Smooth muscle cell recruitment to lymphatic vessels requires PDGFB and impacts vessel size but not identity

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Abstract

Tissue-fluid drains through blind-ended lymphatic capillaries, via smooth muscle cell (SMC)-covered collecting vessels into venous circulation. Both defective SMC recruitment to collecting vessels and ectopic recruitment to lymphatic capillaries are thought to contribute to vessel failure, leading to lymphedema. However, mechanisms controlling lymphatic SMC recruitment and their role in vessel maturation are unknown. Here we demonstrate that platelet-derived growth factor B (PDGFB) regulates lymphatic SMC recruitment in multiple vascular beds. PDGFB is selectively expressed by lymphatic endothelial cells (LECs) of collecting vessels. LEC-specific deletion of Pdgfb prevented SMC recruitment causing dilation and failure of pulsatile contraction of collecting vessels. However, vessel remodelling and identity were unaffected. Unexpectedly, PDGFB overexpression in LECs did not induce SMC recruitment to capillaries. This was explained by the demonstrated requirement of PDGFB extracellular matrix (ECM) retention for lymphatic SMC recruitment, and low presence of PDGFB-binding ECM components around lymphatic capillaries. These results demonstrate a requirement of LEC-autonomous PDGFB expression and retention for SMC recruitment to lymphatic vessels and suggest an ECM-controlled checkpoint preventing SMC investment of capillaries, which is a common feature in lymphedematous skin.
Introduction

The lymphatic vasculature in mammals controls homeostasis by transporting liquid from tissue back to the blood circulation. It is also part of the immune system by filtering the lymph through lymph nodes for immune surveillance. The lymphatic vasculature is organised in a hierarchy of capillaries, pre-collectors and collecting lymphatic vessels, each with specific functional and morphological features (Lutter et al., 2012; Ulvmar and Makinen, 2016). Lymphatic capillaries lack mural cell coverage and are blind-ended vessels with permeable button-like cell junctions, which allow for fluid uptake and immune cell entry from the tissue (Baluk et al., 2007; Pflicke and Sixt, 2009). Downstream of the capillaries are the pre-collectors, which are sparsely covered by smooth muscle cells (SMCs) and drain into the collecting vessels with more extensive SMC coverage. Here, valves and continuous zipper-like endothelial cell junctions ensure an efficient unidirectional lymph transport (Alitalo, 2011; Baluk et al., 2007; Koltowska et al., 2013; Schulte-Merker et al., 2011; Ulvmar and Makinen, 2016).

Impaired lymphatic drainage causes accumulation of fluid in the tissue and leads to swelling, referred to as lymphedema. Long-term symptoms include discomfort, pain and an increased incidence of infections (Williams et al., 2005). Lymphedema can be caused by genetic mutations (primary), or trauma, infections, cancer surgery and/or irradiation (secondary), however the knowledge on the pathological mechanisms is still limited (Szuba and Rockson, 1998). Collecting lymphatic vessels and their valves are known to be crucial for proper lymph drainage but the role of SMCs in this process is only partly understood. For example, SMC contraction has been shown to promote lymph drainage in the larger collecting vessels such as the popliteal vessels (Kunert et al., 2015; Liao et al., 2011), but their importance in relatively smaller collecting vessels such as in the skin that is commonly involved in lymphedema is not known. Nevertheless, deficient SMC function has been suggested to be an integral part of chronic lymphedema in humans (Ogata et al., 2015). In patients suffering from Lymphedema Distichiasis (LD), caused by mutations in the forkhead transcription factor FOXC2, lymphedema is accompanied by valve agenesis but also by aberrant recruitment of SMCs to lymphatic capillaries (Petrova et al., 2004). Genetic deletion of Foxc2 as well as Ang2 or Efnb2 in mice recapitulates this disease phenotype and leads to profound lymphatic remodelling defects characterised by defective
valve formation, SMC recruitment and establishment of collecting vessel and capillary lymphatic endothelial cell (LEC) identities (Gale et al., 2002; Makinen et al., 2005; Norrmen et al., 2009; Petrova et al., 2004; Sabine et al., 2015). Altogether, these studies suggest an important role of SMC- and LEC- interplay in lymphatic morphogenesis and function, but precisely how SMCs regulate these processes is not known.

The successive steps of SMC recruitment to the lymphatic vasculature during development have been characterized in both dermal and mesenteric lymphatic vessels (Lutter et al., 2012; Norrmen et al., 2009). In the mouse ear, SMCs start to colonize the collecting vessels at postnatal day (P) 14, which coincides with down-regulation of lymphatic vessel hyaluronan receptor 1 (LYVE-1). From P16, only lymphatic capillaries maintain Lyve-1 expression and also remain devoid of SMCs throughout life. The reciprocal cellular interaction between SMCs and LECs has thus been suggested to regulate the establishment of capillary versus collecting vessel identity. Supporting this notion, SMC-LEC contact induces secretion and activation of the extracellular matrix glycoprotein Reelin specifically in LECs of collecting vessels, which in turn promotes further SMC recruitment (Lutter et al., 2012). In Reln−/− mice, reduced SMC coverage is accompanied by sustained LYVE-1 expression in collecting vessels. Platelet-derived growth factor B (PDGFB), which is central for mural cell (SMCs and pericytes) recruitment to the blood vasculature (Hellstrom et al., 1999), is also expressed by LECs in vivo and may be similarly involved in recruitment of SMCs to lymphatic vessels (Tammela et al., 2007). For example, mesenchymal overexpression of PDGFB was shown to lead to SMC dissociation both from lymphatic vessels and veins (Tammela et al., 2007). Also, upregulation of PDGFB in lymphatic capillaries of LD patients, proposed to be a consequence of loss of a direct FOXC2 suppressive effect, was suggested to underlie their ectopic SMC coverage (Meinecke et al., 2012; Petrova et al., 2004; Tammela et al., 2007). However, FOXC2 is highly expressed in developing collecting vessels to which SMCs are recruited. Hence FOXC2-mediated inhibition of PDGFB cannot explain the differential SMC recruitment to collecting vessels and capillaries, at least not during development. Altogether these data suggest that SMC recruitment is important for the maturation of vessels into functional collecting vessels, nevertheless the direct role of SMCs in this process is not known.

Herein, we reveal that expression of Pdgfb within the developing dermal lymphatic vasculature is restricted to LECs of collecting vessels but absent from capillaries. Using conditional loss- and gain-of-function models, we show that LEC expression of PDGFB is
required for SMC recruitment to collecting vessels, but that overexpression is insufficient to mediate recruitment to lymphatic capillaries. We find that in addition to PDGFB expression the ligand relies on its binding and retention to the local extracellular matrix to promote SMC recruitment. We also show that while SMCs are crucial for pulsatile contraction of collecting vessels, they are not required for dermal lymphatic vessel remodelling, valve morphogenesis and establishment of capillary versus collecting vessel identity.

**Results**

**PDGFB is selectively expressed by LECs of SMC covered collecting vessels but not by capillary LECs**

SMCs cover arteries and veins of the blood vasculature as well as the collecting lymphatic vessels (Fig. 1A). PDGFB is known to regulate mural cell recruitment to blood vessels but its potential involvement in the analogous process in the lymphatic vasculature has not been demonstrated. To characterize the precise cellular source of PDGFB within the developing vasculature we utilized a double transgenic mouse; Pdgfb-CreER\(^{T2}\)-IRES-egfp;R26-mTmG, carrying a Pdgfb-promoter-driven tamoxifen-inducible Cre-recombinase, a Pdgfb-driven GFP, as well as a conditional allele (loxp flanked stop) for inducible expression of a membrane bound GFP functioning as a lineage tracer (Claxton et al., 2008; Muzumdar et al., 2007). Tamoxifen administration at P14 and analysis by immunostaining for GFP at P21 allowed for identification of cells expressing Pdgfb at either time point, as well as the progeny of PDGFB+ cells in P14 ears. In the mouse ear skin GFP immunoreactivity revealed that Pdgfb was expressed throughout the blood vasculature whereas in the lymphatics it was restricted to LECs of collecting vessels and absent in capillaries (Fig. 1A,B,B’,C,C’). The spatial distribution of SMCs within the lymphatics inversely correlated with the expression of the lymphatic capillary marker LYVE-1 (Fig. 1A,B,B’,C,C’). To relate the expression of the PDGFB ligand to the localisation of cells expressing its main receptor - PDGFRβ, we utilized a transgenic reporter mouse line with Pdgfrb promoter-driven expression of GFP (here denoted Pdgfrβ-GFP). Staining of the ear skin of P21 mice revealed co-localisation of GFP and α-smooth muscle actin (α-SMA), indicative of Pdgfrb expressing SMCs in collecting vessels (Fig. 1D,D’). No GFP signal was observed in LYVE-1+ lymphatic capillaries thereby indicating absence of other potential α-
SMA-Pdgfrβ-GFP+ mural cells, such as pericytes (Fig. 1E,E’). These results demonstrate that unlike the ubiquitous expression of Pdgfb in the blood vasculature, Pdgfb is restricted to collecting vessels within the lymphatic vasculature. The spatial correlation between LECs producing PDGFB and mural cells expressing PDGFRβ suggests that this ligand-receptor pair is involved in lymphatic SMC recruitment.

In addition to recruitment, SMC in situ proliferation may contribute to the final coverage of developing collecting lymphatic vessels. To investigate this we administered EdU to wildtype mice, starting at the time of initial SMC recruitment (P14), and continued at P16 and P18 to cover the time period of collecting vessel maturation. Analyses of SMC coverage and proliferation at P21 (Fig. 1F,G,H,I,I’,J,K) showed EdU+ SMCs along the entire length of collecting vessels. Although SMC coverage was higher in the proximal than in the distal ends of vessels the proximal regions showed a relatively lower degree of SMC proliferation (Fig. 1I,I’,J,K), suggesting that proliferation ceases as vessels mature. Interestingly, within the distal parts of the vessels EdU+ SMCs were confined to regions near vessel branches (Fig. 1I,I’). Together, these data suggest that SMCs not only populate the lymphatics by initial recruitment but also by subsequent proliferation.

**Postnatal LEC-specific deletion of Pdgfb prevents SMC recruitment to dermal collecting lymphatics causing vessel dilation without affecting vessel hierarchy**

To investigate the role of LEC-autonomous PDGFB in the recruitment of SMCs, we generated Prox1-CreERT2; Pdgfblox/lox; R26R-eYFP mice (henceforth denoted PdgfbiLECKO). These mice allowed for tamoxifen-induced LEC-specific deletion of Pdgfb, as well as identification of recombination by YFP expression. Daily tamoxifen administration from P4 to P7 and staining of the ear skin at P21 indicated specific recombination in the lymphatic vasculature, in accordance with previous data (Fig. 2A)(Bazigou et al., 2011). In addition, collecting lymphatic vessels of the PdgfbiLECKO mice displayed a near complete SMC deficiency (1.0% SMC coverage in PdgfbiLECKO versus 44.2% in control) as revealed by image analysis (Fig. 2A,B,C,D; Fig. S1A,B). Immunolabelling for PDGFRβ illustrated the absence of other potential PDGFRβ+;α-SMA- mural cell populations (Fig. S1E,E’,F,F’). No apparent change in blood vascular morphogenesis or mural cell coverage was recorded (Fig. S1C,D). Furthermore the SMC-devoid lymphatic collecting vessels of the PdgfbiLECKO mice displayed increased diameter (Fig. 2E), but showed no alteration in lymphatic capillary morphology.
In the absence of SMCs in the Pdgfb mutant, LYVE-1 was downregulated in the collecting vessels to a similar degree as in controls. (Fig. 2B’,C’,F). Also, the number and architecture of lymphatic valves were unchanged in the Pdgfb^{iLECKO} mice compared to controls (Fig. 2G). We further assessed mural cell coverage of collecting vessels following induced deletion of Pdgfb using the Cdh5(PAC)-CreERT2 mouse that targets both blood and lymphatic vessels (Wang et al., 2010). Tamoxifen induction and analysis of Cdh5(PAC)-CreERT2; Pdgfb^{flox/flox}, R26R-eYFP (Pdgfb^{iECKO}) mice revealed recombination (YFP+) in both blood- and lymphatic endothelial cells (Fig. S2D). Mural cell coverage of blood vessels was not drastically affected, however SMC coverage of lymphatic collecting vessels was reduced (Fig. S2A,B,C), thereby recapitulating the observations from the Pdgfb^{iLECKO} mice. The lack of an obvious effect on blood vessel SMC coverage may reflect a higher degree of maturation of the blood vasculature compared to the lymphatic vessels at the time of induction. Together, these data provide direct genetic evidence for the requirement of LEC-derived Pdgfb in the recruitment of mural cells to the lymphatic collecting vessels and indicate that SMCs are not required for lymphatic vessel remodelling into a hierarchy of collecting vessels and capillaries.

**Establishment of collecting versus capillary LEC identities does not require SMC interaction**

The basement membrane (BM) of collecting vessels is continuous whereas that of capillaries is described as discontinuous (Pflick and Sixt, 2009). To further investigate the potential involvement of SMCs in the maturation of collecting vessels we studied the abundance of BM proteins in lymphatic vessels of the Pdgfb^{iECKO} mice. Immuno-staining for Pan-laminin (Fig. 3A,A’,B,B’) and collagen IV (Fig. 3C,C’,D,D’) revealed no differences between dermal lymphatic vessels in the Pdgfb^{iECKO} and control ears in either intensity or continuity. Also, VEGFR3, a ubiquitous LEC marker, was unchanged in both collecting vessels (Fig. 3E,E’,F,F’) and capillaries (Fig. 3G,G’,H,H’). The expression pattern of the capillary-restricted CCL21 as well as the junction protein Vascular endothelial Cadherin (VE-CAD) was unaffected in the Pdgfb^{iLECKO} mice (Fig. 3J,J’,L,L’) compared to control mice (Fig. 3I,I’,K,K’). However, despite normal levels and junctional localisation of VE-CAD in LECs of collecting vessels of Pdgfb^{iLECKO} mice, individual cells were enlarged compared to LECs from control mice (Fig. 3M,M’,N,N’,O). Together, these data demonstrate that the establishment of lymphatic vessel identity and vascular hierarchy is independent of SMC
interaction but that such interaction affects cellular size. They furthermore indicate that the contribution of SMCs to the major constituents of the BM of collecting vessels is minor.

**PDGFB is required for SMC recruitment to the large diameter collecting vessels of the hind limb and mesentery**

Recruitment of mural cells to larger diameter collecting vessels such as the popliteal and mesenteric collecting vessels occurs during embryogenesis. To study the involvement of PDGFB in these tissues we deleted PDGFB in \textit{Pdgfb}^{iLECKO} embryos by administration of 4-OHT to pregnant females at embryonic day (E)15, E16 and E17, and analysed the embryos at E18.5. SMC coverage in the \textit{Pdgfb}^{iLECKO} mice was reduced in both popliteal (Fig. 4A,A',B,B',C) and mesenteric lymphatic vessels (Fig. 4E,E',F,F',G) compared to controls. Although the diameter of popliteal lymphatic vessels was unaltered (Fig. 4D), mesenteric vessels were enlarged (Fig. 4H), suggesting tissue specific consequences of SMC reduction.

To investigate if PDGFB is also required for expansion and maintenance of SMCs after their initial recruitment, PDGFB deletion was induced at P1 and P2 and lymphatic vessels were compared between \textit{Pdgfb}^{iLECKO} and control mice at P12. SMC coverage of the popliteal vessels was reduced to 73.8% comparing to 93.1% of the control (Fig. 4I,I',J,J',K), while it remained unchanged in the mesenteric vessels (Fig. 4M,M',N,N'). Interestingly, local regions lacking SMC coverage in the popliteal vessels of \textit{Pdgfb}^{iLECKO} were bulging, demonstrating local effects of SMCs in constriction of the vessel (Fig. 4J,J', arrow heads). Nevertheless the average vessel diameter remained unchanged (Fig. 4L). All together, these data revealed a strict requirement of LEC-derived PDGFB in SMC recruitment also to large diameter collecting vessels of the hind limb and mesentery.

**Loss of SMCs causes impaired contraction of dermal collecting vessels**

Despite the near complete absence of dermal lymphatic SMC coverage in the \textit{Pdgfb}^{iLECKO} mice no obvious lymphedema could be observed. Although contraction of large diameter vessels such as popliteal and mesenteric lymphatic vessels has been previously documented, dermal vessel contraction remains poorly studied and has not been correlated to SMC function or presence (Liao et al., 2011; Sabine et al., 2015). To further assess potential effects of mural cell loss on lymphatic properties we studied lymphatic vessel contraction and drainage following tracer injection, utilizing non-invasive in vivo live-imaging of the ear skin. By high-frequency non-invasive imaging pulsatile vessel
contraction was recorded (Movie S1). Dermal collecting vessels displayed a wide range of contractile frequency and amplitude (Fig. 5A,B,C,D). Notably, only a subpopulation of collecting dermal lymphatic vessels was seen to contract under the experimental conditions applied. To investigate the dependence and role of SMCs on this contractile behaviour Pdgfb^{iLECKO} mice (n=6) and control mice (n=10) at an age of one month were imaged and compared. The number of contraction sites per ear was significantly lower in Pdgfb^{iLECKO} mice compared to control mice (Fig. 5E; p<0.05, Mann-Whitney Test; Movie S2,3). Out of 10 control mice analysed, 32 contraction sites were observed in 7 mice and the contraction sites associated with SMC coverage, indicated by immunofluorescence staining of the same ear after live imaging (Fig. 5F,F'; Movie S4). However, only 1 out of 6 Pdgfb^{iLECKO} mice displayed collecting vessel contraction, restricted to 2 sites. Importantly, immunofluorescent staining of this ear revealed local sparse SMC coverage precisely matching the sites observed to contract during live imaging (Fig. 5E, asterisk; Fig. 5G,G'; Movie S5). These observations demonstrate that SMCs are strictly required for pulsatile lymphatic vessel contraction. To test the functionality of the non-contracting and widened lymphatic vasculature a fluorescent tracer was subcutaneously injected into the ear of Pdgfb^{iLECKO} and control mice. Live imaging did however not reveal any difference in the clearance of the injected tracer from tissue over time (Fig. S3).

Postnatal LEC-specific overexpression of PDGFB does not induce ectopic SMC recruitment to lymphatic capillaries

To investigate whether aberrant PDGFB expression within lymphatic capillaries is sufficient to induce SMC recruitment, we crossed Prox1-CreERT2 mice with R26hPDGFB^{+/+} mice (Armulik et al., 2010) allowing for inducible LEC-specific overexpression of human PDGFB. Endothelial cell-specific expression of the R26hPDGFB^{+/+} allele has been demonstrated to rescue embryonic lethality and mural cell recruitment of Pdgfb^{i-} mice (Armulik et al., 2010). Prox1-CreERT2; R26hPDGFB^{+/+} mice (Pdgfb^{iLECOE}) and controls received three doses of tamoxifen between P2 and P8, followed by analysis of the dermal ear lymphatic vasculature at 4 weeks of age. Prox1-CreERT2 mediated recombination driving expression of the human PDGFB was observed throughout the lymphatic vasculature, as indicated by visualisation of the conditional reporter (data not shown; see Fig. 2A). Ectopic expression in lymphatic capillaries was confirmed by detection of human PDGFB transcripts in FACS-sorted LYVE-1+ LECs from dermal ear skin of Pdgfb^{iLECOE} mice.
(Fig. S4A). SMC coverage of the collecting vessels of *Pdgfb*<sup>LECOE</sup> mice was modestly increased compared to littermate controls (Fig. 6A) (44.5% versus 33.4%, *p*<0.05) illustrating functionality of induced overexpression as well as a PDGFB dosage effect on SMC recruitment. Collecting vessels in *Pdgfb*<sup>LECOE</sup> mice further showed a trend towards reduced average diameter (Fig. 6B). However, despite aberrant PDGFB expression in capillary LECs, no α-SMA or PDGFRβ positive cells were recruited to lymphatic capillaries (Fig. 6C,D,E,F). These data demonstrate that, although PDGFB is essential for SMC recruitment to the lymphatic vasculature, LEC expression is not sufficient to mediate recruitment of SMCs to lymphatic capillaries.

**Deficient interaction between PDGFB and extracellular matrix causes defective SMC recruitment to dermal collecting lymphatic vessels**

Mural cell recruitment to the blood vasculature relies not only on endothelial cell production of PDGFB but also on its binding to the extracellular matrix. To investigate whether such interaction is important also for the lymphatic vasculature, we studied mice with a genetically modified PDGFB that lack its Heparan sulphate-binding domain (*Pdgfb*<sup>ret/ret</sup>)(Lindblom et al., 2003). Here, heterozygote *Pdgfb*<sup>ret/wt</sup> served as controls as they showed no alterations of collecting vessels (Fig. 7A). Similar to the situation in *Pdgfb*<sup>LECKO</sup> mice, *Pdgfb*<sup>ret/ret</sup> mice displayed reduced SMC coverage and dilation of dermal ear lymphatic collecting vessels (Fig. 7A,B,C,D), without affecting density or number of branch points of the lymphatic capillaries (Fig. 7E,E’,F,F’). These results show that interaction between extracellular matrix and PDGFB is required for recruitment of SMCs to the lymphatic collecting vessels of the skin. Differential composition of the collecting versus capillary BMs, with respect to PDGFB-binding molecules, may thus contribute to the inability of ectopically expressed PDGFB to induce mural cell recruitment to the lymphatic capillaries. To investigate this we assessed lymphatic vascular expression and deposition of heparan sulphate proteoglycan perlecan as well as collagen IV, which are known to bind PDGFB either via the heparan sulphate chains or directly via the core protein (Gohring et al., 1998). Immunostaining for perlecan and collagen IV revealed high levels of both proteins in dermal collecting vessels (Fig. 7I,I’,K,K’,M,M’) but strikingly lower levels in capillaries (Fig. 7J,J’,L,L’,N,N’). These data suggest that in the absence of PDGFB-binding BM
proteins, PDGFB may not be sufficiently retained in close proximity to the LECs of capillaries to allow for recruitment of mural cells, even following ectopic PDGFB expression.

**Discussion**

Here we generated novel mice allowing for inducible LEC-specific deletion of PDGFB. PDGFB deletion rendered the dermal lymphatic vasculature of the ear completely devoid of SMCs and led to reduced SMC coverage of mesenteric and popliteal vessels, in turn revealing a strict requirement for LEC-derived PDGFB in SMC recruitment to collecting vessels. Utilising this model we provide the first description of the direct role of SMCs in lymph vessel morphogenesis and function, independent of other factors. The data resolve that several key aspects of lymphatic vascular development do not rely on SMCs unlike previously suggested.

SMC recruitment to the collecting vessels has been shown to coincide with vessel remodelling and maturation. Several studies have in addition revealed abnormal SMC coverage on lymphatic vessels in disease as well as in genetic mouse models. Some of these investigations have inferred an inverse correlation between SMC coverage and LYVE-1 expression (Dellinger et al., 2008; Lutter et al., 2012; Meinecke et al., 2012; Petrova et al., 2004; Yu et al., 2016). In support of the role of SMCs in promoting Lyve-1 downregulation, Tammela et al showed that mesenchymal over expression of PDGFB in the mouse ear skin resulted in displacement of collecting vessel SMCs, accompanied by increased expression of LYVE-1 (Tammela et al., 2007). In addition, reduced SMC coverage of Reln-deficient collecting vessels correlated with increased LYVE-1 expression (Lutter et al., 2012). In none of the previous studies, the contribution of potential secondary and systemic effects could be ruled out. Here however we found that collecting vessels in Pdgfb\_LECKO mice displayed normal (low) LYVE-1 levels in spite of the near-complete absence of SMCs. This indicates that SMC contact *per se* does not lead to downregulation of LYVE-1, which is considered a hallmark of establishment of collecting vessel identity. Together with the inability of ectopic expression of PDGFB in lymphatic capillaries to induce recruitment of SMC, these data suggest that lymphatic capillary- versus collecting- vessel identities are not directly dictated by SMC interaction.

The degree of SMC proliferation during the process of SMC coverage within lymphatic vessels had not been investigated. Our analysis revealed a higher ratio of proliferating SMCs in the “younger” distal collecting vessels than the “older” proximal half,
suggesting that proliferation mainly occurs during the collecting vessel maturation process. Although PDGFB likely contributes also to proliferation, the near total absence of SMCs in collecting vessels of Pdgfb\textsuperscript{iLECKO} shows the strict requirement for PDGFB in the recruitment of the initial pool of SMCs. Furthermore the reduced coverage seen in the Pdgfb\textsuperscript{ret/ret} mice, in which the signalling of the mutant PDGFB is not altered but only its ability to be retained within the local extracellular matrix, further suggests that the initial recruitment is dependent on LEC-derived PDGFB.

Pulsatile contraction of perivascular SMCs in major collecting vessels is known to contribute to the efficiency of lymph drainage (Kunert et al., 2015; Zawieja, 2009). However whether such contractions are required during normal physiology, or even in pathology, has not been thoroughly assessed. Furthermore the functional impact of a similar contraction in the skin had not been studied. Here Pdgfb\textsuperscript{iLECKO} mice displayed severely impaired dermal lymphatic vessel contraction due to loss of SMCs, but with no apparent lymphedema and no recordable change in lymphatic drainage in the applied experimental setup (Fig. S3). It should be noted that even genetic mouse mutants that display severe lymphatic vessel defects, such as hypoplasia of lymphatic capillaries in the mouse model of Milroy disease, a form of primary lymphedema (Karkkainen et al., 2001), do not show as severe tissue swelling as human patients with the corresponding genetic defect. It is therefore likely that loss of dermal SMCs would have more dramatic consequences in humans.

Ectopic SMC coverage of the lymphatic capillaries has been observed in both primary and secondary lymphedema (Yu et al., 2016) and is postulated to inhibit lymphatic drainage function. In patients with primary lymphedema, due to FOXC2 loss of function mutations, the cause of ectopic SMC recruitment was suggested to be a consequence of induction of PDGFB expression within the capillaries. Indeed dermal lymphatic capillaries of Foxc2\textsuperscript{-/-} embryos displayed abnormal PDGFB expression, but whether this alteration was sufficient or required in this pathology is not clear. Our data show that genetically induced LEC-specific overexpression of PDGFB is not sufficient to drive recruitment of SMCs to lymphatic capillaries. These data, together with the increased SMC coverage in patients with secondary lymphedema, indicate that other alterations are required in addition to induced PDGFB expression. Our results demonstrate that PDGFB requires the binding to heparan/chondroitin sulfate chains to mediate normal SMC recruitment to the collecting vessels. Whereas collecting vessels have a continuous BM, lymphatic capillaries display
only discontinuous BMs and as shown here with very low levels of the PDGFB-binding perlecan and collagen IV, thereby potentially limiting PDGFB retention to LECs. Interestingly Foxc2−/− mice display increased deposition of BM components within the defective lymphatic capillaries, which may act together with PDGFB to mediate ectopic SMC recruitment (Petrova et al., 2004). Extracellular matrix alterations may also influence integrin-mediated SMC migration and adhesion, adding to the complexity. In addition the retention motif of PDGFB can be cleaved by proteases, with potential differential abundance or activity in capillaries and collecting vessels. Finally, it is possible that SMC recruitment to lymphatic capillaries is actively inhibited under normal physiological conditions. It was shown that Sema3-Nrp1-PlexinA1 signalling prevents recruitment of SMCs to the valve regions of collecting vessels to ensure normal valve morphogenesis and function (Bouvree et al., 2012; Jurisic et al., 2012) and a similar mechanism may exist in lymphatic capillaries.

Precisely how the lymphatic vasculature acquires and maintains its hierarchical structure of capillaries and collecting vessels is not well understood (Schulte-Merker et al., 2011). Herein we demonstrate that the establishment of vessel identity is mainly unaffected by a complete inhibition of lymphatic mural cell recruitment—a process here shown to be strictly dependent on lymphatic endothelial PDGFB expression and pericellular retention. We also clarified that erroneous spatial expression of PDGFB unlikely serves as the single cause of ectopic recruitment of SMCs to lymphatic capillaries in disease. The genetic mouse model of lymph vessel-specific mural cell deficiency provides a novel and specific tool for further studies on the importance of SMCs in lymphatic development and lymphedema.
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Competing financial interests

The authors declare no competing financial interests.

Author contributions

Y.W. conducted experiments and prepared figures. Y.J. and Y.Z. helped with the experiments of SMC proliferation, characterization of lymphatic morphogenesis and data analysis. M.A.M. managed mice breeding and experiments of Tamoxifen induction. H.O. helped with FACS and imaging of lymphatic drainage. C.B. helped with data analysis and discussions. L.J. and T.M. designed the project and analysed data. Y.W., L.J., T.M., and C.B. wrote the paper. L.J. supervised all aspects of the project.

Materials and Methods

Mice and treatments

All animal experiments included male and female mice. Pdgfrβ-eGFP mice (Gensat.org. Line name: Tg(Pdgfrb-eGFP) JN169Gsat/Mmucd) express GFP under the control of the Pdgfrβ promoter and hence function as mural cell reporter. Pdgfb-CreER$^{T2}$ and R26-mTmG mice were previously described (Claxton et al., 2008; Muzumdar et al., 2007) and here combined
by inter-crosses to generate Pdgfb-CreERT2, R26-mTmG mice. To evaluate differential Pdgfb expression within the dermal lymphatic vasculature 4-Hydroxytamoxifen (4-OHT) was injected into the abdominal cavity at P14 and mice were sacrificed at P21 and ear samples were then fixed in 4% PFA. Prox1-CreERT2 and Pdgfb flox/flox mice were previously described (Bazigou et al., 2011; Enge et al., 2002) and herein crossed with the so called Ai3 reporter mouse (B6.Cg-Gt(Rosa)26Sor tm3(CAG-EYFP)Hze/J, Stock Number 007903, The Jackson Laboratory, here denoted R26R-eYFP) to generate the Prox1-CreERT2, Pdgfb flox/flox, R26-eYFP (Pdgfb iLECKO) mice. Recombination was induced from P4 to P7 by oral administration of Tamoxifen (20mg/kg) to the mother. Alternatively, 150 µg of Tamoxifen, dissolved in acetone (10 mg/ml), was applied topically to the abdominal skin of the pups. Mice were sacrificed at P21 to analyse the dermal ear vasculature. To analyse mesenteric lymphatic vessels and popliteal lymphatic vessels of hind limb at E18.5, recombination was induced from E15 to E17 by injection of 1mg 4-OHT, dissolved in peanut oil (10 mg/ml) to the mother. To analyse mesenteric lymphatic vessels and popliteal lymphatic vessels at P12, recombination was induced at P1 and P2 by topical-abdomen treatment of 150 µg tamoxifen. Pdgfb ret/ret and Pdgfb ret/wt mice were previously described (Lindblom et al., 2003). Briefly, Pdgfb ret/ret mice lack the heparan sulphate binding domain of PDGFB, following gene targeting. Single allele knockout mice do not present a phenotype (Pdgfb ret/wt) and were hence used as controls. Mice were analysed at 10 weeks age. The Pdgfb flox/flox mice were also crossed with Cdh5(PAC)-CreERT2 mice(Wang et al., 2010) to generate Cdh5(PAC)-CreERT2, Pdgfb flox/flox mice and then crossed with the R26R-eYFP mice (B6.Cg-Gt(Rosa)26Sor tm3(CAG-EYFP)Hze/J, Stock Number 007903, The Jackson Laboratory). In order to specifically delete Pdgfb in the mouse endothelium these mice received 50 µl Tamoxifen (20 mg/ml) at P17 and sacrificed at 4 months. Animal experiment protocols were approved by the Stockholm North Ethical Committee on Animal Research (Permit number N14/13, N168/14), the Uppsala Ethical Committee on Animal Research (Permit number: C224/12, C225/12, C130/15). All animal experiments were carried out in accordance with their guidelines.

Whole mount immunofluorescence staining

Ears and hind limbs were fixed in 4% PFA at room temperature for two to four hours and then either stored in PBS with 0.01% NaN3 or immediately processed for
immunofluorescence staining. To dissect the ear and expose lymphatic vasculature, hair was removed with fine surgical forceps and scissors (Agnthos) and two layers of superficial skin were separated to expose the dermal layer of the ear. Muscles and fat were carefully trimmed away. To isolate the popliteal vessels from the hind limb of pups at P12, the skin was removed and the exposed blood and lymphatic vessels were dissected together with the underlying muscles. Dissected ears or tissues from hind limbs were then washed with PBS 3 x 10 minutes on a rocking table at room temperature. Samples were then blocked in PBS with 1.5% BSA and 0.5% TritonX-100 for 3 hours at room temperature followed by addition of primary antibodies and incubation overnight at 4°C. Samples were then washed in PBS with 0.25% TritonX-100 three times, one hour each, at room temperature on a rocking table followed by secondary antibody incubation overnight at 4°C. After three washes (one hour each) in PBS with 0.25% TritonX-100 at room temperature on a rocking table, ear samples were flattened on a glass slide and mounted with Prolong Gold (Life Technologies). Mesenteries were fixed in 4% paraformaldehyde at room temperature for two hours. Samples were washed in PBS, permeabilized in 0.3%-Triton X-100 in PBS (PBSTx), and blocked in 3% BSA in PBSTx. Primary antibody incubation was performed at 4°C overnight, followed by washing in PBSTx and incubation with secondary antibodies at room temperature for 2 hours. Samples were then washed in PBSTx and mounted in Prolong Gold for imaging. Primary antibodies include goat anti Podocalyxin (AF1556, R&D Systems), goat anti CD31 (AF3628, R&D System), Rat anti CD31 (553370, BD Pharmingen) rabbit anti ERG (ab92513, Abcam), chicken anti GFP (ab13970, Abcam), mouse anti α-smooth muscle actin (α-SMA) (c6198, Sigma), rabbit anti PDGFRβ (ab32570, Abcam), rat anti PDGFRβ (14-1402-82, eBioscience), rabbit anti PROX-1 (Martinez-Corral et al., 2015), Goat anti NRP-2 (AF567, R&D Systems), rat anti LYVE-1 (#0117, R&D Systems) rabbit anti LYVE-1 (ab14917, Abcam), goat anti CCL21 (AF457, R&D Systems), goat anti VEGFR3 (AF743, R&D Systems), rat anti VE-Cadherin (550548, BD Pharmingen), rabbit anti collagen IV (2150-1470, Bio-Rad), Rat anti Perlecan (ab17848, Abcam) and rabbit anti Pannexinin (L9393, Sigma Aldrich). Secondary antibodies conjugated with Alexa Fluorophores were from Jackson ImmunoResearch Laboratories and Life Technologies.

Assessment of SMC proliferation

To assess proliferation of SMCs, mice received three intraperitoneal injections of 5-ethyl-2-deoxyuridine (EdU, 100 µg/mouse) at P14, P16, P18 and sacrificed at P21 for
immunofluorescence staining of the ear. EdU staining was performed using the Click-iT EdU imaging kit (Life Technologies) apart from a 3 hours incubation of reaction cocktail at room temperature.

**In vivo imaging of dermal ear lymphatic vessel contraction**

For evaluation of dermal lymphatic vessel contraction, mice were anaesthetized with isoflurane and hair on the dorsal side of the ears was removed by a sharp blade. Head and nose of the mouse was fixed to a customized head holder device and the left ear was glued to a customized plastic plate to prevent movement. 1 µl of TRITC-Dextran (10 mg/ml, 500kD, Sigma-Aldrich) was injected subcutaneously with a (30G) insulin syringe (BD Biosciences) or a Hamilton syringe. Mice were then immediately transferred to the imaging stage for time-lapse epifluorescence imaging using either the 20x/1.0 objective on a Leica SP8 laser confocal microscope system (Movie S1, images acquired every 1 second) or Leica M205FA microscope with a PLANAPO 1.0x objective (Leica Microsystems) (Movie S2,3,4,5, images acquired with interval less than 1 second ).

**Imaging acquisition of immunofluorescence stained specimens**

Confocal images of lymphatic vessels of the ear skin, the popliteal lymphatic vessels or mesenteric lymphatic vessels were acquired utilizing a Zeiss LSM 700 system (Carl Zeiss) with a 20x/0.8 objective or a Leica SP8 laser confocal microscope system (Leica Microsystems) with either of 25x/1.0, 25x/0.95, 20x/0.75, 10x/0.3 objectives. The images represent maximum intensity projections of z stacks, that in the case of overviews were stitched from multiple tile scan images, either manually using Adobe Photoshop (Adobe) or automatically by the Leica LAF software. Images were processed with FIJI (Schindelin et al., 2012) or Adobe Photoshop software (Adobe). Intensity Adjustments of Figure 5F’ were applied specifically to the vessel area (dashed line) in order to enhance the visibility of perivascular SMCs. Tiled epi-fluorescence images showing the entire capillary network were acquired using an Axio Observer Z1 system (Carl Zeiss) with a 5x/0.13 objective and images were automatically aligned by Zen blue 2012 software (Carl Zeiss).

**Image analysis**

To quantify EdU+ cells/mm² SMC surface area, pictures of complete lymphatic collecting vessels were created by manual alignment (Adobe Photoshop) of individual high resolution
Cells double positive for EdU and α–SMA were manually counted and SMC surface area of a complete collecting vessel was measured in Volocity (Perkin Elmer). To quantify collecting vessel width and SMC coverage, regions with excessive branches and intersecting blood vessels were excluded and vessel width was measured along the vessel and averaged by number of measurements. SMCs coverage was quantified in Volocity (Perkin Elmer)(dermal ear skin, popliteal lymphatic vessels) or FIJI (Schindelin et al., 2012)(mesenteric lymphatic vessels) and indicated as percentage of vessel area covered by SMC areas. “Capillary density” was measured in Volocity by area of capillaries out of the complete region of interest. Branch points/mm² lymphatic capillaries within a defined region of interest, were measured using ImageJ. Lymphatic vessel contraction was analysed in Volocity (Perkin Elmer) and plotted as vessel area against time in GraphPad Prism5 (GraphPad Software).

Flow cytometry and PCR

Dermal lymphatic endothelial cells were sorted as previously described (Martinez-Corral et al., 2016). Ear skin from adult mice were dissected in cold PBS and digested in collagenase IV (Life technologies) 10 mg/ml and DNase1 (Roche) 0.2 mg/ml in PBS. All digests were incubated for 30–40 min at 37 °C, quenched by adding 2 mM EDTA and filtered through a 70-µm nylon filter (BD Biosciences). Cells were washed with FACS buffer (PBS, 0.5 % FBS, 2 mM EDTA) and immediately processed for immuno-labelling in 96-well plates. Fc receptor binding was blocked with rat anti-mouse CD16/CD32, (eBioscience). Samples were thereafter stained with anti-podoplanin e660, anti-LYVE-1 and anti-CD31 (390) PE-Cy7 (eBioscience). Immune cells and erythrocytes as well as dead cells were excluded using anti-CD45 (30-F11), anti-CD11b (M1/70) and anti-TER-119 (TER-119) e450 (eBioscience), together with the cell death dye Sytox blue (Life technologies), all detected by the violet laser as one dump channel. For compensation, the AbC anti-rat/hamster compensation bead kit (Life Technologies) was used. Cells were analyzed and sorted on a FACSAria cell sorter (BD Biosciences). Single cells were gated using FSC-H/FSC-W and SSC-H/SSC-W followed by exclusion of dead cells and immune cells and erythrocytes in the violet dump channel. (Capillary) LECs were sorted as CD31 +; Podoplanin+; LYVE-1+ cells. Sorted cells were directly transferred to lysis buffer and mRNA was isolated using the RNeasy Micro Kit (74004, QIAGEN), followed by reverse transcription into cDNA using the iScript cDNA synthesis kit (1708891, Bio-Rad). cDNA was amplified using the TaqMan PreAmp Master
Mix Kit (4384267, ThermoFisher Scientific) using TaqMan GeneExpression Assays (ThermoFisher Scientific) of mouse $Cd31$ (Mm01242577_m1), mouse $Lyve-1$ (Mm00475056_m1), and human $PDGFB$ (Hs00966522_m1) using the Applied Biosystems 7300 Real-Time PCR system. PCR products were then visualized following electrophoresis in 3% agarose.

Statistics

Statistical analysis was performed in GraphPad Prism (GraphPad Software) and all differences were determined by unpaired student t test except Fig. 5E was determined by Mann Whitney test. All differences were defined as significant by $p<0.05$. Investigators were not blinded to the group allocation when performing experiments and assessing outcomes.
References


Figure 1. **PDGFB is expressed by collecting but not capillary LECs, and collecting vessel SMCs express PDGFRβ.** (A,B,B’,C,C’) Immuno-stained dorsal ear skin of Pdgfb-CreER<sup>T2,R26-mTmG</sup> mice induced by 4-OHT at P14 and analysed at P21. (A) Overview of the lymphatic ear vasculature, outlined by dashed lines, including the SMC-covered (α-SMA+, red) collecting vessels (green, arrows) and the non-covered lymphatic capillaries (blue, asterisks). GFP indicates Cre-mediated recombination, as a consequence of an active Pdgfb promoter, observed in collecting vessels (arrows) and blood vessels (capillaries, arrowheads). (B,B’) Collecting vessels (Podocalyxin+, LYVE-1-) are GFP positive (green, arrows) and are covered by SMCs (red). (C,C’) Lymphatic capillaries (LYVE-1+, white) are GFP negative (arrows, no expression of Pdgfb) and lack SMC coverage. (D,D’,E,E’) Dorsal ear skin of Pdgfrβ-GFP mice at P21 stained for Podocalyxin (blue), α-SMA (red), and Lyve-1 (white). GFP (green) positivity reveals the presence of Pdgfrβ- expressing SMCs around collecting vessels (D,D’, arrows) and blood vessels (D,D’, arrowheads) but not in lymphatic capillaries (E,E’, dashed lines). (F,G,H,I,I’) Whole-mount staining of P21 mouse ear after EdU injection at P14, P16 and P18 indicates proliferating SMCs (arrows) on the lymphatic vessels with antibodies against ERG 1 (blue), EdU (green) and α-SMA (red). (I,I’) Distribution of EdU+ SMCs (I, arrows in upper panel; I’, schematic green ellipse in lower panel) in a complete collecting vessel. (J,K) Quantification of EdU+ and α-SMA+ cells (K) relating to SMC coverage (J) in the proximal and distal part of the entire collecting vessel (n=3). Error bars indicate mean ± SEM. *p* value is calculated by student’s *t* test. Scale bars, A, 200 µm; B,B’,C,C’,D,D’,E,E’, 50 µm; F, 20 µm; I, I’, 200 µm.
Figure 2. Postnatal LEC-specific deletion of Pdgfb causes severe collecting vessel SMC-deficiency without major effects on morphogenesis. (A,A’,B,B’,C,C’) Whole-mount immunofluorescence of dorsal mouse ear skin of Pdgfb<sup>LECKO</sup> mice (A,C,C’) and control mice (B,B’) at the age of P21 stained for YFP (A,C, green), α-SMA (red), Podocalyxin (B, green) and LYVE-1 (B’,C’, grey). Tiled confocal images (A) indicate successful recombination of the
dermal lymphatic vasculature (YFP, green), including capillaries (A’, lower panel indicates LYVE-1 positivity) and collecting vessels (C). In Pdgfb^{iLECKO} mice only sporadic SMCs locate to collecting vessels (C, arrows) and the vessels are dilated compared to controls (B, arrows). Lymphatic valves appeared normal in the mutants (C, arrowheads) compared to the controls (B, arrowheads). Collecting vessel LYVE-1 down regulation and valve morphology are unchanged in the Pdgfb^{iLECKO} mice (C’) compared to the controls (B’). (D,E) Quantification of collecting vessel SMC coverage (D) and vessel width (E) comparing the control mice (n=3) and the Pdgfb^{iLECKO} mice (n=4). (F,G) Quantification of LYVE-1 negative area (F) and valve density (G) within collecting vessels of the Pdgfb^{iLECKO} mice (n=3) compared to control mice (n=3). Scale bars, A, 2 mm; A’, 100 μm; B,C, 100 μm.
Figure 3. Absence of perivascular SMCs does not affect the establishment of LEC identities. Whole-mount immunofluorescence of dorsal mouse ear skin of control (left) or Pdgfb<sup>LECKO</sup> mice (right) at P21. (A,A’,B,B’) Antibodies against CD31 (blue), α-SMA (red), and Pan-laminin (grey). (C,C’,D,D’) Antibodies against CD31 (Blue), α-SMA (red), and collagen IV (grey). (E,E’,F,F’,G,G’,H,H’) Antibodies against CD31 (blue), LYVE-1 (green), α-SMA (red), and VEGFR3 (grey). (I,I’,J,J’,K,K’,L,L’) Antibodies against VE-CAD (red), α-SMA (green), and
CCL21 (grey). Collecting vessels were indicated by dashed lines (I,I′,J,J′). None of the above proteins were altered following Pdgfb deletion. (M,M′,N,N′) Immunostaining of mouse ear skin with antibodies against CD31 (blue), VE-CAD (green), and α-SMA (red). Individual LEC size is enlarged on the collecting vessel of Pdgfb<sup>LECKO</sup> mice comparing to the control mice. (O) Quantification of individual cell area of LECs (n=72) from two Pdgfb<sup>LECKO</sup> mice and LECs (n=55) from two control mice. Error bars indicate mean ± SEM and p value is calculated by student’s t-test. Scale bars, A,B,C,D,E,F,G,H,I,J,K,L, 50 µm; M,N, 10 µm.
Figure 4. SMC recruitment to large diameter vessels requires PDGFB. Whole-mount immunofluorescence of popliteal and mesenteric collecting vessels from control mice and Pdgfb\textsuperscript{iLECKO} mice with PDGFB deletion induced either before (induced on E15, E16, and E17) or after (induced on P1 and P2) initial SMC recruitment. (A,A',B,B') Popliteal vessels of E18.5 embryos following induction at E15, E16, and E17. Immunostaining of lymphatic vessels (LV, dashed line) with antibodies against CD31 (A, cyan), YFP (B, cyan) and α-SMA (red). (C,D) Quantification of SMC coverage and average vessel width comparing control embryos (n=6) and Pdgfb\textsuperscript{iLECKO} embryos (n=5). (E,F,G,H) Mesenteric lymphatic vessels of E18.5 embryos following induction at E15, E16, and E17. Immunostaining of lymphatic vessels (LV, dashed line) with antibodies against LYVE-1 (blue), CD31 (E, green), YFP (F, green) and α-SMA (red). (G,H) Quantification of SMC coverage and average vessel width comparing control embryos (n=3) and Pdgfb\textsuperscript{iLECKO} embryos (n=4). (I,I',J,J') Popliteal vessels of P12 mice, induced by topical tamoxifen treatment at P1 and P2. Immunostaining of lymphatic vessels (LV, dashed line) with antibodies against CD31 (I, cyan), YFP (J, cyan) and α-SMA (red). Vessel segments lacking SMC coverage in Pdgfb\textsuperscript{iLECKO} mice were enlarged (J,J', arrowheads). (K,L) Quantification of SMC coverage and average vessel width comparing control mice (n=4) and Pdgfb\textsuperscript{iLECKO} mice (n=4). (M,M',N,N') Mesenteric lymphatic vessels of P12 mice treated with tamoxifen at P1 and P2. Immunostaining of lymphatic vessels (LV, dashed line) with antibodies against NRP-2 (blue), PROX-1 (green) and α-SMA (red). All error bars indicate mean ± SEM. *p value is calculated by student’s t-test. Scale bars, A,B, 100 µm; E,F, 200 µm; I,J, 50 µm; M,N, 100 µm.
Figure 5. Pulsatile contraction of dermal lymphatic collecting vessels requires SMC coverage. (A-C) Lymphatic collecting vessels of ears of living mice, highlighted by subcutaneous injections with Tritc-Dextran (grey). Snap shots from live-imaging of lymphatic collecting vessels analysed as two regions of interest (A, red box, and green box). Snap shots of vessels contracting (arrow heads) and relaxing in the respective region of interests (ROIs) (ROI1, red box, magnified in B; ROI2, green box, magnified in C). Pulsatile contractions indicated in (B) and (C) are plotted by amplitude against time. (E) The number of lymphatic vessel contraction sites per number of animals analysed in the $Pdgfb^{iLECKO}$ mice (n=6) and control mice (n=10) (F-G'). The live-imaged contraction sites in control (F, snap shot from movie) or $Pdgfb^{iLECKO}$ (G, snap shot from movie) ears, identified by whole mount immunofluorescence staining (F' and G'). Areas of contraction (dashed line) were covered by SMCs in control mice (arrows, green in F'; intensity adjustments were applied specifically to the vessel area in order to enhance the visibility of perivascular SMCs) as well as in the only two areas of the $Pdgfb^{iLECKO}$ mouse (arrows, green in G'). Asterisk in E indicates a difference of contraction sites between control and $Pdgfb^{iLECKO}$ ($p<0.05$, calculated by Mann-Whitney’s test). Scale bars, A, 20 µm; F,G, 500 µm; F’,G’, 200 µm.
Figure 6. Over-expression of PDGFB throughout the lymphatic vasculature does not cause aberrant SMC recruitment to capillaries. (A,B) Quantification of SMC coverage (A) and average vessel width (B) of collecting vessels from control mice (n=5) and Pdgfb^iLECOE^ mice (n=3) based on analyses of whole-mount immunofluorescent labelling of dorsal ear skin (images refer to Suppl.Fig.4B,C). Error bars indicate mean ± SEM. p value is calculated by student’s t-test. (C,D) Whole-mount immunofluorescence staining of lymphatic vessels (LV, arrow) in the dorsal ear skin of control mice (C) and Pdgfb^iLECOE^ mice (D) at one month of age with antibodies against CD31 (blue), LYVE-1 (green) and α-SMA (red). (E,F) Whole-mount immunofluorescence staining of lymphatic vessels (LV, arrow) in the dorsal ear skin of control mice (E) and Pdgfb^iLECOE^ mice (F) at the age of one month, with antibodies against CD31 (blue), LYVE-1 (green) and PDGFRβ (red). Mural cells expressing PDGFRβ are found on blood vessels (arrows) but not on lymphatic capillaries (dashed line) despite PDGFB overexpression in Pdgfb^iLECOE^ mice. Scale bars, C,D, 100 µm; E, 50 µm; E, high magnification, 20 µm; F, 50 µm; F, high magnification, 20 µm.
Figure 7. Impaired PDGF-B retention to the extra cellular matrix causes defective SMC recruitment to collecting vessels without affecting lymphatic capillaries. (A,B) Dorsal ear skin of mice at 10 weeks of age stained for Podocalyxin (green), α-SMA (red). SMCs form concentric rings in collecting vessels in Pdgfb<sup>ret/wt</sup> mice (A, arrows) but are only sparsely covering collecting vessel in Pdgfb<sup>ret/ret</sup> mice (A', arrows). (C,D) Quantification of SMC coverage (C) and average vessel width (D). (C,C') Whole-mount immunofluorescence staining of capillaries of Pdgfb<sup>ret/wt</sup> mice (E', and E' in high magnification) and Pdgfb<sup>ret/ret</sup> mice (F, and F' in high magnification) with antibody against LYVE-1. (G,H) Quantification of lymphatic capillary density (G) and branch points (I) comparing Pdgfb<sup>ret/wt</sup> mice (n=4) and Pdgfb<sup>ret/ret</sup> mice (n=4). Error bars indicate mean ± SEM. p value is calculated by student’s t-test. (I,I',J',K',L',M',N',N') Dorsal ear skin of the control mouse at 1 month of age with antibodies against CD31 (blue), LYVE-1 (green), α-SMA (red), perlecan (I,I',J', grey), collagen IV (K,K',L', grey), and Pan-laminin (M,M',N', grey) comparing the collecting vessels (I,I',K',M', dashed line) and the capillaries (J',L',L',N',N', dashed line). Scale bars, A,B, 200 µm; E,F, 2 mm; E’F’, 1 mm; I,J,K,L,M,N, 50 µm.
Supplementary Figures

Supplementary figure 1.
Supplementary Figure 1. **LEC-specific deletion of Pdgfb alters collecting vessel morphology but not blood vessel patterning.** (A,B,C,D) Whole-mount immunofluorescence staining of dorsal mouse ear skin from Pdgfb\textsuperscript{iLECKO} and Ctrl mice at four months age with antibodies against Podocalyxin (A,B, green), α-SMA (A,B,C,D, red). Collecting vessels (LV, arrow) from the control (A) and the Pdgfb\textsuperscript{iLECKO} mice (B) are traced respectively. (C,D) Tiled confocal images indicate normal blood vessel morphology of the control mice (C) and the Pdgfb\textsuperscript{iLECKO} mice (D). (E,E’,F,F’) Immuno-stained dorsal ear skin with antibodies against CD31 (blue), α-SMA (red), PDGFRβ (grey) showing collecting vessels (dashed lines) of control mice (E,E’) and Pdgfb\textsuperscript{iLECKO} mice (F,F’). Perivascular SMCs of collecting vessels are identified by expression of both α-SMA (red, arrow) and PDGFRβ (grey). PDGFRβ+/α-SMA- cells (arrowheads) are only found in blood vessels but not lymphatic vessels. Scale bars, A,B, 200 µm; C,D, 2 mm; E,F, 50 µm.
Supplementary Figure 2. Endothelial cell-specific deletion of Pdgfb alters collecting vessel morphology. (A,B) Whole-mount immunofluorescence staining of dorsal ear skin of mice at 4 months age with antibodies against podocalyxin (green), and α-SMA (red). Collecting vessels in control ears are covered by SMCs (A, arrows) forming concentric rings on the endothelium. In Pdgfb^iECKO mouse ears, SMCs sparsely cover collecting vessels (B, arrows) and the vessels are dilated compared to the controls (A). (C) Quantification of vessel width and SMCs coverage of collecting vessels comparing the controls (n=4) and mutants (n=4). Error bars indicate mean ± SEM. p value is calculated by student’s t-test.(D) Vascular specific deletion of Pdgfb indicated by YFP expression is shown in both blood and lymphatic vasculature. Scale bars, A,B,D, 100 μm.
Supplementary Figure 3. Loss of collecting vessel SMC coverage does not affect lymphatic drainage in the ear skin. (A,A’,B,B’) Ears at the time of 0 hour (A,A’) or 2 hours following (B,B’) subcutaneous injection with Tritc-Dextran (white) and imaged by fluorescent microscopy through the intact skin of control ears (n=12) versus Pdgfb^{iLECKO} ears (n=9) over time. Total fluorescence intensity within the area surrounding the injection site are measured (dashed line) at 0 hour (A,A’) and 2 hours (B,B’) post-injection, and plotted (C) as percentage remaining tracer to indicate the draining capacity. Error bars indicate mean ± SEM. Scale bars, A,A’,B,B’, 1mm.
Supplementary Figure 4. Postnatal LEC-specific overexpression of PDGFB increases SMC coverage of collecting vessels but not capillaries

(A) Electrophoresis of amplified cDNA of cd31 (116 bp), Lyve1 (69 bp) and human PDGFB (56 bp) by PCR reactions. Isolated mRNA from FACS sorted capillary LECs (CD31+;Podoplanin+;LYVE1+) from control mice and Pdgfb^{LECOE} mice were reverse transcribed into cDNA, pre-amplified, followed by PCR and products run in an agarose gel. (B,C) Tiled images of complete collecting vessels (dashed line) indicate the morphology of collecting vessels of Pdgfb^{LECOE} mice (C) compared to control mice (B). Scale bars, B,C, 500 µm.
Supplementary Movies

Supplementary Movie 1. *Pulsatile contraction of a dermal lymphatic vessel of the ear.* A wild-type mouse was anaesthetized and subcutaneously injected with Tritc Dextran, followed by time-lapse imaging utilizing a 25x/1.0 water immersion objective with images acquired at a rate of 5 frames/second.
Supplementary Movie 2. *Dermal lymphatic vessels show pulsatile contraction- overview of control.* A wild-type mouse was anaesthetized and the ear was subcutaneously injected with Tritc Dextran, followed by time-lapse imaging utilizing a Leica M205FA microscope with a PLANAPO 1.0x objective. Arrows indicate contracting sites within the lymphatic vasculature. Scale bar, 500 μm.
Supplementary Movie 3. *Dermal lymphatic vessel contraction is lacking in the absence of SMCs coverage of collecting vessels - overview of Pdgfb<sup>ilecko</sup>*. A Pdgfb<sup>ilecko</sup> mouse was anaesthetized and the ear was subcutaneously injected with Tritc Dextran, followed by time-lapse imaging utilizing a Leica M205FA microscope with a PLANAPPO 1.0x objective. Contraction was not observed on the lymphatic vessels. Scale bar, 500 μm.
Supplementary Movie 4. *Dermal lymphatic vessel contraction is associated with SMCs coverage (Control).* A control mouse was anaesthetized and the ear was subcutaneously injected with Tric Dextran, followed by time-lapse imaging for 5 minutes utilizing a Leica M205FA microscope with a PLANAPPO 1.0x objective. A contraction site on the lymphatic vessel is indicated by the arrow. Scale bar, 200 μm.
Supplementary Movie 5.  *Dermal lymphatic vessel contraction is associated with SMCs coverage in the Pdgfb<sup>ilECKO</sup> mouse (mutant).* An anaesthetized Pdgfb<sup>ilECKO</sup> mouse following subcutaneous ear injection with Tritc Dextran, and time-lapse imaged utilizing a Leica M205FA microscope with a PLANAPC 1.0x objective. Arrows indicate contracting sites within the lymphatic vasculature. Scale bar, 200 μm.