INVESTIGATION OF FLT1 AND VEGF SIGNALING IN CONNECTIONS DURING DEVELOPMENTAL SPROUTING ANGIOGENESIS

Jessica E. Nesmith

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in a partial fulfillment of the requirements for the degree of Doctorate of Philosophy in the Curriculum in Genetics and Molecular Biology in the School of Medicine.

Chapel Hill
2016

Approved by:
Victoria L. Bautch
Bob Goldstein
Kathleen M. Caron
Frank L. Conlon
Jiandong Liu
ABSTRACT

Jessica Nesmith: Investigation of Flt1 and VEGF signaling in connections during sprouting angiogenesis
(Under the Direction of Victoria L. Bautch)

Blood vessel formation is essential for vertebrate development and is primarily achieved by angiogenesis, the sprouting of endothelial cells from pre-existing vessels. Vessel networks expand when sprouts form new connections, a process whose regulation is poorly understood. Here we show that vessel anastomosis is spatially regulated by VEGFR1 (Flt1), a VEGF-A receptor that acts as a ligand decoy. Expanding vessel networks in vivo favor interactions with flt-1 mutant endothelial cells. Live imaging in vitro revealed that stable connections are preceded by transient contacts from extending sprouts, suggesting sampling of potential target sites, and reduction of Flt1 reduced transient contacts. Endothelial cells at target sites with elevated protrusive activity and/or reduced Flt1 were more likely to form stable connections with incoming sprouts. Target cells with reduced membrane-localized Flt1 (mFlt1), but not soluble Flt1, recapitulated the bias towards stable connections, suggesting that relative mFlt1 expression spatially influences selection of stable connections. Thus multiple sprout anastomosis parameters are regulated by VEGF signaling, and stable connections are spatially regulated by endothelial cell-intrinsic modulation of mFlt1, suggesting new ways to manipulate how vessel networks form.
To my family, those I was lucky enough to be born with and the ones I have chosen. You have provided support and love during the highs and lows of this work, willingly listened to my scientific excitement and helped me figure out my career.
ACKNOWLEDGEMENTS

This work was accomplished with the help of a great many people over the years, without whom this work and my success would not be possible.

First I want to thank my advisor Vicki Bautch for the guidance and critical analysis of this project since its generation. Your suggestions and help have shaped this story and my scientific goals. The laboratory environment you fostered has helped me find the courage and strength to continue questioning and investigating scientific research. With your mentorship I feel prepared to enter the scientific community as a contributing, well-informed, and active member.

To the members of the Bautch lab, past and present, your input has been invaluable. Specifically, I want to thank John Chappell who was available as a conceptual sound board, for countless moments of troubleshooting, and provided invaluable input in designing realistic experiments and shaping the body of this work. Many discussions with the lab members in our regular meetings and the sounding board you offered for conclusions on data, presentation techniques, and career goals were instrumental in my time here. You all have seen me through tough personal and academic challenges that I faced these past years. Each of you contributed to the rigorous scientific environment which challenged and kept me on my toes and kept a wonderful balance with levity and humor that truly made this a great group to be a part of.

I would also like to thank my thesis committee for their invaluable input and critiques of this work. Without your support my graduate, and post-doctoral, career would not be where they are today, thank you for the time and energy you chose to give my time here at UNC.
And to Jim, the amazing man who has been by my side, giving me the strength and the intellectual sounding board throughout this project. Your willingness to listen to my talks, argue about the meaning of obscure results, and read and edit all of those drafts have made these past four years a better experience. I couldn’t ask for a better partner, I love you.
PREFACE

Portions of the methods in CHAPTER 2 and data in CHAPTER 3 are adapted in part from a paper in revision at Development.


TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................ iii

ACKNOWLEDGEMENTS .............................................................................................................. v

PREFACE ......................................................................................................................................... vii

LIST OF FIGURES ........................................................................................................................ xi

LIST OF TABLES ............................................................................................................................ xii

LIST OF ABBREVIATIONS ............................................................................................................. xiii

CHAPTER 1: Introduction ........................................................................................................... 1

I. Angiogenic blood vessel network formation during development ............................................. 1

II. VEGF signal propagation and ligand specific effects ................................................................. 3

   a. Flk1 during developmental angiogenesis ................................................................................ 4

   b. Flt1 during developmental angiogenesis .............................................................................. 7

   c. VEGF ligand effects in animal models ................................................................................ 9

III. Sprouting angiogenesis during development ....................................................................... 12

   a. Sprout initiation from quiescent vessels ............................................................................. 12

   b. Sprout migration and extension ......................................................................................... 13

   c. Anastomosis to create connections and new vessel conduits .......................................... 15

   d. Formation of a new lumen .................................................................................................. 17

IV. Perspective ............................................................................................................................. 19
CHAPTER 2: Materials and Methods ........................................................................28

I. Experimental Materials and Methods ..................................................................28
   a. Retinal Angiogenesis Imaging and Analysis ..................................................28
   b. Cell Lines and Culture ..................................................................................29
   c. HUVEC Sprouting Angiogenesis Assay Imaging and Analysis ..................29
   d. ES Cell-derived Vessel Imaging and Analysis ..............................................30
   e. Zebrafish Experiments and Husbandry ......................................................31
   f. Protein Quantification ..................................................................................31
   g. Lentivirus and siRNA knockdown ................................................................32
   h. Fluorescence-activated Cell Sorting (FACS) Analysis .............................33
   i. mRNA Preparation and Quantification .......................................................33
   j. Statistical Analysis ......................................................................................33

II. Tables .................................................................................................................34

III. REFERENCES ....................................................................................................37

CHAPTER 3: Blood Vessel Anastomosis is Spatially Regulated by Flt1 During Angiogenesis .................................................................38

I. Introduction .........................................................................................................38

II. Results ................................................................................................................40
   a. Flt1 Influences Retinal Vessel Interactions ...............................................40
   b. Transient Contacts Precede Stable Blood Vessel Connections .............40
   c. Flt1 Regulates the Number of Transient Contacts ................................41
   d. Differential Flt1 isoform Requirements for Sprout Anastomosis .........43

III. Perspective .........................................................................................................45
CHAPTER 4: Flt1 During Sprouting Angiogenesis .............................................63
I. Results .............................................................................................................63
   a. Connection stability and success requires Flt1 .........................................63
   b. Flt1 isoforms differently affect mitosis and protein localization ............66
   c. Flt1 and VEGF signaling influence anastomosis within zebrafish vessels ......69
II. Figures ...........................................................................................................73
III. REFERENCES ...............................................................................................79

CHAPTER 5: Conclusions and Future Directions ..............................................81
I. Summary of Flt1 regulation of blood vessel anastomosis ............................81
II. Future directions for investigating Flt1 regulation of anastomosis ...............83
   a. Mechanism of mFlt1 influence on vessel interaction outcome ..................83
   b. mFlt1 regulation influence on non-endothelial anastomotic regulation ........86
   c. Applications for anastomotic regulation in disease states .......................89
III. Perspective ...................................................................................................90
IV. Figures .........................................................................................................92
V. REFERENCES ...............................................................................................94
LIST OF FIGURES

Figure 3.1: Flt1 Influences Retinal Vessel Interactions In Vivo .................................................48

Figure 3.2: Blood Vessel Stable Connections are Preceded by Transient Contacts .......................50

Figure 3.3: Transient Contact Dynamics during Vessel Anastomosis are Regulated by Flt1 ..........52

Figure 3.4: Both Flt1 Isoforms Reduce Transient Contacts Prior to Connection .......................54

Figure 3.5: Target Vessel Connection Site is Biased by Flt1 .......................................................56

Supplementary Figure 3.1: Flt1 shRNA Validation .................................................................57

Supplementary Figure 3.2: Transient Contacts and Stable Connections in Mosaic Target Vessels .................................................................60

Figure 4.1: Vessel connection outcomes in ES cell-derived sprouting angiogenesis is influenced by Flt1 .................................................................74

Figure 4.2: Isoform specific effects on sprouting human endothelial venous endothelial cells . 76

Figure 4.3: Flt1 and VEGF influence connections in zebrafish intersegmental vessels ...............78

Figure 5.1: Schematic of mFlt1 regulation during blood vessel anastomosis ..............................93
LIST OF TABLES

Table 1: Major Phosphorylation Sites of Flk1 and Cellular Effects/Interactions..........................4

Table 2.1: Antibodies and Nuclear Stains. .................................................................................34

Table 2.2: siRNA sequences targeting Flt1 isoforms.................................................................35

Table 2.3: qRT-PCR primers....................................................................................................36
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJ</td>
<td>adherens junction</td>
</tr>
<tr>
<td>AKT</td>
<td>AK-thymoma/protein kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division control protein 42</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CVP</td>
<td>caudal vein plexus</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DLAV</td>
<td>dorsal longitudinal anastomotic vessel</td>
</tr>
<tr>
<td>Dll4</td>
<td>Delta-like 4</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco modified eagle medium</td>
</tr>
<tr>
<td>dpf</td>
<td>days post-fertilization</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cells</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EPC</td>
<td>endothelial precursor cells</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Flk1</td>
<td>fetal liver kinase 1 (see VEGFR2/Kdr)</td>
</tr>
<tr>
<td>Flt1</td>
<td>fms-like kinase 1 (see VEGFR1)</td>
</tr>
<tr>
<td>Flt4</td>
<td>fms-related tyrosine kinase 1 (see VEGFR3)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GOF</td>
<td>gain of function</td>
</tr>
<tr>
<td>HB</td>
<td>hindbrain</td>
</tr>
<tr>
<td>hpf</td>
<td>hours post-fertilization</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulfate</td>
</tr>
<tr>
<td>hsp</td>
<td>heatshock protein</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical venous endothelial cells</td>
</tr>
<tr>
<td>InDels</td>
<td>insertion or deletion of deoxyribonucleic acid bases</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>ISVs</td>
<td>intersegmental vessels</td>
</tr>
<tr>
<td>JAM</td>
<td>junctional adhesion molecule</td>
</tr>
<tr>
<td>KD</td>
<td>knockdown</td>
</tr>
<tr>
<td>Kdr</td>
<td>kinase insert domain receptor (see Flk1/VEGFR2)</td>
</tr>
<tr>
<td>LOF</td>
<td>loss of function</td>
</tr>
<tr>
<td>MAPK</td>
<td>p38 mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mFlt1</td>
<td>membrane-bound Flt1</td>
</tr>
<tr>
<td>MO</td>
<td>morpholino</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NHLF</td>
<td>normal human lung fibroblasts</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NRP</td>
<td>neuropilin</td>
</tr>
<tr>
<td>Par3</td>
<td>portioning defective homolog 3</td>
</tr>
<tr>
<td>PAK1</td>
<td>p21 activated kinase 1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PECAM</td>
<td>platelet endothelial cell adhesion molecule 1</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>pFlk1</td>
<td>phosphorylated fetal liver kinase 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLCγ</td>
<td>phospholipase C gamma</td>
</tr>
<tr>
<td>PIGF</td>
<td>planctental growth factor</td>
</tr>
<tr>
<td>PODXL</td>
<td>podocalyxin</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radiommunoprecipitation assay</td>
</tr>
<tr>
<td>RNAi</td>
<td>ribonucleic acid interference</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>sFlt1</td>
<td>soluble/secreted Flt1</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SIVs</td>
<td>sub-intestinal vessels</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>Src</td>
<td>Proto-oncogene tyrosine-protein kinase Src</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TJ</td>
<td>tight junction</td>
</tr>
<tr>
<td>TSAd</td>
<td>T-cell specific adapter</td>
</tr>
<tr>
<td>VE-Cad</td>
<td>VE-Cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>vascular endothelial growth factor receptor 1 (see Flt1)</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>vascular endothelial growth factor receptor 2 (see Kdr/Flk1)</td>
</tr>
<tr>
<td>VPF</td>
<td>vascular permeability factor</td>
</tr>
<tr>
<td>VEGFR3</td>
<td>vascular endothelial growth factor receptor 3 (see Flt4)</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-related integration site</td>
</tr>
<tr>
<td>WT</td>
<td>wildtype</td>
</tr>
<tr>
<td>ZO1</td>
<td>zona occludins 1</td>
</tr>
</tbody>
</table>
CHAPTER 1: Introduction

The body of work presented here seeks to understand the impact of regulatory signaling on the formation of a complex, structured blood vessel network. There are a large number of growth factors implicated in the process; however, the vascular endothelial growth factor ligand family is a major main growth factor implicated in directing blood vessel growth and the focus of these studies. Blood vessel expansion into a hierarchical network of arteries and veins connected by capillaries is accomplished through angiogenesis. The varying interactions and interplay of signaling cascades that direct the initiation and coordinated migration of angiogenesis is highly researched but these same cascades are not evaluated during anastomosis, the process of two vessel connecting. Therefore, this work addresses the regulation anastomosis of sprouting angiogenesis through spatio-temporal restriction of growth factor signaling.

I. Angiogenic blood vessel network formation during development

Blood vessels comprise the vascular network and carry oxygen and nutrients within blood and plasma throughout the body in addition to being the primary route of metabolic waste transport. As such, appropriate vascularization of tissues is a critical and highly regulated process. The formation of a branched vascular network is an essential, conserved process in vertebrates. The vascular system is the first organ to develop in the vertebrate embryo, and is functional and carrying blood by 24 hours post-fertilization (hpf) in zebrafish and as early as embryonic day 8 (E8.0) in mice (Drake & Fleming 2000; Isogai et al. 2001). In addition to
forming early, the vascular system is also necessary for continued development. Zebrafish embryos with mutations which cause defective heart muscle, can survive for 5 days post-fertilization (dpf) without a functioning circulatory system using passive oxygen diffusion from the water, but subsequent development requires cardiovascular function (J. N. Chen et al. 1996). Similarly, mouse embryos cannot survive past E9.5 without a functional circulatory system, as is observed in various genetic mutants which fail to form blood vessels (Carmeliet et al. 1996; Ferrara et al. 1996). Thus, in diverse vertebrate lineages embryonic survival and appropriate development relies upon the formation and expansion of an effective vascular network.

Blood vessel development during embryogenesis is a multistep process that begins with primitive vessel formation from endothelial progenitor cells (EPCs) through a process known as vasculogenesis (Risau & Flamme 1995; Drake & Fleming 2000). Vasculogenesis creates the large, primary arteries and veins within the embryo and generates the initial vessel network. Blood vessels are composed of multiple cell types: 1) the endothelium is composed of endothelial cells (EC) that line the luminal side of the vessel; and 2) support cells, including pericytes and the smooth muscle cells of arteries that constrict to pump blood. The initial EPC undergo directed migration and coalesce into a cord that is then remodeled into a tubular structure which later becomes a lumenized vessel (Xu & Cleaver 2011). The vessels that form the initial arteries and veins carry blood throughout the developing embryo.

As the embryo grows a larger and more complex vessel network is needed. Angiogenesis forms the branched, hierarchical vessel networks, comprising capillary beds connecting the arteries and veins that are present throughout life. The expansion and increased complexity of the developing vessel network is driven through sprouting angiogenesis. The de novo creation and subsequent expansion of the vascular system is directed through a multitude of growth factors and signaling
pathways. The primary growth factor implicated in both processes is vascular endothelial growth factor (VEGF) ligand family. VEGF was first described as vascular permeability factor (VPF) and is a secreted protein first identified by its ability to increase vessel density and permeability in tumors and upon exogenous expression (Folkman et al. 1971; Senger et al. 1983). Since its initial description and cloning (Senger et al. 1983; Leung et al. 1989; Ferrara & Henzel 1989), an extensive body of research has investigated the VEGF ligand family and signaling.

II. VEGF signal propagation and ligand specific effects

The VEGF ligand family induces responses in the endothelium that are complex and influenced by a variety of factors, including ligand identity and cofactors. There are 4 known VEGF ligands in the endothelial cells, VEGFA-D, that are differentially expressed and bind with varying affinities to three tyrosine kinase receptors (VEGFR1-3) (Lohela et al. 2009). In regards to EC, VEGF-A ligand binds to two tyrosine kinase receptors, Flk1 (KDR/VEGFR2) and Flt1 (VEGFR1) which initiate and dampen its signaling cascade, respectively (de Vries et al. 1992; Terman et al. 1992; Quinn et al. 1993). Downstream of ligand-receptor binding, tyrosine phosphorylation of Flk1 initiates a host of signaling cascades, which eventually result in increased migration, increased proliferation, and blockade of apoptosis (Holmqvist et al. 2004; Takahashi et al. 2001). Increasing levels of VEGF-A ligand causes activation of EC, leading to angiogenesis (Gerhardt et al. 2003; Kappas et al. 2008). These receptors and their roles in developmental angiogenesis, along with the role of the VEGF ligands during development are discussed in more detail below.
a. Flk1 during developmental angiogenesis

Flk1 contains a conserved selective binding domain for the VEGF family of ligands, and is capable of binding VEGF-A with a $K_d$ of 750 pM (Terman et al. 1992; Quinn et al. 1993; Shinkai et al. 1998). Flk1 bound to VEGF-A triggers mitogenic signaling that increases EC proliferation, chemotaxis, and promotes cell survival; thereby initiating and sustaining sprouting angiogenesis (Koch et al. 2011).

To understand the role Flk1 plays within EC of growing vessels, mechanistic studies investigating the main phosphorylation sites that trigger signaling were mapped. The four main tyrosine/Tyr/Y phosphorylation sites on Flk1 were found to be Y951, Y1054/1059, Y1175, and Y1214 (Olsson et al. 2006). The receptor tyrosine kinase activity is initiated through a phosphorylation cascade, largely downstream of phospholipase C gamma (PLCγ) and phosphoinositide 3-kinase (PI3K). The sites and their effects are summarized in Table 1 and explained in further detail below.

**Table 1: Major Phosphorylation Sites of Flk1 and Cellular Effects/Interactions.**

<table>
<thead>
<tr>
<th>Phosphorylation Site</th>
<th>Signaling pathway/s activated</th>
<th>Main cellular effects</th>
<th>Cellular function</th>
<th>Direct protein interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y951</td>
<td>Src</td>
<td>Migration</td>
<td>Alters adhesion</td>
<td>TSAd</td>
</tr>
<tr>
<td>Y1054/1059</td>
<td></td>
<td></td>
<td>Propagates signaling</td>
<td></td>
</tr>
<tr>
<td>Y1175</td>
<td>PLCγ, PI3K</td>
<td>Proliferation, Anti-apoptosis</td>
<td>Cell division</td>
<td>PLCγ, SHB, Sck</td>
</tr>
<tr>
<td>Y1214</td>
<td>p38/MAPK</td>
<td>Migration</td>
<td>Motility</td>
<td>Nck</td>
</tr>
</tbody>
</table>

Phosphorylation at Y1054/1059 supports further phosphorylation and thought to occur first and propagate signaling through bolstering activity of the other sites (Kendall et al. 1999). This site is proposed to alter conformation of the receptor and change the availability or accessibility of
the activating phosphorylation sites on the Flk1 receptor. Phosphorylation at Y951 alters cell surface localization of VE-Cadherin and cytoskeletal rearrangements that are needed for cellular migration through T-cell-specific adapter molecule (TSAd) (Wu et al. 2000; Matsumoto et al. 2005; Gavard & Gutkind 2006). This phosphorylation event is therefore associated with chemotaxis and an increase in migratory potential. In fact, recent studies demonstrated TSAd is directly linked to junction adhesiveness and migration (Gordon et al. 2016). Phosphorylation at Y1214 also induces migration through activating p38 mitogen-activated protein kinase (MAPK) when phosphorylated (Lamalice et al. 2004; Lamalice et al. 2006). Unlike the other two sites, when Y1175 is phosphorylated it recruits and phosphorylates PLC\(\gamma\), which then induces activation of protein kinase C (PKC) to increase the proliferation (thereby also blocking apoptosis) (Takahashi et al. 1999; Meadows et al. 2001). Active PKC downstream of Flk1 signaling initiates a signaling cascade that is directly linked to and increases DNA synthesis (Xia et al. 1996; Takahashi et al. 1999). This signaling serves to increase the number of EC, generating new EC that can be used to expand the vessel network. Phosphorylated Y1175 can also activate migration through FAK/Cdc42 downstream of PI3K signaling and through Src activation (Xia et al. 1996; Wong & Jin 2005; Holmqvist et al. 2004). The combination of increasing both proliferation and migration results in more cells which are motile within the vessel, the two key components to angiogenesis.

Functional analysis of Flk1 within growing vessels demonstrated specificity to the endothelial lineage and an essential role during vessel generation. \textit{In vivo} genetic deletion of Flk1 causes embryonic lethality by day E8.5, with phenotypes that resemble deletion of the VEGF-A ligand (Ferrara et al. 1996; Shalaby et al. 1997). Specific mutation of Y1175 to F1175 is lethal by E9.5 due to a lack of endothelial and hematopoietic cells, mimicking the
receptor deletion phenotype (Sakurai et al. 2005). These two deletion experiments indicate that Flk1 is required for proliferation and survival of the endothelium, and support a critical role for the Y1175 phosphorylation site. Flk1\(^+/−\) cells within chimeric mouse embryos, generated with Flk1-deficient stem cells, did not contribute to early blood islands which generate the hematopoietic lineage or nascent vascular beds; indicating VEGF-A signaling through Flk1 is essential for ECs to contribute to the growing vasculature (Shalaby et al. 1997). Similarly, the two zebrafish isoforms of Flk1, kdra and kdrb, are both expressed and essential in vessel growth (Bahary et al. 2007). Depletion of either kdr in zebrafish embryos using MO injection results in angiogenic failure, specifically the intersegmental vessels (ISVs) from the common artery do not sprout (Jin et al. 2005; Wiley et al. 2011). In fact, the EC restricted expression of this receptor is specific, such that the kdr promoter driving GFP is commonly used as a transgenic fish line for visualization of blood vessels from as early as 20 hpf (Lawson & Weinstein 2002; Wiley et al. 2011). These in vivo studies demonstrate an intimate link between blood vessel growth and VEGF-A signaling through Flk1.

Binding of VEGF-A to Flk1 causes a series of intracellular signaling events resulting in blood vessel growth through angiogenesis. The combined data from biochemical studies and in vivo analyses indicate how appropriate activation and regulation of this receptor is essential for endothelial growth and quiescence, thus implicating this ligand-receptor cascade in anastomosis. Many of the biochemical and vessel morphology effects are described, however the precise spatio-temporal influences are less understood. These local and time-dependent roles for VEGF-A signaling are likely contributors during anastomosis and warrant closer analysis in that context.
b. Flt1 during developmental angiogenesis

VEGFR1/Flt1 was identified as a tyrosine kinase receptor for members of the VEGF ligand family. VEGF-A binds to the Flt1 receptor with a $K_d$ of 15 pM, a 10-fold higher affinity than Flk1, but exhibits weak kinase activity (Kendall & Thomas 1993; Ito et al. 1998). Additionally, Flt1 is expressed as two alternatively spliced isoforms, a membrane-bound tyrosine kinase transmembrane receptor (mFlt) and a soluble isoform lacking the transmembrane and tyrosine kinase domains (sFlt) (de Vries et al. 1992; Kendall & K. A. Thomas 1993; C. P. Thomas et al. 2010).

Flt1 is expressed selectively in the endothelial lineage of mice, both developmentally and postnatally (Peters et al. 1993). Complete Flt1 deletion in mice ($flt1^{-/-}$) is embryonic lethal by E9.5 due to a poorly organized vasculature and over-proliferation of hemangioblasts over ECs (Fong et al. 1995; Fong et al. 1999). Interestingly, inactivation of the tyrosine kinase domain, which presumably blocks signaling activity while preserves ligand binding activity, results in a developmentally normal mouse; although the mice are more susceptible to pathological challenges, including xenograft tumor growth, and have a faster tumor growth and increased tumor vessel formation (Hiratsuka et al. 1998; Hiratsuka et al. 2001). Combined, these data indicate a reliance on Flt1 for binding VEGF but not as a tyrosine kinase signaling receptor during embryonic murine development. Postnatal VEGF-A signaling is essential for patterning the retinal vasculature, a well-studied vessel bed due to the stereotyped growth and ease of isolation (Gerhardt et al. 2003). Loss of Flt1, either by neutralizing antibody or genetic deletion using temporally controlled Cre recombinase expression, increases filopodial extensions and tip cells in sprouting retinal vessels (Chappell et al. 2009). These studies conclusively show
that despite lacking a strong signaling role during development, Flt1 is required to generate and
pattern EC to expand the vasculature during both vasculogenesis and angiogenesis in mice.

In addition to genetic mouse studies, recent work has explored the role of Flt1 in zebrafish
angiogenesis. Flt1 is expressed in the embryonic fish vasculature, in both the arterial lineage of
the intersegmental vessels (ISVs) and venous in the caudal vein plexus (CVP) (Krueger et al.
2011). Analysis of loss-of-function (LOF) and gain-of-function (GOF) manipulations in the
sub-intestinal vessels (SIVs) demonstrated a regulatory role for Flt1 in angiogenesis.
Specifically, excess and precocious sprouting was in SIVs without Flt1 and dampened after
reintroduction of Flt1 by mRNA injection (Avraham-Davidi et al. 2012). LOF MO and GOF
mRNA injections of VEGF-A and Flt1 also demonstrated that Flt1 influences branching via tip
cell formation in the ISVs; thus mimicking the phenotypes in the postnatal retinal vessels of
mice (Chappell et al. 2009; Krueger et al. 2011). Again these data suggest that Flt1 restricts
angiogenesis, as its deletion results in excess blood vessel growth.

Finally, previous work from our group examined the role of Flt1 and its isoforms in vitro
using mouse embryonic stem (ES) cells that undergo endogenously directed differentiation and
form a multi-cell type bed from which an EC network sprouts and expands (Rylova et al.
2008). The normal pattern of complex vessel branching is lost and proliferation is increased in
flt1−/− mouse ES cell-derived vessels (Kearney et al. 2002; Kearney et al. 2004). The
proliferative effects in vessels are present when either isoform is absent, although the
branching is largely regulated by sFlt1 within these vessels (Kappas et al. 2008). We recently
discovered that fltl−/− mouse ES cell-derived vessels show increased sprout initiations
(Chappell et al. 2016). This finding was in contrast with reduced network complexity found in
flt1−/− vessels of both ES cell-derived and retinal angiogenic vessels during development.
(Kappas et al. 2008; Chappell et al. 2009). These discrepant effects were resolved by the fact that \( \text{flt1}^{-/-} \) vessel connections are less stable over time (Chappell et al. 2016). In total, these experiments suggest a reliance on Flt1, acting as a VEGF-A ligand sink in sprouting angiogenesis, to fine-tune VEGF-A levels during angiogenesis. This role supported by \textit{in vivo} and \textit{in vitro} studies in model systems.

Flt1 has been demonstrated to be a ligand-sink for VEGF-A in many contexts, both vasculogenic and angiogenic. As the angiogenic and developmental role of the receptor has been demonstrated to function outside of its signaling capabilities, the phosphorylation sites and signaling cascades, while understood at a basic level (Koch et al. 2011), are not discussed here. Rather this receptor is of greater interest for its potential use by the EC to determine local levels of VEGF-A signaling, previously hypothesized as a method by which anastomosis is directed during angiogenesis.

c. VEGF ligand effects in animal models

Genetic deletion of even a single VEGFA allele, \textit{vegfa}^{+/-}, causes embryonic lethality by E10.5 in mouse embryos (Carmeliet et al. 1996; Ferrara et al. 1996). VEGFA hemizygous embryos exhibit pronounced endothelial apoptosis and compromised vessel integrity, characterized by a failure in dorsal aorta closure and decreased EC numbers within the vessels of many organ systems (Carmeliet et al. 1996; Ferrara et al. 1996); thus implicating VEGFA in the embryonic vascular system. Following the initial characterization of VEGFA in animal models, a large body of work has explored the role of VEGF ligands in multiple contexts and animal systems.

VEGF-A ligand can be alternatively spliced into at least nine isoforms, which are classified by their molecular weight (Tischer et al. 1991). VEGF-120, VEGF-164, and VEGF-188 are the
three most prevalent isoforms in angiogenic vessels and many of their distinct roles in vessel growth have been elucidated. Mice genetically engineered to express only VEGF-120 survive birth but die by two weeks of age due to cardiorespiratory failure (Carmeliet et al. 1999). Both VEGF-164 and VEGF-188 isoform expressing mice survive to fertility, although expression of only VEGF-188 exhibits reduced survival and non-Mendelian ratios (Stalmans et al. 2002). VEGF-120 and VEGF-188 expressing mice have decreased and disrupted vessel morphology in the retinal of postnatal mice, although the VEGF-120 mice show a more severe phenotype than the VEGF-188 (Stalmans et al. 2002). Thus, regardless of the isoform, VEGF-A ligand interacts with EC to promote vessel growth. In fact, the shared vessel phenotypes in these rescue models suggest an overlapping and conserved ability of VEGF-A to regulate ECs. This conclusion is supported by parallel experiments in zebrafish that express multiple isoforms of VEGF-A, including both VEGF-120 and VEGF-164, within the endothelial lineage of embryos as determined by in situ hybridization (Liang et al. 1998; Liang et al. 2001; Bahary et al. 2007). Over-expression of either of these isoforms, through mRNA injection, results in excessive vessel growth and an increased commitment of cells to the hematopoietic lineage (Liang et al. 2001). Morpholino (MO) injection to block all VEGF-A ligand causes a decrease in vessels throughout the embryo (Nasevicius et al. 2000). Combined, the data from these model systems support a conserved role the VEGF-A ligand in appropriate proliferation and health of the endothelium.

Beyond size, these isoforms also vary in their relative abundance and presentation in the extracellular matrix (ECM). ECM presentation is critical for binding of VEGFR cofactors and modulating signaling strength. Several co-factors that amplify VEGFA signaling have been identified, namely heparan sulfate (HS), neuropilins (NRPs) and integrins (Houck et al. 1992;
Jakobsson et al. 2006; Kawamura et al. 2008). VEGF-A bound to heparan sulfate in the ECM confers the spatial patterning of the blood vessels via ligand sequestration in ECM that generates a growth factor gradient to direct angiogenesis (Ruhrberg et al. 2002; Jakobsson et al. 2010). Complexing of VEGF-A and Flk1 or Flt1 at the cell surface with NRP-1 promotes phosphorylation that augments downstream signaling and increases migration and sprouting of endothelial cells (Wang et al. 2003; Pan et al. 2007). Further increases in signal propagation are accomplished by αVβ3-integrin, which complex with VEGF-A/Flk1/NRP to amplify the signal cascade (Ruhrberg et al. 2002; Robinson et al. 2009). These data demonstrate that VEGF-A signaling activity within EC is not only a product of ligand binding at receptors but also impacted by the complexing of additional cofactors, an intricate and complicated series of binding.

VEGF-B, VEGF-C, and VEGF-D are structurally similar to VEGF-A but exhibit different specificity and binding capacities during development. VEGF-B binds the VEGFR1/Flt1 receptor and influences heart development, but is developmentally dispensable (Aase et al. 1999; Bellomo et al. 2000). VEGF-C and VEGF-D are thought to predominately bind VEGFR3/Flt4 on hematopoietic cells, specifically regulating formation of the lymphatic vessels during lymphangiogenesis (Jeltsch et al. 1997; Schoppmann et al. 2010; Hogan et al. 2009). The VEGF ligand family as a whole is able to alter signaling within the endothelial lineage but VEGF-A, or VEGF within this text, is the primary growth factor implicated in the development and maturation of the blood vasculature.
III. Sprouting angiogenesis during development

Sprouting angiogenesis is initiated by mature ECs within established vessels. After exposure to new growth factors these EC activate proliferative and migratory programs in order to extend processes and initiate a new sprout, followed by migration into the extravascular space along guidance cues, then connection or anastomosis with another vessel, and finally create a continual luminal space to carry blood flow (Chappell & Bautch 2010). Each of these processes is described in more detail below.

a. Sprout initiation from quiescent vessels

Sprouts form in response to a complex interplay of growth factors within the vascularized tissue. The vessels start as stable and quiescent, with the EC contributing to the vessel wall but not actively migratory or proliferative, and surrounding by the supportive pericytes and macrophages along with the basement membrane. Only upon a change in growth factors in the local environment do the EC activate and undergo structural changes.

A wide array of growth factors have been implicated in sprout initiations, including VEGF, Notch, and bone morphogenetic protein (BMP) (Kappas et al. 2008; Wiley et al. 2011; Hellström et al. 2007). Notably, EC with low Notch are primed to respond to growth factor ligands, such as BMP and VEGF, and contribute to a new sprout while their immediate neighboring EC are under the opposite pressure and unresponsive to growth cues (Hellström et al. 2007; Mouillesseaux et al. in press). Through lateral inhibition provided by Notch (Benedito et al. 2009) cell-by-cell heterogeneity in signaling is amplified through interactive regulation of Notch and VEGF-A at the transcriptional level within vessels (Lobov et al. 2007; Wiley et al. 2011; Suchting et al. 2007), creating inherent differences in the activity and responsiveness of
individual ECs. Sprout initiation is a result of the combined effects of these signaling pathways, leading to the EC exiting quiescence. Exit from quiescence requires suppression of Notch signaling, demonstrated by multiple LOF Notch manipulations in mouse vessels that show increased sprout formation (del Toro et al. 2010). For the EC in the sprout to migrate away from the parent vessel and into the ECM, the EC loosens established EC-EC junctions, alter cellular structures and extends filopodia (Chen et al. 2010; Cruys et al. 2016; Gordon et al. 2016; Kushner et al. 2014; Wright et al. 2015), both effects known to occur downstream of VEGF-A signaling (Senger et al. 1983; Keck et al. 1989; Dvorak et al. 1995; Taylor et al. 2010; Zeng & Bautch 2009).

From these data it becomes apparent that the cumulative growth factor effects within each cell contribute to sprout initiation within a vessel. Thus understanding growth factor interactions and EC within the context of whole vessels is essential for a complete understanding of how these signaling pathways initiate sprouting angiogenesis. Once an individual cell has initiated a migratory program and exits the axis of the parent vessel it is a part of the new, active sprout.

b. Sprout migration and extension

To expand the vessel network, the sprout must migrate away from the parent vessel and towards another existing vessel. Cell migration is characterized by the extension of filopodia and selectively EC activation, which are both observed when Notch or VEGF-A signaling are altered (Gerhardt et al. 2003; Hellström et al. 2007; Bentley et al. 2014). This sprouting process requires two components, guidance cues to direct sprout growth and coordinated cell movements.
The signaling and growth cues which direct migration function both locally and over long distances. The local growth factor environment surrounding the cell is influenced by secreted proteins by the EC; for example, Flt1 modulation of the VEGF-A gradient in the ECM directs sprouts to migrate away from the parent vessel (Chappell et al. 2009). Similar to neuronal migration, EC are predicted to use filopodia to sense directional migration along local and long distance cues within the targeted tissue (Kater & Rehder 1995). As they encounter these guidance factors, the sprouts either alter their directional migration to either persist or redirect the trajectory of the vessel. The EC within the sprout undergo collective cell migration and individual cells within the vessel change positions frequently as the sprout extends into the ECM along guidance cues (Arima et al. 2011; Jakobsson et al. 2010). Migration and EC movement within the sprout is facilitated by extended junctions at the sprout front (Pelton et al. 2014), resulting in cells which move in parallel and providing a border that permits cell-cell communication and presumably coordinates EC activity. The ability of EC to communicate and migrate is dependent on multiple signaling pathways, including Notch and VEGF-A. Increased Notch signaling, through genetic deletion of Dll4, or loss of VEGF-A signaling, due to heterozygously deleted Flk1, reduces migratory potential; conversely, increasing VEGF-A signaling, through loss of Flt1, increases migratory potential (Jakobsson et al. 2010).

Through a combination of these external cues and cell communications, the sprout migrates in a directional manner within the extracellular space. From these data a picture of sprout migration emerges. The ECM and existing vessels contain factors to direct the nascent sprout, which is able to follow these sets of cues by continually extending sensory processes, appropriately the rearranging the cells, and migrating into the non-vascular areas of the network.
c. Anastomosis to create connections and new vessel conduits

At the most basic level, anastomosis requires that two cells generate a new, stable, cell junction; appropriately, junction components have been the main consideration in anastomotic studies to date. Within the endothelial cells the creation of new junctions occurs in a stereotyped order. The order is as follows: first is homophilic binding of platelet endothelial cell adhesion molecule 1 (PECAM) on the surface of two EC; second is establishment of the adherens junctions (AJs), composed of multiple proteins including vascular endothelial cadherin (VE-Cad), β-catenin, p120, and plakoglobin; and finally the tight junctions (TJs), comprising proteins like zona occludins 1 (ZO1), claudins, nectins, and junctional adhesion molecules (JAMS) (Dejana 2004). The formation of each of these junctions is briefly discussed below.

The reallocation of junctional proteins to generate new EC-EC connections is essential for anastomosis to occur. PECAM-PECAM homophilic binding is the first to occur, in part due to the non-restricted localization of the protein on the cell surface of EC (Feng et al. 2004). The ability of homophilic PECAM interactions to expand by diffusion-trapping on the cell surface and without a need for PECAM signaling (Sun et al. 2000; Privratsky et al. 2011) bolster the role of these junctional proteins in the first step of creating a new EC junction. These PECAM-PECAM interactions provide a method for EC to identify like cells and begin the process of establishing more permanent junctions. After the EC initially bind, AJ proteins are recruited to the cell surface. Active trafficking of VE-Cad away from the cell-surface and into intracellular vesicles is accomplished through TSAd interactions with VEGFR2 downstream of VEGF-A ligand, such that VE-Cad is more present at junctions in EC with lower VEGF-A signaling (Esser et al. 1998; Gavard & Gutkind 2006). VE-Cad homophilic interactions anchor the
protein at the cell surface and set up the basis for AJ formation. Normal and full expression of VE-Cadherin is essential for new connections (Lenard et al. 2013; Montero-Balaguer et al. 2009). The AJ components, including β-catenin and p120, are then actively recruited and maintained at EC junctions thus stabilizing the new EC-EC interaction through a positive feedback loop of AJ recruitment (Williams et al. 2000; Gaengel et al. 2012). Finally, stable scaffolding junctional proteins, namely ZO1, claudins and occludins, create TJs at the basal side of the junction. These junctions provide structural integrity to the cell in a tissue specific manner, for example the lung vessels have loosely organized TJs to allow exchange but the neurovascular interface has rigid and complex TJ (Gallicano et al. 2001; Saitou et al. 2000). All three types of junctions are detected between EC in established vessels, indicating their essential nature to blood vessels and linking their deposition at sites of connection to anastomosis.

The components in EC that contribute to vessel anastomosis in embryonic zebrafish were recently explored. Two beds within the embryonic zebrafish have been examined during vessel fusion, the intersegmental vessels (ISVs) and the hindbrain (HB). Examination of the ISVs showed a stereotypic change in localization of the junctional proteins during anastomosis, including ZO1 and VE-Cad (Herwig et al. 2011). Genetic loss of VE-Cadherin results in connective failure in the HB vessels, and prevents the stereotypical rearrangements in EC junctions after initial connection that permits lumen ingression and flow after anastomosis (Lenard et al. 2013). Therefore, in order for anastomosis to proceed the formation of new junctions is needed. Additional work showed that blockade of filopodial extensions through pharmacologic inhibitors dampened, but did not abolish, anastomosis in multiple vessel beds (Phng et al. 2013). This indicates that filopodia may assist the formation of new connections.
but are not an obligate component of anastomosis. Another non-obligate contributor was found in the F4/80+ macrophages in angiogenic retinal vessels of postnatal mice. F4/80+ macrophages were coincident with many sprout fronts and branch points, suggesting these cells support the vessel network, and potentially connections, during angiogenesis (Fantin et al. 2010; Outtz et al. 2011).

Combined, these studies suggest that the ability of vessels to form new, stable connections depends on creating new junctions and is assisted by non-EC support cells. However, a large number of questions remain from these data. For instance, whether new connections between EC are directed or stochastic, the signaling changes within sprouts and existing vessels as the two discrete EC form a new junction and which signaling components are most critical for permitting or blocking connections.

As the physical aspects involved in the anastomosis have been described, at least in regards to the generation of new junctions, studying the regulatory and signaling events that direct these physical changes is a high priority. The signaling that directs endothelial cells during terminal guidance and anastomosis is a pressing question which is currently unaddressed by existing studies. The fluctuations between high Notch and high VEGF-A are conducive for coordinated migration of EC as a sprout but no information on how these signaling cascades influence and direct connections is available.

d. **Formation of a new lumen**

Having formed a new connection, the angiogenic vessel needs to transition into quiescence and rejoin the mature vasculature. In order for the newly formed vessel to contribute to the vessel bed, a continual luminal network is needed. The lumen is created at the apical side of EC and forms an open channel which carries blood and plasma through the network. The two
main processes thought to be critical to lumen formation are apical-basal polarity and blood flow.

Apical-basal polarity is established within a cell to define a ‘top’ and ‘bottom’, respectively, in tissues that require this additional structural information for each cell, most notably in epithelium (Datta et al. 2011). This polarity is firmly established within vessels, such as the arterioles, and observed by unequal distribution of proteins like portioning defective homolog 3 (Par3), podocalyxin, and β1 integrin (Horvat et al. 1986; Iruela-Arispe & Davis 2009; Zovein et al. 2010). However, the distinct sidedness must be created or transferred when a new vessel is formed. These components are actively shuttled to and maintained at the appropriate domains of the cell surface by assistive proteins. Cell division control protein 42 (Cdc42) and Ras-related C3 botulinum toxin substrate 1 (Rac1) are known to establish apical-basal polarity in epithelial cells and are also required for EC to create lumens (Koh et al. 2008). Expression of podocalyxin (PODXL) and β1 integrin, the established markers in lumens for apical and basal polarity, respectively, can be found between the leading cells in a sprout (Horvat et al. 1986; Pelton et al. 2014), suggesting EC are primed for lumen formation early on during sprouting. The final stage of lumenization requires joining two formed lumens in relatively closely proximity. Recent work found that this joining is assisted by rearranging the cell morphologies through loosening junctions at the newly formed connection site and then the lumen expands using pressure from blood flow (Sauteur et al. 2014; Lenard et al. 2013). In fact, the EC membranes were observed to actively retreat through a mechanism termed inverse blebbing to permit luminal ingression (Gebala et al. 2016). This pressure driven rearrangement of cell boundaries is directly linked to blood flow at the apical surface of the newly connected vessels.
Once the new, continual lumen is formed, exchange of blood flow between the pre-existing vessels is found. At this point the vessels return to a quiescent state, and sprouting angiogenesis considered completed.

IV. Perspective

While data from our lab and others have explored the cues governing sprout initiation and extension along with lumen formation, relatively little is known about the signaling factors involved with terminal guidance leading to connection and fusion of a sprout with another vessel through anastomosis. Given previous data implicating VEGF-A signaling on a multitude of sprouting angiogenesis stages, a more in depth analysis of VEGF-A, Flt1 and anastomosis was undertaken using live imaging in a 3D in vitro angiogenesis model. The sprouting vessel and connecting vessel were examined prior to forming the new connection. In addition to classifying connective behaviors, we examined the impact of VEGF-A signaling through system wide and cell-autonomous manipulations of Flt1. Given the overall increase in vascularization of vessel beds with increased VEGF-A, we predicted that loss of Flt1 would increase VEGF-A signaling and thereby increase anastomosis.


Cruys, B. et al., 2016. Glycolytic regulation of cell rearrangement in angiogenesis. *Nature*
Communications, 7, p.12240.


Gaengel, K. et al., 2012. The Sphingosine-1-Phosphate Receptor S1PR1 Restricts Sprouting


Kearney, J.B. et al., 2004. The VEGF receptor flt-1 (VEGFR-1) is a positive modulator of vascular sprout formation and branching morphogenesis. Blood, 103(12), pp.4527–4535.


Kendall, R.L. et al., 1999. Vascular endothelial growth factor receptor KDR tyrosine kinase activity is increased by autophosphorylation of two activation loop tyrosine residues. The


Lobov, I.B. et al., 2007. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proceedings of the National Academy of Sciences, 104(9), pp.3219–3224.


Taylor, S.M. et al., 2010. Angiogenic factor signaling regulates centrosome duplication in


CHAPTER 2: Materials and Methods

I. Experimental Materials and Methods

a. Retinal Angiogenesis Imaging and Analysis

*Mice (Mus musculus)* with *flt-1<sup>flox/flox</sup>* alleles (Genentech; San Francisco, CA) were bred with [Tg(UBC-cre/ERT2), JAX #007001] mice that also carried the Cre-mediated recombination reporter gene R26R TdTomato [*Gt(ROSA)26Sor<sup>tm14(CAG-tdTomato)Hze</sup>, JAX #007914*]. Mice were maintained in accordance with the University of North Carolina, Chapel Hill Institutional Animal Care and Use Committee. Mosaic Cre excision of vessels was accomplished by IP injection of 0.5 mg/ml tamoxifen (MP Biomedicals; Santa Ana, CA) at P2. P5 retinas were perfusion fixed with 0.5% PFA/PBS, harvested, and fixed for 2h with 2% PFA. Retinas were rinsed, counterstained for isolectinB4 and DAPI, and mounted using established protocols (Chappell et al. 2009). Images were acquired on a Leica DMI 6000B or Zeiss LSM 880 confocal microscope at 40× magnification, with optimal z-stacks compressed post-acquisition using ImageJ software. All antibody manufacturers and concentrations are listed in Table 2.1.

Sprouts were identified in the angiogenic front using previously established methods (Chappell et al. 2009). Cellular genetic identity was classified from reporter expression during analysis and interactions between sprouts and other sprouts/vessels were identified as overlap of cellular extensions with other extensions/cells in the isolectinB4 channel.

---

1 Chapter 2 is adapted in part from Nesmith, J.E. et al. Blood Vessel Anastomosis is Spatially Regulated by Flt1 During Angiogenesis. *Development*, In review. (2016)
b. Cell Lines and Culture

Human umbilical venous endothelial cells (HUVEC; Lonza, Portsmouth, NH) were maintained in EBM-2 media with the EGM Bullet Kit and 1X antibiotic/antimycotic (Sigma; St Louis MO) according to manufacturer’s directions. Normal human lung fibroblasts (NHLF; Lonza, Portsmouth, NH) were maintained in high-glucose DMEM supplemented with 10% FBS and 1X antibiotic/antimycotic. Mouse embryonic stem cells isolated from WT mice and \(flt-1^\text{--}\) mice (gift from G.H. Fong) were maintained and differentiated as previously described (Rylova et al. 2008).

c. HUVEC Sprouting Angiogenesis Assay Imaging and Analysis

The HUVEC sprouting angiogenesis assay was set up as described (Nakatsu & Hughes 2008). Briefly, HUVEC were detached, combined with Cytodex microcarrier beads (Sigma; St Louis, MO), then incubated with periodic agitation for 4h. Following overnight growth, the HUVEC-coated microcarrier beads were embedded in a 1.5% fibrinogen-thrombin gel that was allowed to polymerize at 37°C for 15-45 min, and then a layer of normal lung fibroblasts were seeded on top of the solidified fibrinogen gel.

Dynamic imaging of d3-5 HUVEC sprouts and vessels was performed on an Olympus VivaView Incubator Fluorescence inverted microscope at 10× magnification with image acquisition at 10 min intervals for 42h. Fixed imaging was performed at d5. After removing fibroblasts, cultures were fixed with 4% PFA for 15 min at RT. Cultures were permeabilized using 0.1% Triton-100 (Sigma; St Louis, MO) in Tris or Phosphate Buffered Saline (TBS and PBS, respectively) for 2-4h at RT and then blocked at 4°C overnight in TBS or PBS with 0.1% Tween and 5% goat serum (blocking buffer). Primary antibodies were incubated as indicated in Table 2.1, washed with blocking buffer overnight and incubated overnight at
4°C with either goat anti-mouse conjugated secondary or goat anti-rabbit conjugated secondary in TBST or PBST (Life Technologies; Grand Island, NY). Confocal images were acquired on an Olympus FV1200 system using a 10× objective (NA 0.40) and optimal Z-stacks, compressed post-acquisition for analysis.

Vessel interactions were scored when two cell bodies overlapped in either the DIC or fluorescent channels, depending upon the experimental set-up. Interactions classified as transient contacts were defined as overlap in a single time frame, therefore lasting <10 minutes. Stable connections were defined as overlap that persisted for >3 time frames, at least 20 min. Target vessel activity was classified as static or protrusive based upon the endothelial cells within 30 µm of the interaction site. Static vessels exhibited no LifeAct protrusions extending away from the vessel axis, while protrusive vessels contained a minimum of 3 extensions, defined as fluorescent protrusions >4 µm.

d. ES Cell-derived Vessel Imaging and Analysis

Generation of ES cell lines expressing PECAM-eGFP was previously reported (Kearney 2004). ES cell culture differentiation was performed as previously reported (Rylova et al. 2008). Dynamic imaging of d7-8 differentiating ES cell cultures was performed as follows: confocal images at either 10× or 20× magnification using an Olympus FluoView FV1000 or FV10i system, both with full environmental chambers, were acquired at 4-10 min intervals for 16-20h. A z-stack, with 4-6 µm between focal planes, of 6-8 images was acquired at each time point. The z-stacks were compressed post-acquisition into a single image for each time point.
e. **Zebrafish Experiments and Husbandry**

Zebrafish (*Danio rerio*) lines were carried on an AB strain background with transgenic insertion of either Tg(kdrl:eGFP) or Tg(kdrl:eGFP); Tg(hsp70l:vegfaa121; cmlc2:GFP) that were maintained as heterozygotes and genotyped through test crosses (Jin 2005; Wiley et al. 2011). Heat shock inducible expression was activated in embryos 20 hpf by incubation for 30 mins at 30°C. All zebrafish maintained in accordance with the University of North Carolina, Chapel Hill Institutional Animal Care and Use Committee.

The Flt1 morpholino was previously designed, published, and verified for vessel defects and effectiveness (Krueger et al. 2011). Flt1 morpholino (3 ng) and control (PBS of equivalent volume) injections were performed on one-cell fertilized embryos. Genotyping of experimental embryos was identified using presence or absence of GFP expression in the cardiac muscle. Embryos were fixed at 48 hpf in 4% PFA for 15 mins for end point analysis. Imaging was performed on an Olympus FV1200 using 10× magnification and optimal z-stacks, approximately 5 µm in width. Analysis was performed using Image J-Fiji software.

All embryos with gross morphological defects were excluded from quantification. ISVs at the base of the yolk were imaged as described above. The confocal images were binned according to their connections along the DLAV. Those vessels which connected with a vessel not at the DLAV or formed multiple connection at the dorsal aspect were considered ‘inappropriate’ or outside of the typical somite boundaries.

f. **Protein Quantification**

Protein analysis was performed on culture medium after concentration (Fisher Scientific; Pittsburgh, PA) or on cell lysates collected in Radiommunoprecipitation Assay (RIPA) Buffer + 1X protease inhibitor cocktail (Cell Signaling; Danvers, MA). Western blot analysis
was performed 48h after lentivirus infection or siRNA transfection. Briefly, protein concentration was quantified by the Bradford reaction (BioRad; Berkeley, CA) and equivalent protein amounts were loaded onto a 7.5% sodium dodecyl sulfate polyacrylamide gel for electrophoresis. Protein was transferred to a polyvinylidene fluoride (PVDF) membrane, stained with Ponceau S solution to visualize transferred protein and then incubated with the appropriate primary antibody in TBS or PBS with 0.5% Triton-100 (Sigma; St Louis, MO). Horseradish peroxidase-conjugated secondary antibody was incubated with membranes for 1h at RT and chemiluminescent detection was performed. All antibody manufacturers, and concentrations are listed in Table 2.1.

g. **Lentivirus and siRNA knockdown**

The Flt1 shRNA construct (RHS3979-201732907; Dharmacon, Lafayette, CO) was modified to include GFP in addition to shFlt1. Lentivirus was produced by the UNC LentiCore Facility. Lentivirus was incubated with endothelial cells for 6-8h with addition of 0.25 µg/mL of polybrene (EMD Millipore; Billerica, MA). Lentiviral infection with LifeAct-GFP or LifeAct-RFP (gifts from Rusty Lansford [Addgene plasmid #51010] and Weiping Han [Addgene plasmid #64048], respectively) allowed visualization of F-actin.

siRNA knockdown was performed by 6h incubation with RNAiMax (Invitrogen; Grand Island, NY), and a pre-designed targeted construct against total Flt1 (Life Technologies; Grand Island, NY) or siRNA constructs, generated with locked nucleic acid (LNA) ends, targeting either the unique portions of sFlt1 or mFlt1 (Exiqon; Woburn, MA). Targeted sequences are listed in Table 2.2. Knockdown efficiency was quantified by Western blot.
h. **Fluorescence-activated Cell Sorting (FACS) Analysis**

HUVEC were washed with PBS, detached using 1X accutase (Sigma; St. Louis, MO) and then fixed in FACS buffer containing 1% PFA. Cells were analyzed by flow cytometry using a BD Accuri™ C6 flow cytometer and CFlow Plus Analysis software (BD Biosciences; San Jose, CA). Samples were manually gated and analyzed using the FloJo v10 software package.

i. **mRNA Preparation and Quantification**

RNA was collected 48h post-treatment using TRIZol® (Life Technologies; Grand Island, NY), according to the manufacturer’s protocol, and converted to cDNA using iScript (BioRad; Berkeley, CA), according to the manufacturer’s protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) \( \Delta\Delta CT \) analysis was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems; Grand Island, NY). Primers for Flt1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to quantify mRNA. Primer sequences are listed in Table 2.3. Data are reported normalized to GAPDH.

j. **Statistical Analysis**

Statistical analyses were performed using GraphPad Prism® 6 software. A p value of <0.05 was considered statistically significant. The statistical methods and significance are noted in the Figure Legends.
II. Tables

Table 2.1: Antibodies and Nuclear Stains.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Company</th>
<th>CAT. #</th>
<th>Use</th>
<th>Western Blot Dilution</th>
<th>Immunofluorescence Dilution/Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Cell Signaling</td>
<td>3700P</td>
<td>HUVEC</td>
<td>1:10,000</td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>Abcam</td>
<td>ab6556</td>
<td>HUVEC</td>
<td>1:7,500</td>
<td></td>
</tr>
<tr>
<td>Flt1</td>
<td>Abcam</td>
<td>ab32152</td>
<td>HUVEC</td>
<td>1:2,000</td>
<td>1:1,000; 48h</td>
</tr>
<tr>
<td>PECAM</td>
<td>Cell Signaling</td>
<td>3528S</td>
<td>HUVEC</td>
<td></td>
<td>1:1,500; 48h</td>
</tr>
<tr>
<td>Conjugated phalloidin</td>
<td>Life Technologies</td>
<td>ab109202</td>
<td>HUVEC</td>
<td></td>
<td>1:50; 48h</td>
</tr>
<tr>
<td>Alexa Flour® 488</td>
<td>Life Technologies</td>
<td>A-21206</td>
<td>HUVEC</td>
<td></td>
<td>1:750; overnight</td>
</tr>
<tr>
<td>Alexa Flour® 555</td>
<td>Life Technologies</td>
<td>A-21428</td>
<td>HUVEC</td>
<td></td>
<td>1:750; overnight</td>
</tr>
<tr>
<td>Alexa Flour® 647</td>
<td>Life Technologies</td>
<td>A-21235</td>
<td>HUVEC</td>
<td></td>
<td>1:750; overnight</td>
</tr>
<tr>
<td>DRAQ-7</td>
<td>Abcam</td>
<td>ab109202</td>
<td>HUVEC</td>
<td></td>
<td>1:1000; 1h</td>
</tr>
<tr>
<td>Isolectin conjugated Alexa488</td>
<td>Life Technologies</td>
<td>I21411 Mouse retina</td>
<td></td>
<td></td>
<td>1:100; overnight</td>
</tr>
<tr>
<td>DAPI</td>
<td>Life Technologies</td>
<td>D1306 Mouse retina</td>
<td></td>
<td></td>
<td>1:1000; 30 mins</td>
</tr>
</tbody>
</table>
Table 2.2: siRNA sequences targeting Flt1 isoforms.

<table>
<thead>
<tr>
<th>Targeted Isoform</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sFlt1</td>
<td>GAGCACTGCAACAAAAAGGCTTTTCTCTCGGATCTC</td>
</tr>
<tr>
<td>mFlt1</td>
<td>GGAAATAGTGGTTTACATA</td>
</tr>
</tbody>
</table>
Table 2.3: qRT-PCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CCTCAAGATCATCAGCAATGCCTCCT</td>
<td>TTGGTATCGTGGAAGGACTCATGACC</td>
</tr>
<tr>
<td>Flt1</td>
<td>AGGGCCTCTGTGATGGTGATTGTTGA</td>
<td>ATGCAGCACTACACATGGGAGCCTA</td>
</tr>
</tbody>
</table>
III. REFERENCES


CHAPTER 3: Blood Vessel Anastomosis is Spatially Regulated by Flt1 During Angiogenesis

I. Introduction

As described in CHAPTER 1, blood vessel formation is an essential, conserved process in vertebrates that provides oxygen and nutrients to tissues and organs (Carmeliet 2005; Adams & Alitalo 2007; Chappell & Bautch 2010). Aberrant angiogenesis is associated with disease; for example, tumor angiogenesis is one of the hallmarks of cancer (Khurana 2005; Hanahan & Weinberg 2011). Blood vessel development during embryogenesis is a multistep process that begins with primitive vessel formation from endothelial progenitor cells through vasculogenesis (Risau & Flamme 1995; Drake & Fleming 2000; Xu & Cleaver 2011), and the subsequent formation of branched vessel networks is called sprouting angiogenesis. Sprouting angiogenesis is initiated by endothelial cells that proliferate, extend processes, migrate into extravascular space, and finally connect, or anastomose, with another vessel (Betz et al. 2016; Blanco & Gerhardt 2013; Larrivee et al. 2009).

Among numerous regulatory signaling pathways, Vascular Endothelial Growth Factor-A (VEGF) signaling is required for sprouting angiogenesis (Shibuya 2013; Simons et al. 2016). VEGF binds to the endothelial cell-expressed receptor tyrosine kinases Flk1 (VEGFR2) and Flt1 (VEGFR1). VEGF-bound Flk1 triggers a signaling cascade that promotes endothelial cell proliferation, chemotaxis, and cell survival, thereby initiating and sustaining sprouting

---

angiogenesis (Khurana 2005; Koch et al. 2011; Hanahan & Weinberg 2011). Flt1 is alternatively spliced to form two isoforms, a membrane-localized tyrosine kinase receptor (mFlt1) and a soluble isoform lacking the transmembrane and tyrosine kinase domains (sFlt1) (Kendall & Thomas 1993). Both isoforms of Flt1 have a 10-fold higher binding affinity for VEGF-A ligand than Flk1 and complete genetic deletion is embryonic lethal in mice (Kendall & Thomas 1993; Fong et al. 1995). Nonetheless, sFlt1 cannot independently signal, and mFlt1 has weak kinase activity that is not required for developmental angiogenesis (Ito et al. 1998; Hiratsuka et al. 1998). Thus Flt1 functions as an endothelial cell-intrinsic decoy receptor or ligand sink to negatively modulate VEGF signaling amplitude during angiogenesis.

Stages of sprouting angiogenesis include sprout initiation, extension, anastomosis, and lumenization (Chappell et al. 2011; Geudens & Gerhardt 2011). Sprout initiation, extension and lumen formation are relatively well understood processes. Recent zebrafish studies revealed a role for endothelial cell filopodia in vessel anastomosis, and found that adherens junction-mediated cell rearrangements subsequent to connection promote lumen formation (Lenard et al. 2013; Phng et al. 2013). However, it is unknown whether the site or timing of sprout anastomosis is regulated.

We recently showed that Flt1 positively affects the stability of new conduits, suggesting that Flt1 may regulate aspects of anastomosis that affect stability (Chappell et al. 2016). Here we show that extending sprouts form transient contacts before establishing stable connections. Flt1 regulates the frequency of transient contacts and the probability of a target site being used for a permanent connection, and this spatial selectivity requires mFlt1. These results indicate that blood vessel anastomosis is temporally and spatially regulated by endothelial cell-intrinsic signaling.
II. Results

a. Flt1 Influences Retinal Vessel Interactions

Global or vascular-selective deletion of flt1 in mouse post-natal retinal vessels increased overall sprouting and filopodia (Chappell et al. 2009; Ho et al. 2012; Chappell et al, in prep). To better understand the role of negative modulation of VEGF-A signaling in sprout anastomosis, we used low-dose tamoxifen to induce mosaic excision of flt1 in retinal vessels, with an excision reporter (Fig 3.3.1).

Sprouts were defined as previously described (Chappell et al. 2009) and interactions between vessels at the angiogenic front were identified (Fig 3.3.1A). The interacting endothelial cells were classified based on the cytoplasmic reporter expression in the endothelial cell from which filopodia extended (Fig 3.3.1B-C). Interestingly, wildtype (WT) sprouts were linked to flt1-/- endothelial cells significantly more often than to WT cells (Fig 3.3.1D), suggesting that Flt1 influences sprout connection parameters. However, further analysis of sprout anastomosis in mouse retinas was hampered by our inability to follow this dynamic process over extended time periods to determine what precedes and follows the static interactions. Moreover, most retinal interactions are at the front and consist of two sprouts intersecting, which does not allow for analysis of target site selectivity.

b. Transient Contacts Precede Stable Blood Vessel Connections

To better understand the dynamics of blood vessel anastomosis, we turned to primary human umbilical vein endothelial cells (HUVEC) in a 3D sprouting angiogenesis assay to model mammalian angiogenesis and anastomosis in vitro (Nakatsu & Hughes 2008). HUVEC coated onto beads and placed in a fibrin matrix form lumenized sprouts that often connect with
targets over 3-7 days. LifeAct-expressing HUVEC were imaged from days 3-5 (d3-5) of sprouting, allowing for visualization of F-actin in live cells and dynamic assessment of endothelial cell behaviors preceding and during anastomosis (Fig 3.3.2A, Movie 1). We were surprised to see two distinct forms of interaction between extending sprouts and potential targets in these movies. We scored brief, limited interactions (present for only one 10 min frame) of cytoplasmic extensions from the sprout that we termed transient contacts (Fig. 2A\textsuperscript{i-iv}). We also documented longer interactions, termed stable connections, which persisted for at least 30 minutes, and were often coincident with a widened sprout front and/or lumen formation (Fig 3.3.2A\textsuperscript{v-viii}). Transient contacts occurred on average four times prior to a sprout forming a stable connection (Fig 3.3.2B). These transient contacts occurred throughout the lifetime of the sprout and at varying distances from the eventual connection site (Fig 3.3.2C).

To further characterize the transient contacts that precede stable sprout connections, we quantified the LifeAct fluorescence intensity at transient contact locations prior to, during and subsequent to the transient contact (Fig 3.3.2D-E). Fluorescence intensity in the contact area was increased at the contact site compared to either side (Fig. 2D) and higher at the contact site during contact than at either pre-contact or post-contact times (Fig. 2E), confirming the transient nature of the contacts. Taken together, these results suggest that endothelial cell cytoplasmic extensions transiently “sample” potential target areas before forming a permanent connection.

c. Flt1 Regulates the Number of Transient Contacts

We next wanted to understand whether blood vessel anastomosis is a regulated or stochastic process, and we hypothesized that VEGF signaling regulates aspects of anastomosis. We manipulated VEGF signaling by reducing levels of a negative modulator, Flt1. We
generated and validated a lentivirus shRNA targeted against both isoforms of Flt1 (Fig S3.1A-B) and showed a high infection efficiency by flow cytometry (Fig S3.1C). We used the 3D sprouting angiogenesis assay to examine the effects of Flt1 knockdown (KD) on branching and sprouting parameters, and found that sprouting from the bead was unaffected but branching was significantly increased with loss of Flt1 (Fig 3.3A-B). These results are similar to effects of Flt1 loss in postnatal retinas.

We used time-lapse imaging in the sprouting angiogenesis assay to assess the effects of Flt1 manipulation on transient contacts and stable connections (Fig 3.3C-G, Movies 2-3). Both control and Flt1 KD sprouts had transient contacts and formed stable connections, but the dynamics were altered with reduced Flt1 levels. Connections from Flt1 KD sprouts formed earlier in the time sequence than controls, as measured from the emergence of a “tip-cell” phenotype (filopodia formation and directed migration away from parent vessel) to stable connection, although the overall distance travelled to the target was unaltered (Fig 3.3E-F). Consistent with the LifeAct analysis, control sprouts made about four transient contacts prior to forming a stable connection, but Flt1 KD sprouts averaged less than two transient contacts before forming a stable connection (Fig 3.3G). We also analyzed sprout behavior in mouse ES cell-derived vessels that differentiate over a week and form interconnected lumenized blood vessel networks (Rylova et al. 2008). ES cells transgenic for PECAM-eGFP and genetically deleted for flt1 (Chappell et al. 2009), also showed earlier connection times, similar connection distances, and reduced numbers of transient contacts prior to stable connection, relative to wild-type controls (Fig 3.3H-L, Movies 4-5). Taken together, these data suggest that sprout anastomosis is a regulated process, and that the number of transient contacts, which may be
exploratory behavior to gather information about potential targets, is reduced when Flt1 levels are reduced or absent.

d. Differential Flt1 isoform Requirements for Sprout Anastomosis

Flt1 regulates angiogenesis through both the membrane-bound decoy receptor and the secreted isoform that also acts as a ligand sink (Kendall & Thomas 1993; Roberts et al. 2004), so we examined the influence of each Flt1 isoform on sprout anastomosis. We generated and validated isoform-specific siRNAs that selectively reduced levels of mFlt1 in cell lysates or sFlt1 in conditioned medium (i.e. secreted sFlt1) (Fig 3.4A-C). In the sprouting angiogenesis assay, HUVEC sprouts with knockdown of total Flt1 via siRNA mimicked the effects of the shRNA knockdown, with no effect on sprouting from the bead but significantly increased branching (Fig 3.4D,E,H-I). Likewise, selective knockdown of either isoform did not affect sprouting, but knockdown of mFlt1 increased branching, while knockdown of sFlt1 did not affect branching in this assay (Fig 3.4F-I). We then used live-imaging to determine the number of transient contacts that occurred before stable connections formed, and found that knockdown of either Flt1 isoform reduced the number of transient contacts prior to stable connection (Fig 3.4J). These results suggest that, despite different ultimate effects on vessel morphology, both Flt1 isoforms are important in regulating the exploratory behavior of sprouts as they approach potential connection targets.

We next examined target sites for transient contacts and stable connections, and asked whether target areas had unique attributes prior to contact or connection. We focused on targets that were part of established vessels or sprouts, as opposed to targets that were other tip cells, so that the incoming (i.e. non-target) sprout in principle had a choice of sites for formation of transient contacts or stable connections. Live-imaging of LifeAct-infected HUVEC in the
sprouting angiogenesis assay revealed that some endothelial cells in the target area exhibited protrusive activity, while others were non-protrusive, or static (Fig 3.5A-B). Analysis of time stamps just prior to transient contact or stable connection showed that subsequent transient contact sites were equally likely to be protrusive or static, but sites for stable connection were more likely to exhibit protrusive activity prior to connection (Fig 3.5C). Thus protrusive behavior correlates with target sites for stable connections, suggesting that this attribute contributes to spatial selectivity in anastomosis via differential cell behaviors prior to stable connection but not transient contacts, consistent with transient contacts being exploratory in nature.

Because protrusive activity is associated with endothelial cells that will subsequently form stable connections, and protrusive activity is associated with loss of Flt1 (Chappell et al. 2016), we hypothesized that Flt1 levels within target endothelial cells would affect the probability of forming a stable connection. We first examined Flt1 expression at target sites. Flt1 levels are heterogeneous in developing blood vessels (Kappas et al. 2008; Chappell et al. 2009), so we predicted that this heterogeneous expression would contribute to target site selection during sprout anastomosis. Fixed-image staining, using PECAM-1 to define cell borders and interactions, revealed that Flt1 intensity was decreased in target endothelial cells that were “touched” by sprouts (note that contacts vs. connections cannot be distinguished absent live-imaging) compared to neighboring cells (Fig 3.5D-E). Next we manipulated Flt1 expression at the target site by generating mosaic HUVEC vessels that were a 50:50 mixture of endothelial cells with Flt1 KD (green) and control endothelial cells (red). These mosaic beads were incubated with control beads coated with unmanipulated endothelial cells without reporter expression (Fig S3.2A). We then live-imaged unmanipulated sprouts approaching
mosaic target areas to analyze their behavior. Controls where neither reporter was linked to Flt1 KD showed no bias in either transient contact or stable connection target site selection, relative to input or to each other (Fig S3.2B). In contrast, in experiments where both control and Flt1 KD endothelial cells were available as targets, there was a significant bias towards Flt1 KD endothelial cells as connection targets relative to contact (Fig 3.5F, Fig S3.2C).

We then examined the role of each Flt1 isoform in the preference for endothelial cells with reduced Flt1 as stable connection sites. We live-imaged mosaic target vessels in which control endothelial cells were mixed with endothelial cells with knockdown of total Flt1, mFlt1 or sFlt1 via siRNA. Similar to total Flt1 knockdown by shRNA, siRNA Flt1 knockdown target endothelial cells were significantly more likely to form stable connections relative to transient contacts, and this bias was recapitulated by selective reduction of mFlt1 from endothelial cells, but not by selective reduction of sFlt1 (Fig 3.5G-I). Taken together, these findings indicate that sprouts sample potential targets in an unbiased way via transient contacts, and form stable connections with target site selectivity influenced by relative mFlt1 levels.

III. Perspective

Here we highlight dynamic features of blood vessel sprout anastomosis, and show that connection site selectivity in developing vessel networks is regulated by levels of the VEGF receptor Flt1. Our results indicate that endothelial cell “sampling” occurs prior to formation of stable connections in expanding vessel networks where sprouts have “choices”. These brief interactions likely impart information to the sprout regarding the appropriateness of a given endothelial cell for stable connection. Initial analysis of mosaic retina vessels mosaic revealed that wildtype sprouts interacted more often with flt1-/- cells. This bias was also found in HUVEC mosaic vessel targets with reduced Flt1. Thus cells with reduced Flt1, and presumably
experiencing more VEGF signaling, are favored as sites of stable connection, and this bias depends on the expression of the membrane bound decoy VEGF receptor, mFlt1.

In summary, this work shows that endothelial sprout anastomosis is a regulated process and implicates VEGF signaling in both the exploratory behavior that precedes formation of stable connections and connection target site choice. These findings suggest that the location and timing of establishing a stable connection is essential in forming a new conduit in vascular beds that do not have an imposed pre-pattern. This requirement for Flt1 in the regulated patterning of anastomoses suggests a novel role for the VEGF signaling pathway in blood vessel patterning, and potentially new ways to regulate vascular network formation with implications for regenerative medicine.
IV. Figures
Figure 3.1: Flt1 Influences Retinal Vessel Interactions In Vivo.

(A) Vascular front of representative P5 retinal vessel with mosaic loss of Flt1. (A) Merge visualized with excision reporter (A, red), vessels (A, green) and nuclei (nuclei, blue). Scale bar, 25 µm. (B-C) Higher magnification of left box (B) and right box (C) outlined with dashed lines in A. Arrow, scored interaction; white dotted lines, the area behind the extension used to define the category. (D) Quantification of mosaic interactions (n=7 retinas, 45 sprouts). Statistics, paired two-tailed Student’s t-test (**, p<0.01). Error bars, mean ± 95% CI.
Figure 3.2: Blood Vessel Stable Connections are Preceded by Transient Contacts.

(A) Live-imaging of HUVEC infected with LifeAct. Representative time-lapse stills of DIC (left) and fluorescence (right) channels over d3-5. (A¹-A⁷) Transient contact. Insets show scan area. Green arrowhead and dotted line, location of pre-contact scan; Red arrowhead and dotted line, location of transient contact scan; blue arrowhead and dotted line, location of post-contact scan. (A⁸-A¹³) Stable connection. Orange arrow, example of stable connection. Scale bar, 25 µm; time (lower left) hr:mins. (B-C) Quantification of transient contact frequency before stable connection (B), and relative time and distance from stable connection site at transient contact (C). n= 28 sprouts from 12 movies. (D) Fluorescence intensity of LifeAct at transient contact site was quantified across the site (dashed line in insets panel A) pre-contact (A², green line), at contact (A³, red line), and post-contact (A⁴, blue line). (E) Fluorescence intensity of LifeAct at transient contact site was quantified pre-contact time (green arrowhead in A²), at contact time (red arrowhead in A³), and post-contact time (blue arrowhead in A⁴). n=14 contacts from 9 movies. Statistics, One-way ANOVA with Bonferroni’s post-hoc correction (*p<0.05, **p<0.01). Error bars, mean ± 95% CI.
Figure 3.3: Transient Contact Dynamics during Vessel Anastomosis are Regulated by Flt1.

(A) Representative images of HUVEC sprouts with indicated treatments at 5d in 3D sprouting angiogenesis assay, stained with phalloidin and depth-encoded. Scale bar, 50 µm; EV, empty vector; shFlt1, lentivirus-expressing Flt1 shRNA. (B) Quantification of indicated parameters at 5d (control, n=41 beads; shFlt1, n=41 beads). (C-D) Representative time-lapse stills of EV (C) and shFlt1 (D) HUVEC sprouts from 3-5d live-imaging. Transient contact, red arrowhead; stable connection, red arrow. Scale bar, 25 µm; time (upper right), hrs:mins. EV control (n=27 sprouts), shFlt1 (n=41 sprouts). (E-G) Quantification of indicated parameters. (H-I) Representative time-lapse stills of WT (H) and flt1-/- (I) ES cell-derived vessels at 6d of differentiation. Transient contact, red arrowhead; stable connection, red arrow. Scale bar, 10 µm; time (upper right), hrs:mins. (J-L) Quantification of indicated parameters (WT, n=88 sprouts; flt1-/-, n=48 sprouts). Statistics, Two-tailed Student’s t-test (**, p<0.01; ***, p <001; ****, p<0.0001; NS, not significant). Error bars, mean ± 95% CI.
Figure 3.4: Both Flt1 Isoforms Reduce Transient Contacts Prior to Connection.

(A) Schematic showing Flt1 isoforms and sequences targeted by indicated siRNAs. (B-C) Western blot of HUVEC lysates (B) and concentrated conditioned media (C) with indicated knockdowns. Fold change from control shown below lanes. (D-G) Representative HUVEC sprouts with indicated treatments, stained for phalloidin at 5d and depth-encoded. Control (n=15 beads); total si-Flt1 (n=12 beads); si-sFlt1 (n=15 beads); si-mFlt1 (n=14 beads). Scale bar, 50 µm. (H-I) Quantification of indicated parameters. Statistics, One-Way ANOVA with Bonferroni’s post-hoc correction (*p<0.05; **, p<0.01; ****, p<0.0001; NS, not significant). Error bars, mean ± 95% CI.
Figure 3.5: Target Vessel Connection Site is Biased by Flt1.

(A-B) Representative images of LifeAct-infected 3D HUVEC sprouts and targets showing transient contacts. Green arrowheads, LifeAct-positive protrusions. Scale bar, 10 µm. (C) Quantification of target cell protrusions (see Methods for definition of “static” and “protrusive”) at time stamp immediately preceding contact (n=63 events) or connection (n=18 events). Statistics, Observed vs. Expected Binomial Test (**, p<0.01). (D) Left, Representative HUVEC vessel at 5d (with indicated staining). Middle and right, Flt1 channel only. Interacting target cell (red outline) and neighboring target cell (blue outline) of representative HUVEC vessels are noted. Scale bar, 25 µm. (E) Relative Flt1 staining intensity between pairs of endothelial cells was quantified (n=29 pairs). Statistics, Unpaired Student’s T-test versus no difference (red line) (*, p<0.05). (F) Representative time-lapse images from d3-5 of WT sprout (no label) and target vessel with 50% Flt1 KD endothelial cells (green) and 50% WT cells (magenta). Scale bar, 25 µm; time (lower right), hrs:mins. Transient contact, red arrowhead; stable connection, red arrow. (G-I) Percentage of transient contacts and stable connections with endothelial cells of indicated type in mosaic target vessels (si-Flt1 vs. WT, n=41 contacts, n= 12 connections; si-mFlt1 vs. WT, n=57 contacts, n=17 connections; si-sFlt1 vs. WT, n=53 contacts, n=17 connections). Statistics, Observed vs. Expected Binomial Test (**, p<0.01; NS, not significant).
Supplementary Figure 3.1: Flt1 shRNA Validation.

(A-C) HUVEC collected 48h post-infection with indicated lentivirus and analyzed by qRT-PCR (A), Western blot (B), or FACS sorted for infection efficiency (via GFP signal) (C). Flt1 mRNA expression was normalized to GAPDH and relative to control. Flt1 protein expression was normalized to β-actin and relative to EV-GFP. Statistics, Unpaired two-tailed Student’s t-test (*, p<0.05; ****, p<0.0001). Error bars, mean ± 95% CI.
Supplementary Figure 3.2: Transient Contacts and Stable Connections in Mosaic Target Vessels.

(A) Schematic of experimental design for mosaic vessel live-imaging analysis. (B-C) Percentage of transient contacts and stable connections with WT sprouts and endothelial cells (EC) with indicated manipulations in mosaic target vessels. (EV-tdTomato vs. EV-GFP, n=19 contacts, n=8 connections; shFlt1 vs. WT, n=95 contacts, n=36 connections). Statistics, Observed vs. Expected Binomial Test (***, p<0.001; NS, not significant).
V. REFERENCES


CHAPTER 4: Flt1 During Sprouting Angiogenesis

These are preliminary data and an addendum to the completed story presented in the previous chapter. As such, the rationale and potential follow-up experiments are included within each section.

I. Results

a. Connection stability and success requires Flt1

Previous studies of Flt1 and VEGF signaling during sprout initiation and extension demonstrated a strong requirement for the ligand and receptor in patterning the vasculature (Chappell et al. 2009; Kappas et al. 2008; Gerhardt et al. 2003). To understand how sprouts contribute to pattern in real time, we performed time-lapse imaging of flt1/− ES cell-derived vessels alongside WT ES cell-derived vessels from the start of sprout initiation to completed anastomosis. These data allow understanding of connective success and the impact of VEGF signaling throughout angiogenesis.

This analysis of ES cell-derived sprouts during sprouting angiogenesis identified and three distinct outcomes for a sprout: retraction, unmaintained connection, and maintained connection (Chappell et al. 2016). Unique in the outcomes, retracted sprouts do not form a connection within the vessel but instead rejoin the parent vessel from which they emerged. The other two outcomes form new conduits but over time exhibit different contributions to the network morphology. An unmaintained connection initially forms a new branch point but eventually
this new connection breaks down and merges with existing vessels, unlike maintained connections which form a new branch point and permanently expand the complexity of the vessel network. In WT ES cell-derived vessels, the sprouts were evenly split into thirds with 37.5%, 33.1% and 29.4% of the sprouts resulting in retraction, unmaintained connections, and maintained connections, respectively (Fig 4.1A). In flt1-/- sprouts, the retractions decreased to 12.6% and unmaintained connections increased to 48.6% (Fig 3.4.1A). The rate of maintained connections was not significantly altered (Fig 4.1A). Given the observed changes in outcome, we conclude that without Flt1 the sprouts which are unable to properly retract may compensate through an alternate method of failure.

We then investigated the unmaintained connections further to understand the mechanism of increased connective failure in flt1-/- ES cell-derived vessel networks. The unmaintained connections in both genetic backgrounds collapsed into the parent vessel in two different fashions. We termed these active collapse and passive collapse, which were distinguished by the presence or absence of protrusive endothelial cells into the newly formed ‘gap area’ (Fig 4.1B). If the gap area closed over time without any protrusions from the surrounding endothelial cells, this indicated a passive collapse but if the gap area is invaded by endothelial cell protrusions during collapse it was determined to be an active collapse (Fig 4.1B-C). WT ES cell-derived vessels showed predominantly passive collapse, at 66% of the time, while active collapse was more prevalent in flt1-/- ES cell-derived vessels, occurring 78.3% of the time (Fig 4.1D). In addition to a different method of collapse, the two genotypes exhibited different timing for collapse as well. Both active and passive collapsed vessels in WT ES cell-derived networks collapsed at the same rate, while in flt1-/- ES cell-derived vessels the passive collapse was 2.0-fold slower than the active collapse (Fig 4.1E). These data suggest that
connections require active maintenance cues that are downstream of Flt1, likely through its role in restricting VEGF.

In *flt1-/-* ES cell-derived vessels, previously demonstrated to exhibit increased pFlk1 (Roberts et al. 2004; Kappas et al. 2008), a higher percentage of sprouts form connections and these connections are more likely to fail, collapsing into the existing vessels and not maintaining their own branch point. In fact, the EC in *flt1-/-* ES cell-derived vessels are overly active and show increased protrusive activities that are correlated with the collapse of vessels after connection. Computational modeling of pFlk1, indicative of active VEGF signaling, predicted that these protrusions result from inappropriately active VEGF (Chappell et al. 2016). These data suggest that after connection Flt1 dampens protrusive activities through preventing pFlk1 activity. However, this has only been demonstrated *in silico* as testing the activity of Flk1 is technically challenging in fixed imaging and not currently feasible in real time. Recent advances in computational analysis of complex staining patterns used to decipher VE-Cad activity, which decipher VE-Cad changes between inactive vesicle localization and active cell surface localization, could be applied to pFlk1 in these vessels (Bentley et al. 2014). Using this type of post-hoc analysis would allow vessels stained for pFlk1 to be analyzed on a sliding scale for regions with higher and lower activity and attempt to corroborate the predictions from *in silico* modeling. These data can elucidate the mechanism by which Flt1 is able to alter behavior of vessels after connection.

Beyond mechanism, the failure in connections could be due to inappropriate selection of a connection site or a result of continued VEGF signaling in the surrounding vessels. While genetic deletion of Flt1 in the ES cell-derived vessel model is very informative for understanding VEGF signaling in angiogenesis, any discrete roles for Flt1 in the separate
stages must be teased apart using an alternate method. In order to isolate these effects, spatiotemporal control of Flt1 deletion is needed. This can be accomplished using recently developed optogenetic techniques (Deisseroth 2011), which takes advantage of light-induction driven by microbial opsins linked to Cre-recombinase would allow targeted, cell-type specific deletion of Flt1 at varying stages of angiogenesis. These methods would allow a separate examination of Flt1 when the vessel is re-entering quiescence, necessary for a complete understanding of the receptor’s ability to promote connective stability and maintain branches within the vessel network.

b. Flt1 isoforms differently affect mitosis and protein localization

Full Flt1 deletion alters intra-cellular signaling and increases proliferation, migration, and survival, a VEGF GOF mimetic. This effect has been demonstrated in whole mouse models, mouse ES cell-derived in vitro models of angiogenesis, and biochemically (Fong et al. 1995; Ito et al. 1998; Kearney et al. 2002; Kearney 2004). The Flt1 isoforms have unique effects on angiogenesis, with sFlt1 influencing proliferation, overall branching and sprout initiation while mFlt1 is only reported to impact proliferation (Kappas et al. 2008; Chappell et al. 2009). How these isoforms are differentially controlled and utilized by the sprouting cells is an open question. To address this we took advantage of HUVEC sprouting vessels to study angiogenesis, and specifically proliferation after depletion of each Flt1 isoform.

Despite the relatively common use for modeling angiogenesis, the proliferative rate within HUVEC sprouts is not well defined (Wiley et al. 2011; Pelton et al. 2014; Kushner et al. 2016). We wanted to confirm that the Flt1 isoforms are able to rescue the proliferative increase reported in mouse ES cell-derived vessels. As the cells in the sprouts are presumed to experience different signaling and external pressures than those coating the microcarrier beads,
we only analyzed HUVEC within sprouts and excluded those on the beads (Fig 4.2A). The mitotic index in controls was 1.2% (Fig 4.2B). siRNA knockdown of Flt increased the mitotic index to 2.1%, while each of the isoforms showed a reduced effect, a mitotic index of 1.8% for si-sFlt1 and 1.6% for si-mFlt1 (Fig 4.2B). These results show equivalent effects on mitosis from either isoform alone and a greater effect when both Flt1 isoforms are reduced. This supports a conserved role for the Flt1 isoforms in sprouting angiogenesis across mouse ES cell-derived vessels and HUVEC vessels.

Previously, the ability of Flt1 to influence sprout migration has been suggested to be a product of its spatial localization. Specifically, the basal secretion of sFlt1 is proposed to act as a spatial coordinating factor, restricting VEGF ligand availability and thereby directing sprout migration in the extracellular space (Chappell et al. 2009). This model was proposed based on studies of chimeric ES cell-derived blood vessels but whether the ligand is selectively secreted or localized has not been examined. The potential biased protein localization of the Flt1 isoforms was examined using HUVEC vessels that were stained for Flt1. Individual cells along lumenized vessels were identified using nuclear markers and PECAM staining. The apical or basal side of the cell was defined as the innermost or outermost third of the cell width (Fig 4.2C). In untransfected cells there is no observable bias in Flt1 staining for the apical or basal domains of the cell (Fig 4.2D). si-sFlt1 HUVEC within lumenized vessels show a trend of more basally localized Flt1 while si-mFlt1 HUVEC within lumenized vessels show a greater apical than basal localization (Fig 4.2D). These data indicate that, surprisingly counter to the predictions from the model, sFlt1 is more apically localized and mFlt1 is more basally localized. However, the data are consistent with the newly proposed role of mFlt1 in sprout connections which occur on the basal side of the cell. These data don’t elucidate the impact of
selective isoform localization in the HUVEC vessels, but they provide a model that can be used
to further investigate any unique and overlapping roles of each Flt1 isoform.

As the isoforms are differently localized, this may indicate unique roles that require
receptor presence at the appropriate cell surface. The apical and basal domains of the EC
interact with very different surroundings. Apically the cell is exposed to constant fluid flow of
the blood passing through the lumen while basally there are stable, permanent interactions with
associated cells like smooth muscle cells, macrophages, and pericytes (Adams & Alitalo 2007;
Chappell & Bautch 2010). These disparate environments require unique cell structures and
signaling. The different domain components have been well classified in stratified epithelium
where there is a clearly defined apical and basal side to the cell, however the endothelium is
not a stratified tissue and these domains are less well understood in this tissue.

Previous data in the lab identified the apical restriction of PODXL and basal restriction of
collagen IV out at the front of the sprout (Pelton et al. 2014). To more precisely identify apical
and basal restriction of Flt1 protein, and specifically each isoform, co-localization with these
known domain restricted proteins is needed. Co-staining for Flt1 and either PODXL or collagen
IV within the HUVEC sprouts should be the first step in investigating the observed trends. Not
only will this provide a quantifiable metric for membrane localization, but these proteins
provide a more restrictive and defined apical and basal compartment. Given the current data, we
predict stronger co-localization of sFlt1 with PODXL and stronger co-localization of mFlt1 and
collagen IV.

A recent study in the hindbrain of rats sought to identify the apical and basal specific
effects of VEGF ligand. These data demonstrated that VEGF was able to induce permeability
when presented basally and found abundant Flk1 protein on the basal side of the EC while Flt1
was more prominent apically (Hudson et al. 2014). These protein localizations were associated
to the spatially restricted effects of the ligand, presuming that the VEGF ligand was likely
present at both sides of the cells but receptor presence limited the activity. The apical
localization doesn’t fit with our proposed model for sFlt1 secretion during sprouting, but is
consistent with the identified trends for sFlt1 localization in HUVEC sprouts. However, these
data are not isoform specific. Therefore, reciprocal experiments in mice using flt1−/− and
isoform selective flt1 mutants are an interesting proposition. If the lack of flt1 contributes to the
biased response to VEGF ligand, then the flt1−/− mice should not exhibit the same bias. The
question would then be whether presence of only the sFlt1 isoform mimics the WT or flt1−/−
endothelial cells.

These preliminary data (Fig 3.4.2) open an area of research examining the intracellular
localization and potential impact of this spatial restriction of Flt1. As a potent modulator of
VEGF signaling, differential location for the receptor could alter our understanding of where the
VEGF growth factor gradient exists and the impact on endothelial cells and angiogenesis.

c. Flt1 and VEGF signaling influence anastomosis within zebrafish vessels

While the data presented in CHAPTER 3 present a compelling case for Flt1 spatial
regulation of connections, an in vivo model which permits live imaging would bolster the
conclusions presented. The embryonic zebrafish provides an in vivo model that can be readily
and easily live imaged during sprouting angiogenesis (Childs et al. 2002; Isogai et al. 2001;
Lenard et al. 2015). The VEGF-dependent vessels which grow from the common artery, form
the intersegmental vessels (ISVs), and eventually connect to generate the dorsal longitudinal
anastomotic vessel (DLAV) are a prime candidate (Wiley et al. 2011; Ghaffari et al. 2015).
Perturbations to the VEGF pathway were accomplished two ways in the embryonic fish. First, Flt1 was depleted by injection of a morpholino (MO) from previous publication, blocking the ATG start site for protein translation and knocking down the protein (Krueger et al. 2011). The Flt1 MO causes some defects in ISV patterning (Fig 4.3A), but a non-significant change in the connections outside the somite boundary, as compared to wild-type embryos (Fig 4.3B). Second, VEGF expression was increased via a heat-shock through whole embryo inducible transgene which is capable of generating ectopic sprouting from the common artery (Wiley et al. 2011). Increased VEGF expression causes ectopic sprout formation in the ISVs. In addition to the excess sprouts, these sprouts also fail to follow the normal guidance cues to generate the DLAV and form inappropriate connections between the somite boundaries (Fig 4.3A). Unlike Flt1 depletion, disrupting VEGF ligand causes a significant 5.6-fold increase in the frequency of inappropriate connections (Fig 4.3B). Most interestingly, these two manipulations have an additive effect and result in an 11.8-fold increase in inappropriate connections (Fig 4.3B).

Therefore, VEGF ligand guides sprout growth in the ISVs and Flt1 influences VEGF ligand effects in these vessels.

Altering VEGF signaling through either method caused a disruption in sprouting, resulting in connections that form outside the normal somite boundaries. Given the strong guidance cues that are present within the somitic boundaries, it is unsurprising that without exogenous VEGF signaling the disruption of Flt1 had no noticeable effect. However, upon both increased VEGF signaling and depletion of Flt1 a dramatic increase in connections outside the somitic boundaries are found. While these data do not address whether Flt1 can function as a predictive marker of vessel interaction outcomes in vivo, they do support a conserved role for the receptor
in restricting anastomosis in a complex environment. These preliminary data have established one model for further investigation of anastomosis in vivo using zebrafish ISVs.

Recent work by the Gerhardt group analyzed anastomoses in angiogenic vessels after blocking filopodial extensions, via pharmacological blockade of actin filaments. This treatment blunted sprouting angiogenesis and inhibited anastomosis in a bed-dependent fashion, namely the ISVs were able to eventually connect but the CVP was not, and concluded that filopodia were dispensable for anastomosis (Phng et al. 2013). Our data indicate that filopodia and cell protrusions are used to regulate and direct connections, therefore we postulate that while connections may form in the absence of filopodia that they are not appropriately regulated. These conclusions were based upon HUVEC sprouts and could be examined using LifeAct zebrafish lines. This line is amenable to live imaging and would enable analysis of anastomoses formed in a variety of angiogenic vessel beds. Additionally, this line can be crossed with VEGF over-expression or Flt1 MO to examine the effects of these signaling pathways on actin dynamics.

The next step with the available tools is to perform live imaging on these fish after manipulating VEGF signaling with heat-shock overexpression and MO knockdown. Given the outcomes in vitro, we predict transient contact between the vessels prior to observing anastomoses. In addition, if increasing VEGF signaling reduces contacts, the fish with over-expressed VEGF and Flt1 MO should exhibit reduced contacts. If this role for VEGF is conserved the next experiment to analyze is ISVs containing a mixture of ECs with and without Flt1 MO. Based upon the data in HUVEC sprouts, the EC with reduced Flt1 should be preferentially targeted for connections. The genetic tools for manipulations of Flt1 isoforms to distinguish between the tyrosine kinase versus ligand sink roles of the receptor are not yet
created in this system. Generating these deletions or mutants could be accomplished using CRISPR targeting to the shared or unique regions of the receptor (Hoshijima et al. 2016). The selective creation of InDels within mFlt1, the essential Flt1 isoform for restriction connections, could be accomplished and used to generate a new line. As mFlt1 is reportedly only essential in pathological angiogenesis (Hiratsuka et al. 1998; Hiratsuka et al. 2001), a true deletion mutant should be both fertile and viable. This fish would allow isolated analysis of mFlt1 during connection and could be combined with GOF VEGF using already existing lines if needed. This set of experiments would nicely demonstrate that the quantified role for Flt1 in vitro is utilized in cells that are experiencing the full complement of signaling from the surrounding tissue and blood flow.

The zebrafish have been live imaged to generate a basic understanding of where and when anastomosis occurs but few mechanistic analyses exist to date. Given the many genetic and chemical manipulations to which zebrafish are amenable, there is a great variety of experimental avenues to pursue in understanding the signaling that regulates anastomosis.
II. Figures

A

WT

fit1-/-

% of sprouts

0 25 50 75 100

maintained
unmaintained
retracted

B

C

D

** active
** passive

% of sprouts

WT

fit1-/-

E

Time to Collapse (mins)

WT collapsed connections

fit1-/- collapsed connections

active

passive

NS

**

NS
Figure 4.1: Vessel connection outcomes in ES cell-derived sprouting angiogenesis is influenced by Flt1.

(A) Extended sprouts were grouped into three categories depending on their connective success, either retracted (no prolonged connection observed), unmaintained (stable connection observed which does not contribute a new branch over time), and maintained (stabled connection that provides a new branch). Quantification and comparison of the percent sprouts within each category in WT and flt1-/- ES cell-derived vessels. Statistics, Two-Way ANOVA with Bonferroni post-hoc correction (*p<0.05). (B) Representative time lapse of sprout connection (red arrow) that undergoes passive collapse, outlined with red dashed line. Scale bar, 50 µm. Time, hours:mins. (C) Representative time lapse of sprout connection (red arrow) that undergoes active collapse, outlined with red dotted line. Green arrows indicate the protrusive events which distinguish active collapse. Scale bar, 25 µm. Time, hours:mins. (D) Comparison collapse type in WT and flt1-/- ES cell-derived vessels. Statistics, Two-Way ANOVA with Bonferroni post-hoc correction (**p<0.01). (E) The time from connection to complete collapse in WT and flt1-/- ES cell-derived vessels was quantified. Statistics, One-Way ANOVA with Bonferroni post-hoc correction (**p<0.01). Error bars, ± 95% CI.
Figure 4.2: Isoform specific effects on sprouting human endothelial venous endothelial cells.

(A) Sprout specific effects on mitosis were examined in fixed D5 sprouts. Representative sprouts stained with phospho-histone H3 (pH3, green), nuclear marker (DRAQ7, blue) and actin (phalloidin, red). Only pH3+ nuclei within sprouts, yellow arrow, not those on the microcarrier bead, yellow arrowhead, were considered in the mitotic index. Scale bar, 50 µm (B) The relative mitotic index of NT, si-Flt1, si-sFlt1, and si-mFlt1 sprouts were quantified. (C) Sprouts were stained for Flt1, PECAM and nuclei. Single HUVEC within lumenized vessels were divided into thirds along lumen axis (dashed lines). Scale bar, 10 µm. (D) Relative Flt1 protein within apical and basal domains in NT (n=12), si-sFlt1 (n=7), and si-mFlt1 (n=6) transfected HUVEC. All statistics, One-way ANOVA with Bonferri’s post-hoc correction (**p<0.01). Error bars, ± 95% CI.
Figure 4.3: Flt1 and VEGF influence connections in zebrafish intersegmental vessels.

(A) 48 hpf fish with eGFP labeled vessels, both with and without excess VEGF ligand and with and without Flt1 MO. Depth encoded along z-axis to distinguish ISVs. Arrows indicate connections outside somite boundaries. Scale bar, 100 µm. (B) Quantification of connections outside somite boundaries per tail under each condition. Statistics, One-Way ANOVA with Bonferroni post-hoc correction (* p<0.05; ** p<0.01, *** p<0.001, **** p<0.0001). Error bars, ± 95% CI.
III. REFERENCES


CHAPTER 5: Conclusions and Future Directions

I. Summary of Flt1 regulation of blood vessel anastomosis

We undertook a thorough analysis of anastomosis using in vitro assays, focusing on endothelial specific signaling directing and during connection. While this level of understanding is largely known for the signaling and growth cues that direct sprout initiation and extension, the signaling pathways that direct anastomosis remain poorly understood.

The first, and one of the more novel and interesting observations, was that not all vessel interactions resulted in connection. Previous reports in multiple angiogenic systems indicated that connection was the obligate outcome of vessels interacting (Blum et al. 2008; Fantin et al. 2010; Lenard et al. 2013). Our data found transient contacts between vessels occur 80% more often than stable connections. This suggests quite the opposite that in fact forming a stable connection requires specific signaling. Therefore vessel interactions have two possible outcomes, a supported interaction or stable connection and unsupported interaction or transient contact (Fig 5.1A). Given that the interaction outcomes have a non-random rate, with connections occurring significantly less frequently than contacts, we propose that transient contacts and stable connections form by a regulated process.

Regulation of transient contacts is supported by their cellular composition. We found actin-containing extensions, as observed through use of an actin reporter line, at the time and location of transient contacts; suggesting the presence of filopodia. Filopodia in sprouting angiogenesis are positively regulated by VEGF-A signaling and correlate with appropriate guidance and
extension (Gerhardt et al. 2003; Chappell et al. 2013). We therefore examined vessel interactions in vessels with decreased Flt1, the ligand sink receptor which normally limits VEGF-A ligand activity. Initially, we looked at the postnatal mouse retina, a well-established model for sprouting angiogenesis and noted that wildtype sprouts had a greater incidence of interaction with flt1-/cells. These data suggested VEGF-A may regulate vessel interactions during anastomosis; however, the retinal model is not conducive to time lapse imaging. Therefore we turned to two discrete in vitro models of sprouting angiogenesis which permit time lapse imaging, transient contacts decreased with increased VEGF-A signaling. In fact, the decrease was to a nearly random rate, equal likelihood of connection or contact, suggesting a strong reliance on Flt1 for vessel interactions to result in transient contact.

Having observed a reliance on Flt1 and alternate outcomes of vessel interactions, we next explored the individual Flt1 isoforms as they have demonstrated unique roles for proliferation and network morphology during vessel development (Kappas et al. 2008). We were able to selectively deplete each isoform in HUVEC using siRNA. These isoform selective knockdowns demonstrated a role during HUVEC sprouting angiogenesis but surprisingly, both were equally required for transient contacts prior to stable connection. This indicates that transient contacts are regulated by VEGF-A ligand and an increase in ligand availability prevents transient contacts.

We hypothesized the reduced contact numbers was due to Flt1 biasing vessel interactions toward stable connection and away from transient contact. To test this, we generated vessels with either WT or Flt1 KD, distinguished by cytoplasmic fluorescence, and assayed the outcome of sprout interactions at these mosaic vessels. Depletion of both Flt1 isoforms, by either lentiviral shRNA or siRNA, resulted in EC that were more likely to result in stable connections and less likely to result in transient contacts. These data suggest that Flt1 does in fact influence vessel
interactions, although whether this is due to its role as a ligand sink or signaling receptor is unclear. To clarify this issue, we then generated mosaic vessels with a single isoform KD. In the isoform selective experiments, the si-sFlt1 cells did not exhibit a difference in interaction outcome however si-mFlt1 cells phenocopied the total Flt1 KD effect. Therefore, we conclude that mFlt1 is the isoform responsible for the bias in vessel interaction outcomes (Fig 5.1A).

This is the first reported mFlt1 specific role in angiogenesis. In fact, all previous studies have suggested that mFlt1 is dispensable for sprouting angiogenesis though implicated in some pathological and gestational scenarios (Hiratsuka et al. 1998; Hiratsuka et al. 2001; Kappas et al. 2008). Thus these data provide not only the first report of a selective process for connection during sprouting angiogenesis but also implicate VEGF-A/mFlt1 signaling as the regulatory pathway in this process.

II. Future directions for investigating Flt1 regulation of anastomosis

a. Mechanism of mFlt1 influence on vessel interaction outcome

The data demonstrate that mFlt1 is the contributing isoform for determining the outcome of vessel interactions and that it acts as a predictive marker for stable connection versus transient contact. Previous research indicates that phosphorylation of mFlt1 occurs downstream of the placental growth factor (PlGF) not VEGF-A, and more importantly tyrosine signaling is not critical for angiogenesis (Hiratsuka et al. 1998). Therefore, while mFlt1 possesses the ability to sequester VEGF-A ligand and act as a tyrosine kinase receptor it has been largely dismissed in the context of angiogenesis (Fig 5.1B) (Nishi et al. 2008; Chappell et al. 2009; Hudson et al. 2014).
As the first report of a unique role for mFlt1 in angiogenesis, the cellular mechanism surrounding this effect should be further explored. Given the known effects of mFlt1 it is more likely that the mFlt1 expression in a cell is indicative of a cell primed for connection. Control of the cellular levels of Flt1 is influenced by the activity of Flk1, which in its active state is phosphorylated to pFlk1 (Roberts et al. 2004). Of the multitude of intracellular changes downstream of pFlk1, there are two candidates that should be investigated first as promoting connection: increased filopodia and loosening of junctional complexes.

We observed increased connections at cells with more filopodia and less mFlt1, thus linking the two cell profiles. Increasing VEGF-A within the neonatal mouse retina, through injection of Flt1 neutralizing antibody, genetic deletion of Flt1 or injection of VEGF-A ligand, leads to increased filopodia from sprouting vessels (Gerhardt et al. 2003; Ho et al. 2012; Chappell et al. 2013). Subsequent work in zebrafish vessels indicated that filopodia promote anastomosis (Phng et al. 2013). Epithelial cells in the early embryo must sheet of cells to protect the developing embryo and tissues. During epithelial cell interactions the existence of ‘filopodial priming’ was postulated as a method to promote the formation of new junctions because filopodia extension was linked to the cadherin-dependent coordinated adherence of epithelial cells (Raich et al. 1999). Filopodial priming could be conserved in endothelial cells and promote the formation of a continual network by directing connections to sites containing filopodia, which would also be primed for the creation of a new junction.

If filopodia are the responsible party in promoting connection, then altering actin dynamics in cells should mimic the observed effects from manipulating mFlt1. As simple removal of F actin or excessive actin polymerization dramatically impact a wide array of cell behaviors, this approach is unlikely to yield information about connection dynamics. A cleaner approach,
though more technically challenging, would be to take advantage of other methods. First, optogenetically engineered actin that could be forcibly activated (Taslimi et al. 2014), in a temporal fashion immediately prior to contact and connection, in mosaic vessels to present cells with excess filopodia. Based upon the current data, this would promote stable connections in these cells. Alternately, laser ablation of fluoresceinly tagged actin within the filopodia prior to contact and connection would prevent filopodia from contributing to the interaction outcome, likely preventing stable connections. Through either of these methods the actin dynamics would only be altered at the relevant time frame and allow other migratory aspects to function normally; thus any observed biases in outcome are associated solely with filopodia presence or absence.

Second, active pFlk1 loosens existing EC-EC junctions by initiating a signaling cascade through Src and p21 activated kinase 1 (PAK1) to internalize VE-Cad (Gavard & Gutkind 2006). As VE-Cad activity is regulated by its localization, the end result of this shuttling is to create a less attached and thereby more motile cell within the vessel (Bentley et al. 2014). The rearranging of VE-Cad to establish a new junction is a proven requirement for anastomosis in zebrafish vessels (Montero-Balaguer et al. 2009; Lenard et al. 2013). Therefore, cells with low mFlt1 and increased pFlk1 may have a greater pool of free VE-Cad that would be ready for reincorporation into a new EC-EC junction at a connection. To test whether VE-Cad availability is responsible for the observed bias in connection selectivity, direct analysis of the protein during contact and connection is needed. A LOF mutation in zebrafish VE-Cad was previously analyzed during anastomosis and resulted in connections that were weakened and malformed (Lenard et al. 2013). Mimicking this deletion in HUVEC or mouse ES cell-derived vessels would allow analysis of VE-Cad function in the context of selectivity during
connection. Supplying LOF VE-Cad in mosaic vessels would allow determination of whether this protein is able to induce a bias in connection sites, presumably decreasing the likelihood of connection.

The increase in pFlk1 and resulting cellular changes are the likely explanation for how mFlt1 alters cellular mechanics to increase connection likelihood; however, if the proposed experiments do not identify a bias, which matches the cellular effects of increased VEGF-A signaling, there may be a signaling role for mFlt1 during connection. Should the formality of a signaling role need to be tested, inactivation of signaling can be accomplished by kinase-dead mutations or full deletion of this section, like the mouse mutant (Hiratsuka et al. 1998). These manipulations leave the VEGF-A ligand sequestration aspect of Flt1 intact, but prevent signaling through the tyrosine kinase receptor. The signaling cascade that is reportedly differentially activated by Flt1 but not Flk1 is AK-thymoma/protein kinase B (AKT) (Hudson et al. 2014). Assessing the activity of AKT within modified mFlt1 systems can validate the manipulations in this pathway as well. Once confirmed, this modified mFlt1 can then be tested for a role in transient contact frequency and its ability to induce connection between vessels.

Low mFlt1 expression indicates cells with high pFlk1 that are more capable of creating a supported, stable connection due to changes in cell structure downstream of VEGF-A. These proposed experiments manipulating filamentous actin and VE-Cad separate VEGF-A signaling from the changes in cell structure to isolate the components that are used to identify a cell that is primed or appropriate for connection.

b. mFlt1 regulation influence on non-endothelial anastomotic regulation

Previous data in mouse retinas suggested that vessel branch points are supported by the presence of tissue macrophages (Fantin et al. 2010). These are the only support cells proposed
Further examination of this macrophage population failed to identify which receptors were responsible for the presence at branches. As a secreted ligand, VEGF-A is available to any cells that express the appropriate receptors and besides endothelial cells these comprise neurons and the hematopoietic lineage, including macrophages (Koch et al. 2011). Additionally, Flt1 is reported to recruit macrophages in a manner dependent on its intracellular signaling, a process which is defective in mice where the bone marrow is replaced with Flt1 kinase-dead marrow (Murakami et al. 2008). Combining this knowledge with our new understanding of anastomotic regulation confounds our understanding of how the macrophages at branch points are recruited. If mFlt1 is reduced in target vessels at the sites of new conduits then the macrophages are not recruited to these points by straightforward Flt1 signaling. Two distinct possibilities resolve the macrophage recruitment signaling conflict, 1) mFlt1 recruits macrophages to the sprout but not the target or 2) site an alternate signaling mechanism recruits F4/80+ macrophages to sites of new connection.

The experiments performed and analyzed in CHAPTER 3, focus on the signaling impact that the targeted vessel has on directing connection sites. As such, the observed bias that influences connection sites in the target vessel may not be the same bias that is used by sprouting EC to direct connection. As both sides of the connection are potentially able to contribute to selectivity, additional experiments to assess this potential are needed. Rather than mixing control sprouts with mosaic target vessels, sprouts containing KD of either complete or isoform selective Flt1 KD will be used. This will address whether Flt1 within the sprout is used in the identification of connection sites in the target vessel. The evaluated bias for transient
contact and stable connection will determine if these manipulations impact the regulation of vessel anastomosis.

There are two candidate pathways for macrophage recruitment during anastomosis. First, Notch1 is reported to both recruit macrophages to vessels and activated at branch points during retinal angiogenesis in mice (Outtz et al. 2011). As high Notch1 signal is coincident with high Flt1 in EC, this is a prime candidate for the signaling, which recruits and educates macrophages at sites of connection. While full over-expression of Notch1 does not support sprouting angiogenesis (Larrivee et al. 2012; Chappell et al. 2013), a mosaic culture system in vivo using zebrafish could be used to address whether this signaling recruits macrophages to connections. Additionally, the myeloid lineage is reportedly recruited to angiogenic vessels by non-canonical Wnt signaling (Stefater et al. 2011). Intriguingly, the Wingless-related integration site (Wnt) signaling is a positive recruiter and is actively suppressed by Flt1 expression. Again, this association fits with the association between low mFlt1 and connection sites. Should the Notch signaling not be implicated, this is the next candidate for investigation. The sequences for the various Wnt players, notably the homologues to Wnt5a and Wnt11 which were previously implicated, are mapped in the zebrafish (Heisenberg et al. 2000; Huang et al. 2011). Examining the role of these signaling pathways in zebrafish will take advantage of the macrophage reporter line and previously identified anastomotic pathways (Fantin et al. 2010). Combining the reporter lines for vessels and macrophages with knockdown of each Wnt candidate and heatshock over-expression of Notch1 will permit analyses of macrophage presence at the site of blood vessel anastomosis.

These experiments, combined with further analysis of zebrafish vessel anastomosis, should generate a complete picture of the various components that are implicated in connection.
data should link the EC signaling that is newly demonstrated to direct connections and the supportive or directive role of macrophages.

c. Applications for anastomotic regulation in disease states

Failure of the vascular and circulatory system is implicated in a host of diseases, including macular degeneration, heart failure, pathogenesis and recovery from stroke and ischemia, and solid tumor growth and metastasis (Carmeliet 2005). Of these, Flt1 has been implicated in stroke incidence and solid tumor growth and pathogenesis. Alterations in Flt1 levels result in de-regulated VEGF-A signaling, which leads to aberrant vessel growth. Misregulated blood vessel growth is strongly implicated in the pathogenesis and the development of tumor blood vessels has been identified as one of the hallmarks of cancer (Khurana et al. 2005; Hanahan & Weinberg 2011). Meta-analysis of multiple cancer types found that expression of PlGF in solid tumors caused overall patient survival to decrease 1.69 fold (Meng et al. 2015). As Flt1 is the activating receptor for PlGF in the endothelium, involvement of Flt1 in progression of these cancers is presumed. In fact, when PlGF-expressing tumor cells are injected into mice, strong tumor-directed angiogenesis is observed that is decreased upon deletion of Flt1 (Hiratsuka et al. 2001). As this ligand-receptor binding interaction involves the mFlt1 isoform over the sFlt1 isoform, the recent data gains greater implications. We demonstrate that mFlt1 receptor presence dictates the involvement of a given cell in forming new conduits. This suggests potential therapeutic advantages for targeting the PlGF/mFlt1 axis of signaling in pathological angiogenesis. As the signaling contribution of Flt1 is limited and non-essential during developmental angiogenesis (Hiratsuka et al. 1998), is highly selective and based upon my data could dramatically impact the formation of new conduits.
The classic pathological condition in which anastomosis is considered are the collateral vessels. These vessels are unused conduits that only carry flow when the preferential route is occluded, through a plaque, ischemia, or experimentally using ligations. Many are thought to form at birth, but there is debate regarding whether new collaterals are generated in response to ischemic stress. The small diameter and number of these collaterals is correlated with survival after ischemia or cardiac events (Seiler et al. 2013). Collateral vessels are believed to be established early in development and manipulations in Notch1/Dll4 alter the density of pial collaterals (Cristofaro et al. 2013). As Notch signaling is strongly connected to VEGF-A signaling in the endothelium, consideration should be given to the impact of Flt1 on collateral vessels, especially given the new information regarding the impact of Flt1 on directing connections in blood vessels. To being with, investigation and quantification of the collaterals in mouse vessels with flt1/- and those with the tyrosine dead mutant, flt1Δ/Δ, are needed. Given the available in vitro data there should be a notable increase in the collateral vessels when Flt1 is decreased that may also be true in the kinase-dead mutant. Should a difference exist, further testing of the response of these mice to further ischemic challenges, such as surgical ligation, would determine if the altered collateral state has pathological implications.

As stroke and solid cancers present a large public health concern, understanding the role of Flt1 as an anastomotic regulatory component is of interest. These avenues of research would tie the novel observations found here with clear clinical directions and therapeutic targets.

III. Perspective

Despite the breadth of knowledge on VEGF-A ligand and its receptors, the known therapeutics demonstrate limited efficacy in solid tumors (Weis & Cheresh 2011). As exemplified here, there are still undiscovered roles for VEGF-A signaling in regulated
angiogenesis. VEGF-A signaling has been clearly shown to regulate the initiation of both vasculogenesis and angiogenesis (Carmeliet et al. 1996; Shalaby et al. 1997), however a role in anastomosis was previously unreported. The implications of varied responses depending upon the angiogenic stage of the vessels may help to explain, the incomplete treatment responses. Identifying temporal cues that govern the varying stages of angiogenesis may allow selective and specific targeting of inappropriate angiogenesis.
IV. Figures

A

interaction unsupported

CONTACT

interaction supported

CONNECTION

reduced protrusions & High mFt1 Expression

Low mFt1 Expression & increased protrusions

B

VEGF + Flk1

PLCγ or AKT

phosphorylation site

mFt1

VEGF ligand
Figure 5.1: Schematic of mFlt1 regulation during blood vessel anastomosis.

(A) Schematic of vessel interactions that result in transient contact, which are not correlated with low mFlt1, and stable connection, which are correlated with low mFlt1. (B) The potential signaling roles of mFlt1 during connection. mFlt1 is known to act as a ligand sink to block VEGF-A binding to Flk1. Alternately, Flt1 preferentially activates intracellular signaling in some published reports, and therefore could be directly signaling for cellular changes which prohibit stable connections. Legends are included within each schematic.
V. REFERENCES


Cristofaro, B. et al., 2013. Dll4-Notch signaling determines the formation of native arterial collateral networks and arterial function in mouse ischemia models. *Development*, 140(8), pp.1720–1729.


Ho, V.C. et al., 2012. Elevated vascular endothelial growth factor receptor-2 abundance contributes to increased angiogenesis in vascular endothelial growth factor receptor-1-deficient mice. *Circulation*, 126(6), pp.741–752.


Raich W.B., Agbunag C., Hardin J., 1999. Rapid epithelial-sheet sealing in the *Caenorhabditis*


