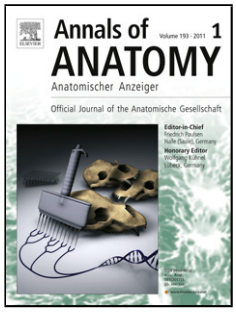

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Key molecules in lymphatic development, function, and identification

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Abstract

While both blood and lymphatic vessels transport fluids and thus share many similarities, they also show functional and structural differences, which can be used to differentiate them. Specific visualization of lymphatic vessels has historically been and still is a pivot point in lymphatic research. Many of the proteins that are investigated by molecular biologists in lymphatic research have been defined as marker molecules, i.e. to visualize and distinguish lymphatic endothelial cells (LECs) from other cell types, most notably from blood vascular endothelial cells (BECs) and cells of the hematopoietic lineage.

Among the factors that drive the developmental differentiation of lymphatic structures from venous endothelium, Prospero homeobox protein 1 (PROX1) is the master transcriptional regulator. PROX1 maintains lymphatic identity also in the adult organism and thus is a universal LEC marker. Vascular endothelial growth factor receptor-3 (VEGFR-3) is the major tyrosine kinase receptor that drives LEC

proliferation and migration. The major activator for VEGFR-3 is vascular endothelial growth factor-C (VEGF-C). However, before VEGF-C can signal, it needs to be proteolytically activated by an extracellular protein complex comprised of collagen and calcium binding EGF domains 1 (CCBE1) protein and the protease A disintegrin and metallopeptidase with thrombospondin type 1 motif 3 (ADAMTS3).

This minireview attempts to give an overview of these and a few other central proteins that scientific inquiry has linked specifically to the lymphatic vasculature. It is limited in scope to a brief description of their main functions, properties and developmental roles.

Keywords

vascular biology; lymphangiogenesis; lymphatic marker; transcription factors; growth factors; cell surface receptors; VEGF-C/VEGFR-3 signaling

Introduction

The lymphatic system is involved in the maintenance of the body fluid balance (Dongaonkar et al., 2009), in immune cell trafficking (specifically in dendritic cell trafficking from tissues to lymph nodes; Miteva et al., 2010; Randolph et al., 2005), and in dietary lipid absorption from the intestine via the blind-ended central lymph vessels in the intestinal villi known as lacteals (Iqbal and Hussain, 2009). Similar to the blood vasculature (Aird, 2012), there is substantial heterogeneity and plasticity within the lymphatic system. Developmental age, function and location of lymphatic

vessels are reflected in their molecular setup (Ulvmar and Mäkinen, 2016). Lymphatic research has entered the molecular era more than two decades ago with the discovery of the first lymphangiogenic growth factor VEGF-C (Joukov et al., 1996) and its receptor VEGFR-3 (Kaipainen et al., 1995), which was also used as the first lymphatic-specific marker. The interest in lymphatic research has been increasing since due to the recognition that lymphatics are integral to many disease processes (Alitalo, 2011). However, the continuing discoveries of molecules that play important roles for lymphatic biology underline that our understanding of the molecular mechanisms of lymphatic development and function under both physiological and pathological settings is far from complete.

Transcription factors SOX18, COUP-TFII, PROX1 and FOXC2

The SOX18 transcription factor is perhaps the earliest involved in the specification of endothelial cells into the lymphatic lineage. It activates the expression of PROX1 (Prospero Homeobox 1) in endothelial cells of the cardinal veins around E9.5 (Francois et al., 2008). SOX18 cannot activate PROX1 alone, but needs cooperation from COUP-TFII, which is expressed throughout the venous system (Srinivasan et al., 2007). Contrary to SOX18 expression, which appears to be needed only for the initiation of LEC specification, COUP-TFII and PROX1 continue to be strongly expressed in established lymphatic vessels (Francois et al., 2011), although only PROX1, but not COUP-TFII is necessary for the maintenance of the lymphatic identity (Johnson et al., 2008; Lin et al., 2010).

PROX1 is expressed by several cell types like liver cells and many stem cells, but in the vascular compartment, it is largely specific for lymphatic endothelial cells, although it can be found in some specialized subpopulations of endothelial cells, e.g. in the

venous (Bazigou et al., 2011) and the cardiac (Rodriguez-Niedenführ et al., 2001) valves. PROX1 is the key transcription factor for the early steps of LEC differentiation from the embryonic veins (Wigle and Oliver, 1999) and remains required for lymphatic identity (Johnson et al., 2008). The over-expression of PROX1 in BECs modifies their expression patterns to resemble LECs (Hong et al., 2002; Petrova et al., 2002), including the upregulation of the gene encoding VEGFR-3, that is seen in the cardinal vein endothelial cells which are committed to LEC differentiation (Wigle et al., 2002).

The FOXC2 transcription factor becomes important during later stages of the lymphatic development. It controls the interaction between pericytes and LECs (Petrova et al., 2004), and is required together with NFATc1 for the lymphatic remodeling and maturation including the formation of lymphatic valves in the precollectors and collectors (Norrmén et al., 2009).

The major mitogenic receptor on LECs: VEGFR-3

VEGFR-3 (previously also called FLT4) is the quintessential lymphatic receptor tyrosine kinase. However, early developing blood vessels (from day 8 to 10) also express significant amounts of VEGFR-3. In mice, loss of VEGFR-3 leads to embryonic death at E10.5 due to cardiovascular defects (Dumont et al., 1998). Unlike VEGFR-3 deletion, the simultaneous deletion of both VEGFR-3 ligands (VEGF-C and VEGF-D) in mouse embryos does not, for the most part, affect blood vessels, with the exception of VEGF-C-induced BEC migration, which is important for the development of the coronary vasculature (Chen et al., 2014a, 2014b). Nevertheless, most of the early embryonic function of VEGFR-3 does apparently not require activation by ligands of the VEGF family (Haiko et al., 2008). Hence it remains unclear, what mechanism underlies the need for VEGFR-3 in the development of the cardiovascular system.

Heterodimerization with VEGFR-2 in response to VEGF-A (Dixelius et al., 2003; Nilsson et al., 2010) could enable VEGFR-3 signaling, but ligandless baseline signaling or alternative activation mechanisms involving integrins and mechanoinduction could also play a role (Galvagni et al., 2010; Planas-Paz et al., 2012; Wang et al., 2001).

VEGFR-3 expression declines on BECs during the period of lymphatic budding from venous endothelium and the establishment of the first lymphatic structures, which starts around E10-E10.5. By day 14.5, it is seen mostly on lymphatics (Kaipainen et al., 1995) with the exception of a few vascular specializations, where it persists into adulthood, such as fenestrated vessels (Partanen et al., 2000) and some high endothelial venules (Kaipainen et al., 1995). Mutations in *Flt4* cause Type IA hereditary lymphedema (Milroy disease), which is the most common form of primary lymphedema in humans. Most of these mutations inactivate the tyrosine kinase activity of VEGFR-3 (Karkkainen et al., 2000) and in mice, functionally analogous mutations result as well in a lymphedema phenotype (Karkkainen et al., 2001).

Both LECs and BECs express VEGFR-2

VEGFR-2 is the tyrosine kinase receptor, which mediates most - if not all - functions of the classic hemangiogenic growth factor VEGF-A (Simons et al., 2016). However, VEGFR-2 can also be activated by the mature forms of VEGF-C and VEGF-D (the different forms of VEGF-C and VEGF-D are discussed in the paragraph *VEGF-D is a dissimilar twin of VEGF-C* and the following). Because VEGFR-2 is also expressed at moderate levels on most LECs, VEGF-A replaces VEGF-C as medium supplement in many LEC culture protocols (Lonza, 2017; PromoCell, 2017). Lymphatic hyperplasia has been induced by VEGF-A (Nagy et al., 2002; Wirzenius et al., 2007) and by the

VEGFR-2-monospecific VEGF-E (Wirzenius et al., 2007). Despite lymphatic hyperplasia, there was no increase in lymphatic numbers in VEGF-E overexpressing mice and in mouse ears transduced with a VEGF-A-expressing adenovirus. This led to the hypothesis that VEGFR-2 signalling causes only circumferential growth of lymphatic vessels, while VEGFR-3 signalling causes the generation of new vessels by sprouting lymphangiogenesis. VEGF-A-induced lymphangiogenesis might also be indirectly mediated by upregulating VEGF-C expression in BECs (Skobe et al., 1999; Skobe & Detmar, 2000) or in macrophages (Harvey and Gordon, 2012), which can be recruited e.g. by VEGF-A via VEGFR-1 (Hiratsuka et al., 1998). However, it remains unclear which mechanisms are involved *in vivo*.

VEGF-C is the primary lymphangiogenic growth factor

VEGF-C is the primary ligand that activates VEGFR-3 (see Figure 1). The sprouting of endothelial cells from the embryonic veins is crucially dependent on VEGF-C. Its absence leads to the failure of lymph sac formation and embryonic death around E16.5 (Hagerling et al., 2013; Karkkainen et al., 2004). Also in the heterozygous state, VEGF-C deficiency leads neonatally to severe complications due to insufficient lacteal function and resulting chylous ascites (Karkkainen et al., 2004). Although rare, mutations in the human *VEGFC* gene have been shown to be responsible for some forms of hereditary lymphedema (Balboa-Beltran et al., 2014; Gordon et al., 2013). VEGF-C is first produced in larger amounts in regions juxtaposed to the prospective locations of lymphatic sprouting (e.g. the mesenchyme around the developing metanephros and in the jugular area; Karkkainen et al., 2004; Kukk et al., 1996) and forms perhaps a gradient, along which the LECs are migrating (Jha et al., 2017; Yang

and Oliver, 2014). However, direct evidence of a VEGF-C gradient formation is lacking, and it is also still unknown how VEGF-C expression is induced.

Some lymphatic networks are not generated by lymphangiogenesis (the growth of lymphatics from pre-existing vessels), but instead by lymphvasculogenesis (the differentiation and assembly from non-venous precursor cells). Lymphvasculogenesis appears to be used in different organs and by different organisms to various degrees, but at least in mice, the lymphvascularization of the heart (Klotz et al., 2015), the mesentery (Stanczuk et al., 2015) and the skin (Martinez-Corral et al., 2015) involves lymphvasculogenesis. The molecular orchestration of this process is under investigation, and similar to lymphangiogenesis, VEGF-C appears to be required.

VEGF-D is a dissimilar twin of VEGF-C

Together with VEGF-D (Achen et al., 1998), which had been first described as c-fos-induced growth factor (FIGF) (Orlandini et al., 1996), VEGF-C forms a subgroup within the protein family of vascular endothelial growth factors. Unlike the other VEGFs, both VEGF-C and VEGF-D are produced as pro-proteins and require a multistep proteolytic cleavage before they become active. The first (C-terminal) cleavage is similarly executed for both VEGF-C and VEGF-D by furin or the proprotein convertases PC5 and PC7 (McColl et al., 2007; Siegfried et al., 2003). While the first cleavage is constitutive, the second (N-terminal) cleavage is tightly regulated and depends on different enzymes for VEGF-C and VEGF-D (Bui et al., 2016).

Both VEGF-C and VEGF-D appear similarly lymphangiogenic in a variety of models like transgenic mice (Jeltsch et al., 1997; Veikkola et al., 2001), adenoviral transduction of skeletal muscle (Rissanen et al., 2003) and the CAM assay (Jeltsch et

al., 2003; Oh et al., 1997). However, unlike *Vegfc*, *Vegfd* can be deleted, at least in mice, without appreciable consequences for the lymphatic system during embryogenesis (Baldwin et al., 2005). However, adult *Vegfd*-deleted mice present with initial dermal lymphatics of reduced size and functionality, implying a role of VEGF-D during adult lymphangiogenesis, specifically perhaps during wound healing (Paquet-Fifield et al., 2013). In several *in vivo* models, VEGF-D shows a stronger and distinct angiogenic effect compared to VEGF-C (Duong et al., 2014; Leppanen et al., 2011; Song et al., 2007; Rissanen, 2003). This agrees with data showing that the maximally processed form of VEGF-D does – differently to VEGF-C – not anymore activate VEGFR-3, but only the angiogenic receptor VEGFR-2 (Leppanen et al., 2011). The difference between VEGF-C and VEGF-D has been pinpointed to a diverging role of the N-terminal α-helix for receptor binding (Davydova et al., 2016). However, many of these binding studies have been performed with truncated and/or mutated forms of VEGF-D, making it difficult to extrapolate to the in-vivo situation. Similarly, in 293EBNA cells, processing of VEGF-D results in 2.5 times more of the VEGFR-3-binding form compared to the VEGFR-2-binding form (Stacker et al., 1999), but it is completely unknown whether and how much of the VEGFR-2-specific form is generated *in vivo* .

VEGF-C activation requires CCBE1 and ADAMTS3

During development, a disintegrin and metalloproteinase with thrombospondin motifs 3 (ADAMTS3) is indispensable for the proteolytic activation of pro-VEGF-C, resulting in the mature, active VEGF-C (Jeltsch et al., 2014). ADAMTS3 was originally assumed to be a procollagen II processing enzyme (Fernandes et al., 2001), but *Adamts3*-deleted mice do not show procollagen processing defects, but instead a prenatally lethal edema phenotype (Bui et al., 2016; Janssen et al., 2015). VEGF-C cleavage by

ADAMTS3 requires the collagen- and calcium-binding EGF domains 1 (CCBE1) protein (Bos et al., 2011; Bui et al., 2016; Jeltsch et al., 2014; Le Guen et al., 2014) and mutations in the *CCBE1* gene can be responsible for *Hennekam Syndrome*, a human hereditary condition characterized by generalized lymphedema (Alders et al., 2013, 2009). Recent studies have delineated details of the molecular requirement of CCBE1 for ADAMTS3 function and shown that also *ADAMTS3* mutations can be the cause of hereditary lymphedema conditions (Brouillard et al., 2017; Jha et al., 2017). Differently to VEGF-C, VEGF-D is not activated by ADAMTS3/CCBE1 (Bui et al., 2016; Jeltsch et al., 2014), but instead by plasmin, indicating that VEGF-D would rather act during inflammation- or wound-healing associated lymphangiogenesis (Bui et al., 2016).

Differences between human and murine VEGFR-3 signaling

The first cDNA of *VEGFC* was isolated from a human library (Joukov et al., 1996). While the early confirmations of its lymphatic function used human proteins (Jeltsch et al., 1997; Oh et al., 1997), most experimental studies about the lymphatic system are performed nowadays in mice. Therefore, it is important to highlight distinct differences between the molecular interactions of the human and the corresponding murine molecules of the VEGFR-3 signaling pathway. While mature human VEGF-D can bind to both human VEGFR-2 and human VEGFR-3, mature mouse VEGF-D has been reported to bind only to mouse VEGFR-3, but not to mouse VEGFR-2 (see Figure 1) (Baldwin et al., 2001). A similarly important difference exists for VEGFR-3. Humans have two functionally diverging splice isoforms: VEGFR-3s (short isoform) and VEGFR-3l (long isoform). This diversity has not been seen so far in any other non-

primate species (Hughes, 2001). However, it remains speculative whether these dissimilarities result in morphological or functional differences.

Unorthodox VEGF-C signaling

While lymphatics and VEGF-C expression can be found in almost all tissues during development, not all VEGF-C/VEGFR-3 signaling targets endothelial cells. During brain development, neuronal progenitor cells in the olfactory bulb and glial precursor cells in the optic nerve respond to VEGF-C exposure with proliferation (Le Bras et al., 2006). Also in adult mice, VEGF-C signaling appears to be able to stimulate neurogenesis (Han et al., 2015), and, in zebrafish, VEGF-C appears crucial for motor neuron axon growth (Kwon et al., 2013). In the eye, corneal epithelial cells express VEGFR-3, where it can act as a decoy receptor removing lymphangiogenic and angiogenic factors thereby maintaining avascularity (Cursiefen et al., 2006). Unsurprisingly, due to their common ancestry, quite a few cells of hematopoietic origin express VEGFR-3 and react to VEGF-C. Such cells include hematopoietic stem cells (Fang et al., 2016; Hamada et al., 2000) and megakaryocyte precursors (Thiele et al., 2012). The expression of VEGFR-3 by corneal dendritic cells (Hamrah et al., 2003) and by conjunctival cells of the monocyte/macrophage lineage (Hamrah et al., 2004) has been suggested to play a role for the immune response in the eye. Macrophages not only can express VEGFR-3, but they also can secrete the VEGFR-3 ligand VEGF-C. In the skin, macrophages intriguingly appear to regulate the salt balance of body fluids by secreting VEGF-C, which in turn has been proposed to regulate the lymphatic volume and gateway function between the hyperosmotic interstitium and normosmolar blood (Machnik et al., 2009). VEGFR-3 can also be expressed by tumor associated macrophages (Schoppmann et al., 2002); and VEGF-C reportedly enhances tumor

cell metastasis (Su et al., 2006) and leukemic cell growth and proliferation (Dias et al., 2002) by signaling through the VEGFR-3 present on the tumor cells. However, this notion has been challenged for solid tumors. Poor antibody specificity is likely responsible for most of the VEGFR-3 signals from tumor cells, while true-positive VEGFR-3 signals originate predominantly from endothelial cells (Petrova et al., 2008; Smith et al., 2010), where VEGFR-3 signaling promotes both tumor angiogenesis (Tammela et al., 2008) and tumor lymphangiogenesis (Mandriota et al., 2001; Karpanen et al., 2001; Skobe et al., 2001), which results in increased metastasis.

Co-receptors

Neuropilin-1 (NRP1) and neuropilin-2 (NRP2) have been first described as transmembrane proteins of neuronal cells, in which they regulate the growth of dendrites and axons together with their different semaphorin ligands, which act either as attractants or repellents (Schwarz and Ruhrberg, 2010). Both NRP1 and NRP2 are also expressed by endothelial cells, with NRP1 more prominently on arteries and NRP2 more prominently on LECs and veins (Herzog et al., 2001; Yuan et al., 2002). They act as co-receptors for VEGF ligands by stabilizing the growth factor/receptor complex, but likely do not exercise a signaling function in endothelial cells (Guo and Vander Kooi, 2015). While virtually all VEGF family members have been seen to interact with NRP1 (and most with NRP2), the VEGF-A/NRP1 (Kawamura et al., 2008) and the VEGF-C/NRP2 (Xu et al., 2010) interactions appear to be significant both *in vivo and in vitro* (Karpanen et al., 2006). Similar to the neuropilins, α5β1 integrin (Wang et al., 2001; Zhang et al., 2005) and syndecan-4 (Johns et al., 2016) are known co-receptors for VEGFR-3. They can enrich the effective cell surface concentration of VEGF-C, stabilize the receptor interaction of VEGF-C and render VEGFR-3 signaling

pressure-sensitive (Planas-Paz et al., 2012). Similarly, VEGFR-2 signalling can be enhanced by interaction with αvβ3 integrin (Soldi et al., 1999) and perlecan (Zoeller et al., 2009).

LYVE-1 and podoplanin

When endothelial structures are immunohistochemically interrogated, the expression of the cell surface glycoproteins LYVE-1 and podoplanin (PDPN) are good indicators of lymphatic nature. Apart from LECs, LYVE-1 is expressed also on liver BECs (Carreira et al., 2001) and on certain macrophages (Schledzewski et al., 2006), but it is generally a useful marker to identify lymphatic capillaries (Banerji et al., 1999). LYVE-1 expression is decreased on lymphatic pre-collectors and absent from collectors (Lutter et al., 2012). LYVE-1 is a receptor for hyaluronic acid and functions in dendritic cell entry into the lymphatics (Johnson et al., 2017). Similar to LYVE-1, PDPN is frequently used in the immunohistochemical detection of lymphatics (Breiteneder-Geleff et al., 1999). PDPN was originally identified from cells of the osteoblastic lineage (Wetterwald et al., 1996) and podocytes, where it is important for the formation of the glomerular filtration barrier of the kidney (Matsui et al., 1999). PDPN is not required for the early steps of lymphatic development and is e.g. also absent from the first LECs that emigrate from the cardinal veins (initial LECs or iLECs; see Figure 2) (Hagerling et al., 2013). PDPN rather appears to play a later role in lymphatic patterning (Schacht et al., 2003) and separation from the blood vasculature (Bertozzi et al., 2010; Uhrin et al., 2010).

Angiopoietin-TIE system

The ANG/TIE system is an important signaling component controlling endothelial cell behavior in both angiogenesis and lymphangiogenesis. Like the VEGF receptors, TIE receptor expression is mostly restricted to endothelial cells with the notable exception of some hematopoietic cells (Batard et al., 1996). No ligand has been identified for TIE1 and it is thus considered to be an orphan receptor. It contributes to signaling in concert with TIE2, which is the receptor for all known angiopoietins (see Figure 1). Genetic targeting of *Tie1* is embryonically lethal and shows lymphatic abnormalities resulting in edema which arises from dysregulated lymph sac formation (D'Amico et al., 2010), defects in lymphatic vessel remodeling, collecting vessel formation and valve morphogenesis (Shen et al., 2014; Qu et al., 2015). In adult mice, however, *Tie1* ablation is well tolerated (D'Amico et al., 2014). Deletion of *Ang2* results in defective collecting lymphatic vessel formation (Dellinger et al., 2008) and a smaller diameter of lymphatic capillaries without any noticeable effect in the early lymphatic development (Shen et al., 2014). Interestingly, *Ang1* can rescue the lymphatic abnormalities observed in the *Ang2* deleted mice (Dellinger et al., 2008). However, no apparent effect on lymphatic vasculature is observed in the *Tie2* deleted mice (Shen et al., 2014).

ANG ligands can signal in two different configurations. The TIE receptors from two cells in close proximity can become ligated by ANG ligands (trans complexes). Alternatively, TIE receptors can form complexes within one cell triggered by e.g. matrix-bound ANG ligands (cis complexes) (Fukuhara et al., 2008; Saharinen et al., 2008). This, and the fact that ANG2 is a partial agonist (Yuan et al., 2009)

demonstrate, that the biological response within the ANG/TIE signaling is complex and context-dependent (reviewed by Eklund et al., 2017).

Conclusions

While the growing interest in lymphatic research has spawned many searches for “lymphatic” molecules (Hirakawa et al., 2003; Nelson et al., 2007; Petrova et al., 2002), the recent discovery of the essential functions of CCBE1 and ADAMTS3 for lymphatic development shows that we likely have not yet identified all important molecules in this field and that some surprises still lie ahead. This minireview is focused on the most central molecules (summarized in Table 1) skipping many essential molecules like those for the interaction of LECs with cells of the immune system (e.g. CCL21 for dendritic cell migration) (Russo et al., 2016; Vaahtomeri et al., 2017; Weber et al., 2013) and those for pathological lymphangiogenesis, in which VEGF-C and VEGF-D are activated differently compared to developmental lymphatic growth (Bui et al., 2016; McColl et al., 2003). The designation of the LEC markers and the identification of lymphatic vessels are not without pitfalls. While the expression of commonly used LEC markers (VEGFR-3, PROX1, LYVE-1, PDPN) is largely restricted to LECs unlike the expression of general endothelial markers like PECAM-1 (CD31) (Parums et al., 1990; Sawa et al., 1998) or VE-cadherin (Baluk et al., 2007; Lampugnani et al., 1992), none of them is entirely exclusive for LECs. Unequivocal identification of lymphatic vessels requires therefore a combination of markers, and guidelines have been published, e.g. for the lymphatics of the human eye (Schrödl et al., 2015; Schroedl et al., 2014). To identify not only lymphatic structures but also their subtypes such as collector, valve and capillary, marker molecules are normally used in combination. E.g. LYVE-1 and

podoplanin are frequently used LEC surface markers, which show a heterogeneous expression depending on the vessel caliber.

The list of molecules with a lymphatic connection will continue to grow over the next years. From the medical perspective, almost every molecule in that list, once sufficiently understood, represents a possibility for therapeutic intervention. A few promising interventions are ongoing at the pharmaceutical level (Eiger BioPharmaceuticals, 2016; Herantis Pharma Plc, 2016). Beyond these, the growing precision of genome editing tools might in the future open the door for the correction of hereditary lymphatic conditions.

A recent in-depth general review beyond the scope of this minireview is Vaahtomeri et al. (2017). Additionally, several review articles treat specific focus areas like the relationship of lymphatics with the cardiovascular system (Aspelund et al., 2016), the embryonic development of the lymphatic system (Koltowska et al., 2013), lymphatic tissue engineering (Schaupper et al., 2016), lymphatic diseases (Wang and Oliver, 2010), therapeutic prospects (Zheng et al., 2014), and VEGF-C (Rauniyar et al., 2018).

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Conflict of interest statements

The authors declare that they have no competing interests.

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Figure 1. Frequently encountered growth factors, receptors and co-receptors in endothelial cell biology. Both the VEGF receptors and the TIE receptors are the major signalling receptors for endothelial cells and they are for the most part specific for endothelial cells. VEGF receptors are supported by neuropilins, integrins and heparan sulfate proteoglycan (HSPG) co-receptors, which stabilize the growth factor receptor interaction and enhance signalling. The cartoon depicts the receptor domain organization, and known ligand-receptor interactions for the VEGF receptor tyrosine kinases and neuropilin co-receptors. From the integrin and HSPG co-receptor groups, not all known interacting co-receptors are included. In addition to the mammalian growth factor ligands also snake venom VEGFs (collectively known as VEGF-F) and viral VEGFs (collectively known as VEGF-E) are shown. Ligand-receptor interactions are not always conserved between orthologs of different mammalian species. The dotted arrows indicate absent interaction in some species or for some isoforms. To enhance clarity, interaction arrows are differently colored according to receptor group. The arrows of HSPG co-receptors connects them to their interacting VEGF receptors since the direct interaction of VEGF ligand and HSPG has not been shown in all cases. All VEGFs are – as well as the VEGF-C activators CCBE1 and ADAMTS3 – ill-suited as marker molecules due to their secreted nature. Note, that the short isoform of VEGFR-3 (VEGFR-3s) has been seen so far only in higher primates including humans. Modified from Wikimedia Commons.

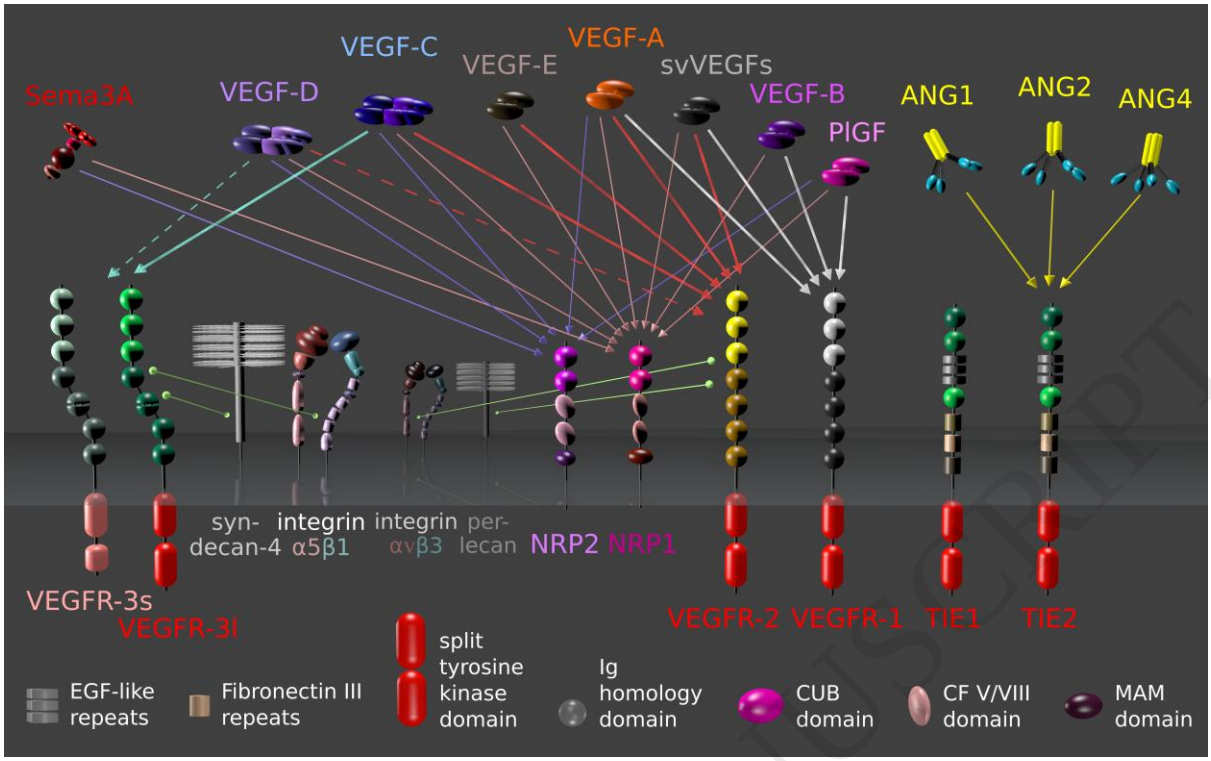

Figure 2. Key and marker molecules in the development of the lymphatic system. In mice, expression of SOX18 and COUP-TFII in the embryonic veins induces PROX1 expression at around E9.5 in a subset of venous endothelial cells. PROX1-expressing lymphatic progenitor cells are specified to become lymphatic endothelial cells. PROX1-expressing venous endothelial cells (shown in purple) emigrate upon VEGFR-3-mediated VEGF-C signals. These initial LECs (iLECs, shown in red) assemble into the earliest lymphatic structures ("lymph sacs", shown in olive), which mature and expand to form a hierarchical lymphatic vascular network with capillaries (in green) and collecting vessels (in cyan). The yellow parts of the lymphatic network are not of venous origin: for a long time, it was thought, that lymphatics develop exclusively from the venous-derived early lymphatic structures by sprouting lymphangiogenesis. However, there is substantial evidence that e.g. the mesenteric lymphatic network is assembled from differentiating, non-venous precursors (lymphvasculogenesis) (Stanczuk et al., 2015). In the skin, both sprouting and

differentiation mechanisms seem to contribute to lymphvascularization (Martinez-Corral et al., 2015). The ruler indicates the approximate starting times of the main developmental events in days of mouse embryonic development, but there are both significant spatial differences and temporal overlaps. The color labeling of the developmental events refers to the main LEC population(s) involved.

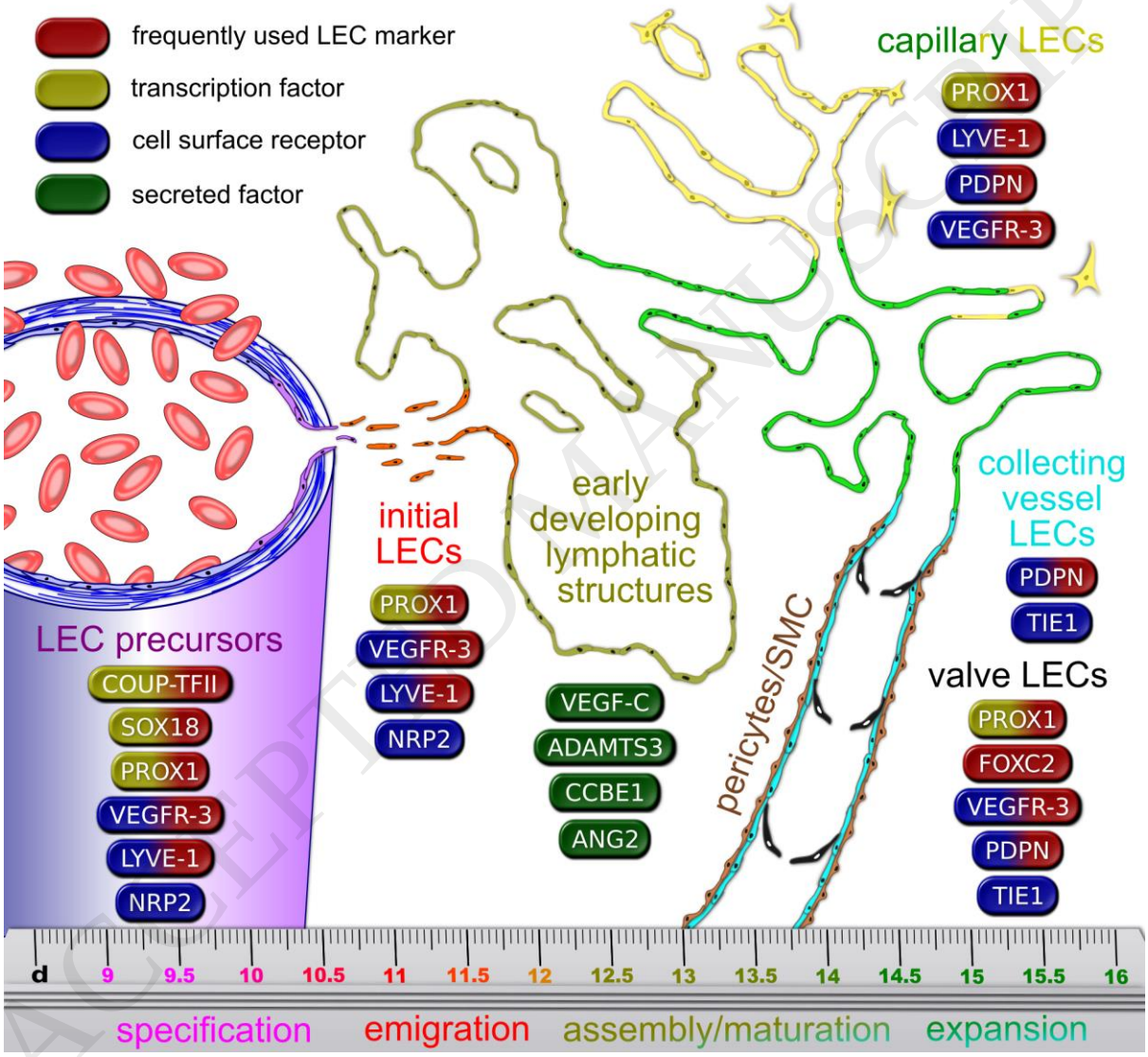

Table 1. Selected key molecules in lymphatic research and their properties. Listed from top to bottom and separated by background color are receptor tyrosine kinases, other transmembrane receptors, secreted growth factors, proteases/protease cofactors and transcription factors.

		Abbrevia-tion	aka	*Gene*	Major EC-related function	Splice isoforms	Major interacting proteins	References (recent review)	Hereditary diseases (OMIM)	KO-phenotype of the mouse gene	Commonly used antibodies	Blocking antibodies	Drugs
Receptor tyrosine kinases	Vascular endothelial growth factor	VEGFR-3	FLT4	*FLT4*	Receptor for VEGF-C, VEGF-D	Isoform 1, VEGFR-3L; isof	VEGF-C, VEGF-D, NRP2, Integrin	(Simons et al., 2016)	Hereditary lymphedema type 1A (153100),	Mice die between E10 and E12.5 due	Anti-human VEGFR-3 (mouse mon	mF4-31C1 (Pytowski et al.,	VGX-300/OPT-302, IMC-3C5

	recept or-3					orm 2, VE GF R-3S; isof orm 3, solu ble VE GF R-3/s VE GF R-3	α5β 1, Syn deca n-4		Capill ary infantil e hema ngiom a (6020 89)	to abno rmal vasc ular devel opme nt and growt h retar datio n (Dum ont et al., 1998)	oclon al, clone 9D9F 9, Jussi la et al., 1998), anti- hum an VEG FR-3 (goat polyc lonal, AF34 9, R&D Syst ems) ,	200 5)	

											anti-mouse VEGFR-3 (goat polyclonal, AF743, R&D Systems)		
	Vascular endothelial growth factor receptor-2	VEGFR-2	KDR, Flk-1, CD309	*KDR*	Receptor for VEGF-A, VEGF-C, VEGF-D	Isoform 1, mb Vegfr-2; isoform 2, sVegfr-	VEGF-A, VEGF-C, VEGF-D, VEGF-E, NRP1, Plexi		Capillary infantile hemangioma (602089)	Mice die between E8.5 and E9.5 due to reduction	Anti-human VEGFR-2 (rabbit monoclonal, #247	DC101 (Prewett et al., 1999), ScFv P1C	Axitinib, Tivozanib, Cediranib

						2; isoform 3, VEGFR2-712	n, Integrin αvβ3			in hematopoietic progenitors and impaired vasculogenesis (Shalaby et al., 1995)	9, Cell Signaling Technology), anti-human VEGR-2 (goat polyclonal, AF357, R&D Systems), anti-mouse	11 (Zhu et al., 1998), 2C3 (Brekken et al., 1998)	

										VEGFR-2 (goat polyclonal, AF644, R&D Systems)		
	Tyrosine kinase receptor with Immunoglobulin and EGF like domains 1	TIE1		*TIE1*	Orphan receptor, regulates TIE2 signaling	3 isoforms	TIE2	(Eklund et al., 2017; Saharinen et al., 2017)		Death at >= E13.5 because of hemorrhages, lymphatic vess	Anti-human TIE1 (goat polyclonal, AF619, R&D Systems)		

										el defects, edema, endothelial integrity, reduced atherosclerosis and tumor growth (D'Amico et al., 2010; Sato			

									et al., 1995)			
	Tyrosine kinase receptor with Immunoglobulin and EGF like domains 2	TIE2	TEK	*TEK*	Receptor for Ang1, Ang2, Ang4	3 isoforms	ANG1, ANG2, ANG4, TIE1, VE-PTP		Dominantly inherited venous malformations (600195), Primary congenital glaucoma-3E (617272)	Death at E9.5-E10.5, reduced myocardial growth, lymphatic vessel defects when delet	Anti-human TIE2 (goat polyclonal, AF313, R&D Systems)	Anti-human TIE2 (AF313, R&D)	

										ed at E12.5 but no effect when deleted at E7 (Dumont et al., 1994; Shen et al., 2014)			
Other transmembrane receptors	Neuropilin-1	NRP1	NP-1, NRP-1, CD304	*NRP1*	Co-receptor for VEGF-A	Isoform, mb-NRP1; isof	VEGF-A, Semaphorin 3A, PlG	(Guo and Van der Kooi,		Mice die prenatally at E10.5,	Anti-human Neuropiln-1 (goat	YW107.4.87 (Liang et al.,	

						orm 2, SNRP 1; isoform 3	F-2, VEGF$_{165}$, VEGFB$_{167}$	2015)		vascular regression in embryos (Kawasaki et al., 1999)	polyclonal, C-19, sc-7239, Santa Cruz)	2007)	
	Neuropilin-2	NRP2	NP-2, NRP-2	*NRP2*	Co-receptor for VEGF-A and VEGF-C	Isoform A22; isoform A0; isoform A17; isof	VEGF-165, VEGF-145, PLGF-2, Semaphorins 3C			Mice are viable, small lymphatic vessels and capillaries	Anti-human Neuropilin-2 (rabbit polyclonal, H-300,	Anti-Nrp2B (Caunt et al., 2008)	

						orm B0; isof orm B5; isof orm s9	and 3F			are reduc ed in size (Yua n et al., 2002)	sc-5542 , Sant a Cruz)		
	Lymp hatic vessel endot helial hyalur onic acid recept or 1	LYV E-1	Cell surfa ce reten tion sequ ence - bindi ng prote in 1 (CR SBP-1),	*LY VE 1*	Rece ptor for hyalu ronic acid			(Jac kson et al., 2001)		No obvio us phen otype (Gale et al., 2007)	Anti-mous e LYV E-1 (rabb it antis erum) (Prev o et al., 2001),	Anti-LYV E-1 (11-034, Angi oBio)	

			Extracellular link domain-containing protein 1								anti-mouse LYVE-1 (rat monoclonal, MAB2125, R&D Systems), anti LYVE-1 (rabbit polyclonal, ab14917,		

										abcam)		
	Podoplanin	PDPN	Aggrus, Glycoprotein 36 (Gp36), PA2.26 antigen, T1-alpha (T1A)	*PDPN*	Ligand for CLEC1B	Isoform 1, hT1alpha-2; isoform 2, hT1alpha-1; isoform 3;	CLEC1B	(Astarita et al., 2012; Ugorski et al., 2016)		Mice die after birth as a result of respiratory failure, reduced lymphatic transport,	Anti-human podoplanin (mouse monoclonal, clone D2-40, Covance), anti-	D2-40	

						isoform 4; isoform 5; isoform 6				lymphedema, dilation of lymphatic vasculature (Schacht et al., 2003)	mouse podoplanin (syrian hamster, monoclonal, clone 8.1.1, eBioscience™)		
Secreted growth	Vascular endothelial growth	VEGF-C	VEGF-2, VRP, Flt4 ligand	*VEGFC*	Ligand of VEGFR-3 and		VEGFR-3, VEGFR-2,	(Rauniyar et al., 2018)	Hereditary lymphedema type 1D	Mice die prenatally between	Anti-human VEGF-C (goat	VGX-100 (Vegenics,	Lymphactin, VGX-100

factors	factor-C		(Flt4-L)		VEGFR-2		ADAMTS3		(615907)	E15.5-E17.5, lack of lymphatic sprout formation from the cardinal vein, lymphedema (Karkkainen et al.,	polyclonal, AF752, R&D Systems), anti-VEGF-C #5 (Baluk et al., 2005)	Opthea Ltd, Australia)	

										2004); defect in fetal eryhtropoiesis when deleted after E7.5 (Fang et al., 2016)			
	Vascular endothelial growth	VEGF-D	FIGF	*VEGFD*	Ligand for VEGFR-2 and		VEGFR-3, VEGFR-2	(Krebs and Jeltsch, 2013		No obvious embryonic phen	Anti-human VEGF-D (VD1	VD1, VD2, VD3,	

	factor-D				VEGFR-3			; Stacker and Achen, 2018)		otype (Baldwin et al., 2005)	) (Achen, 2000); anti-human VEGF-D (goat polyclonal, AF286, R&D Systems)	VD4 (Achen et al., 2000)	
	Angiopoietin 2	ANG2	ANGPT2	*ANGPT2*	Ligand for TIE2	3 isoforms	TIE2	(Eklund et al., 2017;		Mice die 2 weeks after birth,	Anti-human Ang2 (goat polyc	MEDI3617 (Leow,	Nesvacumab, MEDI3617,

								Saharinen et al., 2017)		abnormal lymphatic modelling and postanatal angiogenesis (Dellinger et al., 2008)	lonal, AF623, R&D Systems)	2012)	Vanucizumab, RG7716
Protease	A disintegrin and metalloproteinase	ADAMTS3	Procollagen II N-proteinase	*ADAMTS3*	Activation of VEGF-C		VEGF-C, CCBE1	(Bekhouche and Colige,	Hennekam lymphangiectasia-lymphedem	Mice die prenatally, lack of lymp			

	with thrombospondin motifs 3		(PCII-NP)					2015)	a syndrome 3	hatic vasculature, edema, compromised liver development (Janssen et al., 2016)			
Cofactor	Collagen- and calcium-bindin	CCBE1		*CCBE1*	Protein cofactor for ADA	3 isoforms	ADAMTS3, Vitronectin	(Vahtomeri et al., 2017)	Hennekam lymphangiectasia-lymph	Mice die prenatally, lack of	Anti-human CCBE1 (rabb		

	g EGF domains 1				MTS 3 protease				edema syndrome 1 (HKLLS1, 235510)	lymphatic vasculature (Bos et al., 2011), defective erythropoiesis in fetal liver (Zou et al., 2013)	it polyclonal, HPA041374, Atlas antibodies)		

Transcription factors	SRY-related HMG-box 18	SOX18	HLTS, HLTRS	*SOX18*	Activates PROX1 expression in subpopulations of venous endothelial cells		Myocyte-specific enhancer factor 2C (MEF2C)	(Francois et al., 2011)	Hypotrichosis-lymphedema-telangiectasia syndrome (HLTS, 607823), Hypotrichosis-lymphedema-telangiectasia-	Mice die prenatally after E14.5., complete lack of lymphatic vasculature resulting from failure of LEC differentiat	Anti-Sox18 (rabbit polyclonal, H-140, sc-20100, Santa Cruz)		

									renal defect syndrome (HLTRS, 137940)	ion (Francois et al., 2008)			
	Chicken ovalbumin upstream promoter-transcription factor 2	COUP-TFII	Nuclear receptor subfamily 2, group F, member 2 (NR2F2), Apolipoprotein	*NR2F2*	Transcriptional regulator of NRP2 expression	3 isoforms	PROX1		Congenital heart defects, multiple types, 4 (CHTD4, 615779)	Mice die prenatally around E10. Embryos have compromised growth, edem	Anti-human COUP-TFII (mouse monoclonal, pp-H7147-00, R&D		

			A-I regulatory protein 1 (ARP-1)						a, hemorrhage (Pereira et al., 1999)	Systems)		
	Prospero homeobox protein 1	PROX 1		*PROX 1*	Specifies the lymphatic lineage in venous endothelial cells and		COUP-TFII		Mice die prenatally at E14.5, complete lack of lymphatic structures	Anti-human Prox 1 (goat polyclonal, AF2727, R&D Systems)		

					maintains lymphatic identity					(Wigle et al., 2002)			
	Forkhead box protein C2	FOXC2	Forkhead-related protein FKHL14, Transcription factor FKH-14	*FOXC2*	Controls interaction between pericytes and LECs, regulates lymphatic matura tio		NFATc1		Lymphedema-distichiasis syndrome (153400)	Mice die prenatally > E13.5, cardiovascular abnormalities, some mutants can survi	Anti-mouse FoxC2 (Sheep polyclonal, AF6989, R&D Systems); anti-human		

					n and remodelling					ve with skeletal abnormalities (Winnier et al., 1999, 1997)	FoxC2 (Sheep polyclonal, AF5044, R&D Systems)		