

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 metalloproteinase 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.

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1. 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.

2. 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 mouse embryonic day (E)9.5 (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 pre-collectors and collectors (Norrmén et al., 2009).

3. 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 (E8 to E10) 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,b). 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).

4. 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 and 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.

5. VEGF-C is the primary lymphangiogenic growth factor

VEGF-C is the primary ligand that activates VEGFR-3 (see Fig. 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.

6. 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 et al., 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*.

7. 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 (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).

8. 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 Fig. 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.

9. 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

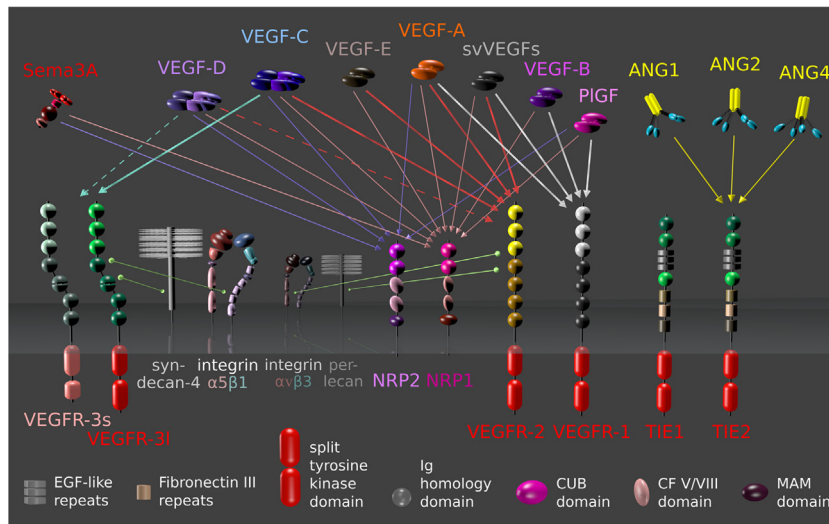


Fig. 1. Frequently encountered growth factors, receptors and co-receptors in endothelial cell biology. Both the VEGF receptors and the TIE receptors are the major signalling receptors for endothelial cells and they are for the most part specific for endothelial cells. VEGF receptors are supported by 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.

(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.

10. 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, $\alpha 5 \beta 1$ 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 $\alpha v \beta 3$ integrin (Soldi et al., 1999) and perlecan (Zoeller et al., 2009).

11. 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 Fig. 2) (Hagerling et al., 2013).

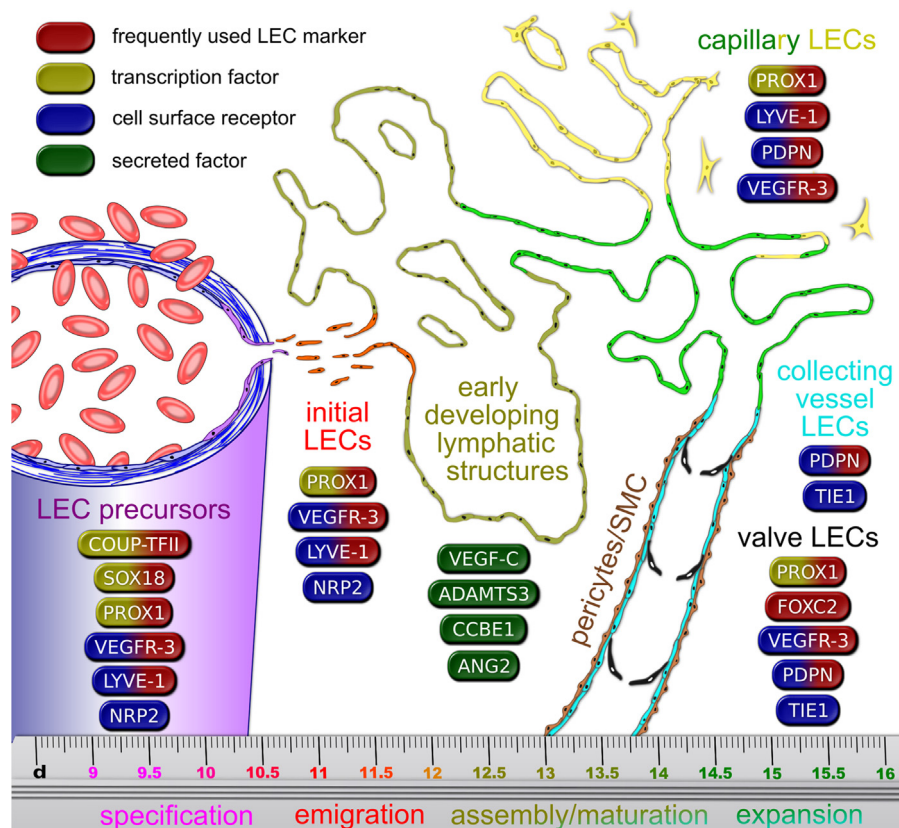


Fig. 2. Key and marker molecules in the development of the lymphatic system. In mice, expression of SOX18 and COUP-TFII in the embryonic veins induces PROX1 expression at around E9.5 in a subset of venous endothelial cells. PROX1-expressing lymphatic progenitor cells are specified to become lymphatic 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 lymphovascularization (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. (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)

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).

12. 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 Fig. 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).

13. 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., 2017a; Weber et al., 2013) and those for pathological lymphan-

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. (Achen et al., 2000; Astarita et al., 2012; Baluk et al., 2005; Bekhouche and Colige, 2015; Brekken et al., 1998; Caunt et al., 2008; Dumont et al., 1994; Gale et al., 2007; Jackson et al., 2001; Jussila et al., 1998; Kawasaki et al., 1999; Krebs and Jeltsch, 2013; Leow, 2012; Liang et al., 2007; Pereira et al., 1999; Prevo et al., 2001; Prewett et al., 1999; Pytowski et al., 2005; Saharinen et al., 2017; Sato et al., 1995; Shalaby et al., 1995; Stacker and Achen, 2018; Ugorski et al., 2016; Winnier et al., 1997; Winnier et al., 1999; Zhu et al., 1998; Zou et al., 2013)

		Abbreviation	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 receptor-3	VEGFR-3	FLT4	FLT4	Receptor for VEGF-C, VEGF-D	Isoform 1, VEGFR-3L; isoform 2, VEGFR-3S; isoform 3, soluble VEGFR-3/sVEGFR-3	VEGF-C, VEGF-D, NRP2, Integrin $\alpha 5 \beta 1$, Syndecan-4	(Simons et al., 2016)	Hereditary lymphedema type 1A (153100), Capillary infantile hemangioma (602089)	Mice die between E10 and E12.5 due to abnormal vascular development and growth retardation (Dumont et al., 1998)	Anti-human VEGFR-3 (mouse monoclonal, clone 9D9F9, Jussila et al., 1998), anti-human VEGFR-3 (goat polyclonal, AF548, R&D Systems), anti-mouse VEGFR-3 (goat polyclonal, AF743, R&D Systems)	mF43-31C1 (Pytowski et al., 2005)	VGX-300/OP1302, IMC-3C5
	Vascular endothelial growth factor receptor-2	VEGFR-2	KDR, Flk-1, CD309	KDR	Receptor for VEGF-A, VEGF-C, VEGF-D	Isoform 1, mbVegfr-2; isoform 2, sVegfr-2; isoform 3, VEGFR2-T12	VEGF-A, VEGF-C, VEGF-D, VEGF-E, NRP1, Plexin, Integrin $\alpha v \beta 3$		Capillary infantile hemangioma (602089)	Mice die between E8.5 and E9.5 due to reduction in hematopoietic progenitors and impaired vasculogenesis (Shalaby et al., 1995)	Anti-human VEGFR-2 (rabbit monoclonal, #2479, Cell Signaling Technology), anti-human VEGFR-2 (goat polyclonal, AF557, R&D Systems), anti-mouse VEGFR-2 (goat polyclonal, AF644, R&D Systems)	DC101 (Prewett et al., 1999), ScFv P1C11 (Zhu et al., 1998), 2C3 (Brekken et al., 1998)	Axitinib, Tivozanib, Cediranib
	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 $\approx$ E13.5 because of hemorrhages, lymphatic vessel defects, edema, endothelial integrity, reduced atherosclerosis and tumor growth (D'Amico et al., 2010; Sato et al., 1995)	Anti-human TIE1 (goat polyclonal, AF619, R&D Systems)		
	Tyrosine kinase receptor with immunoglobulin and EGF-like domains 2	TIE2	TEK	TEK	Receptor for Ang1, Ang2, Ang4	3 isoforms	ANG1, ANG2, ANG4, TIE1, VEG-PTP		Dominantly inherited venous malformations (600195), Primary congenital glaucoma-3E (617272)	Death at E9.5-E10.5, reduced myocardial growth, lymphatic vessel defects when deleted at E12.5 but no effect when deleted at E7 (Dumont et al., 1994; Shen et al., 2014)	Anti-human TIE2 (goat polyclonal, AF313, R&D Systems)	Anti-human TIE2 (AF313, R&D)	
Other transmembrane receptors	Neuropilin-1	NRP1	NP-1, NRP-1, CD304	NRP1	Co-receptor for VEGF-A	Isoform, mb-NRP1; isoform 2, SNRP1; isoform 3	VEGF-A, Semaphorin 3A, PGF-2/VEGF-B ₁₆₇	(Guo and Vander Kooi, 2015)		Mice die perinatally at E10.5, vascular regression in embryos (Kawasaki et al., 1999)	Anti-human Neuropilin-1 (goat polyclonal, C-19, sc-7239, Santa Cruz)	YW107.4.87 (Liang et al., 2007)	
	Neuropilin-2	NRP2	NP-2, NRP-2	NRP2	Co-receptor for VEGF-A and VEGF-C	Isoform A22; isoform A0; isoform A17; isoform B0; isoform B5; isoform B9	VEGF-A ₁₆₅ , VEGF-A ₁₈₁ , PGF-2, Semaphorin 3C and 3F			Mice are viable, small lymphatic vessels and capillaries are reduced in size (Yuan et al., 2002)	Anti-human Neuropilin-2 (rabbit polyclonal, H-300, sc-5542, Santa Cruz)	Anti-Nrp2B (Caunt et al., 2008)	
	Lymphatic vessel endothelial hyaluronate receptor 1	LYVE-1		LYVE1	Receptor for hyaluronic acid			(Jackson et al., 2001)		No obvious phenotype (Gale et al., 2007)	Anti-mouse LYVE-1 (rabbit antiserum) (Prevo et al., 2001), anti-mouse LYVE-1 (rat monoclonal, MAB2125, R&D Systems), anti-LYVE-1 (rabbit polyclonal, ab14917, abcam)	Anti-LYVE-1 (11-034, AngioBio)	
	Podoplanin	PDPN	Aggrecan, Glycoprotein 36 (GP36), PAZ 26 antigen, T1-alpha (T1A)	PDPN	Ligand for CLEC1B	Isoform 1, hT1-alpha-2; isoform 2, hT1-alpha-1; isoform 3; isoform 4; isoform 5; isoform 6	CLEC1B	(Astarita et al., 2012; Ugorski et al., 2016)		Mice die after birth as a result of respiratory failure, reduced lymphatic transport, lymphedema, dilation of lymphatic vasculature (Schacht et al., 2003)	Anti-human podoplanin (mouse monoclonal, clone D2-40, Covance), anti-mouse podoplanin (synian hamster, monoclonal, clone 8.1.1, eBioscience™)	D2-40	
Secreted growth factors	Vascular endothelial growth factor-C	VEGF-C	VEGF-2, VRP, Flk4 ligand (Flk4-L)	VEGFC	Ligand of VEGFR-3 and VEGFR-2		VEGFR-3, VEGFR-2, ADAMTS3	(Rauniyar et al., 2018)	Hereditary lymphedema type 1D (615907)	Mice die perinatally between E15.5-E17.5, lack of lymphatic sprout formation from the cardinal vein, lymphedema (Karkkainen et al., 2004), defect in fetal erythropoiesis when deleted after E7.5 (Fang et al., 2016)	Anti-human VEGF-C (goat polyclonal, AF782, R&D Systems), anti-VEGF-C #5 (Baluk et al., 2005)	VGX-100 (Vegenics, Cythera Ltd, Australia)	Lymphactin, VGX-100
	Vascular endothelial growth factor-D	VEGF-D	FIGF	VEGFD	Ligand for VEGFR-2 and VEGFR-3		VEGFR-3, VEGFR-2	(Krebs and Jeltsch, 2013; Stacker and Achen, 2018)		No obvious embryonic phenotype (Baldwin et al., 2005)	Anti-human VEGF-D (VD1) (Achen, 2000), anti-human VEGF-D (goat polyclonal, AF268, R&D Systems)	VD1, VD2, VD3, VD4 (Achen et al., 2000)	
	Angiopoietin 2	ANG2	ANGPT2	ANGPT2	Ligand for TIE2	3 isoforms	TIE2	(Eklund et al., 2017; Saharinen et al., 2017)		Mice die 2 weeks after birth, abnormal lymphatic modelling and postnatal angiogenesis (Dellinger et al., 2008)	Anti-human Ang2 (goat polyclonal, AF623, R&D Systems)	MED13617 (Leow, 2012)	Nervacumab, MED13617, Vanucizumab, RG7716
Protease	A disintegrin and metalloprotease with thrombospondin motifs 3	ADAMTS3	Procollagen II N-proteinase (PC II-NP)	ADAMTS3	Activation of VEGF-C		VEGF-C, CCBE1	(Bekhouche and Colige, 2015)	Hennekam lymphangiectasia-lymphedema syndrome 3	Mice die perinatally, lack of lymphatic vasculature, edema, compromised liver development (Janssen et al., 2016)			
Cofactor	Collagen- and calcium-binding EGF domains 1	CCBE1		CCBE1	Protein cofactor for ADAMTS3 protease	3 isoforms	ADAMTS3, Vitronectin	(Vaahomeri et al., 2017)	Hennekam lymphangiectasia-lymphedema syndrome 1 (HLLS1, 235510)	Mice die perinatally, lack of lymphatic vasculature (Bos et al., 2011), defective erythropoiesis in fetal liver (Zou et al., 2013)	Anti-human CCBE1 (rabbit 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)		Hydrothorax-lymphedema-lymphedema syndrome (HLS, 607823), Hydrothorax-lymphedema-lymphedema-renal defect syndrome (HLTRS, 137940)	Mice die perinatally after E14.5, complete lack of lymphatic vasculature resulting from failure of LEC differentiation (Francois et al., 2008)	Anti-Sox18 (rabbit polyclonal, H-140, sc-20100, Santa Cruz)		
	Chicken ovalbumin upstream promoter transcription factor 2	COUP-TFII	Nuclear receptor subfamily 2, group F, member 2 (NR2F2), Apolipoprotein A1 regulatory protein 1 (AARP-1)	NR2F2	Transcriptional regulator of NRP2 expression	3 isoforms	PROX1	(Francois et al., 2011)	Congenital heart defects, multiple types, 4 (CHTD4, 615779)	Mice die perinatally around E10. Embryos have compromised growth, edema, hemorrhage (Pereira et al., 1999)	Anti-human COUP-TFII (mouse monoclonal, pp-H7147-00, R&D Systems)		
	Prospero homeobox protein 1	PROX1		PROX1	Specifies the lymphatic lineage in venous endothelial cells and maintains lymphatic identity		COUP-TFII			Mice die perinatally at E14.5, complete lack of lymphatic structures (Wigle et al., 2002)	Anti-human Prox1 (goat polyclonal, AF2727, R&D Systems)		
	Forkhead box protein C2	FOXC2	Forkhead-related protein FKHL14, Transcription factor FKHL14	FOXC2	Controls interaction between pericytes and LECs, regulates lymphatic maturation and remodeling		NFATc1		Lymphedema-distichiasis syndrome (153400)	Mice die perinatally $>$ E13.5, cardiovascular abnormalities, some mutants can survive with skeletal abnormalities (Winnier et al., 1999, 1997)	Anti-mouse FoxC2 (Sheep polyclonal, AF6989, R&D Systems), anti-human FoxC2 (Sheep polyclonal, AF5044, R&D Systems)		

giogenesis, 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 Vaahomeri et al. (2017b). 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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References

- Achen, M.G., Jeltsch, M., Kukk, E., Mäkinen, T., Vitali, A., Wilks, A.F., Alitalo, K., 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. U. S. A.* 95, 548–553.
- Achen, M.G., Roufail, S., Domagala, T., Catimel, B., Nice, E.C., Geleick, D.M., Murphy, R., Scott, A.M., Caesar, C., Makinen, T., Alitalo, K., Stacker, S.A., 2000. Monoclonal antibodies to vascular endothelial growth factor-D block its interactions with both VEGF receptor-2 and VEGF receptor-3. *Eur. J. Biochem.* 267, 2505–2515.
- Aird, W.C., 2012. Endothelial cell heterogeneity. *Cold Spring Harb. Perspect. Med.* 2, a006429.
- Alitalo, K., 2011. The lymphatic vasculature in disease. *Nat. Med.* 17, 1371–1380.
- Alders, M., Hogan, B.M., Gjini, E., Salehi, F., Al-Gazali, L., Hennekam, E.A., Holmberg, E.E., Mannens, M.M.A.M., Mulder, M.F., Offerhaus, G.J.A., Prescott, T.E., Schroor, E.J., Verheij, J.B.G.M., Witte, M., Zwijnenburg, P.J., Vikkula, M., Schulte-Merker, S., Hennekam, R.C., 2009. Mutations in CCBE1 cause generalized lymph vessel dysplasia in humans. *Nat. Genet.* 41, 1272–1274.
- Alders, M., Mendola, A., Ades, L., Al-Gazali, L., Bellini, C., Dallapiccola, B., Ederly, P., Frank, U., Hornshuh, F., Huisman, S.A., Jagadeesh, S., Kayserili, H., Keng, W.T., Lev, D., Prada, C.E., Sampson, J.R., Schmidtke, J., Shashi, V., van Bever, Y., Van der Aa, N., Verhagen, J.M., Verheij, J.B., Vikkula, M., Hennekam, R.C., 2013. Evaluation of clinical manifestations in patients with severe lymphedema with and without CCBE1 mutations. *Mol. Syndromol.* 4, 107–113.
- Aspelund, A., Robciuc, M.R., Karaman, S., Makinen, T., Alitalo, K., 2016. Lymphatic system in cardiovascular medicine. *Circ. Res.* 118, 515–530.
- Astarita, J.L., Acton, S.E., Turley, S.J., 2012. Podoplanin: emerging functions in development, the immune system, and cancer. *Front. Immunol.* 3, 283.
- Balboa-Beltran, E., Fernández-Seara, M.J., Pérez-Muñuzuri, A., Lago, R., García-Magán, C., Couce, M.L., Sobrino, B., Amigo, J., Carracedo, A., Barros, F., 2014. A novel stop mutation in the vascular endothelial growth factor-C gene (VEGFC) results in Milroy-like disease. *J. Med. Genet.* 51, 475–478.
- Baldwin, M.E., Catimel, B., Nice, E.C., Roufail, S., Hall, N.E., Stenvers, K.L., Karkkainen, M.J., Alitalo, K., Stacker, S.A., 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., 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., 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., Ylä-Herttuala, S., Jackson, D.G., Alitalo, K., McDonald, D.M., 2005. Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *J. Clin. Invest.* 115, 247–257.
- Banerji, S., Ni, J., Wang, S.-X., Clasper, S., Su, J., Tammi, R., Jones, M., Jackson, D.G., 1999. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J. Cell Biol.* 144, 789–801.
- Batard, P., Sansilvestri, P., Scheinecker, C., Knapp, W., Debili, N., Vainchenker, W., Bühring, H.J., Monier, M.N., Kukk, E., Partanen, J., Matikainen, M.T., Alitalo, R., Hatzfeld, J., Alitalo, K., 1996. The Tie receptor tyrosine kinase is expressed by human hematopoietic progenitor cells and by a subset of megakaryocytic cells. *Blood* 87, 2212–2220.
- Bazigou, E., Lyons, O.T.A., Smith, A., Venn, G.E., Cope, C., Brown, N.A., Makinen, T., 2011. Genes regulating lymphangiogenesis control venous valve formation and maintenance in mice. *J. Clin. Invest.* 121, 2984–2992.
- Bekhouche, M., Colige, A., 2015. The procollagen N-proteinases ADAMTS 2, 3 and 14 in pathophysiology. *Matrix Biol.* 44–46, 46–53.
- Bertozzi, C.C., Schmaier, A.A., Mericko, P., Hess, P.R., Zou, Z., Chen, M., Chen, C.-Y., Xu, B., Lu, M., Zhou, D., Sebzdza, E., Santore, M.T., Merianos, D.J., Stadtfeld, M., Flake, A.W., Graf, T., Skoda, R., Maltzman, J.S., Koretzky, G.A., Kahn, M.L., 2010. Platelets regulate lymphatic vascular development through CLEC-2–SLP-76 signaling. *Blood* 116, 661–670.
- Bos, F.L., Caunt, M., Peterson-Maduro, J., Planas-Paz, L., Kowalski, J., Karpanen, T., van Impel, A., Tong, R., Ernst, J.A., Korving, J., van Es, J.H., Lammert, E., Duckers, H.J., Schulte-Merker, S., 2011. CCBE1 is essential for mammalian lymphatic vascular development and enhances the lymphangiogenic effect of vascular endothelial growth factor-C in vivo. *Circ. Res.* 109, 486–491.
- Breiteneder-Geleff, S., Soleiman, A., Kowalski, H., Horvat, R., Amann, G., Kriehuber, E., Diem, K., Weninger, W., Tschachler, E., Alitalo, K., Kerjaschki, D., 1999. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am. J. Pathol.* 154, 385–394.
- Brekken, R.A., Huang, X., King, S.W., Thorpe, P.E., 1998. Vascular endothelial growth factor as a marker of tumor endothelium. *Cancer Res.* 58, 1952–1959.
- Brouillard, P., Dupont, L., Helaers, R., Coulie, R., Tiller, G.E., Peeden, J., Colige, A., Vikkula, M., 2017. Loss of ADAMTS3 activity causes Hennekam lymphangiectasia-lymphedema syndrome 3. *Hum. Mol. Genet.* 21, 4095–4104.
- Bui, H.M., Enis, D., Robciuc, M.R., Nurmi, H.J., Cohen, J., Chen, M., Yang, Y., Dhillon, V., Johnson, K., Zhang, H., Kirkpatrick, R., Traxler, E., Anisimov, A., Alitalo, K., Kahn, M.L., 2016. Proteolytic activation defines distinct lymphangiogenic mechanisms for VEGFC and VEGFD. *J. Clin. Invest.* 126, 2167–2180.
- Carreira, C.M., Nasser, S.M., di Tomaso, E., Padera, T.P., Boucher, Y., Tomarev, S.I., Jain, R.K., 2001. LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and down-regulation in human liver cancer and cirrhosis. *Cancer Res.* 61, 8079–8084.
- Caunt, M., Mak, J., Liang, W.-C., Stawicki, S., Pan, Q., Tong, R.K., Kowalski, J., Ho, C., Reslan, H.B., Ross, J., Berry, L., Kasman, I., Zlot, C., Cheng, Z., Le Couter, J., Filvaroff, E.H., Plowman, G., Peale, F., French, D., Carano, R., Koch, A.W., Wu, Y., Watts, R.J., Tessier-Lavigne, M., Bagri, A., 2008. Blocking neuropilin-2 function inhibits tumor cell metastasis. *Cancer Cell* 13, 331–342.
- Chen, H.I., Poduri, A., Numi, H., Kivela, R., Saharinen, P., McKay, A.S., Raftery, B., Churko, J., Tian, X., Zhou, B., Wu, J.C., Alitalo, K., Red-Horse, K., 2014a. VEGF-C and aortic cardiomyocytes guide coronary artery stem development. *J. Clin. Invest.* 124, 4899–4914.
- Chen, H.I., Sharma, B., Akerberg, B.N., Numi, H.J., Kivela, R., Saharinen, P., Aghajanian, H., McKay, A.S., Bogard, P.E., Chang, A.H., Jacobs, A.H., Epstein, J.A., Stankunas, K., Alitalo, K., Red-Horse, K., 2014b. The sinus venosus contributes to coronary vasculature through VEGFC-stimulated angiogenesis. *Development* 141, 4500–4512.
- Cursiefen, C., Chen, L., Saint-Geniez, M., Hamrah, P., Jin, Y., Rashid, S., Pytowski, B., Persaud, K., Wu, Y., Streilein, J.W., Dana, R., 2006. Nonvascular VEGF receptor 3 expression by corneal epithelium maintains avascularity and vision. *Proc. Natl. Acad. Sci. U. S. A.* 103, 11405–11410.
- D'Amico, G., Korhonen, E.A., Anisimov, A., Zarkada, G., Holopainen, T., Hägerling, R., Kiefer, F., Eklund, L., Sormunen, R., Elamaa, H., Brekken, R.A., Adams, R.H., Koh, G.Y., Saharinen, P., Alitalo, K., 2014. Tie1 deletion inhibits tumor growth and improves angiopoietin antagonist therapy. *J. Clin. Invest.* 124, 824–834.
- D'Amico, G., Korhonen, E.A., Waltari, M., Saharinen, P., Laakkonen, P., Alitalo, K., 2010. Loss of endothelial tie1 receptor impairs lymphatic vessel development. *Arter. Thromb. Vasc. Biol.* 30, 207–209.
- Davydova, N., Harris, N.C., Roufail, S., Paquet-Fifield, S., Ishaq, M., Streltsov, V.A., Williams, S.P., Karnezis, T., Stacker, S.A., Achen, M.G., 2016. Differential receptor binding and regulatory mechanisms for the lymphangiogenic growth factors VEGF-C and VEGF-D. *J. Biol. Chem.* 291, 27265–27278.
- Dellinger, M., Hunter, R., Bernas, M., Gale, N., Yancopoulos, G., Erickson, R., Witte, M., 2008. Defective remodeling and maturation of the lymphatic vasculature in Angiopoietin-2 deficient mice. *Dev. Biol.* 319, 309–320.
- Dias, S., Choy, M., Alitalo, K., Rafii, S., 2002. Vascular endothelial growth factor (VEGF)-C signaling through FLT-4 (VEGFR-3) mediates leukemic cell proliferation, survival, and resistance to chemotherapy. *Blood* 99, 2179–2184.
- Dixelius, J., Mäkinen, T., Wirzenius, M., Karkkainen, M.J., Wernstedt, C., Alitalo, K., 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.
- Dongaonkar, R.M., Laine, G.A., Stewart, R.H., Quick, C.M., 2009. Balance point characterization of interstitial fluid volume regulation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 297, R6–R16.
- Dumont, D.J., Gradwohl, G., Fong, G.H., Puri, M.C., Gertsenstein, M., Auerbach, A., Breitman, M.L., 1994. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase tie, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* 8, 1897–1909.
- Dumont, D.J., Jussila, L., Taipale, J., Lymboussaki, A., Mustonen, T., Pajusola, K., Breitman, M., Alitalo, K., 1998. Cardiovascular failure in mouse embryos deficient in vegf receptor-3. *Science* 282, 946–949.
- Duong, T., Koltowska, K., Pichol-Thievend, C., Le Guen, L., Fontaine, F., Smith, K.A., Truong, V., Skoczylas, R., Stacker, S.A., Achen, M.G., Koopman, P., Hogan, B.M., Francois, M., 2014. VEGFD regulates blood vascular development by modulating SOX18 activity. *Blood* 123, 1102–1112.
- Eiger BioPharmaceuticals, 2016. Ubenimex in adult patients with lymphedema of the lower limb (ULTRA). Website: <http://www.eigerbio.com/lymphedema>. (Retrieved 2 February 2018).
- Eklund, L., Kangas, J., Saharinen, P., 2017. Angiopoietin–Tie signalling in the cardiovascular and lymphatic systems. *Clin. Sci. (Lond.)* 131, 87–103.
- Fang, S., Nurmi, H., Heinolainen, K., Chen, S., Salminen, E., Saharinen, P., Mikkola, H.K.A., Alitalo, K., 2016. Critical requirement of VEGF-C in transition to fetal erythropoiesis. *Blood* 128, 710–720.
- Fernandes, R.J., Hirohata, S., Engle, J.M., Colige, A., Cohn, D.H., Eyre, D.R., Apte, S.S., 2001. Procollagen II amino propeptide processing by ADAMTS-3: insights on dermatosparaxis. *J. Biol. Chem.* 276, 31502–31509.
- Francois, M., Caprini, A., Hosking, B., Orsenigo, F., Wilhelm, D., Browne, C., Paavonen, K., Karnezis, T., Shayan, R., Downes, M., Davidson, T., Tutt, D., Cheah, K.S.E., Stacker, S.A., Muscat, G.E.O., Achen, M.G., Dejana, E., Koopman, P., 2008. Sox18 induces development of the lymphatic vasculature in mice. *Nature* 456, 643–647.
- Francois, M., Harvey, N.L., Hogan, B.M., 2011. The transcriptional control of lymphatic vascular development. *Physiology* 26, 146–155.
- Fukuhara, S., Sako, K., Minami, T., Noda, K., Kim, H.Z., Kodama, T., Shibuya, M., Takakura, N., Koh, G.Y., Mochizuki, N., 2008. Differential function of Tie2 at

- cell–cell contacts and cell–substratum contacts regulated by angiopoietin-1. *Nat. Cell Biol.* 10, 513–526.
- Gale, N.W., Prevost, R., Espinosa, J., Ferguson, D.J., Dominguez, M.G., Yancopoulos, G.D., Thurston, G., Jackson, D.G., 2007. Normal lymphatic development and function in mice deficient for the lymphatic hyaluronan receptor LYVE-1. *Mol. Cell. Biol.* 27, 595–604.
- Galvagni, F., Pennacchini, S., Salameh, A., Rocchigiani, M., Neri, F., Orlandini, M., Petraglia, F., Gotta, S., Sardone, G.L., Matteucci, G., Terstappen, G.C., Oliviero, S., 2010. Endothelial cell adhesion to the extracellular matrix induces c-Src-dependent VEGFR-3 phosphorylation without the activation of the receptor intrinsic kinase activity. *Circ. Res.* 106, 1839–1848.
- Gordon, K., Schulte, D., Brice, G., Simpson, M.A., Roukens, M.G., van Impel, A., Connell, F., Kalidas, K., Jeffery, S., Mortimer, P.S., Mansour, S., Schulte-Merker, S., Ostergaard, P., 2013. Mutation in vascular endothelial growth factor-C, a ligand for vascular endothelial growth factor receptor-3, is associated with autosomal dominant milroy-like primary lymphedema. *Circ. Res.* 112, 956–960.
- Guo, H.-F., Vander Kooi, C.W., 2015. Neuropilin functions as an essential cell surface receptor. *J. Biol. Chem.* 290, 29120–29126.
- Hagerling, R., Pollmann, C., Andreas, M., Schmidt, C., Nurmi, H., Adams, R.H., Alitalo, K., Andresen, V., Schulte-Merker, S., Kiefer, F., 2013. A novel multistep mechanism for initial lymphangiogenesis in mouse embryos based on ultramicroscopy. *EMBO J.* 32, 629–644.
- Haiko, P., Makinen, T., Keskkitalo, S., Taipale, J., Karkkainen, M.J., Baldwin, M.E., Stacker, S.A., Achen, M.G., Alitalo, K., 2008. Deletion of vascular endothelial growth factor C (VEGF-C) and VEGF-D is not equivalent to VEGF receptor 3 deletion in mouse embryos. *Mol. Cell. Biol.* 28, 4843–4850.
- Hamada, K., Oike, Y., Takakura, N., Ito, Y., Jussila, L., Dumont, D.J., Alitalo, K., Suda, T., 2000. VEGF-C signaling pathways through VEGFR-2 and VEGFR-3 in vasculogenesis and hematopoiesis. *Blood* 96, 3793–3800.
- Hamrah, P., Chen, L., Cursiefen, C., Zhang, Q., Joyce, N.C., Dana, M.R., 2004. Expression of vascular endothelial growth factor receptor-3 (VEGFR-3) on monocytic bone marrow-derived cells in the conjunctiva. *Exp. Eye Res.* 79, 553–561.
- Hamrah, P., Chen, L., Zhang, Q., 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.
- Han, J., Calvo, C.-F., Kang, T.H., Baker, K.L., Park, J.-H., Parras, C., Levitt, M., Birba, U., Pibouin-Fragner, L., Fragner, P., Bilguvar, R., Duman, R.S., Nurmi, H., Alitalo, K., Eichmann, A.C., Thomas, J.-L., 2015. Vascular endothelial growth factor receptor 3 controls neural stem cell activation in mice and humans. *Cell Rep.* 10, 1158–1172.
- Harvey, N.L., Gordon, E.J., 2012. Deciphering the roles of macrophages in developmental and inflammation stimulated lymphangiogenesis. *Vasc. Cell.* 4, 15.
- Herantis Pharma Plc, 2016. A phase I study with Lymfactivin® in the treatment of patients with secondary lymphedema. Website: <http://herantis.com/pipeline/lymfactivin-for-lymphedema>. (Retrieved 2 February 2018).
- Herzog, Y., Kalchauer, C., Kahane, N., Reshef, R., Neufeld, G., 2001. Differential expression of neuropilin-1 and neuropilin-2 in arteries and veins. *Mech. Dev.* 109, 115–119.
- Hirakawa, S., Hong, Y.-K., Harvey, N., Schacht, V., Matsuda, K., Libermann, T., Detmar, M., 2003. Identification of vascular lineage-specific genes by transcriptional profiling of isolated blood vascular and lymphatic endothelial cells. *Am. J. Pathol.* 162, 575–586.
- Hiratsuka, S., Minowa, O., Kuno, J., Noda, T., Shibuya, M., 1998. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl. Acad. Sci. U. S. A.* 95, 9349–9354.
- Hong, Y.-K., Harvey, N., Noh, Y.-H., Schacht, V., Hirakawa, S., Detmar, M., Oliver, G., 2002. Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev. Dyn.* 225, 351–357.
- Hughes, D.C., 2001. Alternative splicing of the human VEGFR-3/FLT4 gene as a consequence of an integrated human endogenous retrovirus. *J. Mol. Evol.* 53, 77–79.
- Iqbal, J., Hussain, M.M., 2009. Intestinal lipid absorption. *Am. J. Physiol. Endocrinol. Metab.* 296, E1183–E1194.
- Jackson, D.G., Prevost, R., Clasper, S., Banerji, S., 2001. LYVE-1, the lymphatic system and tumor lymphangiogenesis. *Trends Immunol.* 22, 317–321.
- Janssen, L., Dupont, L., Bekhouche, M., Noel, A., Leduc, C., Voz, M., Peers, B., Cataldo, D., Apte, S.S., Dubail, J., Colige, A., 2015. ADAMTS3 activity is mandatory for embryonic lymphangiogenesis and regulates placental angiogenesis. *Angiogenesis* 19, 53–65.
- Jeltsch, M., Jha, S.K., Tvorogov, D., Anisimov, A., Leppanen, V.-M., Holopainen, T., Kivela, R., Ortega, S., Karpanen, T., Alitalo, K., 2014. CCBE1 enhances lymphangiogenesis via a disintegrin and metalloprotease with thrombospondin motifs-3-mediated vascular endothelial growth factor-C activation. *Circulation* 129, 1962–1971.
- Jeltsch, M., Kaipainen, A., Joukov, V., Meng, X., Lakso, M., Rauvala, H., Swartz, M., Fukumura, D., Jain, R.K., Alitalo, K., 1997. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* 276, 1423–1425.
- Jeltsch, M., Tammela, T., Alitalo, K., Wilting, J., 2003. Genesis and pathogenesis of lymphatic vessels. *Cell Tissue Res.* 314, 69–84.
- Jha, S.K., Rauniyar, K., Karpanen, T., Leppanen, V.-M., Brouillard, P., Vikkula, M., Alitalo, K., Jeltsch, M., 2017. Efficient activation of the lymphangiogenic growth factor VEGF-C requires the C-terminal domain of VEGF-C and the N-terminal domain of CCBE1. *Sci. Rep.* 7, 4916.
- Johns, S.C., Yin, X., Jeltsch, M., Bishop, J.R., Schuksz, M., El Ghazal, R., Wilcox-Adelman, S.A., Alitalo, K., Fuster, M.M., 2016. Functional importance of a proteoglycan coreceptor in pathologic lymphangiogenesis. *Circ. Res.* 119, 210–221.
- Johnson, L.A., Banerji, S., Lawrence, W., Gileadi, U., Prota, G., Holder, K.A., Roshorm, Y.M., Hanke, T., Cerundolo, V., Gale, N.W., Jackson, D.G., 2017. Dendritic cells enter lymph vessels by hyaluronan-mediated docking to the endothelial receptor LYVE-1. *Nat. Immunol.* 18, 762–770.
- Johnson, N.C., Dillard, M.E., Baluk, P., McDonald, D.M., Harvey, N.L., Frase, S.L., Oliver, G., 2008. Lymphatic endothelial cell identity is reversible and its maintenance requires Prox1 activity. *Genes Dev.* 22, 3282–3291.
- Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N., 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.
- Jussila, L., Valtola, R., Partanen, T.A., Salven, P., Heikkilä, P., Matikainen, M.T., Renkonen, R., Kaipainen, A., Detmar, M., Tschachler, E., Alitalo, R., Alitalo, K., 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.
- Kaipainen, A., Korhonen, J., Mustonen, T., van Hinsbergh, V.W., Fang, G.H., Dumont, D., Breitman, M., Alitalo, K., 1995. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc. Natl. Acad. Sci. U. S. A.* 92, 3566–3570.
- Karkkainen, M.J., Ferrell, R.E., Lawrence, E.C., Kimak, M.A., Levinson, K.L., McTigue, M.A., Alitalo, K., Finegold, D.N., 2000. Missense mutations interfere with VEGFR-3 signaling in primary lymphoedema. *Nat. Genet.* 25, 153–159.
- Karkkainen, M.J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T.V., Jeltsch, M., Jackson, D.G., Talikka, M., Rauvala, H., Betscholtz, C., Alitalo, K., 2004. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat. Immunol.* 5, 74–80.
- Karkkainen, M.J., Saari, A., Jussila, L., Karila, K.A., Lawrence, E.C., Pajusola, K., Bueler, H., Eichmann, A., Kauppinen, R., Kettunen, M.I., Ylä-Herttua, S., Finegold, D.N., Ferrell, R.E., Alitalo, K., 2001. A model for gene therapy of human hereditary lymphoedema. *Proc. Natl. Acad. Sci. U. S. A.* 98, 12677–12682.
- Karpanen, T., Egeblad, M., Karkkainen, M.J., Kubo, H., Ylä-Herttua, S., Jäättelä, M., Alitalo, K., 2001. Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res.* 61, 1786–1790.
- Karpanen, T., Heckman, C.A., Keskkitalo, S., Jeltsch, M., Ollila, H., Neufeld, G., Tamagnone, L., Alitalo, K., 2006. Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. *FASEB J.* 20, 1462–1472.
- Kawamura, H., Li, X., Goishi, K., van Meeteren, L.A., Jakobsson, L., Cebe-Suarez, S., Shimizu, A., Edholm, D., Ballmer-Hofer, K., Kjellen, L., Klagsbrun, M., Claesson-Welsh, L., 2008. Neuropilin-1 in regulation of VEGF-induced activation of p38MAPK and endothelial cell organization. *Blood* 112, 3638–3649.
- Kawasaki, T., Kitsuoka, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T., Fujisawa, H., 1999. A requirement for neuropilin-1 in embryonic vessel formation. *Development* 126, 4895–4902.
- Klotz, L., Norman, S., Vieira, J.M., Masters, M., Rohling, M., Dubé, K.N., Bollini, S., Matsuzaki, F., Carr, C.A., Riley, P.R., 2015. Cardiac lymphatics are heterogeneous in origin and respond to injury. *Nature* 522, 62–67.
- Koltowska, K., Betterman, K.L., Harvey, N.L., Hogan, B.M., 2013. Getting out and about: the emergence and morphogenesis of the vertebrate lymphatic vasculature. *Development* 140, 1857–1870.
- Krebs, R., Jeltsch, M., 2013. The lymphangiogenic growth factors VEGF-C and VEGF-D part 2: the role of lymphangiogenic growth factors VEGF-C and VEGF-D in lymphatic disorders. *Lymphforsch* 17, 96–104.
- Kukk, E., Lymboussaki, A., Taira, S., Kaipainen, A., Jeltsch, M., Joukov, V., Alitalo, K., 1996. VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vasculature. *Development* 122, 3829–3837.
- Kwon, H.-B., Fukuhara, S., Asakawa, K., Ando, K., Kashiwada, T., Kawakami, K., Hibi, M., Kwon, Y.-G., Kim, K.-W., Alitalo, K., Mochizuki, N., 2013. The parallel growth of motoneuron axons with the dorsal aorta depends on Vegfc/Vegfr3 signaling in zebrafish. *Development* 140, 4081–4090.
- Lampugnani, M.G., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L.P., Dejana, E., 1992. A novel endothelial-specific membrane protein is a marker of cell–cell contacts. *J. Cell Biol.* 118, 1511–1522.
- Le Bras, B., Barallobre, M.-J., Homman-Ludie, J., Ny, A., Wyns, S., Tammela, T., Haiko, P., Karkkainen, M.J., Yuan, L., Muriel, M.-P., Chatzopoulou, E., Bréant, C., Zalc, B., Carmeliet, P., Alitalo, K., Eichmann, A., Thomas, J.-L., 2006. VEGF-C is a trophic factor for neural progenitors in the vertebrate embryonic brain. *Nat. Neurosci.* 9, 340–348.
- Le Guen, L., Karpanen, T., Schulte, D., Harris, N.C., Koltowska, K., Roukens, G., Bower, N.I., van Impel, A., Stacker, S.A., Achen, M.G., Schulte-Merker, S., Hogan, B.M., 2014. Ccbe1 regulates Vegfc-mediated induction of Vegfr3 signaling during embryonic lymphangiogenesis. *Development* 141, 1239–1249.
- Leppanen, V.-M., Jeltsch, M., Anisimov, A., Tvorogov, D., Aho, K., Kalkkinen, N., Toivanen, P., Ylä-Herttua, S., Ballmer-Hofer, K., Alitalo, K., 2011. Structural determinants of vascular endothelial growth factor-D receptor binding and specificity. *Blood* 117, 1507–1515.
- Leow, C., 2012. MEDI3617, a human anti-Angiopoietin 2 monoclonal antibody, inhibits angiogenesis and tumor growth in human tumor xenograft models. *Int. J. Oncol.* 40, 1321–1330.
- Liang, W.-C., Dennis, M.S., Stawicki, S., Chanthery, Y., Pan, Q., Chen, Y., Eigenbrot, C., Yin, J., Koch, A.W., Wu, X., Ferrara, N., Bagri, A., Tessier-Lavigne, M., Watts, R.J., Wu, Y., 2007. Function blocking antibodies to neuropilin-1 generated from a designed human synthetic antibody phage library. *J. Mol. Biol.* 366, 815–829.
- Lin, F.-J., Chen, X., Qin, J., Hong, Y.-K., Tsai, M.-J., Tsai, S.Y., 2010. Direct transcriptional regulation of neuropilin-2 by COUP-TFII modulates multiple steps in murine lymphatic vessel development. *J. Clin. Invest.* 120, 1694–1707.

- Lonza, 2017. Endothelial cell growth medium. Website: <https://www.lonza.com/products-services/bio-research/primary-cells/human-cells-and-media/endothelial-cells-and-media/endothelial-cell-growth-media-kits.aspx>. (Retrieved 28 December 2017).
- Lutter, S., Xie, S., Tatin, F., Makinen, T., 2012. Smooth muscle–endothelial cell communication activates reelin signaling and regulates lymphatic vessel formation. *J. Cell Biol.* 197, 837–849.
- Machnik, A., Neuhofer, W., Jantsch, J., Dahlmann, A., Tammela, T., Machura, K., Park, J.-K., Beck, F.-X., Müller, D.N., Derer, W., Goss, J., Ziomber, A., Dietsch, P., Wagner, H., van Rooijen, N., Kurtz, A., Hilgers, K.F., Alitalo, K., Eckardt, K.-U., Luft, F.C., Kerjaschki, D., Titze, J., 2009. Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism. *Nat. Med.* 15, 545–552.
- Mandriota, S.J., Jussila, L., Jeltsch, M., Compagni, A., Baetens, D., Prevo, R., Banerji, S., Huarte, J., Montesano, R., Jackson, D.G., Orci, L., Alitalo, K., Christofori, G., Pepper, M.S., 2001. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J.* 20, 672–682.
- Martinez-Corral, I., Ulvmar, M.H., Stanczuk, L., Tatin, F., Kizhatil, K., John, S.W.M., Alitalo, K., Ortega, S., Makinen, T., 2015. Nonvenous origin of dermal lymphatic vasculature. *Circ. Res.* 116, 1649–1654.
- Matsui, K., Breitender-Gelleff, S., Soleiman, A., Kowalski, H., Kerjaschki, D., 1999. Podoplanin, a novel 43-kDa membrane protein, controls the shape of podocytes. *Nephrol. Dial. Transplant.* 14, 9–11.
- McColl, B.K., Baldwin, M.E., Roufail, S., Freeman, C., Moritz, R.L., Simpson, R.J., Alitalo, K., Stacker, S.A., Achen, M.G., 2003. Plasmin activates the lymphangiogenic growth factors VEGF-C and VEGF-D. *J. Exp. Med.* 198, 863–868.
- 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., Rogers, P.A.W., Alitalo, K., Stacker, S.A., Achen, M.G., 2007. Proprotein convertases promote processing of VEGF-D, a critical step for binding the angiogenic receptor VEGFR-2. *FASEB J.* 21, 1088–1098.
- Miteva, D.O., Rutkowski, J.M., Dixon, J.B., Kilarski, W., Shields, J.D., Swartz, M.A., 2010. Transmural flow modulates cell and fluid transport functions of lymphatic endothelium. *Circ. Res.* 106, 920–931.
- 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., Dvorak, A.M., Dvorak, H.F., 2002. Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J. Exp. Med.* 196, 1497–1506.
- Nelson, G.M., Padera, T.P., Garkavtsev, I., Shioda, T., Jain, R.K., 2007. Differential gene expression of primary cultured lymphatic and blood vascular endothelial cells. *Neoplasia* 9, 1038–1045.
- Nilsson, I., Bahram, F., Li, X., Gualandi, L., Koch, S., Jarvius, M., Söderberg, O., Anisimov, A., Kholová, I., Pytowski, B., Baldwin, M., Ylä-Herttuala, S., Alitalo, K., Kreuger, J., Claesson-Welsh, L., 2010. VEGF receptor 2/-3 heterodimers detected in situ by proximity ligation on angiogenic sprouts. *EMBO J.* 29, 1377–1388.
- Norrmén, C., Ivanov, K.I., Cheng, J., Zangger, N., Delorenzi, M., Jaquet, M., Miura, N., Puolakkainen, P., Horsley, V., Hu, J., Augustin, H.G., Ylä-Herttuala, S., Alitalo, K., Petrova, T.V., 2009. FOXC2 controls formation and maturation of lymphatic collecting vessels through cooperation with NFATc1. *J. Cell Biol.* 185, 439–457.
- Oh, S.-J., Jeltsch, M.M., Birkenhäger, R., McCarthy, J.E.G., Weich, H.A., Christ, B., Alitalo, K., Wiltgen, J., 1997. VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. *Dev. Biol.* 188, 96–109.
- Orlandini, M., Marconcin, L., Ferruzzi, R., Oliviero, S., 1996. Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. *Proc. Natl. Acad. Sci. U. S. A.* 93, 11675–11680.
- Paquet-Fifield, S., Levy, S.M., Sato, T., Shayan, R., Karnezis, T., Davydova, N., Nowell, C.J., Roufail, S., Ma, G.Z.-M., Zhang, Y.-F., Stacker, S.A., Achen, M.G., 2013. Vascular endothelial growth factor-D modulates caliber and function of initial lymphatics in the dermis. *J. Invest. Dermatol.* 133, 2074–2084.
- Partanen, T.A., Arola, J., Saarioto, A., Jussila, L., Ora, A., Miettinen, M., Stacker, S.A., Achen, M.G., 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.
- Parums, D.V., Cordell, J.L., Mickle, K., Heryet, A.R., Gatter, K.C., Mason, D.Y., 1990. JC70: a new monoclonal antibody that detects vascular endothelium associated antigen on routinely processed tissue sections. *J. Clin. Pathol.* 43, 752–757.
- Pereira, F.A., Qiu, Y., Zhou, G., Tsai, M.J., Tsai, S.Y., 1999. The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. *Genes Dev.* 13, 1037–1049.
- Petrova, T.V., Bono, P., Holnthoner, W., Chesnes, J., Pytowski, B., Sihto, H., Laakkonen, P., Heikkilä, P., Joensuu, H., Alitalo, K., 2008. VEGFR-3 expression is restricted to blood and lymphatic vessels in solid tumors. *Cancer Cell* 13, 554–556.
- Petrova, T.V., Karpanen, T., Norrmén, C., Mellor, R., Tamakoshi, T., Finegold, D., Ferrell, R., Kerjaschki, D., Mortimer, P., Ylä-Herttuala, S., Miura, N., Alitalo, K., 2004. Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasis. *Nat. Med.* 10, 974–981.
- Petrova, T.V., Mäkinen, T., Mäkelä, T.P., Saarela, J., Virtanen, I., Ferrell, R.E., Finegold, D.N., Kerjaschki, D., Ylä-Herttuala, S., Alitalo, K., 2002. Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J.* 21, 4593–4599.
- Planas-Paz, L., Strilić, B., Goedecke, A., Breier, G., Fässler, R., Lammert, E., 2012. Mechanoinduction of lymph vessel expansion. *EMBO J.* 31, 788–804.
- Prevo, R., Banerji, S., Ferguson, D.J., Clasper, S., Jackson, D.G., 2001. Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. *J. Biol. Chem.* 276, 19420–19430.
- Prewett, M., Huber, J., Li, Y., Santiago, A., O'Connor, W., King, K., Overholser, J., Hooper, A., Pytowski, B., Witte, L., Bohlen, P., Hicklin, D.J., 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.
- PromoCell, 2017. Endothelial cell medium MV2. Website: <https://www.promocell.com/products/cell-culture-media/media-for-primary-cells/endothelial-cell-media-mv>. Retrieved (28 December 2017).
- Pytowski, B., Goldman, J., Persaud, K., Wu, Y., Witte, L., Hicklin, D.J., Skobe, M., Boardman, K.C., 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.
- Qu, X., Zhou, B., Baldwin, H.S., 2015. Tie1 is required for lymphatic valve and collecting vessel development. *Dev. Biol.* 399, 117–128.
- Randolph, G.J., Angeli, V., Swartz, M.A., 2005. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat. Rev. Immunol.* 5, 617–628.
- Rauniyar, K., Jha, S.K., Jeltsch, M., 2018. Biology of vascular endothelial growth factor C in the morphogenesis of lymphatic vessels. *Front. Bioeng. Biotechnol.* 6.
- Rissanen, T.T., Markkanen, J.E., Gruchala, M., Heikura, T., Puranen, A., Kettunen, M.I., Kholová, I., Kauppinen, R.A., Achen, M.G., Stacker, S.A., Alitalo, K., Ylä-Herttuala, S., 2003. VEGF-D is the strongest angiogenic and lymphangiogenic effector among vegfs delivered into skeletal muscle via adenoviruses. *Circ. Res.* 92, 1098–1106.
- Rodriguez-Niedenführ, M., Papoutsis, M., Christ, B., Nicolaides, K.H., von Kaisenberg, C.S., Tomarev, S.I., Wiltgen, J., 2001. Prox1 is a marker of ectodermal placodes, endodermal compartments, lymphatic endothelium and lymphangioblasts. *Anat. Embryol.* 204, 399–406.
- Russo, E., Teixeira, A., Vahtomeri, K., Willrodt, A.-H., Bloch, J.S., Nitschké, M., Santambrogio, L., Kerjaschki, D., Sixt, M., Halin, C., 2016. Intralymphatic CCL21 promotes tissue egress of dendritic cells through afferent lymphatic vessels. *Cell Rep.* 14, 1723–1734.
- Saharinen, P., Eklund, L., Alitalo, K., 2017. Therapeutic targeting of the angiopoietin-TIE pathway. *Nat. Rev. Drug Discov.* 16, 635–661.
- Saharinen, P., Eklund, L., Miettinen, J., Wirkkala, R., Anisimov, A., Winderlich, M., Nottebaum, A., Vestweber, D., Deutsch, U., Koh, G.Y., Olsen, B.R., Alitalo, K., 2008. Angiopoietins assemble distinct Tie2 signaling complexes in endothelial cell–cell and cell–matrix contacts. *Nat. Cell Biol.* 10, 527–537.
- Sato, T.N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., Qin, Y., 1995. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376, 70–74.
- Sawa, Y., Yoshida, S., Ashikaga, Y., Kim, T., Yamaoka, Y., Shiroto, H., 1998. Lymphatic endothelium expresses PECAM-1. *Tissue Cell* 30, 377–382.
- Schacht, V., Ramirez, M.I., Hong, Y.-K., Hirakawa, S., Feng, D., Harvey, N., Williams, M., Dvorak, A.M., Dvorak, H.F., Oliver, G., Detmar, M., 2003. T1α/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *EMBO J.* 22, 3546–3556.
- Schapper, M., Jeltsch, M., Rohringer, S., Redl, H., Holnthoner, W., 2016. Lymphatic vessels in regenerative medicine and tissue engineering. *Tissue Eng. B: Rev.* 22, 395–407.
- Schledzewski, K., Falkowski, M., Moldenhauer, G., Metharom, P., Kzhyshkowska, J., Ganss, R., Demory, A., Falkowska-Hansen, B., Kurzen, H., Ugurel, S., Geginat, G., Arnold, B., Goerdt, S., 2006. Lymphatic endothelium-specific hyaluronan receptor LYVE-1 is expressed by stabilin-1+, F4/80+, CD11b+ macrophages in malignant tumours and wound healing tissue in vivo and in bone marrow cultures in vitro: implications for the assessment of lymphangiogenesis. *J. Pathol.* 209, 67–77.
- Schoppmann, S.F., Birner, P., Stöckl, J., Kalt, R., Ullrich, R., Caucig, C., Kriehuber, E., Nagy, K., Alitalo, K., Kerjaschki, D., 2002. Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *Am. J. Pathol.* 161, 947–956.
- Schrödl, F., Kaser-Eichberger, A., Trost, A., Strohmaier, C., Bogner, B., Runge, C., Motloch, K., Bruckner, D., Laimer, M., Heindl, L.M., Reitsamer, H.A., 2015. Lymphatic markers in the adult human choroid. *Investig. Ophthalmol. Vis. Sci.* 56, 7406.
- Schrodl, F., Kaser-Eichberger, A., Schlereth, S.L., Bock, F., Regenfuss, B., Reitsamer, H.A., Luty, G.A., Maruyama, K., Chen, L., Lütjen-Drecoll, E., Dana, R., Kerjaschki, D., Alitalo, K., De Stefano, M.E., Junghans, B.M., Heindl, L.M., Cursiefen, C., 2014. Consensus statement on the immunohistochemical detection of ocular lymphatic vessels. *Investig. Ophthalmol. Vis. Sci.* 55, 6440.
- Schwarz, Q., Ruhrberg, C., 2010. Neupilin, you gotta let me know: should I stay or should I go? *Cell Adh. Migr.* 4, 61–66.
- Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.-F., Breitman, M.L., Schuh, A.C., 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376, 62–66.
- Shen, B., Shang, Z., Wang, B., Zhang, L., Zhou, F., Li, T., Chu, M., Jiang, H., Wang, Y., Qiao, T., Zhang, J., Sun, W., Kong, X., He, Y., 2014. Genetic dissection of Tie pathway in mouse lymphatic maturation and valve development. *Arter. Thromb. Vasc. Biol.* 34, 1221–1230.
- Siegfried, G., Basak, A., Cromlish, J.A., Benjannet, S., Marcinkiewicz, J., Chrétien, M., Seidah, N.G., Khatib, A.-M., 2003. The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. *J. Clin. Invest.* 111, 1723–1732.
- Simons, M., Gordon, E., Claesson-Welsh, L., 2016. Mechanisms and regulation of endothelial VEGF receptor signaling. *Nat. Rev. Mol. Cell Biol.* 17, 611–625.
- Skobe, M., Detmar, M., 2000. Structure, function, and molecular control of the skin lymphatic system. *J. Invest. Dermatol. Symp. Proc.* 5, 14–19.

- Skobe, M., Detmar, M., Brown, L.F., Tognazzi, K., Ganju, R.K., Dezube, B.J., Alitalo, K., 1999. Vascular endothelial growth factor-C (VEGF-C) and its receptors KDR and Flt-4 are expressed in AIDS-associated Kaposi's sarcoma. *J. Invest. Dermatol.* 113, 1047–1053.
- Skobe, M., Hawighorst, T., Jackson, D.G., Prevo, R., Janes, L., Velasco, P., Riccardi, L., Alitalo, K., Claffey, K., Detmar, M., 2001. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat. Med.* 7, 192–198.
- Smith, N.R., Baker, D., James, N.H., Ratcliffe, K., Jenkins, M., Ashton, S.E., Sproat, G., Swann, R., Gray, N., Ryan, A., Jurgensmeier, J.M., Womack, C., 2010. Vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are localized primarily to the vasculature in human primary solid cancers. *Clin. Cancer Res.* 16, 3548–3561.
- Soldi, R., Mitola, S., Strasly, M., Defilippi, P., Tarone, G., Bussolino, F., 1999. Role of alphavbeta3 integrin in the activation of vascular endothelial growth factor receptor-2. *EMBO J.* 18, 882–892.
- Song, M., Yang, H., Yao, S., Ma, F., Li, Z., Deng, Y., Deng, H., Zhou, Q., Lin, S., Wei, Y., 2007. A critical role of vascular endothelial growth factor D in zebrafish embryonic vasculogenesis and angiogenesis. *Biochem. Biophys. Res. Commun.* 357, 924–930.
- Srinivasan, R.S., Dillard, M.E., Lagutin, O.V., Lin, F.-J., Tsai, S., Tsai, M.-J., Samokhvalov, I.M., Oliver, G., 2007. Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. *Genes Dev.* 21, 2422–2432.
- Stacker, S., Achen, M., 2018. Emerging roles for VEGF-D in human disease. *Biomolecules* 8, 1.
- Stacker, S.A., Stenvers, K., Caesar, C., Vitali, A., Domagala, T., Nice, E., Roufail, S., Simpson, R.J., Moritz, K., Karpanen, T., Alitalo, K., Achen, M.G., 1999. Biosynthesis of vascular endothelial growth factor-D involves proteolytic processing which generates non-covalent homodimers. *J. Biol. Chem.* 274, 32127–32136.
- Stanczuk, L., Martinez-Corral, I., Ulvmar, M.H., Zhang, Y., Laviña, B., Fruttiger, M., Adams, R.H., Saur, D., Betsholtz, C., Ortega, S., Alitalo, K., Graupera, M., Mäkinen, T., 2015. cKit lineage hemogenic endothelium-derived cells contribute to mesenteric lymphatic vessels. *Cell Rep.* 10, 1708–1721.
- Su, J.-L., Yang, P.-C., Shih, J.-Y., Yang, C.-Y., Wei, L.-H., Hsieh, C.-Y., Chou, C.-H., Jeng, Y.-M., Wang, M.-Y., Chang, K.-J., Hung, M.-C., Kuo, M.-L., 2006. The VEGF-C/Flt-4 axis promotes invasion and metastasis of cancer cells. *Cancer Cell* 9, 209–223.
- Tammela, T., Zarkada, G., Wallgard, E., Murtomäki, A., 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., Shibuya, M., Pytowski, B., Eichmann, A., Betsholtz, C., Alitalo, K., 2008. Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature* 454, 656–660.
- Thiele, W., Krishnan, J., Rothley, M., Weih, D., Plaumann, D., Kuch, V., Quagliata, L., Weih, H.A., Sleeman, J.P., 2012. VEGFR-3 is expressed on megakaryocyte precursors in the murine bone marrow and plays a regulatory role in megakaryopoiesis. *Blood* 120, 1899–1907.
- Ugorski, M., Dziegiel, P., Suchanski, J., 2016. Podoplanin – a small glycoprotein with many faces. *Am. J. Cancer Res.* 6, 370–386.
- Uhrin, P., Zaujec, J., Breuss, J.M., Olcaydu, D., Chrenek, P., Stockinger, H., Fuertbauer, E., Moser, M., Haiko, P., Fassler, R., Alitalo, K., Binder, B.R., Kerjaschki, D., 2010. Novel function for blood platelets and podoplanin in developmental separation of blood and lymphatic circulation. *Blood* 115, 3997–4005.
- Ulvmar, M.H., Mäkinen, T., 2016. Heterogeneity in the lymphatic vascular system and its origin. *Cardiovasc. Res.* 111, 310–321.
- Vaahhtomeri, K., Brown, M., Hauschild, R., De Vries, I., Leithner, A.F., Mehling, M., Kaufmann, W.A., Sixt, M., 2017a. Locally triggered release of the chemokine CCL21 promotes dendritic cell transmigration across lymphatic endothelia. *Cell Rep.* 19, 902–909.
- Vaahhtomeri, K., Karaman, S., Mäkinen, T., Alitalo, K., 2017b. Lymphangiogenesis guidance by paracrine and pericellular factors. *Genes Dev.* 31, 1615–1634.
- Veikkola, T., Jussila, L., Mäkinen, T., Karpanen, T., Jeltsch, M., Petrova, T.V., Kubo, H., Thurston, G., McDonald, D.M., Achen, M.G., Stacker, S.A., Alitalo, K., 2001. Signaling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J.* 20, 1223–1231.
- Wang, Y., Oliver, G., 2010. Current views on the function of the lymphatic vasculature in health and disease. *Genes Dev.* 24, 2115–2126.
- Wang, J.F., Zhang, X.-F., 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.
- Weber, M., Hauschild, R., Schwarz, J., Moussion, C., de Vries, I., Legler, D.F., Luther, S.A., Bollenbach, T., Sixt, M., 2013. Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science* 339, 328–332.
- Wetterwald, A., Hofstetter, W., Cecchini, M.G., Lanske, B., Wagner, C., Fleisch, H., Atkinson, M., 1996. Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. *Bone* 18, 125–132.
- Wigle, J.T., Harvey, N., Detmar, M., Lagutina, I., Grosveld, G., Gunn, M.D., Jackson, D.G., 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., Oliver, G., 1999. Prox1 function is required for the development of the murine lymphatic system. *Cell* 98, 769–778.
- Winnier, G.E., Hargett, L., Hogan, B.L., 1997. The winged helix transcription factor MFH1 is required for proliferation and patterning of paraxial mesoderm in the mouse embryo. *Genes Dev.* 11, 926–940.
- Winnier, G.E., Kume, T., Deng, K., Rogers, R., Bundy, J., Raines, C., Walter, M.A., Hogan, B.L.M., Conway, S.J., 1999. Roles for the winged helix transcription factors MF1 and MFH1 in cardiovascular development revealed by nonallelic noncomplementation of null alleles. *Dev. Biol.* 213, 418–431.
- Wirzenius, M., Tammela, T., Uutela, M., He, Y., Odorisio, T., Zambruno, G., Nagy, J.A., Dvorak, H.F., Ylä-Herttuala, S., Shibuya, M., Alitalo, K., 2007. Distinct vascular endothelial growth factor signals for lymphatic vessel enlargement and sprouting. *J. Exp. Med.* 204, 1431–1440.
- Xu, Y., Yuan, L., Mak, J., Pardanaud, L., Caunt, M., Kasman, I., Larrivée, B., del Toro, R., Suchting, S., Medvinsky, A., Silva, J., Yang, J., Thomas, J.-L., Koch, A.W., Alitalo, K., Eichmann, A., Bagri, A., 2010. Neuropilin-2 mediates VEGF-C-induced lymphatic sprouting together with VEGFR3. *J. Cell Biol.* 188, 115–130.
- Yang, Y., Oliver, G., 2014. Development of the mammalian lymphatic vasculature. *J. Clin. Invest.* 124, 888–897.
- Yuan, H.T., Khankin, E.V., Karumanchi, S.A., Parikh, S.M., 2009. Angiopoietin 2 is a partial agonist/antagonist of Tie2 signaling in the endothelium. *Mol. Cell. Biol.* 29, 2011–2022.
- Yuan, L., Moyon, D., Pardanaud, L., Bréant, C., Karkkainen, M.J., Alitalo, K., Eichmann, A., 2002. Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* 129, 4797–4806.
- Zhang, X., Groopman, J.E., Wang, J.F., 2005. Extracellular matrix regulates endothelial functions through interaction of VEGFR-3 and integrin $\alpha 5\beta 1$. *J. Cell. Physiol.* 202, 205–214.
- Zheng, W., Aspelund, A., Alitalo, K., 2014. Lymphangiogenic factors, mechanisms, and applications. *J. Clin. Invest.* 124, 878–887.
- Zhu, Z., Rockwell, P., Lu, D., Kotanides, H., Pytowski, B., Hicklin, D.J., Bohlen, P., Witte, L., 1998. Inhibition of vascular endothelial growth factor-induced receptor activation with anti-kinase insert domain-containing receptor single-chain antibodies from a phage display library. *Cancer Res.* 58, 3209–3214.
- Zoeller, J.J., Whitelock, J.M., Iozzo, R.V., 2009. Perlecan regulates developmental angiogenesis by modulating the VEGF-VEGFR2 axis. *Matrix Biol.* 28, 284–291.
- Zou, Z., Enis, D.R., Bui, H., Khandros, E., Kumar, V., Jakus, Z., Thom, C., Yang, Y., Dhillon, V., Chen, M., Lu, M., Weiss, M.J., Kahn, M.L., 2013. The secreted lymphangiogenic factor CCBE1 is essential for fetal liver erythropoiesis. *Blood* 121, 3228–3236.