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The role of notch signaling in vessel maturation and stabilization

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The Role of Notch Signaling in Vessel Maturation and Stabilization

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Molecular Pathology

by

Elizabeth Lea Scheppke Smoot

Committee in charge:

Professor David Cheresh, Chair
Professor Sylvia Evans
Professor Chris Hughes
Professor Randall Johnson
Professor Geert Schmid-Schönbein

2011
The Dissertation of Elizabeth Lea Scheppke Smoot is approved, and is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2011
EPIGRAPH

“Tiger got to hunt, bird got to fly; Man got to sit and wonder, ‘Why, why, why?’ Tiger got to sleep, bird got to land; Man got to tell himself he understand.”

Kurt Vonnegut, *Cat’s Cradle*
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<table>
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<tbody>
<tr>
<td>bFGF</td>
<td>basic FGF</td>
</tr>
<tr>
<td>DAPT</td>
<td>N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butylester (a gamma-secretase inhibitor)</td>
</tr>
<tr>
<td>DLL</td>
<td>Delta-like ligand</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobin G</td>
</tr>
<tr>
<td>Jag</td>
<td>Jagged (ligand)</td>
</tr>
<tr>
<td>Jag1 ECKO</td>
<td>Jagged1 endothelial cell knockout</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartic acid tripeptide sequence (integrin binding recognition site)</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand Factor</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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Association for Research in Vision and Ophthalmology (ARVO), 2004
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Association for Research in Vision and Ophthalmology (ARVO), 2004
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ABSTRACT OF THE DISSERTATION

The Role of Notch Signaling in Vessel Maturation and Stabilization

by

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Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2011

Professor David Cheresh, Chair

Vascular development and angiogenesis initially depend on endothelial tip cell invasion, which is followed by a series of maturation steps, including lumen formation and recruitment of perivascular cells. Many studies have shown that Notch signaling is involved throughout embryonic vascular
development, including the regulation of tip cell formation, artery/vein patterning, and vascular remodeling. However, the role of Notch signaling in the postnatal vasculature has not been well described. Blocking postnatal Notch results in a highly branched, immature vascular plexus. Vascular plexus maturation is partly regulated by vascular smooth muscle cell (VSMC) coverage, and little is known about the role of Notch in the postnatal perivascular compartment. Therefore, the studies in this dissertation proposed to investigate Notch effectors in VSMCs that modulate vessel maturation during postnatal angiogenesis.

To verify that Notch signaling is important for postnatal arterial VSMC coverage and subsequent vessel maturation, the perivascular compartment of pups undergoing retinal angiogenesis was examined following Notch inhibition. This resulted in significantly less arterial VSMC coverage. An angiogenesis-specific microarray panel identified Notch effector genes upregulated in VSMCs in contact with the endothelial Notch ligand Jagged1, which is known to be required for arterial VSMC coverage. One of the upregulated genes was integrin β3, which led to increased surface expression of the integrin heterodimer, αvβ3.

Integrin αvβ3 is important for cell adhesion and cell migration. In the vasculature, the only available ligand for αvβ3 is von Willebrand Factor (vWF). Examination of pups undergoing retinal developmental angiogenesis revealed that VSMC coverage co-patterns with accumulation of vWF in the endothelial
basement membrane. In a 3-dimensional *in vitro* tube formation assay, disruption of Notch, αvβ3, or vWF prevented an association between VSMCs and newly formed endothelial tubes. Genetic or pharmacological disruption of Jagged1, αvβ3, or vWF suppressed VSMC coverage of nascent vessels and arterial maturation during vascular development *in vivo*. Therefore, the findings described in this dissertation define a Notch-mediated interaction between the developing endothelium and VSMCs, leading to adhesion of VSMCs to the endothelial basement membrane and arterial maturation.
Chapter 1

Introduction
1.1 **Angiogenesis**

1.1.1 **Overview of angiogenesis**

Angiogenesis, the growth of new blood vessels, is a physiologic process important in development, wound repair, and pregnancy. It also plays a central role in various pathologies, including cancer, diabetic retinopathy, stroke, and myocardial infarction. Angiogenesis must be highly regulated in order to produce patent vessels with adequate perfusion to support surrounding tissue. Without such regulation, vessels may over-proliferate leading to non-perfusion and poor delivery of nutrients. Alternatively, a breakdown in vessel barrier function may result, leading to increased permeability and subsequent edema in surrounding tissues.

Blood vessels consist of two associated cell types: endothelial cells and perivascular cells, which are referred to as pericytes, vascular smooth muscle cells (VSMCs), or mural cells. Because initial studies of angiogenesis and anti-angiogenic therapeutics focused on endothelial cells, our understanding of pericyte biology has lagged. However, perivascular cells are critical for proper angiogenesis. The absence of perivascular cells leads to leaky vessels, hemorrhage, edema in the surrounding tissue, as well as poor control of blood pressure and flow. This becomes significant in diseases such as diabetic retinopathy and age-related macular degeneration, where edema leads to blindness, and in cancer, where poor blood flow hinders...
intravenous delivery of chemotherapeutics\textsuperscript{4,5}. Perivascular cells also act as gatekeepers, controlling the passage of cells and soluble molecules across the vessel wall. Their relative absence in tumor vasculature allows for metastasis, as tumor cells surrounding vessels denuded of pericytes are more likely to migrate into the vessel lumen and metastasize to distant sites\textsuperscript{6}. Combined, these factors provide a rationale for developing vessel normalization strategies that improve perivascular coverage to treat diseases involving pathological angiogenesis.

1.1.2 Perivascular cells

Morphological analysis of pericytes reveals that they form a sheath around the endothelial tube by extending long processes from their cell body around the abluminal endothelial wall\textsuperscript{2}. While pericytes were once considered cells that simply provide physical support to the endothelium, it is now understood that pericytes and endothelial cells communicate through gap junctions that allow the exchange of ions and small molecules. They also share a common basement membrane to which both cell types contribute\textsuperscript{7}. Gaps in the basement membrane allow for peg-and-socket contacts that transmit mechanical forces from perivascular cell to endothelial cell, regulating blood pressure and flow\textsuperscript{8}.

Pericytes are found throughout the vasculature on arteries, veins, and capillaries, and their characteristics are as diverse as their locations and
corresponding functions. This makes the cells difficult to define, and there is no single marker for all perivascular cells. However, there are a few markers found throughout the perivascular compartment whose expression varies depending on the developmental stage of their associated blood vessel or in a tissue-specific manner. These markers include desmin and alpha-smooth muscle actin (αSMA), which are intracellular contractile proteins, and regulator of G protein signaling 5 (RGS-5), another intracellular protein. Found on the cell surface of perivascular cells are neuron-glial 2 (NG2), a chondroitin-sulfate proteoglycan, and platelet-derived growth factor receptor beta (PDGFRβ), a tyrosine kinase receptor². αSMA is found predominantly on arteries, and may be a marker of more differentiated pericytes⁹. NG2 is expressed early in the angiogenic process, and therefore may be considered a marker of less mature pericytes¹⁰. PDGFRβ is widely expressed in pericytes, and is also found on fibroblasts, astrocytes, and some tumor cells¹¹.

1.1.3 Signaling pathways in angiogenesis

The angiogenic process is controlled by multiple cell-surface proteins, cytokines, and signaling networks. These include VEGF, bFGF, angiopoietin, PDGF-B, integrins, ephrins, cadherins, and recently found to be involved, the Notch signaling pathway¹²-¹⁴. The coordination of endothelial cells, perivascular cells, fibroblasts, and the basement membrane is important in regulating angiogenesis and yielding a functional network of vessels (Figure
1.1 a). The interplay between multiple signaling pathways ensures the organized progress of angiogenesis.

Sprouting angiogenesis occurs when endothelial cells are stimulated by angiogenic growth factors, such as VEGF, secreted by stromal cells, which induces endothelial proliferation and migration towards the angiogenic stimulus\textsuperscript{15,16}. The endothelial cells in turn secrete growth factors to recruit pericytes and promote vessel maturation. These include sphingosine-1-phosphate-1 (S1P1), angiopoietins, and PDGF-B\textsuperscript{17}. Of these, PDGF-B is the most widely studied factor in pericyte recruitment. PDGF-B binds to PDGFR\(\beta\) on perivascular cells, causing pericytes to migrate towards the PDGF-B stimulus and coat the nascent vessel\textsuperscript{18}. Expression of VEGFR2 on perivascular cells attenuates pericyte response to growth factors in the environment through the formation of PDGFR\(\beta\)/VEGFR2 inhibitory complex, which prevents downstream PDGFR\(\beta\) signaling in the perivascular cell and inhibits cell migration\textsuperscript{19}. Once in contact with the newly formed endothelial tube, pericytes stabilize the vessel, preventing its regression and inducing maturation. There is a slight lag in pericyte coverage following initial vessel formation that allows for plasticity in the endothelial response to local angiogenic cues (Figure 1.1 b, c).
Figure 1.1 Angiogenesis

(a) Blood vessels are composed of an inner layer of endothelial cells, a basement membrane shared by the endothelial and mural cells to which both cell types contribute, and a layer of mural cells that stabilizes the vessel and regulates blood flow. (b) Angiogenesis is driven by the secretion of angiogenic growth factors, such as VEGF, from nearby stromal cells. (c) This leads to a breakdown of the extracellular matrix, the detachment of perivascular cells, and new vessel sprouting via tip cell formation. (d) Nascent vessels secrete growth factors, such as PDGF-B, that lead to the recruitment of mural cells (e).
1.2 **Vessel maturation**

1.2.1 **Perivascular cells stabilize vessels**

Following the initial stages of angiogenesis, which include endothelial cell outgrowth, tube formation, and artery/vein patterning, comes the process of vessel maturation. Vessel maturation includes deposition of an endothelial basement membrane, remodeling of the vascular bed, and the recruitment of pericytes and VSMCs to nascent vessels. Mural cell recruitment is required for vessel stabilization. In the absence of perivascular cell coverage, newly formed vessels are subject to regression and are dependent upon growth factor stimulation from the environment for their survival. However, once invested with pericytes, vessels are stabilized and resistant to regression. One reason for this stabilization is the secretion of TGF-β in response to endothelial cell-mural cell contact. TGF-β is a growth factor that acts on both cell populations, inhibiting endothelial cell proliferation and migration while inducing mural cell differentiation.

Pathological animal models and studies of diseased human tissue demonstrate the need for perivascular coverage of newly formed vessels. In wound healing studies, the investment of pericytes within the vascular basement membrane is temporally related to the onset of capillary quiescence. Conversely, the absence of pericyte coverage leads to pathologically unstable vessels. For example, one of the first clinical
hallmarks of diabetic retinopathy is pericyte loss. As another example, tumor vessels characteristically have absent, or loosely associated, perivascular cells, leading to a constantly immature and unstable vasculature. In addition to loosely associated perivascular cells, the tumor endothelium is characterized by an abnormal or absent basement membrane, another indicator of an immature vasculature. Tumor vessels, though they lack such stabilizing features as perivascular cells and an intact basement membrane, are maintained by exposure to high levels of growth factors.

1.2.2 The endothelial basement membrane regulates angiogenesis and vessel maturation

In addition to perivascular cell investment, the formation of the endothelial basement membrane is a critical step in the formation of a mature and functional vasculature. Basement membranes are thin layers (50-100 nm) of specialized extracellular matrix shared by endothelial and epithelial cells which provide structure and support for those cells. The endothelial basement membrane is rich in fibronectin, collagen type IV, laminin, and heparin sulfate proteoglycans. Both collagen type IV and laminin are abundant in the basement membrane and their scaffold forms the basic structure of the basement membrane.

The function of the basement membrane goes beyond scaffolding; it also potentiates bi-directional signaling. In outside-in signaling, ligands such
as collagen type IV promote adhesion, migration, differentiation, and growth for both endothelial cells and NG2\(^+\) perivascular cells\(^{30-32}\). Fibronectin, another ligand, is an adhesive protein with several distinct binding sites for ECM components, as well as an integrin binding domain which promotes endothelial cell proliferation and migration in wound healing and diabetic retinopathy\(^{33}\). The basement membrane also serves as a reservoir for growth factors and other signaling molecules, which are bound up by the matrix and released following proteolytic degradation associated with angiogenesis\(^{29}\). Heparin sulfate proteoglycans potentiate the local effects of VEGF and bFGF signaling during angiogenesis\(^{34}\). In addition to entrapped growth factors, cleaved fragments of the basement membrane also have the ability to influence angiogenesis when proteolyzed\(^{35,36}\).

The ligands and growth factors that compose basement membranes are tissue-specific. The endothelial basement membrane is unique in its accumulation of the protein von Willebrand Factor (vWF), which is derived from endothelial Weibel-Palade body secretions\(^{37}\), is important for clot formation and has recently been shown to play a role in vessel patterning\(^{38}\). Interestingly, the composition of tumor endothelial basement membranes is different from that of the normal endothelium. This abnormal basement membrane contributes to the instability of tumor vessels that are leaky and permit metastasis\(^{29,39}\).
1.2.3 Overarching role of Notch in angiogenesis and vessel maturation

While multiple cell types, secreted proteins, and growth factors coordinately regulate angiogenesis and vessel stabilization, the Notch signaling pathway is unique in that it is involved at multiple stages of angiogenesis, from initial vascular plexus formation and artery/vein patterning, to VSMC recruitment and vascular remodeling. Furthermore, Notch family member receptors and ligands are expressed on both endothelial cells and perivascular cells, and are a mechanism for cell-cell communication between the neighboring cell types.

Because of the importance of perivascular cells in angiogenesis, and the fact that little is known about the effects of Notch signaling in mural cells, this dissertation seeks to further explore the role of Notch signaling specifically as it applies to perivascular cells and vessel maturation.
1.3  **The Notch signaling pathway**

### 1.3.1 Components of the Notch pathway

Notch receptors and ligands are part of an evolutionarily conserved pathway that regulates cell survival and differentiation through lateral interactions between equivalent cells\(^{40,41}\). These interactions occur in tissues from all three germ layers, and Notch has been shown to play a role in neurogenesis\(^{42}\), hematopoiesis\(^{43,44}\), somitogenesis\(^{45}\), adipogenesis\(^{46}\), limb development\(^{47}\), myogenesis\(^{48,49}\), and most recently, angiogenesis\(^{13,50-53}\).

Notch signaling was first described in *Drosophila melanogaster*, where it was understood that the absence of functional Notch signaling led to enhanced neurogenesis. Further studies in both *Drosophila* and in *Caenorhabditis elegans* determined that Notch signaling involves a process of lateral cell-fate decisions, in which one cell differentiates through a Notch receptor-ligand interaction, while the neighboring cells in this interaction remain undifferentiated\(^{40}\). The effects of Notch signaling are complex and context-dependent. Notch promotes cell survival, differentiation, or proliferation, depending on the cell types involved.

*Drosophila* have a single Notch receptor which can be activated by two ligands, Delta and Serrate. In contrast, vertebrate have four Notch receptors (Notch1-4), which can interact with any one of five ligands, (three Delta-like (DLL)1, 3, 4, and two Serrate-like, Jagged (Jag)1, and Jag2) (Figure 1.2 b). These ligands bind in an overlapping pattern to Notch receptors expressed on
adjacent cells, of which there are four (Notch1-4). Notch receptors are single-pass transmembrane receptors consisting of a ligand-binding extracellular domain, a transmembrane domain that is involved in receptor activation, and an intracellular domain that is cleaved and responsible for activating Notch signaling (Figure 1.2 a). Receptor ligation induces a presenilin-dependent proteolytic cleavage that results in the release of the extracellular domain, as well as a γ-secretase-mediated cleavage event adjacent to the transmembrane domain that results in release of the Notch intracellular domain within the receptor-expressing cell\textsuperscript{54,55} (Figure 1.3 a). The Notch intracellular domain translocates to the nucleus, where it activates the major downstream target for Notch (CBF1/RBP-Jκ in mammals) by displacing repressors and recruiting transcriptional co-activators (Figure 1.3 b). The transcriptional complex induces expression of target genes, members of the HES family, which regulate the expression of tissue-specific proteins\textsuperscript{14}. While canonical Notch downstream signaling is regulated through HES family members, it has also been shown that the Notch intracellular domain is able to bind directly to promoters of certain target genes and drive their expression, circumventing the need for transcriptional regulation by HES members\textsuperscript{56}. 
Figure 1.2 Notch receptors and ligands

(a) Representative schematic of a mammalian Notch receptor, of which there are four (Notch1-4). The receptors are all characterized by a large extracellular domain with 29-36 EGF-like repeats (EGF-LR) and 3 LIN Notch repeats (LNR) non-covalently linked to an intracellular domain, containing the RAM23 domain and 7 Ankyrn/CDC10 repeats (ANK). Also intracellular is the nuclear localization sequence (NLS), which is required to target the intracellular domain to the nucleus. The transactivation domain (TAD, found only on Notch1 and 2) is required to activate downstream events. The C-terminal Pro-Glu-Ser-Thr (PEST) domain targets the intracellular domain for degradation. (b) There are 5 mammalian DSL (Delta, Serrate, LAG 2) ligands which bind to Notch receptors. They are Delta-like ligand (DLL)1, 3, 4 and Jagged1, 2 (homologous to Drosophila Serrate). The extracellular domain consists of EGF-like repeats that interact with EGF-like repeats in the receptor, a conserved DSL domain. Jagged-1 and 2 have more EGF-like repeats than DLL ligands, as well as a cysteine rich domain (CRD).
Figure 1.3 Notch signaling

(a) The Notch receptor undergoes a furin-mediated proteolytic cleavage event in the Golgi apparatus (marked by the star, “1”) to become a mature heterodimeric protein. This translocates to the plasma membrane. On binding its cognate ligand, there is a second extracellular cleavage event (marked by the star, “2”) mediated by TNF-α converting enzyme (TACE) and ADAM17 metalloprotease, which is quickly followed by a third, intracellular cleavage mediated by γ-secretase (marked by the star, “3”). This third cleavage releases the Notch intracellular domain (NICD). (b) The NICD translocates to the nucleus, where it displaces co-repressors, binds the CSL protein of target genes and recruits transcriptional co-activators including the mastermind-like nuclear protein (MAML) and leads to the transcription of Notch downstream genes, such as *HES* family members.
1.3.2 Notch in the vasculature

Though Notch ligands and receptors are widely expressed in all germ layers, within each tissue type there are selective expression patterns. In the vasculature, all four Notch receptors are expressed. Notch1 and Notch4 are predominantly expressed in the arterial endothelium\textsuperscript{57-60}, Notch2 is expressed in the pulmonary endothelium\textsuperscript{61}, and Notch3 is predominantly expressed in adult arterial VSMCs\textsuperscript{62,63}. Though all Notch receptors are expressed in the endothelium, the same does not hold true for the Notch ligands. During embryonic development, DLL4 and Jag2 are expressed in arterial endothelial cells\textsuperscript{61}. Jag1 is expressed in both the endothelium and in arterial VSMCs\textsuperscript{61}. DLL1 is expressed postnatally in arteries, veins, and capillary beds; a pattern which sets it apart from other Notch ligands and receptors, which are restricted to the arterial vasculature\textsuperscript{64}. Unlike the other ligands, DLL3 is not expressed in the endothelium\textsuperscript{61}.

1.3.3 A requirement for Notch signaling in angiogenesis and vascular remodeling

Studies in knockout mice demonstrate the requirement for Notch receptors and ligands during angiogenesis. Global knockout of Notch1 alone, or of Notch1 and Notch4 together, results in embryonic lethality with severe defects in vascular remodeling, such that the initially formed vessels fail to remodel into larger vessels and smaller capillaries. This affects the embryo,
yolk sac, and placenta\textsuperscript{65}. Knocking out only Notch4 yields mice that are viable and fertile\textsuperscript{65}, whereas expression of an activated form of Notch4 in the endothelium leads to defects in vascular remodeling and embryonic lethality at E10.5\textsuperscript{66}, indicating that the regulation of Notch signaling is required for vascular patterning. The ligand that Notch1 and Notch4 receptors are likely interacting with is DLL4, based on gene expression patterns\textsuperscript{65}. As such, it is not surprising that haploinsufficiency of \textit{DLL4} is embryonic lethal in mice, with most embryos dying at E10.5 due to vascular defects, though the lethality displays incomplete penetrance and the severity of the phenotype is strain-specific\textsuperscript{51}.

There are developmental requirements for specific expression of other Notch ligands in the vasculature as well. Homozygous mutation of \textit{Jag1}\textsuperscript{67} or \textit{DLL1}\textsuperscript{68} genes result in embryonic lethality due to hemorrhage and defects in vascular remodeling at approximately E10.5 during development. Interestingly, DLL4 expression patterns change from embryonic development to adulthood. During the embryonic stage, DLL4 is highly expressed on major arteries. In adulthood, expression remains arterially restricted, but predominantly shifts to smaller arteries and their associated microvessels\textsuperscript{51}. The continued expression of Notch receptors in the endothelium, and their changing pattern compared with embryonic stages, suggests that Notch signaling plays an important role in both vascular development in the embryo and vessel maintenance and homeostasis in the adult. To date, the role of Notch in the adult vasculature has not been well described.
While there is a clear requirement for Notch signaling in the endothelium, the cell fate decisions that Notch regulates and its downstream mechanisms are not yet well described. What is known, however, reveals a complex interaction between multiple signaling pathways known to play a role in angiogenesis. One example is the role of Notch in regulating endothelial cell sprouting. Tip cells express elevated levels of the ligand DLL4, while neighboring stalk cells express Notch1 and have elevated intracellular Notch signaling levels\textsuperscript{69}. This relationship determines an endothelial tip/stalk cell phenotype. Signaling through VEGFR2 modulates tip cell formation by upregulating DLL4 on tip cells, thereby ensuring their phenotype\textsuperscript{69,70}. Meanwhile, activated Notch drives down VEGFR2 levels in stalk cells, thereby ensuring that a select number of endothelial cells commit to the tip cell phenotype and preventing hypersprouting\textsuperscript{71}. Blocking Notch results in an excess of sprouts, a disorganized and immature vascular plexus, and poor blood flow\textsuperscript{69,72}.

1.3.4 Notch signaling in vascular smooth muscle cells

The importance of Notch in endothelial cells has been better described than the role of Notch signaling in perivascular cells. However, it is known that Notch is required for the formation of VSMCs (Figure 1.4). Expression of the Notch3 receptor, which is restricted to VSMCs in the endothelium, is required for arterial differentiation and VSMC maturation\textsuperscript{62,63}. While Notch3 seems to
be the critical receptor for mural cell differentiation, Jag1 is the corresponding ligand that has been shown to be most important for this process. Endothelial-specific deletion of Jag1 results in severe mural cell defects, whereas the expression of Jag1 on endothelial cells promotes mural cell differentiation. The expression of Notch3 on mural cells is induced by contact with endothelial Jag1. Furthermore, Notch signaling in VSMCs promotes their own expression of Jag1, leading to a positive-feedback loop that ensures expression of Notch3 on VSMCs and Notch signaling within those cells, driven either by contact with the endothelium, or by contact with neighboring VSMCs.

Beyond its role in embryonic developmental angiogenesis, two inherited genetic disorders provide evidence for the role of Notch in VSMC maintenance into adulthood. In Alagille syndrome, mutations in Jag1 lead to cardiac defects resulting from deficient Notch signaling in the neural crest population, preventing their differentiation into smooth muscle cells. CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is caused by mutations in Notch3 and is characterized by loss of VSMC coverage over time, leading to stroke and dementia. Since Notch signaling regulates PDGFRβ expression in VSMCs, mutations in Notch3 interfere with a paracrine signaling mechanism that is required for stabilizing mature vessels. This process of vessel stabilization and maturation is critical for the development of normal vasculature, and its absence is a hallmark of pathological vessels. Beyond Notch3 regulation of
PDGFRβ in VSMCs, other Notch effectors that regulate VSMC coverage and vessel maturation have not been described.

**Figure 1.4 Endothelial Jag1 induces Notch on neighboring VSMCs**

(a) During normal development, the ligand Jag1 on endothelial cells (ECs) binds to Notch3 expressed on neighboring VSMCs, upregulating Notch in VSMCs. This drives a positive feedback loop in which Notch enhances expression of Jag1 on VSMCs, which acts as a ligand to neighboring VSMCs, perpetuating Notch signaling in the arterial mural cell compartment. (b) Notch signaling in VSMCs is required for VSMC coverage. Notch3 KO mice have no arterial VSMC coverage. (c) Notch signaling in VSMCs is initiated by endothelial expression of Jag1, which is required for VSMC coverage.
1.4 **Postnatal retinal angiogenesis assay**

The mouse retina serves as an ideal *in vivo* model system to study the processes of angiogenesis and vessel maturation. Mouse pups are born with an essentially avascular retina. During the first week of life (postnatal days (P)0-7) blood vessels grow out in a single plane from the optic nerve towards the periphery in response to a VEGF gradient established by the underlying astrocytic template\(^{78,79}\) (Figure 1.5 a). As the vessels grow out, they pattern into radially alternating arteries and veins beginning approximately P3\(^80\). Subsequently, the endothelial cells establish a basement membrane and recruit perivascular cells, including pericytes and vascular smooth muscle cells. As the vessels become more mature, beginning approximately P7, the intervening capillary network is pruned back\(^4\). This process establishes the primary vascular plexus (Figure 1.5 b-d).

Two additional vascular plexuses form subsequent to the outgrowth of the primary vascular plexus. Beginning at approximately P5-P7, depending on the mouse strain, microvessels dive down from the primary plexus and then branch to form the deep plexus, a plane of capillaries\(^4\). About one week later, an intermediate vascular plexus of capillaries forms between the primary and deep plexuses.
Figure 1.5  Angiogenesis in the postnatal mouse retina

(a) Representative images of whole-mounted murine retinas with radial cuts depicted schematically. Mice are born with an essentially avascular retina (P0). During the first week of life, blood vessels grow out from the optic nerve towards the periphery in a radially alternating pattern of arteries (red) and veins (blue). (b) The initial outgrowth of vessels forms an immature vascular plexus, with tip cells leading, followed by stalk cells. (c) Remodeling of the primary vascular plexus occurs from approximately P7-P18. During this phase, intervening vessels are pruned back, leading to a mature vascular plexus (d).

The mouse retina serves as an ideal model to study developmental angiogenesis and vessel maturation for several reasons. The pattern of angiogenesis is highly reproducible, the eye is easily accessible, and the
planar nature of the vessels allows for reproducible imaging. Moreover, because retinal angiogenesis occurs postnatally, it is possible to manipulate angiogenesis either by systemic drug treatment or by intraocular injection of angiostatic agents\textsuperscript{81}.

The work described in this dissertation employs the mouse retina to study the role of Notch in vessel maturation. To date, studies investigating the role of Notch in angiogenesis have relied primarily on genetically manipulated mouse models that disrupt or prevent Notch signaling events\textsuperscript{51,67,75,82}. Due to the embryonic lethal nature of the majority of these mouse models, these studies have been limited to observations on the role of Notch during embryogenesis and cellular differentiation. In mice that do survive, for example, Notch3 KO mice, it is hard to tease apart the effects of the gene knockout on cell differentiation from the effects on vessel maturation\textsuperscript{62}.

To address the open question of the role of Notch in vessel maturation, rather than vascular cell differentiation, the studies described in this dissertation manipulated Notch signaling and newly identified Notch-downstream targets during the postnatal period of retinal angiogenesis.
1.5 **Hypotheses**

The following broad hypothesis is addressed in two chapters:

*Notch signaling between endothelial cells and perivascular cells promotes arterial maturation.*

**Chapter 3 Hypothesis:**

*Endothelial Jagged1 drives Notch-downstream effectors in vascular smooth muscle cells that regulate arterial VSMC coverage.*

The effect of blocking Notch signaling on arterial VSMC coverage was examined *in vivo* and micorarray analysis was used to identify Notch-downstream targets in VSMCs *in vitro*.

**Chapter 4 Hypothesis:**

*Notch promotes vascular maturation by inducing integrin-mediated smooth muscle cell adhesion to the endothelial basement membrane.*

VSMC coverage was quantified in vWF KO mice, in mice expressing a kinase-dead knock-in β3, and by pharmacological blockade of integrin β3 *in vivo*. *In vitro* studies confirmed that Notch signaling regulates β3-dependent adhesion of VSMCs to vWF in the endothelial basement membrane.
Chapter 2

Methods
2.1 **In vivo assays**

Mouse experiments were performed under approval by the University of California San Diego Institutional Animal Care and Use Committee. Balb/c mice (Jackson Labs) were used for DAPT treatment, anti-CD61 injection, and the vWF/αSMA time course. DiYF mice were a gift from T. Byzova (The Cleveland Clinic) and vWF KO mice were a gift from D. Wagner (Harvard). Both DiYF and vWF KO mice were on a C57BL/6 background, and age-matched C57BL/6 mice (Jackson Labs) were used as controls.

For injection of anti-CD61, postnatal day 5 Balb/c pups received a single intravitreal injection of purified NA/LE hamster anti-mouse CD61 (BD Biosciences) (0.5 µg in 0.5 ul volume) using a 2.5 µl Hamilton syringe (Hamilton cat# 87942) fitted with a 33 GA 0.5-inch removable needle (point style 4; Hamilton) into one eye. The contralateral eye received a single intravitreal injection of purified NA/LE Hamster IgGκ isotype control. Retinas were harvested on day 8 and processed for immunohistochemistry.

For injection of DAPT, DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butylerster) (Tocris) was dissolved in 20% DMSO and 80% corn oil (Sigma). Balb/c pups were injected subcutaneously at 100 mg/kg in a volume of 10 µl/g daily, from P2-P5.
2.2 Whole mount immunohistochemical staining of retinas

Retinas were harvested from postnatal pups aged P1-P28, fixed in 4% PFA for 10 min and dissected in PBS. The retinas were permeabilized in ice-cold methanol for 10 min then blocked in 20% FBS/20% NGS (or 20% NDS)+0.3% Triton X-100 in PBS for 2 hr. Primary antibodies were added to antibody diluents (10% FBS/10% NGS or 10%NDS + 0.3% Triton X-100 in PBS) overnight at 4°C. The next day, retinas were washed for 2 hr and secondary antibodies were added. Secondary antibodies were incubated with the retinas for 2 hr at RT, then the retinas were washed in PBS for 2 hr. All wash steps (following primary and secondary incubation) involved three initial quick washes, followed by washes spaced 20 minutes apart, with the retinas on an orbital shaker set to medium-low speed. Following all washes, retinas were post-fixed with 2% PFA for 5 minutes and flat-mounted on glass slides with SlowFade Mounting Medium (Invitrogen).

Primary antibodies: Blood vessels were labeled with either isolectin B4 directly conjugated to Alexa 647 (Invitrogen) or with rat anti-mouse CD31/PECAM-1 (BD Biosciences). VSMCs were labeled with mouse-αSMA directly conjugated to either FITC or Cy3 (Sigma-Aldrich). Other primary antibodies used: rabbit anti-human vWF (Millipore), rabbit anti-NG2 chondroitin sulfate proteoglycan (Millipore), rabbit anti-Jagged1 (Santa Cruz), goat anti-Jagged1 (Santa Cruz), goat anti-Notch3 (Santa Cruz). Secondary
antibodies: goat anti-rabbit A568 or A647, goat anti-rat A488, and donkey anti-goat A568 or A647 (Invitrogen). All antibodies were used at 1:200 dilution.

2.3 Immunohistochemistry of retinal frozen sections

Retinas were harvested as described, fixed in 4% PFA in PBS O/N at 4°C, incubated in 20% sucrose in PBS for 2 hr, and embedded in OCT (Tissue-Tek). 10 um thick frozen sections were cut, post-fixed in acetone, rehydrated in PBS, then blocked with 5% BSA, 2% NGS in PBS for 2 hr. Sections were incubated with primary antibodies: rat anti-mouse CD31, 1:200 (BD Biosciences), mouse anti-SMA FITC-conjugated, 1:200 (Sigma-Aldrich), and hamster anti-mouse CD61, 1:10 (BD Biosciences) overnight in 5% BSA, 2% NGS in PBS. After washing, sections were incubated with goat anti-rat A546 and goat anti-hamster A647 (Invitrogen) for 2 hr and washed again then mounted with SlowFade Mounting Medium (Invitrogen).

2.4 Confocal fluorescence microscopy

Imaging was performed on a Nikon Spectral C1 confocal microscope (Nikon C1si with EZC1 acquisition software, Nikon Instruments) with Plan Apo 10×/0.45 air, Plan Apo 20×/0.75 air and Plan Apo 60×/1.40 oil objective lenses (Nikon). All images were recorded with a sequential acquisition of the fluorescent channels to prevent fluorescence bleed-through. Images were analyzed with MetaMorph software (Molecular Devices) for determination of VSMC coverage and with ImageJ for VSMC-endothelial cell co-patterning (RG2B colocalization plugin).
2.5 Transmission electron microscopy

Samples were immersed in modified Karnovsky’s fixative (1.5% glutaraldehyde, 3% paraformaldehyde and 5% sucrose in 0.1 M sodium cacodylate buffer, pH 7.4) for at least 8 hours, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour and stained en bloc in 1% uranyl acetate for 1 hour. Samples were dehydrated in ethanol, embedded in epoxy resin, sectioned at 60 to 70 nm, and picked up on Formvar and carbon-coated copper grids. Grids were stained with uranyl acetate and lead nitrate, viewed using a JEOL 1200EX II (JEOL, Peabody, MA) transmission electron microscope and photographed using a Gatan digital camera (Gatan, Pleasanton, CA).

2.6 Cell culture

HUVECs (Lonza) were cultured in EBM-2 media (Lonza) supplemented with a bullet kit containing endothelial-specific growth factors (Lonza) and 10% FBS (Hyclone). Human aortic vascular smooth muscle cells (VSMCs) from a young donor (Invitrogen, lot #200707) were cultured in Media 231 with smooth muscle cell growth supplement (Invitrogen).

2.7 Jag-1 stimulation of VSMC in vitro

VSMCs between passages 3-7 were cultured on 6-well TCT-plates coated with 5 ug/ml IgG or Jagged1 for 18 hr. The procedure for Jagged-1 coating of wells was based on a previously published protocol by Jin, et al. Briefly, 6-well TCT plates were coated with recombinant Protein G (50 ug/ml in
PBS) (Invitrogen) at room temperature overnight. Wells were washed 3x with PBS, then blocked with 10 mg/ml endotoxin-free BSA in PBS for 2 hr. Wells were washed 3x, with PBS, then coated with 5 ug/ml Jagged1 or IgG (R&D Systems) in PBS + 0.1% endotoxin-free BSA for 2 hr. Wells were washed with PBS and VSMCs were plated at a density of 3x10^5 cells/well.

2.8 **Microarray analysis**

VSMCs were cultured on Jag1 or IgG-coated plates as described above. RNA was extracted using RNA isolation kit (Qiagen). Reverse-transcription PCR was done with RNA-to-cDNA kit (Applied Biosystems). cDNA was run on a TaqMan Low-Density Array, human angiogenesis panel (Applied Biosystems, Cat# 4378725), amplified on a 7900 HT Fast Real Time PCR system (Applied Biosystems) according to manufacturer’s instructions.

2.9 **RNA extraction, reverse transcription PCR**

VSMCs were cultured on Jag1 or IgG-coated plates as described. RNA was harvested with TRizol (Invitrogen) according to manufacturer’s instructions. cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Individual quantitative RT-PCRs were performed with Absolute qPCR mix (Thermo Scientific) and SYBR Green (Invitrogen) on 20 µg cDNA on a SmartCycler (Cepheid) according to the manufacturers’ instructions.
Primers:

**Hey2**: CAACCCCTTGTCGCCTCTC / CCGTGGATGGCATTCCGGAG,
PrimerBank ID# 6912414a3

**Hes1** (CAGGAATGTTTTCACGGAGTCT / AACGTGCTCAGGTTTTTAGCC),
PrimerBank ID# 5031691a2

**Integrin β3** (GTGACCTGAAGGAGAATCTGC / TCACTCACTGGGAACTCGATG, PrimerBank ID# 2443452a3)

**β-actin** (GGAGGAGCTGGAAAGACAGCC / GCTGTGCTACGTCGCCCTG)

2.10 **FACS Analysis**

Human VSMCs were cultured on Jag1 coated plates (as described in 2.7) for 18 hours, trypsinized, and suspended in PBS+2%FBS. 1x10^6 cells per condition were incubated on ice for 1 hr with 1.2 µg primary antibody (LM609 to label integrin β3, mouse IgG for control). Cells were washed and incubated on ice with secondary antibodies (goat anti-mouse FITC) and propidium iodide to label dead cells for 30 min, then washed and analyzed in the Moores Cancer Center FACS analysis facility.

2.11 **Tube formation assay**

Human umbilical vein endothelial cells (HUVECs) at passage 2 were trypsinized and coated onto Cytodex3 microcarrier beads (GE Healthcare) as previously described. In brief, coating was achieved by incubating 10^6
HUVECs with 2500 beads in EGM-2 (Lonza) in a 3 ml Falcon tube and inverting every 20 minutes for 3-4 hr. The coated beads were placed overnight in EGM-2 in the tissue culture incubator (37°C, 5% CO$_2$) in an appropriately sized TCT-dish. Following overnight culture, HUVEC-coated beads were suspended in fibrinogen (Sigma, 2 mg/ml) with aprotinin (Sigma, 0.15 U/ml) in PBS at a concentration of 500 beads/ml. VSMCs were labeled with DilC12(3) (1:1000 in culture media for 2 hr; BD Biosciences) and added to bead solution at $1 \times 10^5$ cells/ml. Fibrin gels were made in 24-well TCT-plates by mixing 0.5 ml fibrinogen solution with thrombin at a final concentration 0.625 U/ml. After gels solidified, 1 ml EGM2 was added to each well with *Ulex europaeus* Agglutinin I conjugated to FITC (1:200; Vector Labs) to visualize endothelial tubes. Beads were imaged daily using the Nikon confocal microscope as previously described, from day 1 to day 8 (Day 0 was considered the day beads were suspended in fibrin).

For IHC, 1 ml gels were made on glass chamber slides following the same procedure described. Fibrin gels were fixed with 4% PFA O/N, permeabilized with methanol (4°C) for 15 min. Staining proceeded as described for the retinas, with all antibody incubations done overnight.

2.12 **VSMC-Endothelial Cell colocalization analysis**

The VSMC coverage of endothelial tubes was analyzed using ImageJ with the RG2B colocalization plugin. Stacks of confocal fluorescent images (described in 2.4) were analyzed using the RG2B plugin. The resultant
colocalization images were converted to 8-bit images and thresholded. These are the “colocalization” images.

Separately, the corresponding RGB confocal images were split into individual color channels and processed to binary. Of these binary images, the channel of interest is that of the endothelial tubes. These are the “EC tubes” images.

For analyzing colocalization in the bead assay, the pixels corresponding to the beads themselves were deleted from all images analyzed because we were interested in colocalization along the tubes. Finally, the ImageJ “analyze particles” function was run on all images, with the readout being Area Fraction.

To determine colocalization, the following calculation was used:

\[
\text{Area Fraction}_{(\text{colocalization})}/\text{Area Fraction}_{(\text{EC tubes})} = \text{colocalization}
\]

2.13 xCelligence adhesion assay

16-well E plates (Roche) were coated with vWF (gift from Z. Ruggeri) at 10 \(\mu\)g/ml overnight and blocked with 1% heat denatured BSA (Hyclone). VSMCs in Opti-MEM with 0.25 mM MnCl\(_2\), 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 20 mM Hepes were added to wells, each condition was run in triplicate. Data was collected on the xCelligence machine (Roche) according to manufacturer’s instructions. Impedance was measured every minute for 2 hrs, then every 5 minutes for another hour, resulting in a cell index number that correlated to VSMC adhesion.
2.14 **Inhibition of Notch, vWF, \(\alpha v\beta3\), and \(\beta3\) in vitro**

A 100 mM stock solution of DAPT (Tocris) in DMSO was added to VSMC culture media at a final concentration of 25 \(\mu\)M, vehicle was DMSO. A vWF blocking antibody specific to the \(\beta3\) RGD binding site on vWF (\(\alpha\)-152B, gift from Z. Ruggeri) was added to the culture media at a final concentration of 30 \(\mu\)g/ml; the \(\alpha v\beta3\) blocking antibody LM609 and the integrin \(\beta1\) blocking antibody P4C10 were each added to the culture media at a final concentration of 20 \(\mu\)g/ml. Vehicle was mouse IgG (BD Biosciences).

2.15 **siRNA knockdown in VSMC**

VSMCs were transfected with siRNAs against Notch3 (Qiagen; Notch3-3) or All Stars control siRNA (Qiagen) using human aortic SMC nucleofector kit (Lonza) in the Amaxa nucleofector system (Lonza) according to manufacturer’s instructions. Knockdown was confirmed by western blot with rabbit anti-Notch3 (Sigma), rabbit anti-Hey2 (ProteinTech Group), mouse anti-HSP60 (Santa Cruz) was used as a loading control.

2.16 **Statistical analysis**

All statistical analyses were performed with Excel (Microsoft). Two-tailed Student’s \(t\) test was used to calculate statistical significance. A \(P\) value < 0.05 was considered to be significant.
Chapter 3

Endothelial Jagged1 drives Notch-downstream effectors in vascular smooth muscle cells, which regulate arterial VSMC coverage
3.1 Abstract

Angiogenesis plays an essential role in development, and aberrations in the angiogenic process contribute to pathologies associated with cancer and retinal disease\textsuperscript{1}. Developing blood vessels undergo a maturation process that depends on the establishment of an endothelial basement membrane and perivascular cell coverage of endothelial tubes. Recent studies suggest that Notch signaling in endothelial and mural cells plays a fundamental role in the establishment of a vascular network and in the differentiation of mural cells into VSMCs. However, little is known about the effects of Notch signaling on the subsequent maturation of the vasculature\textsuperscript{52,86,87}. In this chapter of the dissertation, it is shown that blocking Notch signaling leads to the formation of an immature vascular plexus, marked by poor arterial VSMC coverage, and that endothelial expression of Jag1 is required for both the establishment and maintenance of a mature vasculature. Furthermore, it is established that Jag1 stimulation upregulates angiogenesis-related genes in VSMCs, including αvβ3, a receptor known to be important in cell adhesion and migration.
3.2 Introduction

Angiogenesis is a complex process regulated by a diverse assortment of signaling pathways including VEGF, bFGF, PDGF-B, ephrins, angiopoitens, and Notch, among others. The process of angiogenesis is important in both development and wound healing, as well as in pathologies including cancer, retinal diseases, diseases of ischemia, and restenosis following surgery. By understanding the processes that control all stages of angiogenesis, from initial blood vessel formation to maturation and stabilization of the vascular plexus, it will be possible to develop tailored therapeutics that target angiogenic diseases.

The role of Notch in angiogenesis has been described only recently, and little is known about the role of Notch in perivascular cell biology. Notch is an evolutionarily conserved signaling pathway that regulates cell fate decisions in tissues arising from all three germ layers. Notch signaling is a heterotypic cell-cell interaction between a Notch ligand (DLL1, DLL3, DLL4, Jag1, Jag2)-expressing cell and a Notch receptor (Notch1-4)-expressing cell. In the vasculature, Notch ligand/receptor expression is generally restricted to the arteries

Notch has been shown to regulate vessel patterning during both embryonic angiogenesis and angiogenesis associated with cancer. Blocking Notch during angiogenesis leads to an immature vascular plexus, poor blood
flow\textsuperscript{72}, and embryonic lethality or, in tumor studies, reduced tumor burden\textsuperscript{88}. The resultant immature vascular plexus is due, in part, to enhanced tip cell formation when Notch signaling is blocked\textsuperscript{69,89}. In addition to a highly branched and disorganized capillary network, another hallmark of an immature vascular plexus is a lack of perivascular cell coverage, as is often seen in tumor biology. Therefore, it was hypothesized that Notch signaling also regulates vascular plexus maturation by regulating the recruitment and retention of VSMCs, in addition to the differentiation of those cells, as has been previously shown\textsuperscript{90}.

Studies to date investigating the role of Notch in angiogenesis have focused on embryonic Notch signaling. Therefore, it has not been possible to understand the role of Notch in vessel maturation. By using a postnatal retinal angiogenesis model in which Notch signaling was blocked and a Jag1 endothelial knockout mouse model that is not embryonic lethal, it was possible to examine the role of Notch in postnatal arterial VSMC coverage and, importantly, vessel maturation.
3.3 Results

3.3.1 Notch is required for arterial VSMC coverage in postnatal angiogenesis

To understand the role of Notch signaling in the perivascular compartment during postnatal developmental angiogenesis, vascular mural cell coverage in the developing mouse retina was examined following pharmacological inhibition of Notch signaling activity. The γ-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butylester) inhibits the cleavage and release of the intracellular domain of Notch, which is the critical effector that controls gene expression. DAPT acts as a global Notch inhibitor both in vivo and in vitro. As previously reported, DAPT treatment leads to the formation of an immature vascular plexus in the periphery of the mouse retina (Figure 3.1a, c). In addition to a highly branched capillary plexus, systemic blockade of Notch signaling reduced overall VSMC arterial coverage (Figure 3.1b, d). Measured from the optic nerve as a percentage of the total vascular outgrowth along the arterial tracts, there was a 40% decrease in arterial VSMC coverage on DAPT treatment (P=0.004) (Figure 3.2a, b). Because the effect of Notch inhibition on vessel maturation was of primary interest in these studies, VSMC coverage in the arteries closest to the optic nerve, which are more mature, was examined. There, many areas of incomplete VSMC coverage were observed following
DAPT treatment, indicative of an immature artery (Figure 3.3 a, b).

Interestingly, the effect of Notch inhibition on mural cell coverage was limited to αSMA⁺ perivascular cells and did not impact αSMA⁻/NG2⁺ pericytes (Figure 3.4).

3.3.2 Endothelial Jag1 is required for both initial arterial VSMC coverage and maintenance of arterial VSMC coverage in the adult

Endothelial cell Jag1, a Notch ligand recognized by VSMCs, is predominantly expressed in the arterial endothelium during murine retinal development (Figure 3.5)⁹². It is known that endothelial Jag1 is required for VSMC differentiation during embryogenesis⁷³. Genetic ablation of Jag1 from the endothelium of mice using a Tie2-Cre mouse line resulted in embryonic lethality with severe VSMC defects⁷³. To investigate the role of Jag1 in postnatal arterial maturation, a murine model that bypasses the critical requirement for Jag1 in early endothelial development (VE-Cadherin-Cre; Jag1 ECKO mice)⁹³ was used. In the retina, there was weak arterial αSMA⁺ perivascular staining in P7 pups compared to controls, indicating that VSMC recruitment was deficient, leading to less mature arteries during development (Fig. 3.6 a, b). The use of an alternative marker of adult smooth muscle, SM22α⁹⁴, confirmed that Jag1 is required for arterial VSMC recruitment postnatally, as P8 retinal arteries were nearly devoid of SM22α staining (Figure 3.7 a, b).
Abnormalities in the VSMC compartment continued into adulthood in the Jag1 ECKO mice. There was a loss of VSMCs immediately adjacent to the Jag1-negative endothelium in both the aorta and in the mesentery of the adults (Figure 3.8 a-f). The finding that the VSMC compartment is compromised in Jag1 ECKO mice postnatally, both in mice undergoing active vascular remodeling and in mice with a stable, mature vasculature, suggests a previously unappreciated role for Jag1. Specifically, Jag1 is an endothelial cell Notch ligand capable of regulating arterial maturation and maintaining arteries in a mature state, without loss of VSMCs. The fact that mice lacking endothelial cell Jag1 still maintain capillaries covered with NG2⁺ perivascular cells suggests that only arterial VSMC coverage depends on Jag1.

3.3.3 Culture on Jag1 induces VSMC upregulation of integrin αvβ3

To identify Jag1-dependent Notch-mediated genes that might contribute to arterial maturation, VSMCs were seeded onto plates coated with or without immobilized recombinant Jag1. Eighteen hours later changes in gene expression were analyzed using a low-density array featuring a panel of genes associated with human angiogenesis (Applied Biosystems). Cell interaction with Jag1 altered the expression of several angiogenesis-related genes in VSMCs including KDR, PDGFRβ, and integrin β3, as well as the Notch-downstream gene Hey1 (Figure 3.9 a).
Integrin β3, which associates with αv to form the integrin heterodimer αvβ3, is known to play an important role in vascular cell adhesion, migration, and development\textsuperscript{95,96}. This could explain the observed phenotype in retinas of DAPT-treated pups, where VSMCs appeared loosely attached and more rounded up. As such, Notch regulation of β3 in VSMCs was confirmed by two independent methods. Independent quantitative RT-PCR verified that integrin β3 mRNA expression was increased 5.3-fold (+/0.7 s.e.m.; n=6) when VSMCs were plated on Jag1 for 18 hrs (Figure 3.9 b). By FACS, there was a 3-fold increase in αvβ3 surface expression on VSMCs following culture on Jag1-coated plates compared to culture on control IgG (Figure 3.9 c). In retinal cross-sections of Balb/c P13 pups, integrin β3 staining co-localized with αSMA staining on VSMCs immediately adjacent to the CD31\textsuperscript{+} endothelium (Figure 3.10 a-c).
3.4 **Conclusions**

Endothelial expression of the Notch ligand Jag1 is required for the maturation and maintenance of mature arteries. In the absence of endothelial Jag1, developing arteries lack VSMC coverage, marked by αSMA and SM22α, and the stable arteries of adults lack or have loosely associated VSMCs. VSMC interaction with Jag1 *in vitro* leads to the upregulation of several angiogenesis-related genes. Of those, integrin β3 is an interesting candidate to examine because it is one of the most highly upregulated and it is known to be involved in cell adhesion and cell migration. When neonatal pups undergoing retinal angiogenesis were treated with the γ-secretase inhibitor to pharmacologically block Notch signaling, arterial VSMCs appeared morphologically distinct and more rounded compared to those of vehicle-treated pups. A down-regulation of integrin β3 resulting from the inhibition of Notch signaling could explain the observed VSMC phenotype. Therefore, Notch regulation of integrin αvβ3 on VSMCs, and its role in vessel maturation, will be explored in the subsequent work described in this dissertation.
3.5 Figures

Figure 3.1 Notch is required for arterial maturation *in vivo*

Representative image of P6 retinas, following (a, b) vehicle (DMSO) or (c, d) γ-secretase inhibitor (DAPT) treatment to block Notch signaling from P2-P5, immunostained with antibodies specific to CD31 (red) and αSMA (green). DAPT treatment results in an immature vascular plexus, represented by enhanced vascular branching (arrows) and reduced αSMA⁺ perivascular cell coverage of arteries (arrowheads). Scale bars: 600 μm.
Figure 3.2 Quantification of arterial VSMC coverage

(a) VSMC coverage was quantified by measuring the length of αSMA$^+$ perivascular cell coverage along each artery (green) starting at the optic nerve, as a percentage of the total length of vascular plexus extension from the optic nerve towards the periphery, along the arterial path (dotted line). (b) DAPT treatment prevents arterial maturation, measured as a 40% decrease in arterial VSMC (αSMA$^+$) coverage compared to controls. Values represent means ± s.e.m. DMSO (n=17), DAPT (n=25), where n is the number of arteries analyzed. P=0.004.
Figure 3.3 Notch signaling regulates continuity of arterial VSMC coverage and VSMC morphology *in vivo*

High-magnification images of P6 retinas, following (a) vehicle (DMSO) or (b) γ-secretase inhibitor (DAPT) treatment to block Notch signaling from P2-P5, immunostained with antibodies specific to CD31 (red) and αSMA (green). (a) Vehicle treated pups show continuous VSMC coverage of arteries, marked by αSMA, unlike (b) DAPT-treated pups where individual VSMCs are clearly visible (arrowhead) and arterial coverage is interrupted (arrows). Scale bars: 25µm.
Figure 3.4 Notch signaling does not regulate NG2\(^+\) pericyte coverage \textit{in vivo}

Balb/c pups were treated with the \(\gamma\)-secretase inhibitor DAPT from P2-P5 to block Notch signaling. Retinas were harvested at P6 and immunostained with antibodies against CD31 (red) and NG2 (green). DAPT treatment does not affect NG2\(^+\) pericyte coverage of vessels in developing retina. Scale bar: 300 \(\mu\text{m}\).
Figure 3.5 Jag1 expression is localized to the arterial endothelium

Representative images of the vasculature in P12 (a, b) and P10 (c) mice immunostained with antibodies to CD31 to label the endothelial cells, Jag1 (red) to label the Notch ligand, and αSMA (green) to label VSMCs. Jag1 expression is localized to arteries and adjacent arterioles. Scale bars, a, b, 150 µm; c, 40 µm
Figure 3.6 Endothelial Jag1 is required for $\alpha$SMA$^+$ perivascular cell coverage during retinal angiogenesis

Representative images of the vasculature in P7 retinas from mice (a) with (Jag1 lox/lox VECAD-Cre negative, “wild type”) and (b) without (Jag1 lox/lox VECAD-Cre positive, “JAG1 ECKO”) Jag1 in the endothelium. Retinas were immunostained with antibodies to CD31 (red) and $\alpha$SMA (green). Arterial VSMC coverage as marked by $\alpha$SMA is reduced in the absence of endothelial Jag1. Scale bars, 100 μm.
Figure 3.7 Endothelial Jag1 is required for SM22α⁺ perivascular cell coverage during retinal angiogenesis

Representative images of the vasculature in P8 retinas from mice (a) with (Jag1 lox/lox VECAD-Cre negative, “wild type”) and (b) without (Jag1 lox/lox VECAD-Cre positive, “Jag1 ECKO”) Jag1 in the endothelium. Retinas were immunostained with antibodies to CD31 (green) to label the endothelium and SM22α (red) to label vascular smooth muscle cells. Scale bars, 70 µm.
Figure 3.8 Endothelial Jag1 is required for VSMC maintenance in the vasculature

Large vessels from young adult mice with (a, b; Jag1 lox/lox VECAD-Cre negative, “wild type”) and without (c-f; Jag1 lox/lox VECAD-Cre positive, “Jag1 ECKO”) Jag1 in the endothelium were immunostained with antibodies to CD31 (red), αSMA (green), and TOPRO (blue). Aorta from wild type (a, b) and Jag1 ECKO (c, d) adult mouse. Arrows (d) indicate loss of smooth muscle cells in the layer immediately below the Jag1-negative endothelium. (e, f) Mesenteric vessel from Jag1 ECKO mouse, arrows (f) note lack of smooth muscle cells. Scale bars, a-d, 50 µm; e, 20 µm; f, 10 µm.
Figure 3.9 Jag1 upregulates integrin αvβ3 expression on VSMCs

(a) List of genes upregulated or downregulated more than 3-fold in human aortic VSMCs following culture on a Jag1-coated substrate to induce Notch signaling, compared to culture on control IgG. (b) VSMC culture on a Jag1-coated substrate increased mRNA expression of integrin β3 5.3-fold by qRT-PCR (±0.7; n=6). Culture on Jag1 also upregulated canonical Notch-downstream genes Hes1 (3.4 ± 1.4, n=3) and Hey2 (2.4 ±0.5, n=4). (c) Surface expression of αvβ3 is increased 33% in human aortic VSMCs cultured on Jag1 compared to control IgG as analyzed by FACS. Values represent means ± s.e.m.
Figure 3.10 VSMCs express integrin β3 during vascular plexus development and maturation

(a-c) Balb/c P13 retina in cross-section, immunostained with antibodies to CD31 (blue), αSMA (green), and integrin β3 (CD61, red). (b, c) Higher-magnification image of artery (arrow, a) shows β3 staining peripheral to CD31+ endothelium and co-labeling αSMA+ VSMCs. Scale bars, a, 50 µm; b-c, 20 µm.
Chapter 4

Notch promotes vascular maturation by inducing integrin-mediated vascular smooth muscle cell adhesion to the endothelial basement membrane
4.1 **Abstract**

Vascular development and angiogenesis initially depend on endothelial tip cell invasion, which is followed by a series of maturation steps, including lumen formation and recruitment of perivascular cells. Notch ligands expressed on the endothelium and their cognate receptors expressed on perivascular cells are involved in blood vessel maturation, though little is known regarding the Notch dependent effectors that facilitate perivascular coverage of nascent vessels\textsuperscript{69,73,92}. VSMC recognition of the Notch ligand Jagged1 on endothelial cells leads to expression of integrin \( \alpha v \beta 3 \) on VSMCs, a receptor known to be involved in cell adhesion and migration.

This chapter describes the finding that integrin \( \alpha v \beta 3 \) facilitates VSMC adhesion to von Willebrand Factor (vWF) in the endothelial basement membrane of developing retinal arteries, leading to vessel maturation. Genetic or pharmacological disruption of Jagged1, Notch, \( \alpha v \beta 3 \), or vWF suppresses VSMC coverage of nascent vessels and arterial maturation during vascular development. These findings define a Notch-mediated interaction between the developing endothelium and VSMCs leading to adhesion of VSMCs to the endothelial basement membrane and arterial maturation.
4.2 Introduction

Integrins “integrate” intracellular functions and the extracellular environment by mediating interactions between the actin in the cytoskeleton and the extracellular matrix. They are critically important because they allow cells to physically interact with and respond to their environment and, as such, are ubiquitously expressed.

In their mature and functional form, integrins are transmembrane heterodimeric receptors, consisting of a noncovalent association between an alpha subunit (α), of which there are 24, and a beta subunit (β), of which there are 9. Following activation and binding to its cognate ligand in the extracellular matrix, the integrin β-tail binds several intracellular anchor proteins, which, through a cascade of events, link the integrin to actin in the cytoskeleton and leads to a clustering of the integrins into focal adhesions. This sequence of events is required to enforce a tight binding interaction with the extracellular matrix and promotes cell adhesion and/or cell migration. As such, cells in mice with truncated β-tails or inactive β signaling are unable to bind tightly to the extracellular matrix.

Besides being ubiquitously expressed, integrins are promiscuous receptors. A single integrin heterodimer pair has the ability to bind to several ligands. For example, at least 8 integrins bind fibronectin. Ligand binding specificity is context and cell-type dependent. Specificity can be achieved by
regulating the presence of extracellular divalent cations (Ca$^{++}$ or Mg$^{++}$) in the extracellular matrix or by restricting the available ligands in a tissue-specific manner.

**Figure 4.1 Integrins**

Integrins consist of an alpha-beta heterodimer which binds ligands in the extracellular matrix. Upon integrin ligation, the β tail binds a complex of intracellular anchor proteins which link the activated integrin to the cytoskeleton. Through this cascade of events, integrins facilitate cell adhesion and migration.
Chapter 3 of this dissertation describes the finding that VSMCs upregulate expression of integrin β3 at the mRNA level in response to Notch, resulting in an increase in surface expression of the heterodimer αvβ3. Expression of integrin αvβ3 is highly restricted to activated endothelium, activated vascular smooth muscle cells, tumors, and osteoclasts\textsuperscript{102,103}. Integrin αvβ3 mediates cell adhesion to the extracellular matrix through its interaction with a ligand containing the tripeptide sequence, Arginine-Glycine-Aspartic acid (RGD)\textsuperscript{104}. RGD-containing ligands that αvβ3 is capable of binding include vitronectin, fibrinogen, von Willebrand Factor, osteopontin, and thrombospondin\textsuperscript{105-107}.

While αvβ3 is capable of binding multiple ligands, the only available ligand in the endothelial basement membrane is the adhesive protein von Willebrand Factor (vWF). Therefore, the expression of αvβ3 by a nucleated cell in the vasculature gives it the unique ability to bind to vWF. As endothelial cells mature, they produce vWF, which is either secreted into the blood, or laid down as part of the endothelial basement membrane where it is closely associated with other matrix proteins\textsuperscript{108}. The endothelial basement membrane is common to both endothelial cells and adjacent perivascular cells\textsuperscript{109}. As such, both cell types are able to engage available ligands in the basement membrane via integrin receptor expression.
vWF is known to play two important biological roles. First, when blood vessels are damaged, platelet exposure to vWF initiates the coagulation cascade that leads to thrombus formation and the plugging of torn vessels. Integrin αIIbβ3 is involved in the initial platelet adhesion event to vWF and subsequent cell spreading, aggregation, and clot retraction\footnote{110}. Second, endothelial cells themselves express integrin αvβ3, localized to endothelial cell focal adhesion contacts; this mediates endothelial cell adhesion to and spreading on vWF\textsuperscript{105,98}, in addition to cytoskeletal organization\textsuperscript{105,111}.

In this chapter of the dissertation it is proposed that that Notch promotes arterial maturation by inducing integrin αvβ3-mediated VSMC adhesion to vWF in the endothelial basement membrane. VSMC coverage during postnatal retinal angiogenesis in mice with deficient integrin β3 signaling, in wild type mice with pharmacological blockade of β3, and in vWF knock out mice was examined. In all cases, a paucity of VSMC coverage was observed compared to control mice, reminiscent of that observed in mice treated with a γ-secretase inhibitor to block Notch described in Chapter 3. The requirement for VSMC interaction with vWF in the endothelial basement membrane was confirmed \textit{in vitro}. Additionally, in a time course of retinas from pups undergoing developmental angiogenesis it was noted that arterial VSMC coverage co-patterned with accumulation of vWF in the endothelial basement membrane, suggesting that endothelial cells control vascular maturation through the availability of two ligands, Jag1 and vWF.
4.3 Results

4.3.1 Arterial VSMC coverage is regulated by integrin β3 in vivo

Because VSMCs upregulated expression of αvβ3 in response to enhanced Notch signaling (Figure 3.9), it was considered whether αvβ3 expression on VSMCs might contribute to arterial VSMC coverage during retinal development. Pharmacological and genetic approaches were used to block β3 expression and function in vivo.

First, retinas were characterized from the β3 knockin “DiYF mice,” which express an inactive β3 subunit with two critical tyrosines converted to phenylalanines, producing deficient αvβ3 signaling\textsuperscript{101,112}. Retinas were harvested at P3, P5, and P7 and blood vessels were labeled using an endothelial-specific lectin and αSMA to visualize endothelial cells and VSMCs, respectively (Figure 4.2). VSMC coverage was quantified by measuring the arterial αSMA labeling as a percentage of endothelial outgrowth from the optic nerve to the vascular plexus periphery, along each arterial branch (Figure 3.2 a). Arteries at each time point displayed significantly less αSMA\textsuperscript{+} perivascular cell coverage in DiYF mice compared to wild type mice (Figure 4.2 b). There was a 30% reduction in VSMC coverage at P3, a 22% reduction at P5, and a 14% reduction at P7. Interestingly, arteries in retinas from mice completely deficient in αvβ3 showed a delay in VSMC coverage compared to age-matched controls at P3, yet by P7 normal vascular patterning and maturation
were observed (Figure 4.3). The fact that integrin β3 KO mice showed a relatively minor delay in retinal vascular maturation is not surprising given that these mice are known to show compensatory changes in VEGF signaling due to over-expression of VEGFR2\textsuperscript{99,113}.

To confirm a role for αvβ3 in arterial maturation, a β3 integrin function-blocking antibody was injected intravitreally into wild type mice at P5 and retinas were examined at P8 for the extent of vessel maturation and VSMC arterial coverage (Figure 4.4). Treatment was initiated at P5 because arterial patterning has been established at this developmental age, but arterial maturation is not yet complete. The β3 function-blocking antibody suppressed angiogenesis in the deep vascular plexus of the retina, consistent with previous reports of the anti-angiogenic activity of β3 integrin antagonists\textsuperscript{114,115} (Figure 4.4 b, e). Treatment with anti-β3 significantly reduced αSMA\textsuperscript{+} coverage of the developing arteries of P8 mice relative to control (IgG)-injected mice (Figure 4.4 a, d, c, f). Overall, there was a 47% reduction in arterial VSMC coverage in mice treated with the β3 function-blocking antibody (Figure 4.4 g). Thus, both genetic and pharmacological approaches indicate that αvβ3 expression on VSMCs is required for the coverage and maturation of newly developing arteries.
4.3.2 Arterial VSMC coverage co-patterns with vWF accumulation in the endothelial basement membrane.

During vascular development both endothelial cells\textsuperscript{95} and VSMCs\textsuperscript{116} transiently express integrin $\alpha v \beta 3$, which is a receptor for vWF. Importantly, vWF is an RGD containing adhesion protein that is unique to the vascular basement membrane\textsuperscript{117,118}. Previous studies have revealed that endothelial cells and VSMCs share a common basement membrane in mature arteries\textsuperscript{119,120}. The presence of a shared basement membrane between the endothelium and VSMCs during retinal arterial development in the mouse was verified by transmission electron microscopy (Figure 4.5).

Since endothelial cells and VSMCs both upregulate integrin $\alpha v \beta 3$, a receptor for their shared basement membrane during angiogenesis, it was considered that vWF might regulate VSMC-dependent arterial maturation. To test this \textit{in vivo}, the vWF expression pattern during retinal development in mice from P1 to P21 was characterized. Whole-mount staining revealed that vWF accumulated in the endothelial basement membrane during vessel maturation (Figure 4.6). There was a strong accumulation of vWF in major arteries and veins, but only minimal accumulation in the capillary beds. Importantly, during the first month of development, vWF accumulated along arteries in a pattern that completely co-aligned with the perivascular outgrowth of VSMCs (Figure 4.6). As the endothelium became more mature towards
adulthood, vWF was found throughout the vasculature, in arteries, veins, and capillaries.

4.3.3 vWF regulates arterial VSMC coverage in vivo

To determine the functional role of basement membrane vWF in arterial maturation, retinas from vWF KO mice were examined. Arterial VSMC coverage was quantified by measuring the arterial αSMA labeling as a percentage of endothelial outgrowth, from the optic nerve to the vascular plexus periphery, along each arterial branch (Figure 3.2 a). During the early stages of developmental angiogenesis, vWF KO mice showed significantly less VSMC coverage compared with wild type controls (Figure 4.7 a). There was a 37% reduction in arterial VSMC coverage at P3, a 21% reduction at P5, and a 17% reduction at P7, mirroring the pattern of reduced coverage in DiYF mice (Figure 4.7 b). High magnification serial imaging along the arteries of wild type and vWF KO mice, from the optic nerve towards the periphery, revealed that αSMA staining is less intense along the entire length of the artery in vWF KO mice during developmental angiogenesis (Figure 4.8). Similar to the observations in DiYF mice, early defects in developmental angiogenesis were resolved by three weeks of age, suggesting the existence of compensatory/redundancy mechanisms (Figure 4.9). These findings suggest that vWF regulates an early step in VSMC coverage of developing arteries.
4.3.4 Jag1-stimulated VSMC adhesion to vWF is αvβ3-dependent *in vitro*

To explore the link between Notch signaling in VSMCs and their adhesion to vWF, VSMCs cultured for 18 hours on Jag1 to upregulate Notch-downstream signaling were allowed to attach to a vWF-coated substrate *in vitro*. Adhesion was compared to VSMCs cultured on control IgG before attachment to vWF. Jag1-stimulated VSMCs, which upregulate αvβ3 expression (Figure 3.9 b, c), showed enhanced adhesion to vWF compared with control cells not exposed to Jag1 (Figure 4.10 a). This increased adhesion could be disrupted by exposure of cells to function-blocking antibodies for either αvβ3 or vWF, but not β1 integrin (Fig. 4.10 b, c). Also, inhibition of Notch signaling with DAPT suppressed VSMC adhesion to vWF (Figure 4.10 d). Together, these findings suggest that Jag1 ligation leads to upregulation of integrin αvβ3, allowing VSMCs to adhere to the vWF-containing vascular basement membrane.

4.3.5 VSMCs stabilization of endothelial tubes depends on vWF and Notch

To further examine the functional relevance of vWF in the basement membrane in the context of a VSMC-endothelial cell interaction, an *in vitro* model system was adapted in which vascular maturation could be readily visualized in real time. Microcarrier beads coated with primary human umbilical vein endothelial cells (HUVECs) were suspended in a fibrin gel
containing human aortic VSMCs. The endothelial cells form large lumen-containing vascular networks within the fibrin gel. The vessels recruit adjacent VSMCs to form a perivascular compartment, approximating the relationship between endothelial cells and αSMA⁺ perivascular cells observed in vivo (Figure 4.11). The vascular networks that form deposit vWF into a basement membrane-like structure (Figure 4.12).

The addition of a vWF blocking antibody, anti-152b¹¹⁸ which specifically blocks the αvβ3 RGD binding site on vWF, to the culture media reduced VSMC interaction with outgrowing endothelial tubes by approximately 70% compared to control IgG (Figure 4.13). These findings indicate that vWF can serve as a common adhesion protein for both endothelial cells and VSMCs, providing a mechanism for these cell types to engage a common basement membrane and thereby enable specific perivascular coverage during arterial development.

To further investigate the impact of Notch signaling on the endothelial/VSMC interaction, the effects of blocking Notch signaling on co-patterning during tube formation in vitro were examined. Inhibiting Notch signaling with DAPT decreased co-patterning between VSMCs and endothelial tubes by approximately 50% relative to control cells (Figure 4.14). Specifically blocking Notch signaling in VSMCs by knock-down of Notch3 gene expression (Figure 4.15 f) resulted in a 69% reduction in VSMC co-patterning with endothelial tubes compared to control-transfected VSMCs (Figure 4.15 a-e).
Similar to the observation that vWF was required for VSMC-endothelial interaction, it was found that Notch signaling in VSMCs, which upregulates αvβ3, was also required for this interaction. Taken together, these findings reveal a central role for Notch signaling leading to αvβ3-mediated adhesion between VSMC and vWF in the endothelial basement membrane.
4.4 Conclusions

From their restricted location on the arterial endothelium, Notch ligands and receptors ensure the maturation of arterial vessels. Notch ligands such as Jag1 expressed on the arterial endothelium engage neighboring non-committed stromal cells, thereby locally facilitating their conversion to a VSMC phenotype. Among the Notch-induced phenotypic changes in VSMCs is the expression of integrin αvβ3, which enables VSMC interaction with vWF within the newly formed endothelial cell basement membrane. This process is specific to arterial development since arterial vWF staining and VSMC coverage are coincident, and this is not observed in developing venules in the retina. Additionally, Notch receptor and ligand expression is restricted to the arterial compartment. This cascade ensures that VSMCs are selectively localized to mature arterial regions, rather than to regions that continue to undergo extensive vascular remodeling.
4.5 Figures

Figure 4.2 Integrin β3 is required for VSMC coverage in developmental angiogenesis.

(a) Representative images of retinas undergoing early postnatal retinal developmental angiogenesis at three different time points: P3, P5, and P7. Mice with deficient downstream αvβ3 signaling (DiFYF) have less arterial VSMC coverage at each time point than age-matched wild type (C57BL/6) controls. Retinas were immunostained with isolectin GS-IB4 from Griffonia simplicifolia (lectin, red) to label the endothelium and αSMA to label VSMC (green). (b) VSMC coverage was measured along each artery as a percentage of the outgrowth of the vascular plexus (dotted lines). Arterial coverage is reduced by 30% at p3, 22% at p5, and 14% at P7. Values represent means ± s.e.m. C57BL/6 (n=12 (P3), n=23 (P5), n=19 (P7)), DiFYF (n=15 (P3), n=23 (P5), n=21 (P7)), where n is the number of retinas analyzed. P = 1.4x10^{-4} (P3), P = 7.5x10^{-9} (P5), P = 1.0x10^{-3} (P7). Scale bars, P3, 150 µm; P5, 300 µm; P7, 600 µm.
Figure 4.3 Integrin β3 knockout mice show no difference in overall αSMA⁺ perivascular cell coverage.

Representative whole mount retinal images at two different time points, P4 and P7, of retinas from integrin β3 knockout mice and their littermate controls. Retinas were immunostained with antibodies against CD31 (red) to label endothelial cells and αSMA (green) to label VSMCs. Scale bar, 1 mm.
Figure 4.4 Intravitreal injection of integrin β3 blocking antibody reduces arterial VSMC coverage

(a) Pharmacological blockade of integrin β3 reduces αSMA⁺ arterial VSMC coverage compared to (d) injection with isotype control. Functional blockade of β3 resulted in reduced angiogenesis in the deep plexus (e) compared to control (b). High magnification images of arterial VSMC coverage following anti-β3 injection (f) or control injection (c). Intravitreal injection of a β3 function blocking antibody reduced arterial VSMC coverage by 74% ($P = 0.01$). Values represent means ± s.e.m. Isotype control, $n = 5$; anti-β3, $n = 6$. Scale bars, a-b, d-e: 275 um; c, f: 50 µm.
Figure 4.5 Endothelial cells and perivascular cells share a common basement membrane

(a, b) Electron micrographs depicting the relationship between an endothelial cell (EC) and its adjacent perivascular cell (PC) in the mouse retina. The two cell types share a common basement membrane (BM) (arrows, a). (b) High-magnification image of (a). (c, d) Outline of cell types shown in micrographs a, b, respectively. (L), blood vessel lumen; (BM), basement membrane; (EC), endothelial cell; (PC), perivascular cell. Scale bars: a, 0.5 um; b, 200 nm.
Figure 4.6  Arterial VSMC outgrowth co-patterns with vWF deposition in the endothelial basement membrane

Representative images of retinas from postnatal Balb/c pups harvested over a time course during the process of blood vessel outgrowth and maturation, from postnatal days (P)4-P16. Retinas were immunostained with antibodies against CD31 to label blood vessels, αSMA to label VSMCs (green), and vWF (red). VSMC coverage of arteries is limited to areas of significant vWF accumulation in the arterial basement membrane. Scale bars, 200 µm.
Figure 4.7 vWF is required for VSMC coverage in developmental angiogenesis

(a) Representative images of retinas undergoing early postnatal retinal developmental angiogenesis at three different time points: P3, P5, and P7. Retinas were immunostained with antibodies against CD31 (red) to label the endothelium and αSMA to label VSMC (green). (b) vWF KO mice have significantly less VSMC coverage compared to age-matched controls, measured along each artery as a percentage of the outgrowth of the vascular plexus (dotted lines). Arterial coverage is reduced 37% at P3, 21% at P5, and 17% at P7. Values represent means ± s.e.m. C57BL/6 (n=12 (P3), n=23 (P5), n=19 (P7)), vWF KO (n=14 (P3), n=25 (P5), n=14 (P7)), where n is the number of retinas analyzed. \( P = 9.3 \times 10^{-4} \) (P3), \( P = 1.7 \times 10^{-3} \) (P5), \( P = 7.3 \times 10^{-3} \) (P7). Scale bars, a, 150 µm (P3); 300 µm (P5); 600 µm (P7).
Figure 4.8 High-magnification montages of wild type and vWF KO retinal arteries

Retinas from P6 mice were immunostained with antibodies against CD31 (red) and αSMA (green) and imaged at high magnification from the optic nerve (*) towards the periphery. vWF KO mice have less intense αSMA staining than wild type mice along the length of the artery. Scale bars, 200 µm.
Figure 4.9 Retinal arteries in adult DiYF and vWF KO mice are complete in their VSMC coverage

Retinas from adult mice were immunostained with antibodies against CD31 (red) to label endothelial cells and αSMA (green) to label VSMCs. Compared to wild type mice (a), DiYF mice (b) and vWF KO mice (c) show no difference in overall arterial VSMC coverage. Scale bar, 300 µm.
Figure 4.10 VSMC adhesion to vWF is due to a Notch-dependent upregulation of integrin αvβ3

(a) The ability of VSMCs to adhere to a vWF-coated substrate was quantified following 18-hour culture on control (IgG) or Jag1 coated plates (to stimulate Notch signaling). (b, c) Blocking antibodies specific to αvβ3 (b, LM609) and the RGD-binding site of vWF (c, anti-152B) prevented Notch-dependent adhesion to vWF, whereas a blocking antibody specific to β1 integrins (c, anti-P4C10) did not. (d) Addition of the γ-secretase inhibitor DAPT to block Notch signaling reduced VSMC adhesion to vWF.
Figure 4.11 Time course of endothelial tube outgrowth and VSMC coverage in fibrin bead angiogenesis assay

Representative images of tube formation in the fibrin bead angiogenesis assay, taken in a single field of view over multiple days. HUVECs were immunostained with FITC-labeled Ulex specific to human endothelial cells (green) and VSMCs were labeled with a lipid-based intracellular dye, Dil (red). Tubes begin to form around day 2 (a), VSMCs begin to co-pattern with endothelial tubes at day 3 (b), during days 4 (c) and 5 (d), tubes thicken and VSMCs continue to coat the newly formed tubes. Arrows point to VSMC coverage of endothelial tubes. Scale bars, 150 µm.
Figure 4.12 Endothelial cells produce vWF in fibrin bead tube-formation assay

(a, b) Representative images of *in vitro* fibrin bread angiogenesis assays in which HUVECs were cultured on microcarrier beads and suspended in a fibrin gel with VSMCs. Fibrin gels were fixed and immunostained with a rhodamine-labeled Ulex specific to human endothelial cells (red) and with antibodies to vWF (blue). Endothelial cells in this assay produce a vWF-rich basement membrane. (c, d) Higher magnification images showing vWF production by endothelial cells. Scale bars, a-b, 150 µm; c-d, 50 µm.
Figure 4.13 VSMC interaction with vWF in the endothelial basement membrane is required for VSMC coverage of endothelial tubes *in vitro*

Representative images of *in vitro* fibrin bead angiogenesis assays in which HUVECs (green) were cultured on microcarrier beads and suspended in a fibrin gel with VSMCs (red). The addition of a blocking antibody to vWF (c, d) significantly reduced co-patterning compared to isotype control (a, b). (b, d) High-magnification images of the regions marked with arrows in (a, b), respectively. (e) Co-patterning between VSMCs and endothelial tubes was reduced 66% on addition of anti-vWF to the culture media, as quantified by pixel overlap in ImageJ. Values represent means ± s.e.m. IgG control (n=14); α-152b (n=17); where n is the number of beads analyzed; *P*=2.5×10^{-13} (α-152b). Scale bars, a, c, 325 µm; b, d, 150 µm.
Figure 4.14 Notch signaling in VSMCs enhances the ability of VSMCs to adhere to vWF and to the endothelial basement membrane

(a-d) Representative images of in vitro fibrin bead angiogenesis assays in which HUVECs (green) were cultured on microcarrier beads and suspended in a fibrin gel with VSMCs (red). Addition of the γ-secretase inhibitor DAPT to the culture media (c, d) prevented association of VSMC with endothelial tubes compared to vehicle (a, b). Arrows point to regions magnified, shown in b, d. (e) Blocking Notch signaling with DAPT reduced VSMC-endothelial tube co-patterning 49%. Values represent means ± s.e.m. DMSO control (n=6); DAPT (n=12); where n is the number of beads analyzed; p=0.002. Scale bars, a, c, 325 µm; b, d, 150 µm.
Figure 4.15 VSMC expression of Notch3 is required for VSMC coverage of endothelial tubes in vitro

Representative images of in vitro fibrin bead angiogenesis assays in which HUVECs (green) were cultured on microcarrier beads and suspended in a fibrin gel with VSMCs (red) that were transfected with Notch3 siRNA (c, d) or with All Stars negative control siRNA (a, b). Arrows point to regions magnified, shown in b, d. (e) Co-patterning between VSMCs and endothelial tubes was reduced 69% when VSMCs were transfected with Notch3 siRNA. (f) Notch3 receptor knockdown in VSMCs was confirmed by western blot, as was the resultant decrease in Notch signaling as measured by decreased Hey2 expression, a canonical Notch downstream transcription factor. Values represent means ± s.e.m. Control siRNA (n=23), Notch3 siRNA (n=23); where n is the number of beads analyzed; P=8.4x10^{-13}. Scale bars, a, c, 325 µm; b, d, 150 µm.
Chapter 5

Discussion
5.1 **Notch downstream effectors in VSMCs**

Studies in Notch3 knockout mice indicate that the overall effect of Notch signaling in VSMCs is to promote vessel maturation and arterial VSMC coverage\(^62\). Notch3 knockout mice are viable, though their arteries fail to properly mature postnatally, have thinner walls, and a paucity of VSMCs\(^62\). Importantly, Notch3 expression is restricted to VSMCs in the vasculature\(^64\).

The work described in this dissertation expands the knowledge of the angiogenesis field by demonstrating a novel downstream effect of Notch signaling in mural cells that leads to arterial VSMC coverage and maturation.

It is known that Notch upregulates genes that promote perivascular cell differentiation towards a VSMC phenotype such as smooth muscle myosin heavy chain\(^90\) and αSMA\(^121\). Therefore, Notch ligands expressed by endothelial cells are able to influence the phenotype of nearby mural cells, inducing them to take on the specialized vascular smooth muscle properties required for arteries.

A second known function of Notch in VSMCs is the upregulation of PDGFR\(β\)\(^56\), which is critically involved in perivascular cell recruitment\(^18\). An unexplored function of Notch in the context of vessel maturation is the regulation of PDGFR\(β\) levels relative to VEGFR2 levels. While the data is not included in this dissertation, I found that by increasing PDGFR\(β\), Notch allows VSMCs to overcome the PDGFR\(β\)/VEGFR2 inhibitory complex in the presence of both PDGF-BB and VEGF angiogenic growth factors\(^19\), and
permits downstream PDGFRβ signaling. In this way, Notch may provide an additional level of regulation during normal angiogenesis that allows VSMCs to respond appropriately to PDGF-BB in the environment.

While Notch-induced upregulation of PDGFRβ appears to play a significant role in the initial recruitment of mural cells to arteries, the work described in this dissertation adds to these previously published findings by demonstrating that Notch also induces the upregulation of αvβ3. This dissertation describes that through the expression of αvβ3 and its interaction with vWF, Notch is able to restrict the localization of these specialized VSMCs to the more mature arterial endothelium, which has higher levels of vWF in the endothelial basement membrane. This restricted localization prevents VSMC coverage of immature arteries undergoing continued remodeling, and therefore prevents interference with sprouting angiogenesis and vascular plexus maturation. Notch-induced PDGFRβ upregulation could be the initial recruitment event, while the αvβ3/vWF interaction could regulate VSMC localization and artery maturation.

5.2 Notch in vascular injury

The findings described in this dissertation may explain the biology observed in various pathologies. For example, in addition to its initial role in development, Notch signaling is modulated in repair following vascular injury. Carotid balloon arterial injury in the rat induces an initial downregulation of Notch family members and downstream genes during the first 2-3 days
following vascular injury\textsuperscript{122}, followed by a significant upregulation of Notch 7-14 days following injury, during the repair process\textsuperscript{123,124}. This temporal regulation of Notch could prevent a premature overgrowth of VSMCs around the damaged artery, before the endothelium can heal and undergo a repair process.

When canonical Notch signaling is interrupted due to loss of the Hey2 transcription factor in Hey2 knockout mice, vascular response to arterial wire injury is characterized by decreased neointimal formation compared to that in wild type mice\textsuperscript{125}. This observation, coupled with decreased VSMC proliferation in Hey2 knockout mice and increased VSMC proliferation when Notch3 is induced by Jag1 ligation in VSMCs\textsuperscript{126}, supports a role for Notch in the maintenance and repair of VSMCs in mature vessels.

While Notch signaling is involved in the normal process of blood vessel repair, there are clinical benefits to be realized in preventing neointimal thickening associated with VSMC recruitment\textsuperscript{52,127}. Patients whose arteries have narrowed and hardened due to the accumulation of cholesterol plaques within the lumen can be treated by angioplasty, in which a catheter is inserted into the affected artery and an expanded balloon widens the artery to improve blood flow. Restenosis is the subsequent re-narrowing of the artery following medical intervention that happens in response to the arterial injury induced by the procedure. Restenosis is caused by the accumulation of VSMCs in the tunica intima, and Notch signaling likely plays a role in this recruitment\textsuperscript{128}. In
support of the concept that Notch in VSMCs contributes to restenosis is the observation that increased Notch receptor expression leads to more VSMCs in the intima of murine carotid arteries following vascular injury\textsuperscript{129}, which is complemented by the previously discussed decreased neointimal thickening in Hey2 knockout mice following arterial wire injury\textsuperscript{125}.

Importantly, integrin αvβ3 is upregulated on VSMCs during restenosis, and is considered a therapeutic target to prevent arterial narrowing\textsuperscript{130,131}. The signaling pathways mediating VSMC upregulation of αvβ3 in restenosis have not been described, however the findings described in this dissertation could apply. While the commonly accepted ligand in this pathogenesis is osteopontin\textsuperscript{130}, not vWF as was the focus of this dissertation, Notch signaling may still be the driving factor in the well-described upregulation of αvβ3 on VSMC. In this case, both inhibitors of Notch signaling and inhibitors of αvβ3 are potential therapeutics to consider.

5.3 Applications to tumor vascular biology

In addition to therapeutic applications following angioplasty, the work described in this dissertation is also directly relevant to cancer. Tumor vessels are immature, highly branched, and have poor VSMC coverage or loosely attached VSMCs\textsuperscript{120}. The poor mural cell coverage associated with tumor vasculature is problematic because it leads to leaky vessels and local edema, hence increased inflammation and necrosis. In addition, the tortuous,
immature tumor vessels are poorly perfused, which presents a problem for drug delivery when chemotherapeutics are delivered intravenously.

While initial anti-cancer therapies were designed to completely inhibit angiogenesis with the goal of starving the tumor of essential oxygen and nutrients, an alternate approach is to induce vessel normalization within the tumor vasculature, thereby improving subsequent delivery of anti-cancer agents. Therefore, the finding that Notch regulates vessel maturation and arterial VSMC localization is an important consideration in the design of future anti-cancer therapeutics.

Notch is dysregulated in several different types of cancer\textsuperscript{132}. In breast cancer, high levels of Notch1 and Jag1 correlate with poor survival of breast cancer patients\textsuperscript{133}. In medulloblastoma, high levels of Notch2 and Hes1 correlate with poor patient prognosis\textsuperscript{134}. In pancreatic cancer, Notch receptors and downstream targets are upregulated in pre-neoplastic pancreatic lesions and in invasive pancreatic cancer. The dysregulation of Notch may lead to poorly differentiated cells, typical of this type of cancer, since Notch is known to be required for the maintenance of undifferentiated pancreatic precursor cells in the embryo\textsuperscript{135,136}. Additionally, Notch signaling is upregulated in melanoma, leukemia, and colorectal cancer\textsuperscript{132}.

The role of Notch is tissue-specific, and in many tissues Notch signaling maintains progenitor cells in an undifferentiated state during embryonic development. It is only through the loss of Notch that cells terminally
differentiate. However, in some cell types Notch induces differentiation, as has been described in VSMCs throughout this dissertation. Therefore suppression of Notch, rather than activation, can also be oncogenic. This is true for basal and squamous cell carcinoma\textsuperscript{137,138}, hepatocellular carcinoma\textsuperscript{139} and small cell lung cancer\textsuperscript{140}. The varied effect of Notch signaling between tumor-types complicates our analysis of the role of Notch in cancer and prevents sweeping generalizations from being made regarding Notch as a therapeutic target.

As one would expect, the dysregulation of Notch within the tumor affects the local vasculature, since endothelial and mural cells come into contact with Notch ligands expressed by tumor cells. As an example, Jag1 is over-expressed in head and neck squamous cell carcinoma, leading to increased Notch signaling in local endothelial cells and increased angiogenesis\textsuperscript{141}. Therefore, any drug designed to target Notch signaling by affecting ligand expression will also affect the tumor vasculature.

Some efforts have already been made to interfere with Notch signaling to treat cancers. DLL4 antagonists have been shown to be effective at reducing tumor burden by increasing tip cell formation in the vasculature, leading to a non-functional and highly branched vascular network\textsuperscript{142}. Gamma-secretase inhibitors have been shown effective at increasing apoptosis in Kaposi sarcoma\textsuperscript{143}, and are being tested in glioblastomas, but the off-target
effects of these inhibitors, which include gastrointestinal cytotoxicity, makes them relatively unattractive drug candidates\textsuperscript{144}.

If Notch is to be considered a target for clinical development, it is important to remember that nonspecific blockade of Notch signaling will also affect the endothelial cells and VSMCs. Therefore, if maturing the vasculature becomes an important tool in the treatment of cancer, Notch inhibitors must be specifically targeted to the tumor cells.

\subsection*{5.4 The tumor endothelial basement membrane and VSMC coverage}

One of the driving forces behind tumor vessel abnormalities is the abnormal endothelial basement membrane. The basement membrane that forms as part of tumor angiogenesis is a provisional matrix composed of vitronectin, fibronectin, thrombin, and type I collagen. Because the components of the tumor basement membrane are not in their mature, assembled form, neighboring endothelial cells and mural cells engage the basement membrane in different ways than in normal endothelium\textsuperscript{145}. Different integrins bind, resulting in a tumor basement membrane that acts in an angiogenic capacity rather than as a stabilizing agent\textsuperscript{145,146}.

In addition to the degradation and immature assembly of basement membrane components that is coupled with tumor angiogenesis, tumor
endothelial basement membranes also show decreased accumulation of vWF. This is especially interesting in light of the research described in this dissertation. If vWF is required during the initial stages of VSMC coverage as a ligand for αvβ3 expressed on VSMCs, the lack of vWF in the tumor endothelial basement membrane could explain the relative paucity of VSMCs observed in tumor vasculature. By extension, the lack of vWF could also contribute to the overall immaturity of the tumor vascular plexus.

5.5  **An expanded role for vWF**

Following the formation of a quiescent vasculature, vWF plays an essential role in hemostasis. The lack of vWF, or dysfunctional vWF, leads to the congenital bleeding disorder von Willebrand Disease, highlighting the importance of vWF in clotting. Additionally, vWF in the endothelium acts in the recruitment, adhesion, and migration of leukocytes and therefore plays a role in the inflammatory response.

It was recently reported that endothelial vWF also regulates angiogenesis. Starke, *et al.* found that mice deficient in vWF have increased angiogenesis in a matrigel plug assay. Additionally, siRNA knockdown of vWF in HUVECs resulted in increased VEGFR2-dependent proliferation and migration, decreased αvβ3 expression, and increased Ang2 secretion. The work described in this dissertation adds to these findings, revealing that vWF is acting not only on endothelial cells, but is also an important ligand for
VSMCs in the shared endothelial basement membrane. It also raises the possibility that vWF secreted into the basement membrane could participate in outside-in signaling in the VSMCs. In endothelial cells, the effects of vWF were due both to extracellular and intracellular vWF expression. Therefore, vWF in the basement membrane serves a dual purpose: it regulates both endothelial network formation and VSMC recruitment.

5.6 Summary

Vascular development and angiogenesis depend first on endothelial tip cell invasion, followed by a series of maturation steps, which include lumen formation and the recruitment of perivascular cells. Notch signaling has been linked to endothelial tip and stalk cell formation as well as to VSMC coverage of newly formed arteries. However, the majority of the studies to date reporting on the role of Notch in the vasculature have focused on developmental angiogenesis in the embryo, using genetically modified mouse models in which Notch signaling is inhibited. Therefore, the angiogenesis field is just beginning to understand the role of Notch signaling postnatally, especially since many of the models used are embryonic lethal. As such, the involvement of Notch signaling in vessel maturation, stabilization, and maintenance has not been investigated.

The work in this dissertation describes a new role for Notch that appears to regulate the specific recruitment of VSMCs to developing arteries. Notch signaling in VSMCs due to recognition of the ligand Jag1 on the
endothelium leads to the upregulation of integrin αvβ3, enabling VSMC interaction with vWF, an adhesion protein unique to the endothelial basement membrane\textsuperscript{150}. Therefore, the work presented herein proposes that Notch-induced αvβ3 expression on VSMCs, in concert with vWF deposition in the endothelial basement membrane, regulates vessel maturation.
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