Cardiac lymphatics are heterogeneous in origin and respond to injury

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The lymphatic vasculature is a blind-ended network crucial for tissue-fluid homeostasis, immune surveillance and lipid absorption from the gut. Recent evidence has proposed an entirely venous-derived mammalian lymphatic system. By contrast, here we show that cardiac lymphatic vessels in mice have a heterogeneous cellular origin, whereby formation of at least part of the cardiac lymphatic network is independent of sprouting from veins. Multiple Cre–lox–based lineage tracing revealed a potential contribution from the putative haemogenic endothelium during development, and discrete lymphatic endothelial progenitor populations were confirmed by conditional knockout of Prox1 in Tie2+ and Vav1+ compartments. In the adult heart, myocardial infarction promoted a significant lymphangiogenic response, which was augmented by treatment with VEGF–C, resulting in improved cardiac function. These data prompt the re-evaluation of a century-long debate on the origin of lymphatic vessels and suggest that lymphangiogenesis may represent a therapeutic target to promote cardiac repair following injury.

In 1902, Florence Sabin proposed that the primary lymph sacs originate from the embryonic veins and then give rise to the entire lymphatic vasculature by sprouting and remodelling. An alternative model of lymphatic development was proposed by Huntington and McClure in 1910, who suggested that lymph sacs arise in the mesenchyme, independently of veins, via distinct progenitor cells. More recent evidence has supported Sabin’s model, such that trans-differentiation of venous into lymphatic endothelial cells (LECs) is now widely accepted, with the veins regarded as the sole origin of the entire lymphatic vasculature in mammals. To date, studies which support a venous origin have focused exclusively on the development of the systemic lymphatic vasculature. Organ-based lymphatics have received little attention and in the heart, while the presence of cardiac lymphatic vessels has been described, virtually nothing is known about their role during development or in the healthy or failing adult heart. We therefore sought to characterize the formation of the cardiac lymphatic vessels through developmental stages, to identify their embryonic origin and effect during organogenesis and to assess their response to pathological insult in the adult setting.

Development of the cardiac lymphatic vasculature

Whole-mount staining of murine hearts for early LEC markers VEGFR-3 (ref. 9) and Prox1 (ref. 10), revealed the emergence of lymphatic vessels at embryonic day 12.5 (E12.5), sprouting from extra-cardiac regions proximal to the outflow tract, on the ventral side (Fig. 1a, increased magnification in Fig. 1b). At E14.5, lymphatic vessels were observed on the ventricular surface sprouting from the region of the sinus venosus, on the dorsal side (Fig. 1c, increased magnification in Fig. 1d and Extended Data Fig. 1a, increased magnification in Extended Data Fig. 1b). At E16.5 the major dorsal vessels spread inferiorly from the inflow region (Fig. 1e, increased magnification in Fig. 1f), while ventrally smaller vessels arose between the atria (Extended Data Fig. 1c, d). By E18.5, the vessels continued to expand and projected towards the apex of the heart on both dorsal and ventral surfaces (Fig. 1g, h and Extended Data Fig. 1e, f). From birth (postnatal day 0 (P0)), the vessels developed a more extensive branched network and expanded further over the ventral side of the neonatal heart (Fig. 1i, j). By P10, the cardiac lymphatics provided superficial coverage of the majority of the epicardial surface of the heart (Extended Data Fig. 1g, h) and appeared fully developed by P15 (Extended Data Fig. 1i, j). The lymphatic identity of the VEGFR-3- and Prox1-labelled cardiac vessels (Fig. 1a–j and Extended Data Fig. 1a–n) was further validated by co-immunostaining for the lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1), which also labels tissue macrophages. Coronary LECs within the expanding plexus on both dorsal and ventral sides of the developing heart co-expressed VEGFR-3, Prox1 and Lyve-1 (Extended Data Fig. 1o–v). Cardiac lymphatic vessels aligned with the endomucin (Emcn)-positive coronary veins during late gestation (E15.5–18.5) and established extensive inter-vessel connections analogous to blood vessel anastomosis (Fig. 1n–p). At birth (P0) lateral Lyve-1+ sprouts beneath smooth-muscle-actin-positive coronary veins (Fig. 1q–s) were indicative of a close anatomical relationship between the coronary veins and developing lymphatic vasculature (Fig. 1t).

A venous and non-venous contribution of LECs

Prox1+ LECs did not appear to emerge or bud-off from Emcn-expressing coronary vessels between E12.5–14.5 (Extended Data Fig. 2a–i). Instead, extra-cardiac LECs migrated into the sinus venosus on the dorsal side, and outflow tract on the ventral side of the heart by E12.5 (Extended Data Fig. 2a, b, also Fig. 1a, b) and expanded to form a network proximal to Emcn+ veins from E13.5 and E14.5 (Extended Data Fig. 2d–i) through to E17.5 (Extended Data Fig. 2j–o). Whole embryo staining at E10.5 and E12.5 (Extended Data Fig. 3a–f) revealed a Prox1/VEGFR-3-expressing LEC population emerging from the Emcn+ common cardinal vein and migrating towards the neighbouring sinus venosus and outflow tract (Extended Data Fig. 3a–f).
Figure 1 | Spatiotemporal development of the murine cardiac lymphatic vasculature. a-h, Whole-mount confocal imaging of embryonic hearts stained with VEGFR-3 and Prox1 at E12.5 (a; white box enlarged in b), E14.5 (c; white box enlarged in d), E16.5 (e; enlarged box in f) and E18.5 (g; enlarged box in h). i, j. From birth (P0), lymphatic vessels branch and expand further onto the dorsal epicardial surface of the heart (i; enlarged box in j). Schematics below the images represent the stages of lymphatic vessel development (n = 5 hearts analysed per time point). k, l. Whole-mount staining with Emcn (veins) and Lyve-1 (lymphatics). m, 3,3′-diaminobenzidine (DAB) staining with VEGFR-3. n-p. Enlarged images of box in k stained for Emcn (m), Lyve-1 (o) or both (p). White arrowhead in α highlights a coronary vein. q-s. α-Smooth muscle actin (SMA)- (veins, q) and Lyve-1-stained (lymphatics, r) hearts (white arrowhead in r indicates location of blood vessels) at later stages (P0). s. Merge of SMA and Lyve-1 staining. t. Schematic representation of the dorsal side of the heart at P10 (shown in Extended Data Fig. 1g, h; n = 5 hearts analysed per time point). CA, coronary artery; CV, coronary vein. Scale bars: a, c, e, f, 300 μm; g, 1 mm; i, 2 mm; k–m, 200 μm; p, 10 μm; s, 5 μm.

Fig. 3d–f), suggesting that cardinal-vein-derived endothelial cells may be the venous source of coronary lymphatic vessels, an observation supported by previous studies67.

To investigate the lymphatic cellular origin further, we first performed lineage-tracing experiments using a Tie2-Cre line68 with a R26R-eYFP reporter69, revealing labelling of the embryonic cardinal vein at E10.5 (Extended Data Fig. 4a–d). At E12.5, Emcn+ jugular (cardinal) veins and lymph sacs, contributors to the systemic lymphatic vascular network70, were both YFP+ and Lyve-1+ (Fig. 2a–f). In contrast, E14.5 hearts revealed lymphatic vessels proximal to the outflow tract region which were YFP− (Fig. 2g, h), despite complete Tie2-eYFP recombination and labelling of lymphatics elsewhere in the embryo (Extended Data Fig. 4a–c). The relative incidence of YFP+ versus YFP− lymphatic vessels in the heart was 78 ± 5.5% YFP+ versus 19 ± 3.3% YFP− cells (mean percentage of cells ± s.e.m. per field of view; n = 24 fields of view; six fields of view per heart, four hearts in total) and confirmed by orthogonal z-stack reconstruction

Figure 2 | Incomplete contribution of Tie2+ venous-derived LECs indicates a novel non-venous contribution to the developing cardiac lymphatics. a, b, Tie2-Cre;R26R-eYFP embryos at E12.5 (a) stained with anti-GFP to detect the eYFP reporter expression, -Lyve-1 and -Emcn antibodies (b). c–f, Enlarged images of the area marked by the white box in b. JLS, jugular lymph sac; ILS, jugular vein. Scale bars: a, 200 μm; b–h, j–m, 100 μm.

Fig. 2i), which revealed both YFP+ (Fig. 2j, k) and YFP− vessels (Fig. 2i, m) in the developing heart at E17.5.

To confirm a non-venous contribution to cardiac lymphatic vessels, we analysed tamoxifen-inducible PDGFβ-CreERT2 mice, crossed with either R26R-tdTomato14 or R26R-mTmG13 reporter lines to specifically label endothelial cells lining blood vessels (Extended Data Fig. 4e–o). Incomplete recombination of tdTomato within Lyve-1 lymphatic vessels (Extended Data Fig. 4f) was evident with both tdTomato+/Lyve-1− (Extended Data Fig. 4g–i) as well as tdTomato+/Lyve-1+ lymphatic vessels (Extended Data Fig. 4j–l), indicating a mixed contribution of endothelial- and non-endothelial-derived cardiac lymphatics. This was supported by crosses with an mTmG reporter mouse, where the level of GFP recombination within cardiac lymphatic vessels was mosaic (Extended Data Fig. 4m–o).

A putative haemogenic source of cardiac LECs

We next examined the possibility that an alternate source of LECs might arise from one of three potential cardiac progenitor populations: the epicardium, cardiac mesoderm (early and late stage) or cardiac neural crest by lineage tracing with Wt1-CreERT2, Mesp1-Cre, Nkx2.5-Creβ and Wnt1-Creβ lines crossed with the R26R-eYFP reporter, respectively. There was no contribution of Wt1+ (YFP−) cells to the developing coronary lymphatics, excluding the pro-epicardial organ as a source of LECs (Extended Data Fig. 5a–c)
and neither Mesp1−/− nor Nkx2.5−/− labelled lateral-plate-mesoderm-derived progenitors (Extended Data Fig. 5d–i) nor Wnt1−/− cardiac neural crest cells (Extended Data Fig. 5j–l) contributed to the developing coronary lymphatics. Subsequently, we sought to determine whether there might be a distinct Tie2− endothelial source of LEC progenitors. The haemogenic endothelium represents the site of primitive haematopoiesis in the visceral yolk sac and developing blood islands of the early embryo and while Tie2-Cre does label a significant proportion of cells within the yolk sac, there are aggregations and primitive haematopoietic derivatives which are Tie2-negative21. To potentially capture this Tie2− population, we employed three Cre-driver lines under the control of Vav122–25, Pdgfrβ26 and Csf1r26, in combination with either the R26R-tdTomato or R26R-mTmG reporters. Initially, we excluded reporter labelling of the endothermal of the common cardinal vein by these three drivers at E10.0 (Extended Data Fig. 6a–d and data not shown) and jugular vein at E12.5 (Extended Data Fig. 6e–h), however, we cannot exclude the possibility of tracing a subset of venous-derived cells fated to form LECs before any evidence of Prox1 expression. Extensive labelling of Vav1−tdTomato+ cells was evident in regions of the developing heart at E17.5 (Fig. 3a), including tdTomato+/Lyve-1+ tissue macrophages (Fig. 3a), which were negative for Prox1 and located proximally to the developing vessels (Fig. 3b,c). In contrast to the situation in the heart, Vav1−tdTomato+ cells were not observed in the dermal lymphatics from dorsal skin preparations analysed at E17.5 (n = 4 embryos; Extended Data Fig. 5m–r). Subsequently, we confirmed the presence of tdTomato+ cells within lymphatic vessels which were Prox1+ and Lyve-1+ (Fig. 3a, b, d; 14 ± 5.3% tdTomato+/Prox1+ (mean percentage of cells ± s.e.m. per field of view; n = 20 fields of view; five fields of view per heart, four hearts in total) as confirmed by z-stack reconstruction (Fig. 3a). In Pdgfrβ−Cre;R26R-mTmG embryos, GFP+ cells were observed in the coronary lymphatics at E17.5 (Fig. 3e), which were positive for Prox1 (Fig. 3f, g) and VEGFR-3 (Fig. 3h; 28 ± 4.7% GFP+/Prox1+; mean percentage of cells ± s.e.m. per field of view; n = 18 fields of view; six fields of view per heart, three hearts in total) (Fig. 3e with z-stack) and in hearts derived from Csf1r−CreER;R26R-tdTomato embryos, a contribution of tdTomato+ cells which co-labelled with Prox1 (Fig. 3i with z-stack) and Lyve-1 (Fig. 3j–l with z-stacks), further suggested a yolk-sac progenitor contribution (Fig. 3i–l). The relative incidence of tdTomato+/Prox1+ cells was low (less than 5%) likely reflecting inefficient labelling by the inducible Csf1r−CreER. In order to investigate a yolk-sac contribution to LECs further, we derived ex vivo cultures of explanted Vav1−Cre;R26R-ttdTomato conceptuses at E8.027. Intact yolk sac explants were treated with 100 ng ml−1 of recombinant VEGF-C(C156S)28, a potent selective lymphangiogenic cue that only signals via VEGFR-3 (the C156S mutation prevents binding to VEGFR-2). A tdTomato+ outgrowth from the yolk sac was observed under VEGF-C induction (Extended Data Fig. 6i,j) with specification of Prox1− LECs in culture (Extended Data Fig. 6k–z). Since this stage of development was too early to detect a venous origin or alternate embryonic source, we conclude that these LECs were yolk-sac-derived.

**Prox1 loss of function supports dual LEC origin**

To provide further evidence for both a venous-endothelium and independent source of cardiac LECs, we genetically deleted Prox1 independently in both the Tie2+ blood endothelial and Vav1+ compartments. We first used Prox1 conditional mice29 crossed with the Tie2-Cre mice (Extended Data Fig. 7a). Fluorescence-activated cell sorting of targeted GFP+ cells from isolated Tie2-CreProx1β/β hearts (Extended Data Fig. 7b) revealed appropriate knock-down of Prox1 (Extended Data Fig. 7c; 0.59-fold; n = 5 mutant hearts analysed; P ≤ 0.05), accompanied by knock-down of Vegfr3 (also known as Flt4; Extended Data Fig. 7d; 0.39-fold; n = 5 mutant hearts analysed; P ≤ 0.001) and Lyve1 (Extended Data Fig. 7e; 0.22-fold; n = 5 mutant hearts analysed; P ≤ 0.001). Tie2-CreProx1β/β mutant embryos had gross vascular anomalies, including ectopic surface blood vessels, a disrupted vascular network and apparent haemorrhaging (Extended Data Fig. 7f–i). An initial failure in specification of cardiac LECs was confirmed at E14.5, coincident with the first emergence of the lymphatics on the dorsal surface of the heart (Fig. 1c). GFP− targeted and Lyve-1− LECs were observed at the base proximal to the atrioregional region of the heart in Tie2-CreProx1β/β controls but were absent in the mutant hearts (Extended Data Fig. 8a–f). There was no apparent effect on the coronary vasculature, as determined by comparable whole-mount CD31 staining (Extended Data Fig. 8g, h). At E17.5 Tie2-CreProx1β/β hearts

**Figure 3 | Vav1+, Pdgfrβ+ and Csf1r+ lineages contribute LECs to the developing cardiac lymphatics.** a, Vav1−CreR26R-ttdTomato lineage tracing revealed regions of tdTomato+ recombination throughout the heart at E17.5 (n = 4 hearts analysed). Co-labelled tdTomato+/Prox1+/Lyve-1+ were confirmed by z-stack reconstructions (below a, l–4; 5 and 6 lack Prox1 and are tdTomato+/Lyve-1+ macrophages). b–d, Representative enlarged views of alternative Vav1−Cre;R26R-tdTomato hearts. Left, tdTomato staining; middle, Prox1 staining; right, merged images of tdTomato, Prox1 and Lyve-1 staining. White arrowheads in b indicate cardiac lymphatic vessels. White arrows in c indicate macrophages proximal to the Prox1− LECs that were evident at higher magnification. e–h, Pdgfrβ−Cre;R26R-mTmG hearts at E17.5. White hearts including z-stack reconstruction (e, stacks 1–4 are GFP+/Prox1+; white arrowheads in j; 5 and 6 are singly Prox1+; n = 3 hearts analysed) revealed a heterogeneous contribution of recombinant GFP+ cells that were Prox1− (f, g) or VEGFR-3− (h). i–l, Analyses of E17.5 hearts from Csf1r−CreER;R26R-ttdTomato embryos injected with 4-hydroxytamoxifen at E7.5 (I, n = 5 hearts analysed) revealed contribution of recombinant tdTomato+ cells that were Prox1+ (z-stack projections 1–4 in i, and white arrowheads in j with accompanying z-stacks). j–l, Enlarged views of alternative Csf1r−CreER;R26R-tdTomato hearts. Scale bars: a, c, i, 60 μm; b–d, 30 μm; f, h, j, k, l, 100 μm; g, 200 μm.
were recovered largely devoid of VEGFR-3+ LECs (Extended Data Fig. 9a–d) and were relatively dysmorphic along the apical–basal (long) axis (Extended Data Fig. 9c, d), with smaller chambers and thickening of the ventricular compact layer (Extended Data Fig. 8i, j). Despite these anomalies, endocardial cushions formation appeared unaffected (Extended Data Fig. 8i, j). Relative to Tie2-Cre:Prox1+/lacZ heterozygotes (Extended Data Fig. 9e–h), GFP+/Lyve-1+ lymphatic vessels were either partially or completely absent from the dorsal surface and completely absent from the ventral surface of mutant hearts (Extended Data Fig. 9i–p). The partial and complete loss of LECs correlated with the loss of Prox1 protein expression (Extended Data Fig. 9k, o). Tie2-Cre:Prox1+/lacZ mutant hearts were also recovered largely devoid of VEGFR-3+ LECs and an extensive lymphatic network on the ventral surface as indicated by Lyve-1, with retained Prox1 expression (Extended Data Fig. 9q–s). Tie2-Cre:Prox1+/lacZ mutants revealed no obvious systemic vessel defects (Extended Data Fig. 7j–m). Co-expression of GFP+/Lyve-1+ was observed in LECs (Extended Data Fig. 9t–x), however, specific loss of Lyve-1+ LECs was detected at subcellular resolution that directly correlated with loss of Prox1 and GFP-targeting (Extended Data Fig. 9y, z), supporting a Prox1-dependent Vav1 source of cardiac lymphatics.

**Neo–lymphangiogenesis post–cardiac injury**

Lymphangiogenesis in other settings (most notably during skin infection) has been implicated in antigen clearance and inflammatory resolution30,31. Thus, we determined whether the cardiac lymphatics might attempt compensatory angiogenesis during the pro-inflammatory phase following myocardial infarction (MI)32. We first analysed VEGFR-3 protein levels as a surrogate for an early lymphatic response, and observed a significant increase in VEGFR-3 at all stages from 24 h up to 21 days post-MI (Fig. 4a). Alterations in VEGFR-3 protein levels were recapitulated at the gene-expression level (Fig. 4b) and a general activation of the developmental lymphatic gene program was confirmed by concomitant increased expression of Lyve1 and Prox1 (Fig. 4c, d). At day 7 following injury there was a significant increase in the branching of surface VEGFR-3+ lymphatic vessels (Fig. 4e, f), and alignment of Prox1+ lymphatic sprouting with EPCn+ veins (Fig. 4g). Longitudinal analyses, from days 7 to 35 post-MI, revealed marked spatiotemporal changes in the lymphatic response. In the intact heart there were few superficial lymphatic

**Figure 4 | Myocardial infarction induces a significant cardiac lymphangiogenic response that can be enhanced by VEGF-C-stimulation to promote functional improvement.** a, VEGFR-3 protein levels increased from 24 h to 21 days post-MI, peaking at day 4 (D4) (n = 3 animals analysed per time point; single representative western blot with densitometry). b–d, Real-time analysis of Vegfr3 (b), Lyve1 (c) and Prox1 (d) mRNA all revealed a significant increase in expression levels across the equivalent time-points post-MI (n = 3 animals per time point). e, f, VEGFR-3 whole-mount staining revealed increased lymphangiogenesis in the left ventricle (LV), proximal to the infarct 7 days post-MI (e, f; black arrowheads indicate areas in left ventricle with increased lymphangiogenesis, white arrowheads indicate areas with reduced lymphatic vessel density; n = 3 mice per group). g, Sprouting of Prox1+ lymphatics was observed aligning with EPCn+ veins 7 days post-MI (white asterisk, ligating suture (see Methods)). h–q, Short-axis sections at day 7 post-MI revealed Lyve-1-/+Pdpn+ lymphangiogenesis in the scar region (white boxes in h, j, l, n, p), which was significantly increased relative to the intact heart (h, i) and which expanded through days 14 (m), 21 (o) and 35 with large lymphatic ‘shunts’ evident in the left ventricle (q; n = 5 hearts analysed). i, k, m, o, q, Enlarged images of the boxed areas in h, j, l, n, p, respectively. RV, right ventricle. r–u, Whole-mount X-gal staining of Vegfr3+/Tg (u) hearts after administration of VEGF-C reveals the lymphangiogenic response post-MI (n = 3 per treatment group). r–s, m, i, Lice treated with recombinant human VEGF-C(C156S) exhibited extensive lymphangiogenesis in the injury area (t, black arrowheads and white inset box enlarged in the top image of panel u) compared with vehicle-treated (s, black arrowheads and white inset box enlarged in the bottom image of panel u) or sham-operated mice (r, w, Whole-mount DAB staining of MI hearts with or without VEGF-C administration with Vegfr3- (v) or Prox-1 (w) confirmed the observations in r–u (white asterisks indicate the ligating suture (see Methods)). x, y, Longitudinal MRI analyses of infarcted hearts 21 days after surgery following treatment with either vehicle (x) or VEGF-C (y). z, Ejection fraction measurements revealed a significant improvement in VEGF-C-treated hearts, compared to vehicle, at 14 and 21 days post-MI. n = 8 wild-type mice per treatment group. All graphs show mean ± s.e.m. Data analysed with Student’s t-test; *P < 0.05. Scale bars: e, f: 1 mm; g: 400 μm; h, j, l, n, p, 200 μm; i, k, m, o, q, 400 μm; r–t: 1 mm; u–w: 500 μm; x, y: 2 mm.
Table 1 | Functional parameters from longitudinal MRI of VEGF-C- and vehicle-treated hearts post-MI

|                      | 7 days post-MI | 14 days post-MI | 21 days post-MI | 28 days post-MI |
|----------------------|----------------|-----------------|-----------------|-----------------|
| **Body weight (g)**  | 190 ± 0.6      | 198 ± 0.5       | 196 ± 0.7       | 198 ± 0.5       |
| **Heart rate (b.p.m.)** | 515 ± 10     | 499 ± 19        | 511 ± 6         | 496 ± 19        |
| **Left ventricle**   |                |                 |                 |                 |
| End diastolic volume (µl) | 49.6 ± 4.4   | 68.9 ± 11.1     | 48.3 ± 4.0      | 70.9 ± 10.9     |
| End systolic volume (µl) | 2.3 ± 4.8    | 40.3 ± 9.7      | 21.5 ± 4.1*     | 43.5 ± 10.0*    |
| Stroke volume (µl)   | 26.5 ± 2.3    | 28.6 ± 2.3      | 26.8 ± 1.5      | 27.3 ± 1.5      |
| Ejection fraction (%) | 56 ± 6        | 46 ± 5          | 57 ± 6          | 43 ± 5*         |
| Cardiac output (ml min⁻¹) | 13.5 ± 0.9    | 14.3 ± 1.4      | 13.7 ± 0.6      | 13.6 ± 1.0      |
| Left ventricular mass (g) | 80 ± 5       | 96 ± 8          | 80 ± 4          | 90 ± 7          |
| Absolute infarct size (mm²) | 13.6 ± 5.6    | 36.4 ± 11.4     | 14.2 ± 5.8      | 35.0 ± 10.8     |
| Relative infarct size (%) | 10 ± 4        | 21 ± 5          | 11 ± 4          | 20 ± 5          |

Data presented as mean ± standard error of the mean. Asterisks indicate significant differences between VEGF-C- and PBS-treated hearts; P < 0.05; repeated measures t-test, two-tailed distribution, two-sample equal variance.

Table 1: Functional parameters from longitudinal MRI of VEGF-C- and vehicle-treated hearts post-MI.

The table provides a comparison of functional parameters from longitudinal MRI of VEGF-C- and vehicle-treated hearts post-MI. The parameters include body weight, heart rate, end diastolic and systolic volumes, stroke volume, ejection fraction, cardiac output, left ventricular mass, absolute and relative infarct sizes.

**Discussion**

Our study challenges the unequivocal view of lymphatic vessel development derived from Sabin’s model of venous origin. We reveal that the lymphatic vasculature of the embryonic mouse heart comprises a heterogeneous make-up of cell populations, with contributions derived from both extra-cardiac venous endothelium and a novel source of lymphatic progenitors which may arise from the yolk sac haemogenic endothelium. Targeting of Prox1 in both venous endothelial and non-venous-derived compartments resulted in loss of the cardiac LECs, supporting a dual origin in the developing heart and consistent with previous studies demonstrating that Prox1 is both necessary and sufficient to drive LEC fate specification. Prox1 acts at the decision point between blood and lymphatic endothelial cell specification, such that Prox1-deficient LECs contributing to the systemic blood vasculature resulted in ectopic vessels and haemorrhaging throughout the embryo. However, in Tie2-Prox1 mutant hearts, hypoplasia of the lymphatic vessels did not appear to impact upon the gross development of the coronary blood vessels, highlighting a further unique ontology of the cardiac lymphatics relative to systemic lymphatic vasculature. Previously, Proxl dosage effects underpinned formation of the systemic lymphovenous valves; here partial Proxl knockdown resulted in formation of the cardiac lymphatics but with truncation of the developing plexus and aberrant remodelling suggesting a novel role for Prox1 in maintaining the cardiac lymphatic network.

Insight into the embryological origin and development of the cardiac lymphatics has important implications for understanding cardiovascular fluid homeostasis, injury-induced inflammation and disease. Following MI the cardiac lymphatics underwent a profound angiogenic response, accompanied by an upregulation in the lymphatic development gene program. Significantly, this was enhanced by ectopic VEGF-C stimulation following injury, leading to improvement in cardiac function. Myocardial injury is associated with a robust immune reaction, characterized by sequential mobilization of monocytes involved in inflammatory functions and wound healing. Lymphangiogenesis in inflammatory settings facilitates the resolution of tissue oedema and promotes macrophage mobilization and induction by VEGF-C alleviates inflammation in mouse models. Therefore, mechanisms coupling lymphatic development to immune regulation represent a therapeutic target. Induction of lymphatic vessels could provide a pathway for inflammatory cell efflux to tip the balance in favour of wound healing within the injured adult heart.

**Online Content**

Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions L.K., S.N. and J.M.V. carried out all experiments (except myocardial infarction surgeries and MRI scanning), analysed the data and contributed figures for the manuscript. K.D., S.B., M.M. and M.R. performed all MI surgeries and MRI scanning), analysed the data and contributed conflicting or competing interests.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to P.R. (paul.riley@dpag.ox.ac.uk).
METHODS

Mouse strains. The following mouse strains were used as previously described: Csf1r-CreER<sup>26</sup>, MesP1-Cre<sup>45</sup>, Nkx2.5-Cre<sup>46</sup>, Pdgfrα-CreER<sup>27</sup>, Pdgfrβ-Cre<sup>45</sup>, Prox1<sup>2</sup> (ref. 29), R26R-EYFP<sup>15</sup>, R26R-mTmG<sup>15</sup>, R26R-tdTomato<sup>44</sup>, Tie2-Cre<sup>45</sup>, Vav1-Cre<sup>46</sup>, Wnt1-CreER<sup>27</sup>, Wnt1-Cre<sup>46</sup>, Vegfr3<sup>2</sup> (ref. 34). Breeding was carried out using only Cre<sup>+</sup> males for all Cre strains except the Vav1-Cre where Cre<sup>−</sup> females were used. Pregnant females crossed to inducible Cre male studs were injected intraperitoneally at E7.5 (Csf1r-CreER) or E9.5 (Wnt1-CreER2T2; Pdgfrα-CreER2T2) with 2 mg of 4-hydroxytamoxifen (4-OHT) dissolved in peanut oil. Embryonic staging was determined by the day of the vaginal plug (E0.5). C57BL/6 mice were used for the longitudinal cardiac cine-MRI study. Investigators were blinded to genotype and treatment groups. All animal experiments were carried out according to UK Home Office project license PPL 30/2987 Compliant with the UK Animals (Scientific Procedures) Act 1986.

Quantitative real-time PCR. Total RNA was isolated from hearts using the Qiagen RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized using the Reverse Transcription System (Promega), following the manufacturer’s instructions and used for quantitative real-time PCR using SYBR Green on an ABI 7900 for the following genes: Vegfr3, Prox1, Lyve1. Fold change was determined by applying the 2<sup>-(ΔΔC<sub>T</sub>)</sup> method, following the primer sequences used. Vegfr3, 5′-CCATCGAGAGTCTGGACAGC-3′ reverse; Prox1, 5′-GGGACTTGCAGCTATG-3′ forward, 5′-diaminobenzidine (DAB) staining was performed. Cardiac and epicardial circumferential lengths of the thinned, akinetic region of all fields of view per heart analysed. Western blotting. Heart samples were lysed in RIPA buffer (50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS) supplemented with protease inhibitors (Protease Inhibitor Cocktail Tablet (Roche), 1 mM PMSF (Sigma) and 1 mg ml<sup>−1</sup> aprotinin (Sigma)). The lysate was centrifuged at 13,000g for 15 min at 4°C and the supernatant retained. For SDS-PAGE samples were incubated with an equal volume of 2× Laemmli Buffer/5% 2-mercaptoethanol (Life Technologies) supplemented with 100 ng ml<sup>−1</sup> recombinant human VEGF-C (C156S) (R&D systems) or PBS. Further injections were administered at 2, 3, and 4 days post-surgery. Experimenters were blinded to treatment groups for subsequent cardiac cine-MRI and analysis. Hearts were collected at 1, 2, 4, 7, 14, 21, 28 and 35 days post-MI and either sectioned or left intact and prepared for histology, immunofluorescence, RNA and protein extraction. Mice were housed and maintained in a controlled environment. All surgical and pharmacological procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986, (Home Office, UK).

Cardiac cine-MRI. Cardiac cine-MRI was performed post-LAD ligation as described<sup>46</sup>. In brief, mice were anaesthetized with 2% isoflurane and placed under assisted external ventilation through the insertion of an endotracheal tube. Cardiac injury was induced by permanent ligation of the left descending artery (LAD). LAD-ligation mice were directly compared with sham-operated animals which underwent tracheotomy, opening of the chest and insertion of the needle trough the left ventricle but no suture ligation. Sutureless LAD-ligation (subcutaneous hydrodilution; Vegerise) was delivered as a 0.015 mg ml<sup>−1</sup> solution via intraperitoneal injection at 20 min before the procedure to provide analgesia. On recovery mice were randomly allocated to receive an intraperitoneal injection of 0.1 μg g<sup>−1</sup> recombinant human VEGF-C (C156S) (R&D systems) or PBS. Further injections were administered at 2, 3, 4 and 6 days post-surgery. Experimenters were blinded to treatment groups for subsequent cardiac cine-MRI and analysis. Hearts were collected at 1, 2, 4, 7, 14, 21, 28 and 35 days post-MI and either sectioned or left intact and prepared for histology, immunofluorescence, RNA and protein extraction. Mice were housed and maintained in a controlled environment. All surgical and pharmacological procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986, (Home Office, UK).

Cardiac cine-MRI. Cardiac cine-MRI was performed post-LAD ligation as described<sup>46</sup>. In brief, mice were anaesthetized with 2% isoflurane in O<sub>2</sub> and positioned supine in a purpose-built cradle. ECG electrodes were inserted into the forepaws and a respiration loop was taped across the chest. The cradle was lowered into a vertical-bore, 11.7 T magnetic resonance system (Magnex Scientific) with a 40 mm birdcage coil (Rapid Biomedical) and a Bruker console running Paravision 2.11 (Bruker Medical). A stack of contiguous 1-mm thick true short-axis ECG-gated cine-FLASH images were acquired to cover the entire left ventricle (TE/TR 1.43/4.6 ms; 17.5° pulse; field of view 25.6×25.6 mm; matrix size 128×128 zero filled to 256×256 giving a voxel size of 100×100×1,000 μm; 20 to 30 frames per cardiac cycle). Long-axis two-chamber and four-chamber images were also acquired.

MRI data analysis. Blinded image analysis was performed using ImageJ (NIH). Left ventricular mass, volumes and ejection fraction were calculated as described<sup>46</sup>. The relative infarct size was calculated from the average of the endocardial and epicardial circumferential lengths of the thinned, akinetic region of all slices, measured at diastole, and expressed as a percentage of the total myocardial surface area<sup>46</sup>.

Statistical analysis. No statistical methods were used to predetermine sample size. Statistical difference between groups was evaluated using Student’s t-test (two-tailed) or one-way ANOVA. A P value of <0.05 was considered statistically significant. All values and graphs present the mean value ± s.e.m.

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| VEGFR-3 | E14.5 | E16.5 | E18.5 | P10 | P15 |
|---------|-------|-------|-------|-----|-----|
| dorsal  |       |       |       |     |     |
| ventral |       |       |       |     |     |

| Prox1   | E17.5 |       |
|---------|-------|-------|
| ventral |       |       |
| dorsal  |       |       |

| Prox1 VEGFR-3 LYVE-1 | E17.5 |       |
|----------------------|-------|-------|
|                      |       |       |

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Extended Data Figure 1 | Molecular characterization of the murine cardiac lymphatic vasculature. a–j, Whole-mount DAB staining of hearts (n = 3 per time point) with the lymphatic marker VEGFR-3 revealed cardiac lymphatic vessels first sprout from the region of the sinus venosus, on the dorsal side of the heart at E14.5 (a, white box, enlarged in b). At E16.5, ventrally the first small vessels arose between the atria (c), while the main dorsal vessels spread inferiorly from the sinus venosus at the inflow region of the heart (d). At E18.5 the network appears similar with little expansion (e, f). From birth (P0) lymphatic vessels branch and expand onto the ventral epicardial surface of the heart such that by P10 the network has expanded markedly, coincident with cardiac growth (g, h). Consistent with the systemic lymphatic vasculature, cardiac lymphatic vessels are fully developed by P15 (i, j), with no difference in vessel density at later stages (data not shown). k–n, Whole-mount DAB staining of E17.5 hearts with the lymphatic marker Prox1 (n = 4) further confirmed extensive spread of the sprouting lymphatics inferiorly from the outflow tract region (k) and sinus venosus, at the inflow region of the heart (l). White inset boxes in k and l are shown in m and n, respectively, highlighting the punctate nuclear expression of Prox1 in coronary lymphatics. o–v, Whole-mount confocal imaging of E17.5 hearts (n = 4) stained with VEGFR-3, Prox1 and Lyve-1 confirmed co-labelling of coronary lymphatic vessels. Note that while at this developmental stage VEGFR-3 is restricted to LECs (p, t), Prox1 is also expressed in the underlying myocardium (o, s) and Lyve-1 labels tissue-resident macrophages (q, u). Scale bars: a, 750 μm; b, 300 μm; c, d, 750 μm; e, f, 1 mm; g, h, 2 mm; i, j, 2.5 mm; k, l, 400 μm; m, n, 200 μm; o, s, 100 μm.
Extended Data Figure 2 | Cardiac lymphatic vessels do not emerge from the developing coronary vasculature. a–o, Whole-mount confocal imaging of hearts stained with Emcn (vessels) and Prox1 (lymphatics) revealed sprouting of Prox1⁺ lymphatics from extra-cardiac tissue neighbouring the sinus venosus on the dorsal side of the developing heart at E12.5–13.5 (a, d), but no Prox1⁺ LECs were observed budding from Emcn⁺ coronary vessels (c and f; white arrowheads in b and e highlight Prox1⁺ LECs). Prox1⁺ lymphatics had reached the sinus venosus by E13.5 (white arrow in f) and the outflow tract, on the ventral side of the heart by E14.5 (white arrow in h); no Prox1⁺ LECs were observed emerging from Emcn⁻ vessels on the ventral side at E14.5 (g–i). Background-like labelling on the ventricular surface in c, f and i reflects Prox1 expression in the developing myocardium. Between E15.5–17.5, Prox1⁺ lymphatics aligned with Emcn⁺ coronary veins but no contribution of Prox1⁺ LECs was observed (j, l and n, white boxes enlarged in k, m and o, respectively; n = 5 hearts analysed per time point). Scale bars: a, 550 μm; c, f, i, 250 μm; d, g, 750 μm; j, l, n, 400 μm; k, m, o, 200 μm.
Extended Data Figure 3 | The common cardinal vein contributes LECs that migrate towards the sinus venosus and outflow tract of the developing heart. a–c, Whole-mount confocal analysis of E10.5 embryos stained with Emcn or Prox1 and VEGFR-3 revealed Prox1/VEGFR-3– LECs emerging along the common cardinal vein (a, red box enlarged in b; white box enlarged in c) migrating towards the sinus venosus (white arrowheads in c; n = 3 embryos).

d–f, Whole-mount DAB staining revealed Prox1+ LECs migrating towards the outflow tract, on the ventral surface of the developing heart at E12.5 (d, white inset box enlarged in e; alternative lateral view in f; white arrowheads indicate migrating LECs; n = 4 embryos). ba, branchial arch; ccv, common cardinal vein; fl, forelimb; h, heart; isv, inter-somitic vessel; la, left atrium; lv, left ventricle; oft, outflow tract; paa, pharyngeal artery arch; ra, right atrium; rv, right ventricle. Scale bars: a, 1 mm; b, 500 μm; c, 200 μm; d, 600 μm; e, 400 μm; f, 300 μm.
Extended Data Figure 4 | Tie2-Cre efficiently labels the developing cardinal vein and partial contribution of Pdgfb<sup>1</sup>-derived LECs indicates a non-venous contribution to the developing cardiac lymphatics. a–d, Tie2-Cre;R26R-eYFP lineage tracing revealed recombination and labelling of the cardinal vein and jugular lymph sacs at E10.5 (a; n = 3 embryos analysed). Plane of section to capture jugular lymph sacs is shown in b. White inset box in a is shown at higher magnification and demarcated by GFP (c) and Emcn (d) co-staining. e–o, Schematic (e) to show how embryos were generated by breeding Pdgfb-CreER<sup>17</sup> mice with either R26R-tdTomato (f–l) or R26R-mTmG (m–o) reporter mice and then being injected with 4-hydroxytamoxifen (4-OHT) at E9.5, before venous sprouting. Whole-mount confocal analysis of E17.5 hearts (n = 4) stained with Lyve-1 revealed incomplete tdTomato recombination in cardiac lymphatic vessels (f). Both Pdgfb<sup>1</sup> (g–i) and Pdgfb<sup>2</sup> (j–l; m–o) lymphatic vessels were observed, highlighted by the dotted green outlines (g, j, m), indicating a combined Pdgfb<sup>1</sup> endothelial origin and Pdgfb<sup>2</sup> non-venous source for the cardiac LECs. Scale bars: a, 200 μm; b, 1.5 mm; c, d, 50 μm; f, 400 μm; e, l, o, 100 μm.
Extended Data Figure 5 | Neither the pro-epicardial organ, cardiac mesoderm nor cardiac neural crest contribute LECs to the developing heart and dermal lymphatics are not derived from the Vav1 lineages. a–l, Lineage tracing using WT1-CreERT2;R26R-eYFP (4-hydroxytamoxifen injected at E9.5; a–c), Mesp1-Cre;R26R-eYFP (d–f), Nkx2.5-Cre;R26R-EYFP (g–i) and Wnt1-Cre;R26R-eYFP (j–l; n = 3 hearts analysed per lineage trace) showed no YFP recombination in cardiac lymphatic vessels as marked by Prox1 or Lyve-1, suggesting that neither the pro-epicardial organ/epicardium, cardiac mesoderm (early or late) or cardiac neural crest, respectively, contribute LECs to the developing cardiac lymphatics.

m–o, Embryos generated by breeding Vav1-Cre with R26R-tdTomato reporter mice were subject to whole-mount confocal analysis of E17.5 dorsal skin preparations (n = 4 Vav1-tdTomato+ embryos analysed). tdTomato epifluorescence (m) and Prox1 immunostaining (n) revealed a lack of Vav1-Cre recombination in dermal lymphatic vessels (highlighted by the green dotted lines, m) and a lack of overlap of tdTomato with Prox1 and Lyve-1 expression (o; all Prox1+ nuclei assessed across 5 fields of view per embryonic skin; n = 4 skins in total).

p–r, Higher magnification of inset white box (n) revealed that tdTomato+ cells (p) did not overlap with Prox1+ nuclei in the lymphatic vessels (q) (white arrowhead highlights tdTomato+/Prox1− cell in r). Scale bars: a–l, 100 μm; m–o, 100 μm; p–r, 50 μm.
Extended Data Figure 6 | The Vav1<sup>+</sup> lineage does not contribute to LECs emerging from the common cardinal or jugular veins but contributes to VEGF-C induced LECs emerging from yolk sac explants. a–d, Vav1-Cre;R26R-tdTomato lineage tracing revealed no recombination nor labelling of the nascent LECs budding from common cardinal vein endothelium in E10.0 embryos (a) as confirmed by co-staining for Emcn, Prox1 and tdTomato fluorescence. White inset box in a is highlighted in enlarged panels (b–d; arrows indicate Prox1<sup>+</sup> (blue) LECs delaminating from the common cardinal vein b, c). In the sinus venosus region Vav1<sup>+</sup> cells were evident but lacked Prox1 expression, excluding an LEC identity (d). e–h, Vav1-Cre;R26R-eYFP lineage tracing revealed no recombination nor labelling of LECs forming the jugular lymph sacs in E12.5 embryos (e) as confirmed by co-staining for GFP, Emcn and Prox1. White inset box in e highlighted by individual GFP (f), Emcn (g) and Prox1 (h) staining (n = 3 embryos analysed per time-point). ccv, common cardinal vein; jls, jugular lymph sac; jv, jugular vein; sv, sinus venosus. i, j, Representative staining for Prox1 and native tdTomato fluorescence of ex vivo cultures of explanted Vav1-Cre;R26R-tdTomato conceptuses at E8.0, including the intact yolk sac (i) and outgrowth of tdTomato<sup>+</sup> cells (j). Explants were cultured with 100 ng ml<sup>–1</sup> of recombinant VEGF-C(C156S)<sup>28</sup> (R&D Systems), a potent selective lymphangiogenic cue that only signals via VEGFR-3 (i). k–z, High-resolution images of the specification of tdTomato<sup>+</sup>/Prox1<sup>+</sup> LECs (indicated by white inset boxes) in the yolk sac explants (k–n) and in the surrounding cellular outgrowth (o–z) was observed (tdTomato<sup>+</sup> in red; Prox1<sup>+</sup> in blue; single and merged channels shown). Co-staining was confirmed by z-stack reconstructions for each four high-resolution panel set (n, r, v, z); n = 6 explants analysed. Scale bars: a, e, 50 μm; b–d and f–h, 12.5 μm; i, 100 μm; j, 50 μm; m, q, u, y, 15 μm.
Extended Data Figure 7 | Prox1 knockdown results in significantly decreased Vegfr3 and Lyve1 and Tie2-Cre;Prox1fl/fl mutant embryos exhibit superficial vascular defects whereas Vav1-Cre;Prox1fl/fl mutants have a normal systemic vasculature. a, Prox1 targeting via floxed excision of exon 1 and 2, results in EGFP expression thus labelling targeted cells. b, E17.5 hearts from either Tie2-Cre;Prox1fl/+ control embryos or Tie2-Cre;Prox1fl/fl mutants were grouped and digested to create a single-cell suspension for FACS. A total of 100,000 GFP cells were collected for each sample group. c–e, Relative gene expression was determined by qRT–PCR and revealed significantly decreased Prox1 (c; 0.59 fold), Vegfr3 (d; 0.39 fold) and Lyve1 (e; 0.22 fold) expression; n = 5 hearts per sample group, analysed in triplicate; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. All graphs are mean ± s.e.m. Statistical test used was Student’s t-test. f–i, Dissection of Tie2-Cre;Prox1fl/+ heterozygous (f; n = 6) and Tie2-Cre;Prox1fl/fl mutant (g, h; n = 9) littermate embryos at E17.5 revealed gross vascular anomalies in the double-floxed mutants (three examples shown in g–i), with evidence of ectopic surface blood vessels (g, ectopic vessels highlighted by black arrowheads), a disrupted vascular network (h; black arrowheads indicate blood-filled superficial vessels) and either haemorrhaging (i; bleeding foci highlighted by black arrowheads) or blood-filled lymphatics, compared to littermate Prox1fl/+ controls (f). j–m, Vav1-Cre;Prox1fl/+ heterozygous (j, k; n = 5) and Vav1-Cre;Prox1fl/fl mutants (l, m; n = 8) revealed no obvious systemic vessel defects. Scale bars: g, h, m, 100 μm.
Extended Data Figure 8 | The emergence of cardiac lymphatics at E14.5 is disrupted in Tie2-Cre;Prox1^{fl/fl} mutant hearts, which are dysmorphic and exhibit elevated apoptosis of LECs, however, mutant embryos recover with normal cardiac lymphatics at post-natal stages. a–c, GFP^{+}-targeted and Lyve-1^{+} LECs emerged from the base of the heart in the atrioventricular region at E14.5 in control Tie2-Cre;Prox1^{fl/+} hearts (LECs highlighted by white arrowheads in a and b, n = 3 hearts analysed). d–f, In mutant Tie2-Cre;Prox1^{fl/fl} hearts (n = 7) the GFP^{+} network was absent (d) and Lyve-1 only detected tissue resident macrophages with an absence of lymphatics at the inflow base of the heart (arrows in e, f). g, h, Coronary vessels, as determined by whole mount CD31 staining, were comparable between control Tie2-Cre;Prox1^{fl/+} (g) and Tie2-Cre;Prox1^{fl/fl} hearts (h). i, j, Haematoxylin and eosin staining of paraffin-embedded E17.5 hearts revealed that Tie2-Cre;Prox1^{fl/fl} mutants (j; n = 3 analysed) were grossly smaller compared to control hearts (i), with lack of extension of the ventricles towards the apex, smaller chambers and thickening of the ventricular free wall (j). Normal membranous septation of the mutant ventricle (white asterix) and valve leaflet formation (white arrowhead in j) indicate normal endocardial cushion development. k, l, Whole-mount confocal imaging of hearts stained with GFP, cleaved caspase-3 and Prox1 revealed an increase in apoptotic cells within the termini of mutant coronary lymphatic vessels (white arrowheads in magnified panels), compared to control hearts, supporting the requirement for Prox1 in LEC identity and maintenance. n = 3 hearts analysed for histology and immunostaining. m–p, Whole-mount VEGFR-3 immunostaining of hearts isolated at P7 revealed that Tie2-Cre;Prox1^{fl/+} heterozygotes (m, n) and Tie2-Cre;Prox1^{fl/fl} mutants (o, p) have an equivalent normal cardiac lymphatic vasculature (n = 3 hearts analysed per genotype). As such the lymphatic hypoplasia and disruption of the vessel network, evident in mutant hearts at E17.5 (Extended Data Fig. 10), is rescued during the later stages of development and neonatal period. q–x, In Vav1-Cre;Prox1^{fl/fl} hearts (n = 4) there was evidence of an initial formation of the cardiac lymphatics on both ventral (q, s) and dorsal (u, w) surfaces and the coronary vessels were unaffected (r, t, v, x). Scale bars: f, h, x, 400 μm; j, 1 mm; k, 400 μm; l, 50 μm; m, 500 μm.
Extended Data Figure 9 | Prox1 is essential for Tie2\(^+\) and Vav1\(^+\)-derived cardiac lymphatics. a–d, In control Tie2-Cre;Prox1\(^{fl/1}\) mice at E17.5 \((n = 6)\) there was an extensive lymphatic network on both the dorsal and ventral surfaces, as indicated by whole-mount VEGFR-3 immunostaining (a, b), whereas the lymphatic vessels were virtually absent in mutant Tie2-Cre;Prox1\(^{fl/fl}\) hearts (c, d; \(n = 9\)); a few vessels evident on the dorsal surface was consistent with LECs arising from a non-Tie2-targeted source (c, white arrowheads). Tie2-Cre;Prox1\(^{fl/1}\) mutant hearts were dysmorphic relative to controls (compare c, d with a, b). e, f, GFP\(^+\) staining indicated targeting of Prox1 in Tie2-Cre;Prox1\(^{fl/1}\) mice (e) and an expansive Lyve-1\(^+\) lymphatic network (f). g, h, Prox1 immunostaining confirmed expression in LECs in heterozygote controls (g; inset box shown at higher magnification in h). Co-expression of GFP/Lyve-1 and Prox1 was evident in LECs in addition to Lyve-1/Prox1 double-positive cells not targeted by Tie2-Cre (h, white arrowheads). i–l, In contrast, Tie2-Cre;Prox1\(^{fl/1}\) mutant hearts revealed an absence of the GFP\(^+\) lymphatic network with only a minor contribution of Lyve-1 LECs evident at the base of the heart on the dorsal surface (i, j; white arrowhead in j highlights retained Lyve-1\(^+\) LECs), which were Prox1\(^+\) (k, l). m–p, On the ventral surface there was complete absence of GFP and Lyve-1\(^+\) lymphatic vessels (m); Lyve-1 staining was retained in tissue-resident macrophages (n). Loss of LECs correlated with a loss of Prox1 (o, p). q–t, In control Vav1-Cre;Prox1\(^{fl/1}\) mice at E17.5 \((n = 5)\) there was evidence of appropriate targeting of GFP\(^+\) LECs (q) and an extensive lymphatic network on the dorsal surface as indicated by Lyve-1 (r). Prox1 expression was retained (s), which at higher resolution revealed co-expression of GFP\(^+/\)Lyve-1\(^+/\)Prox1\(^+\) in a subpopulation of LECs, consistent with the lineage trace data (Fig. 3a–d; white inset box in s shown at higher magnification in t). u–x, In Vav1-Cre;Prox1\(^{fl/1}\) mutant hearts \((n = 8)\) there was equivalent GFP\(^+\) targeting (u) and a Lyve-1\(^+\) network (v) with retained Prox1 expression (w, x). y, z, Specific loss of Lyve-1-staining (y) correlated with loss of Prox1 and GFP-targeting (z, left and right panels, respectively, highlighted by white arrowheads). Mosaic levels of Prox1 knockdown accounted for examples of isolated LECs that, despite GFP-targeting, remained Lyve-1\(^+\) (white arrows in y, z). Scale bars: d, g, l, p, s, x, 400 \(\mu\)m; h, t, 40 \(\mu\)m; y, 5 \(\mu\)m.
Extended Data Figure 10 | Prox1 knockdown in Tie2-Cre;Prox1fl/fl mutants results in a hypoplastic and disrupted lymphatic plexus. a–f, Relative to Tie2-Cre;Prox1fl/fl control hearts at E17.5 (a–c), GFP+ lymphatic vessels were thinner and the network truncated along the short axis, having failed to appropriately extend and remodel in Tie2-Cre;Prox1fl/fl hearts with partial knockdown of Prox1 (d–f; see Extended Data Fig. 7c; n = 4 hearts per genotype; representative regions indicated by white inset boxes in a, d). g–l, Higher magnification of the lymphatic plexus in Tie2-Cre;Prox1fl/fl control (g–i) and Tie2-Cre;Prox1fl/fl mutants (j–l) were captured for AngioTool analyses. AngioTool tracing in red of GFP+ vessels and blue for branch points (g–l), enabled quantitative assessment of vessel parameters. m–o, The mutant lymphatic vessels were significantly shorter in overall length (m), more truncated and disorganized with an increased total number of end points (n). Mutant vessels were also significantly reduced in diameter, being thinner on average, compared to controls (o). Scale bars: c, f, 400 μm; l, 30 mm; All graphs show mean ± s.e.m. Student’s t-test; *P ≤ 0.05; **P ≤ 0.001 (n = 4 hearts analysed per genotype).