ANALYSIS OF THE VEGFR1 (FLT-1) ISOFORMS IN VASCULAR DEVELOPMENT

Nicholas Chris Kappas

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Approved by:

Advisor: Victoria L. Bautch

Reader: Frank Conlon

Reader: Robert J. Duronio

Reader: Robert Goldstein

Reader: Lillie L. Searles
ABSTRACT

Nicholas C. Kappas: Analysis of the VEGFR1 (FLT-1) isoforms in vascular development

(Under the direction of Victoria L. Bautch)

While sprouting angiogenesis is critical to blood vessel formation, the cellular and molecular controls of this process are poorly understood. As blood vessels form, the endothelial cells that comprise the vessel must coordinate the processes of cell division and cell migration for proper vascular morphogenesis to occur. Vascular endothelial growth factor-A (VEGF-A), an endothelial cell-specific ligand, is critical for these endothelial cell responses. Two high affinity receptors for VEGF-A, Flk-1 and Flt-1, are responsible for the effects of VEGF-A on vascular development. While VEGF-A signaling through Flk-1 has been shown to stimulate endothelial cell division, migration, and survival, the role of Flt-1 is less clear. Mice lacking the flt-1 receptor die around mid-gestation (E9.5) due to vascular overgrowth and disorganization. It is thought that Flt-1 acts to sequester VEGF-A, thereby inhibiting signaling through Flk-1.

In my thesis work, I have used time-lapse imaging of GFP-expressing vessels derived from mouse embryonic stem (ES) cells, to reveal a role for flt-1 in modulating vascular branch (sprouts) formation and migration. Analysis of time-lapse data also reveals that flt-1 mutant vessels have increased rates of endothelial
cell division. This data supports a model whereby the role of \textit{flt-1} is to positively regulate sprout formation and migration and negatively regulate endothelial cell proliferation.

Flt-1 is expressed as a full-length receptor tyrosine kinase (mFlt-1) and as a soluble isoform (sFlt-1). In order to get a clearer understanding of the role of the flt-1 isoforms in vascular development, I have reintroduced each flt-1 isoform into \textit{flt-1} mutant ES cells. My results reveal that the sFlt-1 isoform rescues the vessel branching morphogenesis defect observed in \textit{flt-1} mutant vessels, and that it does so more efficiently than the mFlt-1 isoform. Furthermore, both flt-1 isoforms appear to partially rescue the cell proliferation defect. These results support a model whereby Flt-1 primarily affects vascular branching morphogenesis via a soluble molecule, sFlt-1, perhaps by affecting the formation of a VEGF-A gradient that normally provides directional information for sprouts. These results also reveal that both Flt-1 isoforms have the ability to regulate endothelial cell proliferation.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LIST OF TABLES</strong></td>
<td>ix</td>
</tr>
<tr>
<td><strong>LIST OF FIGURES</strong></td>
<td>x</td>
</tr>
<tr>
<td><strong>I. GENERAL INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>A. Mechanisms of Blood Vessel Formation</td>
<td>1</td>
</tr>
<tr>
<td>1. Vasculogenesis</td>
<td>3</td>
</tr>
<tr>
<td>2. Angiogenesis</td>
<td>5</td>
</tr>
<tr>
<td>3. Blood Vessel Formation in the Adult</td>
<td>7</td>
</tr>
<tr>
<td>4. In Vitro Differentiation of Embryonic Stem Cells to Form Vessels</td>
<td>8</td>
</tr>
<tr>
<td>B. Molecular Control of Blood Vessel Development</td>
<td>10</td>
</tr>
<tr>
<td>1. Vascular Endothelial Growth Factor-A (VEGF-A)</td>
<td>10</td>
</tr>
<tr>
<td>2. Flk-1 (VEGFR-2)</td>
<td>13</td>
</tr>
<tr>
<td>3. Flt-1 (VEGFR-1)</td>
<td>15</td>
</tr>
<tr>
<td><strong>II. THE VEGF-A RECEPTOR FLT-1 (VEGFR-1) IS A POSITIVE MODULATOR OF VASCULAR SPROUT FORMATION AND BRANCHING MORPHOGENESIS</strong></td>
<td>20</td>
</tr>
<tr>
<td>A. Introduction</td>
<td>20</td>
</tr>
<tr>
<td>B. Materials and Methods</td>
<td>24</td>
</tr>
</tbody>
</table>

Cell culture and *in vitro* differentiation. 24
DNA constructs and electroporation.............................................................. 24

Time-lapse imaging and quantitative analysis............................................. 25

Antibody and β-galactosidase staining......................................................... 26

Embryo manipulations.................................................................................. 27

C. Results ....................................................................................................... 27

Dynamic image analysis of vascular sprout formation ................................. 27

Sprout formation and function is compromised
in the absence of flt-1 ............................................................................... 29

Cell division defects are not immediately upstream
of morphogenesis defects in flt-1 mutant vessels ..................................... 32

Aberrant flt-1−/− vascular morphogenesis is rescued in a
non cell-autonomous manner and with an sflt-1 transgene ...................... 33

D. Discussion .................................................................................................. 35

How does flt-1 affect morphogenesis.......................................................... 35

A model of vascular sprout formation.......................................................... 38

Conclusions .................................................................................................... 41

III. SOLUBLE FLT-1 (VEGFR-1) IS THE PREDOMINANT FLT-1
ISOFORM REGULATING VESSEL BRANCHING ....................................... 57

A. Introduction............................................................................................... 57

B. Materials and Methods ............................................................................. 61

Cell culture and in vitro differentiation ....................................................... 61

DNA constructs and electroporation............................................................ 61

Antibody staining ........................................................................................ 64

Quantitative image analysis ......................................................................... 64

RNA analysis ................................................................................................ 65
In situ hybridization .................................................................................. 66

C. Results ..................................................................................................... 67

Transgene encoding individual flt-1 isoforms
rescue the flt-1⁻⁻ vessel phenotype .................................................... 67

Expression profile of flt-1 isoform
transgene-expression clones ............................................................. 69

The cytoplasmic domain of mflt-1 is not required
for flt-1 to rescue the flt-1⁻⁻ vascular phenotype ......................... 72

Genetic targeting of flt-1 isoform transgenes into the ROSA26
locus: sflt-1 rescues branching morphogenesis more
efficiently than mflt-1 ........................................................................ 72

D. Discussion ............................................................................................. 74

IV. ENDOTHELIAL CELL DIVISION OPERATES INDEPENDENTLY
OF BRANCHING MORPHOGENESIS IN ES CELL-DERIVED
VESSELS ..................................................................................................... 99

A. Introduction .......................................................................................... 99

B. Materials and Methods ........................................................................ 103

ES cell culture and in vitro differentiation ............................................. 103

DNA constructs and electroporation ..................................................... 103

Antibody staining .................................................................................. 104

Quantitative image analysis ................................................................. 104

RNA analysis .......................................................................................... 105

C. Results .................................................................................................. 106

Selection of WT Tg Tie2-p21-GFP clones ............................................. 106

WT Tg Tie2-p21-GFP clones have a reduced vascular area ............... 106

WT Tg Tie2-p21-GFP clones have normal vessel branching ........... 107
WT Tg Tie2-p21-GFP clones have smaller vessel diameters compared to WT vessels ................................................................. 108

D. Discussion ........................................................................................................... 109

V. GENERAL DISCUSSION ....................................................................................... 127

Morphogenesis versus Cell Division ........................................................................ 128

Regulation of sprouting angiogenesis by flt-1 .................................................. 129

Are endothelial cells divided into two distinct subpopulations? ...................... 133

Conclusions ............................................................................................................. 135

REFERENCES ............................................................................................................ 136
### LIST OF TABLES

#### Chapter II.

Table 1: Distribution of endothelial cell divisions during sprouting angiogenesis ........................................... 42

#### Chapter III.

Table 2: Distribution of phenotypes from differentiated flt-1<sup>=/=</sup>
ES cell clones expressing flt-1 transgene ........................................... 82
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Chapter II.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1:</td>
<td>GFP expression allows for visualization of mammalian sprouting angiogenesis</td>
</tr>
<tr>
<td>Figure 2:</td>
<td>Sprout formation and migration are reduced in the absence of <em>flt-1</em></td>
</tr>
<tr>
<td>Figure 3:</td>
<td>Branching morphogenesis is reduced in the absence of <em>flt-1</em></td>
</tr>
<tr>
<td>Figure 4:</td>
<td>Sprouting from the dorsal aorta is reduced in the absence of <em>flt-1</em></td>
</tr>
<tr>
<td>Figure 5:</td>
<td>Endothelial cell divisions are increased in the absence of <em>flt-1</em></td>
</tr>
<tr>
<td>Figure 6:</td>
<td>The <em>flt-1</em> mutant phenotype is partially rescued non cell-autonomously and by a sflt-1 transgene</td>
</tr>
<tr>
<td>Figure 7:</td>
<td>Models of <em>flt-1</em> modulation on VEGF signal – negative modulation results in net positive effect on sprout formation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter III.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 8:</td>
<td>Differentiation of <em>flt-1</em>/* Tg PECAM sflt-1/mflt-1/mflt-1ΔCyt* ES cell Cultures show rescue of the <em>flt-1</em> mutant vessel phenotype</td>
</tr>
<tr>
<td>Figure 9:</td>
<td>Branch point analysis of Primary Morphogenesis Rescue clones</td>
</tr>
<tr>
<td>Figure 10:</td>
<td>Quantitation of vascular area in <em>flt-1</em> isoform transgene-expressing clones</td>
</tr>
<tr>
<td>Figure 11:</td>
<td><em>flt-1</em>/* Tg PECAM <em>flt-1</em> clones express <em>flt-1</em> RNA throughout developmental time-course</td>
</tr>
<tr>
<td>Figure 12:</td>
<td><em>flt-1</em>/* Tg PECAM <em>flt-1</em> clones express <em>flt-1</em> RNA in ES cell-derived vessels</td>
</tr>
<tr>
<td>Figure 13:</td>
<td>Flt-1 isoform transgene expression levels by real-time PCR correlate with <em>flt-1</em> mutant vessel rescue phenotype</td>
</tr>
</tbody>
</table>
Figure 14: \( f_{lt-1}^{-/-} \); Tg ROSA26-PECAM- sflt-1 clone contains more branched vascular networks than \( f_{lt-1}^{-/-} \); Tg ROSA26-PECAM- mflt-1 clone ............................................ 95-96

Figure 15: sflt-1 more efficient than mflt-1 to positively affect branching morphogenesis ................................................... 97-98

Chapter IV.

Figure 16: The cell cycle.......................................................... 115-116

Figure 17: WT \( \text{Tg Tie}^{2}\text{-p21-GFP} \) clones express the p21-GFP fusion transgene.......................................................... 117-118

Figure 18: Quantitation of proliferation rescue.................................................. 119-120

Figure 19: WT \( \text{Tg Tie}^{2}\text{-p21-GFP} \) transgenic clones and WT cultures have equivalent vascular branching ........................................ 121-122

Figure 20: WT \( \text{Tg Tie}^{2}\text{-p21-GFP} \) transgenic clones have reduced vessel Diameters compared to WT vessels ........................................... 123-124

Figure 21: Model of communication between endothelial cell division pathway and morphogenesis pathway during blood vessel development .................................. 125-126
CHAPTER I

GENERAL INTRODUCTION

In this thesis, I describe my work investigating the role of the VEGF-A receptor, flt-1, in murine blood vessel development. Specifically, chapter II describes time-lapse imaging of wild-type and flt-1 mutant ES cell-derived blood vessels. This analysis has revealed both dynamic processes of normal blood vessel development along with morphogenetic and cell division defects in the development of flt-1 mutant blood vessels. Chapter III investigates the role of the two flt-1 receptor isoforms, soluble flt-1 (sflt-1) and membrane-tethered flt-1 (mflt-1), during vascular development. Here, I have demonstrated that sflt-1 is more efficient than mflt-1 in rescuing a flt-1\(^{-/-}\) morphogenesis defect, while both isoforms can affect a rescue of endothelial cell proliferation. This work supports a model whereby flt-1 primarily affects vascular branching morphogenesis via a soluble molecule, sflt-1. Chapter IV describes the relationship between endothelial cell division and vascular branching morphogenesis. I begin with a general introduction on vascular development, followed by what is currently known about how flt-1 exerts its effects on vascular development.

A. Mechanisms of Blood Vessel Formation

The basic constituents of the vascular system, endothelial and blood cells, are determined very early during embryonic development in vertebrates (Flamme et
al., 1997; Olsson et al., 2006; Risau and Flamme, 1995). The closed circulatory system is critical for promoting cellular survival and growth by functioning as a transport system for nutrient distribution, gas exchange, and waste removal to essentially all tissues of the body. This is highlighted by the fact that the cardiovascular system is the first functional organ that develops in the vertebrate embryo. Early vertebrate embryonic life relies on passive diffusion to facilitate oxygen uptake. However, as the size of the embryo increases, passive diffusion is not an effective method of gas exchange. The increasing oxygen demands of the embryo are met by induction of the cardiovascular system, whereby formation of the heart, blood, and blood vessels are initiated.

The initial steps of blood vessel formation require a combination of multiple cellular processes in several cell types to form a mature, branched vascular network. The first steps in developing a primitive branched vascular plexus involve differentiation, migration, cell division, and fusion of endothelial cells. This nascent plexus subsequently matures and strengthens by recruitment of mural cells, such as smooth muscle cells and pericytes, which envelop the primitive endothelial cell vessel lining. In the mouse, the vascular network is connected to the developing heart, and circulation is established upon initiation of the heartbeat around embryonic day 8.5 (E8.5) (Nishii and Shibata, 2006).

During development, blood vessels form through a combination of two processes termed vasculogenesis and angiogenesis (Noden, 1989) review: (Risau, 1997). Vasculogenesis is the in situ differentiation of endothelial cells from mesoderm, and the subsequent coalescence of these endothelial cells into a
primitive cord (Risau and Flamme, 1995). Angiogenesis is the subsequent expansion of blood vessels from a pre-existing primitive cord or blood vessel, by both sprouting and non-sprouting mechanisms (review) (Risau, 1997). Blood vessels are then pruned and remodeled into a functional adult circulatory system.

1. **Vasculogenesis**

   The first step in the process of vasculogenesis involves the differentiation of endothelial precursor cells known as angioblasts. Angioblasts differentiate from multiple mesodermal sources, such as from lateral-plate, paraxial, and yolk sac mesoderm around embryonic day 7.0 (E7.0) of mouse development (Coffin et al., 1991; Risau and Flamme, 1995). Several key experiments have suggested crucial roles for fibroblast growth factor (FGFs) and bone morphogenetic proteins (BMPs) as inductive cues for the angioblast lineage. Dissociated quail epiblast can be induced to form both endothelial cells and hematopoietic cells upon treatment with FGF (Flamme and Risau, 1992). Also, the use of endothelial cell markers in conjunction with lineage tracing suggest the requirement of BMP signaling in hematopoietic and endothelial cell development (Walmsley et al., 2002). The first secreted molecule with specificity for the endothelium during development is vascular endothelial growth factor (VEGF). Angioblasts express markers such as the VEGF receptors Flk-1 (VEGFR-2) and Flt-1 (VEGFR-1), as well as PECAM (CD31) (Bautch et al., 2000; Drake, 2003; Fong et al., 1996; Yamaguchi et al., 1993) following their specification. Subsequent morphogenesis transforms vessel cords into lumenized vessels, and by this time, angioblasts begin to express more
definitive endothelial cell markers such as CD34, VE-Cadherin, and ICAM-2 (Bautch et al., 2000; Drake and Fleming, 2000; Kamei et al., 2006).

Expression of many of the aforementioned markers is not unique to the endothelial cell lineage, since some hematopoietic precursors and other cell types also express these markers. However, as with the hematopoietic lineage, a set of markers defines the endothelial cell lineage. The expression of markers by both endothelial and hematopoietic cells, in addition to the observation that close association of nascent endothelial cells with blood cells occurs in the blood islands of the yolk sac, has led to the hypothesis that both of these cell populations are derived from a common precursor, known as the hemangioblast (Sabin, 1920; Murray, 1932; His, 1990; (Choi, 2002). The hemangioblast model was further supported by the isolation of individual genes that, when mutated, affected the development of both endothelial and hematopoietic lineages (Carmeliet et al., 1996; Damert, 2002; Ferrara et al., 1996; Shalaby et al., 1995). Further evidence for the existence of the hemangioblast was found with the isolation of individual cells that can differentiate into both blood cells and endothelial cells from embryonic stem (ES) cell cultures (Choi et al., 1998; Wang et al., 2004). Additional studies provide evidence for the existence of hemangioblasts. In vitro experiments identify a postnatal hemangioblast in a subset of cells that express CD34, a marker for hematopoietic progenitors, and Flk-1 (Pelosi et al., 2002). There is also evidence that hemangioblasts arise from cells that express the TEK receptor in the aorta-gonad-mesonephros region of the mouse (Hamaguchi et al., 1999). Furthermore, hemangioblast commitment has recently been demonstrated to arise from the
murine primitive streak, before blood island development occurs in the yolk sac (Huber et al., 2004). These results suggest that angioblasts can derive from a hemangioblast precursor; however, it has been suggested that the hemangioblast is not a required intermediate. An example of this can be found in the dorsal aorta, which assembles in the absence of hematopoiesis (Jaffredo et al., 2000).

Once angioblasts coalesce (by either migratory or non-migratory mechanisms) and differentiate to form endothelial cells, these primitive vessels can then become lumenized. Subsequent expansion of a lumenized vascular network is required for establishing the circulation of blood in the embryo.

2. Angiogenesis

During angiogenesis, primitive vessels that form by vasculogenesis expand to form secondary vascular segments such as capillaries and venules (Olsson et al., 2006; Risau, 1997). There are two types of angiogenesis in vivo, they are termed sprouting and non-sprouting (intussusceptive) angiogenesis (Burri, 1992; Burri and Tarek, 1990).

During development, sprouting angiogenesis occurs in both the embryo and in the yolk sac when vessels sprout from an established parent vessel. The sprouting of new vessels follows a well-defined program that includes proteolytic degradation of the basement membrane, endothelial cell proliferation, formation of solid sprouts of endothelial cells connecting to a neighboring vessel via cell migration, and restructuring of a sprout into a lumen, lined by endothelial cells and integrated in the vascular network (Ausprunk and Folkman, 1977; Burri et al., 2004;
Clark and Clark, 1939; Daniel and Abrahamson, 2000). In the embryo, the intersomitic vessels, which branch from the dorsal aorta, form by sprouting angiogenesis. Also, the brain is vascularized by the process of sprouting angiogenesis.

In the process of non-sprouting angiogenesis, a pre-existing vessel is split into two by localized extracellular matrix and tissue invasion. This was first demonstrated in capillary beds of the lung, where intussusception predominates over sprouting angiogenesis (Burri, 1992). During development, non-sprouting angiogenesis occurs simultaneously with sprouting angiogenesis.

Although vasculogenesis and angiogenesis are often times thought of as occurring during two different stages of development, where vasculogenesis occurs early during development, and angiogenesis vascularizes most embryonic organs and all adult blood vessels. However, there is evidence that endothelial precursor cells may contribute to neoangiogenesis in the adult, suggesting that both vasculogenesis and angiogenesis may be functioning throughout life (Asahara et al., 1997).

Once the vascular endothelium has assembled, mural cells such as smooth muscle cells (SMCs) and pericytes are recruited to the vascular endothelium to provide structural support. SMCs and pericytes are important regulators of blood vessel development, maturation, and remodeling (review) (Eklund and Olsen, 2006). Angiopoietin-1 and 2 (Ang1 and Ang2), which are expressed by pericytes/SMCs and endothelial cells, respectively (Maisonpierre et al., 1997), have also been identified as ligands for the receptor tyrosine kinase, Tie-2 (Davis et al., 1994). Signaling
through the endothelial cell-specific receptor, Tie-2, is important for endothelial cell survival, vascular permeability, and may regulate recruitment of pericyte/SMC to the vascular endothelium. Ang1 signaling through Tie-2 has been shown to function in a complementary and coordinated fashion with VEGF-A, and contribute to angiogenesis and the maturation and stabilization of blood vessels (Sato et al., 1995) (Suri et al., 1996). Targeted mutation of Tie-2 or Ang1 in mice results in embryonic lethality between day 9.5 and 12.5 due to a lack of remodeling of the primary capillary plexus (Sato et al., 1995) (Suri et al., 1996). Thus suggesting a requirement for proper maintenance and remodeling of the vasculature in the embryo and adult.

3. Blood Vessel Formation in the Adult

Although it is established that blood vessels form and expand during development, angiogenesis can also occur in adult vertebrates. The formation of new vessels in the adult is essential when there is vessel occlusion, and the body must form collateral vessels to bypass the ischemic area. Vascular sprouting is also essential during wound healing (Folkman and Klagsbrun, 1987). Moreover, adult angiogenesis is also at play during a variety of pathological conditions such as diabetic retinopathy, arthritis, and tumor formation (Folkman, 2003; Frank, 2004).

Although there are other mechanisms of adult angiogenesis, one model involves the recruitment of new vessels to hypoxic tissues. VEGF expression is regulated by oxygen, and most cells secrete VEGF when they experience hypoxic conditions, resulting in the directional growth of vessels to the hypoxic tissue.
Adaptation to hypoxic conditions involves changes in the protein expression and DNA binding activity of a family of transcription factors known as hypoxia inducible factors (HIFs), the most prevalent form being HIF-1\(\alpha\), which can directly promote VEGF expression (Brahimi-Horn and Pouyssegur, 2005). Thus, to recruit these vessels, hypoxic tissues can secrete pro-angiogenic growth factors such as VEGF to invade tissue and supply needed oxygen and nutrients (Maxwell and Ratcliffe, 2002).

The microenvironment of tumors is a good example of the hypoxic model of adult angiogenesis. As tumor cells proliferate, they will typically outgrow their vascular supply, leading to the formation of hypoxic tumor cells that are thought to be sensed by the vascular supply (review) (Verma, 2006). Tumor tissues subsequently secrete VEGF, which leads to the recruitment of host-derived blood vessels (Carmeliet, 2005). These newly recruited blood vessels provide cancerous cells with the nutrients and oxygen necessary for growth and survival, while also providing a mechanism for metastatic cells to migrate to other regions of the body, where they can reinitiate de novo tumorigenesis.

4. **In Vitro Differentiation of Embryonic Stem Cells to Form Vessels**

While the mouse is a powerful genetic tool and model system to examine both embryonic and adult blood vessel formation, mice have the disadvantage that development occurs *in utero* and it is not easily accessible for manipulation. Also, because embryonic lethal phenotypes make it virtually impossible to examine subsequent vascular phenotypes, embryonic stem (ES) cells provide a useful
method to study certain aspects of blood vessel development (Bautch et al., 1996; Wang et al., 1992).

Embryonic stem (ES) cells, which are derived from developing mouse blastocysts, have the capacity to give rise to all cell types in the adult body. The ability of ES cells to do so has opened the door for novel experimental approaches in the field of developmental biology. Under appropriate culture conditions, ES cells will also differentiate and form embryoid bodies (EBs). Upon attachment to a permissive surface, EBs continue a programmed differentiation, where many of the cells that differentiate from the EB in vitro reflect those found in the developing embryo and yolk sac, such as hematopoietic cells, endoderm, and endothelial cells (Bautch, 2002; Wang et al., 1992; Wiles and Keller, 1991). The ES culture system has been used as a model to study blood vessel formation in flt-1-/-, flk-1-/-, and vegf-a-/- genetic backgrounds (Bautch et al., 2000; Kearney et al., 2002; Kearney et al., 2004; Schuh et al., 1999). Endothelial cells that arise during ES cell differentiation have the potential to form primitive blood vessels, comparable to the vessels that first form in vivo, further suggesting that endothelial cell differentiation in this model system mimics the in vivo environment (Bautch, 2002; Bautch et al., 2000; Kearney et al., 2002).
B. Molecular Control of Blood Vessel Development

Many of the genes that are essential for the proper development of blood vessels have already been identified. Vascular Endothelial Growth Factor-A (VEGF-A), VEGFR-2 (Flk-1) and basic Fibroblast Growth Factor (bFGF) are thought to influence angioblast differentiation (Carmeliet et al., 1996; Ferrara, 1999; Ferrara et al., 1996; Shalaby et al., 1997).

Correct modulation of VEGF-A signaling via Flk-1 is required for proper cell division and migration of endothelial cells during blood vessel formation, and it is thought that Flt-1 is a negative regulator of Flk-1 signaling (Kearney et al., 2002; Roberts et al., 2004). In the next section, I will discuss several of the molecules that modulate vascular development. I will begin with a discussion of VEGF-A, and follow with discussion of the VEGF-A receptors, Flk-1 and Flt-1. These molecules comprise a signaling transduction pathway that regulates cell migration, division, and survival, and is essential for proper vascular development (Ferrara and Davis-Smyth, 1997; Shibuya et al., 1999).

1. Vascular Endothelial Growth Factor-A (VEGF-A)

The VEGF family members are part of the PDGF growth factor family of proteins. Although VEGF-A is the family member most extensively studied, there are five total members that make up the VEGF family (VEGF A-D, PLGF). VEGF-A was originally discovered as an efficient and rapid inducer of vascular permeability \textit{in vivo} (Keck et al., 1989) and therefore is also known as vascular permeability factor (VPF). VEGF-A is secreted from non-endothelial cells and preferentially forms
homodimers, although VEGF-A and PLGF heterodimers have been identified (De Falco et al., 2002). Most biological activities of VEGF-A are mediated by its interaction with two high-affinity receptor tyrosine kinases, Flt-1 and Flk-1, which are expressed on endothelial cells. *In vitro* studies have indicated that VEGF-A stimulates endothelial cell migration, division, and survival (Bernatchez et al., 1999; Gerber et al., 1999; Leung et al., 1989). Gene targeting studies have also shed light on the role of VEGF-A, demonstrated by an experiment in which genetic deletion of only a single copy of VEGF-A results in embryonic lethality at mid-gestation (E11) in mice due to severe defects in vasculogenesis and angiogenic sprouting (Carmeliet et al., 1996; Ferrara et al., 1996). Moreover, further analysis revealed that VEGF-A homozygous null mice, generated by aggregation of mutant ES cells with tetraploid embryos, also resulted in embryonic lethality around E9.5-E10, due to severe defects in vascular development. Furthermore, two-fold overexpression of VEGF-A led to embryonic lethality in mice (Miquerol et al., 2000). Likewise, analysis of blood vessel formation from VEGF-A+/− and VEGF-A−/− ES cell cultures (Bautch et al., 2000) demonstrates a dose-dependent requirement for VEGF-A in vascular development.

Murine VEGF-A is transcribed from a single gene that is alternatively spliced to form three mRNA species, encoding three proteins: VEGF_{120}, VEGF_{165}, and VEGF_{188} (Ng et al., 2001a; Shima et al., 1996; Tischer et al., 1991). These isoforms have differing affinities for heparin sulfate proteoglycans, and therefore are predicted to be distributed differentially from VEGF-A-producing cells via differential binding to the extracellular matrix. VEGF_{120}, which lacks a heparin binding domain, is thought
to be diffusible. VEGF$_{188}$ contains two heparin-binding domains, and is thought to be found on the cell surface or bound to the extracellular matrix in close proximity to the VEGF-A producing cell. VEGF$_{165}$, which lacks one of the two heparin-binding domains, is thought to have intermediate properties (Park et al., 1993). Thus, a model has been proposed in which the VEGF-A isoforms establish a morphogen gradient, and that an intact gradient is crucial for proper vessel formation and patterning (Ruhrberg et al., 2002; Stalmans et al., 2002). In support of this model, analysis of retinal vessels of mouse mutants expressing only VEGF$_{120}$ or VEGF$_{188}$ showed defects in vessel size and number, suggesting that proper VEGF-A distribution and availability is critical for normal blood vessel development and patterning (Ruhrberg et al., 2002; Stalmans et al., 2002) (Gerhardt et al., 2003).

The downstream components of the VEGF signaling pathway have also been studied extensively (Olsson et al., 2006). VEGF stimulation of endothelial cells results in the phosphorylation of many signaling proteins including phospholipase C (PLC$_\gamma$), which leads to the activation of mitogen activated protein kinase (MAPK)/ERK1/2 cascade and proliferation of endothelial cells (Takahashi and Shibuya, 2001). Other targets include phosphatidylinositol 3’ kinase (PI3K), which is involved in survival (Gerber et al., 1998); c-Src, which is involved in vascular permeability and migration (Chou et al., 2002; He et al., 1999); and focal adhesion kinase (FAK), which also plays a role in migration (Abedi and Zachary, 1997). These upstream signaling components, along with others, are stimulated via VEGF-A, and eventually lead to changes in gene expression, as well as modulation of other pathways that regulate cell division, morphogenesis, and survival. Much of my own
thesis work revolves around the question of how modulating VEGF-A stimulation of Flk-1, via Flt-1, can affect cell proliferation and migration during vascular morphogenesis.

2. Flk-1 (VEGFR-2)

The Flk-1 receptor belongs to a subfamily of receptor tyrosine kinases (RTKs) implicated in both vasculogenesis and angiogenesis. Flk-1 is first expressed in embryonic lateral mesoderm at E6.5, and subsequently observed at low levels in many mesoderm progenitors (E6.5-8.5) as well as later mesodermal cell types including cardiomyocytes and somite (E8.5) (Ema and Rossant, 2003) (Ema et al., 2006). Flk-1 is also expressed in angioblasts surrounding the blood islands around E7.0 (Shalaby et al., 1995). Flk-1 is subsequently expressed on mature endothelial cells and binds VEGF-A with high affinity (Quinn et al., 1993; Terman et al., 1992). Genetic deletion of Flk-1 results in embryos that die in utero between E8.5 and E9.5 from early defects in hematopoietic and endothelial cell development (Shalaby et al., 1995). Furthermore, targeted deletion of Flk-1 in ES cell cultures resulted in a drastic reduction of vascular endothelium formation, mimicking the phenotype observed in vivo (Shalaby et al., 1997). Moreover, the addition of either a neutralizing antibody against VEGF-A, or a dominant negative form of Flk-1 was able to block angiogenesis (Kim et al., 1993; Millauer et al., 1994). In vitro cell culture experiments have also demonstrated a requirement for Flk-1 on VEGF-A-dependent cell division, migration, and survival (Bernatchez et al., 1999; Gerber et al., 1998; Waltenberger et al., 1994). Thus, Flk-1 is required for proper
hematopoietic and vascular endothelial cell formation, as well as VEGF-A-dependent endothelial cell function.

Flk-1 is an RTK that has seven immunoglobulin-like repeats in the extracellular domain (VEGF-binding domain), a single transmembrane region, a juxta-membrane domain, followed by a cytoplasmic tail with docking sites and a tyrosine kinase domain (Fuh et al., 1998). VEGF-A binding to Flk-1 results in dimerization and activation of the receptor (review) (Schlessinger, 2000). This results in autophosphorylation of Flk-1, leading to the activation of many downstream targets, which is consistent with the idea that Flk-1 mediates many biological effects in response to VEGF-A (review) (Olsson et al., 2006).

VEGF-dependent Flk-1 activation has been shown to activate several signaling pathways in endothelial cells. Briefly, VEGF-A stimulates phospholipase C$_\gamma$ (PLC$_\gamma$), which can in turn activate Protein Kinase C (PKC) to induce cell proliferation via the Raf-MEK-MAP-kinase cascade (Guo et al., 1995; Wellner et al., 1999; Xia et al., 1996). VEGF/Flk-1 activation of phosphoinositide 3-kinase (PI3K) stimulates Akt, a protein involved in endothelial cell survival (Gerber et al., 1998; Gliki et al., 2002). Flk-1 activation and downstream signaling is also important for endothelial cell migration. It is believed that actin reorganization and focal adhesion turnover both contribute to endothelial cell migration. Actin reorganization is potentially mediated by p38 MAPK and Rac (Bernatchez et al., 2001), while focal adhesion turnover is stimulated through focal adhesion kinase (FAK) (Abedi and Zachary, 1997) and c-Src, which regulate the interactions between the extracellular matrix and the actin-
cytoskeleton, thus playing a role in endothelial cell movement (Abedi and Zachary, 1997; Abu-Ghazaleh et al., 2001; Bernatchez et al., 2001; Rousseau et al., 2000).

These studies indicate that VEGF-A exerts most of its growth promoting effects on the vasculature through its association and activation of the Flk-1 receptor. Moreover, although many studies have shown that VEGF-A signaling through Flk-1 promotes the positive effects of vessel morphogenesis (review) (Carmeliet, 2000), chapters II and III of this dissertation will focus on the role of Flt-1 as a positive regulator of blood vessel morphogenesis.

3. Flt-1 (VEGFR-1)

Flt-1 (VEGFR-1), a second high affinity VEGF receptor that is also a receptor tyrosine kinase, is important in blood vessel formation and development. Flt-1 expression is first detected in angioblasts around E6.5-E7.0 of mouse development (Peters et al., 1993). Its expression decreases slightly during late gestation, but is expressed at high levels in most adult vessels. Flt-1 is also expressed on monocytes/macrophages (Barleon et al., 1996; Clauss et al., 1996), smooth muscle cells (Wang and Keiser, 1998), and trophoblasts (Ahmed et al., 1995). Genetic studies in mice have shown that deletion of flt-1 results in embryonic lethality at mid-gestation (E9.5). The phenotype associated with this lethality, however, is very different from a genetic deletion of Flk-1: a highly disorganized vascular endothelium. Flt-1-/- mice also have excess endothelial cells in the yolk sac and heart endocardium (Fong et al., 1995). Studies using mouse ES cell-derived vessels show that the increase in endothelial cell numbers is a result of
increased endothelial cell proliferation (Kearney et al., 2002; Kearney et al., 2004). Thus, the excess numbers of endothelial cells in Flt-1 mutants suggests that Flt-1 negatively regulates vessel development by limiting the number of endothelial cells produced.

Flt-1 is expressed as a full-length receptor tyrosine kinase (mFlt-1) and as a soluble isoform (sFlt-1) that consists of only the first six N-terminal IgG-like extracellular ligand-binding domains (Kendall and Thomas, 1993; Kendall et al., 1996). Soluble Flt-1 is generated by alternative splicing of Flt-1 RNA (Kendall and Thomas, 1993). Full-length Flt-1 is organized into four different domains. The Flt-1 receptor contains an extracellular domain (7 IgG-like domains), a single transmembrane region, a juxta-membrane domain, followed by a cytoplasmic tail with docking sites and a tyrosine kinase domain (Olsson et al., 2006). The extracellular domain binds VEGF-A with a 10-fold stronger affinity for VEGF-A than that of Flk-1 (Kondo et al., 1998; Park et al., 1994).

The molecular mechanisms that underlie Flt-1 function are not completely understood. VEGF binding through the Flt-1 receptor produces a relatively weak tyrosine autophosphorylation in cultured endothelial cells (Seetharam et al., 1995) that does not transduce a mitogenic signal (de Vries et al., 1992; Shibuya et al., 1990; Waltenberger et al., 1994). The Flt-1 intracellular domain contains binding sites for PLC-γ, PI-3’kinase, and Src, suggesting a signaling role for Flt-1 in cell survival and migration (Cunningham et al., 1995; Ito et al., 1998; Waltenberger et al., 1994). In vitro studies have shown interactions between the Flt-1 intracellular
domain and Crk, a protein involved in focal adhesion signaling and cell motility (Ito et al., 1998). However, mice lacking the cytoplasmic domain of the Flt-1 receptor are viable and undergo normal vascular development, indicating that the signaling function of the Flt-1 receptor is not essential during embryonic development (Hiratsuka et al., 1998). The fact that vessels create a soluble form of Flt-1, in conjunction with the data revealing that the cytoplasmic domain is dispensible for proper vascular development, has led to a model in which Flt-1 may act as a decoy molecule to bind and sequester VEGF-A during development. Flt-1-/- ES cell-derived vessels have approximately 3-fold higher levels of activated Flk-1 than do normal vessels, consistent with a role for Flt-1 in ligand sequestration (Roberts et al., 2004). A recent study identified a single amino acid in the kinase domain of Flt-1 that severely reduces kinase activity and signaling, further suggesting its role as a ligand sink (Meyer et al., 2006). Moreover, a study using Flk-1 and Flt-1 chimeric receptors showed that the intracellular juxtamembrane region in Flt-1 suppresses VEGF-A-dependent activation of PI 3'-kinase and subsequent endothelial cell migration (Gille et al., 2000), further suggesting a non-signaling role for Flt-1 during development. Collectively, this data suggests that Flt-1 functions in vascular development as a ligand sink to bind and sequester VEGF-A, and thus regulate signaling through the Flk-1 receptor.

Although Flt-1 signaling during development is not essential for proper vascular development, Flt-1 does appear to function as a positive regulator of ischemic and pathological revascularization (Luttun et al., 2002). Placental Growth Factor (PIGF) and VEGF-B, two Flt-1-specific ligands, are required for these
functions (Bellomo et al., 2000; Olofsson et al., 1998; Park et al., 1994). PLGF also promotes recruitment of hematopoietic stem cells via Flt-1, from a quiescent to a proliferative microenvironment within the bone marrow, favoring differentiation, mobilization, and reconstitution of hematopoiesis (Rafii et al., 2003). Collectively, this data further implicates Flt-1 signaling function primarily in the adult.

Chapter II of this dissertation describes evidence that Flt-1 is a negative regulator of cell division in ES cell-derived vessels, and a positive regulator of vessel branching in ES cell-derived vessels and \textit{in vivo}. However, there are unanswered questions regarding the role of flt-1 during vascular development. Data from chapter II shows evidence to suggest that the secreted form of Flt-1 is important in regulating vessel branching. Here we propose a model in which sFlt-1 may modulate an existing VEGF-A gradient to regulate vessel branching. Although it remains unclear to what extent the mFlt-1 isoform regulates vascular development, it has been suggested that membrane-tethered Flt-1 is required developmentally for the proper delivery of VEGF-A to the endothelial cell membrane (Hiratsuka et al., 2005). This idea is supported by the observation that mice that do not express membrane-tethered Flt-1 succumb to embryonic lethality half of the time due to poor blood vessel development on the C57BL6/J genetic background. The lack of mFlt-1 in these mice results in reduced membrane-targeted VEGF-A to endothelial cells, and the subsequent suppression of Flk-1 phosphorylation on endothelial cells (Hiratsuka et al., 2005). Their conclusion, however, is ambiguous, as there is an observed 99% survival rate in mice lacking mFlt-1 on the 129 genetic background (Hiratsuka et al., 2005). The authors point to increased Flk-1 expression in 129 mice as the reason
for this high survival rate, however, their explanation is not well supported. Thus, there is no conclusive evidence to explain the role of mFlt-1 during development. In chapter III, I have attempted to get a clearer understanding of the role of mFlt-1 and sFlt-1 in vascular development, specifically vessel branching and proliferation, by reintroducing isoform-specific Flt-1 transgenes into flt-1/- ES cell-derived vessels.
A. Introduction

The first step in the formation of most blood vessels is the production of an intricately branched vascular plexus (reviews) (Daniel and Abrahamson, 2000; Drake et al., 1998; Risau, 1997). This plexus is subsequently pruned and remodeled, and in some cases coalesced to form larger vessels. The primary branched plexus forms by several processes, including the initial assembly of vascular precursor cells called vasculogenesis, and the subsequent migration of endothelial cells from the parent vessel called sprouting angiogenesis. The sprouts that form migrate until they reach another sprout or vessel, whereupon they most often form connections that are essential for elaborating and expanding the branched network. Although the formation of vascular sprouts has been described historically (Clark and Clark, 1939; Sabin, 1920), surprisingly little is known of the cellular processes and molecular controls of sprout formation, and even less is known about how these processes are integrated with other ongoing cellular events such as cell division.

The vascular sprouts that form during embryonic development extend from primitive vessels. Individual endothelial cells send out filopodia, then migrate away from the parent vessel without breaking all contacts with surrounding cells, so that
eventually multiple cells comprise the sprout. Cell numbers in the sprout are also increased by cell division behind the leading tip of the sprout (Ausprunk and Folkman, 1977; Clark and Clark, 1939). Formation of a branched vascular plexus depends on the regulated expression of vascular endothelial growth factor-A (VEGF-A) by non-endothelial cells. This signal modulates intracellular signaling pathways that regulate endothelial cell division, migration, and survival (review) (Matsumoto and Claesson-Welsh, 2001a). This regulation is dose-dependent, as modest changes in the amount of available VEGF-A in either direction compromise vascular development, and loss of even one copy of the Vegf-A gene leads to vascular disruption and embryonic lethality (Bautch et al., 2000; Carmeliet et al., 1996; Damert, 2002; Ferrara et al., 1996; Miquerol et al., 2000). Availability also seems to be regulated by the production of different isoforms of VEGF-A via alternative splicing. These isoforms have differing affinities for matrix components, and disruption of individual isoforms affects vessel morphogenesis (Park et al., 1993; Ruhrberg et al., 2002; Stalmans et al., 2002).

VEGF-A signals through two receptor tyrosine kinases, flk-1 (VEGFR2) and flt-1 (VEGFR1). VEGF/flk-1 interactions positively influence endothelial chemotaxis and proliferation, but the outcomes of VEGF/flt-1 interactions are less clear-cut (Bernatchez et al., 1999; Waltenberger et al., 1994). The flt-1 gene encodes both a receptor tyrosine kinase (mflt-1) and a secreted splice variant, sflt-1, that consists of the flt-1 extracellular domain. sflt-1 binds VEGF-A with high affinity and is a potent antagonist of VEGF/flk-1 signaling (Kendall and Thomas, 1993; Kendall et al., 1996). Mouse mutants lacking flt-1 die at embryonic day 9.5 with highly disorganized blood
vessels and excess endothelial cells (Fong et al., 1995), yet a mouse with a modified
\textit{flt-1} gene that lacks most of the cytoplasmic domain is viable (Hiratsuka et al.,
1998), suggesting that sflt-1 is sufficient for the developmental role of \textit{flt-1}. We
recently showed that \textit{flt-1} negatively modulates endothelial cell division (Kearney et
al., 2002), but the role of \textit{flt-1} in endothelial cell migration remains unclear. In vitro
studies, including analysis of chimeric VEGF receptors, failed to demonstrate a role
for \textit{flt-1} in endothelial migration (Bernatchez et al., 1999; Gille et al., 2001; Rahimi et
al., 2000; Waltenberger et al., 1994; Zeng et al., 2001). However, a study in which
VEGF/\textit{flt-1} binding was selectively blocked suggested that \textit{flt-1} promotes VEGF-
dependent actin reorganization and migration (Kanno et al., 2000).

In the course of investigating the effects of a \textit{flt-1} null mutation on blood
vessel formation, we noticed aberrant morphogenesis of \textit{flt-1}\textsuperscript{−/−} embryonic vessels
(Kearney et al., 2002). Thus we reasoned that \textit{flt-1} might modulate the VEGF-
induced endothelial cell migration that occurs during embryonic sprouting
angiogenesis. Our use of confocal time-lapse imaging afforded us the unique ability
to analyze dynamic processes of mammalian vascular development. Vascular
sprout formation was analyzed in wild-type and \textit{flt-1}\textsuperscript{−/−} mutant ES cells induced to
differentiate to form primitive vessels. To our surprise, \textit{flt-1}\textsuperscript{−/−} blood vessels exhibited
decreased sprout formation, and the \textit{flt-1}\textsuperscript{−/−} mutant sprouts that formed had a
reduced migration rate. Moreover, \textit{flt-1}\textsuperscript{−/−} embryos showed defective sprouting of
vessels from the dorsal aorta. This defect is likely not an immediate consequence of
increased endothelial cell division in the mutant background, since the sprouting
defect persisted after endothelial cell division rates returned to normal in \textit{flt-1}\textsuperscript{−/−}
mutant vessels. Rather, rescue of the mutant phenotype with an sflt-1 transgene suggests a model whereby sflt-1 protein interacts locally with VEGF-A. This interaction is predicted to establish or modify a gradient that regulates vascular sprouting and endothelial cell migration.
B. Materials and Methods

Cell culture and in vitro differentiation

Wild type (WT, +/+ ) and flt-1⁻/⁻ ES cells (Fong et al., 1995) containing an eGFP or an sflt-1 transgene under the transcriptional control of the PECAM promoter/intron 2 enhancer element (Robson, P., Ellerstrom, C., Bautch, V.L., and Baldwin, H.S., in preparation) were maintained and differentiated in vitro as described previously (Bautch et al., 1996). Cultures were plated onto slide flasks (Nalge Nunc, Rochester NY) at day 3 of differentiation and cultured at 37°C in 5% CO₂ until time-lapse imaging was performed.

DNA constructs and electroporation

The PECAM promoter/enhancer (gift of H.S. Baldwin, Robson et al., in prep) was cloned into a modified eGFP vector (BD Clontech, Palo Alto CA) for electroporation into WT ES cells, and designated PECAM-eGFP. PGK-HygroR (gift of N. Maeda) was then inserted into the PECAM-eGFP vector for electroporation into flt-1⁻/⁻ ES cells, and designated PECAM-eGFP-HygroR. sflt-1 cDNA was generated by RT-PCR amplification of a 500 bp fragment of sflt-1 (nt 1834-2544) from WT day 8 ES cell cultures, which was cloned via the TA method (Promega) and verified by sequence analysis. A 2.1 kb XbaI fragment from mflt9 (mouse fit-1 cDNA, gift of G. Breier (Breier et al., 1995)) that encompassed nt 1-2134 of full-length fit-1 was cloned into the unique XbaI site at position 2134 of the sflt-1 clone. The sflt-1 cDNA was cloned into the PECAM-HygroR vector and designated PECAM-sflt-1.
20µg of linearized PECAM-eGFP and PECAM-eGFP-Hygro<sup>R</sup> DNAs were electroporated into 2 x 10<sup>7</sup> ES cells using a BioRad GenePulser II electroporator (250V/300µF). WT ES cell selection was in 200 µg/ml G418 (Gibco, Carlsbad CA), and selection of flt-1<sup>-/-</sup> ES cells was in 200 µg/ml hygromycin B (Roche Diagnostics, Indianapolis IN). After 10-12 days, drug resistant ES colonies were picked, expanded, and analyzed. We initially analyzed 3 WT transgenic lines (designated as WT; Tg pecam-egfp) and 4 flt-1<sup>-/-</sup> transgenic lines (designated as flt-1<sup>-/-</sup>; Tg pecam-egfp), and saw no differences except by genotype, so single WT and mutant lines were used. PECAM-eGFP and PECAM-sflt-1 were linearized and electroporated into flt-1<sup>-/-</sup> ES cells at a 1:3 molar ratio as described above. After selection in hygromycin B, drug resistant ES colonies that expressed eGFP were picked and analyzed.

**Time-lapse imaging and quantitative analysis**

Slide flasks containing day 6-8 in vitro differentiated ES cultures were sealed, then placed on a heated stage on a Nikon TE300 inverted microscope with a Perkin Elmer spinning disk confocal head. Confocal images were acquired at 1 min intervals using Metamorph software (version 6.0, Universal Imaging Corp., Downingtown PA) and a Hamamatsu Orca CCD camera with a 20x objective. Quantitative image analysis was performed using Metamorph software. For sprout index measurements, total vessel perimeter was determined by outlining vessels and measuring total line length. Sprouts were defined as endothelial cell projections that included cytoplasm and were at least 10 µm in length. For sprout velocity
measurements, total sprout length was determined by measuring the distance from the tip of the sprout to the point at which the sprout joined the primary vessel wall. For cell division score measurements, average percentage of GFP-positive area was determined for each movie by thresholding and averaging 3 separate frames: the first, middle, and last frame. Significance was determined using the two-tailed student’s T test.

For fixed branch point analysis, cultures labeled with anti-PECAM antibody were photographed using a Nikon eclipse E800 upright microscope at 10x magnification. Vessel length was determined by tracing a vascular skeleton, and measuring total line length using Metamorph software. Branch points were identified by visual scoring of each frame.

**Antibody and β-galactosidase staining**

Following time-lapse imaging, ES cell cultures were rinsed with phosphate buffered saline (PBS) and fixed for 5 minutes in ice-cold methanol:acetone (50:50). Fixed cultures were reacted with rat anti-mouse PECAM at 1:1000 (MEC 13.3; B-D Pharmingen, San Diego CA) and donkey anti-rat immunoglobulin G (IgG; H+L) TRITC cross-absorbed at 1:100 (Jackson Immunoresearch, West Grove PA) antibodies as described previously (Bautch et al., 2000; Bautch et al., 1996). All cultures were viewed and photographed with an Olympus IX-50 inverted microscope outfitted with epifluorescence. PECAM images were aligned with the last frame of each movie using Photoshop version 5.5 (Adobe Systems, San Jose CA). Co-
cultures were fixed and processed for β-galactosidase reactivity as described (Kearney et al., 2002).

**Embryo manipulations**

*Flt-1*+/− mice were intercrossed, and embryos were dissected at 8.5 dpc (days post-coitum), and fixed overnight in 4% PFA at 4°C. Yolk sacs were used for genotyping as described (Kearney et al., 2002). The next day embryos were dehydrated through a MeOH series, then rehydrated and whole mount stained for PECAM-1 as described (Redick and Bautch, 1999). After staining, embryos were visualized and photographed on an Olympus SZH10 dissecting microscope.

**C. Results**

**Dynamic image analysis of vascular sprout formation**

To assay dynamic components of vascular morphogenesis, we generated stable ES cell subclones using a PECAM promoter/enhancer reporter in WT and *flt-1*−/− genetic backgrounds (see Methods for details). A subset of drug resistant subclones expressed eGFP in the endothelial lineage upon differentiation. Based on phenotypic similarities, we chose a single WT line (P27, WT-eGFP) and a single *flt-1*−/− line (GG5-15, *flt-1*−/−-eGFP) for further analysis. To confirm that expression of the reporter recapitulated expression of endogenous PECAM, we fixed day 8 WT-eGFP and *flt-1*−/−-eGFP ES cell differentiation cultures after time-lapse image analysis and stained for PECAM (Fig. 1). These images were overlayed with the last GFP image filmed (Fig. 1Ac, f, i). The congruence of the images showed that the eGFP
reporters were expressed in the same cells that also expressed endogenous PECAM, and in all cases expression was associated with blood vessels. While the majority of PECAM+ cells were endothelial cells lining the vessels, a small number of cells within the vessels also expressed PECAM, and these are likely to be the subset of hematopoietic cells that are PECAM+. The apparent lack of eGFP expression in a subset of PECAM-positive cells results from the comparison of confocal (Fig. 1Aa, d, g) with epifluorescence (Fig. 1Ab, e, h) images, since confocal images had a thinner focal plane. These data also confirm that the vascular phenotypes of the eGFP-expressing ES cell subclones recapitulate the parental vascular phenotypes (Fong et al., 1999; Kearney et al., 2002). Specifically, a branched vascular plexus was seen in WT-eGFP cultures (Fig. 1Aa-c), while both sheets of endothelial cells (Fig. 1Ag-i) and some branched areas (Fig. 1Ad-f) were seen in the flt-1−/−-eGFP cultures. Finally, these results confirm that the conditions of time-lapse imaging do not adversely affect vascular development in the ES cell cultures.

We next asked whether a specific morphogenetic behavior, the formation of a vascular sprout, was recapitulated in ES cell differentiation cultures (Fig. 1B and Movie 1). Day 8 WT-eGFP ES differentiation cultures were imaged for 2-10 hours. The resulting images were examined for sprout formation, as defined by projections from the parent structure that were at least 10 μm in length and were GFP positive. Numerous examples of sprout formation were noted, and one example is shown in Fig. 1B. This sprout initiated, extended, and formed a connection with a sprout from another area within a 145 minute period. The eGFP reporter did not allow for
precise location of cell boundaries, but the sprout length was approximately 20 µm at maximum, suggesting that it was comprised of one or at most two endothelial cells. Most sprouts that formed in WT cultures either joined with another sprout or part of a vessel, or they remained in place as sprouts at the end of filming (Fig. 2A and data not shown). Rarely, sprouts formed and retracted during the period of filming. These results show that ES cell differentiation is an appropriate model system for visualization and analysis of vascular morphogenetic processes in the mammal.

**Sprout formation and function is compromised in the absence of flt-1**

To determine how flt-1 affects vascular morphogenesis at the cellular level, we next compared sprout formation between WT and \( flt-1^{-/-} \) vessels using dynamic image analysis (Fig. 2A-B and Movies 2-3). Because \( flt-1^{-/-} \) ES cell cultures had areas of endothelial sheets, we were careful to score mutant sprout formation only in areas of \( flt-1^{-/-} \) ES cell cultures that contained a vascular plexus (i.e. Fig. 1Ad-f, Fig. 2Ad-f). To measure sprout formation, a sprout index that was normalized for time and vessel perimeter was calculated for multiple movies of each genotype. Since the lack of flt-1 leads to increased rates of endothelial cell division via upregulation of VEGF signaling (Kearney et al., 2002; Roberts et al., 2004), we hypothesized that morphogenetic parameters such as sprout formation would also be increased in the \( flt-1^{-/-} \) genetic background. To our surprise, \( flt-1^{-/-} \) vessels had a reduced sprout index relative to WT vessels. The sprout indices for d6 and d8 WT-eGFP time-lapse movies (Fig. 2B, grey circles) had a wider distribution that ranged much higher than day-matched \( flt-1^{-/-} \)-eGFP movies (Fig. 2B, black circles). Furthermore, \( flt-1^{-/-} \)-eGFP
vessels showed a 2.5 fold decrease in average sprout formation index over day-matched WT controls. These results show that flt-1−/− blood vessels sprout less often than WT blood vessels.

We next determined whether the absence of flt-1 affects the rate of sprout migration. Analysis of time-lapse images was used to determine average sprout velocity (sprout vessel tip displacement/time) for flt-1−/−-eGFP vascular sprouts and WT control sprouts (Fig. 2C-D and Movies 4-5). Sprout vessel tips (Fig. 2C, white arrowheads) were tracked over time using the base of the sprout at each time as the reference point. A plot of the average velocity for individual sprouts (Fig. 2D) showed that flt-1−/−-eGFP sprouts (black circles) displayed a lower range of velocities than WT-eGFP sprouts. Moreover, flt-1−/−-eGFP sprouts had a mean average sprout velocity that was reduced by 36% compared to WT-eGFP sprouts. This result indicates that flt-1 also modulates the speed with which sprouts migrate along a vector once they form.

We confirmed these results with a more traditional assay that is reflective of sprout formation, branch point analysis. Fixed samples of day 8 WT-eGFP and flt-1−/−-eGFP differentiated ES cell cultures were labeled with PECAM to visualize vessels (Fig. 3). flt-1−/−-eGFP and WT-eGFP vascular plexi showed a range of branch point densities (Fig. 3A), but flt-1−/−-eGFP ES cell cultures had less complex networks of blood vessels on average than WT controls (compare Fig. 3Aa-c with d-f). Quantitation showed that flt-1−/−-eGFP ES cell cultures had a 30-40% decrease in branch point formation relative to WT (Fig. 3B). Thus, the fixed branch point data are consistent with the time-lapse data, and they support a model whereby flt-1
normally modulates vessel morphogenesis by positively regulating both the
frequency of endothelial sprout formation and the rate of sprout migration.

To determine if the reduced sprout formation seen in the absence of flt-1 was
also seen in vivo, we examined the formation of intersomitic vessels that arise via
sprouting angiogenesis from the dorsal aorta (Fig. 4A). At 8.5 dpc, wild-type
embryos consistently had several intersomitic vessels, and well-defined vascular
sprouts were seen in register posterior to the vessels (Fig. 4Aa-c). In contrast, day-
matched flt-1-/− embryos had very few intersomitic sprouts, and those that formed
were short and seldom connected to the vertebral vessel (Fig. 4Ad-f). Thus the
sprout defect documented in vascular development in ES differentiation cultures is
also seen in mutant embryos lacking flt-1.

We reasoned that if sprout formation and migration were affected in the
absence of flt-1, the endothelial cells at the tip of the sprout might have an abnormal
morphology. Indeed, our examination of sprouts in flt-1-/− embryos suggested that
sprout tips tend to be blunted in the absence of flt-1 (Fig. 4A, inset e, and f). To
analyze this observation more carefully, we examined the morphologies of WT and
flt-1-/− mutant sprouts in ES cell differentiation cultures (Fig. 4B). WT sprout tips in
general gradually tapered to a point, although rare WT sprouts were less tapered
(Fig. 4Ba-d). In contrast, more of the flt-1-/− mutant sprout tips were blunted (Fig.
4Be-h), consistent with our findings that sprout migration along a vector is reduced.
Cell division defects are not immediately upstream of morphogenesis defects in flt-1/− mutant vessels

We previously showed that flt-1/− ES cultures display a 2-3 fold increase in endothelial cell division on day 6 of differentiation (Kearney et al., 2002). To determine if this difference was also observed with dynamic image analysis, we scored endothelial cell mitoses in flt-1/−-eGFP ES cell differentiation cultures and in WT controls (Fig. 5 and Movies 6-7). Day 6 flt-1/−-eGFP cultures had increased numbers of mitotic endothelial cells compared to WT controls (compare Fig. 5A and 5B). The number of endothelial cell divisions per hour was normalized for vascular area and used to produce a mitotic score that was averaged for multiple movies of both genotypes (Fig. 5C). Day 6 flt-1/−-eGFP vessels had an average mitotic score that was increased over WT-eGFP vessels by greater than 2-fold, consistent with our previous results (Kearney et al., 2002). However, the differences in mitotic score between WT and flt-1/− mutant vessels were resolved by day 8, a time point that was not assayed in our original study.

One explanation for the decreased frequency of sprout formation in the absence of flt-1 is the possibility that the increased rate of cell division precludes sprout formation and migration. The fact that day 8 flt-1/−-eGFP cultures had the same mitotic score as day-matched WT controls, whereas the sprout index and sprout velocity were still significantly decreased at day 8 in the flt-1/− mutant vessels, suggests that this simple explanation is not valid. Time-lapse analysis provided the opportunity to observe the temporal and spatial positioning of endothelial cell divisions relative to sprout formation and maturation (Table 1). The sprout field was
defined as the area of the primary vessel wall that would in the near future give rise to a sprout, and the sprout itself. The rest of the structure was classified as primary vessel. Significant cell divisions occurred in the sprout field, and we even saw rare divisions in the distal-most tip cell of the sprout in both wild-type and \(flt-1^{−/−}\) mutant vessels (data not shown), indicating that cell division and sprout formation/migration are not mutually exclusive over time. Moreover, the distribution of mitoses between the primary vessel and the sprout field was similar between WT and \(flt-1^{−/−}\) mutant vessels on both days, indicating that the \(flt-1\) mutation does not change the distribution of endothelial mitoses.

**Aberrant flt-1\(^{−/−}\) vascular morphogenesis is rescued in a non cell-autonomous manner and with an sflt-1 transgene**

To begin to determine the mechanism whereby \(flt-1\) positively affects vascular morphogenesis, we asked whether sflt-1, the soluble antagonist of VEGF signaling, was generated coincident with vascular morphogenesis during ES cell differentiation. Day 8 wild-type and \(flt-1^{+/−}\) heterozygous ES differentiation cultures expressed sflt-1 RNA and protein, and as predicted both RNA and protein were undetectable in day-matched \(flt-1^{−/−}\) ES differentiation cultures (data not shown). We reasoned that if \(flt-1\) positively regulates sprout formation via effects of the sflt-1 protein, it might be possible to rescue the morphogenetic defect in \(flt-1^{−/−}\) vessels in a non cell-autonomous manner. To test this hypothesis, we co-cultured wild-type and \(flt-1^{−/−}\) mutant embryoid bodies (EBs) in the same wells, by allowing EBs of both genotypes to attach on day 3 and continue differentiation to day 8 (Fig. 6A). In
contrast to $\text{flt-1}^{-/-}$ vessels that consisted of large sheets with a few sprouts at the edges, $\text{flt-1}^{-/-}$ vessels in wells with wild-type EBs (ratio 3 WT: 1 mutant) showed areas of significant rescue and increased branching (compare Fig. 6Aa to 6Ab). Thus the morphogenetic defect of $\text{flt-1}^{-/-}$ vessels can be rescued in a non cell-autonomous manner, suggesting that sflt-1 and/or some other soluble component is critical to normal vascular morphogenesis.

To directly address the role of sflt-1 in vascular morphogenesis, we reintroduced sflt-1 into $\text{flt-1}^{-/-}$ ES cells. A PECAM-sflt-1 transgene was expressed in day 8 $\text{flt-1}^{-/-}\text{PECAM-sflt}$ cultures by RT-PCR (data not shown), and this was accompanied by a rescue of the mutant vascular phenotype (Fig. 6B). A branch point analysis was performed, and the $\text{flt-1}^{-/-}\text{PECAM-sflt}$ cultures had rescued branching, with values that fell between WT and mutant cultures and that were significantly increased from $\text{flt-1}^{-/-}$ cultures (Fig. 6C).
D. Discussion

Our results show that flt-1 modulates vascular sprout formation developmentally. To our surprise, this modulation is positive, and flt-1 is required for proper sprout formation and migration. The data that support this conclusion are: 1) the rate of sprout formation and migration are both decreased in the absence of flt-1, 2) decreased vascular branching accompanies loss of flt-1 during ES cell differentiation and is rescued by an sflt-1 transgene, and 3) decreased sprouting from the dorsal aorta results from loss of flt-1 in vivo. Our data support a model whereby flt-1 affects vascular morphogenesis via a soluble mediator that is likely to be sflt-1, suggesting that formation and/or modulation of a VEGF gradient may be important to the effect of flt-1 on vascular sprout formation (Fig. 7).

How does flt-1 affect morphogenesis?

Since flt-1/- endothelial cells have a higher rate of division than do wild-type endothelial cells (Kearney et al., 2002), it was possible that the cell division defect was immediately upstream of the morphogenetic defect. That is to say, the endothelial cells possibly were too busy dividing to migrate. There is precedent for this model, in that both normal and tumor vessels restrict cell divisions to sprout areas more proximal to the parent vessel (Ausprunk and Folkman, 1977; Clark and Clark, 1939; Gerhardt et al., 2003), suggesting that actively migrating and sensing cells are prevented from dividing. Moreover, in tubulogenesis in the fly trachea, the tracheal precursor cell pool divides to produce all the tracheal cells, which then
migrate and form the tracheal tubes in the absence of further cell division (Metzger and Krasnow, 1999).

Our use of time-lapse image analysis allowed us to determine the relationship between cell division and cell migration with finer resolution than with end point analyses, and it showed that endothelial cells in a migratory mode can also undergo cell division. The cells did not migrate during mitosis or for some time prior to mitosis, but once division was complete the daughter cells often migrated quite rapidly to resume sprout formation. We found that divisions were not excluded from the “sprout field” but in fact were in most cases enriched in the sprouts and their vicinity (N.C.K. and V.L.B., unpub. results), suggesting that sprout formation may stimulate cell division. We even on occasion saw the most distal cell of the sprout undergo mitosis in both WT and \( \text{flt-1}^{-/} \) mutant vessels, although this event did not occur with high frequency. This finding differs from a recent study that found no tip cell division in retinal vessels (Gerhardt et al., 2003). However, retinal vessels track along a VEGF template provided by astrocytes, while in our model and in many developmental vascular beds the source of VEGF is likely to be more diffuse. Thus sprouts may have distinct phenotypes in response to different presentations of agonist. Finally, we saw no difference in the distribution of cell divisions to different areas of the vessel in the \( \text{flt-1}^{-/} \) vessels compared to controls, indicating that \( \text{flt-1} \) does not regulate this parameter of cell division. It is formally possible that aberrant cell division in \( \text{flt-1}^{-/} \) mutant vessels somehow compromises sprout formation, since there are more endothelial cell divisions in the \( \text{flt-1} \) mutant background. However,
the observation that the sprouting defects persist at day 8, when the rate of cell division in mutant vessels has returned to wild-type levels, strongly suggests that the effect of flt-1 on sprout formation and extension is independent of cell division.

*Flt-1* is a negative regulator of VEGF-A/flk-1 signaling (Kendall and Thomas, 1993; Roberts et al., 2004), and VEGF-A and flk-1 are both expressed during ES cell differentiation and required for vessel formation (Bautch et al., 2000; Shalaby et al., 1997). Thus the *flt-1* mutant vessels were expected to have increased sprout formation and migration. Instead, *flt-1* mutant sprouts formed less frequently and had slower migration rates. There are several models that are consistent with this phenotype. Perhaps VEGF signaling through the flt-1 receptor (mflt-1) positively modulates endothelial cell migration developmentally, and the absence of this receptor in *flt-1* mutants blocks this positive migratory pathway. VEGF/flt-1 signaling induces actin reorganization in cultured endothelial cells (Kanno et al., 2000), and the flt-1 kinase domain interacts with proteins involved in cell migration (Ito et al., 1998). However, a mouse model that lacks the flt-1 kinase domain shows normal vascular development, and stimulation of retinal vessels with PIGF, which binds flt-1 but not flk-1, did not affect filopodia formation (Gerhardt et al., 2003; Hiratsuka et al., 1998). These findings suggest that signaling through the flt-1 receptor is not required for proper sprout formation developmentally.

Another possible model to explain reduced sprouting in the absence of *flt-1* is that mflt-1 and/or sflt-1 negatively modulate local interactions between VEGF-A and flk-1 that are important for migration (Fig. 7). Flk-1 mediates VEGF-dependent endothelial cell migration, and VEGF/flk-1 signaling affects FAK and Src, molecules
involved in focal adhesion signaling and turnover (Abedi and Zachary, 1997; Abu-Ghazaleh et al., 2001; Gille et al., 2001; Rousseau et al., 2000). VEGF/flk-1 signaling also upregulates the activity of the small GTPase Rho (van Nieuw Amerongen et al., 2003; Zeng et al., 2002), whose levels and spatial distribution are critical to proper migration as well (Ren et al., 2000; Worthylake et al., 2001). Indeed, recent work shows that cell migration involves the proper balance of adhesion and retraction of a cell, and imbalance in either parameter can impede movement (reviews) (Etienne-Manneville and Hall, 2002; Ridley, 2001). Thus the increased flk-1 signaling in flt-1−/− endothelial cells may lead to deregulation of Rho and/or focal adhesion turnover and thereby inhibit endothelial cell migration and sprout formation.

A model of vascular sprout formation

Our data is most consistent with a model of vascular sprout formation in which flt-1 normally negatively regulates the amount of VEGF-A that is available to interact with the flk-1 receptor, and this regulation provides the proper balance for a net positive effect on sprout formation and migration. Moreover, we show that the flt-1−/− morphogenetic defect can be rescued in a non cell-autonomous manner, that sflt-1 is expressed appropriately to effect this rescue, and that an sflt-1 transgene expressed via the PECAM promoter/enhancer can significantly rescue the aberrant branching. Thus, although we cannot exclude a role for surface-localized mflt-1 in regulating the availability of VEGF-A, it is likely that sflt-1 is the primary molecular mediator of sprout formation from the flt-1 locus (Fig. 7).
In our model, sflt-1 is produced by endothelial cells and secreted into the local milieu, where it can interact with local sources of VEGF-A. VEGF-A secretion from non-endothelial cells induces migration towards the source of the signal. It is possible that sflt-1 forms a protein gradient emanating from endothelial cells that interacts with VEGF-A to set up an effective VEGF gradient, or it could steepen an existing gradient (Fig. 7B). sflt-1 binds heparin via IgG domain 4 (Park and Lee, 1999), so it may bind the matrix and thus form a gradient. Alternatively, sflt-1 could modulate a pre-existing VEGF-A gradient without forming an independent gradient (Fig. 7C). VEGF-A has three major isoforms that result from alternative splicing, and they have different biochemical properties. The VEGF$_{120}$ isoform is completely diffusible, while the VEGF$_{165}$ and VEGF$_{188}$ isoforms are localized to the outer surface and surrounding matrix of VEGF-A producing cells, although a significant fraction of VEGF$_{165}$ diffuses from the cell (Park et al., 1993). Thus these isoforms could produce a VEGF-A gradient by virtue of their differing affinities for matrix, and sflt-1 could impact this gradient. In this scenario, flt-1$^{-/-}$ mutant endothelial cells are inhibited from forming sprouts by exposure to excess VEGF-A (Fig. 7A). Clearly, flt-1$^{+/+}$ mutant blood vessels do form sprouts, so while sflt-1 may modulate sprout formation and migration, it is not absolutely required for sprouting angiogenesis.

What is the evidence for this model? Spatially localized VEGF expression predicts branching morphogenesis in the embryonic lung (Healy et al., 2000). Analysis of retinal vessels in mouse mutants expressing only a single VEGF isoform demonstrates defects in vessel size and number, suggesting that the local availability and diffusion of VEGF-A protein is critical for vascular patterning during
angiogenesis (Stalmans et al., 2002). A further analysis of VEGF isoform-selective mice provides evidence that a VEGF-A gradient set up in the hindbrain is compromised in the absence of matrix-bound VEGF-A (Ruhrberg et al., 2002). Moreover, inappropriate amounts of VEGF-A negatively affect sprout formation. Exogenous VEGF-A$_{165}$ injected into quail embryos inhibits the formation and branching of intersomitic vessels from the dorsal aorta (Drake and Little, 1995), similar to the effects seen in the flt-1$^{-/-}$ mutant embryos in our study. A recent study in the eye showed that increased levels of each VEGF isoform led to reduced filopodia length and reduced expansion of the retinal vascular plexus (Gerhardt et al., 2003). These results suggest that excess VEGF-A leads to a perturbed endothelial migratory response, perhaps by disruption of a VEGF-A gradient, and support a model whereby flt-1 negatively modulates local availability of VEGF-A to positively affect sprout formation.

The use of soluble receptors to modulate the activity of soluble ligands is not exclusive to the VEGF-A pathway. Other growth factors such as FGF, morphogens such as Wingless/Wnt, and vascular modifiers such as the angiopoietins, have soluble receptors of high affinity that can negatively modulate their respective signaling pathways (Guillonneau et al., 1998; Gurdon and Bourillot, 2001; Hanneken et al., 1994; Moon et al., 1997; Reusch et al., 2001). Indeed, the soluble receptor Frzb is thought to form gradients that modulate the availability of Wingless to target cells (Moon et al., 1997). Thus, this molecular mechanism of signal regulation is conserved in other signaling pathways.
Conclusions

We have identified a novel role for \textit{flt-1} in vascular morphogenesis, as a positive regulator of sprout formation and migration. This role is independent of a negative modulatory effect on endothelial cell division. We propose a model in which \textit{flt-1} positively affects sprout formation by negatively controlling the amount of VEGF-A signal that is sensed by endothelial cells, using sflt-1 as the primary mediator of the effect. We also suggest that gradient formation, either by VEGF-A or sflt-1 or both proteins, may be important in modulating sprout formation and migration. In any case, these results have implications for the development of disease therapies and for reconstruction of vessels. Clearly, the local availability of VEGF-A signal is critical to proper morphogenesis, and the regulation of this availability under normal conditions appears complex. Thus a better understanding of how this is accomplished will allow for better design of regimens for vessel reconstitution.
Table 1. Distribution of endothelial cell divisions during sprout formation.

<table>
<thead>
<tr>
<th>Location in Vessel</th>
<th>DAY 6</th>
<th>DAY 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (23*)</td>
<td>flt-1⁻/⁻ (75)</td>
</tr>
<tr>
<td>Primary Vessel</td>
<td>78%</td>
<td>75%</td>
</tr>
<tr>
<td>Total Sprout Field</td>
<td>22%</td>
<td>25%</td>
</tr>
<tr>
<td>Proximal sprout area†</td>
<td>4%</td>
<td>6%</td>
</tr>
<tr>
<td>Distal sprout area</td>
<td>9%</td>
<td>11%</td>
</tr>
<tr>
<td>Nascent sprout area</td>
<td>9%</td>
<td>8%</td>
</tr>
</tbody>
</table>

* denotes total number of cell divisions for each genotype.
† denotes different compartments of the sprout field.
Figure 1. GFP expression allows for visualization of mammalian sprouting angiogenesis. Stable ES cell lines expressing PECAM-GFP were generated (see Methods) and subjected to time-lapse image analysis of vascular processes for 4.5-9.5 h. A) Day 8 WT-eGFP (+/+, a-c) or flt-1^-/-eGFP (-/-, d-i) ES differentiation cultures were imaged by time-lapse at one min intervals for 4.5-9.5 h, then fixed and reacted with an anti-PECAM antibody. The last frame captured (a, d, g) was overlayed (c, f, i) with the PECAM stained image (b, e, h). B) Time-lapse frames of a day 8 WT-eGFP ES differentiation culture showing the formation of a vascular sprout and its fusion with another sprout. Time in min is at lower left of each frame. The white arrowhead denotes sprout formation and extension, while the white arrow shows the fusion of the sprout tip with the second sprout. Scale bar in B is 20 µm.
Figure 2. Sprout formation and migration are reduced in the absence of flt-1.

Day 8 WT-eGFP (+/+, Aa-c, Ca-c) or flt-1^-/-eGFP (-/-, Ad-f, Cd-f) ES differentiation cultures were imaged by time-lapse at one min intervals for the indicated times. A) Over a 10 h period, numerous new WT sprouts (a-c) formed (white arrowheads) and remained or extended and/or fused at later times (black arrowheads), whereas very few flt-1^-/- sprouts (d-f) formed during the same time period. Scale bar is 20 µm. B) Movie frames were analyzed to determine a sprout index (#sprouts/mm/hr) for multiple movies on day 6 (d6) or day 8 (d8) of differentiation. Grey dots = WT-eGFP sprout indices, black dots = flt-1^-/-eGFP sprout indices, and black squares with line = average of each group. * = P < 0.03; ** = P < 0.01. C) Over a 40 min period, a WT sprout formed and extended between 30-40 µm, while over a 100 min period a flt-1^-/- mutant sprout formed and extended only 20 µm. White arrowheads point to sprout tips, and time in min is at lower left. Scale bar = 20 µm. D) Movie frames were analyzed to determine average sprout velocity (µm/hr) for multiple sprouts from different movies on day 8 (d8) of differentiation. Grey dots = WT-eGFP average sprout velocities, black dots = flt-1^-/-eGFP average sprout velocities, and black squares with line = average of each group. *** = P < 0.001.
Figure 3. Branching morphogenesis is reduced in the absence of flt-1. Day 8 WT-eGFP (+/+; Aa-c) or flt-1/-eGFP (-/-; Ad-f) ES differentiation cultures were fixed and reacted with an anti-PECAM antibody. A) Note that while each genotype had a range of branching complexities, on average less complexity was seen in the flt-1/- eGFP cultures. Scale bar is 50 µm. B) The average number of branch points per mm of vessel length was determined using 10 fields of each genotype in 2 experiments, and expressed as a percentage of wild-type branching. *** = P < 0.001.
Figure 4. Sprouting from the dorsal aorta is reduced in the absence of *flt-1*.

A) 8.5 dpc embryos that were WT (+/+, a-b), *flt-1*+/− (+/−, c), or *flt-1*−/− (−/−, d-f), were collected and whole mount stained with an anti-PECAM antibody. a-c), In 3 different normal embryos, intersomitic vessels (white arrows) branch from the dorsal aorta (black arrowheads). A magnified view in C shows several intersomitic sprouts (white arrowheads) in register posterior to the vessels. d-f] 2 different *flt-1*−/− embryos show few branches from the dorsal aorta, and those that are seen are often stunted (black arrows). The inset in e and panel f show stunted sprouts with blunted tips. A = anterior, P = posterior. Original magnification is 5 x. B) Day 8 WT (a-d) and *flt-1*−/− (e-h) ES differentiation cultures were fixed and stained with a PECAM antibody. Representative sprouts were photographed. Note that most WT sprouts are tapered at the distal end (white arrowheads), while most *flt-1*−/− mutant sprouts are blunted (white arrows) at the distal end. Magnification is 60 x.
Figure 5. Endothelial cell divisions are increased in the absence of *flt-1*. Day 6 WT-eGFP (+/+, A) or *flt-1*<sup>-/-</sup>-eGFP (-/-, B) ES differentiation cultures were imaged by time-lapse at one min intervals for the indicated times. Day 8 cultures of both genotypes were also imaged for quantitation (C). A-B) Endothelial cell pre-division rounding up is denoted by white arrows, and cytokinesis is denoted by grey arrows. Time in min is at the lower left. Note that over a similar time span there are more divisions scored in the *flt-1*<sup>-/-</sup> mutant vessels. Magnification is 20 x. C) Time-lapse movies from day 6 WT-eGFP (N=6), day 6 *flt-1*<sup>-/-</sup>-eGFP (N=8), day 8 WT-eGFP (N=8), and day 8 *flt-1*<sup>-/-</sup>-eGFP (N=8) were analyzed for a mitotic score (# endothelial cell divisions/eGFP+ area/hr). *** = P < 0.001.
Figure 6. The $flt-1^{-/-}$ phenotype is partially rescued non cell-autonomously and by a $sflt-1$ transgene. A) Co-culture analysis. Day 3 EBs were plated in wells so that a) 100% were $flt-1^{-/-}$ mutant EBs, or b) 75% were WT and 25% were $flt-1^{-/-}$ mutant EBs, and after attachment and incubation for a further 5 days, cultures were fixed and stained for $\beta$-galactosidase activity. B) Day 8 WT (Ba-b), $flt-1^{-/-}$ transfected with a PECAM-sflt transgene (Bc-d), and $flt-1^{-/-}$ (Be-f) ES differentiation cultures were fixed and reacted with an anti-PECAM antibody. Ba, Bc, Be: 10X exposure; Bb, Bd, Bf: 20X exposure. Note that branching in $flt-1^{-/-}^{PECAM-sflt}$ cultures more closely resembles WT than $flt-1^{-/-}$ cultures. C) The average number of branch points per mm of vessel length was determined using multiple fields of each genotype. ** = $P<0.01$, $flt-1^{-/-}$ vs. $flt-1^{-/-}^{PECAM-sflt}$; *** = $P < 0.001$, WT vs. $flt-1^{-/-}$. 
Figure 7. Models of flt-1 modulation of VEGF signal - negative modulation results in net positive effect on sprout formation. We favor a model of flt-1 action on sprout formation that results from flt-1 binding VEGF-A and preventing binding to flk-1 (see text for details). This could occur in several ways. A) VEGF-A may be deposited in a gradient between the producing tissue and the target vessel, and this unmodified gradient is not conducive to sprout formation in the absence of flt-1. B) sflt-1 may be secreted and form a counter-gradient that modifies and steepens the VEGF-A gradient, leading to increased sprout formation. C) mflt-1 and/or sflt-1 may locally decrease the availability of VEGF-A, leading to increased sprout formation.
CHAPTER III

SOLUBLE FLT-1 IS THE PREDOMINANT FLT-1 ISOFORM
REGULATING VESSEL BRANCHING

A. Introduction

Angiogenesis is an important aspect of blood vessel formation, both developmentally and in physiological and pathological conditions (Ferrara and Alitalo, 1999; Risau, 1997). During angiogenesis, endothelial cells respond to both proliferative signals and morphogenetic cues for vessel sprouting and fusion. In this way, simple vascular structures are extended to form a branching plexus. Many of the factors and pathways important in angiogenesis have been identified, but relatively little is known about how signals are regulated to co-ordinate vessel branching and endothelial cell proliferation during angiogenesis.

The vascular endothelial growth factor-A (VEGF-A) signaling pathway is a crucial mediator of angiogenesis (Olsson et al., 2006; Shibuya and Claesson-Welsh, 2006). Activation and subsequent signaling via VEGF-A regulates endothelial cell division, migration, and survival (Matsumoto and Claesson-Welsh, 2001b). The VEGF-A signaling pathway requires tight dose-dependent regulation for proper morphogenesis. Minor changes in the amount of available VEGF-A adversely affect vascular development, and the loss of even one copy of the Vegf-A gene leads to embryonic lethality (Bautch et al., 2000; Carmeliet et al., 1996; Damert, 2002;
Ferrara et al., 1996; Miquerol et al., 2000). VEGF-A signaling is further modulated by alternative splicing of VEGF-A mRNA to produce three different isoforms (Ng et al., 2001b; Park et al., 1993; Tischer et al., 1991). These VEGF-A isoforms have differing affinities for heparin, and therefore are predicted to be distributed differentially from VEGF-A-producing cells via differential binding to the extracellular matrix. Genetic manipulations that lead to expression of only the VEGF-A120 or the VEGF-A188 isoform from the VEGF-A locus result in vessel dysmorphogenesis (Ruhrberg et al., 2002; Stalmans et al., 2002). Mice that express only the VEGF-A120 isoform, which does not bind heparin, develop to term, but the hindbrain vessels have fewer branches and increased diameter. In contrast, mice expressing only the VEGF-A188 isoform, which has two heparin binding sites, have ectopic vessel branches that are long and thin (Ruhrberg et al., 2002). Recent elegant studies by Gerhardt et al. (2003) in the retina support a model in which the spatial context of VEGF-A presentation to the endothelial cell is important for vessel morphogenesis, while endothelial cell proliferation is regulated by the local VEGF-A concentration in a spatially-independent manner (Gerhardt et al., 2003).

The biological effects of VEGF-A are mediated by two high affinity receptor tyrosine kinases, flk-1 (VEGFR-2) and flt-1 (VEGFR-1), that are expressed on endothelial cells. VEGF-A signaling through flk-1 positively regulates endothelial cell division and migration (Dayanir et al., 2001; Gerhardt et al., 2003; Guo et al., 1995; Kroll and Waltenberger, 1997; Zeng et al., 2001), whereas the function of flt-1 is less clear. Deletion of flt-1 in embryoid bodies or in vivo results in embryonic lethality at mid-gestation, with disorganized blood vessels and over-proliferation of endothelial
cells (Fong et al., 1995; Kearney et al., 2002). Flt-1 mRNA is alternatively spliced to encode both a full-length receptor tyrosine kinase (mflt-1) and a soluble isoform (sflt-1) that contains the VEGF-A-binding extracellular domain (Kendall and Thomas, 1993; Kendall et al., 1996). VEGF-A has a higher affinity for flt-1 than for flk-1, so both flt-1 isoforms can potentially sequester VEGF-A and modulate signaling through flk-1 (Kondo et al., 1998; Park et al., 1994). Flt-1−/− ES cell-derived vessels have approximately 3-fold higher levels of activated flk-1 than do normal vessels, consistent with a role for flt-1 in ligand sequestration (Roberts et al., 2004). Mice lacking the cytoplasmic tail of the flt-1 receptor are viable, indicating that the signaling function of the flt-1 receptor is not essential during embryonic development (Hiratsuka et al., 1998). A recent study identified a single amino acid in the kinase domain of flt-1 that severely reduces kinase activity and signaling (Meyer et al., 2006), and the intracellular juxtamembrane region of flt-1 suppressed VEGF-A-dependent activation of PI 3'-kinase and subsequent endothelial cell migration (Gille et al., 2000). Taken together, this data suggests that flt-1 functions in vascular development as a ligand sink to bind and sequester VEGF-A, and thus regulate signaling through the flk-1 receptor.

We previously showed that flt-1 regulates both endothelial cell proliferation and vessel branching (Kearney et al., 2002; Kearney et al., 2004), but the mechanisms underlying this regulation were unclear. We hypothesized that flt-1, through its two isoforms, can modify both the level and presentation of VEGF-A to endothelial cells in forming vessels. To test this model, we reintroduced isoform-specific flt-1 transgenes into flt-1−/− stem cell-derived vessels. We confirmed that
signaling through flt-1 is not essential for proper vascular development, and we found soluble flt-1 to be more effective at rescuing branching morphogenesis, while both membrane-tethered and soluble flt-1 rescued endothelial cell proliferation. Thus, the ability of soluble flt-1 to disseminate from the endothelial cell appears critical to efficient regulation of morphogenesis, whereas both flt-1 isoforms have the ability to regulate endothelial cell proliferation.
B. Materials and Methods

Cell culture and in vitro differentiation

Wild-type (WT) ES cells, flt-1<sup>-/-</sup> ES cells, and flt-1<sup>-/-</sup> ES cells containing a sflt-1 or mflt-1 transgene under the transcriptional control of the platelet endothelial cell adhesion molecule (PECAM) promoter/intron enhancer element (Tg PECAM-sflt-1 and Tg PECAM-mflt-1) were maintained and differentiated as described previously (Bautch, 2002; Bautch et al., 1996). Embryoid bodies (EBs) were plated onto either slide flasks (Nunc, Rochester, NY), or wells of a 24-well tissue culture dish at day 3 of differentiation and cultured at 37°C in 5% CO<sub>2</sub> until day 8, when cultures were fixed and analyzed.

DNA constructs and electroporation

The PECAM promoter/intron enhancer (gift of P. Robson and H.S. Baldwin) was linked to flt-1 cDNAs: sflt-1 (Kearney et al., 2004); mflt-1 (gift of G. Breier; Breier et al, 1995); or mflt-1<sup>ΔCyt</sup>. The mflt-1 cDNA was first subcloned into the pBluescript vector, digested with *Mlu*I, blunted, then digested with *Sal*I. This fragment was cloned into a PECAM-Hygro vector via *Sal*I and a blunted *Not*I site and designated PECAM-mFlt-1-Hygro. The mflt-1<sup>ΔCyt</sup> cDNA was created by amplifying mFlt-1 using the following primers: forward primer 5’ ACGCGTCGACAGCGCGGAGGCGGACACTCCCGGGAG 3’; reverse primer 5’ CCCAAGCTTTATCATATCTTCTTACCCGCTTCAGTTTTCTGATG 3’. The PCR product was digested with *Sal*I and *Hind*III, while the 3’ UTR was obtained by digesting pBluescript-mFlt-1 with *Hind*III and *Not*I. The two fragments were
ligated together via HindIII and cloned into the PECAM-Hygro vector and designated PECAM-mFlt-1\textsuperscript{ΔCyt}-Hygro.

Fifteen µg of linearized DNA was electroporated into 2 X 10^7 flt-1\textsuperscript{-/-} ES cells using a BioRad GenePulser II electroporator (250V/300µF; BioRad, Hurcules, CA.) Selection of flt-1\textsuperscript{-/-} ES cells was in 200 µg/mL hygromycin B (Roche Diagnostics, Indianapolis, IN). After 12-14 days, drug-resistant ES cell colonies were picked, expanded, and analyzed. Thirty-five flt-1\textsuperscript{-/-} transgenic lines containing PECAM-sFlt-1-Hygro (designated at flt-1\textsuperscript{-/-}; Tg PECAM-sflt-1), 25 flt-1\textsuperscript{-/-} transgenic lines containing PECAM-mFlt-1-Hygro (designated at flt-1\textsuperscript{-/-}; Tg PECAM-mflt-1), and 30 transgenic lines containing PECAM-mFlt-1\textsuperscript{ΔCyt} (designated at flt-1\textsuperscript{-/-}; Tg PECAM-mflt-1\textsuperscript{ΔCyt}) were picked and analyzed further.

Targeted insertion of flt-1 isoform transgene into the ROSA26 genomic locus was carried out using Gateway MultiSite cloning vectors. The targeting vector, pROSA26-1 (gift of Phil Soriano) was modified by adding an MluI site into the lone restriction site of the MCS, XbaI. PacI was also added into the KpnI site of pROSA26-1. The resulting vector was designated Modified pROSA26-1. Modified pROSA26-1 was next transformed into a Gateway destination vector by amplifying pDEST R4-R3 (with flanking MluI sites) using the following primers: forward primer 5' CGACGCGTCAGGAAACAGCTATGAC 3'; reverse primer 5' CGACGCGTTGTAAAACGACGGCCA 3'. pDEST R4-R3 was then cloned into Modified pROSA26-1 using MluI to create pROSA26-1+DEST R4-R3. Three Gateway donor vectors were subsequently created. The first one, pDONR P2R-P3, was made as two different vectors; one containing sflt-1 cDNA, and the other
containing mflt-1 cDNA. Each flt-1 transgene and polyadenylation site was amplified using the following primers: forward primer 5’ GGGGACAGCCTTCTTCTTG TACAAAGTGACCGCGGCCGCGACACTCC 3’; reverse primer 5’ GGGGACAA CTTTGTTATAATGTTGTGAGCTGAAAAATG CTTTTATTGTG 3’. Each PCR product (sflt-1 and mflt-1 transgene) was recombined into pDONR P2R-P3 via attB sites to create: pDONR P2R-P3-sflt-1 and pDONR P2R-P3-mflt-1. The PECAM promoter/intron enhancer was then amplified using the following primers: forward primer 5’ GGGGACAAAGTTTGTACAAAAAAGCAGGCTTCCGCTCT ATGCTATGAGG 3’; reverse primer 5’ GGGGACCACTTTGTACAA GAAAGTGTTGGGTAGCTCTGTAATTTTCCTGAVAATGG 3’. The PCR product was recombined into pDONR-221 via attB sites to create pDONR221-PECAM. We next amplified a PGK-Hygromycin cassette using the following primers: forward primer 5’ GGGGACAACTTTGTATAGAAAAGTTGCCTCGAGGTCGATA TCG 3’; reverse primer 5’ GGGGACTGCTTTTGTACAAACTTGGATCCCCG GGGCTGCAGGAATTC 3’. The PCR product was recombined into pDONR P4-P1R via attB sites to create pDONR P4-P1R-PGK-Hygro. Each flt-1 isoform-specific pDONR-P2R-P3-flt-1 vector was combined with pDONR221-PECAM, pDONR-P4-P1R-PGK-Hygro, and pROSA26-1+DEST R4-R3 to create pROSA26-1-PECAM-sflt-1-Hygro and pROSA26-1-PECAM-mflt-1-Hygro.

Each pROSA26-1-PECAM-flt-1-Hygro DNA was linearized with PacI and electroporated into flt-1−/− ES cells as described above. Resistant ES clones were picked and verified for correct targeting into the ROSA26 locus via PCR using the
following primers: forward primer 5’ CCTAAAGAAGAGGCTGTGCT TTGG 3’; 
reverse primer 5’ CCGATGGCTGTGTAGAAGTACTC 3’.

**Antibody staining**

Day 8 ES cell cultures were rinsed with phosphate-buffered saline (PBS) and fixed for 5 minutes in ice-cold methanol-acetone (50:50). Fixed cultures were reacted with rat anti-mouse PECAM at 1:1000 (MEC 13.3; BD Pharmingen, San Diego, CA) and donkey anti-rat immunoglobulin G (IgG; H+L) conjugated to TRITC at 1:100 (Jackson Immuno research, West Grove, PA). Alternatively, Alexa Fluor 488 goat anti-rat (IgG; H+L) at 1:200 (Molecular Probes-Invitrogen) was used, as described previously (Bautch et al., 2000). Cultures were viewed and photographed with either an Olympus IX-50 inverted microscope (Melville, NY) outfitted with epifluorescence, or with a Leica inverted DMIRB microscope (Bannockburn, IL) and a Hamamatsu OrcaER CCD camera (McHenry, IL).

**Quantitative image analysis**

To quantify the area labeled with PECAM antibody in differentiated ES cell cultures, fixed cultures were reacted with PECAM antibody, photographed, and analyzed as described (Kearney et al., 2002). Percent PECAM area averages for each well were calculated, and the average of 4 wells for each clone was used to determine SD values. Branch point analysis was done on fixed cultures reacted with PECAM antibody as described (Kearney et al., 2004). The average branch point score from 8-12 pictures for each clone was used to determine SD values.
**RNA analysis**

For RT-PCR, total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufactures instructions. Ten µg of total RNA was digested with DNase I (Promega, Madison, WI), and 2 µg of DNase-treated total RNA was in vitro transcribed with Superscript II reverse transcriptase (Invitrogen) in a total volume of 80 µl. 10 µl of the reaction was used for PCR experiments. *Taq* polymerase (Qiagen, Valencia, CA) was used to amplify cDNA using the following primers: Flt-1 forward 5’ ACA CCG CGG TCT TGC CTT AC 3’; Flt-1 reverse 5’ CAG CCT TTT GTC CTC CTG GC 3’, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Forward 5’ AGC CCA TCA CCA TCT TCC 3’; GAPDH reverse 5’ GCC ATC CAC AGT CTT CTG G 3’. PCR reactions were performed for 3 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 59°C, and 1 min at 72°C. The PCR products were electrophoresed on a 3% agarose gel and detected with ethidium bromide.

For real-time PCR, total RNA was isolated using Trizol reagent and stored at -20°C. PCR primers and fluorogenic probes for the target genes were designed by Dr. Hyung-Suk Kim at the University of North Carolina-Chapel Hill Animal Clinical Chemistry and Gene Expression Facility. Sequences of the primer/probe pairs used are as follows: Flt-1 forward 5’ ACA CCG CGG TCT TGC CTT AC 3’; Flt-1 reverse 5’ TTT AAC TTC GAC CCT GAG CCA 3’; Flt-1 probe 5’ f-CT GCT CGG GTG TCT TCT CAC-Q 3’, β-actin forward 5’ CTG CCT GAC GGC CAG GTC 3’; β-actin reverse 5’ CAA GAA GGA AGG CTG GAA AAG A 3’; β-actin probe 5’ f-CA CTA
TTG GCA ACG AGC GGT TCC G-Q 3’ (f, reporter dye, FAM (6-carboxyfluorescein); Q, quencher dye, TAMRA (6-carboxytetramethyl-rhodamine). Relative levels of RNA for flt-1 and β-actin were determined by performing real time RT-PCR in a 96-well plate in a total volume of 30 µl. Each RT-PCR amplification was performed in triplicate: 30 min at 48°C for the RT reaction, then 10 min at 95°C, followed by 40 temperature cycles (15 sec at 95°C and 1 min at 60°C). During the amplification, the fluorescence of FAM was measured with the ABI Prism 7700 Sequence Detection System (PE Biosystems Inc.) at the UNC-Chapel Hill Animal Clinical Chemistry and Gene Expression Facility. The threshold cycle (C_T) value of each target product was determined and normalized to its corresponding β-actin C_T value. The relative quantitative results were used to determine the difference in flt-1 expression in experimental ES cell cultures compared to WT flt-1 RNA.

**In situ hybridization**

Flt-1 antisense RNA probe labeled with digoxigenin-UTP (Boehringer, Mannheim, Germany) was synthesized by reverse transcription of a 1.6 kb PvuII fragment which was derived from the 5' end of flt-1 cDNA and subcloned into pBluescript KS II (gift from Gua-Hua Fong). In situ hybridization was performed by modification of a standard protocol used for whole-mount hybridization (Rosen and Beddington, 1993) as described previously (Bautch et al., 2000). After in situ hybridization of day 8 differentiated cultures, samples were viewed and photographed with an Olympus IX-50 inverted microscope.
C. Results

Transgenes encoding individual flt-1 isoforms rescue the flt-1⁻/⁻ vessel phenotype

ES cell-derived vessels lacking flt-1 have an increased endothelial cell mitotic index (Kearney et al., 2002), and a morphogenetic defect that results in reduced sprouting and branch formation (Kearney et al., 2004). To directly assess the role of the flt-1 isoforms on endothelial cell division and vessel morphogenesis, we generated clones carrying flt-1 cDNA transgenes (Fig. 8A; sflt-1, mflt-1, or mflt-1ΔCyt) randomly integrated into the genome of flt-1⁻/⁻ ES cells. The transgenes were linked to the PECAM promoter/enhancer because it results in expression of transgenes in developing blood vessels (Kearney et al., 2004). After electroporation and selection, flt-1⁻/⁻; Tg PECAM-flt-1 ES cell clones were differentiated to day 8, then labeled with PECAM antibody to visualize vessel area and morphology, or processed for RNA isolation (Fig. 8B). flt-1⁻/⁻; Tg PECAM-flt-1 ES cell clones that were positive for flt-1 RNA by RT-PCR (data not shown) were used for further analysis.

Flt-1⁻/⁻; Tg PECAM-flt-1 vessels exhibited a range of vascular phenotypes compared to flt-1⁻/⁻ vessels. Clones with vessels that were visually indistinguishable from flt-1⁻/⁻ vessels were categorized as having no rescue (Fig. 8C, F, I, Table 2). A second group of clones showed primarily a rescue of vessel morphogenesis (Fig. 8D, G, J, Table 2), and areas of endothelial sheets seen in flt-1 mutant cultures were replaced with areas of branched vascular plexus. A third group of clones had primarily a rescue of endothelial cell proliferation (Fig. 8E, H, K, Table 2), with an overall reduction of vascular area, but little or no rescue of morphogenesis.
80% of flt-1⁻/⁻; Tg PECAM-sflt-1 clones had primarily a rescue of vessel morphogenesis (Table 2). In contrast, only 16% of flt-1⁻/⁻; Tg PECAM-mflt-1 clones had a primary morphogenesis rescue (Table 2). All of the flt-1⁻/⁻; Tg PECAM-sflt-1 clones showed some type of rescue, whereas 63% of flt-1⁻/⁻; Tg PECAM-mflt-1 clones (Table 2) were not rescued. Finally, a similar percentage of both flt-1⁻/⁻; Tg PECAM-sflt-1 and flt-1⁻/⁻; Tg PECAM-mflt-1 clones had primarily a proliferation rescue, 20% and 21%, respectively (Table 2). These data suggest that the sflt-1 transgene rescues vessel branching morphogenesis more efficiently than the mflt-1 transgene, and that the transgenes rescue endothelial proliferation with equivalent efficiency.

We next quantitated the rescue of vessel morphogenesis in a subset of clones using a branch point analysis. Day 8 cultures of WT, flt-1⁻/⁻, and two clones each of flt-1⁻/⁻; Tg PECAM-sflt-1 and flt-1⁻/⁻; Tg PECAM-mflt-1 genotype labeled with PECAM were analyzed (Fig. 9). Flt-1⁻/⁻ cultures, as previously described (Kearney et al., 2004), had a 30% decrease in branch point formation relative to WT. Vessels of clones that express sflt-1 or mflt-1 transgenes, and were categorized as a Primary Morphogenesis rescue, had a higher branch point score (# branch points/mm vessel length) than flt-1⁻/⁻ vessels (Fig. 9B-F, G). Thus, clones grouped as a Primary Morphogenesis rescue by visual analysis also had elevated branch point scores, although no rescued clones attained the branch point score of WT vessels.

To quantitate the rescue of endothelial cell proliferation in the clones expressing flt-1 isoform transgenes, we calculated the amount of vasculature as measured by the percentage of the cellular area that was positive for PECAM-1
antibody reactivity. We previously showed that this parameter reflects the number of endothelial cells, and that it is increased in flt-1 mutant vessels because of an increase in the endothelial mitotic index (Kearney et al., 2002). Two clones, scored as a primary rescue of proliferation by visual observation (flt-1\(^{-/-}\); Tg PECAM-mflt-1#26 and flt-1\(^{-/-}\); Tg PECAM-sflt-1#2), had a percent vascular area of 24% and 28%, respectively, which was near the WT value of 22% (Fig. 10). Flt-1\(^{-/-}\) cultures had a vascular area of 67%, which was a 3-fold increase in vascular area relative to WT, and consistent with previously published data (Fig. 10; (Kearney et al., 2002). Two flt-1\(^{-/-}\); Tg PECAM-mflt-1 clones (flt-1\(^{-/-}\); Tg PECAM-mflt-1#13 and flt-1\(^{-/-}\); Tg PECAM-mflt-1#1), which are phenotypically indistinguishable from flt-1\(^{-/-}\) cultures, had vascular areas of 73% and 78%, respectively, which were similar to the flt-1\(^{-/-}\) value (Fig. 10). Thus, clones placed in the No Rescue category by visual observation also belong there by quantitative criteria. Interestingly, 5 of the 6 clones grouped as primarily a morphogenesis rescue had vascular area values that fell between WT and flt-1\(^{-/-}\) cultures (Fig. 10). Thus, although these clones primarily show a rescue of morphogenesis, they also have a partial rescue of vascular area.

**Expression profile of flt-1 isoform transgene-expressing clones**

Electroporation of PECAM-flt-1 isoform DNA into flt-1\(^{-/-}\) ES cells yielded ES cell clones with flt-1 isoform transgenes integrated randomly at different genomic locations. Random integration of transgenes leads to expression that can differ temporally, spatially, and quantitatively among integration sites (Palmiter and Brinster, 1986). To determine if the phenotypic differences seen among the rescue
clones correlated with differences in flt-1 isoform transgene expression, we analyzed transgene expression parameters in representative rescue clones. Total RNA was collected from WT, flt-1<sup>-/-</sup>, flt-1<sup>-/-</sup>; Tg PECAM-sflt-1 and flt-1<sup>-/-</sup>; Tg PECAM-mflt-1 ES cell cultures at the ES cell stage and every two days during differentiation, and analyzed by RT-PCR using flt-1-specific primers to obtain a developmental expression profile. Flt-1 was detected throughout the time-course in WT controls, and it was not detected in flt-1<sup>-/-</sup> cultures. Similar to WT, all flt-1 isoform transgene clones tested (6 sflt-1 isoform rescue clones and 6 mflt-1 isoform rescue clones) had detectable flt-1 RNA throughout the time-course (Fig. 11 and data not shown).

We next asked whether phenotypic differences among the flt-1 isoform expressing ES cell cultures correlated with differences in the location of transgene expression. Day 8 WT, flt-1<sup>-/-</sup>, and representative flt-1<sup>-/-</sup>; Tg PECAM-sflt-1 and flt-1<sup>-/-</sup>; Tg PECAM-mflt-1 cultures were analyzed by in situ hybridization with an anti-sense probe to flt-1. All flt-1 isoform cultures expressed flt-1 RNA in vessels, albeit at reduced levels compared to the WT control (Fig. 12 and data not shown). Taken together, these data suggest that the different phenotypes observed among the flt-1 isoform-expressing cultures are not due to differences in the timing and localization of transgene expression.

We used real-time PCR to quantitate the level of flt-1 isoform transgene expression in representative flt-1 isoform expressing ES cell cultures. Flt-1 RNA levels were normalized to β-actin and expressed as a percentage of WT flt-1 RNA (Fig. 13). Levels of flt-1 isoform transgene RNA varied among the cultures. Moreover, the levels of flt-1 isoform RNA correlated with the vascular phenotype,
with the categories of No Rescue, Primary Morphogenesis Rescue, and Primary Proliferation Rescue roughly corresponding to increasing levels of transgene RNA. However, isoform-specific differences were also noted. The enhanced ability of the sflt-1 transgene to rescue morphogenesis (Table 2) does not appear to result from increased levels of sflt-1 transgene RNA, since all of the sflt-1 isoform-expressing clones that primarily rescued vascular morphogenesis had lower levels of transgene RNA than the few comparable mflt-1 clones. Moreover, two flt-1−/−; Tg PECAM-mflt-1 cultures that did not rescue the vascular phenotype had transgene RNA levels comparable to the levels of several flt-1−/−; Tg PECAM-sflt-1 clones that rescued vessel morphogenesis (Fig. 13: flt-1−/−; Tg PECAM-mflt-1#1 = 2.0%, flt-1−/−; Tg PECAM-mflt-1#13 = 7.9% versus flt-1−/−; Tg PECAM-sflt-1#27 = 1.3%, flt-1−/−; Tg PECAM-sflt-1#23 = 6.7%, flt-1−/−; Tg PECAM-sflt-1#33 = 8.7%, flt-1−/−; Tg PECAM-sflt-1#36 = 11.7%). Thus, levels of mflt-1 transgene RNA that did not elicit a rescue response were sufficient to rescue morphogenesis if the isoform transgene was sflt-1. A low percentage of mflt-1 transgenes rescued vessel morphology and significantly higher levels of mflt-1 RNA were required to elicit that response (Fig. 13: flt-1−/−; Tg PECAM-mflt-1#8 = 20.7% and flt-1−/−; Tg PECAM-mflt-1#17 = 30.6% compared to sflt-1-expressing clones; 1.3%-11.7%). This trend continued when comparing sflt-1 and mflt-1-expressing clones that rescue proliferation. Flt-1−/−; Tg PECAM-sflt-1#2 is rescued to the same degree as flt-1−/−; Tg PECAM-mflt-1#26 (Fig. 8 D, G and Fig. 10), however, a 3.5-fold increase in mflt-1 RNA compared to sflt-1 RNA is required for this rescue (Fig. 13 flt-1−/−; Tg PECAM-sflt-1#2 = 18.3% versus flt-1−/−; Tg PECAM-mflt-1#26 = 65.0%). These data suggest that the sflt-1 isoform rescues the flt-1−/− phenotype more efficiently than the mflt-1 isoform.
The cytoplasmic domain of mflt-1 is not required for flt-1 to rescue the flt-1\(^{-/-}\) vascular phenotype

To determine if signaling is important in the observed rescue of either endothelial proliferation or vessel morphogenesis by mflt-1, we introduced a flt-1 transgene lacking the cytoplasmic domain (mflt-1\(^{\Delta\text{Cyt}}\)) into flt-1\(^{-/-}\) ES cells (Fig. 8). The mflt-1\(^{\Delta\text{Cyt}}\) isoform transgene used in this study encoded the same amino acids as the flt-1 allele used to conclude that signaling via flt-1 is not required for normal development and angiogenesis in vivo (Hiratsuka et al., 1998). flt-1\(^{-/-}\); Tg PECAM-mflt-1\(^{\Delta\text{Cyt}}\) clones rescue vessel branching and cell proliferation as effectively as full-length flt-1 (Fig. 8I, J, K, table 1), suggesting that signaling through flt-1 is not required to rescue either proliferation or morphogenesis of the flt-1\(^{-/-}\) vascular vessels.

Genetic targeting of flt-1 isoform transgenes into the ROSA26 locus: sflt-1 rescues branching morphogenesis more efficiently than mflt-1.

Random integration of flt-1 isoform transgenes into flt-1\(^{-/-}\) ES cells resulted in a range of transgene expression levels and phenotypes among rescue cultures expressing the same flt-1 isoform. To more directly compare the effects of the isoforms, we targeted the sflt-1 and mflt-1 isoform transgenes into the ROSA26 genomic locus. Attempts to target DNA into the ROSA26 locus of ES cells have been described previously and have proved successful in avoiding complications regarding expression differences due to variation in transgene copy number and integration site (Soriano, 1999) (Srinivas et al., 2001). Targeting constructs were
generated and electroporated into flt-1−/− ES cells (Fig. 14A). Resistant flt-1−/− ES colonies were differentiated, correct targeting by homologous recombination was verified by PCR, and expression of individual flt-1 isoform transgenes was verified by RT-PCR (data not shown). Day 8 cultures of one clone each of flt-1−/−; Tg ROSA26-PECAM-sflt-1 and flt-1−/−; Tg ROSA26-PECAM-mflt-1 genotype labeled with PECAM were initially analyzed (Fig. 14B-C). Vessels that expressed the sflt-1 isoform transgene had an increase in vascular branching complexity compared to vessels that expressed the mflt-1 isoform transgene, and endothelial cell proliferation appeared to be rescued to similar levels in vessels of both cultures. This preliminary data corroborates our initial clonal analysis and supports a model in which the soluble isoform of flt-1 rescues branching morphogenesis more efficiently than the membrane-tethered flt-1 isoform.
D. Discussion

Our results show that the soluble flt-1 isoform rescues the vessel branching morphogenesis defect observed in flt-1−/− ES cell-derived vessels (Kearney et al., 2004), and that it does so more efficiently than the membrane-tethered flt-1 isoform. In support of this conclusion, 80% of flt-1−/−; Tg PECAM-sflt-1 clones rescue branching morphogenesis, whereas only 16% of flt-1−/−; Tg PECAM-mflt-1 clones rescue the flt-1−/− branching defect. Expression analysis revealed that lower levels of sflt-1 transgene RNA are sufficient to rescue morphogenesis compared to the few mflt-1- expressing clones. Furthermore, genetic targeting of the flt-1 isoform transgenes into the ROSA26 genomic locus of flt-1−/− ES cells shows that sflt-1 contributes to more complex vascular branching than mflt-1. This data supports a model whereby flt-1 primarily affects vascular branching morphogenesis via a soluble molecule, sflt-1 (Fig. 15). This indicates that the modulation of VEGF-A presentation to endothelial cells via sflt-1 is important for proper vascular morphogenesis.

What is the evidence for this model? VEGF-A RNA is alternatively spliced to produce multiple VEGF-A isoforms. Due to the differing affinities of the three major VEGF-A isoforms (VEGF-A_{120}, VEGF-A_{164}, VEGF-A_{188}) to bind heparin, it is hypothesized that their spatial distribution in the extracellular matrix regulates vessel patterning and morphogenesis, as illustrated in the embryonic lung (Healy et al., 2000). Evidence for this hypothesis comes from analysis of retinal vessels in mouse mutants expressing only a single VEGF-A isoform. Mice expressing either VEGF-A_{120}, which lacks a heparin-binding domain, or VEGF-A_{188}, with multiple heparin binding domains, have retinas with perturbed migration of vascular tip cell filopodia,
reduced expansion of the retinal vascular plexus, and vessels with aberrant morphogenesis (Ruhrberg et al., 2002) (Gerhardt et al., 2003). Furthermore, analysis of VEGF-A isoform-selective mice provides evidence that a VEGF-A gradient set up in the hindbrain is compromised in the absence of matrix-bound VEGF-A, resulting in reduced vascular branching (Ruhrberg et al., 2002). Thus, it is thought that a proper distribution of VEGF-A is required for correct regulation of vascular morphogenesis.

Our data indicates that sflt-1, secreted from endothelial cells, interacts with VEGF-A to modulate its presentation to endothelial cells in a way important for proper morphogenesis. sflt-1 contains a heparin-binding domain (Park and Lee, 1999), so it likely binds the extracellular matrix upon release from the endothelial cell. The secreted flt-1 could spread uniformly from the endothelial cell and regulate in a quantitative fashion the presentation of VEGF-A which is already established. Alternatively, sflt-1 might establish a counter-gradient that could modulate VEGF-A presentation to endothelial cells qualitatively as well, and this counter-gradient could steepen an existing VEGF-A gradient. How might this happen? VEGF-A protein has been observed to migrate over several cell distances from the hindbrain midline to establish a VEGF-A gradient (Ruhrberg et al., 2002). It is possible that sflt-1 can do this as well. Preliminary studies in quail embryos have suggested that sflt-1 may travel over several cell diameters to modulate VEGF-A, and affect the ingestion of vessels into the neural tube from the peri-neural vascular plexus (J.M.J. and V.L.B., unpublished results). Thus, it is possible that sflt-1 is secreted into the outside environment and moves some distance from the vessel, where it can modulate
VEGF-A presentation for efficient control of vascular branching morphogenesis (Fig. 15).

We have observed that filopodia, which predominantly form from the tips of growing vascular sprouts, can extend 1-2 cell lengths into the extracellular matrix. Analysis of retinal vessels suggest that the tip cells that extend these filopodia, express higher than normal levels of flk-1, implicating these cells as sensors of positional information (Gerhardt et al., 2003). Thus, it is possible that the filopodia emanating from a migrating sprout can detect VEGF-A and subsequently transmit a signal to begin a branching response; which may include polarizing the endothelial cell, followed by enablement of active migration/elongation of the sprout. Therefore, if there is perturbed presentation of VEGF-A to the sensing sprout tip, deregulation of branching morphogenesis may follow. Filopodia have also been implicated in sensory roles in other systems, such as in the neuronal growth cone, where filopodia are thought to guide the growing axon by sensing extracellular cues. In Drosophila, filopodia are extended from terminal branches of tracheal cells in response to FGF from oxygen-deprived cells (Sutherland et al., 1996).

In the present study, while all \( \textit{flt-1}^{-/-}; \text{Tg PECAM-sflt-1} \) clones subjected to branch point analysis partially rescued the \( \textit{flt-1}^{-/-} \) branching defect, no \( \textit{flt-1}^{-/-}; \text{Tg PECAM-sflt-1} \) clone fully restored vascular branch scores to WT levels. It is possible that the reason for the lack of complete rescue in these clones is due to an incomplete flt-1 gradient in the absence of mflt-1. While sflt-1 may be important for modulating VEGF-A at a distance from the vessel, there may be a requirement for mflt-1 to be expressed on the endothelial cell membrane so as to begin the formation of a flt-1
gradient. It is also a possibility that the PECAM promoter used to express the individual flt-1 isoform transgenes does not exactly recapitulate flt-1 expression, leading to inappropriate production of sflt-1, and thus, not a full rescue of the flt-1\textsuperscript{-/-} branching defect.

Although most of the flt-1\textsuperscript{-/-}; Tg PECAM-sflt-1 clones primarily rescued morphogenesis (80%; Table 2), only a small minority of flt-1\textsuperscript{-/-}; Tg PECAM-mflt-1 clones did so (16%; Table 2). The reduced ability of the flt-1\textsuperscript{-/-}; Tg PECAM-mflt-1 clones to primarily rescue morphogenesis is likely due to the flt-1 receptor being anchored to the endothelial cell surface. In these clones, mflt-1 is not free to diffuse into the local milieu, where it can interact with sources of VEGF-A to efficiently modulate branching morphogenesis. There were, however, exceptions to this, since 16% of flt-1\textsuperscript{-/-}; Tg PECAM-mflt-1 clones partially rescued the flt-1\textsuperscript{-/-} branching defect. It is possible that ectopic expression of the mflt-1 transgene led to the primary morphogenesis phenotype. While mflt-1 isoform transgene expression was predominantly limited to the vessels (Fig. 12), we did observe some ectopic mflt-1 isoform transgene expression outside of the vasculature in these clones (data not shown). Thus, it is possible that minor amounts of avascular expression of mflt-1 can modulate VEGF-A presentation for efficient rescue of morphogenesis. It is also a formal possibility that the mflt-1 protein produced in these clones is being proteolytically cleaved from the endothelial cell membrane, thus mimicking the function of sflt-1. Immunostaining experiments, along with ELISA and/or western analysis will be carried out in the future to confirm any of the aforementioned possibilities concerning the efficacy of mflt-1 rescuing branching morphogenesis.
Our results show that both sflt-1 and mflt-1 can rescue the endothelial cell over-proliferation defect observed in \textit{flt-1}^{-/-} ES cell-derived vessels (Kearney et al., 2002; Kearney et al., 2004). However, the way in which flt-1 rescues endothelial cell proliferation is unclear. Nearly equal numbers of \textit{flt-1}^{-/-}; Tg PECAM-sflt-1 clones and \textit{flt-1}^{-/-}; Tg PECAM-mflt-1 clones (20% and 21%, respectively; table 2) primarily rescued endothelial cell proliferation, suggesting that sflt-1 and mflt-1 isoform transgenes rescue proliferation with equivalent efficiency. Preliminary analysis of \textit{flt-1}^{-/-} ES cell-derived vessels with a targeted insertion of the flt-1 isoform transgene into the ROSA26 locus also indicate that mflt-1 and sflt-1 can rescue proliferation equivalently (data not shown). This is in agreement with other models that suggest that the levels of VEGF-A, not distribution, are critical for proper regulation of endothelial cell proliferation. \textit{In vitro} experiments have shown that endothelial cell proliferation is dependent on a local concentration of VEGF-A, and that this response is dose-dependent (Cai et al., 2006; Waltenberger et al., 1994). VEGF-A\textsubscript{120} and VEGF-A\textsubscript{165} promoted in vitro endothelial cell proliferation with equal efficiency, in a dose-dependent manner (Ruhrberg et al., 2002). Moreover, studies in mouse retinas have shown that cells behind the leading tip cell in a sprout (stalk cells) are stimulated to divide in the presence of VEGF-A, regardless of correct VEGF-A distribution, suggesting that endothelial cell proliferation is regulated by the local VEGF-A concentration in a spatially-independent manner (Gerhardt et al., 2003). How may this be occurring? Although some filopodia do extend from other cells within the vessel, most filopodial projections are found on tips of angiogenic sprouts. We and others have observed that the tips of vascular sprouts rarely, if
ever, divide. Therefore, while filopodial projections from sprout tips may be important for sensing a VEGF-A environment for proper sprout formation, it is possible that filopodia are not required to transmit proliferative signals to the vessel. It could be that the cells behind the leading cell in the sprout, which also express flk-1, divide upon sensing any VEGF-A, regardless of gradient or distribution. Thus, if proliferation is not dependent on a gradient of VEGF-A, then it is possible that both sflt-1 and mflt-1 are equally efficient in rescuing endothelial cell proliferation in \textit{flt-1}$^{-/-}$ ES cell-derived vessels.

While it is possible that sflt-1 and mflt-1 are equally efficient in rescuing the proliferation defect in \textit{flt-1}$^{-/-}$ cultures, it is also a possibility that sflt-1 is more efficient than mflt-1 in rescuing endothelial cell proliferation. While sflt-1 and mflt-1 do rescue an equal number of clones in the Primary Proliferation Rescue category, vascular area analysis indicates that all \textit{flt-1}$^{-/-}; \text{Tg PECAM-sflt-1}$ clones that primarily rescued morphogenesis had a partial rescue of \textit{flt-1}$^{-/-}$ vascular area (Fig. 10). Although not all of the \textit{flt-1}$^{-/-}; \text{Tg PECAM-sflt-1}$ clones were analyzed for vascular area, personal observation indicates that 100\% of \textit{flt-1}$^{-/-}; \text{Tg PECAM-sflt-1}$ clones (20/20 sflt-1 isoform transgene clones: 16/20 Primary Morph. Rescue clones plus 4/20 Primary Prolif. Rescue clones; table 2) had some level of proliferation rescue compared to only 37\% of \textit{flt-1}$^{-/-}; \text{Tg PECAM-mflt-1}$ clones (7/19 mflt-1 isoform transgene clones: 3/19 Primary Morph. Rescue clones plus 4/19 Primary Prolif. Rescue clones; table 2). Also, real time PCR analysis of \textit{flt-1}$^{-/-}; \text{Tg PECAM-sflt-1}$ and \textit{flt-1}$^{-/-}; \text{Tg PECAM-mflt-1}$ clones reveal that less sflt-1 isoform transgene RNA is required to achieve the same phenotypic response as mflt-1 (Fig. 13), further suggesting sflt-1 to be more efficient
than mflt-1 in rescuing endothelial cell proliferation. If this is true, then the ability of flt-1 to be secreted away from the endothelial cell must also be crucial for regulating endothelial cell division. How may this be occurring? One possibility is that after sflt-1 modulation of VEGF-A, angiogenic sprouts send out filopodia to receive proliferative signals from the external environment. Since cell divisions are rare at the tips of sprouts, there may be a potential block in cell proliferation of sprout tips; however, through an unknown mechanism, tip cells might be able to transmit a proliferative signal to the rest of the vessel. Thus, if modulation of VEGF-A presentation is an important parameter for sprout tips to sense a proliferative signal, then there may be an increased role for sflt-1 in regulating cell proliferation in vessels. Alternatively, since we have observed some filopodial extensions radiating from non-tip cells, albeit at reduced levels compared to the sprout tip cells, these filopodia could be sensing a VEGF-A environment, and upon correct modulation via sflt-1, can help to properly modulate endothelial cell division.

A recent study has suggested that membrane-tethered flt-1 is required developmentally for the proper delivery of VEGF-A to the endothelial cell membrane (Hiratsuka et al., 2005). This data is supported by the observation that half of the mice that do not express membrane-tethered flt-1 die as embryos due to poor blood vessel development. These results become complicated, as the other half of the mice survive. The conclusion that is reached for a requirement for membrane-tethered flt-1 becomes even more ambiguous, as 99% of mice lacking membrane-tethered flt-1 from a 129 genetic background survive. The authors point to increased flk-1 expression (flk-1 phosphorylation levels were not measured) in these mice as
the reason for the high survival rate; however, their explanation is not well supported. The mice used in this experiment also express higher than normal levels of soluble flt-1, as all RNA transcribed from the locus encodes sflt-1. Thus excess levels of sflt-1, which can sequester VEGF-A, could be contributing to the 50% lethality observed in these mice, as minor changes in the amount of available VEGF-A can adversely affect vascular development. Although these results are complicated, the data does indicate that sflt-1 is sufficient for normal vascular development *in vivo* under certain circumstances. These results suggest that mflt-1 is not necessary to combine with sflt-1 to create a complete gradient of flt-1. Perhaps it is possible that sflt-1 expression alone is sufficient *in vivo*, even though branching morphogenesis may not be completely optimal.

My data indicates that sflt-1 is more efficient than mflt-1 in rescuing the defect of *flt-1*<sup>−/−</sup> vessels in branching morphogenesis. This is especially evident in the high percentage of mflt-1 transgene-expressing clones that did not alter the *flt-1* mutant phenotype (62%, table 2). I have also shown that both sflt-1 and mflt-1 can rescue the aberrant endothelial cell proliferation of *flt-1* mutant vessels, but it is not exactly clear how this is accomplished. Further analysis of flt-1 isoform targeted clones (ROSA 26 locus) may reveal more precisely how flt-1 modulates vascular development.
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<td><strong>Tg PECAM-mflt-1</strong></td>
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Table 2. Distribution of phenotypes from differentiated flt-1<sup>Cre</sup> ES cell clones expressing flt-1 transgenes. Numbers in parenthesis indicate the number of clones with the indicated phenotype divided by the total clones of that genotype analyzed.
Figure 8. Differentiated \( flt-1^{-/-} \); Tg PECAM sFlt-1/mFlt-1/mFlt-1\( \Delta \)cyt ES cell cultures show rescue of the flt-1 mutant vessel phenotype. (A) Map of individual flt-1 isoforms. Soluble flt-1 (sflt-1), full length flt-1 (mflt-1), and cytoplasmic-deleted flt-1 (mflt-1\( \Delta \)Cyt) each contain an extracellular, VEGF-A-binding domain (grey circles). mflt-1 and mflt-1\( \Delta \)Cyt contain a transmembrane domain region (TM), while only mflt-1 contains a tyrosine kinase domain. (B) A transgene consisting of the PECAM promoter/enhancer linked to either sflt-1, mflt-1, or mflt-1\( \Delta \)Cyt cDNA was electroporated into flt-1\( ^{-/-} \) ES cells. Individual hygromycin B-resistant ES cell clones were differentiated to day 8, then stained with PECAM-1 antibody or processed for RNA. (C-K) flt-1\( ^{-/-} \) ES cell clones expressing a PECAM-sflt-1 transgene (flt-1\( ^{-/-} \); Tg PECAM-sflt-1) (C-E), a PECAM-mflt-1 transgene (flt-1\( ^{-/-} \); Tg PECAM-mflt-1) (F-H), or a PECAM-mflt-1\( \Delta \)Cyt transgene (flt-1\( ^{-/-} \); Tg PECAM-mflt-1\( \Delta \)Cyt) (I-K) were grouped into 3 categories based on visual inspection of vessels: No Rescue (C, F, I), Primary Morphogenesis Rescue (D, G, J), or a Primary Proliferation Rescue (E, H, K). Numbers at upper right in panels indicate the clone number. Magnification is 10x.
Figure 9. Branch point analysis of Primary Morphogenesis Rescue clones. (A-F) Day 8 differentiated ES cell cultures were reacted with PECAM antibody to visualize branched vessels. WT (A), flt-1−/− (B), flt-1−/−; Tg PECAM-sflt-1 (C-D), and flt-1−/−; Tg PECAM-mflt-1 (E-F) cultures are at 10 X magnification. Note that the rescue clones (C-F) have more branching vessels than flt-1−/− vessels (B). Clone number is in the upper right of each panel. (G) The average number of branch points per mm of vessel length was determined. ***P< 0.001, WT versus flt-1−/−; ***P< 0.001, flt-1−/− versus flt-1−/−; Tg PECAM-sflt-1#33; **P< 0.015, flt-1−/− versus flt-1−/−; Tg PECAM-sflt-1#26 and flt-1−/− versus flt-1−/−; Tg PECAM-mflt-1#8.
Figure 10. Quantitation of vascular area in flt-1 isoform transgene-expressing clones.

Day 8 differentiated ES cell cultures were analyzed for PECAM-positive area as described (see Materials and Methods). flt-1-/ Tg PECAM-flt-1 clones are grouped by visual vascular phenotypes: No Rescue, Primary Morphogenesis Rescue, and Primary Proliferation Rescue. WT area, white bar; flt-1-/ area, grey bar; flt-1-/ Tg PECAM-sflt-1 area, red bars; flt-1-/ Tg PECAM-mflt-1 area, blue bars. Clone numbers are indicated for each flt-1-/ Tg PECAM-flt-1 clone (s=flt-1-/ Tg PECAM-sflt-1, m=flt-1-/ Tg PECAM-mflt-1). *P< 0.004, flt-1-/ versus WT and flt-1 isoform transgene clones.
Figure 11. *flt-1*−/−; Tg PECAM-*flt-1* clones express *flt-1* RNA throughout development. Total RNA was isolated from wild-type (WT), *flt-1*−/−, *flt-1*−/−; Tg PECAM-*mflt-1*#13, and *flt-1*−/−; Tg PECAM-sflt-1#2 ES cell cultures at the indicated times: day 0, ES cells; day 2, D2; day 4, D4; day 6, D6; day 8, D8; and analyzed by RT-PCR using Flt-1 primers (top panels) or GAPDH primers (bottom panels).
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Figure 12. *flt-1^-/-; Tg PECAM-*flt-1 clones express flt-1 RNA in ES cell-derived vessels.

Day 8 differentiated ES cell cultures were fixed and processed for in situ hybridization and hybridized with an antisense flt-1 probe (A-D). Wild-type (A), *flt-1^-/-* (B), *flt-1^-/-; Tg PECAM-sflt-1#33* (C), and *flt-1^-/-; Tg PECAM-mflt-1#8* (D) cultures are pictured at 40 x magnification.
Figure 13. Flt-1 isoform transgene expression levels by real-time PCR correlate with \textit{flt-1$^{-/}$} vessel rescue phenotype. Flt-1 RNA from day 8 ES cell cultures was normalized to $\beta$-actin RNA and expressed as percentage of wild-type flt-1 RNA (100%). \textit{flt-1$^{-/}$; Tg PECAM-flt-1} clones are grouped by the rescue phenotype: WT, white bar; \textit{flt-1$^{-/}$}, grey bar; \textit{flt-1$^{-/}$; Tg PECAM-sflt-1}, red bars; and \textit{flt-1$^{-/}$; Tg PECAM-mflt-1}, blue bars. Clone numbers are indicated for each \textit{flt-1$^{-/}$; Tg PECAM-flt-1} clone (s= \textit{flt-1$^{-/}$; Tg PECAM-sflt-1}, m= \textit{flt-1$^{-/}$; Tg PECAM-mflt-1}). Each real time PCR was performed in triplicate and data reflects the average of two experiments performed in triplicate. $^*$P $\leq$ 0.05, \textit{flt-1$^{-/}$; Tg PECAM-mflt-1#26} versus \textit{flt-1$^{-/}$; Tg PECAM-sflt-1#2}; $^{**}$P $\leq$ 0.001, \textit{flt-1$^{-/}$; Tg PECAM-mflt-1} clones versus \textit{flt-1$^{-/}$; Tg PECAM-sflt-1} clones or \textit{flt-1$^{-/}$; Tg PECAM-mflt-1} clones versus \textit{flt-1$^{-/}$}. 
Figure 14. flt-1<sup>-/-</sup>; Tg ROSA26-PECAM-sflt-1 clone contains more branched vascular networks than flt-1<sup>-/-</sup>; Tg ROSA26-PECAM-mflt-1 clone. (A) ROSA26 targeting. Top, targeting vector containing 5’ and 3’ ROSA26 sequence for homologous recombination into genomic locus, PECAM-sflt-1/mflt-1 transgene, PGK-Hygro cassette for positive selection, and PGK-DTA for negative selection. Middle, ROSA26 genomic locus. Bottom, targeted insertion of transgene into ROSA26 locus. (B-C) Day 8 differentiated ES cell cultures were reacted with PECAM antibody to visualize branched vessels. flt-1<sup>-/-</sup>; Tg ROSA26-PECAM-sflt-1 (B) and flt-1<sup>-/-</sup>; Tg ROSA26-PECAM-mflt-1 (C) cultures at 10x magnification. Note that sflt-1 isoform-expressing transgene clone (B) has more vascular branching than mflt-1 isoform-expressing transgene clone (C). Clone numbers are indicated in upper right corner of each image.
Figure 15. sflt-1 more efficient than mflt-1 to positively effect branching morphogenesis. Flt-1 is thought to modulate VEGF-A presentation to endothelial cells for proper vascular branching (A). When Flt-1 is not present, we have observed defective sprout formation and migration (Chapter II) (B). We propose a model in which flt-1 is secreted from endothelial cells, to potentially modulate a VEGF-A gradient, leading to increased sprout formation (C). Filopodia, which express flk-1 and are extended from sprout tips, may act as sensors in an environment where VEGF-A presentation is important to properly regulate vascular sprout migration. Improper spatial presentation of VEGF-A (mflt-1-expressing cells), may lead to perturbed ability of sprout tips to sense environment, leading to improper formation of vessel branches (D).
**A**

VEGF-A

Normal vascular branching

*WT endothelial cell*

**B**

VEGF-A

Signal overload vascular branching affected

*fli-1/- endothelial cell*

**C**

Partial rescue of branching morphology

*sFli-1 rescue*

**D**

VEGF-A

Perturbed VEGF-A presentation leads to deregulated vascular branching

*mFli-1 rescue*
The work described in this chapter was done in conjunction with Kim Kallianos, an undergraduate student in the lab. This work was the basis for Kim’s senior honors thesis.

A. Introduction

To understand how blood vessels develop, it is important to determine how the individual components of cell division and cell migration and sprouting (morphogenesis) are integrated. During angiogenesis, cells must process and integrate extra-cellular signals to coordinate cell division, migration, and fusion of endothelial cells from existing vessels to form a branched vascular network, but how these signals are regulated and assimilated to produce a properly branched vascular network is unknown.

The classic paradigm for the relationship of cell division and other cellular processes is that cells compartmentalize these components, so that dividing cells are not competent to differentiate or undergo non-division related morphogenesis (Zhu and Skoultchi, 2001). One example of this can be found in the developing fly trachea, another tubular structure. During tracheal development, the ectodermal cells that comprise the trachea undergo multiple rounds of cellular division first, and then the entire tracheal system will form and extend in the absence of any further
divisions (Metzger and Krasnow, 1999). In contrast, development of the vertebrate vasculature is associated with simultaneous cell division, and the cells divide in the context of a vascular structure (Clark and Clark 1939). Thus, during sprouting angiogenesis, cell division and morphogenesis occur concurrently.

VEGF-A binds two high affinity receptors on endothelial cells, Flk-1 and Flt-1, and modulation of VEGF-A signaling by genetic deletion of either receptor affects endothelial cell division and morphogenesis (Fong et al., 1995; Kearney et al., 2002; Kearney et al., 2004; Shalaby et al., 1997). There is evidence that suggests that different downstream VEGF-A signaling targets influence endothelial cell division and morphogenesis. VEGF-A activation of the Flk-1 signaling pathway stimulates endothelial cell division through the Raf/MEK/ERK pathway, while endothelial cell migration is activated via the p38 pathway (Kroll and Waltenberger, 1997; Rousseau et al., 2000). Also, the overall concentration of available VEGF-A is thought to modulate endothelial cell division, while VEGF distribution, is thought to modulate endothelial cell morphogenesis (Gerhardt et al., 2003; Kearney et al., 2002; Kearney et al., 2004; Ruhrberg et al., 2002).

Work done in our lab suggests that morphogenetic signals may modulate cell division during angiogenesis in vitro and in vivo. An example of this can be observed in ES cell-derived vessels, where endothelial cell division is increased in areas of active morphogenesis (Kearney et al., 2004). Recent data from ES cell-derived vessels and from rat retinas suggest that the plane of endothelial cell division is normally oriented perpendicular to the long axis of the vessel, which can promote vessel lengthening (Zeng et al., submitted). However, cell division
orientation becomes randomized in a \textit{flt-1} mutant background, a mutation that leads to vessel dysmorphogenesis. Thus, there is suggestive evidence that cross-talk between the cell division component and the morphogenesis component travels from morphogenesis to cell division. However, it has not yet been determined if the flow of cross-talk can occur in the opposite direction; that is, can the cell division pathway directly communicate with the morphogenesis pathway? To test this, we have used a genetic approach to negatively modulate cell division in the vascular endothelium of ES cell-derived vessels, and to analyze subsequent effects on morphogenesis.

Most eukaryotic cells proceed through an ordered series of events, constituting the cell cycle, during which the chromosomes are duplicated and one copy of each duplicated chromosome segregates into two daughter cells. Regulation of the cell cycle is critical for normal development. Various cyclin-dependent kinase (CDK)-cyclin complexes trigger progression through different stages of the eukaryotic cell cycle by phosphorylating specific target proteins. Cells employ cyclin-dependent kinase inhibitors to inhibit the kinase activity of CDKs. The p21 Cip/WAF1 family of CDKs is downstream of p53 activity and acts to inhibit Cdk4, 6-cyclinD and Cdk2-cyclinE, thereby arresting the cell cycle during the G$_1$ to S transition (Figure 16) (Dulic et al., 1994; el-Deiry et al., 1993; Gentilini et al., 1999; Kumeda et al., 1999; Schonthal et al., 1999).

It has also been reported that mice expressing p21 specifically in hepatocytes results in a block in cell cycle progression, with no apoptosis observed (Wu et al., 1996). Furthermore, we have expressed p21 in Py-4-1 cells (endothelial cell line),
and observed no obvious cell death over several days (data not shown). Thus, p21 appears to be a good candidate for inducing ectopic endothelial cell growth modulation. Our lab has also used the DNA replication inhibitor, mitomycin C, to treat day 6 \(flt-1\) mutant cultures. \(Flt-1\) mutant cultures have an endothelial cell overgrowth defect, exhibited by an increase in vascular area; however, two days after mitomycin C treatment, a partial rescue of the vascular area is observed (Kearney et al., 2002). However, the caveat exists that mitomycin C treatment has a pervasive effect on all cells in culture. Thus, a transgenic approach to achieve cell cycle modulation is required.

To determine if inhibiting cell division has consequences on endothelial cell morphogenesis, the vascular-specific Tie-2 promoter was linked to a p21-GFP fusion transgene, and expressed in WT ES cell-derived vessels. Our preliminary results indicate that a lack of cell division does not appear to affect the pattern of vessel branching in WT ES cell-derived vessels, suggesting that the cell division pathway does not directly communicate with the morphogenesis pathway.
B. Materials and Methods

**ES cell culture and in vitro differentiation**

Wild-type (WT) ES cells containing a p21-GFP fusion transgene (gift from Dr. Neus Agell) under the transcriptional control of the Tie-2 promoter/intron enhancer element were maintained and differentiated as described previously (Bautch et al., 96; Kearney and Bautch, 03). Embryoid bodies (EBs) were plated onto either slide flasks (Nunc, Rochester, NY), or in wells of a 24-well tissue culture dish at day 3 of differentiation and cultured at 37°C in 5% CO₂ until day 8, when cultures were fixed and analyzed.

**DNA constructs and electroporation**

The Tie-2 promoter/intron enhancer (gift of Tomas Sato) was linked to a p21-GFP fusion transgene. The p21-GFP (GFP fused to N-terminus of human p21 cDNA) transgene was first removed from pBluescript II KS⁺ using EcoRV and NotI. The Tie-2 expression vector containing a neomycin resistance cassette was next digested with SmaI and NotI, and the p21-GFP transgene was subsequently ligated to the Tie-2 expression vector to make Tie-2-p21-GFP-NeoR.

Fifteen µg of linearized DNA was electroporated into 2 X 10⁷ WT ES cells using a BioRad GenePulser II electroporator (250V/300µF; BioRad, Hurcules, CA.) Selection of WT ES cells was in 200 µg/mL geneticin. After 12-14 days, Thirty WT drug-resistant transgenic ES clones containing Tie2-p21-GFP-Neo (designated at WT TgTie2-p21-GFP) were picked, expanded, and analyzed.
Antibody staining

ES cultures at day 8 of differentiation were rinsed with phosphate-buffered saline (PBS) and fixed for 5 minutes in either ice-cold methanol-acetone (50:50) or 4% paraformaldehyde. Fixed cultures were reacted with rat anti-mouse PECAM at 1:1000 (MEC 13.3; BD Pharmingen, San Diego, CA) or anti-mouse ICAM-2 at 1:500 (BD Pharmingen, San Diego, CA), and donkey anti-rat immunoglobulin G (IgG; H+L) conjugated to TRITC at 1:100 (Jackson Immunoresearch, West Grove, PA) as described previously (Bautch et al., 2000). Cultures were viewed and photographed with either an Olympus IX-50 inverted microscope (Melville, NY) outfitted with epifluorescence, or with a Leica inverted DMIRB microscope (Bannockburn, IL) and a Hamamatsu OrcaER CCD camera (McHenry, IL).

Quantitative image analysis

To quantify the area labeled with PECAM antibody in differentiated ES cultures, fixed cultures were reacted with PECAM antibody, and photographed (10X) and analyzed as described (Kearney et al., 2002). Percent PECAM area averages for each well were calculated, and the average of 4 wells for each ES clone was used to determine SD values. Branch point analysis was done on fixed cultures reacted with PECAM antibody and photographed at 20x as described (Kearney et al., 2004). The average branch point score from 8-12 pictures for each ES clone was used to determine SD values.

Vessel diameter analysis was used to calculate the average diameter of vessels photographed using a 20x objective. Measurements were taken from two
different subcategories of vessels: branch-to-branch vessels and branch-to-tip vessels. The average diameter from three measurements of a vessel or vessel segment was calculated (measurements taken at each end of the vessel, and in the middle) for WT and WT\textsuperscript{Tg Tie2-p21-GFP} vessels.

**RNA analysis**

For RT-PCR, total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufactures instructions. Ten µg of total RNA was digested with DNase I (Promega, Madison, WI), and 2 µg of DNase-treated total RNA was in vitro transcribed with Superscript II reverse transcriptase (Invitrogen) in a total volume of 80 µl. 10 µl of the reaction was used for PCR experiments. Taq polymerase (Qiagen, Valencia, CA) was used to amplify cDNA using the following primer sets: (1\textsuperscript{st} set; GFP-specific primers) forward 5' GACGTAAACGGCCACAAGTT 3'; reverse 5' TGCTCAGGTAGTGGTTGTCG 3'. (2\textsuperscript{nd} set; p21-GFP-specific primers) forward 5' CCGACCACTACCAGCAGA 3'; reverse 5' AGACGAAGTTGCCCTCCAG 3'. PCR reactions were performed for 3 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The PCR products were electrophoresed on a 3% agarose gel and detected by ethidium bromide staining.
C. Results

Selection of WT $^{Tg \text{Tie2-p21-GFP}}$ clones

To directly assess the relationship between cell division and cell morphogenesis, we generated ES clones carrying p21-GFP fusion transgenes randomly integrated into the genome of WT ES cells. The transgenes were linked to the Tie-2 promoter/enhancer because it results in expression of transgenes in developing blood vessels (C. Ellerstrom and V.L. Bautch, unpublished results). After electroporation and selection, no visible GFP was apparent in any of the ES clones. Since GFP was not detectable, individual ES clones were picked at random for further analysis. 25 WT $^{Tg \text{Tie2-p21-GFP}}$ ES clones were picked and differentiated to day 8, then processed for RT-PCR using GFP-specific primers (Fig. 17A and data not shown). Next, primers specific for the p21-GFP fusion transgene were used for RT-PCR to identify transgenic ES clones that produced a p21-GFP transcript (Fig. 17B). Transgenic ES clones #26, #27, #29, N5, and N10 were positive for the p21-GFP RNA via RT-PCR using either one or both primer sets, and were selected for further analysis.

$^{WT \text{Tg Tie2-p21-GFP}}$ clones have a reduced vascular area

WT and WT $^{Tg \text{Tie2-p21-GFP}}$ clones (#26, #27, #29, N5, and N10) were differentiated to day 8 and labeled with PECAM-1 or ICAM-2 to visualize vascular morphology. The p21-GFP transgenic clones contained less organized and fragmented vasculature, with an overall reduction of areas containing branched vascular networks compared to WT controls (Fig. 18A-B). To quantify the degree of
reduced vascular area in the transgenic clones, we calculated the percentage of the culture that stains for PECAM-1, which approximates the amount of vasculature (Kearney et al., 2002). WT cultures had between 15% and 22.8% PECAM+ area (Fig. 18C), and WT Tg Tie2-p21-GFP#26 and WT Tg Tie2-p21-GFP#27 cultures had percent PECAM areas that were not significantly different from WT controls. Clones N#26 and N#27 also had phenotypes similar to that of WT cultures (Fig. 18C and data not shown). WT Tg Tie2-p21-GFP#29, WT Tg Tie2-p21-GFP#N5, and WT Tg Tie2-p21-GFP#N10 clones, however, displayed a significant reduction in percent PECAM area compared to WT controls (Fig. 18: WT= 22.74% versus WT Tg Tie2-p21-GFP#29= 10.94% , WT Tg Tie2-p21-GFP#N5= 8.87%; WT=15.3% versus WT Tg Tie2-p21-GFP#N10= 4.7%, WT Tg Tie2-p21-GFP#N5= 5.3%). These results were confirmed after quantifying percent vascular area with a second vascular-specific marker, ICAM-2, for selected clones (Fig. 18D). Thus, three of the five p21-GFP-expressing clones showed a decrease in vascular area.

**WT Tg Tie2-p21-GFP clones have normal vessel branching**

To begin to understand the effects of a p21 transgene on vascular morphogenesis, we next quantified the number of vascular branches using a branch-point assay. Fixed samples of day 8 WT and WT Tg Tie2-p21-GFP (#26, #27, #29, and N5) cultures were labeled with PECAM to visualize vessels (Fig. 19A-F and data not shown). Our results show that there is no significant difference in branch point scores between WT and any of the transgenic clones analyzed (Fig. 19G). Thus, this data suggests that while expression of a p21-GFP transgene reduces the overall vascular area, it does not appear to influence vessel branching.
**WT Tg Tie2-p21-GFP clones have smaller vessel diameters compared to WT vessels**

Since there is a decrease in vascular area to go along with normal branching in the ES clones expressing the p21-GFP transgene, we hypothesized that the resultant vessels would have a smaller diameter than WT vessels. Thus, PECAM-stained samples of WT and two p21-GFP transgenic clones that showed reductions in percent vascular area (#N5 and #N10), but had a normal vessel branch score, were assayed for vessel diameter (Fig. 20A-C).

To calculate vessel diameter, measurements were taken from two different categories of vessels: Branch-to-tip vessels; and branch-to-branch vessels. Branch-to-tip vessels included the length of vessel that extended from a branch point to the vessel tip. Branch-to-branch vessels included the length of vessel that extended between two branch points. Our data shows that WT Tg Tie2-p21-GFP clones (WT Tg Tie2-p21-GFP#N5 and WT Tg Tie2-p21-GFP#N10) have significantly reduced vessel diameters in the branch-to-branch category compared to WT controls (Fig. 20D). In the branch-to-tip category, although the average value in WT was higher than in the transgenic clones, there was no statistically significant difference between transgenic clones and WT in this category (Fig. 20D). Thus, it appears that in the two clones analyzed with decreased vascular area, vessel lumens have decreased vessel diameters. However, vessel tips (sprouts), which normally have very thin vessels, do not have a significant change in vessel diameter.
D. Discussion

The work presented in this chapter suggests that the endothelial cell division pathway does not directly communicate with the morphogenesis pathway. In an attempt to block the cell division pathway in endothelial cells, we have introduced a Tie-2-p21-GFP fusion transgene into WT ES cells. Several experimental clones expressing the p21-GFP transgene had significant reduction in vascular area. However, despite the lack of vasculature, the amount of vascular branching found in these clones appears normal. Moreover, further analysis revealed that two of these transgenic clones, WT^{Tg Tie2-p21-GFP#N5} and WT^{Tg Tie2-p21-GFP#N10}, have thinner vessels than controls. Thus, this data, along with other results from our lab, suggest that signals do not directly travel from the cell division pathway to the cell morphogenesis pathway, but that communication travels from morphogenesis to cell division (Fig. 21).

The vascular-specific promoter, Tie-2, which is expressed by endothelial precursors and by the endothelium of actively growing blood vessels \textit{in vivo} (Dumont et al., 1992; Sato et al., 1993) and in our ES cell model, was used for endothelial-specific expression of a p21-GFP fusion transgene. The p21-GFP fusion protein has been shown to properly localize in the nucleus, and to inhibit cell proliferation in NIH3T3 cells as assessed by BrdU incorporation (Rodriguez-Vilarrupla et al., 2002). Thus, the cell cycle inhibitor p21, which inhibits cell cycle progression from G\textsubscript{1} to S phase, was used to inhibit cell proliferation in developing blood vessels.

While we have not formally shown that the p21-GFP transgene is inhibiting the cell cycle in our clones, we do have some evidence to suggest that the
phenotypes observed in the WT Tg Tie2-p21-GFP cultures are due to the effects of the p21 transgene. First, we previously introduced the Tie-2-p21-GFP transgene into Py-4-1 cells (endothelial cell line). These cells did not form colonies over a 6-8 day period; however, they continued to express GFP, suggesting that these cells were viable but unable to divide (data not shown). Secondly, RT-PCR results indicate that the p21-GFP transgene is being transcribed into RNA in these clones (Fig. 17). Lastly, personal experience with many experiments requiring transgene electroporation into ES cells suggests that the phenotypes observed here; decreased vessel area and diameter, do not normally result from integration of transgenes into genomic DNA or clonal isolation. Taken together, this evidence suggests that the phenotypes that we have observed in this study are most likely due to the effects of a p21 transgene.

After selection of resistant clones, GFP was not detectable in differentiating ES cell cultures. GFP and p21 antibody experiments were performed unsuccessfully in an attempt to determine where the p21-GFP protein was localized. While we do see expression of the transgene RNA by RT-PCR, it remains a possibility that p21-GFP protein is not being produced in these clones. RT-PCR results show modest levels of transgene expression, and our experience with the Tie-2 promoter has revealed it to have relatively weak transcriptional activity (C. Ellerstrom and V.L. Bautch, unpublished results). The Tie-2 promoter was chosen because of its activity in endothelial cells during later stages of vascular development, thus allowing for the formation of stable ES cell colonies. Thus, we
suggest that levels of p21 protein are made that elude detection but lead to a vascular phenotype.

In the future it may behoove us to subject our transgenic clones to FACS analysis to determine endothelial cell numbers. This could then be followed by a cell cycle analysis to determine the degree of cell cycle inhibition. Further evidence to implicate the p21-GFP transgene in inhibiting cell cycle progression would be to look at the activity of certain downstream targets of p21 in the p21-GFP-expressing clones. For example, inhibition of cyclin-dependent kinase (cyclin E/Cdk2, cyclin D/Cdk4, and cyclin A/Cdk2) activity may be a good marker of p21 activity. Unphosphorylated retinoblastoma protein (Rb) may also be a good proxy for p21 transgene activity. While the expression of p21 did not induce any observable apoptosis in the hepatocytes of mice (Wu et al., 1996) or in our endothelial cell line (data not shown), it may be important to assay for cell apoptosis in our p21-GFP-expressing clones. This would help to confirm that the phenotypes observed are not due to cell death of p21-GFP-expressing cells.

Our results show that there is a reduction of vascular area in many of the WT Tg Tie2-p21-GFP experimental clones compared to WT controls. This was determined by calculating the percentage of PECAM and/or ICAM-2-stained area within a culture. Previous work done in our lab using ICAM-2 labeling, along with FACS analysis with PECAM antibody on differentiating ES cultures has indicated that percent PECAM area analysis to be a good measure of endothelial cell numbers (Kearney et al., 2002). Of the five p21-GFP transgenic clones subjected to PECAM or ICAM-2 area analysis, three had a significant reduction of vascular area compared to WT controls.
While all five clones expressed the transgene via RT-PCR, it is not surprising that not all of the clones exhibited the same phenotype. Work described in Chapter III illustrates how electroporation of transgenes into ES cells can result in different expression profiles from one clone to the next, and thus, different phenotypes. Therefore, it may be important to carefully analyze the expression profiles of individual transgenic clones to better understand how p21 contributes to the phenotype of each clone.

To assay the potential affects of inhibiting cell proliferation on vascular morphogenesis, we compared branch point scores between WT and WT \text{Tg}^{\text{Tie2-p21-GFP}} experimental clones. Vascular sprouting analysis done in our lab using time-lapse movies of GFP-labeled vessels along with fixed culture analysis has shown that branch point analysis is a relevant measure of vascular sprout formation (Kearney et al., 2004). Of the four p21-GFP transgenic clones subjected to branch point analysis, two (WT \text{Tg}^{\text{Tie2-p21-GFP#N5}} and WT \text{Tg}^{\text{Tie2-p21-GFP#N10}}) showed reduced vascular area compared to controls (Fig. 19). However, none of the four clones analyzed had a significantly different vascular branching score compared to controls. This data suggests that a potential decrease in the number of available endothelial cells via p21 does not influence vascular branching.

Although vessel branching is normal in transgenic clones with decreased vascular area, these clones (WT \text{Tg}^{\text{Tie2-p21-GFP#N5}} and WT \text{Tg}^{\text{Tie2-p21-GFP#N10}}) have decreased vessel diameters compared to WT controls (Fig. 20), further suggesting that limiting cell numbers via p21 does not affect branching morphogenesis. While
there was a significant difference in vessel diameter between WT and experimental clones WT $^{Tg\, Tie2-p21-GFP\#N5}$ and WT $^{Tg\, Tie2-p21-GFP\#N10}$ in the branch-to-branch category of vessels, there was not a significant difference when measuring the diameter of branch-to-tip vessels. From time-lapse imaging of GFP-labeled vessels, we have evidence that on occasion, albeit rarely, the most distal cell of the sprout (tip cell) undergoes cell division. However, in a study using retinal vessels, tip cells were never observed to divide (Gerhardt et al., 2003). Thus, it seems that tip cells rarely, if ever, divide. Therefore, it is possible that the reason for no significant difference in vessel diameter of branch-to-tip vessels between WT and p21-GFP transgenic cultures can be attributed to the idea that cell division in those regions of the vasculature is inherently less frequent, and that inhibiting cell division should not have a substantial effect on vessel thinning.

The data reported here is consistent with a model in which the cell division compartment does not directly communicate with the morphogenesis compartment. Preliminary work done in our lab suggests, however, that morphogenesis may influence cell division. Live imaging of cell division and sprouting angiogenesis in ES cell-derived vessels showed that cell divisions were not excluded from vascular sprouting areas, but in fact, were in most cases enriched in the sprouts and their vicinity, suggesting that sprout formation may stimulate cell division (Kearney et al., 2004). Furthermore, evidence suggests that the effects of the VEGF-A receptor flt-1 on vascular sprout formation are independent of cell division (Kearney et al., 2004) (Chapter II). Moreover, work done in ES cell cultures and in rat retinas shows that endothelial cell division orientation is normally oriented perpendicular to the long axis...
of the vessel; however, this orientation is perturbed in a \textit{flt-1} mutant background, a mutation that also leads to vessel dysmorphogenesis (Zeng et al., submitted). Thus, there is preliminary evidence that morphogenetic signals may modulate cell division during angiogenesis \textit{in vitro} and \textit{in vivo} (Fig. 21). Activation of VEGF-A signaling can affect both endothelial cell division and morphogenesis, and modulation of VEGF signaling can regulate the rate of endothelial cell division (Kearney et al., 2002; Kroll and Waltenberger, 1997). We and others have also shown that VEGF-A can regulate cell migration and sprout formation (Kearney et al., 2004; Ruhrberg et al., 2002). Thus, it is likely that the VEGF-A signaling pathway is involved in regulating the integration between the cell division and morphogenesis components. However, cross-talk between these two components probably occurs upon activation of targets down-stream of VEGF-A signaling. It will be interesting to see exactly which downstream pathways allow for morphogenesis and cell division to communicate. VEGF-A signaling can affect focal adhesion kinase (FAK) and Src, molecules involved in endothelial cell migration (Abedi and Zachary, 1997; Abu-Ghazaleh et al., 2001; Rousseau et al., 2000). Activation of the small GTPase Rho by VEGF-A/flk-1 has also been implicated in cell migration (van Nieuw Amerongen, Koolwijk et al. 2003), however, it is known that Rho also modulates the actin cytoskeleton and subsequent cell cycle progression (Mettouchi et al., 2001; Welsh et al., 2001). Thus, the intersection for communication between morphogenesis and cell division may begin with this target. Therefore, teasing out the different pathways will be the key to a better understanding of how these two compartments are integrated for proper blood vessel formation.
**Figure 16. The cell cycle.** The cell cycle is divided into four major phases. In replicating somatic cells, chromosomes are replicated during S (synthesis) phase. After progressing through the G₂ (gap) phase, cells begin the process of mitosis (M-phase). Following mitosis, cycling cells enter G₁ phase, the period before DNA synthesis is reinitiated in S-phase. The cell cycle inhibitor, p21, is active during several points during the cell cycle, specifically at the G₁→S transition. Image taken from [www.cancerline.com](http://www.cancerline.com).
Stages of the cell cycle
G₀ - dormant cells
G₁ - resting phase
S - DNA synthesis
G₂ - premitotic phase
M - mitosis

Cell divides (mitosis)
Start of cycle
Cell prepares to divide
Cell replicates its DNA
Restriction point: cell decides whether to commit itself to the complete cycle
Cell enlarges and makes new proteins
Cell rests
Figure 17. WT $^{\text{Tg Tie2-p21-GFP}}$ clones express the p21-GFP fusion transgene.

GFP RNA and p21-GFP fusion RNA was analyzed by RT-PCR. Total RNA (2 $\mu$g) was isolated from wild-type (WT) and WT $^{\text{Tg Tie2-p21-GFP}}$ transgenic ES clones on day 8 of differentiation. RNA was reverse transcribed, and resulting cDNAs were amplified by 30 PCR cycles using GFP primers (A), and primers specific to the p21-GFP fusion construct (B, C). DNA vector was used as positive control (+), while no template was used as a negative control (-). Transgenic clone numbers are indicated above images.
Figure 18. Quantitation of proliferation rescue.

(A-B) Day 8 differentiated ES cultures reacted with ICAM-2 antibody to visualize vasculature. WT (A) and WT $^{Tg}$ Tie2-p21-GFP$^N5$ (B) are 10x magnification. (C-D) Day 8 differentiated WT and WT $^{Tg}$ Tie2-p21-GFP transgenic clones were analyzed for PECAM$^+$ (C) and ICAM-2$^+$ (D) area as described (see Materials and Methods). Clone numbers are indicated along x-axis. *P<0.001, WT versus WT $^{Tg}$ Tie2-p21-GFP transgenic clones.
Figure 19. WT $^{\text{Tg}}$Tie2-p21-GFP transgenic clones and WT cultures have equivalent vascular branching. (A-C) Day 8 differentiated ES cultures reacted with PECAM antibody to visualize branched vessels. WT (A), WT $^{\text{Tg}}$Tie2-p21-GFP#29 (B), and WT $^{\text{Tg}}$Tie2-p21-GFP#N5 (C) are 20x magnification. Clone number is indicated in lower right of each panel. (D) The average number of branch points per mm of vessel length was determined. No p21-GFP transgenic clone had a branch score that was significantly different from WT.
Average Branch Point Score

<table>
<thead>
<tr>
<th>Clone</th>
<th>Branch Score</th>
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<tr>
<td>R1</td>
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<tr>
<td>26</td>
<td>10.1</td>
</tr>
<tr>
<td>27</td>
<td>10.2</td>
</tr>
<tr>
<td>29</td>
<td>10.0</td>
</tr>
<tr>
<td>N5</td>
<td>10.9</td>
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Avg. # branch points/mm length

D

Clone

R1 (WT) 26 27 29 N5

Avg. # branch points/mm length

10.2 10.1 10.2 10.0 10.9
Figure 20. WT $^{Tg\text{Tie2-p21-GFP}}$ transgenic clones have reduced vessel diameters compared to WT vessels. (A-C) Day 8 differentiated ES cultures reacted with PECAM antibody to visualize vasculature. WT (A), WT $^{Tg\text{Tie2-p21-GFP#N5}}$ (B), and WT $^{Tg\text{Tie2-p21-GFP#N10}}$ (C) are 20x magnification. Clone number is indicated in lower right of each panel. (D) The average diameter of vessel was determined in mm (average of three measurements/vessel length). Measurements were made from two different vessel categories in both WT and p21-GFP transgenic clones: branch-to-branch; and branch-to-tip. *$P<0.001$, WT versus WT $^{Tg\text{Tie2-p21-GFP}}$ transgenic clones.
Figure 21. Model of communication between endothelial cell division pathway and morphogenesis pathway during blood vessel development. Previous work done in our lab, in combination with the work presented here, suggests that the flow of communication does not go from the cell division pathway to the morphogenesis pathway, but that information travels from morphogenesis to cell division.
CHAPTER V
GENERAL DISCUSSION

In this dissertation, I present my work investigating the role of the VEGF-A receptor, flt-1, in embryonic vascular development. Specifically, I have (in collaboration with another graduate student, Joseph Kearney) demonstrated that flt-1 negatively modulates endothelial cell division of GFP-labeled ES cell-derived vessels (Chapter II). This is consistent with in vivo and in vitro biochemical and genetic studies indicating that flt-1 functions to sequester VEGF-A and inhibit activation of the flk-1 pathway (Roberts et al., 2004) (reviews) (Olsson et al., 2006; Shibuya and Claesson-Welsh, 2006). I have also, in collaboration with Joseph Kearney, demonstrated that flt-1 positively modulates vascular sprout formation (Chapter II). Additionally, I have investigated the role of the flt-1 isoforms, membrane-bound flt-1 (mflt-1), and soluble flt-1 (sflt-1), in embryonic vascular development (Chapter III), and have demonstrated that both isoforms can modulate endothelial cell proliferation, while sflt-1 is more efficient than mflt-1 in rescuing the flt-1⁻/⁻ vascular morphogenesis defect. These data support a model whereby flt-1 must be secreted from endothelial cells to interact with sources of VEGF-A and modulate the presentation of VEGF-A to developing vessels for proper vessel branching morphogenesis.
**Morphogenesis versus Cell Division.** Historically, endothelial cells have been observed to extend sprouts that migrate through the extracellular matrix of surrounding tissues (Clark and Clark, 1939). Cells within sprouts can migrate and proliferate to establish a new vascular cord. Furthermore, early studies suggested that there were separate migratory and proliferative functions within a developing vessel (Ausprunk and Folkman, 1977). Our time-lapse imaging data shares some consistency with this idea. The observation that since there is more endothelial cell division in the flt-1 mutant background, led to the formal possibility that aberrant cell division in flt-1-/- vessels somehow compromises vascular sprouting, leading to reduced sprout formation. However, the observation that the sprouting defect persisted at day 8 (Chapter II, Fig. 2-3), when the rate of cell division in mutant vessels had returned to WT levels (Chapter II, Fig. 5), strongly suggests that the effect of flt-1 on sprout formation is independent of cell division.

However, we did observe a novel relationship between dividing cells and migrating (sprouting) cells. Dynamic image analysis of day 6 GFP-labeled ES cell-derived vessels demonstrated that 78% of endothelial cell divisions occur within the walls of parent vessels (Primary Vessels), while 22% of cell divisions occur in the “sprout field” (Chapter II, table 1). Image analysis of GFP-labeled vessels indicates that 97% of the vascular area consists of parent vessels, while only 3% of vascular area comprises the sprout field (N. C. K, V. L. B., unpublished results). The finding that 22% of cell divisions occur in 3% of the vascular area indicates that divisions are enriched in vascular sprouts and their vicinity, and suggests that sprout
formation may stimulate endothelial cell division. Preliminary work examining p21-GFP-expressing ES cell-derived vessels suggests that limiting vascular cell numbers via p21 does not affect branching morphogenesis (Chapter IV). Moreover, work done in ES cell cultures and in rat retinas shows that endothelial cell division orientation is normally oriented perpendicular to the long axis of the vessel; however, this orientation is perturbed in a \textit{flt-1} mutant background (Zeng et al., submitted), further suggesting that there may be communication between the morphogenesis compartment and cell division pathway. Taken together, I believe that this work supports a model whereby morphogenesis stimulates endothelial cell division in vascular development. There is precedent for this model in that tumor vessels also restrict cell divisions to sprout areas (Ausprunk and Folkman, 1977).

**Regulation of sprouting angiogenesis by \textit{flt-1}**. Joe Kearney and I have demonstrated that the \textit{flt-1} mutation has an effect on vascular sprouting \textit{in vivo} and in ES cell-derived vessels. \textit{flt-1}\textsuperscript{−/−} vessels had reduced sprout formation and migration compared to wild-type vessels (Chapter II, Fig. 2-4), indicating that flt-1 normally has a positive effect on these processes. We hypothesized that in the absence of flt-1, there would be increased VEGF-A activation of flk-1, and subsequent increase in vascular sprout formation since VEGF-A promotes endothelial cell migration (review) (Olsson et al., 2006). However, administration of exogenous VEGF-A inhibits sprouting of intersomitic vessels from the dorsal aorta in developing quail embryos (Drake and Little, 1995). This is consistent with a model in which increased VEGF-A signaling inhibits vascular sprouting. Therefore, flt-1
may have a role as a ligand sink to sequester VEGF-A and limit signaling via flk-1. While this is in agreement with a model in which flt-1 positively regulates vascular branching via antagonism of VEGF-A, intracellular signaling from the flt-1 receptor has long been thought to have a role in vascular development, possibly in regulating vascular branch formation. In collaboration with Dr. Gefei Zeng, I have demonstrated that expression of a flt-1 isoform transgene lacking the cytoplasmic domain, in flt-1−/− vessels (flt-1−/− Tg PECAM-mflt-1ΔCyt), rescues the flt-1 mutant vascular phenotype as efficiently as full-length mflt-1, suggesting that signaling via flt-1 is not required developmentally (Chapter III; Fig. 8, table 2). This is consistent with a previous study where mice expressing cytoplasmic domain-deleted flt-1 survive and are healthy (Hiratsuka et al., 1998). Taken together, this data indicates that the signaling function of flt-1 is dispensable for proper vascular development.

While we have showed the flt-1 is important in regulating branching morphogenesis, an understanding of the roles of the flt-1 isoforms, sflt-1 and mflt-1, has remained elusive in the field of developmental vascular biology. While flt-1 signaling is critical for macrophage/monocyte migration (Barleon et al., 1996; Clauss et al., 1996), as well as in certain circumstances in the adult (Luttun et al., 2002; Rafii et al., 2003), we and others have shown that it is not required developmentally. Thus, we hypothesized that sflt-1 may be the important isoform involved in vascular development.

The introduction of sflt-1 and mflt-1 isoform transgenes into flt-1−/− vessels has demonstrated that sflt-1 is more efficient than mflt-1 in rescuing the flt-1−/− branching
defect (Chapter III). One extension of the VEGF-A/flt-1 ligand sink model is that sflt-1 aids in the formation of VEGF-A gradients in the extracellular matrix, and that proper modulation of VEGF-A correctly regulates vascular branching morphogenesis. Consistent with this idea, previous results from our lab using western analysis of flt-1 protein suggests that sflt-1 protein is the dominant flt-1 isoform (Frank W. DiPaola, Joseph B. Kearney, unpublished results). In support of this data, other flt-1 antibody studies have shown that sflt-1 protein is expressed at higher levels than mflt-1 protein in vivo (Carmeliet et al., 2001; Hiratsuka et al., 1998). Furthermore, co-culture experiments utilizing wild-type and flt-1-/- ES cells indicates that flt-1 can act in a non-cell autonomous manner (Chapter II, Fig. 6), suggesting that sflt-1 is secreted from endothelial cells during vascular development. Likewise, analysis of VEGF-A isoform-selective mice provides evidence that a proper distribution of VEGF-A is required for correct regulation of vascular morphogenesis (Gerhardt et al., 2003; Ruhrberg et al., 2002; Stalmans et al., 2002). Thus, we propose a model where sflt-1 modulation of VEGF-A presentation to endothelial cells is important for efficient control of vascular branching. I have demonstrated that sflt-1 can partially rescue the branching defect observed in flt-1-/- vessels (Chapter III, Fig. 8-9); however, there may be a requirement for mflt-1 to fully restore the branching score to wild-type levels. Mflt-1, in combination with sflt-1, may be required to establish a complete flt-1 gradient. Thus, introduction of both isoforms into flt-1-/- vessels may be necessary to completely rescue the flt-1 mutant phenotype.
It may be possible to provide experimental evidence for gradients of VEGF-A and sflt-1 in vivo or in differentiated ES cultures by visualization of both VEGF-A and sflt-1 protein. Attempts to carry out immunolocalization experiments using antibodies against VEGF-A or sflt-1 have proved to be unsuccessful in our laboratory. Therefore, a dynamic image analysis approach could provide useful information. The use of different fluorescent molecules, linked to VEGF-A, flt-1, or even flk-1, would enable us to dynamically visualize and study how these molecules regulate vascular development in vivo or in ES cell-derived vessels.

How may improper presentation of VEGF-A, which is thought to occur in flt-1−/− or flt-1−/−; Tg PECAM-mflt-1 vessels, lead to deregulated vascular sprout formation? The migration of cells involves the assembly and disassembly of focal contacts between the actin cytoskeleton and the extracellular matrix (review) (Raftopoulou and Hall, 2004). Therefore, for cells to migrate, cells must be able to break existing focal contacts, while also forming new ones. VEGF-A signaling through flk-1 promotes the migration of endothelial cells by the regulation of focal adhesion kinase (FAK) and Src, which stimulate focal adhesion formation and turnover (Abedi and Zachary, 1995; Chou et al., 2002; Rousseau et al., 2000). The activation of Src has been shown to inhibit the activity of RhoA, a major regulator of focal adhesion signaling and cell migration (Raftopoulou and Hall, 2004), while other studies have demonstrated that inappropriate stimulation or suppression of RhoA has an inhibitory effect on cell migration (Arthur et al., 2002). Thus, inappropriate VEGF-A presentation and subsequent perturbed flk-1 activation, as seen in flt-1−/− or flt-1−/−; Tg
PECAM-mflt-1 vessels, may lead to increased levels of Src activation and inappropriate effects on RhoA, leading to a defect in proper vascular sprout formation.

We have observed sprout tips from flt-1⁻/⁻ vessels to be more blunted, compared to the more tapered wild-type sprout tips (Chapter II, Fig. 4). Closer inspection of flt-1⁻/⁻ or flt-1⁻/⁻: Tg PECAM-mflt-1 vascular sprouts may reveal abnormal distribution of focal adhesion and actin-cytoskeleton regulators such as FAK, Src, and Rho family members. Also, the generation of primary endothelial cell lines from flt-1⁻/⁻ or flt-1⁻/⁻: Tg PECAM-mflt-1 differentiated ES cultures and subsequent analysis of focal adhesion signaling may help to determine if inappropriate RhoA-mediated focal adhesion signaling leads to improper vascular morphogenesis.

Are endothelial cells divided into two distinct subpopulations? Early studies by Ausprunk and Folkman (1977) demonstrated that endothelial cells have separate migratory and proliferative roles during embryonic angiogenesis. More recent observation of vascular development has revealed a possibility that endothelial cells may exist as two different subpopulations (Gerhardt et al., 2003). These two distinct types of endothelial cells include the sprout tip endothelial cell and the main vessel body endothelial cell (hereafter termed “stalk cell”). The sprout tip cell is the leading cell in a vascular sprout, while stalk cells include all of the cells behind a sprout tip cell. We and others have observed several biochemical and cell biological differences between these two different groups of cells. First, increased expression of flk-1 in sprout tip cells compared to stalk cells was observed by Gerhardt et al. (2003). Secondly, while stalk cells do produce filopodial extensions,
we and others have observed increased numbers of filopodia emanating from sprout tip cells. Filopodia carry out such functions as intercellular communication, cell migration, and cell adhesion (Wood and Martin, 2002). Thus, it is plausible to implicate sprout tip cells as sensors of positional information, which is provided in the form of VEGF-A presentation, to promote endothelial cell migration. Therefore, these sensing sprout tip cells can potentially transmit positional information from the outside environment to the sprout tip cell and induce a vascular branching response. Aberrant distribution of VEGF-A has been associated with inappropriate filopodial extension and vascular branch formation (Ruhrberg et al., 2002; Stalmans et al., 2002), suggesting a relationship between sprout tip cell filopodia and VEGF-A distribution.

We and others have observed that while stalk cells divide frequently, sprout tip cells rarely, if ever, divide. This, coupled with the assessment that stalk cells have decreased filopodia and flk-1 expression compared to sprout tip cells, suggests that stalk cells may have more of a proliferative role in vascular development, while sprout tip cells may have a primary role in branching morphogenesis. Remarkably, these processes may be functionally independent since they affect two unique subpopulations of endothelial cells. Stalk cell proliferation can proceed in the absence of sprout tip cell migration (Gerhardt et al., 2003), while we have observed sprout tip cell migration to occur in the absence of cell proliferation. However, since we have suggestive evidence that sprout formation may stimulate cell division, there could be cross-talk in that direction. Since morphogenesis and proliferation are mediated by flk-1, the two endothelial cell subpopulations may interpret the VEGF-
A/flk-1 signals differently. Modulation of VEGF-A presentation by a VEGF-A/flt-1 gradient is interpreted differently in the sprout tip cell, whereas stalk cells may only interpret a concentration of VEGF-A (Gerhardt et al., 2003). To better understand the role of the sprout tip cell and stalk cell, harvested endothelial cells could be grown in culture and induced to form vascular tubes. Sources of VEGF-A could be placed at a distance from a newly formed vessel, so as to stimulate filopodia formation from the sprout tip cell. If possible, microdissection and isolation of sprout tip cells, or possibly, cell sorting experiments selecting for cells with increased flk-1 expression could be employed to isolate this endothelial cell subpopulation. Comparisons between these two cell populations can be carried out using microarray analysis to determine if there are genes that are differentially regulated in the sprout tip cell versus the stalk cell.

**Conclusion:** At the beginning of my studies, relatively little was known about how the flt-1 receptor affected vascular development. My studies have demonstrated that flt-1 has a role in endothelial cell division and sprout formation during embryonic blood vessel formation. In addition to these findings, I have helped to pioneer time-lapse imaging of vascular development in ES cell-derived vessels. Furthermore, I have analyzed the roles of the flt-1 isoforms, sflt-1 and mflt-1, and demonstrated that sflt-1 is more efficient than mflt-1 in regulating vascular branch formation; while sflt-1 and mflt-1 both have a role in regulating endothelial cell proliferation during embryonic vascular development. Thus, flt-1 functions to
shape blood vessel morphology and is critical to the establishment of an intricate network of blood vessels during vertebrate embryogenesis.
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