New Insights into the Molecular Control of the Lymphatic Vascular System and its Role in Disease

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The cutaneous lymphatic system plays an important role in the maintenance of tissue fluid homeostasis, in the afferent phase of the immune response, and in the metastatic spread of skin cancers. However, the lymphatic system has not received as much scientific attention as the blood vascular system, largely due to a lack of lymphatic-specific markers and to the dearth of knowledge about the molecular regulation of its development and function. The recent identification of genes that specifically control lymphatic development and the growth of lymphatic vessels (lymphangiogenesis), together with the discovery of new lymphatic endothelium-specific markers, have now provided new insights into the molecular mechanisms that control lymphatic growth and function. Moreover, studies of several genetic mouse models have set the framework for a new molecular model for embryonic lymphatic vascular development, and have identified molecular pathways whose mutational inactivation leads to human diseases associated with lymphedema. These scientific advances have also provided surprising evidence that malignant tumors can directly promote lymphangiogenesis and lymphatic metastasis, and that lymphatic vessels play a major role in cutaneous inflammation and in the cutaneous response to UVB irradiation.

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Physiology and anatomy

Apart from the cardiovascular system, higher vertebrates also possess a lymphatic system that consists of the lymphatic vessels and the lymphoid organs that include lymph nodes, tonsils, Peyer's patches, spleen, and thymus. Whereas the cardiovascular system forms a closed circle around which blood is pumped by the heart, the lymphatic system comprises a one-way, open-ended network without a central driving force. Lymph, a protein-rich exudate from blood vessels, is taken up by the lymphatic capillaries in the tissue. From there it is returned to the venous circulation via the larger collecting lymphatic vessels and the thoracic duct, which connects the lymphatic system to the inferior vena cava (Figure 1). The pressure gradients to move lymph through the vessels result from skeletal muscle action, respiratory movement, and contraction of smooth muscle cells in vessel walls. The lymphatic system also contributes to the immune surveillance of the body. Lymphatic vessels transport immune cells – including lymphocytes and antigen-presenting dendritic cells – from the skin to regional lymph nodes, where specific immune responses are initiated. In addition, the lacteal lymphatic vessels of the intestine are involved in the uptake of dietary fat and of the fat-soluble vitamins A, D, E, and K from the digestive system. However, recent scientific discoveries have revealed that the lymphatic system also plays a major role in a number of pathologic conditions, including lymphedema, inflammatory diseases, and tumor metastasis.

Lymphatic vessels are present in almost all tissues but are absent from avascular structures such as the epidermis, hair, nails, cartilage, and cornea, and from some vascularized organs such as the brain and retina. In the skin, the superficial lymphatic plexus collects lymph from lymphatic capillaries that can extend into the dermal papillae. These lymphatic capillaries are lined by a single, non-fenestrated layer of overlapping endothelial cells, and – in contrast to blood vessels – lack a continuous basement membrane as well as pericyte or smooth muscle cell coverage. Lymphatic endothelial cells (LECs) are connected to the surrounding extracellular matrix by specialized fibrillin-containing anchoring filaments (Gerli *et al.*, 2000). Upon increase of interstitial fluid pressure, these filaments exert tension on LECs, thereby widening the capillary lumen and opening the overlapping cell junctions, which enables the uptake of fluid, macromolecules, and cells. The larger

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Abbreviations: Ang1, angiopoietin-1; BEC, blood vascular endothelial cell; FGF-2, fibroblast growth factor-2; HGF, hepatocyte growth factor; KS, Kaposi's sarcoma; LEC, lymphatic endothelial cell; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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collecting lymphatic vessels in the lower dermis and upper subcutis are surrounded by a basement membrane and by a layer of smooth muscle cells that contribute to lymph propulsion. They contain luminal valves to ensure unidirectional fluid transport.

Embryonic development

The first description of the lymphatic system dates back to the seventeenth century, when the Italian anatomist Gasparo Aselli identified lymphatic vessels as ''milky veins'' in the mesentery of a ''well-fed'' dog (Asellius, 1627). However, the developmental origin of lymphatic vessels remained unclear until Florence Sabin proposed in 1902 – based upon ink-injection experiments in pigs – that endothelial cells bud off from the veins during early embryonic development and form primitive lymph sacs. The peripheral lymphatic system then originates from these primary lymph sacs by endothelial sprouting into the surrounding tissues and organs, where local capillaries are formed (Sabin, 1902). This model was challenged in 1910 by Huntington and McClure who alternatively suggested that lymph sacs arise – independently of the veins – from mesenchymal precursor cells (lymphangioblasts), with consecutive establishment of venous connections (Huntington and McClure, 1910). Recent studies in genetically engineered mouse models have now provided clear evidence for the origin of the mammalian lymphatic system from embryonic veins (Oliver, 2004), and they have also identified some of the molecular determinants that control the step-wise process of lymphatic competence, commitment, differentiation and maturation (Figure 2).

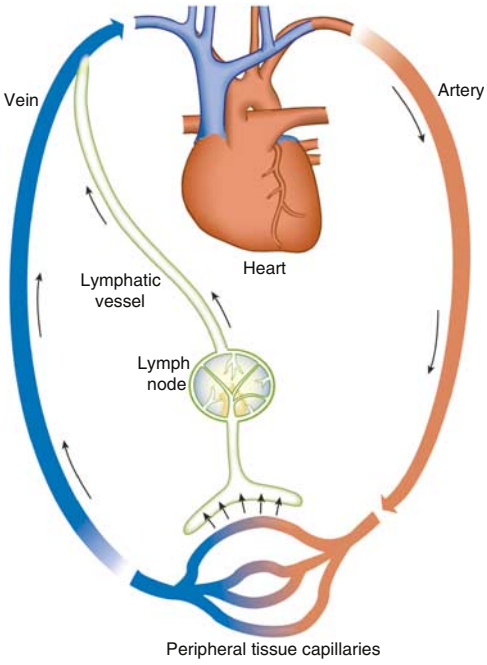

Figure 1. Schematic illustration of the blood vascular and lymphatic system. The blood vascular system is a circular and closed system, whereas the lymphatic system is open-ended and linear. Fluids, macromolecules, and cells extravasated from blood capillaries enter lymphatic capillaries in peripheral tissues and are then transported via the larger collecting lymphatic vessels and the thoracic duct back to the blood vascular system for recirculation.

Novel molecular markers of lymphatic endothelium

Major advances in lymphatic research have been made possible by the recent establishment of defined cultures of blood vascular endothelial cells (BECs) and LECs isolated from human skin (Kriehuber *et al.*, 2001; Makinen *et al.*, 2001; Podgrabinska *et al.*, 2002; Hirakawa *et al.*, 2003). These cells maintain their lineage-specific differentiation even after several passages *in vitro*. Comparative microarray analyses of their specific transcriptomes revealed that the majority of all genes investigated (appr. 98%) were

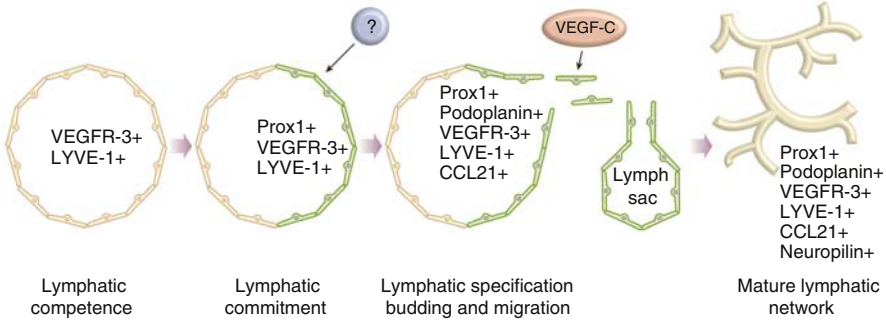

Figure 2. Current model of the stepwise embryonic development of the mammalian lymphatic system. During early vascular development, all endothelial cells of the embryonic cardinal vein express two important lymphatic markers, lymphatic vascular endothelial hyaluronan receptor (LYVE-1) and VEGFR-3, and display lymphatic competence. Stimulation by a yet unidentified inductive mesenchymal signal leads to induction of the transcription factor Prox1 in a subset of endothelial cells that become committed to the lymphatic lineage. These cells bud off from the vein and migrate into the surrounding tissue to form primitive lymph sacs. During this process, they adopt the expression of additional lymphatic lineage markers. The formation of a mature lymphatic network continues through the first postnatal days.

Table 1. Specific markers for LV versus BV

Markers	Function	LV	BV	References
Prox1	Transcription factor	++	–	(Wigle and Oliver, 1999)
Podoplanin	Transmembrane glycoprotein	++	–	(Wetterwald *et al.*, 1996; Breiteneder-Geleff *et al.*, 1999)
LYVE-1	Hyaluronan receptor	++	–	(Banerji *et al.*, 1999)
VEGFR-3	Growth factor receptor	+	–/(+)[1]	(Kaipainen *et al.*, 1995)
Neuropilin-2	Semaphorin and growth factor receptor	+	–/(+)[2]	(Yuan *et al.*, 2002)
Macrophage mannose receptor 1	L-selectin receptor	+	–	(Irjala *et al.*, 2001)
CCL21	CC-chemokine	+	–	(Gunn *et al.*, 1998)
CCL20	CC-chemokine	+ (++)[3]	– (++)[3]	(Kriehuber *et al.*, 2001; Hirakawa *et al.*, 2003)
Desmoplakin	Anchoring protein of adhering junctions	+	–	(Ebata *et al.*, 2001)
Plakoglobin	Connect cadherins to cytoskeleton in cell–cell junctions	+	–	(Petrova *et al.*, 2002; Hirakawa *et al.*, 2003)
Integrin α9	Adhesion molecule, subunit of osteopontin and tenascin receptors, VEGFR-3 coreceptor?	+	–	(Huang *et al.*, 2000; Petrova *et al.*, 2002)
CD44	Hyaluronan receptor	–	+	(Kriehuber *et al.*, 2001)
VEGF-C	Growth factor	–	+	(Kriehuber *et al.*, 2001; Hirakawa *et al.*, 2003)
VEGFR-1	Growth factor receptor	–	+	(Hirakawa *et al.*, 2003)
Neuropilin-1	Semaphorin and growth factor receptor	–	+	(Hong *et al.*, 2002; Petrova *et al.*, 2002)
Endoglin/CD105	Low-affinity receptor for TGF-*β*	–	++	(Hirakawa *et al.*, 2003)
CD34	L-selectin receptor	–/(+)[4]	++	(Young *et al.*, 1995)
IL-8	CXC-chemokine	–	+	(Petrova *et al.*, 2002)
N-cadherin	Adhesion molecule	–	+	(Petrova *et al.*, 2002; Hirakawa *et al.*, 2003)
ICAM-1/CD54	Adhesion molecule	–	+	(Erhard *et al.*, 1996)
Integrin α5	Adhesion molecule, subunit of fibronectin receptor	–	+	(Petrova *et al.*, 2002; Hirakawa *et al.*, 2003)
Collagen IV	Extracellular matrix protein	–/(+)[5]	++	(Hirakawa *et al.*, 2003)
Versican	Chondroitin sulfate proteoglycan	–	+	(Petrova *et al.*, 2002; Hirakawa *et al.*, 2003)
Laminin	Basement membrane molecule	–/(+)[5]	++	(Barsky *et al.*, 1983; Petrova *et al.*, 2002)
Collagen XVIII	Basement membrane molecule	–/(+)[5]	++	(Petrova *et al.*, 2002; Hirakawa *et al.*, 2003)
PAL-E	Caveolae-associated glycoprotein?	–	++	(Schlingemann *et al.*, 1985; Niemela *et al.*, 2005)

BV, blood vessel; CCL, CC chemokine ligand; LV, lymphatic vessel; LYVE-1, lymphatic vascular endothelial hyaluronan receptor-1; PAL-E, pathologische anatomie leiden-endothelium; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.
[1]VEGFR-3 expression was also found on some blood capillaries during tumor neovascularization and in wound granulation tissue (Valtola *et al.*, 1999; Paavonen *et al.*, 2000).
[2]Neuropilin-2 is also expressed in veins (Yuan *et al.*, 2002).
[3]After activation, both blood vascular and lymphatic endothelial cells strongly express CCL20 (Kriehuber *et al.*, 2001).
[4]CD34 expression has also been found on lymphatic endothelial cells (Sauter *et al.*, 1998; Kriehuber *et al.*, 2001).
[5]Peripheral lymphatic vessels sometimes have an incomplete basement membrane, large collecting vessels a complete one.

expressed at comparable levels by the two endothelial cell types (Petrova *et al.*, 2002; Hirakawa *et al.*, 2003), corroborating their close genetic relationship. However, these studies have also identified numerous, previously unknown lineage-specific markers for blood vascular and lymphatic endothelium (Table 1). Whereas the specific function of the majority of the differentially expressed genes still remains unknown, the study of several lymphatic-specific molecules has provided important new insights into the molecular control of lymphatic development and function (Table 2).

Vascular endothelial growth factor receptor-3 (VEGFR-3), also known as Flt4, was the first lymphatic-specific growth factor receptor identified (Kaipainen *et al.*, 1995). VEGFR-3 is a member of the fms-like tyrosine kinase family and specifically binds vascular endothelial growth factor (VEGF)-C and VEGF-D, but not VEGF-A. During early embryonic development, VEGFR-3 is expressed both

Table 2. Genetic mouse models with abnormalities of the lymphatic system

Genes	Function	Models	Phenotype	References
Integrin α9	Adhesion receptor	KO	Respiratory failure caused by pleural fluid (chylothorax), lymphedema	(Huang *et al.*, 2000)
Angiopoietin-1	Growth factor	TG	Hyperplastic lymphatic vessels	(Tammela *et al.*, 2005)
Angiopoietin-2	Growth factor	KO	Chylous ascites and peripheral edema, abnormal patterning of lymphatic vasculature	(Gale *et al.*, 2002)
VEGF-C	Growth factor	TG	Hyperplastic lymphatic vessels	(Jeltsch *et al.*, 1997)
VEGF-C	Growth factor	KO	No lymphatic vasculature (−/−), delayed lymphatic vascular development, lymphatic hypoplasia and lymphedema (+/−)	(Karkkainen *et al.*, 2004)
HGF	Growth factor	TG	Enhanced formation and enlargement of lymphatic vessels	(Kajiya *et al.*, 2005)
VEGFR-3	Growth factor receptor	KO	Cardiovascular failure, defective remodelling of vascular networks	(Dumont *et al.*, 1998)
VEGFR-3	Growth factor receptor	Chy mice (inactivating mutation)	Lymphedema	(Karkkainen *et al.*, 2001)
Neuropilin-2	Growth factor receptor	KO	Absence or severe reduction of small lymphatic vessels and capillaries during development	(Yuan *et al.*, 2002)
Prox1	Transcription factor	KO	No lymphatic vasculature (−/−), adult-onset obesity, chylous ascites (+/−)	(Wigle and Oliver 1999; Harvey *et al.*, 2005)
FOXC2	Transcription factor	KO	Lymphatic hyperplasia (+/−), abnormal patterning and pericyte investment of lymphatic vessels, agenesis of valves, lymphatic dysfunction (−/−)	(Kriederman *et al.*, 2003; Petrova *et al.*, 2004)
Net (Elk3)	Transcription factor	KO	Chylothorax, dilated lymphatic vessels	(Ayadi *et al.*, 2001)
SOX18 (ragged)	Transcription factor	KO (spontaneous missense mutations)	Edema, chyle accumulation in the peritoneum, cardiovascular and hair follicle defects	(Pennisi *et al.*, 2000)
Podoplanin	Membrane glycoprotein	KO	Lymphedema, dilation of lymphatic vessels, abnormal patterning	(Schacht *et al.*, 2003)
Syk and SLP-76	Tyrosine kinase (Syk), adaptor protein (SLP-76)	KO	Abnormal blood-lymphatic connections, chylous ascites	(Abtahian *et al.*, 2003)
Ephrin B2	Ligand of EphB receptors	Mutant lacking PDZ interaction site	Defective remodelling of lymphatic vascular network, hyperplasia, lack of valves, chylothorax	(Makinen *et al.*, 2005)

FOXC2, forkhead box C2; HGF, hepatocyte growth factor; KO, knock-out; PDZ, PSD-95, DISCS-large, and Zo-1; SLP, Src homology 2-domain containing leukocyte protein; SOX18, sex determining region Y-related high mobility group box 18; TG, transgenic; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

in developing venous and in presumptive lymphatic endothelia. In normal adult tissues, VEGFR-3 expression is largely restricted to the lymphatic endothelium (Kaipainen *et al.*, 1995; Partanen *et al.*, 2000). However, VEGFR-3 expression has also been detected on some blood capillaries associated with tumor neovascularization or with wound granulation tissue (Partanen *et al.*, 1999; Paavonen *et al.*, 2000); therefore VEGFR-3 alone is not a sufficiently specific marker for lymphatic vessels.

The transcription factor *Prox1* is a homolog of the *Drosophila* homeobox gene *prospero* (Oliver *et al.*, 1993). At present, it is considered as the most specific lineage marker for lymphatic endothelium – among endothelial cells, it is exclusively detected in lymphatic vessels of adult tissues and tumors (Oliver and Detmar, 2002). During lymphatic development, Prox1 expression is induced in lymphatically ''competent'' endothelial cells at one side of the cardinal vein around mouse embryonic day (E) 9.5–E10.5 by a presently unknown signal, leading to lymphatic commitment and specification (Figure 2). In Prox1 null mice, budding and sprouting of these cells from the veins is arrested prematurely at around E11.5–E12.0, and as a result, these mice completely lack lymphatic vasculature (Wigle and Oliver, 1999) (Table 2). In Prox1 null mice, the budding endothelial cells fail to express lymphatic endothelial markers (Wigle *et al.*, 2002). Conversely, ectopic expression of Prox1 in differentiated BECs is sufficient to reprogram these cells to

adopt a lymphatic phenotype (Hong *et al.*, 2002; Petrova *et al.*, 2002). Prox1 +/− mice die within 2–3 days after birth in all of the tested genetic backgrounds except one (Wigle and Oliver, 1999), in which they develop chylous ascites and – interestingly – adult onset obesity, suggesting a link between impaired lymphatic function and obesity (Harvey *et al.*, 2005).

Lymphatic vascular endothelial hyaluronan receptor-1 (LYVE-1), a homologue of the blood vascular endothelium-specific hyaluronan receptor CD44 (Banerji *et al.*, 1999), was identified as a specific cell surface protein of LECs and macrophages. It is presumably involved in hyaluronan metabolism (Jackson, 2004) but its exact function remains unclear – LYVE-1-deficient mice have no obvious lymphatic vascular malfunctions or morphological abnormalities (G. Thurston, personal communication). LYVE-1 is the first marker of lymphatic endothelial competence during development (Figure 2); in the mature vasculature, LYVE-1 expression remains high in lymphatic capillaries while being downregulated in the collecting lymphatic vessels (Makinen *et al.*, 2005).

Podoplanin, a mucin-type transmembrane glycoprotein, is expressed by lymphatic but not by BECs *in vivo* and *in vitro* (Wetterwald *et al.*, 1996; Breiteneder-Geleff *et al.*, 1999; Kriehuber *et al.*, 2001; Petrova *et al.*, 2002; Hirakawa *et al.*, 2003). Its biological function is currently unknown, although *in vitro* studies indicated that podoplanin may be involved in mediating cell motility by promoting rearrangement of the actin cytoskeleton (Schacht *et al.*, 2003). Podoplanin null mice display lymphedema, dilated lymphatic vessels, and impaired lymphatic transport, and they die at birth of respiratory failure (Ramirez *et al.*, 2003; Schacht *et al.*, 2003). Therefore, to further study the role of podoplanin in postnatal life, it will be essential to generate tissue-specific knockout mice. Human podoplanin is recognized by the D2-40 antibody (Schacht *et al.*, 2005) and is also expressed by several non-endothelial cells (Schacht *et al.*, 2005) and by squamous cell carcinomas and certain germ cell tumors (Martin-Villar *et al.*, 2005; Schacht *et al.*, 2005), suggesting a potential role in tumor progression.

The chemokine *CCL21*, also known as secondary lymphoid chemokine or Exodus-2, is secreted by lymphatic endothelium, but not blood vascular endothelium (Kriehuber *et al.*, 2001), and interacts with the CC chemokine receptor 7. It mediates homing of lymphocytes and migration of antigen-stimulated dendritic cells from the tissues into the lymphatic vessels and the secondary lymphatic organs, thus playing an important role in immunoregulatory and inflammatory processes. CCL21 has also been shown to enhance lymph node metastasis of CCR-7-expressing malignant melanoma xenotransplants (Wiley *et al.*, 2001). *Desmoplakin* is a cytoplasmic anchor protein of lymphatic endothelial adherens junctions that connects intermediate filaments to the plasma membrane. Blood vessels do not express desmoplakin (Ebata *et al.*, 2001).

Using gene array analysis of LECs versus BECs, a large number of previously unknown vascular lineage-specific genes have been identified (Petrova *et al.*, 2002; Hirakawa *et al.*, 2003). Newly identified LEC-specific genes include macrophage mannose receptor 1, plakoglobin, integrin α9, and the chemokine CCL20. The latter, however, becomes strongly expressed in both blood vascular and LECs upon activation (Kriehuber *et al.*, 2001). BEC-specific genes include VEGFR-1, neuropilin-1, the hyaluronan receptor CD44, endoglin, adhesion molecules such as ICAM-1, integrin α5 and N-cadherin, and several basement membrane, and extracellular matrix components (collagen IV, versican, laminin, collagen XIII) (Hirakawa and Detmar, 2004). Other blood vascular markers include PAL-E (Skobe and Detmar, 2000) and CD34 (Hirakawa *et al.*, 2003). The roles of most of these molecules for the functional regulation and physiological maintenance of the two types of vasculature remain to be elucidated.

Newly identified lymphangiogenic growth factors and receptors

VEGF-C and *VEGF-D* were originally cloned as ligands for VEGFR-3 (Joukov *et al.*, 1996; Orlandini *et al.*, 1996; Achen *et al.*, 1998), and are presently the only known ligands for this receptor. VEGF-C promotes proliferation, migration, and survival of cultured human LECs (Makinen *et al.*, 2001). Importantly, transgenic mice overexpressing VEGF-C or VEGF-D in the skin show hyperplasia of cutaneous lymphatic vessels (Jeltsch *et al.*, 1997; Veikkola *et al.*, 2001) (Table 2), whereas VEGF-C null mouse embryos completely lack a lymphatic vasculature and die prenatally of fluid accumulation within the tissues (Karkkainen *et al.*, 2004). In these mice, the lymphatically committed venous endothelial cells express Prox1 but are unable to migrate out to form the initial lymph sacs. These findings indicate that LEC specification and subsequent migration are two separate events, and that VEGF-C signalling is indispensable for the latter (Figure 2). VEGF-D also stimulates lymphangiogenesis in tissues and tumors (Stacker *et al.*, 2001; Veikkola *et al.*, 2001). However, VEGF-D-deficient mice do not exhibit a lymphatic phenotype (Karkkainen *et al.*, 2004; Baldwin *et al.*, 2005), probably because VEGF-D is not expressed at the critical sites of lymph sac formation in the embryo (Avantaggiato *et al.*, 1998; Karkkainen *et al.*, 2004). Inactivation of VEGFR-3 causes cardiovascular failure and embryonic death at E9.5 before the emergence of lymphatic vessels (Dumont *et al.*, 1998), hampering the analysis of its role in lymphatic development. Nonetheless, VEGFR-3 mutations have been identified in *Chy* mutant mice that are characterized by cutaneous lymphedema (Karkkainen *et al.*, 2001), providing support for an important role of this gene in lymphatic development and function. The distinct contributions of VEGFR-3 versus VEGFR-2 towards lymphangiogenesis remain at present unclear, because VEGF-C and VEGF-D – after enzymatic cleavage – also activate VEGFR-2, which is expressed by LECs (Kriehuber *et al.*, 2001; Veikkola *et al.*, 2001; Hirakawa *et al.*, 2003; Hong *et al.*, 2004b). Importantly, however, skin-specific overexpression of a VEGFR-3-specific mutant of VEGF-C (VEGF-C156S; Joukov *et al.*, 1998) in

transgenic mice revealed that activation of VEGFR-3 signal transduction is sufficient to promote lymphangiogenesis (Veikkola *et al.*, 2001).

The possible contribution of *VEGF-A* (also named VEGF or vascular permeability factor), to lymphangiogenesis has been a matter of controversy. VEGF-A was discovered in 1983 as the first member of the VEGF family (Senger *et al.*, 1983) and activates VEGFR-1 and VEGFR-2. Although VEGF-A cannot substitute for VEGF-C to rescue the phenotype of VEGF-C-deficient mice (Karkkainen *et al.*, 2004), several recent studies have established a role of the VEGF-A/VEGFR-2 signalling pathway in lymphangiogenesis. VEGF-A potently induces proliferation of LECs *in vitro* (Hirakawa *et al.*, 2003), and injection of adenoviral murine VEGF-A164 resulted in pronounced and persistent *in vivo* lymphangiogenesis in mouse ear skin (Nagy *et al.*, 2002). Targeted overexpression of murine VEGF-A164 in the skin of transgenic mice enhanced lymphangiogenesis as well as angiogenesis during tissue repair and in skin inflammation (Kunstfeld *et al.*, 2004; Hong *et al.*, 2004b). These effects could be inhibited by a specific VEGFR-2 blocking antibody, indicating that VEGF-A signalling through VEGFR-2 is important for lymphangiogenesis. However, indirect effects of VEGF-A, via attraction of inflammatory cells producing VEGF-C and -D (Cursiefen *et al.*, 2004; Baluk *et al.*, 2005) might also contribute to VEGF-A's lymphangiogenic activity (Hirakawa *et al.*, 2005).

Neuropilins are non-kinase type I transmembrane proteins that play an important role in axon guidance within the nervous system as semaphorin receptors (Neufeld *et al.*, 2002). Whereas neuropilin-1 is predominantly expressed in arterial endothelial cells, *neuropilin-2* is expressed in veins and lymphatic capillaries (Karkkainen *et al.*, 2001; Hong *et al.*, 2002; Yuan *et al.*, 2002). It serves as a co-receptor for several VEGF family members including VEGF-A165, placental growth factor, and VEGF-C (Gluzman-Poltorak *et al.*, 2000; Karkkainen *et al.*, 2001). In addition to neural defects, neuropilin-2-deficient mice show a severe reduction of small lymphatic vessels, whereas they develop normal arteries, veins, and larger collecting lymphatic vessels (Yuan *et al.*, 2002). The finding that neuropilin-2 binds VEGF-C raises the possibility that VEGF-C signalling through VEGFR-3 may be enhanced by neuropilin-2, similar to the Nrp1-mediated promotion of VEGF-A signalling to VEGFR-2 (Soker *et al.*, 1998).

The angiopoietin signalling system is indispensable for normal blood vessel development as demonstrated by several genetic mouse models (Dumont *et al.*, 1994; Suri *et al.*, 1996; Maisonpierre *et al.*, 1997). Angiopoietin-1 (Ang1), an activating ligand for the endothelial-specific receptor tyrosine kinase Tie2 (tyrosine kinase with immunoglobulin-like loop and epidermal growth factor homology domains-2) (Davis *et al.*, 1996), induces lymphangiogenesis in the mouse cornea (Morisada *et al.*, 2005) and – after adenoviral gene transfer – in other adult mouse tissues (Tammela *et al.*, 2005). Transgenic mice overexpressing Ang1 in the skin show cutaneous lymphatic hyperplasia (Tammela *et al.*, 2005). The mechanism of these effects remains unclear. Tie2 is expressed in cultured LECs (Kriehuber *et al.*, 2001; Petrova *et al.*, 2002) and in the lymphatic vessels of mice (Morisada *et al.*, 2005; Tammela *et al.*, 2005), suggesting that Ang1 might exert direct effects on lymphatic endothelium. However, treatment with soluble VEGFR-3 inhibited the effects of virally delivered Ang1 in mice, and Ang1 stimulation of LECs resulted in upregulation of VEGFR-3 *in vitro* and *in vivo* (Tammela *et al.*, 2005), indicating that Ang1 also acts indirectly via the VEGF-C/VEGFR-3 pathway. Angiopoietin-2, considered to be an antagonist of Tie2 (Maisonpierre *et al.*, 1997), also appears to be needed for the normal formation of the lymphatic vasculature because angiopoietin-2-deficient mice have chylous ascites, peripheral edema, and abnormal patterning of lymphatic vessels (Gale *et al.*, 2002). Interestingly, these defects, but not the blood vascular phenotype, can be rescued by Ang1 (Gale *et al.*, 2002). Thus, angiopoietin-2 might act like Ang1 as an agonist of Tie2 in lymphangiogenesis, while it is an (context-dependent) antagonist of Tie2 in angiogenesis.

Hepatocyte growth factor (HGF, also known as scatter factor) was recently identified as a potent lymphangiogenesis factor (Kajiya *et al.*, 2005). HGF promotes proliferation, migration, and tube formation of LECs via its receptor HGF-R. HGF-induced LEC proliferation was abolished by an HGF-R blocking antibody but not by blockade of VEGFR-3, indicating that HGF exerts its effects independently of the VEGFR-3 pathway. The promigratory effects of HGF are in part mediated by the integrin $\alpha 9$, which is specifically expressed by LECs and is required for normal lymphatic function (Huang *et al.*, 2000). Overexpression of HGF in transgenic mice as well as subcutaneous delivery of this growth factor resulted in increased numbers and enlargement of lymphatic vessels. These effects were not inhibited by a VEGFR-3 blocking antibody (Kajiya *et al.*, 2005), demonstrating that HGF can directly promote lymphangiogenesis *in vivo*.

Additional lymphangiogenic factors. Fibroblast growth factor-2 (FGF-2) was one of the first angiogenic factors identified and the role of FGFs in vascular development has been well characterized (Auguste *et al.*, 2003). FGF-2 also promotes lymphatic vessel growth – in addition to blood vessel growth – in the mouse cornea assay by inducing VEGF-C secretion from blood vascular endothelial and perivascular cells (Kubo *et al.*, 2002; Chang *et al.*, 2004). FGF-2 might also directly act via its receptor FGFR-3, which is upregulated by Prox1 in lymphatic endothelium (Shin *et al.*, 2005). FGF-2 enhances migration and proliferation of primary LECs *in vitro,* and the promigratory effect could not be abrogated by neutralization of VEGFR-3, raising the possibility that FGF-2 might also function independently of the VEGF-C/VEGFR-3 pathway (Shin *et al.*, 2005). In addition, recent studies have suggested that platelet-derived growth factor-BB (Cao *et al.*, 2004) and insulin-like growth factors 1 and 2 might also induce lymphangiogenesis

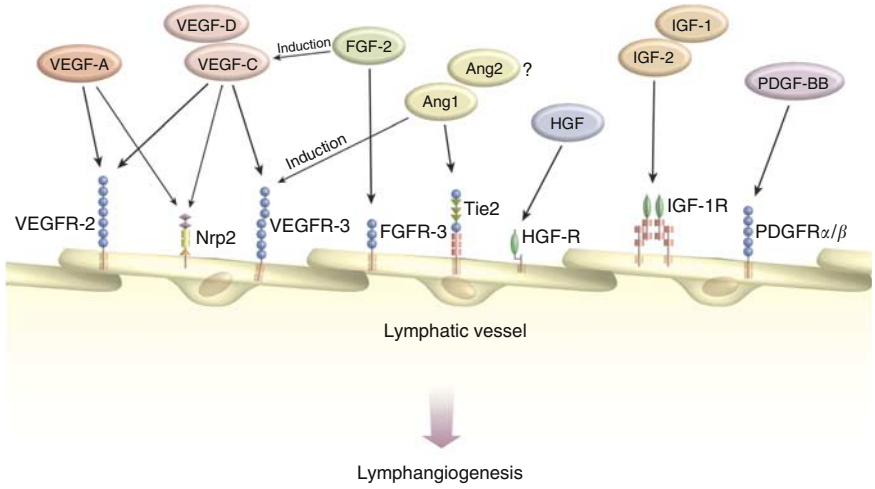

Figure 3. Schematic representation of lymphangiogenic growth factors and their receptors expressed by lymphatic endothelium. Several vascular endothelial growth factors (VEGF-A, VEGF-C, VEGF-D) promote lymphangiogenesis by activation of distinct VEGFRs and Nrp2. FGF-2 acts directly through FGFR-3 and also via induction of VEGF-C. Angiopoietin-1 (Ang1) activates Tie2 and up-regulates VEGFR-3. HGF, insulin-like growth factors (IGF), and platelet-derived growth factor-BB (PDGF-BB) act directly through their respective receptors HGF-R, IGF-1R, and PDGFR.

in the mouse corneal assay (Bjorndahl *et al.*, 2005), but their potential effects on skin lymphangiogenesis remain unclear. Likely, several lymphangiogenic growth factors work together in a complex way, contributing to the process of lymphatic vessel formation and growth in physiological or pathological conditions (Figure 3).

Genetic basis of lymphedema and new molecular therapies

Lymphedema is caused by insufficient lymph transport owing to lymphatic hypoplasia, impaired lymphatic function, or obstruction of lymph flow. Primary lymphedema is characterized by dilated lymphatic capillaries and interstitial accumulation of lymph fluid. In some families, congenital lymphedema is linked to the *VEGFR-3* locus on distal chromosome 5q, and missense mutations in the *VEGFR-3* gene have indeed been identified in several cases of hereditary, early-onset lymphedema (Witte *et al.*, 2001). Recent studies have identified additional mutations in other genes that are associated with different human lymphedema syndromes. In lymphedema-distichiasis, an autosomal-dominant disorder with congenital lymphedema and double rows of eyelashes (distichiasis), inactivating mutations of the *FOXC2* gene, a member of the forkhead/winged-helix family of transcription factors, were identified in several families (Fang *et al.*, 2000). Moreover, mutations of the *SOX18* gene on chromosome 20q13, a SRY-related transcription factor, cause recessive and dominant forms of hypotrichosis–lymphedema–telangiectasia syndrome. Mutations in the DNA-binding domain of SOX18 have been found in the recessive form of the disease whereas the dominant hereditary form is caused by a heterozygous nonsense mutation of the transactivation domain (Irrthum *et al.*, 2003). An involvement of Sox18 in lymphatic vessel development is further supported by the phenotype of ragged mice that develop lymphedema and also show abnormalities of the hair coat (Table 2).

Based on its potent lymphangiogenic effect, VEGF-C has been tested for gene and protein therapy of lymphedema in animal models. Adeno-associated virus-mediated VEGF-C gene therapy promoted lymphatic vessel generation in the skin of *Chy* lymphedema mice (Karkkainen *et al.*, 2001). Importantly, VEGF-C156S, a mutant form of VEGF-C that selectively activates VEGFR-3, successfully induced the formation of a functional cutaneous lymphatic vessel network without blood vessel growth or vascular leakiness, side effects observed with VEGF-C gene therapy due to its activation of VEGFR-2 (Saaristo *et al.*, 2002). Recently, successful regeneration of a lymphatic network was observed after injection of VEGF-C protein in a surgical lymphedema model in the rabbit ear, indicating the potential use of VEGF-C for the treatment of secondary lymphedema (Skobe *et al.*, 2001).

Tumor lymphangiogenesis and metastasis

Tumor metastasis to regional lymph nodes represents the first step of tumor dissemination in most skin cancers and also serves as a major prognostic indicator for disease progression. Little is known, however, about the mechanisms how tumor cells gain entry into the lymphatic system, and it has been generally thought that lymphatic invasion only occurs once infiltrating tumor cells happen upon pre-existing peritumoral lymphatic vessels. Recent studies in animal tumor models have now provided direct experimental evidence that increased levels of VEGF-C and/or VEGF-D promote active tumor lymphangiogenesis and lymphatic tumor spread to regional lymph nodes, and that these effects can be suppressed by blocking VEGFR-3 signalling (Mandriota *et al.*, 2001; Skobe *et al.*, 2001; Stacker *et al.*, 2001; He *et al.*, 2002). Very recently, we have found that VEGF-A also acts as a potent tumor lymphangiogenesis factor and that tumor-derived VEGF-A promotes expansion of the lymphatic network within

draining, sentinel lymph nodes, even before these tumors metastasized (Hirakawa *et al.*, 2005). These novel findings indicate that lymph node lymphangiogenesis might contribute to the further metastatic tumor spread beyond the sentinel lymph node. Importantly, a large number of clinicopathological studies have shown a direct correlation between expression of VEGF-C or VEGF-D by tumor cells and metastatic tumor spread in many human cancers, indicating an important role of lymphangiogenesis also in human tumor progression (Stacker *et al.*, 2002). Our own recent studies in human cutaneous malignant melanomas demonstrated – for the first time – the presence of both intratumoral and peritumoral lymphangiogenesis (Dadras *et al.*, 2003). They also showed that primary melanomas that later metastasized were characterized by increased lymphangiogenesis – as compared to non-metastatic tumors – and that the degree of tumor lymphangiogenesis can serve as a novel predictor of lymph node metastasis and overall patient survival, independently of tumor thickness. Tumor lymphangiogenesis also significantly predicted the presence of sentinel lymph node metastases at the time of surgical excision of the primary melanoma (Dadras *et al.*, 2005). Further studies involving larger numbers of cases are needed to confirm these findings.

New insights into the pathogenesis of Kaposi's sarcoma

Kaposi's sarcoma (KS) is the most frequently occurring malignant tumor in patients infected with the human immunodeficiency virus. KS mainly affects the skin and forms lesions of various types, including early inflammatory and patch stage lesions, and tumors with a predominant population of spindle cells. Infection with KS-associated herpesvirus (also known as human herpesvirus-8) is essential for KS tumor formation. KS has been considered to be a neoplasm of KS-associated herpesvirus-infected lymphatic endothelium, owing to the morphological characteristics of the tumor cells and their expression of several lymphatic lineage-specific genes including VEGFR-3 and podoplanin. Recently, we and others have shown that infection of differentiated BECs with KS-associated herpesvirus leads to their LEC re-programming with induction of approximately 70% of the major lymphatic lineage-specific genes including Prox1, a master regulator of lymphatic development, and downregulation of blood vascular genes (Wang *et al.*, 2004; Hong *et al.*, 2004a). Together, these results provide a molecular explanation for the previously observed controversial results regarding the lineage-specific differentiation of KS cells.

Inflammation and lymphangiogenesis

There is increasing evidence that lymphatic vessels actively participate in acute and chronic inflammation, as well as in the cutaneous response to UVB irradiation. Psoriatic skin lesions are characterized by pronounced lymphatic hyperplasia (Kunstfeld *et al.*, 2004), and chronic skin inflammation in mice is also associated with LEC proliferation and lymphatic hyperplasia (Kunstfeld *et al.*, 2004). Furthermore, kidney transplant rejection is frequently accompanied by lymphangiogenesis, and LEC-derived chemokines such as CCL21 might actively promote the inflammatory process (Kerjaschki *et al.*, 2006). Recently, we found that acute UVB irradiation of the skin results in hyperpermeable, leaky lymphatic vessels that are functionally impaired (Kajiya and Detmar, 2006). Importantly, blockade of VEGFR-3 resulted in prolonged inflammation and edema after UVB irradiation. Together, these results indicate that lymphatic vessels are not only required to drain inflammation-associated tissue edema, but might also actively participate in the maintenance of chronic inflammatory diseases.

Perspectives

Although traditionally neglected as a topic of scientific study, the lymphatic vascular system has recently received tremendous scientific interest (Brown, 2005). Owing to a number of recent discoveries, some of the mechanisms controlling the normal and pathological development of the lymphatic vasculature are now being unravelled, and several genetic defects have been identified in patients with lymphedema. The identification of specific markers and growth factors for lymphatic vessels have been instrumental in this advance. The recently proposed concept of tumor lymphangiogenesis and its role in tumor metastasis is of particular importance for the understanding of cancer progression. Further progress in this field will likely lead to a better diagnosis and treatment of a variety of lymphatic disorders but also of certain types of skin cancer and of inflammatory skin diseases.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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