Notch Signaling Determines Lymphatic Cell Fate and Regulates Sprouting Lymphangiogenesis

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ABSTRACT

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Minji Kim Uh

The lymphatic vascular system is necessary for physiological regulation of tissue fluid homeostasis and absorption of dietary fat. Lymphatics also function in the inflammatory response and are involved in pathological conditions such as wound healing and cancer. We show that the Notch signaling pathway is a regulator of both developmental and pathological lymphangiogenesis.

Notch1 and Notch4 are expressed by the lymphatic endothelium, and Delta-like ligand 4 (Dll4) is the predominantly expressed Notch ligand in the developing lymphatic vessels of the embryonic dermis and pathological lymphatic vessels of the wounded cornea. Dll4 was able to induce Notch activation in human dermal lymphatic endothelial cells (HDLECs), whereas Jagged1 (Jag1) was not. In HDLECs, Notch signaling is activated in response to Vascular Endothelial Growth Factor (VEGF) or Vascular Endothelial Growth Factor-C (VEGF-C) stimulation. In vitro assays demonstrated that Notch activation inhibits HDLEC proliferation, migration, and capillary network formation; these effects were coincident with increased levels of HEY1 and HEY2, biphasic regulation of VEGFR-3, and decreased levels of VEGFR-2.

Using genetic intervention of Notch signaling, we demonstrated that Notch regulates developmental sprouting lymphangiogenesis by restricting growth and sprouting of lymphatics in the murine embryonic dermis. Using pharmacological intervention of Notch signaling, we found that Notch restricted pathological sprouting lymphangiogenesis in the corneal suture.
assay, which models inflammation-induced lymphangiogenesis. However, pharmacological intervention of Notch signaling did not measurably affect pathological sprouting lymphangiogenesis in an orthotopic tumor model of human breast cancer. Our data from analysis of HDLECs, dermis, and sutured cornea support a role for Dll4-driven Notch signaling in restricting sprouting lymphangiogenesis.

Lymphatic specification/separation requires a venous endothelial cell to become a lymphatic endothelial cell, and lymphatic valve formation requires a duct endothelial cell to become a valve endothelial cell. Through analysis of genes regulated by Notch in HDLECs, we demonstrated that Notch determines lymphatic endothelial cell fates. Notch inhibits genes critical for lymphatic specification and separation (PROX1, PDPN), and induces genes important for lymphatic valve formation (FNEIIIA, ITGA9, CX37).

We conclude that Notch is a context-dependent regulator of lymphangiogenesis. Notch functions in the tip/stalk, venous to lymphatic, and duct endothelial to valve endothelial cell fate decisions in lymphatic vasculature. Given the critical functions of the lymphatic vasculature in multiple physiological and pathological settings, understanding Notch functions in the lymphatic vasculature is critical to design treatments for conditions caused by lymphatic malfunction.
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Chapter 1

Introduction
Development of the lymphatic vasculature

The circulatory system, which is composed of both the cardiovascular and lymphatic systems, develops through a series of sequential events, beginning with vasculogenesis\(^\text{[1]}\). In vasculogenesis, multipotent hemangioblasts of mesodermal origin differentiate into endothelial progenitor cells, which subsequently assemble themselves to form a primitive vascular network. The next step, angiogenesis, is the process by which new blood vessels sprout from pre-existing blood vasculature\(^\text{[1]}\). This allows for expansion and remodeling of the primitive vasculature into a hierarchical network of arteries, veins, and intervening capillaries. Smooth muscle cells are recruited to stabilize larger vessels while pericytes are recruited to smaller vessels and capillaries. Once the vascular system has formed, it can function to provide oxygen and nutrients while removing waste throughout the organism.

The venous origin of the lymphatic system (Figure 1)\(^\text{[2]}\), first hypothesized in 1902 by Florence Sabin\(^\text{[3]}\), has been confirmed through lineage tracing studies\(^\text{[4]}\). In the mouse, a subset of blood endothelial cells in the anterior cardinal vein becomes committed to the lymphatic endothelial cell (LEC) fate between embryonic days (E) 9.5-10. The lymphatic master regulator Prox1 is turned on in these LEC progenitor cells in a process dependent on the transcription factors Coup-TFI\(\text{I}\) and Sox18\(^\text{[5-7]}\). Between E11.5-12.5, LEC progenitor cells extrude through a “ballooning” mechanism\(^\text{[8]}\) and migrate dorsolaterally from the cardinal vein to form the primitive lymph sacs\(^\text{[2, 9-12]}\). This process requires Vascular Endothelial Growth Factor Receptor 3 (VEGFR-3), expressed by the lymphatic endothelial cells, and the VEGFR-3 ligand Vascular Endothelial Growth Factor-C (VEGF-C), expressed by mesenchymal cells\(^\text{[13]}\). From E12.5-14.5,
lymph sacs continue to form and undergo sprouting lymphangiogenesis to form the initial lymphatic plexus\textsuperscript{[2, 9-12]}. Further remodeling and maturation of the initial lymphatic plexus forms the complete lymphatic vasculature, which is composed of smaller lymphatic capillaries, intermediate pre-collecting vessels, and larger collecting vessels\textsuperscript{[2, 9-12]}. Smooth muscle cells are recruited to stabilize collecting vessels, and specialized valves begin to develop at E15.5 in the collecting vessels to allow for unidirectional flow of lymph\textsuperscript{[9-12]}. Valves are of particular importance in the lymphatic system, which lacks a central pump and depends on skeletal muscle movement and smooth muscle cell contractions for lymph flow.

Lymphatic capillaries, pre-collecting vessels, and collecting vessels have unique structural characteristics that differentiate them from each other, as well as from the blood vasculature\textsuperscript{[9-12]}. Lymphatic capillaries, unlike blood capillaries, are blind ended and have discontinuous inter-cell junctions. Lymphatic capillaries have a sparse basement membrane, no mural cell coverage, and they connect to the extracellular matrix via anchoring filaments. These characteristics allow for effective absorption of fluid and passage of cells in and out of lymphatic capillaries. Collecting vessels, on the other hand, have continuous basement membrane and smooth muscle cell coverage, and contain valves. These characteristics allow for collecting vessels to move fluid in a unidirectional manner for return to the venous circulation. As an intermediate vessel type, pre-collecting vessels possess characteristics of both capillaries and collecting vessels. The fully developed lymphatic vasculature runs alongside the blood vasculature, and lymphatic vessels are present in all vascularized tissues, excluding the bone marrow and central nervous system\textsuperscript{[11, 12]}. Although the two vascular systems run alongside
each other, they connect only in defined locations to allow for lymph collected by the lymphatic capillaries to be returned to the blood circulation\[^{9-12}\].

**Physiological and pathological roles of the lymphatic vasculature**

Proper function of the lymphatic vasculature is critical for life. Its physiological functions include regulation of tissue fluid homeostasis and absorption of lipids from the digestive tract\[^{9, 11, 12}\]. Fluid and molecules that leak out of the blood vasculature are taken up by lymphatic capillaries, through pre-collecting vessels to the collecting ducts, and returned to the venous circulation. In healthy humans, the lymphatic system collects and returns 1-2 liters of lymph to the venous circulation per day\[^{14}\]. In the small intestine, specialized lymphatic vessels known as lacteals absorb dietary lipids, which are subsequently taken up by the mesenteric collecting ducts and eventually enter the bloodstream for further processing.

Pathological functions of the lymphatic vasculature include resolving wounds, responding to inflammation, and facilitating metastasis of tumor cells\[^{9, 11, 12}\]. Inflammation induces macrophages to release lymphangiogenic factors such as VEGF-C and VEGF-D\[^{15}\]. The lymphatic endothelium can attract various leukocyte populations by releasing chemokines and adhesion molecules\[^{12, 16}\]. Lymphatic capillaries also allow for transport of antigens and antigen-presenting cells from sites of inflammation to the lymph nodes\[^{9, 12, 16}\]. Post-inflammation, the lymphatics function to clear dead cells and fluid\[^{16}\]. In tumors, lymphatics can act as a conduit for metastasis. Tumors secrete a multitude of growth factors, many of which can stimulate lymphangiogenesis and/or angiogenesis\[^{17, 18}\]. By entering into the peritumoral lymphatics,
tumor cells can metastasize to regional lymph nodes\textsuperscript{[17, 18]}. Thus, cancer treatment often involves biopsy and surgical resection of regional lymph nodes, as the presence of metastases in lymph nodes is a prognostic indicator of poor outcome in multiple cancers\textsuperscript{[12, 18, 19]}. Whether regional lymph node metastasis is a bona fide step towards distant metastasis or is simply a characteristic of more aggressive cancers is a highly-debated topic\textsuperscript{[20]}.

In humans, malfunction of the lymphatics can lead to multiple complications\textsuperscript{[9-12]}. Lymphedema, or the build-up of fluid in tissues, results when lymphatic vessels are lost or damaged, compromising their ability to absorb interstitial fluid. Lymphedema most often presents as a secondary condition, resulting from damage to lymphatic vessels or removal of lymph nodes during surgery or radiation. Malfunctioning collecting vessels can lead to chyle leakage into the peritoneal or thoracic cavities, and dilation of the lacteals in the small intestine, also known as intestinal lymphangiectasia, can compromise the body’s ability to absorb fats and protein. Unwanted lymphangiogenesis can also present problems. The avascularity of the cornea is critical for corneal homeostasis, but in pathological conditions, lymphatic and blood vessels can grow into the cornea, compromising visual acuity\textsuperscript{[21, 22]}. Furthermore, lymphangiogenesis in the cornea can exacerbate inflammatory responses in the cornea by facilitating immune cell trafficking to and from the cornea, and increase risk for transplant rejection following corneal transplant\textsuperscript{[21, 22]}. 
The Notch signaling pathway

The Notch signaling pathway (Figure 2)\[^{23}\] is a highly conserved pathway expressed in multiple cell types that plays important roles in determining cell fate, as well as regulating cellular proliferation, differentiation, and apoptosis\[^{24-26}\]. In mammals, the Notch family consists of 4 receptors (Notch1, Notch2, Notch3, and Notch4) that interact with 5 ligands (Delta-like ligands 1, 3, and 4, and Jaggeds 1 and 2). Receptor and ligand are both transmembrane proteins, and signaling occurs through direct contact between cells. Mature Notch is presented at the cell surface as a non-covalently bound heterodimer\[^{27}\]. Upon Notch/ligand binding, a conformational change in Notch allows for cleavage in the extracellular region by ADAM family metalloprotease TACE (S2 cleavage), and cleavage within the transmembrane region by Presenilin/γ-secretase (S3 cleavage). This releases the extracellular domain of Notch (NECD) – which bound to ligand, is endocytosed into the ligand-presenting cell – from the intracellular domain of Notch (NICD). NICD translocates to the nucleus where it binds to CSL (CBF-1/Su(H)/Lag-1), also known as RBP-Jk, displacing proteins that make CSL a transcriptional repressor and recruiting other co-activator proteins, including Mastermind-like proteins\[^{23}\], switching CSL into a transcriptional activator. This complex then activates transcription of downstream target genes, including the HES (hairy/enhancer of split) and HEY (hairy/enhancer-of-split related with YRPW motif) transcriptional repressors\[^{28}\]. This allows Notch to both directly activate some genes and indirectly repress other genes.
**Notch signaling in the blood vasculature**

The Notch family plays a critical role in the blood vasculature\[^{29}\], as evidenced by the vascular phenotypes exhibited by mutants. Mice nullizygous for *Notch1* or *Jag1* die between E9.5-11.5, exhibiting severe vascular abnormalities\[^{30,31}\], while loss of just one copy of *Dll4* is sufficient to cause embryonic death\[^{32}\]. A small number of *Dll4*\(^{+/-}\) mice outbred to the CD1 background survive to birth, and these mice display severe hypersprouting of blood vasculature with increased sprouts, branch points, and vessel density\[^{33}\]. Interestingly, mice with endothelial-specific loss of Jag1 exhibit hyposprouting of blood vasculature with reduced sprouts, branch points, and vessel density\[^{34}\]. Mice nullizygous for *Notch4* are viable and fertile, but *Notch1*\(^{-/-}\);*Notch4*\(^{-/-}\) mice die embryonically with more severe vascular phenotypes than mice nullizygous for *Notch1* alone\[^{30}\]. Mice nullizygous for *Dll1* die shortly after birth with vascular defects\[^{35}\].

The tip/stalk model has been developed to explain how Notch signaling functions in the context of sprouting angiogenesis\[^{36}\] (Figure 3), and explains the severe hypersprouting phenotype seen in *Dll4*\(^{+/-}\) mice\[^{33}\]. The endothelial cell leading the way at the front of the sprout, also known as the “tip cell”, extends filopodia to sense its surroundings and is highly responsive to Vascular Endothelial Growth Factor (VEGF)\[^{37}\]. When VEGF binds VEGF receptor-2 (VEGFR-2) in the tip cell, a signaling cascade is initiated, in which Dll4 expression is induced in the tip cell\[^{33,38,39}\], which activates Notch in the neighboring “stalk cell”. NICD then translocates to the nucleus of the stalk cell and, through its transcriptional effectors, represses VEGFR-2. This ensures that the tip cell, but not the stalk cell, is highly responsive to VEGF stimulation,
preventing hypersprouting. However, tip and stalk cell identities are not static. Competition between cells to adopt the tip or stalk cell positions is a highly dynamic process, and cells are constantly shifting between the two cell types\textsuperscript{[40]}.

**Notch signaling in lymphatic vasculature**

The field of lymphatic vascular biology has benefited in the past fifteen to twenty years from the identification of markers that are specific to or highly expressed by LECs, and the phenotypes of mice lacking these genes have been identified\textsuperscript{[10, 11, 41]}. A list of genes required for lymphatic vascular development and function is provided in Table 1.

Given that lymphatics have a venous origin and that Notch plays important roles in the blood vasculature, interest has recently shifted to understanding Notch signaling in the lymphatic vasculature. Studying the effects of genetic ablation of Notch pathway components in the context of the lymphatic endothelium has become possible through the development of the Prox1-Cre\textsuperscript{ERT2} lymphatic-specific driver line\textsuperscript{[4]}, as global Notch knockouts die due to vascular defects before lymphatic phenotypes can manifest themselves.

We sought to understand the role of Notch signaling in developmental and pathological lymphangiogenesis. We hypothesized that Notch regulates sprouting lymphangiogenesis through regulation of genes important in this process, namely VEGFR-3. Through analysis of cultured LECs, we show that Notch transcriptionally regulates VEGFR-3, a critical player in sprouting lymphangiogenesis. We report that Notch is a negative regulator of HDLEC proliferation, migration, and network formation in *in vitro* assays designed to mimic the
different steps of lymphangiogenesis. We also show that Notch signaling is responsive to VEGF or VEGF-C stimulation. Using pharmacological and genetic tools in in vivo models, we demonstrate that Notch regulates sprouting lymphangiogenesis in both developmental and pathological settings by limiting lymphatic sprouting. Furthermore, we show that Notch plays important roles in lymphatic cell fate decisions. We report that Notch transcriptionally regulates genes required for lymphatic specification/separation and lymphatic valve formation, two settings in which lymphatic endothelial cells must make cell-fate decisions. We conclude that Notch signaling regulates sprouting lymphangiogenesis and determines lymphatic cell fate.
Chapter 1

Figures
Figure 1. Venous origin of the lymphatic vasculature. In the mouse, lymphatic specification from the venous vasculature begins at E9.75 with the polarized expression of Prox1 in the anterior cardinal vein. From E11.5-12.5, Prox1+ cells form primitive lymph sacs. From E12.5, lymph sacs undergo further sprouting, differentiation, and maturation to form the lymphatic plexus. Adapted from Oliver, G. and R.S. Srinivasan, Endothelial cell plasticity: how to become and remain a lymphatic endothelial cell. Development, 2010. 137(3): p. 363-72.
Figure 2. The Notch signaling pathway. The Notch signaling pathway is initiated when two neighboring cells interact via receptors (Notch1-Notch4) and ligands (Delta-like ligand 1,3,4 and Jagged 1,2). Notch is presented at the cell surface as a non-covalently bound heterodimer. Upon receptor/ligand binding, a conformational change in the receptor allows for two cleavages in the extracellular and transmembrane regions, releasing the intracellular domain of the receptor (ICN). ICN translocates to the nucleus, where it binds to CSL/RBP-Jk and recruits Mastermind-like (MAML) and other co-activators (Co-A), displacing co-repressors (Co-R). This switches CSL/RBP-Jk from a transcriptional repressor to a transcriptional activator. Adapted from McElhinny, A.S., J.L. Li, and L. Wu, Mastermind-like transcriptional co-activators: emerging roles in regulating cross talk among multiple signaling pathways. *Oncogene*, 2008. 27(38): p. 5138-47.
Figure 3. The tip/stalk model of sprouting angiogenesis. An angiogenic sprout is composed of the *tip cell*, a cell at the front of the sprout that expresses high levels of VEGFR-2, extends multiple filopodia to sense its surroundings, and migrates toward VEGF; and *stalk cells*, which follow behind the tip cell. When VEGF binds VEGFR-2 in the tip cell, the Notch ligand Dll4 is induced. Dll4 signals to Notch in the neighboring stalk cell, and Notch transcriptionally represses VEGFR-2 in the stalk cell. This results in a highly VEGF-responsive tip cell and poorly-responsive stalk cells, allowing for tightly controlled sprouting. Adapted from Thurston, G. and J. Kitajewski, VEGF and Delta-Notch: interacting signalling pathways in tumour angiogenesis. *Br J Cancer*, 2008. 99(8): p. 1204-9.
Chapter 1

Tables
Table 1. List of genes important in lymphatic vasculature

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression Pattern</th>
<th>Mutant Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prox1</td>
<td>LEC progenitor cells in the cardinal vein early on (E9.5), then by all LECs; hepatocytes, cardiomyocytes, skeletal muscle, lens, retina, pancreatic/lung/intestinal endocrine cells</td>
<td>Prox1-/- is embryonic lethal at E14.5-15 due to complete lack of LECs; surviving Prox1+/ mice display edema and chylous ascites.</td>
</tr>
<tr>
<td>Sox18</td>
<td>LEC progenitor cells in the cardinal vein early on; then by all LECs until E14.5, arterial and venous BECs, hair follicle cells</td>
<td>Sox18-/- is embryonic lethal at E14.5 in C57BL/6 background due to complete lack of LECs; Sox18+/ mice display mild edema and lymphatic vessel patterning defects.</td>
</tr>
<tr>
<td>Coup-TFII</td>
<td>LEC progenitor cells in the cardinal vein early on, then by all LECs; venous BECs, vascular smooth muscle cells</td>
<td>Coup-TFII-/- is embryonic lethal at E10 with abnormal venous development; deletion of Coup-TFII in vasculature (Tie2-Cre; Coup-TFIIfl/fl) results in 80% reduction of Prox1+ in cardinal vein.</td>
</tr>
<tr>
<td>Pdpn</td>
<td>LECs (E11), keratinocytes, podocytes, alveolar cells, osteoblasts</td>
<td>Pdpn-/- is perinatally lethal in 129/SvEv background due to respiratory failure; a fraction of Pdpn-/- mice survive in C57BL/6 background and display blood in lymphatics due to defective platelet activation.</td>
</tr>
<tr>
<td>Lyve1</td>
<td>LEC progenitor cells in the cardinal vein early on (E9), then by all LECs; macrophages; liver/spleen/lymph node sinusoidal BECs</td>
<td>Lyve1-/- mice have no apparent abnormalities.</td>
</tr>
<tr>
<td>Cx37 (Connexin37)</td>
<td>LECs (E13.5), progressively becomes restricted to lymphatic valve ECs postnatally (P3-P7)</td>
<td>Cx37-/- mice are viable but display enlarged jugular lymph sacs and reduced number of valves in collecting ducts.</td>
</tr>
<tr>
<td>Gene</td>
<td>Expression Pattern</td>
<td>Mutant Phenotypes</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><em>FnEIIIA</em></td>
<td>Lymphatic valve ECs, fibroblasts, epithelial cells, etc</td>
<td><em>FnEIIIA</em>−/− mice are viable and fertile but display normal skin wound healing and shortened life span[^48], as well as reduced and abnormal lymphatic valves[^49]</td>
</tr>
<tr>
<td><em>Itga9</em></td>
<td>LECs, with highest expression in lymphatic valve ECs, vascular smooth muscle cells</td>
<td><em>Itga9</em>−/− is perinatally lethal with respiratory failure caused by chylothorax[^50] due to reduced and abnormal lymphatic valves in collecting vessels[^49]</td>
</tr>
<tr>
<td><em>Lama5</em></td>
<td>Cells of the kidney, lung, skin, intestine, skeletal muscle cells, etc</td>
<td><em>Lama5</em>−/− is lethal by E16.5 displaying defects in anterior neural tube closure and digit septation, as well as reduced placental labyrinth and defective placental vasculature[^51]</td>
</tr>
<tr>
<td><em>Vegfr3</em></td>
<td>LECs, BECs early in development but becomes reduced later on</td>
<td><em>Vegfr3</em>−/− is embryonic lethal beginning at E9.5 due to defects in blood vessel remodeling resulting in cardiovascular failure[^52]; mice mutant for the ligand binding domain (<em>Vegfr3ΔLBD/ΔLBD</em>) or tyrosine kinase domain (<em>Vegfr3TKmut/Tkmut</em>) have normal blood vasculature but display defects in development of lymphatic vasculature, leading to edema[^53]</td>
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<tr>
<td><em>Efnb2</em></td>
<td>LECs, with highest expression in lymphatic valve ECs; arterial BECs, vascular smooth muscle cells</td>
<td><em>Efnb2</em>−/− is embryonic lethal before E11.5 due to defects in embryonic vasculature[^54]; mice with mutated PDZ binding sites (<em>Efnb2ΔV/ΔV</em>) have normal blood vasculature but display defects in development of lymphatic vasculature and lack of lymphatic valves[^55]</td>
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Chapter 2

Materials and Methods
Isolation of Human Dermal Lymphatic Endothelial Cells (HDLECs)

Human neonatal foreskins were minced, rinsed in phosphate-buffered saline (PBS, Gibco) to remove blood, then digested in DMEM low glucose medium (Gibco) supplemented with Ca\(^{2+}\)/Mg\(^{2+}\), 2% fetal bovine serum (FBS), 1X pen/strep (100U penicillin/100U streptomycin, Invitrogen), and 1mg/mL collagenase A (Roche) for 30min at 37°C in a rotating incubator. An equal volume of “Buffer A” (PBS supplemented with 5mg/mL bovine serum albumin [BSA] and 0.6% Acid Citrate Dextrose Solution A) was added and tissue was crushed with a cell scraper to release cells (3X). Solution was filtered through a 100μm cell strainer, centrifuged at 1500rpm for 5min at 4°C, and resuspended in Buffer A. CD31\(^+\) cells were isolated using the Dynabead® magnetic bead system (Invitrogen) as per manufacturer’s instructions. CD31\(^+\) cells were expanded, then further purified by fluorescence activated cell sorting (FACS) using the BD FACSaria™ flow cytometer to obtain CD31\(^+\); podoplanin\(^+\); CD34\(^-\) HDLECs (Figure 1, Table 1).

Cell Lines

All cells were maintained at 37°C in a mixture of 5% CO\(_2\) and 95% humidified air. HDLECs were maintained on fibronectin-coated plates in EGM\(^{TM}\)-2 MV Endothelial Cell Growth Media (Lonza) supplemented with FBS (final concentration of 5%) and 10ng/mL VEGF-C (R&D), and used before passage 7. The MDA-MB-231 human mammary tumor cell line (ATCC) was maintained in Eagle’s Minimum Essential Medium (EMEM, ATCC) supplemented with 10% FBS and 1X pen/strep. The luciferase expressing subline MDA-MB-231-luc-D3H2LN\(^{®}\) (Perkin Elmer)
has been described previously\textsuperscript{[56]} and was maintained in the same medium as MDA-MB-231 cells.

**Immunocytochemistry**

HDLECs plated to 90% confluency on fibronectin-coated glass coverslips were fixed with 4% paraformaldehyde (PFA) for 15min at RT, then blocked/permeabilized with PBS containing 0.1% Triton X-100 and 5% serum for 30min at RT. Cells were incubated in PBS with 5% appropriate serum and appropriate primary antibodies (Table 1) for 2hrs at RT, then in PBS with 5% serum and appropriate Alexa Fluor® secondary antibodies at 1:500 for 2hrs at RT. Coverslips were mounted on slides using VECTASHIELD® mounting medium containing DAPI (Vector Laboratories).

**Lentivirus-Mediated Stable Transduction of HDLECs**

1.5x10\textsuperscript{6} 293T packaging cells were calcium phosphate-transfected as previously described\textsuperscript{[57]} with the following plasmids: 3\mu g of pVSVG, 5\mu g of pRRE, 2.5\mu g of pRSV-Rev, and 10\mu g of pCCL lentiviral plasmids encoding genes of interest. We activated Notch using pCCL lentiviral vectors encoding N1IC (Figure 2), N4/int3 (Figure 2), or GFP (ctrl). N1IC comprises the Notch1 intracellular domain, and N4/int3 comprises the Notch4 transmembrane and intracellular domains. We inhibited Notch using a pCCL lentiviral vector encoding N1ECDFc\textsubscript{1-36} (Figure 3) or hFc (ctrl). N1ECDFc\textsubscript{1-36} comprises EGF-like repeats 1-36 in the extracellular domain
of human Notch1 fused to human Fc. 48hrs after transfection, supernatant containing lentiviral particles shed by the 293T cells was collected, passed through a 0.45μm filter, and used to replace normal cell growth medium on HDLECs. 24hrs after infection, medium was replaced with normal cell growth medium. 48hrs after infection (24hrs after medium was replaced), cells were collected for RNA or protein isolation.

Adenovirus-Mediated Infection of HDLECs

1x10^6 HDLECs were infected in suspension with adenovirus at a multiplicity of infection (MOI) of 20, in low serum (2%) media, for 1hr at 37°C. 48hrs after infection, cells were collected for RNA or protein isolation.

Proliferation Assay

1x10^4 HDLECs were plated in triplicate in 24 well plates, using normal growth medium. 2hrs later (“0hr” time point) and 72hrs later, cell number was quantified using the Cell Counting Kit-8 (Dojindo) and VersaMAX microplate reader (Molecular Devices), as per manufacturers’ instructions. This assay was performed a minimum of 3 times.
Migration Assay

HDLECs were plated O/N to confluence (7x10^4) in triplicate in 12 well plates, using normal growth medium. The next day ("0hr" time point), a scratch was made across the diameter of each well using a p200 pipet tip, and medium was changed to serum-free medium (SFM) containing 100ng/mL VEGF-C (R&D). Pictures were taken every 4hrs up to the 25hr time point. Cell migration rate was calculated using TScratch software. This assay was performed a minimum of 3 times.

Network Formation Assay

4x10^4 HDLECs were seeded in triplicate between two collagen gels (Wako) overlaid with SFM containing 100ng/mL VEGF-C (R&D), in 24 well plates. Pictures were taken of cell network formation after 72hrs. This assay was performed a minimum of 3 times.

Co-Culture Notch Reporter Assay

Cells were plated to 90% confluency, then lipofected using Opti-MEM® medium (Invitrogen) and Lipofectamine® 2000 reagent (Invitrogen) as per manufacturer’s instructions. HDLECs were lipofected with the Notch reporter plasmid pGL3.11CSL-luc (containing 11 repeats of a CSL-responsive element) and pGL3.Renilla-luc housekeeping plasmid. HeLa cells were lipofected with pCR3 plasmids expressingDll4-FLAG, Jag1-FLAG, or ctrl. 4hrs after lipofection, medium was replaced with normal cell growth medium. 24hrs after lipofection, HeLa and
HDLECs were co-cultured together at a 1:1 ratio in fibronectin-coated plates and HDLEC medium. 24hrs after co-culture, a luciferase reporter assay was performed using the Dual-Luciferase® Reporter Assay System (Promega) and a TD-20/20 luminometer (Turner Designs), as per manufacturers’ instructions. Each condition was performed in triplicate; due to time constraints this assay was performed only twice.

**Activation of Endogenous Notch with EDTA**

HDLECs plated to confluency were treated with a γ-secretase inhibitor (compound E) at 200nM O/N at 37°C to suppress endogenous Notch signaling. The next day, compound E was removed by washing cells with PBS, and endogenous Notch signaling was activated by treating HDLECs for 15min at 37°C with 10mM EDTA. Cells were collected for RNA isolation at multiple time points.

**Gene Expression Analysis**

RNA was isolated from cultured cells using the RNEasy® Mini Kit (Qiagen), as per manufacturer’s instructions. RNA was reverse transcribed to cDNA using the Verso™ cDNA Synthesis Kit (Thermo Fisher) as per manufacturer’s instructions. Semi-quantitative PCR was performed using 1μL of cDNA, primers specific to genes of interest (Table 2), and Platinum® Taq DNA Polymerase (Invitrogen). Quantitative Real Time PCR (qRT-PCR) was performed in triplicate for each condition, using ABsolute™ Blue QPCR SYBR Green Master Mix (Thermo Fisher) and
the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems), as per manufacturers’ instructions. Primers specific to genes of interest (Table 2) and qRT-PCR standards specific to genes of interest were used. Data was analyzed with Microsoft Excel. Gene expression analysis was performed a minimum of 3 times.

**Protein Expression Analysis**

Cell lysates were isolated from cultured cells in TENT lysis buffer supplemented with Halt™ Protease Inhibitor Cocktail (Pierce). 20μg - 40μg of total cell lysates were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked for 30min in PBS with 0.1% Tween, 2% milk, and 2% BSA at RT, incubated with the appropriate primary antibodies in blocking buffer O/N at 4°C (Table 1), then incubated with the appropriate HRP-conjugated secondary antibodies in blocking buffer at 1:5000 for 30min at RT. Bands were detected using Amersham ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences). Protein expression analysis was performed a minimum of 3 times.

**VEGF and VEGF-C Treatment of HDLECs**

Confluent cultures were serum-starved O/N in EBM-2 basal medium with 0.1% BSA, 1% FBS and subsequently for 5hrs in EBM-2 basal medium with 0.1% BSA, no FBS. Starvation medium was replaced with EBM-2/0.1% BSA with VEGF (100ng/mL) or VEGF-C (100ng/mL) for
1hr. Cells were then collected for RNA isolation. Due to time constraints this assay was performed only once.

**Flow Cytometry**

HDLECs were incubated with appropriate primary antibodies in PCN buffer for 30min at 4°C (Table 1), then incubated with appropriate fluorescent secondary antibodies in PCN buffer at 1:200 for 20min at 4°C. Cells were passed through polystyrene tubes with filter tops, then analyzed with the BD FACSCalibur™ flow cytometer. Flow cytometry analysis was performed a minimum of 3 times.

**Mouse Lines**

$Prox1\text{CreER}^{T2}$ and $DNMAML^{fi/ff}$[58] mice used in embryonic dorsal skin studies have been previously described. 12 week old, wild-type female C57BL/6J mice used in corneal suture studies were obtained from Jackson Laboratories. 6-8 week old, athymic nude female mice used in tumor studies were obtained from Harlan. All experiments using mice followed guidelines and protocols approved by Columbia University’s Institutional Animal Care and Use Committee. Corneal suture assays adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research.
Embryonic Dorsal Skin Studies and Whole-Mount Immunohistochemistry

20mg/mL tamoxifen dissolved in corn oil was administered by oral gavage to pregnant dams at 10mg/40g of body weight on E12.5. At E14.5, dorsal skin was microdissected from each embryo, fixed for 2hrs in 4% PFA, then blocked/permeabilized for 2hrs at RT in PBS containing 10% appropriate serum and 0.3% Triton X-100. Skins were incubated with the appropriate primary antibodies in blocking buffer O/N at 4°C (Table 1), then incubated with the appropriate fluorescent secondary antibodies in blocking buffer at 1:500 O/N at 4°C. Skins were mounted on slides using VECTASHIELD® mounting medium containing DAPI (Vector Laboratories). 3-6 embryos were analyzed per genotype. 1 litter has been analyzed to date.

Corneal Suture Studies and Whole-Mount Immunohistochemistry

11-0 nylon sutures (Covidien) were placed in both corneas of mice. In the first experiment, adenoviruses expressing N1ECDFc1-24 (Figure 3) or hFc (ctrl) were administered by retro-orbital injection (5x10^8 ffu/mouse) on day 0, a single continuous suture covering at least a 180° circumference was placed on day 4, and eyes were collected on day 16. In all other experiments, 3 sutures were placed as knots on the nasal/superior/inferior positions on day 0, adenoviruses expressing Notch1 decoys (N1ECDFc’s, Figure 3) or hFc (ctrl) were administered by retro-orbital injection (5x10^8 ffu/mouse) on day 4, and eyes were collected on day 7 or 10. Both suture methods yielded the same results. Serum was collected regularly from mice to monitor circulating levels of Notch1 decoys by Western blot. 3-4 mice were analyzed per group per experiment. N1ECDFc1-24 treatment was analyzed in 3 separate experiments, N1ECDFc1-13
treatment was analyzed in 2 separate experiments, and N1ECDFc_{10-24} treatment was analyzed in 1 experiment.

Corneas were microdissected from each eye, digested with Proteinase K (20μg/mL) for 5min at RT, permeabilized with 100% methanol for 30min at RT, then blocked/permeabilized O/N at 4°C in PBS with 2.5% BSA, 0.5% Triton X-100. Corneas were incubated with the appropriate primary antibodies in blocking buffer O/N at 4°C (Table 1), then incubated with the appropriate fluorescent secondary antibodies in blocking buffer at 1:500 O/N at 4°C. Corneas were flat-mounted on slides using VECTASHIELD® mounting medium containing DAPI (Vector Laboratories).

**Tumor Studies and Immunohistochemistry**

5x10^5 to 1.5x10^6 cells were suspended in a solution containing 50% PBS, 50% Matrigel™ (BD), and orthotopically implanted into the 4th mammary fat pad of mice. Adenoviruses expressing Notch1 decoys (N1ECDFc’s, Figure 3) or hFc (ctrl) were administered by retro-orbital injection (5x10^8 ffu/mouse) at the beginning and mid-point of the tumor study. Serum was collected regularly from mice to monitor circulating levels of Notch1 decoys by Western blot. Tumor progression was monitored with weekly caliper measurements and luciferase imaging using the IVIS® Imaging System and Living Image Software (Caliper Life Sciences), 10min after intraperitoneal injection of 30mg/mL D-luciferin (Caliper Life Sciences) at 150mg/kg of body weight. *In vivo* monitoring for metastases was performed weekly throughout the experiment and *ex vivo* post-sacrifice with the IVIS® Imaging System and Living Image Software. 4-10 mice
were analyzed per group per experiment. N1ECDFc1-24 treatment was analyzed in 3 separate experiments, while N1ECDFc1-13 and N1ECDFc10-24 treatments were analyzed in 1 experiment.

At end points, mice were sacrificed and tumors, lungs, and axillary lymph nodes were collected. Tumors were fresh frozen in Tissue-Tek® OCT compound (Sakura Finetek), while lungs and axillary lymph nodes were frozen after O/N fixation in 4% PFA at 4°C. 7μm sections were post-fixed in ice-cold acetone for 3min and blocked for 1hr at RT in PBS with 3% BSA and 2% appropriate serum. Tissues were incubated with the appropriate primary antibodies in blocking buffer O/N at 4°C (Table 1), then incubated with the appropriate Alexa Fluor® secondary antibodies in blocking buffer at 1:750 for 30min at RT. Tissues were mounted on slides using VECTASHIELD® mounting medium containing DAPI (Vector Laboratories).

**Image Acquisition, Quantification, and Statistical Analysis**

Images of *in vitro* assays using cultured cells were acquired with a Zeiss Axiovert 40 CSL inverted microscope. Images of immunocytochemistry and immunohistochemistry of tumors were acquired with a Nikon Eclipse E800 and analyzed with Adobe Photoshop. Vessel or macrophage density was calculated by dividing positive pixels of a defined color threshold within a set field by total number of pixels in the field. Images of whole-mount embryonic dorsal skin and corneas were acquired with a Zeiss LSM 510 Meta confocal microscope or Nikon A1 confocal microscope and analyzed with ImageJ or Adobe Photoshop. Vascular coverage was calculated by quantifying area covered by vasculature and dividing by total area of the specimen. Macrophage density was calculated by dividing positive pixels of a defined color
threshold within a set field by total number of pixels in the field. Lymphatic sprouts and branch points were counted per field. Two-tailed student’s t-test was used for statistical analysis and considered significant at p<0.05.
Chapter 2

Figures
Figure 1. HDLEC cell surface marker profile. HDLECs isolated from human neonatal foreskins are CD31+ and podoplanin+. Human umbilical venous endothelial cells (HUVEC) which are blood endothelial cells (BEC), and human dermal microvascular endothelial cells (HDMEC) which are a mix of blood and lymphatic endothelial cells, are used as controls.
Figure 2. Structures of Notches, ligands, and Notch activation constructs. The structures of full-length Notch1, Notch4, Dll4, and Jag1 are shown above. N1IC consists of the intracellular domain of Notch1 and N4/int3 consists of the transmembrane and intracellular domains of Notch4. EGF = Epidermal growth factor; LNR = LIN-12/Notch repeats; RAM = Rbp-associated molecule domain; NLS = Nuclear localization signal; TAD = Transactivation domain; PEST = Proline (P), glutamic acid (E), serine (S) and threonine (T) degradation domain; DSL = Delta/Serrate/Lag-2; CR = Cysteine-rich.
Figure 3. Structures of Notch inhibition constructs. The structure of full-length Notch1 is shown above. Notch1 decoys, also known as N1ECDFCs, function as inhibitors of Notch signaling. N1ECDFc1-36 consists of all 36 EGF-like repeats in the extracellular domain of Notch1 fused to human Fc. It can bind and inhibit both Dll and Jag ligand-induced signaling. N1ECDFc1-24 consists of EGF-like repeats 1-24 in the extracellular domain of Notch1 fused to human Fc. It can bind and inhibit both Dll and Jag ligand-induced signaling. N1ECDFc1-13 consists of EGF-like repeats 1-13 in the extracellular domain of Notch1 fused to human Fc. It can bind and inhibit Dll ligand-induced signaling. N1ECDFc10-24 consists of EGF-like repeats 10-24 in the extracellular domain of Notch1 fused to human Fc. It can bind and inhibit Jag ligand-induced signaling.
Chapter 2

Tables
## Table 1. List of antibodies

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<th>Antibody (host)</th>
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<th>Vendor</th>
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Chapter 3

Inhibition of Notch signaling in lymphatic endothelial cells causes hypersprouting of developing dermal lymphatics
INTRODUCTION

Sprouting lymphangiogenesis is the growth of new lymphatic vessels from pre-existing lymphatic vessels. This process first begins in mouse development at embryonic day (E)12.5, when the primitive lymph sacs that have migrated off of the cardinal vein continue on to form the lymphatic plexus\[9-12\]. After development, sprouting lymphangiogenesis occurs more sparingly, with the majority occurring in pathological conditions, such as in response to inflammation and tumors (Chapters 4 and 5, respectively). Lymphangiogenesis is a multi-step process that requires capillary lymphatic endothelial cells (LECs) to proliferate, directionally migrate, and coalesce to form connected networks. One could imagine that lymphangiogenesis, like angiogenesis, also requires determination of the tip cell, the cell leading the way of a sprout; and stalk cell, the cell following behind the tip cell. This would ensure that sprouting is regulated and proper lymphatic patterning is achieved. Disruption of any step in lymphangiogenesis can result in hypersprouting of lymphatic capillaries or lymphatic hyperplasia\[9-12\]. Hypersprouting is defined by an increase in sprouts and branch points. Hyperplasia is a result of increased LEC proliferation, increased LEC migration, and/or dilation of lymphatic vessels. Alternatively, disruption of lymphangiogenesis can result in hyposprouting, which is a reduction in sprouts and branch points; or hypoplasia, which can result from decreased LEC proliferation and migration\[9-12\]. Any of these disruptions can compromise lymphangiogenesis and lymphatic function.

VEGFR-3 and VEGFR-2 are the primary mediators of sprouting lymphangiogenesis and sprouting angiogenesis\[59, 60\]. Expression of both receptors has been observed in lymphatic and
blood vascular systems\textsuperscript{[59, 60]}. VEGFR-3 is expressed by the blood vasculature prior to the development of the lymphatic vasculature\textsuperscript{[52]}, but its expression is gradually downregulated as it becomes largely restricted to the lymphatic vasculature\textsuperscript{[59, 60]}. There are several exceptions to this expression pattern for VEGFR-3 at later time points during development and in disease. For instance, VEGFR-3 is highly expressed at sites of active sprouting angiogenesis\textsuperscript{[59, 60]}. VEGFR-3 is strongly expressed by angiogenic tip cells in the postnatal retina, as well as by tumor blood vasculature\textsuperscript{[61]}. VEGFR-2 is also strongly expressed by angiogenic tip cells, as well as angiogenic stalk cells\textsuperscript{[59]}. VEGFR-2 is also expressed by the lymphatic vasculature, but at lower levels than in the blood vasculature\textsuperscript{[59]}. VEGF-A (VEGF) is the primary ligand for VEGFR-2 and VEGF-C the primary ligand for VEGFR-3, but VEGF-C can acquire VEGFR-2 activating properties through proteolytic processing\textsuperscript{[62-64]}. Both VEGF and VEGF-C induce formation of VEGFR-2/VEGFR-3 heterodimers in angiogenic sprouts\textsuperscript{[65]}, further intertwining the two pathways. As lymphatic vessels express both VEGFR-3 and VEGFR-2, one could speculate that heterodimers may play a role in lymphangiogenesis. Thus the functions of these two receptors and their ligands are intimately linked and can influence each other.

VEGFR-2 and Notch are the two key players in a feedback loop that has been studied extensively and underlies the process of sprouting angiogenesis\textsuperscript{[33, 38, 39, 59, 60]}. Angiogenic tip cells at the front of a sprouting blood capillary highly express VEGFR-2, and upon activation by VEGF, induce Dll4 in the tip cell\textsuperscript{[59, 60]}. Tip cell Dll4 then activates Notch signaling in the stalk cell, resulting in inhibition of VEGFR-2 expression in the stalk cell\textsuperscript{[59, 60]}. Evidence suggests that VEGFR-3 can regulate Notch signaling as well – deletion of VEGFR-3 in blood endothelial cells (BECs) causes hypersprouting in the retina concurrent with decreased levels of Dll4, suggesting
that loss of VEGFR-3 reduces Dll4/Notch signaling and inhibits angiogenic tip/stalk cell selection\textsuperscript{[66]}.

Notch regulation of VEGFR-3 has been extensively studied in both the lymphatic and blood vasculature, but a uniform model has yet to emerge\textsuperscript{[59, 60]}. In the blood vasculature, Notch is reported to suppress VEGFR-3 expression in zebrafish and mouse models\textsuperscript{[61, 67-69]}. Other reports, including work from our group, demonstrate that Notch induces VEGFR-3 expression in cultured blood endothelial cells (BECs)\textsuperscript{[70, 71]} and in the intersomitic blood vessels of the murine embryo\textsuperscript{[71]}. Reporter assays and chromatin immunoprecipitation performed by our group show direct binding and activation of the VEGFR-3 promoter by the Notch intracellular domain (NICD)/CSL complex in BECs\textsuperscript{[71]}. In cultured human dermal lymphatic endothelial cells (HDLECs), Notch effectors HEY1 and HEY2 repress VEGFR-3 expression\textsuperscript{[72]}. Still other reports demonstrate no changes in VEGFR-3 expression with Notch activation or inactivation\textsuperscript{[73, 74]}. Given the importance of Notch and VEGFR-3 in both lymphatic and blood vasculature, determining how the two regulate each other is a priority.

To date, information on how Notch functions in lymphangiogenesis is limited and lacks consensus. We discuss the results from the three main reports implicating Notch in sprouting lymphangiogenesis below.

In a zebrafish model, Geudens \textit{et al.} found that silencing Dll4 increased the fraction of venous intersomitic vessels (vISVs), decreased the number of arterial intersomitic vessels (aISVs), and stalled sprouting of lymphatic intersomitic vessels (LISVs)\textsuperscript{[70]}. Sprouted LISVs were often misrouted along vISVs, instead of developing along aISVs as expected\textsuperscript{[70]}. The authors
proposed several potential models that could explain these phenotypes: 1)Dll4/Notch silencing had primary effects on blood vessels, leading to secondary effects on lymphatic vessels; 2) Dll4/Notch silencing disabled the arterial endothelium from releasing lymphangiogenic factors; and/or 3) Dll4 expressed by arterial endothelial cells signals in trans to Notch lymphatic endothelial cells to guide their sprouting. The authors concluded that in the zebrafish, Dll4/Notch signaling is necessary for guided sprouting lymphangiogenesis alongside arterial intersomitic templates[70].

Niessen et al. targeted murine sprouting lymphangiogenesis with neutralizing antibodies against Notch1 (αNotch1) or Dll4 (αDll4). They reported that Notch inhibition reduced LYVE1+ lymphatic vessel density (LVD) in physiological sprouting lymphangiogenesis of the postnatal mouse ear and tail dermis[74]. αDll4 treatment also decreased LYVE1+ LVD in a model of pathological lymphangiogenesis (discussed in Chapter 4)[74]. To determine the molecular mechanism by which Notch suppressed sprouting lymphangiogenesis, the authors quantified expression of VEGFR-3 and ephrinB2, a necessary component of VEGFR signaling[75, 76], after Notch inhibition[74]. In tail dermal lymphatics, αDll4 treatment did not alter VEGFR-3 expression, but reduced ephrinB2 expression[74]. Thus the authors postulated that Notch promotes sprouting lymphangiogenesis through indirect regulation of VEGFR-3 signaling[74]. However, Niessen et al.’s method for quantifying LVD may not be adequate, as they also reported that knockdown of Notch1 or Dll4 in cultured HDLECs resulted in significant downregulation of LYVE1 expression[74]. Thus their phenotype may be a result of reduced expression of LYVE1 and not reduced LVD[74]. The authors did not address this possibility or stain with another lymphatic vessel marker to confirm their findings.
Zheng et al. engineered a Dll4 decoy composed of the Dll4 extracellular domain fused to Fc (Dll4-Fc) to inhibit Notch signaling\[^{73}\]. Notch inhibition with Dll4-Fc stimulated sprouting physiological lymphangiogenesis in the postnatal mouse ear\[^{73}\]. Blockade of VEGF signaling inhibited sprouting lymphangiogenesis stimulated by Dll4-Fc, demonstrating that sprouting lymphangiogenesis after Notch inhibition is at least partially achieved through increased VEGF/VEGFR-2 signaling\[^{73}\]. Furthermore, Notch signaling was activated in HDLECs treated with VEGF, placing Notch both upstream and downstream of VEGF signaling\[^{73}\]. The authors postulated that in lymphatics, Notch strongly regulates the VEGF/VEGFR-2 signaling axis but not the VEGF-C/VEGFR-3 signaling axis. To determine the molecular mechanism, VEGFR-2 and VEGFR-3 expression was analyzed in HDLECs treated with Dll4-Fc. Notch inhibition did not alter VEGFR-2 nor VEGFR-3 levels in vitro, and no expression analysis was performed in the mouse model\[^{73}\]. However, the authors observed reduced expression of ephrinB2 in cultured HDLECs treated with Dll4-Fc\[^{73}\]. The authors postulated that Notch suppresses lymphangiogenesis by indirectly inhibiting VEGF/VEGFR signaling. Thus, Zheng et al. and Niessen et al. both implicated Notch regulation of ephrinB2, as a regulator of lymphangiogenesis, but posed opposing mechanisms\[^{73, 74}\]. However, treatment of HDLECs with ephrinB2-blocking antibodies did not recapitulate the enhanced sprouting lymphangiogenesis seen with Dll4-Fc\[^{73}\]. Thus the authors identified interaction between Notch and VEGF signaling in physiological sprouting lymphangiogenesis, but were unable to identify the molecular mechanism by which this occurs.

In summary, Geudens et al. and Niessen et al. concluded that Notch signaling induces sprouting lymphangiogenesis during development\[^{70, 74}\] and wound-healing\[^{74}\], while Zheng et al. concluded that Notch signaling suppresses sprouting lymphangiogenesis during development\[^{73}\].
Thus, the interplay between Notch and VEGF receptors and how they regulate sprouting lymphangiogenesis is yet to be fully defined. Furthermore, a shared limitation of these studies is that none have focused on cell-autonomous functions of Notch in lymphatic endothelial cells. These opposing results and the lack of a molecular mechanism called for further investigation.

We considered that Notch plays important roles in cell-fate determination\(^{[24-26]}\) and sprouting angiogenesis\(^{[33, 59, 60]}\). Given the importance of cell-fate determination in sprouting lymphangiogenesis, and given the parallels between the lymphatic and blood vasculature, we set out to investigate how Notch signaling in lymphatic endothelial cells regulates sprouting lymphangiogenesis. Previous work on Notch in lymphatic and blood vasculature led us to hypothesize that Notch signaling regulates lymphangiogenesis through regulation of VEGFR-3 and VEGFR-2.

**RESULTS**

**Cultured lymphatic endothelial cells express Notch proteins and Notch ligands**

We performed a survey of the expression of Notch proteins and Notch ligands in lymphatic endothelial cells that we isolated from neonatal human dermis. These cells are referred to as human dermal lymphatic endothelial cells (HDLECs). HDLECs are \(\text{CD31}^+/\text{podoplanin}^-/\text{CD34}^\) (Chapter 2, Figure 1), and, as their name suggests, of dermal lymphatic capillary origin. Thus these cells are ideal for the study of sprouting lymphangiogenesis. We detected Notch1, Notch4,Dll4, and Jag1 transcripts (data not shown) and protein (Figure 1) in HDLECs. These are the same receptors and ligands in the Notch family
that are expressed in cultured human umbilical venous endothelial cells (HUVECs, data not shown).

**Murine lymphatic vasculature expresses Notch components**

In order to confirm our expression analysis in cultured HDLECs, we performed expression analysis in murine sprouting lymphatics during development. In the mouse embryonic dermis, the lymphatic vasculature develops in a highly consistent pattern. We whole-mount stained embryonic dermises for LYVE1, a lymphatic endothelial marker, and CD31, a pan-endothelial marker that is expressed strongly by blood vasculature and weakly by lymphatic vasculature (Figure 2). At E14.5 in the dorsal region, two lymphangiogenic capillary fronts were observed approaching the midline in a lateral to medial direction from either side (Figure 2). At this time point, the two angiogenic fronts had already reached the midline and fused to form a dense blood capillary network (Figure 2), indicating that lymphangiogenesis in the dorsal dermis continues after angiogenesis is complete. We concluded that the murine embryonic dermis would be an ideal model for the study of physiological sprouting lymphangiogenesis.

Our group has previously shown expression of Notch1 and Notch4 in dermal lymphatic capillaries\(^7\), using immunohistochemical analysis of postnatal day 4 (P4) murine dermal sections. To build upon these findings we analyzed ligand expression. Whole-mount immunohistochemical analysis revealed Dll4 expression in the developing lymphatics and in the neighboring blood vessels in the embryonic dermis (Figure 3A). Jag1 was not detected in
developing lymphatics, though it was expressed by the neighboring blood vessels (Figure 3B). Consistent with previous publications\textsuperscript{[77-79]}, strong Dll4 expression was observed in the arterial endothelium (Figure 3A) and strong Jag1 expression was observed in arterial endothelium and vascular smooth muscle cells (Figure 3B). Expression of Dll4 in the lymphatic vasculature was lower than in blood vasculature (Figure 3A). We conclude that Notch1, Notch4, and Dll4 are expressed by dermal lymphatics. Based upon the lack of Jag1 expression in this setting, we hypothesize that Jag1 becomes upregulated upon culturing of HDLECs from dermis.

HDLECs overexpressing Dll4 preferentially assume the tip cell position \textit{in vitro}\textsuperscript{[73]}. We investigated whether Dll4 was a lymphatic tip cell marker in the murine lymphatic vasculature. Indeed, upon closer examination of developmental dermal lymphatics, we found enriched Dll4 expression (Figure 3C’) at the leading edge of lymphangiogenic sprouts (Figure 3C) in a pattern reminiscent of Dll4 expression in angiogenic sprouts. Closer examination confirmed that Jag1 (Figure 3D’) was not expressed in lymphangiogenic sprouts (Figure 3D). Thus, we provide the first documentation of Dll4 as a lymphatic tip cell marker in the physiological lymphatic vasculature, suggesting that Dll4 plays an important role in sprouting lymphangiogenesis.

\textbf{Notch activation in cultured HDLECs depends on Dll4}

Notch signaling can occur between two cells of the same type, or heterotypically between two different cell types. Heterotypic Notch signaling between pericytes and BECs has been demonstrated to help stabilize and mature vasculature\textsuperscript{[80, 81]}, while heterotypic Notch signaling between macrophages and angiogenic tip cells has been suggested to aid in vessel
anastomosis. Although our expression analysis did not detect Jag1 in lymphatic vasculature, we wanted to rule out the possibility that Jag1 expressed by another cell type could heterotypically activate Notch signaling in LECs. To determine which ligands can activate Notch in lymphatic endothelial cell, we performed a Notch reporter assay using a luciferase reporter consisting of 11 tandems of a CSL responsive domain (11 CSL-luc). As HDLECs express Notch1, Notch4, Dll4, and Jag1, Notch signaling should occur endogenously in confluent monolayers of HDLECs. To maximize ligand expression levels and to isolate and identify differences between Dll4 and Jag1-induced Notch signaling, we chose to express either Dll4 or Jag1 in HeLa cells, which have low endogenous levels of Notches and ligands. HeLa cells were lipofected with plasmids expressing Dll4, Jag1, or control (ctrl, GFP). Quantitative Real Time PCR (qRT-PCR) confirmed that HeLa cells were strongly expressing the lipofected plasmids (Figure 4B). HDLECs were lipofected with 11 CSL-luc and co-cultured in a 1:1 ratio with the HeLa cells, as shown in the schematic (Figure 4A). Dll4 presented by HeLa cells induced Notch reporter activity over baseline in HDLECs (Figure 4C), demonstrating the ability of Dll4 to activate Notch in HDLECs. Jag1 neither induced nor repressed Notch signaling in HDLECs (Figure 4C). Dll4 and Jag1 presented together achieved the same level of induction as Dll4 alone (Figure 4C). These findings, in conjunction with the results of our expression analysis in dermal lymphatics, suggest that Dll4, and not Jag1, is the primary ligand for Notch signaling in capillary lymphatics.
VEGF receptor activation regulates Notch signaling in HDLECs

In the blood vasculature, it is well known that Notch signaling is induced in response to VEGFR-2 signaling, and it has been suggested that Notch signaling is also induced in response to VEGFR-3 signaling\[^{59, 60}\]. To date, one report has suggested that HDLECs exhibit this same induction response\[^{73}\]. We confirmed this through qRT-PCR analysis of HDLEC cultures treated with equal concentrations of VEGF or VEGF-C, using HUVEC cultures as a control. Both VEGF and VEGF-C induced Dll4, as well as Notch effector HES1 (Figure 5A). At the concentrations used, Dll4 was more strongly induced by VEGF, HEY1 was only induced in response to VEGF, and HEY2 was only induced in response to VEGF-C (Figure 5A). We observed a similar pattern of induction in HUVEC (Figure 5B). Thus, we demonstrate that VEGF receptor signaling can induce Notch signaling in HDLECs. This provided us with a link between these two important signaling pathways, and suggested that both may be critical in regulating LEC behavior.

Notch regulates HDLEC behavior

In sprouting lymphangiogenesis, LECs undergo several cellular processes in order to grow new vessels. LECs proliferate to add to the growing lymphatic sprout, migrate directionally towards VEGF-C and other cues, and fuse with neighboring LECs to form a network. We decided to test whether Notch signaling plays a role in some or all of these steps by subjecting HDLECs to assays designed to mimic these cellular processes. We overexpressed activated forms of Notch (N1IC or N4/int3, Chapter 2, Figure 2) or control (ctrl, GFP) using lentivirus-mediated stable transduction of cultured HDLECs and determined their effects on proliferation, migration,
and network formation. The N1IC lentiviral plasmid, which expresses the intracellular domain of Notch1, and the N4/int3 lentiviral plasmid, which expresses the transmembrane and intracellular domains of Notch4, are both constitutively active. Thus they will be referred to from hereon as “Notch activation”. N1IC and N4/int3 lentiviral plasmids effectively overexpressed active forms of Notch1 and Notch4, respectively (data not shown). We found that Notch activation suppressed HDLEC proliferation over 72hrs (Figure 6A). Notch activation suppressed HDLEC migration over a 24hr period in a monolayer wounding assay (Figure 6B). Notch activation also inhibited lymphatic capillary-like network formation over a 72hr period when HDLECs were seeded between two collagen gel layers (Figure 6C).Dll4/Notch signaling has been shown to be a negative regulator of sprouting in the blood vasculature, at least partly through its ability to regulate VEGFR expression and responsiveness to VEGF ligand stimulation\[59, 60\]. Our results in HDLECs suggest that Notch is also a negative regulator of multiple steps of sprouting lymphangiogenesis, namely proliferation, migration, and network formation.

**Notch regulates VEGFR-3 in a dynamic temporal manner**

In sprouting angiogenesis, the ability of Notch signaling to repress VEGFR-2 is critical\[59, 60\]. However, the data on the role of Notch in sprouting lymphangiogenesis, and whether this involves regulation of VEGFR-3 or VEGFR-2, has yet to reach a consensus. Given the dramatic effects seen on HDLEC proliferation, migration, and network formation with Notch overexpression, and given the critical role VEGFR-3 plays in sprouting lymphangiogenesis, we
set out to explore the possibility that Notch suppresses sprouting lymphangiogenesis through regulation of VEGFR-3 expression. We first validated that Notch activation constructs could induce downstream effectors HEY1 and HEY2 when introduced into HDLECs (Figure 7A). We also validated ectopic expression of HEY1 and HEY2 overexpression constructs (Figure 7A). VEGFR-2 transcripts were repressed by Notch activation (Figure 7B) and by overexpression of Notch effectors HEY1 or HEY2 (Figure 7C) in HDLECs. Repression of VEGFR-2 transcripts upon Notch activation (Figure 7B) agrees with what has been reported in the blood vasculature\textsuperscript{[59, 60]}. In contrast to VEGFR-2 transcripts, VEGFR-3 transcripts were induced by Notch activation in HDLECs (Figure 7B). However, overexpression of Notch effectors HEY1 or HEY2 repressed VEGFR-3 transcripts (Figure 7C). That Notch and its effectors could transcriptionally induce and suppress VEGFR-3 indicated that VEGFR-3 is regulated by multiple mechanisms. It also indicated that tight regulation of VEGFR-3 plays a significant role in LECs. We hypothesized that Notch both induces and represses VEGFR-3 transcripts, and that this may be important in the process of sprouting lymphangiogenesis. To test this hypothesis, we performed time course analysis.

Endogenous Notch signaling was suppressed overnight by treating confluent HDLEC cultures with a γ-secretase inhibitor (compound E). The next day, cells were washed to remove compound E and endogenous Notch signaling was activated (time 0’) by treatment with EDTA, a chelating agent that dissociates the Notch heterodimer and releases the Notch intracellular domain (NICD)\textsuperscript{[27]}. RNA was collected at multiple time points starting at time 0’ and analyzed by qRT-PCR. This experimental design allows for analysis of dynamic time-dependent responses to Notch signaling, which are lost in standard transcript or protein analysis. In this assay, we found VEGFR-3 transcripts to be an early responder to Notch activation, with significant induction as
early as 5 minutes and peaking at 10 minutes after Notch activation (Figure 8A). This supports previous observations from our group that VEGFR-3 is a direct transcriptional target of NICD/CSL in BECs. A second wave of induction was observed between 25 and 30 minutes, which returned to baseline by 120 minutes (Figure 8A).

A bi-phasic response to Notch activation was also observed for HEY1 and HEY2 transcripts, with induction between 5 and 10 minutes after Notch activation, followed by return to baseline levels between 15 and 30 minutes after Notch activation (Figure 8B). A second, more pronounced induction was observed at 60 and 120 minutes (Figure 8B).

Here we show that NICD/CSL and the downstream effectors HEY1/HEY2 can transcriptionally regulate VEGFR-3 in opposing manners. We propose a temporal model, in which NICD/CSL directly induces VEGFR-3 as well as HEY1/HEY2 between 5 to 10 minutes post-activation. Modest HEY1/HEY2 induction at this time point may not be enough to overcome direct induction of VEGFR-3 transcripts by Notch. A second wave of VEGFR-3 induction between 25 and 30 minutes after Notch activation is dampened by VEGFR-3 downregulation by HEY1/HEY2 beginning at 30 minutes after Notch activation. We hypothesize that this dynamic profile of VEGFR-3 transcripts in response to Notch activation may be key in regulating sprouting lymphangiogenesis, perhaps in defining lymphatic tip and stalk cells.
**Notch deficiency induces lymphangiogenesis in embryonic murine dermis**

We turned to the embryonic dorsal dermis to study how Notch deficiency in lymphatic endothelial cells affects sprouting lymphangiogenesis *in vivo*. We utilized the inducible *Prox1CreER<sup>T2</sup>* driver line, which allowed us to target lymphatic endothelial cells without affecting blood endothelial cells[^4]. To block Notch signaling, we used the *DNMAML* transgenic line, which expresses a dominant negative form of MAML1 that binds NICD/CSL but lacks the ability to recruit the transcriptional co-activators necessary to activate transcription of Notch target genes[^58] (Figure 9). Thus, DNMAML blocks signaling mediated by all Notch proteins and Notch ligands, allowing us to inhibit both Notch1 and Notch4 signaling in LECs. Furthermore, DNMAML does not interfere with the role of CSL as a transcriptional repressor prior to Notch activation. We crossed *Prox1CreER<sup>T2</sup>* driver mice with *DNMAML<sup>fl/fl</sup>* mice. *DNMAML<sup>fl/+</sup>* embryos were used as controls (ctrl) and compared to *Prox1CreER<sup>T2</sup>;DNMAML<sup>fl/+</sup>* mutant embryos. To circumvent the effects on early lymphatic specification caused by loss of Notch in LECs[^79], we administered tamoxifen to pregnant females at E12.5, just as lymph sacs are undergoing sprouting lymphangiogenesis. Dorsal skin was isolated at E14.5 for analysis.

Whole-mount immunohistochemistry at E14.5 focusing on the skin in the dorsal thoracic region revealed that Notch deficiency in LECs results in increased lymphangiogenesis (Figure 10B) compared to ctrl (Figure 10A). Unlike Niessen *et al.*, we did not observe changes in LYVE1 expression in LECs deficient for Notch (data not shown), which gave us confidence to use LYVE1 as an immunohistochemical marker for lymphatic vasculature. Overall lymphatic vessel density

[^4]: References
[^58]: References
(LVD) was significantly increased in mutant mice (Figure 10C). Blood vessel density (BVD) was unchanged (Figure 10A',B',D).

To determine what contributes to increased lymphatic vessel area, we divided the dorsal dermis into the lymphatic front, comprising the area occupied by lymphangiogenic sprouts, and the lymphatic plexus, comprising the region following behind the lymphatic front. At the lymphatic front, branch points (Figure 11C) and sprouts (Figure 11D) were significantly increased in mutant mice. Thus, loss of Notch signaling in the developing lymphatic vasculature at E12.5 causes hypersprouting at the lymphatic front (Figure 11A-B) characterized by increases in lymphatic vessel area, branching, and sprouting.

At the lymphatic plexus, we discovered a distinct phenotype from the phenotype at the lymphatic front. We observed an increase in the diameter of the lymphatic vessels in mutant mice (Figure 12B) compared to control mice (Figure 12A). Unlike at the front (Figure 11C), lymphatic branch points in the plexus were unchanged between the two groups (Figure 12C). Thus, loss of Notch signaling in developing lymphatic vasculature at E12.5 causes increased lymphatic vessel diameter at the lymphatic plexus (Figure 12A-B), without affecting sprouting or branching.

In order to determine molecular mechanism by which loss of Notch causes lymphatic hypersprouting, we compared lymphatic VEGFR-3 expression levels in mutant and control mice. There were no obvious differences in overall lymphatic VEGFR-3 expression between control (Figure 13A) and mutant (Figure 13B) mice. We believe that these results are not incompatible with the results of our time-course analysis (Figure 8), as standard immunohistochemistry and
microscopy may lack the sensitivity to detect dynamic and subtle changes in VEGFR-3 expression. Although we have not yet definitively confirmed that Notch dynamically regulates VEGFR-3 in vivo, we can conclude that Notch signaling is necessary to regulate developmental sprouting lymphangiogenesis in the murine dermis.

DISCUSSION

Here, we establish Notch as a negative regulator of developmental sprouting lymphangiogenesis in a lymphatic endothelial cell-autonomous manner. Loss of Notch in LECs results in lymphatic hypersprouting during the formation of the dermal lymphatic capillary network.

Notch1, Notch4, Dll4, and Jag1 have been detected in cultured HDLECs\textsuperscript{73}. In developing lymphatic capillaries of the murine embryonic dermis, we detected Dll4 expression, but did not detect Jag1 expression. High Dll4 expression has been observed in angiogenic tip cells\textsuperscript{33, 38, 39}, and others have observed high Dll4 expression in lymphatic “tip cells” in culture\textsuperscript{73}. We provide the first description of Dll4 enrichment in lymphangiogenic tips in vivo. The similarity in Dll4 expression patterns between lymphangiogenic sprouts and angiogenic sprouts suggests that Dll4 may function similarly in sprouting lymphangiogenesis as in sprouting angiogenesis.

We establish that Dll4 can functionally activate Notch signaling in lymphatics, a property not associated with Jag1. Dll4 activated Notch signaling in HDLECs, but Jag1 neither activated nor inhibited Notch signaling. In the blood vasculature, Jag1/Notch signaling is important, as
loss of Jag1 inhibits angiogenesis, but the mechanism by which this occurs is unclear. One model suggests that Jag1 and Dll4 both activate Notch signaling, but when Notch is post-
translationally modified by the Fringe family of glycosyltransferases, Jag1 antagonizes
Dll4/Notch signaling\cite{34}. Our lab proposed an alternate model suggesting that Jag1 and Dll4 both activate Notch signaling but have unique downstream effectors and thus individually regulate angiogenesis in opposing manners\cite{83}. Both models could explain the hyposprouting phenotype upon Jag1 inhibition\cite{34,83}. Furthermore, although the models propose different mechanisms, both agree that Jag1 can induce or inhibit Notch signaling. Our finding that Jag1 neither activates Notch signaling alone nor inhibits Dll4/Notch signaling, in conjunction with our finding that Jag1 is not expressed in developing lymphatic capillaries \textit{in vivo}, strongly suggest that Jag1 is not a functional ligand within the lymphatic capillary endothelium or in the context of sprouting lymphangiogenesis. Thus, we identify a key difference in Notch signaling in lymphatic vasculature, which does not seem to depend on Jag1.

HDLEC cultures treated with VEGF or VEGF-C both induce the tip cell marker \textit{DLL4}, as well as Notch effectors, placing VEGF receptors upstream of Notch signaling in HDLECs. Although we show that Notch signaling is downstream of VEGF and VEGF-C, several questions remain. VEGF and VEGF-C possess differential binding affinities and kinetics of activation for their cognate receptors\cite{62,84}, thus further investigation is necessary to compare the efficiency of Notch activation elicited by VEGF and VEGF-C. Furthermore, this experiment utilized fully processed VEGF-C (21kD), which strongly activates VEGFR-3 but can also more weakly activate VEGFR-2\cite{62}. Therefore it is possible that VEGF-C activates Notch signaling by activating both VEGFR-3 and VEGFR-2\cite{62}. Finally, VEGF and VEGF-C are capable of inducing VEGFR-2/VEGFR-3
heterodimer formation\textsuperscript{[65]}. Whether signaling downstream of VEGFR heterodimers can induce Notch signaling and how this compares to signaling downstream of VEGFR homodimers will also help us to better understand this feedback mechanism.

Loss of Notch signaling in the lymphatic vasculature during developmental sprouting lymphangiogenesis of the dermis results in increased overall lymphatic vessel density (LVD) with no change in blood vessel density (BVD). We observed hypersprouting at the lymphatic front characterized by increased lymphatic vessel area, sprouting, and branching. In the plexus, sprouting was unaffected, as there was no change in the number of branch points. Rather, an increase in lymphatic vessel diameter was appreciated. Several mechanisms are proposed here that could explain our phenotypes. They are discussed in further detail below: 1) Lymphatic hypersprouting caused by Notch inhibition may correct itself over time in the plexus as the vasculature in this region stabilizes and matures; 2) Notch inhibition may increase proliferation, migration, and network formation of LECs, resulting in hyperplasia at the lymphatic plexus and contributing to hypersprouting at the lymphatic front; 3) Notch inhibition may result in increased expression of VEGFR-2 in the stalk cells of the lymphatic plexus, leading to hyperplasia at the lymphatic plexus; 4) Notch dynamically regulates VEGFR-3 to regulate sprouting lymphangiogenesis, and inhibition of Notch may lead to deregulation of VEGFR-3 expression, causing lymphatic hypersprouting.

\textit{Lymphatic hypersprouting caused by Notch inhibition may correct itself over time in the plexus as the vasculature in this region stabilizes and matures (\#1).} Hypersprouting may be
corrected in the plexus as the lymphatic endothelium in this region becomes stabilized and the “tip” and “stalk” cell identities become less relevant.

*Notch inhibition may increase proliferation, migration, and network formation of LECs, resulting in hyperplasia at the lymphatic plexus and contributing to hypersprouting at the lymphatic front (#2).* Sprouting lymphangiogenesis is a concerted effort that requires LECs to proliferate, migrate, form networks, and identify as tip or stalk cells. We showed that exogenous Notch activation in HDLECs inhibits their proliferation, migration, and network formation. Thus, one explanation for our phenotype is that loss of Notch signaling in the lymphatic vasculature causes hypersprouting due to excessive LEC proliferation. Furthermore, excessive LEC proliferation, migration, and network formation may increase lymphatic vessel diameter. Immunohistochemical analysis of a proliferation marker such as BrdU could confirm whether loss of Notch causes LEC hyperproliferation in the lymphatic front and plexus.

*Notch inhibition may result in increased expression of VEGFR-2 in the stalk cells of the lymphatic plexus, leading to hyperplasia at the lymphatic plexus (#3).* Our transcript analysis in HDLECs showed that Notch activation represses VEGFR-2 transcripts in HDLECs. LECs in the lymphatic plexus are stalk cells; inferring from the sprouting angiogenesis model, Notch may function in these cells to suppress VEGFR-2 expression. It is plausible that when Notch is lost in the lymphatic vasculature, VEGFR-2 expression may increase specifically in plexus LECs, resulting in increased lymphatic vessel diameter. VEGFR-2 has been demonstrated to promote lymphatic vessel thickening without affecting sprouting in dermal lymphatic vasculature\[85\]. This phenotype was induced by VEGFR-2-specific activation, and could not be inhibited by blocking
VEGFR-3 signaling\[^{85}\]. Analysis of changes in VEGFR-2 expression in control and mutant mice will be critical in proving this hypothesis.

**Notch dynamically regulates VEGFR-3 to regulate sprouting lymphangiogenesis, and inhibition of Notch may lead to deregulation of VEGFR-3 expression, causing lymphatic hypersprouting (\#4).** Our transcript analysis in HDLECs showed that VEGFR-3, HEY1, and HEY2 are dynamically regulated by Notch activation in a biphasic manner. VEGFR-3 transcripts are immediately upregulated after Notch activation, likely through direct binding of NICD/CSL to the VEGFR-3 promoter\[^{71}\]. Subsequently, VEGFR-3 is downregulated, likely through the transcriptional repressors HEY1 and HEY2\[^{72,79}\], both of which are induced by Notch. Oscillating expression of HEYs and HES has been reported in other developmental settings in vertebrates\[^{86-89}\]. Therefore it is plausible that the oscillatory patterns of HEY1 and HEY2 we observed after Notch activation in cultured HDLECs are also important in regulating VEGFR-3. Sprouting angiogenesis is a highly dynamic process in which endothelial cells are constantly shifting between tip and stalk cell positions\[^{40}\]. The ability of BECs to differentially express Notch and VEGF receptors was shown to be critical for the dynamics of position shifting\[^{40}\]. We postulate that sprouting lymphangiogenesis is also a dynamic process that requires LECs to shift positions and compete with each other to obtain the tip or stalk cell positions. We suggest that differential regulation of VEGFR-3 may be critical for this process, and that inhibition of Notch in LECs deregulates this process, leading to lymphatic hypersprouting.

Whole-mount immunohistochemistry for VEGFR-3 was unable to capture these dynamics in embryonic dermal lymphatics. However, we believe that these results do not
disqualify a role for Notch in dynamically regulating VEGFR-3 to control sprouting lymphangiogenesis. Our Immunohistochemical methods may not yet be sensitive enough to detect subtle changes in VEGFR-3 expression levels. Live imaging may provide further clues about how loss of Notch signaling affects VEGFR-3 expression in vivo during lymphangiogenesis. Comparing and contrasting phenotypes of Notch inhibition, VEGFR-3 inhibition, and VEGFR-2 inhibition may also provide genetic evidence linking the pathways to each other in the embryonic dermal lymphangiogenesis model.

Taken together, we have demonstrated that Notch signaling suppresses sprouting lymphangiogenesis, and we have identified VEGFR-3 and VEGFR-2 as two potential Notch targets responsible for regulating sprouting lymphangiogenesis. Thus, we propose that sprouting lymphangiogenesis can be explained by the same tip/stalk model that explains sprouting angiogenesis (Figure 14): VEGF-C and VEGF activate VEGFR-3 and VEGFR-2 in the lymphatic tip cell; VEGFR-3 and VEGFR-2 signaling induce Dll4 expression in the lymphatic tip cell; Dll4 expressed by the lymphatic tip cell activates Notch signaling in lymphatic stalk cell; Notch dynamically regulates VEGFR-3 expression and suppresses VEGFR-2 expression in the lymphatic stalk cell.
Chapter 3

Figures
Figure 1. HDLECs express Notches and ligands. Cultured HDLEC express Notch1, Notch4,Dll4, and Jag1 (in green). Cell nuclei are stained with DAPI (blue); scale bars=10μm.
Figure 2. The embryonic murine dermis as a model of sprouting lymphangiogenesis. E14.5 dorsal murine dermis whole-mount stained for blood vasculature (CD31, green) and lymphatic vasculature (LYVE1, red) shows vasculature growing medially towards the midline (marked with dashed white line); scale bars=100μm. LYVE1+ cells with rounded morphologies (yellow arrows) are macrophages.
Figure 3. Physiological lymphatic vasculature expresses Dll4. (A-B) Murine embryonic dermises were whole-mount stained at E14.5 in the dorsal thoracic region for lymphatic vasculature (LYVE1, red), Dll4 or Jag1 (green), and blood vasculature (CD31, blue); scale bars=100μm. (A) Dll4 is detected in murine dermal lymphatics (arrowheads), as well as in neighboring blood vessels (arrows). (B) Jag1 is not detected in murine dermal lymphatics (arrowheads), but is detected in neighboring blood vessels (arrows). (C-D) Boxed areas in A and B; scale bars=100μm. Dll4 (C’) is enriched in lymphatic tip cells (C). Jag (D’) is not detected in lymphatic tip cells (D).
Figure 4. Dll4 activates Notch in HDLECs. (A) HDLEC lipofected with a luciferase Notch reporter containing 11 repeats of CSL-responsive elements (11 CSL-luc) are co-cultured with HeLa lipofected with Dll4, Jag1, or both. (B) Quantitative RT-PCR shows Dll4 and Jag1 are induced in lipofected HeLa. (C) A luciferase reporter assay shows Dll4 activates Notch signaling in HDLECs (p=0.05), while Jag1 neither activates nor antagonizes Notch signaling in HDLECs. Dll4+Jag1 together activate Notch signaling to the same degree as Dll4 alone (p<0.05).
Figure 5. Notch signaling is responsive to VEGF receptor activation. (A) Quantitative RT-PCR with HDLEC cDNA shows Notch ligand DLL4, as well as Notch effectors HEY1 and HES1, are induced in HDLEC cultures treated with VEGF or VEGF-C. Notch effector HEY2 is only induced in HDLEC cultures treated with VEGF-C. (B) Quantitative RT-PCR with HUVEC cDNA shows Notch ligand DLL4, as well as Notch effector HEY1, are induced in HUVEC cultures treated with VEGF or VEGF-C. Notch effector HES1 is only induced in HUVEC cultures treated with VEGF. Error bars represent standard deviation of the mean, *p<0.05, **p<0.001.
Figure 6. Notch regulates HDLEC proliferation, migration, and network formation. (A) Notch activation (N1IC or N4/int3) inhibits HDLEC proliferation over a 72hr period. (B) Notch activation (N1IC or N4/int3) inhibits HDLEC migration in a monolayer scratch assay over a 25hr period; scale bars=5μm. (C) Notch activation (N1IC or N4/int3) inhibits HDLEC network formation on a collagen gel over a 72hr period; scale bars=2.5μm. Error bars represent standard deviation of the mean, *p<0.05, **p<0.001.
Figure 7. Notch induces VEGFR-3, but Notch effectors HEY1 and HEY2 repress VEGFR-3. Quantitative RT-PCR with HDLEC cDNA. (A) qPCR validates that Notch activation lentiviral constructs (N1IC and N4/int3) and HEY overexpression lentiviral constructs (HEY1 and HEY2) increase HEY1 and HEY2 levels. (B) Notch activation represses VEGFR-2 but induces VEGFR-3. (C) Notch effectors HEY1 and HEY repress VEGFR-3 and VEGFR-2 levels. Error bars represent standard deviation of the mean, *p<0.05, **p<0.001.
Figure 8. Notch dynamically regulates **VEGFR-3**. Quantitative RT-PCR with HDLEC cDNA. (A) **VEGFR-3** is an early responder to Notch activation, with significant induction seen as early as 5 minutes after Notch activation. Levels return to below baseline between 20 and 25 minutes. A second wave of induction is seen between 25 and 30 minutes, which returns to below baseline by 120 minutes. (B) **HEY1** and **HEY2** show the strongest induction starting at 30 minutes after Notch activation. Error bars represent standard deviation of the mean, *p<0.05, **p<0.001. (C) Proposed model of transcriptional regulation of VEGF receptors by Notch in lymphatic endothelial cells.
Figure 9. **DNMAML inhibits Notch signaling.** DNMAML can bind Notch IC and CSL, but is missing the domain necessary to recruit co-activators necessary for transcription of Notch target genes. Adapted from McElhinny, A.S., J.L. Li, and L. Wu, Mastermind-like transcriptional co-activators: emerging roles in regulating cross talk among multiple signaling pathways. *Oncogene*, 2008. 27(38): p. 5138-47.
Figure 10. Loss of Notch signaling causes lymphatic hypersprouting in the embryonic murine dermis. (A-B) Tamoxifen was administered to pregnant dams (10mg/kg) at E12.5 and dermises of embryos were whole-mount stained at E14.5 for lymphatic vasculature (LYVE1, red) and blood vasculature (CD31, green). Dashed white line marks the midline; scale bars=1000μm. Mutant mice (Prox1CreER<sup>T2;DNMAML<sup>/+</sup>) (B), display lymphatic hypersprouting when compared to control littermates (DNMAML<sup>/+</sup>) (A). Blood vessel density in mutant mice (Prox1CreER<sup>T2;DNMAML<sup>/+</sup>) (B'), is unchanged when compared to control littermates (DNMAML<sup>/+</sup>) (A'). (C) Quantification of overall lymphatic vessel area. (D) Quantification of overall blood vessel area. Error bars represent standard deviation of the mean, *p<0.05, n=3-6.
Figure 11. Loss of Notch signaling increases branch points and sprouts at the lymphatic front. (A-B) Tamoxifen was administered to pregnant dams (10mg/kg) at E12.5 and dermises of embryos were whole-mount stained at E14.5 for lymphatic vasculature (LYVE1, red) and blood vasculature (CD31, green); scale bars=100μm. Lymphatic sprouts marked with arrowheads, branch points marked with asterisks. Mutant mice (Prox1CreER²; DNMAML⁰/⁺) (B), display increased lymphatic sprouts and branch points at the front compared to control littermates (DNMAML⁰/⁺) (A). Blood vasculature is unaffected (A',B'). (C) Quantification of lymphatic branch points. (D) Quantification of lymphatic sprouts. Error bars represent standard deviation of the mean, *p<0.05, n=3-6.
Figure 12. Loss of Notch signaling does not affect branch points but increases vessel diameter at the lymphatic plexus. (A-B) Tamoxifen was administered to pregnant dams (10mg/kg) at E12.5 and dermises of embryos were whole-mount stained at E14.5 for lymphatic vasculature (LYVE1, red) and blood vasculature (CD31, green); scale bars=100μm. Lymphatic branch points marked with asterisks, vessel width marked with cyan lines. Mutant mice (Prox1CreER\textsuperscript{R2};DNMAML\textsuperscript{fl/+}) (B), display increased lymphatic vessel diameter at the plexus compared to control littermates (DNMAML\textsuperscript{fl/+}) (A). Lymphatic branch points (A,B) and blood vasculature (A',B') are unaffected. (C) Quantification of lymphatic branch points. Error bars represent standard deviation of the mean, *p<0.05, n=3-6.
Figure 13. Loss of Notch signaling does not alter VEGFR-3 expression in developing lymphatic vasculature. (A-B) Tamoxifen was administered to pregnant dams (10mg/kg) at E12.5 and dermises of embryos were whole-mount stained at E14.5 for VEGFR-3 (green), lymphatic vasculature (LYVE1, red), and blood vasculature (CD31, green); scale bars=200μm. Overall VEGFR-3 expression is unchanged between mutant mice (Prox1CreER\textsuperscript{T2};DNMAML\textsuperscript{fl/+}) (B), and control littermates (DNMAML\textsuperscript{fl/+}) (A).
Figure 14. Notch and VEGF feedback loop. (A) Proposed model of the feedback loop between Notch and VEGF in sprouting lymphangiogenesis. (B) Lymphatic hypersprouting occurs upon Notch inhibition with DNMAML.
Chapter 4

Inhibition of Dll/Notch signaling causes hypersprouting of pathological lymphatics in the corneal suture model
INTRODUCTION

Manipulating Notch signaling in cultured HDLECs and in the mouse embryo, we found that Notch functions to suppress developmental sprouting lymphangiogenesis. We identifiedDll4 as a lymphatic tip cell marker, suggesting a specialized function for Dll4 in lymphatic tip cells – possibly to induce Notch signaling in lymphatic stalk cells and thereby suppress sprouting of stalk cells. We also identified a key difference between lymphatic and blood vasculature by finding that Jag1 is not expressed by developing lymphatic vessels in vivo. We found that the Notch and VEGF signaling pathways regulate each other in a feedback loop, suggesting a potential mechanism by which Notch suppresses developmental sprouting lymphangiogenesis. Here we focused on how Notch functions in pathological lymphangiogenesis. Based on our studies of physiological/developmental lymphangiogenesis, we hypothesized that Notch also functions to suppress lymphangiogenesis in pathological conditions, and that loss of Notch signaling would result in hypersprouting, or increased sprouting and branching, of pathological lymphatic vasculature.

To date, only one report has described the effects of Notch inhibition in pathological lymphangiogenesis. Niessen et al., whose work is also described in Chapter 3, utilized a Dll4 neutralizing antibody (αDll4) to inhibit Notch signaling and observed hyposprouting of lymphatics in a wound healing model. Dll4 inhibition has been studied extensively in the blood vasculature and has been shown to cause hypersprouting in physiological angiogenesis in the postnatal retina, as well as in pathological angiogenesis in tumors, thus serving as an internal control in this experiment. As expected, the authors found that αDll4 treatment
increased pathological angiogenesis as determined by staining for CD31\textsuperscript{74}, whereas αDll4 treatment decreased pathological lymphangiogenesis as determined by staining for LYVE1\textsuperscript{74}. Thus they concluded that Notch inhibition suppresses pathological lymphangiogenesis. Niessen et al.’s results suggest that pathological lymphangiogenesis, at least in wound healing, is not a secondary response to primary effects on pathological angiogenesis. Secondly, these results suggest that Dll4/Notch signaling has unique functions in lymphatic and blood vasculatures.

The cornea is a commonly used model for the study of pathological lymphangiogenesis\textsuperscript{21}. It is one of the few tissues in the body that is devoid of vasculature\textsuperscript{22} (Figure 1A,B), which is critical in maintaining corneal transparency and visual acuity\textsuperscript{22}. The neighboring conjunctiva and the limbus, which marks the border between the cornea and conjunctiva, are vascularized with lymphatic and blood vessels\textsuperscript{22} (Figure 1A,B). In pathological settings, such as keratitis (i.e., inflammation of the cornea) or complications following corneal implant surgery, lymphatic and blood vessels from the neighboring limbus invade into the cornea\textsuperscript{21, 22}. This leads to compromised vision, exacerbated inflammation as antigen-presenting cells are provided with easy access between the cornea and draining lymph nodes, and graft rejection in the case of corneal implants\textsuperscript{21, 22}. Thus, understanding how lymphangiogenesis occurs in corneal inflammation may provide insights about pathological lymphangiogenesis in general. It can also provide opportunities for the development of therapeutics.

The murine corneal suture assay mimics human corneal pathologies by inducing vascularization of the cornea (Figure 1A’,B’). Here too, as in the embryonic dermis, a lymphatic
front can be observed, which arrests once the vessels reach the suture. Newly formed lymphatic and blood vessels can be seen in the cornea as early as 2 days after suture placement, and at times, lymphangiogenesis into the cornea can even outpace angiogenesis[21]. Lymphangiogenesis can also occur independently of angiogenesis in the cornea – low doses of FGF-2 in a corneal pellet assay were shown to selectively induce lymphangiogenesis without inducing angiogenesis[95]. These features make the corneal suture assay a useful model for the study of pathological lymphangiogenesis.

Macrophages are important mediators of developmental lymphangiogenesis[9, 11, 12, 96]. They also play a crucial role in corneal suture-induced lymphangiogenesis[22], whether by transdifferentiating into lymphatic endothelial cells[97], or by secreting pro-lymphangiogenic molecules like VEGF-C or VEGF-D[98]. Interestingly, although the stroma of the uninjured cornea is home to many resident macrophages[99, 100], lymphatic and blood vessels are not present in the cornea under normal conditions. This is likely due to the secretion of anti-lymphangiogenic and anti-angiogenic factors by the corneal epithelium[101-103].

VEGF receptors 1, 2, and 3 are expressed endogenously by the corneal epithelium and are critical in maintaining corneal avascularity[101-103]. Soluble VEGFR-1 (sVEGFR-1)[102] and full-length VEGFR-3[103] are expressed by corneal epithelial cells and function as anti-angiogenic factors by competing with blood vessels to bind VEGF and VEGF-C, respectively. Meanwhile, corneal epithelial cells express soluble VEGFR-2, which competitively binds VEGF and VEGF-C but functions solely as an anti-lymphangiogenic factor, with no inhibitory effects on angiogenesis[101]. Loss of expression of any of these three receptor variants causes infiltration of
vasculature into the cornea\textsuperscript{[101-103]}. These observations highlight the importance of VEGF receptors in controlling corneal lymphangiogenesis and angiogenesis, and given that Notch signaling can regulate transcription of VEGFR-3 and VEGFR-2 in HDLECs (Chapter 3), we postulated that this may be a mechanism by which Notch regulates pathological lymphangiogenesis and angiogenesis in the corneal suture assay.

To elucidate the role of Notch signaling in pathological lymphangiogenesis in the corneal suture assay, we utilized pharmacological tools to inhibit Notch signaling. A class of Notch inhibitors known as the Notch1 decoys (N1ECDFc, Chapter 2, Figure 3) were previously designed and created by our group\textsuperscript{[83, 91]}. Notch1 decoys are composed of the EGF-like repeats of the human Notch1 receptor fused to human Fc (hFc) and can bind ligands, but lack the transmembrane and intracellular domains necessary for signal transduction\textsuperscript{[83, 91]}. They thereby act as competitive inhibitors of endogenous Notches. The full-length Notch1 decoy, or N1ECDFc\textsubscript{1-36}, comprises all 36 EGF-like repeats of human Notch1 and has been shown to function as a pan-ligand inhibitor\textsuperscript{[83, 91]}. The smaller N1ECDFc\textsubscript{1-24} variant possesses the same pan-ligand inhibiting capabilities but demonstrates improved extravasation from the blood vessels and diffusion into tissues\textsuperscript{[83]}. The N1ECDFc\textsubscript{1-13} variant possesses specific inhibitory functions against signaling mediated by ligands of theDll class (i.e., Dll1, Dll3, and Dll4)\textsuperscript{[83]}. The N1ECDFc\textsubscript{10-24} variant possesses specific inhibitory functions against signaling mediated by ligands of the Jag class (i.e., Jag1 and Jag2)\textsuperscript{[83]}.

The Notch1 decoys have been tested in multiple settings of physiological and pathological angiogenesis\textsuperscript{[81, 83, 91]}. Treatment with N1ECDFc\textsubscript{1-13} (Dll inhibitor) gives a classical
Dll4 inhibition phenotype\cite{33, 93} and induces angiogenic sprouting in the retina\cite{83} and in multiple tumor types\cite{83}. Treatment with N1ECDFc10-24 (Jag inhibitor) suppresses angiogenic sprouting in the retina\cite{83}, the ovarian corpus luteum\cite{81}, and in multiple tumor types\cite{83}. Although two hypotheses have been posed to explain the angiogenic hyposprouting phenotype\cite{33, 83} (discussed in Chapter 3), the mechanism by which Jag inhibition suppresses angiogenesis is currently unknown. Treatment with N1ECDFc1-24 (Dll/Jag inhibitor) has differential effects on the vasculature that appear to be dependent on the setting. In the retina, treatment with N1ECDFc1-24 induces angiogenic sprouting, suggesting that Dll-mediated signaling is dominant in this setting \textit{(Kangsamaksin, unpublished data)}. In multiple tumor types, treatment with N1ECDFc1-24 suppresses angiogenic sprouting, suggesting that Jag-mediated signaling is dominant in these models\cite{83}.

The lack of information on how Notch signaling regulates pathological lymphangiogenesis called for further investigation. In choosing a pathological model of lymphangiogenesis, we took into consideration the unique features of the cornea, including its avascularity under normal conditions, the rapid growth of lymphatic vasculature in response to suture placement, and the importance of the VEGF signaling pathway in regulating corneal lymphangiogenesis. We utilized pharmacological intervention with Notch1 decoys to inhibit Notch signaling. Our findings in HDLECs and in developmental lymphangiogenesis led us to hypothesize that Dll4 expressed by lymphatic tip cells activates Notch signaling in lymphatic stalk cells to inhibit excessive sprouting during lymphangiogenesis, while Jag1 is not expressed by lymphatics and therefore does not play a significant role in sprouting lymphangiogenesis. We hypothesized that this lymphatic tip/stalk model would be maintained in pathological sprouting
lymphangiogenesis in the corneal suture model. We predicted that treatment with N1ECDFc\textsubscript{1-24} (Dll/Jag inhibitor) and N1ECDFc\textsubscript{1-13} (Dll inhibitor) would induce lymphatic hypersprouting in the corneal suture model, while treatment with N1ECDFc\textsubscript{10-24} (Jag inhibitor) would not affect sprouting lymphangiogenesis.

RESULTS

Characterization of suture-induced lymphatic vasculature

Lymphatic vessels recruited in the corneal suture assay expressed LYVE1 and were not covered by NG2\textsuperscript{+} pericytes (data not shown) or αSMA\textsuperscript{+} smooth muscle cells (data not shown), suggesting a lymphatic capillary phenotype. However, corneal lymphatic vessels contained integrinα\textsubscript{9}\textsuperscript{+} lymphatic valves (data not shown), suggesting a lymphatic collecting duct phenotype. The high levels of LYVE1 expression, lack of mural cell coverage, and presence of valves led us to identify these vessels as hybrids between lymphatic capillaries and pre-collecting lymphatic vessels.

We first sought to establish which Notch proteins and Notch ligands are expressed by corneal suture-induced lymphatics. We performed whole-mount staining sutured corneas for Dll4 or Jag1, LYVE1, a lymphatic endothelial marker, and CD31, a pan-endothelial marker that is expressed strongly by blood vasculature and weakly by lymphatic vasculature (Figure 2). Dll4 was expressed by pathological lymphatic vessels (Figure 2A) and neighboring pathological blood vessels (Figure 2A). Jag1 was not detected in pathological lymphatic vessels (Figure 2B), though
it was expressed by the neighboring pathological blood vessels (Figure 2B). Consistent with previous publications\(^{77-79}\), strong Dll4 expression was observed in the arterial endothelium (Figure 2A) and strong Jag1 expression was observed in arterial endothelium and vascular smooth muscle cells (Figure 2B). Expression of Dll4 in corneal suture-induced lymphatics was lower than Dll4 expression in corneal suture-induced blood vessels (Figure 2A).

Closer examination of pathological lymphatic vasculature revealed enriched Dll4 expression (Figure 2C’) in the lymphatic tip cells at the lymphangiogenic front (Figure 2C). Enriched Dll4 expression could also be appreciated at the angiogenic front (Figure 2A, Figure 2C’). Closer examination confirmed that Jag1 (Figure 3D’) was not expressed in pathological lymphangiogenic fronts (Figure 3D). In summary, our ligand expression analysis of suture-induced lymphatic vasculature reveals the same expression patterns as those we observed in physiological lymphatic capillaries (Chapter 3).

**Notch1 decoys are detected in the circulation after adenovirus administration**

We delivered Notch1 decoys to adult female C57BL/6J mice through retro-orbital injection of adenovirus. Once delivered to the bloodstream, the adenovirus travels to the liver and infects the hepatocytes, which are particularly receptive to adenoviral infection. Large amounts of the protein encoded by the adenovirus are subsequently produced and secreted, without adverse effects on the liver. The protein is shed into the circulation and travels systemically throughout the mouse. Previous work from our lab showed efficient extravasation of Notch1 decoys into the tissue\(^{83}\). After delivery of \(5 \times 10^8\) ffu of adenovirus/mouse, Notch1
decoy variants and hFc (ctrl) were easily detected by Western blot in as little as 0.5μL of serum, and as soon as 2 days post-delivery (Figure 3). Smaller size Notch1 decoy variants were detected at higher levels in the circulation (Figure 3). These results gave us confidence to continue forward with this method of Notch inhibition.

**Inhibition of Dll/Notch signaling induces lymphangiogenesis in the corneal suture model**

We tested ligand-specific Notch1 decoys as well as a pan-ligand inhibitor (N1ECDFc1-24) in the corneal suture model to identify differences resulting from Dll blockade (N1ECDFc1-13) or Jag blockade (N1ECDFc10-24). We whole-mount stained sutured corneas at end point to visualize invading lymphatic vessels (LYVE1) and blood vessels (CD31). We observed pathological lymphangiogenesis (Figure 4A) and angiogenesis (Figure 4A’) into control (ctrl, hFc) sutured corneas. Corneas of mice treated with N1ECDFc1-24 (Dll/Jag inhibitor) revealed significantly increased area vascularized by lymphatic vessels (Figure 4B,E) and blood vessels (Figure 4B’,E) as compared to ctrl. Corneas of mice treated with N1ECDFc1-13 (Dll inhibitor) also revealed significantly increased area vascularized by lymphatic vessels (Figure 4C,F) and blood vessels (Figure 4C’,F) as compared to ctrl. The area vascularized by lymphatic and blood vessels did not differ between N1ECDFc1-13 and N1ECDFc1-24 corneas. Corneas of mice treated with N1ECDFc10-24 (Jag inhibitor) showed no change in area vascularized by lymphatic vessels (Figure 4D), but significantly reduced area vascularized by blood vessels (Figure 4D’,G) as compared to ctrl. Our results suggested that Jag1/Notch signaling does not play a significant role in pathological
lymphangiogenesis in the corneal suture model, while Dll4/Notch signaling functions to suppress pathological lymphangiogenesis in the corneal suture model.

**F4/80^+ macrophage content is unaffected in Notch1 decoy treated corneas**

Macrophages play a crucial role in inducing lymphangiogenesis and angiogenesis in the corneal suture model, and removal of macrophages or inhibition of their function in the corneal suture model can also have secondary effects on the vasculature\(^{[22, 97, 98, 104]}\). Given the promiscuity of Notch1 decoys, we wanted to determine whether N1ECDFc1-13 (Dll inhibitor) or N1ECDFc1-24 (Dll/Jag inhibitor) enhance corneal suture-induced lymphangiogenesis by affecting macrophage recruitment or function. We whole-mount stained sutured corneas at end point to visualize macrophages (F4/80). F4/80 staining revealed no differences in macrophage density upon treatment with N1ECDFc1-24 (Dll/Jag inhibitor, Figure 5B,C) compared to ctrl (Figure 5A,C). Neither N1ECDFc1-13 (data not shown) nor N1ECDFc10-24 (data not shown) affected macrophage density compared to ctrl. We concluded that increased sprouting lymphangiogenesis and angiogenesis in the cornea upon treatment with N1ECDFc1-24 (Dll/Jag inhibitor) or N1ECDFc1-13 (Dll inhibitor) and decreased corneal angiogenesis upon treatment with N1ECDFc10-24 (Jag inhibitor) are not caused by changes in macrophage density.
**N1ECDFc1-24 causes lymphatic hypersprouting in the corneal suture model**

We sought to identify the underlying changes in the lymphatic vasculature that contribute to increased area vascularized by lymphatics upon treatment with N1ECDFc1-24 (Dll/Jag inhibitor) or N1ECDFc1-13 (Dll inhibitor). We quantified lymphatic sprouts and lymphatic branch points in Notch1 decoy-treated corneas and compared them to lymphatic sprouts and lymphatic branch points in ctrl corneas (Figures 6-8). N1ECDFc1-24 (Dll/Jag inhibitor) treatment caused lymphatic hypersprouting (Figure 6B) compared to control (Figure 6A), characterized by an increased number of lymphatic sprouts (Figure 6C) and branch points (Figure 6D). N1ECDFc1-13 (Dll inhibitor) treatment did not alter lymphatic sprouting (Figure 7B) compared to ctrl (Figure 7A), and the number of lymphatic sprouts (Figure 7C) and branch points (Figure 7D) were unchanged with treatment. N1ECDFc10-24 (Jag inhibitor) treatment did not alter lymphatic sprouting (Figure 8B) compared to ctrl (Figure 8A), and the number of lymphatic sprouts (Figure 8C) and branch points (Figure 8D) were unchanged with treatment.

**DISCUSSION**

Here, we have identified a role for Notch signaling in regulating pathological sprouting lymphangiogenesis in the corneal suture assay. We report that suture placement in the murine cornea induces pathological lymphangiogenesis. Corneal suture-induced lymphatics express the Notch ligand Dll4 strongly at lymphatic tips, but do not express Jag1. Corneal suture-induced lymphatic vessels displayed the same expression patterns of Dll4 as developing dermal
lymphatics (Chapter 3), which suggested that Dll4/Notch signaling regulates sprouting lymphangiogenesis similarly in both lymphatic vascular beds.

Treatment with N1ECDFc1-24 (Dll/Jag inhibitor) and N1ECDFc1-13 (Dll inhibitor) comparably increased corneal area occupied by pathological lymphatics, while treatment with N1ECDFc10-24 (Jag inhibitor) did not alter corneal area occupied by pathological lymphatics. Jag1 is not detected in corneal pathological lymphatic vasculature. Furthermore, Niessen et al. reported that Jag1 neutralizing antibodies had no effect on physiological sprouting lymphangiogenesis in tail and ear dermises[74]. Thus we feel comfortable excluding Jag1 as a functional ligand in this model. We postulate that N1ECDFc1-24 (Dll/Jag inhibitor) functions primarily in the corneal suture assay to inhibit Dll/Notch signaling.

We hypothesized that increased corneal area occupied by lymphatics upon N1ECDFc1-24 (Dll/Jag inhibitor) or N1ECDFc1-13 (Dll inhibitor) treatment was a result of lymphatic hypersprouting. Treatment with N1ECDFc1-24 (Dll/Jag inhibitor) caused lymphatic hypersprouting, but treatment with N1ECDFc1-13 (Dll inhibitor) did not. Pan-ligand inhibitor N1ECDFc1-24 has demonstrated increased potency over either ligand-specific inhibitor alone, despite being present in the circulation at lower levels[83]. Thus, the difference in lymphatic sprouting phenotypes between N1ECDFc1-24 (Dll/Jag inhibitor) and N1ECDFc1-13 (Dll inhibitor) treatments may be due to increased potency of N1ECDFc1-24 compared to N1ECDFc1-13 in blocking Dll/Notch signaling. An interesting possibility is that new phenotypes may emerge as Dll4/Notch signaling is increasingly inhibited. Sprouting lymphangiogenesis not only requires tip/stalk cell identification, but also requires LEC proliferation, migration, and network
formation. Thus, it is plausible that weaker inhibition ofDll4/Notch signaling is sufficient to enhance certain LEC behaviors while not affecting other LEC behaviors. For example, weaker inhibition ofDll4/Notch signaling (N1ECDFc1-13) may be sufficient to enhance migration ofLECs, which could account for increased lymphatic vascular coverage. As Dll4/Notch signaling is more strongly inhibited (N1ECDFc1-24), additional phenotypes, such as lymphatic hypersprouting, may be acquired. We would like to test these hypotheses in the future using Notch1 decoys in their purified protein form. Using purified proteins will allow us to develop dose-response curves for LEC behaviors such as proliferation, migration, network formation, or lymphatic tip cell formation.

We cannot definitively conclude that increased pathological sprouting lymphangiogenesis in the corneal suture model upon treatment with N1ECDFc1-24 (Dll/Jag inhibitor) is a LEC-autonomous response. To discern LEC-autonomous functions of Notch signaling would require genetic inhibition of Notch specifically in LECs (Prox1CreERT2). However, the fact that we also observe lymphatic hypersprouting in the embryonic dermis upon LEC-specific inhibition of Notch signaling (Prox1CreERT2;DNMAMLfl/+; Chapter 3) provides evidence to support the claim that N1ECDFc1-24 (Dll/Jag inhibitor) treatment induces pathological sprouting lymphangiogenesis by targeting the lymphatic vasculature. The observation that corneal lymphangiogenesis can occur independently of angiogenesis[95] also strengthens the argument that pathological sprouting lymphangiogenesis in the corneal suture model is not solely a secondary response to pathological sprouting angiogenesis.
We plan to analyze the contribution of VEGFR-3 or VEGFR-2 to the phenotype we found in corneal suture-induced lymphangiogenesis. Based on our observations thus far, we would speculate that Notch inhibition with Notch1 decoy treatment induces lymphangiogenesis through regulation of VEGFR-3 and VEGFR-2. To validate this, future experiments should include careful analysis of VEGFR-3 and VEGFR-2 expression in Notch1 decoy treated corneal lymphatics. Comparing and contrasting the effects of Notch inhibition, VEGFR-3 blockade, and VEGFR-2 blockade in the corneal suture model may provide evidence linking the pathways to each other. As corneal epithelium also strongly express VEGFR-3 and soluble VEGFR-2 (sVEGFR-2), and soluble VEGFR-1 (sVEGFR-1), it would be interesting to compare and contrast how Notch inhibition affects lymphatic vasculature, blood vasculature, and corneal epithelium.

Here we demonstrate that Notch signaling regulates pathological sprouting lymphangiogenesis in the corneal suture model. Taken together, our data suggests that Dll4/Notch signaling functions in a cell-autonomous manner within the lymphatic endothelium to suppress pathological lymphangiogenesis in the corneal suture model.
Chapter 4

Figures
Figure 1. The corneal suture assay as a model of pathological sprouting lymphangiogenesis. Visual acuity requires a transparent and avascular cornea. The conjunctiva is richly vascularized with blood (green) and lymphatic (red) vessels. The limbus, or the border of the cornea, also has vasculature. We used two methods of suture placement (dark gray). In the first method, placement of a single continuous suture covering at least 180° (A), caused lymphatic vessel and blood vessel growth into the cornea (A’). In the second method, placement of 3 sutures in the nasal, superior, and inferior positions (B), also caused lymphatic vessel and blood vessel growth into the cornea (B’).
Figure 2. Suture-induced pathological lymphatic vasculature expresses Dll4. (A-B) Murine adult corneas were whole-mount stained after suture placement (S) for lymphatic vasculature (LYVE1, red), Dll4 or Jag1 (green), and blood vasculature (CD31, blue); scale bars=100μm. (A) Dll4 is detected in murine pathological lymphatics (arrowheads), as well as in neighboring blood vessels (arrows). (B) Jag1 is not detected in murine pathological lymphatics (arrowheads), but is detected in neighboring blood vessels (arrows). (C-D) Boxed areas in A and B; scale bars=100μm. Dll4 (C’) is enriched in lymphatic tip cells (C). Jag (D’) is not detected in lymphatic tip cells (D).
Figure 3. **N1ECDFc’s are detected in circulation after adenovirus administration.** Mice retro-orbitally administered adenoviruses (5x10⁸ ffu/mouse) expressing N1ECDFc’s (Notch1 decoys) or hFc (ctrl). 2 days after adenovirus delivery, blood was collected and 0.5μL serum was resolved by SDS-PAGE, then probed with an Fc antibody. (A) Representative Western blot showing relative circulating levels of hFc, N1ECDFc₁⁻¹₃ (Dll inhibitor) and N1ECDFc₁⁻²₄ (Dll and Jag inhibitor). (B) Representative Western blot showing relative circulating levels of hFc, N1ECDFc₁⁻¹₃ (Dll inhibitor) and N1ECDFc₁₀⁻²₄ (Jag inhibitor).
Figure 4. Dll/Notch blockade enhances pathological lymphangiogenesis. (A-D) Murine adult corneas were whole-mount stained after suture placement for lymphatic vasculature (LYVE1, red) and blood vasculature (CD31, green); scale bars=500μm. Corneas demarcated with white dotted line, lymphatic vessels demarcated with cyan dotted line, blood vessels demarcated with magenta dotted line, yellow asterisk denotes unsutured leaflet. N1ECDFc<sub>1-24</sub> (Dll and Jag inhibitor) treated corneas (B,B') and N1ECDFc<sub>1-13</sub> (Dll inhibitor) treated corneas (C,C') display increased area vascularized by lymphatic vessels and blood vessels compared to hFc (ctrl) corneas (A, A'). N1ECDFc<sub>10-24</sub> (Jag inhibitor) treated corneas (D,D') display no change in area vascularized by lymphatic vessels but decreased area vascularized by blood vessels. Quantification of vascularized area for N1ECDFc<sub>1-24</sub> (E), N1ECDFc<sub>1-13</sub> (F) and N1ECDFc<sub>10-24</sub> (G), as compared to ctrl. Error bars represent standard deviation of the mean, *p<0.05.
Figure 5. N1ECDFc1-24 treatment does not affect macrophage density in the cornea. (A-B) Murine adult corneas were whole-mount stained after suture placement (S) for lymphatic vasculature (LYVE1, red) and macrophages (F4/80, green); scale bars=200μm. There are no dramatic differences in F4/80+ macrophage content between ctrl corneas (A) and N1ECDFc1-24 (Dll and Jag inhibitor) treated corneas (B). (C) Quantification of F4/80+ macrophage density. Error bars represent standard deviation of the mean, n=5-6.
Figure 6. N1ECDFc_{1-24} treatment results in hypersprouting of pathological lymphatics. (A-B) Murine adult corneas were whole-mount stained after suture placement (S) for lymphatic vasculature (LYVE1, red); scale bars=200μm. Sprouts marked with arrowheads and branch points marked with asterisks. N1ECDFc_{1-24} (Dll and Jag inhibitor) treated corneas (B) display increased lymphatic sprouts and lymphatic branchpoints compared to ctrl corneas (A). (C) Quantification of lymphatic sprouts. (D) Quantification of lymphatic branch points. Error bars represent standard deviation of the mean, *p<0.05, n=20-27.
Figure 7. N1ECDFc_{1-13} treatment does not result in hypersprouting of pathological lymphatics. (A-B) Murine adult corneas were whole-mount stained after suture placement (S) for lymphatic vasculature (LYVE1, red); scale bars=200μm. Sprouts marked with arrowheads and branch points marked with asterisks. N1ECDFc_{1-13} (Dll inhibitor) treated corneas (B) display similar numbers of lymphatic sprouts and lymphatic branch points compared to ctrl corneas (A). (C) Quantification of lymphatic sprouts. (D) Quantification of lymphatic branch points. Error bars represent standard deviation of the mean, n=11-15.
Figure 8. N1ECDFc<sub>10-24</sub> treatment does not result in hypersprouting of pathological lymphatics. (A-B) Murine adult corneas were whole-mount stained after suture placement (S) for lymphatic vasculature (LYVE1, red); scale bars=200μm. Sprouts marked with arrowheads and branch points marked with asterisks. N1ECDFc<sub>10-24</sub> (Jag inhibitor) treated corneas (B) display similar numbers of lymphatic sprouts and lymphatic branch points compared to ctrl corneas (A). (C) Quantification of lymphatic sprouts. (D) Quantification of lymphatic branch points. Error bars represent standard deviation of the mean, n=6-7.
Chapter 5

Inhibition of Notch signaling does not alter growth or metastasis of an orthotopic model of human breast cancer
INTRODUCTION

The abilities of malignant tumors to induce angiogenesis and metastasize are two of the ten hallmarks of cancer that were first described by Hanahan and Weinberg\cite{105,106}. The concept of tumor angiogenesis was first suggested in 1971 by Judah Folkman, who postulated that tumors require blood vasculature to grow and to metastasize\cite{107}. Since this first description of tumor angiogenesis, research on this process and how it contributes to cancer progression has thrived. This has lead to the identification of key regulators of tumor angiogenesis, most importantly VEGF\cite{108}.

In addition to recruiting blood vessels, many tumors induce lymphangiogenesis\cite{18,109-112}. While it is known that tumor blood vasculature provides oxygen, nutrients, and a route for metastasis to distant organs, the role that the tumor lymphatic vasculature plays in cancer progression is debated\cite{18,109-112}. Tumor lymphatics provide a route for metastasis to the first lymph nodes that drain the primary tumor, also known as the sentinel lymph nodes\cite{18,109-112}. The incidence and clinical significance of sentinel lymph node metastasis range across different tumor types\cite{113}. Thus, whether sentinel lymph node metastasis is a necessary first step in a series of events that ultimately brings metastatic tumor cells to distant lymph nodes or other organs is a matter of intense debate\cite{18,109-112}. Another possibility is that lymphatic metastasis to sentinel lymph nodes occurs concurrently with hematogenous metastasis to distant organs. This would suggest that the presence of metastases in the sentinel lymph nodes is simply a prognostic indicator of a more aggressive and metastatic tumor\cite{18,109-112}.
In the case of breast cancer, up to 80% of tumors are estimated to first metastasize to sentinel lymph nodes before metastasizing to distant organs\[^{113}\]. A large body of literature correlates tumor VEGF-C expression levels with lymphatic vessel density (LVD) and lymph node metastasis in human breast cancer\[^{111,114-118}\]. Tumor VEGF-C expression, LVD, and lymph node metastasis also correlate with poor prognosis in breast cancer\[^{111,114-118}\]. Multiple tumor models in the mouse have demonstrated a role for VEGF-C in promoting tumor lymphangiogenesis and metastasis to sentinel lymph nodes as well as to distant organs\[^{18,109-112,119-123}\]. Furthermore, blockade of VEGFR-3 signaling or VEGFR-3 interaction with VEGF-C effectively inhibits tumor lymphangiogenesis and metastasis\[^{124-126}\].

Notch signaling has emerged as a target for anti-angiogenic therapy in cancer\[^{83,90-94,127}\]. In developing blood vasculature, genetic or pharmacological blockade of Dll4/Notch signaling consistently induces hypersprouting\[^{33,34,38,83}\]. Genetic and pharmacological blockade of Jag1/Notch signaling in developing blood vasculature has been shown to inhibit retinal angiogenesis\[^{34,83}\]. In agreement with the function of Dll4/Notch signaling in restricting angiogenic tip cell formation during developmental sprouting angiogenesis, Dll4-blockade also causes hypersprouting of tumor blood vasculature\[^{83,90-94}\]. Although Dll4-blockade causes tumor blood vessel hyperplasia, tumor burden is reduced\[^{83,90-94}\]. This is due to the nature of tumor neo-vessels, which are disorganized, not perfused, and thus, non-functional\[^{83,90-94}\]. Notably, Dll4-blockade with a Dll4 decoy (Dll4-Fc) was able to reduce tumor growth in HT1080-RM tumors, a line that is resistant to VEGF inhibition\[^{93}\]. This result is significant, as it suggests that Notch might be an attractive target for tumors refractory to other conventional angiogenic inhibitors. Of the Notch1 decoys developed by our group (Chapter 2, Figure 3), N1ECDFc1-13 (Dll
inhibitor) mimics Dll4-blockade by inducing tumor blood vessel hyperplasia and inhibiting subcutaneous growth of multiple mouse and human tumors in immunocompromised mice[83]. The Jag inhibitor N1ECDFc_{10-24} inhibits tumor angiogenesis and tumor growth[83]. N1ECDFc_{1-36} and N1ECDFc_{1-24}, both of which are pan-ligand inhibitors, inhibit tumor angiogenesis and tumor growth. This suggests that N1ECDFc_{1-36} and N1ECDFc_{1-24} predominantly block Jag/Notch signaling in the tumor models tested.

Given the success of Notch1 decoys in targeting tumor angiogenesis, preliminary studies were performed by our group to determine the effects of Notch1 decoys on tumor lymphangiogenesis. Immunohistochemical analysis of human breast carcinoma samples revealed that Notch1 and Notch4 are expressed by human tumor lymphatics[71]. Mice deficient for Notch4 (Notch4^{-/-}) displayed reduced Prox1^+ lymphatic vessel density (LVD) at the periphery of Colo-38 melanomas orthotopically implanted adjacent to the cecum (Yasuhiro Funahashi and Carrie Shawber, unpublished data). Immunocompromised mice treated with N1ECDFc_{1-36} displayed reduced LYVE1^+ LVD in orthotopically implanted SKNEP kidney tumors (Xing Wang and Carrie Shawber, unpublished data). Thus, our group demonstrated in two tumor models that genetic or pharmacological inhibition of pan-ligand Notch signaling inhibits tumor lymphangiogenesis.

Inhibition of Notch signaling caused lymphatic hypersprouting in the embryonic dermis (Chapter 3) and corneal suture assay (Chapter 4), consistent with a role for Dll4/Notch signaling in restricting lymphangiogenic tip cell formation. These two lymphatic vascular beds expressed Dll4, but not Jag1. The observation that Notch inhibition suppressed sprouting
lymphangiogenesis in Colo-38 and SKNEP tumors was intriguing, as it is not a classical Dll4/Notch inhibition phenotype. We postulated Jag/Notch signaling may possess pro-lymphangiogenic functions in tumors, and that Jag1 expressed by tumor lymphatic endothelial cells (LECs) or tumor cells could activate Notch in tumor lymphatic endothelial cells.

Several observations support our hypothesis that Notch signaling functions in tumor lymphangiogenesis. First, VEGFR-3/VEGF-C signaling has been identified as a critical mediator of tumor lymphangiogenesis and metastasis\(^{18, 109-112, 119-126}\), and we showed that Notch regulates VEGFR-3 in HDLECs (Chapter 3). Second, we showed that Notch signaling functions to suppress sprouting lymphangiogenesis in the embryonic murine dermis (Chapter 3) and the corneal suture assay (Chapter 4). Third, preliminary work suggests that Notch signaling promotes lymphangiogenesis (Yasuhiro Funahashi and Xing Wang, unpublished data). Together, this suggests that Notch1 decoy treatment would affect tumor lymphangiogenesis and metastasis, although it is difficult to predict precisely what the outcome would be. One could hypothesize that Dll4/Notch signaling suppresses tumor lymphangiogenesis, while Jag1/Notch signaling induces tumor lymphangiogenesis. Based on this hypothesis, one would predict that treatment with N1ECDFc\(_{1-13}\) (Dll inhibitor) would cause increased but non-productive tumor lymphangiogenesis and angiogenesis, inhibit tumor growth, and inhibit tumor metastasis. In contrast, treatment with N1ECDFc\(_{10-24}\) (Jag inhibitor) or N1ECDFc\(_{1-24}\) (Dll/Jag inhibitor) would inhibit tumor lymphangiogenesis and angiogenesis, inhibit tumor growth, and inhibit tumor metastasis.
**RESULTS**

**Lymphatics recruited by tumors express Notch proteins and ligands**

MDA-MB-231 is a human breast carcinoma cell line that is commonly used for tumor modeling in immunocompromised mice. MDA-MB-231 cells were stably transfected to express luciferase and be antibiotic resistant, then single cell clones were generated and expanded, followed by several rounds of *in vitro* and *in vivo* selection\[56\]. This ultimately resulted in the MDA-MB-231-luc-D3H2LN subline, which was isolated from a spontaneous lymph node metastasis of a primary tumor implanted in the mammary fat pad of an immunocompromised mouse\[56\]. D3H2LN cells express high levels of VEGF-C and VEGF-D (data not shown), and preferentially metastasize to axillary lymph nodes when implanted into immunocompromised mice\[56\]. These qualities, as well as their stable expression of luciferase, make D3H2LN cells a useful model for studying tumor lymphangiogenesis and metastasis to lymph nodes. For our experiments, we used either the MDA-MB-231 parental cell line (231) or the MDA-MB-231-luc-D3H2LN (D3H2LN) subline.

We first sought to establish which Notch proteins and Notch ligands are expressed in the tumor microenvironment of orthotopically grown 231 tumors. We performed immunohistochemistry on thin (7μm) sections of 231 tumors that had been orthotopically implanted into the mammary fat pads of female immunocompromised mice. Our immunohistochemical analysis demonstrated that Notch1 (Figure 1A, A”), Notch4 (Figure 1B, B”), and Dll4 (Figure 1C, C”) are expressed by tumor lymphatics (Figure 1A’, B’, C’) of 231 tumors grown orthotopically in the mammary fat pads of nude mice. Interestingly, Jag1 (Figure 1D, D”)
was expressed by tumor lymphatics (Figure 1D'). Neighboring tumor blood vessels expressed Notch1 (Figure 1A,A’), Dll4 (Figure 1C,C’), and Jag1 (Figure 1D,D’). As seen in both developmental vasculature (Chapter 3, Figure 3) and pathological vasculature (Chapter 4, Figure 2), expression of Notch components was consistently higher in blood vessels (Figure 1A”,B”,C”,D”) and lower in lymphatics (Figure 1A’,B’,C’,D’) of 231 tumors. Notch1 (Figure 1A”), Notch4 (Figure 1B”), Dll4 (Figure 1C”), and Jag1 (Figure 1D”) were also detected at varying levels of expression in other cells, which we postulate to be 231 tumor cells. In summary, lymphatic vessels of the embryonic dermis, the sutured cornea, and the orthotopically grown 231 tumor express Notch1, Notch4, and Dll4. In contrast, lymphatic vessels of the embryonic dermis and the sutured cornea do not express Jag1, whereas lymphatic vessels of the orthotopically grown 231 tumor express Jag1. Non-endothelial cell types in 231 orthotopic tumors also express Notch1, Notch4, Dll4, and Jag1.

**D3H2LN tumors express Notch proteins and ligands**

Multiple cell types, including tumor cells, express Notch signaling components, and alterations in Notch signaling or expression have been implicated in tumorigenesis of multiple hematological and solid tumors\[^{127, 128}\]. In human breast cancers, Notch1 and Jag1 have been implicated as oncogenic factors, while Notch2 has been implicated as a tumor suppressor\[^{128}\]. Our immunohistochemical analysis revealed expression of Notch1, Notch4, Dll4, and Jag1 by non-endothelial cells in 231 tumors (Figure 1). To determine whether these were 231 tumor cells, and to perform a more thorough analysis of Notches and ligands, we performed a PCR
survey, using cDNA isolated from cultured D3H2LN and 231 cells. Semi-quantitative PCR showed that D3H2LN cells (Figure 2) and 231 cells (data not shown) contain transcripts for \textit{NOTCH1, NOTCH2, NOTCH3, DLL4, JAG1, and JAG2}. Low levels of \textit{NOTCH4} transcripts were detected (Figure 2). D3H2LN cells (Figure 2) had the same expression profile as parental MDA-MB-231 cells (data not shown). These results suggested that 231 tumor cells could be partially responsible for non-endothelial expression of Notch1 (Figure 1A), Dll4 (Figure 1B), and Jag1 (Figure 1C). These results also suggested that the strong non-endothelial expression of Notch4 (Figure 1B) was not coming from 231 tumor cells, but from another cell type.

Given the oncogenic or tumor-suppressive roles of Notch in primary tumors, we tested whether inhibition of Notch signaling affects behavior of 231 cells. We lentivirally transduced 231 cells to express N1ECDFc1-36 (Chapter 2, Figure 3), which comprises EGF-like repeats 1-36 of human Notch1 and inhibits Notch signaling. We validated expression of N1ECDFc1-36 in 231 cells, and found that it did not alter proliferation, migration, or anchorage-independent growth of 231 cells in culture (data not shown). Given the identical Notch expression profiles of 231 cells and D3H2LN cells as determined by PCR (Figure 2), we hypothesized that Notch1 decoy (N1ECDFc) treatment would also not affect D3H2LN behavior in culture or D3H2LN cell growth \textit{in vivo}. Rather, we hypothesized that Notch1 decoys would affect tumor lymphangiogenesis and tumor metastasis.
**N1ECDFc\textsubscript{1-13} and N1ECDFc\textsubscript{10-24} do not affect D3H2LN tumor progression**

We tested ligand-specific Notch1 decoys in the D3H2LN tumor model to identify differences resulting from Dll blockade (N1ECDFc\textsubscript{1-13}) or Jag blockade (N1ECDFc\textsubscript{10-24}). 1.5x10\textsuperscript{6} D3H2LN cells were orthotopically implanted into the 4\textsuperscript{th} mammary fat pad of adult female immunocompromised mice. Tumors were allowed to establish in the mammary fat pad for 8 days before 5x10\textsuperscript{8} ffu of adenovirus/mouse was administered retro-orbitally. Adenoviruses expressing N1ECDFc\textsubscript{1-13} (Dll inhibitor), N1ECDFc\textsubscript{10-24} (Jag inhibitor), or control (ctrl, hFc) were administered on day 9 and day 21 of the tumor study. As early as 2 days post-delivery, Notch1 decoy variants and ctrl were easily detectable in 1\textmu L of serum (Figure 3A). Tumor progression was followed weekly using caliper measurements. Tumor luminescence was measured after intraperitoneal injection of luciferin using the IVIS imaging system. Luminescence imaging allowed us to monitor for axillary lymph node metastases *in vivo* throughout the study. Treatment with N1ECDFc\textsubscript{1-13} (Dll inhibitor) or N1ECDFc\textsubscript{10-24} (Jag inhibitor) did not affect tumor size as determined by caliper measurements (Figure 3B), tumor viability as determined by luminescence measurements (Figure 3C), or tumor weight at time of sacrifice (Figure 3D).

To determine if Notch1 decoys affect metastatic potential of D3H2LN tumors despite not affecting primary tumor growth, we monitored mice for axillary lymph node metastases *in vivo* throughout the study. We also screened for metastases in axillary lymph nodes and lungs at time of sacrifice. N1ECDFc\textsubscript{1-13} (Dll inhibitor) and N1ECDFc\textsubscript{10-24} (Jag inhibitor) had no effect on incidence of metastasis to axillary lymph nodes or lungs (Figure 4A). *Ex vivo* luminescence
measurements of excised axillary lymph nodes at time of sacrifice uncovered no significant differences in average metastatic burden (Figure 4B).

**N1ECDFc₁₋₁₃ and N1ECDFc₁₀₋₂₄ do not affect D3H2LN tumor or axillary lymph node microenvironments**

Although D3H2LN tumor progression and metastasis were not altered under the given conditions, we reasoned that other components of the tumor microenvironment, such as the vasculature, might be affected by Notch inhibition. Thus, we investigated whether Notch1 decoy treatment could induce changes in the tumor microenvironment.

To analyze the tumor lymphatic vasculature, we performed immunohistochemistry on 7μm thin tumor sections for LYVE1. Lymphatic vessels recruited by D3H2LN tumors were sporadic and mostly situated at the tumor periphery across all treatments (Figure 5A). No major changes in lymphatic vessel morphology or distribution were observed upon treatment with N1ECDFc₁₋₁₃ (Dll inhibitor) or N1ECDFc₁₀₋₂₄ (Jag inhibitor) (Figure 5A). Furthermore, quantification of lymphatic vessel density (LVD) did not uncover any differences upon treatment with N1ECDFc₁₋₁₃ (Dll inhibitor) or N1ECDFc₁₀₋₂₄ (Jag inhibitor) (Figure 5B).

Lymph nodes are known to prime themselves to provide a favorable metastatic niche by inducing lymphangiogenesis, sometimes even prior to tumor arrival\textsuperscript{[119, 129]}. To test whether Notch1 decoy treatment could induce changes in lymph node LVD, we performed immunohistochemistry on 7μm thin sections of axillary lymph nodes for LYVE1. Dense
lymphatic vasculature was found in axillary lymph nodes of mice from all treatments (Figure 6A). Neither N1ECDFc1-13 (Dll inhibitor) treatment nor N1ECDFc10-24 (Jag inhibitor) treatment had any effect on lymphatic vessel density or distribution in axillary lymph nodes (Figure 6A,B).

To analyze the tumor blood vasculature, we performed immunohistochemistry on 7μm thin tumor sections for CD31. D3H2LN tumors were densely populated with blood vessels throughout the tumor across all treatments (Figure 7A). We observed no major changes in blood vessel morphology or distribution in D3H2LN tumors upon treatment with N1ECDFc1-13 (Dll inhibitor) or N1ECDFc10-24 (Jag inhibitor) (Figure 7A). Furthermore, quantification of blood vessel density (BVD) did not uncover any differences upon treatment with N1ECDFc1-13 (Dll inhibitor) or N1ECDFc10-24 (Jag inhibitor) (Figure 7B).

Tumor-associated macrophages polarized to the M2 phenotype can play a pro-tumor role by facilitating tumor angiogenesis, lymphangiogenesis, growth, and metastasis\(^{[15]}\). To analyze macrophage content in D3H2LN tumors, we performed immunohistochemistry on 7μm thin tumor sections for F4/80. D3H2LN tumors recruited a large number of F4/80\(^+\) macrophages (Figure 8A). We observed no major changes in macrophage distribution in D3H2LN tumors upon treatment with N1ECDFc1-13 (Dll inhibitor) or N1ECDFc10-24 (Jag inhibitor) (Figure 8A). Quantification of macrophage density did not uncover any differences upon treatment with N1ECDFc1-13 (Dll inhibitor) or N1ECDFc10-24 (Jag inhibitor) (Figure 8B).

In summary, treatment of D3H2LN tumors with adenoviruses expressing ligand-specific Notch1 decoys N1ECDFc1-13 (Dll inhibitor) or N1ECDFc10-24 (Jag inhibitor) did not affect tumor growth, metastasis, lymphangiogenesis, angiogenesis, or macrophage recruitment.
**N1ECDFc_{1-24} does not affect D3H2LN tumor progression**

In both developmental and pathological settings, pan-ligand inhibitor N1ECDFc_{1-24} has at times demonstrated increased potency over either ligand-specific inhibitor alone, despite being present in the circulation at lower levels\(^{[83]}\). Therefore, we tested whether N1ECDFc_{1-24} (Dll/Jag inhibitor) treatment affects D3H2LN tumor progression although treatment with N1ECDFc_{1-13} (Dll inhibitor) or N1ECDFc_{10-24} (Jag inhibitor) did not. In addition, we modified several experimental parameters, implanting fewer cells and starting treatment concurrently with tumor implantation. Implantation of 1.5x10^6 cells in the previous experiment resulted in tumors reaching a size of 700mm^3 in 4 weeks. This was a much faster growth rate than what was previously reported, in which 2x10^6 cells were implanted and reached a size of 700mm^3 in 5 weeks\(^{[56]}\). Thus, we reduced the number of cells implanted to 5x10^5 cells. In the previous experiment, we allowed for tumors to establish in the mammary fat pad before starting treatment. Here we treated tumors concurrently with tumor implantation, and again on day 21.

We tested N1ECDFc_{1-24} (Dll/Jag inhibitor) in the D3H2LN tumor model to determine if it could elicit changes in tumor progression. 5x10^5 D3H2LN cells were orthotopically implanted into the 4\(^{th}\) mammary fat pad of adult female immunocompromised mice. Adenoviruses expressing N1ECDFc_{1-24} (Dll/Jag inhibitor) or hFc (ctrl) were administered retro-orbitally at 5x10^6 ffu of adenovirus/mouse on day 0 and day 21 of the tumor study. 3 days post-delivery, N1ECDFc_{1-24} and hFc were easily detectable in 1\(\mu\)L of serum (Figure 9A). Tumor progression was followed weekly using caliper measurements. Tumor luminescence was measured after intraperitoneal injection of luciferin using the IVIS imaging system. Luminescence imaging
allowed us to monitor for axillary lymph node metastases in vivo throughout the study.

Treatment with N1ECDFc1-24 (Dll/Jag inhibitor) did not affect tumor size as determined by caliper measurements (Figure 9B), tumor viability as determined by luminescence measurements (Figure 9C), or tumor weight at time of sacrifice (Figure 9D).

To determine if N1ECDFc1-24 affects metastatic potential of D3H2LN tumors despite not affecting primary tumor growth, we monitored mice for axillary lymph node metastases in vivo throughout the study. We also screened for metastases in axillary lymph nodes and lungs at time of sacrifice. N1ECDFc1-24 (Dll/Jag inhibitor) had no effect on incidence of metastasis to axillary lymph nodes or lungs (Figure 10A). Ex vivo luminescence measurements of excised axillary lymph nodes at the time of sacrifice uncovered no significant differences in average metastatic burden (Figure 10B).

Our results show that inhibition of Dll/Notch signaling (N1ECDFc1-13), inhibition of Jag/Notch signaling (N1ECDFc10-24), or pan-ligand inhibition (N1ECDFc1-24) does not affect D3H2LN primary tumor progression in this setting. Subsequent analysis will determine whether N1ECDFc1-24 (Dll/Jag inhibitor) treatment can induce changes in the tumor microenvironment. However, results upon N1ECDFc1-13 (Dll inhibitor) treatment and N1ECDFc10-24 (Jag inhibitor) treatment, as well as the lack of change in tumor growth and metastasis with N1ECDFc1-24 (Dll/Jag inhibitor) treatment, suggest that N1ECDFc1-24 will not induce changes in tumor lymphangiogenesis, tumor angiogenesis, or axillary lymph node lymphangiogenesis in the D3H2LN orthotopic model.
DISCUSSION

We report that D3H2LN human mammary carcinoma cells orthotopically implanted into the 4th mammary fat pad of immunocompromised mice recruit lymphatic vessels and metastasize preferentially to the axillary lymph nodes. The MDA-MB-231 tumor microenvironment expressed high levels of Notch components. Given that multiple cell types express Notch family proteins in the D3H2LN tumor microenvironment, Notch signaling could theoretically occur between tumor cells, between tumor lymphatic endothelial cells (LECs), and heterotypically between tumor cells and tumor LECs. MDA-MB-231 tumor lymphatics expressed Notch components. We have identified a difference in 231 tumor lymphatics compared to developmental dermal lymphatics (Chapter 3) and pathological corneal suture lymphatics (Chapter 4) in our observation that 231 tumor lymphatics express Jag1. This suggests that Jag1 may play a specialized role in 231 tumor lymphatics, if not all tumor lymphatics. We suggest several possibilities that may explain why Jag1 is expressed in 231 tumor lymphatics: 1) Notch expression by 231 tumor cells may induce Jag1 expression in tumor lymphatics; 2) 231 tumors may secrete factors that induce Jag1 expression in lymphatics; 3) 231 tumor lymphatics may be mis-specified and thus possess characteristics of blood vessels, including Jag1 expression.

Previous work from our group found that inhibition of Notch signaling suppressed tumor lymphangiogenesis in the Colo-38 model (Yasuhiro Funahashi and Carrie Shawber, unpublished data) and SKNEP model (Xing Wang and Carrie Shawber, unpublished data). We speculate that reduced tumor lymphangiogenesis is a result of reduced Jag1/Notch signaling. Jag1/Notch
signaling may occur between tumor cells and tumor LECs, or among tumor LECs, to induce tumor lymphangiogenesis. It will be necessary to determine whether tumor lymphatics and/or tumor cells in the Colo-38 and SKNEP models express Jag1 to support this hypothesis. More broadly, determining the Notch and ligand expression profiles of various tumors may help us identify the contributions from the tumor cell component, tumor LEC component, and other cell components that activate Notch signaling within the tumor microenvironments. This information may be useful in predicting if a tumor will respond to Notch inhibition, and how it will respond.

Adenovirus-mediated Notch1 decoy treatment had no effect on primary D3H2LN tumors or their microenvironments. We conclude that Notch1 decoys as used in these studies do not affect growth, metastasis, or microenvironment of D3H2LN tumors. But given the strong evidence from our work (Chapters 3 and 4) and others[83, 90-94, 127] (Yasuhiro Funahashi, Xing Wang, and Carrie Shawber, unpublished data) implicating Notch signaling in regulating lymphatic and blood vasculature, we believe the results in the D3H2LN model are an exception. Several possibilities are listed here that may explain why adenovirus-mediated Notch1 decoy treatment did not affect D3H2LN tumor progression, and are described in further detail below:

1) The mammary fat pad microenvironment may provide advantages to tumors that allow them to overcome Notch inhibition; 2) Orthotopic implantation in general may provide advantages over subcutaneous implantation that allows tumors to overcome Notch inhibition; 3) Adenoviral delivery of Notch1 decoys may not achieve the necessary serum levels to affect tumor lymphangiogenesis; 4) Notch1 decoy treatment may need to be used as combination
therapy with another lymphangiogenic inhibitor; 5) D3H2LN tumors may be refractory to Notch inhibition.

The mammary fat pad microenvironment may provide advantages to tumors that allow them to overcome Notch inhibition (#1). Orthotopic implantation in general may provide advantages over subcutaneous implantation that allows tumors to overcome Notch inhibition (#2). The tumor microenvironment undeniably plays a role in tumor progression by providing important cues to tumors. Interaction between tumor cells and other accessory cells (e.g., endothelial cells, immune cells, and fibroblasts, to name a few) provides structural support and can modulate tumor behavior. Cytokines and growth factors secreted by tumor cells or accessory cells contribute to tumor progression, and the extracellular matrix also contains pro and anti-tumor factors that can regulate tumor progression. The majority of the tumor experiments described above using Notch inhibitors were xenografted subcutaneously, with the exception of the Colo-38 and SKNEP tumor models, which were orthotopically implanted. In our studies, however, D3H2LN tumors were orthotopically implanted into the mammary fat pad. One can speculate that the microenvironment in the subcutaneous region differs in multiple ways from the mammary fat pad, and from other microenvironments in the mouse. Experiments comparing D3H2LN implantation in the subcutaneous layer to implantation in the mammary fat pad could elucidate key differences between the two microenvironments that benefit or inhibit D3H2LN tumor progression.
Adenoviral delivery of Notch1 decoys may not achieve the necessary serum levels to affect tumor lymphangiogenesis (#3), D3H2LN tumors may be refractory to Notch inhibition (#5). The Notch1 decoys could be ineffective in the D3H2LN tumor model for reasons specific to how they behave in this model. One possibility is the bioavailability of the Notch1 decoys upon adenovirus injection. While we are able to readily detect circulating Notch1 decoys after adenovirus injection using Western blot, we have yet to quantify these levels. Studies to understand the pharmacokinetics of Notch1 decoys in the mouse system (e.g., absorption, distribution, metabolism, excretion) have not yet been performed. Median effective doses (ED$_{50}$), half maximal inhibitory concentrations (IC$_{50}$), dose limiting toxicities, and other characteristics have not yet been determined. To that end, we believe that the future of Notch1 decoys lies in utilizing them as purified proteins and identifying these characteristics to optimize dosing conditions. It is also possible that D3H2LN tumors are refractory to Notch inhibition treatment. Alternative methods of Notch inhibition, genetic or pharmacological, may help us identify whether D3H2LN growth in the mammary fat pad is unresponsive to Notch inhibition in general, or specifically unresponsive to Notch1 decoy treatment. Determining the benefits and limitations of the Notch1 decoys will be instrumental in bringing them into the clinic in the future.
Chapter 5

Figures
Figure 1. Tumor lymphatic vasculature expresses Notch1, Notch4, Dll4, and Jag1. (A-D) MDA-MB-231 tumors orthotopically implanted into the mammary fat pads of immunocompromised mice were stained for lymphatic vasculature (LYVE1, green) and Notch components (red), nuclei are stained with DAPI (blue); scale bars=20μm. Lymphatic vessels (A',B',C',D') recruited by MDA-MB-231 tumors express Notch1 (A''), Notch4 (B''), Dll4 (C''), and Jag1 (D''). (A-D) Higher expression of Notch components is observed in blood vessels (arrows) than in lymphatics (arrowheads), and expression of Notch components is also detected in non-endothelial cells (asterisks).
Figure 2. D3H2LN tumor cells express Notches and ligands. Semi-quantitative PCR (35 cycles) was performed using 1μL cDNA from cultured D3H2LN cells, which are a subline of the MDA-MB-231 parental human breast carcinoma tumor cell line. D3H2LN tumor cells express Notch1, Notch2, Notch3, Dll4, Jag1, and Jag2.
Figure 3. Neither N1ECDFc_{1-13} nor N1ECDFc_{10-24} affect D3H2LN tumor growth, viability, or weight.

Mice were implanted with 1.5x10^6 D3H2LN tumor cells and retro-orbitally administered adenovirus (5x10^6 ffu/mouse). (A) Western blot showing relative circulating levels of hFc (ctrl), N1ECDFc_{1-13} (Dll inhibitor) and N1ECDFc_{10-24} (Jag inhibitor) in 1μL serum, 2 days after adenovirus delivery. (B) N1ECDFc_{1-13} (Dll inhibitor) and N1ECDFc_{10-24} (Jag inhibitor) treatments do not affect tumor volume. Adenovirus was administered at days 8 and 21 (red squares). (C) N1ECDFc_{1-13} (Dll inhibitor) and N1ECDFc_{10-24} (Jag inhibitor) treatments do not affect tumor luminescence. Adenovirus was administered at days 8 and 21 (red squares). (D) N1ECDFc_{1-13} (Dll inhibitor) and N1ECDFc_{10-24} (Jag inhibitor) treatments do not affect tumor weights at sacrifice. Error bars represent standard deviation of the mean, n=8.
Figure 4. Neither N1ECDFc1-13 nor N1ECDFc10-24 affect D3H2LN tumor metastasis. Mice were implanted with 1.5x10⁶ D3H2LN tumor cells and retro-orbitally administered adenovirus (5x10⁸ ffu/mouse). (A) N1ECDFc1-13 (Dll inhibitor) and N1ECDFc10-24 (Jag inhibitor) treatments do not affect tumor rate of metastasis to axillary lymph nodes (LN) and lungs. (B) N1ECDFc1-13 (Dll inhibitor) and N1ECDFc10-24 (Jag inhibitor) treatments do not affect metastatic burden in axillary lymph nodes. Error bars represent standard deviation of the mean, n=8.
Figure 5. Neither N1ECDFc1-13 nor N1ECDFc10-24 affect D3H2LN tumor lymphatic vessel density. Mice were implanted with 1.5x10^6 D3H2LN tumor cells and retro-orbitally administered adenovirus (5x10^8 ffu/mouse). (A) Tumors were stained for lymphatic vasculature (LYVE1, red), nuclei are stained with DAPI (blue); scale bars=200μm. (B) N1ECDFc_{1-13} (Dll inhibitor) and N1ECDFc_{10-24} (Jag inhibitor) treatments do not affect tumor lymphatic vessel density (LVD). Error bars represent standard deviation of the mean, n=8.
Figure 6. Neither N1ECDFc1-13 nor N1ECDFc10-24 affect axillary lymph node lymphatic vessel density. Mice were implanted with 1.5x10^6 D3H2LN tumor cells and retro-orbitally administered adenovirus (5x10^8 ffu/mouse). (A) Axillary lymph nodes were stained for lymphatic vasculature (LYVE1, red), nuclei are stained with DAPI (blue). (B) N1ECDFc1-13 (Dll inhibitor) and N1ECDFc10-24 (Jag inhibitor) treatments do not affect lymphatic vessel density (LVD) in axillary lymph nodes. Error bars represent standard deviation of the mean, n=8.
Figure 7. Neither N1ECDFc1-13 nor N1ECDFc10-24 affect D3H2LN tumor blood vessel density. Mice were implanted with 1.5x10^6 D3H2LN tumor cells and retro-orbitally administered adenovirus (5x10^8 ffu/mouse). (A) Tumors were stained for blood vasculature (CD31, brown), nuclei are counterstained with hematoxylin (blue); scale bars=50μm. (B) N1ECDFc1-13 (Dll inhibitor) and N1ECDFc10-24 (Jag inhibitor) treatments do not affect tumor blood vessel density (BVD). Error bars represent standard deviation of the mean, n=8.
Figure 8. Neither N1ECDFc<sub>1-13</sub> nor N1ECDFc<sub>10-24</sub> affect D3H2LN tumor macrophage density. Mice were implanted with 1.5x10<sup>6</sup> D3H2LN tumor cells and retro-orbitally administered adenovirus (5x10<sup>8</sup> ffu/mouse). (A) Tumors were stained for macrophages (F4/80), nuclei are stained with DAPI (blue); scale bars=20μm. (B) N1ECDFc<sub>1-13</sub> (Dll inhibitor) and N1ECDFc<sub>10-24</sub> (Jag inhibitor) treatments do not affect tumor macrophage density. Error bars represent standard deviation of the mean, n=8.
Figure 9. N1ECDFc1-24 does not affect D3H2LN tumor growth, viability, or weight. Mice were implanted with 5x10^5 D3H2LN tumor cells and retro-orbitally administered adenovirus (5x10^8 ffu/mouse). (A) Western blot showing relative circulating levels of hFc (ctrl) and N1ECDFc1-24 (Dll and Jag inhibitor) in 1μL serum, 3 days after adenovirus delivery. (B) N1ECDFc1-24 (Dll and Jag inhibitor) treatment does not affect tumor volume. Adenovirus was administered at days 0 and 19 (red squares). (C) N1ECDFc1-24 (Dll and Jag inhibitor) treatment does not affect tumor luminescence. Adenovirus was administered at days 0 and 19 (red squares). (D) N1ECDFc1-24 (Dll and Jag inhibitor) treatment does not affect tumor weights at sacrifice. Error bars represent standard deviation of the mean, n=5-10.
Figure 10. **N1ECDFc1-24 does not affect D3H2LN tumor metastasis.** Mice were implanted with $5 \times 10^5$ D3H2LN tumor cells and retro-orbitally administered adenovirus ($5 \times 10^8$ ffu/mouse). (A) N1ECDFc1-24 (Dll and Jag inhibitor) treatment does not affect tumor rate of metastasis to axillary lymph nodes (LN) and lungs. (B) N1ECDFc1-24 (Dll and Jag inhibitor) treatment does not affect metastatic burden in axillary lymph nodes. Error bars represent standard deviation of the mean, n=5-6.
Chapter 6

Notch signaling suppresses lymphatic specification and separation but induces lymphatic valve formation by regulating lymphatic endothelial cell fate decisions
INTRODUCTION

The field of lymphatic vascular biology has benefited greatly from the identification of genes that are specific to or highly expressed by lymphatic endothelium. The proteins encoded by these genes regulate different stages of lymphatic development, including lymphatic specification and separation of blood and lymphatic vasculature, sprouting lymphangiogenesis, and collecting duct valve formation\[^{10,11,41}\]. In addition to understanding the molecular functions and expression patterns of these lymphatic regulatory genes, it is important to understand how they are transcriptionally regulated. Several of the key players are discussed in this chapter, and a more comprehensive list of the genes required for lymphatic vascular development and function is provided in Chapter 1, Table 1.

Prox1 is the master regulator of lymphatic endothelial cell (LEC) fate\[^{7,72,130-132}\]. Mice nullizygous for Prox1 die between E14.5-15 with no lymphatic specification from the cardinal vein\[^7\]. Ectopic Prox1 expression in the blood endothelium is sufficient to induce a lymphatic endothelial phenotype, while deletion of Prox1 reverts lymphatic endothelium to a blood endothelial phenotype\[^{130-132}\]. Another important player in the separation of the lymphatic vasculature from the blood vasculature is podoplanin (Pdpn), a transmembrane glycoprotein\[^{44}\]. LEC’s express podoplanin to induce platelet aggregation, which is necessary for proper separation of the lymph sacs from the cardinal vein\[^{44}\]. Loss of podoplanin results in defective separation of lymphatic and blood vasculature and thus, blood-filled lymphatics\[^{44}\]. Once the lymph sacs have separated off of the cardinal vein, they go on to form the lymphatic plexus through developmental sprouting lymphangiogenesis (covered in Chapter 3).
After lymphatic specification/separation and sprouting lymphangiogenesis occurs, the primary lymphatic plexus is further remodeled to develop the complete lymphatic vascular system of small capillaries, intermediate pre-collecting ducts, and large collecting vessels. One of the defining features that distinguish lymphatic collecting vessels from lymphatic capillaries is the presence of intraluminal valves, which serve to move lymph in a unidirectional manner\cite{9-12}. In mice, lymphatic valve formation initiates at E15.5 with the clustering of LECs expressing high levels of Prox1 and FoxC2 at future valve sites, followed by local deposition of the extracellular matrix (ECM) proteins Lamininα5 and Fibronectin-EIIIA (FN-EIIIA)\cite{49}. The pre-mRNA of Fibronectin (FN) has three potential sites for alternative splicing (EIIIA, EIIIB, and IIICS) that produce several splice variants\cite{133}. FN-EIIIA, which contains the EIIIA domain, is a ligand of the matrix adhesion protein Integrinα9, which is highly expressed by lymphatic valve endothelial cells as they reorient themselves within the collecting duct and migrate into the lumen to form the lymphatic valves\cite{133}. A properly formed lymphatic valve has two leaflets, each of which consists of two layers of Integrinα9+ LECs tightly associated with an ECM core containing Lamininα5 and FN-EIIIA\cite{49}. Gap junction protein Connexin37 (Cx37), which is first detected in the jugular lymph sacs at E13.5, gradually becomes upregulated in lymphatic collecting ducts and can be detected in lymphatic collecting duct valves at E18.5\cite{47}. Mice lacking any of the aforementioned genes display defective lymphatic valve formation\cite{47,49}.

Prox1 and Pdpn are restricted to the LEC progenitor cell population during lymphatic specification and separation. Meanwhile, Prox1, FoxC2, Lamininα5, FN-EIIIA, Integrinα9, and Cx37 are restricted to or highly expressed by the valve LEC population during lymphatic valve formation. The specific induction of these genes in discrete cell populations suggests that both
lymphatic specification and separation, as well as lymphatic valve formation, require cell fate decisions. During lymphatic specification and separation, LECs need to be defined from the blood endothelium, and during lymphatic valve formation, valve lymphatic endothelial cells need to be defined from the lymphatic collecting duct endothelium. Notch is required for cell-fate decisions in multiple settings, including in the blood vasculature\textsuperscript{[24-26]}. Notch also plays an important role in heart valve development\textsuperscript{[134, 135]}. Furthermore, our work suggests a role for Notch signaling in defining tip and stalk cell fates during sprouting lymphangiogenesis (Chapters 3 and 4). Given the importance of cell-fate determination in the development of the lymphatic vasculature, and given the importance of Notch in cell-fate decisions, we hypothesized that Notch regulates lymphatic specification/separation from the blood vasculature and lymphatic valve formation. We postulated Notch regulates these processes by transcriptional regulation of lymphatic genes important for these processes.

RESULTS

Notch regulates genes critical for development of the lymphatic vasculature

Lymphatic specification from the venous vasculature initiates with polarized expression of the transcription factor Prox1 between E9.5-10. Expression analysis in the developing embryo from our group detected Notch1 in the cardinal vein at E9.75, also with polarized expression but in a pattern opposing that of Prox1\textsuperscript{[79]}. The polarized and opposing expression patterns of Notch1 and Prox1 suggested that Notch1 and Prox1 might possess opposing functions in lymphatic specification. We hypothesized that Notch1 suppresses Prox1 in a subset
of ECs in the cardinal vein so that a different subset of ECs lacking Notch can commit to the LEC fate. To test this hypothesis, we overexpressed activated forms of Notch (N1IC or N4/int3, Chapter 2, Figure 2), HEY overexpression constructs, or the Notch1 decoy inhibitor (N1ECDFc1-36, Chapter 2, Figure 3), using lentivirus-mediated stable transduction of cultured HDLECs. The N1IC lentiviral plasmid, which expresses the intracellular domain of Notch1, and the N4/int3 lentiviral plasmid, which expresses the transmembrane and intracellular domains of Notch4, are both constitutively active. Thus they will be referred to from hereon as “Notch activation”. The N1ECDFc1-36 lentiviral plasmid, which expresses all 36 EGF-like repeats in the extracellular domain of human Notch1 fused to human Fc, will be referred to from hereon as “Notch inhibition”.

We first confirmed that our Notch activation plasmids and HEY overexpression constructs resulted in increased expression of HEY1 and HEY2 (Figure 1A). We also validated that our Notch1 decoy inhibition plasmid represses these effectors in cultured HDLECs (Figure 1B). Next, we used qRT-PCR to determine if Notch has transcriptional control over genes that are important in lymphatic specification. Indeed, activation of Notch1 or Notch4 strongly repressed PROX1 transcripts in HDLECs (Figure 1C)[79]. Overexpression of HEY1 or HEY2 was also able to repress PROX1 transcripts, but not as dramatically as Notch1 or Notch4 (Figure 1C)[79]. This suggested that while downstream effectors HEY1 and HEY2 mediate PROX1 repression upon Notch activation, there might be other effectors involved in repressing PROX1 after Notch activation. We then asked if Notch inhibition results in an increase in PROX1 transcripts. qRT-PCR analysis determined that Notch inhibition in cultured HDLECs does not alter PROX1 transcript levels (data not shown).
Once the lymph sacs have migrated out from the cardinal vein during lymphatic specification, podoplanin is required to keep the blood and lymphatic vessels separate\[^{44}\]. We showed that Notch can suppress Prox1 transcripts, and we hypothesized that Notch may also suppress podoplanin during separation of the lymphatic vasculature from the cardinal vein. To test this hypothesis, we activated or inhibited Notch using the aforementioned lentiviral plasmids (Chapter 2, Figure 2) in cultured HDLECs. Indeed, Notch activation strongly repressed PDPN (podoplanin) transcripts in HDLECs (Figure 6C)\[^{79}\]. Overexpression of HEY1 or HEY2 did not repress PDPN transcripts (Figure 6C)\[^{79}\]. This suggests that PDPN repression upon Notch activation involves alternate effectors instead of or in addition to HEY1 and HEY2. One candidate is Prox1, which has been shown to induce expression of podoplanin in blood endothelial cells (BECs)\[^{136}\]. It is plausible that repression of PDPN transcripts upon Notch activation is secondary to repression of PROX1 transcripts. As such, HEY1 and HEY2 overexpression may be unable to repress PDPN transcripts because they do not repress PROX1 as effectively as does Notch activation. We then asked if Notch inhibition results in increased expression of podoplanin. qRT-PCR analysis determined that Notch inhibition in HDLECs does not alter PDPN transcript levels (data not shown).

**Notch regulates genes critical for maturation of the lymphatic vasculature**

A fully developed lymphatic valve has two leaflets, each consisting of a Lamininα5/FN-EIIIA ECM core surrounded by LECs expressing Prox1, Integrinα9, Connexin37, and FoxC2, and mice lacking the genes for any of these markers exhibit defects in lymphatic valve formation\[^{47,129}\].
Work from our group found that Notch1 is highly expressed in lymphatic valves at E17.5 (Murto{	extasciitilde}maki et al., submitted). Furthermore, a transgenic Notch reporter mouse revealed that Notch signaling was concentrated in lymphatic valve ECs at the same time point (Murto{	extasciitilde}maki et al., submitted).

Given the role we determined for Notch in regulating PROX1 and PDGN, as well as our observations in lymphatic collecting ducts during valve formation, we hypothesized that Notch functions to regulate genes critical for lymphatic valve formation. To test this hypothesis, we activated or inhibited Notch using the aforementioned lentiviral plasmids (Chapter 2, Figure 2) in cultured HDLECs and determined expression of Fibronectin (FN), FN-EIIIA, Connexin37, and Integrinα9.

Notch1 activation induced expression of valve ECM protein FN-EIIIA (Figure 2A). Notch4 activation, though to a lesser degree, also induced expression of FN-EIIIA (Figure 2A). We also observed increased expression of total FN (Figure 2A). Because the total FN antibody recognizes EIIIA-containing as well as EIIIA-lacking FN, our expression analysis could not determine whether increased expression of total FN was solely due to increase in FN-EIIIA, or other FN splice variants. Expression of gap junction protein Connexin37 was also induced by Notch1 activation (Figure 2B). However, we did not observe changes in Connexin37 expression upon Notch4 activation (Figure 2B). Cell surface expression of Integrinα9, a binding partner of FN-EIIIA, was induced upon Notch1 activation (Figure 2C). However, we did not observe changes in cell surface expression of Integrinα9 upon Notch4 activation (Figure 2C). Based on our protein
expression analysis, we concluded that Notch1, and possibly Notch4, induces expression of proteins essential for proper lymphatic valve morphogenesis.

To determine whether Notch induces valve markers by transcriptional regulation, we performed qRT-PCR using primer sets that recognize EIIIA-containing (FN-EIIIA⁺) and EIIIA-lacking (FN-EIIIA⁻) FN transcripts (Figure 3, Murtomaki et al., submitted). We found that Notch1 activation induced both transcripts (Figure 3A). Notch4 activation also induced both transcripts, though to a lesser degree (Figure 3A). Thus, we concluded that Notch induces transcription of FN, which leads to increased levels of both EIIIA-containing (FN-EIIIA⁺) and EIIIA-lacking (FN-EIIIA⁻) splice variants. Overexpression of Notch effectors HEY1 or HEY2 did not give the same response (Figure 3A). Rather, HEY1 repressed FN-EIIIA⁺ and FN-EIIIA⁻ transcripts, while HEY2 repressed FN-EIIIA⁻ transcripts (Figure 3A). This suggests that FN is a direct target of NICD/CSL transcriptional activation. Alternatively, FN induction upon Notch activation could involve alternate effectors instead of or in addition to HEY1 and HEY2.

Notch1 activation induced CX37 transcripts (Figure 3B). Although we did not detect increased protein expression of Connexin37 upon Notch4 activation (Figure 2B), Notch4 activation induced CX37 transcripts, though to a lesser degree than did Notch1 (Figure 3B). Overexpression of Notch effectors HEY1 or HEY2 repressed CX37 transcripts (Figure 3B). This suggests that CX37 is a direct target of Notch. Alternatively, its induction upon Notch activation could involve alternate effectors instead of or in addition to HEY1 and HEY2.

Notch1 activation induced ITGA9 transcripts (Figure 3C). Although we did not detect increased protein expression of Integrinα9 upon Notch4 activation (Figure 2C), Notch4
activation induced ITGA9 transcripts, though to a lesser degree than did Notch1 (Figure 3C). Overexpression of Notch effectors HEY1 or HEY2 did not significantly alter ITGA9 transcripts (Figure 3C). This suggests that ITGA9 is a direct target of NICD/CSL transcriptional activation. Alternatively, its induction upon Notch activation could involve alternate effectors.

Next, we asked if Notch inhibition results in decreased expression of FN, FN-EIIIA, Connexin37, or Integrinα9. Notch inhibition in HDLECs did not affect transcript or protein levels of these valve components (data not shown). As HDLECs are isolated from lymphatic capillaries, they have very low endogenous expression of proteins involved in lymphatic valve formation. Thus, it is not surprising that Notch inhibition could not further reduce expression of these proteins in HDLECs.

**DISCUSSION**

We have demonstrated a role for Notch in transcriptionally suppressing expression of Prox1 and Pdpn during lymphatic specification/separation from the venous vasculature. We showed that activation of Notch1 or Notch4 in HDLECs suppresses both PROX1 and PDPN transcripts. These results agree with *in vivo* observations made by our group, in which ectopic Notch1 activation in Prox1+ LEC progenitors (*Prox1CreER<sup>T2</sup>;N1IC*) at E9.75 resulted in death by E15.5 along with defects in lymphatic vascular formation, blood-filled lymphatics, and edema<sup>[79]</sup>. This also correlated with reduced expression of Prox1<sup>[79]</sup>. Work from our group also found that ectopic Notch1 activation in Prox1+ LEC progenitors (*Prox1CreER<sup>T2</sup>;N1IC*) at E9.75 correlated
with reduced expression of podoplanin\textsuperscript{[79]}. Taken together, these results suggest a role for Notch1 as a negative regulator of lymphatic specification.

Previous studies reported that Prox1 expression was unchanged at E10 upon endothelial-specific removal of the RBP-Jk/CSL component of the Notch transcriptional complex (\textit{Tie2-Cre; Rbpj}^{f/f})\textsuperscript{[6]}. The number of Prox1\textsuperscript{+} cells in the cardinal vein was also unchanged at the same time point\textsuperscript{[6]}. The authors concluded that Notch is not important for Prox1 expression in the cardinal vein. However, several weaknesses should be pointed out regarding this analysis. First, analysis of the \textit{Tie2-Cre; Rbpj}^{f/f} genotype could only be performed in a small number of embryos due to the fact that this genotype was embryonic lethal in most embryos by E10. The time point of lethality and the loss of arterial markers suggested that arterial/venous defects were the cause of death. As such, it would be difficult to determine the contribution of Notch to lymphatic specification without first taking into account the effects on the blood vasculature. Furthermore, RBP-Jk/CSL functions as a transcriptional repressor in the absence of activated Notch\textsuperscript{[25, 26]}. Thus, deletion of RBP-Jk/CSL not only prevents Notch signaling, but also interferes with the intrinsic transcriptional repressor functions of RBP-Jk/CSL independent of Notch. It is for these reasons that our group used tamoxifen inducible drivers and specifically targeted Notch1 or DN-MAML, neither of which alters RBP-Jk/CSL’s repressor functions.

Although the specific contribution of Notch4 in these processes was not analyzed \textit{in vivo}, our transcript analysis in HDLECs suggests that Notch4 may also play a role in suppressing Prox1 and podoplanin \textit{in vivo}. Furthermore, work from our group determined that Jag1 expression overlapped with Notch1 expression in LEC progenitor cells within the cardinal vein. It would be
interesting to overexpress or knock-down of Jag1 or Dll4 in HDLECs to determine which of these two ligands function to suppress PROX1 transcripts. If Jag1 overexpression could suppress PROX1 in HDLECs, it would suggest that Jag1 functions in venous endothelium to suppress Prox1. This would also validate our observations that Jag1 expression is missing in multiple lymphatic vascular beds (Chapters 3 and 4).

We have demonstrated a role for Notch in transcriptionally inducing expression of FN-EIIIA, Connexin37, and Integrinα9 during valve formation in lymphatic collecting ducts. We report that activation of Notch1 or Notch4 in HDLECs induces FN-EIIIA^+, CX37, and ITGA9 transcripts. Overexpression of Notch effectors HEY1 or HEY2 either represses or does not affect these genes. These results agree with in vivo observations made by our group, in which loss of Notch1 in LECs (Prox1CreERT2;N1^fl/fl) at E15.5 resulted in a reduced number of lymphatic valves, as well as reduced expression of FN-EIIIA and Integrinα9 (Murtomaki et al., submitted). Any valves that managed to form were poorly organized and displayed reduced expression of FN-EIIIA and Integrinα9 (Murtomaki et al., submitted). Loss of all NICD/CSL signaling in LECs (Prox1CreERT2;DNMAML^fl/+ ) resulted in a more severe phenotype than loss of Notch1 alone (Murtomaki et al., submitted). Taken together, these results suggest a role for Notch as a positive regulator of lymphatic valve formation.

Our work suggests that Notch activation is critical in suppressing PROX1 transcripts during early lymphatic specification. This would appear to conflict with the co-expression of high levels of Prox1 and Notch1 in lymphatic valve sites. One possibility is that Notch regulates Prox1 differently in larger lymphatic vessels compared to smaller lymphatic vessels. To support
In lymphatic collecting ducts as well, the specific contribution of Notch4 was not analyzed in vivo. However, transcript analysis in HDLECs, as well as the increased severity of the phenotype in Prox1CreERT2;DNMAMLfl/+ embryos compared to Prox1CreERT2;N1fl/fl embryos, suggest that Notch4 or another Notch family member may also play a role in inducing valve markers in vivo. Furthermore, expression of Notch ligands has not been determined in lymphatic collecting ducts. Based on our expression analysis of Notch ligands in other lymphatic vascular beds, however, we would expect that Dll4 is the ligand responsible for activating Notch in lymphatic collecting ducts.

Here we show that Notch functions as a regulator of lymphatic endothelial cell fates in a context-dependent manner. During early lymphatic development, Notch acts as a negative regulator of lymphatic endothelial cell fate by suppressing PROX1 and PDPPN transcripts. Later on, during lymphatic valve formation, Notch acts as a positive regulator of lymphatic valve endothelial cell fate by inducing FN-EIIIA+, CX37, and ITG9A transcripts. Taken together, our data points to the conservation of the role of Notch as a mediator of cell fate decisions in the lymphatic vasculature.
Chapter 6

Figures
Figure 1. Notch transcriptionally represses genes critical for lymphatic vascular specification and separation from venous vasculature. Quantitative RT-PCR with HDLEC cDNA. (A) qPCR validates that Notch1 lentiviral activation construct (N1IC) induces downstream effector HEY2, while Notch4 lentiviral activation construct (N4/int3) induces downstream effectors HEY1 and HEY2. HEY1 and HEY2 lentiviral overexpression constructs result in increase of HEY1 and HEY2 transcripts, respectively. Overexpression of HEY2 significantly represses HEY1 transcripts. (B) Overexpression of Notch1 decoy lentiviral construct (N1ECDFc1-36) represses downstream effectors HEY1 and HEY2. (C) Activation of Notch1 or Notch4 represses PROX1 and PDPN, while overexpression of Notch effectors HEY1 and HEY2 only repress PROX1.

Error bars represent standard deviation of the mean, *p<0.05, **p<0.001.
Figure 2. Notch activation results in overexpression of markers critical for lymphatic valve formation. Analysis of protein expression levels in HDLECs by Western blot (A-B) or flow cytometry (C). (A) Notch1 activation construct (N1IC) strongly induces expression of ECM protein fibronectin (FN), as well as splice variant FN-EIIIA. Notch4 activation construct (N4/int3) results in a modest increase of FN and FN-EIIIA. (B) Notch1 activation construct (N1IC) strongly induces expression of gap junction protein Cx37, while Notch4 activation construct (N4/int3) does not induce expression. (C) Notch1 activation construct (N1IC) strongly induces cell surface expression of FN-EIIIA adhesion protein integrinα9, while Notch4 activation construct (N4/int3) does not induce expression.
Figure 3. Notch transcriptionally induces genes critical for lymphatic valve formation.
Quantitative RT-PCR with HDLEC cDNA. (A) Notch1 adenoviral activation construct (N1IC), and to a lesser extent Notch4 adenoviral activation construct (N4/int3), induce \textit{FNEIIIA}\textsuperscript{+} and \textit{FNEIIIA}\textsuperscript{-} transcripts. HEY1 and HEY2 adenoviral overexpression constructs do not induce \textit{FNEIIIA}\textsuperscript{+} and \textit{FNEIIIA}\textsuperscript{-} transcripts. (B) Activation of Notch1, and to a lesser extent Notch4, induce \textit{CX37} transcripts. HEY1 and HEY2 adenoviral overexpression constructs do not induce \textit{CX37} transcripts. (C) Activation of Notch1, and to a lesser extent Notch4, induce \textit{ITGA9} transcripts. HEY1 and HEY2 adenoviral overexpression constructs do not induce \textit{ITGA9} transcripts.
Error bars represent standard deviation of the mean, *p<0.05, **p<0.001.
Chapter 7

Discussion
Notch signaling suppresses sprouting lymphangiogenesis by restricting sprout formation

Notch functions to restrict sprouting angiogenesis of blood vessels, and here we report that this function is conserved during sprouting lymphangiogenesis. We demonstrate that Notch activation, likely viaDll4, suppresses sprouting lymphangiogenesis. Previous studies found conflicting roles for Notch in sprouting lymphangiogenesis. Pharmacological inhibition of Notch signaling using a Dll4 decoy was shown to enhance developmental sprouting lymphangiogenesis during murine development\cite{73}, while neutralizing antibodies against Dll4 or Notch1 were shown to suppress developmental sprouting lymphangiogenesis\cite{74}. It should be noted that these approaches were limited in that they depended on pharmacological agents, whose activities are not always easily interpreted. In addition, these studies did not conclusively establish whether the effects on lymphangiogenesis were cell-autonomous or secondary effects.

We found that inhibition of Notch signaling in the lymphatic endothelium during developmental sprouting lymphangiogenesis (Prox1CreERT2;DNAMLfr/+ ) increased dermal lymphatic vessel density. Thus, precise genetic manipulation of the Notch pathway in lymphatic endothelium allowed us to definitively demonstrate that Notch must be activated within the lymphatic endothelium to restrict sprouting lymphangiogenesis. We built upon this finding and demonstrated that Dll4 is highly expressed by lymphatic tip cells during developmental sprouting lymphangiogenesis of the murine embryonic dermis. In contrast, Jag1 expression was not detected in the dermal lymphatic vascular bed. The high expression of Dll4 in lymphatic tip cells suggested that these cells have a specialized identity and function. Notch signaling in
cultured HDLECs was induced byDll4 but neither induced nor suppressed by Jag1, further highlighting the importance of Dll4/Notch signaling in the lymphatic endothelium.

The murine embryonic dermis allowed us to follow the function of Notch in newly sprouting lymphatic vessels at the lymphatic front, and in recently established lymphatic vessels at the lymphatic plexus, within the same tissue. At the lymphatic front, Notch inhibition was concurrent with increased sprouts and branch points, while at the lymphatic plexus, Notch inhibition caused no change in branch points. However, Notch inhibition caused an increase in lymphatic vessel diameter.

We propose several mechanisms, which are not mutually exclusive, to explain how Notch signaling suppresses sprouting lymphangiogenesis. First, we found that Notch activation suppresses HDLEC proliferation, migration, and network formation. As each of these processes contribute to sprouting lymphangiogenesis, we propose that Notch inhibition may increase proliferation, migration, and network formation of LECs, resulting in hyperplasia at the lymphatic plexus and contributing to hypersprouting at the lymphatic front. Second, Notch and VEGF receptors display complex cross-regulation (Chapter 3, Figure 14), and we postulate that this may be critical for controlling sprouting lymphangiogenesis. We identified a feedback loop between VEGFR-3/VEGFR-2 and Notch. Notch signaling was activated when HDLECs were treated with VEGF or VEGF-C. In turn, Notch activation suppressed VEGFR-2 transcripts through HEY1/HEY2 in HDLECs. In the lymphatics, VEGFR-2 signaling does not regulate sprout formation but causes lymphatic hyperplasia. Our finding that Notch suppresses VEGFR-2 in HDLECs suggests that increased VEGFR-2 levels upon Notch inhibition may contribute to
hyperplasia at the lymphatic plexus. Notch has been reported to induce\textsuperscript{[70, 71]}, suppress\textsuperscript{[61, 67-69]}, or not affect\textsuperscript{[73, 74]} VEGFR-3 expression. Our work revealed that VEGFR-3 transcripts are regulated by Notch and HEYs in a dynamic manner, resulting in oscillating transcript levels\textsuperscript{[79]} in a manner consistent with a negative regulatory feedback loop. During sprouting angiogenesis, blood endothelial cells (BECs) shuffle between the tip and stalk cell identities by dynamic regulation of VEGFR-2 and VEGFR-1 expression\textsuperscript{[40]}, and we suggest that the ability of Notch to dynamically regulate VEGFR-3 may be important in determining tip and stalk cell identities in sprouting lymphangiogenesis. We postulate that when Notch signaling is inhibited in LECs, VEGFR-3 expression is deregulated, which contributes to hypersprouting at the lymphatic front. Given the dynamic expression patterns of VEGF receptors, live imaging and time course analysis will be necessary in the future to understand Notch/VEGF receptor signaling during sprouting lymphangiogenesis.

Our studies shed new light on the relative roles ofDll4 and Jag1 in sprouting lymphangiogenesis. Dll4 was highly expressed by lymphatic tip cells during pathological sprouting lymphangiogenesis in the sutured murine cornea. Jag1 expression was not detected in this lymphatic vascular bed. Pharmacological inhibition of pan-ligand Notch signaling (N1ECDFc\textsubscript{1-24}) or Dll inhibition (N1ECDFc\textsubscript{1-13}) resulted in increased corneal area occupied by lymphatic vasculature. Increased lymphatic vascular coverage after N1ECDFc\textsubscript{1-24} treatment was concomitant with lymphatic hypersprouting, but increased lymphatic vascular coverage after N1ECDFc\textsubscript{1-13} treatment was not accompanied by hypersprouting. Jag inhibition (N1ECDFc\textsubscript{10-24}) did not affect lymphatic vascular coverage or sprouting. Given that Dll4 was strongly expressed in lymphatic tip cells and Jag1 was not detected in the lymphatic vessels in this setting, we
postulate that N1ECDFc1-24 enhances lymphatic vascular coverage and sprouting lymphangiogenesis by targeting Dll4/Notch signaling. N1ECDFc1-24 has been shown to elicit more dramatic phenotypes than N1ECDFc1-13 or N1ECDFc10-24 alone[83]. We postulate that relatively weak inhibition of Dll4/Notch signaling is sufficient to enhance lymphatic coverage, but stronger inhibition of Dll4/Notch signaling may be necessary to enhance lymphatic sprouting. This may explain why N1ECDFc1-13 was able to enhance lymphatic vascular coverage but unable to induce lymphatic hypersprouting.

Tumor lymphatic vessels are poorly understood and likely differ in fundamental ways from physiological lymphatics and even from other pathological lymphatics (i.e., corneal suture-induced lymphatics). For one, lymphatics often grow in a peritumoral manner, failing to penetrate deeply into tumors, whereas most normal tissues are infused with lymphatic capillaries. We found that pathological lymphatic capillaries recruited by orthotopically implanted D3H2LN human breast tumors express both Dll4 and Jag1, showing a key difference in Notch ligand expression in the D3H2LN lymphatic vascular bed, as compared to non-tumor lymphatics. We suggest several possibilities that may explain why Jag1 is expressed in 231 tumor lymphatics: 1) Notch expression by 231 tumor cells may induce Jag1 expression in tumor lymphatics; 2) 231 tumors may secrete factors that induce Jag1 expression in lymphatics; 3) 231 tumor lymphatics may be mis-specified and thus possess characteristics of blood vessels, including Jag1 expression.

Notch inhibitors, including Notch1 decoys (N1ECDFc), have been used with great success in suppressing angiogenesis, lymphangiogenesis, and metastasis in multiple tumor models[83, 90-]
Of particular interest to us were preliminary studies conducted by our group that showed that Notch inhibition suppresses tumor lymphangiogenesis (Yasuhiro Funahashi and Xing Wang, unpublished data). However, we found that pharmacological inhibition of Notch signaling with N1ECDFc1-13 (Dll inhibitor) or N1ECDFc10-24 (Jag inhibitor) had no effect on pathological sprouting lymphangiogenesis in the D3H2LN orthotopic model of human breast cancer. We suggest several possibilities to explain why adenoviral delivery of Notch1 decoy treatment did not affect D3H2LN tumor progression: 1) The mammary fat pad microenvironment may provide advantages to tumors that allow them to overcome Notch inhibition; 2) Orthotopic implantation in general may provide advantages over subcutaneous implantation that allows tumors to overcome Notch inhibition; 3) Adenoviral delivery of Notch1 decoys may not achieve the necessary serum levels to affect tumor lymphangiogenesis; 4) Notch1 decoy treatment may need to be used as combination therapy with another lymphangiogenic inhibitor; 5) D3H2LN tumors may be refractory to Notch inhibition.

**Notch determines cell fate during lymphatic development**

Notch is known to regulate cell fates in multiple contexts and tissues\(^{24-26}\). In blood vascular development alone, Notch regulates at least two cell fate decisions. Notch signaling induces arterial fate over venous fate, and stalk cell fate over tip cell fate\(^{29}\). Lymphatic vascular formation requires cell fate decisions during early specification and separation from the venous vasculature, as well as during lymphatic valve formation. Initiation of lymphatic development requires cell-fate determination, as the first lymphatic endothelial cells (LECs) are specified
within the cardinal vein by inducing Prox1\textsuperscript{[9-12]}. They then further differentiate and separate from the cardinal vein by inducing podoplanin expression\textsuperscript{[9-12]}. Work from our group showed that within the cardinal vein, Notch1 and Jag1 were expressed in the cells that did not go on to induce Prox1 and podoplanin expression\textsuperscript{[79]}. Work from our group showed that activation of Notch1 in LEC progenitors (Prox1\textsuperscript{CreER\textsuperscript{T2};N1IC}) led to reduced expression of Prox1 and podoplanin, and reduced lymphatic differentiation and separation from the venous vasculature\textsuperscript{[79]}. This suggested a suppressive role for Notch during lymphatic specification and separation. We report that Notch activation in HDLECs suppresses PROX1 and PDPN transcripts\textsuperscript{[79]}. Thus, Notch functions as a negative regulator of lymphatic specification and separation through transcriptional repression of PROX1 and PDPN.

Lymphatic valve formation also requires cell-fate determination, as a subset of LECs within the collecting ducts must become committed to the valve LEC fate. Work from our group showed that Notch1 is expressed throughout lymphatic collectingducts and becomes upregulated at sites of valve formation at E17.5 (Murtomaki et al., submitted). Loss of Notch1 in lymphatic collecting ducts (Prox1\textsuperscript{CreER\textsuperscript{T2};N1\textsuperscript{fl/fl}}) led to reduced lymphatic valve formation, concurrent with reduced expression of FN-EIIIA and Integrin\alpha9 (Murtomaki et al., submitted). This suggested an inductive role for Notch in lymphatic valve formation. We report that Notch activation in HDLECs induces transcripts for FN-EIIIA, ITGA9, and CX37, another important regulator of lymphatic valve formation (Murtomaki et al., submitted). Thus, we conclude that Notch functions as a positive regulator of lymphatic valve formation through transcriptional induction of FN-EIIIA, ITGA9, and CX37.
**Notch signaling in lymphatic vasculature is context-dependent**

The studies described here establish that Notch functions as a regulator of cell-fate decisions in lymphatic vasculature, and that this function is conserved in several steps of lymphatic vascular formation. Notch functions during the early stages of lymphatic specification from the venous vasculature, in sprouting lymphangiogenesis during development and disease, and during lymphatic valve formation. Expression of Notch family proteins and function of Notch signaling, even within the lymphatic vasculature, is highly context-dependent. For example, Jag1 is not expressed in developing lymphatic vessels of the embryonic dermis and pathological lymphatic vessels of the wounded cornea, but is expressed in pathological tumor lymphatic vessels. Notch can both directly induce or indirectly repress VEGFR-3 levels in HDLECs, a distinction that may be critical for context-dependent functions of Notch. Notch inhibition has been shown to induce sprouting lymphangiogenesis in the embryonic dermis and wounded cornea, while it suppresses sprouting lymphangiogenesis in certain tumor models. Notch suppresses Prox1 in the cardinal vein during lymphatic specification but does not suppress Prox1 in valve LECs during lymphatic valve formation. Although Notch functions differently in different contexts, it is undeniably important in the lymphatic vasculature. Identifying these differences may help us to better understand and predict how Notch functions in a context-dependent manner.
Conclusion

Here we show that Notch signaling functions to regulate sprouting lymphangiogenesis. Dll4 was highly expressed in capillary lymphatic tip cells during sprouting lymphangiogenesis, and inhibition of Notch signaling resulted in lymphatic hypersprouting. We demonstrate a feedback loop between Notch and VEGF signaling, which we postulate is important in regulating sprouting lymphangiogenesis. Our results validate the tip/stalk model in sprouting lymphangiogenesis. We also identify Notch as a transcriptional inhibitor of lymphatic specification/separation and transcriptional inducer of lymphatic valve formation. Our work identifies novel roles for Notch signaling as a cell fate determinant in tip/stalk, venous to lymphatic, and duct to valve LEC cell fate decisions in lymphatic vasculature. Given the importance of the lymphatic vascular system in physiological and pathological settings, our work suggests that the Notch signaling pathway may a productive therapeutic target in treating lymphatic vascular diseases.
Chapter 7

Figures
Figure 1. Model. Notch regulates lymphatic endothelial cell fate decisions and sprouting lymphangiogenesis in a context-dependent manner during development and postnatally.
References


Appendix 1

Notch1 functions as a negative-regulator of lymphatic endothelial cell differentiation in the venous endothelium
Notch1 functions as a negative-regulator
of lymphatic endothelial cell
differentiation in the venous endothelium

Short Title: Notch inhibits lymphatic fate

Key words: Notch1, lymphatic endothelial cells, Prox1

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Introduction

In mice, lymphatic vascular development initiates around embryonic day 9.75 (E9.75) with the expression of Prox1 in a subset of the endothelial cells (ECs) of the cardinal vein (CV) (Oliver et al. 1993; Oliver and Harvey 2002). Prox1, a homeobox transcription factor, functions as master regulator of lymphatic endothelial cell (LEC) specification and maintains LEC fate (Wigle et al. 2002). In vitro studies suggest Prox1 functions in LEC progenitors to promote differentiation and upregulate LEC specific genes, such as SLC, neuropilin-2 (Nrp2), podoplanin, and a9 integrin and downregulate blood endothelial cells (BEC) genes, CD34, neuropilin-1 (Nrp1), and endoglin (Petrova et al. 2002; Hong and Detmar 2003). Prox1 deletion in mice results in a loss of LEC progenitor cells in veins and blocked lymphatic vascular development (Wigle and Oliver 1999; Wigle et al. 2002; Johnson et al. 2008). In contrast, ectopic Prox1 expression in the blood endothelium results in enlarged lymphatic sacs (LS) and edema (Kim et al. 2010). Loss of Prox1 after completion of lymphatic development leads to a loss of LEC specific proteins and misexpression of BEC markers, demonstrating that Prox1 is required to maintain LEC identity (Johnson et al. 2008).

At E9.75, expression of Prox1 in the cardinal vein requires Coup-TFII and Sox18 transcription factors (Srinivasan et al. 2007; Francois et al. 2008; Lin et al. 2010). After E13.5, maintenance of Prox1 expression no longer requires either Coup-TFII or Sox18; thus the maintenance of Prox1 transcription occur by different mechanisms (Srinivasan et al. 2007; Srinivasan et al. 2010). Coup-TFII further cooperates with Prox1 to drive the expression of lymphatic endothelial specific genes, such as Nrp2 and VEGFR-3 (Lee et al. 2009; Lin et al. 2010). Recently, it has been
shown that Prox1 and Coup-TFII function together to regulate lymphovenous valve formation in mice (Srinivasan and Oliver 2011).

Migration of Prox1-positive LEC progenitors from the veins and formation of LSs requires the activity of Vascular Endothelial Growth Factor C (VEGF-C) (Karkkainen et al. 2004). VEGF-C through its receptor, VEGFR-3, induces LEC proliferation, survival and migration in culture (Makinen et al. 2001). VEGFR-3 is a tyrosine kinase receptor that, in complex with the co-receptor Nrp2, binds VEGF-C (Favier et al. 2006; Kärpänen T 2006; Xu et al. 2010).

Notch signaling modulates cell-fate decisions via direct cell-cell contact (Andersson et al. 2011). The Notch pathway comprises a family of four transmembrane receptors (Notch 1-4) and five membrane-bound ligands (Delta-like1, 3, 4, Jagged1 and 2). Receptor–ligand interactions release the intracellular domain via proteolysis that translocates to the nucleus, where it binds to CBF1/Su(H)/LAG1 (CSL) (Borggrefe and Liefke 2012). The intracellular Notch peptide recruits a transcriptional activating complex to CSL converting it from a repressor to an activator of transcription, inducing multiple downstream targets such as the transcriptional repressors, HES and Hey.

In the blood vasculature, Notch signaling regulates arterial/venous specification and sprouting angiogenesis. In the arterial endothelium, high Dll4/Notch1 signaling maintains arterial identity and inhibits venous EC differentiation (Gridley 2010). In sprouting angiogenesis, Dll4 in tip cells signal to Notch1 in adjacent stalk cells to restrict the number of tip cells and vascular density (Jakobsson et al. 2009; Tung et al. 2012). Dll4/Notch1 signaling also has a role in lymphatic vascular development. In zebrafish, Dll4 functions in thoracic duct morphogenesis (Geudens et al. 2010). Dll4 expressed on the intersomitic arteries guides secondary
lymphangiogenic sprouting from the posterior CV. In mice, Dll4/Notch1 signaling has been shown to function in sprouting lymphangiogenesis, by acting to control lymphatic tip cell differentiation (Niessen et al. 2011; Zheng et al. 2011). Consistent with this finding, Notch1 and Notch4 are expressed in dermal lymphatic endothelium, when the embryonic lymphatics are actively remodeling (Shawber et al. 2007).

We demonstrate a novel role for Notch in lymphatic vascular development; that of regulating the choice between venous and lymphatic endothelial identity during murine development. Using the Prox1CreERT2 inducible driver (Srinivasan et al. 2007), Notch activity was manipulated in Prox1+ LEC progenitor cells in veins. Conditional loss of Notch1 or inhibition of Notch/CSL transcriptional activation resulted in precocious and excessive LEC differentiation that correlated with misexpression of Prox1 in the veins and dilation of lymphatics. Ectopic Notch1 activation in Prox1+ ECs resulted in misspecified LECs leading to severe edema, blood-filled lymphatics and incorporation of BECs into the peripheral lymphatics.

Materials and Methods

Constructs/Cell Culture

Early passage HdMVECs (Lonza) were infected with 25 pfu/cell adenoviruses encoding the intracellular domain of human Notch1 (N1IC), or LacZ, as previously described (Shawber et al. 2007). HdLECs were isolated from human foreskins (Exemption AAAB-1700) using CD31 conjugated Dynabeads (Invitrogen). Passage 1 cells were FACS sorted for podoplanin (Angiobio), then negatively sorted by FACS for CD34 (Pharmingen). HdLEC were maintained in EGM-
2MV BulletKit (Lonza) with 10 ng/ml VEGF-C (RnD). HdLEC lines were generated by lentiviral infection using pCCL.pkg.wpre vector encoding N1IC, Hey1, Hey2 or GFP (Tung et al. 2009). Transgene expression was confirmed by quantitative (q) RT-PCR and Westerns.

For activation of endogenous Notch signaling, HDLEC grown to confluency on fibronectin-coated dishes were treated overnight with 200nM Compound E, a gamma secretase inhibitor (GSI). The next day, GSI was removed and cells treated with 10mM EDTA/PBS at 37°C for up to 15 minutes. Media was then replaced with EGM-2 MV containing 10 ng/ml VEGF-C (RnD systems).

**RT-PCR/Western Analyses**

RNA (RNeasy Mini Kit; Qiagen) and protein was isolated 24 hours after adenoviral infections and 48 hours after lentiviral infection (Shawber et al. 2007). RT-PCR was performed (Funahashi et al. 2010), and Westerns performed with antibodies against Prox1 (Millipore), or a-tubulin (Sigma). cDNAs were synthesized and qPCR performed with Sybr Green PCR Master Mix (ABI) and 7300 Real-Time PCR System (ABI). PCR amplicons for genes cloned into pDrive (Stratagene) served as reference standards. b-actin qRT-PCR was used to normalize samples. Primers described in Table S1.

**Mice**

*Notch1*+/− (Krebs et al. 2000), *Prox1CreER^T2* (Srinivasan et al. 2007), *Prox1^GFPcre* (Srinivasan et al. 2010; Srinivasan and Oliver 2011), *N1IC* (Buonamici et al. 2009), *Notch1*flo/flo (Yang et al. 2004), and *DNMAML* (Tu et al. 2005) mice are described. *Notch1*flo/flo and *ROSA:LacZ*flo/flo mice were
purchased (Jax Labs). Tamoxifen (5mg/40g or 7mg/40g) dissolved in corn oil was injected intraperitoneally at E9.75, E10.5, E13.5 and E14.5 for N1IC and Notch1fffl studies. 5mg/40g tamoxifen was administered orally at E9.75 for DNMAML studies. 2-7 litters were evaluated for each time-point and described in Table S2.

**Immunohistochemistry**

Immunohistochemistry on 5 mm fixed-frozen/paraffin-embedded and 30 mm fixed-frozen sections was performed (Shawber et al. 2007). Wholemounts were performed as described (Lohela et al. 2008). Antibodies listed in Table S3. MOM kit (Vector) was used with mouse monoclonal antibodies. Secondary antibodies were from Invitrogen (Alexa fluor 488, Alexa fluor 594) and Vector Labs (Biotinylated-anti-goat, biotinylated anti-hamster). Colorimetric staining was hematoxylin counterstained. Nuclei visualized with DAPI in immunofluorescent staining. Images captured using a Nikon SMZ-U Zoom 1:10 microscope and Nikon 4500 digital camera, or Nikon ECLIPSE E800 microscope, Nikon DXM 1200 digital camera, and Image ProPlus software. Confocal microscopy performed with a Zeiss LSM 510 META Confocal Microscope and the LSM software.

**Quantitation/Statistical Analyses**

ImageJ software (NIH) was used for quantitative analyses of images. Prox1+ cells within the CV or LS were normalized by vessel/sac circumference and emerging from CV by area analyzed. LYVE1 vessels were normalized by area analyzed to determine lymphatic vessel density. Significance between two groups determined by T-Test.
Results

Notch1 activation suppresses lymphatic endothelial gene expression in microvascular endothelial cells.

To determine if Notch regulates LEC specific gene expression, a constitutively active Notch1 protein (N1IC) was ectopically expressed in human neonatal dermal microvascular endothelial cells (HdMVEC) by adenoviral infection and human dermal lymphatic endothelial cells (HdLEC) by lentiviral infection. HdMVEC consist of both BECs and LECs, and a subset of HdMVEC express LEC genes, Prox1, LYVE1 and podoplanin. Notch1 activation suppressed Prox1, podoplanin and LYVE1 transcripts in both HdMVEC and HdLEC, and Prox1 protein in HdMVEC (Figs. 1A,B). Notch activation did not affect the expression of Sox18 in HdLEC (Fig. S1A). In HdLEC, ectopic expression of the Notch effectors, Hey1 or Hey2, reduced expression of LEC genes Prox1, LYVE1, and podoplanin (Fig. 1B). Hey2 suppression of Prox1 and LYVE1 was stronger relative to Hey1. Notch1- and Hey2-mediated downregulation of Prox1 and podoplanin in cultured LECs is consistent with previous observations (Kang et al. 2010).

Notch regulates the expression of Vascular Endothelial Growth Factor Receptors (VEGFR) in BECs. Notch downregulates VEGFR-2 (Taylor et al. 2002; Shawber et al. 2007; Funahashi et al. 2010). Notch either induces or suppresses VEGFR-3, depending on the cellular context in vivo (Shawber et al. 2007; Tammela et al. 2008). The effect of activating Notch1 or ectopic Hey1 or Hey2 on VEGFR-2/3 was evaluated in HdLEC. N1IC, Hey1 or Hey2 suppressed VEGFR-2 transcript levels (Fig. 1C,D). N1IC induced VEGFR-3, while ectopic Hey1 or Hey2
suppressed VEGFR-3 (Fig. 1C,D), suggesting that Notch dynamically regulated VEGFR-3 expression.

We previously established that Notch directly induces VEGFR-3 via Notch/CSL transactivation of the VEGFR-3 promoter (Shawber et al. 2007). We hypothesize that VEGFR-3 may be downregulated, secondary to Notch/CSL induction, by the action of Hey proteins, as part of a negative feedback loop. To evaluate this possibility, we determined the effect of activating endogenous Notch on the timing of VEGFR-3 and Hey1/2 induction. HdLEC express Notch1-4, Jagged1 and Dll4 (Fig. S1B). Confluent HdLEC were grown overnight with the gamma secretase inhibitor (GSI), Compound E, to suppress Notch signaling. The following day GSI was removed, endogenous Notch activated by EDTA, and RNA collected at different time-points post-Notch activation. Significant induction of VEGFR-3 was observed after 5 minutes and reached maximum expression at 10 minutes (Fig. 1E). At 20 minutes, VEGFR-3 induction was significantly downregulated, which followed low level Hey1/2 induction observed at 5 and 10 minutes. After Hey1/2 levels went back to baseline at 20 minutes, VEGFR-3 was significantly up at 30 minutes. Strong induction of Hey1/2 observed at 60 minutes correlated with VEGFR-3 downregulation. These data suggest that VEGFR-3 is an immediate response gene downstream of Notch activation and downregulated by the Notch effectors, Hey1 and Hey2.

*Notch1 expression and activity in the cardinal vein during venous/lymphatic endothelial specification.*

LEC differentiation is a dynamic process that initiates around E9.75 with the onset of Prox1 expression and progresses anterior to posterior along the cardinal veins (Oliver et al.
As Notch1 suppressed Prox1 in vitro (Figs. 1A,B), we hypothesized that Notch acts in the venous endothelium to restrict the differentiation of Prox1+ progenitor cells. Expression of Notch proteins (Notch 1-4) and Notch ligandsDll4 and Jagged1, relative to endothelial Prox1 was determined in murine E9.75 and E10.5 wild-type and Notch reporter (TNR) embryos. The transgenic TNR mouse line expresses GFP in response to Notch signal activation (Duncan et al. 2005). We found that the venous endothelium primarily expressed Notch1 and Jagged1 during this developmental period (Figs. 1F-H, S1C-E).

Analysis of E9.75 transverse sections anterior to the common atrial chamber identified a region of the CV where Notch1 expression was opposite of polarized Prox1 (Fig. 1F). This pattern differed from the aorta, where high Notch1 expression was observed throughout the aortic endothelium (Fig. 1F), suggesting distinct expression patterns in arterial versus venous vessels. The Notch ligands, Dll4 and Jagged1 were both expressed in the aortic endothelium at E9.75 (Fig. S1C). Notch activity (GFP) was detected in a subset of Notch1-expressing CV ECs (Fig. 1F), when weak punctate Jagged1 expression was first observed in the E9.75 CV (Fig. S1C). Thus, Jagged1 may function as the ligand for Notch1 in venous endothelium.

Analysis of thick sagittal sections of E10.5 embryos demonstrated that Notch1 expression was discontinuous in the CV and jugular vein (JV) with punctate weak Notch1 expression observed dorsally (Fig. 1G). In serial sections, Prox1+ progenitors resided in the dorsal JV where Notch1 was sometimes co-expressed with Prox1 (Fig. 1G). Dorsally migrating Prox1+ progenitors were negative for Notch. In thick E10.5 transverse sections, Notch1 and Prox1 had both distinct and overlapping expression in the posterior cardinal vein (PCV; Fig. 1H). At this posterior position of the PCV, Prox1+ progenitors were evenly distributed, not yet
polarized to one side. Three distinct expression patterns were observed for Prox1 and Notch1; PCV cells that expressed high Prox1 and low Notch1, cells that expressed low Prox1 and high Notch1 and cells that co-expressed both proteins (Fig. 1H). At E10.5, Jagged1 expression was polarized medially in the anterior CV (Fig. S1D); this region displayed punctate Notch activation (GFP) (Fig. 1I, S2A).

The level of Notch signaling in the CV was much lower than that observed in the aortic endothelium and vascular smooth muscle cells at E10.5 and E11.5 (Fig. S2A,C) which correlated with the level of Notch1 expression (Fig. S2B). Notch1 expression was evaluated in the mesenteric vasculature that consists of arterial, venous and lymphatic vessels (Fig. S2D). At E16.5 and E17.5, Notch1 expression was strongest in the artery, weaker in veins and weakest in lymphatics (Fig. S2D,E). This pattern was quantified, and Notch1 expression in the veins was 51% and 43% of that in the arteries at E16.5 and E17.5, respectively (Fig. S2E). In the lymphatic vessels, Notch1 was expressed 22% at E16.5 and 13% at E17.5 relative to the artery (Fig. S2E). These expression studies suggest that Notch1 expression and activity is found in arterial, venous and lymphatic endothelium, but differs depending on the vessel type and developmental stage.

Loss of Notch signaling in Prox1-positive endothelial cells caused ectopic lymphatic endothelial cell specification and enlarged lymph sacs

To determine the effect of deleting Notch in the Prox1+ LEC progenitors residing in veins, we used the inducible Prox1-CreERT2 driver (Johnson et al. 2008). The Prox1-CreERT2 line drives recombination in Prox1+ ECs in response to tamoxifen without disrupting the endogenous
Prox1 gene. Prox1CreERT2T2 drivers were crossed with mice carrying a floxed allele of Notch1 (N1fl/fl) (Yang et al. 2004), or a dominant negative isoform of Mastermind-like (DNMAML) (Tu et al. 2005). DNMAML forms an inactive transcriptional complex with Notch on CSL without disrupting CSL repressor activity. Prox1CreERT2T2;N1fl/f+ males were crossed with N1fl/f+ females. Prox1CreERT2T2 females were crossed with homozygous DNMAML males. Tamoxifen was administered at E9.75, a time when Prox1 expression initiates and Notch1 and Prox1 are co-expressed in the venous endothelium (Figs. 1F-H). E14.5 Prox1CreERT2T2;N1fl/f+ (Fig. 2A) and Prox1CreERT2T2;DNMAML embryos (data not shown) displayed mild edema and small foci of blood filled dermal lymphatics. Prox1CreERT2T2;N1fl/f+, Prox1CreERT2T2, and N1fl/f+ control embryos were indistinguishable from wild-type littermates (data not shown).

Immunohistochemistry on E14.5 wild-type and Prox1CreERT2T2;N1fl/f+ transverse sections was performed using antibodies against BEC (CD31/endomucin) and LEC (Prox1/LYVE1) specific proteins. Compared to wild-type littermates, Prox1CreERT2T2;N1fl/f+ embryos had enlarged LSs surrounding the CV at the level of the aortic arch (Fig. 2B), and descending thoracic aorta (Fig. 2C), and dermal lymphatic vessels (Fig. 2D). Prox1CreERT2T2;N1fl/f+ LS luminal area was 5.7-fold greater than wild-type littermates and 4.7-fold greater than Prox1CreERT2 littermates (Fig. 2G). The increase in LS size in Prox1CreERT2T2;N1fl/f+ embryos correlated with a 2.5-fold increase in Prox1+ LEC emerging from veins relative to control embryos (Fig. 2E,G), indicative increased LEC differentiation. Similarly, Prox1CreERT2T2;DNMAML lymphatic vessels were enlarged 4.2-fold and lymphatic vessel density increased 2.3-fold relative to DNMAML controls (Figure 2F,H). These data suggest that the increase in Prox1+ progenitors emerging from veins in Notch deficient embryos contributed to lymphatic sac enlargement.
As VEGF-C signaling is necessary for LEC progenitors to migrate from the CV (Favier et al. 2006; Kärpänen T 2006), expression of the VEGF-C receptors, VEGFR-2 and VEGFR-3 was evaluated. Conditional deletion of Notch1 in the Prox1+ ECs resulted in increased VEGFR-2 expression in the venous endothelium, but not the lymphatic (Fig. S3A) and is consistent with a loss of Notch-mediated suppression of VEGFR-2 (Taylor et al. 2002; Shawber et al. 2007). VEGFR-3 expression was relatively unaffected. The increase in migrating Prox1+ cells may be due to changes in LEC VEGFR-2 or VEGFR-3 expression.

Loss of Notch in Prox1+ EC progenitors resulted LECs in veins

As expected, Prox1 expression was restricted to the lymphatic endothelium at E14.5 and rarely observed in the CV of control embryos (Fig. 2E, 3A). Prox1CreER<sup>T2</sup>;N1<sup>fl/fl</sup> tissues had a significant 3.75- and 5.32-fold increase in the number of Prox1+ cells in the CV relative to wild-type and Prox1CreER<sup>T2</sup> embryos, respectively (Fig. 3B). Prox1 expression was observed throughout the CV of Prox1CreER<sup>T2</sup>;N1<sup>fl/fl</sup> tissues at E14.5. This phenotype was also observed in Prox1CreER<sup>T2</sup>;DNMAML embryos that had a 1.89-fold increase in Prox1+ cells in the CV (Fig. 3B). Similar to the conditional Notch loss-of-function mice, Prox1+ ECs residing in the CV was increased 1.58-fold in N1<sup>+/−</sup> heterozygotes at E10, relative to wild-type littermates (Figs. S4A,B). These results suggest that Notch1 activity is necessary to assure that Prox1 is suppressed in venous endothelium.

The presence of Prox1+ ECs in the CV of Notch mutant embryos suggested a specification defect in the CV endothelium. To explore this, we determined if the CV expressed LYVE1 and podoplanin, proteins normally restricted to the lymphatic endothelium at E14.5 (Fig.
In Prox1CreER$^{T2};N1^{fl/fl}$ embryos, LYVE1 and podoplanin were misexpressed in the CV endothelium (Figs. 3A,C,D). Staining for podoplanin revealed that the lymphatic vessels surrounding the CV of Prox1CreER$^{T2};N1^{fl/fl}$ embryos were sometimes merged with the CV (Fig. 3A,C), indicative of a failure to segregate lymphatic and venous vessels. LYVE1 was also misexpressed in the CV of E10.0 $N1^{+/−}$ embryos relative to their wild-type littermates (Fig. S4C). These data indicate that reduced Notch1 activity resulted in an increase of venous Prox1+ ECs coincident with the expression of several key lymphatic proteins within veins, well beyond the time when LECs should have segregated from venous endothelium.

We next examined whether Notch1 loss could rescue the embryonic lethality of Prox1 haploinsufficiency, as might be predicted if Notch1 downregulates Prox1. In a mixed C57Bl and NMRI background, Prox1$^{GFPCre/+}$ mice, in which one allele of Prox1 is disrupted with a GFPCre cassette (Srinivasan et al. 2010; Srinivasan and Oliver 2011), are not viable (Table 1). Prox1$^{GFPCre/+}$ mice were crossed with $N1^{fl/+}$ mice to generate Prox1$^{GFPCre/+;N1^{fl/+}}$ mice where one copy of Notch1 is lost in Prox1+ ECs. Prox1$^{GFPCre/+;N1^{fl/+}}$ were viable and observed at predicted frequency. Endomucin and Prox1 staining of E10.5 Prox1$^{GFPCre/+;N1^{fl/+}}$, Prox1$^{GFPCre/+}$, and $N1^{fl/+}$ embryos was carried out to determine if improved viability correlated with rescued lymphatic endothelial specification. Prox1+ LECs were reduced 1.9-fold in the CV and 3.35-fold emerging out of the CV in Prox1$^{GFPCre/+}$ tissues relative to $N1^{fl/+}$ (Fig. 3E). Prox1+ LEC numbers were not significantly different in the CV of Prox1$^{GFPCre/+;N1^{fl/+}}$ tissues relative to $N1^{fl/+}$ tissues. Prox1+ LECs emerging from the CV were significantly increased 2.41-fold in Prox1$^{GFPCre/+;N1^{fl/+}}$ tissues compared to Prox1$^{GFPCre/+}$ tissues. Thus, losing one copy of Notch1 in the LEC progenitors rescued the LEC specification defect within the CV and viability of Prox1 heterozygous mice.
**Notch1 activation in Prox1+ endothelial cells resulted in edema, blood-filled lymphatics**

To conditionally activate Notch1 within Prox1+ ECs, the *Prox1CreER<sup>T2</sup>* driver was crossed with mice carrying a Cre-responsive, constitutively activated Notch1 (N1IC) transgene downstream (*N1IC*) (Buonamici et al. 2009). Tamoxifen was administered at E9.75 or E10.5. *Prox1CreER<sup>T2</sup>;N1IC* embryos died prior to E15.5. When analyzed at E14.5, *Prox1CreER<sup>T2</sup>;N1IC* embryos displayed severe edema, and an extensive network of blood-filled superficial lymphatics (Figs. 4A, S5A). After fixation, a blood-filled jugular LS was observed in *Prox1CreER<sup>T2</sup>;N1IC* embryos (Fig. 4A). When tamoxifen was administered at E10.5 and embryos isolated at E13.5, *Prox1CreER<sup>T2</sup>;N1IC* embryos displayed mild edema and appeared to undergo normal blood vascular development (Fig. S5A). Thus, the severe lymphatic defects arose between E13.5 and E14.5 when flow begins in the embryonic lymphatics. Staining with an antibody against the intracellular domain of Notch1 confirmed that the N1IC transgene was expressed in the lymphatic endothelium of *Prox1CreER<sup>T2</sup>;N1IC* tissues (Fig. S5B). The *Prox1CreER<sup>T2</sup>* driver was crossed with a mouse carrying a conditional *LacZ* transgene downstream of the ROSA26 promoter to generate *Prox1CreER<sup>T2</sup>;LacZ<sup>fl/fl</sup>* mice that were crossed with *N1IC* mice. *Prox1CreER<sup>T2</sup>;LacZ<sup>fl/+</sup>;N1IC* embryos displayed severe lymphatic vascular defects at E14.5. Staining for b-gal and LYVE1 confirmed recombination occurred in ECs incorporated into the LSs and nearby lymphatic vessels in control *Prox1CreER<sup>T2</sup>;LacZ<sup>fl/+</sup>* and *Prox1CreER<sup>T2</sup>;LacZ<sup>fl/fl</sup>;N1IC* tissues (Fig. S5C). Thus, Notch1 activation in Prox1+ ECs led to severe edema and embryonic lethality, likely related to venous/lymphatic defects.
We hypothesized that the Prox1CreERT2;N1IC phenotype was due to aberrant lymphatic endothelial specification. The lymphatic vascular phenotype was determined by staining for Prox1, podoplanin, and LYVE1. In wild-type tissues, Prox1 expression was absent from the CV endothelium and restricted to the LSs and peripheral lymphatic vasculature at E13.5 and E14.5 (Fig. 4B,C). In E14.5 Prox1CreERT2;N1IC embryos, expression of Prox1 and podoplanin was strongly reduced in the presumptive LSs (Fig. 4B). The presumptive LSs of E14.5 Prox1CreERT2;N1IC consisted of numerous small disorganized blood-filled channels (Fig. 4B, S5D). These abnormal lymphatic channels had spotty Prox1 and weak podoplanin expression (Fig. S5D). Unlike the Notch loss-of-function models, the presumptive LSs clearly segregated from the CV in Prox1CreERT2;N1IC embryos. A less severe lymphatic phenotype was observed in E13.5 Prox1CreERT2;N1IC embryos and correlated with a 3.72-fold decrease in Prox1+ ECs in the lymphatic endothelium where LYVE1 expression was also reduced (Fig. 4C,D). The reduction in Prox1+ ECs was associated with a significant 3.8-fold and 2.31-fold decrease in Prox1+ EC density in Prox1CreERT2;N1IC tissues relative to control tissues at E13.5 and E14.5, respectively (Fig. 4E). To determine if this phenotype was due to an early migration defect, the number of Prox1+ progenitor cells within and emerging from the CV was determined in E10.5 Prox1CreERT2;N1IC and control embryos in which tamoxifen was administered at E9.75. Prox1+ progenitors numbers did not significantly differ between the two groups (Fig. S6). Thus, activation of Notch1 in the Prox1+ progenitor cells suppressed lymphatic specification, opposite to that observed when Notch signaling was inhibited in the lymphatic progenitor ECs.
Notch1 inhibition of Prox1 was restricted to the initiation and early maintenance phase of lymphatic vascular development

The transcription factors, Sox18 and Coup-TFII cooperate to regulate Prox1 expression in the lymphatic progenitor ECs between E9.75 and E13.5 in mice (Francois et al. 2008; Lee et al. 2009; Srinivasan et al. 2010). In the blood vasculature Coup-TFII and Notch function reciprocally to inhibit the other’s expression and maintain venous and arterial EC identity, respectively (You et al. 2005; Diez et al. 2007), suggesting that Notch downregulates Coup-TFII. In fact, Notch via Hey1 and Hey2 directly suppresses Coup-TFII expression in cultured HdLECs (Kang et al. 2010).

As Notch1 did not suppress Sox18 in HdLECs (Fig. S1B), we analyzed Notch regulation of Coup-TFII during lymphatic endothelial specification. On the side of the CV where Prox1+ progenitors resided, Notch activity and Coup-TFII expression did not overlap, but were observed in neighboring cells at E10.5 (Fig. 5A), consistent with Notch functioning to suppress Coup-TFII. To determine if activation of Notch1 in Prox1+ ECs influenced Coup-TFII expression in vivo, E12.5 and E13.5 wild-type and Prox1CreER
t2;N1IC tissues were stained for Coup-TFII. In the wild-type CV, Coup-TFII was expressed throughout the venous and lymphatic endothelium (Fig. 5B). In contrast, Coup-TFII was strongly suppressed in the venous and LS ECs of Prox1CreER
t2;N1IC embryos. The loss of Coup-TFII in the Prox1CreER
t2;N1IC embryos preceded Prox1 downregulation (Fig. 4C), suggesting the loss of Prox1 may be secondary to that of Coup-TFII.

As Coup-TFII is not required to maintain Prox1 expression in LECs after E13.5 (Srinivasan et al. 2010), tamoxifen was administered at E13.5 and E14.5 to Prox1CreER
t2 and N1IC crosses
and embryos isolated at E16.5 and E18.5. When tamoxifen was administered at E14.5, Prox1CreER<sup>T2</sup>;N1IC embryos were indistinguishable from their wild-type and heterozygote littermates (Fig. 5C). Expression of Prox1 and podoplanin was unchanged in the lymphatic endothelium of E16.5 Prox1CreER<sup>T2</sup>;N1IC tissues compared to wild-type tissues (Fig. 5D). When recombination was initiated at E13.5, approximately 40% of E18.5 Prox1CreER<sup>T2</sup>;N1IC embryos displayed mild lymphatic defects (Fig. S7), that included small blood-filled subcutaneous lymphatics around the base of the head. Thus, a developmental window exists from E9.75-E13.5 during which Notch1 functions to suppress endothelial Prox1 in veins, most likely via its suppression of Coup-TFI.

*Notch1 activation suppressed VEGFR-2 and VEGFR-3, but not Nrp2*

Expression of multiple lymphatic specific proteins was reduced in the LEC progenitors in Prox1CreER<sup>T2</sup>;N1IC embryos; however, misspecified LECs still segregated from veins and coalesced at sites of LS formation. LEC migration from veins requires VEGF-C/VEGFR-3/Nrp2 signaling (Karkkainen et al. 2004; Kärpänen T 2006; Xu et al. 2010). Expression of the VEGF-C receptor complexes, VEGFR-2, VEGFR-3 and the VEGFR-3 co-receptor, Nrp2, was evaluated. At E14.5, VEGFR-2 was expressed in the CV and LS endothelium, and VEGFR-3 expressed in the LS of control tissues (Fig. 6A,B; S8A). In contrast, VEGFR-2 and VEGFR-3 expression was strongly reduced in E14.5 Prox1CreER<sup>T2</sup>;N1IC CV and LS. At E13.5, reduced VEGFR-2 expression was observed in LYVE1+ lymphatic vessels of Prox1CreER<sup>T2</sup>;N1IC tissues; while VEGFR-3 expression was absent in the Prox1CreER<sup>T2</sup>;N1IC lymphatic vessels (Fig. S8B,C). A reduction in VEGFR-3 was observed in limb bud peripheral lymphatics of gain-of-function tissues (Fig. S9A). Nrp2
expression within the LS endothelium was unaffected in \textit{Prox1CreER^{T2};N1IC} presumptive lymphatics (Fig. 6C), even though Prox1 and podoplanin expression was reduced (Fig 6D). VEGFR-2 and VEGFR-3 were both strongly downregulated by Notch activation in Prox1+ LEC progenitors, while Nrp2 expression was maintained.

\textit{Notch1 activation in Prox1+ endothelial cells resulted in abnormal thoracic duct and peripheral lymphatic morphogenesis.}

We examined the morphology of the thoracic duct and the peripheral lymphatics in embryos with ectopic Notch1 activation. Staining for LYVE1 or podoplanin revealed a defect in thoracic duct morphogenesis at the level of the descending thoracic aorta at E14.5. Instead of a well-defined thoracic duct observed in wild-type embryos, the thoracic duct of \textit{Prox1CreER^{T2};N1IC} embryos consisted of numerous small vessels (Fig. 7A). This phenotype correlated with discontinuous expression of podoplanin. The dermal lymphatics were also more numerous in the \textit{Prox1CreER^{T2};N1IC} tissues with discontinuous LYVE1 expression and reduced podoplanin levels (Fig. 7B). As podoplanin is necessary to maintain the segregation between BEC and LECs, we evaluated the expression of the blood endothelial markers, CD34 and Nrp1, in the peripheral lymphatics. CD34+ and Nrp1+ ECs were found in the enlarged peripheral lymphatic vessels of \textit{Prox1CreER^{T2};N1IC} embryos, a phenotype not seen in control tissue (Fig. 7C). LYVE1+ ECs were not observed in the peripheral blood vasculature indicating defects were restricted to the lymphatic vasculature. The ectopic appearance of Nrp1 and CD34 in peripheral lymphatics seen upon Notch1 activation could be interpreted as a defect in separation of blood
and LECs during development, which might lead to the abnormal blood-filled lymphatic vasculature.

*Presumptive LS of Prox1CreER\textsuperscript{T2};N1IC embryos did not express blood endothelial cell markers.*

Prox1 downregulates the expression BEC genes (Petrova et al. 2002; Hong and Detmar 2003; Johnson et al. 2008). We determined the expression of the BEC markers, Nrp1 and CD34, both repressed by Prox1 (Wigle et al. 2002; Johnson et al. 2008). In E13.5 and E14.5 Prox1CreER\textsuperscript{T2};N1IC tissues, Nrp1 and CD34 expression was identical to the controls (Fig. S10A,B). Neither Nrp1, nor CD34 was misexpressed in the LS endothelium demonstrating that the LS endothelium of Prox1CreER\textsuperscript{T2};N1IC embryos did not result misexpress BEC specific proteins.

*Notch1 activation in Prox1+ endothelium did not induce arterial endothelial cell proteins.*

Global or endothelial loss of Notch signaling results in venous gene expression in the arterial endothelium, while ectopic Notch activation leads to misexpression of arterial genes in the venous endothelium (Gridley 2010). CV expression of arterial endothelial specific proteins, CD34 and ephrinB2, and vascular smooth muscle cell recruitment was determined in the gain-of-function model. CD34 expression was unaltered in Prox1CreER\textsuperscript{T2};N1IC tissues (Fig. S10B). EphrinB2 expression was restricted to the aortic endothelium, and SMA was expressed in the aortic vascular smooth muscle cells of Prox1CreER\textsuperscript{T2};N1IC and wild-type embryos (Fig. S11A,B). Weak punctate expression of ephrinB2 observed in the wild-type LS was not seen in Prox1CreER\textsuperscript{T2};N1IC LSs (Fig. S11A). Thus, midgestational Notch activation in the venous endothelium did not promote arterial endothelial differentiation, suggesting that there is a
limited time window prior to E9.75 in which Notch functions to regulate arterial/venous endothelial identity.

**Discussion**

We report a novel role for Jagged1/Notch1 signaling in the venous endothelium during lymphatic endothelial specification, whereby Notch suppresses lymphatic endothelial differentiation to maintain venous cell identity. We demonstrated that Notch1 inhibited Prox1 expression within the CV, most likely via suppression of Coup-TFII. Loss of Notch signaling, via either loss of *Notch1* or expression of DNMAML, in the LEC progenitors resulted in Prox1+ ECs in the CV and disrupted lymphatic and CV separation. Loss of Notch signaling led to over-commitment of LEC progenitors resulting in enlarged lymphatic sacs and vessels. Loss of one allele of *Notch1* also rescued embryonic lethality due to Prox1 haploinsufficiency and resulted in a significant increase in Prox1+ EC progenitor cells at E10.5. In contrast, ectopic Notch1 signaling suppressed endothelial expression of Prox1 resulting in perturbed LEC differentiation. These misspecified LECs failed to express LEC specific proteins, podoplanin, LYVE1 and VEGFR-3, but still emerge from veins and form disorganized lymphatic sac-like structures. In these gain-of-function embryos, BECs were found incorporated into the endothelium of blood-filled peripheral lymphatics suggesting a failure in either the segregation of blood and LEC types or maintenance of LEC fate. Taken together, our results demonstrate an essential role for Notch1 in limiting the number of LECs that differentiate from the embryonic veins.
Our studies suggest that Notch has a distinct role in veins relative to arterial endothelium. Unlike the aorta where Notch1, Dll4 and Jagged1 are strongly and uniformly expressed, Notch1 and Jagged1 expression was weak and discontinuous in the E9.75 and E10.5 veins when Prox1+ LEC progenitors arise. Consistent with the low levels of ligand and receptor expression, Notch activity was weaker in the CV as compared to the aorta. In leukemogenesis and T-cell development, different levels of Notch signaling transactivate distinct target genes (Liu et al. 2010). Thus, the low levels of Notch signaling in the venous endothelium may induce unique target genes from those in arteries. In fact, activation of Notch in the Prox1+ progenitors did not lead to the expression of arterial specific proteins in the CV. The ability of Notch to drive arterial specification may be limited to a specific developmental time window. Alternatively, Prox1+ progenitors may be sufficiently committed to the lymphatic fate to override Notch-driven arterialization.

The genetic studies presented demonstrate that Notch1 functions in the venous endothelium to suppress lymphatic endothelial differentiation. Reduction of Notch signaling in Prox1+ venous cells resulted in precocious Prox1 expression in the CV. The extent of Prox1 missexpression in the CV of Notch loss-of-function embryos was much more extensive than that observed for either podoplanin or LYVE1. Therefore, Prox1 expression in the venous endothelium was not sufficient to drive LEC differentiation, and an additional factor with polarized expression may cooperate with Prox1 in LEC specification. One such potential factor, Sox18 is necessary for Prox1 expression and LEC specification (Francois et al. 2008). We found that Notch activation did not alter Sox18 in cultured HdLECs, though the effect of altering Notch1 signaling on Sox18 expression in vivo remains to be determined.
The ability of Notch1 to suppress endothelial Prox1 was limited to E9.75-E13.5. This developmental window overlaps with the requirement of Coup-TFII for Prox1 expression (Srinivasan et al. 2010), suggesting Notch1 inhibited Prox1 via Coup-TFII repression. In HdLECs, Notch via its induction Hey1 and Hey2, inhibited Coup-TFII reporters (Kang et al. 2010). In the E10.5 CV, Notch activity and Coup-TFII expression was in neighboring cells consistent with Notch inhibiting Coup-TFII via lateral inhibition. In fact, ectopic Notch activation in Prox1+ progenitors strongly suppressed Coup-TFII expression in both the venous and lymphatic endothelium. Loss of Coup-TFII was observed at E12.5, two days before strong Prox1 downregulation observed at E14.5. Taken together, the data suggests that Jagged1/Notch1 signaling in a subset of venous ECs downregulates Coup-TFII to suppress LEC differentiation and, by default, maintain venous identity. In cells with little to no Notch signaling, Coup-TFII is upregulated to drive Prox1 in LEC progenitors and repress Notch1 signaling. Thus, we propose that Notch1 regulates venous/lymphatic specification via suppression of the Coup-TFII/Prox1 signaling axis. Whether Notch signaling more directly represses Prox1, possibly at the level of transcription, remains unknown.

Loss and activation of Notch1 signaling both led to a failure to separate the blood and lymphatic vasculature, but by distinct mechanisms. In Prox1CreERT2;N1f/f embryos, lymphatic vessels sometimes failed to separate from the CV, most likely due the persistence of LECs within the CV. In contrast, BECs were incorporated in the affected peripheral lymphatic vessels of Prox1CreERT2;N1IC embryos. This gain-of-function phenotype may arise from the reduced podoplanin expression, as podoplanin is necessary to maintain the segregation of blood and lymphatic vessels (Bertozzi et al. 2010). In support of this hypothesis, Notch1 signaling via the
induction of Hey1 and Hey2 suppressed podoplanin in cultured HdLECs (Fig. 1; Kang et al. 2010).

In murine dermal lymphatics, Notch1 and podoplanin have non-overlapping expression (Kang et al. 2010). The reduction of podoplanin in Prox1CreERT2;N1IC embryos was most likely due to a loss of Prox1, as podoplanin is induced by Prox1 (Johnson et al. 2008; Kim et al. 2010). In fact, we found that the loss of podoplanin expression was coincident with the loss of Prox1.

Loss of Notch signaling resulted in an increase in the number of LECs that emerged from the CV that correlated with lymphatic vessel enlargement and increased lymphatic density. Unexpectedly, misspecified LECs in the Prox1CreERT2;N1IC embryos still separated from veins and formed disorganized lymph sac-like structures, although Notch1 activation suppressed VEGFR-3 in this model. This downregulation of VEGFR-3 may occur via Notch induction of Hey1/2. Alternatively, it may be secondary to Notch suppression of the CoupTFII/Prox1 axis, as VEGFR-3 is a transcriptional target of both Coup-TFII and Prox1 (Lin et al. 2010; Srinivasan and Oliver 2011). VEGFR-3 and its co-receptor Nrp2 have been shown to be necessary to promote LEC migration towards VEGF-C (Karkkainen et al. 2004; Xu et al. 2010). It is unknown if alternative signaling pathways can promote lymphatic endothelial migration independent of VEGFR-3. The expression of VEGFR-3 co-receptor, Nrp2, was unaffected in the LS and vessel endothelium of the gain-of-function embryos. Nrp2 can form complexes with plexin to bind semaphorin3F in cultured LECs (Coma et al. 2011). Thus, Nrp2 may provide signals for migration away from the CV via different ligands, despite the reduced VEGFR-3 levels. Alternatively, our in vitro studies suggest that Notch transiently induces VEGFR-3 in HdLECs, and this may be sufficient for the misspecified LECs to migrate away from veins. In fact, the number of Prox1+ progenitors emerging from the CV did not differ between control and Prox1CreERT2;N1IC
embryos at E10.5. The level of VEGFR-3 necessary for LEC migration may also be below the level of antibody detection and still functional in our model. Finally, a population of Nrp2\textsuperscript{high}/LYVE1\textsuperscript{low} LECs has been identified to migrate directly off the CV, but do not incorporate into lymph sacs and instead go on to form peripheral lymphatic vessels (François et al. 2011). In the Notch gain of function model, the lymphatic channels that arise at lymph sac sites may arise from the migratory Nrp2\textsuperscript{high}/LYVE1\textsuperscript{low} LECs. Thus, the disorganized淋巴 sac-like structures consist of numerous misspecified lymphatic vessels in \textit{Prox1CreER}\textsuperscript{T2};\textit{N1IC} embryos. Whether this Nrp2\textsuperscript{high}/LYVE1\textsuperscript{low} population also expresses VEGFR-3 is unknown.

Our study of venous/lymphatic differentiation in mammals uncovered a unique role for Notch in limiting lymphatic endothelial differentiation in veins. Recent studies in zebrafish suggest that blood endothelial Dll4 signals to the adjacent lymphatic endothelium to regulate lymphatic sprouting angiogenesis and formation of the thoracic duct (Geudens et al. 2010). Our studies do not exclude the possibility, that Notch has a role in lymphatic sprouting or functions in guiding lymphatic growth along arterial vasculature. We observed that Notch signal activation no longer suppressed Prox1 after E14.5, and thus this would allow for Notch to function in the Prox1+ lymphatic endothelium later in development. Using inhibitors of Notch1 or Dll4, it was recently shown Notch regulates postnatal and pathological lymphatic sprouting angiogenesis (Niessen et al. 2011), and Notch has a role in restricting in lymphatic tip cells (Zheng et al. 2011). Consistent with these findings, Notch1 and Notch4 are expressed in postnatal dermal lymphatic vessels and tumor lymphatic vasculature (Shawber et al. 2007). Thus, Notch may have a distinct role in the postnatal lymphatic endothelium.
We concluded that the venous to lymphatic differentiation event represents a unique a Notch-regulated cell fate decision. Once the fate of lymphatic endothelium has been achieved, the differentiated lymphatic endothelium may once again call upon a Notch fate mechanism to further specialize or shape the lymphatic vasculature.
Appendix 1

Figures
Wild-type CreERT2;N1fl/fl

Wild-type CreERT2;N1fl/fl

CD31 Prox1

Wild-type CreERT2;DNMAML

CreERT2;DNMAML

Prox1+-EC out of CV

Lymphatic Area

DNMAML

CreERT2;DNMAML

Lymphatic Area

Lymph Vessel Density
Wild-type CreER\textsuperscript{T2};N1\textsuperscript{fl/fl} A Prox1/Podoplanin Podo

CD31/Podoplanin

Prox1/Podoplanin

C

Wild-type CreER\textsuperscript{T2};N1\textsuperscript{fl/fl}

CD31/Podoplanin

D

Wild-type CreER\textsuperscript{T2};N1\textsuperscript{fl/fl}

CD31/LYVE1

LYVE1

E

Prox1\textsuperscript{+}-EC in CV

Prox1\textsuperscript{+}-EC out CV

N1\textsuperscript{fl/+} Prox1\textsuperscript{CreGFP/+} Prox1\textsuperscript{CreGFP/+;N1\textsuperscript{fl/+}}
Shawber_Fig5.
A  

Wild-type  
CreERT2;N1IC  

LYVE1  
-  

podoplanin  
-  

B  

Wild-type  
CreERT2;N1IC  

LYVE1  
-  

podoplanin  
-  

C  

Wild-type  
CreERT2;N1IC  

LYVE1/Nrp1  

podoplanin  
-  

LYVE1/CD34  
-  

Shawber Fig 7.
Figure Legends

Figure 1. Notch1 regulation of LEC specific genes \textit{in vitro}, and Notch1 expression/activity in E9.75/E10.5 veins. A) Prox1, LYVE1 and b-actin RT-PCR and Prox1 and a-tubulin Westerns of LacZ and N1IC expressing HMVECs. B-D) Prox1, LYVE1, podoplanin, VEGFR-2 and VEGFR-3 qRT-PCR of N1IC, Hey1, Hey2 and GFP expressing HdLECs. E) VEGFR-3, Hey1 and Hey2 qRT-PCR on RNA isolated at different times post EDTA-induced Notch activation. Data represented as mean +/- s.e.m. *p < 0.05, **p < 0.05, ***p<0.005. F) Endomucin (BEC marker), Prox1 (yellow arrowheads), and Notch1 staining of serial E9.75 transverse sections (20X mag.). Endomucin or Notch1 (N1)/GFP staining of E9.75 TNR transverse sections to determine Notch activation (white arrowheads; 50X mag.) G) Endomucin/Notch1, endomucin (endo)/Prox1 or Notch1/Prox1 staining of serial E10.5 sagittal thick sections. Boxed areas enlarged on right (20X mag.). H) Notch1/Prox1 staining of E10.5 sagittal thick sections. White arrowheads highlight Prox-/N1+ ECs, yellow arrowheads Prox+/N1- ECs, blue arrowheads, Prox1+/N1+ within the CV (20X mag.). I) Endomucin/GFP staining of E10.5 TNR transverse section. White arrowhead highlight ECs with Notch activity. aorta (ao), brachial arch (ba), cardinal vein (cv), descending aorta (dao), dorsal (d), jugular vein (jv), posterior cardinal vein (pcv), ventral (v).

Figure 2. E9.75 conditional loss of Notch in Prox1+ ECs increased LEC differentiation at E14.5. A) \textit{Prox1CreER}^{T2};\textit{N1}^{flo/flo} (\textit{CreER}^{T2};\textit{N1}^{flo/flo}) embryos displayed mild edema and occasional blood filled dermal lymphatics (black arrowheads). Boxed areas enlarged below. B) LYVE1 staining of transverse sections at the level of the aortic arch (20X mag.). C) CD31/LYVE1 staining of
transverse sections through the descending thoracic aorta (20X mag.). D) LYVE1 staining of dermal cross-sections (50X mag.) E) CD31/Prox1 staining of transverse sections at the level of the aortic (20X mag.). White boxes enlarged below highlight Prox1+ ECs surrounding the aorta. Yellow boxes enlarged below highlight Prox1+ ECs residing in (white arrowheads) and emerging from CV. G) Quantitation of Prox1+ EC density surrounding the CV (out), and LS luminal area in Prox1CreER\textsuperscript{T2};N1\textsuperscript{flo/flo} and control tissues normalized by area. F) Endomucin/LYVE1 staining of E14.5 DNMAML and Prox1CreER\textsuperscript{T2};DNMAML (CreER\textsuperscript{T2};DNMAML) transverse sections through the level of the brachial arch (upper) and aortic arch (lower;10X mag.). H) Quantitation of LS luminal area and lymphatic vessel density in Prox1CreER\textsuperscript{T2};DNMAML and DNMAML tissues normalized by area. G,H) Data represented as mean +/- s.e.m. *p< 0.05, **p< 0.0005. aorta (ao), cardinal vein (cv), internal jugular vein (ijv), lymph sac (ls), subclavian vein (scv).

**Figure 3. Loss of Notch signaling in Prox1+ ECs resulted in lymphatic specification within the CV at E14.5, and rescued the Prox1 heterozygous phenotype at E10.5.** A) Prox1/podoplanin staining of transverse sections at the level of the aortic arch (20X mag.). B) Quantitation of Prox1+ EC density in the CV. C) CD31/podoplanin staining of transverse sections at the level of the aortic arch. B,C) Podoplanin expression (yellow arrowheads) and non-expression (white arrowheads) was observed in the CV Prox1CreER\textsuperscript{T2};N1\textsuperscript{flo/flo} and wild-type tissues, respectively. D) CD31/LYVE1 staining of transverse sections at the level of the aortic arch. LYVE1 expression (yellow arrowheads) and non-expression (white arrowheads) was observed in the CV. A,C,D) 20X mag. Boxed areas are enlarged below. E) Quantitation of Prox1+ EC density in and emerging from CV of N1\textsuperscript{flo/+}, Prox1\textsuperscript{GFPCre/+}, and Prox1\textsuperscript{GFPCre/+;N1\textsuperscript{flo/+}.} B,E) Prox1+ EC in CV
normalized by CV circumference. Data represented as mean +/- s.e.m. *p< 0.05, **p< 0.005, ***p< 0.0005. aorta (ao), cardinal vein (cv).

**Figure 4. Notch1 activation in Prox1+ LEC progenitors resulted in severe edema, blood-filled lymphatics and loss of Prox1.** A) E14.5 Prox1CreERT2;N1IC embryos displayed severe edema (red arrowheads), blood-filled superficial lymphatics and jugular LS (blue arrowhead). B) Podoplanin/Prox1 staining of transverse sections at the level of the aortic arch. Asterisks mark lymph sac-like structures (20X mag). C) Endomucin/Prox1 (20X mag) or Prox1/LYVE1 (50X mag) staining of transverse sections at the level of the aortic arch. Boxed areas enlarged below. D) Quantitation of Prox1+ ECs in lymphatic sacs normalized by LS circumference. E) Quantitation of Prox1+ EC density at E13.5 and E14.5 normalized by area. D,E) Data represented as mean +/- s.e.m. *p< 0.05, ***p< 0.0005. aorta (ao), cardinal vein (cv), lymph sac (ls)

**Figure 5. Notch activation downregulated venous and lymphatic endothelial Coup-TFII.** A) GFP/Coup-TFII or Prox1 staining of E10.5 TNR transverse sections at the level of the heart stained. Boxed area enlarged below and re-adjusted to highlight expression pattern. White arrowheads highlight Coup-TFII+/GFP- ECs, yellow arrowheads Coup-TFII-/GFP+ ECs, and blue arrowheads Coup-TFII+/GFP+ ECs (20X mag.) B) Coup-TFII/LYVE1 staining of E12.5 transverse sections at the level of jugular vein/LS and E13.5 transverse sections at the level of the aortic arch (20X mag.). E12.5 control-wild-type, E13.5 control-Prox1CreERT2. C) E16.5 and E18.5 wild-type and Prox1CreERT2;N1IC (CreERT2;N1IC) embryos. D) Prox1/podoplanin or CD31/Prox1 staining of E16.5 of peripheral lymphatic vessels (50X mag.) Tamoxifen was administered E10.5
(B) and E14.5 (C,D). Boxed areas enlarged below. aorta (ao), cardinal vein (cv), jugular vein (jv), jugular lymph sac (jls), lymph sac (ls).

**Figure. 6. Notch activation downregulated VEGFR-2 and VEGFR-3.** A) LYVE1/VEGFR-2, B) LYVE1/VEGFR-3 staining of E14.5 transverse sections. Asterisks mark lymph sac-like structures. C) Nrp2/endomucin, D) Prox1/podoplanin staining of E13.5 transverse serial section. Boxed areas are enlarged below. White arrowhead highlight Prox1/podoplanin coincident expression. A-D) Images at the level of the aortic arch (20X mag.). aorta (ao), cardinal vein (cv), lymph sac (ls).

**Figure. 7. Thoracic duct morphogenesis and BEC/LEC segregation defects in Prox1CreERT2:N1IC embryos.** A) LYVE1 or podoplanin staining of E14.5 transverse sections at the level of the descending aorta (left) and esophagus (right). B) LYVE1 or podoplanin staining of dermal cross-sections. Black arrowheads highlight discontinuous LYVE1 in effected dermal lymphatics. C) Nrp1/LYVE1 or CD34/LYVE1 staining of peripheral lymphatics. Yellow arrowheads marked Nrp1 and CD34 BECs incorporated into LYVE1+ vessels. A-C) Boxed areas are enlarged below. (20X mag.) aorta (ao), cardinal vein (cv), esophagus (es), hemiazygos vein (hv), lymph sac (ls), thoracic duct (td), vagal trunk (vt).
References


Appendix 2

Notch signaling functions in lymphatic valve formation
Notch signaling functions in lymphatic valve formation

Keywords: Notch, lymphatic valve morphogenesis, integrin a9

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Introduction

The lymphatic system consists of capillaries that uptake interstitial fluid and collecting ducts that transport the fluid back into the bloodstream. The intraluminal valves of the lymphatic collecting ducts ensure unidirectional flow of fluid. Mature lymphatic valves are made up of leaflets consisting of an extracellular matrix (ECM) core surrounded by specialized lymphatic endothelial cells (LECs). In mice, lymphatic valve formation begins at embryonic day (E) 15.5. LECs expressing high levels of transcription factors, Prox1 (Prox1\textsuperscript{high}) and Foxc2, begin clustering at putative valve sites, becoming clearly distinguishable from ductal LECs expressing lower Prox1 (Prox1\textsuperscript{low}). At the same time, the first valve-specific ECM component, laminin a5 is detected in the clustered LECs (Sabine et al. 2012). The gap junction protein Connexin 37 (Cx37) is induced in clustered LECs and is required for valve morphogenesis (Kanady et al. 2011, Sabine et al. 2012). A more organized ring-like structure forms and constricts the collecting duct, followed by invagination of valve forming LECs into the duct lumen. During these stages, a valve leaflet ECM core consisting of laminin a5, collagen IV and fibronectin-EIIIA is formed, enabling leaflet elongation and maturation. Integrin α9 expression is increased in valve LECs protruding into the lumen allowing them to attach to the ECM core and form mature valve leaflets (Bazigou et al. 2009, Sabine et al. 2012).

The molecular mechanism of valve initiation and maturation are beginning to be understood. Lymphatic genes Prox1, Foxc2, integrin a9, fibronectin splice variant containing the EIIIA domain (FN-EIIIA) and Connexin37 are all highly expressed in lymphatic valves (Bazigou et al. 2009, Kanady et al. 2011, Norrmen et al. 2009, Petrova et al. 2004). Integrin a9 interaction with its ligand FN-EIIIA is necessary for proper valve leaflet ECM core assembly and subsequent...
leaflet elongation while Connexin37 is thought to facilitate the coordination of valve-forming LECs into a more organized valve structure (Bazigou et al. 2009, Sabine et al. 2012). It is unclear how a select group of LECs in these lymphatic vessels is induced to form a valve while the surrounding duct LECs do not adopt the valve fate.

Notch signaling requires direct cell-cell contact between Notch ligand expressing cells and receptor presenting cells (Andersson et al. 2011). Upon ligand binding proteolytic cleavage releases the Notch intracellular domain allowing it to translocate to the nucleus where it forms a transcriptional activation complex with CSL, Mastermind-like and coactivators (Borggrefe and Liefke 2012). The Notch signaling pathway regulates lymphatic sprouting events, both in physiological and pathological settings (Niessen et al. 2011, Zheng et al. 2011). Notch has recently been shown to function in embryonic venous endothelium to restrict the number of venous ECs that go on to adopt the lymphatic fate (Murtomaki et al. 2013). As the Notch pathway mediates bi-potential cell fate decisions in multiple biological settings, we hypothesized that Notch may regulate lymphatic valve fate decisions. Consistent with this hypothesis, Notch1 expression and Notch activation was observed in the valve forming LECs.

To assess Notch function in lymphatic valve morphogenesis we used the Prox1CreERT2 inducible driver (Srinivasan et al. 2007) to delete Notch1 or inhibit Notch signaling by expressing a dominant negative Mastermind-like transgene (Tu et al. 2005) in the lymphatic endothelium at the initiation of valve formation. Lymphatic endothelial-specific loss of Notch1 or of Notch signaling resulted in abnormal Prox1$^{\text{high}}$- Prox1$^{\text{low}}$ expression pattern, reduced integrin a9 and FN-EIIIA expression in valve LECs, and abnormal valve morphology. In human dermal lymphatic endothelial cells (HdLECs), ectopic activation of Notch1 or Notch4 resulted in significant
induction of integrin α9, FN-EIIIA, and Cx37 expression. Thus, we report a novel role for Notch in lymphatic valve formation through its regulation of the Prox1 expression pattern and integrin α9 and FN-EIIIA expression.

**Materials and Methods**

*Mouse lines*

Prox1CreER<sup>T2</sup> (Srinivasan et al. 2007), Notch1<sup>fl/fl</sup> (Yang et al. 2004) and DNMAML (Tu et al. 2005) mice were described. Notch1<sup>fl/fl</sup>, ROSA:LacZ<sup>fl/fl</sup> and TNR mice from Jax Labs. Tamoxifen (20mg/ml) in corn oil was administered via oral gavage or intraperitoneal injection (10mg/40g) to pregnant females at E15.5. 2-6 litters were analyzed for each time-point. 3-6 embryos were analyzed for each genotype.

*Immunohistochemistry*

Mesenteries collected at E16.5, E17.5 and E18.5 were stained as described (Lohela et al. 2008). Samples were fixed in 4% paraformaldehyde, washed, blocked in 5% donkey serum, 0.2% BSA, 0.5% Triton-X100, PBS and incubated overnight with primary antibodies. The following day mesenteries were washed and incubated with secondary antibodies diluted in 0.2% BSA, 0.3% Triton-X100, PBS. Samples were washed and mounted with Vectashield with DAPI (Vector Labs). MOM kit (Vector Labs) was used with mouse monoclonal antibodies. Primary antibodies are listed in Table S1. Secondary Alexa Fluor antibodies from Invitrogen.
Constructs and Cell Culture

HdLECs were isolated from human neonatal foreskins (Exemption AAAB-1700) as described (Murtomaki et al. 2013) and maintained in EGM-2MV BulletKit (Lonza) with VEGF-C (10 ng/ml, RnD Systems). HdLECs were infected with adenovirus expressing the intracellular domain of Notch1 (N1IC) and Notch4 (N4/int-3), HEY1, HEY2, Prox1, LacZ or GFP (Shawber et al. 2007, Tung et al. 2009) with expression verified by qRT-PCR and/or Western analyses.

RT-PCR and Western analyses

RNA was isolated 48h after adenoviral infections using RNeasy Mini Kit (Qiagen) and cDNA synthesized with Verso cDNA synthesis kit (Fisher Scientific). qPCR was performed with Sybr Green Master Mix (ABI) or Absolute Blue qPCR SYBR Green ROX Mix (Fisher Scientific) using 7300 Real-Time PCR System (ABI). Gene specific PCR products were generated and cloned into pDrive (Stratagene) for reference standards. b-actin was used to normalize qRT-PCRs. Primer sequences listed in Table S2. Protein was isolated 48h after adenoviral infections in TENT lysis buffer (Shawber et al. 2007) and Western analyses were performed with antibodies listed in Table S1.

Flow Cytometry

48h after adenoviral infection HdLECs were harvested, incubated with anti-integrin a9b1 antibody (abcam), washed, labeled with anti-rabbit-APC (Jackson Immunoresearch) and flow cytometry performed. 10,000 cells per group were counted using FACSCalibur and CellQuestPro acquisition software (BD Biosciences).
Statistical Analysis and Imaging

Quantification of Prox1+ area was done with ImageJ software, normalizing to duct length. Statistical significance was determined using 2-tailed Student’s T-test, with P-value of 0.05 or less statistically significant. Data presented is representative of three or more independent experiments. Images were acquired using either laser scanning confocal Zeiss LSM 510 Meta microscope and LSM software, or Nikon A1 confocal microscope and NIS Elements software.

Results and Discussion

Notch1 expression and Notch activity becomes enriched in lymphatic valves

Notch1 expression was analyzed in lymphatic ducts and valves of the embryonic mesenteries during development. At E16.5, Notch1 was uniformly expressed in the mesenteric collecting lymphatic ductal endothelium (Fig. 1A). At E17.5, Notch1 expression was highest at putative valve sites, identified as clusters of lymphatic endothelial cells (LECs) expressing high levels of Prox1 (Prox1$^{\text{high}}$; Fig. 1A). By E18.5, Notch1 expression was enriched in valve forming LECs and weak Notch1 expression was seen in the lymphatic collecting ducts (Fig. 1A, S1). Notch1 expression was seen in adjacent blood vessels and capillaries at all time-points.

We assessed Notch signaling in developing mesenteric lymphatic vessels and valves using the transgenic Notch reporter mouse (TNR), which expresses GFP in response to CSL-dependent Notch signaling. TNR mesenteries were stained for GFP and a lymphatic marker, VEGFR-3 or integrin α9 (α9). At E17.5, Notch signal activation was seen in clusters of cells at the
putative valve sites (Fig. 1B). LECs in the developing collecting ducts and blood vessels adjacent to the ducts also expressed GFP (data not shown). At E18.5, GFP expression was mainly detected in the a9+ LECs of the lymphatic valves (Fig. 1B). The initial expression of Notch1 and Notch reporter protein in the mesenteric collecting ducts and subsequent restriction of expression to the lymphatic valves suggested Notch1 functions in lymphatic valve development.

**Notch1 is required for lymphatic collecting duct valve morphogenesis**

We examined the effect of lymphatic endothelial specific loss of Notch1 on lymphatic valve development. A \textit{Prox1CreERT2} driver was crossed with mice carrying a floxed allele of \textit{Notch1} (\textit{N1}^{fl/fl}) to generate \textit{Prox1CreERT2};\textit{N1}^{fl/fl} (LOF) embryos. Recombination was induced with tamoxifen at E15.5, when murine valve morphogenesis begins, and embryonic mesenteries analyzed at E18.5 (Fig. S2A-C). \textit{Prox1CreERT2};\textit{N1}^{fl/fl} embryos were indistinguishable from control littermates (data not shown). Control mesenteric valve forming LECs expressed high levels of Prox1 (Prox$^{1\text{high}}$), while the duct LECs expressed lower levels of Prox1 (Prox$^{1\text{low}}$) (Fig. 2A). Prox$^{1\text{high}}$ valve LECs had reoriented themselves perpendicularly to the duct walls in control mesenteries (Fig. 2B). The Prox$^{1\text{high}}$ LECs of the \textit{Prox1CreERT2};\textit{N1}^{fl/fl} mesenteric lymphatic ducts failed to reoriented perpendicularly to the ductal LECs (Fig. 2B). LOF mesenteric lymphatics had fewer valves and those that were observed were poorly organized. Prox1 expression in mesenteries of LOF embryos was more uniform in LECs along the duct with an overall significant increase in Prox$^{1\text{high}}$ LECs over the length of a duct (Fig. 2C). Clusters of valve forming LECs were reduced in the dermal lymphatics of LOF embryos (Fig. S3). The increase in the number of Prox$^{1\text{high}}$ LECs suggests that Notch1 functions to restrict the number of LECs that go
on to become Prox$^{\text{high}}$. Even when Prox$^{\text{high}}$ LECs in LOF mice come together their failure to reorient may perturb subsequent steps of valve formation, a phenotype similar to that described for Connexin37 mouse mutants (Kanady et al. 2011, Sabine et al. 2012).

Integrin $\alpha 9$ and Fibronectin $\text{EIIIA}$ expression are dependent on Notch in the mesenteric valve LECs

Formation of the lymphatic valve leaflets requires the expression of integrin $\alpha 9$ and its ligand fibronectin $\text{EIIIA}$ (FNEIIIA). The expression of these proteins was evaluated in the disorganized lymphatic valves in Prox1CreER$^{T2}$;N1$^{fl/fl}$ and compared to control embryos. At E18.5, expression of integrin $\alpha 9$ and FNEIIIA and high levels of Prox1 (Prox1$^{\text{high}}$) was observed in the maturing mesenteric valves of control embryos (Fig. 2D). In the poorly organized LOF valves in which Prox1$^{\text{high}}$ LECs had migrated into the lumen, integrin $\alpha 9$, and FNEIIIA expression was reduced, demonstrating that Notch1 regulates integrin $\alpha 9$, and FNEIIIA expression in valve LECs.

We speculated that additional Notch proteins compensate for the loss of Notch1, partly based upon incomplete loss of integrin $\alpha 9$ and FNEIIIA expression observed in the LOF embryos. We addressed this hypothesis using a transgenic mouse line to express a dominant-negative Master-mind like-GFP fusion protein (DNMAML) (Tu et al. 2005) in Prox1-expressing LECs. DNMAML forms an inactive complex with Notch bound to CSL and thus inhibits canonical Notch/CSL signaling. $\text{DNMAML}^{fl/fl}$ mice were crossed with the Prox1CreER$^{T2}$ driver to generate Prox1CreER$^{T2}$;DNMAML$^{fl/+}$ embryos. Recombination was induced at E15.5 and mesenteries collected at E18.5. GFP expression was detected in Prox1CreER$^{T2}$;DNMAML$^{fl/+}$ (Fig. S4A-C), but not control (DNMAML$^{fl/+}$) mesenteries (data not shown). Prox1CreER$^{T2}$;DNMAML$^{fl/+}$ embryos
had a valve morphogenesis phenotype that was more severe (Fig. 2D, 3A) than the Prox1CreER^{T2};N1^{fl/fl} embryos (Fig. 2D,). Many of the Prox1CreER^{T2};DNMAML^{fl/+} embryos had no identifiable lymphatic valves. Where partial valves were present these structures usually only expressed one of the two valve markers, integrin a9 or FNEIIIA (Fig. 3A). Integrin a9 was also expressed by smooth muscle cells around arteries in the mesentery and this was not affected in Prox1CreER^{T2};N1^{fl/fl} or Prox1CreER^{T2};DNMAML^{fl/+} mesenteries (Figs. 2D, 3A). Integrin a9 and its ligand FNEIIIA are indispensable for valve leaflet formation. As Integrin a9 and FNEIIIA expression is reduced in LOF mesenteries with defective valves, we propose that Notch functions in valve morphogenesis by inducing expression of these proteins.

Notch induces integrin a9, fibronectin EIIIA and Connexin37 in human dermal lymphatic endothelial cells in vitro.

Notch functions by eliciting transcriptional responses and our data implicate Notch1, and possibly Notch4, in promotion of lymphatic valve formation. We sought to determine if these Notch proteins could regulate the expression of lymphatic valve proteins in LECs by expressing constitutively active Notch1 intracellular domain (N1IC) or a form of constitutively active Notch4, N4/int-3 (Shawber et al. 2007, Tung et al. 2009). Activated Notch1 and Notch4 were expressed in human dermal lymphatic endothelial cells (HdLECs) by adenoviral infection and LEC transcript and protein levels evaluated. N1IC was a more potent inducer of Notch downstream effectors Hey1 and Hey2 than N4/int-3 (Fig. S5). N1IC and N4/int-3 induced integrin a9 transcripts (Fig. 4A), while only N1IC resulted in increased integrin a9 surface expression (Fig. 4A). The absence of increased integrin a9 surface in N4/int-3 expressing HdLECs
may be due to its reduced activity relative to N1IC (Fig. S5). The transcriptional repressors Hey1 and Hey2 did not affect integrin α9 levels suggesting the α9 gene is a direct target of Notch or is regulated by other Notch induced proteins.

Mammalian fibronectin consists of various isoforms, created through alternative splicing (Muro et al. 2003). Fibronectin EIIIA splice variant is expressed in the developing lymphatic valve ECM core (Bazigou et al. 2009). We evaluated fibronectin transcript and protein levels for fibronectin with (FN-EIIIA+) or without (FN-EIIIA-) the EIIIA domain. Expression of N1IC or N4/int-3 increased both FN-EIIIA+ and FN-EIIIA- transcripts (Fig. 4B). Western blot analyses showed increased total fibronectin levels and FN-EIIIA+ in both N1IC and N4/int-3 samples. The antibody used to detect total fibronectin recognizes both FN-EIIIA+ and FN-EIIIA- proteins, thus the increase in total fibronectin levels may be due to increased FN-EIIIA+ levels or a combination of increased FN-EIIIA+ and FN-EIIIA- levels.

Gap junction protein Connexin37 (Cx37) is specifically expressed in developing lymphatic valves and required for valve formation in mice (Kanady et al. 2011, Sabine et al. 2012). We evaluated Cx37 transcript and protein levels following Notch signal activation in HdLECs and observed strong induction of both Cx37 transcripts and protein by either N1IC or N4/int-3. As Cx37 is required for reorientation of Prox1^{high} LECs in valve morphogenesis, the reorientation phenotype in the LOF models may be due to a loss of Cx37 expression.

We demonstrate that lymphatic specific loss of either Notch1 or a block of canonical Notch signaling using DNMAML disrupted lymphatic valve morphogenesis. Loss of Notch altered proper levels of Prox1 expression along the duct, caused abnormal valve morphology and reduced integrin α9 and FN-EIIIA expression by valve forming LECs. Either Notch1 or
Notch4 activated alleles induced integrin α9, FN-EIIIA+ and Cx37 transcript and protein expression. We conclude that Notch functions to induce the expression of both FN-EIIIA and integrin α9 in valve LECs. Notch induced their expression in vitro and their expression was reduced when Notch signaling was disrupted in vivo. We propose that Notch signaling functions in multiple steps of valve formation. Notch regulates Prox1<sup>high/low</sup> ratios, reorientation of Prox1<sup>high</sup> valve-forming LEC and induction FN-EIIIA and integrin α9 in valve LECs. We conclude that Notch signaling is essential for LECs to properly adopt the valve LEC fate.
Appendix 2

Figures
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<th>Control</th>
<th>CreER(^{T2}), DN-MAML(^{fl/+})</th>
<th>α9 FNEIII A Prox1</th>
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**A** Integrin α9 Transcripts

![Graph showing fold change to LacZ for different samples.]

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<th>Sample</th>
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<th>% in M2</th>
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<td>LacZ</td>
<td>93.2</td>
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<td>N4int3</td>
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**B** FN-EIIIA+ Transcripts

![Graph showing fold change to LacZ for different samples.]

**C** Cx37 Transcripts

![Graph showing fold change to LacZ for different samples.]

**FN-EIIIA- Transcripts**

![Graph showing fold change to LacZ for different samples.]

Sample % in M1 % in M2
LacZ 93.2 6.8
N1IC 83.6 16.4
N4int3 93.0 7

**WB**

- FN-EIIIA
- total FN
- α-tubulin
Figure Legends

Figure 1. Notch1 expression pattern and Notch signaling activity in embryonic lymphatic ducts and valves. A) E16.5, E17.5 and E18.5 wild-type mesenteries were stained for Notch1 and Prox1. Boxed areas enlarged on right. Lymphatic ducts marked with white dotted lines. White arrows indicate lymphatic valves. Yellow asterisks mark blood capillaries. Scale bars 100 µm. B) TNR mesenteries were stained for GFP to detect Notch activity and VEGFR-3 (E17.5) or integrin a9 (E18.5). Lymphatic ducts marked with white dotted lines. Artery (A), lymphatic collecting duct (L) and vein (V). Scale bars 50 µm.

Figure 2. Lymphatic endothelial specific loss of Notch1 resulted in defective valve morphogenesis and decreased valve LEC integrin a9 and FN-EIIIA expression. Notch1 was deleted in LECs at E15.5 by tamoxifen administration and embryonic mesenteries isolated at E18.5. A) Control (N1fl/+ ) and CreER<sup>T2;</sup>N1<sup>fl/fl</sup> (Prox1CreER<sup>T2;</sup>N1<sup>fl/fl</sup>) mesenteries stained for Prox1. Arrows point at Prox1<sup>high</sup> valves, and bracket denotes expansion of Prox1<sup>high</sup> cells within the duct walls in Prox1CreER<sup>T2;</sup>N1<sup>fl/fl</sup> mesenteries. Lymphatic collecting duct (L). Scale bars 100 µm. B) Control (N1<sup>fl/+ </sup>) and CreER<sup>T2;</sup>N1<sup>fl/fl</sup> (Prox1CreER<sup>T2;</sup>N1<sup>fl/fl</sup>) mesenteries stained for Prox1 and DAPI. White arrowheads indicate Prox1<sup>high</sup> valve forming LECs in control valve, reoriented perpendicularly to duct wall. Yellow arrowheads indicate Prox1<sup>high</sup> LECs that are poorly oriented in LOF valve. Scale bars 50 µm. C) Quantification of Prox1<sup>high</sup> area normalized by duct length. Note increased Prox1<sup>high</sup> area, seen as more uniform expression along LOF ducts. Error bars indicate s.d. *p<0.05. D) Control (N1<sup>fl/fl</sup>) and CreER<sup>T2;</sup>N1<sup>fl/fl</sup> (Prox1CreER<sup>T2;</sup>N1<sup>fl/fl</sup>) mesenteries stained for Prox1, integrin a9 and FN-EIIIA. Note abnormal location and morphology of valves in
LOF embryos. Lymphatic collecting ducts marked by white dotted line. White arrows indicate valves magnified in upper left corner. Asterisk marks a cluster of Prox1<sup>high</sup> cells magnified in bottom right corner. Artery (A), lymphatic collecting duct (L) and vein (V). Scale bars 100 µm.

**Figure 3. Lymphatic endothelial specific loss of Notch signaling resulted in defective valve formation with decreased integrin α9 and FN-EIIIA expression.** DNMAML expression was induced in LECs at E15.5 by tamoxifen and embryonic mesenteries isolated at E18.5. A) DNMAML<sup>fl/+</sup> (Control) and Prox1<sup>CreER<sup>T2;DNMAML<sup>fl/+</sup> (CreER<sup>T2;DNMAML<sup>fl/+</sup> mesenteries stained for Prox1, integrin α9 and FN-EIIIA. Middle row shows LOF mutant with mild phenotype, showing reduced integrin α9 and FN-EIIIA expression. Bottom row shows severely affected mesentery with uniform Prox1 expression and complete loss of both integrin α9 and FN-EIIIA. Lymphatic collecting ducts marked with white dotted lines. White arrows indicate valves magnified in upper left corner. Artery (A), lymphatic collecting duct (L) and vein (V). Scale bars 100 µm.

**Figure 4. Ectopic activation of Notch1 or Notch4 induces integrin α9, FN-EIIIA and Connexin37 expression in HdLECs.** HdLECs were adenovirally infected with N1IC, N4/int-3, HEY1, HEY2, Prox1 or LacZ coding viruses and RNA and protein were isolated 48h after infection. A) qRT-PCR and FACs for integrin α9. B) qRT-PCR of fibronectin transcripts containing EIIIA (FN-EIIIA+) and without EIIIA (FN-EIIIA-). Western analyses of FN-EIIIA, total fibronectin or α-tubulin. C) qRT-PCR and western analyses of Connexin37. A-C) All values are means and error bars indicate s.d. *p ≤ 0.05 and **p ≤ 0.005.
References


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