Segmental territories along the cardinal veins generate lymph sacs via a ballooning mechanism during embryonic lymphangiogenesis in mice

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Introduction

The lymphatic vascular system plays a critical role in normal fluid homeostasis and in pathologies including lymphedema and tumour metastasis (Witte et al., 2001). Despite this, our understanding of the molecular, cellular and morphogenetic events surrounding the initial establishment of the lymphatic system in the embryo remains rudimentary. Recent molecular studies have revealed that the transcription factors SOX18 and COUPTFII are expressed throughout the rudimentary. Recent molecular studies have revealed that the transcrip-

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While simple and attractive, this model alone fails to account for several theoretical issues, including: 1) How a long, continuous precursor tissue, the cardinal vein, yields a series of non-continuous outgrowths from the cardinal veins (Van Der Putte, 1975); although reported over 35 years ago, this phenomenon has received little attention, perhaps because it is difficult to reconcile with the current model of lymph sac formation. Thirdly, even though VEGF-C is thought to be a critical morphogen that promotes exit of lymphatic endothelial progenitor cells from the cardinal veins, VEGF-R3 mutant mice lacking the VEGF-C binding domain appear to show lymphatic endothelial sprouting defects but display normal lymph sac formation (Zhang et al., 2010).

In the present study, we exploit a suite of newly developed imaging tools to uncover the initial morphogenetic events during formation of the lymphatic vasculature in the mouse embryo. Using optical projection tomography (OPT), confocal microscopy and in vivo live imaging, we show that PROX1 expression in the cardinal veins at 11.5 dpc is not uniform, but that metamer clusters of PROX1-high cells are apparent along the anteroposterior axis; we term these “pre-lymphatic clusters” (PLCs) of lymphatic endothelial progenitor cells. These PLCs generate a primitive lymph sac via a ballooning mechanism. As a consequence, lymph sacs maintain a transient connection to the cardinal veins and are therefore typically filled with blood. In addition, we identify a population of NRP2-positive lymphatic endothelial cells migrating directly from the cardinal veins independent of lymph sac formation. This finding reveals that lymphatic vascular morphogenesis proceeds via a combination of ballooning and sprouting events. Our 3-dimensional description of early lymphatic vascular development in the mouse embryo provides a revised model of the early events during lymphatic vascular morphogenesis, explains theoretical and experimental phenomena that were not catered for by existing dogma, and provides a framework in which to investigate the source and identity of guidance cues that direct the construction of the lymphatic vasculature.

Materials and methods

Mouse strains

Mouse embryos were obtained from wild type CD1 or C57Bl/6 mice and collected at different time points. Staging was performed by somite count or, for later-stage embryos, days post detection of a vaginal plug. PROX1–EGFP BAC transgenic mice (Tg (Prox1–EGFP)221Gsat/Mmcd) were purchased from the Mutant Mouse Regional Resource Centres (Choi et al., 2011). All procedures involving animals conformed to institutional guidelines (University of Queensland and SA Pathology/CHN Animal Ethics Committees).

Optical projection tomography

Stained embryos were embedded in warm 1% low-melting-point agarose and left until set, adjusting the orientation as necessary. Set agarose blocks were glued to aluminium–magnetic mounts. Specimens were then dehydrated in 50% methanol for 18 h with 3 graduated changes of methanol to 100%, and then cleared overnight in benzyl alcohol: benzyl benzoate mixed at a ratio of 1:2. Once clear, samples were imaged in a Bioptonics 3001 OPT scanner (Bioptonics, UK). Images were acquired at 0.9° intervals and reconstructed. Stacks were rendered for presentation using Drishti volumetric rendering software (http://sf.anu.edu.au/Vizlab/drishti/).

Immunofluorescence

Immunofluorescence was performed on cryosections as previously described (Wilhelm et al., 2005). Embryos were dissected and fixed overnight in 4% PFA. Primary antibodies in blocking solution (100 mM Maleic acid, 10% horse serum, 1% DMSO in PBS-Trition-X 0.1%) were added and incubated for 24 h at 4°C. Samples were washed for 6 h in washing solution (100 mM Maleic acid, 10% horse serum, 1% DMSO in PBS-Trition-X 0.1%) and incubated for 16 h with secondary antibodies in blocking solution. Whole mount immunostaining of 10.0–11.5 dpc embryos for confocal microscopy analysis was performed as follows: using sharpened tungsten needles, embryos were dissected in half along the longitudinal axis through the midline. Samples were washed twice in PBS-Trition-X 0.3%, incubated for at least 2 h in blocking solution (PBS-Trition-X 0.3% containing 1% bovine serum albumin) and then overnight at 4°C in primary antibody diluted in blocking solution. Samples were washed five times for 1 h each in PBS-Trition-X 0.3% at room temperature and then incubated overnight at 4°C with secondary antibodies in blocking solution. Samples were washed for 5 h in PBS-Trition-X 0.3% at room temperature, then overnight at 4°C and fixed for 30 min (4% formaldehyde in PBS). For visualization, embryos were dehydrated in methanol and cleared in benzyl alcohol:benzyl benzoate as described for optical projection tomography. Images were acquired with a 10X lens on a Biorad Radiance 2100 confocal microscope equipped with 3 lasers (488 nm Argon ion, 543 nm Green HeNe and 637 nm Red Diode), attached to an Olympus IX70 inverted microscope (Olympus). Adobe Photoshop CS5 version 12.0 (Adobe) was used for subsequent image processing.

Antibodies

Antibodies were used in the following dilutions: rabbit polyclonal anti-mouse PROX1 (Angiobio), 1:1000; goat polyclonal anti-human PROX1 (R&D Systems), 1:250; rabbit polyclonal anti-mouse LYVE-1 (Fitzgerald Industries, Concord, MA), 1:1000; rabbit polyclonal anti-mouse LYVE-1 (Angiobio), rat anti-mouse CD31 (BD Pharmingen), 1:500; rat monoclonal anti-PECAM antibody (BD Pharmingen), 1:200; hamster polyclonal anti-Podoplanin (Fitzgerald Industries, Concord, MA), 1:500; rat polyclonal anti-CD34 antibody (kindly provided by Prof. E. Dejana, IFOM, Milan, Italy) 1:30; hamster polyclonal anti-ICAM antibody (CD54) (Millipore, Australia) 1:200; rat monoclonal anti-Endoglin (CD105) (clone MJ7/18, Becton Dickinson) 1:200; goat polyclonal anti-Neuropilin-2 (R&D Systems) 1:500; 1:200 goat polyclonal anti-GFP antibody (R&D Systems); rat monoclonal anti-endomucin (Santa Cruz Biotechnology Inc) 1:500. Secondary antibodies anti-rat IgG Alexa 555, anti-rat IgG Alexa 488, anti-rabbit IgG Alexa 488, anti-hamster IgG Alexa 488, anti-rat IgG Alexa 647, anti-mouse IgG Alexa 594, anti-goat Alexa 647, anti-rabbit IgG Alexa 555 and anti-rabbit IgG Alexa 647 (Molecular Probes) used at 1:200 or 1:500 for whole mount immunostaining.
Live in vivo imaging

Transgenic Prox1-EGFP embryos were dissected at 11.5dpc and cultured in a glass-bottom MatTek dishes over a period of 12 hrs in a humidified chamber with 5% CO₂ at 37 °C, as previously described (Bowles 2010 Dev Cell). Time lapse and Z-stack images of the cardinal vein were captured using an inverted confocal microscope (Zeiss LSM-710 FCS) over 1 min with a 5 min interval. Data sets were analysed using IMARIS software.

Intracardiac dye injection

Embryos with yolk sac and placenta intact were dissected and placed immediately into PBS at 4 °C for 5–10 min. Small volumes (4–6 μl) of 10% India ink were injected into the left ventricle. After 5 min at room temperature, injected embryos were returned to 4 °C for at least 20 min before analysis.

Histology

Embryos for histological examination were fixed in 4% paraformaldehyde at 4 °C overnight and embedded in paraffin or OCT. Blocks were then sectioned (6–10 μm) and stained with haematoxylin and eosin.

Results

Segmental expression of lymphatic markers along the length of the cardinal veins

In the mouse embryo, lymphatic vessels arise from SOX18/PROX1-expressing lymphatic endothelial cell precursors first found in the dorso-lateral walls of the paired cardinal veins around 9.5 dpc (Francois et al., 2008; Wigle and Oliver, 1999). While lymphatic endothelial cell (LEC) precursors have been extensively mapped in transverse sections of the mouse embryo, their arrangement along the antero-posterior (longitudinal) axis of the cardinal vein has not been described. To address this question, we used a combination of two-dimensional approaches (coronal sections) and three-dimensional imaging technologies (optical projection tomography —OPT—and confocal microscopy) to visualize the formative steps in lymphangiogenesis in wild type mouse embryos.

Whole mount immunostaining and OPT analysis revealed that at 11.5 dpc, large numbers of PROX1-positive cells were visible in the anterior cardinal veins, progressively decreasing in abundance towards the posterior region of these vessels (Supp. video 1). Still images from the video are represented in Fig. 1A, B and C. Rather than staining uniformly for PROX1, clusters of cells exhibiting high...
PROX1 levels were observed along the length of the cardinal veins; we term these “pre-lymphatic clusters” (Fig. 1A: PLC). Three-dimensional imaging of the CV from an anterior position (Fig. 1C) confirmed that PLCs are positioned dorso-laterally in the cardinal vein (Wigle and Oliver, 1999).

The spatial organisation of pre-lymphatic clusters was further investigated by in vivo live imaging using transgenic Prox1-EGFP reporter mice (Choi et al., 2011) (Supp. movie 2). Quantitative analysis of PROX1-EGFP-positive nuclei in the walls of the cardinal veins revealed distinct areas where the density of LEC precursors was significantly higher, mirroring the pre-lymphatic clusters observed in fixed embryos (Supp. video 3). Still images from the video are represented in Fig. 1D–I. In vivo imaging of LEC precursors in the cardinal vein of 11.5 dpc Prox1-EGFP embryos showed that PROX1-EGFP positive LEC progenitors dynamically congregated in the vessel wall to assemble as PLCs (Supp. video 2 see also Supp. Fig. 1). Measurement of EGFP signal intensity in individual PROX1-EGFP positive venous endothelial cells revealed that PROX1-EGFP levels progressively increased over time (Supp. Fig. 1C–F). By approximately 11.75 dpc (6 h post-recording), EGFP signal intensity in PROX1EGFP-positive endothelial cells within pre-lymphatic clusters had doubled (Supp. Fig. 1C–F and Supp. Fig. 2A and B), reflecting elevated Prox1 transcription. These data reveal that Prox1 expression in the cardinal veins is both spatially and temporally regulated and suggest that the same signals regulating Prox1 expression are likely to be locally restricted.

Immunostaining of coronal sections of 11.5 dpc wild type embryos (Fig. 2A) corroborated the OPT and live imaging data; PROX1-high (Fig. 2B), LYVE-1-positive (Fig. 2C) cells were clustered in anteroposterior domains rather than being uniformly distributed along the length of the cardinal veins. Furthermore, a series of coronal sections from the ventral to the dorsal aspect of the embryo (Fig. 2D, E, F) revealed that clusters of PROX1-high cells sometimes localise to venous branchpoints. For example, PROX1-positive pre-lymphatic clusters could be observed at the level of the forelimb bud where the cardinal vein diverts to form a lateral branch (Fig. 2E and Fig. 2F: Lat. Br. white lines). These data suggest that signals responsible for promoting vascular branching might also pattern PLCs and that venous endothelial cells other than those residing within the cardinal veins might be competent to respond to signals that specify LEC fate.

Lymph sacs arise adjacent to the pre-lymphatic clusters in the cardinal vein

The identification of pre-lymphatic clusters along the anteroposterior axis of the CV prompted us to investigate the positional relationship between these clusters and developing lymph sacs in the embryo. Due to the curvature of the cardinal vein, only a subset of coronal sections allowed observation of relative positioning of pre-lymphatic clusters and developing lymph sacs (Fig. 2A, plane C and Fig. 2C). In order to better visualize pre-lymphatic clusters in relation to lymph sacs, we analysed a series of transverse sections of 11.5dpc embryos by immunofluorescence using antibodies to podoplanin and either LYVE1 (Fig. 3A–C) or PROX1 (Fig. 3D–F), in combination with the pan-endothelial cell marker PECAM. Lymph sacs were observed immediately adjacent to the pre-lymphatic clusters (Fig. 3A–F, brackets), suggesting that each pre-lymphatic cluster might generate a corresponding lymph sac.

Fig. 2. Lymph sacs develop adjacent to pre-lymphatic clusters and are directly connected to the cardinal vein. (A) Schematic representation of coronal sections showing the position of images shown in panels B and C. (B) Coronal section of an 11.5dpc wild-type embryo labelled with Prox1 (green) and Pecam1 (blue) reveals lymphatic endothelial cells in the venous wall organised in a pre-lymphatic cluster (PLC). (C) Coronal section of an 11.5dpc wild type embryo labelled withLyve1 (red), Pecam1 (blue) and Icam1 (green) shows the presence of a subset of venous endothelial cells that are tightly associated with a developing lymph sac. (D–F) Triple immunofluorescence on coronal sections of an 11.5dpc wild type embryo using the blood vascular marker CD34 (blue) and lymphatic specific markers Prox1 (red) and Podoplanin (green) shows that Prox1, but not podoplanin, is expressed in lymphatic endothelial precursor cells within the venous wall. Lymphatic endothelial cell precursors that have delaminated from the cardinal vein express both Prox1 and Podoplanin (asterisks). Cells expressing a high level of Prox1 in the wall of the cardinal vein are often found in the vicinity of a developing lymph sac. Coronal serial sections shown from D to F show progression from the dorsal to ventral side of the embryo. Connection of the cardinal vein with a lateral branch is observed in E (site of opening, SO) and the vessel wall of the lateral branch is seen in F (dashed line). (G–I) Coronal sections of an 11.5dpc embryo stained for Prox1 (red) and the blood cell marker Ter119 (blue) confirmed that lymph sacs (dashed lines) are filled with blood cells. (J) Whole mount embryo at 11.5 dpc showing Indian ink distribution after intracardiac injection. Dye accumulated in the developing lymph sac (white arrowhead) and could be detected in the cephalic blood vasculature (white arrow), (n=5). (K) Sagittal bisection of injected embryo uncovered the cardinal vein and a lymph sac filled with Indian ink. (L) Hamatoxylin and eosin staining of transverse section of injected embryo validated the presence of ink in the developing lymph sac. CV, cardinal vein; DA, dorsal aorta; LS, lymph sac; SO, site of opening of the lateral branch in the cardinal vein; PLC, pre-lymphatic clusters; A, anterior; M, medial; Scale bars, D 500 μm; F 200 μm; G 1 mm; L, lateral; D, dorsal; Scale bars A–C 500 μm; D–F and G–I 40 μm; J, 1 mm; K, 250 μm and L, 20 μm. Number of embryos for A–F: n=10 in total.

Previous studies have provided histological evidence that the venous system and lymph sacs are directly connected and communicate via a small aperture (Uhrin et al., 2010; Van Der Putte, 1975), and that lymph sacs in some cases contained blood (Uhrin et al., 2010; Van Der Putte, 1975). In the course of our experiments, we also observed that primary lymph sacs were typically filled with red blood cells at 11.5 dpc (Fig. 2G–I and Fig. 3, asterisks). Immunofluorescence analysis using Prox1 together with the specific red blood cell marker Ter119 further confirmed that the cells observed in lymph sacs included nucleated erythrocytes (Fig. 2G–I). To confirm a physical connection between the cardinal vein and the nascent lymphatic vasculature, we analysed the distribution of India ink after intracardiac injection in mouse embryos (Fig. 2J–L, n=6). The presence of the ink in the blood circulation was detectable in the cephalic vessels (Fig. 2J, white arrow) and due to the blind-ended structure of the lymph sacs, accumulated in the developing lymph sacs and outlined their organization (Fig. 2J, white arrowhead). Sagittal bisection of embryos and gross morphological analysis enabled us to visualize a nascent lymph sac filled with ink directly attached to the cardinal vein (Fig. 2K, dashed line). The presence of India ink in the lymph sac was further validated on transverse sections of injected embryos (Fig. 2L, black arrows). The presence in developing lymph sacs of ink that was injected into the blood circulation demonstrates a physical connection between the two vascular networks.

In order to further characterise the physical connection between the cardinal veins and the lymph sacs, we performed wholemount immunofluorescent immunostaining on 10.0–11.5 dpc wild type embryos that were then imaged by confocal microscopy (Fig. 4, S) and OPT analysis (Supp. video 4). A schematic representation of the CV and lymph sac, stained for Prox1 and Pecam, is shown in Fig. 4A. A series of optical coronal sections of this lymph sac is shown in Fig. 4C–L. Analysis of this series revealed continuity between the Pecam/Prox1 positive lymph sac endothelium and the cardinal vein endothelium (Fig. 4E–I, white arrowhead). In the most medial sections, the connecting point of the cardinal vein with a lymph sac was observed (Fig. 4J–L, asterisks). Further analysis by OPT also revealed a lymph sac growing outwards directly from the vessel wall (Fig. 4M), and the view from a lateral perspective exposed a connecting point between the vein and the lymph sac (Fig. 4N, c.p. and Supp. movie 4).
Further evidence of segmented connections between lymph sacs and the cardinal vein was obtained by viewing whole mount immunostained embryos from the lateral aspect. Confocal imaging of embryos stained with antibodies to LYVE-1, NRP2, PROX1 and endomucin (Fig. 5A–H), revealed the existence of discrete, stalk like connections between the cardinal vein and lymph sacs at 10.5 dpc and 11.5 dpc (Fig. 5A–H, arrows).

In summary, we have utilized four independent methods to show that development of the initial lymph sacs involves direct swelling and outgrowth of PLCs from the cardinal vein, a process that we refer to as ballooning. This ballooning is followed by a pinching as the ballooning and extrusion progresses, resulting in a connecting aperture that becomes progressively smaller until the lymph sac separates completely. The ballooning model provides a
ready explanation for the presence of circulating blood cells in the lymph sacs.

**Lymphatic vascular morphogenesis proceeds via a combination of ballooning and sprouting**

Despite robust evidence for a ballooning and pinching mode of generating lymph sacs from PLCs in the cardinal vein, we routinely also observed a plexus of PROX1-positive, Nrp2-positive lymphatic endothelial cells sprouting in a dorso-lateral direction towards the embryo periphery (Fig. 5E–H). In addition, Prox1-positive, NRP2-positive cells were observed migrating directly from the cardinal veins prior to lymph sac formation (Fig. 5I–P, open arrowheads). Moreover, whole mount immunostaining studies revealed that the levels of LYVE-1 and Nrp2 were distinct on the cardinal vein, lymph sacs and migrating lymphatic plexus; LYVE-1 was prominent on Prox1-positive cells in the CV and lymph sacs (Fig. 5A–D), but was reduced on migrating lymphatic endothelial cells, while Nrp2 levels were low on the CV and lymph sacs, but high on actively migrating lymphatic endothelial cells (Fig. 5E–H). Taken together, our data reveal that lymphatic vascular development proceeds via a dual mode of morphogenesis; sprouting of Prox1-positive, Nrp2-positive migrating cells and ballooning of Prox1-positive, LYVE-1-positive lymph sacs.

**Discussion**

Despite several decades of morphological observation and a more recent resurgence of interest in the analysis of lymphangiogenesis using molecular tools, several of the key findings of this study had either not been described before, or had been considered inexplicable in terms of available models. These are: 1) Metameric clusters of PROX1-high, LYVE-1-positive lymphatic endothelial progenitor cells that we term “pre-lymphatic clusters” dynamically aggregate along the length of the cardinal veins at ~11.5 dpc; 2) Lymph sac morphogenesis occurs through ballooning from pre-lymphatic clusters, followed by pinching off, enveloping blood cells in the lymph sacs; 3) In parallel, a molecularly distinct population of lymphatic endothelial cells sprouts directly from the cardinal veins independently of lymph sac formation. These findings lead us to propose a revised model for the early events in lymphatic vascular patterning and morphogenesis, as illustrated in Fig. 6. Features of this model are discussed in the following sections.

**Pre-lymphatic clusters in the cardinal vein**

During embryonic patterning and organogenesis, territories of competent precursor cells typically prefigure the formation of a particular tissue. Here we extend this principle to the genesis of the lymphatic vasculature, revealing that the lymphatic plexus arises from metameric territories along the length of the cardinal vein endothelium. What positional cues instruct the segmental expression of PROX1 in the cardinal veins? The molecular machinery responsible for the organization of PLCs might involve classical morphogenetic pathways involved in antero-posterior patterning. Given recent data implicating the morphogen retinoic acid in the induction of lymphatic endothelial cell competence (Marino et al., 2011), it is tempting to speculate a role for RA in PLC formation. This possibility is consistent with the established role of retinoic acid in directly inducing or repressing the expression of homeobox (Hox) genes (Marshall et al., 1996) to control both antero-posterior and dorso-ventral patterning during central nervous system morphogenesis (Lumsden and Krumlauf, 1996). Signals emanating from segmented structures such as the somites and inter-somitic blood vessels may also be responsible for patterning PLCs. These possibilities call for further investigation.

**A ballooning model for the origin of lymph sacs**

The observation that nascent lymph sacs are commonly filled with blood cells is entirely consistent with our model of ballooning of cardinal vein endothelial cells, transition of endothelial phenotype from vascular to lymphatic, and finally segregation from the blood vasculature by pinching (Fig. 6). Although the presence of blood cells in lymph sacs has been noted in passing in several studies (Uhrin et al., 2010; Van Der Putte, 1975), it has not been possible to...
reconcile with the prevailing model of migration of lymphatic precursors from the cardinal veins to subsequently reassemble in a more peripheral location as lymph sacs. Our study provides the first direct evidence of the mechanism by which primitive lymph sacs form.

The ballooning and pinching model suggested by our work is indirectly supported by studies showing that platelets need to physically interact with LEC precursors in order to complete a proper separation of the lymphatic and venous systems (Uhrin et al., 2010). This interaction proceeds after ballooning has occurred at 12.5dpc and relies on binding between CLEC2 on platelets and podoplanin on the surface of the lymphatic endothelial cells (Uhrin et al., 2010). Since podoplanin is not expressed on lymphatic endothelial precursor cells while they are present in the venous wall (Fig. 2), a ballooning mechanism provides an opportunity for contact between lymphatic endothelial cells and platelets after podoplanin expression is upregulated.

Fig. 4. Lymph sacs form by ballooning from the cardinal vein. (A) Schematic representation of a developing lymph sac according to a ballooning and pinching model. Dashed lines indicate series of z-stack images shown from C to L in panels below. The arrow indicates the axis of imaging (dorsal to ventral axis), the pre-lymphatic cluster is shown in red. (B) Wholemount fluorescent immunostaining of 11.5dpc embryo using antibodies to PECAM1 (green) and PROX1 (red) showing that lymph sacs develop in the vicinity of pre-lymphatic clusters and maintain connections to the cardinal vein. (C–L) Images extracted from Z-stack series illustrated in (B); White arrowheads indicate a direct connection of the lymph sac with the cardinal vein, asterisks indicate a connecting point between the lumen of the cardinal vein and a lymph sac. (M and N) Double staining of a PROX1-EGFP embryo at 11.5dpc with PROX1 and GFP (yellow, n = 5) reveals a ballooning lymph sac towards the dorso-lateral side of the embryo. Anterior and medial projections generated using optical projection tomography (OPT) show lymph sac ballooning from the cardinal vein towards the dorso-lateral side of the embryo (M, dashed line). (N) Medial view uncovers a connection point between the venous wall and the forming lymph sac (c.p.). CV, Cardinal Vein; LS, lymph sac; PLC, pre-lymphatic cluster; LS, lymph sac; CSG, cervical sympathetic ganglia; M, medial; V, ventral; P, posterior; scale bars, A–L, 50 μm; M and N, 250 μm.
Further, it is also possible that intravenous blood pressure may play a mechanical role in the ballooning process, by providing the necessary force to inflate the nascent lymph sacs.

Lymphatic vascular morphogenesis proceeds via a combination of ballooning and sprouting events

Our data indicate that ballooning is not the only mechanism by which the lymphatic vasculature is generated in the embryo, and that lymph sac morphogenesis is preceded, and accompanied, by the more conventionally described sprouting mechanism involving migration of individual or groups of lymphatic endothelial precursor cells away from the cardinal veins beginning around 10.0 dpc (Fig. 5). LYVE-1 was present at high levels on lymph sacs and more weakly on migrating cells, while NRP2 showed an opposite profile.

In addition, endomucin staining was prominent on blood vessels and barely visible on lymph sacs or on migrating lymphatic endothelial cells. This differential expression of markers in LECs may reflect differing degrees of dependence on particular lymphangiogenic signals for the genesis of each type of lymphatic vascular structure (e.g. lymph sacs or migrating cells). The two distinct mechanisms of lymphatic vascular morphogenesis might also reflect two different fates for LEC precursors, one that generates deep internal lymphatic vessels via outgrowth from lymph sacs, and another that generates the superficial lymphatic capillary network via cell migration towards the dorso-lateral edge of the embryo. In support of this hypothesis, it has recently been shown that NRP2 plays a key role in lymphatic vascular sprouting (Xu et al., 2010) and that the jugular lymph sacs and thoracic duct form normally in Nrp2<sup>−/−</sup> mice, while the development of lymphatic capillaries is abrogated (Yuan et al., 2002). On the basis
of our data, we would predict that lymph sac ballooning would proceed normally in Nrp2−/− mice, while LEC sprouting from the cardinal veins is likely to be restricted. Further studies will allow these hypotheses to be tested.

A striking feature of migrating lymphatic endothelial cells was the presence of discrete clusters and chains of migrating cells ahead of the developing lymphatic vascular plexus (Fig. 5E–H, arrowheads). This observation suggests that in contrast to prevailing dogma, lymphatic vessels may not grow solely by proliferating behind a sprouting tip cell pioneer, as has been established for sprouting angiogenesis. Our present findings suggest that lymphatic and blood vascular networks extend via fundamentally different mechanisms. Migrating lymphatic endothelial cells may be capable of detaching from one another, exploring their environment and then making connections with one another in response to the appropriate cues. Alternatively, isolated LEC clusters might represent a pool of lymphatic endothelial cells derived from a progenitor cell source distinct from the cardinal vein (e.g. perhaps from veins other than the cardinal veins). Analysis of lymphatic vascular morphogenesis in real time will allow us to distinguish between these possibilities.

The genesis of the lymphatic vasculature has remained mysterious for over 100 years since first described by Florence Sabin (Sabin, 1902). Our revised model for the generation of lymphatic vessels accounts for all existing observations, and lays a foundation for identification of molecular cues governing morphogenesis of the lymphatic vasculature.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.12.032.

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References


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