VEGF REGULATES CENTROSOME DUPLICATION AND DIVISION ORIENTATION IN ENDOTHELIAL CELLS OF DEVELOPING BLOOD VESSELS

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ABSTRACT

Sarah Maria Taylor: VEGF REGULATES CENTROSOME DUPLICATION AND DIVISION ORIENTATION IN ENDOTHELIAL CELLS OF DEVELOPING BLOOD VESSELS (Under the direction of Victoria L Bautch)

Blood vessel formation is critical for vertebrate development and is required for the progression of many diseases, including cancer. Thus, understanding how blood vessels form and function is a necessary prerequisite to treating and preventing human disease. Vascular Endothelial Growth Factor-A (VEGF) activates signaling cascades in endothelial cells to promote survival, migration and proliferation; and elevated VEGF signaling yields overgrown, dysfunctional vessels. Tumor vessels experience high VEGF signaling and are abnormal in many ways. Of interest, tumor vessels have excess centrosomes and display aneuploidy; and they are tortuous and over-grown. I hypothesized that these abnormalities result from elevated VEGF signaling. I used developmental models of elevated VEGF signaling to test my hypothesis. I showed that VEGF gain-of-function flt-1⁻/⁻ embryonic stem (ES) cell-derived vessels and in vivo yolk sac vessels display centrosome over-duplication and aneuploidy. Moreover, VEGF signals through MEK/ERK and AKT to cyclin E/Cdk2 to promote centrosome over-duplication. Interestingly, cells with excess centrosomes are enriched at the leading edge of in vitro scratch wounds, indicating that endothelial cells with excess centrosomes have a migratory advantage. I also wondered if elevated VEGF signaling affects endothelial cell division orientation...
to disrupt proper vascular morphogenesis. In collaboration with Dr. Gefei Zeng, I showed that endothelial cell divisions are normally oriented to increase the long axis of developing vessels in ES cell-derived vessels and in vivo in neonatal retinal vessels. Furthermore, \textit{flt-1}\textsuperscript{-/-} endothelial cell divisions are randomly oriented compared to wildtype divisions. Randomized division orientations lead to dysmorphogenesis in other tissue types and it is possible that randomized endothelial cell divisions contribute to vascular dysmorphogenesis. My data describe new roles for VEGF signaling during developmental blood vessel formation and suggest novel mechanisms as to how pathological vessels, such as tumor vessels, become abnormal.
For Mom and Dad
ACKNOWLEDGEMENTS

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<th>Description</th>
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<tr>
<td>aPKC</td>
<td>Atypical Protein Kinase C</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>bFGF-2</td>
<td>basic Fibroblast Growth Factor-2</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
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<tr>
<td>Cdk2</td>
<td>Cyclin-dependent Kinase-2</td>
</tr>
<tr>
<td>CS</td>
<td>Chromosome</td>
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<tr>
<td>CycE KD</td>
<td>cyclin E Knockdown</td>
</tr>
<tr>
<td>Daam</td>
<td>Dishevelled-Associated Activator of Morphogenesis</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Dsh</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>E9.5</td>
<td>Embryonic Day 9.5</td>
</tr>
<tr>
<td>EBs</td>
<td>Embryoid Bodies</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced Green Fluorescence Protein</td>
</tr>
<tr>
<td>EGM-2</td>
<td>Endothelial Growth Media-2</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Regulated Kinase</td>
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<tr>
<td>ES</td>
<td>Embryonic Stem</td>
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<tr>
<td>EV</td>
<td>Empty Vector</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<td>Flt-1</td>
<td>FMS-Like Tyrosine Kinase-1</td>
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<td>Frizzled</td>
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<td>Gap 1</td>
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<td>Gap 2</td>
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<td>HDAC</td>
<td>Histone Deacetylase</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
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<tr>
<td>HNE</td>
<td>4-Hydroxynonenal</td>
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<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
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<tr>
<td>I</td>
<td>Inhibitor</td>
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<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
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<tr>
<td>LGN</td>
<td>Leu-Gly-Asn Repeat-Enriched Protein</td>
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<tr>
<td>M</td>
<td>Mitosis</td>
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<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<td>MDCK</td>
<td>Madin-Darby Canine Kidney</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-Activated Protein Kinase Kinase</td>
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<tr>
<td>mflt-1</td>
<td>Membrane-bound Flt-1</td>
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<tr>
<td>MTOC</td>
<td>Microtubule Organizing Center</td>
</tr>
<tr>
<td>NPM</td>
<td>Neucleophosmin-1</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NRP-1</td>
<td>Neuropilin-1</td>
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<tr>
<td>NuMA</td>
<td>Nuclear Mitotic Apparatus</td>
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<tr>
<td>P-</td>
<td>Phosphorylated</td>
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<tr>
<td>PLGF</td>
<td>Placental Growth Factor</td>
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<tr>
<td>p21</td>
<td>Tumor Protein 21</td>
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<tr>
<td>p53</td>
<td>Tumor Protein 53</td>
</tr>
<tr>
<td>PAR</td>
<td>Partitioning Defective</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
</tr>
<tr>
<td>PCM</td>
<td>Pericentriolar Material</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar Cell Polarity</td>
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<tr>
<td>PECAM-1</td>
<td>Platelet Endothelial Cell Adhesion Molecule-1</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
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<tr>
<td>Poly</td>
<td>Polyploidy</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho Kinase</td>
</tr>
<tr>
<td>S</td>
<td>Synthesis</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>sflt-1</td>
<td>Soluble Flt-1</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short Hairpin Ribonucleic Acid</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethyl Rhodamine Isothiocyanate</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor-A</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>Vascular Endothelial Growth Factor Receptor-1</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>Vascular Endothelial Growth Factor Receptor-2</td>
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VEGFR-3  Vascular Endothelial Growth Factor Receptor-3
Wnt    Wingless/Mammary Tumor Virus Integration
WT     Wildtype
CHAPTER I
GENERAL INTRODUCTION

Proper blood vessel formation and function is critical for the survival of vertebrate organisms, and aberrant blood vessel function can result in tissue death. Indeed, the top three causes of death in the United States of America (heart disease, cancer and stroke) either result from dysfunctional existing blood vessels (heart disease and stroke) or depend on new blood vessel formation for disease progression (cancer).\(^1\) Thus, understanding how blood vessels form and function is a necessary prerequisite to treating and preventing several debilitating human diseases. The data described herein focus on understanding the morphological and cellular processes that occur during normal blood vessel formation, as well as how these processes are perturbed when developing blood vessels are exposed to high Vascular Endothelial Growth Factor-A (VEGF) signaling. Because VEGF is a critical regulator of blood vessel formation during embryonic development and pathogenesis, these data are a significant contribution toward understanding normal and pathological vascular biology.

A. Mechanisms of blood vessel formation

Early blood vessel formation occurs via two sequential processes: vasculogenesis and angiogenesis. Vasculogenesis is the process whereby
mesoderm-derived angioblasts differentiate into endothelial cells and coalesce to form primitive blood vessels. Angiogenesis is the process by which existing blood vessels branch and form new vessel connections to yield a branched vascular plexus. During angiogenesis, vessels become lumenized and the onset of blood flow initiates vascular remodeling. During remodeling, the existing plexus reorganizes so that large vessels connect to progressively smaller vessels to ultimately reach every cell in the body. My research addresses regulation of angiogenesis using developmental models of blood vessel formation.

Blood vessel formation occurs in response to growth-promoting signaling proteins that are secreted by surrounding tissues. Such signaling proteins include, but are not limited to, basic Fibroblast Growth Factor-2 (bFGF), Bone Morphogenetic Protein (BMP), Transforming Growth Factor beta (TGF-β) and, perhaps the most critical vascular signaling molecule, VEGF.

B. Vascular Endothelial Growth Factor-A

The requirement for Vascular Endothelial Growth Factor-A (VEGF) signaling during blood vessel formation is evident by the fact that deletion of VEGF or VEGF receptors is lethal due to severe embryonic vascular defects. Endothelial cells express numerous VEGF receptors, including Flk-1 (VEGFR-2) and Flt-1 (VEGFR-1). Flk-1 contains an extracellular VEGF-binding domain, a transmembrane domain and a tyrosine kinase domain. It binds VEGF as a homodimer or as a Flk-1/Neuropilin-1 heterodimer. VEGF/Flk-1 binding initiates protein signaling cascades in endothelial cells to promote endothelial survival and vascular permeability (AKT signaling), migration (p38MAPK signaling) and
proliferation (MEK/ERK signaling). Thus, VEGF signaling through Flk-1 promotes angiogenesis. Consistent with this model, Vegfa deletion or heterozygosity, or flk-1 deletion result in avascular embryos and early embryonic death.

The VEGF receptor Flt-1 is expressed by endothelial cells and is present as two differentially spliced isoforms, membrane-bound Flt-1 (mFlt-1) and soluble/secerted Flt-1 (sFlt-1). mFlt-1 is similar to Flk-1 with regard to protein structure. However, during development, VEGF binding to mFlt-1 does not elicit a downstream signaling response that is required for blood vessel formation. Likewise, VEGF binding to sFlt-1 does not elicit a downstream signaling response in endothelial cells, because sFlt-1 contains only the extracellular Flt-1 domain and is secreted into the extracellular matrix (ECM).

Our lab and others proposed a model in which Flt-1 acts as a VEGF sink to inhibit VEGF/Flk-1 binding. In this scenario, endothelial cells can self-regulate the level of Flk-1 activation by VEGF. Consistent with Flt-1’s function as a VEGF sink, deletion of flt-1 is a VEGF gain-of-function perturbation, and results in dramatic vascular overgrowth and embryonic death, due to increased endothelial proliferation and decreased vessel branching. Work from our lab showed that restoration of sFlt-1 function in flt-1-/- vessels rescues vascular morphology more efficiently than mFlt-1 restoration, suggesting that Flt-1-mediated sequestration of VEGF protein in the ECM may be more effective than sequestration at the endothelial cell membrane.

VEGF-A is primarily expressed as three differentially-spliced isoforms: VEGF120, VEGF164 and VEGF188, with superscript designations referring to the
The number of amino acids present in the mouse protein isoforms. The isoforms differ in the number of heparin sulfate proteoglycan-binding domains present, and thus the ability to bind heparin in the ECM. VEGF$^{120}$ does not contain heparin-binding domains and is thought to diffuse the farthest distance from VEGF-secreting cells. VEGF$^{188}$ contains two heparin-binding domains and is thought to have very limited diffusion capability because heparin binding “tethers” VEGF$^{188}$ near VEGF-secreting cells. VEGF$^{164}$ contains one heparin-binding domain and is thought to diffuse at an intermediate distance relative to VEGF$^{120}$ and VEGF$^{188}$. Thus, simultaneous expression of all three VEGF isoforms is thought to contribute to the establishment of a VEGF gradient, which endothelial cells sense (based on localized Flk-1 activation) and migrate toward.

Interestingly, the three VEGF isoforms are rarely expressed at equal levels. In fact, genetically manipulated mice that express only one of the three isoforms are viable, save for VEGF$^{120/120}$-expressing mice, which die soon after birth from cardiac abnormalities. Vascular phenotypes in single VEGF isoform-expressing mice are apparent in the mouse neonatal retina. VEGF$^{120/120}$ retinal vessels are thick and less branched compared to wildtype vessels. This phenotype is thought to result from the inability of endothelial cells to properly sense a VEGF gradient due to high VEGF$^{120}$ diffusion. Conversely, VEGF$^{188/188}$ retinal vessels are thin and highly branched compared to wildtype vessels. This phenotype is thought to result from numerous small VEGF gradients that are established due to VEGF$^{188}$ heparin “tethering”. VEGF$^{165/165}$ retinal vessels are similar to wildtype vessels due to intermediate diffusion of VEGF$^{165}$ protein. The phenotypes of single isoform-expressing mice are consistent with isoform
expression patterns in wildtype animals. For example, in lungs, where VEGF is the dominantly expressed isoform, vessels are thin and highly branched.

In addition to the VEGF-A/(flk-1/flt-1) mode of endothelial cell signaling, there are a number of other VEGF ligands and receptors which regulate a variety of cellular processes. For example, VEGF-B and PIGF bind Flt-1 in macrophages to affect migration. VEGF-C and -D bind Flk-1 and Flt-4 to regulate lymphangiogenesis. A number of parapox virus open reading frames share sequence homology with traditional VEGFs and these are grouped together as VEGF-E. VEGF-E is not present in vertebrates, but can bind Flk-1. NRP-1 and -2 can act as co-receptors for VEGF-A, -C and -D. Each VEGF/VEGFR/NRP combination is important for a specific regulatory function in a specific cell type. I am primarily interested in investigating VEGF-A/(Flk-1/Flt-1) interactions because these ligands/receptors seems to be the most important for regulating blood vessel formation.

In addition to critical roles in normal angiogenesis, altered VEGF signaling is also causal for a number of pathological processes. Cancer is perhaps the most well-characterized disease that requires VEGF signaling. Tumors can only grow a few millimeters in size until they must recruit blood vessels to obtain oxygen and nutrients. To recruit new vessels, tumor cells secrete very high levels of VEGF. High VEGF signaling causes nearby established blood vessels to become angiogenic and migrate toward the VEGF signal (the tumor cells). Once a tumor is vascularized, it can grow and spread to other organs via the blood vessels, a process termed metastasis.
For many years, it was assumed that tumor endothelial cells were genetically the same as normal endothelial cells. This assumption lead to the development of a number of anti-tumor therapies aimed at inhibiting tumor angiogenesis. The majority of anti-angiogenesis therapies used to date are anti-VEGF therapies that are surprisingly ineffective at inhibiting tumor angiogenesis in long-term clinical trials.\textsuperscript{21} Recently, a number of tumor vessel abnormalities were described. It is possible that the ineffectiveness of anti-VEGF treatments is due to these tumor vessel irregularities.

Tumor vessels are abnormal in a number of ways. They are abnormal morphologically, in that there is little to no hierarchical organization (large vessels vs. capillaries) and the vessels are abnormally dilated.\textsuperscript{21-23} At the cellular level, tumor endothelial cells display increased permeability (leakiness), have excess centrosomes, and exhibit aneuploidy compared to normal endothelial cells.\textsuperscript{21-25} Elevated VEGF signaling promotes vessel permeability and abnormal vessel morphology, indicating that elevated VEGF signaling in tumors may lead to the abnormalities observed in tumor vessels. My work describes roles for VEGF signaling in regulating two critical cellular processes: centrosome duplication and cell division orientation. Elevated VEGF signaling disrupts both processes, resulting in vascular abnormalities. These novel discoveries provide insight into how elevated VEGF signaling, such as that found in tumors, could lead to vascular irregularities during development and disease.
C. Overview of centrosome duplication

In 1888, Theodor Boveri first described the centrosome as the “special organ of cell division”.\textsuperscript{26} Using a light microscope, he was able to determine that the centrosomes bundled microtubules to regulate chromosome allocation during mitosis. Later, Boveri used sea urchin embryos to demonstrate that embryos with excess centrosomes displayed aneuploidy and abnormal development. He also speculated that aneuploidy would contribute to cancer.\textsuperscript{26} Although Boveri observed how important proper centrosome number is to cellular fidelity over 100 years ago, we are only recently beginning to understand how centrosome number is regulated.

Centrosomes are cellular organelles that act as the microtubule organizing center (MTOC) during interphase and mitosis. Centrosomes are composed of two orthogonal barrel-shaped microtubular structures called centrioles, which are surrounded by a cloud of microtubule-nucleating proteins, termed pericentriolar material (PCM). Just prior to mitosis, two closely associated centrosomes are present in the cell. Upon mitotic entry, the centrosomes separate and migrate to opposite ends of the cell to form the spindle poles. Following mitosis and cytokinesis, each daughter cell contains one centrosome. The centrosome duplicates during S phase of the cell cycle to ensure that only two centrosomes are present during the subsequent mitosis.\textsuperscript{27}

Centrosome duplication, like cell division, is regulated by cell cycle cues. Genetic knockout of the tumor suppressor gene p53 results in centrosome over-duplication in cultured mouse fibroblasts, and p53 loss of function is associated with centrosome over-duplication \textit{in vivo} in tumor cells.\textsuperscript{28} During the G1/S cell
cycle transition and throughout S phase, p53 is activated in response to DNA damage. Activated p53 blocks cell cycle progression via transcriptional regulation of cell cycle regulating proteins, thus allowing DNA repair machinery sufficient time to repair damaged DNA.\textsuperscript{28} During the G1/S cell cycle transition, activated p53 promotes transcription of p21. p21 inhibits the cell cycle promoting complex cyclin E/Cdk2; thus, p53 is an inhibitor of cyclin E/Cdk2.\textsuperscript{28} Interestingly, cyclin E overexpression, like p53 loss-of-function, leads to centrosome over-duplication in mouse fibroblasts, and cyclin E is often overexpressed in tumors that display centrosome over-duplication.\textsuperscript{29-33}

To promote centrosome duplication, cyclin E/Cdk2 phosphorylates a protein called Nucleophosmin-1 (NPM).\textsuperscript{34} Prior to cyclin E/Cdk2-mediated NPM phosphorylation, NPM is located at the centrosome, where it is thought to stearically inhibit centrosome duplication. Upon phosphorylation, NPM leaves the centrosome and translocates to the nucleus, where it is involved in ribosome biogenesis. Inhibition of cyclin E/Cdk2-mediated NPM phosphorylation inhibits centrosome duplication, and genetic loss of NPM leads to centrosome over-duplication.\textsuperscript{34}

In endothelial cells, integrin activity seems to be important for centrosome duplication. Focal Adhesion Kinase (FAK) deletion or expression of a Serine-732 non-phosphorylatable version of FAK leads to centrosome over-duplication in cultured endothelial cells.\textsuperscript{35} A number of additional molecules play roles in centrosome duplication, including Separase and Polo-like Kinase 4; however, if and how these molecules regulate cyclin E/Cdk2 activity is unclear.\textsuperscript{36}
Centrosome over-duplication is observed in many tumors and is typically accompanied by aneuploidy. Tumor blood vessels also display aneuploidy and have excess centrosomes. This suggests that the presence of excess centrosomes in tumor endothelial cells contributes to tumor blood vessel dysfunction. Cells containing excess centrosomes can form aberrant spindles during mitosis, resulting in aneuploidy. Aneuploid cells are typically abnormal due to changes in gene expression levels. Additional abnormalities associated with excess centrosomes have not been described. Here, I show that VEGF signals through MEK/ERK and AKT to cyclin E/Cdk2 to promote endothelial centrosome duplication, and elevated VEGF signaling promotes centrosome over-duplication. I also show that endothelial cells with excess centrosomes form aberrant mitotic spindles and display aneuploidy and abnormal migration. These data are a significant contribution toward understanding how elevated VEGF signaling contributes to centrosome over-duplication and vascular dysfunction.

D. Overview of cell division orientation

Regulated cell division orientation is important for proper morphogenesis in a number of developing tissues. For example, elongation of the zebrafish body axis during gastrulation and extension of the avian primitive streak are dependent on regulated division orientation. Additionally, shaping of the neural tube in avian and mouse models is associated with oriented division. In these cases, oriented cell divisions increase the length of the axis in which they divide to establish tissue shape. When division orientation is randomized, tissue morphology is abnormal. In the mature mouse aorta and in vitro, endothelial cell
division orientation is established in response to blood flow, such that the cleavage plane forms perpendicular to the direction of flow;\(^{41}\) however, it is unclear if endothelial cell division orientation is regulated independent of flow by other signaling inputs.

Cell division orientation is regulated by a number of mechanisms. One signaling pathway that regulates division orientation is planar cell polarity (PCP) signaling. PCP signaling was described early on in \textit{D. melanogaster} as the signaling pathway responsible for polarizing wing hairs, such that all hairs are in the same location and face the same direction in each cell.\(^{42}\) PCP regulates symmetric divisions during zebrafish body elongation and asymmetric divisions in \textit{C. elegans}, fly and mouse.\(^{42}\) In some cases, the PCP pathway is initiated following Frizzled (Frz) receptor activation by a Wnt ligand; however, Wnt ligand activity is not necessary. Activated Frz interacts with Disheveled (Dsh) and Daam proteins at the cell membrane to initiate a protein signaling cascade that modulates Jun Kinase (JNK) and Rho Kinase (ROCK).\(^{42}\) JNK and ROCK are thought to regulate cytoskeletal activity to affect division orientation.\(^{42}\) Whether or not PCP signaling is important in regulating vascular morphogenesis is unknown.

Thery, \textit{et al.} (2005) performed very elegant \textit{in vitro} experiments which demonstrated that extracellular matrix patterning can regulate cell division orientation. Briefly, the group micro-patterned glass slides with varying patterns of the ECM protein Fibronectin and placed a single cell on each pattern to assess division orientation. They showed that cell shape during interphase (as regulated by the Fibronectin pattern) directly regulates the orientation of cell division during
mitosis. There is no known role for the ECM in regulating endothelial cell division orientation, but it is conceivable that ECM modulation might affect endothelial cell division orientation. In collaboration with Gefei Zeng, I showed that endothelial cell divisions are oriented to increase the length of developing blood vessels in the absence of blood flow. Elevated VEGF signaling leads to randomized endothelial cell division orientation and vascular dysmorphogenesis. Furthermore, we showed that PCP signaling may be involved in regulating endothelial cell division orientation.
E. References


CHAPTER II

ANGIOGENIC FACTORS REGULATE CENTROSONE DUPLICATION IN ENDOTHELIAL CELLS OF DEVELOPING BLOOD VESSELS

The majority of the work described in this chapter was performed by me. Cdk2 activity assays were performed in collaboration with Jean Cook and Kathleen Nevis at UNC. An undergraduate researcher, Hannah Park, who I mentored, assisted with western blotting. Chromosome number analysis was performed by Karyologic, Inc. Substantial intellectual input was provided by Steve Rogers and Greg Rogers at UNC and the University of Arizona, respectively.
A. Introduction

Blood vessels supply both normal and diseased tissues with the oxygen and nutrients necessary for growth and survival. Thus, proper blood vessel formation and expansion is critical for normal development, and for the progression of diseases such as cancer. Blood vessel networks expand via angiogenesis, a process whereby vessels form by sprouting migration from pre-existing vessels. Angiogenic expansion requires regulated endothelial cell division. Endothelial cell division in developing vessels, as in other cells, is a tightly regulated process ensuring that DNA goes through only one round of replication per cell cycle. The centrosome that comprises the microtubule organizing center during interphase also replicates only once per cell cycle, to provide two centrosomes that facilitate mitotic spindle assembly during mitosis. Cell cycle regulation is well characterized in terms of timing, checkpoints, and regulation of DNA replication. However, regulation of centrosome duplication is less well understood in general, and even less is known about how this critical cellular process is regulated in endothelial cells. Centrosome over-duplication is associated with elevated cyclin E/Cdk2 activity in other cell types; loss of p53, which can inhibit cyclin E accumulation, also promotes centrosome over-duplication. Tumor endothelial cells have excess centrosomes and are aneuploid, but the signaling pathways responsible for this phenotype are unknown.

Endothelial cell proliferation and migration are normally tightly regulated to form proper vessels, and angiogenic factors, such as Vascular Endothelial Growth Factor-A (VEGF), play a central role in these processes. Developing vessels express several VEGF receptors, including Flk-1 (VEGFR-2) and Flt-1
(VEGFR-1). Genetic loss of VEGF pathway components leads to vessel perturbations and embryonic lethality, but the phenotypes differ. Homozygous loss-of-function for flk-1 or heterozygosity for Vegfa results in dramatically reduced blood vessel formation, since VEGF binding to Flk-1 positively activates downstream signaling that promotes endothelial proliferation, migration and survival. In contrast, loss of flt-1 leads to vessel over-growth and dysmorphogenesis that results from both increased endothelial cell proliferation and decreased vessel branching. We and others have shown that Flt-1 functions developmentally as a VEGF sink to negatively modulate VEGF-mediated signaling through Flk-1, and the flt-1/- mutation thus behaves like a gain-of-function perturbation in VEGF signaling. Tumor endothelial cells are exposed to elevated VEGF levels produced by tumor cells, and tumor vessels also express low levels of Flt-1 compared to normal vessels, suggesting that multiple inputs promote excess VEGF signaling in tumor endothelial cells.

We hypothesized that signaling of angiogenic factors such as VEGF regulates centrosome number in endothelial cells, and that elevated VEGF mis-regulates endothelial cell centrosome duplication. Our hypothesis predicts that endothelial cells with aberrant centrosome numbers are not restricted to tumor vessels, but are found more generally in any environment with elevated endothelial VEGF signaling. Here, we show that elevated VEGF or FGF signaling leads to excess centrosomes in endothelial cells. This mis-regulation utilizes both MEK/ERK and AKT signaling pathways downstream of VEGF-A to enhance cyclin E/Cdk2 activity. Furthermore, endothelial cells with excess centrosomes survive and form aberrant spindles during cell division, and vessels exposed to high VEGF
signaling have elevated levels of endothelial cell aneuploidy. These are the first data to link regulation of endothelial cell centrosome duplication to upstream VEGF signaling, and they highlight a novel mechanism that is likely to contribute to the dysfunction of vessels exposed to elevated angiogenic factor signaling.

B. Materials and methods

Cell lines, yolk sacs and VEGF manipulations

Wildtype (WT) and flt-1−/− mouse embryonic stem (ES) cells were differentiated for eight days as described previously.22,23 For analysis of differentiated and dissociated ES cell cultures, WT and flt-1−/− ES cell lines expressing H2B-eGFP downstream of the PECAM-1 promoter/enhancer were used.24 Cultures were dissociated for 20 min in trypsin-EDTA (Gibco 25300, Carlsbad, CA, USA), strained to eliminate clumps (70 µm Nylon strainer, BD Biosciences 352350, San Jose, CA, USA), and plated on 0.1% gelatin-coated dishes for 18 hr prior to fixation. Human umbilical vein endothelial cells (HUVEC, Lonza Group Ltd., cc2519, Basel, Switzerland) were maintained in Endothelial Growth Medium-2 (EGM-2) as suggested by Lonza Group Ltd (cc-3162).

For centrosome counting experiments, HUVEC were cultured in low growth factor (EGM-2) medium, high growth factor (EGM-2 + 200 ng/mL growth factor) medium, or high growth factor medium + inhibitor. Supplemented growth factors included VEGF165 and FGF-2 (Peprotech 100-20 and 100-18B, Rocky Hill, NJ, USA). Inhibitors included U0126 (MEK inhibitor), AKT inhibitor, SB 203580 (p38 MAPK inhibitor), and Bisindolylmaleimide I (PKC inhibitor) (Calbiochem 662005, 124005, 559398, and 203290, Gibbstown, NJ, USA) and were applied at 5 µM,
10 mM, 10 µM and 10 µM, respectively. Medium was replaced daily for 4 or 10 days, and cells were maintained at 30-70% confluency.

For HUVEC division rate analysis, HUVEC were plated at 10^3 cells/dish and cultured overnight in normal medium. The next day (Day 0), cells were treated with low VEGF or high VEGF for 4 days, with medium replaced daily. Cell number was scored daily for 4 days. For signaling pathway analysis, HUVEC were serum-starved for 12 hr in Endothelial Basal Medium-2 (Lonza Group Ltd., cc-3156) supplemented with 0.1% fetal bovine serum (Gibco, 35-010-CV), then treated with low VEGF, high VEGF or high VEGF + inhibitor for 5 min, 1 hr or 12 hr. Prior to inhibitor treatments, cells were incubated in serum starvation media + inhibitor for 1 hr. For apoptosis experiments, HUVEC were cultured in high VEGF for 4 days with or without the addition of 20 µM 4-hydroxy Nonenal (HNE, Cayman Chemical, 32100, Ann Arbor, MI, USA) for the final 24 hr of the 4 day treatment, to induce apoptosis as described previously.25

For \textit{flt-1}^{-/-} embryonic yolk sac analysis, \textit{flt-1}^{+/+} mice were inter-crossed and embryos were harvested on E9.5. WT and \textit{flt-1}^{-/-} littermate yolk sacs were used for analysis after embryos were genotyped as described previously.15 Animal experiments were approved by the IACUC Committee at the University of North Carolina.

\textbf{Immunofluorescence and microscopy}

Differentiated ES cell cultures, cells dissociated from differentiated ES cell cultures, or HUVEC were fixed in ice cold 50% methanol/50% acetone for 5 min, and stained as described previously.22,23 Yolk sacs were fixed and stained as
described previously. Primary antibodies were raised against human protein sequences unless otherwise indicated and included rabbit anti-γ-tubulin (1:1000, Sigma T3559, St. Louis, MO, USA), mouse anti-pericentrin (1:1000, Abcam 28144, Cambridge, MA, USA), rabbit anti-nucleophosmin-1 (NPM) (1:500, Santa Cruz Biotechnology, sc-6013-R, Santa Cruz, CA, USA), rat anti-mouse PECAM-1 (1:1000, BD Pharmingen, 553370), rabbit anti-active Caspase 3 (1:500, Abcam Cat. #ab2302), rabbit anti-Caspase 3 (1:500, Abcam 44976), and rabbit anti-α-tubulin (1:500, Abcam 15246). Cy3-conjugated anti-γ-tubulin antibody was used for yolk sac labeling (1:250, Sigma C7604). Secondary antibodies were used at 1:250 and include goat anti-rat, donkey anti-rabbit, donkey anti-goat or goat anti-mouse Alexa 488 (Molecular Probes, A11006, A21206, A11055 and A11029, Carlsbad, CA, USA) and donkey anti-rabbit Alexa 594 (Molecular Probes, 21207). Cells were stained with the DNA dye DRAQ5 for 30 min at RT (1:1000, Biostatus Limited, DR50050, Leicestershire, UK). Confocal images were acquired using a Zeiss LSM 5 Pascal microscope. For flow cytometry analysis, HUVEC were treated with low or high VEGF for 4 days as described above, fixed and stained with propidium iodide, and fluorescence activated cell sorting (FACS) was performed as described.

**Western Blots and Cdk2 Activity Assay**

Western blot analysis was performed as previously described, with slight modifications. Briefly, HUVEC lysates were collected using Mammalian Cell Lysis Buffer (Fermentas, K0301, Burlington, Ontario, Canada) per product instructions and proteins were separated on a 10% SDS-polyacrylamide gel and
transferred to a polyvinylidene fluoride membrane (GE Healthcare, RPN303F, Hillsborough, NC, USA). Primary antibodies raised against human protein sequences were used and included rabbit anti-cyclin E (1:500, Santa Cruz Biotechnology, sc-481), goat anti-actin (1:500, Santa Cruz Biotechnology, sc-1615), rabbit anti-phospho-NPM (Thr199) (1:1000, Abcam, ab59353), rabbit anti-NPM (1:1000, Abcam, ab15440), rabbit anti-phospho-ERK1/2 (Thr202/Tyr204) (1:1000, Cell Signaling Technology, 9101S, Danvers, MA, USA), mouse anti-ERK 2 (1:1000, Santa Cruz Biotechnology, sc-154), rabbit anti-phospho-AKT (Ser473) (1:1000, Cell Signaling Technology, 4060S), and rabbit anti-AKT (1:1000, Cell Signaling Technology, 9272). Signal was detected with HRP anti-rabbit (GE Healthcare, NA934V), HRP anti-mouse (GE Healthcare, NA931V) or HRP anti-goat (Santa Cruz Biotechnology, sc2020), and imaged via enhanced chemiluminescence (GE Healthcare, RPN2132). For the Cdk2 activity assay, HUVEC were serum-starved and treated with low or high VEGF for 12 hr as described above. The Cdk2 activity assay was performed as described previously. Briefly, 12 hr lysates were subjected to immunoprecipitation with anti-Cdk2 (Santa Cruz Biotechnology, sc-163). Immune-complex kinase reactions were carried out in 25 µl kinase buffer containing 5 µg Histone H1 (Sigma, H4524), 1 µM ATP, and 5 µCi [γ-32P]ATP (Perkin Elmer, BLU002A250UC, Waltham, MA, USA) and incubated at 30°C for 30 min. After the reaction was stopped, proteins were separated on a 10% SDS-polyacrylamide gel, and gels were dried and autoradiographed.
**Lentivirus transfection**

Human cyclin E-targeted shRNA vectors were obtained from the Open Biosystems TRC1 shRNA pLKO1 vector library (Open Biosystems, Huntsville, AL, USA). Targeted sequences were TRCN0000045298, TRCN0000045299, TRCN0000045300, TRCN0000045301 and TRCN0000045302. Virus was produced in 293T cells and collected at 36-60 hr post-transfection at a minimum of 1x10^6 IU/mL. Cells were transfected with lentivirus for 6 hr at 37°C and then treated with low or high VEGF medium for 4 days as described above. Virus lacking a target sequence (empty vector) was used as a control.

**Chromosome number analysis**

Wildtype and flt-1^-/- ES cells were differentiated for 8 days, dissociated for 30-45 min in 2 mg/ml collagenase (Worthington 46S9287), and endothelial cells were isolated via magnetic bead isolation per product instructions (Invitrogen sheep anti-rat Dynabeads, 110.35). Rat anti-mouse PECAM-1 antibody was conjugated to magnetic beads for endothelial cell isolation (BD Pharmingen, #553370). Following isolation, endothelial cells were cultured overnight, then treated with 0.1 µg/ml colcemid (Invitrogen, #15210) for 12 hr to halt cells in metaphase. The cells were trypsinized, fixed in 1:3 MeOH: acetic acid and analyzed for chromosome number. Chromosome analysis was performed by KaryoLogic, Inc. of Research Triangle Park, NC, according to published protocols.29
**Scratch wound assay**

Cells exposed to low or high VEGF for 10 d were grown to ~80% confluency and a 1/8 inch scratch was made in the middle of the dish. Twenty-four hrs later, cells were fixed and centrosomes were antibody labeled as described above. Centrosome numbers in the cells at the leading edge of the migrating front were compared to centrosome numbers in “static” cells that were distant from the leading edge.

**Statistical Analysis**

The two-tailed Fisher’s Exact Test was used to determine statistical significance in all cases. Error bars represent standard deviation between experiments.

**C. Results**

**High VEGF signaling increases the frequency of endothelial cells with excess centrosomes in developing vessels**

Centrosome duplication is tightly regulated by cell cycle cues, and excess centrosomes can promote errors in chromosome segregation during mitosis, leading to the production of aneuploid daughter cells and aberrant cellular behaviors.\(^{30,31}\) During early G1, cells contain a single centrosome, composed of a mother-daughter centriole pair surrounded by pericentriolar material. By the G1/S transition, the two centrioles have separated, and each nucleates the growth of a new centriole. Centriole growth continues through S phase and early G2. At the onset of mitosis, centrosomes move to opposite ends of the cell and
initiate the formation of a bipolar spindle. After cytokinesis, each daughter cell contains one centrosome. Thus, cells containing greater than one centrosome in G1 or greater than two centrosomes thereafter have excess centrosomes (Fig. 2.1A).³

Murine endothelial cells isolated from xenograft tumors have an increased frequency of excess centrosomes, but the reason for this is unclear.⁶ Because tumor vessels are often exposed to high levels of angiogenic factors such as VEGF secreted from tumor cells, we hypothesized that the presence of excess centrosomes in tumor endothelial cells not unique to tumor endothelial cells, but is a general consequence of elevated VEGF signaling. Thus we asked whether loss of the VEGF receptor flt-1 led to excess centrosomes in endothelial cells of developing vessels, because flt-1⁻/⁻ vessels experience increased VEGF signaling.¹⁷-¹⁹ We first counted centrosome numbers in WT and flt-1⁻/⁻ mutant vessels that form during mouse embryonic stem (ES) cell differentiation. Pluripotent ES cells induced to differentiate give rise to a variety of cell types, including 3-dimensional, lumenized vessels in a VEGF signaling context that mimics developmental scenarios.²³,³² Differentiated flt-1⁻/⁻ ES cell cultures displayed dramatic vascular overgrowth compared to WT ES cell cultures, and we observed endothelial cells with excess centrosomes in flt-1⁻/⁻ ES cell-derived vessels (Fig. 2.2A-D). To rigorously score centrosome numbers in ES cell-derived endothelial cells, we dissociated differentiated ES cell cultures carrying a H2B::eGFP transgene linked to a PECAM-1 enhancer-promoter that is expressed in endothelial cells.²⁴ After a short attachment period, centrosomes were labeled with anti-γ-tubulin, and centrosome numbers in H2B::eGFP-
expressing cells were quantified. Anti-γ-tubulin antibody co-localized with several distinct centrosomal markers in endothelial cells, confirming the specificity of the staining (Fig. 2.1B-J). We found that endothelial cells from \textit{flt-1}−/− ES cell-derived vessels had a significantly increased frequency of excess centrosomes compared to endothelial cells from WT vessels (Fig. 2.2E-G).

We next sought to determine if \textit{flt-1}−/− vessels had excess centrosomes \textit{in vivo}. We counted centrosomes in endothelial cells of WT and \textit{flt-1}−/− yolk sac vessels at embryonic day 9.5 (E9.5). Like \textit{flt-1}−/− mutant ES cell-derived vessels, \textit{flt-1}−/− yolk sac vessels display dysmorphogenesis and a vascular overgrowth phenotype.\textsuperscript{14} Consistent with our findings in ES cell-derived vessels, \textit{in vivo} \textit{flt-1}−/− mutant yolk sac vessels had significantly more endothelial cells with excess centrosomes than WT vessels (Fig. 2.2H-J). Together, these data show that loss of \textit{flt-1} results in an increased frequency of endothelial cells with excess centrosomes.

Because \textit{flt-1}−/− endothelial cells experience abnormally high VEGF signaling, we hypothesized that elevated VEGF signaling promotes mis-regulation of centrosome duplication in endothelial cells. We directly assessed the effects of elevated VEGF signaling on endothelial cells by culturing human umbilical vein endothelial cells (HUVEC) in low VEGF (Endothelial Growth Medium-2, Lonza Group Ltd.) or high VEGF (EGM-2 + 200 ng/mL VEGF165) for 4 days, followed by centrosome number analysis. We found that treatment of HUVEC with high VEGF resulted in a significant increase in the frequency of cells with greater than two centrosomes compared to low VEGF-treated controls (Fig. 2.2K-M). These data are consistent with a model whereby elevated VEGF signaling, due to either
loss of \textit{flt-1} or exposure to excess VEGF, leads to excess centrosomes in proliferating endothelial cells.

To determine whether the observed centrosome phenotype was unique to elevated VEGF signaling, or a more general feature of elevated angiogenic factor signaling, we assessed centrosome duplication in the presence of elevated Fibroblast Growth Factor-2 (FGF-2). HUVEC incubated in high FGF-2 had a significant increase in the frequency of cells with excess centrosomes that was similar to the frequency seen with high VEGF treatment (Fig. 2.3A). However, incubation in both high VEGF and high FGF-2 did not lead to a further increase in the frequency of endothelial cells with excess centrosomes, suggesting that the growth factor effects are not additive. Since both growth factors activate similar signaling pathways in endothelial cells, this result indicates that these pathways are likely activated at maximum capacity with the addition of one growth factor.

\textit{The VEGF-induced excess centrosome phenotype in endothelial cells is not downstream of elevated proliferation or cytokinesis defects}

To begin to determine the mechanism(s) responsible for the VEGF-induced centrosome over-duplication phenotype, we first determined whether HUVEC exposed to high VEGF proliferated more during the time course than controls, since more cell cycle transits might produce centrosome defects in a non-specific manner. The number of cell doublings was independent of VEGF levels over the time course. Cells divided every 26.5 hr on average in low VEGF and every 26.2 hr on average in high VEGF, suggesting that the number of centrosome duplication cycles that occurred during the time course was equivalent in the two
treatments (Fig. 2.3B). “Low VEGF” is the manufacturers recommended medium to support HUVEC growth and expansion, so it is likely that endothelial cell division is optimized under these conditions. These results show that the excess centrosome defect observed in high VEGF-treated endothelial cells is not due to increased cell proliferation, and they suggest that the elevated VEGF levels in “high VEGF” treatment selectively affect centrosome numbers.

We reasoned that excess centrosomes could result from direct mis-regulation of pathways that regulate centrosome duplication, or they could be downstream of a cytokinesis defect. Failure to complete cytokinesis during cell division leads to daughter cells with two centrosomes that duplicate in the next S phase to produce cells with 4 centrosomes. To test whether high VEGF signaling promotes incomplete cytokinesis in endothelial cells, we analyzed DNA content in HUVEC incubated in low or high VEGF conditions, because polyploidy is an expected consequence of incomplete cytokinesis. FACS sorting for DNA content showed that high VEGF-treated cells had similar levels of polyploid cells compared to low VEGF-treated cells (Fig. 2.4A-B). We also analyzed the distribution of centrosome number in endothelial cells with greater than two centrosomes, as incomplete cytokinesis is predicted to result in a preponderance of aberrant cells with 4 centrosomes. We found that centrosome number distribution in high VEGF treated endothelial cells with greater than two centrosomes peaked at 3 centrosomes and was not skewed to 4 centrosomes (Fig. 2.4C). Taken together, our findings suggest that high VEGF-treated endothelial cells do not acquire excess centrosomes as a result of incomplete cytokinesis.
High VEGF signaling promotes endothelial centrosome over-duplication via hyper-activation of cyclin E/Cdk2

To test the hypothesis that abnormally high VEGF signaling directly promotes mis-regulation of centrosome duplication in endothelial cells, we investigated signaling downstream of VEGF in HUVEC. Cyclin E/Cdk2 activity initiates centrosome duplication beginning at the G1/S cell cycle transition, and abnormally high levels are associated with centrosome over-duplication in other cell types.\textsuperscript{33-37} Cyclin E/Cdk2 phosphorylates Nucleophosmin-1 (NPM) at the centrosome, and this phosphorylation is required for the initiation of centrosome duplication.\textsuperscript{38} However, upstream signaling factors that feed into this pathway have not been elucidated. To determine whether increased cyclin E/Cdk2 activity in endothelial cells is responsible for VEGF-induced centrosome over-duplication, we first examined cyclin E levels. HUVEC were serum-starved to synchronize the cells in a G1-like arrest, then exposed to low or high VEGF conditions for 12 hr, because at that time the synchronized cells are at the G1/S transition that signals the onset of centrosome duplication (data not shown). We found increased levels of cyclin E protein in high VEGF-treated endothelial cells relative to low VEGF-treated cells (Fig. 2.5A). To determine whether the increase in cyclin E levels was accompanied by an increase in cyclin E/Cdk2 activity, we analyzed Cdk2 activity. Cdk2 was isolated by immunoprecipitation from the same lysates used to quantify cyclin E levels, and its ability to phosphorylate histone H1 in an immune-complex kinase assay was assessed. Cdk2 activity was elevated in high VEGF-treated endothelial cells relative to controls, at the 12 hr time point and at earlier and later time points as well (Fig.
2.5B and data not shown). These data show that abnormally high VEGF signaling leads to elevated cyclin E/Cdk2 activity in endothelial cells.

To determine if the increase in cyclin E levels induced by elevated VEGF signaling is required for mis-regulation of centrosome duplication in endothelial cells, we reduced cyclin E levels in high VEGF-incubated HUVEC using lentivirus-delivered shRNA. Cells were cultured in high VEGF for 96 hr after infection with either cyclin E shRNA virus or empty vector control. High VEGF incubation for 96 hr increased cyclin E protein levels, similar to the 12 hr analysis described above, and cyclin E shRNA infection reduced cyclin E protein levels in high VEGF-treated HUVEC to approximately the levels seen in low VEGF-treated cells (Fig. 2.5D). HUVEC incubated in high VEGF with reduced cyclin E levels had a reduced frequency of endothelial cells with excess centrosomes relative to incubation with high VEGF and elevated cyclin E levels (Fig. 2.5E). These data show that elevated cyclin E levels downstream of abnormally high VEGF signaling contribute substantially to centrosome over-duplication in endothelial cells, and they suggest that elevated cyclin E/Cdk2 activity is responsible for the VEGF-induced mis-regulation of endothelial centrosome duplication.

Increased cyclin E/Cdk2 activity results in increased NPM phosphorylation at Serine-199 in other cell types. This modification initiates centrosome duplication by promoting translocation of NPM from the centrosome, where it inhibits centrosome duplication, to the nucleus. Analysis of NPM phosphorylation in HUVEC following serum-starvation and incubation in low or high VEGF for 12 hr showed that P-NPM (Ser199) levels were elevated in high VEGF-treated cells relative to controls (Fig. 2.5C). Taken together, these results are consistent with
a model whereby abnormally high VEGF signaling leads to increased cyclin E levels and cyclin E/Cdk2 hyper-activation, which in turn leads to increased P-NPM and centrosome over-duplication.

**MEK/ERK and AKT promote cyclin E accumulation and centrosome over-duplication downstream of elevated VEGF signaling**

We next investigated signaling between the initial VEGF signal and the cyclin E/Cdk2 hyper-activity that promotes centrosome over-duplication in endothelial cells. VEGF activates a MEK/ERK signaling cascade, and MEK/ERK signaling promotes cyclin E accumulation in other cell types. Thus we hypothesized that elevated VEGF signals through MEK/ERK to elevate cyclin E levels and promote centrosome over-duplication in endothelial cells. We assessed ERK1/2 phosphorylation in HUVEC as a proxy for ERK activation, after serum-starvation and exposure to low or high VEGF for 5 min, and found that P-ERK levels were increased in high VEGF-treated cells relative to controls (Fig. 2.6A). Next, we asked whether MEK inhibition rescued centrosome over-duplication. HUVEC incubated in high VEGF with a MEK inhibitor (U0126) had reduced P-ERK activation, reduced cyclin E levels, and significantly rescued centrosome numbers relative to HUVEC incubated in high VEGF without inhibitor (Fig. 2.6A-C). Thus MEK inhibition rescued pathway hyper-activation induced by high VEGF and also rescued the centrosome over-duplication defect, indicating that MEK/ERK signaling downstream of VEGF activation contributes to mis-regulation of centrosome duplication in endothelial cells. PKC also acts downstream of VEGF to promote ERK activation, and treatment with a PKC inhibitor also
partially rescued high VEGF-induced centrosome over-duplication (Fig. 2.7). High FGF treatment also lead to elevated ERK activation and cyclin E levels, suggesting that FGF also promotes centrosome over-duplication via signaling through MEK/ERK to cyclin E (Fig. 2.8).

AKT is a second signaling arm downstream of VEGF that also affects cyclin E levels. To determine whether elevated AKT activity downstream of VEGF signaling in endothelial cells also contributes to centrosome over-duplication, HUVEC were serum-starved and treated with low or high VEGF for 1 hr, then AKT phosphorylation was assessed. We found elevated P-AKT in high VEGF-treated HUVEC (Fig. 2.6B). Additionally, HUVEC were incubated in high VEGF with an AKT inhibitor (1L-6-Hydroxymethyl-chiro-inositol 2-(R)-O-methyl-3-O-oxtadecylcarbonate). AKT inhibitor treatment significantly reduced cyclin E levels and the percentage of cells with excess centrosomes relative to high VEGF-treated HUVEC without inhibitor (Fig. 2.6C-D). As predicted, activated ERK levels induced by high VEGF treatment were not affected by treatment with AKT inhibitor (Fig. 2.6A), suggesting that VEGF signaling through AKT to cyclin E represents an additional mechanism whereby VEGF modulates centrosome duplication in endothelial cells. We also asked if VEGF signals through p38 MAPK to affect centrosome number, and found that 96 hr incubation in high VEGF + p38 MAPK inhibitor did not rescue centrosome number, suggesting that VEGF does not signal through p38 MAPK to affect centrosome duplication (Fig. 2.7). Taken together, these data suggest that VEGF signals through both MEK/ERK and AKT to promote cyclin E/Cdk2 hyper-activity and centrosome over-duplication in endothelial cells.
Endothelial cells containing excess centrosomes survive and divide, and display aneuploidy and increased migration

To begin to assess the biological consequences of centrosome over-duplication, we examined whether endothelial cells containing excess centrosomes survived or underwent programmed cell death. High VEGF-treated HUVEC were stained for centrosomes and activated caspase 3, a marker of apoptotic cells. Although control cells with 4-hydroxynonenal (HNE)-induced apoptosis were positive for activated caspase 3, there was no detectable activated caspase 3 reactivity in HUVEC with excess centrosomes (0/15 cells with excess centrosomes were activated caspase-3 positive) (Fig. 2.9A-H). We also analyzed the frequency of endothelial cells with excess centrosomes as a function of exposure time to high VEGF, reasoning that significant “drop-out” would result in a constant percentage over time, while survival of endothelial cells with excess centrosomes would lead to increased percentages over time. A significantly higher percentage of endothelial cells had excess centrosomes when treated for 240 hr in high VEGF compared to the normal 96 hr treatment (Fig. 2.9M). These data indicate that endothelial cells with excess centrosomes do not undergo apoptosis at significant frequencies.

We next asked whether endothelial cells with excess centrosomes were capable of cell division, since mitosis in the presence of excess centrosomes can lead to aneuploidy. Examination of high VEGF-treated HUVEC revealed examples of bi-polar spindle formation (Fig. 2.9I-J). While cells with 2 centrosomes had normal spindles (Fig. 2.9I), cells with excess centrosomes displayed aberrant spindles (Fig. 2.9J). The cells with excess centrosomes are...
predicted to complete mitosis but have an increased probability of aneuploidy due to uneven pulling forces from the spindles.\textsuperscript{31,43} We also observed mitotic endothelial cells with multipolar spindles (Fig. 2.9K-L). These cells are also predicted to complete mitosis with high frequency but with gross aneuploidy as a result of having more than two spindles.

Since endothelial cells with excess centrosomes survive and divide, we hypothesized that developing vessels containing endothelial cells with excess centrosomes would also exhibit increased levels of aneuploidy. Thus we assayed endothelial cells isolated from ES cell-derived vessels for chromosome number. As predicted, \textit{flt-1}\textsuperscript{-/-} endothelial cells isolated from ES cell-derived vessels, which had excess centrosomes, also displayed increased aneuploidy (average 48 chromosomes/cell, normal=40) compared to endothelial cells isolated from WT vessels (average 42 chromosomes/cell) (Fig. 2.10A-C). \textit{Flt-1}\textsuperscript{-/-} endothelial cells also had chromosomal aberrations, including chromosome breaks and triradials, that were not detected in WT endothelial cells (Fig. 2.10B). These results suggest that vessels containing endothelial cells with excess centrosomes accumulate aneuploid cells that contribute to vessel dysfunction.

\textbf{Endothelial cells that contain excess centrosomes are enriched at in vitro scratch wounds}

We hypothesized that excess centrosomes might affect additional endothelial cell functions. Because centrosomes are the microtubule organizing centers in cells, and microtubules play a critical role in migration, we wondered if excess centrosomes would perturb endothelial cell migration. Thus we performed a
scratch/wound assay to assess migration in endothelial cells with excess centrosomes. To our surprise, endothelial cells at the leading migratory front had a higher frequency of excess centrosomes compared to cells that were distant from the migratory front (Fig. 2.11). The differences were significant for low VEGF treated endothelial cells, and the same trends were seen with high VEGF treatment (Fig. 2.11C). These data suggest that centrosome over-duplication leads to abnormal migration in endothelial cells by increasing their migratory capacity.

D. Discussion

Our work demonstrates that elevated angiogenic factor signaling promotes centrosome over-duplication in the endothelial cells of developing vessels, and it provides a mechanistic understanding of this phenotype. We also show that endothelial cells with excess centrosomes are not restricted to tumor vessels, but are a hallmark of vessels exposed to elevated VEGF signaling in several contexts, and are associated with aberrant cell divisions and aneuploidy. Thus, mis-regulation of centrosome duplication is a novel aspect of de-regulated angiogenic factor signaling that impacts the phenotype and potentially the function of endothelial cells in blood vessels.

Developing vessels lacking flt-1 function have an elevated frequency of endothelial cells with aberrant centrosome numbers in both ES cell-derived vessels and in vivo, in the developing vessels of the embryonic yolk sac. These vessels also have an elevated mitotic index, suggesting that flt-1 mutant endothelial cells have a shorter cell cycle progression time relative to WT
endothelial cells.\textsuperscript{15} It is possible that a low “normal” frequency of centrosome over-duplication is amplified in the flt-1 mutant background due to this acceleration of the cell cycle. However, the difference in mitotic indices between WT and flt-1\textsuperscript{+/−} endothelial cells is small (1.4\% (WT) vs. 2.8\% (flt-1\textsuperscript{−/−}) for yolk sac endothelial cells\textsuperscript{15}) relative to the difference in the frequency of endothelial cells with excess centrosomes (0\% (WT) vs. 11\% (flt-1\textsuperscript{−/−}), this study), suggesting that centrosome over-duplication in endothelial cells of flt-1\textsuperscript{−/−} developing vessels is not solely a consequence of an accelerated cell cycle. Moreover, we demonstrate a significant increase in centrosome over-duplication in HUVEC exposed to elevated VEGF signaling, that is not accompanied by an overall increase in doubling time. These findings indicate that mis-regulation of centrosome duplication is a primary effect of abnormally high VEGF signaling.

Elucidation of signaling downstream of VEGF that promotes centrosome over-duplication in endothelial cells provides a mechanistic basis for the phenotype, and it also supports a model whereby aberrant centrosome numbers are a direct effect of perturbed VEGF signaling. We show that downstream of elevated VEGF, both MEK/ERK signaling and AKT signaling are elevated, as expected from previous studies of VEGF signaling in endothelial cells.\textsuperscript{40,41} Both MEK/ERK and AKT feed into regulation of the cell cycle regulator cyclin E/Cdk2 activity in other cell types, and cyclin E/Cdk2 is implicated in regulation of centrosome duplication.\textsuperscript{33-37} Thus, we investigated cyclin E/Cdk2 in VEGF-stimulated endothelial cells and found that both cyclin E levels and Cdk2 activity were increased, consistent with a model whereby VEGF signaling exerts a direct effect on cyclin E/Cdk2. Moreover, blockade of any component of these
signaling axes downstream of VEGF significantly reduced the VEGF-induced centrosome over-duplication defect, showing that these activities are required for the phenotype. Both MEK/ERK and AKT signaling have numerous effects on endothelial cells, but the finding that attenuated signaling through either cassette also reduced the high VEGF-induced elevated levels of cyclin E suggests that direct effects from VEGF through MEK/ERK and AKT to cyclin E/Cdk2 regulate centrosome duplication in endothelial cells (Figure 2.12). Blockade of PKC, but not p38 MAPK, also partially rescued centrosome number, suggesting that several, but not all, signaling cassettes downstream of VEGF affect centrosome over-duplication in endothelial cells. Phosphorylation of the centrosome-associated protein NPM downstream of cyclin E/Cdk2 occurs in high VEGF-stimulated endothelial cells, and this phosphorylation can affect centrosome duplication. The microtubule-binding protein ninein has a complex localization pattern in endothelial cells that is regulated by phosphorylation downstream of VEGF, so it may also be a downstream effector of VEGF effects on centrosomes. FAK mutant endothelial cells or endothelial cells expressing Ser-732-mutated FAK protein were reported to have excess centrosomes, suggesting that both proper adhesion to substrates and VEGF signaling are both important for regulation of centrosome numbers in endothelial cells.

What are the consequences of aberrant centrosome numbers in endothelial cells of developing vessels? Endothelial cells with excess centrosomes as a result of elevated VEGF signaling do not undergo apoptosis; rather, they appear to survive and accumulate. Moreover, we demonstrate that endothelial cells with excess centrosomes can form aberrant spindles during mitosis, an abnormality
that is predicted to lead to endothelial aneuploidy. Consistent with this hypothesis, endothelial cells isolated from \textit{flt-1}^{-/-} mutant developing vessels that experienced elevated VEGF signaling had an increased frequency of aneuploidy, as assayed by abnormal chromosome number, and they also had chromosome breaks and triradials, which result from asymmetrical sister chromatid exchange. The centrosome clustering and multipolar spindle formation that result from excess centrosomes are predicted to induce chromosome gain and/or loss as well as chromosome breaks at mitosis due to unequal pulling forces on the chromosomes, so the cytogenetic abnormalities seen in endothelial cells from \textit{flt-1}^{-/-} mutant vessels likely result from the increased frequency of excess centrosomes. Our data also provides a mechanism for the finding that isolated tumor endothelial cells, which presumably experience elevated VEGF signaling, have an increased frequency of excess centrosomes and elevated levels of aneuploidy.\textsuperscript{5,6} We predict that aneuploid endothelial cells are likely to contribute to the aberrant vascular phenotypes associated with elevated VEGF signaling, via gain or loss of chromosomes that encode genes involved in proliferation, migration, and cell-cell adhesion. Endothelial cells with excess centrosomes are also enriched at the leading edge of \textit{in vitro} scratch wounds, suggesting that they might migrate more rapidly than normal endothelial cells, and this cellular phenotype may also contribute to vessel dysfunction.

Our work has implications for regulation of angiogenesis in non-developmental contexts. Elevated VEGF signaling is associated with aberrant angiogenesis in several pathologies, such as diabetes and cancer. Moreover, tumors express numerous growth factors, and elevated FGF also leads to over-
duplication of centrosomes in endothelial cells. Tumor vessels differ substantially from normal vessels – they are tortuous in phenotype and leaky in function, and thus poor at oxygen delivery.\textsuperscript{46-48} Additionally, endothelial cells isolated from some tumors appear to be more “progenitor-like”, and capable of differentiating into other mesodermal lineages.\textsuperscript{49} It is tempting to speculate that the introduction of aneuploidy downstream of centrosome duplication defects contributes to these changes. In any case, our demonstration of a clear link between VEGF signaling and centrosome duplication in endothelial cells of developing vessels suggests that this cellular phenotype contributes to the aberrant angiogenesis that accompanies elevated VEGF signaling.
E. References


Figure 2.1. Centrosomes duplicate once per cell cycle and can be visualized in endothelial cells. (A) Diagram of the cell cycle and centrosome duplication cycle. (B-G) HUVEC were fixed and stained for centrosome markers in endothelial cells: (B-D) γ-tubulin (red) co-localizes with Pericentrin (green). (E-G) γ-tubulin (red) co-localizes with Nucleophosmin-1 (NPM, green). DNA is labeled with DRAQ5 (blue). Arrowheads point to co-localization. Scale bar=5µm.
A

Bi-polar spindle formation

Cell Cycle

Centrosome duplication

B γ-tubulin

C pericentrin

D Merge DNA

E γ-tubulin

F NPM

G Merge DNA
Figure 2.2. High VEGF signaling leads to an increased frequency of excess centrosomes in endothelial cells of developing vessels. (A-B) Day 8 ES cell-derived wildtype (WT) and flt-1^-/- mutant vessels were stained for PECAM-1 (red); note the vessel overgrowth and dysmorphogenesis in panel B. (C-J) Endothelial cells of WT and flt-1^-/- mutant vessels were analyzed for centrosome numbers. (C-D) Day 8 ES cell cultures were fixed and stained for γ-tubulin (red), PECAM-1 (green) and DRAQ5 nuclear dye (blue). (F-G) Day 8 ES cell cultures that were WT or flt-1^-/- and carried a PECAM-H2B::GFP transgene (green) were dissociated and attached to tissue culture dishes prior to fixation and staining for γ-tubulin (red). (E) Percentage of PECAM-H2B::GFP positive cells with >2 centrosomes (WT, n=159; flt-1^-/-, n=336). (I-J) WT and flt-1^-/- embryos were harvested at E9.5, and yolk sacs were stained for γ-tubulin (red), PECAM-1 (green) and DRAQ5 nuclear dye (blue). (H) Percentage of PECAM positive yolk sac endothelial cells with >2 centrosomes (WT, n=88; flt-1^-/-, n=180). (L-M) HUVEC were incubated for 96 hr in low or high VEGF, then stained for γ-tubulin (red) and DRAQ5 nuclear dye (blue). (K) Percentage of HUVEC with >2 centrosomes (low VEGF, n=2393; high VEGF, n=3011). Arrows point to areas of cells with >2 centrosomes, and arrowheads point to areas of cells with 1-2 centrosomes. Insets (panels D, I, J, M) show centrosomes at higher magnification. All experiments were performed at least three times. Scale bar = 50 µm (A-B); 5 µm (C-M). *, p<0.05; ***, p<0.0001, low VEGF vs. high VEGF.
Figure 2.3. VEGF and FGF increase the frequency of excess centrosomes in endothelial cells independent of proliferative changes. (A) Percentage of HUVEC with >2 centrosomes in indicated conditions (low VEGF/low FGF, n=1594; high VEGF, n=1717; high FGF, n=1563; high VEGF/high FGF, n=866). **, p < 0.001; ***, p < 0.0001 vs. low VEGF/low FGF. (B) HUVEC growth curves in low and high VEGF, expressed as fold increase in cell number relative to t=0. Blue line, low VEGF conditions; red line, high VEGF conditions. All experiments were performed at least 3 times.
Figure 2.4. The high VEGF-induced excess centrosome defect does not correlate with incomplete cytokinesis. (A-B) Fluorescence activated cell sorting (FACS)-generated cell cycle profiles of HUVEC treated for 96 hr in low (A) or high (B) VEGF and labeled with propidium iodide (G0/G1, S and G2/M represent cell cycle phases; Poly represents polyploid cells). (C) Distribution of centrosome number in HUVEC containing >2 centrosomes following high VEGF treatment. All experiments were performed at least 3 times.
Figure 2.5. VEGF signals through cyclin E/Cdk2 to promote mis-regulation of centrosome duplication in endothelial cells. (A-C) HUVEC were serum starved, then treated for 12 hr with low or high VEGF. (A) Western blot of lysates hybridized with anti-cyclin E, normalized to actin. (B) Autoradiogram of Cdk2 immune-complex kinase assay normalized to total Cdk2 immunoprecipitation (IP). (C) Western blot of lysates hybridized with anti-P-NPM, normalized to total NPM. (D-E) HUVEC were infected with empty vector (EV) or cyclin E shRNA (CycE KD) lentivirus for 6 hr, then incubated in low or high VEGF for 96 hr. (D) Western blot of lysates hybridized to anti-cyclin E and normalized to actin. (E) Following lentiviral infection and VEGF treatment, cells were fixed, stained for γ-tubulin and DRAQ5, and centrosome numbers were counted (low VEGF, n=656; high VEGF, n=750; high VEGF + empty vector (EV), n=717; high VEGF + cyclin E knockdown (CycE KD), n=696). All experiments were performed at least 3 times. ***, p<0.0001 vs. low VEGF; #, p<0.05 vs. high VEGF.
Figure 2.6. VEGF signals through MEK/ERK and AKT to mis-regulate centrosome duplication in endothelial cells. HUVEC were serum starved, then incubated with low or high VEGF. (A) HUVEC treated for 5 min without inhibitor or with U0126 (MEKI) or 1L-6-Hydroxymethyl-chiro-inositol 2-(R)-O-methyl-3-O-oxtadecylcarbonate (AKTI) were lysed, hybridized to anti-P-ERK and normalized to total ERK2. (B) HUVEC treated with low or high VEGF for 1 h were lysed, hybridized to anti-P-AKT and normalized to total AKT. (C) HUVEC treated with indicated levels of VEGF for 12 hr without inhibitors or with MEKI or AKTI were lysed, hybridized to anti-cyclin E and normalized to actin. (D) HUVEC treated with indicated levels of VEGF for 96 hr without inhibitors or with MEKI or AKTI were fixed, stained for γ-tubulin and DRAQ5, and centrosome numbers were counted (low VEGF, n=1036; high VEGF, n=1369; high VEGF + MEKI, n=1152; high VEGF + AKTI, n=586). All experiments were performed at least 3 times. *, p<0.05 vs. low VEGF; **, p<0.001 vs. low VEGF; ***, p<0.0001 vs. low VEGF; #, p<0.05 vs. high VEGF; ##, p<0.001 vs. high VEGF.
Figure 2.7. VEGF signals through PKC but not p38 MAPK to promote centrosome over-duplication. HUVEC were treated with Low VEGF (EGM-2 medium), High VEGF (EGM-2 + 200 ng/ml VEGF), High VEGF + p38 MAPK Inhibitor (p38I) or High VEGF + PKC Inhibitor (PKCI) for 12 hr following serum starvation (A) or 96 hr (B). A) Western blot analysis of cyclin E levels relative to total actin. B) Bar graph showing % cells with excess (>2) centrosomes following 96 hr of the indicated treatments. *, p<0.05 vs. 96 hr low VEGF; **, p<0.001 vs. 96 hr low VEGF; #, p<0.05 vs. 96 hr high VEGF.
Figure 2.8. High VEGF or FGF signaling leads to elevated ERK activation and cyclin E levels. HUVEC were serum-starved for 12 hr, then treated with Low VEGF (EGM-2 medium), High VEGF (EGM-2 + 200 ng/ml VEGF) or High FGF (EGM-2 + 200 ng/ml FGF-2) for 5 min (P-ERK) or 12 hr (cyclin E). A) Western blot analysis of ERK phosphorylation relative to total ERK2. B) Western blot analysis of cyclin E levels relative to total actin. Data are representative of at least 3 experiments.
Figure 2.9. Mis-regulation of centrosome duplication does not lead to apoptosis in endothelial cells. (A-H) HUVEC were stained with anti-activated caspase 3 (red), pericentrin (green) and DRAQ5 nuclear dye (blue) following treatment with high VEGF for 96 hr, with (A-D) or without (E-H) the apoptosis-promoting drug HNE for the final 48 hr. Inset in F shows centrosomes at higher magnification. HNE-treated cells were positive for activated caspase 3, but high VEGF-treated HUVEC containing excess centrosomes did not stain for activated caspase 3. (I-L) HUVEC were stained with anti-pericentrin (green), anti-α-tubulin (red) and DRAQ5 to visualize mitotic figures. Bipolar spindles containing 2 centrosomes (I) or >2 centrosomes (I-J) were observed in addition to multipolar spindles (K-L) containing >2 centrosomes. (M) HUVEC were incubated with low or high VEGF for the indicated times, then fixed and stained for γ-tubulin and DRAQ5 for centrosome counts (96 hr low VEGF, n=890; 240 hr low VEGF, n=976; 96 hr high VEGF, n=1023; 240 hr high VEGF, n=897). All experiments were performed at least 3 times. Scale bar = 5µm. **, p<0.001 vs. 96 hr low VEGF; ***, p<0.0001 vs. 96 hr low VEGF; #, p<0.05 vs. 96 hr high VEGF.
Figure 2.10. VEGF gain-of-function flt-1⁻/⁻ endothelial cells from developing vessels display aneuploidy and chromosome aberrations. Endothelial cells from WT and flt-1⁻/⁻ ES cell-derived vessels were isolated via magnetic bead isolation and analyzed for chromosome number and abnormalities. (A-B) Giemsa stained WT (A) and flt-1⁻/⁻ (B) endothelial cell metaphase spreads with 40 and 47 chromosomes (CS), respectively (M. musculus 2n=40). Arrows in B point to abnormal tri-radial chromosome configurations. (C) Scatter plot showing chromosome number in WT vs. flt-1⁻/⁻ endothelial cells from developing vessels. Each dot represents one cell, red dots represent cells with 40 CS, blue dots represent aneuploid cells. WT, n=25; flt-1⁻/⁻, n=25. Experiments were performed in triplicate. *p≤0.05.
**Figure 2.11.** Endothelial cells with excess centrosomes are enriched at the leading edge of a scratch wound. HUVEC were incubated for 10 d in low or high VEGF. Then a scratch wound was made 24 hr. prior to analysis. Cells were stained for α-tubulin (C,G), pericentrin (D,H) and the DNA dye DRAQ5 (E,I). (A) Cells at the leading edge of a scratch wound in high VEGF treated cells; single cells in insets (a) and (b) have excess centrosomes and are imaged at higher magnification in (C-F) and (G-J), respectively. Insets in (D) and (H) are zoomed-in images of centrosomes. (B) Quantification of centrosome numbers at the “migratory” leading edge vs. “static” cells away from the edge in the indicated conditions (low VEGF static, n=354; low VEGF leading edge, n=338; high VEGF static, n=307; high VEGF leading edge, n=293). All experiments were performed at least 3 times. (F) and (J) are merged images of (C-E) and (G-I), respectively (α-tubulin, red; pericentrin, green; DRAQ5, blue). Scale bar = 5mm. ***, p≤0.0001 vs. low VEGF static.
Figure 2.12. Model for VEGF regulation of endothelial centrosome duplication. A schematic of proposed signaling to regulate centrosome duplication in endothelial cells. In this model, VEGF promotes MEK/ERK and AKT signaling, leading to increased levels of cyclin E. Cyclin E binds Cdk2 to activate cyclin E/Cdk2 activity and promote centrosome duplication, perhaps via phosphorylation of NPM. Elevated VEGF levels lead to abnormally high Cdk2/cyclin E activity and centrosome over-duplication.
VEGF

AKT  MEK  ERK

CycE  Cdk2

Centrosome over-duplication

Endothelial aneuploidy & cellular dysfunction
CHAPTER III
ORIENTATION OF ENDOTHELIAL CELL DIVISION IS REGULATED BY VEGF SIGNALING DURING BLOOD VESSEL FORMATION

This work was performed in collaboration with Dr. Gefei Zeng, a former Bautch lab post-doctoral researcher, among others. Susan Whitfield of the UNC Biology Department made the image in Fig. 3.1. Gefei performed the experiments described in Figs. 3.2, 3.3, and 3.6. Gefei analyzed movies produced by Joe Kearney and Nick Kappas, former Bautch lab graduate students, for Fig. 3.4. Gefei and I each performed 50% of the work described in Figs. 3.5 and 3.9. I performed the experiments described in Figs. 3.7 and 3.8. All retinal analyses were performed with substantial intellectual input from Jan McColm and ME Hartnett at UNC. The majority of this work was published in Blood:

A. Introduction

Blood vessels form and expand in both development and disease, via processes that include vasculogenesis, angiogenesis, and intussusception.\textsuperscript{1-3} Sprouting angiogenesis is the coordinated migration of groups of endothelial cells from vessels and their subsequent fusion to form new interconnections. In this way, simple vascular tubes are ramified and extended to form a primitive vascular plexus. This vessel plexus forms at numerous sites in the embryo, including the yolk sac, the head mesenchyme, and surrounding the neural tube. The primitive vascular plexus is then remodeled under the influence of blood flow and interactions with mural cells. Thus, the initial pattern of vessels serves as a template for remodeling that leads to a mature vasculature.

During formation of the primitive vascular plexus, several cellular processes must be regulated and integrated. Specifically, endothelial cells respond to some morphogenetic cues by sprouting, while actively dividing to expand the pool of endothelial cells. One level of integration occurs via the signaling pathways that promote angiogenesis, as many impact both endothelial cell division and morphogenesis. The VEGF signaling pathway is an example of this mode of integration, as it regulates both cell division and branching morphogenesis.\textsuperscript{4-8} VEGF-A (VEGF) binds two high affinity receptors on endothelial cells, Flk-1 (VEGFR-2) and Flt-1 (VEGFR-1), and perturbation of VEGF signaling by genetic deletion of either receptor affects both endothelial cell division and morphogenesis.\textsuperscript{9-12} Several lines of evidence, however, suggest that different regulatory nodes in the VEGF signaling pathway influence endothelial cell division and morphogenesis. VEGF signaling through Flk-1 promotes endothelial cell division through the
Raf/MEK/ERK pathway, whereas endothelial cell migration is stimulated through p38 MAPK and adaptor proteins such as Shb and Nck that lead to regulation of the actin cytoskeleton. Additionally, the overall level of available VEGF is thought to regulate the rate of endothelial cell division, while VEGF presentation, perhaps through the formation of a gradient, is thought to regulate sprouting angiogenesis and the formation of filopodia. Moreover, other pathways selectively impact endothelial cell division or morphogenesis. For example, signaling through p27/kip1 regulates the endothelial cell cycle, while the netrin-UNC and semaphorin-plexin pathways regulate endothelial branching morphogenesis and guidance. Thus it is likely that endothelial cell division and morphogenesis are integrated at multiple levels during angiogenesis, but little is known about how this is achieved.

The orientation of the cleavage plane of cell division, which positions the daughter cells relative to other embryonic axes, is regulated in numerous developing tissues. The basis for this regulation is the interaction of the mitotic spindle with molecules differentially localized on the inner side of the plasma membrane (the cortex). The cortical cues include the PAR proteins first identified in C. elegans, atypical protein kinases (aPKCs), and molecules such as LGN, NuMA, and Inscuteable, that appear to link microtubules to the cortex. The spatial regulation of these cues is complex and not completely understood, but the actin cytoskeleton and its interactions with the cortex are required for proper positioning of the cues and in turn the mitotic spindle. The actin cytoskeleton in turn is acted upon by numerous inputs, among them growth factor signaling.

Complex structures such as epithelial sheets and tubes can exhibit a polarity of division orientation. MDCK cells form tubes in culture in response to hepatocyte
growth factor (HGF)/scatter factor signaling, and this signaling can re-orient MDCK cell divisions so that daughter cells leave the epithelial sheet. Kidney tubules expand via cell divisions that are oriented to extend the length of the tubule. Epidermal cleavages oriented parallel to the long axis of the sheet resulted in daughter cells that formed a new layer during embryonic skin development, and loss of several pathways, including those involving β1 integrins, lead to increased randomization of divisions and aberrant stratification. Oriented cell divisions are also associated with flower bud formation, elongation of the avian primitive streak, shaping of the neural plate in avians and mouse, and extension of the zebrafish body axis at gastrulation.

Endothelial cells orient their actin cytoskeleton and microtubule network in response to shear stress, such as that produced by blood flow in vivo. However, despite the fact that endothelial cells actively divide while undergoing morphogenesis, the orientation of endothelial cell cleavages during normal and perturbed angiogenesis has not been investigated. We asked whether the orientation of endothelial division was regulated in a flow-independent model of dynamic angiogenesis in culture and in retinal vessels in vivo. Here we show that endothelial cell cleavage is normally oriented perpendicular to the long axis of the vessel, which can promote vessel lengthening, a hallmark of these expanding vascular plexuses. Moreover, we show that orientation is randomized by a mutation that disrupts VEGF signaling and leads to vessel dysmorphogenesis, and it is rescued by genetic rescue of the mutation. These data indicate that endothelial cell division orientation is regulated by flow-independent morphogenetic cues, and that endothelial cell division is normally oriented by a process that involves VEGF
signaling. Our findings also suggest that the integration of proliferative and morphogenetic processes is critical to proper vessel morphogenesis.

B. Materials and methods

**Cell culture and in vitro differentiation**

Embryonic stem (ES) cell lines used consisted of wild-type (WT; +/-) and flt-1^{-/-} ES cells containing a transgene consisting of enhanced green fluorescent protein (eGFP) fused to histone 2B (H2B) under the transcriptional control of the platelet endothelial cell adhesion molecule (PECAM) promoter/intron enhancer element (Tg PECAM-H2B-GFP), (12 and this report) as well as WT ES cells containing a PECAM-eGFP transgene (Tg PECAM-GFP). Additional lines were WT, flt-1^{-/-}, and two sflt1 rescue lines, flt-1^{-/-};Tg PECAM-sflt-1#33 and flt-1^{-/-};Tg PECAM-sflt-1#26 that did not contain a GFP transgene. All ES cell cultures were maintained and differentiated in vitro as described previously. Embryoid bodies (EBs) were plated onto slide flasks (Nunc, Rochester, NY) at day 3 of differentiation and cultured at 37°C in 5% CO₂ until day 7-8, when time-lapse imaging was performed or they were fixed and stained.

**DNA constructs and electroporation**

The Sal I/Not I H2B-eGFP fragment was cut from pBOS-H2BGFP (BD Pharmingen, San Diego CA) and cloned into the PECAM promoter-enhancer vector for electroporation into WT ES cells (designated PECAM-H2B-GFP). The same fragment was also cloned into PECAM-Hygro for electroporation into flt-1^{-/-} ES cells and designated PECAM-H2B-GFP-Hygro. DNA was
electroporated into ES cells as described previously. Briefly, 20 µg of linearized PECAM-H2B-GFP or PECAM-H2B-GFP-Hygro DNAs were electroporated into 2 x 10^7 ES cells using a BioRad GenePulser II electroporator (250 V/300 µF; BioRad, Hercules, CA). WT ES cell selection was in 200 µg/mL G418 (Gibco, Carlsbad, CA), and selection of flt-1^−/− ES cells was in 200 µg/mL hygromycin B (Roche Diagnostics, Indianapolis, IN). After 12 to 14 days, drug resistant ES colonies were picked, expanded, and analyzed by in vitro differentiation and fluorescence imaging. We initially analyzed 5 WT transgenic lines (designated as WT;Tg PECAM-H2B-GFP) and 4 flt-1^−/− transgenic lines (designated as flt-1^−/−;Tg PECAM-H2B-GFP) that expressed the transgene in vessels and saw no differences except by genotype, so single WT and mutant lines were used for time-lapse imaging. Generation of the sflt-1 rescue lines has been previously described.

**Time-lapse imaging**

Slide flasks containing day-7 to -8 in vitro-differentiated ES cell cultures were sealed, then placed on a heated stage on a Nikon TE300 inverted microscope (Melville, NY) with a Perkin Elmer spinning disk confocal head (Shelton, CT). Confocal images were acquired at 1-minute intervals using Metamorph software (version 6.0; Universal Imaging Corp, Downing-town, PA) and a Hamamatsu Orca CCD camera (McHenry, IL) with 20X or 10X objectives as described.
**Antibody staining**

Following time-lapse imaging, ES cell cultures were rinsed with phosphate-buffered saline (PBS) and fixed for 5 minutes in ice-cold methanol-acetone (50:50). Fixed cultures were reacted with rat anti-mouse PECAM at 1:1000 (MEC 13.3; BD Pharmingen, San Diego, CA) and donkey anti-rat immunoglobulin G (IgG; H+L) conjugated to FITC or TRITC at 1:200 (Jackson Immunoresearch, West Grove, PA) as described previously. In some cases PECAM stained cultures were labeled with phosphohistone H3 as described, using rabbit anti-phosphohistone at 1:500 (Upstate Biotechnology) and donkey anti-rabbit immunoglobulin G (IgG; H + L) conjugated to TRITC at 1:200 (Jackson Immunoresearch). All cultures were viewed and photographed with an Olympus IX-50 inverted microscope (Melville, NY) outfitted with epifluorescence, or a Zeiss 510 confocal microscope. PECAM-stained images were aligned with the last frame of each movie using Photoshop version 7.0 (Adobe Systems, San Jose, CA).

**Retina dissection and staining**

Rat or mouse pups (P3-5) were weighed and anaesthetized by intraperitoneal injection of ketamine (20mg/kg) and xylazine (6 mg/kg) (rats) or by isofluorane inhalation (mice). Paraformaldehyde (PFA) was directly perfused (0.5 ml of 0.5% PFA) into the right ventricle, after which the pups were euthanized by intracardiac injection of Nembutol (80 mg/kg) (rats) or a thorectomy was performed (mice). Both eyes were enucleated and whole eyes were fixed in 2% PFA for 2 hours.
before being washed in PBS. The retinas were dissected using a modification of a described method.\textsuperscript{49,50}

The flat mounted retinas were incubated in ice cold ethanol for 30 minutes, then permeabilized with 1% Triton X-100 in PBS for 30 minutes. The retinas were reacted with isolectin GS-I B4 conjugated to Alexa Fluor 488 at 1:100 (Invitrogen, Carlsbad, CA) overnight at 4°C. The samples were washed once with PBS, then blocked in 1% Triton X-100 and 5% goat serum in PBS for 1 hour. Antibodies and reaction conditions used were: rabbit polyclonal anti-phospho-histone H3 at 1:500 (Upstate Biotechnology), overnight at 4°C, donkey anti-rabbit immunoglobulin G (IgG; H + L) conjugated to TRITC at 1:100 (Jackson Immunoresearch), 3 hours at 37°C. All incubations were done in a humidity chamber and samples were washed 3 times with PBS after each antibody reaction. Retinas were mounted in PBS:glycerol (2:1) and scanned on a Leica SP2 AOBS or a Zeiss 510 confocal microscope using a 40x 1.25 NA Apochromat objective.

\textbf{Quantitative image analysis}

Quantitative image analysis was performed using Metamorph software. Cell division planes were easily identified in H2B-GFP labeled vessels by bisection of the separating chromosomes during anaphase, and in anti-phospho-histone H3 stained vessels by visualization of the metaphase or anaphase chromosomes. Lines were drawn along the division plane and along the long axis of the blood vessel for each mitotic division, and the angle between these two lines was calculated. Angles of 0° are divisions whose cleavage planes are parallel to the
long axis of blood vessel, whereas angles of 90° are divisions whose cleavage planes are perpendicular to the long axis of blood vessel. The angles were then grouped to every 10 degrees ranging from 0-90°. Microsoft Office Word (2003) was used to generate line drawings of the angles. Daughter cell separation was tracked for at least 60 minutes after division using time-lapse imaging and Metamorph software.

C. Results

**Endothelial cell divisions are oriented perpendicular to the vessel long axis in ES cell-derived vessels**

We reasoned that oriented endothelial cell divisions during angiogenic expansion of a vascular plexus might affect vessel morphogenesis by increasing either vessel length or vessel diameter (Fig. 3.1). The cleavage plane was identified as a line between and parallel to the two groups of anaphase chromosomes (Fig. 3.2C). Thus a division oriented so that the plane of cytokinesis is perpendicular to the long axis of the vessel would effectively lengthen the vessel, while a division plane oriented parallel to the vessel long axis would increase the vessel diameter. To determine if endothelial cell divisions are oriented in a flow-independent manner, we utilized a model of vascular development in which mouse ES cells undergo a programmed differentiation. This process generates multiple embryonic cell types, including endothelial cells that form vessels and undergo sprouting angiogenesis.⁴⁷,⁵¹ Although primitive hematopoietic cells also differentiate and the vessels form lumens in this 3-dimensional model of vessel development, there is no flow through the vessels.⁴⁶ WT ES cells were generated with a stably integrated
transgene encoding histone H2B fused to GFP (H2B-GFP) linked to the PECAM enhancer/promoter (Tg PECAM-H2B-GFP). Upon differentiation, these cultures expressed H2B-GFP in the developing primitive vessels, as shown by overlay with PECAM antibody stain (Fig. 3.2A). The cultures were imaged for short periods (3-6 hr) on day 7-8 of differentiation, when sprouting angiogenesis peaks, then fixed and stained for PECAM reactivity. Endothelial divisions were scored during anaphase, and the angle of cleavage relative to the vessel long axis was determined (Fig. 3.2B). Analysis of the movies showed that the majority (56%) of the endothelial divisions were oriented within 10 degrees of perpendicular, and that 76% of divisions were within 20 degrees of perpendicular to the long axis of the vessel (Fig. 3.2B-D). This result shows that the endothelial cleavage plane is oriented relative to the long axis of the vessel, suggesting that oriented endothelial cell division participates in net lengthening of vessels in a developing vascular plexus.

**Daughter cells maintain their division orientation as they migrate and divisions orient relative to the nearest vessel axis**

To determine if the initial relationship of daughter cells following cleavage was maintained, the daughter nuclei produced by endothelial divisions were tracked for the duration of the movies (Fig. 3.3). Divisions whose cleavage angle was 70-90 degrees relative to the long axis of the vessel all had daughter nuclei that moved away from the cleavage plane along the long axis of the vessel (100%, 61/61) (Fig. 3.3A). Of the few divisions whose cleavage plane was 0-45 degrees relative to the vessel long axis, 70% (7/10) of daughter nuclei moved essentially perpendicular to the long axis of the vessel (Fig. 3.3B), while 30% (3/10)
reoriented and moved along the vessel long axis (Fig. 3.3C). These results indicate that the orientation of endothelial cell divisions in vessels affects the subsequent spatial relationship of the daughter cells and thus can modulate vessel morphogenesis.

We next asked whether the orientation of endothelial cell division is altered in and near forming sprouts. To analyze sprout divisions, we utilized ES cell-derived vessels that contain a PECAM-eGFP transgene.12 This GFP reporter localized to the cytoplasm of endothelial cells, and it efficiently outlined the developing sprouts and endothelial cell divisions (Fig. 3.4). We found that the orientation of endothelial division remained perpendicular to the vessel long axis when the division occurred in the parent vessel, whether prior to initiation of sprout formation (2/2, Fig. 3.4A) or subsequent to the initiation of sprout formation (6/6, Fig. 3.4B). However, divisions within the sprout oriented perpendicular to the long axis of the sprout (5/5, Fig. 3.4C). Although the number of scored events was small, the uniform cleavage orientations suggest that endothelial divisions are normally oriented perpendicular to the current vessel axis, and not to a former or future vessel axis.

**Endothelial cell divisions are oriented perpendicular to the vessel long axis in retinal vessels in vivo**

To test the hypothesis that endothelial cell division orientation is also oriented *in vivo*, we examined post-natal rat retinas. Vessel development occurs in a circumferential wave from the optic nerve in a single plane during the early post-natal stages, and the leading edges of this vascular plexus were associated with high levels of division in cat retinas.49 Retinas were double stained with the *Griffonia*
B4 isolectin to visualize vessels, and anti-phospho-histone H3, which reacts with a histone epitope present on mitotic cells.\textsuperscript{52} This allows for visualization of condensed chromosomes during metaphase and anaphase, and by inference the division cleavage plane (Fig. 3.5). Analysis of mitotic endothelial cells in which the cleavage plane could be scored (Fig. 3.5A) showed that 62\% of the divisions were within 10 degrees of perpendicular to the vessel long axis, and a total of 92\% were within 20 degrees of perpendicular (Fig. 3.5B-C). These results indicate that the orientation of endothelial cell divisions perpendicular to the vessel long axis is recapitulated \textit{in vivo}, in a developing vascular bed exposed to blood flow.

\textbf{VEGF signaling regulates endothelial cell division orientation in developing blood vessels}

Growth factor signaling is a key mediator of morphogenesis, and the HGF/scatter factor signaling pathway affects the division orientation of MDCK cells undergoing chain extension in 3-dimensional cultures.\textsuperscript{36} Thus we reasoned that VEGF signaling might be involved in the regulation of endothelial cell division orientation in developing vessels. Proper regulation of signaling through VEGF-A is crucial to proper vessel morphogenesis, and excessive signaling through the VEGF-A receptor Flk-1 leads to vessel dysmorphogenesis. We analyzed the plane of endothelial cell division in ES cell-derived vessels mutant for \textit{flt-1}, which is a gain-of-function for VEGF signaling through flk-1.\textsuperscript{53} These vessels also expressed PECAM-H2B-GFP to allow for visualization of cleavage orientation (Fig. 3.6). Although the vessel dysmorphogenesis associated with the mutation produces endothelial sheets with time, the edges of the sheets have distinguishable vessels whose long axis can
be scored (Fig. 3.6A).\textsuperscript{12} This analysis showed that the orientation of endothelial divisions in $\text{flt-1}^{-/-}$ vessels was randomized relative to WT vessels (Fig. 3.6B-C). Only 24\% of divisions were within 10 degrees of perpendicular, as opposed to 56\% of WT divisions. Moreover, tracking of nuclei showed that 50\% (8/16) of parallel divisions resulted in daughter nuclei that maintained the relationship they had at the time of division, indicating that these divisions can increase vessel diameter over length (data not shown). These data show that perturbation of VEGF signaling leads to increased randomization of endothelial cell division orientation in developing vessels.

To determine if the $\text{flt-1}$ mutation was responsible for the orientation phenotype, we asked whether the randomization of endothelial cell division orientation was rescued by a genetic rescue of the $\text{flt-1}$ mutation. We previously showed that addition of a PECAM-sflt-1 transgene, that expresses the soluble isoform of flt-1 in developing vessels, rescued the reduction in branching morphogenesis seen in $\text{flt-1}^{-/-}$ mutant vessels.\textsuperscript{12} We thus examined the orientation of endothelial cell division in two of the sflt-1 rescue lines, by double staining fixed cultures with anti-PECAM-1 to visualize vessels and anti-phospho-histone H3 to visualize condensed chromosomes for cleavage plane angle calculations (Fig. 3.7). These data show that both $\text{flt-1}^{-/-};\text{Tg PECAM-sflt-1}$ lines rescued the randomized orientation of endothelial cell division seen in $\text{flt-1}^{-/-}$ mutant vessels (Fig. 3.7A-C). In the $\text{flt-1}^{-/-};\text{Tg PECAM-sflt-1#33}$ and the $\text{flt-1}^{-/-};\text{Tg PECAM-sflt-1#26}$ vessels, 43\% and 60\% of endothelial cell divisions were within 10 degrees of perpendicular relative to the vessel long axis (Fig. 3.7B). This compared to WT vessel values of 53\% and $\text{flt-1}^{-/-}$ mutant vessel values of 17\%. Thus a genetic manipulation to rescue the
dysmorphogenesis of \textit{flt-1}^{-/-} mutant vessels rescues the orientation of endothelial cell division almost to WT levels.

Next, we asked if mice expressing only VEGF$^{120}$ had randomized division orientation. Briefly, VEGF is expressed as three primary isoforms: VEGF$^{120}$, VEGF$^{188}$ and VEGF$^{165}$. The isoforms differ in the ability to bind heparin sulfate proteoglycans in the extracellular space.\textsuperscript{17} VEGF$^{188}$ and VEGF$^{165}$ have heparin binding domains, and heparin binding “tethers” them near VEGF-expressing cells. VEGF$^{120}$ does not bind heparin and is thought to diffuse far from VEGF-expressing cells, making it difficult for endothelial cells to sense a VEGF gradient.\textsuperscript{17} Mice expressing only one isoform have differing vascular phenotypes that are apparent in neonatal retinas. Similar to \textit{flt-1}^{-/-} vessels, VEGF$^{120/120}$ mice have thick vessels and decreased branching compared to wildtype vessels (Fig. 3.8A-B).\textsuperscript{17} We found that the difference between wildtype and VEGF$^{120/120}$ retinal vessel division orientation was not significantly different, suggesting that the amount of VEGF signaling, as opposed to the VEGF pattern in the ECM, is important for regulation of endothelial division orientation (Fig. 3.8C).

Interphase cell shape can regulate cell division orientation,\textsuperscript{31} and we wondered if perturbations in cell shape lead to randomized division orientation in \textit{flt-1}^{-/-} vessels. To assess cell shape, we determined the ratio of length/width of ES cell-derived endothelial cells and found that there is no detectable difference in cell shape between wildtype and \textit{flt-1}^{-/-} endothelial cells (data not shown), suggesting that the randomized division orientations observed in \textit{flt-1}^{-/-} vessels are not a result of abnormal cell shape.
Inhibition of JNK or Rho Kinase leads to randomized orientation of endothelial cell divisions

Planar cell polarity (PCP) signaling regulates cell division orientation in a number of cell types, so we asked if inhibition of molecules that act downstream of PCP affected endothelial cell division orientation. We found that inhibition of JNK or Rho Kinase resulted in randomized endothelial cell divisions in wildtype ES cell-derived vessels (Fig. 3.9), suggesting that JNK and Rho Kinase, perhaps acting downstream of PCP, are important for proper endothelial cell division orientation.

D. Discussion

This study shows, for the first time, that endothelial cell division is normally oriented in developing vascular beds so that the cleavage plane is usually perpendicular to the long axis of the vessel. This finding, along with evidence that daughter nuclei maintain the spatial relationships set up by the cleavage, indicates that oriented cell division is a novel mechanism contributing to vessel morphogenesis. The oriented divisions can effectively extend vessel length. Since developing vascular beds normally expand rapidly via the formation of many thin vessels, the bias of endothelial cell division orientation towards vessel lengthening is consistent with the overall morphogenetic program.

Our finding that endothelial cell divisions orient perpendicular to the vessel long axis in both ES cell-derived vessels and in the post-natal retina indicates that regulated endothelial division orientation is a common attribute of developing vascular beds. Moreover, the tight linkage between endothelial division orientation and the long axis of ES cell-derived vessels is striking, because there is no blood
flow in this model. Numerous studies have linked aspects of endothelial cell polarity to the direction of shear stress produced by flow, including the orientation of the actin cytoskeleton, the microtubule network, and the position of the microtubule organizing center (MTOC).\textsuperscript{54-56} Recent work shows that endothelial cells can transduce mechanical shear stress to cell polarity pathways through a sensor that is comprised of PECAM-1, VE-cadherin, and VEGFR-2.\textsuperscript{57} Moreover, BrdU-labeled daughter cells are positioned to suggest division with cleavage perpendicular to the flow vector in rabbit carotid arteries, although cleavage orientation was not directly measured.\textsuperscript{58}

Our data show that endothelial cell division orientation is regulated in a flow-independent manner, at least during the beginning stages of vessel extension. Developing vessels and sprouts do not form lumens capable of sustaining blood flow until later in the angiogenic process, and even then the shear stress values are significantly lower than those found in adult arteries,\textsuperscript{59} so a flow-independent mechanism to regulate endothelial division orientation might be predicted. Additionally, most vessels never experience the levels of shear stress found in major arteries, and the microcirculation has a low flow velocity.\textsuperscript{60,61} Thus, it seems plausible that a major component of endothelial cell division orientation operates independent of blood flow in vessels other than major arteries. We found that retinal vessels, which have blood flow but not the shear stress of major vessels, had 92% of endothelial divisions within 20 degrees of perpendicular, and ES cell-derived vessels with no flow had 76% of endothelial divisions in the same category. These data are consistent with a model in which flow-independent regulation of endothelial division orientation contributes substantially to vascular pattern formation.
Analysis of the divisions that occur in or near a sprout, the “sprout field”, shows that endothelial cells orient their cleavage plane perpendicular to the long axis of the structure in which they reside. We did not find divisions within a parent vessel that oriented parallel to the long axis, as was seen in MDCK cells exposed to HGF and in mouse skin epithelium. Moreover, once a sprout formed we did not score divisions within the parent vessel that oriented with the sprout axis. However, once an endothelial cell was in a sprout, its division oriented to the sprout axis. Thus it appears that endothelial cells, unlike some other epithelial cells, do not use division orientation to initiate new morphogenetic structures, but rather to reinforce new sprouts once they have initiated.

How is endothelial division orientation regulated? Growth factor signaling is critical to this regulation in other models, and recently Wnt signaling was shown to be a positional cue for spindle orientation in early C. elegans embryos. Thus we asked whether VEGF signaling regulated endothelial division orientation in developing vessels, by examining vessels deleted for flt-1, which acts as a gain-of-function mutation in VEGF signaling. Our analysis of flt-1 mutant vessels revealed that endothelial division orientation was randomized relative to the vessel long axis, consistent with a role for VEGF signaling in this process. Moreover, introduction of a sflt-1 transgene that rescues branching morphogenesis in the flt-1 mutant background also rescued the increased randomization of endothelial division orientation. These data strongly support a role for VEGF signaling in the regulation of endothelial orientation in vessels. Elevated VEGF signaling regulates the rate of endothelial cell division, and our data provide the first evidence showing that VEGF signaling also regulates the orientation of endothelial cell division.
Interestingly, we show that endothelial cell division orientation is not randomized in VEGF\textsuperscript{120/120} mice, suggesting that the amount of VEGF signaling, and not VEGF presentation, is important for regulation of division orientation. It is well established that VEGF impacts vessel morphogenesis by regulating migration associated with sprout formation\textsuperscript{4,12,17} and our data indicate that VEGF also affects the morphogenetic program via its ability to regulate endothelial cell division orientation.

Our finding that endothelial division orientation is regulated indicates that integration between the endothelial cell division and morphogenesis programs occurs in developing vessels, and it suggests that morphogenetic signals regulate cell division. While the VEGF signal itself is one point of integration, it is likely that morphogenesis and cell division are coordinated via cross-talk at multiple places in the downstream pathways. In other organisms and tissues, division is oriented by the placement of the astral microtubules that emanate from the spindle poles on the cortex. This placement is regulated by polarity determinants, and the polarity determinants in turn are spatially organized by the actin cytoskeleton. Thus the VEGF signaling pathway is likely to intersect with one or more polarity pathways. It is possible that VEGF signaling leads to differences in gene expression that affect polarity, but this is considered unlikely since most polarity information is imparted by spatial organization within the cell. One likely intersection point is the actin cytoskeleton itself, since VEGF regulates actin dynamics\textsuperscript{14,15} Thus the ability of VEGF to locally influence polymerization/depolymerization of the actin filaments could lead to spatial organization of polarity cues at the cortex. The VEGF signaling pathway is also likely to intersect a planar cell polarity pathway, which orients cells in the plane of an epithelial sheet, since planar axis orientation must be regulated to
obtain the cleavage angles that we scored. Moreover, one such pathway involving non-canonical Wnt signaling is implicated in the regulation of division orientation during zebrafish gastrulation. This intersection might occur at the level of the small GTPase Rho, since Rho is downstream of both pathways. Interestingly, we showed that treatment with JNK or Rho Kinase inhibitors randomizes endothelial cell division orientation, suggesting that VEGF may act through JNK and Rho to regulate endothelial cell division orientation.

We have shown that endothelial cell division orientation is regulated early in vascular development in a flow-independent manner, and that this regulation can affect vessel morphogenesis. This indicates that endothelial morphogenesis and cell division are integrated in developing vessels. Moreover, disruption of the integration via perturbed VEGF signaling correlates with vessel dysmorphogenesis, suggesting that cross-talk between morphogenesis and division orientation is critical to proper vessel morphogenesis. Thus endothelial cell division orientation represents another cellular process that is disrupted in dysmorphogenic vessels, and could therefore be a therapeutic target.
E. References


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Figure 3.1. Regulated endothelial cell division orientation may impact vessel morphogenesis – a model. This model shows how endothelial cell divisions whose orientation is regulated relative to the long axis of the vessel could affect vessel shape. Endothelial divisions oriented perpendicular to the vessel long axis (blue vessel on top) would effectively lengthen the vessel, whereas divisions oriented parallel to the vessel long axis (yellow vessel on bottom) would effectively increase the vessel diameter. Microtubules and spindles are shown in red, and DNA/chromosomes are brown.
Figure 3.2. Endothelial cell divisions are oriented perpendicular to the vessel long axis in ES cell-derived vessels. Mouse ES cells (WT; Tg PECAM-H2B-GFP) differentiated to day 7-8 were imaged for several hours prior to fixation and staining for PECAM-1. A) A confocal image showing H2B-GFP signal in green (a), the same field with PECAM-1 stain in red after fixation (b), and the overlay of the two images (c), showing the H2B-GFP signal and PECAM-1 stain in the same cells. B) The top portion of the images in A, showing time lapse images from 0 minutes (a) to 292 minutes (f). Panel (d) was used to calculate the angle of division relative to the vessel axis, according to the drawn yellow lines. The numbers in the lower right represent elapsed time in minutes. C) Calculation of division angles relative to the vessel long axis. Top panel colorized as in Figure 1, except the chromosomes are blue. 90° division angle is perpendicular to the vessel long axis, and 0° division angle is parallel to the vessel long axis. n = 125 divisions. D) Representation of endothelial division angles, with the vessel long axis diagrammed by the long horizontal lines. Each shorter line represents 3 angle measurements that were close or equivalent to each other. See Fig2video1.mov in Supplemental Data for video of panel B.
A

B

C

D

96
Figure 3.3. Daughter cells maintain their division orientation as they migrate after most divisions. ES cell-derived vessels (WT; Tg PECAM H2B-GFP) were imaged, and after divisions were scored (a panels) the two daughter nuclei (pink) were followed for 1-2 hours further. The numbers in the lower right represent elapsed time in minutes. In all cases b panels show the final image scored. c panels diagram the movement of each daughter nucleus (shown in pink) after division: one daughter nucleus is tracked with a green line and the other with a pink line. The vessel is shown by black lines, the vessel long axis is shown by the broken blue line, and the division angle is shown by the broken orange line. A) A division perpendicular to the vessel long axis, where the daughter cells maintained the division orientation after 1 hour (n = 61/61). B) A division parallel to the vessel long axis, where the daughter cells maintained the division orientation after 1 hour (n = 7/10). C) A division parallel to the vessel long axis, where the daughter cells changed position relative to the division orientation after 102 minutes (n = 3/10).
Figure 3.4. Endothelial cell divisions orient perpendicular to the nearest long axis. ES cell-derived vessels (WT; Tg PECAM-eGFP) were imaged for various lengths of time, and divisions in or around sprouts scored for division orientation. In all series, the yellow cell is the scored endothelial cell just prior to division, the pink cells are the daughter cells, and the numbers in the lower right represent elapsed time in minutes. In all cases panel e diagrams the division that was scored, with the parent vessel long axis the broken blue line, the sprout long axis the dotted green line, and the division angle the broken orange line. A) An endothelial division that occurred prior to nearby sprout formation. Although the sprout (arrows in panels c and d) migrated almost perpendicular to the vessel long axis, the division was oriented perpendicular to the parent vessel long axis (n = 2/2). B) An endothelial division that occurred in the sprout field, at the base of a formed sprout but in the parent vessel. These divisions also oriented perpendicular to the parent vessel long axis (n = 6/6). C) In contrast, an endothelial division that occurred within a formed sprout oriented perpendicular to the sprout vessel long axis (n = 5/5).
Figure 3.5. Endothelial cell divisions are oriented perpendicular to the vessel long axis in retinal vessels in vivo. Rat retinas were harvested on days P3-5 and processed for staining as described with *Griffonia* B4 isolectin (green) to visualize vessels and phosphohistone H3 (red) to visualize DNA in mitotic cells. A) Several examples of divisions in retinal vessels that were scored for division angle. B) Calculation of division angles relative to vessel long axis. $90^\circ$ is perpendicular to the vessel long axis, and $0^\circ$ is parallel to the vessel long axis. $n = 86$ divisions. C) Representation of endothelial division angles, with the vessel long axis diagrammed by the long horizontal lines. Each shorter line represents 3 angle measurements that were close or equivalent to each other.
Figure 3.6. Endothelial cell divisions are randomly oriented \textit{flt}-1\textsuperscript{-/-} ES cell-derived vessels. Mouse ES cells (\textit{flt}-1\textsuperscript{-/-}; Tg PECAM-H2B-GFP) differentiated to day 7-8 were imaged for several hours prior to fixation and staining for PECAM-1. A) Time lapse images of a representative movie from 0 minutes (a) to 240 minutes (f), showing the H2B signal in vessels. Panel (c) was used to calculate the angle of division relative to the vessel axis, according to the drawn yellow lines. The numbers in the lower right represent elapsed time in minutes. B) Calculation of division angles relative to the vessel long axis. WT endothelial division angles are shown in blue, and \textit{flt}-1\textsuperscript{-/-} endothelial division angles are shown in purple. 90\degree is perpendicular to the vessel long axis, and 0\degree is parallel to the vessel long axis. n = 125 divisions for WT vessels (same data as shown in Fig. 3.2) and n = 93 divisions for \textit{flt}-1\textsuperscript{-/-} vessels. C) Representation of endothelial division angles, with the vessel long axis diagrammed by the long horizontal lines. Each shorter line represents 3 angle measurements that were close or equivalent to each other.
Figure 3.7. Randomized endothelial division orientation is rescued by a sflt-1 transgene that rescues flt-1⁻/⁻ vessel dysmorphogenesis. ES cell-derived vessels were wild-type (WT), flt-1⁻/⁻, or flt-1⁻/⁻ with a sflt-1 transgene (flt-1⁻/⁻;Tg PECAM sflt-1#26 (sflt26) or flt-1⁻/⁻;Tg PECAM sflt-1#33 (sflt33)). Cultures were differentiated to day 8, fixed, and stained for PECAM-1 (green) and phosphohistone H3 (red). A) Representative vessels of the indicated genotypes, with white arrows pointing to endothelial divisions that were scored. B) Graphic representation of division angles from the different genetic backgrounds. 90° is perpendicular to the vessel long axis, and 0° is parallel to the vessel long axis. WT is blue (n = 19), flt-1⁻/⁻ is purple (n = 29), sflt26 is yellow (n = 25), and sflt33 is light green (n = 23). C) Representation of endothelial division angles, with the vessel long axis diagrammed by the long horizontal lines. Each shorter line represents a single angle measurement.
Figure 3.8. Endothelial cell division orientation is not randomized in VEGF^{120/120} retinal vessels. Postnatal day 5 VEGF^{120/120} and wildtype littermate mouse retinas were analyzed for endothelial cell division orientation. (A-B) VEGF^{120/120} retinal vessels are overgrown compared to wildtype vessels. Vessels are labeled with Isolectin (green) and mitotic DNA is labeled with anti-phospho-histone H3 (red). Arrows in B point to thick vessels. (C) Quantification of cell division orientation in VEGF^{120/120} vs. wildtype retinal endothelial cells.
Figure 3.9. Inhibition of JNK or Rho Kinase results in randomization of endothelial division orientation. Histone H2B::eGFP-expressing wildtype ES cells were differentiated and treated with JNK or Rho Kinase inhibitor for 4 hr. while movies were made. Divisions were randomly oriented following (A) JNK inhibition (Untreated n=125 (same data as Fig. 3.2); JNK I n=78; \( p \leq 0.0001 \)) or (B) Rho Kinase inhibition (Untreated n=4, Rho K I, n=18; \( p \leq 0.0001 \)). Blue bars represent untreated conditions, red bars represent inhibitor treatment.
A. VEGF and endothelial cell centrosome duplication

In chapter two, I showed that elevated VEGF signaling through MEK/ERK and AKT to cyclin E/Cdk2 promotes centrosome over-duplication in endothelial cells. These findings are the first to illustrate a role for VEGF signaling in promoting centrosome duplication and, to our knowledge, the first to link growth factor signaling in general to centrosome duplication. Because a number of debilitating diseases, including cancer, are dependent on or caused by elevated VEGF signaling, understanding how increased VEGF signaling affects blood vessel form and function is important for disease treatment and prevention.

I showed that elevated VEGF signaling leads to centrosome over-duplication in \(flt-1^{-/-}\) ES cell-derived vessels, \(flt-1^{-/-}\) \textit{in vivo} yolk sac vessels and cultured primary HUVEC. I used HUVEC to dissect the signaling pathways linking VEGF and centrosome duplication. High VEGF-treated HUVEC had increased cyclin E levels, Cdk2 activity and NPM phosphorylation compared to low VEGF-treated HUVEC, suggesting that VEGF activates cyclin E/Cdk2 to affect centrosome duplication. My data showing that cyclin E knockdown in the presence of high VEGF signaling inhibits centrosome over-duplication confirm this model. I also showed that high VEGF-treated HUVEC had increased ERK phosphorylation
compared to low VEGF-treated cells, and that inhibition of MEK signaling in the presence of high VEGF inhibited cyclin E accumulation and centrosome over-duplication, suggesting that VEGF signals through MEK/ERK to affect cyclin E levels and centrosome duplication. Additionally, VEGF is known to promote endothelial cell survival via signaling through AKT, and I showed that elevated cyclin E accumulation and centrosome over-duplication were blocked in HUVEC treated with high VEGF+AKT inhibitor. Treatment with AKT inhibitor did not block high VEGF-induced ERK phosphorylation, suggesting that AKT regulates cyclin E accumulation and centrosome duplication independent of MEK/ERK signaling. Prior to my work, it was clear that cyclin E/Cdk2 activity is important for promoting centrosome duplication; and, my data are the first to link VEGF signaling and cyclin E/Cdk2-regulated centrosome duplication. Further dissection of the pathways that act downstream of VEGF to regulated cyclin E/Cdk2 activity will be an important step toward understanding centrosome duplication in endothelial cells and other cell types. For example, the signaling pathway from ERK to cyclin E that regulates DNA replication is relatively well characterized, but whether or not cyclin E is also regulated by molecules specific to centrosome duplication signaling is unknown. It is possible that there exist centrosome duplication-specific molecules that act downstream of ERK to promote cyclin E transcription or cyclin E/Cdk2 activity and centrosome duplication.

I showed that high VEGF-treated HUVEC with excess centrosomes formed aberrant spindles during mitosis, and that flt-1+/- ES cell-derived endothelial cells displayed increased aneuploidy compared to wildtype cells. Interestingly, tumor
endothelial cells, which experience very high VEGF signaling, have excess centrosomes and display aneuploidy.\textsuperscript{1,2} Aneuploidy leads to abnormal cellular behaviors in other cell types and aneuploid endothelial cells likely behave abnormally as well. For example, endothelial cells that contain excess copy numbers of cell cycle-promoting genes may display increased cell proliferation and vascular overgrowth, similar to \textit{flt-1\textsuperscript{-/-}} vessels. Over time, aneuploidy that is advantageous to tumor growth and survival can lead to clonal tumor cell populations,\textsuperscript{3} in which most of the tumor cells contain the same aneuploidy patterns. The cells are clonal because they out-compete (proliferate faster, survive longer) other tumor cells. Advantageous aneuploidy in tumor endothelial cells could also lead to clonal tumor endothelial cell populations. For example, clonal endothelial cell populations that have a proliferative advantage may form blood vessels faster than normal endothelial cells, thus allowing the tumors to grow and metastasize faster. I did not observe clonal aneuploidy in 8 day differentiated \textit{flt-1\textsuperscript{-/-}} ES cell-derived endothelial cells (data not shown); however, it is possible that aneuploid endothelial populations in tumor blood vessels, which form over months or years, have sufficient time to become clonal. In the future, it will be interesting to determine if tumor endothelial cell populations are clonal, and if so, whether aneuploidy patterns are consistent from one tumor to the next. Such studies would shed light on which genes are important for endothelial cell function in tumors, and they would potentially provide additional targets for anti-tumor blood vessel therapies.

Not only did \textit{flt-1\textsuperscript{-/-}} endothelial cells display increased aneuploidy, they also displayed chromosomal aberrations that were not present in wildtype cells,
including chromosome breaks and triradials, which are thought to result from asymmetrical chromatid exchange. It is unclear why these gross chromosomal abnormalities are present in \textit{flt-1$^{-/-}$} endothelial cells. It is possible that excess VEGF signaling in \textit{flt-1$^{-/-}$} endothelial cells promotes cyclin E accumulation (as it does in HUVEC) and that elevated cyclin E accumulation bypasses the DNA damage checkpoint during S phase, resulting in chromosomal aberrations; or, that VEGF affects DNA repair pathways independent of cyclin E. Another possibility is that anaphase spindle abnormalities, such as centrosome clustering and multipolar spindle formation, induce chromosomal abnormalities due to unequal pulling forces on the chromosomes. Whatever the case, in the future, it will be interesting to determine how \textit{flt-1$^{-/-}$} endothelial cells obtain these chromosomal aberrations and whether or not the same abnormalities are observed in tumor vessels and other endothelial cells that experience high VEGF signaling.

We predicted that wildtype ES cell-derived endothelial cells would not display aneuploidy, but we were surprised to find that 13/25 (52\%) wildtype endothelial cells (vs. 21/25, 84\% in \textit{flt-1$^{-/-}$} cells) were aneuploid. It is possible that wildtype ES cell-derived vessels display high aneuploidy because the blood vessels develop in an \textit{in vitro} system, and the pressures to maintain proper endothelial cell chromosome number (and ultimately cellular function) may not be as strong as those \textit{in vivo}. In the future, it will be important to assess aneuploidy in wildtype vs. \textit{flt-1$^{-/-}$} vessels \textit{in vivo}, such as those in developing yolk sac.

I also showed that endothelial cells with excess centrosomes are enriched at the leading edge of \textit{in vitro} scratch wounds, suggesting that endothelial cells
containing excess centrosomes may have a migratory advantage compared to cells with normal centrosome numbers. How excess centrosomes affect migration is unclear, but two likely scenarios emerge. First, increased migration of endothelial cells containing excess centrosomes may be linked to centrosome-mediated microtubule dynamics. Centrosomes act as the microtubule nucleating centers in the cell. It is possible that having excess centrosomes allows for increased microtubule nucleation and increased migration. Second, it is possible that endothelial cells containing excess centrosomes are largely aneuploid, and that endothelial cells containing excess copy numbers of genes that promote migration may have a migratory advantage. Understanding if and how centrosome number affects cell migration will be critical, both in terms of understanding how endothelial cells with excess centrosomes migrate, but also in terms of understanding the process of migration as a whole.

I showed that elevated FGF signaling also promotes centosome over-duplication in endothelial cells. This result is very intriguing because it suggests that angiogenic factors other than VEGF can regulate centrosome duplication. Tumor vessels that are exposed to VEGF inhibitors regress for a short time, but eventually re-grow and retain morphological and functional abnormalities.\textsuperscript{4-6} My results suggest that in tumors, centosome over-duplication might occur even in the absence of VEGF signaling due to elevated levels of other angiogenic factors such as FGF. Furthermore, while VEGF signaling is specific to endothelial cells, FGF activates signaling pathways in a number of different cell types.\textsuperscript{7} It is possible that FGF and other molecules that activate MEK/ERK and/or AKT signaling promote centrosome duplication in other cell types. In any case, my
finding that VEGF and FGF lead to excess centrosome numbers in endothelial cells is important because it provides novel mechanisms to explain how vascular abnormalities occur in the presence of high growth factor signaling.

In the big picture, regulation of endothelial cell centrosome number may be important for the development of anti-tumor vessel therapies. As previously mentioned, anti-tumor vessel therapies only cause vessel regression for a short time period, after which endothelial cells (likely those with proliferative/survival advantages) re-populate the tumor. Recently, a number of groups proposed that tumor vessels must first be normalized before they will robustly respond to anti-vessel therapies.\textsuperscript{4-6} One way to normalize endothelial cells is to target endothelial cells that are abnormal, such as those containing excess centrosomes. Ganem, \textit{et al} (2009) and Silkworth, \textit{et al} (2009) showed that cells that contain excess centrosomes die if they divide with multipolar spindles, as opposed to clustered centrosome spindles.\textsuperscript{8-9} Thus, if endothelial cells containing excess centrosomes are forced to divide with multipolar spindles, it might be possible to kill off the cells that contain excess centrosomes. Interestingly, Ganem, \textit{et al} (2009) showed that knockdown of histone deacetylase (HDAC) gene expression forced cells containing excess centrosomes to divide with multipolar spindles, resulting in cell death. It is possible that inhibition of HDAC in tumor endothelial cells will cause endothelial cells containing excess centrosomes to die following multipolar cell divisions, ultimately yielding normalized tumor vessels.
B. VEGF and endothelial cell division orientation

In chapter three, Dr. Gefei Zeng and I characterized a role for VEGF signaling in regulating endothelial cell division orientation. We showed that endothelial cell divisions are oriented to increase the length of developing blood vessels in ES cell-derived vessels and in vivo in retinal vessels, and that division orientation is blood flow-independent. We also showed that endothelial cell division orientation was randomized in vessels exposed to elevated VEGF signaling via the \textit{flt-1}^{-/}\textit{mutation}.

Mis-oriented cell divisions are associated with abnormal morphology in other tissue types. For example, oriented divisions are required for zebrafish body elongation, and randomized divisions result in stunted embryos.\textsuperscript{10} We showed that endothelial cells divide to increase the long axis of blood vessels undergoing active angiogenesis in ES cell-derived vessels and neonatal retinal vessels. We also showed that elevated VEGF signaling randomized division orientation in \textit{flt-1}^{-/}\textit{vessels}. Here, randomized division orientation was associated with dysmorphogenic vascular overgrowth, and it is possible that mis-oriented divisions contribute to the dysmorphogenic phenotype. In the future, it will be important to determine if developing blood vessels exposed to high VEGF signaling \textit{in vivo}, such as \textit{flt-1}^{-/}\textit{yolk sac vessels or tumor vessels}, also have mis-oriented cell divisions.

How VEGF regulates cell division orientation remains unclear. I assessed division orientation in VEGF\textsuperscript{120/120} neonatal mouse retinal vessels because the vessels are thick compared to wildtype vessels. Endothelial cell division orientation was not random in VEGF\textsuperscript{120/120} retinal vessels. Because VEGF\textsuperscript{120} is
expressed form the endogenous VEGF locus in VEGF^{120/120} mice, it is assumed that VEGF presentation (localization in the ECM), but not VEGF concentration is disrupted. This suggests that the amount of VEGF signaling, and not VEGF presentation, is important for the regulation of endothelial cell division orientation. It is possible that VEGF feeds into a planar cell polarity (PCP) signaling pathway that is important for regulating division orientation in other cell types.\textsuperscript{11} Consistent with this hypothesis, inhibition of molecules that act downstream of PCP signaling, JNK and Rho Kinase, leads to randomization of endothelial cell division orientation. Alternatively, VEGF signaling may affect endothelial cell division orientation independent of PCP signaling. In any case, understanding how VEGF regulates endothelial cell division orientation will be an exciting and important contribution to the field of vascular biology.

Does centrosome number affect endothelial cell division orientation? I showed that elevated VEGF signaling leads to both centrosome over-duplication and mis-oriented cell divisions in endothelial cells, and that centrosome number can affect mitotic spindle formation. Thus, in high VEGF conditions, it is tempting to speculate that mis-oriented endothelial cell divisions result from excess centrosome-induced clustered or multipolar spindles. In this model, a normal endothelial cell with two centrosomes forms a bipolar spindle to increase the length of an existing vessel, while an endothelial cell with three centrosomes may form a tripolar spindle, in which two spindles cluster in a random orientation prior to anaphase. Using a DNA label (like we did in Chapter 2) to assess division orientation, the spindle would appear to be a mis-oriented bipolar spindle. However, as opposed to VEGF playing a direct role in regulating endothelial cell
division orientation, the mis-orientation would be an indirect consequence of VEGF-induced centrosome over-duplication. We showed that 76% of \textit{flt-1}^{-/-} endothelial cell divisions were mis-oriented, while 11% had excess centrosomes. If centrosome over-duplication were the sole cause of mis-oriented divisions in \textit{flt-1}^{-/-} vessels, then I would expect there to be an equal or higher percentage of cells with excess centrosomes compared to mis-oriented divisions. Thus, it is unlikely that all of the mis-oriented cell divisions observed in \textit{flt-1}^{-/-} endothelial cells are a result of centrosome over-duplication; however, it is possible that centrosome over-duplication partially contributes to mis-oriented divisions.

In conclusion, I described two novel roles for VEGF signaling in regulating endothelial cell function and vascular morphology. I showed that elevated VEGF signaling promotes centrosome over-duplication in endothelial cells, and endothelial cell centrosome over-duplication is associated with aneuploidy and cellular dysfunction. I also showed that elevated VEGF signaling randomizes endothelial cell division orientation, which likely contributes to vascular overgrowth in vessels exposed to high VEGF signaling. These novel contributions to the field of vascular biology are important for understanding how VEGF signaling affects both normal and pathological blood vessel formation.
C. References


