Helsinki University Biomedical Dissertations No. 111

VEGFR-3 and Tie pathways in vascular network formation

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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 May 16, 2008 at 2 p.m.

Helsinki 2008

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ISBN: 978-952-10-4687-2 (nid.)
ISBN 978-952-10-4688-9 (PDF)
ISSN: 1457-8433
http://ethesis.helsinki.fi
Yliopistopaino
Helsinki 2006

To Katja

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ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which have been assigned the following roman numerals:

I **Tammela T**, Saaristo, AS, Lohela M, Morisada T, Tornberg J, Norrmén C, Pajusola K, Thurston G, Suda T, Yla-Herttuala S, and Alitalo K. Angiopoietin-1 induces lymphatic sprouting and hyperplasia.
Blood 105:4642-4648 (2005)

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

III **Tammela T***, He Y*, Lyytikkä J, Jeltsch M, Markkanen J, Yla-Herttuala S, Pajusola K, and Alitalo K. Distinct architecture of lymphatic vessels induced by a chimeric VEGF-C/VEGF heparin-binding domain fusion protein.
Circulation Research 100:1460-147 (2007)

IV **Tammela T**, Zarkada G, Murtomäki A, Wallgard E, Suchting S, Wirzenius M, Waltari M, Hellström M, Schomber T, Peltonen R, Freitas C, Duarte A, Isoniemi H, Laakkonen P, Christofori G, Ylä-Herttuala S, Eichmann A, Betsholtz C, and Alitalo K. Inhibiting VEGFR-3 suppresses angiogenic sprouting and vascular network formation.
Nature, in press

* The authors contributed equally to the study.

ABBREVIATIONS

AAV	adeno-associated virus
Ad	adenovirus
Akt	V-akt murine thymoma viral oncogene homolog-1
Alk	activin receptor-like kinase
AMD	age-related macular degeneration
Ang	angiopoietin
Angpt1	mouse angiopoietin-1 gene
Bcr-Abl	breakpoint cluster region – Abelson leukemia virus homologue
bHLH	basic helix-loop-helix
BM	basement membrane
CA65	VEGF-C/VEGF-exon 7-8 chimera
CA89	VEGF-C/VEGF-exon 6a-8 chimera
CCL	cysteine-cysteine chemokine ligand
CD	cluster of differentiation
COUP-TFII	chicken ovalbumin upstream promoter transcription factor II
CSL	CBF-1/Suppressor of hairless/Lag-1
C-terminal	carboxyterminal
CXCL	cysteine-X-cysteine chemokine ligand
CXCR	CXCL-receptor
Dll	delta-like ligand
DNA	deoxyribonucleic acid
E	embryonic day … of mouse development (here)
EC	endothelial cell
ECM	extracellular matrix
EDG	endothelial differentiation gene
Efb4	zebrafish EphB4 gene (here)
EGF	epidermal growth factor
EGFR	EGF-receptor
Eng	mouse endoglin gene
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
Eph	erythropoietin-producing hepatocellular carcinoma tyrosine kinase
ErbB	erythroblastic leukemia viral oncogene homolog B
FGF	fibroblast growth factor
Flk	fetal liver kinase
Flt	fms-like tyrosine kinase
FN	fibronectin
FoxC2	forkhead box C2
HBD	heparin-binding domain
HB-EGF	heparin-binding EGF
Hes	Hairy/Enhancer of Split-1
Hey	Hes-related (Hesr) with YRPW
HGF	hepatocyte growth factor
HIF-1α	hypoxia-inducible factor-1α
HLTS	hypotrichosis-lymphedema-teleangiectasia syndrome
HSPG	heparan sulfate proteoglycan
Ig	immunoglobulin
IGF	insulin-like growth factor
IL-7Rα	interleukin-7-receptor-α
K14	keratin-14
kDa	kilodalton
KDR	kinase-insert domain receptor
LD	lymphedema distichiasis

LEC	lymphatic endothelial cell
LT	lymphotoxin
LTβR	LTβ-receptor
LTi	lymphoid tissue inducer
LYVE-1	lymphatic vessel hyaluronan receptor-1
MAML-1	mastermind-like-1
MAPK	mitogen-activated protein kinase
mRNA	messenger-RNA
MT1-MMP	membrane-type-1 matrix metalloproteinase (MMP-14)
N-cadherin	neural cadherin
NF-κB	nuclear factor kappa-B
NICD	Notch intracellular domain
NP	neuropilin
N-terminal	aminoterminal
OL-EDA-ID	anhidrotic ectodermal dysplasia with immunodeficiency, osteopetrosis, and lymphedema
OMIM	online Mendelian inheritance of man
PC	proprotein convertase
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDFGR	PDGF-receptor
PDZ	post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (DlgA), and zonula occludens-1 protein (ZO-1) homology domain
PlGF	placenta growth factor
PGC-1α	peroxisome-proliferator-activated receptor-gamma coactivator-1α
Prox1	prospero-related homeobox-1
Rac	Ras-related C3 botulinum toxin substrate
RBP-J	recombination signal binding protein for immunoglobulin kappa J region
Rbpsuh	recombining binding protein – suppressor of hairless
RNA	ribonucleic acid
Robo	roundabout
S1K	sphingosine-1-kinase
S1P	sphingosine-1-phosphate
S1P1	S1P-receptor-1
SCD	superclustering domain
Slp76	SH2 domain containing leukocyte protein of 76 kDa
Smad5	smoothened mothers against decapentaplegy-5
SMC	smooth muscle cell
SOX18	SRY (sex determining region Y)-box 18
Src	sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
Syk	spleen tyrosine kinase
TβrII	mouse TGF-ß-receptor II gene
TGF-β	transforming growth factor-β
Tgfb1	mouse TGF-ß1 gene
Tie	tyrosine kinase with immunoglobulin and EGF homology domains
TKI	tyrosine kinase inhibitor
TNF-α	tumor necrosis factor-α
TRANCE	TNF-related activation induced cytokine
Unc5b	*C. elegans* unc-5 homolog B
VCAM-1	vascular-cell adhesion molecule-1
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR	VEGF-receptor
VHL	von Hippel-Lindau
VPF	vascular permeability factor

ABSTRACT

Angiogenesis, the growth of new blood vessels from pre-existing vasculature, is uncontrolled in tumor growth and insufficient in tissue ischemia. Similarly, the growth of lymphatic vessels, or lymphangiogenesis, is involved in human disease. In particular, insufficient lymphatic vessel function is responsible for development of lymphedema, a debilitating condition characterized by chronic tissue edema and impaired immunity. Vascular endothelial growth factors (VEGFs) stimulate angiogenesis and lymphangiogenesis by activating VEGF receptor (VEGFR) tyrosine kinases in endothelial cells. VEGFR-3 is present on all endothelia during development, but in the adult its expression becomes restricted to the lymphatic endothelium. VEGF-C and VEGF-D are ligands for VEGFR-3, and primarily induce lymphangiogenesis in adult tissues.

The objective of this study was to evaluate the potential of natural and engineered growth factors in inducing therapeutic lymphatic vessel growth, as well as to elucidate the function of VEGFR-3 in angiogenesis in mouse models. Angiopoietin-1 (Ang1), a ligand for the endothelial receptor tyrosine kinases Tie1 and Tie2, induced growth of lymphatic vessels when overexpressed in the skin. A new mouse model of secondary lymphedema was established, which was used to show that collecting lymphatic vessels could be regenerated after lymph node removal using adenoviral gene transfer of VEGF-C or VEGF-D. Notably, the growth factor therapy greatly improved the outcome of lymph node transplantation, including functional reconstitution of the barrier function for tumor metastasis. In order to accelerate the process of lymphatic vessel maturation, novel heparin-binding forms of VEGF-C were engineered. These chimeric growth factors induced formation of lymphatic vessels directly along basement membranes that are rich in heparan sulfate, leading to formation of lumenized lymphatic vessels more efficiently than wild-type VEGF-C.

VEGFR-3 expression was observed specifically in blood vessels undergoing angiogenesis in adult tissues. Notably, VEGFR-3 was highly expressed in angiogenic sprouts, and blocking VEGFR-3 resulted in decreased blood vessel growth in a variety of physiological and pathological settings. Stimulation of VEGFR-3 augmented VEGF-induced angiogenesis and sustained angiogenesis even in the presence of VEGFR-2 inhibitors, whereas blocking both VEGFR-3 and VEGFR-2 in combination resulted in additive inhibition of angiogenesis and tumor growth. Furthermore, these studies indicated that the Notch signaling pathway down-regulates VEGFR-3 in blood vascular endothelial cells, rendering the cells less sensitive to angiogenic signals.

These results constitute the first report of growth factor-induced lymphatic vessel maturation in adults, as well as a novel strategy to accelerate the process by using engineered heparin-binding forms of VEGF-C. The approach of combining growth factor expression with lymph node transplantation provides a basis for future treatment of lymphedema, whereas VEGF/VEGF-C chimeras and angiopoietin-1 could be used to improve and augment VEGF-C/D therapy. Furthermore, these results implicate VEGFR-3 as a novel regulator of sprouting angiogenesis along with its role in regulating lymphatic vessel growth. Targeting VEGFR-3 may provide additional efficacy for anti-angiogenic therapies, especially towards vessels that are resistant to VEGF/VEGFR-2 inhibitors.

REVIEW OF THE LITERATURE

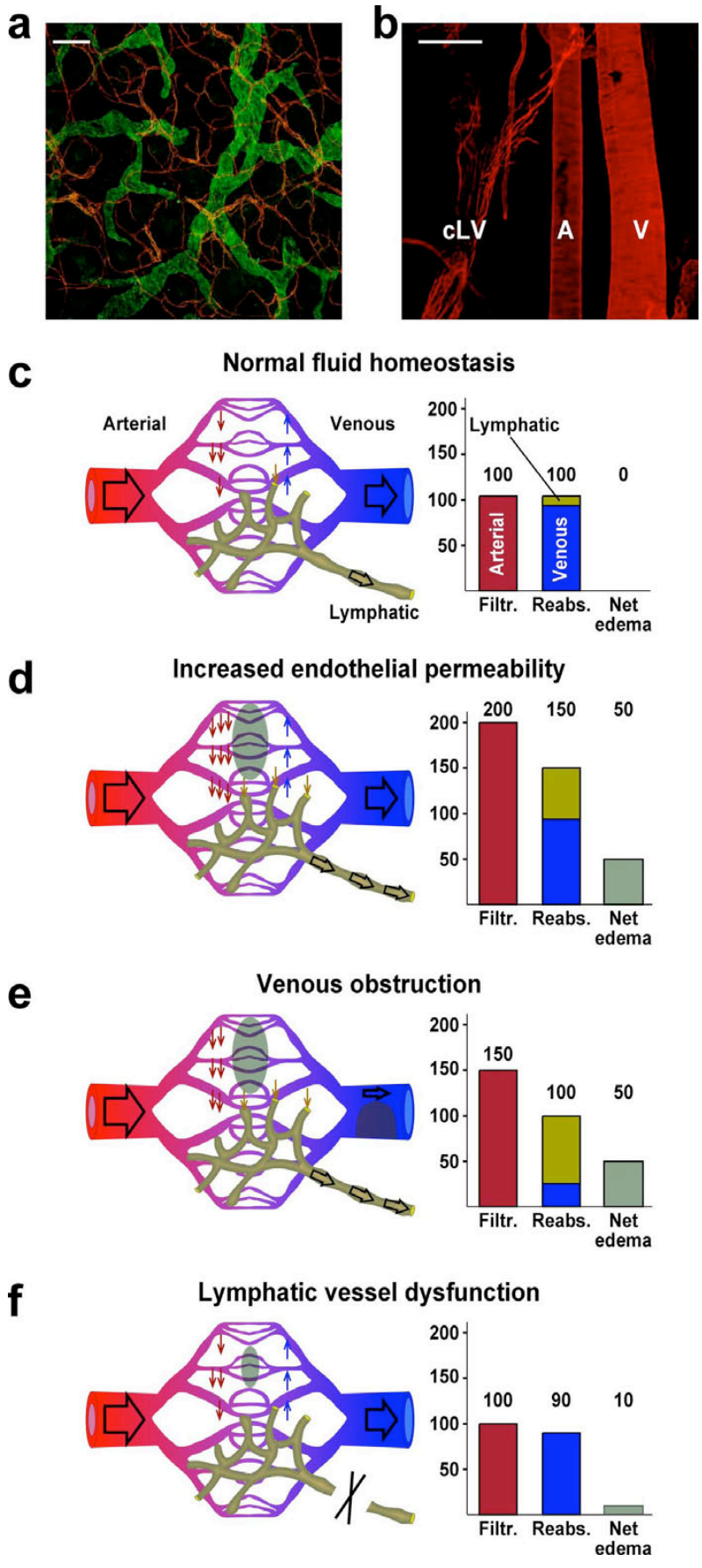
1. Development and function of the cardiovascular and lymphatic systems

Large multicellular organisms such as humans have developed a circulatory system, the blood vascular system, to distribute oxygen, nutrients, hormones and even cells to tissues, as well as to collect carbon dioxide and other metabolic waste products. The principal components of blood are red blood cells (erythrocytes), white blood cells (leukocytes), platelets (thrombocytes), immunoglobulins, as well as a variety of colloid proteins that help maintain the water content of blood higher than in the surrounding tissues. The heart pumps the blood through arteries into a fine network of blood vessel capillaries, 10-20 μm in diameter, which connect with veins that return the blood back to the heart. The exchange of gases from the blood to surrounding tissues occurs through the capillaries, which are found within 200 μm of any cell, with the exception avascular tissues such as cartilage, as well as the lens and cornea of the eye (Ambati et al., 2006). Blood pressure causes plasma proteins accompanied by water molecules to filtrate continuously from the arterial side of the capillary bed into the interstitial space. Approximately 90% of the extravasated water is reabsorbed at the venous side of the capillary bed, where the colloid osmotic pressure of the blood exceeds blood pressure, but the remaining 10% results in a net excess of protein-rich fluid in the interstitial space (**Figure 1**).

The main function of the lymphatic vasculature is to return this excess fluid back to the blood circulation system. Fluid, macromolecules, and cells enter blind-ended lymphatic capillaries in tissues. The lymph is further transported towards collecting lymphatic vessels and is returned to the blood circulation through the lymphatico-venous junctions at the subclavian veins. The collecting lymphatic vessels connect with chains of lymph nodes. Therefore the lymphatic vascular system also plays an important role in immune responses by serving as a conduit for extravasated leukocytes and activated antigen-presenting cells. In the small intestine, lacteal lymphatic vessels inside the intestinal villi absorb dietary lipids released by intestinal epithelial cells in the form of chylomicrons. Lymphatic vessels are typically found in all vascularized tissues, with the notable exception of bone marrow and the

central nervous system, although some connections between the lymphatic vascular and cerebrospinal fluid systems exist (Johnston et al., 2004). Besides the fat-adsorbing small intestine, tissues that frequently become in contact with foreign antigens, such as the skin and mucous membranes, are particularly rich in lymphatic vessels. The lymphatic vascular system is found at least in vertebrates such as teleost fish, amphibians, reptiles and mammals (Ny et al., 2005; Kuchler et al., 2006; Yaniv et al., 2006; reviewed in Jeltsch et al., 2003; and in Ny et al., 2006), whose complex cardiovascular system and relatively large body size require the presence of a secondary vascular system for the maintenance of fluid balance (Figure 1).

Figure 1. Contributions of the blood and lymphatic vascular systems to tissue fluid homeostasis. (**a**) Blood vessels (red) and lymphatic vessels (green) in the mouse ear skin visualized by PECAM-1 and LYVE-1 immunostaining, respectively. Note that here the intense LYVE-1 staining overlies weak PECAM-1 staining in the lymphatic vessels. (**b**) An artery (A), a vein (V), and a collecting lymphatic vessel (cLV) in the mouse ear. Smooth muscle cells are visualized by staining for smooth muscle α-actin (red). (**c-f**) Mechanisms leading to tissue edema. Normal fluid homeostasis in tissues is shown in (**c**): Colloid proteins and associated water are constantly filtrated from the arterial side of the capillary bed into the interstitial space (red arrows). 90% of the filtrate is reabsorbed into the capillaries on the venous side of the capillary bed (blue arrows), whereas 10% is collected by the lymphatic vessels (brown arrows). (**d**) In conditions of increased blood vascular permeability, such as in inflammation, the amount of filtrate is dramatically increased. Although the lymphatic vessels have a remarkable capacity to increase their drainage, net edema remains. (**e**) Obstruction of the veins, e.g. due to venous thrombosis, will impair reabsorption and increase blood pressure within the capillary bed, leading to increased filtration. Again, the lymphatic vessels are capable of increasing drainage, yet net edema is generated. (**f**) Inherited or acquired damage to the lymphatic vessels, such as surgery or radiation therapy, block lymphatic drainage. This will lead to gradual accumulation of edematous fluid in tissues. Reabs: reabsorption; Filtr: filtration. The units in the bar graphs are arbitrary. Note: Only the underlying reasons for edema formation are given in each figure, and secondary effects due to e.g. increased interstitial fluid pressure in edematous conditions are not accounted for. Scalebars: 100 μm.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d
Increased endothelial permeability
200
150
50
e
Venous obstruction
150
100
50
f
Lymphatic vessel dysfunction
100
90
10

The blood vascular system

The luminal surface of blood vessels is lined by a monolayer of endothelial cells (ECs), which are adjoined by tight junctions and the more plastic adherens junctions (reviewed in Risau, 1998). Far from being passive bystanders, ECs have multiple functions: They regulate blood flow by releasing nitric oxide to relax smooth muscle that constricts vessels; act as gatekeepers for cells and macromolecules in between the blood and the interstitium; and respond to growth factors that stimulate the formation of new blood vessels. Besides adopting arterial or venous identity in their gene expression pattern (reviewed in Hirashima and Suda, 2006), ECs exhibit a wide range of functional and morphological differences. For example, the capillary endothelium of endocrine organs, such as the thyroid and adrenal glands, and the β-islets of the pancreas, is fenestrated, allowing a high rate of transport across the EC monolayer (reviewed in Risau, 1998). On the other hand, ECs of the central nervous system form part of the blood-brain barrier, which allows only very selective passage of molecules and cells. The high-endothelial venules found in secondary lymphoid organs (with the exception of the spleen) are specialized for cell transmigration, allowing entry of lymphocytes into the lymphoid tissues. Furthermore, even ECs that appear ultrastructurally similar have unique tissue-specific molecular fingerprints, which has facilitated the discovery of tissue-specific vascular targets (reviewed in Ruoslahti and Rajotte, 2000; and in Trepel et al., 2002).

The basolateral side of the blood vascular EC monolayer, with the exception of blood vessels with a discontinous endothelium found in the liver, spleen and bone marrow, is lined with a basement membrane (BM), an approximately 50-100 nm thick sheet of extracellular matrix (ECM). The EC monolayer is also supported by mesenchymal mural cells called pericytes, which share the BM with the ECs. The pericytes become in contact with the ECs at focal points called peg-socket contacts, conferring survival and stability signals to the endothelium, and providing physical support against hemodynamic stress (reviewed in Armulik et al., 2005; and in von Tell et al., 2006). Arteries are also surrounded by a tunica media composed of a concentric ring of contractile smooth muscle cells (SMCs), which regulate blood pressure and flow, and an outer connective tissue layer, the tunica adventitia. The return of the blood to the heart via the veins is powered by contractions of the

surrounding skeletal muscle and venous SMCs, as well as by arterial pulsations, whereas intraluminal valves positioned at intervals help to prevent venous backflow.

During embryogenesis, the cardiovascular system is the first organ system to develop. The hematopoietic and EC lineages differentiate from a common precursor, the hemangioblast. The early blood vessels of the embryo and yolk sac in mammals develop by aggregation of EC precursors, or angioblasts, which are derived from hemangioblasts, into a primitive vascular plexus (reviewed in Coultas et al., 2005). This assembly of a vascular network by ECs that have differentiated *de novo* from stem cells is termed vasculogenesis. The early vascular plexus undergoes a complex expansion process that is characterized by migration, sprouting and proliferation of ECs. This formation of new blood vessels by sprouting from pre-existing vasculature is denoted angiogenesis. Angiogenic sprouting involves specification of subpopulations of ECs into tip cells, that respond to guidance cues in the surrounding microenvironment, and stalk cells that follow the tip cells and proliferate to form a lumenized vascular network (Gerhardt et al., 2003).

The ECs of the newly formed blood vessels adopt an arterial, venous, or capillary identity, while the nascent vascular network is remodeled by pruning of excess vessels and by the recruitment of pericytes. Interestingly, angiogenesis and organogenesis are closely linked processes, as evidenced by the crucial role of ECs in the induction of e.g. the liver and the pancreas (Lammert et al., 2001; Matsumoto et al., 2001; LeCouter et al., 2003; Yoshitomi and Zaret, 2004; reviewed in Lammert et al., 2003). During pre- and postnatal development after organogenesis, the blood vessel networks meet the requirements of growing organs by expanding within the tissues by angiogenic sprouting as well as by splitting, intussuception, and circumferential enlargement, which are processes that remain poorly understood (Djonov et al., 2000).

Physiological neovascularization is uncommon in stabile adult tissues. However, new blood vessels form in response to tissue hypertrophy in e.g. skeletal muscle or fat, during wound healing, and during the estrus cycle in fertile females both in developing ovarian follicles as well as in the uterine endometrium (Zimmermann et al., 2003; reviewed in Carmeliet, 2005). The neovascularization of adult tissues is thought to occur primarily by angiogenesis, but the possibility of adult vasculogenesis by bone-marrow derived endothelial precursor cells (EPCs) has also been suggested in the literature (reviewed in Aicher et al., 2005; and in Kopp et al., 2006). Pathological angiogenesis occurs in a variety of conditions,

such as in tumors and in proliferative retinal vasculopathies, like age-related macular degeneration (AMD) and in late-stage diabetic retinopathy (reviewed in Gariano and Gardner, 2005). Conversely, angiogenesis is frequently insufficient in ischemic tissues e.g. following arterial occlusion in the heart or the lower limb.

Arteriogenesis, or remodeling of angiogenic blood vascular capillaries or small arterioles into larger caliber vessels that acquire a thick SMC coating, is known to occur in ischemic conditions (reviewed in Schaper and Scholz, 2003). Circumferentially directed stress and shear stress acting on the endothelium are key forces that drive arteriogenesis, and changes in fluid flow have been shown to regulate gene expression in both blood and lymphatic vascular ECs (Garcia-Cardena et al., 2001; Ng et al., 2004; reviewed in Schaper and Scholz, 2003). Furthermore, reactive inflammation of the vessel wall and recruitment of monocytes/macrophages are important for arteriogenesis (Ito et al., 1997; Arras et al., 1998; Pipp et al., 2003)

The lymphatic vascular system

The lymphatic capillaries are thin-walled vessels of approximately 30-80 μm in diameter, and composed of a single layer of non-fenestrated lymphatic endothelial cells (LECs), which are not ensheathed by pericytes or SMCs, and have little or no BM (Leak and Burke, 1966; Leak and Burke, 1968; Leak, 1970). The LEC-LEC junctions in lymphatic capillaries are discontinuous, and the interjunctional gaps act as sites of leukocyte entry into the vessels (Baluk et al., 2007). Collecting lymphatic vessels are characterized by the presence of a SMC layer, a basement membrane, continuous interendothelial junctions, and valves (reviewed in Alitalo et al., 2005). The intrinsic contractility of SMCs, as well as the contraction of surrounding skeletal muscles and arterial pulsations are necessary for lymph propulsion, whereas valves prevent lymph backflow, implying an analogous structure-function relationship between collecting lymphatic vessels and veins. The LECs are terminally differentiated cells distinct from blood vascular ECs. Both cell types remain distinct also in culture conditions (Kriehuber et al., 2001; Mäkinen et al., 2001; Wick et al., 2007), which has facilitated the discovery of lymphatic vascular specific molecular targets that are used for identification of lymphatic vessels in tissues, as well as for finding targets

for the specific induction or inhibition of lymphatic vessel growth in pathological conditions (reviewed in Saharinen et al., 2004). The most commonly used lymphatic vessel markers are the prospero-related homeodomain transcription factor Prox1, the membrane glycoprotein podoplanin, vascular endothelial growth factor receptor-3 (VEGFR-3), and lymphatic vessel hyaluronan receptor-1 (LYVE-1) (reviewed in Saharinen et al., 2004). Notably, LYVE-1 expression is restricted to the lymphatic capillaries and it is not found in the collecting vessels (Makinen et al., 2005).

Genetic experiments in mice have validated that mammalian lymphatic vessels originate from embryonic veins (Wigle and Oliver, 1999; Wigle et al., 2002; Kärkkäinen et al., 2004; Srinivasan et al., 2007), as postulated by the American immunologist Florence Sabin already in 1902 (Sabin, 1902). Several timely papers utilizing dynamic imaging in developing zebrafish embryos have elegantly demonstrated that this process is conserved in evolution (Kuchler et al., 2006; Yaniv et al., 2006). According to this model of lymphatic vessel development, a subset of ECs in the large central veins of the embryo begin to express Prox1, signifying commitment to the LEC lineage, and sprout laterally to form primordial lymphatic vascular structures, the lymph sacs, which expand by sprouting to form the lymphatic vascular network (Wigle and Oliver, 1999; Petrova et al., 2002; reviewed in Oliver, 2004). Compared to the emergence of blood vessels, lymphatic vessels develop considerably later, at around embryonic weeks 6-7 in humans and at embryonic day (E) 10.5 in mice. However, it is not known whether lymphatic endothelium can differentiate in the embryonic mesenchyme in mammals, as has been suggested by experiments in chicks (Wilting et al., 2000; Wilting et al., 2006).

During the course of development, connections between the lymphatics and veins are lost, except the sites where lymph enters the blood in the subclavian veins, although additional lymphatico-venous communications may exist in other peripheral locations (reviewed in Jeltsch et al., 2003). Mice with homozygous mutations in either the tyrosine kinase Syk or the adaptor protein Slp76 display arterio-venous shunts and abnormal lymphatico-venous communications (Abtahian et al., 2003). Syk and Slp76 are expressed almost exclusively in hematopoietic cells, suggesting that these cells contribute to the separation of the two vascular systems (Abtahian et al., 2003; Sebzda et al., 2006).

In adults, lymphangiogenesis occurs physiologically during inflammation, ovarian growth, and wound healing (Pullinger and Florey, 1937; Otsuki et al., 1986; Paavonen et al.,

2000; Baluk et al., 2005). Lymphatic vessel growth is also associated with a number of pathological conditions, including tumor metastasis and transplant rejection (Cursiefen et al., 2004; Kerjaschki et al., 2004; reviewed in Stacker et al., 2002; and in Alitalo et al., 2005). Adult lymphangiogenesis occurs primarily by sprouting from pre-existing vessels (Saaristo et al., 2002a; He et al., 2004; He et al., 2005), although bone-marrow derived cells, such as macrophages, have been suggested to transdifferentiate into lymphatic endothelium at least in human kidney transplants (Kerjaschki et al., 2006), and in a mouse model of corneal injury (Maruyama et al., 2005).

Remodeling of the blood vasculature into arteries, capillaries, and veins is known to play a critical role in the development of a functional blood vessel network. Although the lymphatic vasculature also undergoes significant remodeling after its initial establishment, the molecular mechanisms involved in these processes are largely unknown. Developmental remodeling of the lymphatic vasculature includes sprouting of lymphatic capillaries from the primary lymphatic plexus, while deeper lymphatic vessels recruit SMCs and develop lymphatic valves, acquiring a collecting vessel phenotype (reviewed in Kärpanen and Alitalo, 2007). The ephrins and their Eph receptors have been implicated in repulsive axon guidance in the nervous system, and in controlling blood vessel remodeling (reviewed in Adams, 2002; and in Coultas et al., 2005). Mutant mice lacking the PDZ domain of ephrinB2 develop normal blood vasculature, but display hyperplasia of the collecting lymphatic vessels, lack of luminal valve formation, and failure to remodel the primary lymphatic capillary plexus (Makinen et al., 2005). The forkhead transcription factor FoxC2 is highly expressed in the developing lymphatic vessels as well as in lymphatic valves in adults (Dagenais et al., 2004; Petrova et al., 2004). The early development of lymphatic vessels proceeds normally in the absence of *Foxc2*, but the collecting lymphatic vessels in *Foxc2*-/- mice lack valves, whereas the lymphatic capillaries acquire an ectopic coverage by BM components and SMCs, indicating that FoxC2 controls the specification of the lymphatic capillary versus collecting lymphatic vessel phenotype (Petrova et al., 2004).

Lymph nodes

The first lymph nodes begin to develop as protrusions of connective tissue into the lymph sacs at around E12.5. Lymph node induction is initiated by lymphoid tissue inducer (LTi) cells of hematopoietic origin, which express interleukin-7-receptor-α (IL-7Rα), CD45, and CD4, but lack CD3 (Mebius et al., 1997; reviewed in Mebius, 2003). These cells differentiate from CD45-/CD4-/CD3- precursor cells in response to tumor necrosis factor(TNF)-related activation induced cytokine (TRANCE) (Kim et al., 2000a). Signaling via IL-7Rα induces the LTi cells to produce lymphotoxin-α1β2 (LTα1β2), a member of the TNF family, and associate with stromal cells expressing vascular-cell adhesion molecule-1 (VCAM-1) and the LTβ-receptor (LTβR) (Yoshida et al., 2002). Both LTα1β2 and LTβR are absolutely required for lymph node development, which highlights the importance of this signaling pathway in lymphoid organogenesis (De Togni et al., 1994; Rennert et al., 1998).

The lymphoid chemokine CXCL13 activates CXCR5 in the LTi cells, leading to increased CXCL13 production from the surrounding cells in a positive feedback loop, as well as expression of activated α4β1 integrin, a cell-ECM adhesion molecule, in the LTi cells (Ansel et al., 2000; Finke et al., 2002). The stromal cell VCAM-1 activates integrin α4β1, resulting in increased expression of adhesion molecules and secreted chemokines, such as CCL19, CCL21, CXCL12, and CXCL13 (Finke et al., 2002). This will lead to the amplification of both the LTi and the stromal cell populations, and presumably also to the differentiation of resident blood vessels into high-endothelial venules (reviewed in Mebius, 2003). The emergence of these vessels allows T- and B-cells, attracted by CCL 19, CCL21, CXCL12, and CXCL13 signals, to enter the lymph node from the bloodstream (Cyster, 1999; Okada et al., 2002). These chemokines are also required for the organization of the lymph node into B-cell follicles, which become surrounded by T-cell areas (Forster et al., 1996; Forster et al., 1999; Gunn et al., 1999; Ansel et al., 2000; Luther et al., 2000)

Continuous influx of antigen-presenting cells through the afferent lymphatics is required for the maintenance of organized lymph nodes (Mebius et al., 1991). Although lymph nodes are highly plastic organs in adults, it is not known whether lymph nodes can form spontaneously after embryogenesis. Interestingly, artificial lymph nodes composed of collagen scaffolds have been shown to attract lymphocytes, which elicit immune responses in mouse models, suggesting an approach for the replacement of damaged lymph nodes or for

the augmentation of regional immune responses (Okamoto et al., 2007). So-called tertiary lymphoid organs consisting of clonally expanding B-cell follicles and T-cells are commonly found at sites of chronic inflammation. The organization of these structures may involve many of the same chemokines involved in lymph node development (reviewed in Mebius, 2003; and in Drayton et al., 2006).

2. Molecular mechanisms of angiogenesis and lymphangiogenesis

Vascular endothelial growth factors and their receptors

The mammalian vascular endothelial growth factor (VEGF) gene family comprises five dimeric glycoproteins: VEGF, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF). VEGFs bind and activate their cognate high-affinity VEGF-receptor (VEGFR) tyrosine kinases (**Figure 2**), but also interact with neuropilins (NPs) and several integrin class cell adhesion molecules (reviewed in Ferrara et al., 2003; and in Tammela et al., 2005a).

VEGFRs are composed of seven extracellular immunoglobulin (Ig) homology domains, a single transmembrane region, and a C-terminal intracellular tyrosine kinase domain that is interrupted by a kinase-insert domain (Figure 2). Binding of the VEGF ligand induces receptor dimerization and autophosphorylation, as well as C-terminal phosphorylation of the receptors by intracellular tyrosine kinases. This is followed by the docking and subsequent activation of various intracellular downstream signaling molecules. The signals from tyrosine kinase receptors such as VEGFRs typically converge at mitogen-activated protein kinases (MAPKs), actin cytoskeleton-activating, proteins and the serine/threonine kinase Akt, which promote cell proliferation, migration, and survival, respectively (reviewed in Olsson et al., 2006). The outcome of VEGFR signals is context-dependent, i.e. determined by the gene expression profile and the activity of other signaling pathways withing the cell, as well as by the surrounding microenvironment (reviewed in Olsson et al., 2006).

Over the past two decades, the VEGFs and their receptors have been shown to be essential regulators of vasculogenesis, angiogenesis and lymphangiogenesis (reviewed in Ferrara, 2005; and in Tammela et al., 2005a). This signaling system also plays a role in immune function, and recent evidence indicates that it may also regulate neurogenesis and motoneuron survival (Oosthuyse et al., 2001; reviewed in Carmeliet and Tessier-Lavigne, 2005; Greenberg and Jin, 2005; and in Carvalho et al., 2007).

VEGF

VEGF (also known as VEGF-A) was the first member of the *VEGF* gene family to be discovered (Senger et al., 1983; Ferrara and Henzel, 1989; Leung et al., 1989). Later studies demonstrated that VEGF is a ligand for VEGFR-1 and VEGFR-2 (De Vries et al., 1992; Quinn et al., 1993), as well as for NP-1 and NP-2 (Soker et al., 1998; Gluzman-Poltorak et al., 2000), and that it induces EC proliferation, sprouting, migration and tube formation (reviewed in Ferrara, 2005; and in Olsson et al., 2006). VEGF is also a survival factor for ECs during physiological and tumor angiogenesis, as it induces the expression of anti-apoptotic proteins (Benjamin and Keshet, 1997; Gerber et al., 1998). VEGF was originally denoted vascular permeability factor (VPF), as it potently increases blood vascular permeability via VEGFR-2 and Src-mediated phosphorylation of VE-cadherin, and subsequent disintegration of endothelial adherens junction complexes (Senger et al., 1983; reviewed in Bazzoni and Dejana, 2004; and in Gavard and Gutkind, 2006). VEGF also causes vasodilation through the induction of the endothelial nitric oxide synthase (eNOS) and subsequent increase in nitric oxide production, which leads to the relaxation of vascular SMCs (Hood et al., 1998; Kroll and Waltenberger, 1999).

Although VEGF is ubiquitously expressed during embryonic development, the expression is more pronounced at sites of active vasculogenesis and angiogenesis (reviewed in Weinstein, 1999). Mice lacking even a single VEGF allele die by E12.5 from defects in blood island formation, EC development, and vascular assembly, which highlights the central role of VEGF in angiogenesis (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF is strongly induced in hypoxic conditions via hypoxia-inducible factor (HIF)-regulated elements of the VEGF gene promoter (reviewed in Pugh and Ratcliffe, 2003). In hypoxic conditions, the constitutive degradation of HIF-1α is blocked by the inactivation of oxygen-dependent prolyl hydroxylases, followed by stabilization of HIF-1α, its binding to hypoxia-responsive elements in the promoters of hypoxia-inducible genes involved in glucose transport, glycolysis, erythropoiesis and angiogenesis (reviewed in Pugh and Ratcliffe, 2003). Interestingly, VEGF was recently shown to also be induced independently of HIF-1α by the lack of nutrients via induction of the transcriptional coactivator PGC-1α (peroxisome-proliferator-activated receptor-gamma coactivator-1α), a potent metabolic sensor and regulator (Arany et al., 2008).

Overexpression of VEGF or application of recombinant VEGF leads to robust angiogenesis in a multitude of biological model systems (reviewed in Tammela et al., 2005a). Interestingly, adenoviral or transgenic overexpression of VEGF in the skin was shown to induce lymphatic vessel enlargement, but not lymphatic vessel sprouting (Nagy et al., 2002; Saaristo et al., 2002a; Wirzenius et al., 2007). At least in midgestation mouse embryos, VEGF-C but not VEGF had the capacity to induce migration of ECs committed to the lymphatic endothelial lineage (Kärkkäinen et al., 2004).

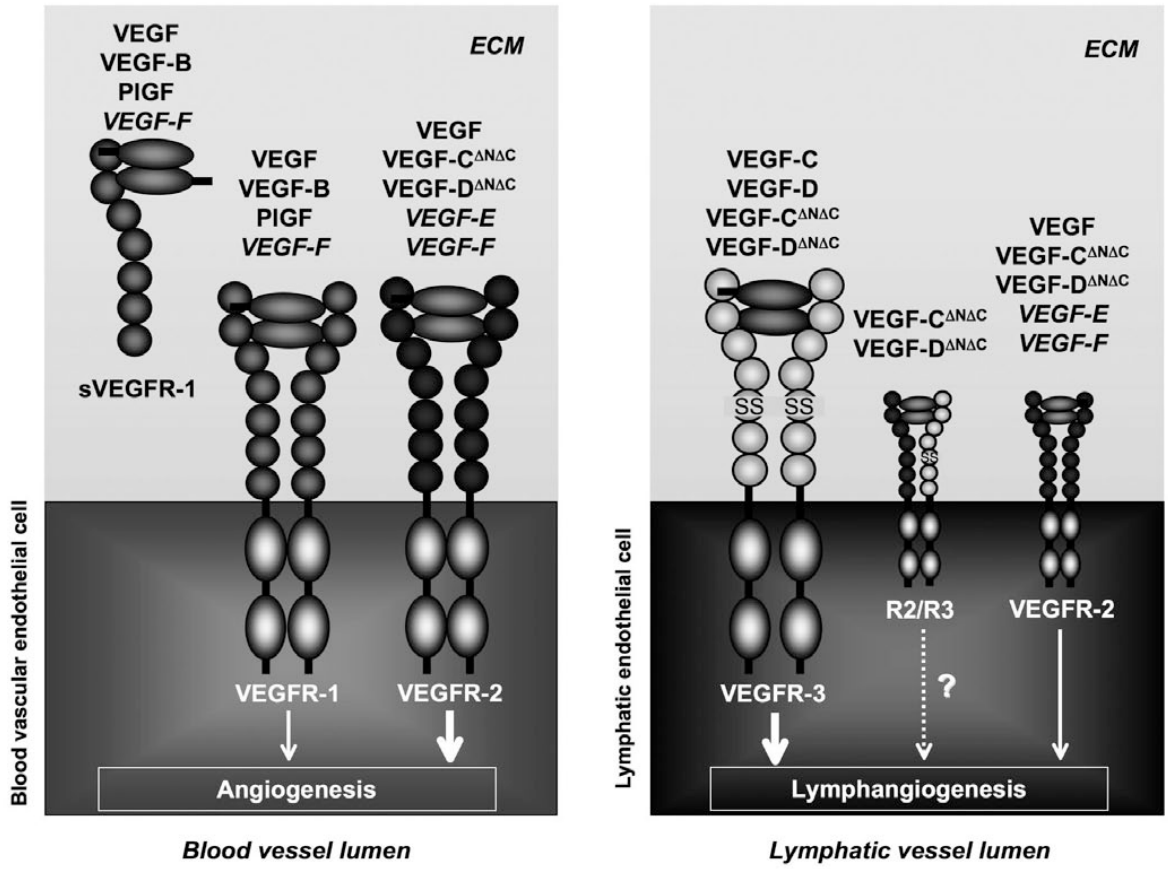


Figure 2. Vascular endothelial growth factors (VEGFs) and VEGF receptors (VEGFRs). Schematic representation of the VEGF family growth factors (ovals), and the VEGFR tyrosine kinases. The VEGFs are antiparallel dimers, which induce dimerization and activation of their cognate receptors upon binding. VEGFRs are composed of seven immunoglobulin-like domains (spheres) and a split tyrosine kinase domain (double ovals). The fifth Ig-like domain of VEGFR-3 is proteolytically cleaved, leaving the fourth and sixth Ig-loops attached by a disulfide bond (SS). In adult tissues, VEGFR-1 and VEGFR-2 are predominantly expressed in blood vascular endothelial cells, whereas VEGFR-3 is expressed in lymphatic vascular endothelium. Low levels of VEGFR-2 are found in lymphatic endothelial cells, and therefore VEGFR-3/VEGFR-2 heterodimers may form in these cells. VEGF-E and VEGF-F (in italics) are not mammalian growth factors, being derived from Orf viruses and snake venoms, respectively. ECM: extracellular matrix; sVEGFR-1: soluble VEGFR-1; $^{\Delta N\Delta C}$: Fully processed forms of VEGF-C and VEGF-D lacking the N- and C-terminal propeptides.

VEGF-C and VEGF-D

VEGF, PlGF and VEGF-B isoforms are formed through alternative splicing, whereas different forms of VEGF-C and VEGF-D are the result of proteolytic processing. Both growth factors are produced as precursor proteins, which are activated by intracellular secretory proprotein convertases (PC) such as furin, PC5, and PC7 (Joukov et al., 1997; Siegfried et al., 2003; McColl et al., 2007). The secreted, disulphide-linked 31/29 kD VEGF-C subunits only bind VEGFR-3, but the factor is further proteolyzed in the extracellular environment by plasmin and other proteases to generate a 21 kD non-disulfide-linked homodimeric protein with high affinity for both VEGFR-2 and VEGFR-3 (Joukov et al., 1997) (**Figure 3**a). VEGF-C induces proliferation, migration, and survival of ECs (reviewed in Tammela et al., 2005b). During development, VEGF-C is expressed predominantly in regions where lymphatic vessels develop (Kukk et al., 1996; Kärkkäinen et al., 2004). In adults, VEGF-C expression remains high in the lymph nodes (Lymboussaki et al., 1999), arterial SMCs (Partanen et al., 2000), and cortical regions of the brain (P. Haiko and T. Tammela, unpublished). Overexpression of VEGF-C potently induces lymphangiogenesis with little angiogenesis as demonstrated by early experiments in the chick chorioallantoic membrane, and in transgenic mice overexpressing VEGF-C in the skin (Jeltsch et al., 1997; Oh et al., 1997). Adenoviral VEGF-C or VEGF-D gene transduction has been shown to induce growth of lymphatic capillaries in several animal models, although the mature form of human VEGF-D also potently promotes angiogenesis at least in rabbit skeletal muscle (Rissanen et al., 2003). Human VEGF-C and VEGF-D also increase blood vascular permeability by activating VEGFR-2 (Veikkola et al., 2001; Saaristo et al., 2002b). Specific activation of VEGFR-3 with a mutant form of VEGF-C (VEGF-C^{C156S}) is sufficient to induce lymphangiogenesis *in vivo* without effects on blood vessels (Joukov et al., 1998; Veikkola et al., 2001; Saaristo et al., 2002b; Wirzenius et al., 2007). Mouse VEGF-D binds only VEGFR-3, suggesting a more limited function for VEGF-D in mice when compared to humans (Baldwin et al., 2001).

Homozygous deletion of *Vegfc* leads to the complete absence of a lymphatic vascular system in mouse embryos, whereas *Vegfc*+/- mice display severe lymphatic hypoplasia, indicating an analogous requirement of VEGF-C for lymphangiogenesis as has been described for VEGF in angiogenesis (Kärkkäinen et al., 2004). In mice, *Xenopus laevis*

tadpoles, and zebrafish where *Vegfc* has been inactivated, LECs initially differentiate in the embryonic cardinal veins, but fail to migrate and form the primary lymph sacs (Kärkkäinen et al., 2004; Ny et al., 2005; Kuchler et al., 2006). In contrast, deletion of *Vegfd* does not affect development of the lymphatic vasculature in mice, although exogenous VEGF-D protein rescues the impaired vessel sprouting in *Vegfc*-/- embryos (Kärkkäinen et al., 2004; Baldwin et al., 2005).

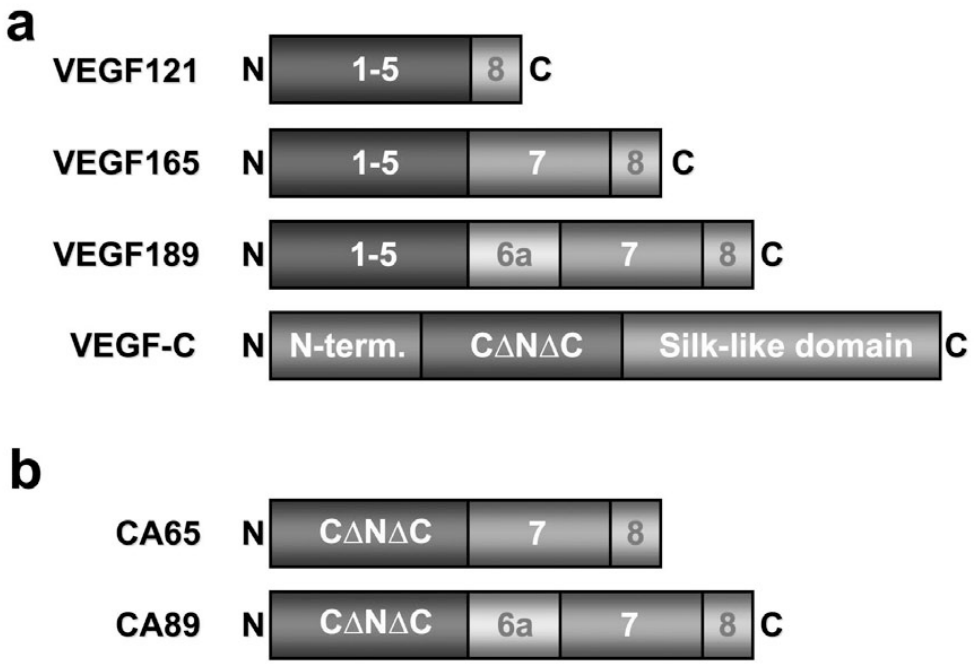


Figure 3. The primary structure of VEGF, VEGF-C, and the VEGF-C/VEGF chimeras used in the study. (**a**) The predominant human VEGF isoforms VEGF121, VEGF165, and VEGF189 are formed as a result of alternative splicing. The numbers refer to VEGF exons. VEGF165 and VEGF189 contain exon 7 with neuropilin and heparin-binding sequences. In addition, VEGF189 contains exon 6a, which increases the affinity of the growth factor towards heparin. VEGF-C is produced as a single prepropeptide, which is proteolytically cleaved in intracellular processing compartments to yield the secreted form that contains an aminoterminal propeptide (N-term.), as well as a carboxyterminal silk-like propeptide. The propeptides are cleaved in the extracellular environment by proteases to yield the mature growth factor, VEGF-CΔNΔC (CΔNΔC). (**b**) The chimeric growth factors have been generated by replacing the receptor-binding domains of VEGF165 and VEGF189 with VEGF-CΔNΔC to generate CA65 and CA89, respectively. These factors bind VEGFR-2 and VEGFR-3, like VEGF-CΔNΔC, but in addition have the capacity to bind heparin and neuropilins (III).

VEGF-C mRNA is induced in tumor cells by a variety of growth factors and oncogenes, but not hypoxia, indicating that VEGF-C does not collaborate with VEGF in the initiation of angiogenic responses (Enholm et al., 1997). On the other hand, VEGF-C is induced in ECs in response to pro-inflammatory cytokines (Ristimäki et al., 1998), suggesting that VEGF-C could regulate lymphatic vessel function during inflammation, possibly reflecting the role of the lymphatic vasculature in the control of immune function and leukocyte trafficking. Accordingly, both VEGF-C and VEGF-D are produced by leukocytes, particularly macrophages, at sites of inflammation (Baluk et al., 2005).

Placenta growth factor and VEGF-B

Placenta growth factor (PlGF) and VEGF-B homodimers bind VEGFR-1 and NP-1, and *Plgf* or *Vegfb* gene-targeted mice survive and lead apparently normal lives (Bellomo et al., 2000; Carmeliet et al., 2001a). However, *Plgf* null mice recover poorly from experimental myocardial infarction, and show impaired collateral formation in response to hind limb ischemia (Carmeliet et al., 2001a). Overexpression of PlGF in the skin of transgenic mice results in marked hyperplasia of cutaneous blood vessels with increased inflammatory and permeability responses, while local administration of PlGF potently promotes arteriogenesis (Luttun et al., 2002; Odorisio et al., 2002; Oura et al., 2003; Pipp et al., 2003). The effects of PlGF are thought to be mediated by displacement of VEGF from VEGFR-1, leading to more VEGF available for the activation of VEGFR-2, and by the recruitment of VEGFR-1 expressing monocytes/macrophages, which are also important for the development of tumor stroma (Carmeliet et al., 2001b; Pipp et al., 2003; Fischer et al., 2007). The biological role of VEGF-B is poorly characterized. Overexpression of VEGF-B does not lead to angiogenesis or to the recruitment of bone-marrow-derived cells. However, as VEGF-B is highly expressed in striated muscle, myocardial muscle, and brown fat, its function may be linked to high cellular energy metabolism (Enholm et al., 1997; Salven et al., 1998).

VEGFR-1

VEGFR-1 (fms-like tyrosine kinase-1, Flt1) transmits weak mitogenic signals in ECs, but it can heterodimerize with VEGFR-2, forming a complex with altered signaling properties when compared to VEGFR-1 or VEGFR-2 homodimers (Fong et al., 1995; Carmeliet et al., 2001b; Huang et al., 2001). VEGFR-1 is expressed in ECs and in monocytes/macrophages (Zachary and Gliki, 2001). In fact, VEGFR-1 mediated angiogenesis and arteriogenesis have been shown to be dependent on monocytes, and angiogenesis in experimental tumors is at least partially inhibited by anti-VEGFR-1 antibodies (Luttun et al., 2002; Pipp et al., 2003). *Vegfr1* gene targeted mice die between E8.5 and E9.5 due to disorganization of blood vessels and excessive commitment of mesenchymal stem cells to the hemangioblast lineage (Fong et al., 1995; Fong et al., 1999). Mice lacking only the intracellular tyrosine kinase domain of VEGFR-1 are normal except for impaired angiogenesis during pathological conditions, suggesting a VEGF-trapping function at least during the early stages of vascular development (Hiratsuka et al., 1998; Hiratsuka et al., 2001). The naturally occurring soluble VEGFR-1 ectodomain (sVEGFR-1) potently blocks VEGF-induced angiogenesis (Carmeliet et al., 2001b; Gerhardt et al., 2003). High levels of circulating sVEGFR-1 correlate with the incidence of pre-eclampsia, a syndrome where hypertension arises in pregnancy in association with significant proteinuria (Levine et al., 2004; Levine et al., 2006).

VEGFR-2

VEGFR-2 is also known as fetal liver kinase-1 (Flk1) and kinase-insert domain receptor (KDR) (reviewed in Ferrara et al., 2003). The binding affinity of VEGF towards VEGFR-2 is approximately 10-fold lower than for VEGFR-1, yet VEGFR-2 is the primary receptor transducing VEGF signals in ECs, including the induction of vascular permeability responses (Meyer et al., 1999; Wise et al., 1999; Gille et al., 2001). VEGF-E (viral VEGF) is a VEGFR-2 specific ligand that is encoded by the parapoxviruses Orf-NZ2 and Orf-NZ7 (Ogawa et al., 1998; Meyer et al., 1999). Certain viper venoms contain VEGF-like proteins, collectively termed as VEGF-F, which bind both VEGFR-2 and VEGFR-1 (Suto et al., 2005;

Tokunaga et al., 2005; Yamazaki et al., 2005a; Yamazaki et al., 2005b). VEGF-E and VEGF-F lack the C-terminal heparin-binding domain found in other heparin-binding VEGFs, and differ in the structure of their receptor binding domain from other VEGFs (Ogawa et al., 1998; Meyer et al., 1999; Suto et al., 2005).

VEGFR-2 gene targeted mice die at E8.5-E9.5 due to lack of development of the blood islands, embryonic vasculature and hematopoietic cells (Shalaby et al., 1995; Gille et al., 2001). VEGFR-2 expression is downregulated in the adult blood vascular ECs, but low levels of VEGFR-2 and autocrine VEGF signaling are required for EC survival even in quiescent vessels (Partanen et al., 1999; Lee et al., 2007). Furthermore, constitutive VEGFR-2 expression maintains ECs responsive to angiogenic VEGF signals, which upregulate VEGFR-2 in the endothelium (Gerhardt et al., 2003; Suchting et al., 2007). During angiogenic sprouting, VEGFR-2 is particularly strongly expressed in the endothelial tip cells, rendering the tip cells most sensitive to VEGF signals (Gerhardt et al., 2003).

Low levels of VEGFR-2 are also found in lymphatic endothelium, but VEGFR-2 signals alone are not sufficient for inducing sprouting lymphangiogenesis (Nagy et al., 2002; Saaristo et al., 2002a; Wirzenius et al., 2007). In fact, careful dissection of the VEGFR-2 and VEGFR-3 pathways utilizing receptor-specific ligands and VEGFR-blocking antibodies demonstrated that VEGFR-3 signals are required for lymphatic vessel sprouting, whereas VEGFR-2 activation leads to circumferential enlargement of the vessels (Wirzenius et al., 2007).

VEGFR-3

Following biosynthesis, VEGFR-3 (fms-like tyrosine kinase 4, Flt4) undergoes proteolytic cleavage of the fifth Ig-homology domain, and the resulting polypeptide chains remain linked via a disulfide bond (Figure 2) (Pajusola et al., 1994; Lee et al., 1996). VEGFR-3 can form heterodimers with VEGFR-2 upon stimulation with the mature forms of VEGF-C and VEGF-D, which may lead to unique downstream signals due to asymmetry between the intracellular domains of the two receptors (Dixelius et al., 2003; reviewed in Olsson et al., 2006). VEGFR-3 is present in all endothelia during development but in the adult it becomes restricted to LECs and fenestrated blood vessels in endocrine organs such as

the thyroid, the adrenal glands, and pancreas (Partanen et al., 2000). However, VEGFR-3 is upregulated in blood vascular ECs in pathological conditions such as in tumors and in wounds (Valtola et al., 1999; Paavonen et al., 2000; Bando et al., 2004; Grau et al., 2007). Interestingly, blood vascular VEGFR-3 expression has been demonstrated to positively correlate with a high tumor grade (Grau et al., 2007). *Vegfr3* gene-targeted mice exhibit embryonic lethality at E9.5 from defective arterio-venous remodeling of the primary vascular plexus and disturbed hematopoiesis (Dumont et al., 1998; Hamada et al., 2000). Correspondingly, knock-down of the VEGFR-3 homologue *Flt4* in zebrafish using morpholino oligonucleotides results in defective segmental artery morphogenesis (Covassin et al., 2006). Transgenic mice overexpressing a soluble VEGFR-3 immunoglobulin G VEGFR-3-Ig fusion protein in the skin from E14.5 onwards lack dermal lymphatic vessels and have hypoplastic deeper lymphatic vessels, but blood vessels in these mice appear normal (Makinen et al., 2001a), suggesting that VEGFR-3 signaling is not required for expansion of the blood vascular networks after organogenesis. Missense mutations in VEGFR-3 have been linked to hereditary lymphedema in humans, as well as in a mouse model of lymphedema (Irrthum et al., 2000; Kärkkäinen et al., 2000; Kärkkäinen et al., 2001).

VEGFR-3 is also expressed in a subpopulation of monocytes/macrophages, and VEGF-C has been shown to promote homing of these cells into tumors and wounds (Skobe et al., 2001b; Saaristo et al., 2006). Interestingly, VEGFR-3 is also expressed in antigen-presenting dendritic cells, and blocking VEGFR-3 suppresses the induction of corneal alloimmunity by inhibiting trafficking of these cells (Hamrah et al., 2003; Chen et al., 2004).

Neuropilins

Neuropilins (NP-1 and NP-2) are transmembrane receptor glycoproteins that do not have enzymatic activities (Takagi et al., 1991; Kolodkin et al., 1997). Neuropilins bind class 3 semaphorins, which are secreted proteins that mediate repulsive signals during neuronal axon guidance, but they also function as receptors for certain VEGFs, and thereby play a role in angiogenesis as well as lymphangiogenesis (reviewed in Carmeliet and Tessier-Lavigne, 2005; and in Klagsbrun and Eichmann, 2005). NP-1 acts as a co-receptor enhancing VEGF-VEGFR-2 interactions, while it also forms complexes with VEGFR-1 (Soker et al., 2002). *Np1* gene-targeted mice die at E13.5 from vascular defects such as insufficient development of yolk sac vascular networks, deficient neural vascularization, and transposition of large vessels (Kawasaki et al., 1999). Interestingly, NP-1 is required for the guidance of angiogenic sprouts (Gerhardt et al., 2004), suggesting that maximal sensing of VEGF by the endothelial tip cells is crucial for the formation and patterning of vascular networks (Gluzman-Poltorak et al., 2000; Soker et al., 2002).

NP-2 is expressed in veins and lymphatic vessels, and *Np2* mutant mice exhibit lymphatic capillary hypoplasia (Yuan et al., 2002). Interestingly, both VEGF-C and VEGF-D bind to NP-2, which co-internalizes with VEGFR-3 upon ligand stimulation (Kärpänen et al., 2006a). This indicates an analogy between the VEGF/NP-1/VEGFR-2 and the VEGF-C/NP-2/VEGFR-3 signaling pathways in angiogenesis and lymphangiogenesis, respectively, and suggests that NP-2 may be important in lymphatic vessel sprouting.

Notch signaling

The Notch signaling pathway regulates cell fate specification, growth, differentiation, and patterning processes in multicellular organisms. In mammals, four Notch receptors (Notch1–Notch4) interact with five membrane-bound ligands, Delta-like 1 (Dll1), Dll3, Dll4, Jagged1, and Jagged2 (reviewed in Roca and Adams, 2007). Both receptors and ligands are EGF repeat-containing transmembrane proteins (Weinmaster, 2000; Lai, 2004; Le Borgne et al., 2005; Bray, 2006; Hurlbut et al., 2007). In analogy with ephrins, the Notch ligands also contain an intracellular PDZ-binding domain that is conserved in evolution, suggesting the possibility of retrograde signaling.

Upon ligand binding, Notch becomes susceptible to processing by the intracellular protease γ-secretase (presenilin), which releases the Notch intracellular domain (NICD) (Weinmaster, 2000; Selkoe and Kopan, 2003; Schweisguth, 2004; reviewed in Nichols et al., 2007). Following translocation of the NICD into the nucleus, its interaction with the DNA-binding protein CSL (RBP-J) and the coactivator MAML-1 leads to the displacement of corepressor proteins and associated chromatin-modifying factors from CSL, which triggers the transcription of Notch target genes such as the basic helix–loop–helix (bHLH) proteins Hes1, Hey1 (Hesr1), and Hey2 (Hesr2), which typically act as repressors of downstream genes (Lai, 2002; Fischer and Gessler, 2003; Iso et al., 2003; reviewed in Ehebauer et al., 2006).

Notch signaling plays a role in arterial specification, and several Notch receptors and ligands are expressed specifically in arteries in mice and in zebrafish (Shutter et al., 2000; Mailhos et al., 2001; Lawson et al., 2001; Leslie et al., 2007; Siekmann and Lawson, 2007). Furthermore, Notch1 signaling activated by Dll1 is critical for postnatal arteriogenesis (Limbourg et al., 2007; Takeshita et al., 2007). In contrast, the orphan nuclear receptor COUP-TFII promotes venous EC differentiation by suppressing Notch signaling during development (You et al., 2005). Disruption of Notch signaling in zebrafish leads to loss of arterial markers such as ephrinB2, and ectopic expression of EphB4, an ephrinB2 receptor that is prominently expressed in veins (Lawson et al., 2001). Conversely, ectopic activation of the Notch pathway represses expression of *Efb4* (EphB4) and *Flt4* (VEGFR-3) and thereby imposes a more artery-like gene expression profile on veins (Lawson et al., 2001), although

NICD was recently shown to bind to the *VEGFR3* promoter and induce VEGFR-3 expression in mammals (Shawber et al., 2007).

The formation of the tracheal system in *Drosophila melanogaster* embryos by growth factor-induced guided migration and self-limiting branching of the tracheal epithelial cells has served as a model for the formation of vascular networks in higher organisms (Zelzer and Shilo, 2000; Ghabrial and Krasnow, 2006). In *Notch* mutant *Drosophila* embryos, an excess of cells attempt to lead the tracheal branches and compete for the lead position, whereas expression of constitutively active Notch prevents outgrowth due to the lack of leading tip cells (Ikeya and Hayashi, 1999; Llimargas, 1999). The Dll–Notch signal also prevents the stalk epithelium from becoming terminal or fusion cells (Steneberg et al., 1999; Zelzer and Shilo, 2000).

Notch signals play a strikingly similar role in the specification of endothelial tip cells, which express the Notch ligand Dll4, and stalk cells, in which Notch signaling becomes activated (Hellstrom et al., 2007; Roca and Adams, 2007). *Dll4* gene-targeted mouse embryos die by E10.5 due to severe defects in arterial-venous remodeling of the embryonic vasculature, whereas the *Dll4* heterozygous embryos have a similar, but attenuated phenotype, and a small proportion survive to adulthood (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). The *Dll4* heterozygous mice and mice treated with Dll4-blocking antibodies or small-molecular γ-secretase inhibitors exhibit excessive numbers of tip cells (Noguera-Troise et al., 2006; Ridgway et al., 2006; Hellstrom et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). Furthermore, disruption of the Notch downstream signaling component *Rbpsuh* in zebrafish results in increased sprouting and high *Flt4* expression (Siekmann and Lawson, 2007). These studies indicate that Dll4 is the key Notch ligand in the process, and Dll4/Notch appears to limit tip cell behavior in the stalk cells by suppressing VEGFR-2 and inducing VEGFR-1, rendering the stalk cells less responsive to VEGF than the tip cells, which express low levels of VEGFR-1 and high levels of VEGFR-2 (Ridgway et al., 2006; Hellstrom et al., 2007; Lobov et al., 2007; Suchting et al., 2007). Befitting this role, VEGF has been shown to induce Dll4 in ECs (Liu et al., 2003).

The angiopoietin/Tie system

The angiopoietin (Ang) family of growth factors includes Ang1 and Ang2, as well as Ang3 and Ang4, which are mouse and human orthologues, respectively (Suri et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). The angiopoietins bind to the Tie2 receptor tyrosine kinase, expressed almost exclusively in ECs, and regulate interactions between ECs and pericytes (reviewed in Thurston, 2003; Brindle et al., 2006; and in Shim et al., 2007). The Tie receptors (Tie1 and Tie2) are composed of an extracellular domain containing three immunoglobulin homology domains, three EGF-like repeats, three fibronectin-type III-like repeats, a transmembrane domain, and a split tyrosine kinase domain (**Figure 4**) (Partanen et al., 1992; Dumont et al., 1993; Ziegler et al., 1993). Ang1 and Ang3/Ang4 are obligate agonists of Tie2, whereas Ang2 can act either as an agonist or an antagonist, depending on the cell type and the surrounding microenvironment (Davis et al., 1997; Maisonpierre et al., 1997; Teichert-Kuliszewska et al., 2001). Tie2 activation promotes EC survival and migration (reviewed in Thurston, 2003; Brindle et al., 2006; and in Shim et al., 2007). Ang1 and Ang4 were shown to stimulate the activation of Tie1, a Tie2 homologue with a highly similar expression pattern, although the presence of Tie2 is required for maximal Tie1 phosphorylation (Saharinen et al., 2005) .

All angiopoietins share a similar overall structure with a short amino-terminal motif followed by a coiled-coil domain and carboxyterminal fibrinogen-like domain (Figure 4) (Suri et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). Tie2 binding is mediated by the fibrinogen-like domain, while the short supercoiling domain (SCD) within the coiled-coil domain is responsible for the formation of angiopoietin homodimers and higher order homo-oligomers (Procopio et al., 1999; Barton et al., 2005; Barton et al., 2006). The linker peptide between the two major domains of Ang1 has been shown to associate Ang1 with the extracellular matrix, rendering Ang1 less soluble in tissues when compared to Ang2 (Thurston et al., 2000; Xu and Yu, 2001).

Ang1 expression in the mouse embryo occurs first in the myocardium, and later in a more widespread manner around the developing vessels (Davis et al., 1997). Ang2 is expressed in the embryonic dorsal aorta and the major aortic branches, and in adults in tissues that are undergoing vascular remodeling (Maisonpierre et al., 1997; Gale et al., 2002). Gene targeting experiments have indicated that Ang1 is necessary for maintaining maximal

interactions between ECs and pericytes and the ECM (Suri et al., 1996). Loss of *Tie2* recapitulates the *Angpt1* null phenotype, with embryonic lethality at E12.5 due to vessel rupture and lack of periendothelial support, whereas *Tie1* gene-targeted mice succumb to similar defects by E14.5 (Puri et al., 1995; Sato et al., 1995; Partanen et al., 1996). Ang1 promotes the integrity of EC monolayers in culture (Wang et al., 2004; Gavard et al., 2008), and exogenously provided Ang1 prevents leakage of plasma components into the interstitium caused by potent vascular permeability agents, such as VEGF, in adult tissues (Thurston et al., 2000). A recent report has indicated that this activity occurs through the suppression of Src activation (Gavard et al., 2008). Conversely, release of Ang2 from Weibel-Palade bodies in ECs upon stimulation with proinflammatory cytokines leads to rapid destabilization of the endothelium and extravasation of plasma components (Fiedler et al., 2004; Fiedler et al., 2006b).

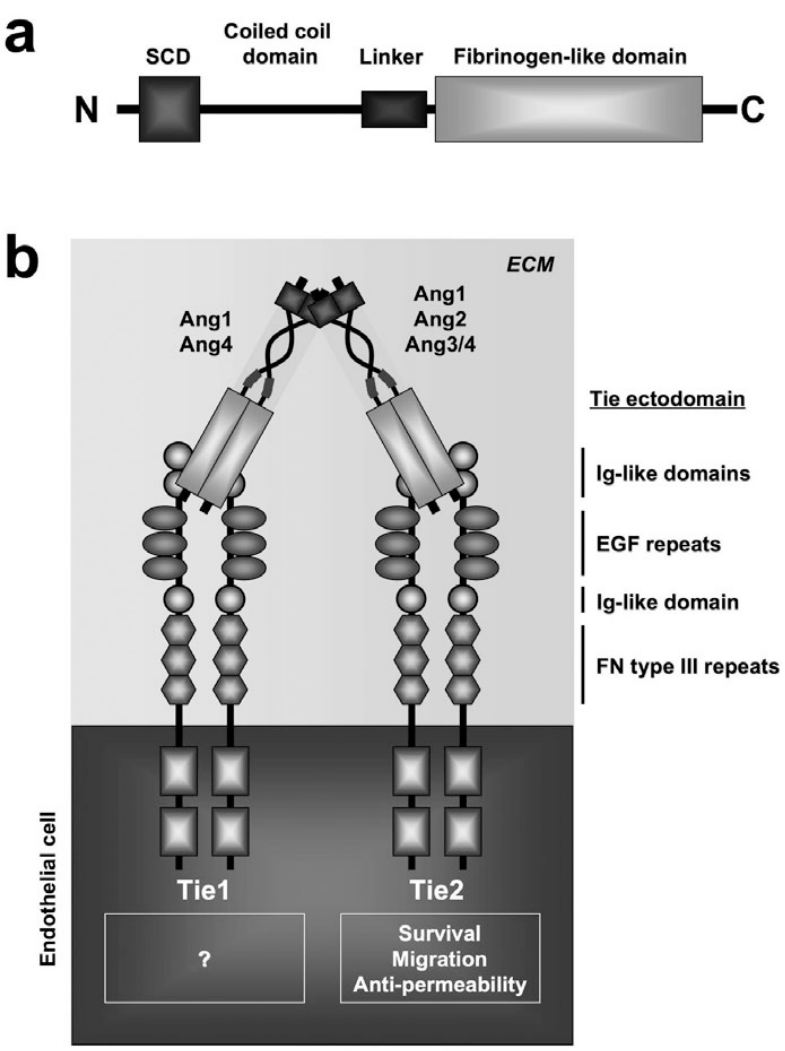


Figure 4. The angiopoietins and the Tie receptors. (**a**) The domain structure of angiopoietin-1 (Ang1). Ang1 migrates at 70 kDa. The N-terminal part of the coiled-coil domain contains the superclustering domain (SCD) responsible for formation of Ang homodimers and higher order oligomers. Ang1 must form at least tetramers in order to cluster and activate Tie receptors. The linker domain associates Ang1 with the extracellular matrix. The C-terminal fibrinogen-like domain is required for the activation of Tie receptors. (**b**) Tie1 and Tie2 are composed of two Ig-loops, followed by three EGF-like repeats, an Ig-loop, three fibronectin (FN) type III repeats, a single transmembrane domain and an intracellular tyrosine kinase domain (double rectangles) that is interrupted by a kinase-insert. Tie2 signals mediate endothelial cell survival and migration, as well as counteract vascular permeability responses.
ECM: extracellular matrix.

Abundant evidence suggests that members of the angiopoietin and VEGF families collaborate during different stages of angiogenesis. Ang2 is expressed at sites of pericyte detachment and blood vessel remodeling in conjunction with VEGF, whereas in the absence of VEGF, Ang2 activity leads to EC apoptosis (Maisonpierre et al., 1997; Goede et al., 1998; Holash et al., 1999). In addition, factors that induce angiogenesis *in vivo*, such as hypoxia and VEGF, have been shown to upregulate Ang2 in ECs (Mandriota and Pepper, 1998).

The role of angiopoietins in lymphangiogenesis has remained unclear, although Tie2 and Tie1 mRNA and protein have been detected at least in cultured LECs (Kriehuber et al., 2001; Makinen et al., 2001b). Ang2 knockout mice have defects in regression of hyaloid blood vessels as well as lymphatic vessel maturation, suggesting that Ang2 may be needed for lymphatic vessel stabilization (Gale et al., 2002). Notably, replacement of the Ang2 gene with a cDNA encoding Ang1 was sufficient to rescue the lymphatic phenotype but not the blood vascular phenotype (Gale et al., 2002).

Endothelial cell – pericyte interactions

Stabilization of the endothelium requires pericyte recruitment, and the lack of pericytes leads to EC hyperplasia, formation of aberrant EC-EC junctions, vessel rupture and embryonic lethality (reviewed in Armulik et al., 2005; and in von Tell et al., 2006). The *de novo* induction of vascular SMCs around the first blood vessels is stimulated by transforming growth factor-ß (TGF-ß) and possibly other factors that remain to be characterized. Genetic inactivation of *Tgfb1* in mice and genes encoding its receptors, activin-receptor-like kinase-1 (*Alk1*), *Alk5,* TGF-ß receptor II (*tßrII*), and *Eng* (endoglin, a TGF-ß co-receptor), as well as its downstream effector *Smad5* all lead to comparable cardiovascular defects and embryonic lethality (Dickson et al., 1995; Oshima et al., 1996; Li et al., 1999; Yang et al., 1999; Oh et al., 2000; Urness et al., 2000; Larsson et al., 2001). TGF-ß has context-dependent effects on ECs, as signaling through ALK1/Smad1/5 promotes EC proliferation, whereas ALK5/Smad2/3 stimulates differentiation (Goumans et al., 2002). TGF-ß signaling in ECs promotes TGF-ß expression, synthesis, and release by these cells, which, in turn, induces differentiation of SMCs from surrounding mesenchymal cells, but also auto-induces TGF-ß expression in the ECs themselves (Carvalho et al., 2004). The role of TGF-ß signaling in

lymphangiogenesis and lymphatic vessel maturation is not known, although it is conceivable that this pathway is involved in the differentiation of the SMCs surrounding the collecting vessels.

Platelet-derived growth factor-B (PDGF-B) signaling via PDGF-receptor-ß (PDGFR-ß) plays a critical role in the recruitment of pericytes to newly formed vessels (reviewed in Hoch and Soriano, 2003; and in Betsholtz, 2004). During angiogenesis, sprouting ECs secrete PDGF-B, which signals through PDGFR-ß expressed by pericytes, resulting in proliferation and migration of pericytes during vessel maturation (reviewed in Armulik et al., 2005). Loss of *Pdgfb* or *Pdgfrb* leads to similar phenotypes and perinatal death caused by vascular dysfunction due to the lack of pericytes (Leveen et al., 1994; Lindahl et al., 1997; Crosby et al., 1998; Hellstrom et al., 1999). Gene-targeting studies have demonstrated that PDGF-B/PDGFR-ß signaling is not required for the induction of pericytes, but it is crucial for the expansion of the pericyte population (Hellstrom et al., 1999). The ectopic SMC coverage observed in lymphatic capillaries of *Foxc2* null mice is presumably due to failure in suppression of *Pdgfb* transcription in LECs (Petrova et al., 2004).

Sphingosine-1-phosphate (S1P) is a secreted sphingolipid synthesized by sphingosine-1-kinase (S1K) in ECs, and involved in cell-cell communication through five G-protein coupled receptors ($S1P_1$-$S1P_5$, also known as EDG1-EDG5) (Shu et al., 2002; Limaye et al., 2005). $S1P_1$, $S1P_2$ and $S1P_3$ are expressed in SMCs/pericytes, and single or compound inactivation of these genes leads to embryonic lethality with severe defects in vascular pericyte investment (Liu et al., 2000b; Kono et al., 2004). S1P1 signaling through the small G-protein Rac promotes trafficking of N-cadherin to polarized plasma membrane domains in ECs, which enforces contacts with mural cells (Paik et al., 2004). N-cadherin-based adherence junctions are located at peg–socket contacts between ECs and pericytes (Gerhardt et al., 2000; reviewed in Armulik et al., 2005; and in von Tell et al., 2006), and N-cadherin is functionally important for the maintenance of these junctions (Gerhardt et al., 2000; Paik et al., 2004). Interestingly, Ang1 was recently shown to activate S1K and stimulate S1P production, suggesting that Ang1 produced by the pericytes can enhance EC-pericyte interactions via this pathway along with having direct effects on ECs (Li et al., 2008). Furthermore, Ang1 has been reported to stimulate pericyte recruitment by inducing heparin-binding epidermal growth factor (HB-EGF) in ECs, although *Hb-egf/Egfr* gene-targeted mice do not display pericyte defects (Iivanainen et al., 2003; Iwamoto et al., 2003).

The extracellular matrix

ECs in quiescent blood vessels are insulated from the surrounding tissue environment by a basement membrane (BM), which is composed of type IV and XVIII collagens, laminin, fibronectin, nidogen (entactin), and heparan sulfate proteoglycans (HSPGs) (reviewed in Davis and Senger, 2005). The BM is required for the stability of blood vessels (Thyboll et al., 2002), as well as for the polarization of ECs (Drake et al., 1995; reviewed in Davis and Senger, 2005). Interestingly, lymphatic capillary ECs express a truncated laminin-α chain that is unable to assemble into networks, which may explain the very sparse BM surrounding lymphatic capillaries (Vainionpää et al., 2007).

At the onset of angiogenic sprouting, ECs utilize a number of proteases to invade through the BM (Pepper, 2001; Davis et al., 2002; Heissig et al., 2003), and become exposed to type I collagen, which stimulates tube formation and vessel morphogenesis (reviewed in Davis and Senger, 2005). The ECM acts as a reservoir for HSPG-binding growth factors, which can be released by proteolysis (reviewed in Lundkvist et al., 2007). PDGF-B produced by sprouting ECs is bound to HSPGs at the EC surface, which provides a high local growth factor concentration for optimal pericyte guidance (reviewed in Armulik et al., 2005; and in von Tell et al., 2006). In fact, loss of the HSPG retention motif in PDGF-B, or defective N-sulfation of HSPGs leads to impaired pericyte recruitment, which highlights the importance of the ECM in generating growth factor gradients and limiting their effects to the local microenvironment (Lindblom et al., 2003; Nystrom et al., 2006; Abramsson et al., 2007; reviewed in Lundkvist et al., 2007).

Three predominant VEGF isoforms of variable amino acid number, VEGF121, VEGF165, and VEGF189, are produced through alternative splicing (reviewed in Robinson and Stringer, 2001; and in Ferrara et al., 2003). After secretion, VEGF121 is freely diffusible in tissues, and does not bind to the neuropilins, while larger forms of VEGF retain the ability to bind NP-1 and NP-2, and are progressively less diffusible as their molecular weight increases (Soker et al., 1998; Gluzman-Poltorak et al., 2000). Except for VEGF121, all the larger forms of VEGF contain a heparin-binding domain (HBD) encoded by exon 6a and/or exon 7, and remain bound to the cell surface and the ECM (Gitay-Goren et al., 1996). The heparin-binding capacity in VEGF exon 7 is conveyed by a cationic polypeptide sequence (44 amino acids) (Poltorak et al., 1997), whereas other parts of the domain enable its binding

to NP-1 (Soker et al., 1996). VEGF165 contains the exon 7-encoded domain, and binds to cell surface HSPGs with intermediate affinity, while VEGF189 contains both exon 6a- and exon7-endcoded domains, remaining almost completely sequestered by HSPGs in the ECM (Figure 3a) (reviewed in Lundkvist et al., 2007). Other members of the VEGF family that contain a HBD include VEGF-B167 and PlGF-2 (Hauser and Weich, 1993; Maglione et al., 1993; Mäkinen et al., 1999).

Optimal three-dimensional distribution of VEGF isoforms is required for the guided migration of endothelial tip cells, which lead the outgrowth of blood vessel sprouts during angiogenesis (Ruhrberg et al., 2002; Gerhardt et al., 2003). Expression of a protease-resistant mutant form of VEGF in tissues was shown to result in excessive vascular sprouting, indicating that, besides mRNA splicing, post-translational modifications of VEGF in the extracellular environment are important for regulating its activity and bioavailability (Houck et al., 1992; Lee et al., 2005). Interestingly, HSPGs provided by pericytes *in trans* prolong VEGF-mediated activation of VEGFR-2, and suffice for normal VEGF signaling in ECs (Jakobsson et al., 2006). VEGF-C and VEGF-D do not bind heparan-sulfate (Joukov et al., 1996; Achen et al., 1998). However, the C-terminal domains of these factors are homologous to silk proteins, and thus may aggregate into stabile fibrils in the extracellular environment (Figure 3a) (reviewed in Dicko et al., 2006).

Integrins are dimeric membrane-bound adhesion molecules composed of α and β subunits, which attach cells to the ECM via focal-adhesion complexes that are active in cell survival and motility signaling (reviewed in Schlaepfer and Mitra, 2004; Mitra and Schlaepfer, 2006; and in Romer et al., 2006). Interestingly, integrins physically interact with several growth factors and receptors that stimulate angiogenesis and lymphangiogenesis (reviewed in Davis and Senger, 2005; Brindle et al., 2006; and in Kärpänen and Alitalo, 2007). *In vitro* studies show that VEGFR-2 may be involved in integrin-dependent migration of ECs, as it forms a complex with integrin αvβ3 upon binding VEGF (Soldi et al., 1999; Hutchings et al., 2003). Furthermore, upon binding to matrix fibronectin, ß1 integrin interacts with VEGFR-3 and induces weak activation of its tyrosine kinase, whereas integrin α5ß1 potentiates VEGFR-3 activation in the presence of VEGF-C (Wang et al., 2001; Zhang et al., 2005a). Integrin α9 binds VEGF-C and inactivation of *Itga9* in mice results in accumulation of lymphatic fluid in the thoracic cavity (chylothorax), although the mechanism remains unresolved (Huang et al., 2000; Vlahakis et al., 2005). Furthermore, Kaposi sarcoma herpes

virus envelope glycoprotein gB can activate both VEGFR-3 and α3ß1 integrin, which results in increased EC proliferation and migration (Zhang et al., 2005b). In addition, Ang1 can bind several different integrins, including α2ß1, α5ß1, αvß3, and αvß5 (Carlson et al., 2001; Cascone et al., 2005; Dallabrida et al., 2005; Weber et al., 2005). The abundance of interactions between integrins and endothelial-specific tyrosine kinase receptors indicate that integrin function is required for EC migration and adhesion during ligand-activated angiogenesis.

3. Angiogenesis and lymphangiogenesis in human disease - the therapeutic horizon

Insufficient or undesired angiogenesis is involved in a plethora of human diseases, and future pro- or anti-angiogenic therapies are estimated to benefit approximately 500 million people worldwide (reviewed in Carmeliet, 2005). Awareness on the role of the lymphatic vascular system in human disease is growing, as connections between the lymphatics and tumor metastasis, edema formation, transplant rejection, fat metabolism, and wound healing continue to be made (Paavonen et al., 2000; Kerjaschki et al., 2004; Alitalo et al., 2005; Harvey et al., 2005; Saaristo et al., 2006). Angiogenesis and lymphangiogenesis are particularly important for tumor growth and metastasis, respectively. An overview of the strategies available for blocking the function of angiogenic/lymphangiogenic molecules is given in **Figure 5**.

Tumor progression

Tumor angiogenesis

Growing tumors rely on sustained angiogenesis for the delivery of oxygen and nutrients (reviewed in Folkman, 1971; and in Hanahan and Weinberg, 2000). Neoplastic lesions are unable to grow beyond a small size without engaging a gene expression program that initiates angiogenesis, termed “the angiogenic switch” (Folkman et al., 1989; reviewed in Ferrara and Kerbel, 2005). Blood vessels in tumors lack hierarchial organization and are leaky, leading to sluggish blood flow and high interstitial fluid pressure within the tumor (reviewed in Jain, 2003; McDonald and Choyke, 2003; and in Jain, 2005). Hypoperfusion within the tumor perpetuates hypoxia and VEGF production, while high intratumoral fluid pressure hampers the delivery of therapeutic agents (reviewed in Jain, 2003; and in Jain, 2005). As tumor growth is dependent on angiogenesis, and as vascular cells, unlike tumor cells, are less likely to become resistant to therapeutics, targeting the tumor vasculature is an attractive strategy to treat cancer patients (reviewed in Folkman, 1971; and in Hanahan and Weinberg, 2000). On the other hand, it is conceivable that anti-angiogenic therapies promote the dedifferentiation of tumor cells by increasing hypoxic stress (reviewed in Axelson et al., 2005).

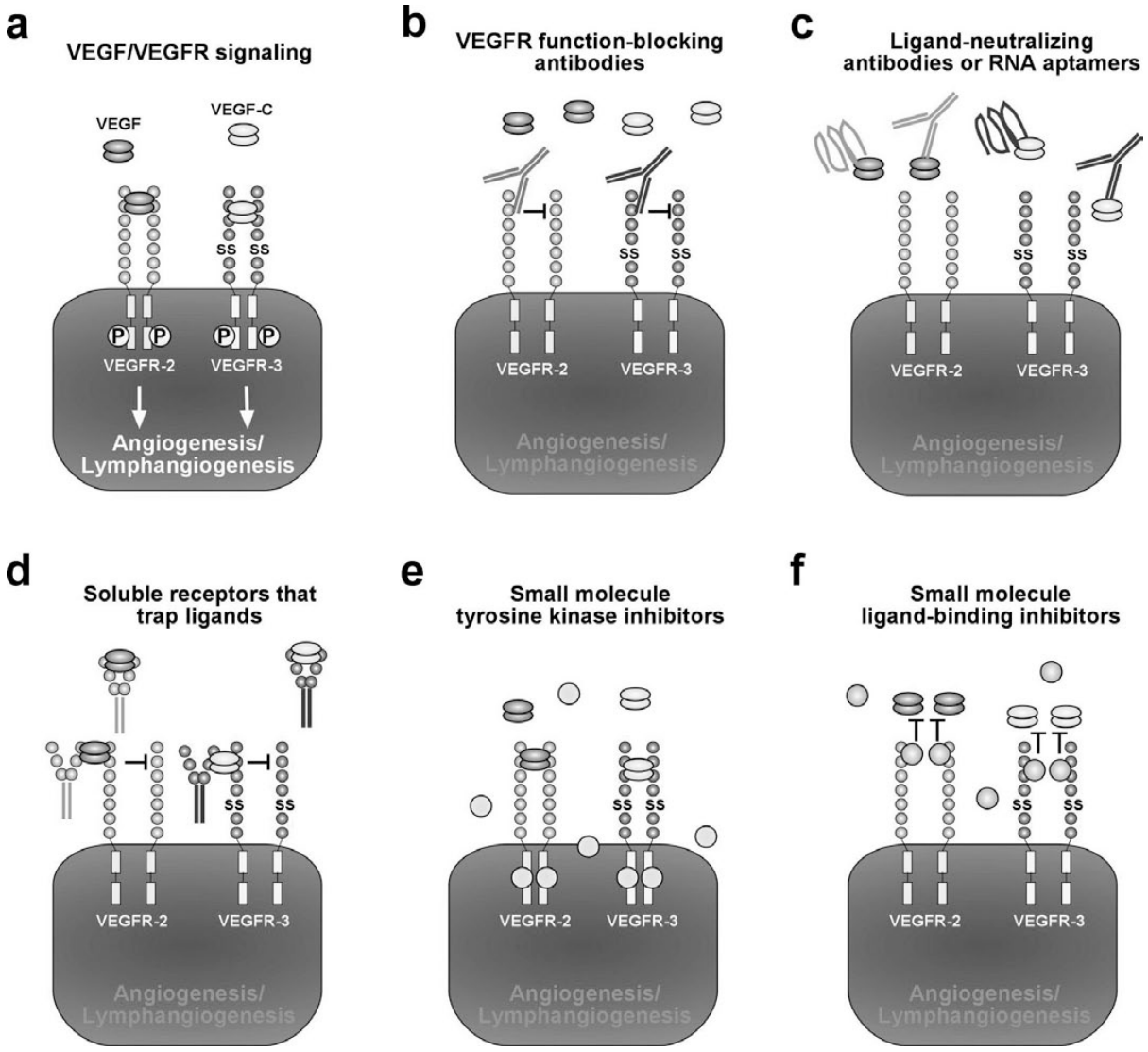


Figure 5. Strategies to inhibit VEGF/VEGFR signaling. (**a**) Ligand-activated VEGFR signaling. (**b**) VEGFR activation can be inhibited by monoclonal antibodies that prevent receptor dimerization and/or the ligand from binding the receptor. (**c**) VEGF ligands can be specifically neutralized using monoclonal antibodies or RNA aptamers. (**d**) Soluble decoy receptors containing the ligand-binding domain(s) of a given VEGFR, typically fused to the Fc domain of immunoglobulin G, trap ligands and may also interfere with receptor dimerization. (**b**) and (**c**) are the most specific means available, while (**d**) offers relatively specific blocking. All of these inhibitors must be administered parenterally, and are expensive to produce with current methods. (**e**) VEGFR tyrosine kinase activation can be blocked with small molecules that bind the intracellular kinase domain either at the ATP-binding site or at an allosteric site. (**f**) Small molecules can also be designed to block ligand-binding to the receptor ectodomain. Small molecule inhibitors are less specific, as they typically block multiple tyrosine kinases. However, these compounds may be administered orally and are inexpensive to synthesize.

Interestingly, many of the genes that are required for the development of the blood vascular system re-adopt their developmental function during tumor angiogenesis, which has led to the discovery of tumor-specific, and therefore potentially safe, vascular targets. Furthermore, tumor cell interactions with ECs promote the expression of surface proteins unique for the tumor endothelium, which may be utilized for specific therapeutic targeting (Arap et al., 1998; Joyce et al., 2003; reviewed in Ruoslahti and Rajotte, 2000).

Reflecting its paramount role in developmental angiogenesis, the VEGF/VEGFR-2 system appears to be the most important regulator of blood vessel growth in tumors (reviewed in Ferrara et al., 2007), and elevated levels of VEGF mRNA are found in most human tumors (reviewed in Dvorak, 2002). Besides hypoxia, a number of oncogenes are associated with VEGF production, including k-Ras, ErbB2, activated ErbB1, VHL, and Bcr-Abl (Rak et al., 1995; reviewed in Kerbel and Folkman, 2002). Blocking VEGF/VEGFR-2 signaling inhibits angiogenesis and growth of tumors, and even premalignant lesions, in experimental models without direct effects on tumor cells *in vitro* (Kim et al., 1993; Millauer et al., 1994; Prewett et al., 1999; Holash et al., 2002; Inoue et al., 2002; Korsisaari et al., 2007). Long-term experiments have shown that adult mice are highly resistant to the adverse effects of VEGF/VEGFR-2 inhibitors, unlike neonatal or adolescent mice (Kitamoto et al., 1997; Gerber et al., 1999; Eremina et al., 2003; Baffert et al., 2004). Nevertheless, the administration of VEGF axis inhibitors leads to apoptotic death of a small number of ECs, loss of EC fenestrations, resulting in subclinical hypothyroidism, and proteinuria even in adult mice (Baffert et al., 2004; Inai et al., 2004; Kamba et al., 2006; Lee et al., 2007; reviewed in Kamba and McDonald, 2007).

In 2004 the monoclonal VEGF-neutralizing antibody bevacizumab became the first anti-angiogenic agent to be approved for use in patients (Kabbinavar et al., 2003; Hurwitz et al., 2004). Bevacizumab combined to the standard chemotherapy regimen has shown marked clinical benefit in the treatment of metastatic colorectal cancer, non-small cell lung cancer, and breast cancer (Hurwitz et al., 2004; Miller et al., 2005; Sandler et al., 2006; Miller et al., 2007), and a positive result was obtained in a recent phase III trial in renal cell cancer (Escudier et al., 2007b). Further examples of specific VEGF axis inhibitors that are currently evaluated in phase II/III trials include the soluble VEGFR-1/VEGFR-2/immunoglobulin G fusion protein aflibercept (VEGF-Trap), and VEGFR-2 neutralizing antibodies (Lau et al., 2005; Baka et al., 2006; Youssoufian et al., 2007). Two small-molecular tyrosine kinase

inhibitors, sunitinib and sorafenib, which potently block the activation of VEGFRs and other tyrosine kinases, have recently been shown to be effective in the treatment of renal cell cancer and other malignancies (Demetri et al., 2006; Escudier et al., 2007a; Llovet et al., 2007; Motzer et al., 2007). Besides inhibiting angiogenesis and further tumor growth, VEGF/VEGFR-2 inhibitors are thought to improve tumor microcirculation by pruning excess vessels and reducing vascular leakage, facilitating the delivery of chemotherapeutics (reviewed in Jain, 2003; and in Jain, 2005). VEGF axis inhibitors are generally well tolerated in patients, although the combined use of chemotherapeutics often accentuates the adverse effects, which include hypertension, proteinuria, hemorrhage, gastrointestinal perforations, thromboembolism, and delayed wound healing (reviewed in Kamba and McDonald, 2007).

The fact that VEGF/VEGFR-2 inhibitors are not effective in all tumor types has suggested the existence of overlapping signaling pathways that drive tumor angiogenesis. Antibodies that block PlGF inhibit tumor growth and neovascularization synergistically with VEGFR-2 antibodies by suppressing macrophage recruitment leading to a poorly developed tumor stroma (Fischer et al., 2007). Interestingly, VEGFR-3 neutralizing antibodies also reduce tumor angiogenesis and growth (Roberts et al., 2006; Laakkonen et al., 2007). NP-1 function-blocking antibodies suppress the growth and angiogenesis of experimental tumors, and, interestingly, synergized with VEGF antibodies, which may indicate that other NP-1 ligands are important for tumor angiogenesis (Liang et al., 2007; Pan et al., 2007). Other neural guidance ligand/receptor systems that have been implicated in the growth and angiogenesis of experimental tumors include Robo1/Slit-2, netrin-1/Unc5b, and ephrinB2/EphB4 (Wang et al., 2003; Lu et al., 2004; Martiny-Baron et al., 2004; Larrivee et al., 2007; reviewed in Carmeliet and Tessier-Lavigne, 2005; and Klagsbrun and Eichmann, 2005).

Interestingly, Dll4 inhibitors were recently shown to promote excessive but non-productive tumor angiogenesis that impaired tumor perfusion and suppressed tumor growth, which has led to the emergence of a novel concept in anti-angiogenesis biology (Noguera-Troise et al., 2006; Ridgway et al., 2006; reviewed in Thurston et al., 2007).

In analogy with embryonic vascular development, blood vessels in tumors also recruit pericytes, which have been shown to protect tumor ECs from VEGF withdrawal (Liu et al., 2000a; Bergers et al., 2003; reviewed in von Tell et al., 2006). Thereapeutic targeting of the PDGF-B/PDGFR-β signaling pathway results in a decreased number of pericytes and in

reduced tumor vascularity, whereas targeting both PDGFRs and VEGFRs with kinase inhibitors leads to synergistic anti-angiogenic and anti-tumor activity (Bergers et al., 2003; Pietras and Hanahan, 2005; Sennino et al., 2007; Pietras et al., 2008). Blocking Tie2 has also been shown to reduce the growth of tumor xenogafts, which may be due to endothelial destabilization (Lin et al., 1997; Lin et al., 1998; Popkov et al., 2005). On the other hand, specific neutralization of Ang2 reduces tumor vascularization and growth via a poorly understood mechanism (Oliner et al., 2004).

Lymphatic metastasis

Metastatic spread of tumor cells via blood or lymphatic vessels occurs in many forms of human cancer, and patients with lymph node metastases have a radically less favorable prognosis when compared to patients with local disease (reviewed in Stacker et al., 2002). The first regional lymph node to be colonized by metastatic tumor cells is denoted a sentinel lymph node, and further dissemination may occur to other nodes and distant organs from this location.

Lymphangiogenesis has been observed in a variety of human tumors, as well as in experimental tumors (reviewed in Stacker et al., 2002). At least in animal models intratumoral lymphatic vessels may not be completely functional, because they collapse in conditions of high intratumoral pressure (Padera et al., 2002). Peritumoral lymphatics were recently shown to originate from the preexisting lymphatic vasculature without any detectable contribution from circulating lymphatic EPCs (He et al., 2004; He et al., 2005). The lymphatic vessels of tumors, and even premalignant lesions, express specific markers, which represent potential targets of anti-metastatic therapeutics (Laakkonen et al., 2002; Laakkonen et al., 2004; Fiedler et al., 2006a; Zhang et al., 2006).

Interestingly, several clinical studies have shown a positive correlation between VEGF-C or VEGF-D expression and vascular invasion, lymphatic vessel and lymph node involvement, distant metastasis, and, in some instances, poor clinical outcomes (reviewed in Stacker et al., 2002). VEGF-C expression in tumor cells may be induced by oncogenes, growth factors or proinflammatory cytokines, whereas some of the VEGF-C may be derived from stromal inflammatory cells (reviewed in Alitalo et al., 2005). Forced expression of

VEGF-C or VEGF-D in tumor cells enhances lymphatic metastasis in various experimental models, which applies even to tumors that normally do not have this propensity (Skobe et al., 2001a; Stacker et al., 2001; Kärpänen et al., 2001; Mandriota et al., 2001; He et al., 2002). Conversely, inhibition of lymphangiogenesis with a soluble form of VEGFR-3 or monoclonal antibodies that neutralize this receptor have been shown to inhibit lymphatic metastasis by 50-70% in preclinical animal models (He et al., 2002; Lin et al., 2005; Roberts et al., 2006). Importantly, although the lymphatic vessels are dependent on VEGFR-3 signaling during the first two postnatal weeks, blocking VEGFR-3 has no effects on normal lymphatic vessels in adult mice (Lin et al., 2005; Kärpänen et al., 2006b).

Interestingly, primary tumors were shown to elicit lymphangiogenesis in the sentinel lymph nodes already before the arrival of the first metastatic tumor cells (Hirakawa et al., 2005). A similar response occurs following immunization, and this promotes trafficking of antigen-presenting dendritic cells, suggesting that tumor cells utilize similar mechanisms as the immune cells to reach the lymph nodes (Angeli et al., 2006). In both cases VEGF was implicated as the key growth factor, although further studies have shown that tumors that produce VEGF-C can also initiate lymph node lymphangiogenesis (Hirakawa et al., 2005; Angeli et al., 2006; Hirakawa et al., 2007). Fibroblast growth factor(FGF)-2, insulin-like growth factor(IGF)-1, IGF-2, hepatocyte growth factor (HGF), and PDGF-B also induce lymphangiogenesis, and at least PDGF-B promotes lymph node metastasis of experimental tumors, but most of these effects may be secondary to the induction of VEGF-C and VEGF-D in a variety of cell types (Kubo et al., 2002; Tang et al., 2003; Cao et al., 2004; Chang et al., 2004; Bjorndahl et al., 2005; Kajiya et al., 2005; Cao et al., 2006).

Lymphedema

Impairment of lymphatic transport capacity due to abnormal vessel development or obstruction or obliteration of the lymphatic vessels causes stagnation of proteins and associated water in the interstitium, resulting in lymphedema, usually a progressive and lifelong condition for which curative treatments are at present not available (Figure 1f). The protein-rich interstitial fluid initiates an inflammatory reaction, leading to fibrosis, impaired

immune responses, and accumulation of subcutaneous fat. Lymphedema is classified into primary (hereditary) lymphedema, and secondary (acquired) lymphedema, based on the mechanism of pathogenesis (reviewed in Rockson, 2001; and in Warren et al., 2007).

Although primary lymphedema is a rare condition, identification of the underlying genetic causes has provided valuable insight into the molecular mechanisms regulating the development and function of the lymphatic vasculature. Heterozygous tyrosine kinase-inactivating point mutations of the *VEGFR3* gene have been identified as a major cause of Milroy disease (OMIM #153100), a form of lymphedema due to hypoplasia of lymphatic capillaries, and typically present at birth (Irrthum et al., 2000; Kärkkäinen et al., 2000; Kärkkäinen et al., 2001). Mutations in the transcription factor *FOXC2* have been linked to lymphedema-distichiasis (LD, OMIM #153400), characterized by late-onset lymphedema, a double row of eyelashes, and varicose veins (Fang et al., 2000; Bell et al., 2001; Finegold et al., 2001; Mellor et al., 2007). Analysis of *Foxc2* mutant mice revealed that LD is due to ectopic SMC coverage of the lymphatic capillaries and loss of valves in the collecting vessels, and similar defects were also observed in samples obtained from LD patients (Petrova et al., 2004).

Dominant-negative mutations of the homeobox transcription factor *SOX18* have been linked with hypotrichosis-lymphedema-telangiectasia syndrome (HLTS, OMIM #607823) (Irrthum et al., 2003). Although the precise molecular mechanisms leading to the phenotype have not been discovered, a mouse model that recapitulates most hallmarks of the syndrome is available for the elucidation of the molecular pathogenesis (Pennisi et al., 2000; James et al., 2003). Lymphatic vessels express constitutively high levels of nuclear factor kappa-B (NF-κB), and mutations in the NF-κB regulatory protein *NEMO* associate with a rare and complex syndrome involving lymphedema (anhidrotic ectodermal dysplasia with immunodeficiency, osteopetrosis, and lymphedema, OL-EDA-ID, OMIM #300301) (Döffinger et al., 2001; Saban et al., 2004).

Over 99% of lymphedema cases worldwide are secondary to acquired damage to the lymphatic vessels (reviewed in Rockson, 2001; and in Warren et al., 2007). Filariasis (elephantiasis) is an infection of the lymphatics by the parasitic worms *Wuchereria bancrofti* or *Brugia malayi,* which leads to obstruction and scarring of lymphatic vessels, and chronic lymphedema of the lower limbs or genital organs. Filariasis is the principal cause of lymphedema worldwide, affecting approximately 100 million people, whereas breast cancer

surgery is the leading cause for secondary lymphedema in industrialized countries (reviewed in Rockson, 2001; and in Alitalo et al., 2005). Metastatic tumor cells frequently spread to the lymph nodes, necessitating radical surgery and radiotherapy, which destroy the lymphatic vessel network and lead to impairment of afferent lymphatic flow (reviewed in Rockson, 2001; and in Alitalo et al., 2005). For example, approximately 20-30% of patients that have undergone radical axillary lymph node dissection develop lymphedema of the upper limb later on (Mortimer et al., 1996; reviewed in Clark et al., 2005; and in Warren et al., 2007). Damage to the lymphatics may also result from bacterial infections of the skin (e.g. erysipelas) or the lymphatic vessels (lymphangitis) (reviewed in Rockson, 2001; and in Warren et al., 2007).

Unlike primary lymphedema, secondary lymphedema is typically due to damage to the collecting lymphatic vessels. Spontaneous recanalization of collecting vessels may occur in minimal lesions, but formation of new lymphatic vessels is typically not observed in lymphedema patients, although pre-existing vessels dilate to accommodate the increased fluid (Ikomi et al., 2006; Tabibiazar et al., 2006). Hypoxia is a ubiquitous stimulus for the initiation of angiogenesis, but it is not known whether intrinsic edema-induced mechanisms for engaging lymphangiogenic gene expression programs exist. The treatment of lymphedema is currently based on physiotherapy, compression garments, liposuction, and occasionally surgery (reviewed in Rockson, 2001; Warren et al., 2007), but means to reconstitute the collecting lymphatic vessels and cure the condition are rarely successful (Baumeister et al., 1981; Olszewski, 1988; Becker et al., 2006). VEGF-C gene transfer via adenoviruses (Ad), adeno-associated viruses (AAV), or naked plasmids, as well as the application of recombinant VEGF-C protein have been shown to stimulate the formation of new lymphatic capillaries and alleviate edema in preclinical animal models of lymphedema (Kärkkäinen et al., 2001; Szuba et al., 2002; Yoon et al., 2003; Saaristo et al., 2004), pointing to a promising means to restore lymphatic vessels in lymphedema patients.

AIMS OF THE STUDY

This study was undertaken to study the potential of Ang1, VEGF-C, VEGF-D, and chimeric heparin-binding forms of VEGF-C in inducing therapeutic lymphatic vessel growth, as well as to elucidate the function of VEGFR-3 in angiogenesis under normal and pathological conditions.

The specific aims were to elucidate:

I The role of angiopoietin-1 in lymphatic vessel sprouting and growth in adult tissues.

II The potential of VEGF-C and VEGF-D to regenerate collecting lymphatic vessels and improve the outcome of lymph node transplantation in a mouse model of secondary lymphedema.

III The analysis of the biological activity and vascular patterns induced by heparin-binding chimeric VEGF/VEGF-C fusion proteins *in vitro* and *in vivo.*

IV The function of VEGFR-3 in angiogenesis in developmental, physiological, and pathological settings.

MATERIALS AND METHODS

The materials and methods are described in detail in the original publications. A list of the most relevant materials and methods used in the studies is provided below.

1. Materials

Mouse line	Description	Source or reference	Used in
Dll4$^{+/LacZ}$	The *LacZ* gene has been inserted into the *Dll4* locus resulting in *Dll4* inactivation	(Duarte et al., 2004)	IV
DsRed	Expresses the *Discosoma* coral red fluorescent protein DsRed in all cells under the control of the chicken β-actin promoter	(Vintersten et al., 2004)	II
K14-Ang1	Overexpresses Ang1 in basal epidermal keratinocytes	(Thurston et al., 1999)	I
K14-VEGF-E	Overexpresses VEGF-E in basal epidermal keratinocytes	(Kiba et al., 2003)	IV
K14-VEGF165	Overexpresses VEGF165 in basal epidermal keratinocytes	(Zheng et al., 2006)	IV
K14-VEGFR-3-Ig	Overexpresses the VEGFR-3-Ig fusion protein in basal epidermal keratinocytes	(Makinen et al., 2001a)	IV
Nu/nu	Immunodeficient athymic nude mice	Taconic	I, II, III, IV
Rip1Tag2	Expresses the oncogenic simian virus 40 large T antigen in pancreatic β-cells under the control of the rat insulin promoter	(Hanahan, 1985)	IV
Vegfc$^{+/LacZ}$	The *LacZ* gene has been inserted into the *Vegfc* locus resulting in *Vegfc* inactivation	(Kärkkäinen et al., 2004)	IV
Vegfr3$^{LacZ/LacZ}$, *Vegfr3*$^{+/LacZ}$	The *LacZ* gene has been inserted into the *Vegfr3* locus resulting in *Vegfr3* inactivation	(Dumont et al., 1998)	IV

Recombinant AAV	Description	Source or reference	Used in
AAV-Ang1	Encodes human Ang1	I	I
AAV-CA65	Encodes the chimeric VEGF-CΔNΔC-VEGF(exons 7-8) fusion protein	III	III
AAV-CA89	Encodes the chimeric VEGF-CΔNΔC-VEGF(exons 6a-8) fusion protein	III	III
AAV-EGFP	Encodes the green fluorescent protein from the jellyfish *Aequorea victoria*	(Kärkkäinen et al., 2001)	I, III
AAV-VEGF-B167	Encodes the human VEGF-B167 isoform	III	III
AAV-VEGF-C	Encodes human full-length VEGF-C	(Kärkkäinen et al., 2001)	I, III
AAV- VEGF-CΔNΔC	Encodes the mature form of human VEGF-C	III	III

Recombinant adenovirus	Description	Source or reference	Used in
AdAng1	Encodes human Ang1	I	I
AdCA65	Encodes the chimeric VEGF-CΔNΔC-VEGF(exons 7-8) fusion protein	III	III
AdCA89	Encodes the chimeric VEGF-CΔNΔC-VEGF(exons 6a-8) fusion protein	III	III
AdLacZ	Encodes *E. coli* β-galactosidase	(Laitinen et al., 1997)	I, II, III, IV
AdmVEGF-D	Encodes mouse full-length VEGF-D	IV	IV
AdPDGF-B	Encodes human PDGF-B	From Dr P. Korpisalo and Dr. S. Ylä-Herttuala	II
AdVEGF165	Encodes the human VEGF165 isoform	(Enholm et al., 2001)	IV
AdVEGF-B186	Encodes the human VEGF-B186 isoform	II	II
AdVEGF-C	Encodes human full-length VEGF-C	(Enholm et al., 2001)	I, II, III, IV
AdVEGF-CΔNΔC-5'UTR	Encodes the mature form of human VEGF-C (contains the 5' untranslated region of *VEGFC*)	III	III
AdVEGF-DΔNΔC	Encodes the mature form of human VEGF-D	(Rissanen et al., 2003)	II
AdVEGF-E	Encodes the VEGF homologue VEGF-E originating from the Orf-NZ7 virus	(Wirzenius et al., 2007)	IV
AdVEGFR-3-Ig	Encodes the VEGFR-3-Ig fusion protein	(Kärpänen et al., 2001)	I, IV

Cell line	Description	Source or reference	Used in
B16	Murine skin melanoma line derived from the inbred C57/black/6 mouse strain	(Riley, 1963)	IV
G401	Human renal cancer cell line	Americn Type Culture Collection (ATCC)	IV
HDMEC	Primary human dermal microvascular endothelial cells	Promo Cell	I, II
HEK-293T	Human embryonic kidney fibroblast line expressing the simian virus 40 large T antigen	ATCC	I, III, IV
HeLa	Human cervical cancer line	ATCC	I, II, III, IV
MKN45	Human gastric carcinoma cell line	(Sakai et al., 1987)	IV
NCI-H460-LNM35-Luciferase	Subline of NCI-H460-N15, a human large-cell lung carcinoma. Selected *in vivo* for the propensity for lymph node metastasis. Tagged with *Photinus pyralis* (firefly) luciferase.	(Kozaki et al., 2000) (He et al., 2005)	II, IV
LLC	Murine lung carcinoma, derived from the inbred C57/black/6 mouse strain	ATCC	IV

Recombinant protein	Description	Source or reference	Used in
Jag1	A 17mer peptide consisting of the receptor-activating motif of the Notch-ligand Jagged1	(Weijzen et al., 2002)	IV
NP-1-Ig	Human NP-1 extracellular domain and immunoglobulin G1 fusion protein	(Kärkkäinen et al., 2001)	III
NP-2-Ig	Human NP-2 extracellular domain and immunoglobulin G1 fusion protein	(Kärkkäinen et al., 2001)	III
PDGFR-β-Ig	Human PDGFR-β- extracellular domain and immunoglobulin G1 fusion protein	R&D Systems	II
Tie2-Ig	Human Tie2- extracellular domain and immunoglobulin G1 fusion protein	R&D Systems	I
SC-Jag1	A scrambled 17mer peptide consisting of the same amino acids as Jag1	(Weijzen et al., 2002)	IV
VEGF165	Human VEGF165	R&D Systems	IV
VEGF-CΔNΔC	Human VEGF-C consisting of amino acids 103-215 and a H6 tag	(Kärpänen et al., 2006a)	III, IV
VEGFR-1-Ig	Consists of the first five Ig homology domains of human VEGFR-1 fused to the Fc region of human IgG1	(Makinen et al., 2001a)	III
VEGFR-2-Ig	Consists of the first three Ig homology domains of human VEGFR-2 fused to the Fc region of human IgG1	(Uutela et al., 2004)	III
VEGFR-3-Ig	Consists of the first three Ig homology domains of human VEGFR-3 fused to the Fc region of human IgG1	(Makinen et al., 2001a)	III

Antigen	Antibody	Source or reference	Used in
Ang1 C-terminus (human)	Goat polyclonal	Santa Cruz Biotech.	I
Ang1 N-terminus (human)	Goat polyclonal	Santa Cruz Biotech.	I
Bromodeoxyuridine	Mouse monoclonal-Alexa 594	Molecular Probes/ Invitrogen	IV
CD11b (mouse)	Rat monoclonal (clone M1/70)	BD Biosciences	IV
Cytokeratin 7 (human)	Rabbit polyclonal	AbCam	II
Dll4 (mouse)	Goat polyclonal	R&D Systems	IV
F4/80 (mouse)	Rat monoclonal (clone BM8)	Acris antibodies	III, IV
FITC	Rabbit polyclonal	Zymed/Invitrogen	IV
GFAP (cow)	Rabbit polyclonal	DAKO	IV
LYVE-1 (mouse)	Rabbit polyclonal	(Petrova et al., 2004)	I, II, III, IV
LYVE-1 (mouse)	Rat monoclonal (clone ALY7)	(Morisada et al., 2005)	I
MECA-32 (mouse endothelial cell antigen)	Rat monoclonal (clone MECA-32)	BD Biosciences	IV

Antigen	Antibody	Source or reference	Used in
N-cadherin (mouse)	Rabbit polyclonal	From Dr. M. Takeichi and Dr. H. Semb	II
NG2 (mouse)	Rabbit polyclonal	Chemicon/Millipore	IV
PCNA (human)	Mouse monoclonal-biotin	Zymed/Invitrogen	I
PDGFR-α (mouse)	Goat polyclonal	R&D Systems	IV
PDGFR-β (mouse)	Rat monoclonal (clone APB5)	eBioscience	IV
PECAM-1 (mouse)	Hamster monoclonal (clone 2H8)	Chemicon/Millipore	II, III, IV
PECAM-1 (mouse)	Rat monoclonal (clone MEC 13.3)	BD Biosciences	II, III, IV
Phosphohistone H3 (mouse)	Rabbit polyclonal	Upstate/Millipore	I, III
Pimonidazole adducts	Mouse monoclonal-FITC	Chemicon/Millipore	IV
Podoplanin (human)	Rabbit polyclonal	(Kriehuber et al., 2001)	II, IV
Prox1 (mouse)	Rabbit polyclonal	(Kärkkäinen et al., 2004)	II
SMA (human)	Mouse monoclonal-Cy3 (clone 1A4)	Sigma	II, IV
Tie2 (mouse)	Rat monoclonal (clone TEK4)	eBioscience	I
VE-cadherin (mouse)	Rat monoclonal (clone 11D4.1)	BD Biosciences	II, IV
VEGF-C (human)	Rabbit polyclonal (#6)	(Baluk et al., 2005)	I, IV
VEGF-CΔNΔC (human)	Rabbit polyclonal (#3 and #4)	III	III
VEGFR-1 (mouse)	Rat monoclonal (clone 5B12)	ImClone	IV
VEGFR-2 (mouse)	Goat polyclonal	R&D Systems	IV
VEGFR-2 (mouse)	Rat monoclonal (clone AVAS-12α1)	BD Biosciences	IV
VEGFR-2 (mouse)	Rat monoclonal (DC101)	(Prewett et al., 1999)	IV
VEGFR-3 (mouse)	Goat polyclonal	R&D Systems	I, II, III, IV
VEGFR-3 (mouse)	Rat monoclonal (clone AFL4)	eBioscience	IV
VEGFR-3 (mouse)	Rat monoclonal (clone mF4-31C1)	(Pytowski et al., 2005)	IV
VEGFR-3 (human)	Mouse monoclonal (clone 9D9)	(Jussila et al., 1998)	IV
von Willebrand factor (human)	Rabbit polyclonal	DAKO	IV
ZO-1 (mouse)	Rat monoclonal	Chemicon/Millipore	II

2. Methods

Method	Used in
AAV transduction of cells or mice	I, III
Adenoviral transduction of cells or mice	I, II, III, IV
Bioassay for growth factor–mediated cell survival	III
Bioluminescence imaging	II, IV
Cell culture	I, II, III, IV
Confocal microscopy	I, II, III, IV
Dissection and transplantation of mouse lymph nodes	II
DNA subcloning	I, II, III, IV
Immunofluorescence	I, II, III, IV
Immunohistochemistry	I, II, III, IV
Immunoprecipitation	I, III, IV
Implantation of tumors into mice	II, IV
Mating and screening of genetically modified mice	II, IV
Metabolic labeling	I, III, IV
Microlymphangiography (tomato lectin, dextran, Evans blue)	II
Northern blotting	I
Polymerase chain reaction (PCR)	I, III, IV
Preparation of human tissues	II, IV
Preparation of mouse tissues	I, II, III, IV
Quantitative analysis and vessel morphometry	I, II, III, IV
RNA extraction	I, IV
Real-time quantitative PCR	IV
Superovulation of mice	IV
Stimulation of mouse embryos with recombinant growth factors	IV
Transduction of cells	I, III, IV
Transfection of cells	III
Transmission electron microscopy	IV
Western blotting	IV
X-gal staining of tissues	IV

RESULTS AND DISCUSSION

1. Angiopoietin-1 induces lymphangiogenesis in adult tissues (I)

Analysis of gene-targeted mice has shown that Ang2 is essential for the proper patterning of lymphatic vessels, while Ang1 is able to rescue the lymphatic phenotype in Ang2 gene-targeted mice, suggesting that either ligand acts as a receptor agonist in LECs (Gale et al., 2002). In order to expand the repertoire of available pro-lymphangiogenic growth factors, it was rational to study whether Ang1 could induce lymphangiogenesis in adult tissues. For this purpose, adenoviral and adeno-associated virus (AAV) vectors encoding human Ang1 were transduced into the skin of adult mice, as this tissue is rich in lymphatic vessels. Ang1 induced lymphatic endothelial proliferation, vessel enlargement and formation of long LEC filopodia that eventually fused, leading to new sprouts over a period of four days. At two weeks, new lymphatic vessels were observed. Lymphatic capillary hyperplasia was also detected in the skin of K14-Ang1 transgenic mice, which express human Ang1 in the basal epidermal keratinocytes under the control of the keratin-14 promoter. Later studies have corroborated these findings, as Ang1, Ang2, and Ang3/Ang4 have now been shown to promote lymphangiogenic sprouting, with Ang1 being the most potent lymphangiogenic factor (Kim et al., 2007). Furthermore, an engineered Ang1 with enhanced biological activity and solubility was shown to promote wound healing through enhanced angiogenesis, lymphangiogenesis, and blood flow in a diabetic mouse model (Cho et al., 2006).

The Ang1 receptor Tie2 is expressed in cultured LECs (Kriehuber et al., 2001; Makinen et al., 2001b; Morisada et al., 2005), and Tie2 expression was observed also in cutaneous lymphatic vessels *in vivo*. Interestingly, stimulation of LECs with Ang1 resulted in upregulation of VEGFR-3 *in vivo* and *in vitro*. Furthermore, the lymphatic vascular effects of Ang1 were blocked by a soluble VEGFR-3-Ig fusion protein *in vivo*, suggesting that the Ang1/Tie2 and VEGF-C/VEGFR-3 pathways interact in the molecular regulation of lymphatic vessel growth and survival. It is possible that by upregulating VEGFR-3, Ang1 sensitizes the lymphatic vessels to VEGF-C and VEGF-D signals emanating from e.g. vascular SMCs, which have been shown to express both VEGF-C and VEGF-D (Partanen et al., 2000; Achen et al., 2001).

The upregulation of VEGFR-3 in response to Tie2 activation in the LECs could account for at least part of the observed increase in the rate of LEC proliferation, as VEGFR-3 signals have been shown to be sufficient for lymphatic vessel growth *in vivo* (Veikkola et al., 2003; Wirzenius et al., 2007). Ang1 may also exert direct effects promoting LEC proliferation and sprouting, as it has been reported to promote the survival and migration of blood vascular ECs (Koblizek et al., 1998; Hayes et al., 1999; Papapetropoulos et al., 2000; Kim et al., 2000b; Cho et al., 2004). Furthermore, Ang1 may also play a role in early lymphatic vessel development, as it was shown to expand colonies of LECs isolated from developing mouse embryos (Morisada et al., 2005). However, biochemical evidence for the cooperation of the Tie2 and VEGFR-3 signaling systems in lymphangiogenesis is lacking. Interestingly, Ang1 has been reported to bind to integrin β1, even in the absence of Tie2, and to stimulate cell adhesion to fibronectin via ß1 integrin (Takakura et al., 1998; Carlson et al., 2001). On the other hand, VEGFR-3 has been shown to form a ligand-independent signaling complex with integrin β1 (Wang et al., 2001). It is thus possible that Ang1 complexes with integrin β1 - VEGFR-3 clusters, in addition to activating of Tie2, suggesting the possibility of a VEGF-C/VEGF-D-independent mechanism. Finally, Ang1 and Ang4 have also been shown to activate Tie1 in cultured LECs, which may be an additional explanation for the lymphangiogenic activity of Ang1 (Saharinen et al., 2005).

Ang1 is an important regulator of vascular permeability, being capable of preventing plasma leakage even after stimulation of blood vessels with highly potent permeabilizing agents, such as VEGF (Thurston et al., 2000; Thurston, 2002). These findings show that Ang1 can also be implicated as a lymphangiogenic factor, and further promote the utility of this factor in the management of tissue edema. Ang1 therapy could be applied in settings of edema, provoked by e.g. inflammation or allergens (Figure 1 d), in order to restore the integrity of EC monolayers in blood vessels, but also to promote the activation of lymphangiogenesis in response to VEGF-C and VEGF-D, which are produced by leukocytes in inflammatory infiltrates (Baluk et al., 2005). Ang1 therapy could also be applied in patients at a risk of secondary lymphedema in order to decrease the volume of extravasated fluid, thus reducing the drainage requirement of the lymphatic vessels that are still functional. Furthermore, Ang1 has been shown to protect ECs from radiation-induced injury, and it could also support LEC survival under stress (Cho et al., 2004b). Ang1 could be applied in patients suffering from reduced activity of the VEGFR-3 signaling pathway, such as Milroy

patients, although this would require evidence of VEGFR-3-independent lymphangiogenic activity of Ang1.

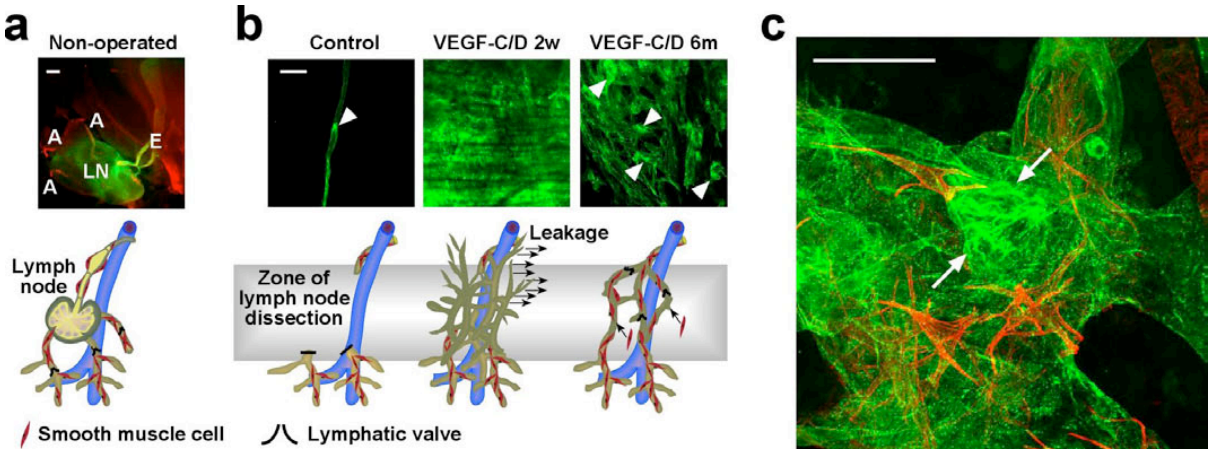
The low solubility of native Ang1 allows for localized treatment in order to minimize adverse effects, and to generate growth factor gradients. However, Ang1 is constantly produced by pericytes, and it is thus unlikely that even intravascularly administered Ang1 would lead to dramatic adverse effects. Systemic administration of Ang1 could be achieved with variants that have been engineered to form predominantly tetramers or pentamers instead of the highly insoluble aggregates formed by native Ang1 (Thurston et al., 2000; Cho et al., 2004a). Further solubility may be achieved by deletion of the linker peptide (Xu and Yu, 2001). Prolonged stimulation with such soluble forms of Ang1 has been reported to induce dilation of postcapillary venules, and to promote blood flow in these vessels, but other systemic effects have not been reported (Baffert et al., 2004; Cho et al., 2005).

2. VEGF-C/VEGF-D therapy restores collecting lymphatic vessels and improves the outcome of lymph node transplantation (II)

Surgery or radiation therapy of metastatic cancer often damages lymph nodes, leading to secondary lymphedema, a highly prevalent and debilitating condition (Figure 1f). Previous studies have demonstrated that VEGF-C and VEGF-D stimulate lymphangiogenesis in adult tissues, but they have been limited to analysis of lymphatic capillaries identified by markers such as LYVE-1 and VEGFR-3, while a comprehensive molecular analysis of the lymphatic vessel phenotype and especially the collecting lymphatic vessels, most commonly damaged in secondary lymphedema, has been lacking. (Jeltsch et al., 1997; Enholm et al., 2001; Kärkkäinen et al., 2001; Veikkola et al., 2001; Saaristo et al., 2002b; Yoon et al., 2003; Rissanen et al., 2003; Saaristo et al., 2004; Kärpänen et al., 2006b).

In order to study secondary lymphedema in a clinically relevant setting, a mouse model involving the dissection of all axillary lymph nodes and collecting vessels, frequently damaged in humans by breast cancer treatments, was established. This was followed by administration of adenoviral vectors into the surrounding tissues. Mice treated with control adenoviruses developed lymphedema of the paws, and demonstrated impaired return of lymph from the paw to the bloodstream. In contrast, mice treated with AdVEGF-C or AdVEGF-DΔNΔC displayed decreased edema and restoration of lymphatic drainage, which

continued to improve over time. Histological analysis of axillas treated with adenoviral VEGF-C or VEGF-D demonstrated robust growth of the lymphatic capillaries, which gradually underwent an intrinsic remodeling, differentiation, and maturation program into functional collecting lymphatic vessels. These vessels acquired all hallmarks of collecting vessels, including formation of uniform endothelial cell-cell junctions and intraluminal valves, although the vessels remained smaller in diameter than in normal non-operated axillas even at 6 months (**Figure 6**). Importantly, the vessels also acquired a coating of SMCs, which coincided with the formation of N-cadherin-mediated junctions and down-regulation of the lymphatic capillary marker LYVE-1. Conversely, the data indicated that the detachment of SMCs upon PDGF-B stimulation re-induced LYVE-1 in the collecting lymphatic vessels, indicating that SMC contact is required for maintenance of the collecting vessel phenotype. Besides N-cadherin, LEC-SMC signaling may be regulated by the ephrinB2/EphB4, Ang/Tie, S1P/S1P receptor, and TGF-β/TGF-βR pathways, as in blood vessels during development (Gale et al., 2002; Makinen et al., 2005; reviewed in Armulik et al., 2005).



Figue 6. Maturation of the lymphatic vessels formed in response to VEGF-C/D therapy. (**a**) In a normal non-operated mouse axilla the lymph nodes (LN) take up fluorescent dextran (green), which is transported to the lymph node via the afferent lymphatic vessels (A) after injection to the footpad of the mouse. The efferent lymphatic vessel (E) transports the tracer onwards from the lymph node. SMCs are stained with smooth muscle α-actin (red). (**b**) In mice that underwent lymph node dissection only a few collecting lymphatic vessels remain (Control). Abundant leaky lymphatic vessels are observed at two weeks (2 w) following adenoviral gene transduction of VEGF-C or VEGF-D to the axillary tissues, while functional collecting lymphatics containing intraluminal valves (arrowheads) are seen at 6 months (6 m). (**c**) A magnified image of the lymphatic vascular plexus at 6 months. The vessel is in contact with SMCs (red), and contains an intraluminal valve (arrows). *Lycopersicon esculentum* lectin lymphangiography (green) was used to visualize lymphatic vessels in (**b-c**). Scalebars: 100 μm.

According to these results, the process of lymphatic vessel maturation is analogous to arteriogenesis, which can be induced by prolonged stimulation with VEGF or PlGF, and by shear stress due to increased flow in the vessels (Garcia-Cardena et al., 2001; Dor et al., 2002; Pipp et al., 2003; reviewed in Schaper and Scholz, 2003). Interestingly, VEGF-C is a chemoattractant for monocytes/macrophages, which are important for arteriogenesis, and these cells may also play a role in lymphatic vessel maturation (Ito et al., 1997; Arras et al., 1998; Skobe et al., 2001b; Pipp et al., 2003; Saaristo et al, 2006). Prolonged VEGF-C stimulation may also directly promote LEC differentiation. In fact, the lymphatic vessels formed in response to VEGF-C stimulation resemble the lymphatic vascular plexus that forms early on during development, and it is likely that the intrinsic developmental mechanisms governing lymphatic vessel maturation are reactivated in these vessels. The molecular players regulating this process are poorly known, but it is likely that flow of the lymph in the nascent vessels contributes to the remodeling (Ng et al., 2004). The transcription factor FoxC2 is likely to be involved in the maturation process, as it regulates the formation of lymphatic and venous valves, as well as other characteristics of collecting lymphatic vessels during development (Petrova et al., 2004; Mellor et al., 2007).

In order to comprehensively restore the anatomy of the axilla following surgery, combined AdVEGF-C therapy with lymph node transplantation was applied. Such an approach has been previously undertaken without growth factor therapy, but in these experiments the autologously transplanted lymph nodes incorporated into existing lymphatic vasculature at a low frequency (Rabson et al., 1982; Becker et al., 2006). The lymph nodes transduced with AdVEGF-C survived, formed connections with the pre-existing lymphatic vessel network, and could even trap metastatic tumor cells, whereas the majority of control-treated nodes regressed, indicating that VEGF-C therapy can improve the success rate of lymph node transplantation (**Figure 7**a). These findings demonstrate for the first time that growth factor therapy can be used to generate functional and mature collecting lymphatic vessels. VEGF-C combined with lymph node transplantation allows for complete restoration of the lymphatic system in damaged tissues, and provides a model for future treatment of lymphedema in patients.

These findings are based on a mouse model, which has several limitations when considering direct extrapolation to the human patient setting. Firstly, the hydrostatic conditions are dramatically different in mice, as humans are considerably larger. This also

means that the absolute area damaged by axillary lymph node dissection in humans is greater in size, and the regenerating lymphatic vessels must span a longer distance in order to form anastomoses with both the distal and the proximal ends of the lymphatic vascular tree. However, this gap could be bridged by the transplantation of chains of lymph nodes from another location in the patient, whereas VEGF-C could be used to form the microvascular anastomoses. On the other hand, maturation of the lymphatic vessel plexus induced by VEGF-C therapy could be more complete in humans due to a longer life span.

VEGF-C has been shown to accelerate wound healing by promoting lymphangiogenesis, angiogenesis, and macrophage recruitment (Saaristo et al., 2004; Saaristo et al., 2006). Furthermore, VEGF-C could be used to augment immune responses by promoting antigen-presenting cell migration (Chen et al., 2004), as well as by increasing the number of lymphatic vessel routes available for these cells. On the other hand, VEGF-C has been shown to promote lymphatic metastasis of tumor cells (reviewed in Alitalo et al., 2005), to increase blood vascular permeability (Saaristo et al., 2002), as well as to stimulate extravasation of lymph from the lymphatic vessels (II). In light of these findings, patient safety is an important issue, which must be considered when identifying patients for future clinical trials.

3. Altered tissue distribution of VEGF-C by fusion to heparin-binding domains of VEGF produces distinct lymphatic vascular patterns

VEGF-C and VEGF-D have an N-terminal propeptide, as well as a C-terminal domain homologous to certain silk proteins. VEGF-C does not contain heparin-binding motifs, and it is unknown how, if at all, VEGF-C associates with the ECM (Joukov et al., 1996; Joukov et al., 1997). In order to study the effects of altered distribution of VEGF-C in tissues, a gain-of-function approach was adopted, comprising fusion of the exon 6a-8 or exon 7-8 encoded domains from VEGF to the C-terminus of the fully processed VEGF-CΔNΔC, denoted CA89 and CA65, respectively (Figure 3b). The VEGF-C/VEGF-HBD chimeras were produced, and shown to activate VEGF-C receptors, as well as to bind to NP-1 and NP-2. Both CA65 and CA89 stimulated lymphangiogenesis *in vivo* when expressed in tissues via adenovirus or AAV vectors. However, both chimeras induced a distinctly different pattern of lymphatic vessels when compared with the wild type VEGF-C. The trophism of the AAV serotype 2 vectors towards skeletal muscle was utilized to target expression into this tissue. The vessels induced by VEGF-C were initially a thick network of small diameter vessels that reorganized and matured slowly, but the lymphatic vessels induced by the chimeric growth factors were wider, less complex, and tended to form directly along tissue borders, along basement membranes that are rich in heparan sulfate (Figure 7b).

The altered biological activity of CA89 and CA65 in comparison to VEGF-C likely results from redistribution of the growth factors by binding of the HBD to pericellular matrix structures that are rich in HSPGs, which are typically present in basal laminae and on the surface of certain cells. Mice lacking the VEGF isoforms corresponding to VEGF165 and VEGF189 display disturbed vascular patterning, with a dramatic reduction in the number of vascular sprouts and branching points, highlighting the importance of the exon 6a- and 7-encoded sequences for normal VEGF function (Carmeliet et al., 1999; Ruhrberg et al., 2002). Conversely, protease-resistant large molecular-weight forms of VEGF produce abnormal vascular patterns characterized by excessive sprouting and branching (Lee et al., 2005). Consistent with the capacity of VEGF-CΔNΔC to activate VEGFR-2, both chimeras and VEGF-CΔNΔC induced angiogenesis, although the effects on blood vessels were not pronounced. Importantly, changes in lymphatic vessels were not observed after gene transfer of VEGF-B167, which contains similar high-affinity heparin- and neuropilin-binding

domains as VEGF189 and CA89 (Mäkinen et al., 1999), indicating that heparin and neuropilin binding capacities alone are not sufficient to stimulate either angiogenesis or lymphangiogenesis unless the factor is able to activate VEGFR-2 or VEGFR-3, respectively.

An inverse chimeric protein was generated, consisting of the minimal receptor-activating domain of VEGF flanked by the N- and C-terminal propeptides of VEGF-C. Interestingly, overexpression of this factor primarily stimulated the formation of very thin blood vessel capillaries, which was not observed by overexpressing VEGF165 or the minimal receptor-activating domain alone, suggesting a unique tissue distribution pattern for VEGF-C when compared to heparin-binding species of the VEGF family (Keskitalo et al., 2007).

These findings indicate that the heparin-binding forms of VEGF-C can be immobilized in a given tissue, and therefore the danger of obtaining aberrant side effects at distant sites is minimized. The matrix-binding domain of VEGF can target VEGF-C activity to heparin-rich basement membrane structures, which may prove useful in guiding the growth of lymphatic vessels into desired locations, such as transplanted lymph nodes (II), as well as in accelerating the formation of lymphatic vessel anastomoses. (Figure 7c).

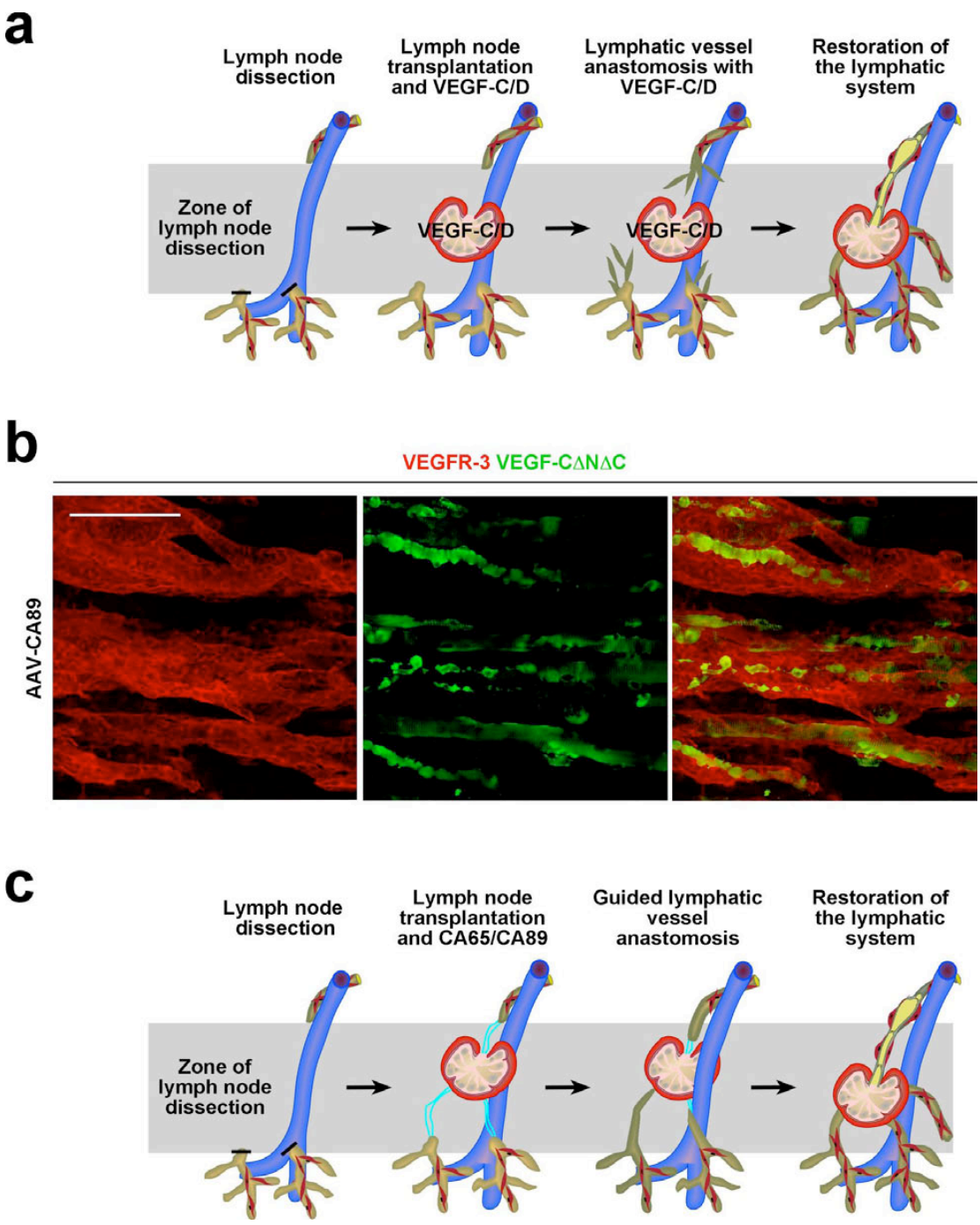


Figure 7. The therapeutic application of engineered heparin-binding and wild type forms of VEGF-C. (**a**) A schematic representation of lymph node transplantation combined to VEGF-C therapy. Lymph node transplants transduced with AdVEGF-C attract lymphatic vessels to form anastomoses with the lymph node (a summary of the findings reported in II). (**b**) Skeletal muscle transduced with AAV2 vectors encoding CA89, a chimera consisting of VEGF-CΔNΔC and exons 6a-8 of VEGF, in the mouse ear. Muscle fibers transduced with the vector are visualized by antibodies that recognize VEGF-CΔNΔC (green). Note that CA89 (green) is immobilized to the muscle fibers. Lymphatic vessels, visualized by LYVE-1 immunostaining (red) have formed longitudinally alongside the transduced muscle fibers. (**c**) Growth of the lymphatic vessels towards the transplanted lymph node could possibly be accelerated by heparin-containing filaments (light blue) coated with CA65 and/or CA89. Scalebar: 100 μm.

4. Blocking VEGF-3 suppresses angiogenic sprouting, vascular network formation and tumor growth (IV)

Angiogenesis is a key process in several pathological conditions, including tumor growth and AMD (reviewed in Carmeliet, 2005). VEGF potently promotes angiogenesis and is indispensable for vascular development (Carmeliet et al., 1996; Ferrara et al., 1996), while VEGFR-2 is the primary receptor transmitting VEGF signals in ECs (Shalaby et al., 1995; Gille et al., 2001). VEGFR-3 is present in all endothelia during development, but in the adult it becomes restricted to the lymphatic endothelium (Kaipainen et al., 1995). However, VEGFR-3 is upregulated in the microvasculature of tumors and wounds (Valtola et al., 1999; Paavonen et al., 2000). The fact that *Vegfr3/Flt4* gene-targeted mice and zebrafish exhibit severe blood vascular defects, and that VEGFR-3 function-blocking antibodies suppress tumor angiogenesis (Dumont et al., 1998; Covassin et al., 2006; Laakkonen et al., 2007), prompted an investigation into the mechanisms of how VEGFR-3 contributes to angiogenesis.

During late embryogenesis and in the adult, blood vessels form primarily by angiogenesis, i.e. sprouting from pre-existing vessels. Angiogenic sprouting involves EC specification into leading tip cells, which respond to VEGF guidance cues, and stalk cells that follow the tip cells (Gerhardt et al., 2003). The findings presented here demonstrated that VEGFR-3 is highly expressed in the tip cells of angiogenic sprouts, and genetic deletion of VEGFR-3 or blocking VEGFR-3 signaling with monoclonal antibodies results in decreased sprouting, vascular density, vessel branching, and EC proliferation *in vivo*. On the other hand, stimulation of VEGFR-3 augmented VEGF-induced angiogenesis and sustained angiogenesis even in the presence of VEGFR-2 inhibitors, whereas antibodies against VEGFR-3 and VEGFR-2 in combination resulted in additive inhibition of angiogenesis and tumor growth.

Expression of the VEGFR-3 ligand VEGF-C was detected in ECs of the developing intersomitic vessels and in retinal leukocytes. In addition, many tumors are known to produce VEGF-C, and this has been correlated with increased propensity for lymphatic metastasis (Stacker et al., 2002). The VEGF-C signal is therefore present for the activation of VEGFR-3 in all angiogenic settings studied (**Figure 8**). However, VEGF-C was not expressed in a gradient, which is typically thought to be required for the guided migration of cells (reviewed in Lundkvist et al., 2007). It is therefore possible that VEGF-C/VEGFR-3 signaling regulates

other aspects of vascular network formation, such as sprout fusion, or provides autocrine survival signals to the ECs, as has been described for VEGF (Lee et al., 2007).

Due to its silk-like domain, VEGF-C may aggregate into filaments with a long half-life in tissues (reviewed in Dicko et al., 2006). On the other hand, the endothelial tip cells express a variety of proteases, such as membrane-type-1 matrix metalloproteinase (MT1-MMP), to invade through the ECM (Yana et al., 2007). One possibility is that VEGF-C is a substrate of such proteases, leading to the release of mature VEGF-C, which potently activates both VEGFR-2 and VEGFR-3 homodimers, as well as VEGFR-2/VEGFR-3 heterodimers in the tip cells (Figure 8). This would indicate that the tip cells are capable of generating VEGF-C gradients by proteolysis. In analogy, certain tumor cells have been shown to generate chemotactic gradients by autocrine release of cytokines, which direct cell migration along lines of interstitial fluid flow (Shields et al., 2007).

However, the final explanation may not involve the canonical VEGFR-3 ligands at all: *Vegfc*-/-;*Vegfd*-/- double null embryos do not recapitulate the *Vegfr3*-/- null phenotype, and rather resemble *Vegfc*-/- single knock-outs, suggesting that alternative ligands for VEGFR-3 exist at least during early embryonic development (Haiko et al., 2008). It is possible that other VEGFs, such as VEGF, can promote endogenous activation of the VEGFR-3 signaling pathway in analogy to EGF-like growth factors that activate ErbB receptors by promoting receptor-mediated homodimerization or, alternatively, by the formation of heterodimers with the orphan receptor ErbB2 (reviewed in Olayioye et al., 2000). Furthermore, VEGF is present at very high concentrations in hypoxic tissue microenvironments (reviewed in Lundkvist et al., 2007), which may provide a stoichiometrically favorable ratio for VEGF-VEGFR-3 interactions that may previously have been overlooked *in vitro*.

Recent evidence indicates that VEGF induces Dll4 in the tip cells, which leads to suppression of excess sprouts in adjacent ECs (Noguera-Troise et al., 2006; Ridgway et al., 2006; Hellstrom et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007; reviewed in Roca and Adams, 2007). The data presented here indicate that VEGF signaling via VEGFR-2 also induces VEGFR-3 in the blood vascular endothelium. Furthermore, VEGFR-2 signals have been shown to be required for the maintenance of VEGFR-3 expression in the fenestrated endothelium under normal conditions (Kamba et al., 2006).

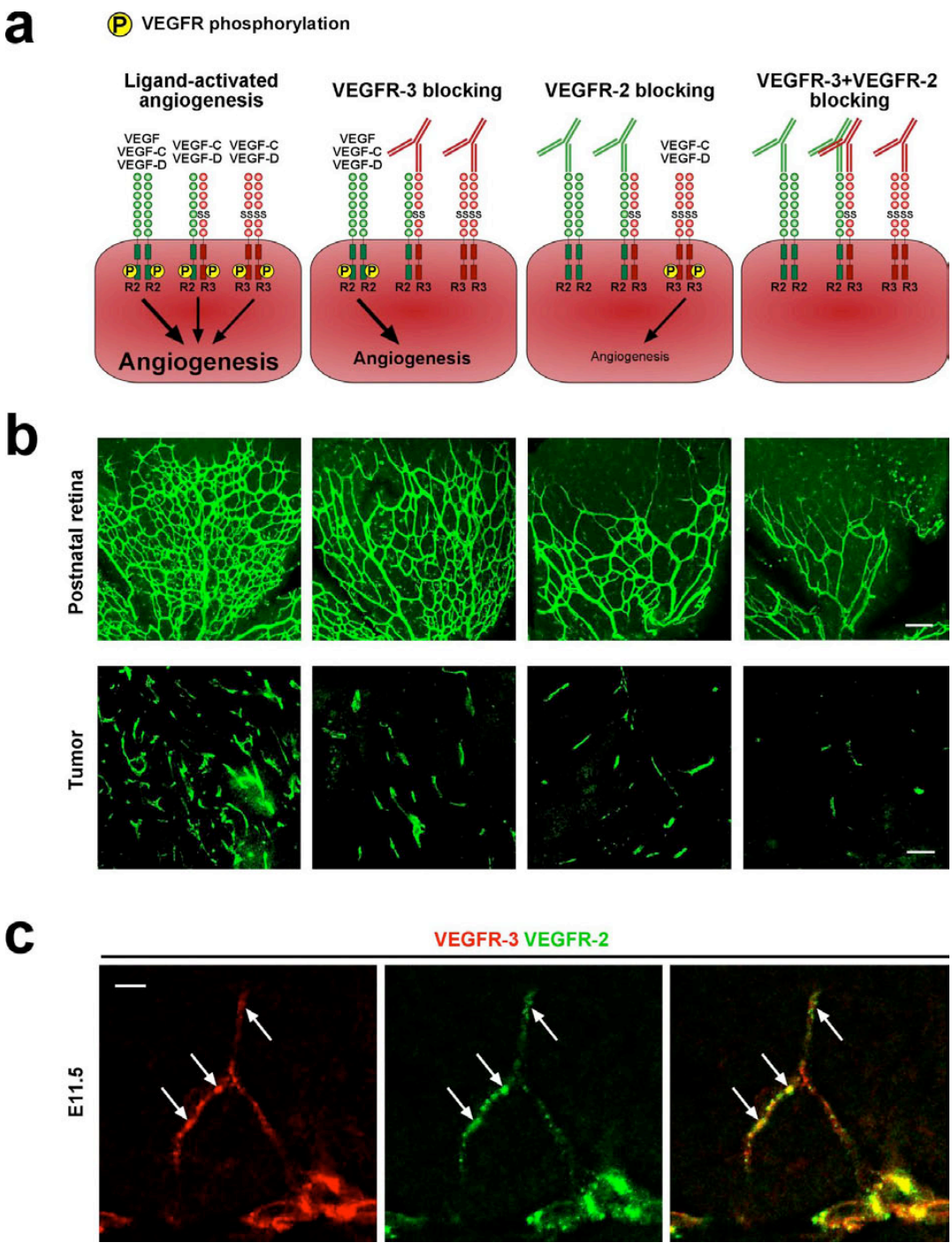


Figure 8. The role of VEGFR-3 in angiogenesis. (**a**) VEGFR-3 is not expressed in quiescent blood vascular endothelial cells. However, VEGFR-2 activation will induce VEGFR-3 in the blood vascular endothelium. Specific blocking of VEGFR-3 with monoclonal antibodies will attenuate angiogenic signaling in the endothelial cells, whereas blocking VEGFR-2 is more effective in blocking angiogenic signals. Blocking both VEGFR-2 and VEGFR-3 leads to additive inhibition of angiogenesis. (**b**) Angiogenesis in the mouse retina on postnatal day 5, and pathological angiogenesis in human tumor xenografts were studied (IV). Blood vessels were visualized by isolectin B4 staining and PECAM-1 immunostaining, respectively. (**c**) Arrows indicate co-localization of VEGFR-3 (red) and VEGFR-2 (green) in filopodial extensions of intersomitic vessel sprouts at E11.5. Scalebars: 100 μm (b); 10 μm (c).

A further objective of this study was to determine whether Dll4/Notch regulates VEGFR-3 expression in the angiogenic ECs. The data indicated that genetic or pharmacological disruption of the Notch signaling pathway led to widespread endothelial VEGFR-3 expression and excessive sprouting, which was inhibited by blocking VEGFR-3 signals. According to the results of this study, at the onset of angiogenesis VEGFR-3 becomes upregulated in the tip cells (by VEGF) and suppressed in the stalk cells (by Notch), indicating that VEGFR-3 functions as a positive regulator of endothelial sprouting and tip cell guidance. Interestingly, VEGFR-2 appears to be regulated in much the same way during angiogenesis (Gerhardt et al., 2003; Ridgway et al., 2006; Suchting et al., 2007; Lobov et al., 2007), suggesting that these two receptors collaborate, possibly via VEGFR-2/VEGFR-3 heterodimer signaling, in the tip cells (Figure 8c). The Notch signal is known to be high in the arteries during development, and these results may help explain why VEGFR-3 first becomes downregulated in arterial ECs during embryonic development (Dumont et al., 1998; Shutter et al., 2000; Lawson et al., 2001; Mailhos et al., 2001; Leslie et al., 2007; Siekmann and Lawson, 2007). A previous study has reported that NICD is capable of inducing *VEGFR3* transcription (Shawber et al., 2007), suggesting that *Vegfr3* repression by Notch is not direct and rather occurs via downstream effectors, such as the Hes and Hey transcription factors. A further mechanism downregulating VEGFR-3 in the nascent vessels may involve EC-pericyte interactions (Veikkola et al., 2003).

In addition to mature lymphatic vessels that do not require VEGFR-3 signals for survival (Makinen et al., 2001a), VEGFR-3 is only present in a few fenestrated endothelia and in angiogenic endothelium in adults (Kaipainen et al., 1995; Valtola et al., 1999; Paavonen et al., 2000; Partanen et al., 2000). As VEGF/VEGFR-2 pathway inhibitors have been shown to cause nephrosis and proteinuria in both humans and in animal models, the effects of VEGFR-3 blockers on kidney function and histology were thoroughly analyzed. Ultrastructural analysis of kidneys of all antibody-treated mice, as well as the K14-VEGFR-3-Ig transgenic mice by transmission electron microscopy did not reveal any hallmarks of glomerular damage, when compared to several examples in the literature (Gerber et al., 1999; Cingel-Ristic et al., 2005; Elliot et al., 2007). Furthermore, nephrosis or pathological albuminuria was not observed in mice that were treated with VEGFR-3 or VEGFR-2 antibodies, or both in combination, indicating normal kidney function (Sugimoto et al., 2003). These findings suggest that VEGFR-3 could be safely targeted in patients.

Surprisingly, even VEGFR-2 function-blocking antibodies did not induce kidney damage in our experiments. These data are in line with the literature showing that adult mice are more resistant to the adverse effects of VEGF/VEGFR-2 inhibitors (Baffert et al., 2004), unlike neonatal or adolescent mice (Kitamoto et al., 1997; Gerber et al., 1999; Eremina et al., 2003).

These results implicate VEGFR-3 as a novel regulator of angiogenic sprouting and vascular network formation, and suggest that VEGFR-3 can be safely targeted in adult patients to provide additional efficacy for anti-angiogenic therapies, especially towards vessels that are resistant to VEGF/VEGFR-2 inhibitors.

CONCLUSIONS

The blood and lymphatic vascular systems are essential for life, but they may become harnessed for sinister purposes in pathological conditions. For example, tumors learn to grow a network of blood vessels, securing a source of oxygen and nutrients for sustained growth. On the other hand, damage to the lymph nodes and the collecting lymphatic vessels may lead to lymphedema.

The Ang1/Tie2 pathway has previously been implicated in promoting endothelial stability and integrity of EC monolayers. The studies presented here elucidate a novel function for Ang1 as a lymphangiogenic factor. Ang1 is known to decrease the permeability of blood vessels, and could thus act as a more global antagonist of plasma leakage and tissue edema by promoting growth of lymphatic vessels and thereby facilitating removal of excess fluid and other plasma components from the interstitium. These findings reinforce the idea that Ang1 may have therapeutic value in conditions of tissue edema.

VEGF-C and VEGF-D are potent lymphangiogenic factors, with direct and remarkably specific effects on the lymphatic endothelium in adult tissues. VEGF-C and VEGF-D therapy restored the collecting lymphatic vessels in a novel orthotopic mouse model of breast cancer-related lymphedema. These results introduce a novel approach to improve VEGF-C/VEGF-D therapy by using engineered heparin-binding forms of VEGF-C, which induced the rapid formation of organized lymphatic vessels. Importantly, VEGF-C therapy also greatly improved the survival and integration of lymph node transplants. The combination of lymph node transplantation and VEGF-C therapy provides a basis for future therapy of lymphedema.

In adults, VEGFR-3 expression is restricted to the lymphatic endothelium and the fenestrated endothelia of certain endocrine organs. However, these results show that VEGFR-3 is induced in the leading endothelial tip cells at the onset of angiogenesis, providing a tumor-specific vascular target. VEGFR-3 acts downstream of VEGF/VEGFR-2 signals, but, once induced, can sustain angiogenesis when VEGFR-2 signaling is inhibited. The data presented here implicate VEGFR-3 as a novel regulator of sprouting angiogenesis along with its role in regulating lymphatic vessel growth. Targeting VEGFR-3 may provide added efficacy to currently available anti-angiogenic therapies, which typically target the VEGF/VEGFR-2 pathway.

ACKNOWLEDGEMENTS

This work was carried out in the Molecular/Cancer Biology Laboratory, University of Helsinki during 2002-2008. I thank the staff at Biomedicum Helsinki and the Haartman Institute for a pleasant and productive working environment with special thanks to Olli Jänne, Eero Saksela, Pekka Häyry, and Seppo Meri for excellent research facilities.

I would like to express my deep gratitude to my thesis supervisor Kari Alitalo for providing me the opportunity to work in his group of outstanding scientists. The expertise in the lab spans all key aspects of biomedical research, and Kari is ever keen on adding to the resources available. I respect Kari's dedication to science and his enthusiasm to pursue the most challenging questions. I am grateful for his support, mentorship, and understanding especially during the times when I had to work under extreme stress.

I thank all of my co-authors without whom the work would not have been at all possible. I am especially indebted to Anne for adopting me right from the start, for key contributions to the science, and for cheering me up with that positive attitude. Yulong is thanked for the very professional and significant contribution to original contribution III, and for help and guidance during my early days in the lab. My students Aino, Gina, Johannes, and Anna K. are thanked for their enthusiasm, and the hard work on the various projects over the years. I have also received help and guidance from Bänä, Camilla, Caroline, Denis, Hanna H., Maria, Marika K., Marika W., Marja, Michael, Karri, Katri, Paula Haiko, Salla, Tanja, Tanya, and Wolf. Thank you all! I am grateful to Seppo Ylä-Herttuala and his group for outstanding adenoviral vectors, which were used in all of the papers discussed here. I would also like to acknowledge the valuable contribution of the very many collaborators from laboratories outside Finland, especially Peter Baluk, Mats Hellström, Tohru Morisada, Steven Suchting, and Elisabet Wallgard.

I am grateful to Tomi Mäkelä, Aija Kaitera, Päivi Ojala, and Elina Värtö for the opportunities provided by the Helsinki Biomedical Graduate School. Tomi Mäkelä (again) and Kirsi Sainio are thanked for serving in my Thesis Committee, and for providing encouragement and valuable advice. I would like to express my sincere gratitude to Sirpa Jalkanen and Tomi Mäkelä (yet again) for the very professional review of my thesis on an extremely tight schedule.

I warmly thank the past and present staff of M/CBL for the significant help and support I have received over the years, as well as for the humor that spiced up the routines in the lab. Guys' nights out with Alun, Antti, Bänä, Iiro, Jarkko, Juho, Marko, Nikke, Karri, Ron, Tapio, and Ville were much enjoyed. Besides knowing where everything is (and why), Tapio is thanked for his friendship and memorable accounts of lab history. I am grateful to Anna M., Mari, Paula Hyvärinen, Riikka, Sanna L., Sanna W., Sari, and Tanja for their friendly and professional assistance, which has been invaluable for this work. Seija, Merja, and Marjatta are acknowledged for instrument care. I would like to thank Anita, Anu, Leena, Paula T., Tarja, and other employees of the Meilahti Experimental Animal Facility for excellent animal care. Elisa, Juhani, Miia, Samu, and Tuomas L. are thanked for administrative help.

I would like to thank all of my friends for their support and understanding, and, most importantly, for every occasion they dragged me out of the lab. Thanks to you guys I don't feel like I missed out on anything, were it parties, trips, or just hanging out. I am especially grateful to Jere, Jesse, Kimmo, Lauri K. and Lauri P. for always being there for me, and for programming all kinds of fun in my life. Special thanks go to Calle and Samuel for their friendship, as well as for help with grant applications and clinical pharmacology, respectively.

I am grateful to my parents Outi and Teuvo for their love and support, as well as for clearing up a path, which was easy for me to follow. I thank Elias for being the best brother, and for cheering me up along the way. I would like to thank my grandparents Jorma, Marjatta, Tellervo, and Urho for their love and valuable insight on life.

I dedicate this thesis to Katja, who continues to challenge me, knows me better than anyone, and lights up my day.
I love you.

I have been financially supported by grants from the Biomedicum Helsinki Foundation, the Emil Aaltonen Foundation, the Farmos Research Foundation, the Finnish Cancer Organizations, the Finnish Cultural Foundation, the Finnish Medical Foundation, Finska Läkaresällskapet, the Ida Montin Foundation, the K. Albin Johansson Foundation, Nylands Nation, the Paulo Foundation, the Oskar Öflund Foundation, the Sigrid Juselius Foundation, and the University of Helsinki Fund, which are sincerely acknowledged.

Helsinki, May 5, 2008

REFERENCES

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.

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., Williams, R. A., Minekus, M. P., Thornton, G. E., Stenvers, K., Rogers, P. A., Lederman, F., Roufail, S., and Stacker, S. A. (2001). Localization of vascular endothelial growth factor-D in malignant melanoma suggests a role in tumour angiogenesis. J Pathol *193*, 147-154.

Adams, R. H. (2002). Vascular patterning by Eph receptor tyrosine kinases and ephrins. Semin Cell Dev Biol *13*, 55-60.

Aicher, A., Zeiher, A. M., and Dimmeler, S. (2005). Mobilizing endothelial progenitor cells. Hypertension *45*, 321-325.

Alitalo, K., Tammela, T., and Petrova, T. V. (2005). Lymphangiogenesis in development and human disease. Nature *438*, 946-953.

Ambati, B. K., Nozaki, M., Singh, N., Takeda, A., Jani, P. D., Suthar, T., Albuquerque, R. J., Richter, E., Sakurai, E., Newcomb, M. T., *et al.* (2006). Corneal avascularity is due to soluble VEGF receptor-1. Nature *443*, 993-997.

Angeli, V., Ginhoux, F., Llodra, 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.

Ansel, K. M., Ngo, V. N., Hyman, P. L., Luther, S. A., Forster, R., Sedgwick, J. D., Browning, J. L., Lipp, M., and Cyster, J. G. (2000). A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature *406*, 309-314.

Arany, Z., Foo, S. Y., Ma, Y., Ruas, J. L., Bommi-Reddy, A., Girnun, G., Cooper, M., Laznik, D., Chinsomboon, J., Rangwala, S. M., *et al.* (2008). HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. Nature *451*, 1008-1012.

Arap, W., Pasqualini, R., and Ruoslahti, E. (1998). Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science *279*, 377-380.

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

Arras, M., Ito, W. D., Scholz, D., Winkler, B., Schaper, J., and Schaper, W. (1998). Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. J Clin Invest *101*, 40-50.

Axelson, H., Fredlund, E., Ovenberger, M., Landberg, G., and Pahlman, S. (2005). Hypoxia-induced dedifferentiation of tumor cells--a mechanism behind heterogeneity and aggressiveness of solid tumors. Semin Cell Dev Biol *16*, 554-563.

Baffert, F., Thurston, G., Rochon-Duck, M., Le, T., Brekken, R., and McDonald, D. M. (2004). Age-related changes in vascular endothelial growth factor dependency and angiopoietin-1-induced plasticity of adult blood vessels. Circ Res *94*, 984-992.

Baka, S., Clamp, A. R., and Jayson, G. C. (2006). A review of the latest clinical compounds to inhibit VEGF in pathological angiogenesis. Expert Opin Ther Targets *10*, 867-876.

Baldwin, M. E., Catimel, B., Nice, E. C., Roufail, S., Hall, N. E., Stenvers, K. L., Kärkkäinen, M. J., Alitalo, K., Stacker, S. A., and Achen, M. G. (2001). 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 dispensable for development of the lymphatic system. Mol Cell Biol *25*, 2441-2449.

Baluk, P., Fuxe, J., Hashizume, H., Romano, T., Lashnits, E., Butz, S., Vestweber, D., Corada, M., Molendini, C., Dejana, E., and McDonald, D. M. (2007). Functionally specialized junctions between endothelial cells of lymphatic vessels. J Exp Med *204*, 2349-2362.

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.

Bando, H., Brokelmann, M., Toi, M., Alitalo, K., Sleeman, J. P., Sipos, B., Grone, H. J., and Weich, H. A. (2004). Immunodetection and quantification of vascular endothelial growth factor receptor-3 in human malignant tumor tissues. Int J Cancer *111*, 184-191.

Barton, W. A., Tzvetkova, D., and Nikolov, D. B. (2005). Structure of the angiopoietin-2 receptor binding domain and identification of surfaces involved in Tie2 recognition. Structure *13*, 825-832.

Barton, W. A., Tzvetkova-Robev, D., Miranda, E. P., Kolev, M. V., Rajashankar, K. R., Himanen, J. P., and Nikolov, D. B. (2006). Crystal structures of the Tie2 receptor ectodomain and the angiopoietin-2-Tie2 complex. Nat Struct Mol Biol *13*, 524-532.

Baumeister, R. G., Seifert, J., and Hahn, D. (1981). Autotransplantation of lymphatic vessels. Lancet, 147.
Bazzoni, G., and Dejana, E. (2004). Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. Physiol Rev *84*, 869-901.

Becker, C., Assouad, J., Riquet, M., and Hidden, G. (2006). Postmastectomy lymphedema: long-term results following microsurgical lymph node transplantation. Ann Surg *243*, 313-315.

Bell, R., Brice, G., Child, A. H., Murday, V. A., Mansour, S., Sandy, C. J., Collin, J. R. O., 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.

Benjamin, L. E., and Keshet, E. (1997). Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. Proc Natl Acad Sci USA *94*, 8761-8766.

Bergers, G., Song, S., Meyer-Morse, N., Bergsland, E., and Hanahan, D. (2003). Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest *111*, 1287-1295.

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

Bjorndahl, M., Cao, R., Nissen, L. J., Clasper, S., Johnson, L. A., Xue, Y., Zhou, Z., Jackson, D., Hansen, A. J., and Cao, Y. (2005). Insulin-like growth factors 1 and 2 induce lymphangiogenesis in vivo. Proc Natl Acad Sci USA *102*, 15593-15598.

Bray, S. J. (2006). Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol *7*, 678-689.

Brindle, N. P., Saharinen, P., and Alitalo, K. (2006). Signaling and functions of angiopoietin-1 in vascular protection. Circ Res *98*, 1014-1023.

Cao, R., Bjorndahl, M. A., Gallego, M. I., Chen, S., Religa, P., Hansen, A. J., and Cao, Y. (2006). Hepatocyte growth factor is a lymphangiogenic factor with an indirect mechanism of action. Blood *107*, 3531-3536.

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.

Carlson, T. R., Feng, Y., Maisonpierre, P. C., Mrksich, M., and Morla, A. O. (2001). Direct cell adhesion to the angiopoietins mediated by integrins. J Biol Chem *276*, 26516-26525.

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.* (2001a). Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med *7*, 575-583.

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

Carmeliet, P., Ng, Y. S., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Ehler, E., Kakkar, V. V., Stalmans, I., Mattot, V.*, et al.* (1999). Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Nat Med *5*, 495-502.

Carmeliet, P., and Tessier-Lavigne, M. (2005). Common mechanisms of nerve and blood vessel wiring. Nature *436*, 193-200.

Carvalho, J. F., Blank, M., and Shoenfeld, Y. (2007). Vascular endothelial growth factor (VEGF) in autoimmune diseases. J Clin Immunol *27*, 246-256.

Carvalho, R. L., Jonker, L., Goumans, M. J., Larsson, J., Bouwman, P., Karlsson, S., Dijke, P. T., Arthur, H. M., and Mummery, C. L. (2004). Defective paracrine signalling by TGFbeta in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia. Development *131*, 6237-6247.

Cascone, I., Napione, L., Maniero, F., Serini, G., and Bussolino, F. (2005). Stable interaction between alpha5beta1 integrin and Tie2 tyrosine kinase receptor regulates endothelial cell response to Ang-1. J Cell Biol *170*, 993-1004.

Chang, L. K., Garcia-Cardena, G., Farnebo, F., Fannon, M., Chen, E. J., Butterfield, C., Moses, M. A., Mulligan, R. C., Folkman, J., and Kaipainen, A. (2004). Dose-dependent response of FGF-2 for lymphangiogenesis. Proc Natl Acad Sci USA *101*, 11658-11663.

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.

Cho, C. H., Kammerer, R. A., Lee, H. J., Steinmetz, M. O., Ryu, Y. S., Lee, S. H., Yasunaga, K., Kim, K. T., Kim, I., Choi, H. H.*, et al.* (2004a). COMP-Ang1: a designed angiopoietin-1 variant with nonleaky angiogenic activity. Proc Natl Acad Sci USA *101*, 5547-5552.

Cho, C. H., Kammerer, R. A., Lee, H. J., Yasunaga, K., Kim, K. T., Choi, H. H., Kim, W., Kim, S. H., Park, S. K., Lee, G. M., and Koh, G. Y. (2004b). Designed angiopoietin-1 variant, COMP-Ang1, protects against radiation-induced endothelial cell apoptosis. Proc Natl Acad Sci USA 101, 5553-5558.

Cho, C. H., Sung, H. K., Kim, K. T., Cheon, H. G., Oh, G. T., Hong, H. J., Yoo, O. J., and Koh, G. Y. (2006). COMP-angiopoietin-1 promotes wound healing through enhanced angiogenesis, lymphangiogenesis, and blood flow in a diabetic mouse model. Proc Natl Acad Sci USA *103*, 4946-4951.

Cingel-Ristic, V., Schrijvers, B. F., van Vliet, A. K., Rasch, R., Han, V. K., Drop, S. L., and Flyvbjerg, A. (2005). Kidney growth in normal and diabetic mice is not affected by human insulin-like growth factor binding protein-1 administration. Exp Biol Med (Maywood) *230*, 135-143.

Clark, B., Sitzia, J., and Harlow, W. (2005). Incidence and risk of arm oedema following treatment for breast cancer: a three-year follow-up study. Qjm *98*, 343-348.

Coultas, L., Chawengsaksophak, K., and Rossant, J. (2005). Endothelial cells and VEGF in vascular development. Nature *438*, 937-945.

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 USA *103*, 6554-6559.

Crosby, J. R., Seifert, R. A., Soriano, P., and Bowen-Pope, D. F. (1998). Chimaeric analysis reveals role of Pdgf receptors in all muscle lineages. Nat Genet *18*, 385-388.

Cursiefen, C., Cao, J., Chen, L., Liu, Y., Maruyama, K., Jackson, D., Kruse, F. E., Wiegand, S. J., Dana, M. R., and Streilein, J. W. (2004). Inhibition of hemangiogenesis and lymphangiogenesis after normal-risk corneal transplantation by neutralizing VEGF promotes graft survival. Invest Ophthalmol Vis Sci *45*, 2666-2673.

Cyster, J. G. (1999). Chemokines and the homing of dendritic cells to the T cell areas of lymphoid organs. J Exp Med *189*, 447-450.

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.

Dallabrida, S. M., Ismail, N., Oberle, J. R., Himes, B. E., and Rupnick, M. A. (2005). Angiopoietin-1 promotes cardiac and skeletal myocyte survival through integrins. Circ Res *96*, e8-24.

Davis, G. E., Bayless, K. J., and Mavila, A. (2002). Molecular basis of endothelial cell morphogenesis in three-dimensional extracellular matrices. Anat Rec *268*, 252-275.

Davis, G. E., and Senger, D. R. (2005). Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circ Res *97*, 1093-1107.

Davis, S., Aldrich, T. H., Jones, P. F., Acheson, A., Compton, D. L., Jain, V., Ryan, T. E., Bruno, J., Raziejewski, C., Maisonpierre, P. C., and Yancopoulos, G. D. (1997). Isolation of Angiopoietin-1, a Ligand for the TIE2 receptor, by Secretion-Trap Expression Cloning. Cell *87*, 1161-1169.

De Togni, P., Goellner, J., Ruddle, N. H., Streeter, P. R., Fick, A., Mariathasan, S., Smith, S. C., Carlson, R., Shornick, L. P., Strauss-Schoenberger, J., and et al. (1994). Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science *264*, 703-707.

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.

Demetri, G. D., van Oosterom, A. T., Garrett, C. R., Blackstein, M. E., Shah, M. H., Verweij, J., McArthur, G., Judson, I. R., Heinrich, M. C., Morgan, J. A.*, et al.* (2006). Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. Lancet *368*, 1329-1338.

Dicko, C., Kenney, J. M., and Vollrath, F. (2006). Beta-silks: enhancing and controlling aggregation. Adv Protein Chem *73*, 17-53.

Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S., and Akhurst, R. J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development *121*, 1845-1854.

Dixelius, J., Makinen, T., Wirzenius, M., Kärkkäinen, 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.

Djonov, V., Schmid, M., Tschanz, S. A., and Burri, P. H. (2000). Intussusceptive angiogenesis: its role in embryonic vascular network formation. Circ Res *86*, 286-292.

Döffinger, R., Smahi, A., Bessia, C., Geissmann, F., Feinberg, J., Durandy, A., Bodemer, C., Kenwrick, S., Dupuis-Girod, S., Blanche, S.*, et al.* (2001). X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-κB signaling. Nat Genet *27*, 277-285.

Dor, Y., Djonov, V., Abramovitch, R., Itin, A., Fishman, G. I., Carmeliet, P., Goelman, G., and Keshet, E. (2002). Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy. EMBO J *21*, 1939-1947.

Drake, C. J., Cheresh, D. A., and Little, C. D. (1995). An antagonist of integrin alpha v beta 3 prevents maturation of blood vessels during embryonic neovascularization. J Cell Sci *108 (Pt 7)*, 2655-2661.

Drayton, D. L., Liao, S., Mounzer, R. H., and Ruddle, N. H. (2006). Lymphoid organ development: from ontogeny to neogenesis. Nat Immunol *7*, 344-353.

Duarte, A., Hirashima, M., Benedito, R., Trindade, A., Diniz, P., Bekman, E., Costa, L., Henrique, D., and Rossant, J. (2004a). Dosage-sensitive requirement for mouse Dll4 in artery development. Genes Dev *18*, 2474-2478.

Dumont, D. J., Gradwohl, G. J., Fong, G.-H., Auerbach, R., and Breitman, M. L. (1993). The endothelial-specific receptor tyrosine kinase, *tek*, is a member of a new subfamily of receptors. Oncogene *8*, 1293-1301.

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.

Dvorak, H. F. (2002). Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. J Clin Oncol *20*, 4368-4380.

Ehebauer, M., Hayward, P., and Martinez-Arias, A. (2006). Notch signaling pathway. Sci STKE *2006*.

Elliot, S. J., Berho, M., Korach, K., Doublier, S., Lupia, E., Striker, G. E., and Karl, M. (2007). Gender-specific effects of endogenous testosterone: female alpha-estrogen receptor-deficient C57Bl/6J mice develop glomerulosclerosis. Kidney Int *72*, 464-472.

Enholm, B., Kärpänen, 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.

Enholm, B., Paavonen, K., Ristimaki, A., Kumar, V., Gunji, Y., Klefstrom, J., Kivinen, L., Laiho, M., Olofsson, B., Joukov, V.*, et al.* (1997). Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. Oncogene *14*, 2475-2483.

Eremina, V., Sood, M., Haigh, J., Nagy, A., Lajoie, G., Ferrara, N., Gerber, H. P., Kikkawa, Y., Miner, J. H., and Quaggin, S. E. (2003). Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. J Clin Invest *111*, 707-716.

Escudier, B., Eisen, T., Stadler, W. M., Szczylik, C., Oudard, S., Siebels, M., Negrier, S., Chevreau, C., Solska, E., Desai, A. A.*, et al.* (2007a). Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med *356*, 125-134.

Escudier, B., Pluzanska, A., Koralewski, P., Ravaud, A., Bracarda, S., Szczylik, C., Chevreau, C., Filipek, M., Melichar, B., Bajetta, E.*, et al.* (2007b). Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double-blind phase III trial. Lancet *370*, 2103-2111.

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. (2005). The role of VEGF in the regulation of physiological and pathological angiogenesis. Exs, 209-231.

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 Henzel, W. J. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun *161*, 851-855.

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

Ferrara, N., Mass, R. D., Campa, C., and Kim, R. (2007). Targeting VEGF-A to treat cancer and age-related macular degeneration. Annu Rev Med *58*, 491-504.

Fiedler, U., Christian, S., Koidl, S., Kerjaschki, D., Emmett, M. S., Bates, D. O., Christofori, G., and Augustin, H. G. (2006a). The sialomucin CD34 is a marker of lymphatic endothelial cells in human tumors. Am J Pathol *168*, 1045-1053.

Fiedler, U., Reiss, Y., Scharpfenecker, M., Grunow, V., Koidl, S., Thurston, G., Gale, N. W., Witzenrath, M., Rosseau, S., Suttorp, N., *et al.* (2006b). Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat Med *12*, 235-239.

Fiedler, U., Scharpfenecker, M., Koidl, S., Hegen, A., Grunow, V., Schmidt, J. M., Kriz, W., Thurston, G., and Augustin, H. G. (2004). The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. Blood *103*, 4150-4156.

Finegold, D. N., Kimak, M. A., Lawrence, E. C., Levinson, K. L., Cherniske, E. M., Pober, B. R., Dunlap, J. W., and Ferrell, R. E. (2001). Truncating mutations in FOXC2 cause multiple lymphedema syndromes. Hum Mol Genet *10*, 1185-1189.

Finke, D., Acha-Orbea, H., Mattis, A., Lipp, M., and Kraehenbuhl, J. (2002). CD4+CD3- cells induce Peyer's patch development: role of alpha4beta1 integrin activation by CXCR5. Immunity *17*, 363-373.

Fischer, A., and Gessler, M. (2003). Hey genes in cardiovascular development. Trends Cardiovasc Med *13*, 221-226.

Fischer, C., Jonckx, B., Mazzone, M., Zacchigna, S., Loges, S., Pattarini, L., Chorianopoulos, E., Liesenborghs, L., Koch, M., De Mol, M., *et al.* (2007). Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. Cell *131*, 463-475.

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

Folkman, J., Watson, K., Ingber, D., and Hanahan, D. (1989). Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature *339*, 58-61.

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.

Forster, R., Mattis, A. E., Kremmer, E., Wolf, E., Brem, G., and Lipp, M. (1996). A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. Cell *87*, 1037-1047.

Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E., and Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell *99*, 23-33.

Gale, N. W., Dominguez, M. G., Noguera, I., Pan, L., Hughes, V., Valenzuela, D. M., Murphy, A. J., Adams, N. C., Lin, H. C., Holash, J., *et al.* (2004). Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc Natl Acad Sci USA *101*, 15949-15954.

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.

Garcia-Cardena, G., Comander, J., Anderson, K. R., Blackman, B. R., and Gimbrone, M. A., Jr. (2001). Biomechanical activation of vascular endothelium as a determinant of its functional phenotype. Proc Natl Acad Sci USA *98*, 4478-4485.

Gariano, R. F., and Gardner, T. W. (2005). Retinal angiogenesis in development and disease. Nature *438*, 960-966.

Gavard, J., and Gutkind, J. S. (2006). VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nat Cell Biol *8*, 1223-1234.

Gavard, J., Patel, V., and Gutkind, J. S. (2008). Angiopoietin-1 prevents VEGF-induced endothelial permeability by sequestering Src through mDia. Dev Cell *14*, 25-36.

Gerber, H.-P., Hillan, K. J., Ryan, A. M., Kowalski, J., Keller, G.-A., Rangell, L., Wright, B. D., Radtke, F., Aguet, M., and Ferrara, N. (1999). VEGF is required for growth and survival in neonatal mice. Development *126*, 1149-1159.

Gerber, H. P., Dixit, V., and Ferrara, N. (1998). Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem *273*, 13313-13316.

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.

Gerhardt, H., Ruhrberg, C., Abramsson, A., Fujisawa, H., Shima, D., and Betsholtz, C. (2004). Neuropilin-1 is required for endothelial tip cell guidance in the developing central nervous system. Dev Dyn *231*, 503-509.

Gerhardt, H., Wolburg, H., and Redies, C. (2000). N-cadherin mediates pericytic-endothelial interaction during brain angiogenesis in the chicken. Dev Dyn *218*, 472-479.

Ghabrial, A. S., and Krasnow, M. A. (2006). Social interactions among epithelial cells during tracheal branching morphogenesis. Nature *441*, 746-749.

Gille, H., Kowalski, J., Li, B., LeCouter, J., Moffat, B., Zioncheck, T. F., Pelletier, N., and Ferrara, N. (2001). Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. J Biol Chem *276*, 3222-3230.

Gitay-Goren, H., Cohen, T., Tessler, S., Soker, S., Gengrinovitch, S., Rockwell, P., Klagsbrun, M., Levi, B. Z., and Neufeld, G. (1996). Selective binding of VEGF121 to one of the three vascular endothelial growth factor receptors of vascular endothelial cells. J Biol Chem *271*, 5519-5523.

Gluzman-Poltorak, Z., Cohen, T., Herzog, Y., and Neufeld, G. (2000). Neuropilin-2 and neuropilin-1 are receptors for the 165-amino acid form of vascular endothelial growth factor (VEGF) and of placenta growth factor-2, but only neuropilin-2 functions as a receptor for the 145-amino acid form of VEGF. J Biol Chem *275*, 18040-18045.

Goede, V., Schmidt, T., Kimmina, S., Kozian, D., and Augustin, H. G. (1998). Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest *78*, 1385-1394.

Goumans, M. J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P., and ten Dijke, P. (2002). Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J *21*, 1743-1753.

Grau, S. J., Trillsch, F., Herms, J., Thon, N., Nelson, P. J., Tonn, J. C., and Goldbrunner, R. (2007). Expression of VEGFR3 in glioma endothelium correlates with tumor grade. J Neurooncol *82*, 141-150.

Greenberg, D. A., and Jin, K. (2005). From angiogenesis to neuropathology. Nature *438*, 954-959.

Gunn, M. D., Kyuwa, S., Tam, C., Kakiuchi, T., Matsuzawa, A., Williams, L. T., and Nakano, H. (1999). Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. J Exp Med *189*, 451-460.

Haiko, P., Makinen, T., Keskitalo, S., Taipale, J., Kärkkäinen, M., Baldwin, M. E., Stacker, S. A., Achen, M. G., and Alitalo, K. (2008). Deletion of VEGF-C and VEGF-D is not equivalent to VEGFR-3-null in mouse embryos. Mol Cell Biol *In press*.

Hamada, K., Oike, Y., Takakura, N., Ito, Y., Jussila, L., Dumont, D. J., Alitalo, K., and Suda, T. (2000). VEGF-C signaling pathways through VEGFR-2 and VEGFR-3 in vasculoangiogenesis and hematopoiesis. Blood *96*, 3793-3800.

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.

Hanahan, D. (1985). Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature *315*, 115-122.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Harvey, N. L., Srinivasan, R. S., Dillard, M. E., Johnson, N. C., Witte, M. H., Boyd, K., Sleeman, M. W., and Oliver, G. (2005). Lymphatic vascular defects promoted by Prox1 haploinsufficiency cause adult-onset obesity. Nat Genet *37*, 1072-1081.

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.

Hayes, A. J., Huang, W. Q., Mallah, J., Yang, D., Lippman, M. E., and Li, L. Y. (1999). Angiopoietin-1 and its receptor Tie-2 participate in the regulation of capillary-like tubule formation and survival of endothelial cells. Microvasc Res *58*, 224-237.

He, Y., Kozaki, K., Kärpänen, 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., Ilmonen, M., Makinen, T., Kärkkäinen, M. J., Haiko, P., Salven, P., and Alitalo, K. (2004). Preexisting lymphatic endothelium but not endothelial progenitor cells are essential for tumor lymphangiogenesis and lymphatic metastasis. Cancer Res *64*, 3737-3740.

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.

Heissig, B., Hattori, K., Friedrich, M., Rafii, S., and Werb, Z. (2003). Angiogenesis: vascular remodeling of the extracellular matrix involves metalloproteinases. Curr Opin Hematol *10*, 136-141.

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.

Hellstrom, M., Phng, L. K., Hofmann, J. J., Wallgard, E., Coultas, L., Lindblom, P., Alva, J., Nilsson, A. K., Karlsson, L., Gaiano, N.*, et al.* (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature *445*, 776-780.

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.

Hirashima, M., and Suda, T. (2006). Differentiation of arterial and venous endothelial cells and vascular morphogenesis. Endothelium, 137-145.

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.

Holash, J., Davis, S., Papadopoulos, N., Croll, S. D., Ho, L., Russell, M., Boland, P., Leidich, R., Hylton, D., Burova, E.*, et al.* (2002). VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci USA *99*, 11393-11398.

Holash, J., Maisonpierre, P. C., Compton, D., Boland, P., Alexander, C. R., Zagzag, D., Yancopoulos, G. D., and Wiegand, S. J. (1999). Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science *284*, 1994-1998.

Hood, J. D., Meininger, C. J., Ziche, M., and Granger, H. J. (1998). VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. Am J Physiol *274*, H1054-1058.

Houck, K., Leung, D., Rowland, A., Winer, J., and Ferrara, N. (1992). Dual regulation of vescular 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 alpha9beta1. Mol Cell Biol *20*, 5208-5215.

Hurlbut, G. D., Kankel, M. W., Lake, R. J., and Artavanis-Tsakonas, S. (2007). Crossing paths with Notch in the hyper-network. Curr Opin Cell Biol *19*, 166-175.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E.*, et al.* (2004). Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med *350*, 2335-2342.

Hutchings, H., Ortega, N., and Plouet, J. (2003). Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion, migration, and survival through integrin ligation. FASEB J *17*, 1520-1522.

Iivanainen, E., Nelimarkka, L., Elenius, V., Heikkinen, S. M., Junttila, T. T., Sihombing, L., Sundvall, M., Maatta, J. A., Laine, V. J., Yla-Herttuala, S.*, et al.* (2003). Angiopoietin-regulated recruitment of vascular smooth muscle cells by endothelial-derived heparin binding EGF-like growth factor. FASEB J *17*, 1609-1621.

Ikeya, T., and Hayashi, S. (1999). Interplay of Notch and FGF signaling restricts cell fate and MAPK activation in the Drosophila trachea. Development *126*, 4455-4463.

Ikomi, F., Yokoyama, Y., Ogiwara, N., Sasaki, K., Mizuno, R., and Ohhashi, T. (2006). Recanalization of the collecting lymphatics in rabbit hind leg. Microcirculation *13*, 365-376.

Inai, T., Mancuso, M., Hashizume, H., Baffert, F., Haskell, A., Baluk, P., Hu-Lowe, D. D., Shalinsky, D. R., Thurston, G., Yancopoulos, G. D., and McDonald, D. M. (2004). Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. Am J Pathol *165*, 35-52.

Inoue, M., Hager, J. H., Ferrara, N., Gerber, H. P., and Hanahan, D. (2002). VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. Cancer Cell *1*, 193-202.

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., Kärkkäinen, 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.

Iso, T., Kedes, L., and Hamamori, Y. (2003). HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol *194*, 237-255.

Ito, W. D., Arras, M., Winkler, B., Scholz, D., Schaper, J., and Schaper, W. (1997). Monocyte chemotactic protein-1 increases collateral and peripheral conductance after femoral artery occlusion. Circ Res *80*, 829-837.

Iwamoto, R., Yamazaki, S., Asakura, M., Takashima, S., Hasuwa, H., Miyado, K., Adachi, S., Kitakaze, M., Hashimoto, K., Raab, G.*, et al.* (2003). Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. Proc Natl Acad Sci USA *100*, 3221-3226.

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

Jain, R. K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science *307*, 58-62.

Jakobsson, L., Kreuger, J., Holmborn, K., Lundin, L., Eriksson, I., Kjellen, L., and Claesson-Welsh, L. (2006). Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis. Dev Cell *10*, 625-634.

James, K., Hosking, B., Gardner, J., Muscat, G. E., and Koopman, P. (2003). Sox18 mutations in the ragged mouse alleles ragged-like and opossum. Genesis *36*, 1-6.

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.

Jeltsch, M., Tammela, T., Alitalo, K., and Wilting, J. (2003). Genesis and pathogenesis of lymphatic vessels. Cell Tissue Res *314*, 69-84.

Johnston, M., Zakharov, A., Papaiconomou, C., Salmasi, G., and Armstrong, D. (2004). Evidence of connections between cerebrospinal fluid and nasal lymphatic vessels in humans, non-human primates and other mammalian species. Cerebrospinal Fluid Res *1*, 2.

Joukov, V., Kumar, V., Sorsa, T., Arighi, E., Weich, H., Saksela, O., and Alitalo, K. (1998). A recombinant mutant vascular endothelial growth factor-C that has lost vascular endothelial growth factor receptor-2 binding, activation, and vascular permeability activities. J Biol Chem *273*, 6599-6602.

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.

Joyce, J. A., Laakkonen, P., Bernasconi, M., Bergers, G., Ruoslahti, E., and Hanahan, D. (2003). Stage-specific vascular markers revealed by phage display in a mouse model of pancreatic islet tumorigenesis. Cancer Cell *4*, 393-403.

Jussila, L., Valtola, R., Partanen, T. A., Salven, P., Heikkila, P., Matikainen, M. T., Renkonen, R., Kaipainen, A., Detmar, M., Tschachler, E.*, et al.* (1998). Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. Cancer Res *58*, 1599-1604.

Kabbinavar, F., Hurwitz, H. I., Fehrenbacher, L., Meropol, N. J., Novotny, W. F., Lieberman, G., Griffing, S., and Bergsland, E. (2003). Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol *21*, 60-65.

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.

Kajiya, K., Hirakawa, S., Ma, B., Drinnenberg, I., and Detmar, M. (2005). Hepatocyte growth factor promotes lymphatic vessel formation and function. EMBO J *24*, 2885-2895.

Kamba, T., and McDonald, D. M. (2007). Mechanisms of adverse effects of anti-VEGF therapy for cancer. Br J Cancer *96*, 1788-1795.

Kamba, T., Tam, B. Y., Hashizume, H., Haskell, A., Sennino, B., Mancuso, M. R., Norberg, S. M., O'Brien, S. M., Davis, R. B., Gowen, L. C., *et al.* (2006). VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature. Am J Physiol Heart Circ Physiol *290*, H560-576.

Kärkkäinen, 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.

Kärkkäinen, 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.

Kärkkäinen, 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.

Kärpänen, T., and Alitalo, K. (2007). Molecular Biology and Pathology of Lymphangiogenesis. Annu Rev Pathol.

Kärpänen, T., Egeblad, M., Kärkkäinen, M. J., Kubo, H., Yla-Herttuala, S., Jaattela, M., and Alitalo, K. (2001). Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. Cancer Res *61*, 1786-1790.

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

Kärpänen, T., Wirzenius, M., Makinen, T., Veikkola, T., Haisma, H. J., Achen, M. G., Stacker, S. A., Pytowski, B., Yla-Herttuala, S., and Alitalo, K. (2006b). 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.

Kerbel, R., and Folkman, J. (2002). Clinical translation of angiogenesis inhibitors. Nat Rev Cancer *2*, 727-739.

Kerjaschki, D., Huttary, N., Raab, I., Regele, H., Bojarski-Nagy, K., Bartel, G., Krober, 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., Kärpänen, 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.

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.

Kim, D., Mebius, R. E., MacMicking, J. D., Jung, S., Cupedo, T., Castellanos, Y., Rho, J., Wong, B. R., Josien, R., Kim, N., *et al.* (2000a). Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. J Exp Med *192*, 1467-1478.

Kim, I., Kim, H. G., So, J. N., Kim, J. H., Kwak, H. J., and Koh, G. Y. (2000b). Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'-Kinase/Akt signal transduction pathway. Circ Res *86*, 24-29.

Kim, K. E., Cho, C. H., Kim, H. Z., Baluk, P., McDonald, D. M., and Koh, G. Y. (2007). In vivo actions of angiopoietins on quiescent and remodeling blood and lymphatic vessels in mouse airways and skin. Arterioscler Thromb Vasc Biol *27*, 564-570.

Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature *362*, 841-844.

Kitamoto, Y., Tokunaga, H., and Tomita, K. (1997). Vascular endothelial growth factor is an essential molecule for mouse kidney development: glomerulogenesis and nephrogenesis. J Clin Invest *99*, 2351-2357.

Klagsbrun, M., and Eichmann, A. (2005). A role for axon guidance receptors and ligands in blood vessel development and tumor angiogenesis. Cytokine Growth Factor Rev *16*, 535-548.

Koblizek, T. I., Weiss, C., Yancopoulos, G. D., Deutsch, U., and Risau, W. (1998). Angiopoietin-1 induces sprouting angiogenesis in vitro. Current Biology *8*, 529-532.

Kolodkin, A. L., Levengood, D. V., Rowe, E. G., Tai, Y. T., Giger, R. J., and Ginty, D. D. (1997). Neuropilin is a semaphorin III receptor. Cell *90*, 753-762.

Kono, M., Mi, Y., Liu, Y., Sasaki, T., Allende, M. L., Wu, Y. P., Yamashita, T., and Proia, R. L. (2004). The sphingosine-1-phosphate receptors S1P1, S1P2, and S1P3 function coordinately during embryonic angiogenesis. J Biol Chem *279*, 29367-29373.

Kopp, H. G., Ramos, C. A., and Rafii, S. (2006). Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue. Curr Opin Hematol *13*, 175-181.
Korsisaari, N., Kasman, I. M., Forrest, W. F., Pal, N., Bai, W., Fuh, G., Peale, F. V., Smits, R., and Ferrara, N. (2007). Inhibition of VEGF-A prevents the angiogenic switch and results in increased survival of Apc+/min mice. Proc Natl Acad Sci USA *104*, 10625-10630.

Kozaki, K., Miyaishi, O., Tsukamoto, T., Tatematsu, Y., Hida, T., Takahashi, T., and Takahashi, T. (2000). Establishment and characterization of a human lung cancer cell line NCI-H460-LNM35 with consistent lymphogenous metastasis via both subcutaneous and orthotopic propagation. Cancer Res *60*, 2535-2540.

Krebs, L. T., Shutter, J. R., Tanigaki, K., Honjo, T., Stark, K. L., and Gridley, T. (2004). Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev *18*, 2469-2473.

Kriehuber, E., Breiteneder-Geleff, S., Groeger, M., Soleiman, A., Schoppmann, S. F., Stingl, G., Kerjaschki, D., and Maurer, D. (2001). Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. J Exp Med *194*, 797-808.

Kroll, J., and Waltenberger, J. (1999). A novel function of VEGF receptor-2 (KDR): rapid release of nitric oxide in response to VEGF-A stimulation in endothelial cells. Biochem Biophys Res Commun *265*, 636-639.

Kubo, H., Cao, R., Brakenhielm, E., Makinen, T., Cao, Y., and Alitalo, K. (2002). Blockade of vascular endothelial growth factor receptor-3 signaling inhibits fibroblast growth factor-2-induced lymphangiogenesis in mouse cornea. Proc Natl Acad Sci USA *99*, 8868-8873.

Kuchler, 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.

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.

Laakkonen, P., Akerman, M. E., Biliran, H., Yang, M., Ferrer, F., Kärpänen, T., Hoffman, R. M., and Ruoslahti, E. (2004). Antitumor activity of a homing peptide that targets tumor lymphatics and tumor cells. Proc Natl Acad Sci USA *101*, 9381-9386.

Laakkonen, P., Porkka, K., Hoffman, J. A., and Ruoslahti, E. (2002). A tumor-homing peptide with a targeting specificity related to lymphatic vessels. Nat Med *8*, 751-755.

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.

Lai, E. C. (2002). Keeping a good pathway down: transcriptional repression of Notch pathway target genes by CSL proteins. EMBO Rep *3*, 840-845.

Lai, E. C. (2004). Notch signaling: control of cell communication and cell fate. Development *131*, 965-973.

Laitinen, M., Zachary, I., Breier , G., Pakkanen, T., Hakkinen, T., Luoma, J., Abedi, H., Risau, W., Soma, M., Laakso, M*., et al.* (1997). VEGF gene transfer reduces intimal thickening via increased production of nitric oxide in carotid arteries. Hum Gene Ther *8*, 1737-1744.

Lammert, E., Cleaver, O., and Melton, D. (2001). Induction of pancreatic differentiation by signals from blood vessels. Science *294*, 564-567.

Lammert, E., Cleaver, O., and Melton, D. (2003). Role of endothelial cells in early pancreas and liver development. Mech Dev *120*, 59-64.

Larrivee, B., Freitas, C., Trombe, M., Lv, X., Delafarge, B., Yuan, L., Bouvree, K., Breant, C., Del Toro, R., Brechot, N*., et al.* (2007). Activation of the UNC5B receptor by Netrin-1 inhibits sprouting angiogenesis. Genes Dev *21*, 2433-2447.

Larsson, J., Goumans, M. J., Sjostrand, L. J., van Rooijen, M. A., Ward, D., Leveen, P., Xu, X., ten Dijke, P., Mummery, C. L., and Karlsson, S. (2001). Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. EMBO J *20*, 1663-1673.

Lau, S. C., Rosa, D. D., and Jayson, G. (2005). Technology evaluation: VEGF Trap (cancer), Regeneron/sanofi-aventis. Curr Opin Mol Ther *7*, 493-501.

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.

Le Borgne, R., Bardin, A., and Schweisguth, F. (2005). The roles of receptor and ligand endocytosis in regulating Notch signaling. Development *132*, 1751-1762.

Leak, L. V. (1970). Electron microscopic observations on lymphatic capillaries and the structural components of the connective tissue-lymph interface. Microvisc Res *2*, 361-391.

Leak, L. V., and Burke, J. F. (1966). Fine structure of the lymphatic capillary and the adjoining connective tissue area. Am J Anat *118*, 785-809.

Leak, L. V., and Burke, J. F. (1968). Ultrastructural studies on the lymphatic anchoring filaments. J Cell Biol *36*, 129-149.
LeCouter, J., Moritz, D. R., Li, B., Phillips, G. L., Liang, X. H., Gerber, H. P., Hillan, K. J., and Ferrara, N. (2003). Angiogenesis-independent endothelial protection of liver: role of VEGFR-1. Science *299*, 890-893.

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.

Lee, S., Chen, T. T., Barber, C. L., Jordan, M. C., Murdock, J., Desai, S., Ferrara, N., Nagy, A., Roos, K. P., and Iruela-Arispe, M. L. (2007). Autocrine VEGF signaling is required for vascular homeostasis. Cell *130*, 691-703.

Lee, S., Jilani, S. M., Nikolova, G. V., Carpizo, D., and Iruela-Arispe, M. L. (2005). Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. J Cell Biol *169*, 681-691.

Leslie, J. D., Ariza-McNaughton, L., Bermange, A. L., McAdow, R., Johnson, S. L., and Lewis, J. (2007). Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis. Development *134*, 839-844.

Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. Science *246*, 1306-1309.

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.

Levine, R. J., Maynard, S. E., Qian, C., Lim, K. H., England, L. J., Yu, K. F., Schisterman, E. F., Thadhani, R., Sachs, B. P., Epstein, F. H., *et al.* (2004). Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med *350*, 672-683.

Levine, R. J., Qian, C., Maynard, S. E., Yu, K. F., Epstein, F. H., and Karumanchi, S. A. (2006). Serum sFlt1 concentration during preeclampsia and mid trimester blood pressure in healthy nulliparous women. Am J Obstet Gynecol *194*, 1034-1041.

Li, D. Y., Sorensen, L. K., Brooke, B. S., Urness, L. D., Davis, E. C., Taylor, D. G., Boak, B. B., and Wendel, D. P. (1999). Defective angiogenesis in mice lacking endoglin. Science *284*, 1534-1537.

Li, X., Stankovic, M., Bonder, C. S., Hahn, C. N., Parsons, M., Pitson, S. M., Xia, P., Proia, R. L., Vadas, M. A., and Gamble, J. R. (2008). Basal and angiopoietin-1-mediated endothelial permeability is regulated by sphingosine kinase-1. Blood.

Liang, W. C., Dennis, M. S., Stawicki, S., Chanthery, Y., Pan, Q., Chen, Y., Eigenbrot, C., Yin, J., Koch, A. W., Wu, X., *et al.* (2007). Function blocking antibodies to neuropilin-1 generated from a designed human synthetic antibody phage library. J Mol Biol *366*, 815-829.

Limaye, V., Li, X., Hahn, C., Xia, P., Berndt, M. C., Vadas, M. A., and Gamble, J. R. (2005). Sphingosine kinase-1 enhances endothelial cell survival through a PECAM-1-dependent activation of PI-3K/Akt and regulation of Bcl-2 family members. Blood *105*, 3169-3177.

Limbourg, A., Ploom, M., Elligsen, D., Sorensen, I., Ziegelhoeffer, T., Gossler, A., Drexler, H., and Limbourg, F. P. (2007). Notch ligand Delta-like 1 is essential for postnatal arteriogenesis. Circ Res *100*, 363-371.

Lin, J., Lalani, A. S., Harding, T. C., Gonzalez, M., Wu, W. W., Luan, B., Tu, G. H., Koprivnikar, K., VanRoey, M. J., He, Y., *et al.* (2005). Inhibition of lymphogenous metastasis using adeno-associated virus-mediated gene transfer of a soluble VEGFR-3 decoy receptor. Cancer Res *65*, 6901-6909.

Lin, P., Buxton, J. A., Acheson, A., Radziejewski, C., Maisonpierre, P. C., Yancopoulos, G. D., Channon, K. M., Hale, L. P., Dewhirst, M. W., George, S. E., and Peters, K. G. (1998). Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2. Proc Natl Acad Sci USA *95*, 8829-8834.

Lin, P., Polverini, P., Dewhirst, M., Shan, S., Rao, P. S., and Peters, K. (1997). Inhibition of tumor angiogenesis using a soluble receptor establishes a role for Tie2 in pathologic vascular growth. J Clin Invest *100*, 2072-2078.

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

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.

Liu, W., Ahmad, S. A., Reinmuth, N., Shaheen, R. M., Jung, Y. D., Fan, F., and Ellis, L. M. (2000a). Endothelial cell survival and apoptosis in the tumor vasculature. Apoptosis *5*, 323-328.

Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C. X., Hobson, J. P., Rosenfeldt, H. M., Nava, V. E., Chae, S. S., Lee, M. J., *et al.* (2000b). Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J Clin Invest *106*, 951-961.

Liu, Z. J., Shirakawa, T., Li, Y., Soma, A., Oka, M., Dotto, G. P., Fairman, R. M., Velazquez, O. C., and Herlyn, M. (2003). Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. Mol Cell Biol *23*, 14-25.

Llimargas, M. (1999). The Notch pathway helps to pattern the tips of the Drosophila tracheal branches by selecting cell fates. Development *126*, 2355-2364.

Llovet, J., Ricci, S., Mazzaferro, V., and Group, e. a. f. t. S. I. S. (2007). Sorafenib improves survival in hepatocellular carcinoma: results of a phase III randomized, placebo-controlled trial. . 2007 Meeting of the American Society of Clinical Oncology, Chicago.

Lobov, I. B., Renard, R. A., Papadopoulos, N., Gale, N. W., Thurston, G., Yancopoulos, G. D., and Wiegand, S. J. (2007). Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc Natl Acad Sci USA.

Lu, X., Le Noble, F., Yuan, L., Jiang, Q., De Lafarge, B., Sugiyama, D., Breant, C., Claes, F., De Smet, F., Thomas, J. L.*, et al.* (2004). The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. Nature *432*, 179-186.

Lundkvist, A., Lee, S., Iruela-Arispe, L., Betsholtz, C., and Gerhardt, H. (2007). Growth factor gradients in vascular patterning. Novartis Found Symp *283*, 194-201; discussion 201-196, 238-141.

Luther, S. A., Tang, H. L., Hyman, P. L., Farr, A. G., and Cyster, J. G. (2000). Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. Proc Natl Acad Sci USA *97*, 12694-12699.

Luttun, A., Tjwa, M., Moons, L., Wu, Y., Angelillo-Scherrer, A., Liao, F., Nagy, J. A., Hooper, A., Priller, J., De Klerck, B.*, et al.* (2002). Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. Nat Med *8*, 831-840.

Lymboussaki, A., Olofsson, B., Eriksson, U., and Alitalo, K. (1999). Vascular endothelial growth factor (VEGF) and VEGF-C show overlapping binding sites in embryonic endothelia and distinct sites in differentiated adult endothelia. Circ Res *85*, 992-999.

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.

Mailhos, C., Modlich, U., Lewis, J., Harris, A., Bicknell, R., and Ish-Horowicz, D. (2001). Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis. Differentiation *69*, 135-144.

Maisonpierre, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S. J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T. H., Papadopoulos, N.*, et al.* (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science *277*, 55-60.

Makinen, T., Adams, R. H., Bailey, J., Lu, Q., Ziemiecki, A., Alitalo, K., Klein, R., and Wilkinson, G. A. (2005). PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. Genes Dev *19*, 397-410.

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

Mäkinen, T., Olofsson, B., Kärpänen, 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.

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

Makinen, T., Veikkola, T., Mustjoki, S., Kärpänen, T., Catimel, B., Nice, E. C., Wise, L., Mercer, A., Kowalski, H., Kerjaschki, D.*, et al.* (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.

Mandriota, S. J., and Pepper, M. S. (1998). Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. Circ Res *83*, 852-859.

Martiny-Baron, G., Korff, T., Schaffner, F., Esser, N., Eggstein, S., Marme, D., and Augustin, H. G. (2004). Inhibition of tumor growth and angiogenesis by soluble EphB4. Neoplasia *6*, 248-257.

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.

Matsumoto, K., Yoshitomi, H., Rossant, J., and Zaret, K. S. (2001). Liver organogenesis promoted by endothelial cells prior to vascular function. Science *294*, 559-563.

McColl, B. K., Paavonen, K., Karnezis, T., Harris, N. C., Davydova, N., Rothacker, J., Nice, E. C., Harder, K. W., Roufail, S., Hibbs, M. L., *et al.* (2007). Proprotein convertases promote processing of VEGF-D, a critical step for binding the angiogenic receptor VEGFR-2. FASEB J *21*, 1088-1098.

McDonald, D. M., and Choyke, P. L. (2003). Imaging of angiogenesis: from microscope to clinic. Nat Med *9*, 713-725.

Mebius, R. E. (2003). Organogenesis of lymphoid tissues. Nat Rev Immunol *3*, 292-303.

Mebius, R. E., Rennert, P., and Weissman, I. L. (1997). Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. Immunity *7*, 493-504.

Mebius, R. E., Streeter, P. R., Breve, J., Duijvestijn, A. M., and Kraal, G. (1991). The influence of afferent lymphatic vessel interruption on vascular addressin expression. J Cell Biol *115*, 85-95.

Mellor, R. H., Brice, G., Stanton, A. W., French, J., Smith, A., Jeffery, S., Levick, J. R., Burnand, K. G., and Mortimer, P. S. (2007). Mutations in FOXC2 are strongly associated with primary valve failure in veins of the lower limb. Circulation *115*, 1912-1920.

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.

Millauer, B., Shawver, L. K., Plate, K. H., Risau, W., and Ullrich, A. (1994). Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. Nature *367*, 576-579.

Miller, K., Wang, M., Gralow, J., Dickler, M., Cobleigh, M., Perez, E. A., Shenkier, T., Cella, D., and Davidson, N. E. (2007). Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. N Engl J Med *357*, 2666-2676.

Miller, K. D., Chap, L. I., Holmes, F. A., Cobleigh, M. A., Marcom, P. K., Fehrenbacher, L., Dickler, M., Overmoyer, B. A., Reimann, J. D., Sing, A. P., *et al.* (2005). Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. J Clin Oncol *23*, 792-799.

Mitra, S. K., and Schlaepfer, D. D. (2006). Integrin-regulated FAK-Src signaling in normal and cancer cells. Curr Opin Cell Biol *18*, 516-523.

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.

Mortimer, P. S., Bates, D. O., Brassington, H. D., Stanton, A. W. B., Strachan, D. P., and Levick, J. R. (1996). The prevalence of arm oedema following treatment for breast cancer. Qjm *89*, 377-380.

Motzer, R. J., Hutson, T. E., Tomczak, P., Michaelson, M. D., Bukowski, R. M., Rixe, O., Oudard, S., Negrier, S., Szczylik, C., Kim, S. T., *et al.* (2007). Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med *356*, 115-124.

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.

Ng, C. P., Helm, C. L., and Swartz, M. A. (2004). Interstitial flow differentially stimulates blood and lymphatic endothelial cell morphogenesis in vitro. Microvasc Res *68*, 258-264.

Nichols, J. T., Miyamoto, A., and Weinmaster, G. (2007). Notch signaling--constantly on the move. Traffic *8*, 959-969.

Noguera-Troise, I., Daly, C., Papadopoulos, N. J., Coetzee, S., Boland, P., Gale, N. W., Lin, H. C., Yancopoulos, G. D., and Thurston, G. (2006). Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. Nature *444*, 1032-1037.

Ny, A., Autiero, M., and Carmeliet, P. (2006). Zebrafish and Xenopus tadpoles: small animal models to study angiogenesis and lymphangiogenesis. Exp Cell Res *312*, 684-693.

Ny, A., Koch, M., Schneider, M., Neven, E., Tong, R. T., Maity, S., Fischer, C., Plaisance, S., Lambrechts, D., Heligon, C., *et al.* (2005). A genetic Xenopus laevis tadpole model to study lymphangiogenesis. Nat Med *11*, 998-1004.

Nystrom, H. C., Lindblom, P., Wickman, A., Andersson, I., Norlin, J., Faldt, J., Lindahl, P., Skott, O., Bjarnegard, M., Fitzgerald, S. M., *et al.* (2006). Platelet-derived growth factor B retention is essential for development of normal structure and function of conduit vessels and capillaries. Cardiovasc Res *71*, 557-565.

Odorisio, T., Schietroma, C., Zaccaria, M. L., Cianfarani, F., Tiveron, C., Tatangelo, L., Failla, C. M., and Zambruno, G. (2002). Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability. J Cell Sci *115*, 2559-2567.

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.

Oh, S. P., Seki, T., Goss, K. A., Imamura, T., Yi, Y., Donahoe, P. K., Li, L., Miyazono, K., ten Dijke, P., Kim, S., and Li, E. (2000). Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. Proc Natl Acad Sci USA *97*, 2626-2631.

Okada, T., Ngo, V. N., Ekland, E. H., Forster, R., Lipp, M., Littman, D. R., and Cyster, J. G. (2002). Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. J Exp Med *196*, 65-75.

Okamoto, N., Chihara, R., Shimizu, C., Nishimoto, S., and Watanabe, T. (2007). Artificial lymph nodes induce potent secondary immune responses in naive and immunodeficient mice. J Clin Invest *117*, 997-1007.

Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J *19*, 3159-3167.

Oliner, J., Min, H., Leal, J., Yu, D., Rao, S., You, E., Tang, X., Kim, H., Meyer, S., Han, S. J*., et al.* (2004). Suppression of angiogenesis and tumor growth by selective inhibition of angiopoietin-2. Cancer Cell *6*, 507-516.

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

Olsson, A. K., Dimberg, A., Kreuger, J., and Claesson-Welsh, L. (2006). VEGF receptor signalling - in control of vascular function. Nat Rev Mol Cell Biol *7*, 359-371.

Olszewski, W. L. (1988). The treatment of lymphedema of the extremities with microsurgical lympho-venous anastomoses. Int Angiol *7*, 312-321.

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.

Oshima, M., Oshima, H., and Taketo, M. M. (1996). TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. Dev Biol *179*, 297-302.

Otsuki, Y., Magari, S., and Sugimoto, O. (1986). Lymphatic capillaries in rabbit ovaries during ovulation: an ultrastructural study. Lymphology *19*, 55-64.

Oura, H., Bertoncini, J., Velasco, P., Brown, L. F., Carmeliet, P., and Detmar, M. (2003). A critical role of placental growth factor in the induction of inflammation and edema formation. Blood *101*, 560-567.

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., Kadambi, A., di Tomaso, E., Carreira, C. M., Brown, E. B., Boucher, Y., Choi, N. C., Mathisen, D., Wain, J., Mark, E. J*., et al.* (2002). Lymphatic metastasis in the absence of functional intratumor lymphatics. Science *296*, 1883-1886.

Paik, J. H., Skoura, A., Chae, S. S., Cowan, A. E., Han, D. K., Proia, R. L., and Hla, T. (2004). Sphingosine 1-phosphate receptor regulation of N-cadherin mediates vascular stabilization. Genes Dev *18*, 2392-2403.

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.

Pan, Q., Chanthery, Y., Liang, W. C., Stawicki, S., Mak, J., Rathore, N., Tong, R. K., Kowalski, J., Yee, S. F., Pacheco, G*., et al.* (2007). Blocking neuropilin-1 function has an additive effect with anti-VEGF to inhibit tumor growth. Cancer Cell *11*, 53-67.

Papapetropoulos, A., Fulton, D., Mahboubi, K., Kalb, R. G., O'Connor, D. S., Li, F., Altieri, D. C., and Sessa, W. C. (2000). Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. J Biol Chem *275*, 9102-9105.

Partanen, J., Armstrong, E., Mäkelä, T. P., Korhonen, J., Sandberg, M., Renkonen, R., Knuutila, S., Huebner, K., and Alitalo, K. (1992). A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains. Mol Cell Biol *12*, 1698-1707.

Partanen, J., Puri, M. C., Schwartz, L., Fischer, K.-D., Bernstein, A., and Rossant, J. (1996). Cell autonomous functions of the receptor tyrosine kinsae TIE in a late phase of angiogenic capillary growth and endothelial cell survival during murine development. Development *122*, 3013-3021.

Partanen, T. A., Alitalo, K., and Miettinen, M. (1999). Lack of lymphatic vascular specificity of vascular endothelial growth factor receptor 3 in 185 vascular tumors. Cancer *86*, 2406-2412.

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.

Pennisi, D., Gardner, J., Chambers, D., Hosking, B., Peters, J., Muscat, G., Abbott, C., and Koopman, P. (2000). Mutations in Sox18 underlie cardiovascular and hair follicle defects in ragged mice. Nat Genet *24*, 434-437.

Pepper, M. S. (2001). Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arterioscler Thromb Vasc Biol *21*, 1104-1117.

Petrova, T. V., Kärpänen, 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 Journal *21*, 4593-4599.

Pietras, K., and Hanahan, D. (2005). A multitargeted, metronomic, and maximum-tolerated dose "chemo-switch" regimen is antiangiogenic, producing objective responses and survival benefit in a mouse model of cancer. J Clin Oncol *23*, 939-952.

Pietras, K., Pahler, J., Bergers, G., and Hanahan, D. (2008). Functions of paracrine PDGF signaling in the proangiogenic tumor stroma revealed by pharmacological targeting. PLoS Med *5*, e19.

Pipp, F., Heil, M., Issbrucker, K., Ziegelhoeffer, T., Martin, S., van den Heuvel, J., Weich, H., Fernandez, B., Golomb, G., Carmeliet, P.*, et al.* (2003). VEGFR-1-selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanism. Circ Res *92*, 378-385.

Poltorak, Z., Cohen, T., Sivan, R., Kandelis, Y., Spira, G., Vlodavsky, I., Keshet, E., and Neufeld, G. (1997). VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. J Biol Chem *272*, 7151-7158.

Popkov, M., Jendreyko, N., McGavern, D. B., Rader, C., and Barbas, C. F., 3rd (2005). Targeting tumor angiogenesis with adenovirus-delivered anti-Tie-2 intrabody. Cancer Res *65*, 972-981.

Prewett, M., Huber, J., Li, Y., Santiago, A., O'Connor, W., King, K., Overholser, J., Hooper, A., Pytowski, B., Witte, L.*, et al.* (1999). Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res *59*, 5209-5218.

Procopio, W. N., Pelavin, P. I., Lee, W. M., and Yeilding, N. M. (1999). Angiopoietin-1 and -2 coiled coil domains mediate distinct homo-oligomerization patterns, but fibrinogen-like domains mediate ligand activity. J Biol Chem *274*, 30196-30201.

Pugh, C. W., and Ratcliffe, P. J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med *9*, 677-684.

Pullinger, B. D., and Florey, H. W. (1937). Proliferation of lymphatics in inflammation. J Pathol Bact, 157-170.

Puri, M. C., Rossant, J., Alitalo, K., Bernstein, A., and Partanen, J. (1995). The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. EMBO J *14*, 5884-5891.

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.

Quinn, T., Peters, K., DeVries, C., Ferrara, N., and Williams, L. (1993). Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. Proc Natl Acad Sci USA *90*, 7533-7537.

Rabson, J. A., Geyer, S. J., Levine, G., Swartz, W. M., and Futrell, J. W. (1982). Tumor immunity in rat lymph nodes following transplantation. Ann Surg *196*, 92-99.

Rak, J., Mitsuhashi, Y., Bayko, L., Filmus, J., Shirasawa, S., Sasazuki, T., and Kerbel, R. S. (1995). Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. Cancer Res *55*, 4575-4580.

Rennert, P. D., James, D., Mackay, F., Browning, J. L., and Hochman, P. S. (1998). Lymph node genesis is induced by signaling through the lymphotoxin beta receptor. Immunity *9*, 71-79.

Ridgway, J., Zhang, G., Wu, Y., Stawicki, S., Liang, W. C., Chanthery, Y., Kowalski, J., Watts, R. J., Callahan, C., Kasman, I.*, et al.* (2006). Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature *444*, 1083-1087.

Riley, V. (1963). Enzymatic determination of transmissible replicating factors associated with mouse tumors. Ann N Y Acad Sci *100*, 762-790.

Risau, W. (1998). Development and differentiation of endothelium. Kidney Int Suppl *67*, S3-6.

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.

Ristimaki, A., Narko, K., Enholm, B., Joukov, V., and Alitalo, K. (1998). Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-C. J Biol Chem *273*, 8413-8418.

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.

Robinson, C. J., and Stringer, S. E. (2001). The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J Cell Sci *114*, 853-865.

Roca, C., and Adams, R. H. (2007). Regulation of vascular morphogenesis by Notch signaling. Genes Dev, 2511-2524.

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

Romer, L. H., Birukov, K. G., and Garcia, J. G. (2006). Focal adhesions: paradigm for a signaling nexus. Circ Res *98*, 606-616.

Ruhrberg, C., Gerhardt, H., Golding, M., Watson, R., Ioannidou, S., Fujisawa, H., Betsholtz, C., and Shima, D. T. (2002). Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev *16*, 2684-2698.

Ruoslahti, E., and Rajotte, D. (2000). An address system in the vasculature of normal tissues and tumors. Ann Rev Immunol *18*, 813-827.

Saaristo, A., Tammela, T., Farkkila, 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., Hytonen, M., Arola, J., Pajusola, K., Turunen, P., Jeltsch, M., Kärkkäinen, 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., Kärkkäinen, M. J., Pajusola, K., Bueler, H., Yla-Herttuala, S., and Alitalo, K. (2002b). Lymphangiogenic gene therapy with minimal blood vascular side effects. J Exp Med *196*, 719-730.

Saban, M. R., Memet, S., Jackson, D. G., Ash, J., Roig, A. A., Israel, A., and Saban, R. (2004). Visualization of lymphatic vessels through NF-kappaB activity. Blood *104*, 3228-3230.

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.

Saharinen, P., Kerkela, K., Ekman, N., Marron, M., Brindle, N., Lee, G. M., Augustin, H., Koh, G. Y., and Alitalo, K. (2005). Multiple angiopoietin recombinant proteins activate the Tie1 receptor tyrosine kinase and promote its interaction with Tie2. J Cell Biol *169*, 239-243.

Saharinen, P., Tammela, T., Kärkkäinen, M. J., and Alitalo, K. (2004). Lymphatic vasculature: development, molecular regulation and role in tumor metastasis and inflammation. Trends Immunol *25*, 387-395.

Sakai, K., Takiguchi, M., Mori, S., Kobori, O., Morioka, Y., Inoko, H., Sekiguchi, M., and Kano, K. (1987). Expression and function of class II antigens on gastric carcinoma cells and gastric epithelia: differential expression of DR, DQ, and DP antigens. J Natl Cancer Inst *79*, 923-932.

Salven, P., Lymboussaki, A., Heikkila, P., Jaaskela-Saari, H., Enholm, B., Aase, K., von Euler, G., Eriksson, U., Alitalo, K., and Joensuu, H. (1998). Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. Am J Pathol *153*, 103-108.

Sandler, A., Gray, R., Perry, M. C., Brahmer, J., Schiller, J. H., Dowlati, A., Lilenbaum, R., and Johnson, D. H. (2006). Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med *355*, 2542-2550.

Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature *376*, 70-74.

Schaper, W., and Scholz, D. (2003). Factors regulating arteriogenesis. Arterioscler Thromb Vasc Biol *23*, 1143-1151.
Schlaepfer, D. D., and Mitra, S. K. (2004). Multiple connections link FAK to cell motility and invasion. Curr Opin Genet Dev *14*, 92-101.

Schweisguth, F. (2004). Regulation of notch signaling activity. Curr Biol *14*, R129-138.

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.

Selkoe, D., and Kopan, R. (2003). Notch and Presenilin: regulated intramembrane proteolysis links development and degeneration. Annu Rev Neurosci *26*, 565-597.

Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science *219*, 983-985.

Sennino, B., Falcon, B. L., McCauley, D., Le, T., McCauley, T., Kurz, J. C., Haskell, A., Epstein, D. M., and McDonald, D. M. (2007). Sequential loss of tumor vessel pericytes and endothelial cells after inhibition of platelet-derived growth factor B by selective aptamer AX102. Cancer Res *67*, 7358-7367.

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.

Shawber, C. J., Funahashi, Y., Francisco, E., Vorontchikhina, M., Kitamura, Y., Stowell, S. A., Borisenko, V., Feirt, N., Podgrabinska, S., Shiraishi, K., *et al.* (2007). Notch alters VEGF responsiveness in human and murine endothelial cells by direct regulation of VEGFR-3 expression. J Clin Invest, 3369-3382.

Shields, J. D., Fleury, M. E., Yong, C., Tomei, A. A., Randolph, G. J., and Swartz, M. A. (2007). Autologous chemotaxis as a mechanism of tumor cell homing to lymphatics via interstitial flow and autocrine CCR7 signaling. Cancer Cell 11, 526-538.

Shim, W. S., Ho, I. A., and Wong, P. E. (2007). Angiopoietin: a TIE(d) balance in tumor angiogenesis. Mol Cancer Res *5*, 655-665.

Shu, X., Wu, W., Mosteller, R. D., and Broek, D. (2002). Sphingosine kinase mediates vascular endothelial growth factor-induced activation of ras and mitogen-activated protein kinases. Mol Cell Biol *22*, 7758-7768.

Shutter, J. R., Scully, S., Fan, W., Richards, W. G., Kitajewski, J., Deblandre, G. A., Kintner, C. R., and Stark, K. L. (2000). Dll4, a novel Notch ligand expressed in arterial endothelium. Genes Dev *14*, 1313-1318.

Siegfried, G., Basak, A., Cromlish, J. A., Benjannet, S., Marcinkiewicz, J., Chretien, M., Seidah, N. G., and Khatib, A. M. (2003). The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. J Clin Invest *111*, 1723-1732.

Siekmann, A. F., and Lawson, N. D. (2007). Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. Nature *445*, 781-784.

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. (2001a). Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med *7*, 192-198.

Soker, S., Fidder, H., Neufeld, G., and Klagsbrun, M. (1996). Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain. J Biol Chem *271*, 5761-5767.

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.

Soldi, R., Mitola, S., Strasly, M., Defilippi, P., Tarone, G., and Bussolino, F. (1999). Role of alphavbeta3 integrin in the activation of vascular endothelial growth factor receptor-2. EMBO J *18*, 882-892.

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. (2002). 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.

Steneberg, P., Hemphala, J., and Samakovlis, C. (1999). Dpp and Notch specify the fusion cell fate in the dorsal branches of the Drosophila trachea. Mech Dev *87*, 153-163.

Suchting, S., Freitas, C., le Noble, F., Benedito, R., Breant, C., Duarte, A., and Eichmann, A. (2007). The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proc Natl Acad Sci USA.

Sugimoto, H., Hamano, Y., Charytan, D., Cosgrove, D., Kieran, M., Sudhakar, A., and Kalluri, R. (2003). Neutralization of circulating vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1) induces proteinuria. J Biol Chem *278*, 12605-12608.

Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1996). Requisite role of Angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell *87*, 1161-1169.

Suto, K., Yamazaki, Y., Morita, T., and Mizuno, H. (2005). Crystal structures of novel vascular endothelial growth factors (VEGF) from snake venoms: insight into selective VEGF binding to kinase insert domain-containing receptor but not to fms-like tyrosine kinase-1. J Biol Chem *280*, 2126-2131.

Szuba, A., Skobe, M., Kärkkäinen, 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.

Tabibiazar, R., Cheung, L., Han, J., Swanson, J., Beilhack, A., An, A., Dadras, S. S., Rockson, N., Joshi, S., Wagner, R., and Rockson, S. G. (2006). Inflammatory Manifestations of Experimental Lymphatic Insufficiency. PLoS Med *3*, e254.

Takagi, S., Hirata, T., Agata, K., Mochii, M., Eguchi, G., and Fujisawa, H. (1991). The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors. Neuron *7*, 295-307.

Takakura, N., Huang, X. L., Naruse, T., Hamaguchi, I., Dumont, D. J., Yancopoulos, G. D., and Suda, T. (1998). Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. Immunity *9*, 677-686.

Takeshita, K., Satoh, M., Ii, M., Silver, M., Limbourg, F. P., Mukai, Y., Rikitake, Y., Radtke, F., Gridley, T., Losordo, D. W., and Liao, J. K. (2007). Critical role of endothelial Notch1 signaling in postnatal angiogenesis. Circ Res *100*, 70-78.

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

Tammela, T., Petrova, T. V., and Alitalo, K. (2005b). Molecular lymphangiogenesis: new players. Trends Cell Biol *15*, 434-441.

Tang, Y., Zhang, D., Fallavollita, L., and Brodt, P. (2003). Vascular endothelial growth factor C expression and lymph node metastasis are regulated by the type I insulin-like growth factor receptor. Cancer Res *63*, 1166-1171.

Teichert-Kuliszewska, K., Maisonpierre, P. C., Jones, N., Campbell, A. I., Master, Z., Bendeck, M. P., Alitalo, K., Dumont, D. J., Yancopoulos, G. D., and Stewart, D. J. (2001). Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2. Cardiovascular Research *49*, 659-670.

Thurston, G. (2002). Complementary actions of VEGF and angiopoietin-1 on blood vessel growth and leakage. Journal of Anatomy *200*, 575-580.

Thurston, G. (2003). Role of Angiopoietins and Tie receptor tyrosine kinases in angiogenesis and lymphangiogenesis. Cell Tissue Res *314*, 61-68.

Thurston, G., Noguera-Troise, I., and Yancopoulos, G. D. (2007). The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth. Nat Rev Cancer *7*, 327-331.

Thurston, G., Rudge, J. S., Ioffe, E., Zhou, H., Ross, L., Croll, S. D., Glazer, N., Holash, J., McDonald, D. M., and Yancopoulos, G. D. (2000). Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med *6*, 460-463.

Thurston, G., Suri, C., Smith, K., McClain, J., Sato, T. N., Yancopoulos, G. D., and McDonald, D. M. (1999). Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science *286*, 2511-2514.

Tokunaga, Y., Yamazaki, Y., and Morita, T. (2005). Specific distribution of VEGF-F in Viperinae snake venoms: isolation and characterization of a VGEF-F from the venom of Daboia russelli siamensis. Arch Biochem Biophys *439*, 241-247.

Trepel, M., Arap, W., and Pasqualini, R. (2002). In vivo phage display and vascular heterogeneity: implications for targeted medicine. Curr Opin Chem Biol *6*, 399-404.

Urness, L. D., Sorensen, L. K., and Li, D. Y. (2000). Arteriovenous malformations in mice lacking activin receptor-like kinase-1. Nat Genet *26*, 328-331.

Uutela, M., Wirzenius, M., Paavonen, K., Rajantie, I., He, Y., Kärpänen, 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.

Vainionpää, N., Butzow, R., Hukkanen, M., Jackson, D. G., Pihlajaniemi, T., Sakai, L. Y., and Virtanen, I. (2007). Basement membrane protein distribution in LYVE-1-immunoreactive lymphatic vessels of normal tissues and ovarian carcinomas. Cell Tissue Res *328*, 317-328.

Valenzuela, D. M., Griffiths, J. A., Rojas, J., Aldrich, T. H., Jones, P. F., Zhou, H., McClain, J., Copeland, N. G., Gilbert, D. J., Jenkins, N. A.*, et al.* (1999). Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. Proc Natl Acad Sci USA *96*, 1904-1909.

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.

Veikkola, T., Jussila, L., Makinen, T., Kärpänen, 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 *6*, 1223-1231.

Veikkola, T., Lohela, M., Ikenberg, K., Makinen, T., Korff, T., Saaristo, A., Petrova, T., Jeltsch, M., Augustin, H. G., and Alitalo, K. (2003). Intrinsic versus microenvironmental regulation of lymphatic endothelial cell phenotype and function. FASEB Journal *17*, 2006-2013.

Vintersten, K., Monetti, C., Gertsenstein, M., Zhang, P., Laszlo, L., Biechele, S., and Nagy, A. (2004). Mouse in red: red fluorescent protein expression in mouse ES cells, embryos, and adult animals. Genesis *40*, 241-246.

Vlahakis, N. E., Young, B. A., Atakilit, A., and Sheppard, D. (2005). The lymphangiogenic vascular endothelial growth factors VEGF-C and -D are ligands for the integrin alpha9beta1. 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.

Wang, B., Xiao, Y., Ding, B. B., Zhang, N., Yuan, X., Gui, L., Qian, K. X., Duan, S., Chen, Z., Rao, Y., and Geng, J. G. (2003). Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity. Cancer Cell *4*, 19-29.

Wang, J. F., Zhang, X. F., and Groopman, J. E. (2001). Stimulation of beta 1 integrin induces tyrosine phosphorylation of vascular endothelial growth factor receptor-3 and modulates cell migration. J Biol Chem *276*, 41950-41957.

Wang, Y., Pampou, S., Fujikawa, K., and Varticovski, L. (2004). Opposing effect of angiopoietin-1 on VEGF-mediated disruption of endothelial cell-cell interactions requires activation of PKC beta. J Cell Physiol *198*, 53-61.

Warren, A. G., Brorson, H., Borud, L. J., and Slavin, S. A. (2007). Lymphedema: a comprehensive review. Ann Plast Surg *59*, 464-472.

Weber, C. C., Cai, H., Ehrbar, M., Kubota, H., Martiny-Baron, G., Weber, W., Djonov, V., Weber, E., Mallik, A. S., Fussenegger, M., *et al.* (2005). Effects of protein and gene transfer of the angiopoietin-1 fibrinogen-like receptor-binding domain on endothelial and vessel organization. J Biol Chem *280*, 22445-22453.

Weijzen, S., Velders, M. P., Elmishad, A. G., Bacon, P. E., Panella, J. R., Nickoloff, B. J., Miele, L., and Kast, W. M. (2002). The Notch ligand Jagged-1 is able to induce maturation of monocyte-derived human dendritic cells. J Immunol *169*, 4273-4278.

Weinmaster, G. (2000). Notch signal transduction: a real rip and more. Curr Opin Genet Dev *10*, 363-369.

Weinstein, B. M. (1999). What guides early embryonic blood vessel formation? Dev Dyn *215*, 2-11.

Wick, N., Saharinen, P., Saharinen, J., Gurnhofer, E., Steiner, C. W., Raab, I., Stokic, D., Giovanoli, P., Buchsbaum, S., Burchard, A., *et al.* (2007). Transcriptomal comparison of human dermal lymphatic endothelial cells ex vivo and in vitro. Physiol Genomics *28*, 179-192.

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.

Wilting, J., Papoutsi, M., Schneider, M., and Christ, B. (2000). The lymphatic endothelium of the avian wing is of semitic origin. Dev Dyn *217*, 271-278.

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

Xu, Y., and Yu, Q. (2001). Angiopoietin-1, unlike angiopoietin-2, is incorporated into the extracellular matrix via its linker peptide region. J Biol Chem *276*, 34990-34998.

Yamazaki, Y., Tokunaga, Y., Takani, K., and Morita, T. (2005a). C-terminal heparin-binding peptide of snake venom VEGF specifically blocks VEGF-stimulated endothelial cell proliferation. Pathophysiol Haemost Thromb *34*, 197-199.

Yamazaki, Y., Tokunaga, Y., Takani, K., and Morita, T. (2005b). Identification of the heparin-binding region of snake venom vascular endothelial growth factor (VEGF-F) and its blocking of VEGF-A165. Biochemistry *44*, 8858-8864.

Yana, I., Sagara, H., Takaki, S., Takatsu, K., Nakamura, K., Nakao, K., Katsuki, M., Taniguchi, S., Aoki, T., Sato, H., et al. (2007). Crosstalk between neovessels and mural cells directs the site-specific expression of MT1-MMP to endothelial tip cells. J Cell Sci 120, 1607-1614.

Yang, X., Castilla, L. H., Xu, X., Li, C., Gotay, J., Weinstein, M., Liu, P. P., and Deng, C. X. (1999). Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5. Development *126*, 1571-1580.

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.

Yoshida, H., Naito, A., Inoue, J., Satoh, M., Santee-Cooper, S. M., Ware, C. F., Togawa, A., Nishikawa, S., and Nishikawa, S. (2002). Different cytokines induce surface lymphotoxin-alphabeta on IL-7 receptor-alpha cells that differentially engender lymph nodes and Peyer's patches. Immunity *17*, 823-833.

Yoshitomi, H., and Zaret, K. S. (2004). Endothelial cell interactions initiate dorsal pancreas development by selectively inducing the transcription factor Ptf1a. Development *131*, 807-817.

You, L. R., Lin, F. J., Lee, C. T., DeMayo, F. J., Tsai, M. J., and Tsai, S. Y. (2005). Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. Nature *435*, 98-104.

Youssoufian, H., Hicklin, D. J., and Rowinsky, E. K. (2007). Review: monoclonal antibodies to the vascular endothelial growth factor receptor-2 in cancer therapy. Clin Cancer Res *13*, 5544s-5548s.

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

Zachary, I., and Gliki, G. (2001). Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovasc Res *49*, 568-581.

Zelzer, E., and Shilo, B. Z. (2000). Cell fate choices in Drosophila tracheal morphogenesis. Bioessays *22*, 219-226.

Zhang, L., Giraudo, E., Hoffman, J. A., Hanahan, D., and Ruoslahti, E. (2006). Lymphatic zip codes in premalignant lesions and tumors. Cancer Res *66*, 5696-5706.

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

Zhang, X., Wang, J. F., Chandran, B., Persaud, K., Pytowski, B., Fingeroth, J., and Groopman, J. E. (2005b). Kaposi's sarcoma-associated herpesvirus activation of vascular endothelial growth factor receptor 3 alters endothelial function and enhances infection. J Biol Chem *280*, 26216-26224.

Zheng, Y., Murakami, M., Takahashi, H., Yamauchi, M., Kiba, A., Yamaguchi, S., Yabana, N., Alitalo, K., and Shibuya, M. (2006). Chimeric VEGF-ENZ7/PlGF Promotes Angiogenesis Via VEGFR-2 Without Significant Enhancement of Vascular Permeability and Inflammation. Arterioscler Thromb Vasc Biol, 2019-2026.

Ziegler, S. F., Bird, T. A., Schneringer, J. A., Schooley, K. A., and Baum, P. R. (1993). Molecular cloning and characterization of a novel receptor protein tyrosine kinase from human placenta. Oncogene *8*, 663-670.

Zimmermann, R. C., Hartman, T., Kavic, S., Pauli, S. A., Bohlen, P., Sauer, M. V., and Kitajewski, J. (2003). Vascular endothelial growth factor receptor 2-mediated angiogenesis is essential for gonadotropin-dependent follicle development. J Clin Invest *112*, 659-669.