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

Paula Haiko

Molecular/Cancer Biology Laboratory
Haartman Institute and Biomedicum Helsinki
University of Helsinki
Finland

Academic dissertation

To be publicly discussed, with the permission of the Faculty of Medicine, University of Helsinki, in Lecture Hall 2, Biomedicum Helsinki, Haartmaninkatu 8, Helsinki on August 8, 2008 at 12 p.m.

Helsinki 2008

Supervised by

Kari Alitalo, M.D., Ph.D.
Research Professor of the Finnish Academy of Sciences
Molecular/Cancer Biology Laboratory
Haartman Institute and Biomedicum Helsinki
University of Helsinki
Finland

Reviewed by

Hannu Sariola, M.D., Ph.D.
Professor
Institute of Biotechnology
University of Helsinki
Finland

and

Klaus Elenius, M.D., Ph.D.
Professor
MediCity Research Laboratory
University of Turku
Finland

Thesis opponent

Sirpa Jalkanen, M.D., Ph.D.
Professor
MediCity Research Laboratory
University of Turku
Finland

CONTENTS

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)
FOXC2	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
PlGF	Placenta growth factor
Prox-1	*prospero*-related homeobox protein 1
SMA	α- 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.

I   Karkkainen, M. J., **Haiko, P.**, Sainio, K., Partanen, J., Taipale, J., Petrova, T. V., Jeltsch, M., Jackson, D. G., Talikka, M., Rauvala, H., Betsholtz, C., and Alitalo, K. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nature Immunology,* 5, 74-80 (2004).

II   **Haiko, P.**, Makinen, T., Keskitalo, S., Taipale, J., Karkkainen, M. J., Baldwin, M. E., Stacker, S. A., Achen, M. G., and Alitalo, K. Deletion of VEGF-C and VEGF-D is not equivalent to VEGFR-3-null in mouse embryos. *Molecular and Cellular Biology, in press.*

III   **Haiko, P.**, Tammela, T., Uutela, M., Soronen, J., Yla-Herttuala, S., Betsholtz, C., and Alitalo, K. PDGF-B regulates embryonic lymphatic development but does not induce lymphangiogenesis in adults. *Manuscript in preparation.*

The articles are reprinted with the kind permission of their copyright holders.

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-ß 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 CO_2 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-ß (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-ß. Sprouting endothelial tip cells secrete PDGF-B that promotes recruitment of PDGFR-ß 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-ß 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).
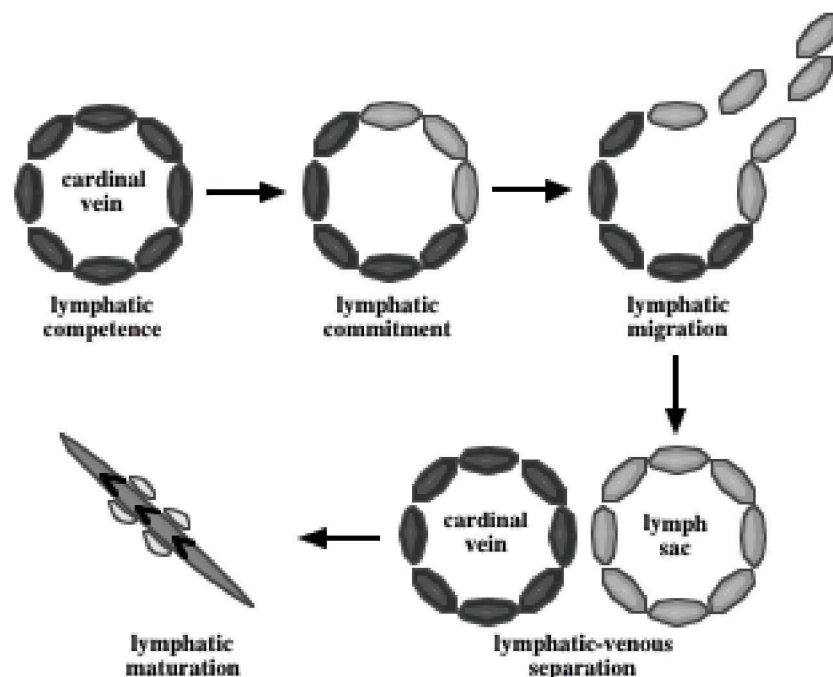

Figure 1. Steps of lymphatic vascular development.

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 ß1 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 α9ß1 regulates lymphatic development remains unclear, but *in vitro* data have shown that in the presence of specific extracellular matrix components, ß1 integrin can induce phosphorylation of VEGFR-3 and modulate cell migration, proliferation and survival (Wang et al., 2001; Zhang et al., 2005). Futhermore, VEGF-C and VEGF-D were shown to bind to integrin α9ß1 (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 lympatic 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 (PlGF), 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 PlGF 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.
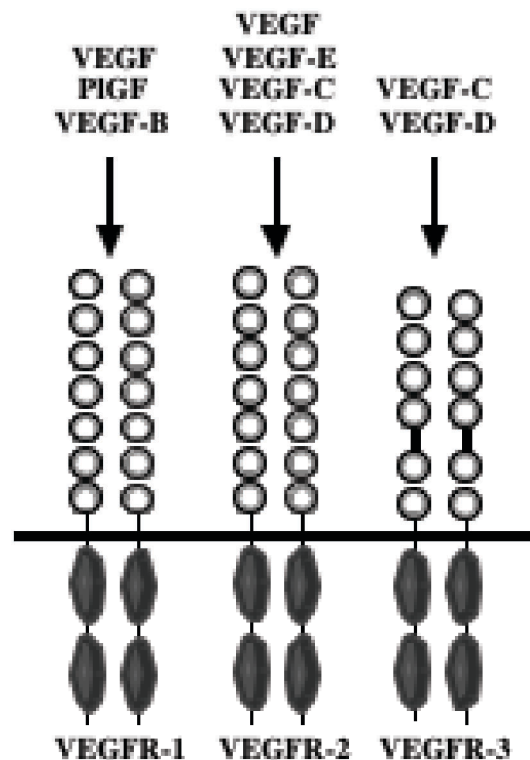

Figure 2. Receptor binding specificity of VEGFs.

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, $VEGF_{121}$, 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 $VEGF_{165}$ remains bound to the extracellular matrix due to binding to heparan sulfate proteoglycans (Houck et al., 1992). The longest isoforms, $VEGF_{189}$ and $VEGF_{206}$, 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, $VEGF-B_{167}$ and $VEGF-B_{186}$, with $VEGF-B_{167}$ 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).

PlGF

PlGF and is most abundantly expressed in the placenta and exists in four different isoforms, PlGF-1, PlGF-2, PlGF-3 and PlGF-4 (Maglione et al., 1991; Hauser and Weich, 1993; Maglione et al., 1993; Cao et al., 1997). PlGF homodimers bind to VEGFR-1 but VEGF/PlGF heterodimers can also bind to VEGFR-2 (Park et al., 1994; DiSalvo et al., 1995; Cao et al., 1996). Additionally, PlGF binds to neuropilins and heparin. PlGF 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). PlGF 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 ß-galactosidase as a marker for endogenous *Vegfc* expression in *Vegfc$^{+/LacZ}$* 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 (Partanen 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). Trangenic 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 (Baldwin 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$_{D1701}$ and VEGF-E$_{NZ2}$, showing homology to VEGF and PlGF, and VEGF-E$_{NZ7}$, 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$_{NZ7}$ in skin keratinocytes of transgenic mice induces a strong angiogenic response (Kiba et al., 2003). Also, chimeric VEGF-ENZ7/PlGF molecules promote angiogenesis in ischemic tissues and wounds (Inoue et al., 2007; Zheng et al., 2006; Zheng et al., 2007). Furthermore, VEGF-E$_{NZ7}$ 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 Ig 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 (Makinen 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 (Makinen 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 $VEGF_{165}$, $VEGF-B_{167}$, $VEGF-B_{186}$, PlGF-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 PlGF-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 (Takashima 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-ß, that appear in three PDGF receptor variants, PDGFR-$\alpha\alpha$, PDGFR-αß and PDGFR-ßß. 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-$\alpha\alpha$ while PDGF-AB, PDGF-BB and PDGF-CC bind to PDGFR-αß, and PDGF-BB and PDGF-DD bind to PDGFR-ßß (Figure 3).
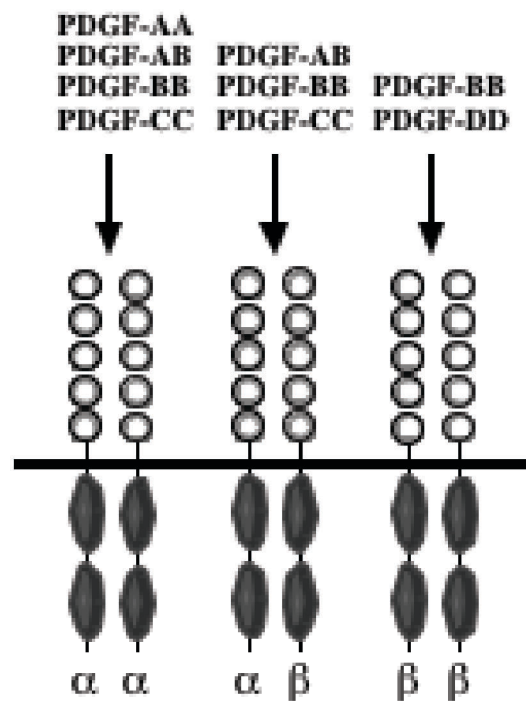

Figure 3. Receptor binding specificity of PDGFs.

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 *Pdgfrα* deficient mice display reduced number of intestinal villi (Karlsson et al., 2000).

PDGF-B/PDGFRß signaling promotes mural cell proliferation and recruitment to the vessel wall. *Pdgfb* and *Pdgfrß* 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 (Enge et al., 2002; Bjarnegard et al., 2004). Lack of pericytes induces endothelial hyperplasia in *Pdgfb* and *Pdgfrß* deficient mice suggesting that pericytes can negatively control endothelial cell proliferation (Hellstrom et al., 2001). Furthermore, activation of PDGFR-ß on the hemangioprecursor cells also accelerates differentiation of endothelial cells (Rolny et al., 2006) *Pdgfb* and *Pdgfrß* 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 PDGFR-ß 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 *Pdgfrß* suggest that PDGF-B signals mainly through PDGFR-ßß 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 PDFG-D only form homodimers (Li et al., 2000; Bergsten et al., 2001). PDGF-CC primarily binds to PDGFR-αα but can also activate PDGFR-αß, while PDGF-D is a specific ligand to PDGFR-ßß (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 *Pdgfrα* deficient mice, whereas double-deficient mice for *Pdgfa* and *Pdgfc* phenocopy the *Pdgfrα* 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-ß

PDGF receptors α and ß 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-ß, 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-ß 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 cells types (Heldin and Westermark, 1999).

Pdgfrα 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). *Pdgfrß* 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 lymphedemas can be divided into primary and secondary lymphedema. Primary lymphedemas are rather rear 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 trancription factor *SOX18* gene have been described in hypotrichosis-telangiectasia-lymphedema syndrome (Irrthum et al., 2003).

Mouse models for human lymphedema have greatly facilited 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 (Rockson, 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 adenoviral 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 ß-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 fuctions 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 $Vegfc^{+/-}$ 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 agaraose 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 $Vegfr3^{neo}$ 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.

ß-galactosidase staining (I, II, III)

Analysis of endogenous *Vegfc* or *Vegfr3* expression was performed using whole-mount staining for β-galactosidase activity in *Vegfc^{LacZ}* and *Vegfr3^{LacZ}* 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-ß (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)

1-5×10^8 pfu of adenoviruses encoding ß-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-ß-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 transcripton 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*$^{+/-}$ pups showed chylous ascites after birth indicating defective intestinal lymphatic vasculature. Analysis revealed that *Vegfc*$^{+/-}$ 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 VEGFR-3 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*$^{+/-}$ 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 hypoplastia 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 patway 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^neo* allele showed that lymphatic vessel development and function is compromised in these mice. The *Vegfr3^{neo/neo}* mice appeared swollen during embryogenesis, lacked cutaneous lymphatic vessels and died perinatally. *Vegfr3^{+/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^{+/-}* 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^{neo/neo}* and *Vegfr3^{+/neo}* mice, suggesting that higher levels of VEGFR-3 signaling are required to sustain embryonic lymphangiogenesis than angiogenesis. Interestingly, the *Vegfr3^neo* allele causes more severe perturbation of lymphatic development than the *Vegfr3^{LacZ}* allele, as *Vegfr3^{+/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^{+/Neo}* mice were first crossed with mice expressing FLPe recombinase under the β-actin promoter (Rodríguez et al., 2000). The resulting *Vegfr3^{+/lx}* and *Vegfr3^{lx/lx}* mice showed an apparantly normal phenotype. To test the functionality of the conditional allele, the *Vegfr3^{lx/lx}* 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^{lx/LacZ}* 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^{+/-};K14-VEGF-D* mice showed that VEGF-D is able to rescue the hypoplastic lymphatic phenotype of the *Vegfc^{+/-}* 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-ß 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.

Pdgfb 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). Furhermore, endothelium-specific *Pdgfb* 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 *Pdgfb* 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 *Pdgfb* deficient embryos were dilated, tortuous and filled with blood. In order to visualize lymphatic vessels in *Pdgfb* deficient embryos using a genetic ß-galactosidase marker, we crossed *Pdgfb* deficient mice with *Vegfr3*$^{+/LacZ}$ mice (Dumont et al., 1998). Interestingly, lymphatic vessel morphology in *Pdgfb*$^{+/-}$;*Vegfr3*$^{+/LacZ}$ double heterozygous embryos showed lymphatic vascular defects reminiscent of *Pdgfb*$^{-/-}$ embryos, suggesting that these two pathways interact in regulation of lymphatic vessel growth. Similar defects have been reported in *Foxc2*$^{+/-}$;*Vegfr3*$^{+/LacZ}$ compound heterozygous embryos (Petrova et al., 2004) implying a more complicated interaction of different pathways in regulation of lymphangiogenesis. Lymphatic capillaries in *Pdgfb* knockout embryos also showed an abnormal investment of smooth muscle cells similarly to observations from *Foxc2* deficient embryos (Petrova et al., 2004). *Pdgfb* expression was reported to be upregulated in *Foxc2* deficient embryos (Petrova et al., 2004), but abnormal investment of SMCs to the lymphatic capillaries in *Pdgfb* 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 *Pdgfb* 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.

Pdgfb 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) Ibα, 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 reassesment 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.

ACKNOWLEDGEMENTS

REFERENCES

Aase, K., von Euler, G., Li, X., Ponten, A., Thoren, P., Cao, R., Cao, Y., Olofsson, B., Gebre-Medhin, S., Pekny, M*., et al.* (2001). Vascular endothelial growth factor-B-deficient mice display an atrial conduction defect. Circulation *104*, 358-364.

Abramsson, A., Kurup, S., Busse, M., Yamada, S., Lindblom, P., Schallmeiner, E., Stenzel, D., Sauvaget, D., Ledin, J., Ringvall, M*., et al.* (2007). Defective N-sulfation of heparan sulfate proteoglycans limits PDGF-BB binding and pericyte recruitment in vascular development. Genes Dev *21*, 316-331.

Abramsson, A., Lindblom, P., and Betsholtz, C. (2003). Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. J Clin Invest *112*, 1142-1151.

Abtahian, F., Guerriero, A., Sebzda, E., Lu, M. M., Zhou, R., Mocsai, A., Myers, E. E., Huang, B., Jackson, D. G., Ferrari, V. A*., et al.* (2003). Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. Science *299*, 247-251.

Achen, M. G., Jeltsch, M., Kukk, E., Makinen, T., Vitali, A., Wilks, A. F., Alitalo, K., and Stacker, S. A. (1998). Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA *95*, 548-553.

Achen, M. G., McColl, B. K., and Stacker, S. A. (2005). Focus on lymphangiogenesis in tumor metastasis. Cancer Cell *7*, 121-127.

Achen, M. G., Roufail, S., Domagala, T., Catimel, B., Nice, E. C., Geleick, D. M., Murphy, R., Scott, A. M., Caesar, C., Makinen, T*., et al.* (2000). Monoclonal antibodies to vascular endothelial growth factor-D block its interactions with both VEGF receptor-2 and VEGF receptor-3. Eur J Biochem *267*, 2505-2515.

Adams, R. H., Wilkinson, G. A., Weiss, C., Diella, F., Gale, N. W., Deutsch, U., Risau, W., and Klein, R. (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev *13*, 295-306.

Alam, A., Herault, J. P., Barron, P., Favier, B., Fons, P., Delesque-Touchard, N., Senegas, I., Laboudie, P., Bonnin, J., Cassan, C*., et al.* (2004). Heterodimerization with vascular endothelial growth factor receptor-2 (VEGFR-2) is necessary for VEGFR-3 activity. Biochem Biophys Res Commun *324*, 909-915.

Alitalo, K., and Carmeliet, P. (2002). Molecular mechanisms of lymphangiogenesis in health and disease. Cancer Cell *1*, 219-227.

André, P., Denis, C. V., Ware, J., Saffaripour, S., Hynes, R. O., Ruggeri, Z. M., and Wagner, D. D. (2000). Platelets adhere to and translocate on von Willebrand factor presented by endothelium in stimulated veins. Blood *96*, 3322-3328.

Angeli, V., Ginhoux, F., Llodrà, J., Quemeneur, L., Frenette, P. S., Skobe, M., Jessberger, R., Merad, M., and Randolph, G. J. (2006). B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization. Immunity *24*, 203-215.

Armulik, A., Abramsson, A., and Betsholtz, C. (2005). Endothelial/pericyte interactions. Circ Res *97*, 512-523.

Autiero, M., Waltenberger, J., Communi, D., Kranz, A., Moons, L., Lambrechts, D., Kroll, J., Plaisance, S., De Mol, M., Bono, F*., et al.* (2003). Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat Med *9*, 936-943.

Avantaggiato, V., Orlandini, M., Acampora, D., Oliviero, S., and Simeone, A. (1998). Embryonic expression pattern of the murine figf gene, a growth factor belonging to platelet-derived growth factor/vascular endothelial growth factor family. Mech Dev *73*, 221-224.

Backhed, F., Crawford, P. A., O'Donnell, D., and Gordon, J. I. (2007). Postnatal lymphatic partitioning from the blood vasculature in the small intestine requires fasting-induced adipose factor. Proc Natl Acad Sci U S A *104*, 606-611.

Baldwin, M. E., Catimel, B., Nice, E. C., Roufail, S., Hall, N. E., Stenvers, K. L., Karkkainen, M. J., Alitalo, K., Stacker, S. A., and Achen, M. G. (2001a). The specificity of receptor binding by vascular endothelial growth factor-D is different in mouse and man. J Biol Chem *276*, 19166-19171.

Baldwin, M. E., Halford, M. M., Roufail, S., Williams, R. A., Hibbs, M. L., Grail, D., Kubo, H., Stacker, S. A., and Achen, M. G. (2005). Vascular endothelial growth factor-D is dispensible for development of the lymphatic system. Mol Cell Biol *25*, 2441-2449.

Baldwin, M. E., Roufail, S., Halford, M. M., Alitalo, K., Stacker, S. A., and Achen, M. G. (2001b). Multiple forms of mouse vascular endothelial growth factor-D are generated by RNA splicing and proteolysis. J Biol Chem *276*, 44307-44314.

Baluk, P., Tammela, T., Ator, E., Lyubynska, N., Achen, M. G., Hicklin, D. J., Jeltsch, M., Petrova, T. V., Pytowski, B., Stacker, S. A*., et al.* (2005). Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. J Clin Invest *115*, 247-257.

Banerji, S., Ni, J., Wang, S.-X., Clasper, S., Su, J., Tammi, R., Jones, M., and Jackson, D. G. (1999). LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell Biol *144*, 789-801.

Bell, R., Brice, G., Child, A. H., Murday, V. A., Mansour, S., Sandy, C. J., Collin, J. R., Brady, A. F., Callen, D. F., Burnand, K.*, et al.* (2001). Analysis of lymphoedema-distichiasis families for FOXC2 mutations reveals small insertions and deletions throughout the gene. Hum Genet *108*, 546-551.

Bellomo, D., Headrick, J. P., Silins, G. U., Paterson, C. A., Thomas, P. S., Gartside, M., Mould, A., Cahill, M. M., Tonks, I. D., Grimmond, S. M.*, et al.* (2000). Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. Circ Res *86*, E29-E35.

Bergsten, E., Uutela, M., Li, X., Pietras, K., Ostman, A., Heldin, C. H., Alitalo, K., and Eriksson, U. (2001). PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor. Nat Cell Biol *3*, 512-516.

Betsholtz, C. (2004). Insight into the physiological functions of PDGF through genetic studies in mice. Cytokine Growth Factor Rev *15*, 215-228.

Bhardwaj, S., Roy, H., Gruchalan, M., Viita, H., Kholova, I., Kokina, I., Achen, M. G., Stacker, S. A., Hedman, M., Alitalo, K., and Yla-Herttuala, S. (2003). Angiogenic responses of vascular endothelial growth factors in periadventitial tissue. Hum Gene Ther *14*, 1451-1462.

Bjarnegard, M., Enge, M., Norlin, J., Gustafsdottir, S., Fredriksson, S., Abramsson, A., Takemoto, M., Gustafsson, E., Fassler, R., and Betsholtz, C. (2004). Endothelium-specific ablation of PDGFB leads to pericyte loss and glomerular, cardiac and placental abnormalities. Development *131*, 1847-1857.

Bollinger, A. (1993). Microlymphatics of human skin. Int J Microcirc Clin Exp *12*, 1-15.

Bollinger, A., Isenring, G., Franzeck, U. K., and Brunner, U. (1983). Aplasia of superficial lymphatic capillaries in hereditary and connatal lymphedema (Milroy's disease). Lymphology *16*, 27-30.

Boström, H., Willetts, K., Pekny, M., Levéen, P., Lindahl, P., Hedstrand, H., Pekna, M., Hellström, M., Gebre-Medhin, S., Schalling, M.*, et al.* (1996). PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. Cell *85*, 863-873.

Breiteneder-Geleff, S., Matsui, K., Soleiman, A., Meraner, P., Poczewski, H., Kalt, R., Schaffner, G., and Kerjaschki, D. (1997). Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. Am J Pathol *151*, 1141-1152.

Breiteneder-Geleff, S., Soleiman, A., Kowalski, H., Horvat, R., Amann, G., Kriehuber, E., Diem, K., Weninger, W., Tschachler, E., Alitalo, K., and Kerjaschki, D. (1999). Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. Am J Pathol *154*, 385-394.

Brice, G., Child, A. H., Evans, A., Bell, R., Mansour, S., Burnand, K., Sarfarazi, M., Jeffery, S., and Mortimer, P. (2005). Milroy disease and the VEGFR-3 mutation phenotype. J Med Genet *42*, 98-102.

Brice, G., Mansour, S., Bell, R., Collin, J. R., Child, A. H., Brady, A. F., Sarfarazi, M., Burnand, K. G., Jeffery, S., Mortimer, P., and Murday, V. A. (2002). Analysis of the phenotypic abnormalities in lymphoedema-distichiasis syndrome in 74 patients with FOXC2 mutations or linkage to 16q24. J Med Genet *39*, 478-483.

Byzova, T. V., Goldman, C. K., Jankau, J., Chen, J., Cabrera, G., Achen, M. G., Stacker, S. A., Carnevale, K. A., Siemionow, M., Deitcher, S. R., and DiCorleto, P. E. (2002). Adenovirus encoding vascular endothelial growth factor-D induces tissue-specific vascular patterns in vivo. Blood *99*, 4434-4442.

Cao, R., Bjorndahl, M. A., Religa, P., Clasper, S., Garvin, S., Galter, D., Meister, B., Ikomi, F., Tritsaris, K., Dissing, S.*, et al.* (2004). PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. Cancer Cell *6*, 333-345.

Cao, R., Bråkenhielm, E., Li, X., Pietras, K., Widenfalk, J., Ostman, A., Eriksson, U., and Cao, Y. (2002). Angiogenesis stimulated by PDGF-CC, a novel member in the PDGF family, involves activation of PDGFR-alphaalpha and -alphabeta receptors. FASEB J *16*, 1575-1583.

Cao, Y., Chen, H., Zhou, L., Chiang, M.-K., Anand-Apte, B., Weatherbee, J. A., Wang, Y., Fang, F., Flanagan, J. G., and Tsang, M. L.-S. (1996). Heterodimers of placenta growth factor/vascular endothelial growth factor. J Biol Chem *271*, 3154-3162.

Cao, Y., Ji, W. R., Qi, P., Rosin, A., and Cao, Y. (1997). Placenta growth factor: identification and characterization of a novel isoform generated by RNA alternative splicing. Biochemical & Biophysical Research Communications *235*, 493-498.

Cao, Y., Linden, P., Farnebo, J., Cao, R., Eriksson, A., Kumar, V., Qi, J.-H., Claesson-Welsh, L., and Alitalo, K. (1998). Vascular endothelial growth factor C induces angiogenesis in vivo. Proc Natl Acad Sci USA *95*, 14389-14394.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. Nat Med *6*, 389-395.

Carmeliet, P. (2003). Angiogenesis in health and disease. Nat Med *9*, 653-660.

Carmeliet, P. (2005). Angiogenesis in life, disease and medicine. Nature *438*, 932-936.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Ebenhardt, C.*, et al.* (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature *380*, 435-439.

Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernolle, V., De Mol, M., Wu, Y., Bon, F., Devy, L., Beck, H.*, et al.* (2001). Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med *7*, 575-583.

Chen, H., Bagri, A., Zupicich, J. A., Zou, Y., Stoeckli, E., Pleasure, S. J., Lowenstein, D. H., Skarnes, W. C., Chedotal, A., and Tessier-Lavigne, M. (2000). Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. Neuron *25*, 43-56.

Chen, L., Hamrah, P., Cursiefen, C., Zhang, Q., Pytowski, B., Streilein, J. W., and Dana, M. R. (2004). Vascular endothelial growth factor receptor-3 mediates induction of corneal alloimmunity. Nat Med *10*, 813-815.

Claesson-Welsh, L., Eriksson, A., Westermark, B., and Heldin, C.-H. (1989). cDNA cloning and expression of the human A-type platelet-derived growth factor (PDGF) receptor establishes structural similarity to the B-type PDGF receptor. Proc Natl Acad Sci USA *86*, 4917-4921.

Covassin, L. D., Villefranc, J. A., Kacergis, M. C., Weinstein, B. M., and Lawson, N. D. (2006). Distinct genetic interactions between multiple Vegf receptors are required for development of different blood vessel types in zebrafish. Proc Natl Acad Sci U S A *103*, 6554-6559.

Crnic, I., Strittmatter, K., Cavallaro, U., Kopfstein, L., Jussila, L., Alitalo, K., and Christofori, G. (2004). Loss of neural cell adhesion molecule induces tumor metastasis by up-regulating lymphangiogenesis. Cancer Res *64*, 8630-8638.

Cursiefen, C., Chen, L., Borges, L. P., Jackson, D., Cao, J., Radziejewski, C., D'Amore, P. A., Dana, M. R., Wiegand, S. J., and Streilein, J. W. (2004). VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. J Clin Invest *113*, 1040-1050.

Dagenais, S. L., Hartsough, R. L., Erickson, R. P., Witte, M. H., Butler, M. G., and Glover, T. W. (2004). Foxc2 is expressed in developing lymphatic vessels and other tissues associated with lymphedema-distichiasis syndrome. Gene Expr Patterns *4*, 611-619.

Dale, R. F. (1985). The inheritance of primary lymphoedema. J Med Genet *22*, 274-278.

De Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T. (1992). The *fms*-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science *255*, 989-991.

Debinski, W., Slagle-Webb, B., Achen, M. G., Stacker, S. A., Tulchinsky, E., Gillespie, G. Y., and Gibo, D. M. (2001). VEGF-D is an X-linked/AP-1 regulated putative onco-angiogen in human glioblastoma multiforme. Mol Med *7*, 598-608.

Dellinger, M. T., Hunter, R. J., Bernas, M. J., Witte, M. H., and Erickson, R. P. (2007). Chy-3 mice are Vegfc haploinsufficient and exhibit defective dermal superficial to deep lymphatic transition and dermal lymphatic hypoplasia. Dev Dyn *236*, 2346-2355.

Ding, H., Wu, X., Boström, H., Kim, I., Wong, N., Tsoi, B., O'Rourke, M., Koh, G. Y., Soriano, P., Betsholtz, C.*, et al.* (2004). A specific requirement for PDGF-C in palate formation and PDGFR-alpha signaling. Nat Genet *36*, 1111-1116.

DiSalvo, J., Bayne, M. L., Conn, G., Kwok, P. W., Trivedi, P. G., Soderman, D. D., Palisi, P. M., Sullivan, K. A., and Thomas, K. A. (1995). Purification and characterisation of a naturally occurring vascular endothelial growth factor.placenta growth factor heterodimer. J Biol Chem *270*, 7717-7723.

Dixelius, J., Makinen, T., Wirzenius, M., Karkkainen, M. J., Wernstedt, C., Alitalo, K., and Claesson-Welsh, L. (2003). Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. J Biol Chem *278*, 40973-40979.

Dreyer, G., Norões, J., Figueredo-Silva, J., and Piessens, W. F. (2000). Pathogenesis of lymphatic disease in bancroftian filariasis: a clinical perspective. Parasitol Today *16*, 544-548.

Dumont, D. J., Jussila, L., Taipale, J., Lymboussaki, A., Mustonen, T., Pajusola, K., Breitman, M., and Alitalo, K. (1998). Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science *282*, 946-949.

Ebos, J. M., Bocci, G., Man, S., Thorpe, P. E., Hicklin, D. J., Zhou, D., Jia, X., and Kerbel, R. S. (2004). A naturally occurring soluble form of vascular endothelial growth factor receptor 2 detected in mouse and human plasma. Mol Cancer Res *2*, 315-326.

Enge, M., Bjarnegard, M., Gerhardt, H., Gustafsson, E., Kalen, M., Asker, N., Hammes, H. P., Shani, M., Fassler, R., and Betsholtz, C. (2002). Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. Embo J *21*, 4307-4316.

Enholm, B., Karpanen, T., Jeltsch, M., Kubo, H., Stenback, F., Prevo, R., Jackson, D. G., Yla-Herttuala, S., and Alitalo, K. (2001). Adenoviral expression of vascular endothelial growth factor-C induces lymphangiogenesis in the skin. Circ Res *88*, 623-629.

Etchevers, H. C., Vincent, C., Le Douarin, N. M., and Couly, G. F. (2001). The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. Development *128*, 1059-1068.

Evans, A. L., Bell, R., Brice, G., Comeglio, P., Lipede, C., Jeffery, S., Mortimer, P., Sarfarazi, M., and Child, A. H. (2003). Identification of eight novel VEGFR-3 mutations in families with primary congenital lymphoedema. J Med Genet *40*, 697-670.

Evans, A. L., Brice, G., Sotirova, V., Mortimer, P., Beninson, J., Burnand, K., Rosbotham, J., Child, A., and Sarfarazi, M. (1999). Mapping of primary congenital lymphedema to the 5q35.3 region. Am J Hum Genet *64*, 547-555.

Fang, J., Dagenais, S. L., Erickson, R. P., Arlt, M. F., Glynn, M. W., Gorski, J. L., Seaver, L. H., and Glover, T. W. (2000). Mutations in FOXC2 (MFH-1), a forkhead family transcription factor, are responsible for the hereditary lymphedema-distichiasis syndrome. Am J Hum Genet *67*, 1382-1388.

Ferrara, N. (1999). Molecular and biological properties of vascular endothelial growth factor. J Mol Med *77*, 527-543.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hilan, K. J., and Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature *380*, 438-442.

Ferrara, N., Gerber, H. P., and LeCouter, J. (2003). The biology of VEGF and its receptors. Nat Med *9*, 669-676.

Ferrara, N., and Kerbel, R. S. (2005). Angiogenesis as a therapeutic target. Nature *438*, 967-974.

Ferrell, R. E., Levinson, K. L., Esman, J. H., Kimak, M. A., Lawrence, E. C., Barmada, M. M., and Finegold, D. N. (1998). Hereditary lymphedema: evidence for linkage and genetic heterogeneity. Hum Mol Genet *7*, 2073-2078.

Folkman, J. (1971). Tumour angiogenesis: therapeutic implications. N Engl J Med *285*, 1182-1186.

Fong, G.-H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature *376*, 66-70.

Fong, G.-H., Zhang, L., Bryce, D.-M., and Peng, J. (1999). Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. Development *126*, 3015-3025.

Foo, S. S., Turner, C. J., Adams, S., Compagni, A., Augyn, D., Kogata, N., Lindblom, P., Shani, M., Zicha, D., and Adams, R. H. (2006). Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly. Cell *124*, 161-173.

Frenette, P. S., Denis, C. V., Weiss, L., Jurk, K., Subbarao, S., Kehrel, B., Hartwig, J. H., Vestweber, D., and Wagner, D. D. (2000). P-Selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet-endothelial interactions in vivo. J Exp Med *191*, 1413-1422.

Frenette, P. S., Johnson, R. C., Hynes, R. O., and Wagner, D. D. (1995). Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin. Proc Natl Acad Sci U S A *92*, 7450-7454.

Fruttiger, M., Karlsson, L., Hall, A. C., Abramsson, A., Calver, A. R., Boström, H., Willetts, K., Bertold, C. H., Heath, J. K., Betsholtz, C., and Richardson, W. D. (1999). Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. Development *126*, 457-467.

Fuh, G., Garcia, K. C., and de Vos, A. M. (2000). The interaction of neuropilin-1 with vascular endothelial growth factor and its receptor flt-1. J Biol Chem *275*, 26690-26695.

Furuhashi, M., Sjoblom, T., Abramsson, A., Ellingsen, J., Micke, P., Li, H., Bergsten-Folestad, E., Eriksson, U., Heuchel, R., Betsholtz, C*., et al.* (2004). Platelet-derived growth factor production by B16 melanoma cells leads to increased pericyte abundance in tumors and an associated increase in tumor growth rate. Cancer Res *64*, 2725-2733.

Gale, N. W., Baluk, P., Pan, L., Kwan, M., Holash, J., DeChiara, T. M., McDonald, D. M., and Yancopoulos, G. D. (2001). Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. Dev Biol *230*, 151-160.

Gale, N. W., Prevo, R., Espinosa, J., Ferguson, D. J., Dominguez, M. G., Yancopoulos, G. D., Thurston, G., and Jackson, D. G. (2007). Normal lymphatic development and function in mice deficient for the lymphatic hyaluronan receptor LYVE-1. Mol Cell Biol *27*, 595-604.

Gale, N. W., Thurston, G., Hackett, S. F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M. H., Jackson, D*., et al.* (2002). Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role Is rescued by Angiopoietin-1. Dev Cell *3*, 411-423.

Galland, F., Karamysheva, A., Mattei, M.-G., Rosnet, O., Marchetto, S., and Birnbaum, D. (1992). Chromosomal localization of *FLT4*, a novel receptor-type tyrosine kinase gene. Genomics *13*, 475-478.

Gerety, S. S., Wang, H. U., Chen, Z.-F., and J., A. D. (1999). Symmetrical Mutant Phenotypes of the Receptor EphB4 and Its Specific Transmembrane Ligand ephrin-B2 in Cardiovascular Development. Mol Cell *4*, 403-414.

Gerhardt, H., and Betsholtz, C. (2003). Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res *314*, 15-23.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D., and Betsholtz, C. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol *161*, 1163-1177.

Giger, R. J., Cloutier, J. F., Sahay, A., Prinjha, R. K., Levengood, D. V., Moore, S. E., Pickering, S., Simmons, D., Rastan, S., Walsh, F. S*., et al.* (2000). Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. Neuron *25*, 29-41.

Gilbertson, D. G., Duff, M. E., West, J. W., Kelly, J. D., Sheppard, P. O., Hofstrand, P. D., Gao, Z., Shoemaker, K., Bukowski, T. R., Moore, M*., et al.* (2001). Platelet-derived growth factor C (PDGF-C), a novel growth factor that binds to PDGF alpha and beta receptor. J Biol Chem *276*, 27406-27414.

Gille, H., Kowalski, J., Yu, L. L., Chen, H., Pisabarro, M. T., Davis-Smyth, T., and Ferrara, N. (2000). A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR-1) constitutively inhibits vascular endothelial growth factor-dependent phosphatidylinositol 3 '-kinase activation and endothelial cell migration. EMBO J *19*, 4064-4073.

Gluzman-Poltorak, Z., Cohen, T., Herzog, Y., and Neufeld, G. (2000). Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165. J Biol Chem *275*, 18040-18045.

Gluzman-Poltorak, Z., Cohen, T., Shibuya, M., and Neufeld, G. (2001). Vascular endothelial growth factor receptor-1 and neuropilin-2 form complexes. J Biol Chem *276*, 18688-18694.

Goldman, J., Rutkowski, J. M., Shields, J. D., Pasquier, M. C., Cui, Y., Schmökel, H. G., Willey, S., Hicklin, D. J., Pytowski, B., and Swartz, M. A. (2007). Cooperative and redundant roles of VEGFR-2 and VEGFR-3 signaling in adult lymphangiogenesis. FASEB J *21*, 1003-1012.

Hamrah, P., Chen, L., Zhang, Q., and Dana, M. R. (2003). Novel expression of vascular endothelial growth factor receptor (VEGFR)-3 and VEGF-C on corneal dendritic cells. Am J Pathol *163*, 57-68.

Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M., and Taketo, M. M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. EMBO J *18*, 5931-5942.

Hattori, K., Heissig, B., Wu, Y., Dias, S., Tejada, R., Ferris, B., Hicklin, D. J., Zhu, Z., Bohlen, P., Witte, L*., et al.* (2002). Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nat Med *8*, 841-849.

Hauser, S., and Weich, H. A. (1993). A heparin-binding form of placenta growth factor (PlGF-2) is expressed in human umbilical vein endothelial cells and in placenta. Growth Factors *9*, 259-268.

He, Y., Kozaki, K., Karpanen, T., Koshikawa, K., Yla-Herttuala, S., Takahashi, T., and Alitalo, K. (2002). Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. J Natl Cancer Inst *94*, 819-825.

He, Y., Rajantie, I., Pajusola, K., Jeltsch, M., Holopainen, T., Yla-Herttuala, S., Harding, T., Jooss, K., Takahashi, T., and Alitalo, K. (2005). Vascular endothelial cell growth factor receptor 3-mediated activation of lymphatic endothelium is crucial for tumor cell entry and spread via lymphatic vessels. Cancer Res *65*, 4739-4746.

Heldin, C. H., and Westermark, B. (1999). Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev *79*, 1283-1316.

Hellstrom, M., Gerhardt, H., Kalen, M., Li, X., Eriksson, U., Wolburg, H., and Betsholtz, C. (2001). Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. J Cell Biol *153*, 543-553.

Hellstrom, M., Kalen, M., Lindahl, P., Abramsson, A., and Betsholtz, C. (1999). Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development *126*, 3047-3055.

Herzog, Y., Kalcheim, C., Kahane, N., Reshef, R., and Neufeld, G. (2001). Differential expression of neuropilin-1 and neuropilin-2 in arteries and veins. Mech Dev *109*, 115-119.

Hirakawa, S., Brown, L. F., Kodama, S., Paavonen, K., Alitalo, K., and Detmar, M. (2007). VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. Blood *109*, 1010-1017.

Hirakawa, S., Kodama, S., Kunstfeld, R., Kajiya, K., Brown, L. F., and Detmar, M. (2005). VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. J Exp Med *201*, 1089-1099.

Hiratsuka, S., Maru, Y., Okada, A., Seiki, M., Noda, T., and Shibuya, M. (2001). Involvement of Flt-1 tyrosine kinase (vascular endothelial growth factor receptor-1) in pathological angiogenesis. Cancer Res *61*, 1207-1213.

Hiratsuka, S., Minowa, O., Kuno, J., Noda, T., and Shibuya, M. (1998). Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proc Natl Acad Sci USA *95*, 9349-9354.

Hoch, R. V., and Soriano, P. (2003). Roles of PDGF in animal development. Development *130*, 4769-4784.

Hong, Y. K., Harvey, N., Noh, Y. H., Schacht, V., Hirakawa, S., Detmar, M., and Oliver, G. (2002). Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. Dev Dyn *225*, 351-357.

Houck, K., Leung, D., Rowland, A., Winer, J., and Ferrara, N. (1992). Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem *267*, 26031-26037.

Huang, K., Andersson, C., Roomans, G. M., Ito, N., and Claesson-Welsh, L. (2001). Signaling properties of VEGF receptor-1 and -2 homo- and heterodimers. Int J Biochem Cell Biol *33*, 315-324.

Huang, X. Z., Wu, J. F., Ferrando, R., Lee, J. H., Wang, Y. L., Farese, R. V., Jr., and Sheppard, D. (2000). Fatal bilateral chylothorax in mice lacking the integrin α9β1. Mol Cell Biol *20*, 5208-5215.

Hughes, D. C. (2001). Alternative splicing of the human VEGFGR-3/FLT4 gene as a consequence of an integrated human endogenous retrovirus. J Mol Evol *53*, 77-79.

Hungerford, J. E., and Little, C. D. (1999). Developmental biology of the vascular smooth muscle cell: building a multilayered vessel wall. J Vasc Res *36*, 2-27.

Huntington, G. S., and McClure, C. F. W. (1908). The anatomy and development of the jugular lymph sac in the domestic cat (*Felis domestica*). Anat Rec *2*, 1-19.

Inoue, N., Kondo, T., Kobayashi, K., Aoki, M., Numaguchi, Y., Shibuya, M., and Murohara, T. (2007). Therapeutic angiogenesis using novel vascular endothelial growth factor-E/human placental growth factor chimera genes. Arterioscler Thromb Vasc Biol *27*, 99-105.

Irjala, H., Johansson, E. L., Grenman, R., Alanen, K., Salmi, M., and Jalkanen, S. (2001). Mannose receptor is a novel ligand for L-selectin and mediates lymphocyte binding to lymphatic endothelium. J Exp Med *194J Exp Med*. , 1033-1042.

Irrthum, A., Devriendt, K., Chitayat, D., Matthijs, G., Glade, C., Steijlen, P. M., Fryns, J. P., Van Steensel, M. A., and Vikkula, M. (2003). Mutations in the transcription factor gene SOX18 underlie recessive and dominant forms of hypotrichosis-lymphedema-telangiectasia. Am J Hum Genet *72*, 1470-1478.

Irrthum, A., Karkkainen, M. J., Devriendt, K., Alitalo, K., and Vikkula, M. (2000). Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. Am J Hum Genet *67*, 295-301.

Jackson, D. G., Prevo, R., Clasper, S., and Banerji, S. (2001). LYVE-1, the lymphatic system and tumor lymphangiogenesis. Trends Immunol *22*, 317-321.

Jain, R. K. (2003). Molecular regulation of vessel maturation. Nat Med *9*, 685-693.

Jeltsch, M., Kaipainen, A., Joukov, V., Meng, X., Lakso, M., Rauvala, H., Swartz, M., Fukumura, D., Jain, R. K., and Alitalo, K. (1997). Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science *276*, 1423-1425.

Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N., and Alitalo, K. (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J *15*, 290-298.

Joukov, V., Sorsa, T., Kumar, V., Jeltsch, M., Claesson-Welsh, L., Cao, Y., Saksela, O., Kalkkinen, N., and Alitalo, K. (1997). Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J *16*, 3898-3911.

Junqueira de Azevedo, I. L., Farsky, S. H., Oliveira, M. L., and Ho, P. L. (2001). Molecular cloning and expression of a functional snake venom vascular endothelium growth factor (VEGF) from the Bothrops insularis pit viper. A new member of the VEGF family of proteins. J Biol Chem *276*, 39836-39842.

Kaipainen, A., Korhonen, J., Mustonen, T., van Hinsbergh, V. W. M., Fang, G.-H., Dumont, D., Breitman, M., and Alitalo, K. (1995). Expression of the *fms*-like tyrosine kinase FLT4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA *92*, 3566-3570.

Karkkainen, M. J., Ferrell, R. E., Lawrence, E. C., Kimak, M. A., Levinson, K. L., McTigue, M. A., Alitalo, K., and Finegold, D. N. (2000). Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. Nat Genet *25*, 153-159.

Karkkainen, M. J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T. V., Jeltsch, M., Jackson, D. G., Talikka, M., Rauvala, H., *et al.* (2004). Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol *5*, 74-80.

Karkkainen, M. J., Saaristo, A., Jussila, L., Karila, K. A., Lawrence, E. C., Pajusola, K., Bueler, H., Eichmann, A., Kauppinen, R., Kettunen, M. I., *et al.* (2001). A model for gene therapy of human hereditary lymphedema. Proc Natl Acad Sci USA *98*, 12677-12682.

Karlsson, L., Lindahl, P., Heath, J. K., and Betsholtz, C. (2000). Abnormal gastrointestinal development in PDGF-A and PDGFR-(alpha) deficient mice implicates a novel mesenchymal structure with putative instructive properties in villus morphogenesis. Development *127*, 3457-3466.

Karpanen, T., and Alitalo, K. (2008). Molecular Biology and Pathology of Lymphangiogenesis. Annu Rev Pathol *3*, 367-397.

Karpanen, T., Egeblad, M., Karkkainen, M. J., Kubo, H., Jackson, D. G., Ylä-Herttuala, S., Jäättelä, M., and Alitalo, K. (2001). Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. Cancer Res *61*, 1786-1790.

Karpanen, T., Wirzenius, M., Makinen, T., Veikkola, T., Haisma, H. J., Achen, M. G., Stacker, S. A., Pytowski, B., Yla-Herttuala, S., and Alitalo, K. (2006). Lymphangiogenic growth factor responsiveness is modulated by postnatal lymphatic vessel maturation. Am J Pathol *169*, 708-718.

Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T., and Fujisawa, H. (1999). A requirement for neuropilin-1 in embryonic vessel formation. Development *126*, 4895-4902.

Kendall, R. L., and Thomas, K. A. (1993). Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci USA *90*, 10705-10709.

Kerjaschki, D. (2005). The crucial role of macrophages in lymphangiogenesis. J Clin Invest *115*, 2316-2319.

Kerjaschki, D., Huttary, N., Raab, I., Regele, H., Bojarski-Nagy, K., Bartel, G., Kröber, S. M., Greinix, H., Rosenmaier, A., Karlhofer, F*., et al.* (2006). Lymphatic endothelial progenitor cells contribute to de novo lymphangiogenesis in human renal transplants. Nat Med *12*, 230-234.

Kerjaschki, D., Regele, H. M., Moosberger, I., Nagy-Bojarski, K., Watschinger, B., Soleiman, A., Birner, P., Krieger, S., Hovorka, A., Silberhumer, G*., et al.* (2004). Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. J Am Soc Nephrol *15*, 603-612.

Keskitalo, S., Tammela, T., Lyytikka, J., Karpanen, T., Jeltsch, M., Markkanen, J., Yla-Herttuala, S., and Alitalo, K. (2007). Enhanced capillary formation stimulated by a chimeric vascular endothelial growth factor/vascular endothelial growth factor-C silk domain fusion protein. Circ Res *100*, 1460-1467.

Kholová, I., Koota, S., Kaskenpää, N., Leppänen, P., Närväinen, J., Kavec, M., Rissanen, T. T., Hazes, T., Korpisalo, P., Gröhn, O., and Ylä-Herttuala, S. (2007). Adenovirus-mediated gene transfer of human vascular endothelial growth factor-d induces transient angiogenic effects in mouse hind limb muscle. Hum Gene Ther *18*, 232-244.

Kiba, A., Sagara, H., Hara, T., and Shibuya, M. (2003). VEGFR-2-specific ligand VEGF-E induces non-edematous hyper-vascularization in mice. Biochem Biophys Res Commun *301*, 371-377.

Kitsukawa, T., Shimizu, M., Sanbo, M., Hirata, T., Taniguchi, M., Bekku, Y., Yagi, T., and Fujisawa, H. (1997). Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. Neuron *19*, 995-1005.

Kitsukawa, T., Shimono, A., Kawakami, A., Kondoh, H., and Fujisawa, H. (1995). Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. Development *121*, 4309-4318.

Komori, Y., Nikai, T., Taniguchi, K., Masuda, K., and Sugihara, H. (1999). Vascular Endothelial Growth Factor VEGF-like Heparin-Binding Protein from the Venom of *Vipera aspis aspis* (Aspic Viper). Biochemistry *38*, 11796-11803.

Kriederman, B. M., Myloyde, T. L., Witte, M. H., Dagenais, S. L., Witte, C. L., Rennels, M., Bernas, M. J., Lynch, M. T., Erickson, R. P., Caulder, M. S*., et al.* (2003). FOXC2 haploinsufficient mice are a model for human autosomal dominant lymphedema-distichiasis syndrome. Hum Mol Genet *12*, 1179-1185.

Krishnan, J., Kirkin, V., Steffen, A., Hegen, M., Weih, D., Tomarev, S., Wilting, J., and Sleeman, J. P. (2003). Differential in vivo and in vitro expression of vascular endothelial growth factor (VEGF)-C and VEGF-D in tumors and its relationship to lymphatic metastasis in immunocompetent rats. Cancer Res *63*, 713-722.

Kubo, H., Fujiwara, T., Jussila, L., Hashi, H., Ogawa, M., Shimizu, K., Awane, M., Sakai, Y., Takabayashi, A., Alitalo, K*., et al.* (2000). Involvement of vascular endothelial growth factor receptor-3 in maintenance of integrity of endothelial cell lining during tumor angiogenesis. Blood *96*, 546-553.

Kukk, E., Lymboussaki, A., Taira, S., Kaipainen, A., Jeltsch, M., Joukov, V., and Alitalo, K. (1996). VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development *122*, 3829-3837.

Kunstfeld, R., Hirakawa, S., Hong, Y. K., Schacht, V., Lange-Asschenfeldt, B., Velasco, P., Lin, C., Fiebiger, E., Wei, X., Wu, Y*., et al.* (2004). Induction of cutaneous delayed-type hypersensitivity reactions in VEGF-A transgenic mice results in chronic skin inflammation associated with persistent lymphatic hyperplasia. Blood *104*, 1048-1057.

Küchler, A. M., Gjini, E., Peterson-Maduro, J., Cancilla, B., Wolburg, H., and Schulte-Merker, S. (2006). Development of the zebrafish lymphatic system requires VEGFC signaling. Curr Biol *16*, 1244-1248.

Kärpänen, T., Heckman, C. A., Keskitalo, S., Jeltsch, M., Ollila, H., Neufeld, G., Tamagnone, L., and Alitalo, K. (2006). Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. FASEB J *20*, 1462-1472.

Laakkonen, P., Waltari, M., Holopainen, T., Takahashi, T., Pytowski, B., Steiner, P., Hicklin, D., Persaud, K., Tonra, J. R., Witte, L., and Alitalo, K. (2007). Vascular endothelial growth factor receptor 3 is involved in tumor angiogenesis and growth. Cancer Res *67*, 593-599.

Lallemand, Y., Luria, V., Haffner-Krausz, R., and Lonai, P. (1998). Maternally expressed PGK-Cre transgene as a tool for early and uniform activation of the Cre site-specific recombinase. Transgenic Res *7*, 105-112.

LaRochelle, W. J., Jeffers, M., McDonald, W. F., Chillakuru, R. A., Giese, N. A., Lokker, N. A., Sullivan, C., Boldog, F. L., Yang, M., Vernet, C.*, et al.* (2001). PDGF-D, a new protease-activated growth factor. Nat Cell Biol *3*, 517-521.

Lawson, N. D., Scheer, N., Pham, V. N., Kim, C. H., Chitnis, A. B., Campos-Ortega, J. A., and Weinstein, B. M. (2001). Notch signaling is required for arterial-venous differentiation during embryonic vascular development. Development *128*, 3675-3683.

Lederle, W., Stark, H. J., Skobe, M., Fusenig, N. E., and Mueller, M. M. (2006). Platelet-derived growth factor-BB controls epithelial tumor phenotype by differential growth factor regulation in stromal cells. Am J Pathol *169*, 1767-1783.

Lee, J., Gray, A., Yuan, J., Luoh, S.-M., Avraham, H., and Wood, W. I. (1996). Vascular endothelial growth factor-related protein: A ligand and specific activator of the tyrosine kinase receptor Flt4. Proc Natl Acad Sci USA *93*, 1988-1992.

Leveen, P., Pekny, M., Gebre-Medhin, S., Swolin, B., Larsson, E., and Betsholtz, C. (1994). Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. Genes Dev *8*, 1875-1887.

Li, X., Pontén, A., Aase, K., Karlsson, L., Abramsson, A., Uutela, M., Bäckström, G., Hellström, M., Boström, H., Li, H.*, et al.* (2000). PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor. Nat Cell Biol *2*, 302-309.

Lindahl, P., Hellstrom, M., Kalen, M., Karlsson, L., Pekny, M., Pekna, M., Soriano, P., and Betsholtz, C. (1998). Paracrine PDGF-B/PDGF-Rbeta signaling controls mesangial cell development in kidney glomeruli. Development *125*, 3313-3322.

Lindahl, P., Johansson, B. R., Leveen, P., and Betsholtz, C. (1997a). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science *277*, 242-245.

Lindahl, P., Karlsson, L., Hellström, M., Gebre-Medhin, S., Willetts, K., Heath, J. K., and Betsholtz, C. (1997b). Alveogenesis failure in PDGF-A-deficient mice is coupled to lack of distal spreading of alveolar smooth muscle cell progenitors during lung development. Development *124*, 3943-3953.

Lindblom, P., Gerhardt, H., Liebner, S., Abramsson, A., Enge, M., Hellstrom, M., Backstrom, G., Fredriksson, S., Landegren, U., Nystrom, H. C.*, et al.* (2003). Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. Genes Dev *17*, 1835-1840.

Lyttle, D. J., Fraser, K. M., Fleming, S. B., Mercer, A. A., and Robinson, A. J. (1994). Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. J Virol *68*, 84-92.

Maglione, D., Guerriero, V., Viglietto, G., Delli-Bovi, P., and Persico, M. G. (1991). Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proc Natl Acad Sci USA *88*, 9267-9271.

Maglione, D., Guerriero, V., Viglietto, G., Ferraro, M. G., Aprelikova, O., Alitalo, K., Del Vecchio, S., Lei, K.-J., Chou, J. Y., and Persico, M. G. (1993). Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. Oncogene *8*, 925-931.

Makinen, T., Adams, R. H., Bailey, J., Lu, Q., Ziemiecki, A., Alitalo, K., Klein, R., and Wilkinson, G. A. (2005). PDZ interaction site in ephrin B2 is required for the remodeling of lymphatic vasculature. Genes Dev *19*, 397-410.

Makinen, T., Jussila, L., Veikkola, T., Karpanen, T., Kettunen, M. I., Pulkkanen, K. J., Kauppinen, R., Jackson, D. G., Kubo, H., Nishikawa, S.-I.*, et al.* (2001a). Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. Nat Med *7*, 199-205.

Makinen, T., Olofsson, B., Karpanen, T., Hellman, U., Soker, S., Klagsbrun, M., Eriksson, U., and Alitalo, K. (1999). Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to neuropilin-1. J Biol Chem *274*, 21217-21222.

Makinen, T., Veikkola, T., Mustjoki, S., Karpanen, T., Catimel, B., Nice, E. C., Kowalski, H., Kerjaschki, D., Stacker, S. A., Achen, M. G., and Alitalo, K. (2001b). Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. EMBO J *20*, 4762-4773.

Mandriota, S. J., Jussila, L., Jeltsch, M., Compagni, A., Baetens, D., Prevo, R., Banerji, S., Huarte, J., Montesano, R., Jackson, D. G.*, et al.* (2001). Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. EMBO J *20*, 672-682.

Maruyama, K., Ii, M., Cursiefen, C., Jackson, D. G., Keino, H., Tomita, M., Van Rooijen, N., Takenaka, H., D'Amore, P. A., Stein-Streilein, J.*, et al.* (2005). Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. J Clin Invest *115*, 2363-2372.

Matsui, T., Heideran, M., Miki, T., Popescu, N., La Rochelle, W., Kraus, M., Pierce, J., and Aaronson, S. A. (1989). Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. Science *243*, 800-803.

McCarty, M. F., Somcio, R. J., Stoeltzing, O., Wey, J., Fan, F., Liu, W., Bucana, C., and Ellis, L. M. (2007). Overexpression of PDGF-BB decreases colorectal and pancreatic cancer growth by increasing tumor pericyte content. J Clin Invest *117*, 2114-2122.

Means, A. L., Chytil, A., Moses, H. L., Coffey, R. J. J., Wright, C. V., Taketo, M. M., and Grady, W. M. (2005). Keratin 19 gene drives Cre recombinase expression throughout the early postimplantation mouse embryo. Genesis *42*, 23-27.

Meyer, M., Clauss, M., Lepple-Wienhues, A., Waltenberger, J., Augustin, H. G., Ziche, M., Lanz, C., Böttner, M., Rziha, H.-J., and Dehio, C. (1999). A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signaling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. EMBO J *18*, 363-374.

Migdal, M., Huppertz, B., Tessler, S., Comforti, A., Shibuya, M., Reich, R., Baumann, H., and Neufeld, G. (1998). Neuropilin-1 is a placenta growth factor-2 receptor. J Biol Chem *273*, 22272-22278.

Morisada, T., Oike, Y., Yamada, Y., Urano, T., Akao, M., Kubota, Y., Maekawa, H., Kimura, Y., Ohmura, M., Miyamoto, T.*, et al.* (2005). Angiopoietin-1 promotes LYVE-1-positive lymphatic vessel formation. Blood *105*, 4649-4656.

Nagy, J. A., Vasile, E., Feng, D., Sundberg, C., Brown, L. F., Detmar, M. J., Lawitts, J. A., Benjamin, L., Tan, X., Manseau, E. J.*, et al.* (2002). Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. J Exp Med *196*, 1497-1506.

Neufeld, G., Cohen, T., Shraga, N., Lange, T., Kessler, O., and Herzog, Y. (2002). The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. Trends Cardiovasc Med *12*, 13-19.

Ny, A., Koch, M., Schneider, M., Neven, E., Tong, R. T., Maity, S., Fischer, C., Plaisance, S., Lambrechts, D., Héligon, C.*, et al.* (2005). A genetic Xenopus laevis tadpole model to study lymphangiogenesis. Nat Med *11*, 998-100.

Ober, E. A., Olofsson, B., Mäkinen, T., Jin, S. W., Shoji, W., Koh, G. Y., Alitalo, K., and Stainier, D. Y. (2004). Vegfc is required for vascular development and endoderm morphogenesis in zebrafish. EMBO Rep 2004 *5*, 78-84.

Ogawa, S., Oku, A., Sawano, A., Yamaguchi, S., Yazaki, Y., and Shibuya, M. (1998). A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. J Biol Chem *273*, 31273-31282.

Oh, S.-J., Jeltsch, M. M., Birkenhager, R., McCarthy, J. E., Weich, H. A., Christ, B., Alitalo, K., and Wilting, J. (1997). VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. Dev Biol *188*, 96-109.

Ohl, L., Mohaupt, M., Czeloth, N., Hintzen, G., Kiafard, Z., Zwirner, J., Blankenstein, T., Henning, G., and Förster, R. (2004). CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. Immunity *21*, 279-288.

Oliver, G. (2004). Lymphatic vasculature development. Nat Rev Immunol *4*, 35-45.

Olofsson, B., Korpelainen, E., Pepper, M. S., Mandriota, S. J., Aase, K., Kumar, V., Gunji, Y., Jeltsch, M. M., Shibuya, M., Alitalo, K., and Eriksson, U. (1998). Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. Proc Natl Acad Sci USA *95*, 11709-11714.

Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Pettersson, R. F., Alitalo, K., and Eriksson, U. (1996a). Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc Natl Acad Sci USA *93*, 2576-2581.

Olofsson, B., Pajusola, K., von Euler, G., Chilov, D., Alitalo, K., and Eriksson, U. (1996b). Genomic organization of the mouse and human genes for vascular endothelial growth factor B (VEGF-B) and characterization of a second splice isoform. J Biol Chem *271*, 19310-19317.

Oosthuyse, B., Moons, L., Storkebaum, E., Beck, H., Nuyens, D., Brusselmans, K., Van Dorpe, J., Hellings, P., Gorselink, M., Heymans, S.*, et al.* (2001). Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat Genet *28*, 131-138.

Orlandini, M., Marconcini, L., Ferruzzi, R., and Oliviero, S. (1996). Identification of a c-*fos*-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. Proc Natl Acad Sci USA *93*, 11675-11680.

Orlandini, M., and Oliviero, S. (2001). In fibroblasts Vegf-D expression is induced by cell-cell contact mediated by cadherin-11. J Biol Chem *276*, 6576-6581.

Paavonen, K., Puolakkainen, P., Jussila, L., Jahkola, T., and Alitalo, K. (2000). Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. Am J Pathol *156*, 1499-1504.

Padera, T. P., Stoll, B. R., Tooredman, J. B., Capen, D., di Tomaso, E., and Jain, R. K. (2004). Pathology: cancer cells compress intratumour vessels. Nature *427*, 695.

Pajusola, K., Aprelikova, O., Armstrong, E., Morris, S., and Alitalo, K. (1993). Two human FLT4 receptor tyrosine kinase isoforms with distinct carboxy terminal tails are produced by alternative processing of primary transcripts. Oncogene *8*, 2931-2937.

Pajusola, K., Aprelikova, O., Pelicci, G., Weich, H., Claesson-Welsh, L., and Alitalo, K. (1994). Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. Oncogene *9*, 3545-3555.

Park, J. E., Chen, H. H., Winer, J., Houck, K. A., and Ferrara, N. (1994). Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, *in vitro* and *in vivo*, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem *269*, 25646-25654.

Park, J. E., Keller, G.-A., and Ferrara, N. (1993). The vascular endothelial growth factor (VEGF) isoforms: Differential deposition into the subepithelial extracellular matrix-bound VEGF. Mol Biol Cell *4*, 1317-1326.

Partanen, T. A., Arola, J., Saaristo, A., Jussila, L., Ora, A., Miettinen, M., Stacker, S. A., Achen, M. G., and Alitalo, K. (2000). VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. FASEB J *14*, 2087-2096.

Persaud, K., Tille, J. C., Liu, M., Zhu, Z., Jimenez, X., Pereira, D. S., Miao, H. Q., Brennan, L. A., Witte, L., Pepper, M. S., and Pytowski, B. (2004). Involvement of the VEGF receptor 3 in tubular morphogenesis demonstrated with a human anti-human VEGFR-3 monoclonal antibody that antagonizes receptor activation by VEGF-C. J Cell Sci *117*, 2745-2756.

Peters, K. G., De Vries, C., and Williams, L. T. (1993). Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. Proc Natl Acad Sci USA *90*, 8915-8919.

Petrova, T. V., Karpanen, T., Norrmen, C., Mellor, R., Tamakoshi, T., Finegold, D., Ferrell, R., Kerjaschki, D., Mortimer, P., Yla-Herttuala, S*., et al.* (2004). Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasis. Nat Med *10*, 974-981.

Petrova, T. V., Makinen, T., Makela, T. P., Saarela, J., Virtanen, I., Ferrell, R. E., Finegold, D. N., Kerjaschki, D., Yla-Herttuala, S., and Alitalo, K. (2002). Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. EMBO J *21*, 4593-4599.

Pfister, G., Saesseli, B., Hoffmann, U., Geiger, M., and Bollinger, A. (1990). Diameters of lymphatic capillaries in patients with different forms of primary lymphedema. Lymphology *23*, 140-144.

Plate, K. H., Breier, G., Weich, H. A., and Risau, W. (1992). Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. Nature *359*, 845-848.

Polgar, J., Matuskova, J., and Wagner, D. D. (2005). The P-selectin, tissue factor, coagulation triad. J Thromb Haemost *3*, 1590-1596.

Prevo, R., Banerji, S., Ferguson, D. J. P., Clasper, S., and Jackson, D. G. (2001). Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. J Biol Chem *276*, 19420-19430.

Pytowski, B., Goldman, J., Persaud, K., Wu, Y., Witte, L., Hicklin, D. J., Skobe, M., Boardman, K. C., and Swartz, M. A. (2005). Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. J Natl Cancer Inst *97*, 14-21.

Reigstad, L. J., Varhaug, J. E., and Lillehaug, J. R. (2005). Structural and functional specificities of PDGF-C and PDGF-D, the novel members of the platelet-derived growth factors family. FEBS J *272*, 5723-5741.

Risau, W. (1997). Mechanisms of angiogenesis. Nature *386*, 671-674.

Risau, W., and Flamme, I. (1995). Vasculogenesis. Annu Rev Cell Dev Biol *11*, 73-91.

Rissanen, T. T., Markkanen, J. E., Gruchala, M., Heikura, T., Puranen, A., Kettunen, M. I., Kholova, I., Kauppinen, R. A., Achen, M. G., Stacker, S. A*., et al.* (2003). VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. Circ Res *92*, 1098-1106.

Roberts, N., Kloos, B., Cassella, M., Podgrabinska, S., Persaud, K., Wu, Y., Pytowski, B., and Skobe, M. (2006). Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. Cancer Res *66*, 2650-2657.

Rockson, S. G. (2001). Lymphedema. Am J Med *110*, 288-295.

Rodríguez, C. I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A. F., and Dymecki, S. M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat Genet *25*, 139-140.

Rolny, C., Nilsson, I., Magnusson, P., Armulik, A., Jakobsson, L., Wentzel, P., Lindblom, P., Norlin, J., Betsholtz, C., Heuchel, R*., et al.* (2006). Platelet-derived growth factor receptor-beta promotes early endothelial cell differentiation. Blood *108*, 1877-1886.

Romo, G. M., Dong, J. F., Schade, A. J., Gardiner, E. E., Kansas, G. S., Li, C. Q., McIntire, L. V., Berndt, M. C., and López, J. A. (1999). The glycoprotein Ib-IX-V complex is a platelet counterreceptor for P-selectin. J Exp Med *190*, 803-814.

Saaristo, A., Tammela, T., Farkkilā, A., Kärkkäinen, M., Suominen, E., Yla-Herttuala, S., and Alitalo, K. (2006). Vascular endothelial growth factor-C accelerates diabetic wound healing. Am J Pathol *169*, 1080-1087.

Saaristo, A., Tammela, T., Timonen, J., Yla-Herttuala, S., Tukiainen, E., Asko-Seljavaara, S., and Alitalo, K. (2004). Vascular endothelial growth factor-C gene therapy restores lymphatic flow across incision wounds. FASEB J *18*, 1707-1709.

Saaristo, A., Veikkola, T., Enholm, B., Hytönen, M., Arola, J., Pajusola, K., Turunen, P., Jeltsch, M., Karkkainen, M. J., Kerjaschki D*, et al.* (2002a). Adenoviral VEGF-C overexpression induces blood vessel enlargement, tortuosity, and leakiness but no sprouting angiogenesis in the skin or mucous membranes. FASEB J *16*, 1041-1049.

Saaristo, A., Veikkola, T., Tammela, T., Enholm, B., Karkkainen, M. J., Pajusola, K., Bueler, H., Ylä-Herttuala, S., and Alitalo, K. (2002b). Lymphangiogenic gene therapy with minimal blood vascular side effects. J Exp Med *196*, 719-730.

Sabin, F. R. (1902). On the origin of the lymphatic system from the veins and the development of the lymph hearts and thoracic duct in the pig. Am J Anat *1*, 367-391.

Sainio, K. (2003). Experimental methods for studying urogenital development In The Kidney. From normal development to congenital disease, P. D. Vize, A. S. Woolf, and J. B. L. Bard, eds. (San Diego: Elsevier Science), pp. 327-342.

Sakurai, Y., Ohgimoto, K., Kataoka, Y., Yoshida, N., and Shibuya, M. (2005). Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. Proc Natl Acad Sci U S A *102*, 1076-1081.

Salmi, M., Koskinen, K., Henttinen, T., Elima, K., and Jalkanen, S. (2004). CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium. Blood *104*, 3849-3845.

Schacht, V., Ramirez, M., Hong, Y., Hirakaw, S., Feng, D., Harvey, N., Williams, M., Dvorak, A. M., Dvorak, H. F., Oliver, G., and Detmar, M. (2003). T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. EMBO J *22*, 3546-3556.

Schneider, M., Othman-Hassan, K., Christ, B., and Wilting, J. (1999). Lymphangioblasts in the avian wing bud. Dev Dyn *216*, 311-319.

Schoppmann, S. F., Birner, P., Stöckl, J., Kalt, R., Ullrich, R., Caucig, C., Kriehuber, E., Nagy, K., Alitalo, K., and Kerjaschki, D. (2002). Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. Am J Pathol *161*, 947-956.

Sebzda, E., Hibbard, C., Sweeney, S., Abtahian, F., Bezman, N., Clemens, G., Maltzman, J. S., Cheng, L., Liu, F., Turner, M.*, et al.* (2006). Syk and Slp-76 mutant mice reveal a cell-autonomous hematopoietic cell contribution to vascular development. Dev Cell *11*, 349-361.

Shalaby, F., Ho, J., Stanford, W. L., Fischer, K. D., Schuh, A. C., Schwartz, L., Bernstein, A., and Rossant, J. (1997). A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell *89*, 981-990.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., and Schuh, A. C. (1995). Failure of blood island formation and vasculogenesis in Flk-1-deficient mice. Nature *376*, 62-66.

Shibuya, M. (2001). Structure and dual function of vascular endothelial growth factor receptor-1 (Flt-1). Int J Biochem Cell Biol *33*, 409-420.

Shibuya, M., Yamaguchi, S., Yamane, A., Ikeda, T., Tojo, A., Matsushime, H., and Sato, M. (1990). Nucleotide sequence and expression of a novel human receptor type tyrosine kinase gene (*flt*) closely related to the *fms* family. Oncogene *5*, 519-524.

Shin, D., Garcia-Cardena, G., Hayashi, S., Gerety, S., Asahara, T., Stavrakis, G., Isner, J., Folkman, J., Gimbrone, M. A. J., and Anderson, D. J. (2001). Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. Dev Biol *230*, 139-150.

Sholto-Douglas-Vernon, C., Bell, R., Brice, G., Mansour, S., Sarfarazi, M., Child, A. H., Smith, A., Mellor, R., Burnand, K., Mortimer, P., and Jeffery, S. (2005). Lymphoedema-distichiasis and FOXC2: unreported mutations, de novo mutation estimate, families without coding mutations. Hum Genet *117*, 238-242.

Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature *359*, 843-848.

Skobe, M., Hamberg, L. M., Hawighorst, T., Schirner, M., Wolf, G. L., Alitalo, K., and Detmar, M. (2001b). Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. Am J Pathol *159*, 893-903.

Skobe, M., Hawighorst, T., Jackson, D. G., Prevo, R., Janes, L., Velasco, P., Riccardi, L., Alitalo, K., Claffey, K., and Detmar, M. (2001). Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med *7*, 192-198.

Soker, S., Miao, H. Q., Nomi, M., Takashima, S., and Klagsbrun, M. (2002). VEGF165 mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF165-receptor binding. J Cell Biochem *85*, 357-368.

Soker, S., Takashima, S., Miao, H. Q., Neufeld, G., and Klagsbrun, M. (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell *92*, 735-745.

Song, M., Yang, H., Yao, S., Ma, F., Li, Z., Deng, Y., Deng, H., Zhou, Q., Lin, S., and Wei, Y. (2007). A critical role of vascular endothelial growth factor D in zebrafish embryonic vasculogenesis and angiogenesis. Biochem Biophys Res Commun *357*, 924-930.

Soriano, P. (1994). Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. Genes Dev *8*, 1888-1896.

Soriano, P. (1997). The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. Development *124*, 2691-2700.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet *21*, 70-71.

Srinivasan, R. S., Dillard, M. E., Lagutin, O. V., Lin, F. J., Tsai, S., Tsai, M. J., Samokhvalov, I. M., and Oliver, G. (2007). Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. Genes Dev *21*, 2422-2432.

Stacker, S. A., Achen, M. G., Jussila, L., Baldwin, M. E., and Alitalo, K. (2002b). Lymphangiogenesis and cancer metastasis. Nat Rev Cancer *2*, 573-583.

Stacker, S. A., Caesar, C., Baldwin, M. E., Thornton, G. E., Williams, R. A., Prevo, R., Jackson, D. G., Nishikawa, S., Kubo, H., and Achen, M. G. (2001). VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med *7*, 186-191.

Szuba, A., Skobe, M., Karkkainen, M. J., Shin, W. S., Beynet, D. P., Rockson, N. B., Dakhil, N., Spilman, S., Goris, M. L., Strauss, H. W*., et al.* (2002). Therapeutic lymphangiogenesis with human recombinant VEGF-C. FASEB J *16*, 1985-1987.

Takahashi, T., Fournier, A., Nakamura, F., Wang, L. H., Murakami, Y., Kalb, R. G., Fujisawa, H., and Strittmatter, S. M. (1999). Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. Cell *99*, 59-69.

Takashima, S., Kitakaze, M., Asakura, M., Asanuma, H., Sanada, S., Tashiro, F., Niwa, H., Miyazaki Ji, J., Hirota, S., Kitamura, Y*., et al.* (2002). Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. Proc Natl Acad Sci U S A *99*, 3657-3662.

Tallquist, M. D., and Soriano, P. (2003). Cell autonomous requirement for PDGFRalpha in populations of cranial and cardiac neural crest cells. Development *130*, 507-518.

Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G. I., Song, H., Chedotal, A., Winberg, M. L., Goodman, C. S., Poo, M*., et al.* (1999). Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell *99*, 71-80.

Tammela, T., Enholm, B., Alitalo, K., and Paavonen, K. (2005). The biology of vascular endothelial growth factors. Cardiovasc Res *65*, 550-563.

Tammela, T., He, Y., Lyytikkä, J., Jeltsch, M., Markkanen, J., Pajusola, K., Ylä-Herttuala, S., and Alitalo, K. (2007a). Distinct architecture of lymphatic vessels induced by chimeric vascular endothelial growth factor-C/vascular endothelial growth factor heparin-binding domain fusion proteins. Circ Res *100*, 1468-1475.

Tammela, T., Saaristo, A., Holopainen, T., Lyytikkä, J., Kotronen, A., Pitkonen, M., Abo-Ramadan, U., Ylä-Herttuala, S., Petrova, T. V., and Alitalo, K. (2007b). Therapeutic differentiation and maturation of lymphatic vessels after lymph node dissection and transplantation. Nat Med *13*, 1458-1466.

Tammela, T., Zarkada, G., Wallgard, E., Murtomaki, A., Suchting, S., Wirzenius, M., Waltari, M., Hellstrom, M., Schomber, T., Pelkonen, R*., et al.* (2008). Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature *in press*.

Uutela, M., Wirzenius, M., Paavonen, K., Rajantie, I., He, Y., Karpanen, T., Lohela, M., Wiig, H., Salven, P., Pajusola, K*., et al.* (2004). PDGF-D induces macrophage recruitment, increased interstitial pressure, and blood vessel maturation during angiogenesis. Blood *104*, 3198-3204.

Valtola, R., Salven, P., Heikkila, P., Taipale, J., Joensuu, H., Rehn, M., Pihlajaniemi, T., Weich, H., deWaal, R., and Alitalo, K. (1999). VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. Am J Pathol *154*, 1381-1390.

van der Putte, S. C. (1975a). The early development of the lymphatic system in mouse embryos. Acta Morphol Neerl Scand *13*, 245-286.

van der Putte, S. C. J. (1975b). The development of the lymphatic system in man. Adv Anat Embryol Cell Biol *51*, 3-60.

Wang, H. U., Chen, Z. F., and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell *93*, 741-753.

Wang, J. F., Zhang, X.-F., and Groopman, J. E. (2001). Stimulation of β1 integrin induces tyrosine phosphorylation of VEGF receptor-3 and modulates cell migration. J Biol Chem *276*, 41950-41957.

Veikkola, T., Jussila, L., Makinen, T., Karpanen, T., Jeltsch, M., Petrova, T. V., Kubo, H., Thurston, G., McDonald, D. M., Achen, M. G.*, et al.* (2001). Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. EMBO J *20*, 1223-1231.

Wigle, J. T., Harvey, N., Detmar, M., Lagutina, I., Grosveld, G., Gunn, M. D., Jackson, D. G., and Oliver, G. (2002). An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. EMBO J *21*, 1505-1513.

Wigle, J. T., and Oliver, G. (1999). Prox1 function is required for the development of the murine lymphatic system. Cell *98*, 769-778.

Wilting, J., Aref, Y., Huang, R., Tomarev, S. I., Schweigerer, L., Christ, B., Valasek, P., and Papoutsi, M. (2006). Dual origin of avian lymphatics. Dev Biol *292*, 165-173.

Vincent, L., and Rafii, S. (2004). Vascular frontiers without borders: multifaceted roles of platelet-derived growth factor (PDGF) in supporting postnatal angiogenesis and lymphangiogenesis. Cancer Cell *6*, 307-309.

Wirzenius, M., Tammela, T., Uutela, M., He, Y., Odorisio, T., Zambruno, G., Nagy, J. A., Dvorak, H. F., Ylä-Herttuala, S., Shibuya, M., and Alitalo, K. (2007). Distinct vascular endothelial growth factor signals for lymphatic vessel enlargement and sprouting. J Exp Med *204*, 1431-1440.

Wise, L. M., Veikkola, T., Mercer, A. A., Savory, L. J., Fleming, S. B., Caesar, C., Vitali, A., Makinen, T., Alitalo, K., and Stacker, S. A. (1999). Vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and neuropilin-1. Proc Natl Acad Sci USA *96*, 3071-3076.

Witte, M. H., Erickson, R., Bernas, M., Andrade, M., Reiser, F., Conlon, W., Hoyme, H. E., and Witte, C. L. (1998). Phenotypic and genotypic heterogeneity in familial Milroy lymphedema. Lymphology *31*, 145-155.

Witzenbichler, B., Asahara, T., Murohara, T., Silver, M., Spyridopoulos, I., Magner, M., Principe, N., Kearney, M., Hu, J.-S., and Isner, J. M. (1998). Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. Am J Pathol *153*, 381-394.

Vlahakis, N. E., Young, B. A., Atakilit, A., and Sheppard, D. (2005). The lymphangiogenic growth factors VEGF-C and D are ligands for the integrin alpha 9beta 1. J Biol Chem *280*, 4544-4552.

von Tell, D., Armulik, A., and Betsholtz, C. (2006). Pericytes and vascular stability. Exp Cell Res *312*, 623-629.

Vrancken Peeters, M. P., Gittenberger-de Groot, A. C., Mentink, M. M., and Poelmann, R. E. (1999). Smooth muscle cells and fibroblasts of the coronary arteries derive from epithelial-mesenchymal transformation of the epicardium. Anat Embryol (Berl) *199*, 367-378.

Wu, X., and Ding, H. (2007). Generation of conditional knockout alleles for PDGF-C. Genesis *45*, 653-657.

Yaniv, K., Isogai, S., Castranova, D., Dye, L., Hitomi, J., and Weinstein, B. M. (2006). Live imaging of lymphatic development in the zebrafish. Nat Med *12*, 711-716.

Yoon, Y. S., Murayama, T., Gravereaux, E., Tkebuchava, T., Silver, M., Curry, C., Wecker, A., Kirchmair, R., Hu, C. S., Kearney, M.*, et al.* (2003). VEGF-C gene therapy augments postnatal lymphangiogenesis and ameliorates secondary lymphedema. J Clin Invest *111*, 717-725.

Yuan, L., Moyon, D., Pardanaud, L., Bréant, C., Karkkainen, M. J., Alitalo, K., and Eichmann, A. (2002). Abnormal lymphatic vessel development in neuropilin-2 mutant mice. Development *129*, 4797-4806.

Zhang, X., Groopman, J. E., and Wang, J. F. (2005). Extracellular matrix regulates endothelial functions through interaction of VEGFR-3 and integrin alpha5beta1. J Cell Physiol *202*, 205-214.

Zheng, Y., Murakami, M., Takahashi, H., Yamauchi, M., Kiba, A., Yamaguchi, S., Yabana, N., Alitalo, K., and Shibuya, M. (2006). Chimeric VEGF-E(NZ7)/PlGF promotes angiogenesis via VEGFR-2 without significant enhancement of vascular permeability and inflammation. Arterioscler Thromb Vasc Biol *26*, 2019-2026.

Zheng, Y., Watanabe, M., Kuraishi, T., Hattori, S., Kai, C., and Shibuya, M. (2007). Chimeric VEGF-ENZ7/PlGF specifically binding to VEGFR-2 accelerates skin wound healing via enhancement of neovascularization. Arterioscler Thromb Vasc Biol *27*, 503-501.

Ziche, M., Maglione, D., Ribatti, D., Morbidelli, L., Lago, C. T., Battisti, M., Paoletti, I., Barra, A., Tucci, M., Parise, G.*, et al.* (1997). Placenta growth factor-1 is chemotactic, mitogenic, and angiogenic. Lab Invest *76*, 517-531.

Ziegler, B. L., Valtieri, M., Porada, G. A., De Maria, R., Muller, R., Masella, B., Gabbianelli, M., Casella, I., Pelosi, E., Bock, T.*, et al.* (1999). KDR receptor: a key marker defining hematopoietic stem cells. Science *285*, 1553-1558.