Nonvenous Origin of Dermal Lymphatic Vasculature

Ines Martinez-Corral, Maria H. Ulvmar, Lukas Stanczuk, Florence Tatin, Krishnakumar Kizhatil, Simon W.M. John, Kari Alitalo, Sagrario Ortega, Taija Makinen

Rationale: The formation of the blood vasculature is achieved via 2 fundamentally different mechanisms, de novo formation of vessels from endothelial progenitors (vasculogenesis) and sprouting of vessels from pre-existing ones (angiogenesis). In contrast, mammalian lymphatic vasculature is thought to form exclusively by sprouting from embryonic veins (lymphangiogenesis). Alternative nonvenous sources of lymphatic endothelial cells have been suggested in chicken and Xenopus, but it is unclear whether they exist in mammals.

Objective: We aimed to clarify the origin of the murine dermal lymphatic vasculature.

Methods and Results: We performed lineage tracing experiments and analyzed mutants lacking the Proxl transcription factor, a master regulator of lymphatic endothelial cell identity, in Tie2 lineage venous–derived lymphatic endothelial cells. We show that, contrary to current dogma, a significant part of the dermal lymphatic vasculature forms independently of sprouting from veins. Although lymphatic vessels of cervical and thoracic skin develop via sprouting from venous-derived lymph sacs, vessels of lumbar and dorsal midline skin form via assembly of non–Tie2-lineage cells into clusters and vessels through a process defined as lymphvasculogenesis.

Conclusions: Our results demonstrate a significant contribution of nonvenous-derived cells to the dermal lymphatic vasculature. Demonstration of a previously unknown lymphatic endothelial cell progenitor population will now allow further characterization of their origin, identity, and functions during normal lymphatic development and in pathology, as well as their potential therapeutic use for lymphatic regeneration. (Circ Res. 2015;116:1649-1654. DOI: 10.1161/CIRCRESAHA.116.306170.)

Key Words: developmental biology ■ endothelial cells ■ endothelial progenitor cells ■ lymphangiogenesis ■ lymphatic vessels

Lymphatic vasculature was traditionally considered a passive drainage system responsible for removal of fluid, molecules, and cells from tissues. However, emerging evidence shows active roles of lymphatic vessels in inflammation, immunity, lipid metabolism, blood pressure regulation, and metastasis, and consequent involvement in common diseases such as autoimmune diseases, atherosclerosis, and cancer.1 Despite recent discoveries, our knowledge about the mechanism regulating lymphatic vessel formation and function is limited.

According to a widely accepted theory, originally proposed by Florence Sabin in the early 20th century, lymphatic vessels form during embryogenesis by sprouting from the veins. An alternative theory by Huntington and McClure suggested that lymphatic vessels develop from mesenchymal lymphangioblasts. Recent studies using molecular biological and real-time in vivo imaging techniques provide support for the concept of transdifferentiation of venous into lymphatic endothelial cells (LECs) and suggest veins as the sole origin of the entire mammalian lymphatic vasculature.2,3 Alternative nonvenous sources of LECs have been suggested in chicken and Xenopus,3,5 but whether they exist in mammals is unclear.

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Here, we investigated the development of murine dermal lymphatic vessels that are thought to form by sprouting from venous-derived primitive lymphatic vessels, the peripheral longitudinal lymphatic vessel, and primordial thoracic duct, also referred to as jugular lymph sacs (JLS).2,6,7 We provide genetic lineage tracing data and functional evidence to

Original received February 5, 2015; revision received February 23, 2015; accepted March 2, 2015. In February 2015, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.9 days.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.116.306170/-/DC1.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.116.306170
Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<td>E</td>
<td>embryonic day</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>JLS</td>
<td>Jugular lymph sac</td>
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<td>LEC</td>
<td>Lymphatic endothelial cell</td>
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They demonstrate that, contrary to current dogma, a significant part of the dermal lymphatic vasculature forms independent of Tie2 lineage venous–derived LECs.

**Methods**

Detailed Methods section is available in the Online Data Supplement.

**Results**

Region-Specific Differences in the Development of the Lymphatic Vessels in the Skin

To visualize dermal lymphatic vessel formation, we used the Vegfr3−lacZ reporter mice (Vegfr3\(^{-}\)). Consistent with previous data, we observed that the first lymphatic vessel sprouts reaching the skin emanated from the JLS at embryonic day (E) 12.5 (Figure 1A; Online Figure IA). Concomitant with the extension of sprouts dorsally, rapid emergence of dermal lymphatic vessels was observed on the lateral side of the embryo between E12.5 and E13.5 (Figure 1A and 1B; Online Figure IA and IB). Whole-mount analysis of the skin showed the presence of scattered cells and discontinuous vessel networks that were not connected to JLS in the lateral skin at lumbar region (Figure 1B). Analysis of skin at E15.5 further revealed isolated clusters of Vegfr3\(^{-}\)-positive cells in the dorsal midline (Figure 1C), where vessels from contralateral sides anastomose by E17.5 (Online Figure IB). Notably, dermal lymphatic vessels at the lumbar region seemed to develop independently from subcutaneous lymphatic vessels until E17.5 when connections between the 2 networks were formed (Online Figure IB). The latter developed along major arteries and veins, whereas superficial dermal lymphatic vessels did not show an apparent alignment with blood vessels (Online Figure IB, data not shown). These results suggest that lymphatic vessels in different regions of the skin (cervical versus lumbar, dermis versus subcutis) develop via different mechanisms. In particular, emergence of LEC clusters without connection to vessel sprouts from the JLS suggests a novel mechanism of vessel formation and potentially a different cellular origin.

We next analyzed the expression of Prox1, the first known marker of differentiated LECs,\(^{3}\) and the established lymphatic markers Nrp2, LYVE-1, and VEGFR3 in the dermal vasculature. Immunofluorescence analysis of lumbar skin from E13.5 Prox1\(^{-}\)-green fluorescent protein (GFP) embryos confirmed the presence of a discontinuous network and isolated clusters of GFP and Nrp2-positive LECs (Figure 1D). Some clusters were interconnected via long membrane protrusions, whereas others were isolated (Figure 1D; Online Movies I and II). Immunofluorescence for Prox1 similarly highlighted the Nrp2\(^{-}\) LEC clusters (Figure 1D). Surprisingly, most LEC clusters appeared LYVE-1\(^{-}\), whereas developing vessels showed a weak LYVE-1 immunoreactivity (Figure 1E). Analysis of Vegfr3\(^{-}\)-GFP reporter embryos allowed visualization of isolated GFP\(^{-}\) LECs already at E11.5 (Figure 1F).

**Formation of Lymphatic Vessels in the Lumbar Skin Is Independent of Tie2 Lineage Venous–derived LECs**

To investigate the origin of dermal lymphatic vessels, we used Cre/loxP-based lineage tracing. A transgenic mouse line expressing Cre recombinase under the control of the (blood) endothelial/hematopoietic-specific Tie2 promoter was crossed with the R26-mTmG double reporter line to allow irreversible marking of Tie2-expressing cells and their descendants with GFP, whereas all other cell types express the red fluorescent protein Tomato (Figure 2A). To first assess the efficiency of Cre-mediated recombination in venous LYVE-1\(^{-}\} LEC progenitors and LECs forming the primordial thoracic duct/peripheral longitudinal...
Figure 2. Contribution of nonvenous-derived cells to dermal lymphatic vessels. A and E, Schematic of the Cre transgene and R26-mTmG reporter for lineage tracing of blood endothelial cells (BECs). Timing of primitive lymphatic vessel (primordial thoracic duct/peripheral longitudinal lymphatic vessel [pTD/PLLV], ie, jugular lymph sac [JLS]) formation and time points for analyses are indicated. B, Fluorescence-activated cell sorting (FACS) analysis of endothelial cells (ECs) from Tie2-Cre;R26-mTmG embryos showing efficient Cre-mediated recombination in venous lymphatic endothelial cell (LEC) progenitors and venous-derived LECs. Representative FACS plots and gating scheme (shown for embryonic day [E] 11) and graph of all results are shown. Dots show % of green fluorescent protein (GFP)+ cells in individual embryos, horizontal lines represent mean (n=3–5). C and D, Immunofluorescence of transverse vibratome sections of Tie2-Cre;R26-mTmG embryos. Note GFP+ lymph sac (LS) and cardinal vein (CV). F, Whole-mount immunofluorescence of E12.5 Tie2-Cre;R26-mTmG skin. Arrowhead points to GFP+ (non-Tie2-lineage) LECs, blood vessels are GFP+. Single channel images of boxed areas are shown. G, A single confocal section of E15.5 Tie2-Cre;R26-mTmG skin. Tomato (red) shows cells that have not undergone Cre-mediated recombination. H, FACS analysis of ECs from E13 Tie2-Cre;R26-mTmG embryos showing a significant GFP+ LEC (podoplanin [PDPN]/LYVE-1−) population. Representative FACS plots and gating scheme and graph of all results are shown. Dots show % of GFP+ cells in individual embryos, horizontal lines represent mean (n=8). Graph colors are as in B. Scale bars: 50 μm (C and G), 200 μm (D), 20 μm (F). DA indicates dorsal aorta; IF, immunofluorescence; and PECAM-1, platelet endothelial cell adhesion molecule 1.
lymphatic vessel, we performed fluorescence-activated cell sorting (FACS) analysis of ECs from Tie2-Cre;R26-mTmG embryos at E9, E10, and E11 (Figure 2A). At E9 ≥89% of the LYVE-1− ECs expressed GFP, increasing to ≥95% at E10 and E11, indicating efficient targeting by the Tie2-Cre transgene (Figure 2B; Online Figure IIA). LYVE-1− ECs, including LEC progenitors generated from the superficial venous plexus, also showed highly efficient Tie2-Cre-mediated recombination at E11 (98.8±0.2%; n=4; Online Figure IIB). A major proportion of ECs were also efficiently recombinated at E11 (98.1±0.4% at E11; n=3 and 99.4±0.7% at E13; n=8), suggesting reporter gene expression but lack of recombination in these cells and their progenitors. A large proportion (35.7±3.5%; n=8) of dermal LECs coexpressed Tomato and GFP (Figure 2H), demonstrating recent upregulation of Tie2. The proportion of GFP+ LECs increased at later stages of development, suggesting progressive induction of Tie2 in the developing vessels (Online Figure IID and data not shown). Importantly, analysis of venous-derived LECs at E11 (Figure 2B; Online Figure IIB) and blood endothelial cells at E13 (Figure 2H), at the stage of LEC cluster emergence, showed efficient targeting by the Tie2 transgene (98.1±0.4% at E11; n=3 and 99.4±0.7% at E13; n=8), thus excluding these cells as the origin of dermal LECs.

We next sought functional evidence for the venous origin of dermal lymphatic vessels by deleting Prox1, the master regulator of LEC fate, in blood endothelia using a conditional Prox1floxed allele (Online Figure IIIA–IID) in combination with the Tie2-Cre transgene. If lymphatic vessels were formed entirely by transdifferentiation of Tie2 lineage ECs, which are currently expressed in blood endothelia, no or few lymphatic vessels were expected to form in the Prox1floxed;Tie2-Cre embryos. In agreement with previous data,3 we indeed found that E14.5 Prox1floxed;Tie2-Cre embryos showed subcutaneous edema and failure of lymphatic vessel formation in the cervical skin (Figure 3A; Online Figure IVA). However, we observed blood-filled dermal lymphatic vessels and isolated LEC clusters in the lumbar skin (Figure 3A). Most LEC clusters were not targeted by the Tie2-Cre transgene and thus expressed Prox1 in the mutant embryos (Online Figure IVB). These data demonstrate that the formation of lymphatic vessels in the lumbar skin is independent of Tie2 lineage venous-derived LECs. Most lymphatic structures were however lost in Prox1floxed;Tie2-Cre skin by E17.5 (Online Figure IVC), likely because of progressive induction of Tie2 in the dermal lymphatic vasculature (Online Figure IID, data not shown) and consequent loss of Prox1 that is required for lymphatic vessel maintenance.11

Tracing of Prox1-Expressing Cells Suggests Continuous LEC Differentiation During Dermal Lymphatic Vessel Formation

To further investigate the origin of the dermal lymphatic vasculature, we used a tamoxifen-inducible Cre line to allow genetic labeling of cells expressing Prox1 (Online Figure V). In contrast to a previous report,2 we found that injection of 4-OHT to pregnant females at E10.5 or E11.5 led to a nearly complete absence of GFP+ LECs in the skin (Online Figure VIA and VIB) and JLS (data not shown). It was possible that cells were not labeled because of continuous LEC differentiation between E10 and E122 and a short 24-hour time-window of 4-hydroxytamoxifen (4-OHT) activity in our study, in comparison with the longer period of activity after administration with tamoxifen in the previous study2 (Online Figure VIIIA and VIIIB). When 4-OHT was instead administered at E12.5 (Online Figure VIB) or E13.5 (data not shown), JLS was well labeled (Online Figure VIF). If lymphatic vessels were derived through continuous migration and proliferation of LECs sprouting from the GFP+ JLS, they would be expected to express GFP particularly at the tips of the sprouts. Surprisingly, however, dermal lymphatic vasculature showed a high proportion of GFP+ cells at the distal end of the vasculature (Figure 3B; Online Figure VIB). In addition, 4-OHT administration at E14.5 or E15.5 resulted in isolated GFP+ LEC clusters at the midline, despite efficient recombination in the rest of the vasculature (Figure 3B; Online Figure VIB). Low GFP labeling selectively at vessel tips and isolated LEC clusters suggests incorporation of newly differentiated cells into the growing vessels at the vascular front.

In addition to visualization of lymphatic vessels, we observed a small population of scattered GFP+ cells in the Prox1CreER2;R26-mTmG embryos that were also positive for LYVE-1 and markers of the macrophage lineage (Online Figure VIIIA and VIIIB). Lineage tracing using Vav-Cre mice however excluded definitive hematopoietic lineage cells as a source of LECs (Online Figure VIIIC), as previously reported.12

Taken together, our data demonstrate the existence of a population of LEC progenitors that is distinct from venous-derived LECs and contributes to the formation of the dermal lymphatic vasculature (Figure 3C).

Discussion

This study demonstrates that a large part of the superficial dermal lymphatic vasculature does not form via transdifferentiation and sprouting of Tie2 lineage venous ECs, which are currently thought to be the sole origin of the mammalian lymphatic vasculature. We found that lymphatic vessels of the dorsal midline and lumbar regions of the skin instead form from venous-derived progenitors through a lymphovasculogenesis process involving assembly of LECs into clusters and their further coalescence to continuous vessel networks. The Prox1CreER2 lineage tracing defined E12.5–E14.5 as the critical time-window when venous-derived LEC progenitors most actively incorporate into dermal lymphatic vessels, which is in agreement with the rapid emergence of these vessels at around E13.5.
The embryonic origin of lymphatic vessels has been controversial until recently. Genetic lineage tracing experiments in mouse and real-time imaging in zebrafish confirmed Sabin’s theory on the venous origin of lymphatic vessels. These experiments did not, however, exclude the existence of alternative sources of LECs. Earlier observations interestingly showed that avian lymphatic vasculature has a dual origin, with JLSs originating from veins and superficial dermal
lymphatic vessels from an unidentified nonvenous-derived precursors of mesodermal origin. Together with our findings, this suggests evolutionarily conserved origins of LECs and mechanisms of lymphatic vessel formation.

Given the distinct origins, molecular mechanisms regulating lymphatic vessel formation in different regions of the skin are likely different. To understand these mechanisms, it is now of critical importance to first clarify the cell of origin of dermal LECs and identify their potential contribution to lymphatic vessels in other organs. Here, we excluded Tie2 lineage endothelial/hematopoietic cells and Vav lineage definitive hematopoietic cells as the source of dermal LECs. Interestingly, our recent study identified hemogenic endothelium-derived cells as an alternative nonvenous origin of lymphatic vessels in the mesentery. However, the origin of nonvenous-derived LECs in the skin and mesentery seems to be different because, unlike dermal LECs, mesenteric LECs are derived from Tie2-lineage cells (unpublished data). Additional cell-type–specific lineage tracing experiments are required to identify the cellular source of nonvenous-derived LEC progenitors, which will allow further studies on their potential therapeutic use for lymphatic regeneration in disease in the future.

Acknowledgments

We thank Dimitris Kioussis (National Institute for Medical Research, London) for providing Vav-Cre mice, transgenic services at London Research Institute (LRI) for help with establishing mouse lines, staff at LRI and Uppsala University animal units for animal husbandry and Henrik Ortsäter for help with mice. Anna Caldwell at King’s College London is acknowledged for mass spectrometry analysis of 4-OHT in sera. We thank the Light Microscopy unit at LRI and BioVis at Uppsala University for advice and help with experiments.

Sources of Funding

This study was supported by Cancer Research UK (I. Martinez-Corral, L. Stanczuk, F. Tatin, and T. Makinen), EMBO Young Investigator Programme, Swedish Research Council and the Kjell and Märta Beijer Foundation (T. Makinen), Fundación Alfonso Martin Escudero (I. Martinez-Corral), Howard Hughes Medical Institute (K. Kizhath, S.W.M. John), EY11721 (S.W.M. John), European Research Council (ERC-2010-AdG-268804) and the Leducq Foundation (11CVD03; K. Allitato), and Ministry of Science and Innovation of Spain (grants BIO2009-09488 and SAF2010-18765; S.O.). S.W.M. John is an investigator of the Howard Hughes Medical Institute.

Novelty and Significance

What Is Known?
- The mammalian lymphatic vasculature forms by sprouting from embryonic veins.

What New Information Does This Article Contribute?
- Part of the mammalian dermal lymphatic vasculature originates from an alternative nonvenous source.
- Nonvenous-derived lymphatic endothelial progenitors form vessels through a novel process defined as lymphvasculegenesis.

Endothelial cells forming the lymphatic vasculature have been described to originate through transdifferentiation and sprouting of venous endothelial cells. Alternative nonvenous sources of lymphatic endothelial cells have been suggested, although never demonstrated to exist in mammals. In this study, we reinvestigated the origin of the lymphatic vasculature by fate mapping. We found that lymphatic vessels in different regions of the skin have different origins and mechanisms of development. Vessels in the neck region form from veins through a sprouting process, as demonstrated in previous studies. However, in the lumbar region of the skin, lymphatic vessels develop from an alternative nonvenous source via a different process that we define as lymphvasculegenesis. Our data provide fundamental novel insight into the mechanism of lymphatic vessel formation by demonstrating the existence of a previously unknown lymphatic endothelial cell progenitor. Identification and characterization of the nonvenous lymphatic endothelial cell progenitors will allow further studies on their potential therapeutic use for lymphatic regeneration in disease in the future.

Disclosures

None.

References

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Circ Res. 2015;116:1649-1654; originally published online March 3, 2015;
doi: 10.1161/CIRCRESAHA.116.306170

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/116/10/1649

Data Supplement (unedited) at:
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SUPPLEMENTAL MATERIAL

Detailed Methods

Mice

R26-mTmG mice were obtained from the Jackson Laboratory\(^1\). Vegfr3\(^{2,3}\), Vegfr3-EGFP-Luc, Tie2-Cre, and Vav-Cre mice were described previously\(^2,5\). Proxl-GFP BAC transgenic mouse sperm (Tg[Proxl-GFP]/KY221Gsat/Mmcd, cryo-archived)\(^7\) was purchased from the Mutant Mouse Regional Resource Centers (MMRRC, UC Davis). A colony was established at the Jackson Laboratory following rederivation by in vitro fertilization of C57BL/6J oocytes. ES cell line containing ‘knockout-first’ Proxl allele (Proxl\(^{m1at(EUCOMM)Wtsi}\)) was obtained from The European Conditional Mouse Mutagenesis Program (EUCOMM). After obtaining germ line transmission, Proxl\(^{Iz-neo-fl/+}\) mice were bred to CD-1 background due to reduced viability of Proxl haploinsufficient mice on C57BL/6J background\(^8\). LacZ-neo cassette was removed by crossing with FlpO deleter strain\(^9\), followed by mating to C57BL/6J at least three generations. For endothelial specific deletion of Proxl, Tie2-Cre\(^{+}\) males (Proxl\(^{flox/+};Cre^{+}\)) were crossed with Cre\(^{-}\) females (Proxl\(^{flox/-}\) or Proxl\(^{flox/+}\)), to avoid inheritance of a null allele when transmitted through the female germ line\(^10\). Proxl\(^{Iz-neo-fl/+}\) or Proxl\(^{flox/\cdot}\) mice were crossed with the PGK-Cre animals to generate germline heterozygous mice Proxl\(^{Iz/\cdot}\) or Proxl\(^{flox/\cdot}\) respectively. Proxl\(^{flox}\) and Proxl\(^{Iz/\cdot}\) mice were genotyped using primers a (TGCTGAAGATGGCTGAAGG), b (GGCTTTTCTGTGCTGAAGG) and c (CTGAACTGATGGCGAGCTGAC). Proxl-CreERT\(^2\) were generated as described\(^11\) and tested by timed matings with Cre reporter strain followed by 4-hydroxytamoxifen (4-OHT) administration for specificity and efficiency of Cre-mediated recombination. For embryonic induction, 4-OHT, dissolved in peanut oil (10 mg/ml), was administered to pregnant females by intraperitoneal injection at indicated developmental stages. For lineage tracing experiments, a single intraperitoneal injection of 2 mg of 4-OHT was used. Early postnatal mice were administered with a single intraperitoneal injection of 50 µg of 4-OHT, dissolved in Ethanol, at P1. At adult stages Cre recombination was induced by feeding mice with tamoxifen-containing diet (Harlan) for two weeks, or by subcutaneous implantation of 15 mg slow-release tamoxifen pellets for 3 weeks (Innovative Research of America). Staging of E9 and E10 embryos was done by counting somite pairs. Embryos harvested before 10 am were typically of stages Eday.0-Eday.25. For staging of embryos older than E11, the morning of vaginal plug detection was therefore considered as E0. All strains were maintained and analysed on C57BL/6J background except for Proxl\(^{Iz-neo-fl/\cdot}\) that was on a mixed C57BL/6J x CD-1 background. Experimental procedures were approved by the United Kingdom Home Office and the Uppsala Laboratory Animal Ethical Committee.

Immunofluorescence and X-Gal staining

For whole-mount immunostaining, tissue was fixed in 4% paraformaldehyde (PFA) for 2h at RT, permeabilised in 0.3% Triton-X100 in PBS (PBSTx) and blocked in PBSTx plus 3% milk. Primary antibodies were incubated at 4°C overnight in blocking buffer. After washing in PBSTx, the samples were incubated with fluorocrome-conjugated secondary antibodies in blocking PBSTx plus 1% milk, before further washing and mounting in Mowiol. For visualization of cardinal veins and lymph sacs, 150 µm vibratome cross sections of E10.5, E11.5 and E12.5 Tie2-Cre;R26-mTmG or Proxl\(^{Iz-neo-fl/\cdot}\) embryos were used for staining as described above. The following antibodies were used: rat anti-mouse LYVE-1 (R&D systems), rat anti-mouse PECAM-1 (BD), hamster anti-mouse PECAM-1 (Millipore), rabbit anti-human Proxl (generated against human Proxl C-terminus (S677-T737aa), Proxl-GST construct provided by Dr. T. Petrova, University of Lausanne), rabbit anti-GFP (Invitrogen), chicken anti-GFP (Abcam), rat anti-mouse Endomucin (Santa Cruz Biotechnology), hamster anti-mouse Podoplanin (Developmental Studies Hybridoma Bank), goat anti-mouse VEGFR-2, VEGFR-3 or Neuropilin-2 (all from R&D Systems), rat anti-mouse F4/80 and CD169b (both from AbD Serotec).
or rat anti-mouse CD45 (BD). Secondary antibodies conjugated to DyLight 405, AF488, Cy3 or Cy5 were obtained from Jackson ImmunoResearch. Staining for β-galactosidase activity in the lymphatic vessels in Vegfr3 \(^{β⁺} \) and Proxl\(^{-}\)neo\(^{−}\) mice was done using the substrate X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) using standard protocols.

Flow cytometry

Embryonic back skin and whole embryos were harvested and the tissues were cut into smaller pieces for digestion in Collagenase IV (Life Technologies) 4 mg/ml (skin) or 2 mg/ml (embryos), and DNase I (Roche) 0.2 mg/ml in PBS with 10% FBS at 37 °C under constant rotation for 8-20 min. Digests were 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 staining in 96 well plates. Fc receptor binding was blocked by rat-anti mouse CD16/CD32 (93) (eBioscience). Skin samples were stained with anti-CD31/PECAM-1 (390) PE-Cy7, anti-LYVE-1 (ALY7) eF660 (E13 and E14), rat-anti podoplanin (PDPN) eF660 (eBio8.1.1) (E13 only) and anti-CD45 (30-F11), PERCP-Cy5.5 (all E14 only). Dump channel included markers to exclude macrophages, anti-F4/80 (BM8), other myeloid cells, anti-CD11b (M1/70) and red blood cells, anti-TER-119 (TER-119); all conjugated with e450 (eBioscience); together with Sytox blue (Life Technologies) for dead cell exclusion. E13 LECs and BECs were gated in three steps; 1. PECAM1 \(^{β⁺} \), dump channel negative cells. 2. CD45 \(^{−} \) negative cells. 2. CD45 \(^{−} \) negative cells. 3. LYVE-1/PDPN \(^{−} \) positive (LECs) LYVE-1/PDPN \(^{−} \) positive (BECs). The use of both PDPN and LYVE-1 in the same channel ensures that all LECs are gated at E13 when LYVE-1 expression is low or undetectable in developing lymphatic vessels (Figure 1E). For E14 skin, CD45 was included in the dump channel by using eF450 conjugated anti-CD45 (30-F11) antibody. Whole embryos were stained with anti-CD31/PECAM-1 (390) PE-Cy7, anti-LYVE-1 (ALY7) eF660, anti-CD45 (30-F11), PERCP-Cy5.5, using the same dump channel as for skin. Venous progenitors were defined as PECAM1 \(^{β⁺} \), LYVE-1 \(^{−} \) positive cells. The anti-rat/hamster compensation bead kit (Life Technologies) was used for compensation controls, with the addition of Tomato positive tissue and GFP positive tissue for Tomato and GFP compensation. The cells were analyzed on a FACSDiva cell sorter with the FACS DIVA software (all from BD biosciences). Data were processed using FlowJo software (TreeStar). Single cells were gated using FSC-A/SSC-A followed by SSC-H/FSC-W and SSC-H/SSC-W. FMO controls were used to setup the subsequent gating schemes.

For analysis of Proxl-GFP \(^{β⁺} \) macrophages (Online Figure VIIIB), back skin from E17.5 Proxl-GFP embryos was cut into small pieces and digested in Collagenase IV (10 mg/ml) for 30-45 minutes at 37°C, followed by passage through a 70 μm cell strainer (BD Falcon). Erythrocytes were lysed using RBC lysis buffer (eBioscience) for 5 minutes at RT. Single cell suspensions were incubated with rat anti-mouse CD45 (BD Pharmigen) and rat anti-mouse F4/80 (AbD Serotec) for 15 minutes followed by incubation with goat anti-rat AF647 (Invitrogen). Samples were analysed on a LSR II Analyser (BD Biosciences) and data analysis was done using FlowJo version 9.4.10.

Image acquisition

All confocal images except Figure 2G, Online Figure IIC (lower panels) and Online Figure VIIIC (E13.5) represent maximum intensity projections of Z-stacks of single tile or multiple tile scan images that were acquired using Zeiss LSM 700 or 710 confocal microscope and Zeiss Imager.Z2 and Zen 2009 software. Image acquisition details are specified in Online Table I. Stereomicroscope images of tissues were acquired with Leica MZ16F fluorescence microscope equipped with Leica DFC420C camera and Leica Microsystems software.
Quantification and image processing

Quantification of GFP+ vessels in the Proxl-CreERT2 lineage tracing experiment was done using MetaMorph (Molecular Devices) software. Each skin was divided into two halves along the dorsal midline, which were further divided into four regions from lateral (a) to medial (d), as depicted in Online Figure VI.B. In each region lymphatic vessels were selected based on positive Nrp2 staining as a region of interest (ROI) and the % of GFP staining over LYVE-1/Nrp2 staining on maximum intensity projection images was measured. Results are shown as mean ± SEM. 3D reconstruction and surface rendering movies of an isolated Nrp2+ LEC cluster were created from z-stack confocal images using Imaris (Bitplane Scientific Software).

Analysis of serum levels of the active 4-OHT metabolite

A single dose of 4-OHT or Tamoxifen was administered to C57BL/6J females (age = 10-11 weeks, weight = 20.1±1.5 g). 200 µl of blood was collected from the tail vein at different time points (6 h, 12 h, 24 h, 36 h, 48 h and 72 h). A maximum of three blood samples were taken from each mouse. Extraction of serum, sample preparation and analysis was performed following the protocol described in12.
Supplemental Figures and Figure Legends

Online Figure I. Visualisation of the developing dermal lymphatic vasculature by X-Gal staining of Vegfr3β embryos.

(A) Lateral and dorsal views of E12.5, E13.5 and E15.5 embryos showing sprouting from the JLS in the cervical/thoracic regions and emergence of discontinuous network of vessels and isolated cell clusters in the lateral skin at the lumbar level and in the dorsal midline. Panels showing lateral view of E12.5 and E13.5 embryos are also shown in Figure 1A. Asterisks indicate the level of forelimbs. Arrowhead points to a subcutaneous lymphatic vessel (sc). Weak X-Gal staining can be observed in blood vessels.

(B) Visualization of superficial and subcutaneous dermal lymphatic vessels in Vegfr3β embryos at E13.5 and E17.5. Subcutaneous vessels in the cervical/thoracic and sacral region connect to the superficial network of sprouting vessels (arrowheads) while in lumbar skin the two networks form separately and establish connections at E17.5 (arrows). Boxed area showing subcutaneous lymphatic vessels forming connections to the superficial lymphatic capillaries in the lumbar region is magnified on the right.

Scale bars = 1 mm (B, top panels), 2 mm (all other).

Online Figure II. Characterisation of the Tie2-Cre line for lineage tracing of venous derived LECs.

(A) FACS analysis of ECs from E9 and E10 Tie2-Cre;R26-mTmG embryos. Representative FACS plots and gating scheme are shown. Summary of all results is shown in Figure 2B.

(B) FACS analysis of all ECs in E11 Tie2-Cre;R26-mTmG embryos, defined by expression of the endothelial markers PECAM1 and VEGFR2. GFP expression, which reflects Tie2-Cre driven recombination, is evaluated in the venous LYVE1+ LEC progenitors and LECs, as well as the LYVE1+ BECs and LEC progenitors. Representative FACS plots and gating scheme and graph of all results are shown. Dots show % of GFP+ cells in individual embryos, horizontal lines represent mean (n=4).

(C) Immunofluorescence of a transverse vibratome section of E12.5 Tie2-Cre;R26-mTmG embryo using antibodies against GFP (green), LYVE-1 (red; marker of lymphatic EC) and Endomucin (blue; marker of venous EC). Note uniform GFP-labeling of the cardinal vein (CV) and lymph sac (LS). DA = dorsal aorta. Boxed area is magnified in Figure 2D.

(D) FACS analysis of ECs from E14 Tie2-Cre;R26-mTmG skin showing a significant GFP LEC population. Representative FACS plots and gating scheme and graph of all results are shown. Dots show % of GFP+ LECs in individual embryos, horizontal line represents mean (n=5).

Scale bars = 200 μm (C).

Online Figure III. Characterisation of the conditional Prox1 line.

(A) Schematic representation of the Prox1 wild-type (Wt) allele, targeted ‘knockout-first’ allele, conditional allele (floxed) and knock-out allele (KO). The ‘knockout-first’ allele contains an IRES:lacz cassette and a floxed neo cassette inserted into the first intron of Prox1, disrupting gene function and allowing monitoring of promoter activity by X-Gal staining. Flp converts the ‘knockout-first’ allele to a conditional allele, restoring gene activity. In the conditional allele, Cre deletes the floxed exon 2 of the Prox1 gene. The locations of PCR genotyping primers are indicated by arrows.

(B) X-Gal staining of 3-weeks old Prox1floxed/+ shows β-gal activity in known Prox1 expressing tissues, including lymphatic vessels and the heart.

(C) Immunofluorescence of vibratome sections of E11.5 Prox1floxed/embryos showing lack of JLS and Prox1 expressing LECs in the homozygous embryo. Note smaller lymph sacs and reduced Prox1 expression in the heterozygous embryo, as previously reported[13,14].

(D) Images of E15.5, E16.5 and E17.5 Prox1lox/lox (left) or Prox1lox/PGK-Cre (right) (i.e. germline Prox1 heterozygous) embryos showing edema and blood-filled lymphatic vessels.

Scale bars: 100 μm (C), 1 mm (B), 2 mm (D).
Online Figure IV. Characterisation of Proxl\textsuperscript{flox};Tie2-Cre embryos.
(A) Gross phenotype of Proxl\textsuperscript{flox/+};Tie2-Cre and Proxl\textsuperscript{floxflox};Tie2-Cre embryos (on C57BL/6J background) at different stages of development. Proportion of embryos showing phenotype is indicated, and % of expected Mendelian frequency is shown in parentheses.
(B) Whole-mount immunofluorescence of E13.5 Proxl\textsuperscript{floxflox};Tie2-Cre;R26-mTmG lumbar skin for Proxl (red) and Nrp2 (blue; upper panels). Note lack of GFP expression, indicating lack of recombination, in most lymphatic vessels. Proxl expression is lost in a targeted GFP\textsuperscript{+} cell (arrowhead), while GFP (open arrowhead) as well as most GFP\textsuperscript{+} cells (arrows) are Proxl\textsuperscript{+} at this stage, suggesting recent upregulation of Tie2. Asterisks indicate red blood cells.
(C) On the left: E17.5 Proxl\textsuperscript{floxflox};Tie2-Cre embryo showing only a few blood-filled lymphatic vessels (arrowheads) but no edema. On the right: whole-mount immunofluorescence of thoracic and lumbar skin (regions indicated in the image on the left) from Proxl\textsuperscript{floxflox};Tie2-Cre and control embryos for Nrp2. Note the complete absence of lymphatic vessels in the mutant with only a few fragmented vessels remaining in the lumbar region.
Scale bars: 20 \textmu m (B, upper panel), 100 \textmu m (B, lower panel), 200 \textmu m (C, left panels), 2 mm (C, embryo)

Online Figure V. Characterisation of the efficiency and specificity of the Proxl-CreER\textsuperscript{T2} line.
Visualisation of reporter gene expression in Proxl-CreER\textsuperscript{T2};R26-mTmG (GFP; A-H, J, L-M') or Proxl-CreER\textsuperscript{T2};R26R (\beta-gal; I, K) mice after 4-OHT administration at indicated stages during embryogenesis (single injection of 2 mg of 4-OHT at E10.5 (A-C, M) or at E14.5 (D-F) or postnatally (single injection of 50 \mu g of 4-OHT at P1, or tamoxifen administered in the diet (J, L) or via slow-release pellet (K) for three weeks). (H) represents a confocal micrograph of ear skin co-stained using Podoplanin antibodies (red), (M) shows transverse vibratome cross section of E10.5 embryo at the level of the cardinal vein. A = aorta, CV = cardinal vein. Boxed area in (M) is magnified in (M'). All other images were taken using a (fluorescence) stereomicroscope. Efficient Cre-mediated recombination was observed in Proxl expressing tissues, including the eye (A, D, G, J), embryonic nervous system (A, B), the heart (C, F, L), the liver (F, L) and the lymphatic vasculature (E, H, I, K), including a subset of venous ECs in the cardinal vein and venous-derived lymphatic sprouts (M, M') as indicated.
Scale bars: 250 \mu m (C-E, H, K), 500 \mu m (A, B, F, I, J, L), 1 mm (G), 100 \mu m (M), 40 \mu m (M').

Online Figure VI. Genetic tracing of Proxl lineage cells during dermal lymphatic vessel development.
(A) Schematic of the inducible Cre transgene and R26-mTmG reporter construct for lineage tracing of Proxl positive LECs (on the left). 4-OHT was administered to induce Cre activity at different developmental stages (arrowheads).
(B) Tile scans of E17.5 Proxl-CreER\textsuperscript{T2};R26-mTmG whole-mount skins, taken from embryos that were administered with a single dose of 4-OHT at indicated stages (E10.5 to E15.5), and stained with antibodies against GFP (green) and Nrp2 (red). Panels on the right show single channel images for GFP. Panels on the left show dermal lymphatic vasculature at the stage of 4-OHT induction.
(C) Immunofluorescence of a vibratome section from E13.5 Proxl-CreER\textsuperscript{T2};R26-mTmG embryo that was administered with 4-OHT at 12.5 showing efficient recombination (GFP; green) in the JLS (stained for Nrp2; red).
Scale bars: 500 \mu m (B), 100 \mu m (C).

Online Figure VII. Kinetics of 4-OHT and Tamoxifen induced Cre-activity.
(A) Analysis of 4-OHT metabolite levels in serum after administration of a single dose of Tamoxifen or 4-OHT to mice at indicated time points. Doses used in this study (2mg of 4-OHT) or in a previous study (5mg of Tamoxifen; ref 4) were tested. A single dose of 4-OHT showed a 24-hour time window for Cre activity, while Tamoxifen administration led to up to a 3-day window of activity.
(B) Upper panel: Schematic of the inducible Cre transgene and R26-mTmG reporter construct for lineage tracing of Proxl positive LECs (on the left). 5mg of Tamoxifen or 1mg of 4-OHT was administered to pregnant females to induce Cre at specified developmental stages (on the right, arrowheads). Time-line of Cre activity, based on the analysis of serum levels of the active 4-OHT
metabolite, with each treatment is shown. Lower panel: Tile scans of E15.5 Prox1-CreER\textsuperscript{T2};R26-mTmG whole-mount skin, taken from embryos that were treated as indicated. Administration of 5mg of Tamoxifen at E10.5 led to an equivalent labelling of dermal lymphatic vessels than 1mg of 4-OHT administered at E12.5, due to longer period of Cre activity upon Tamoxifen treatment. Scale bars: 200 µm.

Online Figure VIII. Characterisation of the Prox1-positive scattered cells and the contribution of macrophages to dermal lymphatic vessels.
(A) Whole-mount immunofluorescence staining of E14.5 skin from Prox1-CreER\textsuperscript{T2};R26-mTmG embryos. Skin was stained after administration of 4-OHT at E12.5 and E13.5, using antibodies against LYVE-1 (blue) and indicated macrophage markers (red). Note co-localisation of GFP fluorescence (i.e. Prox1 expression, green) with F4/80 and CD169b in single cells (arrowheads). Single channel images are shown on the right.
(B) FACS analysis of dermal cell suspensions from E17.5 Prox1-GFP skin showing a low proportion of Prox1-GFP\textsuperscript{+} macrophages (Mø).
(C) Whole-mount immunofluorescence of E17.5 (left panels) and E13.5 (right panels) Vav-Cre;R26-mTmG skin stained with antibodies against GFP (green) and Nrp2 (red). Single channel images showing GFP staining only are shown on the right. No GFP expression is detected in lymphatic vessels (arrowhead) indicating lack of contribution of macrophages to dermal lymphatic vessels. Dotted line outlines lymphatic vessels. Scale bars: 20 µm (A), 50 µm (C).
A

Prox1 wildtype allele

5’arm (5747bp)

targeted allele

1  FRT  En2 SA  RES  lacZ  pA  neo  FRT  loxP  34

Floxed allele

FRT loxP

1  FRT  34

Knock-out allele

FRT loxP

1  FRT  LacZ  pA  loxP  34

lacZ knock-in/knockout allele

Cre recombination

Flop recombination

Cre recombination

P21 Prox1* XGal

Ear skin  Intestine  Diaphragm  Heart

Endogenous Prox1 Lyve1

CV

CV

Prox1

Prox1

Prox1

Prox1

Prox1

Online Figure III
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B. Prox1 flox/flox; R26-mTmG, Tie-2-Cre, GFP (Tie2 lineage) Prox1 Nrp2

C. E17.5 Prox1 flox+/; Tie-2-Cre+ E17.5 Prox1 flox/flox; Tie-2-Cre WT

Online Figure IV
Supplemental Tables

Online Table I. Image acquisition details.
Image acquisition details for confocal micrographs

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Online Table I
Supplemental References


Legends for the Video files

**Online Video I. 3D reconstruction of a dermal LEC cluster.**
3D movie created from confocal images of E14.5 skin stained with antibodies against Nrp2 (red) and PECAM1 (green). Note an isolated Nrp2+ LEC cluster in the middle of the image.

**Online Video II. 3D surface rendering of a LEC cluster**
3D movie created from confocal images of E14.5 skin stained with antibodies against Nrp2 (red) and PECAM1 (green).