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Development and aging of the lymphatic vascular system

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ABSTRACT

The lymphatic vasculature has a pivotal role in regulating body fluid homeostasis, immune surveillance and dietary fat absorption. The increasing number of *in vitro* and *in vivo* studies in the last decades has shed light on the processes of lymphatic vascular development and function. Here, we will discuss the current progress in lymphatic vascular biology such as the mechanisms of lymphangiogenesis, lymphatic vascular maturation and maintenance and the emerging mechanisms of lymphatic vascular aging.

Contents

1. Overview of the lymphatic vasculature

The lymphatic vasculature forms a unidirectional network that drains interstitial fluid (IF) from most organs and returns it to blood circulation, thus maintaining body fluid homeostasis. Lymphatic vessels (LVs) also transport antigens and immune cells to lymph nodes (LNs),

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Nomenclature

Species, Gene symbol, Protein symbol
Homo sapiens, VEGFR3, VEGFR3
Mus musculus, Vegfr3, VEGFR3
Danio rerio (zebrafish), *vegfr3,* vegfr3
Gene and protein symbol nomenclature used ("vascular endothelial growth factor receptor 3″ gene).

contributing to immunosurveillance of peripheral tissues [1,2]. Additionally, LVs perform a range of organ-specific functions: in the gut LVs are critical for dietary fat absorption, in the LNs they mediate immune cell trafficking and antigen storage and presentation, whereas central nervous system-associated lymphatics emerge as a potential route for cerebrospinal fluid outflow [3–7,29].

LVs are lined with lymphatic endothelial cells (LECs), a distinct endothelial cell (EC) lineage with specific transcriptional and metabolomic profiles [8–12]. At the molecular level, and distinct from blood endothelial cells (BECs), LECs express high levels of the transcription factor PROX1, important for the establishment and maintenance of LEC identity [13,14], as well as the transmembrane *O*-glycoprotein podoplanin, vascular endothelial growth factor receptor 3 (VEGFR3), lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1) and neuropilin-2 [15,16]. Vascular endothelial growth factor C (VEGF-C) and its receptor tyrosine kinase VEGFR-3 are the major effectors of developmental and pathological lymphangiogenesis [17].

In addition to a variety of biochemical signals, LVs are continuously subjected to intrinsic and extrinsic mechanical forces resulting from the uptake and propulsion of lymph, vessel wall stretching and exposure to changing tissue stiffness. Biomechanical signalling has been shown to contribute to both lymphangiogenic response and lymphatic vascular maturation during development [18–20].

In pathological situations, such as lymphedema, absence or dysfunction of LVs impairs local IF balance and immune response leading to tissue swelling, fibrosis and inflammation [21,22]. Furthermore, LVs regulate immune responses during acute infection and chronic inflammation [23–25]. In solid cancers, peritumoral LVs serve as routes for tumor cell dissemination to regional LNs and play multiple roles in the tumor microenvironment [26–28]. Additional emerging functions of LVs include regulation of neuroinflammation, neurodegeneration, adipose tissue expansion, blood pressure and cardiovascular diseases [29,30].

2. Structure and function of lymphatic vessels

Mature lymphatic vascular network is comprised of blind-ended capillaries that take up IF and cells, and collecting LVs that transport lymph to the LNs and then back to the blood circulation. Lymphatic capillaries and collecting vessels are structurally specialized to carry out these distinct functions (Fig. 1).

ECs in LYVE1+ lymphatic capillaries have discontinuous "button-like" and overlapping junctions, sparse basement membrane and no supporting mural cells ensuring optimal surveillance of the interstitial space (Fig. 1). Sprouting lymphatic capillaries harbour continuous cell-cell junctions, thus "button-like" junctions are hallmarks of quiescent and mature lymphatic capillary endothelium [31]. Capillary LECs (capLECs) produce high levels of the chemokine CCL21, which attracts CCR7+ dendritic cells (DCs) to migrate to lymphatic capillaries (reviewed by [2,32]). DCs pass through the pores of the discontinuous basement membrane and arrive into the lumen of lymphatic capillaries, from where they migrate towards draining LNs [33,34]. Lymph is drained from capillaries into precollector LVs that morphologically resemble lymphatic capillaries with oak leaf–shaped LECs, but have lymphatic valves as collecting LVs. In addition to DCs, a variety of other CCR7+ immune populations have been shown to migrate *via* initial LVs, such as CD4 + T effector memory cells, Tregs and ILC3 subtype of innate lymphoid cells [35–37].

Collecting LVs harbour a continuous basement membrane and are surrounded by contractile smooth muscle cells (SMCs). ECs of collecting

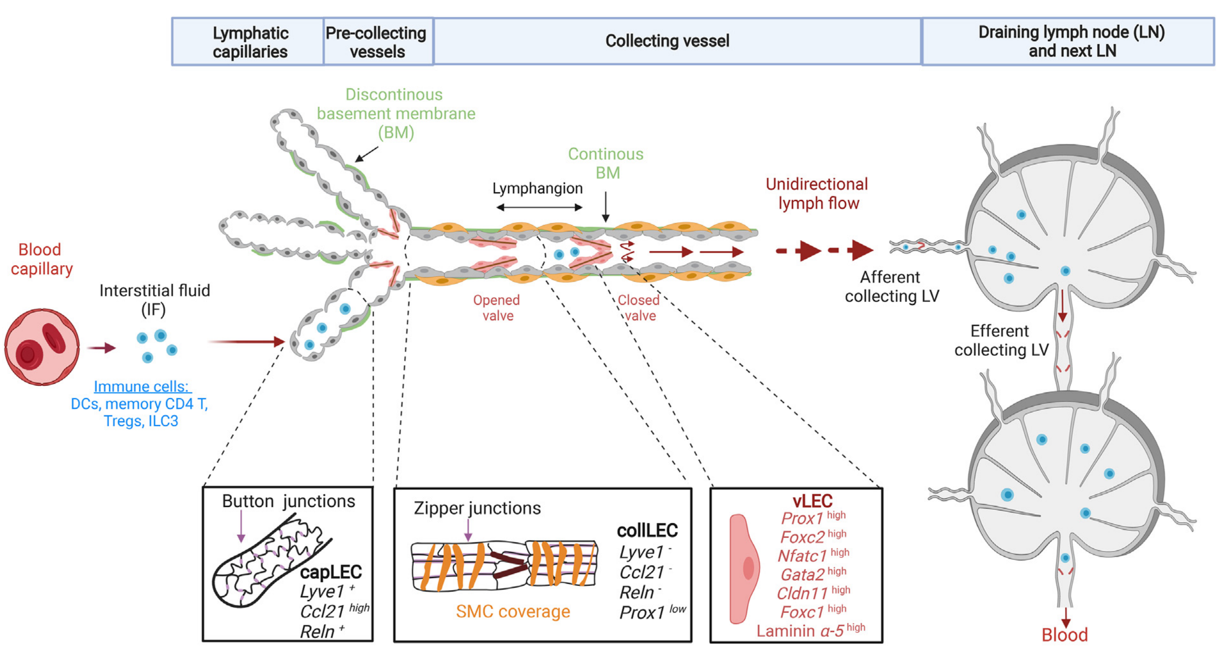

Fig. 1. Structural and molecular features of lymphatic vessels. Blind-ended lymphatic capillaries remove fluid and immune cells from the interstitial space. Capillary LECs (capLECs) harbour button-like intercellular junctions and discontinuous basement membrane (BM). Pre-collecting and collecting vessels efficiently transport lymph towards the draining lymph node due to zipper-like LEC junctions, continuous basement membrane, and intraluminal valves. Contractile smooth muscle cells surround collecting LVs. The selected molecular markers of capLECs, collecting (collLECs) and valve LECs (vLECs) are shown. From the draining lymph node (LN) the lymph is transported to the next LNs until it reaches the blood circulation.

LVs are elongated and connected by continuous zipper-like junctions [31] (Fig. 1). All of these properties facilitate lymph transport through the body and prevent lymph leakage. Collecting LVs contain numerous valves, composed of two semilunar leaflets, covered by a specialized endothelium anchored to an extracellular matrix core [38,39]. Valve opening and closing depend on sequential fluid pressure periods: high lymph pressure upstream of a valve opens the valve enabling lymph flow, whereas reverse flow pushes the leaflets against each other, therefore closing the valve. Lymphatic valves ensure unidirectional flow and subdivide collecting LVs into structural units, called "lymphangions". Due to SMC coverage and in response to the movement of surrounding tissues, such as breathing or skeletal muscle contractions, lymphangions sequentially contract and expand in coordination with the intraluminal valves that open and close to propel lymph unidirectionally through the body [1,40,41] (Fig. 1).

3. Lymphatic vascular development

LVs start to form around mid-gestation when blood circulation is already well established. The majority of LECs are derived from major veins, such as the cardinal vein, after trans-differentiation from venous ECs [13,42,43]. The expanding lymphatic vascular network is also supplemented by LECs originating from alternative sources, which vary in an organ-specific manner. The origin of mesenteric LECs was traced to blood-forming hemogenic endothelium [44], whereas LVs of the dorsal skin are supplemented by a non-vein-derived cell population and local progenitors derived from blood capillaries [45,46]. Additional LEC sources proposed for developing cardiac LVs include hematopoietic cells [47] and the second heart field for the ventral heart lymphatics [48,49]. Interestingly, a recent study indicated that most venous endothelium from which LECs are derived is of a somitic origin [50]. LEC progenitors further coalesce and expand to form the primitive capillary lymphatic plexus [42,51], from which a more definitive lymphatic vascular tree, containing capillaries and collecting LVs, is formed (Fig. 2A).

3.1. Formation of the primary lymphatic vascular plexus

To date, several signalling pathways have been identified as crucial for the early stages of lymphatic vascular development. Independently of their origin, all LECs require the expression of transcription factor

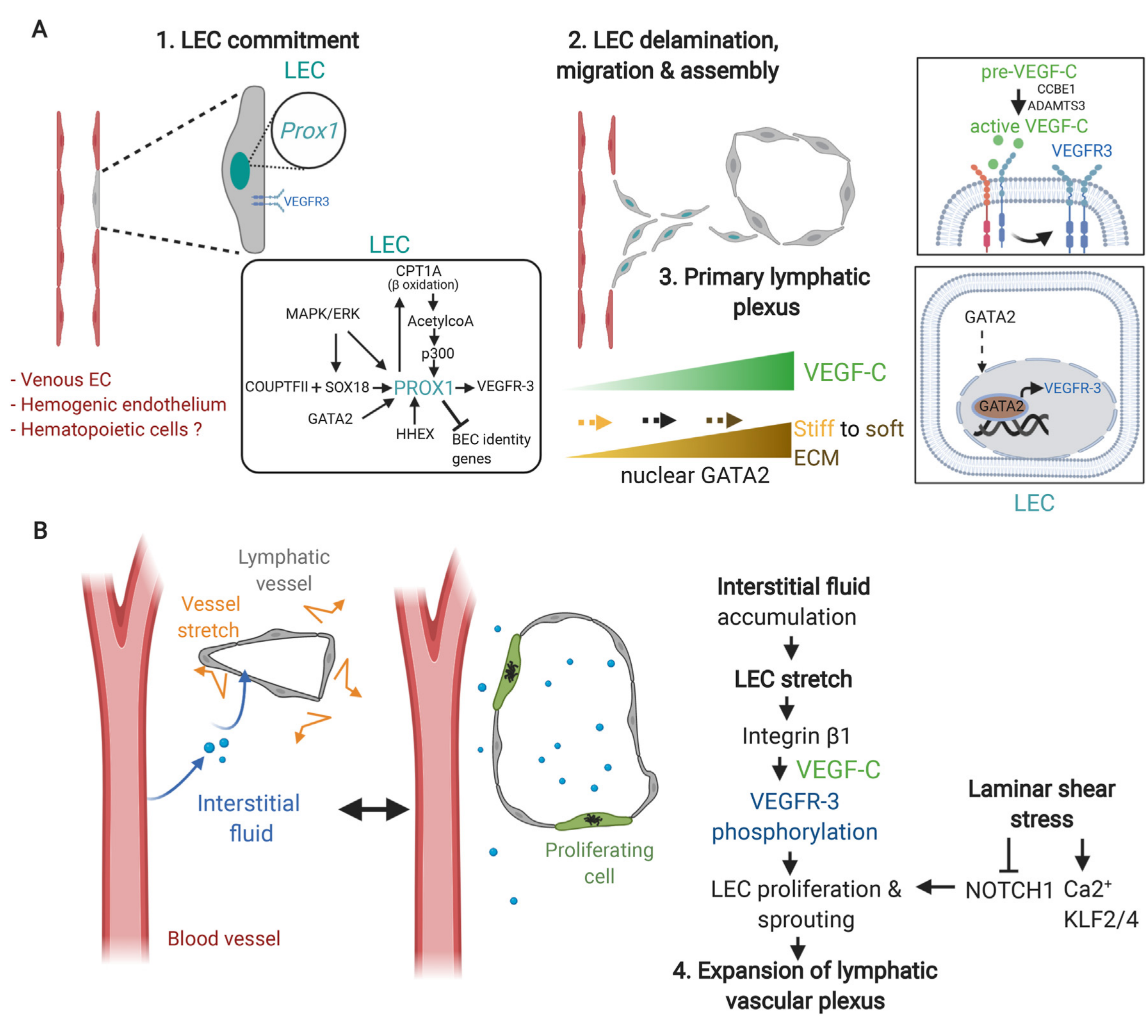

Fig. 2. Molecular mechanisms of lymphatic vascular development. A). The majority of lymphatic endotehlial cells (LEC) arise by trans-differentiation from venous endothelial cells (venous EC). Additional LEC sources include hemogenic endothelium or, potentially, hematopoietic cells. Committed LECs express the transcription factor PROX1, which promotes the expression of VEGFR-3 and represses blood endothelial cells (BEC) transcriptional program. PROX1 expression is induced by several transcription factors (SOX18, COUP TFII, HHEX and GATA2) and ERK signalling, while fatty acid ß-oxidation (CTP1A) enhances PROX1 activity. VEGF-C/VEGFR-3 signalling is essential for LEC delamination, migration and assembly. Extracellular protein CCBE1 and ADAMTS3 metalloprotease are necessary for the generation of lymphangiogenic forms of VEGF-C. The extravasation of LECs from stiff to soft mechanical environment promotes nuclear translocation of GATA2 and further increase in VEGFR-3 levels. B). Fluid filtrating from growing blood vessels accumulates in the interstitial space leading to increased interstitial fluid pressure. Following pressure gradient, fluid then is transferred from the interstitial space into the lumen of the lymphatic plexus stretching the LECs and expanding the plexus as indicated by the arrows. Mechanical stretch activates integrin ß1, enhances VEGF-C dependent VEGFR-3 phosphorylation and potentiates LEC proliferation and subsequent lymphatic vessel expansion. Intraluminal flow *via* NOTCH1 inhibition and induction of Ca^{2+} and KLF2/4 also promotes LEC proliferation and sprouting.

PROX1 for establishment and maintenance of the LEC identity [13,14,46]. PROX1 expression and activity has been shown to be regulated by several transcription factors, such as SOX18 [52], COUP TFII [53], HHEX [54], GATA2 [55], as well as by fatty acid ß-oxidation [11]. In addition, persistent activation of ERK signalling in the developing blood vasculature increases the commitment of venous ECs to the lymphatic fate [56] (Fig. 2A).

Further migration of LECs and expansion of the lymphatic vasculature are directed by signalling *via* lymphangiogenic growth factor VEGF-C and its receptor tyrosine kinase VEGFR-3 (reviewed by [17,57,58]). While *Vegfr3* is expressed at low levels by developing blood vessels, *Prox1* sustains high expression of VEGFR-3 in LECs [8,59]. VEGF-C is initially produced as a pre-propeptide with long N- and C-terminal accessory peptides, which need to be proteolytically cleaved to produce biologically active VEGF-C capable of binding and activating VEGFR-3 ([60], reviewed by [58]). Extracellular protein CCBE1 and the metalloprotease ADAMTS3 form a trimeric complex with VEGF-C and play an essential role in the generation of lymphangiogenic forms of VEGF-C [61–64]. VEGF-C/VEGFR3 signalling is necessary and sufficient for lymphangiogenesis: genetic ablation of any of the above components prevents lymphatic vascular development in mice [65–67], whereas delivery of exogenous VEGF-C promotes the physiological and pathological lymphangiogenic response [68–72].

In line with the essential role of VEGFC-VEGFR-3 pathway in lymphangiogenesis, *VEGFR3*, *VEGFC*, *CCBE1* and *ADAMTS3* are mutated in human congenital lymphedemas, Milroy disease and Hennekam syndrome [73–78]. Mutations in atypical protocadherin *FAT4* and E3 ligase *FBXL7*, which genetically interacts with FAT homolog in Drosophila have also been described in Hennekam syndrome [79,80]. However, *Fat4* inactivation impaired polarization of LECs in response to flow *in vitro* and led to enlarged LVs and reduced number of valves in collecting LVs, suggesting that FAT4 action in these models is uncoupled from the lymphangiogenic VEGFR-C-VEGFR-3 cascade [81,82].

Studies in zebrafish demonstrated that specific fibroblast subtypes and neuronal structures serve as cellular hubs, defining local bioavailabilty of vegf-c and establishing the migration pattern of developing lymphatics [83]. In mice VEGF-C is produced by BECs, vascular and visceral SMCs and selected fibroblast populations suggesting similar guiding roles for these cell types during mammalian lymphangiogenesis [67,84,85]. During tissue injury, VEGF-C is also produced and released by platelets [86]. In addition, during inflammation and tumoral expansion, macrophages have been shown to produce VEGF-C and thus induce local sprouting of pre-existing lymphatic vessels [24,87–89].

Only a combined inactivation of *adamts3* and related *adamts14* led to lymphatic vascular defects in zebrafish [83]. In addition, plasmin, thrombin, cathepsin D and kallikrein 3 also produce mature human VEGF-C [86,90,91], Thus, while *Adamts3* is essential for embryonic lymphangiogenesis in mammals, alternative proteases likely assist in generating bioactive VEGF-C during postnatal and pathological lymphangiogenesis.

VEGF-D is another member of vascular endothelial growth family that is capable of binding and activating VEGFR-3. VEGF-D is dispensable for mammalian lymphangiogenesis [92], however high sequence similarity between species suggests a conserved evolutionary function. In zebrafish vegf-d is essential for normal facial lymphangiogenesis and, together with vegf-c, cooperatively controls the whole body lymphangiogenesis, suggesting that it has context-specific and compensatory functions [93].

3.2. Role of mechanotransduction in lymphangiogenesis

Mechanical forces participate and cooperate with VEGF-C/VEGFR-3 signalling during early lymphatic vascular development. Elegant atomic force microscopy measurements demonstrated that mouse LEC progenitors extravasating from cardinal vein at E10.5-E11 transit from a stiff (cardinal vein) to soft (surrounding mesenchyme) mechanical environment [19]. *In vitro* exposure of LECs to soft matrix promoted cell cycle exit and induced coordinated expression of transcripts associated with cell migration. Importantly, soft matrix also promoted nuclear accumulation of GATA2, which then contributed to increased VEGFR-3 expression (Fig. 2A). In line with these observations, lymphatic endothelial-specific inactivation of *Gata2* decreased VEGFR-3 in LECs and inhibited lymphatic sprouting [19]. The growth arrest of cultured LECs exposed to soft matrix is in agreement with *in vivo* observations that LECs leaving blood vessels in mouse and zebrafish embryos initially do not proliferate [51,94]. In addition, studies of zebrafish revealed that such cell cycle arrest is paradoxically imposed by especially high levels of mitogenic VEGFC/VEGFR3/ERK signalling [94]. Taken together, these results indicate that interplay of tissue stiffness and VEGF-C signalling may lead to highly diverse functional effects.

Subsequent LEC proliferation during expansion of the lymphatic vascular plexus was proposed to result from the combined effect of biochemical VEGF-C/VEGFR3 signalling and LEC stretch, caused by increased tissue hydrostatic pressure due to fluid leakage from expanding blood vasculature in the rapidly growing embryo [95]. *In vitro* mechanical stretch of LECs facilitated interaction of integrin ß1 with VEGFR-3, which in turn enhanced VEGF-C-induced VEGFR-3 phosphorylation and LEC proliferation. Conversely, decreased IF pressure *in vivo* or lymphatic endothelial-specific deletion of *Itgb1* limited lymphangiogenic responses, while increased IF volume or inactivation of integrin-linked kinase, preventing the interaction of ITGB1 with VEGFR-3, resulted in a lymphatic overgrowth phenotype [95] [266].

In addition to LEC stretch, laminar shear stress has been shown to enhance proliferation and sprouting of cultured LECs through inhibition of NOTCH1 and activation of Ca^{2+} signalling and KLF2/4 [96,97], suggesting that intraluminal lymph flow also contributes to lymphangiogenic response (Fig. 2B). Furthermore, studies in cultured BECs and LECs showed that VEGFR-3 regulates the fluid shear stress “set point”, a preferred range of shear stress that induces physiologically relevant endothelial responses to flow, such as cell alignment or suppression of inflammation [98].

Collectively, these results indicate that pro-lymphangiogenic VEGF-C/VEGFR-3 signalling is also highly mechanosensitive. They support a model, in which differential tissue stiffness and VEGF-C bioavailability first establish a pro-migratory program of quiescent LECs to promote their delamination from the cardinal vein. Subsequently, IF accumulation, vessel stretch and intraluminal fluid flow favour VEGF-C-dependent LEC proliferation and further sprouting, thus linking tissue demand for fluid drainage with the expansion of the lymphatic network. The model raises a number of further questions, notably, whether mechanical forces acting in veins contribute to trans-differentiation of venous ECs into LECs prior to their delamination and the role of tissue stiffness, extracellular matrix (ECM) composition and IF in the morphogenesis of organ-specific lymphatic vascular beds. Recently, VEGF-C expression in intestinal murine fibroblasts was shown to be induced downstream of mechanosensitive signalling by YAP/TAZ [99], highlighting the need for further investigations on the role of intestinal stromal cells in maintaining lacteal gut homeostasis.

3.3. Mechanisms of lymphatic collecting vessel and valve development

Once the lymphatic plexus has formed, the blood and the lymphatic vascular systems continue to develop separately with only few connections maintained to allow the return of lymph to the blood circulation. Such connections are protected from blood reflux by lympho-venous valves [100].

The primary lymphatic plexus is remodelled to specialize into capillary and collecting vessels. The timing of collecting vessel formation is tissue-dependent. In mouse mesentery and skin this process is initiated around E15.5 and it continues during postnatal growth period [18,20,101,102]. Formation of collecting LVs includes pruning of capillaries, development of intraluminal lymphatic valves and acquisition of SMC coverage (Fig. 3A). Molecularly, developing collecting LVs LECs reduce expression of capillary markers, such as CCL21 and LYVE1, and display membranous localization of VEGFR-3, indicative of reduced lymphangiogenic signalling [101,103,104].

Lymph flow sensing plays a critical role in the formation of lymphatic collecting vessels and valves (Fig. 3B). Lymphatic valves develop at vessel bifurcations, associated with flow recirculation [20,105,106] and genetic loss of the shear stress sensor Ca^{2+}-channel PIEZO1, as well as the components of endothelial junctional mechanosensitive complex PECAM-1 and VE-cadherin impair lymphatic valve development [107–112]. Forkhead transcription factor FOXC2 is highly expressed in developing valve cells and, to a lesser extent, in

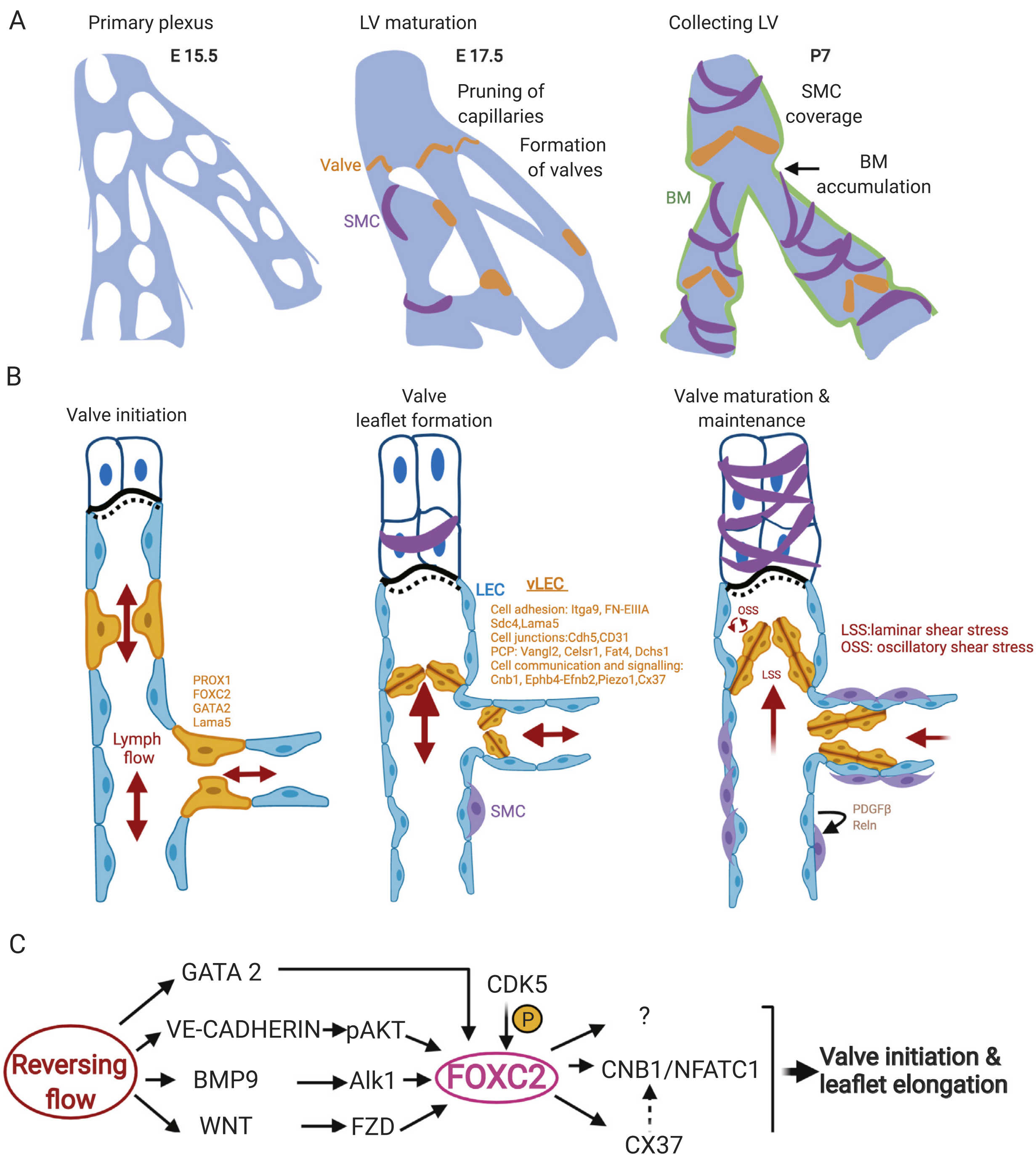

Fig. 3. Formation of collecting lymphatic vessels. A). Formation of collecting LVs includes pruning of capillaries, development of intraluminal lymphatic valves and acquisition of smooth muscle cell (SMC) coverage and continuous basement membrane (BM). B). Valve-forming cells collectively migrate, deposit extracellular matrix and form a mature bi-leaflet intraluminal structure, generating unidirectional lymph flow. Fully formed valve leaflets are subjected to pulsatile laminar shear stress (LSS), whereas valve sinuses lymphatic endothelial cells (LECs) experience oscillatory shear stress (OSS). The markers of valve-forming cells and cellular processes and selected molecular regulators of valve development are shown. For complete list of genes involved in lymphatic valve development see Table 1. PCP, planar cell polarity. C). The mechanosensitive transcription factor FOXC2 is highly expressed in valve-forming and mature valve cells. The transcription factor GATA2, Akt signalling downstream of VE-cadherin sensing of shear stress, Alk1 activation by BMP9 and activation of WNT pahtway have been proposed to induce *Foxc2* expression in valve-forming LECs. FOXC2 transcriptional activity is modulated by Cdk5-dependent phosphorylation. The gap junction protein CX37 and calcineurin/NFATC1 signalling are known effectors needed for collective behaviour of valve-forming LECs and leaflet elongation. Question mark: additional pathways should exist as *Cx37*$^{-/-}$ and calcineurin-deficient mice display milder valve development defects as compared to *Foxc2*-deficient mice.

lymphangion LECs [20,101,113]. *Foxc2* is induced by oscillatory shear stress in cultured LECs, where it controls expression of 1/3 of all shear-stress responsive genes [18,20]. AKT signalling downstream of VE-cadherin shear stress sensing, paracrine and autocrine activation of the WNT pathway and Alk1 activation by BMP9 have been proposed as mechanisms inducing *Foxc2* expression in lymphatic valve-forming cells [109,114,115]. In addition to the control at the transcriptional level, FOXC2 activity is also modulated by phosphorylation, which enables FOXC2 binding to DNA in the context of native chromatin [116,117] (Fig. 3C). *Foxc2* plays a central role in a hierarchy of events leading to collecting LV specialization, as inactivation of *Foxc2* completely prevents formation of lymphatic valves and collecting vessels [18,20,101].

Gap junction communication *via* CX37 and Ca^{2+}-calcineurin/NFAT signalling are downstream effectors of FOXC2, involved in later stages of lymphatic valve formation [20,101,118]. Loss of *Cx37* prevents assembly of PROX1^{high}/FOXC2^{high} valve-forming cells leading to a greatly reduced number of lymphatic valves [20]. Knockdown of *Cx37* impairs the directional migration of BECs under flow [119], suggesting a similar mechanism for the observed valve defects.

NFAT/calcineurin signalling has been proposed to play a central role in developmental and pathological (lymph)angiogenesis [120,121], however results from genetic models support its function as a regulator of blood and lymphatic vascular maturation processes [20,122]. Notably, FOXC2-bound enhancers in LECs are enriched in NFAT binding sites and LEC-specific deletion of the phosphatase calcineurin, which activates NFATs, impairs elongation of the developing valve leaflets [20,101].

In addition to its role in initial sprouting of LECs, the transcription factor GATA2 is also important for lymphatic valve development. GATA2 is highly expressed in valve cells *in vivo* and similar to FOXC2, GATA2 expression is induced in LECs subjected to oscillatory shear stress, whereas inactivation of *Gata2* in LECs impairs the development of lymphatic valves [55]. Loss of *Gata2* prevented the induction of *Foxc2* in LECs in response to shear stress *in vitro* [55], suggesting that *Gata2* is an upstream regulator of *Foxc2*. However, the *in vivo* phenotype of *Gata2* loss in collecting LVs appears to be less severe than that of *Foxc2* deficient mice [18,55] and Mahamud et al. reported that *Gata2* is necessary for the valve maintenance and morphogenesis rather than its initiation [123]. These results indicate non-overlapping functions of these two transcription factors in LECs. Inactivating mutations in *FOXC2* and *GATA2* have been demonstrated in patients with lymphedema-distichiasis syndrome *(FOXC2)* and Emberger and MonoMAC syndromes (*GATA2*, [124–128]), underscoring the key roles for these transcription factors in lymphatic vascular development.

Lymphatic valve development includes collective migration of valve-forming cells, deposition of specialized ECM and culminates in the formation of a mature bi-leaflet intraluminal structure capable of efficiently blocking lymph reflux [129,130]. Multiple regulators of cell polarity, adhesion and migration and cell-cell junctional communication regulate lymphatic valve development and many of these genes have been associated with defects of lymphatic vascular development and function in humans (Table 1). Lymphatic valves exist in zebrafish and inactivation of genes required for mammalian valve morphogenesis led to defective zebrafish lymphatic valve formation, indicating an evolutionary conservation of the underlying mechanisms [131].

Uniquely, BMP9, a BMP/TGFß family member and circulating ligand of ALK1, was shown to be important for lymphatic collecting vessel and valve development [115]. Collecting LVs of *Bmp9*-deficient mice displayed fewer and immature lymphatic valves and expressed high levels of the lymphatic capillary marker LYVE1 [115]. Studies in BECs demonstrated that fluid shear stress potentiates BMP9-ALK1 signalling providing a plausible model of how changes in circulating BMP9 levels can have a localized effect on lymphatic valve development [132].

Mature collecting LVs are surrounded by SMCs, which encircle lymphangions and play a critical role in lymph propulsion [41]. Similar to the blood vasculature, SMCs are recruited to collecting LVs in response to increased production of PDGFß by LECs [133]. LEC-specific inactivation of *Pdgfb* prevented SMC recruitment to collecting LVs, however ectopic overexpression of PDGFß did not attract mural cells to lymphatic capillaries, likely because capLECs do not produce the ECM necessary for efficient PDGFß retention [133]. LECs produce reelin (RELN), a large secreted ECM glycoprotein, previously shown to regulate neuronal migration. Absence of RELN leads to reduced recruitment of SMCs to collecting LVs [102].

SMC coverage is reduced in the valve areas of collecting LVs [102,113]. Inhibition of SEMA3A/NRP1 signalling results in abnormal coverage of the valve area by SMCs, indicating a role for repulsive guidance signals in patterning of lymphatic SMCs [134,135]. SMCs are absent in collecting LVs in rodent and human lungs, where lymph flow is driven by respiration and changes in the thoracic pressure [136]. These results reveal the importance of the biomechanical organ environment in establishing mural coverage of collecting vessels and suggest that lung LVs may be especially vulnerable under conditions of impaired lymph flow [136].

3.4. Role of lymphatic vessels in secondary lymphoid organ development

Collecting LVs connect the chains of LNs, which harbour highly specialized LN-LEC populations. LN-LECs contribute to compartmentalization, trafficking and functions of LN-immune cells (reviewed in [27]). LNs develop during embryogenesis in pre-determined locations at the bifurcations of large blood vessels [165–167]. Previous studies established the critical importance of cross-talk between hematopoietic lymphoid tissue inducer (LTi) cells and fibroblastic lymphoid tissue organizer (LTo) cells in the initiation of LN development [166,167].

Initial accumulation of LTi cells at the developing LN anlagen occurs in the absence of LVs [168,169]. However, LVs are indispensable for expansion of growing LNs through the transport and retention of LTi cells [169–171]. VEGF-C/VEGFR-3 blockade, inactivation of *Rank* or *Ltbr* together with the NFK**ß** inducing kinase (*Nik*) in LECs or arrest of lymphatic vascular maturation in *Foxc2*-LEC deficient mice prevented normal LN development [169–171] (Fig. 4).

Interestingly, while peripheral lymphatic vasculature expands *via* sprouting of NRP2^{+} LECs [172], the LN capsule is formed *via* a distinct morphogenetic process, during which sheets of collectively migrating NRP2^{low} LECs engulf the growing LN anlagen [169]. *Foxc2* inactivation in LECs disrupts formation of the LN capsule and impairs local interstitial fluid flow (IFF) which is necessary to potentiate LTß-dependent production of the chemokine CXCL13 by LTo cells, and to recruit CXCR5-expressing LTi cells [169]. Collectively, these studies underscore the importance of the embryonic lymphatic vasculature transport function and IFF in pre-natal LN formation (Fig. 4).

Recent analyses of adult LNs revealed the existence of multiple specialized LN LEC subsets; thus, it will be important in the future to study the molecular mechanisms leading to their emergence and maintenance [173–175]. Other secondary lymphoid organs, such as Peyer's patches are also richly supplied with LVs during embryogenesis 176], however the role of lymphatics in the development of non-encapsulated secondary lymphoid organs remains to be investigated.

4. Lymphatic vascular maturation and maintenance

4.1. Maturation of capillary LEC junctions

The characteristic organization of adult capLECs with an oak-leaf shape and discontinuous “button-like” junctions and flap valves is optimized for the efficient uptake of IF and transmigration of immune cells, while also maintaining the integrity of the lymphatic capillary wall [177]. ECs of embryonic LVs are connected by continuous “zipper” cell-cell junctions; however, these are replaced by “button-like” like junctions after birth [178]. The greatly increased functional load of LVs

Table 1
Mouse models displaying defective lymphatic valve development.

Gene Name	Model	Phenotype	Human hereditary diseases (MIM number, * if lymphatic defects)
Transcriptional and chromatin regulators			
Foxc1	*Foxc1*$^{f/f}$;*Tie2*-Cre [137] *Foxc1*$^{f/f}$; *Cdh5*-Cre^{ERT} *Foxc1*$^{f/f}$; *Prox1*-Cre^{ERT2} [138]	Reduced number of valves (embryonic) Reduced valve leaflet formation (postnatal)	Axenfeld-Rieger syndrome (602482)
Foxc2	*Foxc2* −/− *Foxc2*$^{f/f}$; *Prox1*-Cre^{ERT2} *Foxc2*$^{f/f}$; *Flt4*-Cre^{ERT2} [18,101,113,118]	Valve agenesis, failure to form collecting vessels (embryonic); valve regression, loss of collecting LV integrity, chylothorax, chylous ascites (postnatal)	Lymphedema-distichiasis syndrome (153400 *)
Gata2	*Gata2*$^{f/f}$; *Prox1*-Cre^{ERT2} [19,55]	Reduced initiation of valve development, dilated collecting vessels (embryonic) Valve regression, dilated collecting vessels (postnatal)	Lymphedema, primary, with myelodysplasia (614038*)
Hdac3	*Hdac3*$^{f/f}$; *Tie2*-Cre *Hdac3*$^{f/f}$; *Cdh5*-Cre *Hdac3*$^{f/f}$: *Lyve1*-Cre [139]	Reduced number of valves, dilated collecting vessels, abnormal SMC recruirment (postnatal)	n.d.
Ppp3ir1 (*Cnb1*)	*Cnb1*$^{f/f}$; *Prox1*-Cre^{ERT2} *Cnb1*$^{f/f}$; *Cdh5*-Cre^{ERT2} [20]	Arrested valve development, failure of leaflet formation in embryos Valve regression (postnatal)	n.d.
Planar cell polarity			
Celsr1	*Celsr1*$^{f/f}$;*Prox1*-Cre^{ERT2} *Celsr1/3*$^{f/f}$; *Prox1*-Cre^{ERT2} *Csrh* mutant [140]	Arrested valve development Defective cell re-orientation for leaflet formation (postnatal) Normal valve maintenance	Primary lymphedema* [141]
Dchs1 *Fat4*	*Dchs1* −/−, *Fat4* −/− [81] *Fat4*$^{f/f}$;*CMV*-Cre *Fat4*$^{f/f}$;*Prox1*-Cre^{ERT2} [82]	Arrested valve development, defective cell re-orientation for leaflet formation, reduced number of valves (embryonic)	Mitral valve prolapse-2 (607829) Van Maldergem syndrome-1 (601390) Hennekam lymphangiectasia-lymphedema syndrome-2 (616006*)
Vangl2	*Looptail* mutant [140]	Arrested valve development, defective cell re-orientation for leaflet formation (embryonic)	n.d.
Adhesion receptors and ECM components			
Emilin1	*Emlin1* −/− [142]	Ring-like valves (embryonic)	n.d.
FnEIIIa	*Fn-EIIIA* −/− [39]	Ring-like valves (embryonic and postnatal)	n.d.
Itga9	*Itga9* −/− *Itga9*$^{f/f}$;*Tie2*-Cre *Itga9*$^{f/f}$;*Cdh5*-Cre^{ERT2} [39]	Ring-like valves (embryonic and postnatal), chylothorax	Congenital chylothorax* [143]
Svep1	*Svep1*$^{-/-}$ [144] *Svep*$^{Lacz/Lacz}$ [145]	Agenesis of lymphatic valves, defective LV remodeling (embryonic)	n.d.
Endothelial cell-cell junctions			
Pecam1	*Pecam1*$^{-/-}$ [110]	Arrested valve development, failure of leaflet formation, defective LV remodeling (embryonic)	n.d.
Cdh5	*Cdh5*$^{f/f}$;*Prox1*-Cre^{ERT2} [109,112]	Normal valve initiation, defective leaflet formation (embryonic), reduced number of valves, ring-like valves, chylous ascites (postnatal); atrophy of mesenteric but not dermal valves (adult)	n.d.
Ctnnb1	*Ctnnb1*$^{f/f}$;*Lyve1*-Cre [146]	Agenesis of valves (embryonic)	n.d.
Cell surface receptors and their ligands			
Bmp9	*Bmp9*$^{-/-}$ [115]	Arrested valve development, failure of leaflet formation (embryonic and postnatal)	Hereditary hemorrhagic telangiectasia type 5 (615506)
Angpt2	*Angpt2*$^{LacZ/Lacz}$ [147] *Angpt2*$^{f/f}$; *EIIa*-Cre [148] ANGPT2 blocking antibodies [149]	Impaired maturation of collecting vessels, agenesis of lymphatic valves, disruption of "zipper"-like junctions in collecting vessels (embryonic) No effect on valve maintenance	Primary lymphedema* [150]
Tie1	*Tie1*$^{f/f}$; *Nfatc1*-Cre [151] *Tie1-ICD*$^{f/f}$;*EIIa*-Cre *Tie1-ICD*$^{f/f}$; *Ubc*-Cre^{ERT2} [148]	Defective valve development, increased recruitment of SMCs (embryonic) Impaired valve maintenance in ear pre-collectors, reduced valve number (postnatal)	n.d.
Notch1	*Notch1*$^{f/f}$; *Prox1*-Cre^{ERT2} *R26-DNMAML*$^{f/+}$; *Prox1*-Cre^{ERT2} [152]	Arrested valve development, failure of valve cell clustering and polarization (embryonic)	Aortic valve disease-1 (109730), Adams-Oliver syndrome-5 (616028)

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Table 1 (*continued*)

Gene Name	Model	Phenotype	Human hereditary diseases (MIM number, * if lymphatic defects)
Efnb2 *Ephb4*	*EphrinB2*$^{\Delta V/\Delta V}$ (mutant lacking PDZ-interaction site) [103] *EphrinB2*$^{6YF\Delta V/6YF\Delta V}$ (defective EphB4 forward signalling) α-EPHB2 blocking antibody α-EPHB4 blocking antibody	Agenesis of lymphatic valve, dilated collecting vessels, defective valve development in embryos and in neonates is rescued by an agonistic α-EphB4 antibody	Lymphatic malformation-7 (617300*), capillary malformation-arteriovenous malformation-2 (618196)
	NVP-BHG712 EphB4 kinase inhibitor [153] *Ephb4*$^{f/f}$; *Prox1*-CreERT2 *Ephb4*$^{f/f}$;*Pdgfb*-Cre^{ERT2} *Efnb2*$^{f/f}$;*Prox1*-Cre^{ERT2} *Ephb4*$^{f/f}$;*Pdgfb*-Cre^{ERT2} [154,155]	Loss of valves, disruption of collecting LEC cell-cell junctions (juvenile and adult mice)	
Sdc4	*Sdc4* −/−	Arrested valve development, failure of leaflet formation, defective LV remodeling (embryonic)	n.d.
	Sdc4 −/−; *Pecam1* −/− [110]	Aggravated defects of LV remodeling, excessive SMC coverage (embryonic)	
Gap junctions			
Gja4	*Gja4*$^{-/-}$ [20,118]	Normal valve initiation, but defective formation of ring-like regions (embryonic) Almost complete absence of valves (adult)	n.d.
Gja1	*Gja1*$^{-/-}$ [118] *Gja1*$^{f/f}$;*Lyve1*-Cre [156]	Reduced number of valves (embryonic); Reduced number of valves, short valve leaflets, chylothorax (postnatal and adult).	Oculodentodigital dysplasia (164200* [157]
Membrane ion channels			
Piezo1	*Piezo1*$^{f/f}$;*Tie2*-Cre; *Piezo1*$^{f/f}$;*Lyve1*-Cre; [107] *Piezo1*$^{f/f}$; *Prox1*-Cre^{ERT2}; *Piezo1*$^{f/f}$;*Cdh5*-Cre^{ERT2} [108]	Reduced number of valves (postnatal) Regression of valves (adult)	Lymphatic malformation-6 (generalized lymphatic dysplasia, 616843*), dehydrated hereditary stomatocytosis (194380*)
Axon guidance molecules			
Sema3a *PlexinA1* *Nrp1*	*Sema3a*$^{-/-}$ *Plxna1*$^{-/-}$ *Nrp1*$^{sema/sema}$ [135] α-NRP1 antibody blocking SEMA3A binding [134]	Abnormal SMC coating of valve regions Reduced valve leaflet length	n.d.
Platelet coagulation cascade			
Clec2	*Clec2* −/− *Clec2*^{fl}/−; *Pf4*-Cre [158]	Reduced valve initiation and valve number, increased SMC coverage	n.d.
Intracellular signalling			
Akt1	*Akt1*$^{-/-}$ [159]	Lack of valves in hypoplastic pre-collectors of adult ears	n.d.
Pik3r1	*Pik3r1*$^{-/-}$ [160]	Lack of mesenteric valves in newborns	SHORT syndrome, (269880), Immunodeficiency-36 (616005)
Rasa1	*Rasa1*$^{f/f}$; *Ubb3*-Cre^{ERT2} *Rasa1*$^{fl/R780Q}$ *Ubb3*-Cre^{ERT2} *Rasa1*$^{f/f}$; *Prox1*-Cre^{ERT2} [161]	Normal valve initiation followed by apoptosis of valve cells (embryonic) Valve leaflet atrophy, Impaired valve function, chylothorax (adult)	Capillary malformation-arteriovenous malformation-1 (608354)
Cdk5	*Cdk5*$^{f/f}$; *Tie2*-Cre *Cdk5*$^{f/f}$; *Cdh5*-Cre^{ERT2} [117]	Arrested valve development Reduced number of lymphatic valves (embryonic)	n.d.
Epsin 1 & 2	*Epsin1*$^{f/f}$;*Epsin2*$^{-/-}$; *Lyve1*-Cre *Epsin1*$^{f/f}$;*Epsin2*$^{-/-}$; *Prox1*-Cre^{ERT2} [104]	Ring-like valves in neonates, rescued by small molecule inhibitor of Vegfr-3	n.d.
Cdc42	*Cdc42*$^{f/f}$;*Prox1*-Cre^{ERT2} *Cdc42*$^{f/f}$;*Cdh5*-Cre [162]	Agenesis of lymphatic valve Arrested collecting vessel maturation (embryonic)	n.d.

Table 1 (*continued*)

Gene Name	Model	Phenotype	Human hereditary diseases (MIM number, * if lymphatic defects)
Rasip1	*Rasip1*$^{f/f}$; *Prox1*-Cre^{ERT2} [163]	Reduced number of valves, dilated collecting vessels (embryonic)	n.d.
Metabolism			
ApoE	*Apoe*$^{-/-}$ [164]	Decreased number of valves, decreased SMC coverage (adult)	Alzheimer's disease-2 (104310)
miRs			
Mir126	*Mir126*−/− [123]	Absent lymphatic valves (embryonic)	n.d.

immediately before and after birth, for example to ensure clearance of amniotic fluid and inflation of lungs [179], is a likely reason for the zipper-to-button transformation of LEC junctions.

Mechanisms of capLEC junctional maturation are not completely understood and may involve exposure to transmural flow, which promotes re-distribution of junctional proteins in LECs *in vitro* [180] and ANGPT2 signalling which induces VE-cadherin phosphorylation [149]. Lymphatic button junctions are highly plastic and are transformed back to zippers during inflammation [178]. Dexamethasone administration accelerates button junction formation after birth and during inflammation. This effect is potentially mediated *via* both the direct activation of glucocorticoid signalling in LECs [178] and anti-inflammatory action of dexamethasone on blood vascular endothelium and immune cells [181].

Junctional organization of LECs is also a subject to organ-specific regulatory mechanisms. Intestinal lacteals harbour a mix of zipper-like and button junctions, as they need to both continuously regenerate in a challenging intestinal microenvironment and efficiently transport chylomicrons and immune cells [3,182]. Regeneration of lacteals is driven by VEGF-C-VEGFR-3-DLL4 signalling, while the nearby blood capillaries have high levels of VEGF-A/VEGFR-2 signalling [3,182,183]. LEC-selective loss of *Dll4* decreased the proportion of functional button junctions and led to lacteal regression and defective fat absorption [182]. Deletion of VEGF-A receptors *Flt1* and *Nrp1* on intestinal blood capillaries generated a local excess of VEGF-A, which induced loss of button junctions in intestinal lacteal LECs *via* activation of VEGFR-2 and reduced dietary fat transport and resistance to high fat diet induced obesity [184]. These results highlight a need for VEGF-A and VEGF-C

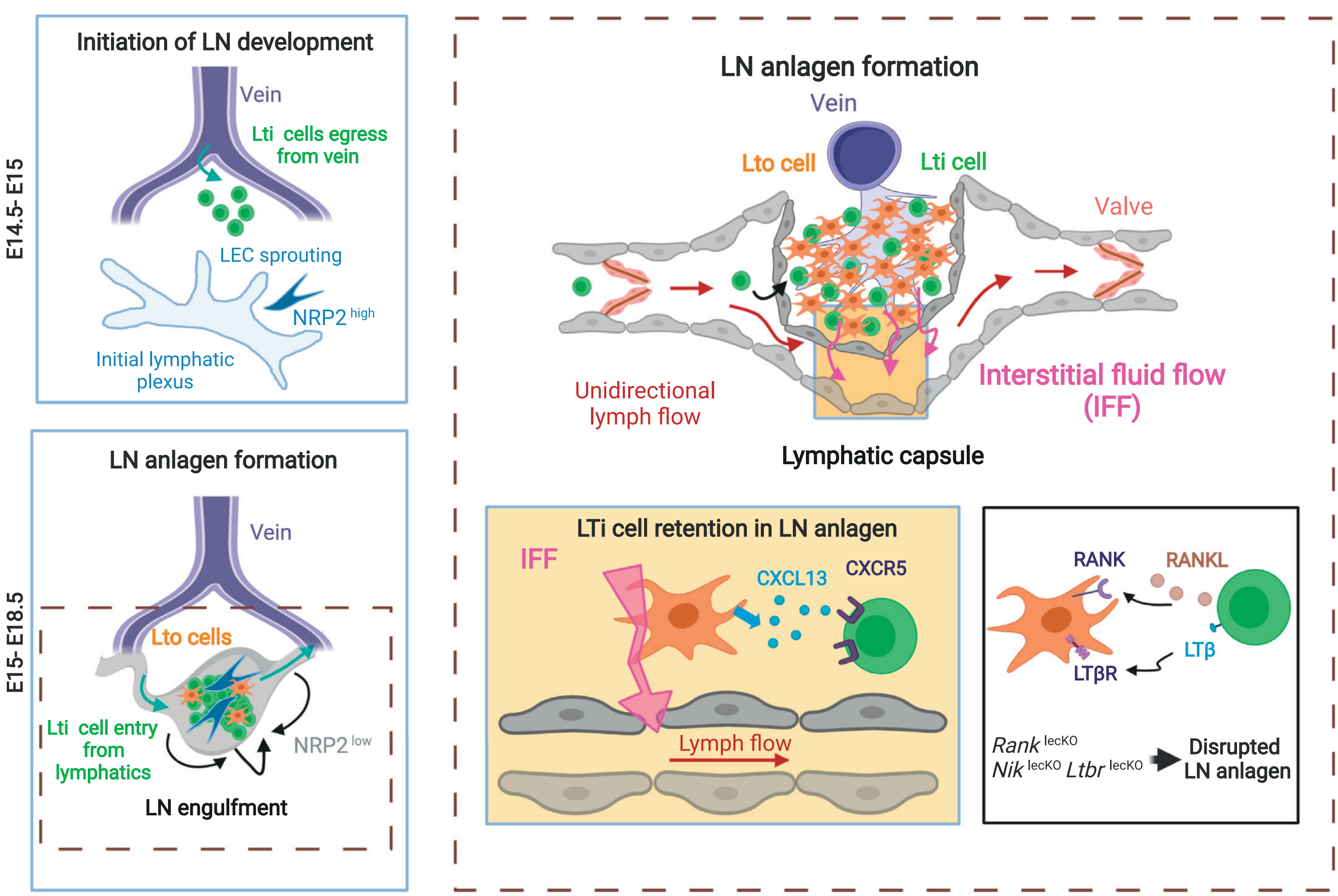

Fig. 4. Lymphatic vessels in lymph node development. Initiation of lymph node (LN) development: LNs start to develop at the bifurcation sites of large blood vessels at the sites of egress of hematopoietic lymphoid tissue inducer (LTi) cells from immature veins. LN anlagen formation: Extravasated LTis are taken up by lymphatic vessels (LVs) that transport them to the LN anlagen contributing to their expansion, while perinodal LVs continue to reorganize into a LN capsule. During this process, sheets of collectively migrating NRP2^{low}LECs engulf growing LN anlagen, while peripheral lymphatic vessels expand *via* sprouting of NRP2^{high} LECs. The zoomed image: the interstitial fluid absorbed by lymphatic vessels generates interstitial fluid flow (IFF) that together with the lymph flow in the subcapsular sinus is needed for the formation of the lymph nodes. Highlighted square: IFF potentiates the expression of CXCL13 by fibroblastic lymphoid tissue organizer (LTo) cells to retain the CXCR5-expressing LTi cells. Inactivation of *Rank* or *Nik* and *Ltbr* in LECs prevents the formation of the LN anlagen.

signalling compartmentalisation for the organ-specific functions of intestinal LVs and call for further investigation of the mechanisms maintaining button junctions in other adult lymphatic vascular beds.

4.2. Maintenance of collecting lymphatic vessel specialization

LV functional and structural specialization into collecting vessels needs to be maintained throughout life, however our knowledge of such mechanisms is still fragmentary. Mice with postnatal LEC-specific *Gata2* inactivation have a reduced number of malformed lymphatic valves and mispatterned collecting vessels [55]. Postnatal inactivation of *Foxc2* results in lymphatic valve degeneration, collecting LEC (collLEC) junction disruption, lymph leakage and rapid lethality within a few days [18]. Mechanistically, *Foxc2* is necessary for cell-cell junction maintenance and quiescence of LECs subjected to reversing flow *in vitro*. *Foxc2* loss leads to excessive collLEC proliferation and apoptosis due to abnormal activation of TAZ signalling [18], indicating that continuous expression of *Foxc2* is critical for maintenance of collecting LV integrity and function. A closely related transcription factor FOXC1 is also expressed in postnatal lymphatic valves, with especially high levels found in a subset of LECs at the free edge of lymphatic valve leaflets [138]. Postnatal *Foxc1* deletion further aggravated *Foxc2* knockout phenotype, indicating complementary functions of these transcription factors in collecting LV maintenance [138]. Lymphatic valve degeneration is also observed in mice with postnatal inactivation of *Piezo1*, which codes for a synonymous mechanosensitive protein [108] and *VE-cadherin* [112], indicating that maintenance of collecting LV integrity and, especially, valves, is an active process which requires multiple inputs by mechanosensitive networks.

Mature collecting LVs are much less permeable than lymphatic capillaries [185], which is important for efficient transport of lymph to the LNs. The permeability of collecting vessels is a subject of both cell-autonomous and paracrine control. EphrinB2-EphB4 signalling is necessary for homeostatic maintenance of LEC junctional integrity, as inactivation of either molecule results in the compromised barrier function of collecting LVs [155]. IRF4-dependent DCs, residing in adipose tissue surrounding adult collecting vessels, interact with the collecting vessel. The disruption of this interaction in *Ccr7*-deficient mice increased leakiness of collecting vessels and augmented collagen deposition [186]. In contrast, transfer of Tregs in an experimental lymphedema model reduced tissue fibrosis and improved lymphatic transport function [187]. Mast cells have been shown to localize close to collecting LVs and were proposed to modulate both trafficking of immune cells and SMC contractility *via* release of inflammatory and vasoactive mediators [188]. These data indicate that specific immune populations may be involved in the maintenance of basal permeability and repair of lymphatic collectors.

5. Aging and lymphatic vessels

Aging, which is broadly defined as the progressive decline of physiological functions of the organism, is driven by alterations in specific molecular and cellular processes, such as genomic stability, proteostasis, mitochondria function, cellular senescence survival and intercellular communication [189]. Understanding of the aging process necessitates better knowledge of its relative impact on different organ systems and the underlying molecular mechanisms.

5.1. Characteristics of aged lymphatic vessels

Lymphatic draining function declines with age, both in rodent models and in humans, as a result of decreased lymphatic capillary density and transport capacity of collecting LVs [190–195]. Such a functional decline was demonstrated for thoracic duct, skin, meningeal and mesenteric LVs [6,196–200], while the impact of aging on other organ-specific LVs remains to be investigated.

Aged-related lymphatic capillary rarefaction (Fig. 5) may be linked to the declining production of lymphangiogenic factors, diminished regenerative capacity of LECs and alterations in the stiffness and composition of ECM, which accompany aging in many organs [201,202]. Functions of lymphatic capillaries can be also impaired by factors accelerating aging, such as UV-B light for skin. In mice, UV-B exposure was shown to reduce VEGF-C expression, promote macrophage infiltration and increase lymphatic permeability [203]. Supplementation of VEGF-C improved both UV-B induced skin edema and the function of aged meningeal LVs in mice and promoted lymphangiogenesis in chronic models of inflammation [200,204,205], demonstrating that aged or damaged LVs remain responsive to pro-lymphangiogenic therapy.

Aged collecting LVs are enlarged and functionally characterized by increased permeability and decreased lymphatic contractility [191,206]. Proteomics analysis uncovered decreased glycocalyx, cell junctional molecules, and ECM in aged collecting LVs [195]. Glycocalyx and cell junctions transmit mechanical stimuli in ECs [105], thus in addition to direct effect on vascular permeability aging may impair normal sensing of lymph flow by collLECs and further contribute to deterioration of collecting vessel function. Aging also reduces collecting LV SMC coverage, especially in perivalvular locations and is associated with strongly reduced pumping capacity due to reduction in lymphatic contractile frequency [206,207] (Fig. 5).

5.2. Cross-talk of immune and lymphatic vascular systems in aging

A dysregulated immune response characterized by increased immune cell infiltration is a hallmark of organ deterioration in aging [208]. LVs are both affected by and contribute to age-related immune imbalance. Under normal conditions, collecting vessels have been shown to be associated with DCs and mast cells [186,209], which regulate LV permeability and the SMC contractility [210]. Chronic activation of peri-lymphatic mast cells during aging has been proposed to underlie the diminished functionality of collecting LV SMCs and their weak response to LPS-induced acute inflammation [209,210] (Fig. 5).

An individual's history of infections and metabolic diseases may be an additional factor contributing to the deterioration of LV function with age. Fonseca et al. demonstrated that intestinal infection with *Yersinia pseudotuberculosis* leads to the long term “immunological scarring” of mesenteric collecting LVs, manifested as increased vessel permeability, which persists after infection clearance [23]. Exposure to bacterial exotoxins, such as those found in a methicillin-resistant strain of *Staphylococcus aureus*, causes selective death of lymphatic SMCs and chronic lymphatic function impairment [211]. The chronicity of lymphatic vascular impairment after infections suggests a rather low regeneration capacity of adult lymphatic SMCs.

Collecting LVs are deteriorated in hyperlipidemic *Apoe* −/− mice and in mouse models of type 2 diabetes, and aged LVs display signs of increased oxidative stress [164,195,212,213]. Thus, hyperlipidemia and diabetes, which afflict a significant proportion of the elderly human population, combined with increased reactive oxygen species production [214] potentially contributes to the decline of lymphatic function with age.

Studies in animal models indicate that alterations of lymphatic function can directly contribute to both the development of autoimmune disorders and decline of protective immune functions observed during aging [215]. Notably, aged K14-VEGF-3-Ig mice, which lack dermal lymphatic capillaries have increased IgG and IgA serum titers and display antibody deposition in the skin [216]. An impaired function of mesenteric LVs also prevented normal trafficking of intestinal DCs and compromised mucosal tolerance and protective immunity [23]. Furthermore, increased permeability of aged collecting LVs may also increase penetration of antigens and bacterial products in the surrounding adipose tissue, leading to activation of adipose tissue immune cells [195,217]. Specialized populations of LN LECs regulate establishment

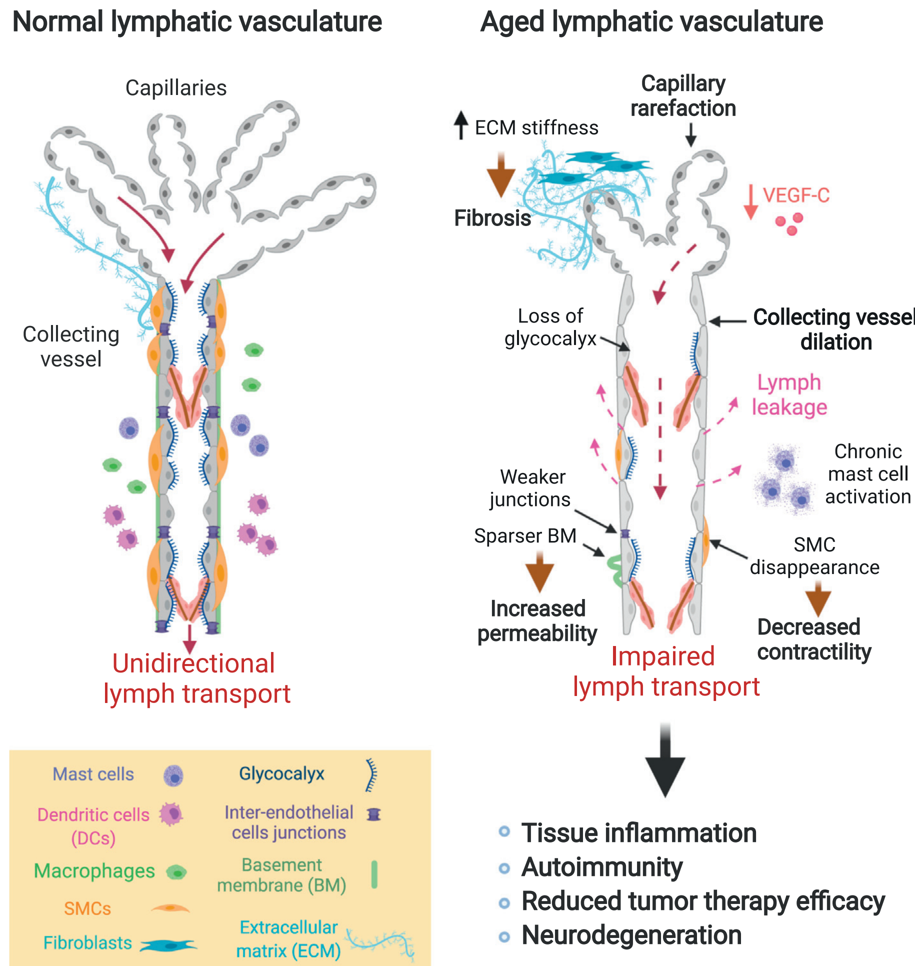

Fig. 5. Defects of lymphatic vessels in aging. During aging, the stiffness of the extracellular matrix (ECM) and increased inflammation leads to tissue fibrosis; the potentially declined production of lymphangiogenic factors (VEGF-C) induces capillaries rarefaction. Aged collecting LVs are dilated, they present an altered lymph flow and loss of glycocalyx and their permeability is increased as consequence of loss of cellular junctions and basement membrane proteins. Aged collecting vessels are also less contractile due to reduced coverage of SMCs and chronic activation of associated mast cells that producevasoactive substances such as NO and histamine. Potential consequences of structural and functional defects of aged LVs include tissue inflammation, autoimmunity and a reduced tumor therapy efficacy. In case of meningeal LVs defective clearance of misfolded amyloid ß and tau proteins may contribute to neurodegeneration in Alzheimer's disease.

of tolerance *via* multiple mechanisms [4,27,218], therefore it will be also important to study the impact of aging on LN LEC subsets.

5.3. Impact of lymphatic vessel dysfunction in cancer

Age is the biggest risk factor for cancer. The role of lymphatic vascular aging in the etiology of cancer has not been extensively investigated, however multiple studies pinpointed both detrimental and beneficial roles of tumor-associated LVs.

Metastasis of solid tumors is frequently mediated by peritumoral LVs that disseminate cancer cells to regional lymph nodes. Commonly, high levels of lymphangiogenic growth factors such as VEGF-C/D are released and therefore tumor-associated lymphangiogenesis actively contributes to metastasis and is usually associated with a poor prognosis in multiple cancers (reviewed by[28][221]). These growth factors also contribute to regional LN lymphangiogenesis that has been related with poor clinical outcomes (reviewed by [221]). Experimentally, inhibition of VEGFR-3 signalling in rodent models decreases tumor spread to LNs [263][264][265]. Recent studies in animal models established that tumor cells reaching sentinel LNs can extravasate into LN blood vessels and disseminate systemically to the lungs [223,224].

Recently, several studies on tumor lymph composition uncovered that lymph derived from melanoma tumors presents a high content of bile acids [225], cancer biomarkers [226] and oleic acid that protects cancer cells from ferroptosis [227] and therefore their ability to survive.

LVs were considered for years mere conduits in tumor metastasis; however, much evidence now supports a role for LVs as immunoregulators of the tumor microenvironment (TME). Inflamed tumoral lymphatic vasculature produces PD-L1, inducible nitric oxidase synthase, indoleamine 2,3 dyoxigenase, and TGFß that inhibit DC maturation and limit cytotoxic lymphocyte function, thus favouring an immunosuppressive TME [26]. Moreover, tumor-associated LVs act as a nook for cancer cell growth by secreting chemokines, such as CCL21 and CXCL12 [228,229]. In the lungs, tumor-associated LVs produce S1P that induces NK and T cells to escape, thus favouring metastatic spread [230]. Conversely, transport of tumor antigens by DCs that migrate through LVs to draining LNs is necessary for proper anti-tumor immunity [231,232]. Therapeutically, intratumoral induction of lymphangiogenesis in primary tumors induces accumulation of CD8 T cells thereby improving responses to immunotherapy in melanoma models [233].

Reduced production of HAPLN1 (hyaluronan and proteoglycan link protein 1), which crosslinks hyaluronan to the extracellular matrix, enhanced metastatic dissemination in aged melanoma patients and in aged mice through increased permeability of peritumoral LVs [234]. Defective tumoral and peritumoral lymphatic transport function is also expected to reduce the efficiency of chemo- and immunotherapies through maintenance of elevated intra-tumoral pressure and reduced transport of tumor antigens and antigen-presenting cells [235–237].

Altogether, although growing LVs facilitate metastasis spread and present immunosuppressive features in advanced tumors, a functional lymphatic vasculature is needed for proper anti-tumor immunity to occur and for efficient responses to immune therapies. Therefore, future work needs to decipher the best lymphatic therapeutic venues for different cancer types.

5.4. Impact of lymphatic vessel dysfunction in other age-related diseases

Defective proteostasis leads to the accumulation of misfolded and aggregated proteins in neurons and is a significant cause of age-related neurological conditions, such as Alzheimer's and Parkinson's diseases [238]. Meningeal LVs may participate in ß-amyloid clearance, as photoablation of meningeal LVs increased brain amyloid ß burden and promoted deposition of meningeal amyloid ß in mouse models of Alzheimer's disease [200]. Furthermore, clearance of aggregated tau protein was impaired in K14-C-VEGFR-3-Ig mice, which lack meningeal lymphatics [239]. These data suggest that age-related decline in meningeal LV function potentially contributes to the development of Alzheimer's disease, and perhaps other organ pathologies caused by defective proteostasis [27,29]. Further research is needed on the routes and therefore, also on the molecular mechanisms that drive the clearance of aggregated proteins in neurologic diseases. Meningeal lymphatics are one potential drainage route, but other cell types such as glial or BECs have also been proposed [240,241].

LVs have been proposed as important players in regulating cholesterol removal from arteries. Adventitial LVs are found in atherosclerotic arteries, which are characterized by inflammation, lipid accumulation and vascular remodeling [242]. Moreover, a lack of LVs in mice alters proper lipoprotein metabolism and vascular homeostasis, leading to accelerated atherosclerosis [243]. Mice lacking apolipoprotein E harbour dysfunctional and degenerated LVs [164]. On the other hand, inhibition of VEGFR-3 in a cardiac allograft model diminishes atherosclerosis [244], while the rescue of lymphatic function by VEGF-C therapy has been shown to limit the progression of atherosclerosis in *Ldrl* knock-out mice [245].

Heart LVs are indeed crucial to remove the excess of fluid generated by transmural pressures due to cardiac contraction [30]. Defective cardiac lymphatics can induce myocardial edema leading to impaired cardiac functioning [246], thus showing an important role of cardiac lymphatics in heart homeostasis. Moreover, in models of infarcted and dilated heart, lymphangiogenic therapy improves resolution of myocardial edema and inflammation, and prevents cardiac dysfunction and fibrosis [247,248].

Diabetic patients suffer cardiovascular pathologies as result of blood vasculature inflammation and decreased nitric oxide (NO) levels due to chronic hyperglycemia [249] but LVs in these patients are also affected. Type 2 diabetes patients harbour increased dermal LV density and LECs from these patients are remodelled and inflamed [250]. In a mouse model of type 2 diabetes, LVs have been shown to be leaky due to low NO availability [213]; moreover lymph leakage has been associated with the development of obesity, atherosclerosis, and oedema [17].

Several studies in humans have linked *PROX1* expression to the development of hyperlipidemia, obesity, and diabetes [251,252]. Experimentally, mice with a heterozygous *Prox1*-inactivating mutation present leaky LVs and develop obesity and inflammation with features associated with late-onset obesity in humans, such as higher levels of leptin and insulin and fatty liver [253]. Mechanistically, it was proposed that leaky lymph from disrupted LVs induced ectopic fat growth through stimulation of adipocyte differentiation and increased lipid storage [253]. Restoration of lymphatic vasculature was capable of rescuing the obese phenotype in *Prox1* $^{+/-}$ mice [254], therefore showing a direct connection between LV dysfunction and obesity development. Furthermore, *Vegfc* haploinsufficient mice (so-called Chy mice) display lymphedema and chronic tissue inflammation in association with abnormal fat accumulation [255]. Perilymphatic accumulation of inflammatory cells, production of cytokines, and increased expression of iNOS in obese mice have been proposed to induce LV permeability and inflammation (reviewed in [256]).

Patients with rheumatoid arthritis (RA), as well as mouse models of the disease, commonly present defective lymphatic functioning, lymphadenopathy and induced lymphangiogenesis [257,258]. VEGF-C treatment is able to reverse joint inflammation [205]; furthermore, excessive NO produced by iNOS+ innate immune cells during inflammation inhibits collecting LV contraction[267], therefore selective inhbition of iNOS may be useful to restore lymphatic function in RA[268].

An outstanding question is whether impaired function of aged lymphatic vasculature can be restored *in vivo*. A pro-lymphangiogenic therapy *via* delivery of VEGF-C already entered clinical trials (NCT03658967) and aged LVs conserve the ability to regrow in response to VEGF-C [200,204,205]. Interestingly, defective collagen IV transport in ECs of mice with inactivation of the negative Ras regulator *Rasa1* could be rescued by combined administration of chemical chaperones [260]. Small molecule-mediated activation of AKT or PIEZO1 signalling was able to promote formation of lymphatic valves *in vivo* [108,109] and lymphatic valve degeneration in *Foxc2*-deficient mice could be partially rescued by treatment with a ROCK inhibitor [138]. These results indicate that new therapeutic approaches can be envisaged for boosting defective ECM production or stimulating the repair of lymphatic valves in order to improve function of aged collecting LVs.

6. Conclusions and perspectives

The field of lymphatic vascular biology has gained recognition during the past decades, shedding light not only to its crucial role in homeostasis, but also in various disease conditions. Here, we have described the general mechanisms of LV development and functioning, however a number of important questions remain to be answered. These questions include the impact of developmental origins in responses of LECs to pathophysiological stimuli; characterization of organotypic features of LECs; the cross-talk of LVs with other cell types in the maintenance of organ functions and the role of LVs in immunological functions beyond the transport of antigen-presenting cells. Lymphatic vascular diseases, such as lymphedema, exhibit a pronounced sexual dimorphism, with predominance of female patients [261], therefore future studies should also address the mechanisms underlying such sex-specific response of LVs. Finally, incorporating LVs into studies of age-related conditions, including neurodegeneration, cancer and cardiovascular diseases, will be necessary for better understanding of underlying pathophysiological mechanisms and development of targeted therapeutic interventions.

Declaration of Competing Interest

TVP receives research funding from Roche

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