> WT
**Figure 3.14 - VEGF in the labyrinth.** (A) No significant change in *Vegf* mRNA was observed in the labyrinth when compared to the whole placenta at E9.5. (B,C) Significant decreases in VEGF$_{120/164}$ and VEGF$_{165b}$ protein were observed at E13.5 relative to the whole placenta at E9.5. Levels remained low near term as well. Significance is indicated by different letters above the error bars (one-way ANOVA, p<0.05). N’s are indicated by the numbers below the time points. (D) LacZ histology of the labyrinth at E17.5. Based on morphology, *Vegf* mRNA (blue stain with arrow) appeared to be expressed primarily by endothelial cells. Hematoxylin was used as a background stain. (E) Negative control (WT littermate) stained with beta-galactosidase. No visible LacZ expression was observed. Scale bars represent 50 µm in D and E. Hematoxylin was used as a background stain. *Vegf*-LacZ histology was obtained from CD1 females mated with *Vegf* hi males.
Figure 3.15 – VEGF in the chorionic plate. (A) No significant change in Vegf mRNA was observed in the chorionic plate when compared to the primitive placenta at E9.5. (B) Increased VEGF_{120/164} protein was observed between E13.5 and E17.5 in the chorionic plate. (C) A significant increase in VEGF_{165b} protein expression was observed in the chorionic plate between E9.5 and E13.5, where levels remained elevated until near term. Significance is indicated by different letters above the error bars (one-way ANOVA, p<0.05). N’s are indicated by the numbers below the time points. (D,E) LacZ histology of the chorionic plate at E17.5. Vegf mRNA (blue stain) is expressed by cells near blood spaces (D) and endothelial cells (E) indicated by the arrows. (F, G) Negative control (WT littermate) chorionic plates at E17.5. No visible LacZ staining was observed in the chorionic plate. Hematoxylin and Eosin (D, F) and hematoxylin (E,G) were used as a background stains. Vegf-LacZ histology was obtained from CD1 females mated with Vegf^{hi} males.
LacZ histology showed that spongiotrophoblast cells are the primary cell type expressing \( \text{Vegf} \) mRNA in the junctional zone at E17.5 (Figure 3.13). LacZ expression was observed in the glycogen trophoblast cells, but it was not as prominent as the spongiotrophoblast cells. LacZ was not observed in the parietal trophoblast giant cells that are located in the junctional zone next to the deciduas (not shown). In the labyrinth (Figure 3.14), a punctate pattern of expression consistent with previous findings (Breier et al., 1992) was observed. We could not identify all the cell types expressing \( \text{Vegf} \), but immunohistochemistry showed that endothelial cells, highlighted by the CD34 antibody, appeared to be the primary expressing cell type (Figure 3.16). No obvious relationship between the pattern of LacZ expression and sinusoidal trophoblast giant cells (Cytokeratin antibody; Figure 3.16), Hofbauer cells (F4/80 antibody; Figure 3.17), or hematopoietic cells (CD45 antibody, Figure 3.17) was observed. In the chorionic plate (Figure 3.14), \( \text{Vegf} \) mRNA did not significantly change during pregnancy. Morphology from LacZ histology showed that \( \text{Vegf} \) was expressed by endothelial cells or cells near blood spaces.

It should be noted that unlike maternal organs, different cell types expressed \( \text{Vegf} \) mRNA as the placenta matured. This can be explained by the fact that unlike maternal organs, which are already mature before pregnancy, different cell types inhabit the placenta as it matures.

LacZ histology was conducted using \( \text{Vegf}^{\text{hi}} \) mice, and these mice are abnormal as they over-express VEGF (Miquerol et al., 1999). To determine whether the pattern of \( \text{Vegf} \) mRNA expression in the placenta was normal, in situ hybridization (ISH) was conducted on placenta at E9.5 and E13.5, and the kidney, which was used as a positive control. Vegf-ISH showed similar patterns of \( \text{Vegf} \) mRNA expression in the kidney, E9.5 placenta, and E13.5 placenta as the Vegf-LacZ samples (Figure 3.18). Morphology suggested that the kidney expressed \( \text{Vegf} \) mRNA in the podocytes of the glomerulus. Placental expression was observed in primary trophoblast giant cells at E9.5, spongiotrophoblast cells in the junctional zone at E13.5, and cells near blood spaces in the labyrinth at E13.5. Thus, both methods (LacZ and ISH) showed the same pattern of \( \text{Vegf} \) mRNA expression in the placenta and kidney.
CD34 and Cytokeratin Immunohistochemistry of the Labyrinth

Figure 3.16 – Labyrinths of E17.5 placentas stained with (A) CD34 and (B) Cytokeratin. LacZ (blue/green dots) indicates Vegf mRNA. CD34 stains primarily for endothelial cells (brown) and cytokeratin indicates presence of trophoblast cells (brown). (A) CD34 IHC stains primarily for endothelial cells (brown). Co-expression suggested that some Vegf expressing cells were endothelial (arrow). (B) Cytokeratin stain showed that sinusoidal trophoblast giant cells (indicated by red *) in the labyrinth do not express Vegf mRNA, but some cells of trophoblast lineage do (arrows). Scale bar in (A) represents 10µm and in (B) 20µm. Vegf<sup>hi</sup> histology was performed on tissue from CD1 females mated with Vegf<sup>hi</sup> males.
Figure 3.17 – Labyrinths of E17.5 placentas stained with F4/80 and CD45. (A,C) Labyrinths and (B,D) deciduas of E17.5 Vegf<sup>hi</sup> placentas stained by IHC for (A,B) F4/80 and (C,D) CD45. LacZ (blue dots) indicate Vegf mRNA. F4/80 stains for placental macrophages (brown) and CD45 stains for hematopoietic cells (brown). Cells expressing LacZ in the labyrinth were not positive for placental macrophages or hematopoietic cells. Positive controls (decidua samples) are given on the right. Scale bars in A and B represent 50 µm. Vegf<sup>hi</sup> histology was performed on tissue from CD1 females mated with Vegf<sup>hi</sup> males.
Vegf-ISH in the Placenta and Kidney

A – E9.5

B – E13.5

C – E13.5

D – E13.5

E – E13.5
Figure 3.18 – ISH of Vegf mRNA expression in the placenta and kidney. (A) Primary trophoblast giant cells (arrow) expressed Vegf mRNA at E9.5. (B) Whole placental Vegf mRNA expression in the junctional zone (*) and labyrinth (**). (C) Cells near blood spaces express Vegf in the labyrinth (arrow) and (D) spongiotrophoblast cells appeared to express Vegf in the junctional zone (arrow). (E) Morphology suggests that the glomerulus (arrow) of the kidney expressed Vegf mRNA. Blue stain indicates Vegf mRNA expression. The background stain was Neutral Red and tissue was obtained from CD1 females mated with CD1 males. Scale bars represent 100 µm in A, 1 mm in B, and 50 µm in C-E.
3.4.2.4 VEGF\textsubscript{120/164} Protein in Maternal Organs

The concentration of VEGF\textsubscript{120/164} protein increased in the maternal circulation during pregnancy. To determine the possible sources for this increase, examining Vegf mRNA does not suffice because transcription does not always correlate with translation. With this in mind, we decided to determine the changes in VEGF\textsubscript{120/164} protein in the organs examined. VEGF\textsubscript{120/164} protein was expressed per mg of total protein.

**Increased VEGF\textsubscript{120/164} Protein**

**Ovarian** (Figure 3.5) VEGF\textsubscript{120/164} protein increased during pregnancy despite constant expression of total Vegf mRNA. A 7-fold increase at E13.5 relative to non-pregnant and E9.5 levels was observed, and expression remained elevated at E17.5. VEGF\textsubscript{120/164} protein in the spleen (Figure 3.4) also increased during pregnancy, 65% at E17.5 relative to non-pregnant levels, matching the time course observed by Vegf mRNA, but contrasting with organ weight which peaked at E13.5. In the brain (Figure 3.19), a significant increase was observed in VEGF\textsubscript{120/164} protein between E9.5 and E13.5, but expression did not change after that. The decidua (Figure 3.7) showed a biphasic pattern of expression. A 6-fold increase in VEGF\textsubscript{120/164} protein at E9.5 relative to the non-pregnant endometrium occurred and then expression regressed to below NP levels at E13.5 and remained low until near term.

**No Changes in VEGF\textsubscript{120/164} Protein**

**Kidney** (Figure 3.3), liver (3.6), lung (Figure 3.8), and heart (Figure 3.9) samples showed no significant changes in VEGF\textsubscript{120/164} protein expression during pregnancy.

3.4.2.5 VEGF\textsubscript{120/164} Protein in the Placenta

Similar to Vegf mRNA, VEGF\textsubscript{120/164} protein expression was highest in the primitive placenta at E9.5 (Figure 3.10). Compared to the E9.5 placenta, expression in the whole placenta decreased 16-fold by E13.5 and remained low near term. In the enriched regions, the labyrinth (Figure 3.14) expressed similar VEGF protein levels to the junctional zone (Figure 3.13) at E13.5 and E17.5, but both showed decreased VEGF\textsubscript{120/164} protein expression when compared to the primitive placenta at E9.5. The chorionic plate expressed high amounts of VEGF\textsubscript{120/164} protein relative to the labyrinth and junctional
Figure 3.19 - VEGF in the maternal brain. A) A significant increase in VEGF_{120/164} protein was observed at E13.5 compared to E9.5, but no significant changes were observed at other time points. B) No changes in VEGF_{165b} protein were observed during pregnancy. Significance is indicated by different letters above the error bars (one-way ANOVA, p<0.05). N’s are indicated by the numbers below the time points.
Figure 3.20 – Cross-gestational comparison of VEGF$_{120/164}$ protein in the three placental regions. The chorionic plate expressed significantly higher VEGF$_{120/164}$ protein than the labyrinth and junctional zone at E13.5 and E17.5. VEGF$_{120/164}$ protein expression significantly increased in the chorionic plate between E13.5 and E17.5, whereas there were no significant changes in the junctional zone or labyrinth between the two time points. Significance is indicated by the stars above the bars and was determined using a two-way ANOVA. * = p<0.05, **= p<0.001. N=5 for all tissues at all time points.
zone (Figure 3.20), and a significant increase was witnessed between E13.5 and E17.5 (Figure 3.15).

3.5 VEGF\textsubscript{165}b Protein Expression during Pregnancy

3.5.1 Rationale

Through alternative splicing, many isoforms of VEGF are possible. Recently identified was the VEGF\textsubscript{xxx}b isoforms (Bates et al., 2002). Current in vitro (Bevan et al., 2008) and in vivo neo-vascular and tumor growth models have shown that VEGF\textsubscript{165}b is anti-angiogenic (Bates et al., 2002; Konopatskaya et al., 2006; Woolard et al., 2004). Therefore the aim of this study was to characterize the expression of VEGF\textsubscript{165}b in maternal organs and the placenta throughout pregnancy. I anticipate that expression of the anti-angiogenic isoform should decrease in highly angiogenic tissue, such as the ovary and placenta, as pregnancy advances.

3.5.2 Results

3.5.2.1 VEGF\textsubscript{165}b in Maternal Organs during Pregnancy

The ovary (Figure 3.3) showed a significant decrease in VEGF\textsubscript{165}b by E13.5 and E17.5 of gestation. This pattern would be anticipated to augment the effects of the simultaneous increases in VEGF\textsubscript{120/164} protein in the ovary. In the decidua (Figure 3.4), VEGF\textsubscript{165}b protein was markedly decreased throughout gestation relative to the non-pregnant endometrium. Kidney (Figure 3.5), spleen (Figure 3.4), lung (Figure 3.8), heart (Figure 3.9), liver (Figure 3.6) and brain (Figure 3.19) tissue showed no significant changes in VEGF\textsubscript{165}b protein expression throughout pregnancy.

3.5.2.2 VEGF\textsubscript{165}b in the Placenta during Pregnancy

VEGF\textsubscript{165}b protein expression decreased 2-fold in the whole placenta (Figure 3.10) from E9.5 to E13.5, and levels then remained low until near term. The junctional zone (Figure 3.13) and labyrinth (Figure 3.14) also showed similar decreases at E13.5 and E17.5 when compared to the primitive placenta at E9.5. The chorionic plate (Figure 3.15) however showed a significant 2-fold increase in VEGF\textsubscript{165}b protein between E9.5 and E13.5, and levels then remained elevated until near term.
3.6 Summary/Discussion

Free sFlt1 in the maternal circulation increased significantly with advancing gestation in mice, as did free VEGF<sub>120/164</sub> protein. When comparing the two proteins at parallel time-points, it was interesting to find that concentrations of sFlt1 were ~25-150x greater than VEGF<sub>120/164</sub>. sFlt1 and VEGF<sub>120/164</sub> in fetal plasma were comparable to their respective values in non-pregnant female adults, indicating the proteins were preferentially localized in the maternal circulation during pregnancy.

To determine the possible source(s) of increased free VEGF<sub>120/164</sub> protein in the maternal circulation, maternal organs and placental tissues were examined for VEGF mRNA and protein expression. Of the maternal organs examined, the ovary was the only one that showed significant increases in VEGF<sub>120/164</sub> protein matching the time course witnessed in the maternal circulation, and during pregnancy, it expressed higher levels of VEGF<sub>120/164</sub> when compared to the placenta and decidua (Figure 3.21). The ovary also exhibited significant decreases in VEGF<sub>165b</sub> protein. This decrease may promote vascularization in the ovary during pregnancy, and thereby enhance its function. This idea is supported by the literature (Ferrara, 1993; Ferrara, 2004), which shows that intensive proliferation of endothelial occurs in the corpus luteum during early pregnancy (Tamura, 1987). Human studies have also shown that Vegf mRNA and protein in the corpus luteum of pregnant women is higher than during the mid-luteal phase (Sugino et al., 2000; Wulff et al., 2002). The elevated expression of Vegf mRNA and protein during gestation suggests that the initial angiogenic process during the early luteal phase of a non-pregnant female is not enough and that further VEGF and angiogenesis is needed to maintain the functions of the ovary during pregnancy (Kacmarek et al., 2005).

In determining the possible sources of increased VEGF<sub>120/164</sub> witnessed in the maternal circulation, we speculated that growing organs would require a growth in their vasculature and an increase in blood flow, and as a result VEGF<sub>120/164</sub> would play an increased role in those organs during pregnancy. We found however that there was no correlation with increased tissue weight and increased VEGF<sub>120/164</sub> expression. The liver, which showed the greatest increase in weight during pregnancy, expressed stable amounts
Comparison of VEGF_{120/164} Protein
In the Placenta, Decidua, and Ovary

Figure 3.21 – Cross-gestational comparison of VEGF_{120/164} protein in the placenta, decidua, and ovary. At E9.5, the decidua expressed the greatest levels of VEGF_{120/164} protein, and the placenta and ovary contained similar levels. At E13.5 and E17.5 however, the ovary significantly increased in VEGF_{120/164} protein expression and expressed greater amounts compared to the placenta and decidua. VEGF_{120/164} protein expression in the decidua decreased relative to E9.5 and levels were similar to the placenta. Significance is indicated by the stars above the bars and was determined using a two-way ANOVA.

***=p<0.001. N=5 for all tissues at all time points.
of VEGF mRNA and protein. VEGF\textsubscript{165b} expression did decrease significantly at E17.5, but this was after the greatest gain in weight, which had already occurred by E13.5. Heart, lung, spleen, and placental weight also increased in weight during pregnancy, but no correlation was observed with VEGF\textsubscript{120/164} protein expression.

The placenta was the primary suspect for the increased secretion of VEGF\textsubscript{120/164} witnessed in the maternal circulation during pregnancy. However, placental VEGF\textsubscript{120/164} protein decreased with gestation and the mature placenta at E13.5 and E17.5 did not express higher levels of VEGF\textsubscript{120/164} protein when compared to any of the maternal organs examined (Figure 3.22). This was surprising because the placenta is a highly vascularized organ and it becomes even more highly vascular late in gestation (Coan et al. 2004). The chorionic plate expressed greater amounts of VEGF\textsubscript{120/164} protein relative to the labyrinth or junctional zone, but it should be noted that the maternal blood does not perfuse the chorionic plate, therefore it is an unlikely source for VEGF\textsubscript{120/164} found in the maternal circulation. The highest levels of VEGF\textsubscript{120/164} protein in the placenta were found at E9.5, but qRT-PCR and LacZ histology showed that low levels of total Vegf were present. Low Vegf mRNA expression in the ectoplacental cone and chorion suggests that VEGF promoting vascularity in the placenta at E9.5 could be of decidual origin. We found that VEGF\textsubscript{120/164} protein expression was 5-fold higher in the decidua at E9.5 relative to the ectoplacental region, indicating that VEGF protein from the neighbouring decidua may be the source of VEGF protein measured in the placenta at E9.5. The opposite may be true in the decidua after E13.5, when glycogen trophoblast cells from the junctional zone migrate into the decidua (Georgiades et al., 2001). This migration of fetal glycogen cells may contribute to VEGF protein levels in the decidua.

In the whole placenta, VEGF\textsubscript{120/164} expression decreased at a time when vascularity markedly increases (Coan et al., 2004; Rennie et al., 2007). This seems to be contradictory, but there was a significant decrease in the inhibitory isoform, VEGF\textsubscript{165b}, which may have aided in the formation of the vasculature. Overall, decreased expression of VEGF\textsubscript{165b} protein was shown in tissues where increased angiogenesis occurs, such as
Figure 3.22 – Comparison of VEGF_{120/164} and VEGF_{165b} protein across all maternal organs during pregnancy. (A) The placenta, decidua, and maternal ovary were the only organs examined to show changes in VEGF_{120/164} protein during pregnancy. Of those, the ovary was the only organ to show increased VEGF_{120/164} protein at E17.5 relative to its non-pregnant (NP) control. (B) The placenta, decidua, ovary, and liver all decreased in VEGF_{165b} protein expression at E17.5 relative to their NP or E9.5 controls. Significance (p<0.05) was determined by Two-way ANOVA and significance is indicated by stars above the error bars. N=5 for all tissues at all time points.
the decidua, ovary, and placenta. In organs where high level of angiogenesis does not occur during pregnancy, such as the brain, no changes in VEGF<sub>165</sub>b protein were witnessed. I have also shown that the chorionic plate was unusual in that VEGF<sub>165</sub>b protein expression increased during pregnancy. This may be because the chorionic plate is not a significant site angiogenesis after E9.5, unlike the labyrinth region of the placenta. It was also observed that the expression of VEGF<sub>165</sub>b in mice was consistently greater than VEGF<sub>120/164</sub>. For example, my data shows that VEGF<sub>165</sub>b comprised ~67% of the total VEGF in the lung. This trend was also observed in human lung tissue, where VEGF<sub>165</sub>b comprised 60% of total VEGF (Woodlard et al, 2009). In the mouse placenta however, this pattern of expression was not observed. VEGF<sub>165</sub>b made up greater than than 80% the total VEGF protein, while in humans VEGF<sub>165</sub>b made up less than 10% of total VEGF protein (Woolard et al., 2009). This suggests that the pattern of expression of the inhibitory isoform of VEGF may not be the same across species. One caveat of the VEGF<sub>165</sub>b data is that the ELISA assay used to measure the protein was designed for human tissue, and therefore may result in imprecise quantification in mice. The murine VEGF<sub>165</sub>b isoform was not used to generate the calibration curve for the ELISA because this protein is not commercially available.

My results also show that there is often a discrepancy between changes in Vegf mRNA and VEGF<sub>120/164</sub> protein expression during pregnancy (e.g. Vegf mRNA does not change but VEGF<sub>120/164</sub> protein increases in the ovary), and there may be several possible reasons for this. First, the primers were designed to measure total Vegf mRNA. Transcription of specific Vegf isoforms may increase while others were decrease but no overall change in Vegf mRNA expression would be detected. For example, in the ovary we may see no change in Vegf mRNA because, even though Vegf<sub>120/164</sub> mRNA increases, Vegf<sub>165</sub>b mRNA decreases. This would be consistent with the observed increase in VEGF<sub>120/164</sub> and decrease in VEGF<sub>165</sub>b measured by the ELISA assays. Secondly, a discrepancy in mRNA and protein concentrations could suggest 1) that VEGF protein is made somewhere else, thus low mRNA and high protein, or 2) that the VEGF made is mostly of the soluble form, VEGF<sub>120</sub>, therefore we would see high mRNA and low protein levels. For example, my results show that the ovary expresses increasing amounts of free soluble VEGF protein during pregnancy, while Vegf mRNA remains constant. It is possible that the ovary expresses increasing amounts of free VEGF<sub>120/164</sub> protein not
because it is producing more, but rather because it is receiving the soluble isoforms from another nearby organ, such as the placenta, which is known to interact with the ovary during pregnancy (Soares et al., 1998) with hormones such as placental lactogen I and II (Thordarson et al., 1997). Thirdly, there can be discrepancies between Vegf mRNA expression and protein levels due to the translational control of the gene. Translational regulation allows for the fine-tuning of gene expression, and targeting initiation factors, mRNA, and ribosomes are some of the ways to regulate translation (reviewed in Lackner and Bahler, 2008). The general translation of proteins in a cell can be controlled by regulating the formation of the ribosomal complexes; and gene specific translation can be regulated by the use of factors which bind to the 5’ and 3’ untranslated regions (UTRs) of mRNA. MicroRNAs are an example of such factors that can bind to the 3’ UTR of mRNA to suppress or upregulate translation (reviewed in Lackner and Bahler, 2008). Finally, a discrepancy between mRNA and protein expression may exist because the mRNA message may be degraded more quickly, and therefore may not be present in the tissue while the protein still persists.

The reproductive biology of humans and mice is not identical, but similarities do exist between the two mammals. My results confirm prior reports in mice that a similar pattern of expression in maternal circulating sFlt1 occurs between mice and humans during pregnancy (McKeeman et al., 2004). These similarities however are not observed when trying to quantify free VEGF. Free VEGF increases in the maternal circulation during pregnancy in mice, whereas no changes or undetectable amounts are observed in human studies (Levine et al., 2004; Molskness et al., 2004). Nevertheless, similarities between the two animals can be found in the localization of Vegf expression. Mouse kidneys for example primarily express Vegf mRNA in glomerular cells, and this is also witnessed in humans (Simon et al., 1995).

Observing these similarities between mice and humans is important because it will help us to use mice as appropriate models to better understand the pathologies and pathways of certain diseases in humans. In pre-eclampsia for example, one of the symptoms is proteinuria, and this is caused by glomerular dysfunction in the maternal kidney. The VEGF pathway is believed to play a role in this, and studies by Eremina et al. (2003) have shown that reducing VEGF expression in the podocytes of rodent kidneys can result in proteinuria. The ovary is another example where similarities between human
and mouse models occur. In the VEGF pathway, the corpus luteum appears to be the primary site of expression in both species (Kaczmarek et al., 2005).

Overall, my results from this chapter suggest that the maternal ovary is a likely source for the increased VEGF witnessed in the maternal circulation during pregnancy. The evidence is that free VEGF$_{120/164}$ protein expression increases significantly in the ovary during the same time points of increases observed in the maternal circulation. The placenta however should not be ruled out. It may still be a contributing organ according to the following rationale. All analyses were done to normalize VEGF protein expression to milligram of total protein. If we consider the increase in the total amount of VEGF$_{120/164}$ present in the placenta, it will be much greater than the increase in expression by any maternal organ as there are approximately 10-15 growing placentas per normal pregnancy. Examining the data with this in mind, there is a significant increase in total VEGF$_{120/164}$ protein expression in the whole placentas of all conceptuses between E13.5 and E17.5 (Figure 3.23), and the increase in levels is much greater than any maternal organ examined. Additionally, if the placenta produces primarily VEGF$_{120}$, then the protein may be secreted into the circulation and therefore not accumulate in the placental tissue.

In summary, the maternal circulating VEGF$_{120/164}$ levels increase with advancing gestation. The maternal ovary and placenta seem to be prime candidates in contributing to this increase in VEGF protein observed, as they expressed the greatest amounts of VEGF$_{120/164}$ per mg of total protein (ovary) and total VEGF$_{120/164}$ protein (placenta). Whether one or both organs contribute to this increase is still to be determined.
Figure 3.23 – Picograms of VEGF$_{120/164}$ protein from all of the placenta in a pregnancy. A significant increase in VEGF$_{120/164}$ protein expression was observed between E13.5 and E17.5. As placental weight increased, so did the total amount of VEGF$_{120/164}$ protein produced. Significance (p<0.05) was determined by t-test.
4 Results – The effects of VEGF overexpression in the Concepti

4.1 Experimental Overview

To determine if higher than normal Vegf expression in the concepti could influence maternal VEGF levels, CD1 females were crossed with Vegfhi males. This resulted in pregnancies where approximately half of the concepti would be expected to carry the Vegfhi transgene. To verify that the transgene was effective, circulating levels of fetal VEGF120/164 from Vegfhi mutants was compared to that of WT littermates, to determine whether levels were elevated as in a prior report (Miquerol et al., 1999). Prior work also showed that overexpression of Vegf in Vegfhi mutants was not constantly observed in all organs (Miquerol et al., 1999). Therefore, placental tissue was also collected to determine if increased Vegf translation and hence VEGF protein were detected in that organ. Maternal plasma and organs were collected for VEGF120/164 and/or sFlt1 protein analysis by ELISA. Protein levels were compared to that of WT pregnancies at E17.5.

4.2 VEGF120/164 Protein Expression in Fetal Plasma

4.2.1 Rationale

With the objective of showing whether the Vegfhi transgene in certain concepti affects VEGF protein expression in the maternal circulation and/or organs, fetal circulating levels of VEGF120/164 from the Vegfhi mutants and WT littermates had to be tested in order to show there was a difference in VEGF protein levels when the transgene was present compared to the WT littermates. It was expected that circulating VEGF120/164 protein would be higher in the fetal blood of Vegfhi mutants compared to their WT littermates, and that levels of the WT littermates would be similar to that of WT controls (CD1 x CD1).

4.2.2 Methods

Virgin CD1 females, aged 8-12 weeks, were mated with Vegfhi males. No problems were encountered when breeding these two strains together, and an expected
50:50 (WT:Vegf\textsuperscript{hi}) ratio was observed during pregnancies (55:43, p>0.05). Average litter sizes in the CD1xVegf\textsuperscript{hi} were also not significantly different when compared to CD1xCD1 pregnancies (13.6±1.4 vs 13.9±2.1, p>0.05. N= 8 and 10 respectively).

At E17.5, a fetal trunk blood was obtained via decapitation (see section 2.2.4). In order to genotype the fetuses, a sample of fetal tissue was incubated with beta-galactosidase. The presence of VEGF protein overexpression was marked by tissue staining blue due to LacZ expression by the transgene. Once the genotypes were determined, plasma from fetuses of the same genotype in the same litter was pooled. Free VEGF\textsubscript{120/164} protein was quantified using a VEGF ELISA (see section 2.5.3). No dilution of fetal plasma samples was required for samples to be within the standard curve of the ELISA assay. Significance (p<0.05) was determined using a one-way ANOVA.

4.2.3 Results

A significantly higher fetal free VEGF\textsubscript{120/164} protein was observed in the plasma of Vegf\textsuperscript{hi} mutant mice compared to their WT littermates. It was interesting to note however that in the Vegf\textsuperscript{hi} mutants and the WT littermates, VEGF\textsubscript{120/164} protein levels in plasma were significantly lower than WT fetal levels at E17.5 (Figure 4.1).

4.2.4 Summary/Discussion

Increased circulating levels of VEGF\textsubscript{120/164} protein in Vegf\textsuperscript{hi} mutant fetuses indicated that the transgene was effective, and that overexpression of Vegf in certain concepti did have an effect when compared to the WT littermates where the transgene was absent. Unexpectedly, fetal plasma VEGF\textsubscript{120/164} levels in the Vegf\textsuperscript{hi} mutants and WT littermates were significantly lower than WT fetal levels. This is unlikely a direct consequence of the insertion of the Vegf\textsuperscript{hi} transgene since the WT littermates also exhibited lower circulating VEGF\textsubscript{120/164} levels. An indirect cause however could possibly explain lowered VEGF protein expression in all of the fetuses. The Vegf\textsuperscript{hi} mutants could be secreting a factor into the maternal circulation that then affects the mother in some way, possibly hormonally, and this in turn is affecting VEGF expression in all of the concepti.
Figure 4.1 – Circulating fetal levels of VEGF_{120/164} at E17.5. A significant increase in VEGF_{120/164} protein was observed in Vegf\textsuperscript{hi} mutant mice compared to their WT littermates. Unexpectedly, WT levels were significantly higher than those of Vegf\textsuperscript{hi} mutants and the WT littermates. WT = CD1 x CD1, Vegf\textsuperscript{hi} = mutants overexpressing VEGF, Vegf\textsuperscript{hi} WT = fetuses in same litter as Vegf\textsuperscript{hi} mutants but that don’t express the transgene. Significance (p<0.05) from one-way ANOVA is indicated by different letters above the data. N’s are indicated by the numbers below the group labels.
4.3 VEGF_{120/164} Protein Expression in the Placenta and Maternal Circulation (VEGF_{120/164} and sFlt1) at E17.5

4.3.1 Rationale

Results showed that VEGF_{120/164} protein was overexpressed in the fetal circulation of mutants relative to their WT littermates. The next step was to determine if the placenta’s of these mutants was an organ that overexpressed VEGF_{120/164} protein, and whether this overexpression would correlate with changes in maternal circulating VEGF_{120/164} levels. In experimental pregnancies (CD1 female x Vegf^{hi} male), the placentas of transgenic fetuses were the only organs perfused by maternal blood that overexpressed VEGF protein. Therefore, if the placenta was a potential source for the increased VEGF_{120/164} witnessed in the maternal circulation (Figure 3.1), then it would be expected that circulating VEGF_{120/164} would be elevated relative to control maternal circulating levels at E17.5, where all fetuses are WT. Maternal organ expression of VEGF was not expected to differ because the mother was WT in all pregnancies.

4.3.2 Methods

Placental samples, whole and enriched regions (junctional zone, labyrinth, chorionic plate), were collected at E17.5 from CD1 female mice (aged 8-12 weeks) mated with Vegf^{hi} males. Tissue was collected and snap-frozen for protein analysis by ELISAs (see section 2.5).

In a separate series of pregnancies, maternal blood was collected at E17.5 from the experimental mice. Blood was collected from the uterine vein and then the left ventricle (see section 2.2.3). Blood was collected from the left ventricle second because when collected from first, blood pressure was too low in the uterine vein to get enough plasma volume for the VEGF_{120/164} ELISA assay. Plasma was isolated and VEGF_{120/164} and sFlt1 protein was quantified (see section 2.2.3). Plasma samples were diluted 3x in the VEGF ELISA assay and 40x for the sFlt1 ELISA assay so that concentrations would be within the standard curve.

4.3.3 Results

Protein quantification by ELISA showed that the placentas of Vegf^{hi} mutants generally expressed similar amounts of VEGF_{120/164} protein as WT littermates. The
exception was VEGF\textsubscript{120/164} protein in the whole placenta of Veg\textsuperscript{\textit{hi}} mutants where levels were significantly \textit{less} than WT littermates and not significantly different than WT controls (Figure 4.2). Levels of VEGF\textsubscript{120/164} protein in the labyrinth and junctional zone were similar in Veg\textsuperscript{\textit{hi}} mutants and WT littermates, and were approximately two-fold higher than WT control levels (Figure 4.3). In the chorionic plate, VEGF\textsubscript{120/164} protein did not significantly differ between Veg\textsuperscript{\textit{hi}} mutants and WT littermates but Veg\textsuperscript{\textit{hi}} mutants had significantly \textit{lower} levels of VEGF\textsubscript{120/164} protein than WT controls (Figure 4.3).

With a generalized increase in VEGF\textsubscript{120/164} protein expression in the placentas of both Veg\textsuperscript{\textit{hi}} and Veg\textsuperscript{\textit{hi}} WT conceptuses, we next measured maternal circulating levels of VEGF\textsubscript{120/164} (Figure 4.4). Contrary to our hypothesis, we observed a significant decrease in plasma levels of VEGF\textsubscript{120/164} in the maternal systemic arterial circulation (i.e. left ventricle) in WT females carrying Veg\textsuperscript{\textit{hi}} conceptuses. We also found that VEGF\textsubscript{120/164} in the uterine vein was not higher in pregnancies carrying Veg\textsuperscript{\textit{hi}} conceptuses than WT control pregnancies. In addition, VEGF\textsubscript{120/164} (p<0.0001) was higher in the systemic circulation than in the uterine vein in WT control pregnancies but this gradient was absent in pregnancies carrying Veg\textsuperscript{\textit{hi}} conceptuses.

Circulating sFlt1 was then measured (Figure 4.5) to determine why VEGF\textsubscript{120/164} was not elevated in plasma from the uterine vein or left ventricle of the experimental group. We hypothesized that high maternal plasma sFlt1 levels may cause low free VEGF\textsubscript{120/164} concentrations in maternal plasma. As hypothesized, significant increases (p=0.0002) in circulating sFlt1 were observed in the uterine vein of WT mothers mated with Veg\textsuperscript{\textit{hi}} males when compared to WT females mated with WT males. No significant differences in sFlt1 however were observed between the two groups when blood was sampled from the left ventricle. In WT pregnancies, sFlt1 concentration was higher in plasma obtained from the left ventricle (i.e. the systemic circulation) than in plasma from the uterine vein (p=0.003). This difference was not seen in WT mothers mated with Veg\textsuperscript{\textit{hi}} males.
Figure 4.2 –VEGF protein in the whole placenta at E17.5. (A) A significant increase in VEGF$_{120/164}$ protein expression was observed in the Vegf$^\text{hi}$-WT littermate placentas when compared to WT levels. No significant difference in expression was observed between the Vegf$^\text{hi}$ and WT placentas. (B) Decreased expression of VEGF$_{165b}$ protein was observed in Vegf$^\text{hi}$ and WT littermate placentas when compared to WT levels. WT = CD1 x CD1, Vegf$^\text{hi}$ = mutants overexpressing Vegf, Vegf$^\text{hi}$ WT = fetuses in same litter as Vegf$^\text{hi}$ mutants but that don’t express the transgene. Significance (p<0.05) from one-way ANOVA is indicated by different letters above the whiskers (95th percentiles). N’s are indicated by the numbers below the group types.
Junctional Zone, Labyrinth, and Chorionic Plate

**VEGF_{120/164} Protein**

- **A** Junctional Zone
  - WT
  - Vegf^{hi} WT
  - Vegf^{hi}

- **C** Labyrinth
  - WT
  - Vegf^{hi} WT
  - Vegf^{hi}

- **E** Chorionic Plate
  - WT
  - Vegf^{hi} WT
  - Vegf^{hi}

**VEGF_{165b} Protein**

- **B** Junctional Zone
  - WT
  - Vegf^{hi} WT
  - Vegf^{hi}

- **D** Labyrinth
  - WT
  - Vegf^{hi} WT
  - Vegf^{hi}

- **F** Chorionic Plate
  - WT
  - Vegf^{hi} WT
  - Vegf^{hi}
Figure 4.3 – VEGF_{120/164} and VEGF_{165b} protein in the junctional zone, labyrinth, and chorionic plate at E17.5. (A,C) The junctional zone and labyrinth showed significantly higher levels of VEGF_{120/164} protein between WT and Vegf^{hi} placentas. Significantly higher levels were also observed between WT and Vegf^{hi} WT placentas. (E) The chorionic plate however showed significantly lower levels of VEGF_{120/164} protein in the Vegf^{hi} placentas compared to the WT controls. (B,D,F) Decreased expression of VEGF_{165b} protein was observed in all three enriched regions of the Vegf^{hi} and WT littermate placentas when compared to WT controls. WT = CD1 x CD1, Vegf^{hi} = mutants overexpressing Vegf, Vegf^{hi} WT = fetuses in same litter as Vegf^{hi} mutants but that don’t express the transgene. Significance (p<0.05) from one-way ANOVA is indicated by different letters above the whiskers (95th percentiles). N’s are indicated by the numbers below the group types.
Circulating free VEGF$_{120/164}$ in the Uterine Vein and Left Ventricle

**Figure 4.4**—Circulating free VEGF$_{120/164}$ in the maternal uterine vein and left ventricle of normal and WT$x$VEGF$^{	ext{hi}}$ pregnancies. A significant increase in circulating VEGF$_{120/164}$ was observed between the UV and LV in WT$x$WT pregnancies. This difference was no longer observed in the WT$x$Vegf$^{	ext{hi}}$ male pregnancies. Circulating levels of VEGF$_{120/164}$ from the LV in WT$x$Vegf$^{	ext{hi}}$ male pregnancies were significantly lower than the WT$x$WT pregnancies. Significance (p<0.05) from one-way ANOVA is indicated by different letters above the whiskers (95th percentiles). N’s are indicated by the numbers below the group types. (UV = uterine vein, LV = left ventricle, WT$x$WT = normal pregnancy, WT$x$Vegf$^{	ext{hi}}$ male = CD1 female mated with Vegf$^{	ext{hi}}$ male).
Figure 4.5 – Circulating free sFlt1 in the maternal uterine vein and left ventricle of normal and WT x Vegf<sup>hi</sup> pregnancies. A significant increase in circulating sFlt1 was observed in the UV between the normal and WT x Vegf<sup>hi</sup> male pregnancies. In normal pregnancies, the UV expresses lower levels of circulating sFlt1 when compared to samples from the LV. Significance (p<0.05) from one-way ANOVA is indicated by different letters above the whiskers (95th percentiles). N’s are indicated by the numbers below the group types. (UV = uterine vein, LV = left ventricle, WT x WT = normal pregnancy, WT x Vegf<sup>hi</sup> male = CD1 female mated with Vegf<sup>hi</sup> male).
To determine whether the decreased free VEGF$_{120/164}$ in the maternal systemic circulation affected VEGF levels in the maternal organs, we examined the decidua, ovary, and kidney for VEGF protein expression. The decidua was examined because even though it is fused to the placenta, it is still a maternal organ, therefore VEGF protein expression in the maternal decidual cells should not be altered. The kidney was selected because of its stable VEGF protein expression during pregnancy; therefore it also was not expected to change in the experimental group. Lastly, the ovary was examined because of its high levels of VEGF expression at E17.5 (Figure 3.3). If the ovary was a possible target for VEGF protein produced by the placenta, then ovarian levels would be lower in the experimental group.

As hypothesized, the decidua (Figure 4.6) and kidney (Figure 4.7) did not show significant differences in VEGF$_{120/164}$ protein levels when compared to the WT levels at E17.5. The ovary (Figure 4.8) however did show significantly lower levels (p=0.02) of VEGF$_{120/164}$ protein expression when compared to WT levels at E17.5.

4.4 Maternal Circulating Progesterone Levels

4.4.1 Rationale

With decreased ovarian VEGF$_{120/164}$ protein in the WT females carrying Vegf$^{hi}$ conceptuses, we wanted to determine whether this decrease had a physiological role. The ovary is responsible for secreting progesterone into the maternal circulation during pregnancy, and data from Chapter 3 showed that VEGF was expressed in the corpus luteum of the ovary, the hormone-producing site of the ovary. Therefore, circulating progesterone was measured and it was hypothesized that progesterone levels would be lower in the experimental group compared to the WT controls.
Figure 4.6 – VEGF protein in the decidua at E17.5. (A) No change in VEGF$_{120/164}$ was observed between the Vegf$^h$ or Vegf$^h$ WT deciduas when compared to the WT deciduas. (B) VEGF$_{165b}$ expression significantly decreased however between the WT and two experimental groups. WT = CD1 × CD1, Vegf$^h$ = mutants overexpressing VEGF, Vegf$^h$ WT = fetuses in same litter as Vegf$^h$ mutants but that don’t express the transgene. Significance (p<0.05) from one-way ANOVA is indicated by the different letters above the whiskers. N’s are indicated by the numbers below the group types.
Figure 4.7 – VEGF protein in the maternal kidney at E17.5. No changes in VEGF$_{120/164}$ (A) or VEGF$_{165b}$ (B) were observed between the WT and WT x Vegf$^{hi}$ groups. WT = CD1 x CD1, WT x Vegf$^{hi}$ = CD1 female mated with Vegf$^{hi}$ male. Significance (p<0.05), indicated by the different letters above the error bars was determined by t-test. N’s are indicated by the numbers below the group types.
Figure 4.8 – VEGF protein in the maternal ovary at E17.5. Significant decreases in VEGF_{120/164} (A) and VEGF_{165b} (B) were observed between the WT and WT x Vegf^{hi} ovaries. WT = CD1 x CD1, WT x Vegf^{hi} = CD1 female mated with Vegf^{hi} male. Significance (p<0.05), indicated by the different letters above the error bars was determined by t-test. N’s are indicated by the letters below the group types.
4.4.2 Methods

300 µl of plasma from section 4.3.2 was sent to the Animal Health Diagnostic Center at Cornell University, where a radioimmunoassay (RIA) was conducted to measure circulating progesterone levels.

4.4.3 Results

Circulating progesterone levels at E17.5 in WT female mice carrying Vegfhi concepti were similar to those of WT female mice carrying WT concepti (Figure 4.9a). Compared to non-pregnant circulating progesterone levels however, the experimental group contained significantly less circulating progesterone than the control group at E17.5. A correlation between ovarian VEGF_{120/164} protein and circulating progesterone showed that as ovarian VEGF_{120/164} increased, so did circulating progesterone levels (Figure 4.9b).

4.5 VEGF_{165b} Protein Expression in the Placenta, Maternal Decidua, Kidney, and Ovary at E17.5

4.5.1 Rationale

With increased VEGF expression observed in the Vegfhi mutant placentas, we sought to determine whether this affected the VEGF_{xxxb} isoform as well. It was hypothesized that VEGF_{165b} protein expression in the placenta would decrease, as the insertion of the IRES-LacZ transgene after exon 8 would disrupt the expression of this isoform. In the maternal organs of the experimental group, we hypothesized that VEGF_{165b} protein expression would follow the trend of VEGF_{120/164} protein expression. No significant change in the decidua or kidney was expected, but ovarian VEGF_{165b} protein expression was expected to be lower than that of WT controls.
Ovarian VEGF$_{120/164}$ vs Circulating Progesterone

**Figure 4.9 – Maternal circulating progesterone vs maternal circulating and ovarian VEGF$_{120/164}$ expression at E17.5.** (A) Progesterone levels in the maternal circulation. No significant difference was observed between non-pregnant and E17.5 progesterone levels in WT pregnancies. When a WT female was mated with a Vegf$^h$ male however, a significant decrease in circulating progesterone was observed. Significance (p<0.05) from one-way ANOVA is indicated by the different letters above the whiskers. N’s are indicated by the numbers below the group types. (B) A strong positive correlation was seen between circulating progesterone and ovarian VEGF$_{120/164}$ levels at E17.5.
4.5.2 Results

Protein quantification through ELISAs determined that the levels of VEGF\textsubscript{165b} in the whole placenta were significantly lower in Vegf\textsuperscript{hi} and Vegf WT conceptuses (p=0.01) compared to WT conceptuses (Figure 4.2). The labyrinth (Figure 4.3), junctional zone (Figure 4.3) and chorionic plate (Figure 4.3) also all displayed significant decreases (p=0.0006, p=0.005, and p=0.004 respectively) in VEGF\textsubscript{165b} levels relative to WT levels. Again, it was interesting to note that the WT littermates expressed similar amount of VEGF\textsubscript{165b} protein as the Vegf\textsuperscript{hi} mutants in the respective placental regions. Similar to VEGF\textsubscript{120/164} protein levels, VEGF\textsubscript{165b} protein did not change significantly relative to WT levels in the deciduala (Figure 4.6) or kidney (Figure 4.7). The ovary (Figure 4.8) was once again affected; there was a significant decrease (p=0.005) in VEGF\textsubscript{165b} protein levels relative to WT levels. Interestingly, whereas reciprocal changes in VEGF\textsubscript{120/164} and VEGF\textsubscript{165b} protein levels were observed in the ovary during pregnancy in chapter 3, in this situation parallel changes in the two proteins were observed.

4.6 Summary/Discussion

Contrary to our hypothesis, the up-regulation of VEGF in approximately half the concepti actually decreased maternal systemic arterial circulating VEGF\textsubscript{120/164} levels. We also found that uterine vein concentrations of VEGF\textsubscript{120/164} did not change between the experimental and control groups, and this may be due to elevated sFlt1 levels observed in the uterine vein of the experimental group. Since uterine vein sFlt1 levels were increased, and free VEGF\textsubscript{120/164} was not decreased compared to the controls, this indicates that increased VEGF may have been secreted from the placenta into the uterine vein, but by binding to sFlt1, we saw no significant increase in free levels of VEGF\textsubscript{120/164}.

My results also show that in WT pregnancies, circulating VEGF\textsubscript{120/164} and sFlt1 concentrations were lower in the uterine vein when compared to the left ventricle, which represents systemic circulation. A likely reason for the observed difference is that the ELISA assays used in the study measure ‘free’ VEGF\textsubscript{120/164} and sFlt1 protein. Therefore, if the placenta produced and secreted VEGF\textsubscript{120/164} and/or sFlt1 into the uterine vein, then binding of the ligand to its soluble receptor would interfere with, and reduce, the total amount of VEGF\textsubscript{120/164} and sFlt1 measured. In the experimental group (CD1 female x Vegf\textsuperscript{hi} male) however, this difference between uterine vein and systemic circulating
VEGF\textsubscript{120/164} and sFlt1 is absent. This again may be attributed to elevated sFlt1 levels in the uterine vein. If higher than normal sFlt1 is produced and secreted by the placenta into the maternal circulation, then it will bind to circulating VEGF\textsubscript{120/164}, thus decreasing the amount of free VEGF\textsubscript{120/164} detected. PIGF is able to bind to sFlt1 in the circulation, but it is unlikely to alter the results in this chapter as the sFlt1 ELISA assay is not affected by PIGF in the circulation (R&D systems MMV00 manual). Whether the binding of VEGF\textsubscript{xxx}b affects free sFlt1 levels in this assay is yet to be determined, but it is a possibility since it has the same binding properties as VEGF\textsubscript{xxx}.

Of the maternal tissues analyzed, the ovary was the only organ where VEGF\textsubscript{120/164} protein levels decreased, a trend observed in the maternal circulation. To determine if decreased ovarian VEGF\textsubscript{120/164} had any physiological affect, we measured progesterone levels in the maternal circulation (Figure 4.9a). Progesterone was measured because it is produced and secreted into the maternal circulation by the corpus luteum, a highly vascularized structure within the ovary (Kaczmarek et al., 2005). If less VEGF\textsubscript{120/164} protein was available to act on the ovary, then it is possible that the corpus luteum would not be vascularized as much, and this would decrease the secretion of progesterone into the maternal circulation. We found that relative to non-pregnant values, progesterone concentrations at E17.5 were significantly lower in WT mothers of the experimental cross, while this difference was not significant with the mothers of WT pregnancies. Lower levels of progesterone were expected due to proximity to labour but not below non-pregnant levels (Doring et al., 2006). Preliminary results suggest a strong positive correlation between ovarian VEGF\textsubscript{120/164} protein and progesterone levels in the maternal circulation (Figure 4.9b). However, further studies are required to establish this relationship as a high correlation is only observed because of the one point with high levels of ovarian VEGF\textsubscript{120/164} protein and circulating progesterone. This preliminary data suggesting an ovarian-VEGF\textsubscript{120/164} and progesterone connection is supported by the literature, where the VEGF pathway has been shown to play a critical role in the regulation of angiogenic events in the corpus luteum of pregnancy. Pauli et al. (2005) showed that administration of anti-VEGFR2 antibody during pre- and post-implantation periods in rodents disrupted maternal ovarian function, by eliminating preexisting luteal blood vessels. The decrease in luteal size was reflected by a significant decline in ovarian progesterone secretion.
The ovarian VEGF$_{120/164}$ data in this chapter of my thesis produced some interesting results. We saw a statistically significant decrease in VEGF$_{120/164}$ protein in the ovary in the experimental group compared to the WT pregnancies. However, four of the 10 mothers analyzed exhibited low levels of VEGF in the ovary (average of 84 pg of VEGF$_{120/164}$/mg of total protein), whereas the other 6 mothers averaged 477 pg of VEGF$_{120/164}$/mg of total protein, which was similar to the WT control level of 500 pg of VEGF$_{120/164}$/mg of total protein. These four mothers also exhibited the lowest levels of circulating progesterone. The reason I believe that these results may not necessarily be accurate is that it is known that as the end of pregnancy nears, circulating progesterone levels decrease (Doring et al., 2006) in order to initiate certain steps necessary for labour. With this in mind, it is possible that the four pregnancies that exhibited abnormal ovarian VEGF and progesterone levels were not at E17.5 as originally believed. We assume the female mice plug at midnight, and therefore label noon of that day as E0.5. But if the female was plugged earlier, and the samples were collected at ~12:00pm as usual, then the mothers may be further into their pregnancy than assumed. This advancement in pregnancy could cause many physiological changes, and the expression of ovarian VEGF and consequently circulating progesterone could be two of the changes. In future studies it would be desirable to look earlier in pregnancy, to avoid this potential problem. A possible time point to look at would be E15.5, where progesterone levels are at their peak in mouse pregnancies (Doring et al., 2006).

Another interesting finding in this study was that the WT littermates, which do not have the VEGF overexpression transgene, expressed similar amounts of VEGF$_{120/164}$ and VEGF$_{165\text{b}}$ protein as the Vegf$_h$ mutants. A possible reason behind this similarity in protein levels could be that the mothers sense overexpression of VEGF protein by the mutant placentas, and this triggers a response in the mother, possibly hormonal. Hormones such as epinephrine, which are known to increase VEGF expression (Lutgendorf et al., 2003), may act on all placentas equally, therefore causing all of the placentas to produce the same amount of VEGF.

We also found that the insertion of the IRES-LacZ cassette in the Vegf$_h$ transgenic mice disrupted the expression of VEGF$_{165\text{b}}$. Significant decreases in placental and ovarian VEGF$_{165\text{b}}$ protein were observed, the two organs where VEGF$_{120/164}$ levels was altered due to the insertion of the transgene. Reasons behind decreased VEGF$_{165\text{b}}$ protein levels
in the placenta are unlikely to be directly attributed to the insertion of the transgene however, as WT littermate placentas also expressed decreased levels. As with VEGF_{120/164}, this decrease observed in the Vegf^{hi} mutant and WT littermate placentas may be due to an indirect cause. The maternal ovaries from the WT mothers also exhibited decreased VEGF_{165b} protein levels, but it was interesting to find that the four mothers who exhibited low levels of VEGF_{120/164} did not have low levels of VEGF_{165b}.

In summary, VEGF_{120/164} overexpression in the placenta was achieved by crossing Vegf^{hi} males with WT females. However, in contrast to the hypothesis, maternal circulating levels of VEGF_{120/164} decreased when compared to the WT controls. This may be explained by elevated sFlt1 levels in the circulation, possibly produced by the placenta as a compensatory mechanism in response to VEGF_{120/164} overproduction. The physiological effects of decreased VEGF_{120/164} levels in the maternal circulation may be affecting the ovary, but further studies need to be conducted to show this. Preliminary results suggest a correlation exists between ovarian VEGF_{120/164} and circulating progesterone, but again, further evidence is needed in linking placental expression of VEGF_{120/164} to ovarian levels of VEGF_{120/164} protein.
5  Summary/Future Directions

5.1  Summary

Vascular endothelial growth factor (VEGF) is a potent stimulator of vascular development but the exact function of this protein during pregnancy and in organs such as the placenta is unknown. Understanding the placental function of VEGF is important because excess placental sFlt1, a receptor of VEGF, is thought to be a potential contributor to pre-eclampsia, a life-threatening complication of human pregnancy. Treatments targeting VEGF activity may be considered in pre-eclampsia, but such treatments should be approached with caution, given our lack of basic understanding of the roles of VEGF in pregnancy. The overall goal of this thesis was to build a foundation for us to understand where and when VEGF is expressed, and whether its expression changes in specific organs during pregnancy.

The first goal of my thesis was to do a cross-gestational profile of VEGF expression in the placenta and maternal organs during pregnancy in mice. In this thesis, we confirmed that free sFlt1 protein and free VEGF_{120/164} protein increase significantly in the maternal circulation of mice with advancing gestation. The source of increased VEGF may have been the maternal ovaries because the increase in ovarian VEGF_{120/164} protein matched the time course witnessed in the maternal circulation (Figure 3.3). The placenta was not excluded as a possible suspect in secreting VEGF into the circulation however. Although it expressed low levels of VEGF protein per unit organ weight in late gestation, there was a large increase in total placental weight over this interval. Thus the placenta may nevertheless contribute increasing amounts to the maternal circulation as gestation advances (Figure 3.22). The corpus luteum was the primary site of expression of Vegf mRNA in the ovary, and multiple cell types expressed Vegf mRNA in the placenta during its maturation.

Another novel finding was the pattern of expression between pro- and anti-angiogenic isoforms of VEGF in the ovary and placenta during pregnancy in mice. The ovary was the only organ examined to show increased VEGF_{120/164} protein while VEGF_{165b} levels decreased; both changes would be anticipated to promote angiogenesis. The placenta was the only organ examined to show decreases in both the pro- and anti-angiogenic isoforms of VEGF. The decrease in VEGF_{165b} may promote the formation of
the placental vasculature despite decreased VEGF\textsubscript{120/164} protein expression during the same time points. The levels of VEGF\textsubscript{165b} were on average 30 times greater than VEGF\textsubscript{120/164} in the mature placenta, therefore a significant decrease in the anti-angiogenic isoform of VEGF could promote angiogenesis.

The second aim of this dissertation focused on investigating the effects on pregnancy of over-expressing placental VEGF. The most notable and striking finding was that when VEGF\textsubscript{120/164} was over-expressed in the placenta in Vegf\textsuperscript{hi} transgenic conceptuses, circulating VEGF\textsubscript{120/164} in the uterine vein of their WT mothers did not significantly change, and maternal circulating levels actually decreased. Elevated sFlt1 protein in the uterine vein of the experimental group may explain why VEGF\textsubscript{120/164} levels remained constant. This indicated that a possible compensatory increase in sFlt1 production by the placenta may have occurred in response to excess VEGF\textsubscript{120/164} production. We also found in this model that as circulating VEGF\textsubscript{120/164} protein in the maternal circulation decreased, so did VEGF\textsubscript{120/164} protein levels in the ovary. A correlation between ovarian VEGF\textsubscript{120/164} and progesterone secretion was also found, and this corresponded with the ovarian LacZ histology results, where Vegf mRNA was predominantly expressed in the corpus luteum, the hormone-producing site of the ovary. These findings together suggest that during mouse pregnancies, VEGF participates in placental-ovarian and ovarian-progesterone communications.

Overall, the experiments summarized in this thesis provide novel evidence for an important role of the ovary and placenta in affecting maternal circulating VEGF\textsubscript{120/164} levels during pregnancy. How much the ovary, placenta, or both, contribute to increased VEGF\textsubscript{120/164} protein in the plasma of the maternal circulation is still unknown, but will be investigated next.

5.2 Future Directions

The results reported in this thesis support the ovary and placenta as possible sources for increased VEGF\textsubscript{120/164} in the maternal circulation during pregnancy. However, there are further studies that will heighten our understanding of their roles and potential contributions to VEGF\textsubscript{120/164} in the maternal circulation during pregnancy.
5.2.1 Ovary, VEGF, and progesterone

To determine if the ovary, placenta, or both, are the source(s) of VEGF_{120/164} secreted into the maternal circulation during pregnancy, I propose to ovariectomize mice during pregnancy. Conducting the surgery should not be difficult, as it has successfully been done before (Walter et al., 2010; Walter et al., 2005). The removal of the ovaries will result in a cessation of progesterone and estrogen production in the female mouse, therefore daily hormonal supplements will be injected (4 mg of progesterone subcutaneously). This has also been done successfully before (Walter et al., 2010) therefore we do not anticipate any major problems in this regard. Circulating VEGF_{120/164} will then be measured as well as placental VEGF concentrations.

There are three potential outcomes that can result from this procedure. First, circulating VEGF_{120/164} levels decrease compared to the controls, indicating that the ovary plays a role in increasing maternal circulating VEGF_{120/164} during pregnancy. Secondly, VEGF_{120/164} levels may remain similar to those of the controls, indicating that the ovary is not the primary contributor to VEGF_{120/164} in the maternal circulation during pregnancy. This would point to the placenta as the likely source, as it was the only other organ in this thesis that had high levels of and significantly increased VEGF_{120/164} protein during late pregnancy (Figure 3.22). Thirdly, a slight decrease in circulating VEGF_{120/164} levels occurs, indicating that the ovary and placenta both contribute to VEGF_{120/164} secretion into the maternal circulation.

To determine the relationship between circulating VEGF_{120/164}, ovarian VEGF_{120/164} and circulating progesterone, further studies need to be done across different time points. Mouse progesterone levels peak at E16.0 (Doring et al., 2006) therefore further studies will be done before, during, and after that time point.

5.2.2 Vegf, Vegf_{xxx}, b,flt1 and sflt1 mRNA by qRT-PCR

In my thesis, I characterized total Vegf mRNA expression in the placenta and maternal organs. In tissues such as the placenta, ovary, and decidua, no change in Vegf mRNA expression was observed, while VEGF_{120/164} and VEGF_{165b} protein levels changed with gestation. Post-translational regulation may account for this discrepancy. Additionally, difference in mRNA and protein isoforms detected by the assays may result
in the discrepancies. For example, if Vegf<sub>120/164</sub> mRNA increased and Vegf<sub>188</sub> mRNA decreased, this would result in no significant overall change in mRNA expression but increased VEGF protein production of the soluble isoforms, which the ELISA assay detects. Also, the primers for total Vegf also picked up Vegf<sub>xxx</sub>b mRNA expression, therefore if Vegf<sub>xxx</sub> mRNA increased and Vegf<sub>xxx</sub>b mRNA decreased, no overall change in total Vegf expression would be observed. This may explain the changes observed in the ovary (Figure 3.3). Post-translational modifications can also occur, and this may also explain why patterns in Vegf mRNA expression differed from VEGF protein levels. Therefore, to further our understanding of Vegf expression in these organs, isoform specific changes of Vegf using qRT-PCR as previously published (Walter et al., 2010; Zhang et al., 2002) will be done. If we find that the placenta or ovary for example produces mostly Vegf<sub>120</sub>, then it would strengthen their cases as contributors to increased VEGF in the maternal circulation, as it is the most soluble isoform and is unlikely to remain in tissue. Down-regulation of Vegf<sub>120</sub> however would indicate that those organs are not likely to contribute to the increased VEGF in the maternal circulation.

In addition to quantifying the different isoforms of Vegf mRNA, mRNA for the anti-angiogenic isoform (Vegf<sub>xxx</sub>b) will also be quantified in the placenta and ovary during pregnancy. Quantifying changes in Vegf<sub>xxx</sub>b mRNA has been done before in humans (Bates et al., 2002), but has not been done in mice. Through personal communication with Dr. Bates, he has informed me that attempts to quantify Vegf<sub>xxx</sub>b mRNA have been done in his lab, but they were unsuccessful. Therefore, in order to characterize the expression of the inhibitory isoform, I will try using newly designed primers that have not been used by Dr. Bates’s lab.

In chapter 4 of my studies, pregnancies with placentas which over-expressed VEGF<sub>120/164</sub> protein showed no significant change in circulating VEGF<sub>120/164</sub> in the uterine vein when compared to the control pregnancies. We did find however that sFlt1 protein was elevated in the uterine vein of these mothers, indicating that the placenta could be over-producing sFlt1 to compensate for excess VEGF protein produced. In order to better understand the source for this elevated sFlt1 protein in the plasma in the uterine vein, qRT-PCR will be done to measure flt1 and sflt1 mRNA in CD1 placentas as well as Vegf<sup>hi</sup> placentas. I hypothesize that the Vegf<sup>hi</sup> placentas and possibly the Vegf<sup>hi</sup> WT littermates will be producing more sflt1 mRNA than the WT placentas.
5.2.3 VEGF and Flt1 Protein by Western Blot

To measure VEGF\textsubscript{120/164} and sFlt1 protein in my thesis, I used ELISA assays from R&D systems. The VEGF\textsubscript{120/164} assay only detects free VEGF\textsubscript{120/164} and VEGF\textsubscript{120/164} bound to KDR, while the sFlt1 assay only picks up free sFlt1. Therefore, if VEGF\textsubscript{120/164} were bound to Flt1 in tissue or sFlt1 in the circulation, then the detected levels of VEGF\textsubscript{120/164} and sFlt1 would be lower than the total amount present. To avoid this issue and get a better understanding of total VEGF levels present in the placenta, ovary, and decidua during pregnancy, western blots using antibodies that measure total VEGF protein (e.g. ab46154, a rabbit polyclonal VEGF antibody) should be done.

5.2.4 Vegf Histology

Regarding the specific cells that express Vegf mRNA, I was unable to confirm the identity of all of the cell types expressing Vegf mRNA in the placenta at E13.5 and E17.5 as well as the decidua at E9.5. It is possible that the antibodies for Hofbauer cells and hematopoietic stem cells originally used were not effective or as specific as needed, therefore different antibodies to those same proteins will be tested. Testing for new possible cell types, such as fibroblasts (Ito et al., 2007), and stromal cells (Amano et al., 2003), which are known to express VEGF in mice, will also be tried. We also have to consider the possibility that standard IHC will not work on the Vegf\textsuperscript{hi} tissue because of the fixation they endured for LacZ staining. Gluteraldehyde was used as a fixative in the LacZ protocol, and due to the strong fixative nature of gluteraldehyde, antigen retrieval during IHC staining can become hard. Therefore, as an alternative, fluorescent IHC on newly collected tissue can be done. As one antibody will target the LacZ gene, the second antibody will target our cell type of interest, and co-localization of the two antibodies will help us identify possible cell types expressing Vegf in the placenta and decidua.

Overall, the results of my thesis have shown that the ovary and placenta are the most likely sources for increased VEGF witnessed in the maternal circulation during pregnancy. Techniques such as ovariectomies, Vegf isoform specific qRT-PCR, western blots for total VEGF protein, and fluorescent IHC, will help us to further understand the role of VEGF in the placenta and maternal organs during pregnancy.
Appendix 1.1 – Mean Ct values for housekeeping genes β-actin and Gapdh. (A) In the placenta, the housekeeping genes had significantly higher Ct values from E9.5 to E13.5. (B) β-actin and Gapdh in the non-pregnant ovaries had significantly higher Ct values then during pregnancy. N=5 for both tissues and all time points. Significance (P<0.05) was determined by one-way ANOVA and is shown by different letters above the error bars.
6.2 Appendix 2 - Protocols

6.2.1 E17.5 Fetal Blood Collection (for VEGF and sFlt1 ELISA)

1) Mouse weight taken
2) Mouse put down via cervical dislocation
3) Skin cut at the abdominal area and abdomen uncovered
4) Uterine horns removed
5) Count and note number of implantation sites in total, and in the left and right horn
6) Remove both horns and place them in ice-cold PBS
7) Dispose of the mother
8) Cut off one implantation site and place it in a petri-dish.
9) Remove the uterus, yolk sac, and amniotic membrane from the conceptus. Do not sever the umbilical cord, leave placenta attached.
10) Warm up the embryo using warm PBS until you can see the heart beating
11) Using gauze, grasp the embryo, and cut the head off with scissors. As soon as the mouse is decapitated, obtain a heparin-coated capillary tube (Fisher Scientific, 70 µl capacity) and collect blood.
12) Once the tube is full or blood flow ceases, use a stopper to push the blood out of the capillary into a 1.5 ml microcentrifuge tube. Place the capped tube in ice water until all the blood from all embryos has been collected (the blood from all embryos is pooled together).
13) After all the blood from the embryos has been collected, the volume of blood collected is recorded and placed into a 4 °C centrifuge for 20 minutes at 2000g.
14) After centrifugation, two distinct layers are seen (clear plasma on top, blood on bottom).
15) Using a pipette, acquire as much plasma as possible without disturbing the cell layers and aliquot the plasma into new 1.5 ml microcentrifuge tubes.
16) Place the capped tubes with plasma into the -20 °C freezer until needed for analysis.
6.2.2 Tissue Collection for LacZ Histology

1) Mouse weight taken.
2) Once weighed, the mouse was sacrificed via cervical dislocation.
3) An incision was made in the abdominal area, allowing for the visibility of the concepti.
4) The number of concepti present in each side of the horn was counted. The number of resorption sites was also noted.
5) The uterus was removed and placed in ice cold PBS (without Ca/Mg).
6) The maternal organs (brain, heart, lungs, kidney, spleen, liver, and ovaries) were removed and placed at room temperature into 4% PFA for one hour on a rocker.
7) One by one, an implantation site was cut off weighed. An incision was carefully made in the anti-mesometrial site and the embryo was removed and weighed (For E13.5 and E17.5). Once weighed, the implantation site and embryo were placed at room temperature into 4% PFA on a rocker for 1 hour.
   a. For E9.5, a small incision is then made where the embryo sits, allowing for fixative and solution to penetrate the primitive placental region and ectoplacental cone. The concepti are then placed into 4% PFA on a rocker for 1 hour.
8) After one hour of fixation, the maternal organs were cut into thin slices to allow for LacZ fixation and staining solutions to penetrate. Embryos were cut into half along the ventral side from the anterior (head) to the posterior (tail). Once cut, the tissue was placed into LacZ fixative for 30min on a rocker inside the cold room (4 °C).
9) The implantation site was also cut into half from the umbilical cord towards the decidua. This gave a lateral view of the placenta, with the different regions showing (chorionic plate, labyrinth, and junctional zone). Once cut, the two halves of the placenta were placed into LacZ fixative for 30 min on a rocker inside a cold room (4 °C)
   a. For E9.5 concepti, they are cut into half, giving a lateral view of the decidua, primitive placenta, and ectoplacental cone. Once cut, the concepti are placed into LacZ fixative for 30 min on a rocker inside a cold room (4 °C)
10) Samples were removed from the LacZ fixative and washed at room temperature three times, at 15 minutes per wash, using the wash buffer (0.4 ml of 1M MgCl2, 2.0 ml of 1% Sodium deoxycholate, 2.0 ml of 2% Nonidet-P40, 195.6ml 0.1M Phosphate Buffer or PBS. Total volume is 200 ml.).
11) Samples were then placed into X-gal (Fermentes, Cat#R0401) and stained overnight in the dark on a rocker at room temperature.
12) The following day, the tissue was washed using PBS (without Ca/Mg) three times, at 10 minutes per wash, on a rocker at room temperature.
13) Tissue was then placed into 10% formalin, covered with foil, and placed into the fridge (4 °C) until it was embedded.
6.2.3 Tissue Collection for mRNA and Protein Analysis

1) Mouse weight taken
2) Anesthetize mouse using isoflurane in conjunction with pure oxygen. The oxygen was set at 1000 ml/min and the isoflurane was set to 5%.
3) Once the mouse is anesthetized, maternal blood was collected through a cardiac puncture guided by ultrasound.
4) Blood collection is done through a heparin coated needle, and it is placed in a 1.5 ml microcentrifuge tube. The tube is then spun at room temperature for 20 min at 2000 g (according to ELISA kit from R&D Systems Cat# MMV00). Plasma (top aqueous layer) is then isolated and aliquoted into 1.5ml microcentrifuge tubes and stored at -20 °C.
5) The mouse is taken to a sterile fume hood cleaned with RNAse Zap. All surgical tools are also sterilized.
6) The mouse is then placed on ice, and an incision is made from the chest cavity down to the abdominal cavity.
7) The uterus (with concepti) are removed and placed into ice cold PBS (without Ca/Mg).
8) Maternal organs (brain, heart, lungs, kidney, spleen, liver, and ovaries) are removed, placed into a 2 ml microcentrifuge tube and flash frozen using liquid nitrogen. In cases where there is two of the same organ (i.e. left and right kidney), the left organ was used for protein analysis and the right organ was used for mRNA. For organs where there is only one (i.e. heart), it was cut into half and then placed into their respective tubes and flash frozen.
9) Placental collection varies depending on age. If at E9.5, then the decidua and primitive placenta are enriched for via microdissection. Due to the small size of the tissues, they are pooled from the same mother to create a single ‘N’.
   a. For E13.5 and E17.5, the whole placenta as well as enriched regions were collected.
      i. Whole Placenta: 3 whole Placentas (without decidua) are pooled for protein and 3 whole placentas (without decidua) are pooled for mRNA. They are placed in a 2 ml microcentrifuge and flash frozen. Their respective deciduas are also pooled, placed in a 2 ml microcentrifuge, and flash frozen for protein and mRNA analysis.
      ii. Enriched Regions: In addition to whole placenta analysis, the individual regions (Chorionic Plate, Labyrinth, and Junctional Zone) are also studied. Three placentas were microdissected and tissue was pooled for analysis. Once pooled, it was placed into a 2 ml microcentrifuge and flash frozen using liquid nitrogen. The same procedure was done on tissue for mRNA analysis.
10) All tissue was stored at -80 °C.
6.2.4  Protein Purification using RIPA

1) Samples were taken from the -80 °C freezer and placed onto dry ice
2) The mortar and pestle were soaked and cleaned with methanol
3) Liquid nitrogen was poured into mortar and tissue was then placed into mortar (always kept in liquid nitrogen so tissue does not thaw)
4) Using the pestle, the tissue was crushed into a fine powder. The powdered tissue was then placed into a new tube and kept on dry ice.
5) Steps 2-4 were repeated for all the samples being done in that session.
6) Make RIPA +++: To 10ml of RIPA buffer (Thermo Scientific, Cat#89901), add 1 pellet COMPLETE protease inhibitor cocktail (Roche, Cat#11836170001) and 10 µl sodium vanadate (100mM).
7) Once all the samples were crushed and in powder form, RIPA+++ was added to each sample (0.2ml per 0.1g of tissue, with a minimum of 0.2ml added) and homogenized for 20 seconds on regular crushed ice. The homogenizer was cleaned with rounds of DEPC water and ethanol between each sample.
8) Once all the samples were homogenized with RIPA+++, they were allowed to sit in ice for 30 min to 1 hour to allow all the bubbles to dissipate.
9) The samples were then spun in a centrifuge for 15 min at 4 °C at 14000 rpm.
10) The samples were then aliquoted and stored in the -80 °C freezer.

6.2.5  Protein Quantification – Bradford Assay

1) Samples were removed from the freezer and kept on ice to thaw.
2) A BSA (Thermo Scientific, Cat#SH30574.01) standard curve in deionized water (0, 50, 100, 200, 300, 400, 500 µg/ml) was created in separate tubes
3) 10 µl of the standard or sample is pipetted into each well of the 96 well microplate. Samples were done in duplicates. 10 µl of deionized water was used as the blank.
4) After all the samples are loaded, the Bradford reagent (Bio-Rad, Cat#500-0006) is diluted 1:5 with deionized water. 200 µl of the diluted Bradford reagent was added to each well using a multi-channel pipette.
5) The plate was incubated for 5 minutes at room temperature and absorbance was then measured at 595 nm (by Magellan6 software on a Tecan, infinite M200).
6.2.6 Paraffin Embedding and Sectioning

1) Once wholemonut pictures were taken, the tissue was dehydrated using a series of ethanol washes (70%, 80%, 90%, 95%, 100%, 100%) for 10 minutes in each step. Ethanol was diluted using PBS (without Ca/Mg).
2) The tissue was then placed into xylene (Fisher Scientific, Cat#X3S-4) for 30 minutes, and then through three paraffin stages, each for 20 minutes.
3) The tissue was then embedded and 5um sections were made using a microtome.
4) Paraffin sections were placed on Fisherbrand Superfrost slides (Cat#12-550-15) and Fisherbrand coverslips (Cat#12-545E) were mounted using Cytoseal XYL (Richard-Allan Scientific, Cat#8312-4) to protect the sections.
5) Slides with paraffin sections were dried overnight at room temperature.

6.2.7 Neutral Red Background Staining

1) Neutral Red (Fisher N129-25) stock is made to 0.1% using deionized water.
2) Ethanol gradient is created using deionized water and ethanol (Fisher Scientific, Cat#A962P-4)
3) Slides are placed into a holder and go through a series of xylene (Fisher Scientific, Cat#X3S-4) and hydration steps as follows:
   a. Xylene 1 – 5 minutes
   b. Xylene 2 – 5 minutes
   c. Xylene 3 – 5 minutes
   d. 100% Ethanol – 3 minutes
   e. 100% Ethanol – 3 minutes
   f. 95% Ethanol – 3 minutes
   g. 90% Ethanol – 3 minutes
   h. 80% Ethanol – 3 minutes
   i. 70% Ethanol – 3 minutes
   j. 50% Ethanol – 3 minutes
   k. Deionzied water – 5 minutes
   l. 0.1% Neutral Red – 45 seconds
   m. Rinse with deionized water
   n. 50% Ethanol – 5 seconds
   o. 70% Ethanol – 5 Seconds
   p. 80% Ethanol – 5 Seconds
   q. 90% Ethanol – 5 Seconds
   r. 95% Ethanol – 5 Seconds
   s. 100% Ethanol – 5 Seconds
   t. Xylene 2 – 2 minutes
   u. Xylene 3 – 2 minutes
4) Coverslip is placed onto slide and allowed to dry for 2 hours at room temperature
6.2.8 Hematoxylin and Eosin Staining

1) Ethanol gradient is created using deionized water and ethanol.
2) Acid ethanol is made: 70% Ethanol, 1% glacial acetic acid (Caledon, Cat#64-19-7), 29% deionized water
3) Slides are placed into a holder and go through a series of xylene and hydration steps as follows:
   a. Xylene 1 – 5 minutes
   b. Xylene 2 – 5 minutes
   c. Xylene 3 – 5 minutes
   d. 100% Ethanol – 3 minutes
   e. 100% Ethanol – 3 minutes
   f. 95% Ethanol – 3 minutes
   g. 90% Ethanol – 3 minutes
   h. 80% Ethanol – 3 minutes
   i. 70% Ethanol – 3 minutes
   j. 50% Ethanol – 3 minutes
   k. Deionized water – 5 minutes
   l. Hematoxylin (Sigma, Cat#HHS16) – 10 seconds
   m. Rinse with deionized water
   n. Tap water – 5 minutes
   o. 8-12 dips in acid ethanol
   p. Tap water – 2 minutes
   q. Tap water – 2 minutes
   r. Deionized water – 2 minutes
   s. Eosin (Sigma, Cat#HT110316) – 30 seconds
   t. Rinse with tap water
   u. 90% Ethanol – 5 Seconds
   v. 95% Ethanol – 5 Seconds
   w. 100% Ethanol – 5 Seconds
   x. Xylene 2 – 2 minutes
   y. Xylene 3 – 2 minutes
5) Coverslip is placed onto slide and allowed to dry for 2 hours at room temperature
6.2.9 Real Time RT-PCR – From RNA extraction to PCR

1) Before beginning, all equipment to be used was cleaned with RNase Zap.

2) Samples were taken from the -80 °C freezer and placed onto dry ice

3) The mortar and pestle were soaked and cleaned with methanol

4) Liquid nitrogen was poured into mortar and tissue was then placed into mortar (always kept in liquid nitrogen so tissue does not thaw)

5) Using the pestle, the tissue was crushed into a fine powder. The powdered tissue was then placed into a new tube and kept on dry ice.

6) Steps 2-4 were repeated for all the samples being done in that session.

7) Samples were then homogenized in 1 ml TRIzol (Invitrogen, Cat#15596-026) and placed on ice for 15 minutes

8) 200 µl of RNase-free chloroform (Sigma, Cat#C2432) was added to each sample and the tube was shaken for 15 seconds until the solution went ‘milky’

9) Samples were let to stand for 3 minutes at room temperature and then spun at 12,600 rpm for 15 minutes at 4 °C

10) The top clear phase was then collected in a new 1.5 ml microcentrifuge tube

11) 500 µl of isopropanol (RNase-free) was added and the tube was inverted to mix the solutions

12) Samples were placed into the -20 °C freezer for overnight

13) The following day, the RNA samples were removed from the freezer and spun at 12,600 rpm for 15 minutes at 4 °C to pellet the RNA.

14) The isopropanol was vacuumed out and 500 µl of ice-cold 70% EtOH-DEPC was added to the sample to wash the pellet.

15) After a short vortex, the samples were spun at 7500 rpm for 10 minutes at 4 °C to pellet the RNA.

16) The EtOH was vacuumed out and 87.5 µl of RNase-free water was added to the tube to dissolve the RNA

17) DNA removal was now conducted using the RNase-Free DNase Set (Quiagen #79254). In the tube containing 87.5 µl of RNase-free water and RNA, 10 µl of buffer and 2.5 µl of DNase stock solution was added, making a 100 µl mixture. This mixture was incubated at room temperature for 10 minutes.

18) RNA cleanup was no conducted on the samples using the RNeasy MinElute Cleanup Kit (Qiagen #74204).

19) 350 µl of buffer RLT/B-ME was added to the 100 µl solution from step 17 and mixed thoroughly using a pipette

20) 250 µl of 100% EtOH (RNase-free) was then added to each sample and mixed thoroughly using a pipette

21) 700 µl of that mixture was then added to a column in a 2 ml collection tube (pink tubes provided in kit). The column was placed on a vacuum and suctioned the mixture was suctioned through the column filter.

22) With the columns still on the vacuum (and vacuum left on), 500 µl of Buffer RPE was added to each sample.

23) 800 µl of 80% EtOH was then added to the columns

24) The vacuum was turned off, and the columns were removed and placed back into the collection tubes. The tubes were then centrifuged at 14,000 rpm for 5 minutes at room temperature.
25) The columns were then removed from the collection tubes and placed in a new 1.5 ml microcentrifuge tube. 14 µl of RNase-free water was added to each column to elute transfer of the RNA. The tubes were left to stand for 3 min at room temperature and then centrifuged at 14,000 rpm for 1 minute.

26) Step 25 was repeated

27) Quality of the RNA was then measured through optical density using nanodrop

28) Now that the RNA has been isolated, it was reverse transcribed into cDNA.

29) Using the TaqMan Reverse Transcription Reagents (Applied Biosystems/Roche #N808-0234), a master mix of the following proportions in a 0.2 ml microcentrifuge was made:
- 2 µl 10x buffer
- 2 µl MgCl
- 1 µl Random hexamers
- 2 µl dNTP
- 0.5 µl RNase Inhibitor
- 2 µl dTdT at 0.1M
- 0.2 µl reverse transcriptase

30) 1 µg of RNA was diluted in 10 µl of water in a new 0.2 µl tube. A concentration of 1 µg/10 µl is now present.

31) 9.7 µl of the master mix was then added to 10 µl (1 µg) of RNA. The final volume is now 19.7 µl.

32) The reverse transcription reaction was run with the following program specifics:
- 25 °C for 5 min
- 42 °C for 30 min
- 95 °C for 5 min
- 4 °C hold

33) It is now time to run the Real Time PCR.

34) The cDNA was diluted to 25 ng/µl
- 19.7 µl cDNA (50 ng/µl)
- 177.3 µl of water
- final volume is 197 µl

35) A master mix with the following proportions was made in a 1.5 ml microcentrifuge tube:
- 12.5 µl SYBER Green
- 0.25 µl forward primer (use primers at 5 µM concentrations)
- 0.25 µl reverse primer
- 2 µl water
- final volume per sample is 15 µl

36) To each PCR plate well, 15 µl of the master mix and 5 µl cDNA (25 ng/µl) was added

37) The PCR transcription reactions were run with the following program:
- 95 °C for 10 min
- 95 °C for 15 sec
- 58 °C for 1 min
- X 45 cycles
- 95 °C for 15 sec
- 60 °C for 15 sec
- Slowly heat up to 95 °C (20 min)

6.2.10 Protein Quantification – Bradford Assay

1) Samples were taken from the -80 °C freezer and thawed on ice
2) A BSA standard curve (50,100,200,300,400,500 µg/ml), diluted with water, was created in separate microcentrifuge tubes and kept on ice. Deionized water was used as a blank.
3) Samples were diluted with deionized water at various folds depending on the amount of tissue present. (i.e. at 40x dilution, 2 µl of sample and 78 µl of deionized water was loaded). Diluted samples were kept on ice
4) After a thorough mixing with the pipette, 10 µl of either the sample or standard was loaded, in doubles, in the wells of a 96 well plate (Sarstedt, #82.1581.001).
5) After the loading of the blank, standards, and samples, 200 µl of the Bio-Rad Protein Assay (Bio-Rad, #500-0006), diluted 1:5 with deionized water, was added to each well using a multichannel pipette
6) The plate was incubated at room temperature for 5 minutes.
7) Absorbance was measured at 595nm on Magellan6 software on an Infinite M200, both by Tecan.

6.2.11 VEGF_{120/164} (R&D Systems, MMV00) ELISA

Reagent Preparation

- Mouse VEGF Control – the control was reconstituted with 1.0mL of deionized water. The vial was then placed on a shaker at room temperature to allow for the powder to completely dissolve
- Wash Buffer – for each plate, 25 mL of Wash Buffer Concentrate was added to 600 mL of deionized water, making a final volume of 625 mL.
- Substrate Solution – Colour Reagents A and B were mixed together in equal volumes right before their use.
- Mouse VEGF Standard – the standard was reconstituted with 5.0 mL of Calibrator Diluent RD5T. This created a stock solution of 500 pg/mL. The vial was put on a shaker at room temperature to allow for the powder to completely dissolve.

Assay Procedure

1) In 1.5 ml Microcentrifuge tubes (Sarstedt), the samples were diluted with the Calibrator Diluent RD5T. Samples were loaded with a known quantity of protein (i.e. 50 mg of protein) that was within the standard curve.
2) The standard curve was created through a series of 2-fold dilutions. The highest point was 500 pg/mL and the lowest point was 7.8 pg/mL.
3) The plate was removed from the seal and 50ul of Assay Diluent RD1N was added to each well using a multi-channel pipette
4) 50 µL of Standard, control, or sample was then added to each well. The plate was tapped gently on the frame for 1 minute to allow for the solutions to mix well. An adhesive strip was then used to cover the plate and it was left to incubate at room temperature for 2 hours.

5) After two hours, each well of the plate was aspirated washed using the wash buffer. The Hydroflex autowasher by Tecan was used. Tecan’s Hydrocontrol 2.0 software was also used. A total of five washes were done, with 400uL of wash buffer added to each well each wash.

6) 100 µL of mouse VEGF Conjugate was then added to each well using a multi-channel pipette. A new adhesive strip was used to cover the plate and the plate was left at room temperature to incubate for 2 hours.

7) The aspiration/wash as in step 5 was repeated.

8) 100 µL of Substrate Solution was added to each well using a multi-channel pipette. The plate was incubated at room temperature for 30 minutes while covered with foil.

9) 100 µL of Stop Solution was added to each well using a multi-channel pipette. Pipetting the Stop Solution up and down three times ensured thorough mixing.

10) The optical density of each well was determined at 450 nm using an Infinite M200 micropate reader by Tecan. The software used was Magellan6, also by Tecan. Wavelength correction was set to 570 nm.

6.2.12 VEGF\textsubscript{165b} (R&D Systems, DY3045) ELISA

**Reagents Used that were not in the kit**

- Wash Buffer (R&D Systems, #WA126) - 0.05% Tween 20 in PBS, pH 7.2-7.4 – 20 mL of Wash Buffer Concentrate was diluted in deionized water to create a 500 mL mixture.
- Substrate Solution (R&D Systems, #DY999) – 1:1 mixture of Colour Reagents A (H\textsubscript{2}O\textsubscript{2}) and Clour Reagent B (Tetramethylbenzidine).
- Reagent Diluent (R&D Systems, #DY995) – 1% BSA in PBS, pH 7.2-7.4, 0.2um filtered.
- Stop Solution (R&D Systems, #DY994) – 2 N H\textsubscript{2}SO\textsubscript{4}

**Reagents Used**

- Capture Antibody (Part 842336) – 360 µg/mL of mouse anti-human VEGF\textsubscript{165b} when reconstituted with 1.0 mL of PBS. A working concentration of 2 µg/mL was used and created by diluting in PBS (without Ca/Mg).
- Detection Antibody (Part 842337) – 45 µg/mL of biotinylated mouse anti-human VEGF\textsubscript{165b} when reconstituted with 1.0 mL of Reagent Diluent. A working concentration of 250 ng/mL was created and used by diluting in Reagent Diluent.
Streptavidin-HRP (Part 890803) – 1.0 mL of streptavidin conjugated to horseradish peroxidase was diluted to working concentration (1:200 dilution) using the Reagent diluent.

- Standard - the standard was reconstituted with 0.5 mL of Reagent Diluent, creating a stock solution of 120 ng/mL. The solution was then diluted with Reagent Diluent when needed to create a 7-point standard curve. The highest point on the curve was at 4000 pg/mL and the lowest point was at 62.5 pg/mL.

**Assay Procedure**

1) On day one, the capture antibody was diluted to the working concentration in PBS. A 96-well microplate (R&D Systems, #DY990) was coated with the capture antibody, with 100 µL per well. The plate was sealed and incubated overnight at room temperature.

2) The following day, each well was aspirated and washed (400 µL) with wash buffer three times using the Hydroflex autowasher.

3) Adding 300 µL of Reagent Diluent to each well then blocked the plate. The plate was incubated at room temperature for one hour with the diluent.

4) The aspiration/wash in step 2 was repeated.

5) In 1.5 ml Microcentrifuge tubes (Sarstedt), the samples were diluted with the Reagent Diluent. Samples were loaded with a known quantity of protein (i.e. 50 mg of protein) that was within the standard curve.

6) The standard curve was created through a series of 2-fold dilutions. The highest point was 4000 pg/mL and the lowest point was 62.5 pg/mL.

7) 100 µL of Standard, control, or sample was then added to each well. An adhesive strip was then used to cover the plate and it was left to incubate at room temperature for 2 hours.

8) After two hours, each well of the plate was aspirated washed using the wash buffer. The Hydroflex autowasher by Tecan was used. Tecan’s Hydrocontrol 2.0 software was also used. A total of three washes were done, with 400 µL of wash buffer added to each well each wash.

9) 100 µL of the Detection Antibody was then added to each well using a multi-channel pipette. A new adhesive strip was used to cover the plate and the plate was left at room temperature to incubate for 2 hours.

10) The aspiration/wash as in step 9 was repeated.

11) 100 µL of the working dilution of Streptavidin-HRP was added to each well using a multi-channel pipette. The plate was covered and incubated at room temperature for 20 minutes.

12) The aspiration/wash as in step 9 was repeated.

13) 100 µL of Substrate Solution was added to each well. The plate was incubated at room temperature for 20 minutes while covered with foil.

14) 50 µL of Stop Solution was added to each well using a multi-channel pipette. Pipetting the Stop Solution up and down three times ensured thorough mixing.

15) The optical density of each well was determined at 450 nm using an Infinite M200 micropate reader by Tecan. The software used was Magellan6, also by Tecan. Wavelength correction was set to 570 nm.
6.2.13  sVEGFR1 (sFlt1) ELISA (R&D Systems, #MVR100) ELISA

Reagent Preparation

- Mouse VEGFR1 Control – the control was reconstituted with 1.0mL of deionized water. The vial was then placed on a shaker at room temperature to allow for the powder to completely dissolve.
- Wash Buffer – for each plate, 25 mL of Wash Buffer Concentrate was added to 600 mL of deionized water, making a final volume of 625 mL.
- Substrate Solution – Colour Reagents A and B were mixed together in equal volumes right before their use. 100 µL of the resultant mixture is used per well.
- Mouse VEGFR1 Standard – the standard was reconstituted with 2.0 mL of Calibrator Diluent RD5-3. This created a stock solution of 8000 pg/mL. The vial was put on a shaker at room temperature to allow for the powder to completely dissolve.

Assay Procedure

1) In 1.5 ml Microcentrifuge tubes (Sarstedt), the samples were diluted with the Calibrator Diluent RD5T. Samples were loaded with tested dilutions that fell within the standard curve.
2) The standard curve was created through a series of 2-fold dilutions. The highest point was 8000pg/mL and the lowest point was 125pg/mL.
3) The plate was removed from the seal and 50 µl of Assay Diluent RD1-21 was added to each well using a multi-channel pipette.
4) 50 µL of Standard, control, or sample was then added to each well. An adhesive strip was then used to cover the plate and it was left to incubate at room temperature for 2 hours on a shaker.
5) After two hours, each well of the plate was aspirated washed using the wash buffer. The Hydroflex autowasher by Tecan was used. Tecan’s Hydrocontrol 2.0 software was also used. A total of five washes were done, with 400 µL of wash buffer added to each well each wash.
6) 100 µL of mouse VEGF R1 Conjugate was then added to each well using a multi-channel pipette. A new adhesive strip was used to cover the plate and the plate was left at room temperature to incubate for 2 hours on a shaker.
7) The aspiration/wash as in step 5 was repeated.
8) 100 µL of Substrate Solution was added to each well using a multi-channel pipette. The plate was incubated at room temperature for 30 minutes on the benchtop while covered with foil.
9) 100 µL of Stop Solution was added to each well using a multi-channel pipette. Pipetting the Stop Solution up and down three times ensured thorough mixing.
10) The optical density of each well was determined at 450 nm using an Infinite M200 micropate reader by Tecan. The software used was Magellan6, also by Tecan. Wavelength correction was set to 570nm.
References


Clapp JF, III and Capeless E. Cardiovascular function before, during, and after the first and subsequent pregnancies. 1997. Am J Cardiol 80: 1469-1473

Coan PM, Ferguson-Smith AC and Burton GJ. Developmental dynamics of the definitive mouse placenta assessed by stereology. 2004. Biol Reprod 70: 1806-1813


Davis-Smyth T, Chen H, Park J, Presta LG, Ferrara N. that it binds with a lower affinity compared to VEGFR-1 (220).The second immunoglobulin-like domain of the VEGF tyrosine kinase receptor Flt-1 determines ligand binding and may initiate a signal transduction cascade. 1996. EMBO J, 15:49’9-4927


Ferrara, N. Vascular endothelial growth factor. 1993. Trends in Cardiovascular Medicine, 3:244-250


Gille H, Kowalski J, Yu L, Chen H, Pisabarro MT, Davis-smuth T, Ferrara N. A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR-1) constitutively inhibits VEGF-dependent PI3 kinase activation and endothelial cell migration. 2000. EMBO J, 19:4064-4073


Ito TK, Ishii G, Chiba H, Ochiai A. The VEGF angiogenic switch of fibroblasts is regulated by MMP-7 from cancer cells MMP-7 activates latent VEGF from fibroblasts. 2007. Oncogene, 26:7194-7203


Maru Y, Yamaguchi S, Shibuya M. Flt-1, a receptor for vascular endothermal growth factor, has transforming and morphogenic potentials. 1998. Oncogene, 16:2585-2595


McKeeman GC, Ardill JE, Caldwell CM, Hunter AJ, McClure N. Soluble vascular endothelial growth factor receptor-1 (sFlt-1) is increased throughout gestation in patients who have pre-eclampsia develop. 2004. Am J Obstet Gynecol, 191:1240-6


Myatt L. Role of Placenta in Pre-eclampsia. 2002. Endocrine, 19:103-111


Soh EY, Sobhi SA, Wong MG, Meng YG, Siperstein AE, Clark OH, Duh QY. Thyroid-stimulating hormone promotes the secretion of vascular endothelial growth factor in thyroid cancer cell lines. 1996. Surgery 120:944–947


**Wulff C, Wilson H, Wiegand SJ, Rudge JS, Fraser HM.** Prevention of thecal angiogenesis, antral follicular growth, and ovulation in the primate by treatment with vascular endothelial growth factor Trap R1R2. 2002. Endocrinology, 143;2797-2807


