VEGF-C/VEGFR-3 AND PDGF-B/PDGFR-ß PATHWAYS IN EMBRYONIC LYMPHANGIOGENESIS

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Academic dissertation

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ABBREVIATIONS

Ad  Adenovirus
Ang  Angiopoietin
E   Embryonic day
Flk1 Fetal liver kinase 1 (mouse VEGFR-2)
Flt1 Fms-like tyrosine kinase 1 (VEGFR-1)
Flt4 Fms-like tyrosine kinase 4 (VEGFR-3)
FOXO2 Forkhead box C2
HIF-1 Hypoxia-inducible factor 1
Ig  Immunoglobulin
K14 Keratin-14
K19 Keratin-19
kb Kilobase
kDa Kilodalton
KDR Kinase insert domain containing receptor (human VEGFR-2)
LYVE-1 Lymphatic endothelial hyaluronan receptor 1
P  Postnatal day
PDGF Platelet-derived growth factor
PDGFR Platelet-derived growth factor receptor
PECAM-1 Platelet endothelial cell adhesion molecule 1
PIGF Placenta growth factor
Prox-1 prosporo-related homeobox protein 1
SMA  a-smooth muscle actin
SMC Smooth muscle cell
Tie Tyrosine kinase with Ig and EGF homology domains (Tie-1)
VEGF Vascular endothelial growth factor
VEGFR Vascular endothelial growth factor receptor
WT Wildtype
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original research articles, which are referred to in the text by their Roman numerals.


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ABSTRACT

The circulatory system comprises the blood vascular system and the lymphatic vascular system. These two systems function in parallel. Blood vessels form a closed system that delivers oxygen and nutrients to the tissues and removes waste products from the tissues, while lymphatic vessels are blind-ended tubes that collect extravasated fluid and cells from the tissues and return them back to blood circulation. Development of blood and lymphatic vascular systems occurs in series. Blood vessels are formed via vasculogenesis and angiogenesis whereas lymphatic vessels develop via lymphangiogenesis, after the blood vascular system is already functional. Members of the vascular endothelial growth factor (VEGF) family are regulators of both angiogenesis and lymphangiogenesis, while members of the platelet-derived growth factor (PDGF) family are major mitogens for pericytes and smooth muscle cells and regulate formation of blood vessels.

Vascular endothelial growth factor C (VEGF-C) is the major lymphatic growth factor and signaling through its receptor vascular endothelial growth factor receptor 3 (VEGFR-3) is sufficient for lymphangiogenesis in adults. We studied the role of VEGF-C in embryonic lymphangiogenesis and showed that VEGF-C is absolutely required for the formation of lymph sacs from embryonic veins. VEGFR-3 is also required for normal development of the blood vascular system during embryogenesis, as Vegfr3 knockout mice die at mid-gestation due to failure in remodeling of the blood vessels. We showed that sufficient VEGFR-3 signaling in the embryo proper is required for embryonic angiogenesis and in a dosage-sensitive manner for embryonic lymphangiogenesis. Importantly, mice deficient in both VEGFR-3 ligands, Vegfc and Vegfd, developed a normal blood vasculature, suggesting VEGF-C- and VEGF-D-independent functions for VEGFR-3 in the early embryo.

Platelet-derived growth factor B (PDGF-B) signals via PDGFR-β and regulates formation of blood vessels by recruiting pericytes and smooth muscle cells around nascent endothelial tubes. We showed that PDGF-B fails to induce lymphangiogenesis when overexpressed in adult mouse skin using adenoviral vectors. However, mouse embryos lacking Pdgfb showed abnormal lymphatic vessels, suggesting that PDGF-B plays a role in lymphatic vessel maturation and separation from blood vessels during embryogenesis.

Lymphatic vessels play a key role in immune surveillance, fat absorption and maintenance of fluid homeostasis in the body. However, lymphatic vessels are also involved in various diseases, such as lymphedema and tumor metastasis. These studies elucidate the basic mechanisms of embryonic lymphangiogenesis and add to the knowledge of lymphedema and tumor metastasis treatments by giving novel insights into how lymphatic vessel growth could be induced (in lymphedema) or inhibited (in tumor metastasis).
REVIEW OF THE LITERATURE

1. THE TWO VASCULAR SYSTEMS

Blood vascular system

The survival of all tissues is dependent on supply of oxygen and nutrients. The cardiovascular system consists of the heart, arteries, veins, arterioles, venules and capillaries that carry oxygen and nutrients to the tissues and remove CO₂ and waste products from the tissues. The cardiovascular system also facilitates communication between distant organs. The heart pumps oxygenated blood from the pulmonary circulation to the arteries that transport it to the tissues via the systemic circulation. Exchange of gases and metabolites occurs in the blood capillary bed, where the blood pressure is sufficiently low and blood vessel walls are thin enough for the exchange of gases and metabolites. Veins return deoxygenated blood back to the heart that pumps it to the pulmonary circulation to be oxygenated once again. The luminal surface of blood vessels is lined by blood vascular endothelial cells that are connected to each other by specific junctions depending on the functional demands of the organs and tissues. Generally, blood vessels show a wide variety of specialized features that meet the metabolic and functional demands of different organs and tissues. The thin blood endothelial cell lining is surrounded and supported by a basement membrane that is composed of collagens, fibronectin, laminins and heparan sulfate proteoglycans. Blood vessels are also supported by mural cells, such as pericytes and vascular smooth muscle cells, which are recruited into the vessel wall. Pericytes surround capillaries and small vessels (arterioles and venules) and are embedded in the basement membrane in direct contact with the endothelial cells through long cytoplasmic processes, whereas vascular smooth muscle cells surround larger vessels and are separated from the endothelial cells and basement membrane by a layer of mesenchymal cells and extracellular matrix. Vascular smooth muscle cells form a separate layer in the blood vessel wall.

Lymphatic vascular system

The lymphatic vascular system consists of capillaries, large ducts, collecting vessels and lymphoid organs (spleen, thymus, tonsils and lymph nodes). Lymphatic capillaries collect excess extracellular fluid and immune cells from the tissues, transport them via lymph nodes, collecting lymphatic vessels, the thoracic duct and the right lymphatic duct, and return them to the venous circulation. Lymphatic vessels also absorb dietary fats from the intestine, and together with lymphoid organs contribute to the immune surveillance of the body. Lymphatic capillaries are truncated, lack pericytes and contain discontinuous basal lamina. These features facilitate the uptake of fluid and macromolecules. Lymphatic endothelial cells also have openings between them and they contain anchoring filaments that connect lymphatic endothelial cells to the extracellular matrix and pull them apart upon increased interstitial pressure, which enhances fluid uptake and maintains the patency of the vessels. The intrinsic contractility of the vascular smooth muscle cells that surround larger collecting vessels, skeletal muscle movements and arterial pulsation all help to compress lymphatic vessels and pump the lymph forward, while valves secure the unidirectional flow of the lymph.
2. DEVELOPMENT OF BLOOD AND LYMPHATIC VASCULAR SYSTEMS

Vasculogenesis

Formation of the blood vascular system is one of the earliest events to occur in an organism during embryogenesis in order to meet the requirements of adequate oxygen diffusion. A blood vasculature is required when tissues grow beyond the limit of oxygen diffusion. Development of the blood vascular system begins in the early embryo de novo in a process called vasculogenesis. In vasculogenesis, precursor cells of blood endothelial cells, angioblasts, differentiate from the mesoderm and fuse together to form the primary capillary plexus (Risau and Flamme, 1995). Angioblasts are derived from hemangioblasts that form blood islands and are common precursor cells for both endothelial and hematopoietic cells. The cells in the interior of the blood islands differentiate into hematopoietic stem cells and the cells in the periphery of the blood islands differentiate into angioblasts. Hematopoietic stem cells later give rise to the cells of hematopoietic lineage and angioblasts differentiate into blood vascular endothelial cells.

Angiogenesis

The primary capillary plexus formed during vasculogenesis is further remodeled into a sophisticated vessel hierarchy composed of veins, arterioles, venules and capillaries in a process called angiogenesis. New blood vessels form from the pre-existing ones by sprouting or non-sprouting angiogenesis (Risau, 1997). In sprouting angiogenesis, the extracellular matrix surrounding endothelial cells is disrupted to allow proliferation and migration of endothelial cells to form new blood vessels. In non-sprouting angiogenesis (intussusceptive growth) new vessels are formed by splitting of the blood vessels by transcapillary pillars of extracellular matrix. The requirements of neovascularization are determined by organ-specific hemodynamic forces, signals from the endothelium and the surrounding environment. Vascular endothelial growth factor (VEGF) is the major angiogenic growth factor, and mice lacking even one allele of Vegf show aberrant blood vessel formation in the yolk sac and embryo proper resulting in death in utero (Carmeliet et al., 1996; Ferrara et al., 1996). Hypoxia stimulates hypoxia-inducible factor-1 (HIF-1) that upregulates VEGF expression in ischemic tissues (Plate et al., 1992; Shweiki et al., 1992).

Blood vascular specification and maturation

Establishment of artery-vein specification was previously thought to arise from hemodynamic forces within vessels, but later evidence supported genetic control mechanisms for vessel identity. EphrinB2-EphB4 signaling plays a critical role in establishment of arterial-venous boundaries. The transmembrane ligand ephrinB2 is expressed in the arteries and smooth muscle cells while its receptor EphB4 is expressed in veins (Adams et al., 1999; Gale et al., 2001; Gerety et al., 1999; Shin et al., 2001; Wang et al., 1998). The Notch pathway, comprising ligands Delta-like-4, Jagged-1 and Jagged-2 and receptors Notch-1, Notch-3 and Notch-4 also promotes an arterial specification of endothelial cells, by repressing venous differentiation and maintaining arteries (Lawson et al., 2001).

The newly formed blood vessels either regress or persist and mature. Blood flow (shear stress) is the mechanical force that regulates angiogenesis by determining which of the nascent vessels regress and which persist. Unperfusion of the vessels induces regression while sufficient perfusion by blood flow promotes persistence. Persisting vessels undergo
maturation, which is also referred to by the term arteriogenesis (Carmeliet, 2000). Nascent endothelial tubes mature and are stabilized by formation of the basement membrane beneath them and investment of mural cells to cover the vessel wall (Jain, 2003; Armulik et al., 2005; von Tell et al., 2006). Mural cells form a very heterogeneous and plastic population of cells that is characterized by the capacity to differentiate into other mesenchymal cell types, such as fibroblasts. Most mural cells are of mesenchymal origin, but some brain pericytes are derived from the neural crest and mural cells in coronary vessels are derived from the epicardium (Hungerford and Little, 1999; Vrancken Peeters et al., 1999; Etchevers et al., 2001). Common markers for mural cell lineage are α-smooth muscle actin (SMA), desmin, NG2, Rgs5 and PDGFR-β (Gerhardt and Betsholtz, 2003). Vascular smooth muscle cells are recruited to large vessels and their contractile ability controls blood vessel lumen diameter. Conversely, pericytes surround small vessels and signal directly to endothelial cells through specific focal contacts that they form with endothelial cells. Mural cells sense angiogenic stimuli from surrounding tissues and from shear stress within the vessels and control endothelial cell proliferation, differentiation and extracellular matrix deposition or degradation accordingly (Gerhardt and Betsholtz, 2003). PDGF-B is the major mitogen for pericytes and smooth muscle cells and it regulates formation of blood vessels by signaling via PDGFR-β. Sprouting endothelial tip cells secrete PDGF-B that promotes recruitment of PDGFR-β expressing pericytes and vascular smooth muscle cells to the endothelium (Gerhardt et al., 2003; Hoch and Soriano, 2003; Betsholtz, 2004). Other signaling pathways playing important roles in mural cell recruitment and vessel maturation and stabilization include Angiopoietin-Tie2, Edg1-sphingosine-1-phosphate, and TGF-β signaling (Armulik et al., 2005; von Tell et al., 2006).

Pathological angiogenesis

New blood vessels are generated by angiogenesis throughout life in many physiological and pathological conditions when neovascularization is needed. Physiological angiogenesis is required during pregnancy, the female reproductive cycle and wound healing, while pathological angiogenesis contributes to malignant, ischemic, inflammatory, infectious and immune disorders (Carmeliet, 2003). A strategy to treat cancer by inhibiting angiogenesis was proposed already at 1971 (Folkman, 1971). Tumor growth is dependent on adequate oxygen diffusion just as is tissue growth in normal physiology. Accordingly, blood vessel growth is required for the tumor to grow beyond the size of only few cubic millimeters. The angiogenic switch, the balance between positive and negative regulators of angiogenesis, determines if blood vessel growth is induced or suppressed. Due to a critical role in inducing angiogenesis, VEGF is an important target for inhibition of vascular growth in tumors (Carmeliet, 2005; Ferrara and Kerbel, 2005). Blood vessels formed during normal physiology are stable and quiescent whereas tumor blood vessels appear unstable, fragile, leaky and dysfunctional. The unstable appearance of the tumor vasculature, however, enables selective targeting of the tumor vasculature with anti-angiogenic drugs, as they are less resistant to these drugs. The challenge, however, remains in targeting only the tumor vessels while leaving normal vessels intact.
Lymphangiogenesis

Lymphatic vessels are formed in a process called lymphangiogenesis (Oliver, 2004; Karpanen and Alitalo, 2008) (Figure 1). At the time the lymphatic vessels emerge the blood vasculature is already functional. According to the current view, the first lymphatic endothelial cells differentiate from venous endothelial cells and migrate to form the primary lymph sacs. Peripheral lymphatic vessels then form by centrifugal sprouting from the lymph sacs and large collecting lymphatic vessels undergo maturation by accumulation of mural cells and formation of valves. The theory of a venous origin for lymphatic vessels was proposed already a century ago by Dr. Florence Sabin who was able to visualize lymphatic vessels in pig embryos using intradermal ink injections (Sabin, 1902). According to an alternative theory by Huntington and McClure (Huntington and McClure, 1908), lymph sacs arise independently of veins from mesenchymal precursor cells and later during development establish venous connections.

In humans the first lymph sacs are found in six to seven week old embryos whereas in mice the development of lymphatic vessels starts at midgestation (van der Putte, 1975a; van der Putte, 1975b). Recently, lymphatic vessels have also been described in zebrafish and frogs (Ny et al., 2005; Küchler et al., 2006; Yaniv et al., 2006). A recent elegant lineage-tracing experiment in mice support the hypothesis that lymphatic vessels arise solely from embryonic veins (Srinivasan et al., 2007). In zebrafish, lymphatic endothelial cells were also shown to have a venous origin using lineage-tracing experiments and contribution of mesenchymal precursor cells to lymphatic vessels development was not found (Yaniv et al., 2006). However, studies on *Xenopus* tadpole and avian models suggest that mesoderm-derived precursors cells called lymphangioblasts may also contribute to lymphangiogenesis (Schneider et al., 1999; Ny et al., 2005; Wilting et al., 2006). Moreover, circulating endothelial progenitor cells have been suggested to contribute to developmental lymphangiogenesis in mouse embryos (Sebzda et al., 2006) and to pathological lymphangiogenesis in rejected renal transplants in humans (Kerjaschki et al., 2006).

![Figure 1](image_url)  
**Figure 1.** Steps of lymphatic vascular development.
Lymphatic competence

Expression of LYVE-1 in endothelial cells lining anterior cardinal veins in E9.0-E9.5 mouse embryos is the first indication of blood endothelial cell competence to differentiate into lymphatic endothelial cell in response to lymphatic-inducing signals. LYVE-1 is a lymphatic endothelium specific receptor for hyaluronan that is involved in the uptake of hyaluronan from the tissues to the lymph (Banerji et al., 1999; Jackson et al., 2001; Prevo et al., 2001). LYVE-1 deficient mice appear normal, suggesting that LYVE-1 is dispensable for lymphatic development and function (Gale et al., 2007). Currently the signal that promotes lymphatic endothelial cell competence to respond to lymphatic-inducing signals is still unknown.

Lymphatic commitment

The Prospero-related homeobox protein 1 (Prox1) is the master regulator of lymphatic endothelial cell differentiation. Expression of Prox1 in cultured primary blood vascular endothelial cells induces expression of lymphatic endothelial cell specific genes and downregulates blood vascular endothelial specific genes (Hong et al., 2002; Petrova et al., 2002). Soon after LYVE-1 expression is initiated, Prox1 is expressed on one side of the cardinal vein in a subpopulation of LYVE-1 positive endothelial cells (Wigle and Oliver, 1999). These Prox1 positive cells are the first committed lymphatic endothelial cells that will migrate from the cardinal vein to form the primary lymph sacs. Prox1 activity on these first committed endothelial cells is required for differentiation towards a lymphatic lineage and maintenance of their migration (Wigle et al., 2002). Prox1 is required for the formation of the primitive lymph sacs as Prox1 deficient mice completely lack these structures (Wigle and Oliver, 1999; Wigle et al., 2002). In Prox1 deficient mice, sprouting of lymphatic endothelial cells from veins is arrested and these cells fail to express lymphatic endothelial cell markers, but continue to express blood vascular markers (Wigle et al., 2002). Accordingly, Prox1 knockdown causes lymphatic defects in zebrafish and frogs (Ny et al., 2005; Yaniv et al., 2006).

Lymphatic migration

VEGFR-3 is expressed almost exclusively in the lymphatic endothelium in adults and during late development, but in midgestation embryos it is required for remodeling of the blood vascular networks as Vegfr3 deficient mice die around E10.5 due defects in blood vessel remodeling, prior to the emergence of lymphatic vessels (Kaipainen et al., 1995; Dumont et al., 1998). In adult human tissues VEGFR-3 is also expressed in certain fenestrated and discontinuous blood vessel endothelia (Partanen et al., 2000). The VEGFR-3 ligand VEGF-C is expressed in the mesenchyme at the sites where lymphatic vessels are forming, close to the VEGFR-3 expressing cardinal vein, suggesting paracrine signaling (Kukk et al., 1996; Karkkainen et al., 2004). Accordingly, the first Prox1 expressing committed lymphatic endothelial cells sprout dorsally and bilaterally upstream of a VEGF-C gradient from the cardinal vein, to form the primary lymph sacs in E11.0-E11.5 mouse embryos (Karkkainen et al., 2004). VEGF-C is required for migration and survival of the first committed lymphatic endothelial cells, as in Vegfc deficient mice lymph sac formation fails (Karkkainen et al., 2004). These mice display complete absence of lymphatic endothelium and die during embryogenesis due to fluid accumulation in tissues but lack blood vascular defects (Karkkainen et al., 2004). Mice heterozygous for Vegfc deletion show delayed development of lymphatic vessels and severe lymphatic hypoplasia in the skin, suggesting that both alleles are required for normal lymphatic vessel formation (Karkkainen et al., 2004). Interestingly,
mice deficient for the other VEGFR-3 ligand, VEGF-D, exhibit only a minor lymphatic defect, involving a reduced number of lymphatic vessels in the lungs, suggesting that VEGF-D is dispensable for lymphatic development (Baldwin et al., 2005).

Adhesion and migration of lymphatic endothelial cells is essential in formation of lymphatic vessels. Podoplanin is an integral glomerular podocyte membrane glycoprotein that in addition to podocytes is also highly expressed on the lymphatic endothelium from E11.5 onwards (Breiteneder-Geleff et al., 1997; Breiteneder-Geleff et al., 1999). Podoplanin deficient mice die soon after birth due to respiratory failure and exhibit defects in lymphatic vessel patterning that leads to dysfunctional lymph drainage and swelling of the limbs (Schacht et al., 2003). In vitro experiments on cultured endothelial cells suggested that podoplanin could stimulate endothelial cell migration and adhesion (Schacht et al., 2003).

Integrin subunits α9 and β1 together form a receptor for extracellular matrix proteins, such as vascular endothelial cell adhesion molecule-1, tenascin C and osteopontin, and is highly expressed in lymphatic endothelial cells (Petrova et al., 2002). Mice deficient for integrin α9 develop a chylothorax soon after birth, suggesting that integrin α9 is required for normal lymphatic development (Huang et al., 2000). The exact mechanism by which integrin α9β1 regulates lymphatic development remains unclear, but in vitro data have shown that in the presence of specific extracellular matrix components, β1 integrin can induce phosphorylation of VEGFR-3 and modulate cell migration, proliferation and survival (Wang et al., 2001; Zhang et al., 2005). Furthermore, VEGF-C and VEGF-D were shown to bind to integrin α9β1 (Vlahakis et al., 2005).

VEGF-C and VEGF-D also bind to neuropilin-2 which is a non-tyrosine kinase co-receptor that mediates semaphorin signals together with plexins in axon guidance (Karkkainen et al., 2001; Kärpänen et al., 2006). Neuropilin-2 internalizes with VEGFR-3 in vitro, and is suggested to modulate VEGFR-3 signaling (Kärpänen et al., 2006). Neuropilin-2 is expressed in lymphatic vessels, and Neuropilin-2 deficient mice lack small lymphatic vessels while large vessels appear intact (Yuan et al., 2002).

**Lymphatic-venous separation**

When the primary lymph sacs have been formed, they remain separated from the blood vessels. Blood and lymphatic vascular systems are connected only at few sites where major lymphatic vessels empty into the central veins. Tyrosine kinase Syk and adaptor protein Slp-76 are haematopoietic intracellular signaling proteins that are involved in the separation of lymphatic endothelium from the blood endothelium (Abtahian et al., 2003; Sebzda et al., 2006). Mice deficient of Syk or SLP-76 show embryonic hemorrhage, arteriovenous shunting and abnormal connections between blood and lymphatic vessels leading to blood-filled lymphatic vessels (Abtahian et al., 2003). Syk and SLP-76 are not expressed in endothelial cells but in hematopoietic cells suggesting that a hematopoietic signaling pathway might be required in the separation process. To support this hypothesis, overexpression of Slp-76 in a subset of hematopoietic cells under the GATA1 promoter rescued the lymphatic defects in Slp76 null mice and studies with chimeric embryos showed that Syk and Slp-76 function cell-autonomously in haematopoietic cells that may contribute directly to vascular development as endothelial precursors (Sebzda et al., 2006). Although it has been postulated that Syk and Slp-76 are required for migration of putative endothelial progenitor cells through vessel walls, the mechanism by which Syk and Slp-76 function in separation of lymphatic endothelium from blood endothelium remains unknown.
**Lymphatic maturation**

Remodeling of the nascent lymphatic vessel network and maturation of the large collecting lymphatic vessels by deposition of basement membrane, investment of mural cells and formation of valves are crucial events in formation of a functioning lymphatic vasculature. Several molecules have been implicated in lymphatic vessel maturation, but the molecular mechanisms of these processes have only recently begun to elucidate.

EphrinB2 signaling via EphB4 is important in establishment of the arterial and venous identities but EphrinB2-EphB4 signaling has also been implicated in lymphatic vessel remodeling (Wang et al., 1998; Adams et al., 1999; Makinen et al., 2005). EphB4 is expressed in collecting lymphatic vessels and in lymphatic capillaries, while EphrinB2 is only expressed in collecting lymphatic vessels in adults (Makinen et al., 2005). The mutant mice lacking the PDZ domain binding motif of EphrinB2 display lymphatic defects including chylothorax, hyperplasia of collecting lymphatic vessels, agenesis of valves and abnormal recruitment of smooth muscle cells into lymphatic capillaries, but do not show blood vessel defects (Makinen et al., 2005). Interestingly, vascular mural cell specific ablation of EphrinB2 also leads to abnormal migration of smooth muscle cells into lymphatic capillaries (Foo et al., 2006).

Forkhead transcription factor Foxc2 is highly expressed in developing lymphatic vessels in embryos and lymphatic valves in adults (Dagenais et al., 2004; Petrova et al., 2004). Also in Foxc2 deficient embryos lymphatic capillaries are abnormally covered with smooth muscle cells, appear irregularly patterned and collecting lymphatic vessels lack valves (Petrova et al., 2004). Lymphatic capillaries in embryos lacking Foxc2 also show upregulation of PDGF-B and endoglin, both important regulators of mural cell recruitment, and increased deposition of basement membrane protein collagen IV (Petrova et al., 2004). These data suggest that Foxc2 is required for lymphatic vessel maturation (Petrova et al., 2004). Foxc2 may interact with the VEGFR-3 pathway in lymphatic development, as embryos heterozygous for both Foxc2 and Vegfr3 show a similar phenotype as the Foxc2 null embryos (Petrova et al., 2004).

Angiopoietin ligands and Tie receptor tyrosine kinases expressed in both blood and lymphatic endothelia are essential regulators of blood vessel remodeling and maturation, but are also involved in lymphangiogenesis. Overexpression of Ang1 induces lymphatic vessel formation in a VEGFR-3 dependent manner (Morisada et al., 2005; Tammela et al., 2005). Ang2 deficient mice exhibit chylous ascites and edema of the paws due to lymphatic dysfunction (Gale et al., 2002). In Ang2 deficient mice collecting lymphatic vessels appear disorganized with loss of tight smooth muscle cell contacts and lymphatic capillaries appear hypoplastic (Gale et al., 2002). Ang1 rescues the lymphatic defect in Ang2 deficient mice but not the blood vascular defects, suggesting that Ang2 acts as a agonist in lymphatic vessels and as an antagonist in blood vessels (Gale et al., 2002).
3. VASCULAR ENDOTHELIAL GROWTH FACTORS AND THEIR RECEPTORS

The vascular endothelial growth factor (VEGF) family includes VEGF (also called VEGF-A), placenta growth factor (PIGF), VEGF-B, VEGF-C and VEGF-D; as well as the VEGF homologues encoded by Orf viruses (collectively called VEGF-E) and VEGF-like proteins in snake venoms. VEGFs are secreted dimeric growth factors that are important regulators of angiogenesis and lymphangiogenesis and signal via three vascular endothelial growth factor receptors: VEGFR-1 (also called Flt1), VEGFR-2 (also called Flk1 in mice and KDR in humans) and VEGFR-3 (also called Flt4). VEGF receptors are receptor tyrosine kinases consisting of seven extracellular immunoglobulin homology domains, a transmembrane domain and an intracellular tyrosine kinase domain. Upon ligand binding, VEGF receptors dimerize and trans-autophosphorylate. VEGFs bind to VEGFRs with different specificities (Figure 2). VEGF binds to VEGFR-1 and VEGFR-2, while PIGF and VEGF-B only bind to VEGFR-1, and VEGF-E only binds to VEGFR-2. In human VEGF-C and VEGF-D are ligands for VEGFR-2 and VEGFR-3 whereas in mouse VEGF-D is a VEGFR-3 specific ligand.

![Figure 2. Receptor binding specificity of VEGFs.](image)

**VEGF**

VEGF (also referred to as vascular permeability factor) is the major angiogenic growth factor both in physiological and pathological situations signaling through VEGFR-1 and VEGFR-2 (Ferrara, 1999). The VEGF_{165} isoform also binds to neuropilin-1 (Soker et al., 1998). Vegf expression is prominent widely in areas of active vessel formation, and is regulated by tissue oxygen tension. The mediator of hypoxia-induced transcription of Vegf is HIF-1. Hypoxia reversibly induces VEGF expression by increasing Vegf transcription and mRNA
stabilization (Plate et al., 1992; Shweiki et al., 1992). In mice deletion of the hypoxia response element in the Vegf promoter leads to progressive motor neuron degeneration (Oosthuyse et al., 2001). Inactivation of a single VEGF allele in mice results in embryonic lethality as blood islands, endothelial cells, and major vessels fail to develop (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF induces lymphatic hyperplasia (Nagy et al., 2002; Hirakawa et al., 2005).

In human, VEGF is expressed in eight different isoforms having size of 121, 145, 148, 162, 165, 183, 189 and 206 amino acid residues. In mice, these isoforms are shorter by one amino acid residue. The bioavailability of different VEGF isoforms is regulated by different heparin-binding ability. The shortest isoform, VEGF121, is a freely soluble protein failing to bind to heparan sulfate proteoglycans in the extracellular matrix (Houck et al., 1992). In contrast, a significant portion of secreted VEGF165 remains bound to the extracellular matrix due to binding to heparan sulfate proteoglycans (Houck et al., 1992). The longest isoforms, VEGF189 and VEGF206, show highest affinity to heparin and are bound to the extracellular matrix almost completely but can be released by proteolytic cleavage facilitating rapid local increase in VEGF concentration in response to tissue growth demand (Park et al., 1993).

**VEGF-B**

VEGF-B exists in two different isoforms, VEGF-B167 and VEGF-B186, with VEGF-B167 being predominant (Olofsson et al., 1996a; Olofsson et al., 1996b). VEGF-B binds to VEGFR-1 and neuropilin-1 (Makinen et al., 1999; Olofsson et al., 1998). Expression of VEGF-B is most abundant in the heart and skeletal muscle in adult tissues (Olofsson et al., 1996a). VEGF-B deficient mice appear normal and healthy, but exhibit atrial conduction defects, reduced heart size and impaired recovery and vascular function after myocardial ischemia (Bellomo et al., 2000; Aase et al., 2001).

**PIGF**

PIGF and is most abundantly expressed in the placenta and exists in four different isoforms, PIGF-1, PIGF-2, PIGF-3 and PIGF-4 (Maglione et al., 1991; Hauser and Weich, 1993; Maglione et al., 1993; Cao et al., 1997). PIGF homodimers bind to VEGFR-1 but VEGF/PIGF heterodimers can also bind to VEGFR-2 (Park et al., 1994; DiSalvo et al., 1995; Cao et al., 1996). Additionally, PIGF binds to neuropilins and heparin. PIGF was shown to induce growth and migration of endothelial cells from bovine coronary postcapillary venules and from human umbilical veins and to induce angiogenesis in the rabbit cornea and the chick chorioallantoic membrane assays (Ziche et al., 1997). PIGF deficient mice show no developmental angiogenic defects, but show impaired angiogenesis, plasma extravasation and collateral growth during ischemia, inflammation, wound healing and cancer (Carmeliet et al., 2001).

**VEGF-C**

VEGF-C was cloned from human prostatic carcinoma cells and discovered as the first ligand for VEGFR-3 (Joukov et al., 1996; Lee et al., 1996). VEGF-C expression patterns have been studied using in situ hybridization analysis and β-galactosidase as a marker for endogenous Vegfc expression in Vegfc<sup>−/−</sup> mice (Kukk et al., 1996; Karkkainen et al., 2004). These analyses showed that Vegfc is expressed in mouse embryos from E8.5 onwards at sites of lymph sac formation in mesenchymal cells adjacent to VEGFR-3 expressing endothelial
cells, suggesting paracrine ligand-receptor signaling mechanism (Kukk et al., 1996; Karkkainen et al., 2004). In human, VEGF-C is expressed in neuroendocrine cells, such as the α cells of the islets of Langerhans, prolactin-secreting cells of the anterior pituitary, adrenal medullary cells and dispersed neuroendocrine cells of the gastrointestinal tract (Partanan et al., 2000).

The receptor binding affinity of VEGF-C is regulated by proteolytic cleavage. VEGF-C is synthesized as a preproprotein comprising a signal sequence, N- and C-terminal propeptides and receptor-binding VEGF-homology domain. Proteolytic processing of VEGF-C increases its affinity for VEGFR-3 and only the fully processed form can bind to VEGFR-2 (Joukov et al., 1997). Recently, the chimeric VEGF-C/VEGF heparin-binding domain fusion proteins were shown to activate VEGF-C receptors and stimulate lymphangiogenesis (Tammela et al., 2007a). Additionally, the chimeric fusion proteins composed of amino- and carboxy-terminal propeptides of VEGF-C fused to receptor-activating core domain of VEGF bound to VEGFR-1 and VEGFR-2 and induced capillary angiogenesis (Keskitalo et al., 2007).

VEGF-C induces migration of capillary endothelial cells in vitro (Joukov et al., 1996) and various in vivo models have established VEGF-C as an important regulator of lymphatic endothelial cells. Overexpression of VEGF-C in skin keratinocytes in transgenic mice leads to hyperplasia of cutaneous lymphatic vessels (Jeltsch et al., 1997). Transgenic mice overexpressing a VEGFR-3 specific mutant form of VEGF-C (VEGF-C156S) also show severe hyperplasia of the dermal lymphatic vessels, suggesting that signaling via VEGFR-3 is sufficient for lymphangiogenesis (Veikkola et al., 2001). VEGF-C was also shown to mediate lymphangiogenesis in the chick chorioallantoic membrane (Oh et al., 1997). Vegfc deficient mice lack lymphatic vessels and heterozygous mice lacking one Vegfc allele exhibit lymphatic hypoplasia, indicating the critical function of VEGF-C in lymphangiogenesis (Karkkainen et al., 2004). Accordingly, knockdown of Vegfc in frog and zebrafish leads to lymphatic vessel defects and edema suggesting that this pathway is conserved in vertebrate evolution (Ny et al., 2005; Küchler et al., 2006; Yaniv et al., 2006).

In addition to lymphangiogenesis, VEGF-C induces angiogenesis via signaling through VEGFR-2. VEGF-C stimulated physiological and pathological angiogenesis has been shown in the mouse cornea and in limb ischemia models (Cao et al., 1998; Witzenbichler et al., 1998). VEGF-C is also required for developmental vasculogenesis and angiogenesis in zebrafish (Ober et al., 2004) and adenoviral delivery of VEGF-C in mouse skin induced not only lymphangiogenesis but also induced blood vessel leakiness (Enholm et al., 2001; Saaristo et al., 2002a). Furthermore, deletion of Vegfc induces aberrant blood vessel formation in frogs (Ny et al., 2005).

**VEGF-D**

VEGF-D has been identified as a ligand for VEGFR-2 and VEGFR-3 (Achen et al., 1998). In mouse, VEGF-D is a specific ligand for VEGFR-3 and there are two VEGF-D isoforms generated by alternative splicing (Baldwin et al., 2001a; Baldwin et al., 2001b). VEGF-D expression is detected by in situ hybridization in various body structures and organs in mouse, including limb bud, acoustic ganglion, teeth, heart, anterior pituitary, lung and kidney mesenchyme, liver, derma, and periosteum of the vertebral column (Avantaggiato et al., 1998). In human, VEGF-D expression is most abundant in the heart, lung, skeletal muscle, colon, and small intestine (Achen et al., 1998). VEGF-D expression is induced by
transcription factors c-Fos and Fra-1 as well as by cell-cell contact mediated by cadherin-11 (Orlandini et al., 1996; Debinski et al., 2001; Orlandini and Oliviero, 2001).

VEGF-D promotes lymphangiogenesis and angiogenesis. Overexpressing of VEGF-D in the skin of transgenic mice induces lymphatic hyperplasia (Veikkola et al., 2001). Adenoviral delivery of VEGF-D in mouse skin, ischemic rabbit hind limb skeletal muscle and inflammatory mouse respiratory tract also promotes lymphangiogenesis (Byzova et al., 2002; Rissanen et al., 2003; Baluk et al., 2005). VEGF-D is a potent angiogenic factor in the rabbit hind limb skeletal muscle, rat cremaster muscle and rabbit carotid artery in vivo models (Byzova et al., 2002; Bhardwaj et al., 2003; Rissanen et al., 2003; Kholová et al., 2007). Overexpression of zVEGF-D in zebrafish causes blood vascular defects including misguidance of intersegmental vessels and abnormal connections between dorsal aorta and caudal vein (Song et al., 2007). Vegfd deficient mice, surprisingly, exhibit only a minor lymphatic defect involving a decreased number of lymphatic vessels around bronchioles in the lungs where Vegfd expression is prominent (Baladin et al., 2005).

**VEGF-E**

VEGF homologues encoded by Orf viruses and pseudocowpox viruses that cause highly vascularized lesions are collectively termed VEGF-E (Lyttle et al., 1994; Ogawa et al., 1998; Meyer et al., 1999; Wise et al., 1999). VEGF-like proteins have also been found in snake venoms (Komori et al., 1999; Junqueira de Azevedo et al., 2001). Virus-encoded VEGFs are separated into two groups: VEGF-E_P170 and VEGF-E_N22, showing homology to VEGF and PIGF, and VEGF-E_N27, showing homology to VEGF-C and VEGFD. Virus-encoded VEGF homologues bind to and induce autophosphorylation of VEGFR-2 (Ogawa et al., 1998; Meyer et al., 1999; Wise et al., 1999). Overexpression of VEGF-E_N27 in skin keratinocytes of transgenic mice induces a strong angiogenic response (Kiba et al., 2003). Also, chimeric VEGF-ENZ7/PIGF molecules promote angiogenesis in ischemic tissues and wounds (Inoue et al., 2007; Zheng et al., 2006; Zheng et al., 2007). Furthermore, VEGF-E_N27 signaling via VEGFR-2 induces circumferential lymphatic vessel hyperplasia but essentially no lymphatic sprouting in the mouse skin when delivered by adenoviral vectors or transgene (Wirzenius et al., 2007).

**VEGFR-1**

VEGFR-1 (Flt1) exists as a transmembrane glycoprotein or as a short soluble form that consists of only the six first extracellular lg homology domains (Shibuya et al., 1990; De Vries et al., 1992; Kendall and Thomas, 1993). During embryogenesis, VEGFR-1 is first expressed in the angioblasts at E8.5 (Peters et al., 1993; Fong et al., 1995). In adults, VEGFR-1 expression is most abundant in blood vessel endothelium, vascular smooth muscle cells, monocytes and macrophages, dendritic cells, trophoblasts, osteoclasts and hematopoietic stem cells (Shibuya, 2001).

Vegfr1 deficient mice show increased hemangioblast commitment leading to overgrowth of endothelial-like cells and disorganization of blood vessels and lethality at E8.5 (Fong et al., 1995; Fong et al., 1999). Deletion of only the intracellular domain of VEGFR-1 does not affect normal blood vessel development, but impairs tumor angiogenesis (Hiratsuka et al., 1998; Hiratsuka et al., 2001). VEGFR-1 also regulates monocyte migration in response to VEGF and recruitment of bone-marrow derived hematopoietic stem cells, suggesting that
VEGFR-1 signaling could play an important role in inflammatory conditions (Hiratsuka et al., 1998; Hattori et al., 2002).

Results from the Vegfr1 gene-targeted mice suggest that VEGFR-1 functions as a decoy receptor regulating VEGF signaling through VEGFR-2 by competing for the ligand. Accordingly, the tyrosine kinase activity of VEGFR-1 is weaker than that of VEGFR-2, possibly due to the repressor sequence in the VEGFR-1 juxtamembrane domain (Gille et al., 2000). VEGFR-1 also positively adjusts VEGFR-2 signaling by heterodimerization with VEGFR-2 (Carmeliet et al., 2001; Huang et al., 2001; Autiero et al., 2003).

**VEGFR-2**

VEGFR-2 (KDR in human and Flk1 in mouse) is the main signal transducer for angiogenesis via its tyrosine kinase activity mediating endothelial cell proliferation, migration and survival (Ferrara et al., 2003). A naturally occuring soluble form of VEGFR-2 has been found in mouse and human plasma (Ebos et al., 2004). VEGFR-2 also plays a role in lymphangiogenesis inducing lymphatic hyperplasia (Nagy et al., 2002; Hirakawa et al., 2005; Wirzenius et al., 2007). During embryogenesis, VEGFR-2 is the key marker for hemangioblasts and angioblasts (Shalaby et al., 1995). In adults, VEGFR-2 is expressed in the blood vessel endothelial cells and hematopoietic stem cells (Ziegler et al., 1999). VEGFR-2 expression in the tip cells of the angiogenic sprouts confers VEGF sensitivity and guided migration (Gerhardt et al., 2003).

Vegfr2 deficient mice die at E8.5-E9.5 and display impaired hematopoiesis and endothelial cell development suggesting that VEGFR-2 is essential in yolk-sac blood island formation and vasculogenesis (Shalaby et al., 1995). Additionally, analysis of chimeric mice confirmed that VEGFR-2 is cell-autonomously required for extraembryonic and intraembryonic endothelial cell development and for hematopoiesis (Shalaby et al., 1997). The phenotype of the Vegfr2 mutant mice with a tyrosine-to-phenylalanine substitution at position 1173 resembles the phenotype of Vegfr2 deficient mice suggesting that Y1173 is the most important tyrosine residue in VEGFR-2 signaling for endothelial and hematopoietic development (Sakurai et al., 2005).

**VEGFR-3**

VEGFR-3 (Flt4) was cloned from human placenta and a human leukemia cell line (Galland et al., 1992; Pajusola et al., 1992). VEGFR-3 exists in two splice variants (Pajusola et al., 1993). Integration of a human retrovirus between the last two exons explains why the shorter transcript does not exist in mouse (Hughes, 2001). In contrast to VEGFR-1 and VEGFR-2, VEGFR-3 is proteolytically processed within the fifth Ig homology domain, resulting in polypeptides that are linked with a disulfide bond (Pajusola et al., 1994).

During embryogenesis, VEGFR-3 is initially expressed in blood endothelial cells but is downregulated first in arteries and then in veins and finally becomes restricted to lymphatic endothelium (Kaipainen et al., 1995). In addition to lymphatic endothelium Vegfr3 is expressed in some fenestrated blood vessels in adults (Partanen et al., 2000). Vegfr3 deficient mice die at midgestation as a consequence of cardiovascular failure and defective remodeling of the primary blood capillary plexus, showing the important role of VEGFR-3 in blood vessel development (Dumont et al., 1998).
VEGFR-3 mediates proliferative and migratory signals to the lymphatic endothelium via the p42/p44 MAPK and Akt pathways, respectively (Makininen et al., 2001b). The important role of VEGFR-3 in lymphangiogenesis has also been shown in various mouse models. Signaling via VEGFR-3 is sufficient for lymphangiogenesis in transgenic mouse models (Jeltsch et al., 1997; Veikkola et al., 2001). Transgenic or adenoviral delivery of soluble VEGFR-3 that competes for the ligands with the endogenous receptor induces lymphatic vessel regression during late embryogenesis and the first two postnatal weeks (Makininen et al., 2001a; Karpanen et al., 2006). Heterozygous missense mutations in Vegfr3 gene that inactivate the tyrosine kinase domain are linked to lymphedema in human as well as in Chy mice carrying such mutation in their germ line (Karkkainen et al., 2000; Karkkainen et al., 2001).

An important aspect to address is the possible co-operation between VEGFR-2 and VEGFR-3 signaling in formation of blood and lymphatic vessels. Fine-tuning of lymphangiogenesis may involve heterodimerization of VEGFR-2 and VEGFR-3 (Dixelius et al., 2003; Alam et al., 2004; Goldman et al., 2007). Likewise, zebrafish Flt4 affects artery morphogenesis together with the Vegfr2 homologue Kdr, suggesting that distinct interactions between different receptors are required for formation of vessels (Covassin et al., 2006).

**Neuropilins**

NRPs are transmembrane receptors originally characterized as semaphorin receptors involved in axon guidance, but they also serve as co-receptors for several VEGFs (Neufeld et al., 2002). Neuropilin-1 and -2 exist in several splice isoforms and also as soluble forms. Neuropilins do not possess signaling capabilities but utilize other associated receptors, such as plexins, for signal transduction (Takahashi et al., 1999; Tamagnone et al., 1999).

Neuropilin-1 binds to VEGF_{165}, VEGF-B_{167}, VEGF-B_{186}, PIGF-2, VEGF-E, VEGF-C and VEGF-D (Migdal et al., 1998; Soker et al., 1998; Makinen et al., 1999; Wise et al., 1999; Kärpänen et al., 2006) while neuropilin-2 binds to PIGF-2, VEGF_{145}, VEGF_{165} and VEGF-C and VEGF-D (Gluzman-Poltorak et al., 2000; Karkkainen et al., 2001; Kärpänen et al., 2006). The modulation of the signal transduction activity may depend on complex formation between neuropilins and VEGF receptors. VEGFR-1 and VEGFR-3 interact with neuropilin-1 and neuropilin-2, whereas VEGFR-2 interacts only with neuropilin-1 (Fuh et al., 2000; Gluzman-Poltorak et al., 2001; Soker et al., 2002; Kärpänen et al., 2006). Both neuropilins are initially expressed in yolk sac endothelial cells. Later neuropilin-1 becomes abundant in arterial endothelial cells and neuropilin-2 first in the venous endothelium then in lymphatic endothelium (Herzog et al., 2001; Yuan et al., 2002).

Neuropilins are required for normal neural development (Chen et al., 2000; Giger et al., 2000; Kitsukawa et al., 1997). Both neuropilins are also important in normal vascular development as shown by mouse models. Overexpression of neuropilin-1 in chimeric mice leads to blood vascular defects including excess capillaries and blood vessels, dilation of blood vessels and malformed heart (Kitsukawa et al., 1995). Neuropilin-1 was also shown to be required for normal blood vascular formation in a knockout mouse model (Kawasaki et al., 1999). Mice deficient for Nrp2 show absence or severe reduction of the lymphatic capillaries, while blood vessels and large collecting lymphatic vessels appear normal, suggesting that neuropilin-2 is selectively required for formation of small lymphatic vessels (Yuan et al., 2002). Mice double-deficient for neuropilin-1 and -2 show severe defects in early embryonic angiogenesis (Takahshima et al., 2002).
4. PLATELET-DERIVED GROWTH FACTORS AND THEIR RECEPTORS

Platelet-derived growth factors (PDGFs) are secreted dimeric growth factors composed of homo- or heterodimeric polypeptide chains: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. PDGFs are major mitogens for several cell types of mesenchymal origin, such as smooth muscle cells, pericytes and fibroblasts (Heldin and Westermark, 1999). PDGFs signal via two platelet-derived growth factor receptors, PDGFR-α and PDGFR-β, that appear in three PDGF receptor variants, PDGFR-αα, PDGFR-αβ and PDGFR-ββ. PDGF receptor tyrosine kinases consist of five extracellular immunoglobulin homology domains, a transmembrane domain and an intracellular tyrosine kinase domain. Upon ligand binding, PDGF receptors dimerize and trans-autophosphorylate. PDGF-AA, PDGF-AB, PDGF-BB and PDGF-CC bind to PDGFR-αα while PDGF-AB, PDGF-BB and PDGF-CC bind to PDGFR-αβ, and PDGF-BB and PDGF-DD bind to PDGFR-ββ (Figure 3).

![Figure 3. Receptor binding specificity of PDGFs.](image)

**PDGF-A and PDGF-B**

PDGF was purified from platelets, thus gaining its name. PDGF consists of A- and B-chains of which PDGF-A exists in two splice isoforms, that form homo- and heterodimers (Heldin and Westermark, 1999). PDGF-A and PDGF-B are synthesized as precursors that undergo proteolytic processing in the aminoterminus, and in the case of PDGF-B also in the carboxyterminus, to produce the mature active forms. PDGF-A and -B are synthesized by various cell types, including vascular smooth muscle cells, endothelial cells, fibroblasts, macrophages and platelets/megakaryocytes and the synthesis is often increased by external stimuli (Heldin and Westermark, 1999).

Studies on knockout mice have revealed the important roles of PDGF-A and PDGF-B in development. *Pdgfa* deficient mice lack lung alveolar smooth muscle cells, exhibit reduced
deposition of elastin fibres in the lung parenchyma, and develop lung emphysema due to complete failure of alveogenesis that is coupled to lack of distal spreading of alveolar smooth muscle cell progenitors during lung development (Boström et al., 1996; Lindahl et al., 1997b). Pdgfa deficient mice also show reduced numbers of oligodendrocytes and tremor (Fruttiger et al., 1999). Furthermore, Pdgfa and Pdgfraα deficient mice display reduced number of intestinal villi (Karlsson et al., 2000).

PDGF-B/PDGFRβ signaling promotes mural cell proliferation and recruitment to the vessel wall. Pdgfb and Pdgfrβ deficient mice display fragile blood vessels and subsequent lethal microaneurysms and hemorrhaging owing to a lack of microvascular pericytes (Leveen et al., 1994; Soriano, 1994; Lindahl et al., 1997a; Hellstrom et al., 1999). Endothelium-specific Pdgfb knockout mice similarly show pericyte deficiency, indicating that the endothelium is the most important source of PDGF-B in pericyte recruitment (Engé et al., 2002; Bjarnegard et al., 2004). Lack of pericytes induces endothelial hyperplasia in Pdgfb and Pdgfrβ deficient mice suggesting that pericytes can negatively control endothelial cell proliferation (Hellstrom et al., 2001). Furthermore, activation of PDGFR-β on the hemangioprecursor cells also accelerates differentiation of endothelial cells (Rolny et al., 2006) Pdgfb and Pdgfrβ deficient mice also lack mesangial cells leading to poor filtration of the glomeruli (Leveen et al., 1994; Soriano, 1994). Expression of PDGF-B in endothelial cells and PDGF-B in closely located mesangial cells suggests a short-range paracrine signaling mechanism similar to other capillaries (Lindahl et al., 1998). The similar phenotypes of mice lacking Pdgfb or Pdgfrβ suggest that PDGF-B signals mainly through PDGFR-β during embryonic development. The diffusion range of PDGF-B is regulated by the retention motif that confers retention of the secreted growth factor within the pericellular space. Deletion of PDGF-B retention motif or heparan sulfate in mice leads to deficient pericyte recruitment in the microvessels, suggesting that retention of PDGF-B is essential for proper investment and organization of pericytes (Lindblom et al., 2003; Abramsson et al., 2007).

**PDGF-C and PDGF-D**

PDGF-C and PDGF-D are the most recent members of the PDGF family. In comparison to PDGF-A and PDGF-B, PDGF-C and PDGF-D only form homodimers (Li et al., 2000; Bergsten et al., 2001). PDGF-CC primarily binds to PDGF-αα but can also activate PDGF-αβ, while PDGF-D is a specific ligand to PDGF-ββ (Li et al., 2000; Bergsten et al., 2001; Gilbertson et al., 2001; LaRochelle et al., 2001; Cao et al., 2002). Both PDGF-C and PDGF-D are broadly expressed. PDGF-C plays a role in development of the heart, ear, central nervous system and kidney, and in adults, is active in the kidney and central nervous system while PDGF-D is active in the development of the kidney, eye and brain (Reigstad et al., 2005).

PDGF-C signaling is implicated in palatogenesis as Pdgfc deficient mice display a complete cleft of the secondary palate (Ding et al., 2004). This defect is not, however, as severe as in Pdgfraα deficient mice, whereas double-deficient mice for Pdgfa and Pdgfc phenocopy the Pdgfraα deficient mice showing cleft palate, subepidermal blistering, deficiency of renal cortex mesenchyme, spina bifida and skeletal and vascular defects, suggesting that PDGF-C signaling via PDGFR-α regulates development of craniofacial structures, the neural tube and mesodermal organs (Ding et al., 2004). Global Cre-mediated deletion of PDGF-C in conditional Pdgfc deficient mice results in similar defects as seen in Pdgfc null embryos (Wu and Ding, 2007). A Pdgfd deficient mouse has not been reported.
**PDGFR-α and PDGFR-β**

PDGF receptors α and β are transmembrane tyrosine kinase receptors that are composed of five extracellular immunoglobulin-like domains, a transmembrane domain and an intracellular tyrosine kinase domain (Claesson-Welsh et al., 1989; Matsui et al., 1989). The receptors dimerize upon binding of the dimeric PDGF ligands and form both hetero- and homodimers (Heldin and Westermark, 1999). The classical target cells of PDGFs, fibroblasts and smooth muscle cells, express both receptors but usually higher levels of PDGFR-β, while some other cell types express only PDGFR-α. The expression level of PDGF receptors is, however, not constant but can be regulated in response to tissue demand. Activation of PDGFR-β stimulates chemotaxis of cells whereas activation of PDGFR-α inhibits chemotaxis of some cell types including fibroblasts and smooth muscle cells and stimulates chemotaxis of certain other cell types (Heldin and Westermark, 1999).

*Pdgfra* deficient mice die during embryonic development and exhibit incomplete cephalic closure, increased apoptosis of neural crest cells and deficient myotome formation (Soriano, 1997). The requirement of PDGFR-α signaling in development of cranial and cephalic neural crest cells is cell-autonomous (Tallquist and Soriano, 2003). *Pdgfrb* deficient mice display a very similar phenotype as the *Pdgfb* deficient mice as discussed above.

5. **LYMPHATIC VESSELS IN DISEASE**

**Lymphedema**

Lymphedema is characterized by chronic, disabling swelling of the limbs due to insufficient lymphatic drainage, which causes accumulation of excessive amounts of protein-rich interstitial fluid in tissues (Rockson, 2001). In lymphedema lymphatic drainage is compromised due to defective transport activity of the lymphatic vessels. Chronic swelling causes fibrosis, adipose and connective tissue accumulation, increased susceptibility to infections and impaired wound healing (Rockson, 2001). Chylous ascites and chylothorax characterized by accumulation of chyle in the abdominal or thoracic cavity, respectively, are also pathological conditions caused by abnormal lymphatic vessel development or trauma.

Etiologically lymphedemmas can be divided into primary and secondary lymphedema. Primary lymphedemas are rather rare hereditary disorders that are often classified according to the age at which the tissue edema arises. About one in six thousand newborns are estimated to develop primary lymphedema (Dale, 1985). Congenital lymphedema is an early onset lymphedema that is usually present at birth while lymphedema praecox is detected around puberty. Lymphedema tarda occurs after age of 35 years. Secondary lymphedema is acquired when lymphatic vessels are damaged and lymphatic vessel function compromised as a result of infection, radiation therapy or post-surgical trauma. Secondary lymphedema caused by mastectomy is the most common form of lymphedema in industrialized countries. Globally the most common form of lymphedema is lymphatic filariasis caused by parasitic infection of the lymphatic vessels affecting more than a hundred million people mainly in tropical areas (Dreyer et al., 2000).

Milroy’s disease (early onset congenital lymphedema) has been linked to mutations in the *Vegfr3* gene that inhibit the biological activity of VEGFR-3 (Ferrell et al., 1998; Witte et al., 1998; Evans et al., 1999; Evans et al., 2003; Brice et al., 2005). Missense mutations in *Vegfr3* abolish the tyrosine kinase activity of the receptor and result in a dominant negative mutant
receptor with an extended half-life that diminishes the VEGFR-3 signaling level (Irrthum et al., 2000; Karkkainen et al., 2000). Accordingly, similar inactivating mutations in the tyrosine kinase domain of VEGFR-3 in Chy mice result in swelling of the paws. In Milroy’s disease the superficial lymphatic capillary network is hypoplastic or aplastic while in late onset lymphedemas the superficial lymphatic vessels are larger than in controls (Bollinger et al., 1983; Pfister et al., 1990; Bollinger, 1993). Similarly, various lymphedema mouse models show defects in subcutaneous lymphatic vessels and subsequent lymphedema (Karkkainen et al., 2001; Makinen et al., 2001a; Gale et al., 2002; Yuan et al., 2002; Schacht et al., 2003; Karkkainen et al., 2004; Dellinger et al., 2007). Some lymphedema mouse models also show defects in intestinal lymphatic vessels, resulting in accumulation of chylous ascites (Wigle and Oliver, 1999; Karkkainen et al., 2001; Gale et al., 2002; Karkkainen et al., 2004; Dellinger et al., 2007). The intestinal lymphatic vasculature often re-grows after the early postnatal period, whereas lymphatic defects in the skin persist in these mice. Currently it is still unclear why the lymphatic vasculature is heterogeneous and why some lymphatic vascular beds are more vulnerable than others to changes in the level of lymphangiogenic signaling.

In addition to Vegfr3, other genetic loci are involved in lymphedema syndromes as well. Mutations in the transcription factor FOXC2 gene are linked to the autosomal dominant lymphedema-distichiasis syndrome, characterized by a double row of eye lashes in addition to lymphedema (Fang et al., 2000; Bell et al., 2001; Sholto-Douglas-Vernon et al., 2005). In lymphedema-distichiasis subcutaneous lymphatic vessel distribution is normal or even hyperplastic but dysfunctional (Brice et al., 2002). Foxc2 is expressed in lymphatic vessels and in other sites associated with lymphedema-distichiasis (Dagenais et al., 2004). Mice heterozygous for Foxc2 serve as an experimental model for lymphedema-distichiasis (Kriederman et al., 2003). Foxc2 null mice exhibit abnormal mural cell recruitment to the lymphatic vessels, agenesis of lymphatic valves and lymphatic dysfunction (Petrova et al., 2004). Interestingly, mice heterozygous for both Foxc2 and Vegfr3 also show abnormal mural cell recruitment to the lymphatic vessels (Petrova et al., 2004). Moreover, mutations in the transcription factor SOX18 gene have been described in hypotrichosis-telangiectasia-lymphedema syndrome (Irrthum et al., 2003).

Mouse models for human lymphedema have greatly facilitated the study of lymphedema and treatments for lymphedema. Currently lymphedema is managed by compression bandages and stockings, massage and physiotherapy but curing treatment is still lacking (Rocksón, 2001). Stimulation of the VEGFR-3 pathway with the ligands VEGF-C and VEGF-D to induce lymphangiogenesis in affected areas has provided a promising basis for lymphedema therapy. Lymphatic vessel function is improved and lymphangiogenesis induced in rabbit ear and a mouse tail models of lymphedema via recombinant or adeno viral delivery of VEGF-C (Szuba et al., 2002; Yoon et al., 2003). Adenoviral VEGF-C gene therapy in mouse skin induces a strong lymphangiogenic response (Enholm et al., 2001), but also blood vascular leakiness, possibly via VEGF-C stimulated VEGFR-2 on the blood endothelium (Saaristo et al., 2002a). Adenovirus mediated VEGF-C transfer also induces growth of functional lymphatic vessels in the skin of lymphedema Chy mice (Karkkainen et al., 2001). Adeno associated virus-mediated VEGF-C gene therapy stimulates formation of lymphatic vessels in Chy lymphedema mice, which persisted for a longer time period (Saaristo et al., 2002b). Furthermore, VEGF-C gene therapy restores lymphatic flow across incision wounds in mice (Saaristo et al., 2004) and improves wound healing in diabetic mice by inducing both angiogenesis and lymphangiogenesis (Saaristo et al., 2006). Moreover, VEGF-C-induced lymphatic vessels undergo maturation and they are successfully regenerated after lymph node
damage (Tammela et al., 2007b). These preclinical models encourage development of lymphangiogenic growth factors as therapeutic targets for future treatment of lymphatic insufficiency.

**Tumor metastasis**

Tumor metastasis occurs when tumor cells disseminate via various routes, including blood or lymphatic vessels, to regional lymph nodes and to distant organs. Generally, tumor cells enter lymphatic vessels more easily than blood vessels due to their greater permeability. Metastasizing tumor cells detach from the primary tumor mass, invade stromal tissues, intravasate blood or lymphatic vessels, spread via them, extravasate, and finally attach, survive and proliferate in secondary sites (Alitalo and Carmeliet, 2002). Association of VEGF-C and VEGF-D expression and enhanced metastasis and tumor lymphangiogenesis has been shown in various experimental and human tumors (Stacker et al., 2002b; Achen et al., 2005). Studies of transgenic or xenotransplantation tumor models suggest that expression of VEGF-C or VEGF-D in tumors induces tumor lymphangiogenesis and enhances metastasis via lymphatic vessels. Accordingly, inhibition of the VEGFR-3 lymphangiogenic signaling pathway could be beneficial for inhibiting tumor lymphangiogenesis and metastatic spread via lymphatic vessels.

RipVEGF-C transgenic mice that overexpress VEGF-C in the β-cells of the endocrine pancreas driven by rat insulin promoter (Rip) show a dense network of lymphatic vessels around the islets of Langerhans. Tumors in RipVEGF-C mice crossed with Rip1Tag2 mice that develop pancreatic β-cell tumors, are surrounded by large lymphatic vessels that contain tumor cell aggregates of β-cell origin. Tumors in these double-transgenic mice also frequently metastasize while tumors in Rip1Tag2 mice are generally non-metastatic (Mandriota et al., 2001). However, adenoviral expression of a soluble VEGFR-3 that functions as a VEGF-C/D trap, blocks VEGF-C/D function, and results in reduced tumor lymphangiogenesis in NCAM-deficient Rip1Tag2 mice that exhibit tumors in which VEGF-C and VEGF-D are up-regulated (Crnic et al., 2004). In other mouse tumor models, human breast cancer cells that ectopically express VEGF-C, induce lymphatic vessel growth in and around orthotopic tumors and tumor lymphangiogenesis promotes lymph node metastasis (Karpanen et al., 2001; Skobe et al., 2001). Tumor metastasis to lymph nodes could, however, be inhibited when VEGF-C interaction with VEGFR-3 is blocked with soluble VEGFR-3 fusion proteins (Karpanen et al., 2001). Tumor lymphangiogenesis and metastasis to regional lymph nodes are also inhibited with soluble VEGFR-3 in other cancer models (He et al., 2002; Krishnan et al., 2003).

Lymphatic vessels situated at the tumor margin are probably more important in tumor cell metastasis than intratumoral lymphatic vessels that frequently collapse due to high intratumoral pressure (Padera et al., 2004). Tumor cells secreting VEGF-C or VEGF-D stimulate the sprouting of lymphatic vessel at the tumor margin, which facilitates metastasis (He et al., 2005). Lymphatic endothelial cells send filopodia towards tumor cells, allowing easier entry of the tumor cells into the vessel lumen. Draining lymphatic vessels also dilate as a result of endothelial proliferation, allowing easier transit for the tumor cells inside the vessels (He et al., 2005). This sprouting and vessel dilation is inhibited with soluble VEGFR-3 (He et al., 2005). Interestingly, VEGF-C also promotes tumor metastasis by inducing expansion of lymph node lymphatic vessels before the onset of metastasis in tumor-bearing mice (Hirakawa et al., 2007).
In addition to VEGF-C, expression of VEGF-D in tumor cells induces tumor growth, angiogenesis and lymphangiogenesis as well as tumor metastasis via lymphatic vessels in a mouse tumor model. The metastatic spread of tumor cells via lymphatic vessels, tumor angiogenesis and lymphangiogenesis is, however, inhibited with VEGF-D specific antibodies (Achen et al., 2000; Stacker et al., 2001). Yet another way to inhibit VEGFR-3 signaling pathway in cancer includes blocking antibodies against VEGFR-3. Neutralizing antibodies against mouse VEGFR-3 induce micro-hemorrhages and suppress tumor growth by inhibiting angiogenesis in a mouse tumor model (Kubo et al., 2000). Additionally, neutralizing antibodies against human VEGFR-3 decrease tumor angiogenesis and lymphangiogenesis without significantly effecting pre-existing blood vessels or lymphatics and suppress regional and distant metastasis (Persaud et al., 2004; Pytowski et al., 2005; Roberts et al., 2006; Laakkonen et al., 2007).

### Inflammation

Lymphatic vessels actively regulate inflammatory responses by transporting leukocytes from the site of inflammation to the secondary lymphoid organs. Chemokine receptor CCR7 expressed in dermal dendritic cells is required for dendritic cell entry into afferent lymphatic vessels expressing the CCR7 ligand, chemokine CCL21/SLC (Ohl et al., 2004). Other molecules that play important roles in controlling inflammatory cell traffic across the lymphatic endothelium include common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1) mediating leukocyte transmigration through both blood vascular and lymphatic endothelia and the mannose receptor expressed in lymph node sinuses and bound by L-selectin on lymphocytes (Irjala et al., 2001; Salmi et al., 2004).

Several model systems suggest that lymphangiogenesis is stimulated by lymphangiogenic signals from inflammatory cells, especially macrophages, in inflamed tissue. Tumor-associated macrophages express VEGF-C and VEGF-D; and VEGF-A stimulates lymphangiogenesis by recruiting VEGF-C and VEGF-D expressing macrophages in inflamed mouse cornea (Skobe et al., 2001b; Schoppmann et al., 2002; Cursiefen et al., 2004). Dendritic cells also express VEGF-C and VEGFR-3 in inflamed mouse cornea (Hamrah et al., 2003).

Lymphatic vessels proliferate in response to inflammation. Massive lymphangiogenesis associated with inflammatory infiltrates was detected in human kidney transplants undergoing rejection (Kerjaschki et al., 2004). Lymphatic hyperplasia was also detected in psoriatic lesions (Kunstfeld et al., 2004). *Mycoplasma pulmonis* infection of mouse airway epithelium stimulates massive lymphangiogenesis that is induced by inflammatory cells expressing VEGF-C and VEGF-D and can be blocked with a VEGF-C/D trap (Baluk et al., 2005). Ocular administration of a VEGF-C/D trap also blocks trafficking of dendritic cells to lymph nodes, induction of delayed-type hypersensitivity and graft rejection in mouse corneal transplants (Chen et al., 2004). Migration of dendritic cells from the periphery into the lymph nodes is enhanced in response to B-lymphocyte-dependent expansion of the lymphatic vessel network within lymph nodes, suggesting a more extensive role for lymph node lymphatic vessels (Angeli et al., 2006). Interestingly, a macrophage contribution to lymphangiogenesis was recently also suggested to be via transdifferentiation of macrophages into lymphatic endothelial cells and subsequent incorporation into lymphatic vessels (Kerjaschki, 2005; Maruyama et al., 2005; Kerjaschki et al., 2006). In general, inflammatory cells can both induce lymphangiogenesis and respond to signals.
AIMS OF THE STUDY

The aim of this study was to elucidate the role of VEGF-C/VEGFR-3 and PDGF-B/PDGFR-β pathways in lymphatic development.

The specific aims of the study were:

I  To investigate the role of VEGF-C in embryonic lymphangiogenesis
II To study the role of VEGFR-3 in embryonic angiogenesis and lymphangiogenesis
III To characterize the lymphatic vascular effects of PDGF-B
MATERIALS AND METHODS

The materials and methods used in these studies are summarized here. The original publications are referred to by Roman numerals.

Analysis of lymphatic vessel function by lymphangiography (I)

Lymphatic vessel function in Vegfc<sup>+/−</sup> versus wildtype mice was tested using lymphangiography. The function of small lymphatic vessels in the skin was analyzed by injecting high-molecular-weight fluorescent dextran intradermally into the mouse ears. The progressive staining of the lymphatic vessel network due to uptake of the dye (or lack of it) from the interstitial space was then followed by fluorescence microscopy. Analysis of the larger deep lymphatic vessel was performed by injecting Evans blue dye intradermally into the hind footpads of the mice. Subsequently, regions of the ischiatic vein in the limb were exposed by removal of the skin and the appearance of the dye was detected in the deep collecting lymphatic vessels running alongside ischiatic vein and further in the para-aortic lymph nodes.

Organ culture and agarose bead experiments (I)

The formation of lymph sacs in Vegfc knockout mice was analyzed using isolated whole-mount explants of the axial vascular system from E10.5-E13 embryos. To prepare the whole-mount explants, all dorsal structures with endodermal and intermediate mesodermal derivatives were microdissected from the embryos (Sainio, 2003). The explants were cultures on Track-tech nucleopore filters in a Trowell-type organ culture system (Sainio, 2003) at 37°C 1-2 hours to attach the explants onto the filters and used for whole-mount immunostaining.

Agarose bead experiments were used to study if Prox1-positive lymphatic endothelial cells could be rescued in Vegfc knockout embryos by VEGF-C protein. These experiments were performed by placing agaraseose beads that had been incubated with the proteins at 38°C for 45 min on whole-mount explants close to the jugular veins. Subsequently, explants were cultured for 48 hours and used for whole-mount immunostaining.

Transgenic and knockout mice (I, II, III)

The transgenic and knockout mice used in these studies were:

Conditional Vegfr3<sup>cre</sup> knockout mice (II), Vegfc (Karkkainen et al., 2004), Vegfd (Baldwin et al., 2005), Vegfr3 (Dumont et al., 1998), and Pdgfb knockout mice (Leveen et al., 1994), K14-PDGF-D (Uutela et al., 2004), K14-humanVEGF-D (Veikkola et al., 2001), K14-mouseVEGF-D (II), β-actinFLPe (Rodríguez et al., 2000), K19Cre (Means et al., 2005), PGKCre (Lallemand et al., 1998), and ROSA26Cre transgenic mice (Soriano, 1999).

All animal experiments were approved by the Committee for Animal Experiments of the District of Southern Finland.
β-galactosidase staining (I, II, III)

Analysis of endogenous Vegfc or Vegfr3 expression was performed using whole-mount staining for β-galactosidase activity in Vegfc<sup>LacZ</sup> and Vegfr3<sup>LacZ</sup> mice. Whole embryos or embryonic or adult tissues were dissected and fixed in 0.2% glutaraldehyde for 30 min, rinsed and stained for β-galactosidase activity using X-Gal substrate at 37°C overnight. After the staining, the samples were rinsed and fixed in 4% PFA. Analysis of the samples was performed whole-mount or they were dehydrated through ethanol series, embedded in paraffin and cut into 6 µm sections that were used for immunostaining.

Immunostaining (I, II, III)

For immunostaining, embryos or adult tissues were fixed in 4% PFA. Whole-mount analysis was performed by immunoperoxidase staining using Vectastain ABC Kit or by immunofluorescence staining. For analysis on sections, tissues we dehydrated through an ethanol series, embedded in paraffin and cut into 6 µm sections. Sections were used for immunofluorescence staining or immunoperoxidase staining using Tyramide Signal Amplification kit. Peroxidase activity was detected with 3-amino-9-ethyl carbazole or 3,3′-diaminobenzidine.

Antibodies used for immunostaining were goat anti-mouse VEGFR-3 (R&D Systems), rat anti-mouse VEGFR-3 (Kubo et al., 2000), rabbit anti-humanProx1 (Karkkainen et al., 2004), rabbit anti-mouse LYVE-1 (Petrova et al., 2004), rat anti-mouse PECAM-1 (PharMingen), Cy3-conjugated mouse monoclonal anti-SMA (Sigma), rat anti-mouse TER119 (PharMingen), rat anti-mouse PDGFR-β (eBioscience), and goat anti-mouse P-selectin (R&D Systems). Secondary antibodies used in immunofluorescence staining were conjugated with fluorescent labels and secondary antibodies used in immunoperoxidase staining were conjugated with biotin.

Adenoviral experiments (III)

1-5x10<sup>8</sup> pfu of adenoviruses encoding β-galactosidase (LacZ) (Enholm et al., 2001), VEGF-C (Enholm et al., 2001) or PDGF-B (Tammela et al., 2007b) were injected intradermally into nu/nu mouse ears. Two, four or fifteen days after adenoviral gene transfer the mice were anesthetized with 10mg/kg xylazine and 50mg/kg ketamine and perfusion-fixed with 1% PFA through the left ventricle. The ears were fixed in 4% PFA and used for immunostaining.

AdPDGF-B viruses were tested in vitro by infecting 293T cells with the viruses (multiplicity of infection 20) for 2 hours in serum-free high glucose DMEM medium. Following over night incubation, the cells were metabolically labeled with S35-methionine and cysteine for 6h and the media was subjected to a binding assay using soluble PDGFR-β-Ig fusion proteins. The bound proteins were precipitated with protein A Sepharose, separated in 12.5% SDS-PAGE and analyzed by autoradiography.
RESULTS AND DISCUSSION

The results of these studies are summarized and discussed here. The original publications are referred to by Roman numerals.

VEGF-C in embryonic lymphangiogenesis (I)

VEGF-C is the major lymphangiogenic growth factor and it signals through VEGFR-2 and VEGFR-3. In vitro, VEGF-C induces migration of capillary endothelial cells (Joukov et al., 1996). In vivo, overexpression of VEGF-C or the VEGFR-3 specific mutant form of VEGF-C (VEGF-C156S) induces formation of hyperplastic dermal lymphatic network, suggesting that VEGFR-3 signaling is sufficient for lymphangiogenesis in adults (Jeltsch et al., 1997; Veikkola et al., 2001). However, contribution of VEGF-C to embryonic lymphangiogenesis has remained unknown, partly due to early lethality of the Vegfr3 deficient mice that die prior to formation of lymphatic vasculature due to failure in remodeling of the blood vasculature (Dumont et al., 1998). Therefore, we studied the function of VEGF-C in embryonic lymphangiogenesis by generating and analyzing Vegfc deficient mice, in which Vegfc was replaced by LacZ marker gene (Karkkainen et al., 2004).

Vegfc deficient embryos displayed edema from E12.5 onward, suggesting a lymphatic vascular defect. Accordingly, endogenous Vegfc expression pattern analysis using LacZ as marker gene revealed that VEGF-C was expressed from E8.5 onward in the jugular area in mesenchyme dorsolateral to the VEGFR-3 positive cardinal veins, which give rise to lymph sacs, suggesting that VEGF-C is needed for lymphatic development and signals in a paracrine manner. Previously, in situ hybridization analysis in mouse embryos similarly showed that Vegfc is expressed from E8.5 onward at sites of lymph sac formation in mesenchymal cells adjacent to VEGFR-3 positive endothelial cells, suggesting paracrine ligand-receptor signaling mechanism (Kukk et al., 1996). Indeed, Vegfc deficient mice completely lacked lymphatic endothelium and died around E16.5 due to fluid accumulation in tissues, suggesting that paracrine VEGF-C signaling is absolutely required for formation of the lymph sacs from cardinal veins and indicating the critical function of VEGF-C in the initial steps of lymphangiogenesis in mice. Recently, lymphatic vessels have also been described in frog and zebrafish (Ny et al., 2005; Küchler et al., 2006; Yaniv et al., 2006). Similarly as in mice, knockdown of Vegfc in frogs and zebrafish induced lymphatic vascular defects and edema suggesting that this pathway is conserved in vertebrate evolution (Küchler et al., 2006; Yaniv et al., 2006).

In mice lymphatic vessel formation starts at E10.5 when a subset of blood endothelial cells in the cardinal vein differentiate into lymphatic endothelial cells that express homeobox transcription factor Prox1 and sprout to form the lymph sacs, which are clearly present at E13.5. Prox1 is absolutely required for the formation of lymph sacs as mice deficient in Prox1 lack these structures (Wigle and Oliver, 1999). We observed that in Vegfc deficient mice endothelial cells commit to a lymphatic lineage expressing Prox1, but fail to sprout and form lymphatic vessels, suggesting that VEGF-C is required for migration and survival of lymphatic endothelial cells. VEGFR-3 specific migration of lymphatic endothelial cells toward VEGF-C gradient was indicated by a rescue experiment as migration was rescued by VEGF-C and VEGF-D, but not by VEGF. Our data support the theory that lymphatic vessels arise from veins, a theory proposed already a century ago (Sabin, 1902). An alternative hypothesis suggests that lymph sacs are formed from mesenchymal precursor cells
independently of veins (Huntington and McClure, 1908). Recent lineage-tracing experiments in mice and zebrafish also support the theory of venous origin for the lymphatic vessels and no contribution of mesenchymal precursor cells in formation of lymphatic vessels was found in these models (Yaniv et al., 2006; Srinivasan et al., 2007). However, circulating endothelial progenitor cells has been suggested to contribute to separation of lymphatic and venous endothelium in mouse embryos (Sebzda et al., 2006). Interestingly, in frog and avian models, the mesoderm-derived precursor cells called lymphangioblasts have been shown to contribute to lymphangiogenesis (Schneider et al., 1999; Ny et al., 2005; Wilting et al., 2006).

VEGF-C signaling through VEGFR-3 is crucial in regulation of lymphangiogenesis, but VEGF-C also induces angiogenesis by signaling through VEGFR-2. Physiological and pathological angiogenesis was stimulated by VEGF-C in mouse cornea and in limb ischemia models, respectively (Cao et al., 1998; Witzenbichler et al., 1998). The role of VEGF-C in developmental vasculogenesis and angiogenesis has been shown in zebrafish and in frogs, in which knockdown of Vegfc induced aberrant blood vessel formation (Ober et al., 2004; Ny et al., 2005). Furthermore, adenoviral delivery of VEGF-C in mouse skin induces blood vessel leakiness in addition to lymphangiogenesis (Enholm et al., 2001; Saaristo et al., 2002a). Although mice deficient in VEGF-C receptors, VEGFR-2 and VEGFR-3, show severe blood vascular defects (Shalaby et al., 1995; Dumont et al., 1998), we observed no blood vascular defects in Vegfc deficient embryos, suggesting that VEGF-D or some yet unidentified ligand for VEGFR-3 is able to compensate for the loss of Vegfc during embryonic angiogenesis. VEGF-D, however, failed to compensate for VEGF-C in embryonic lymphangiogenesis, probably due to different expression pattern in embryos and lack of expression at the critical sites of lymphatic development, thus failing in paracrine signaling through VEGFR-3 in areas of lymph sac formation (Avantaggiato et al., 1998). VEGF-C also binds to non-tyrosine kinase co-receptor Neuropilin-2 and mice deficient in Nrp2 show lack or reduction of small lymphatic vessels (Karkkainen et al., 2001; Yuan et al., 2002). Neuropilin-2 was suggested to modulate VEGFR-3 signaling as Neuropilin-2 is internalized with VEGFR-3 in vitro (Kärpänen et al., 2006). Neuropilin-2 can possibly enhance VEGFR-3 signaling, similarly to what has been shown to Neuropilin-1 enhanced signaling through VEGFR-2 (Soker et al., 1998). Therefore, phenotype of Vegfc deficient mice might result from lack of both VEGFR-3 and Neuropilin-2 signals in lymphatic endothelial cells.

Analysis of mice lacking one Vegfc allele showed that haplo-insufficiency of VEGF-C results in hypoplasia of the lymphatic vessels, suggesting that a normal concentration of VEGF-C is required for normal formation of lymphatic vessels. Vegfc<sup>−/−</sup> pups showed chylous ascites after birth indicating defective intestinal lymphatic vasculature. Analysis revealed that Vegfc<sup>+/−</sup> pups displayed lymphatic hypoplasia in various other organs as well. However, lymphatic vessels re-grew within a few post-natal weeks in the intestine and all other organs except in the skin where lymphatic hypoplasia persisted, suggesting heterogeneity between different vascular beds. Interestingly, adenoviral or transgenic expression of a soluble VEGF-R3 competing for VEGF-C and VEGF-D binding with the endogenous receptor induces lymphatic vessel regression in late-gestation embryos and during the two first postnatal weeks but not during later development, showing that lymphatic vessels are plastic during the early postnatal period but not thereafter (Makinen et al., 2001a; Karpanen et al., 2006).

Lymphatic vessel function was tested with lymphangiography by injecting high-molecular-weight fluorescent dextran into the ears of the mice or by injection of Evans blue dye into the hind limbs. In wildtype mice the dye was collected by the lymphatic vessels, whereas in Vegfc<sup>+/−</sup> mice the dye persisted at site of injection and was not taken up by the lymphatic
vessels. Consistently, adult Vegfc+/- mice displayed lymphedema due to compromised lymphatic vessel function. Heterozygous missense mutations in the Vegfr3 gene inactivating the tyrosine kinase in the encoded protein have been linked to lymphedema in mice and in humans (Karkkainen et al., 2000; Karkkainen et al., 2001). Chy lymphedema mice carry an inactivating Vegfr3 mutation in their germ line and show hypoplasia of dermal lymphatic vessels and subsequent swelling of the limbs (Karkkainen et al., 2001). Chy-3 mice that carry a large chromosomal deletion that includes Vegfc also exhibit dermal hypoplasia and lymphedema (Dellinger et al., 2007). Vegfc heterozygous mice also serve as a preclinical lymphedema animal model and they should be useful for testing of therapeutic agents to regenerate hypoplastic lymphatic vessels.

In conclusion, our results show that VEGF-C is absolutely required for migration and survival of lymphatic endothelial cells and the subsequent formation of lymph sacs from embryonic veins and that both Vegfc alleles are required for normal lymphatic development.

**VEGFR-3 in lymphatic and blood vascular development during embryogenesis (II)**

VEGFR-3 is expressed in blood vessel endothelium and required for blood vascular development during embryogenesis, as Vegfr3 knockout mice die at mid-gestation due to failure in remodeling of the blood vessels (Dumont et al., 1998). After the emergence of lymphatic vessels, VEGFR-3 becomes restricted to the lymphatic endothelium in late-gestation embryos and in adults (Kaipainen et al., 1995). However, VEGFR-3 is upregulated in microvasculature in wounds and tumors (Valtola et al., 1999; Paavonen et al., 2000). Interestingly, while Vegfd knockout mice display only a minor lymphatic phenotype involving a decrease in the number of lymphatic vessels in the lungs, Vegfc knockout mice lack lymphatic vessels completely (Karkkainen et al., 2004; Baldwin et al., 2005). We wanted to clarify the molecular mechanisms underlying the role of the VEGFR-3 signaling in embryonic angiogenesis and lymphangiogenesis in more detail.

We crossed Vegfc knockout mice with Vegfd knockout mice in order to study the combination phenotype of mice deficient in both VEGFR-3 ligands and the role of VEGFR-3 signaling pathway in embryonic angiogenesis. Analysis of blood vessels in Vegfc;Vegfd double knockout embryos at E11.5 revealed a normal blood vasculature. At this time-point Vegfr3 knockout embryos are already dead (Dumont et al., 1998) and thus, surprisingly, the double knockout phenotype did not reproduce the Vegfr3 knockout phenotype. Analysis of the lymphatic vessels in the double knockout mice showed failure in lymph sac formation in these embryos, resembling the Vegfc null phenotype. These data suggest that VEGF-C and VEGF-D are dispensable for embryonic angiogenesis and raise the possibility that another yet unidentified ligand for VEGFR-3 might be required for embryonic angiogenesis. Alternatively, VEGFR-3 may be able to signal in a ligand-independent manner, possibly by forming heterodimers with VEGFR-2 (Dixelius et al., 2003; Alam et al., 2004). In zebrafish the Vegfr3 homolog Flt4 was shown to act in cooperation with Vegfr2 homolog Kdr in regulation of artery morphogenesis (Covassin et al., 2006) and overexpression of VEGF-D induced severe misguidance of intersegmental blood vessels and abnormal connection between dorsal aorta and caudal vein (Song et al., 2007). Interestingly, VEGFR-3 is highly expressed in endothelial tip cells of angiogenic sprouts, where it positively regulates angiogenic sprouting and is induced by inhibition of Notch signals (Tammela et al., 2008).
Analysis of the gene targeted mice with a neomycin cassette inserted between the first two Vegfr3 exons revealed that neomycin insertion produced a hypomorphic allele. Analysis of mice carrying the hypomorphic Vegfr3\textsuperscript{neo} allele showed that lymphatic vessel development and function is compromised in these mice. The Vegfr3\textsuperscript{2neo/neo} mice appeared swollen during embryogenesis, lacked cutaneous lymphatic vessels and died perinatally. Vegfr3\textsuperscript{+/-neo} mice survived to adulthood but showed transient accumulation of chylous ascites after birth similarly to what has been observed in many lymphedema mouse models including Vegfc\textsuperscript{+/-} mice, Chy-3 mice that carry a large chromosomal deletion including Vegfc gene, and Chy mice that carry inactivating missense mutation in Vegfr3 gene (Karkkainen et al., 2001; Karkkainen et al., 2004; Dellinger et al., 2007). These data suggest that a tight regulation of VEGFR-3 signaling level is a prerequisite for normal lymphatic development during embryogenesis. Blood vessels, however, developed normally in Vegfr3\textsuperscript{neo/neo} and Vegfr3\textsuperscript{+/-neo} mice, suggesting that higher levels of VEGFR-3 signaling are required to sustain embryonic lymphangiogenesis than angiogenesis. Interestingly, the Vegfr3\textsuperscript{2neo} allele causes more severe perturbation of lymphatic development than the Vegfr3\textsuperscript{lacZ} allele, as Vegfr3\textsuperscript{+/-lacZ} mice appear essentially normal (Dumont et al., 1998), possibly due to variations in background strains.

In order to study whether defective placental morphogenesis contributes to the Vegfr3 null phenotype, we used the conditional Vegfr3 knockout mice to specifically delete Vegfr3 gene in the epiblast. Vegfr3 was deleted in the epiblast by crossing the conditional Vegfr3 knockout mice with keratin 19 (K19) Cre mice that specifically drives Cre expression in early postimplantation embryos in cells giving rise to all embryonic tissues while the extraembryonic tissues are not targeted (Harada et al., 1999; Means et al., 2005). To remove the Neo cassette, the Vegfr3\textsuperscript{+/-neo} mice were first crossed with mice expressing FLPe recombinase under the \(\beta\)-actin promoter (Rodríguez et al., 2000). The resulting Vegfr3\textsuperscript{+/-} and Vegfr3\textsuperscript{lacZ} mice showed an apparently normal phenotype. To test the functionality of the conditional allele, the Vegfr3\textsuperscript{lacrZ} mice were next crossed with PGKCre mice (Lallemand et al., 1998) for uniform deletion of Vegfr3. Germ-line deletion of Vegfr3 resulted in Vegfr3 null phenotype and no VEGFR-3 protein expression was detected in the Cre-positive embryos, suggesting that the conditional allele is functional. Epiblast-restricted ablation of Vegfr3 resulted in a phenotype identical to Vegfr3 null embryos, indicating that the phenotype is due to defects in the embryo proper and not in the placenta. Some of K19Cre;Vegfr3\textsuperscript{2lacZ} embryos and mice displayed no apparent phenotype and showed only partial recombination, whereas all embryos displaying the null phenotype showed 100% recombination. Similarly, analysis of Cre activity using ROSA26 reporter mice (Soriano, 1999) showed mosaic pattern, with some embryos showing lower levels of recombination, as previously reported (Means et al., 2005).

Lymphatic vessel growth can be stimulated in normal mouse skin using VEGF-C or VEGF-C156S gene therapy (Enholm et al., 2001; Saaristo et al., 2002b). Lymphatic vessel growth can be stimulated and lymphatic vessel hypoplasia rescued in also in Chy lymphedema mice by gene therapy or crossing these mice with K14-VEGF-C156S mice, that display severe hyperplasia of the dermal lymphatic vasculature (Karkkainen et al., 2001). Although our data suggest that VEGF-D is dispensable for lymphatic and blood vascular development during embryogenesis, analysis of the Vegfc\textsuperscript{+/-};K14-VEGF-D mice showed that VEGF-D is able to rescue the hypoplastic lymphatic phenotype of the Vegfc\textsuperscript{+/-} mice and compensate for the loss of VEGF-C when expressed at sufficient in basal skin keratinocytes under the K14-promoter, implying that VEGF-D is a potent lymphangiogenic factor in pathological situations. We analyzed lymphatic vessels in the skin of K14-humanVEGF-D and K14-mouseVEGF-D and
observed that while both transgenic mouse models showed severe hyperplasia of the cutaneous lymphatic vessels, K14-humanVEGF-D mice showed an even more severe phenotype than the K14-mouseVEGF-D mice. This difference may be explained by the fact that mouse VEGF-D is a specific ligand for VEGFR-3, while human VEGF-D signals through both VEGFR-2 and VEGFR-3 (Baldwin et al., 2001a). Furthermore, VEGFR-2 may contribute to lymphangiogenic signaling or lymphangiogenic signals could be transmitted through VEGFR-2/VEGFR-3 heterodimers (Dixelius et al., 2003; Alam et al., 2004; Goldman et al., 2007; Wirzenius et al., 2007).

In summary, we showed that sufficient VEGFR-3 signaling in the embryo proper is required for blood vessel formation during embryogenesis. We also showed that VEGFR-3 signaling is required in a dose-sensitive manner for embryonic lymphangiogenesis. Furthermore, our data suggest that VEGF-D might have evolved to induce pathological rather than developmental lymphangiogenesis. Importantly, VEGF-D does not compensate for the loss of VEGF-C in embryonic angiogenesis or lymphangiogenesis suggesting possible ligand-independent signaling mechanisms or the existence of additional ligands for VEGFR-3.

**Lymphatic vascular effects of PDGF-B (III)**

PDGF-B regulates angiogenesis via PDGFR-β by recruiting pericytes and smooth muscle cells around nascent endothelial tubes. Previous results suggest that PDGF-B binding to PDGFR-β expressed on lymphatic endothelial cells can stimulate lymphangiogenesis in a mouse cornea model and in tumors, promoting lymphatic metastasis independent of VEGF-C/VEGFR-3 signaling (Cao et al., 2004; Vincent and Rafii, 2004). These studies imply the potential of PDGF-B to induce lymphangiogenesis in adult and tumor tissues, but the role of PDGF-B in lymphatic vessel development and maturation during embryogenesis has not been investigated. We studied lymphatic vascular effects of PDGF-B during embryonic lymphangiogenesis using the Pdgfb knockout mice and also tested the lymphatic potential of PDGF-B in adult mice.

To study the lymphatic vascular effects of PDGF-B in adults, we used K14-PDGF-D transgenic mouse model to mimic the effects of PDGF-B, as PDGF-D similarly to PDGF-B binds to PDGFR-β. In these mice PDGF-D is overexpressed in basal skin keratinocytes under the K14-promoter (Uutela et al., 2004). Analysis of dermal lymphatic vessels by whole-mount staining for LYVE-1 did not reveal an increase in the number of lymphatic vessels in these mice suggesting that PDGF-D or PDGF-B does not directly induce lymphangiogenesis in normal mouse skin. Previous analysis of K14-PDGF-D mice revealed increased interstitial fluid pressure and macrophage recruitment in normal skin and during wound healing, but the number of lymphatic vessels in the punch biopsy wounds was not increased either (Uutela et al., 2004). In order to further determine whether PDGF-B overexpression could stimulate lymphangiogenesis in normal skin, we transduced mouse ears with adenoviral vectors encoding human PDGF-B. Analysis of lymphatic vessels in mouse ears transduced with adenoviral PDGF-B did not show PDGF-B-induced lymphatic vessel sprouting. Furthermore, in contrast to previous results by Cao et al. (Cao et al., 2004) we did not detect PDGFR-β expression in lymphatic vessels but predominantly in the vascular pericytes/SMCs. Although we did not detect any direct effects of PDGF-B on the lymphatic endothelium, previous results show that adenoviral PDGF-B expression leads to dissociation of smooth muscle cells from the collecting lymphatic vessels, which may render the vessels more permissive for the
entry of metastatic tumor cells (Tammela et al., 2007b). Furthermore, in tumors PDGF-B has been shown to regulate stromal cells, pericyte abundance and recruitment as well as angiogenesis (Abramsson et al., 2003; Furuhashi et al., 2004; Lederle et al., 2006; McCarty et al., 2007). Our results suggest, however, that PDGF-B fails to directly stimulate lymphangiogenesis in adults.

Pdgb and Pdgfrβ deficient mice lack microvascular pericytes leading to endothelial hyperplasia and show subsequent lethal microaneurysms (Leveen et al., 1994; Soriano, 1994; Lindahl et al., 1997a; Hellstrom et al., 2001). Furthermore, endothelium-specific Pdgb knockout mice display pericyte deficiency (Enge et al., 2002; Bjarnegard et al., 2004). Moreover, retention of PDGF-B on endothelial cell surface is crucial in pericyte recruitment as knockout of the heparan sulfate proteoglycan retention motif in PDGF-B or defective N-sulfation of heparan sulfate proteoglycans leads to deficient pericyte investment of the microvessels (Lindblom et al., 2003; Abramsson et al., 2007). Previous studies have described blood vascular defects in these mice but left open the question whether PDGF-B is required for embryonic lymphangiogenesis. Our analysis of Pdgb gene-targeted mouse embryos revealed no lymphatic hypoplasia in disagreement with previous speculations (Vincent and Rafii, 2004). Instead, we observed that the cutaneous lymphatic capillaries in late-stage Pdgb deficient embryos were dilated, tortuous and filled with blood. In order to visualize lymphatic vessels in Pdgb deficient embryos using a genetic β-galactosidase marker, we crossed Pdgb deficient mice with Vegfr3<sup>−/−</sup> mice (Dumont et al., 1998). Interestingly, lymphatic vessel morphology in Pdgb<sup>−/−</sup>;Vegfr3<sup>−/−</sup> double heterozygous embryos showed lymphatic vascular defects reminiscent of Pdgb<sup>−/−</sup> embryos, suggesting that these two pathways interact in regulation of lymphatic vessel growth. Similar defects have been reported in Foxc2<sup>−/−</sup>;Vegfr3<sup>−/−</sup> compound heterozygous embryos (Petrova et al., 2004) implying a more complicated interaction of different pathways in regulation of lymphangiogenesis. Lymphatic capillaries in Pdgb knockout embryos also showed an abnormal investment of smooth muscle cells similarly to observations from Foxc2 deficient embryos (Petrova et al., 2004). Pdgb expression was reported to be upregulated in Foxc2 deficient embryos (Petrova et al., 2004), but abnormal investment of SMCs to the lymphatic capillaries in Pdgb knockout embryos may also be due to general disturbed migration of SMCs that are detaching from blood vessels in these mice. Generally, lymphatic vessel abnormalities observed in Pdgb deficient embryos resemble the lymphatic vessel patterning, remodeling and maturation defects observed in mice deficient for Podoplanin, Ang2, Foxc2, or PDZ domain in ephrinB2 (Gale et al., 2002; Schacht et al., 2003; Petrova et al., 2004; Makinen et al., 2005). Ang2 deficient mice and mice lacking the PDZ domain in ephrinB2 show a postnatal lymphangiogenic remodeling defect (Gale et al., 2002; Makinen et al., 2005). Moreover, ectopic SMCs on lymphatic vessels were detected in Pdgfrβ deficient mice and in mural cell specific ephrinB2 knockout mice (Foo et al., 2006). Additionally, Podoplanin and Foxc2 gene targeted mice show defects in lymphatic vessel patterning (Schacht et al., 2003; Petrova et al., 2004). Our data suggest that PDGF-B is yet another regulator of lymphatic vessel remodeling, patterning and maturation.

Pdgb deficient embryos showed blood inside the lymph sacs at E14.5, suggesting that lymphatic vessel separation from blood endothelium fails in these mice. Previously, mice lacking hematopoietic signaling proteins Slp-76 or Syk were reported to display a lymphatic separation defect (Abtahian et al., 2003; Sebzda et al., 2006). Slp-76 and Syk gene targeted mice display direct communications between blood and lymphatic vessels suggesting that a hematopoietic signaling pathway is required in the lymphatic vessels to separate from blood vessels. Fasting-induced adipose factor (Fiaf or Angptl4) has also been shown to be required
in postnatal intestinal lymphatic vessel separation from the blood vessels (Backhed et al., 2007). The mechanism of lymphatic vessel separation from blood vessels is, however, still not clear.

P-selectin is an adhesion receptor for leukocytes which is expressed in platelets and endothelial cells upon stimulation, suggesting an important role in inflammation and coagulation (Polgar et al., 2005). Similarly to leukocytes, platelets roll on activated endothelium. This rolling process is supported by von Willenbrand factor or P-selectin (André et al., 2000; Frenette et al., 1995). P-selectin ligand, PSGL-1 (P-selectin glycoprotein ligand 1), is expressed on platelets and it can mediate P-selectin-dependent platelet rolling (Frenette et al., 2000). Additionally, the VWF receptor, glycoprotein (GP) Ibδ, is a counter-receptor for P-selectin and promotes platelet rolling on P-selectin (Romo et al., 1999). P-selectin deficient mice have a 40% prolongation in bleeding time and the contribution of P-selectin to thrombus formation has been shown in many studies (Polgar et al., 2005). We observed downregulation of P-selectin at the site of lymphatic and blood vessel separation in Pdgfb knockout embryos suggesting that impaired local platelet function and resulting defective blood clotting at the critical site of lymphatic vessel separation from the blood endothelium may be required for the separation to occur, and that this clot formation fails in Pdgfb knockout embryos.

In conclusion, our analysis of lymphatic potential of PDGF-B using loss-of-function model in embryos showed abnormal lymphatic vessels that failed to separate from blood vessels, whereas gain-of-function models in adults showed no stimulation of lymphangiogenesis. Our results imply that PDGF-B fails to directly induce lymphangiogenesis in adults, but suggest a role for PDGF-B in regulation of lymphatic vessel patterning, maturation and lymphatic-venous separation in embryos. Our study implies that reassessment of the lymphangiogenic potential of PDGF-B is required.
CONCLUDING REMARKS

Although the lymphatic vasculature has been described as long as a century ago, knowledge of the molecular mechanisms that control lymphangiogenesis has been lacking. Since the discovery of the regulators of lymphatic endothelium, our understanding of lymphatic vessel growth and development has greatly advanced at the molecular level. Intensive research during past decade has shed light on the molecular regulation of lymphangiogenesis, but gaps in our knowledge still exist and unanswered questions remain. What is the signal that triggers lymphatic endothelial cell commitment? How do the pathways involved in lymphangiogenesis interact? What are the characteristics of lymphatic vessel heterogeneity in different vascular beds? What are the molecular mechanisms of lymphatic vessel separation and maturation?

Despite unanswered questions still remaining, studies on lymphangiogenesis have given novel insights into how lymphatic vessels affect diseases and opportunities have arisen to control lymphangiogenesis for the benefit of patients. Lymphedema patients should benefit from therapeutic stimulation of lymphatic vessel growth in the near future. A challenge remains in inhibiting lymphatic metastasis in cancer patients. Mechanisms of lymphatic metastasis need to be clarified in more detail in order to achieve a therapeutic intervention for metastasis in the future. Furthermore, lymphatic vessels play an active role in immune reactions transporting inflammatory cells in lymphoid organs and in the peripheral tissues, but a lot remains to be explored in the involvement of lymphatic vessels in inflammatory diseases.
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