VEGFR3 AND NOTCH SIGNALING IN ANGIOGENESIS

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ACADEMIC DISSERTATION

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To my family
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AJs</td>
<td>Adherens junctions</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
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<tr>
<td>ANG</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic proteins</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorioallantoic membrane</td>
</tr>
<tr>
<td>CCBE1</td>
<td>Collagen- and calcium-binding EGF domains 1 protein</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COUP-TFII</td>
<td>Chicken ovalbumin upstream promoter transcription factor 2</td>
</tr>
<tr>
<td>CV</td>
<td>Cardinal vein</td>
</tr>
<tr>
<td>CX47</td>
<td>Connexin 47</td>
</tr>
<tr>
<td>DA</td>
<td>Dorsal aorta</td>
</tr>
<tr>
<td>DLL4</td>
<td>Delta like ligand 4</td>
</tr>
<tr>
<td>DR</td>
<td>Diabetic retinopathy</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGFL7</td>
<td>Epidermal growth factor-like domain 7</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FIGF</td>
<td>c-fos-induced growth factor</td>
</tr>
<tr>
<td>Flk1</td>
<td>Fetal liver kinase-1</td>
</tr>
<tr>
<td>Flt1</td>
<td>Fms-like-TK-1</td>
</tr>
<tr>
<td>GJC2</td>
<td>Gap junction protein gamma-2</td>
</tr>
<tr>
<td>HEVs</td>
<td>High endothelial venules</td>
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<tr>
<td>HSCs</td>
<td>Hematopoietic stem cells</td>
</tr>
<tr>
<td>HSPGs</td>
<td>Heparan-sulphate proteoglycans</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>ISVs</td>
<td>Intersomitic vessels</td>
</tr>
<tr>
<td>JAM-A</td>
<td>Junctional adhesion molecule A</td>
</tr>
<tr>
<td>KDR</td>
<td>Kinase-insert domain receptor</td>
</tr>
<tr>
<td>LAM</td>
<td>Lymphangioleiomyomatosis</td>
</tr>
<tr>
<td>LECs</td>
<td>Lymphatic endothelial cells</td>
</tr>
<tr>
<td>LOF</td>
<td>Loss of function</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalproteinases</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NP</td>
<td>Neurophilin</td>
</tr>
<tr>
<td>NRARP</td>
<td>Notch-regulated ankyrin repeat protein</td>
</tr>
<tr>
<td>NVU</td>
<td>Neurovascular unit</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>Platelet-derived growth factor BB</td>
</tr>
<tr>
<td>PLGF</td>
<td>Placenta growth factor</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>RBP-J</td>
<td>Recombination signal sequence-binding protein J</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RVO</td>
<td>Retinal vein occlusion</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology-2</td>
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<tr>
<td>SMCs</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>TACE</td>
<td>TNFα-converting enzyme</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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<tr>
<td>TAMs</td>
<td>Tumor associated macrophages</td>
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<tr>
<td>TEMs</td>
<td>TIE2 expressing macrophages</td>
</tr>
<tr>
<td>TIE</td>
<td>Tyrosine kinase with immunoglobulin-like and EGF-like domains</td>
</tr>
<tr>
<td>TJs</td>
<td>Tight junctions</td>
</tr>
<tr>
<td>TK</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>VEC</td>
<td>Vascular endothelial cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VE-PTP</td>
<td>Vascular endothelial protein tyrosine phosphatase</td>
</tr>
<tr>
<td>vSMCs</td>
<td>Vascular smooth muscle cells</td>
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</table>
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals. Unpublished data (study III) are also presented.


III  Zarkada G, Heinolainen K, Makinen T, Alitalo K. VEGFR3 does not sustain angiogenesis without VEGFR2. Manuscript

*Equal contribution

Publication I was included in the doctoral thesis of Tuomas Tammela (2008).

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ABSTRACT

Blood and lymphatic vessels form extensive networks throughout the body, which function in order to deliver oxygen and nutrients to the tissues, to remove extravasated fluid and to absorb dietary lipids. The formation of new blood and lymphatic vessels (termed angiogenesis and lymphangiogenesis) is critical during embryonic development and in the adult, and is regulated by multiple signaling pathways. Vascular Endothelial Growth Factors (VEGFs) and their receptors (VEGFRs), as well as the Notch signaling system, are key governors of blood and lymphatic endothelial cell fate, and regulate angiogenesis and lymphangiogenesis in health and disease. Despite the numerous recent advances in the field of vascular biology, many steps in the complex processes of angiogenesis and lymphangiogenesis remain unclear. In this study we investigated the role of VEGFR3 signaling in blood endothelial cells, tip cell specification, as well as the interplay of the receptor with the VEGFR2 and the Notch signaling pathways during angiogenesis.

VEGFR3 is a tyrosine kinase receptor that is mainly expressed in lymphatic endothelial cells in the adult. We observed VEGFR3 expression in sprouts that guide the blood vascular endothelium in angiogenic conditions. VEGFR3 blockade with a monoclonal antibody displayed synergistic properties with simultaneous VEGFR2 targeting in reducing angiogenesis and inhibiting tumor growth. Furthermore we found that Notch signaling suppresses VEGFR3 expression in endothelial cells, identifying VEGFR3 as a novel tip cell marker, which is normally repressed by Notch activation.

In the next step we employed a combination of genetic and in vitro models to show that loss of VEGFR3 results in a hypervascular phenotype, accompanied by loss of Notch signaling. VEGFR3 could be stimulated by VEGFC and activate Notch in blood endothelial cells. Our results point towards a mechanism where VEGFC produced by macrophages at the vascular front acts via VEGFR3 to activate Notch and turn tip into stalk cells; thus promoting the formation of stable vascular loops. Furthermore we identified the transcription factor FOXC2 as the downstream target of the VEGFC/VEGFR3/Notch signaling cascade. These data reinforce the idea that VEGFR3 has two distinct signaling modalities, one ligand-dependent and one ligand-independent, and that different perturbations in VEGFR3 expression and function result in diverse vascular phenotypes.

Subsequently we investigated the interplay of VEGFR2 and VEGFR3 in postnatal angiogenesis and lymphangiogenesis, using a genetic approach of conditional mutagenesis. Various combinations of genetic ablation of VEGFRs and pharmacological inhibition of Notch showed that VEGFR2 is irreplaceable during sprouting angiogenesis, also in endothelial cells with low Notch signaling, and that it acts upstream of VEGFR3 expression in angiogenic settings. On the other hand VEGFR3 suppressed VEGFR2 expression in a negative feedback loop. Finally we employed for the first time lymphatic endothelial specific deletion of VEGFRs in postnatal conditions and found no significant role for VEGFR2 in lymphatic vessel growth or remodeling, while VEGFR3 signaling was indispensable.
Our results unravel previously unknown roles for VEGFR3 in sprouting angiogenesis and provide new insight into the signaling cross-talk of the receptor with other important regulators of blood vessel development. Increasing our understanding of the molecular mechanisms underlying this process is crucial in order to comprehend the pathophysiology of angiogenesis-related diseases, characterized by excessive or insufficient blood vessel growth, and to promote the emergence of novel angiogenic therapies.
INTRODUCTION

The formation of new blood and lymphatic vessels, termed angiogenesis and lymphangiogenesis respectively, are essential processes for numerous physiological and pathological processes. During the past decades major advances have been made in the field of vascular biology, including the identification of multiple novel molecular players that regulate angiogenesis and lymphangiogenesis. Subsequently some of these regulators have been targeted successfully in preclinical studies and more recently in clinical settings.

Vascular Endothelial Growth Factors (VEGFs) and their receptors (VEGFRs), as well as the Notch signaling system, are major regulators of endothelial cell fate, and their signaling is commonly distorted in a number of pathologies, including tumor growth. It is now becoming clear that these molecules interact on multiple levels to control blood vessel development and growth. Knowledge of these interactions is required for the therapeutic manipulation of the vascular system, in order to treat cancer and other angiogenesis-related diseases.

In this study we investigated the role of VEGFR3 in blood vessel morphogenesis, plus the interplay of this receptor with the main regulator of angiogenesis, VEGFR2, as well as the versatile Notch signaling pathway. Our results, combined with other scientific contributions made over the past years, improve our understanding of the molecular signaling underlying the complex process of sprouting angiogenesis.
REVIEW OF THE LITERATURE

1. ANATOMY AND FUNCTION OF THE VASCULAR SYSTEMS

1.1 The blood vascular system

All large multicellular organisms require a vascular system in order to supply oxygen and nutrients to tissues and to remove carbon dioxide and metabolic waste products. Vertebrates have a closed blood circulatory system, where the heart acts as a pump that circulates highly oxygenated blood to the body via the systemic circulation, and deoxygenated blood from the right side of the heart to the lungs, through the pulmonary circulation. All tissues are vascularized, with the exception of cartilage and the cornea. Blood vessels are lined by endothelial cells (ECs) and are hierarchically organized into arteries, arterioles, capillaries, venules and veins. Capillaries, which are 5-10 µm in diameter and connect arterioles with venules, represent the major site of exchange of water, gases, electrolytes, hormones, cells, nutrients and waste products between the blood and the interstitial fluid.

1.1.1 Blood vessels structure and physiology

In vertebrates, large blood vessels such as arteries, are composed in general from three layers; an interior layer composed of ECs (tunica intima), an intermediate layer composed of vascular smooth muscle cells (vSMCs), nerves and elastin (tunica media), and an external layer composed of collagen and fibroblasts (tunica externa). The composition of blood vessel wall varies, depending on the size and function of the vessel. For example capillaries consist only of a single layer of ECs, pericytes and basal lamina. As the diameter of the blood vessels increases, they become associated with SMCs (arteries, arterioles, veins). Furthermore many veins have valves, composed of two luminal leaflets of two layers of ECs separated by an extracellular matrix (ECM) core, that prevent blood backflow (Bazigou and Makinen 2013).

The basement membrane (BM) is a sheet-like structure of 50–100 nm thickness, which lies basolateral to the endothelium (Vracko 1974). The main components of the BM are type IV collagen, laminin, heparan-sulphate proteoglycans (HSPGs) and nidogen/entactin. The exact composition of the BM varies depending on the tissue. These components have the unique capacity to self-assemble in order to form the BM, which provides structural support and regulates cell behavior. For example matrix metalloproteinases (MMPs) and integrins are key mediators of pro- and anti-angiogenic behavior of the vascular endothelium, and different structural configuration of the components of the BM may have a direct impact on EC behavior during quiescent and angiogenic conditions (Kalluri 2003).

1.1.1.1 Regulation of vascular permeability

Endothelial cells form the inner lining of blood vessels and are important regulators of several functional, metabolic and synthetic functions. ECs are connected to each other with intercellular junctions, termed adheres junctions (AJs), and tight junctions (TJs), which are however not as strictly spatially organized as in epithelial cells (Bazzoni and Dejana 2004). These connections function to regulate
vascular permeability and integrity, as well as vascular homeostasis, by controlling the passage of substances between the intravascular and extravascular compartments. Vascular Endothelial Cadherin (VEC) is the most prominent cadherin at the AJs, and binds to and interacts with VEGFR2 (Shay-Salit, Shushy et al. 2002, Giannotta, Trani et al. 2013) and VE-PTP (Nawroth, Poell et al. 2002). Tight junctions are formed by occludin, claudins and JAM-A, as well as intracellular components like ZO proteins and actin cytoskeleton.

The barrier function of the endothelium is not only regulated at the aforementioned paracellular level (between ECs), but also by transcellular pathways, where materials are transferred through ECs. The morphology of the vascular endothelium is not uniform throughout the vascular tree, and is classified into three different types: continuous, fenestrated and discontinuous. In continuous endothelia (for example in large arteries, lung vessels, heart, skeletal muscle, central nervous system and skin) ECs form an uninterrupted barrier between blood and tissues. Fenestrated endothelia occur in endocrine glands, intestinal tract, pancreas and kidney, where the ECs have round openings around 70 nm in diameter that allow the diffusion of fluids and small solutes. The fenestrae are spanned by thin (5-6 nm) nonmembranous diaphragms. Finally sinusoidal endothelia are a special type of fenestrated capillaries, with larger openings in the ECs (100-200 nm) and a discontinuous basement membrane that allows the transfer of cells and serum proteins across the basal lamina. Sinusoidal endothelia are found in liver, spleen and bone marrow (Aird 2007).

1.1.1.2 Regulation of flow

Blood vessels are covered with mural cells, a term that refers to vSMCs and pericytes. Single or multiple layers of vSMCs cover large arteries and veins, while capillaries are surrounded by pericytes that are embedded in the same basement membrane as the endothelium. Arterioles and venules are covered by mural cells with intermediate properties between typical vSMC and pericytes (Gerhardt and Betsholtz 2003). vSMCs support the large vessels and through their contractile properties regulate vasoconstriction and vasodilation, and subsequently blood flow and blood pressure. Pericytes are also contractile in vitro, in vivo and ex vivo and some studies suggest that they regulate blood flow at the capillary level (Peppiatt, Howarth et al. 2006, Yemisci, Gursoy-Ozdemir et al. 2009, Fernandez-Klett, Offenhauser et al. 2010). The distribution of pericytes in several capillary beds varies greatly throughout the body (Sims 1991) and the CNS vasculature is considered to be the most pericyte covered (Mathiisen, Lehre et al. 2010). In the brain, pericytes are part of the neurovascular unit (NVU), which also includes ECs, astrocyte endfeet, microglia and neurons. Pericytes play a major role in the formation of the blood-brain-barrier (BBB) by regulating brain vessel permeability and endothelial transcytosis (Armulik, Genove et al. 2010, Daneman, Zhou et al. 2010).

1.1.1.3 Organogenesis and tissue regeneration

During embryo organogenesis the blood vasculature acts as a passive conduit of oxygen and nutrients. Moreover, the developing tissues experience inductive interactions from the ECs (Lammert, Cleaver et al. 2001, Matsumoto, Yoshitomi et al.
In the adult, numerous studies demonstrate the involvement of the vasculature in stem cell homeostasis and regeneration, and in tissue regeneration. Endothelial cells of individual organs have a unique and distinct phenotypic and functional signature (Ruoslhahti and Rajotte 2000, Carmeliet 2005, Aird 2007, Aird 2007, Red-Horse, Crawford et al. 2007, Butler, Nolan et al. 2010, Nolan, Ginsberg et al. 2013), and through the production of tissue-specific paracrine trophogens, known as angiocrine factors, they support adult organ regeneration in lung (Ding, Nolan et al. 2011), liver (Ding, Nolan et al. 2010), and bone marrow (Butler, Nolan et al. 2010, Kobayashi, Butler et al. 2010). It has also been suggested that pericytes act as multipotent stem/progenitor cells, which may constitute mesenchymal stem cells (MSCs) (Davidoff, Middendorff et al. 2004, Crisan, Yap et al. 2008, Feng, Mantesso et al. 2011), white adipocyte progenitor cells (Tang, Zeve et al. 2008, Olson and Soriano 2011), muscle stem cells (Dellavalle, Sampaolesi et al. 2007), and neural stem cells (Dore-Duffy, Katychev et al. 2006). However, the lack of absolutely pericyte-specific markers has limited the fate mapping of these cells, raising the possibility that they derive from other perivascular progenitors (Armulik, Genove et al. 2011).

1.2 The lymphatic vascular system

The lymphatic vascular system is an unidirectional system that runs parallel to the blood vascular system and consists of lymphatic capillaries, precollecting and collecting lymphatic vessels. Due to the high blood pressure within the arteries, plasma and macromolecules filtrate continuously from the blood compartment into the interstitial space. About 90% of this protein rich fluid is reabsorbed by venous capillaries and post-capillary venules (Jeltsch, Tammela et al. 2003, Levick and Michel 2010). The main function of the lymphatic vasculature is to return the remaining extravasated fluid from the interstitial space into the blood circulation in an unidirectional fashion. Initially fluid, proteins, lipids, immune cells and large macromolecules (termed as lymph) enter blind–ended lymphatic capillaries that drain into precollecting and collecting lymphatic vessels. The lymph is returned to the venous circulation either through the thoracic duct at the left subclavian vein, or via the right lymphatic duct at the junction of the subclavian and internal jugular veins. The lymphatic vasculature permeates all vascularized tissues with the exception of CNS, retina, cornea, cartilage and bone marrow, although recently an analogous “glymphatic” system has been discovered to mediate waste clearance in the brain (Iliff, Wang et al. 2012).

1.2.1 Lymphatic vessels structure and physiology

Lymphatic capillaries are highly permeable, thin blind-ended vessels, 30-80 µm of diameter. They are composed of oak-leaf-shaped lymphatic endothelial cells (LECs) sitting on a discontinuous BM. The capillary LECs have overlapping flaps that function as “microvalves” that prevent fluid escape into the interstitium (Trzewik, Mallipattu et al. 2001). The junctions between LECs in lymphatic capillaries are discontinuous or “button” like (Baluk, Fuxe et al. 2007), and are the sites of leucocyte entry into the lymphatic vessels. The lymphatic capillaries are attached to the ECM by elastic anchoring filaments that prevent the collapse of
capillaries due to increased interstitial pressure, and allow opening of their lumen in conditions of tissue swelling (Leak and Burke 1966, Leak and Burke 1968). After lymph is taken up from lymphatic capillaries, it is directed into contractile precollecting and collecting vessels that are covered by basement membrane and pericytes/SMCs. The collecting lymphatic vessels, specialized for lymph transport, have continuous “zipper” like junctions (Baluk, Fuxe et al. 2007) and they also contain luminal valves. The bileaflet lymphatic valves consist of two semilunar leaflets (Lauweryns and Boussauw 1973) that open and close depending on the fluid pressure within the lymphatic vessels in order to prevent retrograde lymph flow. The unidirectional lymph movement is regulated by the lymphatic valves, together with other intrinsic forces (like SMCs contractions) and extrinsic forces (like cardiac and arterial pulsation, skeletal muscle contractility and respiration) (Alitalo 2011).

1.2.1.1 Main functions of the lymphatic system

In addition to tissue fluid homeostasis, the lymphatic system is also responsible for the trafficking of antigen presenting cells (APCs) to regional lymph nodes, where they encounter and interact with lymphocytes; thus the lymphatic vessels form an important element of the immune system. Furthermore the lacteal lymphatic vessels in the villi of the small intestine are responsible for the absorption and transportation of dietary lipids (chylomicrons) that are released by intestinal enterocytes (Tammela and Alitalo 2010). Finally, lymphatic capillaries in the subcutaneous interstitial compartment may participate in blood pressure regulation during excess salt intake, via a VEGFC/VEGFR3 dependent mechanism (Machnik, Neuhofer et al. 2009, Wiig, Schroder et al. 2013).

2. BLOOD AND LYMPHATIC VESSEL DEVELOPMENT AND GROWTH

2.1 Embryonic vessel development

2.1.1 Vasculogenesis and angiogenesis

The cardiovascular system is the first functional system than develops in vertebrates. The initial step in blood vessel formation is the differentiation of a subset of primitive mesodermal cells into endothelial cells (termed angioblasts). Angioblasts originate from the posterior primitive streak. They are positive for VEGFR2/Fetal liver kinase 1 (Flk1), the earliest marker of developing endothelial cells that can be detected already at embryonic day (E) 7.0 (Dumont, Fong et al. 1995). These precursors migrate to the yolk sac, where they form clusters of endothelial and hematopoietic progenitors, known as blood islands. The blood islands fuse and generate a primary capillary plexus. The assembly of vascular progenitors into the primitive plexus is termed vasculogenesis (Risau and Flamme 1995), while the formation of new blood vessels from preexisting ones, either by sprouting or by splitting (intussusception) is called angiogenesis (Risau 1997, Djonov, Schmid et al. 2000; Burri, Hlushchuk et al. 2004). Different developing organs rely to different extent on vasculogenesis and angiogenesis for their vascularization, depending on whether they contain angioblasts (Ribatti, Vacca et al. 2001).
Following the formation of the primitive endothelium, the next step in blood vessel development is remodeling and maturation by endothelial cell proliferation, regression, migration and branching. Several factors orchestrate this complex process including genetic programming, growth and other modulating factors, mechanotransduction, blood flow and hypoxia (Coultas, Chawengsaksophak et al. 2005). ECs are assigned arterial or venous identities (Wang, Chen et al. 1998, Adams, Wilkinson et al. 1999, Herzog, Guttmann-Raviv et al. 2005), establish their position along the body axis, and form new capillaries (Coultas, Chawengsaksophak et al. 2005). VEGF and Notch signals are the key regulators of these early patterning events (Krebs, Xue et al. 2000, Lawson, Scheer et al. 2001, Hogan and Bautch 2004, Kearney, Kappas et al. 2004). Finally the new vessels become covered by pericytes and SMCs that provide strength and stability.

2.1.2 Lymphangiogenesis

The lymphatic system in the mouse starts to develop after the establishment of the blood circulation. LECs arise by differentiation from BECs in the cardinal vein (CV) at E9.0, at the time when the SRY-related HMG domain transcription factor SOX18 is expressed in ECs lining the dorsolateral area of the CV (Francois, Caprini et al. 2008). SOX18 cooperates with the orphan nuclear transcription factor COUP-TFII (Srinivasan, Geng et al. 2010) as a developmental switch, and activates the transcription of PROX1 as early as E9.5 (Wigle and Oliver 1999). At E10.5, PROX1 positive LEC precursors bud from the dorsal side of the CV in a polarized manner and form the primitive lymph sacs, which can be found along the anteroposterior axis of the mouse embryo. Interestingly, a second source of LECs distinct from the CV, which is located at the lower edge of the superficial venous plexus, has been identified in mouse embryos (Hagerling, Pollmann et al. 2013). BECs in Prox1 null embryos fail to adopt a lymphatic cell phenotype and do not develop a lymphatic vasculature (Wigle, Harvey et al. 2002). LEC migration away from the CV is mainly orchestrated by VEGFC/VEGFR3 signaling (Karkkainen, Haiko et al. 2004).

Following the formation of the primitive lymphatic capillary plexus, the peripheral lymphatic vessels form by sprouting lymphangiogenesis and mature into capillaries, pre-collecting and collecting vessels. Several molecules play essential roles in the maturation process, including the forkhead transcription factor FOXC2 (Petrova, Karpanen et al. 2004), the nuclear factor of activated T cell (NFAT) c1 transcription factor (Norren, Ivanov et al. 2009), EphrinB2 (Makinen, Adams et al. 2005), the angiopoietins ANG1 and ANG2 (Gale, Thurston et al. 2002, Shimoda, Bernas et al. 2007, Dellinger, Hunter et al. 2008), and integrin a9 (Bazigou, Xie et al. 2009).

The venous origin of the lymphatic system, suggested by the medical scientist Florence Sabin already in 1902 (Sabin 1902), has been confirmed not only in mice, but also in zebrafish (Kuchler, Gjini et al. 2006, Yaniv, Isogai et al. 2006). Yet other models suggest that separate mesenchymal lymphangioblasts might contribute to the formation of the lymphatic vascular system in birds (Schneider, Othman-Hassan et al. 1999, Papoutsi, Tomarev et al. 2001, Wilting, Papoutsi et al. 2001, He, Papoutsi et al. 2003) and amphibians (Ny, Koch et al. 2005). To which extent these cells
contribute to murine embryonic lymphangiogenesis remains controversial (Buttler, Kreysing et al. 2006, Buttler, Ezaki et al. 2008, Bertozzi, Schmaier et al. 2010).

2.1.3 Separation of the two vascular systems

The separation of the lymphatic from the venous blood circulation depends largely on the interaction between the endothelial and hematopoietic systems. Failure of this separation results in blood filled lymphatic vessels and cutaneous hemorrhages. Several studies have shown that platelets mediate lymphatic/blood separation through activation of their receptor CLEC-2 by the transmembrane protein podoplanin, which is presented on LECs. Activation of CLEC-2 results in downstream activation of the tyrosine kinase SYK, the adaptor protein SLP-76, and PLCγ2 in platelets (Bertozzi, Hess et al. 2010). The exact mechanism of how platelet aggregation mediates separation of venous and lymphatic endothelial systems is thus far unknown, but it seems to involve a hemostatic blood clot (Hess, Rawnsley et al. 2014).

2.2 Postnatal angiogenesis and lymphangiogenesis

2.2.1 Postnatal angiogenesis

2.2.1.1 Retinal angiogenesis and the “tip” vs. “stalk” cell concept

Angiogenesis is mainly a developmental process, which continues during the early postnatal growth of tissues. One of the most studied blood vascular angiogenic beds is the rodent retina, which is immature and avascular at birth and becomes vascularized and fully mature during the first three weeks of life (Figure 1). The mouse retina is a powerful in vivo model for the study of vascular development in mice, because it becomes vascularized in a very tightly regulated and organized pattern (Fruttiger 2007), it is easily accessible, and allows reliable detection of any developmental abnormality (Stahl, Connor et al. 2010). Furthermore, it allows the analysis of various aspects of blood vessel growth at different stages of postnatal life, including EC sprouting, proliferation, remodeling and maturation (Pitulescu, Schmidt et al. 2010).

Figure 1. Mouse retina at postnatal day 6. Whole mount immunohistochemistry for Isolectin B4 (iB4, in white). A: artery, V: Vein. Scalebar: 200 µm.
During sprouting angiogenesis, ECs acquire specialized phenotypes that contribute to the formation of new vascular networks in different ways. The “tip” cells are located at the forefront. Then filopodia towards the angiogenic stimulus, such as VEGF gradients. The tip cells lead the directional migration in order to invade the surrounding tissues, they are highly polarized and motile and do not proliferate. When the filopodia of adjacent tip cells meet, they connect and anastomose forming vascular loops (Herwig, Blum et al. 2011). Macrophages at the anastomosis sites are considered to release angiogenic factors and act as “bridges” between the tip cells (Fantin, Vieira et al. 2010). Just behind the tip cells follow the “stalk” cells that proliferate, remodel and form the vascular lumen (Gerhardt, Golding et al. 2003, Ruhrberg 2003). Further away from the angiogenic front, the quiescent lumenized endothelium consists of non-proliferating “phalanx” cells (Mazzone, Dettori et al. 2009). Tip, stalk and phalanx cells have different molecular signatures. For example, tip cells express high levels of VEGFR2 and platelet-derived growth factor (PDGF)-BB (Gerhardt, Golding et al. 2003), UNC5B (Lu, Le Noble et al. 2004), DLL4 (Suchting, Freitas et al. 2007), neuropilin-1 (NP1) (Gerhardt, Ruhrberg et al. 2004), and MT1-MMP (Yana, Sagara et al. 2007), while stalk cells express VEGFR1 (Krueger, Liu et al. 2011), Notch-regulated ankyrin repeat protein (NRARP) (Phng, Potente et al. 2009), Notch ligands Jagged1 and DLL1 (Roca and Adams 2007), ROBO4 (Jones, London et al. 2008), and VCAM1 (Harrington, Sainson et al. 2008, De Smet, Segura et al. 2009). Transcriptome analysis of ECs of DLL4+/− retinas, which are considered to contain an enriched tip cell population, and microarray analysis of retinal ECs isolated by laser capture microdissection, have shown that tip and stalk cells differ in the expression of several genes involved in ECM degradation, ECM proteins and transcription factors (del Toro, Prahst et al. 2010, Strasser, Kaminker et al. 2010). Furthermore, it has been suggested that the metabolic profile of ECs and the levels of glycolysis define EC behavior, and that overexpression of PFKFB3, a potent stimulator of glycolysis, promotes the formation of tip cells in some conditions (De Bock, Georgiadou et al. 2013, De Bock, Georgiadou et al. 2013).

2.2.1.2 Angiogenesis in the adult

Adult ECs are generally quiescent; with a turnover time of hundreds of days they are among the most genetically stable cells in the body (Folkman and D’Amore 1996). Yet there are some processes that require angiogenic growth also in adulthood. These include neoangiogenesis in the female reproductive system, especially during corpus luteum formation in the ovary (Robinson 2013), during embryo implantation, and in the placenta during pregnancy (Zygmunt, Herr et al. 2003). Moreover angiogenesis in the adult occurs during fat mass expansion (Cao 2007), skeletal muscle hypertrophy in response to exercise (Gustafsson 2011), and wound healing (Tonnesen, Feng et al. 2000). Remarkably, ECs in the blood vessel wall may not all have the same angiogenic potential, since the endothelium contains a small population of CD117+ ECs with proliferative and clonogenic properties that could be the source of new ECs during cell maintenance and in angiogenic conditions (Fang, Wei et al. 2012).

2.2.2 Postnatal lymphangiogenesis
During postnatal development lymphatic vessels grow, remodel, mature, alter their expression of LEC-specific markers and recruit SMCs (Dellinger, Hunter et al. 2008, Norrmen, Ivanov et al. 2009, Svingen, Francois et al. 2012). Interestingly, lymphatic vessels rely on VEGFR3 signaling only for the first two postnatal weeks in mice, and become resistant to VEGFR3 blockade thereafter (Karpanen, Wirzenius et al. 2006).

In adults, physiological lymphangiogenesis happens during wound healing and acute inflammation (following for example bacterial infections) (Paavonen, Puolakkainen et al. 2000, Baluk, Tammela et al. 2005), during corpus luteum development (Brown and Russell 2014) and during pregnancy (Rutkowski, Ihm et al. 2013). Lymphangiogenic sprouting is the principal mechanism of lymphatic vessel growth in adults (He, Rajantie et al. 2004, He, Rajantie et al. 2005), although some transdifferentiation of bone marrow derived cells into lymphatic vessels during inflammation has been shown in a cornea transplantation model (Religa, Cao et al. 2005), after kidney transplantation (Kerjaschki, Huttary et al. 2006), in peritumoral and adult lymphangiogenesis (Zumsteg, Baeriswyl et al. 2009, Lee, Park et al. 2010), in human onchocerciasis (Attout, Hoerauf et al. 2009), and in idiopathic pulmonary fibrosis (El-Chemaly, Malide et al. 2009, El-Chemaly, Pacheco-Rodriguez et al. 2009).

### 3. BLOOD AND LYMPHATIC VESSELS IN DISEASE

#### 3.1 Pathological angiogenesis

##### 3.1.1 Tumor angiogenesis

The ability to sustain angiogenesis is an acquired capability of tumor cells and is considered one of the hallmarks of cancer progression (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). Tumors alter their gene expression pattern to change the balance between angiogenesis inducers (like VEGF and FGF) and inhibitors (like thrombospondin-1, β-interferon and platelet factor-4) (Hanahan and Folkman 1996). In collaboration with environmental stimuli, such as metabolic/mechanical stress and immune responses, (Carmeliet and Jain 2000) the tumors activate the “angiogenic switch” (Folkman, Watson et al. 1989). Turning the switch “on” allows to dormant cancerous lesions to generate the required blood supply to grow beyond 1-2 mm in size and to metastasize. Most tumor vascularization occurs via sprouting angiogenesis and intussusception, however EC precursors mobilized from the bone marrow can be incorporated in the tumor blood vasculature as well, in a process termed postnatal vasculogenesis (Lyden, Hattori et al. 2001, Rafii, Meeus et al. 2002). Furthermore alternative types of nonangiogenic vascularization have been reported in tumors, including vessel co-option (Donnem, Hu et al. 2013), vasculogenic mimicry (Maniotis, Folberg et al. 1999, Seftor, Hess et al. 2012), EC generation from cancer stem-like cells (Wang, Chadalavada et al. 2010), and formation of mosaic vessels by incorporation of cancer cells to the vessel wall. The tumor vasculature is chaotic, consisting of irregularly shaped, tortuous and dilated blood vessels that demonstrate vascular shunting (Pries, Hopfner et al. 2010), show poorly connected or overlapping ECs (Hashizume, Baluk et al. 2000) that lack perivascular cells (Benjamin, Golijanin et al. 1999, Abramsson, Lindblom et al. 2003).
VEGFR3 and Notch signaling in angiogenesis

(Morikawa, Baluk et al. 2002), and have an abnormal BM (Baluk, Morikawa et al. 2003). As a result tumor vasculature is heterogeneous, leaky and hemorrhagic, and the blood flow is irregular.

3.1.2 Other angiogenesis-related diseases

Excessive angiogenesis has been coupled together with chronic inflammation in several conditions, including rheumatoid arthritis, (Szekanecz, Besenyei et al. 2010), osteoarthritis (Ashraf and Walsh 2008), psoriasis (Detmar, Brown et al. 1994), Crohn’s disease (Danese, Sans et al. 2006), ocular neovascularization (Miller, Adamis et al. 1994), obesity (Lemoine, Ledoux et al. 2013), and atherosclerotic plaque progression (Moreno, Purushothaman et al. 2006). Other vascular anomalies include benign vascular tumors (hemangiomas) and vascular malformations (Vikkula, Boon et al. 1998). The contribution of endothelial progenitor cells (EPCs) to the endothelium has been suggested in some of these conditions, but this mechanism remains still under debate (Bautch 2011).

3.2 Lymphatic dysfunction

3.2.1 Lymphedema

The chronic accumulation of fluid in the interstitium results in disabling tissue swelling due to impaired drainage and is called lymphedema. The protein-rich fluid triggers an inflammatory reaction that leads to fibrosis, accumulation of adipose tissue and impaired wound healing (Warren, Brorson et al. 2007). Lymphedema may be congenital or acquired. A number of somatic mutations account for the cases of inherited lymphedema. These include mutations in the intracellular tyrosine kinase domain of VEGFR3 resulting in inhibited autophosphorylation of the receptor (Milroy disease) (Ferrell, Levinson et al. 1998, Irrthum, Karkkainen et al. 2000, Karkkainen and Petrova 2000, Butler, Dagenais et al. 2007, Ghalamkarpour, Holnthoner et al. 2009, Gordon, Spiden et al. 2013), nonsynonymous mutations in gap junction protein gamma-2 (GJC2) encoding connexin-47 (CX47) (“four limb lymphedema”) (Ferrell, Baty et al. 2010, Ostergaard, Simpson et al. 2011), insertions/deletions/nonsense mutations of the transcription factor FOXC2 leading to protein loss of function (LOF) (Lymphedema-Distichiasis) (Fang, Dagenais et al. 2000, van Steensel, Damstra et al. 2009), recessive or dominant mutations in SOX18 (hypotrichosis-lymphedema-telangiectasia syndrome) (Irrthum, Devriendt et al. 2003), homozygous or compound heterozygous mutations of CCBE1 (collagen and calcium-binding EGF domain-1) (Hennekam syndrome) (Hennekam, Geerdink et al. 1989, Connell, Kalidas et al. 2010), mutations in the GATA-binding protein 2 (GATA2) (Emberger syndrome) (Hahn, Chong et al. 2011, Ostergaard, Simpson et al. 2011), hypomorphic mutations in the gene IKBKG that result in impaired NF-kappaB signaling (OL-EDA-ID syndrome) (Doffinger, Smahi et al. 2001), as well as mutations in PTPN14 (protein tyrosine phosphatase, non-receptor type 14) (Au, Hernandez et al. 2010), in KIF11 (kinesin family member 11) (Ostergaard, Simpson et al. 2012), and VEGFC (Gordon, Schulte et al. 2013). Finally, the genetic cause underlying primary non-syndromic lymphedema (Meige disease) is so far unknown (Rezaie, Ghoroghchian et al. 2008).
Primary lymphedema is a rare disease, affecting 1.15/100,000 individuals under the age of 20 (Rockson and Rivera 2008). Most lymphedema cases are secondary. Infection with filaria worms affects currently 120 million people worldwide, of which approximately one third experience clinically overt lymphedema (Nutman 2013). In Western societies lymphedema usually follows surgical removal and/or irradiation of lymph nodes during cancer therapy. Breast cancer is a common case, with 21% of patients developing lymphedema in the arm post-operatively, and with higher rates correlating with axillary lymph node dissection compared to sentinel lymph node biopsy (DiSipio, Rye et al. 2013). Another cause of secondary lymphedema is inflammation of the lymphatic channels after infection of the skin with microorganisms (lymphangitis) (Rockson 2001).

3.2.2 Tumor lymphangiogenesis and metastasis

Lymphatic vessels may act as a getaway for lymphatic metastasis, which is suggested by many studies as the most common pathway for metastatic tumor spread (Leong 2011). Although intratumoral lymphatic vessels are dysfunctional due to high intratumoral pressure (Fukumura, Duda et al. 2010), peritumoral lymphatics correlate positively with lymph node and distal metastasis (Alitalo and Detmar 2012). Lymphangiogenesis around the tumor and in the draining lymph nodes is triggered by overexpression of lymphangiogenic factors, such as VEGFC and VEGFD, which are produced by the tumors cells or by tumor associated macrophages (TAMs) (Tammela and Alitalo 2010). With mechanisms that are currently not well understood, tumor cells migrate and enter the lymphatic vessel lumen and are transferred to the sentinel lymph nodes by lymphatic flow. Subsequently tumor cells spread further away to distal lymph nodes and organs (Alitalo and Detmar 2012).

3.2.3 Lymphatic vessels in other pathologies

Lymphangiogenesis is associated with the pathogenesis of diseases involving chronic inflammation, such as psoriasis (Kunstfeld, Hirakawa et al. 2004), rheumatoid arthritis (Zhang, Lu et al. 2007), idiopathic pulmonary fibrosis (El-Chemaly, Malide et al. 2009), and Crohn’s disease (von der Weid, Rehal et al. 2011). Interestingly, tissue inflammation accompanying obesity, obesity associated metabolic disease and atherosclerosis, has been linked to lymphatic dysfunction, although the underlying molecular mechanisms are still poorly known (Alitalo 2011). The lymphatic vasculature can give rise to lymphovascular tumors, including benign lymphangiomas and malignant lymphangiosarcomas, the latter developing on the ground of chronic lymphedema (Morrison 2003), as well as Kaposi sarcoma, following infection with a gammaherpesvirus (KSHV) that reprograms BECs to LECs (Wang, Trotter et al. 2004). Finally, lymphatic vessels are involved in the pathogenesis of the rare disease lymphangioleiomyomatosis (LAM) (Harari, Torre et al. 2011).

4. MOLECULAR REGULATORS OF THE VASCULAR SYSTEMS
The blood and lymphatic vasculature show a significant overlap in the expression of several molecular markers, signifying the close developmental, structural and functional relationship between those two systems.

4.1 VEGFs and their receptors (VEGFRs)

The VEGF family consists of VEGFA, VEGFB, VEGFC, VEGFD and placenta growth factor (PLGF) (Figure 2). Furthermore two non-mammalian homologues have been identified, the parapox virus open reading frame (VEGFE) (Ogawa, Oku et al. 1998, Meyer, Clauss et al. 1999) and the snake venom-derived polypeptides (VEGFF) (Yamazaki, Tokunaga et al. 2005). VEGFs are secreted glycoproteins containing a cystine knot motif (Iyer and Acharya 2011), consisting of antiparallel covalently linked homodimeric polypeptides; although heterodimers of VEGF and PLGF (DiSalvo, Bayne et al. 1995), as well as VEGF and VEGFB (Olofsson, Pajusola et al. 1996) have been described. VEGFRs belong to class V receptor tyrosine kinases (RTKs). They are composed of seven immunoglobulin (Ig) extracellular domains, a transmembrane domain and a C-terminal intracellular kinase domain (Stuttfeld and Ballmer-Hofer 2009). Upon ligand binding the receptors undergo homo- and heterodimerization and auto- or transphosphorylation of tyrosine residues, resulting in the recruitment of intracellular signaling mediators with Src-homology-2 (SH2) or phosphotyrosine binding domains, and the activation of distinct downstream signaling pathways (Koch and Claesson-Welsh 2012).

4.1.1 VEGF

Originally described as “tumor vascular permeability factor” (Senger, Galli et al. 1983), VEGF was isolated and cloned in 1989 (Ferrara and Henzel 1989). Human VEGF exists in at least eight alternatively spliced isoforms (Takahashi and Shibuya 2005). The most common forms, named VEGFA_{121}, VEGFA_{165}, VEGFA_{189} and VEGFA_{206} show different abilities to bind VEGFRs, heparate sulfate and NPs. For example VEGF_{121} lacks heparin-binding and is highly diffusible, while VEGF_{189} and VEGF_{206} are highly basic and heparin-binding proteins that are tightly bound to the extracellular matrix; VEGF_{165} has intermediate properties (Houck, Ferrara et al. 1991). Although VEGF binds to VEGFR1 with an affinity that is 10-fold higher compared to VEGFR2 binding, VEGFR2 is the main VEGF signaling mediator in ECs (Takahashi and Shibuya 2005). Some VEGF isoforms also bind to VEGFR2 co-receptors NP1 (Soker, Takashima et al. 1998) and NP2 (Gluzman-Poltorak, Cohen et al. 2000).

VEGF is indispensable for embryonic development, and inactivation of a single VEGF allele results in death at mid-gestation (E11-E12) (Carmeliet, Ferreira et al. 1996, Ferrara, Carver-Moore et al. 1996). At the same time a modest increase in VEGF expression leads to severe heart developmental defects and embryonic death at E12.5 (Miquerol, Langille et al. 2000). Taken together these results highlight the importance of VEGF gene-dosage control during development. In the mouse embryo, VEGF is detected as early as at E7.0, and is later expressed in several organs and systems, including endocrine glands, kidney glomerulus, lymphoid aggregates, neural structures, the choroid plexus, bones and myocardium. VEGF expression sites are mainly juxtaposed to VEGFR2 expression (Dumont, Fong et al. 1995).
Figure 2. VEGFRs, their co-receptors and their ligands. The main interactions between the ligands and receptors are shown. The dashed lines indicate the proteolytic processing required for VEGFC and VEGFD, which increases binding affinity towards VEGFR2 (Koch and Claesson-Welsh 2012, Jeltsch, Leppanen et al. 2013).

In the adult, most cell types produce VEGF, including fibroblasts (Pertovaara, Kaipainen et al. 1994), hematopoietic stem cells (HSCs) (Bautz, Rafii et al. 2000), keratinocytes (Frank, Hübner et al. 1995), cardiac myofibroblasts (Chintalgattu, Nair et al. 2003), neuronal cells (Haigh, Gerber et al. 2000), macrophages (McLaren, Prentice et al. 1996), neutrophils (Taichman, Young et al. 1997), T cells (Freeman, Schneck et al. 1995), renal mesangial cells (Iijima, Yoshikawa et al. 1993) and endothelial cells (Namiki, Brogi et al. 1995). VEGF acts in a paracrine way, although an autocrine mode of action has been described for ECs and HSCs (Gerber, Malik et al. 2002, Lee, Chen et al. 2007). VEGF expression is controlled at transcriptional, post-transcriptional and translational levels, by growth factors and cytokines, as well as by oncogenes like p53, Src, c-Myc, Fos and Bcl-2 (Xie, Wei et al. 2004). Hypoxia is a key stimulator of VEGF expression (Plate, Breier et al. 1992, Shweiki, Itin et al. 1992) and the VEGF mRNA transcription is induced in hypoxic microenvironments by hypoxia-inducible-factor (HIF-1) (Pugh and Ratcliffe 2003).
VEGF acts mainly on ECs, promoting their survival through the phosphatidylinositol (PI)-3 kinase–Akt pathway and the expression of Bcl-2 and A1, and their growth (Ferrara, Gerber et al. 2003). Furthermore, VEGF induces vascular permeability (Dvorak, Brown et al. 1995) and is associated with the development of fenestrations in ECs (Roberts and Palade 1995, Esser, Wolburg et al. 1998). Consequently adenoviral administration or transgenic overexpression of VEGF results in robust angiogenic effects in several tissues, and this is accompanied by vascular leakage and inflammation (Detmar, Brown et al. 1998, Larcher, Murrillas et al. 1998, Thurston 2002, Baluk, Tammela et al. 2005). Although VEGF is needed for the postnatal survival and growth, the dependence on VEGF signaling is gradually lost as mice mature and reach adulthood (Gerber, Hillan et al. 1999). In addition VEGF signaling is important also in some non-endothelial cell types; for example it promotes survival, growth and migration of neural cells in the CNS and peripheral nervous system (PNS) (Zachary 2005, Rosenstein, Krum et al. 2010, Ruiz de Almodovar, Fabre et al. 2011), plays a critical role in fetal lung maturation by stimulating the production of surfactant proteins by type two pneumonocytes (Compernolle, Brusselmans et al. 2002), boosts bone formation by directly stimulating osteoblast differentiation, migration and growth (Midy and Plouet 1994, Mayr-Wohlfart, Waltenberger et al. 2002, Hiltunen, Ruuskanen et al. 2003), as well as chondrocyte development and survival (Maes, Stockmans et al. 2004). Finally it has multiple regulatory effects on bone-marrow derived cells (Ferrara, Gerber et al. 2003). VEGF is expressed in solid tumors by cancer cells, ECs and infiltrating TAMs, and plays a central role in the expansion of the tumor vascular bed and the growth of solid tumors (Kim, Li et al. 1993, Claesson-Welsh and Welsh 2013).

4.1.2 VEGFB and PLGF

VEGFB (Olofsson, Pajusola et al. 1996) and PLGF (Maglione, Guerriero et al. 1991) both function as ligands for VEGFR1 (Park, Chen et al. 1994, Olofsson, Korpelainen et al. 1998) and NP1 (Migdal, Huppertz et al. 1998, Makinen, Olofsson et al. 1999). VEGFB occurs in two alternative spliced isoforms in humans and mice (VEGFB167 and VEGFB168) (Olofsson, Pajusola et al. 1996). PLGF is made as four alternatively spliced isoforms with different heparin binding properties in humans, and as one isoform in mice (De Falco 2012). In the developing embryo, VEGF can be detected already at E8.5, and is most prominent in the heart and skeletal muscle, as well as bones and cartilage (Aase, Lymboussaki et al. 1999); yet VEGF gene deleted mice are viable without major abnormalities (Aase, von Euler et al. 2001). In adults, VEGFB localizes mainly in heart, skeletal muscle, brown fat and pancreas (Nash, Baca et al. 2006). On the other hand, PLGF is mainly expressed in the placenta throughout gestation (Achen, Gad et al. 1997, Vuorela, Hatva et al. 1997), but also in other tissues (De Falco 2012). Similarly to VEGFB, PLGF is not essential for embryonic development (Carmeliet, Moons et al. 2001).

Despite binding to the same receptors, VEGFB and PLGF have different biological activities. A possible explanation for these differences could lie in the unique structure of loop 1 of VEGFB compared to PLGF, and its inability to interact with the Ig homology domain 3 of VEGFR1 (Anisimov, Leppanen et al. 2013). VEGFB is a poor angiogenic inducer (Bhardwaj, Roy et al. 2003, Rissanen,
Markkanen et al. 2003, Karpanen, Bry et al. 2008). One strain of VEGFB-/- mice exhibits decreased heart size (Bellomo, Headrick et al. 2000), and VEGFB overexpression has been shown to promote cardiac hypertrophy with concurrent coronary growth and ischemia resistance, stemming from metabolic shifting to macromolecular synthesis and glucose oxidation (Karpanen, Bry et al. 2008, Bry, Kivela et al. 2010, Kivela, Bry et al. 2014). Furthermore VEGFB has been claimed to increase fatty acid transport in organs with high metabolic rate, such as the heart, by regulating the transcription of fatty acid transport proteins (Hagberg, Falkevall et al. 2010). On the other hand, PLGF is proangiogenic (Ziche, Morbidelli et al. 1997, Odorisio, Schietroma et al. 2002), but mainly in pathological settings, such as ischemia, inflammation and wound healing (Carmeliet, Moons et al. 2001, Luttun, Tjwa et al. 2002, Pipp, Heil et al. 2003, Rakic, Lambert et al. 2003). At the same time the inhibition of PLGF in several disease conditions improved the associated pathological status (De Falco 2012). Finally although VEGFB expression can be detected in several tumor types, little is known about its significance in tumor progression (Fischer, Mazzone et al. 2008, Albrecht, Kopfstein et al. 2010); also the role or PLGF remains still controversial (Fischer, Jonckx et al. 2007, Bais, Wu et al. 2010, Van de Veire, Stalmans et al. 2010).

4.1.3 VEGFC and VEGFD

VEGFC and VEGFD are produced as precursor proteins that can bind to and activate only VEGFR3 (Joukov, Pajusola et al. 1996, Lee, Gray et al. 1996). Both human proteins are cleaved by furin or the proprotein convertases PC5 and PC7 (McColl, Baldwin et al. 2003, Siegfried, Basak et al. 2003, McColl, Paavonen et al. 2007). This processing increases the binding affinity to VEGFR3. In addition, VEGFC is further proteolytically cleaved at the N-terminus into a 21 kDA polypeptide, which represents the mature form of the protein (ΔNΔC) which is able to activate also VEGFR2 (Joukov, Pajusola et al. 1996). Yet, although the human fully processed VEGFD (ΔNΔC) is able to bind to and activate VEGFR2 (Joukov, Sorsa et al. 1997, Achen, Jeltsch et al. 1998, Stacker, Stenvers et al. 1999), mouse VEGFD is not capable of binding to mouse VEGFR2 (Baldwin, Roufail et al. 2001).

4.1.3.1 VEGFC

VEGFC was the first ligand for VEGFR3 to be discovered (Joukov, Pajusola et al. 1996). It exists in three mRNA splice variants in humans (Lee, Gray et al. 1996) and two in mice. The shortest mRNA forms are rare and little is known about their biological activity. VEGFC is required for embryonic lymphatic vascular development. In VEGFC null mice, LECs remain trapped into the venous vasculature and fail to sprout from the CV; as a result the embryos do not develop a lymphatic vasculature and die at E15.5-17.5 (Karkkainen, Haiko et al. 2004, Hagerling, Pollmann et al. 2013). Mice mutant for the Collagen- and calcium-binding EGF domains 1 protein (CCBE1) phenocopy the VEGFC full knockout embryos. CCBE1 does not have any lymphangiogenic properties of its own, and it seems to act as a critical permissive modulator of VEGFC in lymphangiogenesis by enhancing the N-terminus cleavage of pro-VEGFC into mature VEGFC, by the metalloprotease ADAMTS3 (Bos, Caunt et al. 2011, Jeltsch, Jha et al. 2014, Le Guen, Karpanen et al.
Although VEGFC+/- mice are viable, they show signs of lymphatic dysfunction, including chylous ascites, hypoplastic cutaneous lymphatic vessels and paw edema (Karkkainen, Haiko et al. 2004). In zebrafish VEGFC signaling is also indispensable for lymphatic system development (Kuchler, Gjini et al. 2006).

During development, VEGFC is produced starting at E8.5 by mesenchymal cells in the peritoneal cavity, axillary and jugular regions, proximal to the areas where lymphatic vessels sprout (Kukk, Lymboussaki et al. 1996, Karkkainen, Haiko et al. 2004). In adults VEGFC is expressed close to the areas of VEGFR3 expression, suggesting a paracrine mode of action in LECs (Kukk, Lymboussaki et al. 1996, Lymboussaki, Olofsson et al. 1999). VEGFC is also present in platelets (wartiovaara, Salven et al. 1998), and produced by BECs in the developing zebrafish ISVs, where it has been proposed to function in a cell autonomous manner to support BEC migration and angiogenesis (Villefranc, Nicoli et al. 2013). VEGFC produced in the murine brain, induces the proliferation of VEGFR3+ neural progenitors in the olfactory bulb and oligodendrocyte precursor cells in the optic nerve (Le Bras, Barallobre et al. 2006), and supports survival of dopaminergic neurons (Piltonen, Planken et al. 2011). In the zebrafish, VEGFC is produced by the dorsal aorta (DA) and activates VEGFR3 to promote and guide axon growth of motoneurons (Kwon, Fukuhara et al. 2013). Several sites of VEGFC production have also been described in humans (Partanen, Arola et al. 2000).

VEGFC signaling via VEGFR3 is critical for LEC growth, survival and migration, via PKC dependent activation of p42/p44 MAPK, and PI3 kinase dependent Akt phosphorylation (Makinen, Veikkola et al. 2001). As a result, VEGFC is strongly lymphangiogenic. It induces sprouting lymphangiogenesis when overexpressed in the skin of transgenic mice (Jeltsch, Kaipainen et al. 1997) or with the use of viral vectors in the skin and mouse airways (Enholm, Karpanen et al. 2001, Saaristo, Veikkola et al. 2002, Baluk, Tammela et al. 2005), and when applied in the avian chorioallantoic membrane (CAM) (Oh, Jeltsch et al. 1997). VEGFC mediates lymphangiogenesis also in tumors, and its expression is associated with lymphatic metastasis to sentinel nodes and distal locations (Valtola, Salven et al. 1999, Karpanen, Egeblad et al. 2001, Mandriota, Jussila et al. 2001, Skobe, Hawighorst et al. 2001a, Mattila, Ruohola et al. 2002, Hirakawa, Brown et al. 2007). On the other hand, VEGFC is angiogenic when overexpressed in transgenic mouse embryos before E16.5 (Lohela, Helotera et al. 2008), in the adult mouse cornea and in the CAM assay (Cao, Linden et al. 1998), in the myocardium after myocardial ischemia (Patila, Ikonen et al. 2006), and in skeletal muscle (Anisimov, Alitalo et al. 2009). VEGFC is also required for vasculogenesis and angiogenesis in zebrafish (Ober, Olofsson et al. 2004).

4.1.3.2 VEGFD

VEGFD was discovered in 1996 as a c-fos-induced growth factor (FIGF) (Orlandini, Marconcini et al. 1996), and identified in 1998 as a novel ligand for VEGFR2 and VEGFR3 (Achen, Jeltsch et al. 1998). Alternative splicing in exon 6 produces two distinct VEGFD isoforms (VEGF-D_{358} and VEGF-D_{326}) with different C-termini (Baldwin, Roufail et al. 2001).
In the mouse embryo VEGFD is detected starting at E7.5 and is expressed in several tissues (Avantaggiato, Orlandini et al. 1998). VEGFD deficient mice develop normally and do not show signs of lymphatic abnormality, apart from a minor reduction in the number of lymphatic vessels in the lungs (Baldwin, Halford et al. 2005). In zebrafish, VEGFD has been implied in normal embryonic angiogenesis, as overexpression of VEGFD results in abnormal connections between the DA and the caudal vein, and truncated intersomitic vessels (ISVs) (Song, Yang et al. 2007). Furthermore it has been shown that VEGFD modulates SOX18 activity via the MEK-ERK pathway, in order to determine arteriovenous differentiation in zebrafish, and early vascular development (starting at E11.5) in mouse embryos (Duong, Koltowska et al. 2014). In human adult tissues VEGFD is expressed mostly in the lung, heart, skeletal muscle, colon, and small intestine (Achen, Jeltsch et al. 1998).

VEGFD is a lymphangiogenic factor, and transgenic overexpression of VEGFD in the basal cells of the epidermis under the K14 promoter, or adenoviral delivery of VEGFD, induce lymphatic hyperplasia in mouse skin (Veikkola, Jussila et al. 2001, Byzova, Goldman et al. 2002). Adenoviral delivery of VEGFD produces lymphatic hyperplasia also in the airway epithelium of mice (Baluk, Tammela et al. 2005). VEGFD is upregulated in many tumors and promotes lymphangiogenesis and lymphatic metastasis in mouse models and human cancers (Achen, Williams et al. 2002, Von Marschall, Scholz et al. 2005, Kopfstein, Veikkola et al. 2007). VEGFD is able to produce angiogenic responses when administered via adenoviruses in rabbit hindlimb skeletal muscle and carotid arteries (Bhardwaj, Roy et al. 2003, Rissanen, Markkanen et al. 2003), porcine heart (Rutanen, Rissanen et al. 2004), and mouse cremaster and skeletal muscle (Byzova, Goldman et al. 2002, Anisimov, Alitalo et al. 2009).

### 4.1.4 VEGFR1

Initially known as Fms-like-TK-1 (Flt-1), VEGFR1, discovered in 1990 (Shibuya, Yamaguchi et al. 1990), was shown to be a receptor for VEGF shortly thereafter (de Vries, Escobedo et al. 1992). VEGFR1 is a 180 kDA transmembrane glycoprotein that binds VEGF, VEGFB and PLGF. Although binding VEGF with an affinity that is an order of magnitude larger than VEGFR2 (Sawano, Takahashi et al. 1996), the kinase activity of VEGFR1 is ten times lower that VEGFR2, suggesting that VEGFR1 acts as a “decoy” receptor during angiogenesis. Indeed VEGFR1-/- mice die at E8.5-9, due to excessive EC growth (Fong, Rossant et al. 1995), while conditional global deletion of VEGFR1 leads to increased angiogenesis in postnatal and adult mice, due to increased VEGFR2 protein accumulation and activation (Ho, Duan et al. 2012). Further supporting this function for VEGFR1, mice lacking the tyrosine kinase domain of the receptor (Flt1 TK-/−) develop normally (Hiratsuka, Minowa et al. 1998). Thus, a major function of VEGFR1 is to restrict VEGFR2 activity and signaling, also in a spatial local manner (Kappas, Zeng et al. 2008).

VEGFR1 exists both as a full length receptor and as an alternative spliced soluble shorter variant (sFlt1) (Kendall and Thomas 1993). sFlt1 acts as a VEGF trap and contributes to corneal avascularity (Ambati, Patterson et al. 2007). Furthermore, during angiogenesis in the retina sFlt1 is required for EC sprout guidance (Chappell, Taylor et al. 2009). sFlt1 is produced by trophoblast cells in the placenta and has
been implicated in the pathogenesis of preeclampsia (Koga, Osuga et al. 2003, Maynard, Min et al. 2003, Levine, Maynard et al. 2004). In podocytes and perivascular cells in the kidney sFlt1 supports the integrity of the glomerular barrier (Jin, Sison et al. 2012). Interestingly, both VEGFR1 and sVEGFR1 are able to form heterodimers with VEGFR2 (Kendall, Wang et al. 1996, Huang, Andersson et al. 2001), altering the signal transduction properties of the receptors.

In the developing embryo, VEGFR1 is expressed already at E7.5 in the yolk sac mesoderm and around E9.5 it starts to be expressed also in the capillaries of the developing organs (Breier, Clauss et al. 1995). In adults, VEGFR1 is expressed in ECs (Peters, De Vries et al. 1993), but also in many non-endothelial cells, such as myeloid hematopoietic cells (monocytes and macrophages) (Barleon, Sozzani et al. 1996, Clauss, Weich et al. 1996), where it mediates VEGF induced chemotaxis (Hiratsuka, Minowa et al. 1998, Lyden, Hattori et al. 2001). This well documented function of VEGFR1 on monocyte migration is associated with inflammatory conditions, such as rheumatoid arthritis (Murakami, Iwai et al. 2006), and tumor associated inflammation (Schwartz, Rowinsky et al. 2010) and growth (Muramatsu, Yamamoto et al. 2010). VEGFR1 is required for bone marrow formation (Niida, Kondo et al. 2005), megakaryocytic precursors maturation (Casella, Feccia et al. 2003), and has been implicated the establishment of the pre-metastatic niche in tumor models (Kaplan, Riba et al. 2005). VEGFR1 is able to promote angiogenesis via the expression of other growth factors by recruited macrophages (Kerber, Reiss et al. 2008, Murakami, Zheng et al. 2008). Furthermore VEGFR1 expression in neuronal cells exerts several protective effects in different disease models (Li, Zhang et al. 2008, Poesen, Lambrechts et al. 2008, Falk, Zhang et al. 2009, Wittko, Schanzer et al. 2009, Dhondt, Peeraer et al. 2011).

4.1.5 VEGFR2

VEGFR2, a transmembrane glycoprotein of 200 kDa, is the main VEGF receptor in ECs. It is also known as fetal liver kinase-1 (Flk1) in mice, and kinase-insert domain receptor (KDR) in humans (Koch and Claesson-Welsh 2012). VEGFR2 binds VEGF (Quinn, Peters et al. 1993), as well as the fully processed forms of VEGFC (ΔNΔC) and human VEGFD (ΔNΔC) (Joukov, Sorsa et al. 1997, Achen, Jeltsch et al. 1998, Stacker, Stenvers et al. 1999). The viral VEGF (Ogawa, Oku et al. 1998, Meyer, Clauss et al. 1999) and the snake venom VEGFF (Yamazaki, Tokunaga et al. 2005) are also specific ligands for VEGFR2. VEGFR2 is the main effector of VEGF signaling, regulating EC differentiation, survival, proliferation, migration, vascular permeability and vessel dilation (Koch and Claesson-Welsh 2012). Interestingly recent reports have suggested ligand-independent activation of VEGFR2, by reactive oxygen species (ROS) (Warren, Ziyad et al. 2014).

VEGFR2 exist as one isoform, however a soluble form of the receptor has been detected in human and mouse plasma (sVEGFR2) (Ebos, Bocci et al. 2004). The biological significance of this form is as yet unknown.

VEGFR2 is expressed early during development (E7.0) in extraembryonic and embryonic mesoderm and trophoblasts (Millauer, Wizigmann-Voos et al. 1993, Dumont, Fong et al. 1995). VEGFR2 is highly expressed in embryonic ECs during gestation and is required for the migration of mesodermal precursors of
hematopoietic and endothelial cells to the extraembryonic region of the embryo, where they further differentiate from a common precursor (termed “hemangioblast”) and make blood islands. Embryos deficient for VEGFR2 die at E8.5-9.5 due to developmental defects in vasculogenesis and hematopoiesis (Dumont, Fong et al. 1995, Shalaby, Rossant et al. 1995, Shalaby, Ho et al. 1997). VEGFR2 becomes downregulated in ECs as the vasculature matures, but low-level autocrine VEGF/VEGFR2 signaling remains essential for EC homeostasis (Matsumoto and Claesson-Welsh 2001, Lee, Chen et al. 2007). Apart from the the ECs, VEGFR2 is also expressed in hematopoietic cells (Larsson-Blomberg and Dzierzak 1994, Katoh, Tauchi et al. 1995), hematopoietic stem cells (Ziegler, Valtieri et al. 1999), platelets (Selheim, Holmsen et al. 2002), as well as neurons in the CNS and PNS, where it plays a supportive/neuroprotective role (Zachary 2005). VEGFR2 expression in LECs can drive enlargement of lymphatic vessels after VEGF or VEGF stimulation (Hong, Lange-Asschenfeldt et al. 2004, Wirzenius, Tammela et al. 2007). However it is not known whether such effects are secondary to changes in the blood vascular compartment, or recruitment of monocytes expressing VEGFC (Cursiefen, Chen et al. 2004, Baluk, Tammela et al. 2005). Yet, lymphatic specific ablation of the VEGFR2 gene leads to impaired lymphangiogenesis in mouse embryos and adult mice (Dellinger, Meadows et al. 2013). VEGFR2 becomes upregulated in angiogenic conditions and is a critical component of tumor vascularization (Claesson-Welsh and Welsh 2013).

4.1.6 VEGFR3

Fms-like tyrosine kinase 4 (FLT4) was cloned from the human HEL erythroleukemia cell cDNA library (Pajusola, Aprelikova et al. 1992). VEGFR3 is a 195 kDa transmembrane tyrosine kinase whose fifth Ig-like domain is proteolytically cleaved, and the subunits are linked together by a disulphide bond (-S-S-) (Pajusola, Aprelikova et al. 1994, Lee, Gray et al. 1996). VEGFR3 has two ligands, VEGFC and VEGFD. It was reported that stimulation with VEGFC or VEGF is able to induce the formation of VEGFR2/VEGFR3 heterodimers in vitro that display an altered phosphorylation-site pattern compared to VEGFR3 homodimers (Dixelius, Makinen et al. 2003, Nilsson, Bahram et al. 2010). VEGFR3 phosphorylation by c-Src can be triggered by adhesion to the ECM through integrin β1, in a ligand-independent way (Wang, Zhang et al. 2001, Galvagni, Pennacchini et al. 2010).

VEGFR3 exists in two spliced variants (Pajusola, Aprelikova et al. 1993), as well as in a truncated, soluble form in the mouse cornea, that has been suggested to play a critical role in the lack of corneal lymphatic vessels (Singh, Tiem et al. 2013).

During embryogenesis VEGFR3 is expressed in BECs starting at E8.5 and is required for the remodeling and maturation of the developing vascular plexus; as a result embryos deficient for VEGFR3 die at E9.5 (Dumont, Jussila et al. 1998). Surprisingly, embryos deleted for both VEGFR3 ligands (VEGFC and VEGFD) do now show any blood vascular defects, but rather phenocopy the VEGFC deleted embryos (Haiko, Makinen et al. 2008). This suggests either that another ligand for VEGFR3 exists, or that the receptor functions in a ligand-independent manner during an early developmental stage. As development proceeds, VEGFR3 expression becomes gradually downregulated in BECs and at around E14.5-15.5
becomes restricted to LECs (Kaipainen, Korhonen et al. 1995). Inhibition of VEGFR3 signaling during late embryonic development or during the first two postnatal weeks in mice results in reversible lymphatic vessel regression, however the lymphatic vasculature of adult mice does not rely on ligand-dependent activation of VEGFR3 (Makinen, Jussila et al. 2001, Karpanen, Wirzenius et al. 2006). In the zebrafish, VEGFR3 drives sprouting angiogenesis in the developing intersegmental arteries (Covassin, Villefranc et al. 2006).

In the adult, VEGFR3 is expressed in LECs and transduces proliferation, migration, and survival signals (Makinen, Veikkola et al. 2001). Furthermore VEGFR3 is expressed in high endothelial venules (HEVs) and fenestrated capillaries of several organs including the bone marrow, spleen, liver, kidney glomerulus and endocrine glands (thyroid, parathyroid, adrenals and adrenohypophysis) (Lymboussaki, Partanen et al. 1998, Partanen, Arola et al. 2000). VEGFR3 expression becomes upregulated in angiogenic endothelia, for example in the tumor and wound vasculature, or following VEGF stimulation (Valtola, Salven et al. 1999, Paavonen, Puolakkainen et al. 2000, Partanen, Arola et al. 2000, Witmer, van Blijswijk et al. 2001, Laakkonen, Waltari et al. 2007). VEGFR3 expression in blood endothelia has been implicated in the maintenance of blood vessel integrity during angiogenesis (Kubo, Fujiwara et al. 2000) by modulating sensitivity to VEGFR2 (Matsumura, Hirashima et al. 2003). Other sites of VEGFR3 expression are neurons and astrocytes in the mouse and rat (Le Bras, Barallobre et al. 2006, Hou, Shin et al. 2011), as well as mouse neural stem cells, where it promotes neurogenesis after VEGFC stimulation (Calvo, Fontaine et al. 2011). VEGFR3 is expressed in motoneurons in zebrafish (Kwon, Fukuhara et al. 2013), where it transmits trophic/guidance signals and may participate in the pathophysiology of central/peripheral nervous system (CNS/PNS) diseases. VEGFR3 is expressed in dendritic cells during corneal inflammation (Hamrah, Chen et al. 2003), in conjunctival monocytes/macrophages (Hamrah, Chen et al. 2004) and in recruited monocytes/macrophages in tumors (Skobe, Hamberg et al. 2001b) and in wounds (Saaristo, Tammela et al. 2006). Finally VEGFR3 can be found in megakaryocytic progenitor cells, where it may regulate megakaryopoiesis (Thiele, Krishnan et al. 2012) and in the corneal epithelium, contributing to corneal avascularity (Cursiefen, Chen et al. 2006). Given the importance of VEGFR3 signaling in lymphangiogenesis, VEGFR3 is critically involved in tumor-associated lymphangiogenesis and lymphatic metastasis (He, Rajantie et al. 2005, Lin, Lalani et al. 2005, Roberts, Kloos et al. 2006), representing an attractive target for anti-lymphangiogenic therapies in cancer.

4.2 Notch

Notch signaling is an evolutionarily conserved pathway that plays a central role in cell fate specification and differentiation, and has pleiotropic effects during embryonic development and adult life (Artavanis-Tsakonas, Matsuno et al. 1995). The Notch gene was originally identified and characterized in Drosophila melanogaster (Mohr 1919, Wharton, Johansen et al. 1985). In mammals the Notch signaling pathway consists of four Notch receptors (Notch1-4) and five ligands (Jagged1-2 and Delta-like [DLL] 1-3). In the blood vascular system ECs express
Notch 1 and 4, Jagged1-2, DLL1 and DLL4, while SMCs express Notch1 and 3 and Jagged1 (Kume 2009).

Notch ligands and receptors are large single-pass transmembrane proteins containing epidermal-growth-factor (EGF) repeats in their extracellular domains. Notch ligands contain an N-terminal DSL domain that is required for interactions with their receptors, while the intracellular domain of Notch receptors consists of a RAM domain, six ankyrin (Ank) repeats and a C-terminal PEST domain, as well as nuclear localization signals. Notch signaling requires contacts between neighboring cells and upon ligand binding the Notch receptor is proteolytically cleaved twice, first just outside of the plasma membrane by the ADAM17 metalloprotease (also known as TNFα-converting enzyme, TACE) and then in the cytoplasmic side by a γ-secretase complex, releasing the Notch intracellular domain (NICD). The NICD then translocates to the nucleus and forms a trimeric complex with the DNA-binding protein CSL (also known as recombination signal sequence-binding protein J, RBP-J), and the coactivator MAM. In the absence of NICD, CSL functions as a transcriptional repressor but interaction with NICD turns CSL to an activator by corepressor displacement and recruitment of coactivators, resulting in derepression of transcription of several Notch target genes, including the basic helix-loop-helix (bHLH) transcriptional factors hairy/enhancer of split (HES, HEY) (Borggrefe and Oswald 2009) (Figure 3). Apart from the aforementioned “canonical” Notch pathway, a “non-canonical” Notch pathway also exists, which is CSL independent and involves negative regulation of Wnt/β-catenin signaling by Notch (Le Gall, De Mattei et al. 2008, Andersen, Uosaki et al. 2012).

**Figure 3. The Notch signaling pathway.** Both Notch ligands and receptors are transmembrane proteins expressed in adjacent cells. Upon receptor/ligand interaction, the ADAM metalloproteinase TACE cleaves the extracellular portion of the Notch protein. The extracellular domain together with the ligand are then endocytosed by the signal-sending cell. A γ-secretase cleaves the remaining part of the Notch protein in the signal-receiving cell and releases the Notch intracellular domain, which activates the transcription factor CSL, resulting in the transcription of Notch target genes such as HEY, HES and NRARP. CoR: co-repressors, HDAc: Histone deacetylase, MAML: Mastermind-like, HAc: Histone acetyltransferase, NECD: Notch extracellular domain, NICD: Notch intracellular domain. Modified from (Phng and Gerhardt 2009).
Notch signaling is indispensable for the proper development of the vasculature (Hofmann and Iruela-Arispe 2007). Notch1 mutants die at E9.5 due to cardiovascular and other defects (Swiatek, Lindsell et al. 1994, Conlon, Reaume et al. 1995, Krebs, Xue et al. 2000, Limbour, Takeshita et al. 2005) and Notch3 null mice show arterial differentiation defects (Domenga, Fardoux et al. 2004). While Notch4-/- mice are phenotypically normal, compound loss of Notch1 and Notch4 results in a more severe vascular phenotype than Notch1 homozygous LOF, indicating overlapping functions of the two receptors during vascular development (Krebs, Shutter et al. 2004). On the other hand, loss of Notch ligands Jagged1 and DLL1 results in embryonic death between E10.5-E12, due to vascular defects and hemorrhage (Hrabe de Angelis, McIntyre et al. 1997, Xue, Gao et al. 1999). DLL4 haploinsufficient mice fail to remodel their primitive vascular plexus and form arteries, and die at E9.5. DLL4 haploinsufficiency is strain dependent and highlights the dosage-sensitive importance in Notch signaling (Duarte, Hirashima et al. 2004, Gale, Dominguez et al. 2004).

4.2.1 Notch signaling in arterial-venous specification

Arteries and veins have different gene expression profiles, with arterial ECs expressing Notch1, NP1 and EphrinB2, and venous ECs expressing EphB4 and NP2 (Wang, Chen et al. 1998, Herzog, Kalcheim et al. 2001). Arterial and venous identities are established early in development, before the initiation of hemodynamic flow, indicating the existence of a genetic program, which defines EC identity (le Noble, Fleury et al. 2005). Notch signaling plays a critical role in arterial specification, by repressing venous fate. Mutations in Notch pathway components result in loss/misexpression of artery/vein specific markers, defective artery formation and arteriovenous shunts (Roca and Adams 2007). In mice, the nuclear receptor COUP-TFII suppresses Notch signaling and promotes venous differentiation (You, Lin et al. 2005), while DLL4 heterozygosity results in lack of arterial marker expression and artery malformation (Gale, Dominguez et al. 2004, Krebs, Shutter et al. 2004). In zebrafish, Sonic Hedgehog induces VEGF expression, which in turn upregulates Notch in the aorta and controls arterial differentiation (Lawson, Scheer et al. 2001, Lawson, Vogel et al. 2002). Furthermore, the FOXC transcription factors FOXC1 and FOXC2 have been shown to act upstream of Notch in the regulation of arterial specification, and to activate directly the DLL4 and HEY2 promoters (Seo, Fujita et al. 2006, Hayashi and Kume 2008). Finally Notch signaling is required for proper formation and patterning of postnatal mouse arteries, as well as blood vessel maturation, especially with respect to veins and perivenous capillaries (Ehling, Adams et al. 2013).

4.2.2 Notch signaling in tip-stalk cell specification

Notch is a major determinant of cell fate and of the establishment of tip/stalk cell identity during sprouting angiogenesis. In the mouse retina the VEGF gradient produced by astrocytes provides a scaffold for tip cells to extend their filopodia towards higher VEGF concentrations. Although all ECs are exposed to the VEGF gradient, tip cells form only at intervals in the vascular front, based on DLL4/Notch activity. VEGF and hypoxia upregulate DLL4 expression in some ECs, which
preferentially acquire the tip cell position. Tip cells activate Notch signaling in their neighboring cells that adopt the stalk cell fate. Subsequently, Notch activation suppresses DLL4 expression (Gerhardt, Golding et al. 2003, Patel, Li et al. 2005, Noguera-Troise, Daly et al. 2006, Ridgway, Zhang et al. 2006, Williams, Li et al. 2006, Lobov, Renard et al. 2007, Suchting, Freitas et al. 2007, Roukens, Alloul-Ramdhani et al. 2010). Hence, tip cells express high levels of DLL4 while stalk cells express Notch target genes, like HEY1, HEY2, HES and NRARP (Claxton and Fruttiger 2004, Hellstrom, Phng et al. 2007, Hofmann and Iruela-Arispe 2007). This feedback loop, called lateral inhibition, allows the DLL4 expressing cell to further increase ligand production, intensifying initial small differences in ligand expression and resulting in a “salt and pepper” distribution of cells with diverse identities (Lewis 1998, Suchting, Freitas et al. 2007, Jakobsson, Bentley et al. 2009). The acquisition of the tip cell phenotype relies on randomly determined differences in VEGFR2 expression and other tip/stalk cell-specific genes, or small differences in the local VEGF microenvironment. As a result ECs expressing higher levels of VEGFR2 and DLL4 (or lower levels of VEGFR1, Notch1, NRARP and Jagged1) have an advantage in maintaining the tip cell position (Blanco and Gerhardt 2013). Yet tip and stalk cells continuously and dynamically swap their positions, indicating a constant “battle for the lead” that depends on relative VEGFR2 levels and DLL4 expression (Bentley, Gerhardt et al. 2008, Bentley, Mariggi et al. 2009, Jakobsson, Franco et al. 2010). Another player in tip cell selection is Jagged1. Jagged1 is a weak Notch agonist that localizes mainly to stalk cells. It promotes tip cell formation by antagonizing the ability of DLL4 to activate Notch. It has been suggested that glycosylation of Notch by the glycosyltransferase Fringe enhances or reduces the response of Notch stimulation by DLL4 or Jagged1 binding respectively (Benedito, Roca et al. 2009). NRARP is also expressed in stalk cells where it limits Notch activity at vessel branching points in order to promote EC proliferation and stability (Phng, Potente et al. 2009). Notch has been shown to specify stalk cell identity by cooperating with the bone morphogenetic protein (BMP) signaling (Larrivee, Prahst et al. 2012, Moya, Umans et al. 2012).

Given the significance of Notch signaling in restricting tip cell behavior, loss of Notch signaling by pharmacological or genetic means leads to increased filopodia formation, sprouting and branching, while Notch activation has the opposite effects (Hellstrom, Phng et al. 2007, Lobov, Renard et al. 2007, Suchting, Freitas et al. 2007). VEGFR2 upregulation and VEGFR1 downregulation following Notch LOF could partially explain the observed hypervascular phenotypes.

4.2.3 Notch and VEGFR signaling

multiple lines of evidence that VEGF/VEGFR2 signaling is required upstream of DLL4 upregulation and Notch activation, a recent study that utilized conditional endothelial specific deletion of VEGFR2 in neonatal mice suggested that DLL4 is still expressed in ECs lacking VEGFR2 signaling, and that VEGFR2 levels are not altered upon Notch inhibition. In this study, ECs without VEGFR2 could still hypersprout under low Notch signaling conditions, and inhibition of VEGFR3 tyrosine kinase was able to restrict excessive sprouting associated with Notch LOF (Benedito, Rocha et al. 2012).

Notch regulates also the expression of other VEGFRs. VEGFR1 and sVEGFR1 are induced by Notch activation (Suchting, Freitas et al. 2007, Harrington, Sainson et al. 2008, Funahashi, Shawber et al. 2010, Benedito, Rocha et al. 2012). VEGFR3 is downregulated by Notch in vivo in zebrafish, and Notch LOF upregulates VEGFR3 expression (Siekmann and Lawson 2007). However some in vitro studies show that VEGFR3 is upregulated by Notch (Shawber, Funahashi et al. 2007). Discrepancies between in vivo and in vitro results highlight the importance of the cellular context in which Notch signaling is studied.

4.3 Other VEGF receptors
4.3.1 Neuropilins

Neuropilins are single transmembrane proteins with a small cytoplasmic domain and no catalytic function (Fujiwara, Kitsukawa et al. 1997). Originally identified as receptors for class III semaphorins mediating repulsive signals during axonal growth (Raper 2000), neuropilins were later found to form complexes with VEGFRs. NP1 is a co-receptor for VEGFR2, enhancing its activity in the presence of VEGF165 (Whitaker, Limberg et al. 2001, Soker, Miao et al. 2002). Interestingly in trans VEGFR2/NP1 complex formation has been shown to inhibit angiogenesis and to promote endothelial quiescence (Koch, van Meeteren et al. 2014). NP1 can act also as a co-receptor for VEGFR1 (Soker, Miao et al. 2002) and VEGFR3 (Karpanen, Heckman et al. 2006). PLGF, VEGFB, VEGFC and VEGFD are also able to bind NP1 (Migdal, Huppertz et al. 1998, Makinen, Olofsson et al. 1999, Gluzman-Poltorak, Cohen et al. 2000, Karpanen, Heckman et al. 2006). NP1 is mainly expressed in arterial endothelium (Herzog, Kalcheim et al. 2001, Moyon, Pardanaud et al. 2001) and NP1 deleted mice, as well as endothelial-specific NP1 mutants, die at midgestation due to defects in vessel formation (Kawasaki, Kitsukawa et al. 1999, Gu, Rodriguez et al. 2003). Although mice lacking the cytoplasmic domain of NP1 are viable, displaying only minor arteriovenous patterning defects, NP1 has been shown to act in an cell-autonomous way to support tip cell function in ECs (Fanti, Schwarz et al. 2011, Fantin, Vieira et al. 2013). Mice carrying a mutation of tyrosine 297 in NP1 show reduced affinity to VEGF binding, impaired NP1/VEGFR2 complex formation and attenuated NP1 expression. Surprisingly these NP1 hypomorphic mutants display a milder phenotype than the NP1 knockout mice, suggesting that NP1 has also VEGF-independent roles during development (Fanti, Herzog et al. 2014).

NP2 binds several VEGF family members, including VEGF and PLGF (Neufeld, Cohen et al. 2002), as well as VEGFC and VEGFD (Karpanen, Heckman et al. 2006). NP2 interacts with VEGFR3 (Karpanen, Heckman et al. 2006) and regulates lymphatic vessel sprouting (Xu, Yuan et al. 2010). NP2 is mainly expressed in veins
and lymphatic vessels, and homozygous NP2 mutants display reduced number and size of small lymphatic vessels (Yuan, Moyon et al. 2002). Remarkably, compound inactivation of both NP1 and NP2 results in embryonic death at E8.5 due to severe defects in vascular plexus formation (Takashima, Kitakaze et al. 2002), implying redundant NP signaling during early vascular development.

4.4 Integrins and the ECM

Integrins are transmembrane heterodimers, composed of α and β subunits, that bind to matrix proteins, such as collagen, fibronectin, vitronectin and laminin (Silva, D’Amico et al. 2008). VEGFR2 forms complexes with αβ3 integrin upon VEGF stimulation, and this is crucial for full VEGFR2 activity and angiogenesis. VEGFA induces phosphorylation of the β3 subunit, resulting in integrin activation, interaction with VEGFR2, amplification of VEGFR2 phosphorylation, and activation of Src (Pampori, Hato et al. 1999, Masson-Gadais, Houle et al. 2003, Mahabeleshwar, Feng et al. 2006, Mahabeleshwar and Byzova 2008, Mahabeleshwar, Chen et al. 2008). Potentiation of VEGFR3 signaling may happen via interaction of the receptor with integrin α5β1 after VEGFC stimulation (Zhang, Groopman et al. 2005). VEGF, VEGFC and VEGFD can bind directly to integrin α9β1, contributing to pathological angio- and lymphangiogenesis, and mice deficient for integrin α9β1 die after birth due to chylothorax (Vlahakis, Young et al. 2005). Finally, stimulation of integrin β1 by collagen or fibronectin promotes VEGFR3 phosphorylation in a ligand-independent manner, through Src (Wang, Zhang et al. 2001, Galvagni, Pennacchini et al. 2010).

Fibronectin is an adhesion and migration ligand for the ECs. Mice deficient in astrocytic fibronectin production display reduced EC migration and abnormal filopodia projections in the retina, due to inhibition of VEGF binding to fibronectin (Wijelath, Rahman et al. 2006), and to the loss of filopodia interactions with the fibronectin network through integrin α5β1 (Stenzel, Franco et al. 2011). Moreover, fibronectin, as well as collagen type I, associate with the vascular-specific secreted factor epidermal growth factor-like domain 7 (EGFL7), which has been shown to interact with Notch receptors and antagonize DLL4/Jagged1/Notch interactions (Schmidt, Paes et al. 2007, Nichol, Shawber et al. 2010).

Laminin 411 is mainly expressed at the vascular front, where it restricts tip cell formation via interactions with α-β1 integrin heterodimers. This results in the transcriptional activation of DLL4 and Notch (Stenzel, Franco et al. 2011). In vitro, laminin 111 has been found to interact with α-β1 integrins and to regulate DLL4 expression in the ECs. Interestingly, integrin α2β1 stimulation by laminin triggers the expression of the transcription factor FOXC2 (Estrach, Cailleteau et al. 2011). On the other hand FOXC2 regulates the expression of integrin β3 (Hayashi, Sano et al. 2008).

Heparan sulfate proteoglycans in the ECM also have important roles in vascular patterning. Heparin binding domains on VEGF165 and VEGF189, encoded by exons 6 and 7, allow binding of VEGF to the ECM, and the establishment of concentration gradients in tissues that provide patterning cues for the developing vasculature (Park, Keller et al. 1993). PLGF and VEGFB167 also contain heparin-
VEGFR3 and Notch signaling in angiogenesis

binding domains (Hauser and Weich 1993). Interestingly while tip cells require controlled VEGF distribution in order to orient their filopodia and guide the angiogenic sprout, stalk cells rely principally on local VEGF concentrations for proliferation (Ruhrberg, Gerhardt et al. 2002). Binding of VEGF to ECM modifies its downstream signaling properties, as well as its ability to interact with the β1 integrins (Chen, Luque et al. 2010). Finally, MMPs have been implicated in the cleavage of VEGF and its release from the ECM (Lee, Jilani et al. 2005).

4.5 Other regulators of vascular development

Several other factors are involved in the process of angiogenesis and lymphangiogenesis (Adams and Alitalo 2007, Coso, Bovay et al. 2014). These include the angiopoietin/TIE system that plays an essential role in vessel growth, maturation and stability (Eklund and Saharinen 2013), the fibroblast growth factors (FGFs) that signal through fibroblast growth factor receptors (FGFRs) to promote angiogenic activity (Presta, Dell'Era et al. 2005), transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) signaling (Cai, Pardali et al. 2012), and platelet-derived growth factor B (PDGF-B)/platelet-derived growth factor receptor-β (PDGFR-β) signaling that is crucial for the recruitment of pericytes (Armulik, Abramsson et al. 2005). In addition, many neural axon guidance molecules are indispensable for capillary growth and vascular patterning; these include the Robo/Slit, Netrin/UNC5B, Semaphorin/Plexin and Ephrins/Eph RTKs signaling pathways (Adams and Eichmann 2010). Apart from the aforementioned “classic” factors several “nonclassic” angiogenesis stimulators have been identified as well, such as erythropoietin (EPO), angiotensin II (ANGII), endothelins (ETs) and others (Ribatti, Conconi et al. 2007). Finally various miRNAs are expressed specifically in the ECs and play key roles in vessel development (Liu, Krueger et al. 2011, Dang, Lawson et al. 2013).

5. THERAUPETIC VIEWPOINTS

5.1 Anti- and pro-angiogenic therapies

5.1.1 Tumor angiogenesis modulation

Antiangiogenic therapies are based on the concept of blocking tumor vascularization (Folkman 1971). Given the central role of VEGF signaling in tumor angiogenesis, it is not surprising that all currently FDA approved angiogenesis inhibitors target the VEGF pathway (http://www.fda.gov/). These inhibitors include the VEGF inhibiting antibody bevacizumab (Avastin), the VEGFR1/VEGFR2 fusion protein aflibercept that acts as a soluble decoy receptor, and multi-target tyrosine kinase inhibitors (including sorafenib, sunitinib, pazopanib caboazantinib, regorafenib, vandetanib and axitinib) that target principally VEGFRs and PDGFRβ (Welti, Loges et al. 2013). Despite the largely successful preclinical testing, angiogenesis inhibitors have had only modest efficacy in the clinical setting, posing new questions and challenges to the research community. Anti-angiogenic therapies often result in a transient clinical improvement, which is followed by tumor relapse.
and regrowth (Ebos and Kerbel 2011). Several mechanisms of resistance to anti-angiogenesis treatments have been proposed. Tumors may utilize alternative pro-angiogenic signaling pathways, such as FGF2 or PLGF (Batchelor, Sorensen et al. 2007, Kopetz, Hoff et al. 2010), may recruit bone-marrow pro-angiogenic cells that express pro-angiogenic cytokines (such as TAMs, TIE2 expressing macrophages [TEMs], VEGFR1+ haematopoietic progenitors, and CD11b+Gr1+ myeloid cells) (Pollard 2004, De Palma, Venneri et al. 2005, Kaplan, Riba et al. 2005, Shojaei, Wu et al. 2007), may “normalize” the vasculature by increasing pericyte coverage (Bergers, Song et al. 2003), and promote local invasion in order to gain access to normal tissue vasculature (Ebos, Lee et al. 2009, Narayana, Kelly et al. 2009, Paez-Ribes, Allen et al. 2009). These alternative ways to sustain tumor growth independently of VEGF signaling underlie “evasive resistance” (Bergers and Hanahan 2008). On the other hand not all tumors are sensitive to anti-angiogenic treatments, thus showing “intrinsic resistance” (Shojaei and Ferrara 2007, Olson and Joyce 2013).

The exact mode of function of antiangiogenic therapies remains unclear. Apart from inhibiting angiogenesis, pruning of the tumor vasculature and depriving the cancer cells from oxygen and nutrients, they may block recruitment of bone marrow derived cells into the tumor (Ellis and Hicklin 2008) and reduce the amount and self-renewal potential of cancer stem cells (Beck, Driessens et al. 2011). Importantly, angiogenesis inhibitors may decrease excessive intratumoral VEGF levels, leading to “normalization” of the vasculature. The concept of the vasculature normalization could explain why VEGF inhibitors improve survival in cancer patients only when used in combination with chemotherapy/radiotherapy/immunotherapy (Inai, Mancuso et al. 2004, Tong, Boucher et al. 2004, Dickson, Hamner et al. 2007, Goel, Duda et al. 2011). Based on the vascular normalization hypothesis, anti-VEGF treatment may improve tumor blood vessel morphology and functionality, resulting in reduced vessel permeability (Yuan, Chen et al. 1996), reduced hypoxia (Lee, Heijn et al. 2000), and improved blood flow within the tumor (Wildiers, Guetens et al. 2003); thus permitting delivery of other anticancer drugs and preventing cancer cell intravasation and hematogenous metastasis (Jain 2005). Yet angiogenesis inhibitors may also affect the normal vasculature, and they demonstrate a large spectrum of adverse effects that include hypertension (Sane, Anton et al. 2004), renal dysfunction that manifests itself usually as proteinuria (Hayman, Leung et al. 2012), arteriovenous thrombotic events (Nalluri, Chu et al. 2008), cardiomyopathy and congestive heart failure (Ky, Vejpongsa et al. 2013), hemorrhage, and impaired wound healing (Chen and Cleck 2009).

Another way to counteract tumor vascularization involves inhibition of Notch signaling, which results in the formation of a hyperbranched, poorly perfused and non-functional vascular network, leading to tumor ischemia (Noguera-Troise, Daly et al. 2006, Ridgway, Zhang et al. 2006, Scehnet, Jiang et al. 2007, Thurston, Noguera-Troise et al. 2007). However, long term blockade of DLL4 has been shown to cause liver hemangiomas in mice (Li, Jubb et al. 2010). On the other hand, a soluble extracellular domain of Notch1 (“Notch1 decoy”) has been shown to block VEGF-induced angiogenesis, and to inhibit tumor angiogenesis and growth in mice (Funahashi, Hernandez et al. 2008), plus showing synergistic effects with VEGF
blockade in reducing tumor vascularization and viability (Hernandez, Banerjee et al. 2013). Yet, deregulation of Notch signaling may contribute to carcinogenesis in multiple ways. Compounds inhibiting Notch signaling (including blocking antibodies and γ-secretase inhibitors) are currently in Phase I clinical trials (Espinoza and Miele 2013).

A plethora of novel antiangiogenesis inhibitors that target VEGF/VEGFR signaling and multiple other angiogenic pathways such as integrins, angiopoietins, TGFβ and fibroblast growth factor receptor (FGFR) (Clarke and Hurwitz 2013), are currently in various phases of clinical trials for the treatment of cancer (http://www.cancer.gov/clinicaltrials). Multitargeted and combination treatments could maximize the effectiveness of anti-cancer therapies and reduce the possibility of resistance development (Limaverde-Sousa, Sternberg et al. 2014).

5.1.2 Ocular disease

Blocking VEGF has proved to be a successful approach for the treatment of neovascular eye diseases, including age-related macular degeneration (AMD), diabetic retinopathy (DR) and retinal vein occlusions (RVO) (Kim and D’Amore 2012). Agents used or being currently in clinical trials include the VEGF neutralizing antibodies bevacizumab and ranibizumab, as well as the VEGF-trap aflibercept. These treatments have been a breakthrough in the field of intraocular vascular diseases due to their high efficacy and safety (Miller, Le Couter et al. 2013).

5.1.3 Other angiogenesis-related diseases

Modulation of angiogenesis could be a valuable therapeutic approach for multiple conditions. Preclinical studies have shown that adenoviral delivery or intracerebroventricular infusion of VEGF could slow down the progression of amyotrophic lateral sclerosis (ALS), and a Phase I/II clinical trial utilizing VEGF as a treatment for ALS is currently ongoing (Keifer, O’Connor et al. 2014). Gene vector delivery of VEGFs has shown promising results in the ischemic myocardium and peripheral skeletal muscle of small and large animals, and several Phase I/II clinical studies have been completed during the past years (Yla-Herttuala 2013). Proangiogenic gene transfer therapies have been successfully validated for use in revascularization of the CNS in mice (Gaal, Tammela et al. 2013). Finally, inhibition of angiogenesis associated with inflammation is widely investigated as a novel therapeutic approach to treat rheumatic diseases, atherosclerosis, inflammatory bowel disease and the metabolic syndrome (Costa, Incio et al. 2007).

5.2 Anti- and pro-lymphangiogenic therapies

5.2.1 Lymphatic metastasis

It has been estimated that 80% of solid tumor metastasis occurs via the lymphatic system (Warren, Ziyad et al. 2014). Since most cancer patients die due to metastatic disease (Coghlin and Murray 2010), the development of anti-lymphangiogenic cancer therapies should be of importance. Tumors, perivascular cells and TAMs express lymphangiogenic factors such as VEGFC and VEGFD and induce lymphatic vessel sprouting, enlargement of peritumoral lymphatics and
lymph node lymphangiogenesis. Tumor associated lymphatics act as a conduit for tumor cells to disseminate and metastasize to sentinel lymph nodes and potentially to distant organs (Alitalo and Detmar 2012). Consequently, experimental overexpression or blocking of VEGFC or VEGFD signaling in animal models, promotes or suppresses respectively lymph node metastasis (Thiele and Sleeman 2006). Elevated levels of VEGFC and VEGFD in human tumors correlate with high incidence of lymph node and distal metastasis, and poor patient prognosis (He, Karpanen et al. 2004, Hirakawa, Brown et al. 2007, Sleeman and Thiele 2009). On the other hand, while sentinel lymph node involvement has a high prognostic and staging value in some types of cancer, such as breast cancer and melanoma (Cousins, Thompson et al. 2014), it has been recently shown that systematic local nodal dissection does not have survival benefits in early breast cancer or in melanoma, respectively (Gershenwald and Ross 2011, Salhab, Patani et al. 2011, Meiers, Cil et al. 2013, Rao, Euhus et al. 2013). Overall, it is still unclear how lymph nodes and the lymphatic route are involved in the formation of distal metastasis (Sleeman, Nazarenko et al. 2011) and further research is required to evaluate the potential of anti-lymphangiogenic therapies in cancer metastasis (Alitalo and Detmar 2012). Monoclonal antibodies targeting VEGFR3 and VEGFC are currently in Phase I clinical trials for anti-angiogenic tumor therapy (IMC-3C5, NCT01288989 and VGX-100, NCT01514123 respectively). Finally, targeting tumor cell-aggregates within the tumor-draining lymphatic vessels using the cytotoxic compound verteporfin in combination with photodynamic therapy (that activates the dye) has been shown to prevent metastasis in preclinical models (Tammela, Saaristo et al. 2011).

5.2.2 Lymphedema

Current treatments of secondary lymphedema include pressure garments, manual lymphatic drainage, intermittent pneumatic compression devices, low-level laser therapy, and exercise (Oremus, Dayes et al. 2012). Surgical procedures like liposuction, flap transfer, lymphatic bypass and lymph node transplantation are also utilized in the treatment of lymphedema (Mehrara, Zampell et al. 2011). Lymph node transfer in combination with growth factor therapy with VEGFC has increased the efficiency of incorporation of the lymph nodes into the lymphatic vasculature, providing a better preservation of the function of the transplanted lymph nodes in mice and pigs (Becker, Assouad et al. 2006, Tammela, Saaristo et al. 2007, Lahteenvuo, Honkonen et al. 2011, Honkonen, Visuri et al. 2013).
AIMS OF THE STUDY

This study aims to elucidate the role of VEGFR3 in physiological and pathological angiogenesis, as well as the signaling interplay between VEGFR3, VEGFR2 and Notch during vessel formation. The specific aims of this thesis are:

I To study the function of VEGFR3 in embryonic, postnatal and pathological angiogenesis

II To elucidate the molecular mechanisms underlying VEGFR3 signaling, in relation to Notch signaling

III To clarify the relationship between VEGFR2, VEGFR3 and Notch signaling during postnatal vessel development
MATERIALS AND METHODS

A summary of the materials and methods used in these studies is given below. Detailed description is provided in the following text or in the original publications, indicated here by their assigned Roman numbers.

1. MATERIALS

Table I. Mouse lines

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Description</th>
<th>Reference/Source</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/c nu/nu, NMRI nu/nu</td>
<td>Deletion of the Foxn1 gene, resulting in absence or thymus and immunodeficiency</td>
<td>Taconic</td>
<td>I</td>
</tr>
<tr>
<td>Cdh5CreER&lt;sup&gt;T2&lt;/sup&gt;</td>
<td>Inducible Cre recombinase under the control of the Cdh5 gene promoter</td>
<td>(Wang, Nakayama et al. 2010)</td>
<td>III</td>
</tr>
<tr>
<td>Csf1&lt;sup&gt;op/op&lt;/sup&gt;</td>
<td>Mice currying the osteopetrosis spontaneous mutation (Csf1&lt;sup&gt;op&lt;/sup&gt;)</td>
<td>(Wiktor-Jedrzejczak, Ahmed et al. 1982)</td>
<td>II</td>
</tr>
<tr>
<td>Dll4&lt;sup&gt;+/LacZ&lt;/sup&gt;</td>
<td>The β-galactosidase (lacZ) gene has been inserted into the Dll4 locus, resulting in gene inactivation</td>
<td>(Duarte, Hirashima et al. 2004)</td>
<td>I</td>
</tr>
<tr>
<td>Foxc2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Deletion of the Foxc2 gene</td>
<td>(Iida, Fujita et al. 1997)</td>
<td>II</td>
</tr>
<tr>
<td>K14-VEGF-E</td>
<td>Overexpression of VEGF-E(NZ-7) under the control of the Keratin-14 promoter.</td>
<td>(Kiba, Sagara et al. 2003)</td>
<td>I</td>
</tr>
<tr>
<td>K14-VEGF&lt;sub&gt;165&lt;/sub&gt;</td>
<td>Overexpression of human VEGF&lt;sub&gt;165&lt;/sub&gt; under the control of the Keratin-14 promoter</td>
<td>(Zheng, Murakami et al. 2006)</td>
<td>I</td>
</tr>
<tr>
<td>K14-VEGFR-3-Ig</td>
<td>Overexpression of human VEGFR-3-Ig fusion protein under the control of the Keratin-14 promoter</td>
<td>(Makinen, Jussila et al. 2001)</td>
<td>I</td>
</tr>
<tr>
<td>NMRI, CD1, C57Bl, FVB/n</td>
<td>Wild type outbred mice</td>
<td>Taconic</td>
<td>I, II</td>
</tr>
<tr>
<td>PdgfbCreER&lt;sup&gt;T2&lt;/sup&gt;</td>
<td>Inducible Cre recombinase under the control of the Pdgfb gene promoter</td>
<td>(Claxton, Kostourou et al. 2008)</td>
<td>II, III</td>
</tr>
<tr>
<td>Prox1CreER&lt;sup&gt;T2&lt;/sup&gt;</td>
<td>Inducible Cre recombinase under the control of the Prox1 gene promoter</td>
<td>(Bazigou, Lyons et al. 2011)</td>
<td>III</td>
</tr>
<tr>
<td>Rip1Tag2</td>
<td>Expression of the oncogenic SV40 Large T antigen transgene (TAg) in beta cells of the pancreatic islets, under the rat insulin promoter</td>
<td>(Hanahan 1985)</td>
<td>I</td>
</tr>
<tr>
<td>ROSA26-R</td>
<td>Expression of the β-galactosidase (lacZ) gene after Cre-recombination</td>
<td>(Soriano 1999)</td>
<td>II</td>
</tr>
<tr>
<td>tdTomato</td>
<td>Expression of the red fluorescent protein tdTomato, after Cre-recombination</td>
<td>(Madisen, Zwingman et al. 2010)</td>
<td>III</td>
</tr>
<tr>
<td>Vegf&lt;sup&gt;+/LacZ&lt;/sup&gt;</td>
<td>The β-galactosidase (lacZ) gene has been inserted into the Vegfc locus, resulting in gene inactivation</td>
<td>(Karkkainen, Haiko et al. 2004)</td>
<td>I, II</td>
</tr>
<tr>
<td>Vegfd&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Deletion of the Vegfd gene</td>
<td>(Baldwin, Halford et al. 2005)</td>
<td>II</td>
</tr>
<tr>
<td>Vegfr2&lt;sup&gt;+/LacZ&lt;/sup&gt;</td>
<td>The β-galactosidase (lacZ) gene has been inserted into the Vegfr2 locus, resulting in gene inactivation</td>
<td>(Shalaby, Rossant et al. 1995)</td>
<td>III</td>
</tr>
<tr>
<td>Vegfr2&lt;sup&gt;/loxlox&lt;/sup&gt;</td>
<td>Vegfr2 conditional mouse</td>
<td>(Haigh, Morelli et al. 2003)</td>
<td>III</td>
</tr>
</tbody>
</table>
**VEGFR3 and Notch signaling in angiogenesis**

- Vegfr3<sup>+/KD</sup> mice currying a heterozygous A3157T mutation in the tyrosine kinase domain of Vegfr3 (Karkkainen, Saaristo et al. 2001)
- Vegfr3<sup>+/LacZ</sup> The β-galactosidase (lacZ) gene has been inserted into the Vegfr3 locus, resulting in gene inactivation (Dumont, Jussila et al. 1998)
- Vegfr3<sup>lox/lox</sup> Vegfr3 conditional mouse (Haiko, Makinen et al. 2008)

### Table II. Antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Reference/Source</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU (Bromodeoxyuridine)</td>
<td>Mouse monoclonal, Alexa Fluor 594 conjugated</td>
<td>Invitrogen, B35132, clone MoBU-1</td>
<td>I, II</td>
</tr>
<tr>
<td>CD11b, mouse</td>
<td>Rat monoclonal, Fitc conjugated</td>
<td>BD Biosciences, 550282, clone M1/70</td>
<td>I</td>
</tr>
<tr>
<td>Collagen IV, mouse</td>
<td>Rabbit polyclonal</td>
<td>Cosmo Bio (LB, 1403)</td>
<td>II</td>
</tr>
<tr>
<td>DLL4, mouse</td>
<td>Goat polyclonal</td>
<td>R&amp;D Systems, AF1389</td>
<td>I, III</td>
</tr>
<tr>
<td>Endomucin, mouse</td>
<td>Rat monoclonal</td>
<td>Biotechnology, sc-65495, V.7CV</td>
<td>II</td>
</tr>
<tr>
<td>F4/80, mouse</td>
<td>Rat monoclonal</td>
<td>Acris antibodies, BM4007, clone BM8</td>
<td>I</td>
</tr>
<tr>
<td>F4/80, mouse</td>
<td>Rat monoclonal</td>
<td>AbD Serotec, MCA497R</td>
<td>II</td>
</tr>
<tr>
<td>FITC</td>
<td>Rabbit polyclonal</td>
<td>Zymed/Invitrogen, 71-1900</td>
<td>I, II</td>
</tr>
<tr>
<td>FOXC2, mouse</td>
<td>Rat monoclonal</td>
<td>(Furumoto, Miura et al. 1999)</td>
<td>II</td>
</tr>
<tr>
<td>GFAP, cow</td>
<td>Rabbit polyclonal</td>
<td>Dako, Z0334</td>
<td>I</td>
</tr>
<tr>
<td>GFP</td>
<td>Rabbit polyclonal</td>
<td>Torrey Pined Biolabs, TP401</td>
<td>II, III</td>
</tr>
<tr>
<td>hF4-3C5, human</td>
<td>Mouse humanized monoclonal</td>
<td>ImClone Systems, (Persaud, Tille et al. 2004)</td>
<td>II</td>
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<tr>
<td>LYVE1, mouse</td>
<td>Rabbit polyclonal</td>
<td>(Petrova, Karpanen et al. 2004)</td>
<td>I</td>
</tr>
<tr>
<td>MECA-32, mouse</td>
<td>Rat monoclonal</td>
<td>BD Biosciences, 550563, clone MECA-32</td>
<td>I</td>
</tr>
<tr>
<td>NG2, mouse</td>
<td>Rabbit polyclonal</td>
<td>Chemicon/Millipore, AB5320</td>
<td>I</td>
</tr>
<tr>
<td>PECAM-1, mouse</td>
<td>Hamster monoclonal</td>
<td>Chemicon/Millipore, MAB1398Z, clone 2H8</td>
<td>I</td>
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<tr>
<td>PECAM-1, mouse</td>
<td>Rat monoclonal</td>
<td>BD Biosciences, 557355, clone MEC 13.3</td>
<td>I, II, III</td>
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<tr>
<td>PECAM-1, mouse</td>
<td>Rat monoclonal, Fitc conjugated</td>
<td>BD Biosciences, 553372, clone MEC 13.3</td>
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<tr>
<td>PDGFR-β, mouse</td>
<td>Rat monoclonal</td>
<td>eBioscience, 14-1402-82, clone AP85</td>
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<tr>
<td>Pimonidazole</td>
<td>Mouse monoclonal, Fitc conjugated</td>
<td>Chemicon, HP2-1000</td>
<td>I</td>
</tr>
<tr>
<td>Podoplanin, human</td>
<td>Rabbit polyclonal</td>
<td>(Kriehuber, Breiteneder-Geleff et al. 2001)</td>
<td>I</td>
</tr>
<tr>
<td>PROX1, mouse</td>
<td>Rabbit polyclonal</td>
<td>(Karkkainen, Haiko et al. 2004)</td>
<td>III</td>
</tr>
<tr>
<td>SMA, human</td>
<td>Mouse monoclonal, Cy-3 conjugated</td>
<td>Sigma, C6189, clone 1A4</td>
<td>I</td>
</tr>
<tr>
<td>TIE2, mouse</td>
<td>Rat monoclonal</td>
<td>eBioscience, CD202b, Clone: TEK4</td>
<td>II</td>
</tr>
<tr>
<td>VE-cadherin, mouse</td>
<td>Rat monoclonal</td>
<td>BD Biosciences, 550548, clone 11D4.1</td>
<td>I</td>
</tr>
<tr>
<td>VEGFC, human</td>
<td>Rabbit polyclonal, #6</td>
<td>(Baluk, Tammela et al. 2005)</td>
<td>I, II</td>
</tr>
</tbody>
</table>
VEGFR3 and Notch signaling in angiogenesis

<table>
<thead>
<tr>
<th>Recombinant virus</th>
<th>Description</th>
<th>Reference/Source</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdLacZ</td>
<td>Adenovirus that encodes β-galactosidase</td>
<td>(Laitinen, Zachary et al. 1997)</td>
<td>I</td>
</tr>
<tr>
<td>AdVEGF&lt;sub&gt;165&lt;/sub&gt;</td>
<td>Adenovirus that encodes human VEGF&lt;sub&gt;165&lt;/sub&gt;</td>
<td>(Enholm, Karpanen et al. 2001)</td>
<td>I, II</td>
</tr>
<tr>
<td>AdVEGF&lt;sub&gt;B167&lt;/sub&gt;</td>
<td>Adenovirus that encodes human VEGF&lt;sub&gt;B167&lt;/sub&gt;</td>
<td>(Lahteenvuor, Lahteenvuoro et al. 2009)</td>
<td>II</td>
</tr>
<tr>
<td>AdVEGFC</td>
<td>Adenovirus that encodes human VEGFC (full length)</td>
<td>(Enholm, Karpanen et al. 2001)</td>
<td>I</td>
</tr>
<tr>
<td>AdmVEGFD</td>
<td>Adenovirus that encodes mouse VEGFD (full length)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdVEGFE</td>
<td>Adenovirus that encodes VEGF from the Orf(NZ7) virus</td>
<td>(Wirzenius, Tammela et al. 2007)</td>
<td>I</td>
</tr>
<tr>
<td>AdVEGFR3-Ig</td>
<td>Adenovirus that encodes human VEGFR3-Ig fusion protein</td>
<td>(Karpanen, Egeblad et al. 2001)</td>
<td>I</td>
</tr>
<tr>
<td>pMX-Dll4-ECTM-EGFP</td>
<td>pMX retrovirus that encodes mouse Dll4-ECTM-EGFP</td>
<td>(Zheng, Li et al. 2011)</td>
<td>II</td>
</tr>
<tr>
<td>pMX.VEGFR3-StreptagII</td>
<td>pMX retrovirus that encodes VEGFR3-StreptagII AdVEGFR3-Ig</td>
<td>(Ghalamkarpour, Holnthoner et al. 2009)</td>
<td>II</td>
</tr>
</tbody>
</table>

Table III. Recombinant adenoviruses

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>Description</th>
<th>Reference/Source</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dll4-Fc</td>
<td>Extracellular domain (ECD) of mDll4 fused to Fc domain of human IgG fusion protein</td>
<td>(Zheng, Tammela et al. 2011)</td>
<td>II</td>
</tr>
<tr>
<td>Jagged1</td>
<td>A Notch activating synthetic peptide, corresponding to the Δ/Serrate/LAG-2 domain of hJagged1</td>
<td>(Weijzen, Velders et al. 2002)</td>
<td>I, II</td>
</tr>
<tr>
<td>SC-Jagged1</td>
<td>A synthetic peptide with the scrambled sequence of the Jagged peptide</td>
<td>(Weijzen, Velders et al. 2002)</td>
<td>I, II</td>
</tr>
<tr>
<td>VEGF&lt;sub&gt;165&lt;/sub&gt;</td>
<td>Recombinant human VEGF&lt;sub&gt;165&lt;/sub&gt;</td>
<td>R&amp;D Systems</td>
<td>II</td>
</tr>
<tr>
<td>VEGFC&lt;sub&gt;DNAC&lt;/sub&gt;</td>
<td>Recombinant VEGFC mimicking the human fully processed form of VEGFC</td>
<td>(Karpanen, Heckman et al. 2006)</td>
<td>II</td>
</tr>
</tbody>
</table>

Table IV. Recombinant proteins
Table V. Cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Reference/Source</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16</td>
<td>C57BL/6 Mouse skin melanoma cells</td>
<td>(Riley 1963)</td>
<td>I</td>
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<td>DR4 mouse embryonic fibroblasts</td>
<td>Primary mouse embryonic fibroblast</td>
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<td>G401</td>
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2. METHODS

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3D cultures of embryoid bodies

Embryonic stem cells were cultured on a layer of irradiated DR4 mouse embryonic fibroblasts in the presence of leukemia inhibitory factor (LIF). Cells were cultured for two passages without feeders, trypsinized, depleted of LIF, followed by mixing of
wild-type (DsRed) and Vegfr3+/LacZ cells in a 1:1 ratio and then left in suspension (day (D) 0). On D4, embryoid bodies were embedded in a polymerized collagen I gel with the addition of 30 ng/ml mVEGF164 (Peprotech) with dimethylsulphoxide or DAPT (5 μM, Sigma-Aldrich). Medium was changed on D6 and every day thereafter.

**Adenoviral transduction of mice**

Adenoviruses encoding human VEGF165, VEGFB167, human full-length VEGFC, mouse VEGFD, the Orf(NZ7) virus-encoded VEGFE, or LacZ were injected intradermally into the ears of mice. A total of 2×10^8 plaque-forming units of each virus were injected in a volume of 50 μl. The mice were euthanized and perfusion-fixed 5 (II) or 6 (I) days after injection, and the ears were collected and processed for whole-mount analysis or immersed in OCT medium (Tissue Tek).

**Analysis of VEGFR3 phosphorylation following ECs adhesion to collagen I**

HDBECs were transfected with pMX retrovirus encoding VEGFR3–StreptagII, detached using Accutase (PAA Laboratories) and plated on Collagen I or poly-L-lysine (both 4μg/cm^2). Cells were incubated for the indicated times with 1 μg/ml hF4-3C5, and 1 nM cediranib (Astra Zeneca) or 1 mM PP2 (Calbiochem). VEGFR3 was precipitated from PLCLB lysates using Strep-Tactin beads (IBA). Proteins were analyzed by western blotting using antibodies to pTyr or VEGFR3.

**Cell culture**

NCI-H460-LNM35 cells (LNM35) (a gift from T. Takahashi), MKN45 cells (a gift from A. Ristimäki) were maintained in RPMI-1640 medium, whereas B16-F10, B16-F10–Luc2–G5 cells, LLC cells and the G401 kidney cancer cells (CRL-1441) were grown in DMEM. Both media were supplemented with 2 mM l-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (Autogen Bioclear). Zeocin was added as a selection marker to the cultures of B16–F10–Luc2–G5 cells at the final concentration of 0.3 mg/ml. HDBECs (PromoCell) were maintained in endothelial cell growth medium (ECGM, PromoCell, C22120) with supplements provided by the manufacturer.

**Cell transfections**

For gene silencing experiments, HDBECs were transfected with human VEGFR3 or control siRNA (Thermo Scientific Dharmacon siGENOME ON-TARGETplus SMARTpool reagents), using oligonucleotide-Oligofectamine™ complexes (Invitrogen).

**Human samples**

Patients diagnosed with disseminated colon adenocarcinoma underwent a standard chemotherapy treatment, followed by partial resection of the liver to eradicate metastases. Samples from the interface between normal and tumor tissue were immersed in 4% paraformaldehyde overnight at +4 °C, washed in PBS, incubated in 25% sucrose overnight at +4 °C, embedded in optimal cutting temperature (OCT) medium and frozen. The study was approved by the Ethical Committee of the
Hospital District of Helsinki and Uusimaa according to the guidelines of the Helsinki declaration. Informed consent was provided by the patients.

**Generation and transduction of retroviruses**
An empty pMX vector or pMX vector encoding mDll4-ECTM-EGFP was transfected into 293-GPG (VSV-G) packaging cells using Fugene6 (Roche) according to the manufacturer’s instructions. Next day, the medium was changed and the viral supernatant was collected 4-7 days after the transfection. The supernatant was filtered with 0.45 µm filters (Millex) and stored at -70 °C. 150,000 HDBECs per well were plated on 6-well plates one day before transduction, exposed to 1 ml viral supernatant with 8 µg/mL Polybrene (Sigma) for 4 h, and incubated overnight with 1.5 ml complete ECGM. The media was changed the following day and the cells were used the day thereafter.

**Immunofluorescence/Immunohistochemistry**
After euthanizing the mice, tissues were immersed in 4% paraformaldehyde, washed in phosphate buffered saline (PBS), and then processed for whole-mount staining (I, II, III) or immersed in OCT medium (Tissue Tek) (I, II). Whole mount retinas were stained with biotinylated *Griffonia simplicifolia* lectin (Vector Laboratories) in PB-Lec (10% MnCl₂, 1mM, 0.01% MgCl₂ 1M, 0.01% CaCl₂ 1M, in PBS with 0.1% Triton-X), followed by incubation with Alexa Fluor® streptavidin conjugates (Molecular Probes), to visualize the vasculature. For whole mount immunostaining of retinas, mesenteries, skins, tracheas, embryos and embryonic hindbrains were blocked in immunomix (5% non-immune donkey serum, 0.2% bovine serum albumin and 0.05% NaN3 in PBS with 0.3% Triton-X), incubated with primary antibodies, and washed with 0.3% PBS-Triton-X. 50 µm sections of tumors (I, II) or ovaries (I), 80 µm cross-sections of the duodenum (I), and 7-10 µm sections of skin (I, II) were fixed with cold acetone, washed with PBS and blocked with TNB (PerkinElmer). Following incubation with primary antibodies, the sections were washed with TNT buffer. The primary antibodies were detected with the appropriate Alexa 488, 594 or 647 secondary antibody conjugates (Molecular Probes/Invitrogen). All fluorescently labeled samples were mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Alternatively retinas, embryos or tumor sections were incubated in 5% hydrogen peroxide in methanol, followed by incubation with primary antibodies, biotinylated secondary antibodies (Vector Laboratories), and signal detection with the Avidin–Biotin Complex kit (Vector Laboratories) or tyramide signal amplification (Perkin Elmer). Diaminobenzidine (Sigma or Chemicon) was used as the chromogen in both protocols (I). Stained mouse embryos were embedded in paraffin and sectioned in horizontal or sagittal orientation. Nuclear counterstaining was carried out with nuclear red (I).

**Immunoprecipitation and immunoblotting**
Equal amounts of cleared lysates from embryos, lungs or HDBECs were separated in 7.5% or 10% Mini-PROTEAN TGX Precast gels (BIORAD). After blotting to
polyvinylidene fluoride membranes (Immobilon-P PVDF, Millipore), the proteins were detected using specific primary antibodies. The blots were probed using horseradish peroxidase-labeled secondary antibodies (Dako, Glostrup, Denmark) and the signal was visualized using the SuperSignal West Pico Chemiluminescent Substrate or the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA).

Mice and treatments
All studies were approved by the Committee for Animal Experiments of the District of Southern Finland. Transgenic mice were genotyped using suitable primers and standardized PCR protocols. Cre was induced by subcutaneous implantation of sustained release pellets (21 days) containing tamoxifen (25 mg, Innovative Research) (II), while newborn pups were injected intragastrically with 20 (II) or 50 µg (III) of 4-hydroxyamoxifen dissolved in 97% ethanol using a 10 µl Hamilton syringe. Pregnant females were given 2.5 mg of 4-OHT dissolved in 40% ethanol and 60% sunflower seed oil (Sigma), using a feeding needle at E10.5 or/and E11.5 (II). Successful deletion of the genes of interest was validated by immunohistochemistry, Western Blot and RT-qPCR. The mice received intraperitoneal injections of anti-VEGFR3 and anti-VEGFR2 antibodies, or non-specific rat IgG (Dako) (30-40 mgkg⁻¹ in I for adults and 50 mgkg⁻¹ in II for pups), and 60 mgkg⁻¹ pimonidazole (I) or 0.2 mg of BRDU (I, II). For the analysis of blood vessel density and blood vessel functionality mice were tail-vein-injected with 100 µl of 1 mgml⁻¹ fluorescein-labeled Lycopersicon esculentum lectin (Vector Laboratories) under inhalation-anesthesia with isoflurane (Minrad Inc.) (I). Alternatively mice were intravenously administered with adenoviruses (1×10⁹ p.f.u. per mouse) (I). DAPT (75-100 mgkg⁻¹d⁻¹) or control vehicle was dissolved in 10% ethanol and 90% sunflower oil and administered intraperitoneally to adult mice (I), and subcutaneously to pups (II, III). The small peptide mimetic of the Notch ligand Jagged1 (Jag1) or scrambled control peptide (SC-Jag1) was dissolved in 50% dimethylsulphoxide/50% sterile water, and administered subcutaneously at 10 mgkg⁻¹ (I, II). The mice were anaesthetized with intraperitoneal injections of xylazine (10 mgkg⁻¹) and ketamine (80 mgkg⁻³).

Microscopy
Fluorescently labeled samples were imaged using a confocal microscope (Zeiss LSM 780, air objectives: x10 with numerical aperture [NA] 0.45, oil objectives: x40 with NA 1.3 and x63 with NA 1.4, or Zeiss LSM 5 Duo, air objectives x10 with NA 0.45, oil objectives x40 with NA 1.3 and x63 with NA 1.4, or Zeiss LSM 510 Meta, air objectives: x10 with NA 0.5, oil objectives x40 with NA 1.3 and x63 with NA 1.4), by using multichannel scanning in frame mode. Three-dimensional projections were constructed digitally from confocal z stacks. Signal co-localization was assessed from single confocal optical sections. Images of whole retinas, whole embryos and skin were acquired using tile scanning with a pinhole diameter >3.0 Airy units. X-gal and chromogen stainings were analyzed with a Leica DM LB transmitted light microscope (objectives ×10 with NA 0.25 and ×20 with NA 0.4). 10 µm thick sections of Rip1Tag2 tumors were analyzed with a Nikon Diaphot 300 immunofluorescence
microscope using Openlab 3.1.7. Software (Improvision) (I). Images were edited using PhotoShop software (CS4, CS5, Adobe).

**Mouse embryos stimulation with growth factors**

E10.5–E11.5 NMRI wild-type embryos were excised from amniotic sacs and placed in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.2% bovine serum albumin (BSA) on ice. The embryos were injected through the cardiac outflow tract with 0.5 ml of DMEM containing 100 ng ml\(^{-1}\) recombinant human VEGF\(_{165}\) (R&D Systems), 200 ng ml\(^{-1}\) VEGF\(_{C}\)Δ\(_{N}\)Δ\(_{C}\), or 0.2% BSA. In each group, 10-15 embryos were used altogether. Embryos were placed in DMEM containing the same concentration of growth factors, incubated at 37 °C for 20 min and lysed in 1% Triton-X-100, 40 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM Na\(_3\)VO\(_4\), 100 µM phenylmethylsulphonyl fluoride, 50 mM NaF and 10 µg ml\(^{-1}\) of both aprotinin and leupeptin. Insoluble materials were removed by centrifugation at 14,000 g for 15 min.

**PI3K activity assay**

PI(3)K activity was evaluated in HDBECs using FACE PI3-kinase p85 ELISA Kit (Active Motif) according to the manufacturer’s protocol. The signal was normalized to cell numbers by crystal violet staining. PI(3)K activity was measured with a microplate reader (Thermo Labsystems Multiscan Ascent).

**Production and purification of Dll4-Fc**

293T cells were transfected with Dll4-Fc or HSA (Fugene6 Transfection Reagent; Roche Diagnostics), and cultured in serum-free medium for 24 hours after transfection. After that, the supernatant was collected, concentrated 10 times with Centrifugal Filter Units (membrane size: 10 kDa; Millipore), aliquoted and frozen.

**Real-time quantitative PCR**

Total RNA was extracted and isolated from mouse lungs or retinas, and from HDBECs using RNeasy Mini Kit (Qiagen), or NucleoSpin® RNA II Kit (Macherey-Nagel). Samples were quality-controlled using a Nanodrop ND-1000 or BioSpec-nano spectrophotometer. Reverse transcription into cDNA was performed using the DyNAmo™ cDNA Synthesis Kit (F-470L, Finnzymes), or iScript™ cDNA Synthesis Kit (Bio-Rad). Real-time quantitative PCR (RT-qPCR) was performed using TaqMan Gene Expression Assays (Applied Biosystems) and the DyNAmo™ Probe qPCR Kit (F-450S, Finnzymes), or iQ™ Supermix kit (Bio-Rad). RT-qPCR was carried out using a BIO-RAD C1000 Thermal cycler according to a standardized protocol. At least three independent experiments per condition were analyzed, and fold changes were calculated using the comparative CT method.

**Statistical analysis**

Statistical analysis was performed using IBM SPSS Statistics 18.0 or 20. A two-tailed Student’s t-test, paired Student’s t-test, One-Way ANOVA (Post-Hoc Test: Tukey HSD), Two-Way ANOVA (Post-Hoc Test: Tukey HSD), or a Holm-Sidak test were used for statistical analysis, and a P value of less than 0.05 was considered to be
statistically significant.

**Superovulation**

24 day-old female mice received one intraperitoneal injection of 5 international units (IU) pregnant mare serum gonadotropin (National Hormone and Peptide Program, Harbor-UCLA Medical Centre), followed by an injection of 5 IU human chorionic gonadotropin (Pregnyl) 47 h later. 72 or 84 h after stimulation of superovulation the mice were killed and their ovaries were collected for analysis.

**TEM**

The mice were perfusion-fixed with 4% formaldehyde in 100 mM phosphate buffer, pH 7.4, and slices of the kidneys were incubated in the same fixative overnight (16 h) at +10 °C. The samples were fixed with 2% glutaraldehyde in the same buffer for 1 h, and post-fixed with 1% buffered osmium tetroxide for 1 h, dehydrated and embedded in epon at room temperature (+22 °C). Sections were post-stained with uranyl acetate and lead and examined with a Jeol EX1200 II TEM operating at 60 kV. Images were acquired with an ES500W CCD camera (Gatan Corp.).

**Tumor experiments**

Tumor xenografts or syngeneic grafts were made by injecting 0.5–5×10⁶ cells into the subcutaneous space in the abdominal flank of immunodeficient or transgenic mice respectively. Tumors were allowed to reach a volume of approximately 50 mm³, after which the mice were randomized into treatment groups. Tumors were measured in x, y and z dimensions using a digital caliper, and volumes were calculated using the ellipse formula \( V = \frac{xyz}{2} \). The experiments were terminated when the tumors reached a maximal size of 20 mm in diameter.

**Vessel morphometric analysis**

The vascular surface area in retinas was quantified as the isolectin B4-positive area from 10x (I, II) or 0.7x (III) confocal micrographs acquired of all intact quarters of the retina and at a similar distance from the optic nerve using Image J software (NIH, USA). Vessel branching points, sprouts and filopodia were counted manually from fluorescent micrographs of retinas using Image J (I, II, III). BrdU-positive endothelial cells were counted manually from 10x confocal z-stacks of BrdU and isolectin B4 double-stained retinas (I, II). Mouse embryos were staged at E8.5–E9.5 according to the Edinburgh Mouse Atlas criteria, and the number of vessel branching points was manually counted from 10x confocal stacks in the head vasculature. The length and area of intersomitic vessels from between somites 12 and 15 was quantified from 10x micrographs obtained with the pinhole open (I). For hindbrain analysis (II), the number of sprouting vessels on the pial side and the number of branching points on the subventricular zone were determined in 3-6 randomly chosen 0.85 mm² fields. Tumor vasculature surface area was analyzed from at least three 0.81 mm² (I) or 1.69 mm² (I, II) micrographs from regions of uniform staining intensity, and quantified using Image J, while sprouts were counted manually. Lectin-stained vessels were counted from Rip1Tag2 tumor sections. PECAM-1-positive vessels in the ears or the LYVE1-positive vessel area in the intestines were quantified in a similar manner (I,
II). PROX1-positive lymphatic vessels from postnatal skins and tracheas (III) were quantified from 7.64 or 0.849 mm² micrographs respectively, from regions of uniform staining intensity, and in a similar manner. DLL4 staining was manually quantified from 0.092 mm² micrographs using Image J (III). All images used for quantification were three-dimensional projections of confocal z-stacks. The images used to quantify PROX1-positive lymphatic vessels in the skin were acquired using tile scanning mode with a pinhole diameter >3 Airy Units (III).
RESULTS AND DISCUSSION

1. VEGFR3 is a novel regulator of angiogenic sprouting (Study I)

Although VEGFR3 is mainly expressed in the lymphatic endothelium in the adult (Alitalo, Tammela et al. 2005), Vegfr3 -/- mouse embryos die at E9.5 because of cardiovascular failure, before the formation of lymphatic vessels (Dumont, Jussila et al. 1998), and VEGFR3 is expressed in some fenestrated endothelia in the adult (Partanen, Arola et al. 2000). Furthermore VEGFR3 is upregulated in the tumor blood vasculature and in wounds (Valtola, Salven et al. 1999, Paavonen, Puolakkainen et al. 2000), and blocking VEGFR3 with monoclonal antibodies has been shown to suppress tumor angiogenesis (Laakkonen, Waltari et al. 2007). These data implied that VEGFR3 is involved in angiogenesis, urging us to investigate the VEGFR3-mediated angiogenic mechanisms.

VEGFR3 expression was detected in several angiogenic vascular beds, for example in mouse and human tumors, in the developing embryonic vasculature, in the postnatal mouse retina, and in the ovarian follicles of superovulated female mice. VEGFR3 was mainly localized in endothelial tip cells, as has been previously shown for the developing segmental arteries in zebrafish (Siekmann and Lawson 2007). When VEGFR3 signaling was blocked using a blocking ligand-binding monoclonal antibody (31C1), angiogenic sprouting was inhibited in the retina and in tumor xenografts, and when this inhibitor was combined with an antibody blocking VEGFR2 signaling (DC101; representing the “golden standard” treatment in mouse tumor angiogenesis inhibition), this resulted in additive inhibition in tumor growth.

To investigate the relationship between VEGFR2 and VEGFR3 signaling, angiogenesis was triggered in the mouse skin by overexpression of VEGFR2 specific ligands under the control of the keratin-14 promoter (K14-VEGF165 or K14-VEGF6). VEGFR3 was upregulated in the angiogenic blood vessels, suggesting that VEGFR2 activation induced VEGFR3 expression (see also study III). Moreover, analysis of compound K14-VEGF165-K14-VEGFR3-Ig mice showed that simultaneous overexpression of VEGFR3-Ig, which binds to VEGFC and VEGFD and inhibits VEGFR3 activation, attenuated the angiogenic phenotype; although it did not have any profound effect on quiescent blood vessels in K14-VEGFR3-Ig mice. Interestingly, VEGFR3 stimulation by mouse VEGFD, a VEGFR3 specific ligand, which was delivered to the mouse ear skin by adenoviral gene transfer vector, did not promote sprouting angiogenesis unless combined with VEGFR2 stimulation. In the latter case the overexpression of ligands for both receptors resulted in a greater angiogenic effect than VEGFR2 stimulation alone. This suggests that VEGFR3 dependent angiogenic signaling becomes significant in blood vessels only after VEGFR3 expression is primed by VEGFR2 activation (see also Study III).

Inhibition of Notch signaling in the zebrafish embryo has been shown to upregulate flt4 expression in segmental arteries suggesting that Notch activation normally suppresses flt4 expression (Siekmann and Lawson 2007). In order to investigate the relationship between Notch and VEGFR3 signaling in mammals, we inhibited or activated Notch signaling in newborn mouse pups, using a γ-secretase
inhibitor (DAPT), or a small peptide that mimics the Notch ligand Jagged-1, respectively (Suchting, Freitas et al. 2007, Benedito, Roca et al. 2009). Loss of Notch signaling resulted in increased VEGFR3 mRNA and protein expression in the mouse retina, while Notch activation resulted in diminished expression of VEGFR3. Similarly, DLL4+/- retinas that have reduced Notch signaling, displayed increased levels of VEGFR3. Our results show that Notch activation downregulates VEGFR3 in endothelial tip cells, in a similar manner as reported previously for VEGFR2 (Lobov, Renard et al. 2007, Suchting, Freitas et al. 2007) (Figure 4). A similar loop has been described in zebrafish mutants lacking Notch ICD signaling (mind bomb mutants) or rbpsuh morphants (Lawson, Scheer et al. 2001, Siekmann and Lawson 2007), but not in dll4 morphants (Hogan, Herpers et al. 2009). These results highlight the significant differences that LOF of different components of the Notch pathway yield. Although Notch activity was already altered six hours after Notch inhibition, the expression of VEGFR3 was not changed before twelve hours, suggesting that intermediate effectors are required for Notch-mediated regulation of VEGFR3, or that Notch regulates VEGFR3 at the post-transcriptional level. This possibility is further supported by the notion that VEGFR3 protein levels are strongly upregulated in Rbpj knockout retinas, while VEGFR3 mRNA levels are only modestly increased (Benedito, Rocha et al. 2012).

![Figure 4](image-url)

**Figure 4. Schematic illustration of the role of VEGFR3 in sprouting angiogenesis.** VEGFR3 is highly expressed in endothelial tip cells, and becomes downregulated in stalk cells following Notch activation. However in conditions of low Notch signaling (for example in DLL4+/- mice or after treatment with γ-secretase inhibitors), the expression of VEGFR3 is upregulated in the whole vasculature. Adapted from study I.
In this study we used a variety of *in vivo* models to investigate the role of VEGFR3 in sprouting angiogenesis in mammals. We have shown that blocking VEGFR3 inhibits angiogenesis and displays additive properties with VEGFR2 blockade, a finding that could be exploited in the context of multi-targeted cancer therapies. Later studies have confirmed the superiority of combined targeting of VEGFR2 and VEGFR3 in tumor angiogenesis inhibition (Matsumoto, Roufail et al. 2013). Yet, one important aspect to consider is the possible side effects of VEGFR targeting. For example bevacizumab causes proteinuria and hypertension in humans (Hayman, Leung et al. 2012). Although VEGFR3 is mainly expressed in quiescent fenestrated endothelia in the adult, and thus represents potentially a safer anti-angiogenic target, we performed an anatomical and functional screening of kidney function in K14-VEGFR3-Ig mice and in mice treated with VEGFR3 inhibitors. We did not observe any sign of kidney pathology at the ultrastructural level, neither pathological albuminuria or nephrosis. Although these preclinical data are very encouraging, mice and humans differ in multiple aspects with respect to their physiology; therefore clinical trials would be required to assess the safety of VEGFR3 inhibitors. In early 2011, a humanized blocking antibody against VEGFR3 entered a Phase 1 clinical trial for patients with solid tumors by ImClone/EliLilly&Co (NCT01288989); this trial will address the safety of VEGFR3 inhibition in humans within the next years. Targeting VEGFR3 could be very useful clinically, as it could potentially block simultaneously tumor angiogenesis and lymph node metastasis, especially when used in combination with VEGFR2 inhibition (Matsumoto, Roufail et al. 2013).

2. VEGFC/VEGFR3 signaling activates Notch signaling and facilitates the formation of stable vascular loops (Study II)

VEGFR3 is expressed in mouse embryos already at E8.5 (Kukk, Lymboussaki et al. 1996) and VEGFR3 deficient embryos die at E9.5, due to defective vascular remodeling and maturation (Dumont, Jussila et al. 1998). Thus it has been impossible to study Vegfr3 deficient mice postnatally. So far LOF studies relied principally on systematic administration of blocking antibodies or inhibiting soluble receptors that can affect all cells expressing the receptor. In order to study the functions of VEGFR3 exclusively in blood vessels, and to circumvent the embryonic lethality observed in Vegfr3/- embryos, we employed conditional mutagenesis. We produced mice with conditional VEGFR3 alleles that express the Cre recombinase under the endothelial specific PDGFB promoter. Tamoxifen or 4-OH-tamoxifen administration was then used to activate Cre recombinase and to initiate recombination events in all vascular endothelia (Claxton, Kostourou et al. 2008, Haiko, Makinen et al. 2008).

Surprisingly, endothelial specific deletion of VEGFR3 (Vegfr3iΔEC) resulted in a hypervascular phenotype with excessive filopodia formation in the postnatal mouse retina, the embryonic hindbrain, as well as in the tumor vasculature. Although our previous studies indicated that VEGFR3 is an angiogenesis activator, our subsequent results implied that it acts as an inhibitor. However the methods
used to disrupt VEGFR3 signaling in those studies have been qualitative different: in the first case we used monoclonal antibodies that inhibited ligand binding but left the intracellular domain of the receptor intact, while in the latter we removed the receptor completely from the BECs. Previous publications have shown that VEGFR3 can be phosphorylated \textit{in vitro} by collagen or fibronectin through their binding to β1 integrin (Wang, Zhang et al. 2001). The phosphorylation of VEGFR3 by the ECM is ligand independent, since it occurs in the presence of a kinase inhibitor (MAZ51), or in a mutant, kinase dead receptor, and is mediated by Src. In this case Src produces a different phosphorylation pattern compared to the one induced by ligand activation of VEGFR3 (Galvagni, Pennacchini et al. 2010). \textit{In vivo} deletion of both VEGFR3 ligands (VEGFC and VEGFD) did not recapitulate the VEGFR3-/- phenotype, but instead these embryos mimicked the VEGFC null embryos and died due to lymphatic defects (Haiko, Makinen et al. 2008). This implies that in this developmental context the ligand-dependent functions of VEGFR3 are dispensable.

We were able to confirm ligand-independent phosphorylation of the receptor in HDBECs that were stably transduced with the pMX–VEGFR3–StreptagII retrovirus. \textit{In vivo}, the retinas of Vegfr3KDlox mice, which exhibit VEGFR3 hypophosphorylation (Karkkainen, Saaristo et al. 2001), showed a similar hypervascular phenotype, suggesting that the observed phenotype does not result from perturbed ligand-dependent VEGFR3 signaling, further indicating that the receptor exhibits two modes of action, which are ligand-dependent or ligand-independent.

Since the phenotype resulting from endothelial specific VEGFR3 deletion resembled the Notch LOF phenotype, we explored possible perturbations of Notch signaling in our mutants. Indeed, several Notch target genes were downregulated in the Vegfr3iΔEC retinas, suggesting that Notch LOF underlies the phenotype. To confirm this, we performed a rescue experiment by administering a Notch agonist to Vegfr3iΔEC pups (Benedito, Roca et al. 2009). This was able to restore the retinal vasculature back to normal. We also tested the ability of VEGFR3 to activate Notch signaling in mosaic embryoid bodies and retinas consisting of VEGFR3+/LacZ and wild type endothelial cells (Jakobsson, Franco et al. 2010). The VEGFR3+/LacZ cells occupied preferentially the tip cell position in the developing sprouts, which is in agreement with reduced Notch signaling in these cells. Importantly, Notch signaling was unaltered in the retinas of pups treated with antibodies blocking VEGFR3, confirming that the ligand-dependent VEGFR3 signaling does not involve (canonical) Notch activation.

To investigate whether VEGFR3 ligands are involved in VEGFR3 activation in angiogenic endothelium, we analyzed VEGFC and VEGFD heterozygous mice (Karkkainen, Haiko et al. 2004, Baldwin, Halford et al. 2005). VEGFD mutant retinas were indistinguishable from those of wild type littermates; however VEGFC heterozygotes displayed a hypovascular phenotype that was characterized by excessive vessel regression. Vessel regression is a normal process during maturation of vascular networks and results in the formation of empty “sleeves” of basement membrane that can be identified by collagen IV staining (Baluk, Morikawa et al. 2003). Increased vessel regression, as seen in our VEGFC mutants, is likely a sign of vessel instability and inefficient fusion of tip cells to form vascular loops. Yet what
could be the source of VEGFC in the retina? In the developing mouse and zebrafish embryo the ECs have been shown to produce VEGFC (study I). Furthermore resident microglia has been implicated in vascular branching in the embryonic hindbrain and mouse retina, although these angiogenic effects are not mediated by VEGF expression by microglial cells (Fantin, Vieira et al. 2010, Rymo, Gerhardt et al. 2011). We identified a subpopulation of TIE2 positive macrophages in P5 retinas that expressed VEGFC and were localized at sites of vascular remodeling behind the angiogenic front. TEMs have been shown to promote angiogenesis in tumors and remodeling tissues (De Palma, Venneri et al. 2005, Pucci, Venneri et al. 2009, Coffelt, Tal et al. 2010, Capobianco, Monno et al. 2011, Mazzieri, Pucci et al. 2011, Patel, Smith et al. 2013). Phenotypic analysis of op/op mice that lack macrophages revealed a similar phenotype as in the VEGFC heterozygotes, confirming the previously demonstrated role of these cells in branching anastomosis (Kubota, Takubo et al. 2009). Interestingly, both the VEGFC+/- and the op/op mice showed reduced levels of Notch activation, showing that ECs with low Notch signaling activity may be still unable to hypersprout in some conditions (see also III).

To identify the molecular players downstream of VEGFR3 signaling, we stimulated HDBECs with VEGFC in the presence of a soluble Notch inhibitor (Dll4-Fc), and found non-canonical induction of the Notch target genes HEY1, HEY2 and NRARP, indicating Notch activation. On the other hand, we did not detect Notch activation in the presence of a PI3K inhibitor. Silencing of VEGFR3 eliminated PI3K induction by VEGFC stimulation. This suggested that PI3K acts downstream of VEGFR3 to activate Notch in endothelial cells. PI3Ks are lipid kinases that are activated downstream of VEGFR2 and VEGFR3 in BECs and LECs, respectively (Graupera and Potente 2013), while PI3K activation by VEGFR3 has been previously shown in BECs only in the zebrafish (Herbert, Huisken et al. 2009).

We subsequently identified the transcription factor FOXC2 as a further downstream component of the signaling pathway. FOXC2 was induced by VEGFC stimulation in HDBECs, and it was downregulated following VEGFR3 silencing. Vegfr3+/+;Foxc2+/- compound heterozygous mice recapitulated the phenotype of Vegfr3K/Dlox mice, while single heterozygotes showed no defects, suggesting a possible genetic interaction of VEGFR3 and FOXC2 in developing blood vessels. FOXC2 is a member of the Forkhead transcription factor family that has been implicated in vascular development and disease (Kume 2008). FOXC2 has been shown to directly activate DLL4 and HEY2 (Hayashi and Kume 2008). This is in agreement with the hypothesis that VEGFR3 can activate Notch in a non-canonical way, via this transcription factor.

In this study we used in vivo tools to describe a novel mode of action of VEGFR3. β1 integrin and ECM have been shown to trigger ligand-independent activation of VEGFR3 through Src kinase. Our results support a model were ligand binding activation of VEGFR3 generates pro-angiogenic signals, while ligand-independent activation induces Notch signaling and restricts angiogenic sprouting. In addition, VEGFC/VEGFR3 signaling is able to activate Notch in a non-canonical manner at sites of vascular anastomosis and to mediate the phenotypic conversion of tip cells to stalk cells, and the formation of stable vascular loops (Figure 5). Our
VEGFR3 and Notch signaling in angiogenesis

studies identified the TIE2+ positive macrophages in the retina as the key cells producing VEGFC. Interestingly, VEGFC is produced by proangiogenic TIE2+ macrophages in corpus luteum during the early stages of pregnancy in mice (Care, Diener et al. 2013). Yet the VEGFC+/− and op/op retinas did not display the typical Notch LOF phenotype, rather they were hypoplastic. This result is in agreement with VEGFR3-ligand-blocking studies, showing that the ligand-dependent arm of VEGFR3 promotes angiogenesis. In addition, macrophages produce a plethora of angiogenic factors, including VEGF (Xiong, Elson et al. 1998). Therefore loss of macrophages would disrupt VEGF/VEGFR2 signaling, which is a key pathway in blood vessel growth.

Figure 5: Summary of the proposed function of VEGFC/VEGFR3 signaling in endothelial tip cells. (a) VEGFC expressing macrophages (purple) act as bridges between two tip cells, by activating Notch signaling and decreasing VEGF sensitivity. VEGFR3 is expressed in the tip cells, which upon VEGFR3 LOF fail to sense VEGFC, activate Notch signaling and become stalk cells. This results in excessive amount of tip cells and a hypersprouting phenotype. Adapted from study II. (b) A schematic showing the “active”-ligand-dependent and the “passive”-ligand-independent signaling arms of VEGFR3 in ECs.

3. VEGFR3 cannot replace VEGFR2 in sprouting angiogenesis, while VEGFR2 is not essential for early postnatal lymphangiogenesis (Study III)

VEGFR2 is a key element of Notch signaling, and has been shown to act upstream of DLL4 expression in endothelial tip cells. DLL4 subsequently activates Notch in adjacent stalk cells, which downregulate VEGFR2 expression in a negative feedback loop. In our previous studies (I and II), we demonstrated a similar mode of action of VEGFR3/Notch signaling in the mouse retina. On the other hand, the receptors form VEGFR2/VEGFR3 heterodimers in LECs and BECs, which appear to be important for angiogenic sprouting in mice and arteriogenesis in zebrafish (Dixelius, Makinen et al. 2003, Covassin, Villefranc et al. 2006, Nilsson, Bahram et al. 2010). VEGFR2 activation induces VEGFR3 expression in mouse blood vessels (Study I), and genetic deletion of VEGFR3 prolongs VEGFR2 phosphorylation in BECs (Study II). To investigate the crosstalk of the receptors, and to circumvent the embryonic lethality of Vegfr2−/− and Vegfr3−/− mice, we deleted VEGFR2 and/or
VEGFR3 conditionally in newborn pups and analyzed postnatal angiogenesis and Notch signaling in the mouse retina.

Endothelial specific deletion of VEGFR2 (Vegfr2iΔEC) in postnatal retina resulted in a hypoplastic vasculature, as previously published (Benedito, Rocha et al. 2012). However, since the amounts of residual VEGFR2 protein expression in this model were not negligible (minimum 20% in lungs), we decided to increase the deletion efficiency by analyzing mice in which one VEGFR2 allele was constitutively inactivated and the other one was conditionally targeted (Vegfr2iΔEC/LacZ). Vegfr2+/LacZ retinas were indistinguishable from those in wild type littersmates, but the Vegfr2iΔEC/LacZ retinas were severely hypovascularized, and showed areas of EC clustering. This 3-dimensional accumulation of ECs in the retina could be a sign of disrupted EC competition as has been previously reported in conditions of VEGF gradient loss (Jakobsson, Franco et al. 2010). It could be also a sign of the inability of the ECs to sense the VEGF gradient and migrate towards the retina periphery. The Vegfr2iΔEC/LacZ mice shower reduced Notch signaling, indicating that VEGFR2 is required for Notch activation.

To investigate if VEGFR3 LOF supports ECs sprouting even in the absence of VEGFR2, we generated Vegfr2;Vegfr3iΔEC mice, and deleted both receptors in postnatal blood vessels. The compound deleted mice showed no difference in vascular density compared to Vegfr2iΔEC mice, suggesting that VEGFR2 signals upstream of VEGFR3 (see also study I) and that it is necessary to induce VEGFR3 expression in angiogenic BECs. Interestingly, the Vegfr3iΔEC mice showed elevated levels of VEGFR2, indicating that VEGFR3 suppresses VEGFR2 (see also study II). Otherwise the Vegfr2;Vegfr3iΔEC retinas displayed abnormal ECs connections, as the VEGFC+/- retinas (study II).

We next sought to determine the interplay between VEGFRs and Notch signaling. A recent study by Benedito et al. suggested that hypersprouting following Notch LOF is still possible via VEGFR3 signaling, even when VEGFR2 is missing (Benedito, Rocha et al. 2012). In their study VEGF2 LOF had no effect in Notch LOF mediated hypersprouting, while inhibition of VEGFR3 kinase activity by the kinase inhibitor MAZ51 was able to reduce excess filopodia and sprout formation upon DAPT treatment. Based on these results the authors suggested that VEGFR3 kinase activity, but not ligand binding-dependent signaling, is proangiogenic in ECs with low Notch signaling. A similar finding has been reported earlier in the zebrafish rbpsuh morphant, in which hypersprouting could be partially rescued by flt4 morpholinos, and in the mutant expando, that carries a loss-of-function mutation in the kinase region of flt4, and rescues the dll4 LOF arterial hyperbranching phenotype (Hogan, Herpers et al. 2009). However, we were unable to detect any hyperspouting following Notch inhibition in our VEGFR2 mutants. Furthermore, genetic deletion of VEGFR3 did not restrict hyperspouting upon Notch LOF; on the contrary it exacerbated the phenotype. We could detect reduced numbers of tip cells in all conditions where VEGFR2 signaling was absent; there were fewer filopodia and less DLL4 staining, indicating that VEGFR2 is required by ECs to acquire the tip cell phenotype (Gerhardt, Golding et al. 2003, Patel, Li et al. 2005, Noguera-Troise,
Daly et al. 2006, Ridgway, Zhang et al. 2006, Williams, Li et al. 2006, Lobov, Renard et al. 2007, Suchting, Freitas et al. 2007, Jakobsson, Franco et al. 2010, Roukens, Alloul-Ramdhani et al. 2010). Importantly, although we have previously observed reduced DLL4 mRNA levels in retinas of Vegfr3iΔLEC pups (see study II), further analysis by immunohistochemistry showed that the DLL4 protein levels remain unaltered following VEGFR3 genetic deletion. This finding reinforces the idea that Notch activation by VEGFR3 occurs in a DLL4 independent manner. Another possibility is that DLL4 upregulation happens via VEGFR2-dependent post-transcriptional regulation.

Different perturbations in expression and activity of Notch ligands/receptors and VEGFRs could produce diverse, context-dependent EC responses and biological effects, depending on the particular ligands and receptors involved. Interestingly, other molecules such as laminins have been suggested to upregulate DLL4 expression in BECs (Estrach, Cailleteau et al. 2011, Stenzel, Franco et al. 2011). Yet the specificity of the kinase inhibitor used by Benedito at al (MAZ51) has never been validated at the kinome level, and it has been shown to inhibit for example VEGFR2 phosphorylation (Kirkin, Mazitschek et al. 2001). On the other hand the comparison of Vegfr2iΔEC and Vegfr2iΔEC/LacZ retinas implies that small amounts of VEGFR2 are able to sustain angiogenesis to some extent, and could provide a gateway for hypersprouting upon DAPT treatment in the case of incomplete deletion.

Although the ECs had low Notch levels, this did not result in the typical hypervascular phenotype accompanying Notch LOF, in the absence of the VEGFR2. This highlights the diverse fundamental functions of VEGFR2 signaling in ECs, including PLC-γ1 activation leading to EC proliferation via the MAP kinase pathway (Takahashi, Yamaguchi et al. 2001), PI3K activation leading to EC proliferation, differentiation and survival (Gerber, McMurtrey et al. 1998, Dayanir, Meyer et al. 2001, Meyer, Latz et al. 2003), and phosphorylation of the adapter molecule Shb that drives EC migration (Holmqvist, Cross et al. 2004). Decreased EC numbers and reduced Notch activity have been also observed in the op/op mice, which lack macrophages that are a source of VEGF (Xiong, Elson et al. 1998), and in VEGFC+/−-mice (see study II). In line with these observations, Vegfr2;Vegfr3iΔEC compound mice phenocopied the Vegfr2iΔEC mutants in terms of vascular density. Yet qualitative differences reminiscent of VEGFC+/−-mice, like EC detachment from the vascular plexus, could be observed in the compound mutants, suggesting that some aspects of VEGFR3 angiogenic signaling are independent of VEGFR2.

We also studied the functions of VEGFRs in postnatal lymphatic vessel growth and maturation. VEGFR3 is important for lymphatic vessel growth during the two first postnatal weeks in pups (Karpanen, Wirzenius et al. 2006), however the role of VEGFR2 in lymphatic vessels has remained unclear (Hong, Lange-Asschenfeldt et al. 2004, Kajiya, Hirakawa et al. 2006, Wirzenius, Tammela et al. 2007). We were able to confirm the importance of VEGFR3 by its genetic deletion exclusively in lymphatic vessels by using the lymphatic specific deleter Prox1iCreERT2 (Bazigou, Lyons et al. 2011) and by producing mice deleted of VEGFR3, VEGFR2, or both (Vegfr2iΔLEC, Vegfr3iΔLEC, and Vegfr2;Vegfr3iΔLEC respectively). The Vegfr3iΔLEC mice had fewer lymphatic vessels compared to their
wild type littermates, and were unable to sprout and mature normally. On the contrary the Vegfr2iΔLEC mice did not show any lymphatic vascular defects, suggesting that VEGFR2 signaling in LECs is not crucial at this developmental stage. Interestingly, a recent report by Dellinger et al. showed that conditional lymphatic deletion of VEGFR2 resulted in hypoplastic but functional lymphatic vessels in embryos and adult mice (Dellinger, Meadows et al. 2013). However, in this study constitutive VEGFR2 deletion was initiated already during embryonic development, resulting also in blood vessel defects, raising the possibility that the observed defects in adult mice originate from disruption of VEGFR2 signaling during lymphatic vessel development. In contrast, in our studies, lymphatics developed normally until birth. We performed a time-course analysis of VEGFR2 protein expression on postnatal lymphatic vessels, which showed that although the protein was completely absent at the time of analysis at P6, it is downregulated gradually with some minor expression still detected at P3. Taken together, these data raise the possibility that conditional mutants experienced VEGFR2 LOF for a period that was too short to produce a significant phenotype. Nevertheless, considering the dramatic changes following conditional VEGFR3 LOF in the same context, it is safe to conclude that VEGFR2 has a minor role -if any- in early postnatal lymphatic growth. Yet we cannot exclude the possibility that VEGFR2 deletion could have an effect on the lymphatic vessels at later timepoints or in adult mice. Further studies are required to investigate the role of VEGFR2 in late postnatal lymphatic growth, and lymphatic vessel maintenance in adults.

In this study we used state of the art genetic tools to investigate the molecular interplay between VEGFRs and Notch in sprouting angiogenesis. Although conditional mouse models are characterized by a high degree of precision and reliability, our results highlight the importance of residual protein expression after Cre-recombinase induction that could obscure the interpretation of the results, especially in experiments that are performed during a short period, like in postnatal retina studies. A recent paper by D'Amico et al. reported a similar finding with conditional EC-specific deletion of TIE1; in this study 10% residual expression of TIE1 receptor rescued the hypovascular phenotypes observed in Tie1iΔEC/LacZ mice, which showed maximum deletion levels (D'Amico, Korhonen et al. 2014). On the other hand, we established a functional loop in which VEGFR2 activates VEGFR3 expression in blood vessels, which subsequently suppresses VEGFR2 expression, producing a negative feedback loop. Finally, we showed for the first time that VEGFR2 expression on the lymphatic vasculature is dispensable during postnatal growth and remodeling of lymphatic vessels, and that it cannot replace VEGFR3 during this developmental time.
CONCLUSIONS AND FUTURE PROSPECTS

The discovery of VEGFR3 as a LEC-specific marker in the mid 1990s was a groundbreaking finding that initiated a whole new era of lymphatic research. Yet the role of VEGFR3 in blood endothelia has remained elusive over the years, also because of the early embryonic lethality observed in VEGFR3 null mice. However, it has become clear that VEGFR3 signaling is essential in angiogenic conditions in physiological and pathological settings, for example in solid tumors.

In the present study, we explored the mechanisms of VEGFR3 signaling in sprouting angiogenesis and elucidated a plethora of novel functions for this receptor. We identified VEGFR3 as a novel tip cell marker and a novel regulator of sprouting angiogenesis that possesses ligand-dependent and ligand-independent signaling properties in the blood vascular endothelium, and we clarified some of the signaling relationships of VEGFR3 to VEGFR2 and Notch pathways. Our findings highlight the importance of VEGFR3 signaling in tumor angiogenesis, since we showed that VEGFR3 collaborates with VEGFR2 in the formation of new vessels and is able to sustain angiogenesis even in the presence of VEGFR2 blockers; thus providing a signaling gateway for tumors in which VEGF inhibitors are used to block angiogenesis.

Although our results improved our understanding on how VEGFR3 contributes to blood vessel development and growth, several more questions remain to be addressed: To which extent does the VEGFR3/Notch signaling output depend on the endothelial cell microenvironment? Why interference with different components of the Notch signaling cascade results in different phenotypes in mice and zebrafish, and how could these results be translated clinically? How could we maximize the precision of genetic manipulation of mouse models and the interpretation of the experimental results? Would VEGFR3 inhibition in humans be effective in all tumor types? What would be the determinants of responsiveness to anti-VEGFR3 treatment and how could combinational strategies maximize the clinical benefit? Could VEGFR3 blockade provide dual inhibition of tumor angiogenesis and lymph node metastasis in cancer patients? Finally, since VEGFR3 is expressed in angiogenic endothelia in the adult, it would be exciting to use the models established in these studies in order to investigate the contribution of VEGFR3 in other angiogenesis-related diseases, such as cardiovascular and inflammatory disorders.

More than 40 years ago it was suggested for the first time that angiogenesis supports tumor growth. On the other hand, more recently, a series of significant discoveries have established the importance of the lymphatic system in human physiology, introducing a new exciting research area to the scientific community. During the past years a tremendous amount of work has significantly increased our knowledge and expanded the field of vascular biology into several directions. The challenge for the future would be to further comprehend the molecular mechanisms underlying angiogenesis and lymphangiogenesis, and develop more efficient therapeutic strategies to target these processes in human diseases.
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