Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature

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The origin of the mammalian lymphatic vasculature has been debated for more than 100 years. Whether lymphatic endothelial cells have a single or dual, venous or mesenchymal origin remains controversial. To resolve this debate, we performed *Cre/loxP*-based lineage-tracing studies using mouse strains expressing *Cre* recombinase under the control of the *Tie2*, *Runx1*, or *Prox1* promoter elements. These studies, together with the analysis of *Runx1*-mutant embryos lacking definitive hematopoiesis, conclusively determined that from venous-derived lymph sacs, lymphatic endothelial cells sprouted, proliferated, and migrated to give rise to the entire lymphatic vasculature, and that hematopoietic cells did not contribute to the developing lymph sacs. We conclude that the mammalian lymphatic system has a solely venous origin.

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The lymphatic vasculature returns extravasated fluids to the blood circulation, thereby maintaining tissue fluid homeostasis. It also facilitates immune surveillance and lipid absorption from the intestine. Furthermore, the lymphatic vasculature is a major route for tumor metastasis (Oliver and Alitalo 2005). Recently, the identification of lymphatic markers has greatly increased our understanding of the genes and mechanisms that regulate the development of the lymphatic vasculature (lymphangiogenesis) (Oliver and Alitalo 2005). Despite this progress, our knowledge of the lymphatic system is still rudimentary, and some aspects remain unresolved.

A century-old debate persists about the origin of embryonic lymph sacs, the structures from which the lymphatic vasculature is derived. Studies performed in the early 1900s proposed that early during development, lymph sacs originate from budding venous endothelial cells (ECs); from these initial structures, the entire lymphatic system then spreads into surrounding tissues and organs (Sabin 1902). An alternative view proposed that lymph sacs arise from mesoderm-derived endothelial precursors and secondarily establish venous connections (Huntington and McClure 1910).

We previously showed that in mice, starting around embryonic day 9.5 (E9.5), the homeobox gene *Prox1* is expressed in a subpopulation of blood ECs (BECs) in the anterior cardinal vein (Wigle and Oliver 1999). On the basis of our expression and functional analyses, we favored Sabin's venous model and proposed that Prox1-expressing ECs bud from the veins and form embryonic lymph sacs and the lymphatic vasculature (Wigle and Oliver 1999). In addition, our finding that *Prox1*-null embryos are devoid of lymphatic vasculature (Wigle and Oliver 1999) due to a failure in lymphatic cell-type specification (Wigle et al. 2002) definitively determined the crucial role of *Prox1* in developmental lymphangiogenesis. Although these initial studies demonstrated that *Prox1* activity confers a lymphatic endothelial cell (LEC) phenotype on venous LEC progenitors (Oliver and Detmar 2002; Wigle et al. 2002), they did not determine the origin of the Prox1-expressing progenitors, nor did they exclude the possibility that sources other than Prox1-expressing venous LEC progenitors contribute to mammalian lymphangiogenesis.

Recent work in different model organisms has provided mixed results about the origin of LECs. In chicken and frog embryos, LECs arise from venous-derived ECs and mesenchymal lymphangioblasts (Wilting et al. 2000, 2006; Ny et al. 2005). In zebrafish, time-lapse imaging revealed that LECs of the main thoracic duct-like vessel arise from primitive veins (Yaniv et al. 2006). However,

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this mosaic analysis did not determine whether the entire zebrafish lymphatic vasculature is solely venous derived or has other contributing sources (e.g., mesenchyme-derived lymphangioblasts). Furthermore, unlike mammals, zebrafish do not appear to have lymph sacs; thus, the critical steps leading to the formation of the entire lymphatic networks probably differ in these two model systems. In mammals, the current data propose that venous-derived LECs, hematopoietic cell-derived circulating endothelial progenitors (CEPs), and transdifferentiating leukocytes and macrophages are putative sources of LECs during embryonic and adult lymphangiogenesis (in health and disease) (Wigle and Oliver 1999; Wigle et al. 2002; Salven et al. 2003; Maruyama et al. 2005; Religa et al. 2005; Buttler et al. 2006; Kerjaschki et al. 2006; Sebzda et al. 2006). These results indicate that the source(s) of LECs and the mechanisms of lymphatic vasculature formation are species specific, and they highlight the importance of resolving this century-long question concerning the origin(s) of the lymphatic system to facilitate our understanding of normal and pathological lymphangiogenesis.

To this end, we used tamoxifen (TM)-inducible *Cre*/*LoxP*-based tracing systems to genetically label early Prox1-expressing murine LECs and determine their origin and fate. Fate-mapping studies were also performed to evaluate the contribution(s) of venous ECs and hematopoietic cells to the developing lymphatic vasculature and to elucidate the stepwise mechanisms of lymphangiogenesis.

Results

Generation of the Prox1-CreER^{T2} *mouse strain*

In the mouse, Prox1 expression in ECs initiates around E9.5 in the anterior cardinal vein (Wigle and Oliver 1999). To irreversibly mark and follow the fate of Prox1$^+$ cells that contribute to developmental lymphangiogenesis, we genetically labeled Prox1-expressing cells with a TM-inducible *Cre-ER^{T2}*/*LoxP*-based tracing system (Danielian et al. 1998; Indra et al. 1999). Gene targeting inserted *Cre-ER^{T2}* into the mouse *Prox1* locus (Supplementary Fig. 1) to generate the *Prox1-CreER^{T2}* allele. To generate the targeting construct and avoid the haploinsufficiency phenotype observed in *Prox1$^{+/LacZ}$* mice (Wigle and Oliver 1999; Harvey et al. 2005), we inserted at the second intron of *Prox1* a cassette containing a fusion of a synthetic splice acceptor site, a fragment of *Prox1* cDNA containing exons 3 and 4, an internal ribosome entry site, *Cre-ER^{T2}*, and a poly(A) transcription-stop signal (Supplementary Fig. 1). This modified *Prox1* allele should express *Prox1* and *Cre-ER^{T2}* from a single bicistronic transcript, thereby recapitulating the normal pattern of Prox1 expression. Targeted embryonic stem cells were used to generate the *Prox1-CreER^{T2}* mouse strain. As expected, mice that were either heterozygous or homozygous for the modified *Prox1* allele were viable and showed no obvious phenotypic alteration.

To evaluate whether the modified allele functioned as expected, we used the *R26R* reporter line (Soriano 1999) to monitor the activation of β-galactosidase (*lacZ*) in descendants of Prox1-expressing cells at different time points. No *lacZ* labeling was detected prior to TM administration (data not shown); instead, most Prox1-expressing cells were *lacZ*$^+$ after TM exposure (Supplementary Fig. 2).

Next, we determined the optimal TM dose required to visualize the progeny of Prox1-expressing cells in the developing lymphatics. We determined that intraperitoneal injection of 3–5 mg of TM per 40 g of body weight into pregnant dams was sufficient to label embryonic cells without affecting embryonic viability. As expected, the higher the dose of TM, the more efficient and rapid the extent of cell labeling (data not shown). Accordingly, our choice of TM dose depended on the goal of the particular experiment: To label the maximum number of LECs, we used 5 mg of TM, and to label the Prox1-expressing LEC lineage within the narrowest window of time, we used 3 mg of TM. Therefore, in an initial experiment to determine whether the lymphatic expression of Prox1-CreER^{T2} recapitulates that of endogenous Prox1, we exposed *Prox1-CreER^{T2};R26R* embryos to 5 mg of TM at different embryonic stages and compared their X-gal expression patterns with those of comparably staged control *Prox1$^{+/LacZ}$* embryos (Supplementary Fig. 2).

In addition to the developing lymphatics, we also determined the extent of *lacZ* labeling in other Prox1-expressing embryonic cell types (data not shown). With the exception of the heart and CNS, where only a few *lacZ*$^+$ cells were detected, all other cell types appeared to faithfully recapitulate the Prox1 expression profile (Supplementary Fig. 2; data not shown). Similar to what was reported for other inducible *Cre* strains (Dor et al. 2004; Zhang et al. 2005), the efficiency of cell labeling was variable. Therefore, the pattern of Prox1 expression was mosaic in the generated *Prox1-CreER^{T2}* mouse strain. This feature was most likely caused by multiple factors such as small experimental variations in the effective dose of TM available at any certain time to any particular cell of interest, variations in the susceptibility of the specific locus, and the transient nature of the cell's access to TM. Nevertheless, the labeled domain was reproducible with only small variations in the percentage of marked cells. The reduced number of *lacZ*$^+$ cells detected in the heart and CNS could be explained by the fact that most Prox1-expressing cells in those tissues are post-mitotic.

Having determined the optimal dose of TM, we next performed detailed cell-labeling time-course studies to determine the kinetics of cell labeling mediated by *Prox1-CreER^{T2}*. We have previously shown that Prox1-expressing LECs are normally detected in and near the cardinal vein at around E9.5 (Wigle and Oliver 1999). Considering 9 a.m. of the day the vaginal plug was detected as E0.5, we have now precisely determined that Prox1 expression initiates in the anterior cardinal vein at E9.75. No *lacZ*$^+$ LECs were detected in E10.5 embryos exposed to 3 mg of TM that was administered to pregnant *Prox1-CreER^{T2}* × *R26R* females at E9.0 (Supple-

mentary Fig. 3A). This finding suggested that 3 mg of TM mediates Cre-ER^{T2}-mediated cell labeling for <24 h. Instead, *lacZ*$^+$ LECs were detected in and around the cardinal vein of E10.5 embryos exposed to TM at E9.5 (Supplementary Fig. 3B,C, arrows). Next, to precisely determine the kinetics of Cre-mediated *R26R* activation, we administered 3 mg of TM at E10.5, when Prox1 is strongly expressed in the cardinal vein, and isolated embryos 4, 6, 8, and 12 h later. The *lacZ*$^+$ LECs were first detected 6 h after TM exposure; labeling was found in and near the cardinal vein (Supplementary Fig. 3D; data not shown).

Venous ECs are the earliest Prox1-expressing LEC progenitors

An initial step in developmental lymphangiogenesis is the formation of primitive lymph sacs. Based on expression analysis, we previously proposed that in mice these sacs arise from Prox1$^+$ LECs located in the cardinal veins (Wigle and Oliver 1999). Therefore, we first wanted to conclusively determine whether mammalian primitive lymph sacs are formed by Prox1-expressing venous ECs. To do this, we first aimed to exclusively label the earliest Prox1$^+$ LECs located in the anterior cardinal vein at E9.75. We performed a kinetic analysis (similar to the one described above) by exposing embryos to 3 mg of TM at E9.5 (6 h prior to the first appearance of Prox1$^+$ ECs in the anterior cardinal vein). In agreement with the above results, we first detected scattered *lacZ*$^+$ LECs in some embryos 12 h after TM administration (or 6 h after *Prox1-CreER^{T2}* locus expression); by 24 h and once TM was no longer active, *lacZ*$^+$ LECs were detected in or near the cardinal vein in all embryos (Supplementary Fig. 3C,E). These results demonstrate that administration of 3 mg of TM at E9.5 exclusively labels the earliest Prox1$^+$ LECs located in the embryonic veins.

Next, for fate-mapping experiments, we administered 3 mg of TM at E8.5 or E9.5 to *Prox1-CreER^{T2}* mice to activate the *R26R* reporter. The earliest contribution of Prox1-expressing cells to the developing lymphatics was assessed by the presence of *lacZ*$^+$ cells in the forming jugular lymph sacs and peripheral (superficial) lymphatics of E13.5 embryos. No *lacZ*$^+$ cells were detected in these structures when TM was administered at E8.5 (Fig. 1A,D); scattered *lacZ*$^+$ cells were seen only in the liver (Fig. 1A). However, *lacZ*$^+$ cells were detected in those lymphatic regions when TM was administered at E9.5 (Fig. 1B,E). These results indicate a relation between the initiation of Prox1 expression in the anterior cardinal vein and the formation of the primitive lymph sacs. They argue that ECs in the anterior cardinal veins at around E9.75 are the earliest source of Prox1-expressing LEC progenitors that contribute to the developing lymph sacs and peripheral lymphatics.

The LEC identity of the *lacZ*-labeled cells was determined using appropriate molecular markers (Supplementary Fig. 4). The LEC identity of *lacZ*$^+$ cells agreed with our earlier characterization of Prox1 expression during developmental lymphangiogenesis (Wigle and Oliver 1999; Wigle et al. 2002). At E13.5, the number of *lacZ*$^+$ cells in the peripheral lymphatics and lymph sacs increased with the sequential administration of TM at later developmental stages (Fig. 1C,F), which supports the proposal that embryonic lymphangiogenesis requires a continuous source of Prox1$^+$ venous LEC progenitors. Furthermore, in agreement with van der Putte (1975), who proposed that lymphatic structures develop in a temporal rostrocaudal manner, our analysis of E13.5 embryos showed that TM exposure at E9.5 labeled *lacZ*$^+$ LECs mostly in the anterior part of the embryo (Fig. 1B, arrow); TM exposure at E10.5 labeled these cells as well as cells around the caudal perimesonephric region (Fig. 1C, arrowhead).

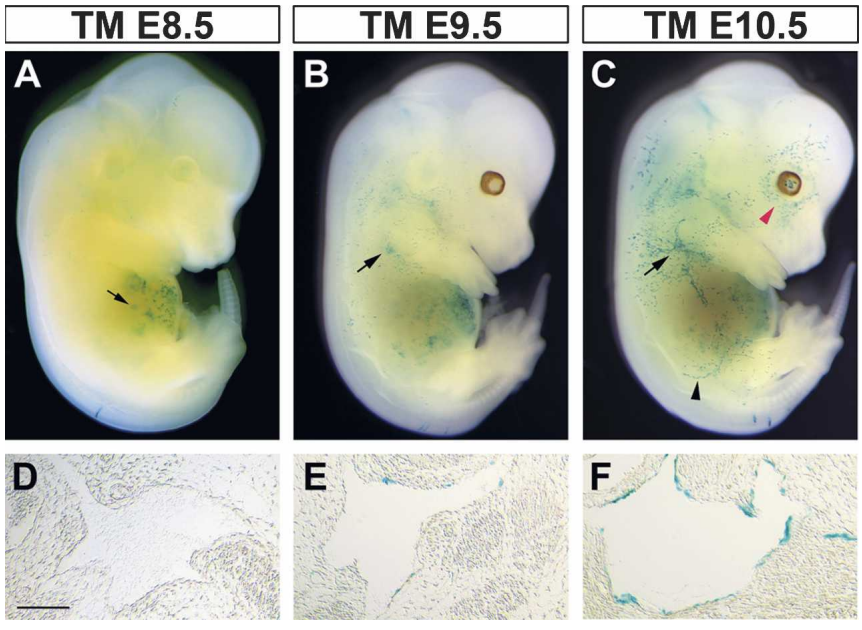

Figure 1. Venous ECs are the earliest LEC progenitors. *Prox1-CreER^{T2};R26R* pregnant dams were injected with 3 mg of TM at different times and X-gal-stained at E13.5. (*A*) TM administration at E8.5 labeled *Prox1* descendent cells only in the liver (arrow); no *lacZ*$^+$ LECs were detected in superficial lymphatics or lymph sacs (shown in *D*). (*B*) Following TM administration at E9.5, the first superficial *lacZ*$^+$ LECs were identified around the jugular region (arrow) and the jugular lymph sacs (shown in *E*). (*C*) Upon TM administration at E10.5, the number of *lacZ*$^+$ LECs increased in the jugular region (arrow) and extended toward the periorbital region (red arrowhead) and the perimesonephric region (black arrowhead). (*F*) The number of *lacZ*$^+$ LECs in the jugular lymph sacs also increased. Bar, 100 µm.

These results support the proposal that in the developing mouse embryo, Prox1$^+$ LEC progenitors are not present prior to the appearance of the Prox1-expressing venous ECs in the anterior cardinal vein (around E9.75). In addition, sequential, stage-dependent administration of TM correlates with an increasing number of *lacZ*$^+$ cells in the lymph sacs and peripheral lymphatics, suggesting that ECs in the cardinal veins are a continuous source of Prox1-expressing LECs, at least until the lymph sacs form.

The lymphatic vasculature arises by sprouting, proliferation, and migration of LECs

Once we determined that Prox1-expressing venous ECs are the earliest LEC progenitors contributing to the developing lymph sacs and peripheral lymphatics, we aimed to assess the mechanisms that control LEC sprouting into peripheral tissues, thereby giving rise to the whole lymphatic vasculature. To do this, we performed similar lineage-tracing studies using *Prox1-CreER^{T2};R26R* embryos. TM (5 mg) remains active for <2.5 d; therefore, to efficiently label as many Prox1$^+$ cells as possible in the forming lymph sacs, TM was administered at E10.5, and embryos were isolated and analyzed daily between E11.5 and E15.5.

At E11.5, most superficial *lacZ*$^+$ cells were located anterior to the developing forelimbs; a few were scattered more caudally (Fig. 2A). Most *lacZ*$^+$ cells were LECs that had originated from the anterior (Supplementary Fig. 5A) and posterior cardinal veins and the iliac veins (data not shown). Therefore, these *lacZ*$^+$ cells were the earliest descendants of Prox1-expressing venous ECs. At E12.5, the number of *lacZ*$^+$ cells along the anteroposterior axis had increased (Fig. 2B), and the cells were easily detected in the lymph sacs (Supplementary Fig. 5B). At E13.5, *lacZ*$^+$ cells were sprouting from the lymph sacs (Supplementary Fig. 5C). Many more *lacZ*$^+$ descendents were observed around the jugular, perimesonephric, and eye regions (Fig. 2C), where they form the rostral periorbital lymphatics. Later the number of superficial *lacZ*$^+$ cells derived from Prox1-expressing E10.5 progenitors increased substantially, particularly around the jugular and perimesonephric regions (Fig. 2D,E), and the expression pattern resembled that of E15.5 *Prox1$^{+/LacZ}$* embryos (Fig. 2F). The steady expansion of *lacZ*$^+$ cells along the anteroposterior axis is consistent with the continuous sprouting, proliferation, and migration of LECs from the lymph sacs.

Next, we determined the spatiotemporal contribution of LEC progenitors to the deeper lymphatic plexus. First, and in order to label the maximum number of LECs in the deeper lymphatics, *Prox1-CreER^{T2};R26R* embryos were exposed to 5 mg of TM at E10.5, and their lungs and intestines were isolated and X-gal-stained at E16.5. The *lacZ*$^+$ cells were detected in the lungs and mesenteric lymphatics (Fig. 3B,F) in a pattern similar to that in *Prox1$^{+/LacZ}$* embryos (Fig. 3A,E). We speculated that like superficial lymphatics (Fig. 1), the appearance of the deeper lymphatics also follows an anteroposterior, spatiotemporal order. To test this possibility, we exposed *Prox1-CreER^{T2};R26R* embryos to 3 mg of TM (to label Prox1$^+$ cells within a 24-h time frame) at either E9.5 or E10.5; their lungs and mesenteries were X-gal-stained at E16.5. As previously explained, 3 mg of TM less efficiently, and therefore less intensely, labeled Prox1$^+$ LECs than did the 5-mg dose; however, we observed *LacZ*$^+$ LECs in the lungs of E16.5 embryos that were exposed to 3 mg of TM at either E9.5 (Fig. 3C) or E10.5 (Fig. 3D). It

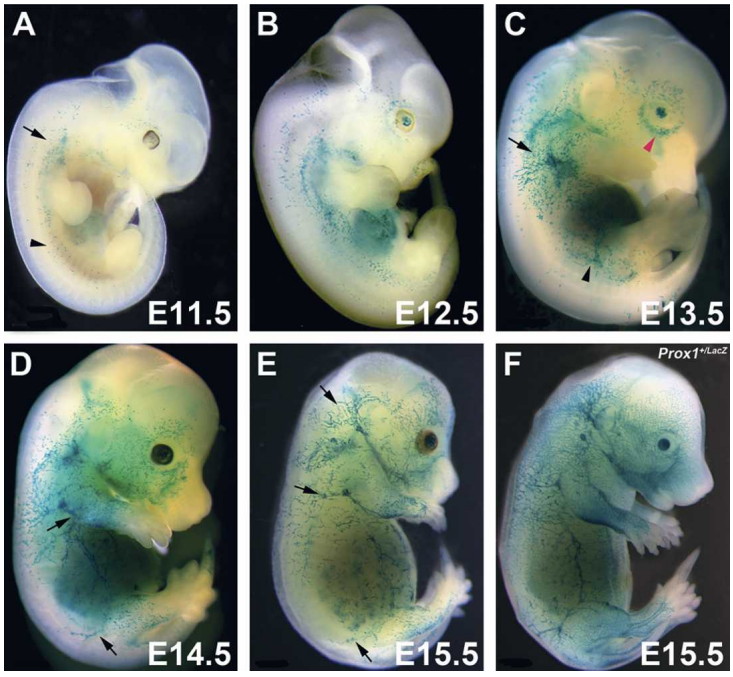

Figure 2. The superficial lymphatic vasculature arises by continuous sprouting, proliferation, and migration of LECs. *Prox1-CreER^{T2};R26R* pregnant dams were injected with 5 mg of TM at E10.5, and embryos were analyzed by X-gal staining at later time points. (*A*) At E11.5, most *lacZ*$^+$ LECs are anterior to the forelimb (arrow); however, cells are also scattered along the anteroposterior axis (arrowhead). (*B*) At E12.5, the number of *lacZ*$^+$ LECs has uniformly increased along the embryonic axis. (*C*) At E13.5, the largest accumulations of *lacZ*$^+$ LECs are seen around the jugular (black arrow), perimesonephric (black arrowhead), and periorbital (red arrowhead) regions. (*D*) At E14.5, the initial lymphatic network appears to sprout, and more superficial *lacZ*$^+$ LECs are present in the jugular and perimesonephric regions (arrows). (*E*) At E15.5, the pattern of superficial lymphatics appears identical to the one seen in similarly staged *Prox1$^{+/LacZ}$* embryos (shown in *F*). Lymphatic vessel sprouting can be clearly seen (arrows).

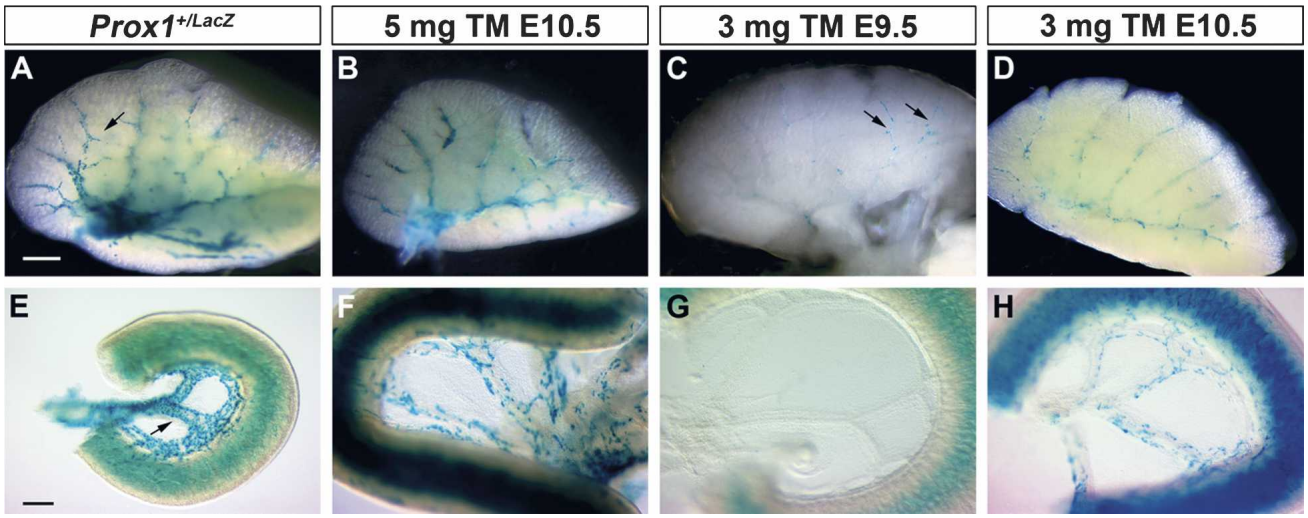

Figure 3. The deeper lymphatic vasculature arises by continuous sprouting, proliferation, and migration of LECs. *Prox1-CreER^{T2};R26R* pregnant dams were injected with 3 or 5 mg of TM at E9.5 or E10.5, and the lungs and mesentery were analyzed by X-gal staining at E16.5. The normal distribution of *lacZ*$^+$ LECs (arrows) in the lung (*A*) and mesentery (*E*) of an E16.5 *Prox1*$^{+/LacZ}$ embryo is shown. Blue staining inside the intestine reflects background trapping. This X-gal staining pattern is similar to the one observed in the lung (*B*) and mesentery (*F*) of E16.5 *Prox1-CreER^{T2};R26R* embryos exposed to 5 mg of TM at E10.5, which demonstrates that E10.5 Prox1$^+$ LEC progenitors contribute to the deeper lymphatic vasculature. (*C*) After reduced TM (3 mg) exposure at E9.5, *lacZ*$^+$ LECs are still detected (although their number is reduced) in the lungs of E16.5 *Prox1-CreER^{T2};R26R* embryos (arrows). (*G*) No *lacZ*$^+$ LECs are detected in the mesentery. An increase in the number of *lacZ*$^+$ LECs can be seen in the lungs (*D*) and mesentery (*H*) of E16.5 *Prox1-CreER^{T2};R26R* embryos exposed to 3 mg of TM at E10.5. Bars: *A–D*, 500 µm; *E–H*, 200 µm.

is worth mentioning that the lungs of embryos exposed to TM at E10.5 contained more *LacZ*$^+$ LECs (Fig. 3D) than did those exposed at E9.5. This result supports our previous results (Fig. 1) indicating that ECs in the cardinal veins are a continuous source of Prox1$^+$ LECs. On the other hand, in the analyzed mesenteric lymphatics, we observed *LacZ*$^+$ LECs only in E16.5 embryos exposed to TM at E10.5 (Fig. 3G,H). Together, these results support the proposal that rostral deep lymphatics appear first and are derived from rostrally located LEC progenitors, and caudal deep lymphatics form later from posteriorly located LEC progenitors.

The rostral jugular lymph sacs near the junction of the subclavian and anterior cardinal veins were the first to arise from the earliest Prox1$^+$ LEC progenitors in the anterior cardinal vein at around E9.75. In contrast, LEC progenitors of the more posterior lymph sacs near the junction of the primitive iliac veins and the posterior cardinal veins appeared around E10.5 (Fig. 1). We have now demonstrated that local lymph sacs are the source of LECs that by sprouting, proliferation, and migration give rise to the lymphatic vasculature of nearby tissues and organs. Jugular lymph sacs form earlier and give rise to the lymphatics of the lungs and most likely other nearby tissues; posterior lymph sacs form later and give rise to the lymphatics of the mesentery and posterior organs. These lineage-tracing results support earlier anatomical descriptions (Sabin 1904; Heuer 1909); i.e., from the jugular lymph sacs, lymphatic capillaries spread toward anterior regions (head, neck, arms, and thorax), and from the posterior lymph sacs, lymphatics spread to posterior regions (abdominal wall, pelvis, and legs).

Lymphatic vasculature is of venous origin

Due to the mosaic labeling observed when using the *Prox1-CreER^{T2}* mouse strain, we cannot exclude the possibility that other sources contribute to the formation of the lymph sacs. To conclusively demonstrate that mammalian lymph sacs and, therefore, the whole lymphatic vasculature are exclusively venous derived, we complemented the studies presented above by performing similar analyses using the available mouse strains *Tie2-Cre*, *Prox1$^{flox/+}$*, *Coup-TFII$^{flox/+}$*, *Runx1-MER-Cre-MER*, and *Runx1*.

Tie2-Cre mice Tie2 is a receptor tyrosine kinase expressed in BECs and hematopoietic cells (Sato et al. 1993; Takakura et al. 1998). The interaction of Tie2 with its ligand angiopoietin-1 (*Ang1*) helps remodel and stabilize primitive vasculature (Sato et al. 1993; Suri et al. 1996). The phenotype of mice lacking another Tie2 ligand, *Ang2*, suggested that Tie2 also functions during postnatal lymphatic remodeling (Gale et al. 2002); however, its expression has not been observed in LECs (Motoike et al. 2000; Saban et al. 2004; Wilting et al. 2006), except in restricted regions of adult lymphatics (Morisada et al. 2005; Tammela et al. 2005). Hence, Tie2-independent, integrin-mediated signaling might mediate angiopoietin function in the lymphatic vasculature (Morisada et al. 2005).

Our in situ hybridization and immunohistochemical (GFP) analyses of sections from E11.5, E13.5, and E15.5 *Tie2-GFP* transgenic mouse embryos failed to detect *Tie2* expression in budding LECs, lymph sacs, developing lymphatics, or mesenchymal cells (Supplementary

Fig. 6; data not shown). Furthermore, flow cytometric analysis failed to detect Tie2$^+$ LECs in dissociated cells from E12.5 *Tie2-GFP* embryos (Supplementary Fig. 6B). Therefore, we reasoned that Tie2 expression in BECs of the cardinal vein and its absence from embryonic LECs and mesenchymal cells support using the *Tie2-Cre* strain (Kisanuki et al. 2001) for cell-lineage analysis. *Tie2-Cre* mice were crossed with the *R26R* reporter line, and embryos were isolated at different stages. At E11.5, Prox1$^+$ cells in the anterior cardinal vein and those budding from it were *lacZ*$^+$ (Fig. 4A). Similarly, all E13.5 (Fig. 4B) and E14.5 (data not shown) Prox1$^+$ ECs in the lymph sacs were *lacZ*$^+$. These results support the proposal that mammalian lymph sacs are venous-derived.

Tie2-Cre;Prox1$^{flox/+}$ mice We previously demonstrated that deletion of *Prox1* from a conditional mouse strain (*Tie2-Cre;Prox1$^{flox/+}$*) (Harvey et al. 2005) resulted in conditional-heterozygous mice with lymphatic defects similar to those of standard *Prox1$^{+/LacZ}$* mice (Wigle and Oliver 1999). This result suggested that early deletion of *Prox1* from LEC progenitors in embryonic veins caused those lymphatic defects (Harvey et al. 2005). We used *Tie2-Cre* to generate conditional *Prox1*-null embryos (*Tie2-Cre;Prox1$^{flox/LacZ}$*). We reasoned that if the lymphatic vasculature is exclusively venous derived, lymphangiogenesis in the conditional-null embryos should be severely compromised. To this end, *Prox1$^{+/LacZ}$* females were bred with *Tie2-Cre;Prox1$^{flox/+}$* males, and E11.5 *Tie2-Cre;Prox1$^{flox/LacZ}$*-null embryos were sectioned and analyzed using anti-Prox1 antibody. At this stage, wild-type and *Prox1$^{+/LacZ}$* embryos showed Prox1$^+$ LECs budding from the anterior cardinal vein (Fig. 5A); however, the conditional *Tie2-Cre;Prox1$^{flox/LacZ}$* mutant embryos showed very few Prox1-expressing LECs in or around the anterior cardinal vein (Fig. 5B). Similarly, whole-mount X-gal staining of E15.5 conditional *Tie2-Cre;Prox1$^{flox/LacZ}$* mutant embryos revealed the presence of occasional scattered superficial *lacZ*$^+$ LECs (Fig. 5D); many of the remaining superficial *lacZ*$^+$ cells were not LECs as they are also detected in E14.5 standard *Prox1*-null embryos (Supplementary Fig. 7, arrows; Wigle and Oliver 1999). We must emphasize that using *Cre* lines results in variable efficiency in deletion of the gene of interest. As a consequence, the efficiency of deleting Prox1-expressing LEC progenitors in the cardinal vein was variable among embryos, and occasional superficial *lacZ*$^+$ LECs were identified in the conditional mutant embryos. Importantly, no deep lymphatic vasculature was identified (Fig. 5E,F); only a few occasional scattered *lacZ*$^+$ LECs were visualized.

Together, the defects in lymphatic patterning and integrity observed in conditional *Tie2-Cre;Prox1$^{flox/+}$*-heterozygous mice (Harvey et al. 2005) and the drastic reduction in the number of LECs present in the conditional *Tie2-Cre;Prox1$^{flox/LacZ}$* mutant embryos further validate our argument that in the developing mouse Prox1-expressing venous ECs are the major (if not the sole) source of LEC progenitors.

Tie2-Cre;Coup-TFII$^{flox/+}$ mice *Coup-TFII* is an orphan nuclear receptor transcription factor, and in its absence, mouse embryos die around E10.0 due to several defects, including abnormal venous development (Pereira et al. 1999). Conditional deletion of *Coup-TFII* from embryonic veins causes abnormal venous expression of arterial markers and the subsequent death of embryos at around E11.0 (You et al. 2005). We speculated that if veins are the single source of LEC progenitors, the change in fate of venous ECs in *Coup-TFII*-mutant embryos should affect lymphatic development. Therefore, we conditionally deleted *Coup-TFII* from the vascular system of E11.0 embryos by using the *Tie2-Cre* strain. This deletion activated a *lacZ* reporter gene (Takamoto et al. 2005), allowing us to follow the lineage of *Coup-TFII*-expressing venous ECs. As controls, E11.0 conditional *Coup-TFII*-heterozygous (*Tie2-Cre;Coup-TFII$^{flox/+}$*) embryos were used.

Normally at E11.0, Prox1-expressing cells are detected in and budding from the anterior cardinal vein (Fig. 6A). *Tie2-Cre;Coup-TFII$^{flox/flox}$* embryos had ~80% fewer Prox1-expressing LECs in and around the cardinal vein than did their heterozygous littermates (Fig. 6B,C). Furthermore, the lymphatic marker Lyve-1 (Banerji et al.

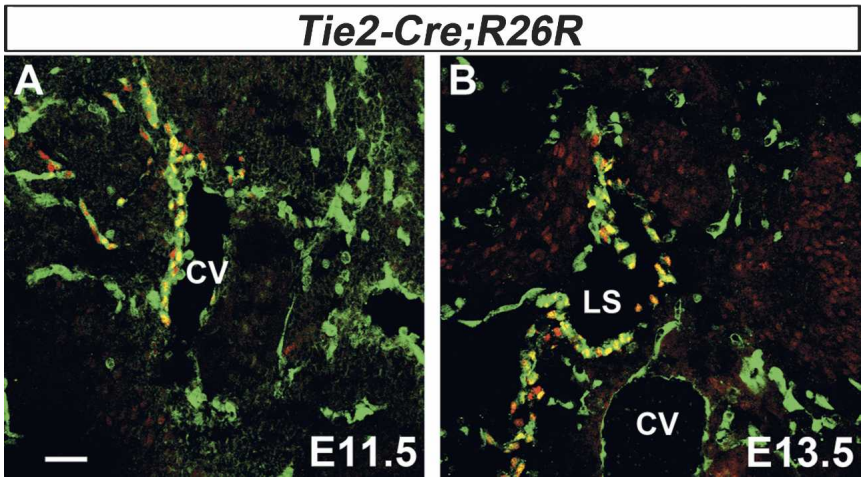

Figure 4. Lineage tracing using the BEC-specific *Tie2-Cre* strain reveals the venous origin of LECs. Transverse sections of *Tie2-Cre;R26R* embryos were analyzed using antibodies against Prox1 (red) and β-gal (green) at E11.5 (*A*) and E13.5 (*B*). Nearly all Prox1$^+$ cells in and around the anterior cardinal vein (CV) colocalized with β-gal, which indicated that they originated from Tie2$^+$ venous ECs. (LS) Lymph sac. Bar, 100 μm.

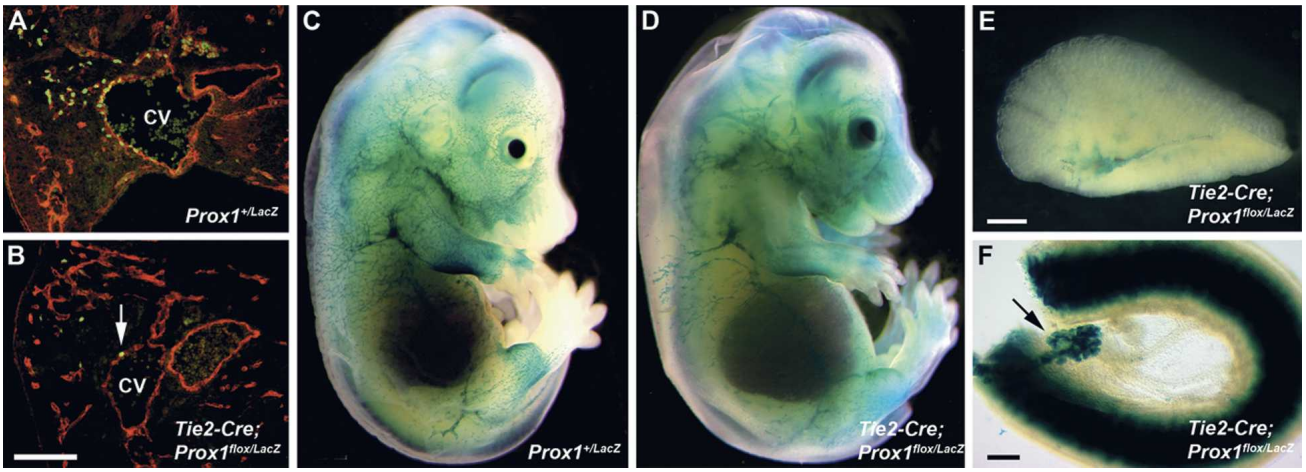

Figure 5. Conditional deletion of *Prox1*-expressing venous progenitors severely reduces the number of LECs. (*A*) Transverse section of an E11.5 *Prox1*$^{+/LacZ}$ embryo showing normal Prox1 expression (green) in LECs located in and around the cardinal vein (CV). (*B*) The number of Prox1-expressing LECs in a *Tie2-Cre;Prox1*$^{flox/LacZ}$ conditional mutant littermate was severely reduced in (arrow) and around the CV. The presence of a few remaining Prox1^{+} cells in the CV suggests that the Cre-mediated deletion of *Prox1* was incomplete. Red staining labels PECAM. (*C*,*D*) The drastic reduction in the number of LEC progenitors affected the overall development of the lymphatic vasculature at later stages. (*C*) The network of superficial lymphatics is vast in an X-gal-stained E15.5 control *Prox1*$^{+/LacZ}$ embryo. (*D*) Instead, only a few scattered *lacZ*$^{+}$ LECs are detected in a conditional *Tie2-Cre;Prox1*$^{flox/LacZ}$ mutant embryo. (*E*) No *lacZ*$^{+}$ LECs were present in the lungs (*E*) or mesentery (*F*) of E16.5 conditional *Tie2-Cre;Prox1*$^{flox/LacZ}$ mutant embryos. The observed blue staining corresponds to Prox1 expression in neuroendocrine cells of the lung (*E*) or pancreas (arrow in F) and nonspecific trapping in the intestinal tract (*F*). Bars: *A*,*B*, 100 µm; *E*, 500 µm; *F*, 200 µm.

1999), which is normally expressed in the anterior cardinal vein at this stage, was absent in the mutant embryos (data not shown), supporting the proposal that venous identity is a necessary prerequisite for LEC specification in the cardinal veins.

The presence of Prox1-expressing cells in the conditional *Coup-TFII*-null embryos was probably the consequence of inefficient Cre activity and, thus, of residual venous identity as previously reported (You et al. 2005). This conclusion is supported by the fact that most of the Prox1^{+} cells in and around the cardinal vein in the E11.0 conditional *Coup-TFII*-heterozygous or -null embryos were also *lacZ*$^{+}$ (data not shown), which indicates that they originated from the vein. These results indicate that venous identity is required for embryonic lymphangiogenesis and that Prox1-expressing venous ECs are the main source of LECs in the developing mammalian embryo. However, a contribution of hematopoietic-derived LEC progenitors to the developing lymphatics has also been suggested (Buttler et al. 2006; Sebzda et al. 2006). Therefore, we further addressed whether hematopoietic-derived LECs (lymphangioblasts) contribute to the mammalian embryonic lymphatic vasculature in another mouse model.

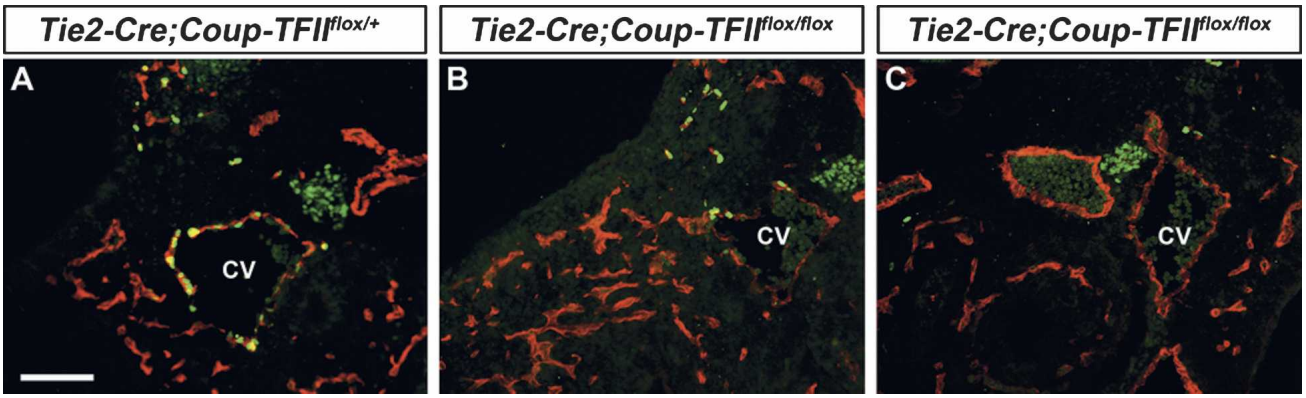

Figure 6. Defective venous identity compromises lymphatic vasculature development. (*A*) Prox1-expressing LECs (green) are seen in and around the anterior cardinal vein (CV) in transverse sections of control E11.0 *Tie2-Cre;Coup-TFII*$^{flox/+}$ mice. The number of Prox1-expressing LECs is drastically reduced (*B*) or almost undetected (*C*) in conditional *Tie2-Cre;Coup-TFII*$^{flox/flox}$ mutant littermates. Red staining labels PECAM. Bar, 100 µm.

Runx1 mice Definitive hematopoiesis is defective in *Runx1* mice, and *Runx1*-null mice die at around E12.5 (Okuda et al. 1996), when the first lymph sacs form. We hypothesized that if hematopoietic precursors contribute to the lymphatic vasculature, then initial stages of LEC budding and lymph sac formation should be affected in E12.5 *Runx1*-null embryos. If the sacs appeared normal, then hematopoietic precursors make little or no contribution to lymphangiogenesis.

In E12.0 *Runx1*$^{-/-}$ embryos labeled with anti-Lyve-1 antibodies, morphologically normal lymph sacs formed, but were blood-filled (Fig. 7B). Defects in lympho–venous separation may have caused this phenotype as *Slp-76* and *Syk*, which are expressed in hematopoietic cells, are essential for lympho–venous separation (Abtahian et al. 2003; Sebzda et al. 2006).

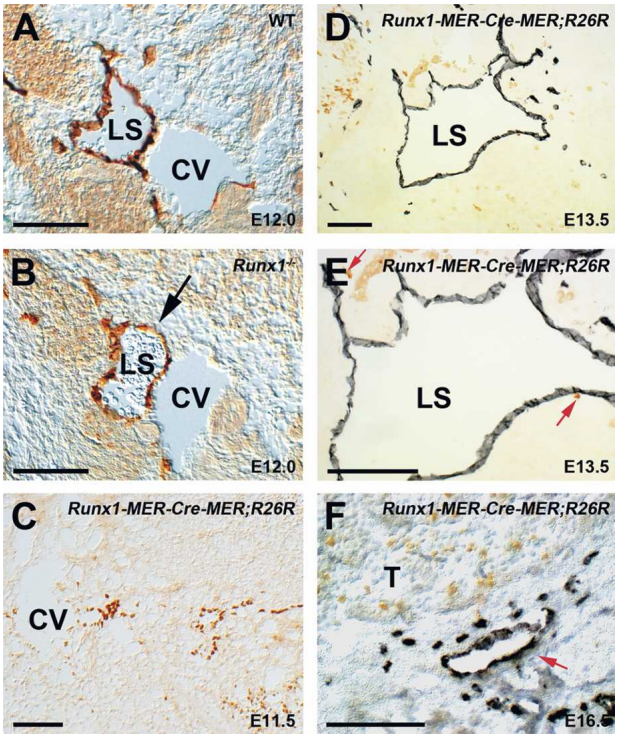

Figure 7. Hematopoietic cells do not contribute to the lymphatic vasculature. (*A*) Transverse section (10 µm) of an E12.0 wild-type embryo showing a Lyve-1$^+$ jugular lymph sac (LS) beside the anterior cardinal vein (CV). (*B*) Transverse section of a *Runx1*$^{-/-}$ littermate showing no obvious difference in the appearance of the jugular lymph sac, but the sac is blood-filled (arrow). *Runx1-MER-Cre-MER;R26R* embryos exposed to TM at E9.5 were analyzed at E11.5 (*C*), E13.5 (*D*,*E*), and E16.5 (*F*). (*C*) An X-gal-stained E11.5 embryo was sectioned and immunostained for Prox1 (brown). No blue cells (descendents of Runx1$^+$ cells) are observed in the field. (*D*,*E*) Lower- and higher-magnification pictures of a section from an E13.5 embryo coimmunostained for β-gal (brown) and Lyve-1 (purple). β-gal$^+$ cells (red arrows) can be seen around the lymph sacs (LS) but do not colocalize with Lyve-1 staining. (*F*) E16.5 embryo coimmunostained for β-gal (brown) and Lyve-1 (purple). β-Gal$^+$ cells are seen in the thymus (T) but not in the lymphatic vessel (arrow). Bar, 100 µm.

Runx1-MER-Cre-MER mice A TM-inducible *Cre* line under the control of the *Runx1* promoter was recently generated (Samokhvalov et al. 2007). Uniform labeling of the descendants of all definitive hematopoietic stem cells was achieved by crossing this strain with the *R26R* reporter line and administering TM at E9.5 (Samokhvalov et al. 2007). Following this approach, we analyzed in detail E11.5, E13.5, and E16.5 *Runx1-MER-Cre-MER;R26R* embryos exposed to 5 mg of TM at E9.5 (Fig. 7C–F; Supplementary Fig. 8). The *lacZ*$^+$ cells were randomly distributed throughout the embryos; some were located around and occasionally in contact with the lymph sacs (Fig. 7D,E). None of the *lacZ*$^+$ cells were double-labeled for any LEC-specific marker (Fig. 7C–F). This result agrees with other reports of early segregation of the hematopoietic compartment and EC compartment with no contribution of the former to the latter (Stadtfeld and Graf 2005; Samokhvalov et al. 2007).

Regarding the blood-filled lymph sacs observed in E12.0 *Runx1*$^{-/-}$ embryos, the lineage-tracing analysis performed using the generated *Runx1-MER-Cre-MER; R26R* embryos ruled out a cell-autonomous mechanism as being responsible for that phenotype. In fact, work performed using *Runx1*$^{-/-}$ mice has previously demonstrated that hematopoietic stem cells regulate angiogenesis in a non-cell-autonomous manner (Takakura et al. 2000). Accordingly, the blood-filled lymph sac phenotype is most likely secondary to a primary angiogenic defect. Interestingly, angiogenic defects (abnormal artero–venous shunting) are also present in *Slp-76*$^{-/-}$ embryos displaying a blood-filled lymphatic phenotype (Abtahian et al. 2003).

We conclude that hematopoietic-derived cells do not significantly contribute to the development of embryonic lymph sacs or to the formation of the lymphatic vasculature. Therefore, veins are the main if not sole source of LECs in the developing mammalian embryo.

Discussion

Here we conclusively demonstrated that in the developing mammalian embryo, Prox1-expressing LEC progenitors are of venous origin. Using detailed lineage-tracing analyses, we not only validated Sabin's original proposal (Sabin 1902) but also van der Putte's anatomical descriptions (van der Putte 1975). We corroborated that the rostral lymphatics form first and arise from the earliest Prox1-expressing LEC progenitors located in the anterior cardinal vein; the more posterior lymphatics form later from LEC progenitors in the posterior veins. Furthermore, we determined that local lymph sacs are the source of LECs that by sprouting, proliferation, and migration give rise to the lymphatic vasculature of nearby tissues and organs. The jugular lymph sacs give rise to the lymphatics of the lungs and most likely other nearby tissues; the posterior lymph sacs give rise to the lymphatics of the mesentery and posterior organs.

Using lineage tracing, we also determined that no descendants of Prox1$^+$ cells were present in embryos exposed to TM prior to E9.75, thereby conclusively dem-

onstrating that at around E9.75, ECs in the anterior cardinal vein are the earliest source of Prox1-expressing LEC progenitors. To demonstrate that the mammalian lymphatic vasculature has a solely venous origin, we used several mouse strains.

Tie2-Cre;Coup-TFII$^{flox/flox}$-mutant embryos showed that venous identity is required at least during the early stages of lymphatic development. Conditional *Tie2-Cre;Prox1$^{flox/LacZ}$* mutant embryos showed that the number of LECs was significantly reduced and that venous-derived *Tie2* progeny contribute to the forming lymphatics. These results support the argument that LECs have a venous origin and that Prox1-expressing venous ECs are the main (if not the sole) source of LEC progenitors in developing mice.

Although our results show that developing lymphatic vasculature arises mostly from venous-derived Prox1-expressing progenitor cells, two recent reports have claimed that other cell types contribute to developing lymphatics in mice. Buttler et al. (2006) proposed that scattered mesenchymal cells with leukocyte and lymphoendothelial characteristics that are first detected after E10.5 eventually integrate into the lymphatics. Sebzda et al. (2006) identified a subpopulation of *Syk*- and *Slp-76*-expressing hematopoietic-derived CEPs that acquire a lymphatic fate. However, two other reports found no evidence of hematopoietic contribution to the mammalian endothelial compartment (Stadtfeld and Graf 2005; Samokhvalov et al. 2007).

We did not identify descendents of Prox1$^+$ cells in embryos exposed to TM prior to E9.5. This result not only demonstrated that E9.75 venous Prox1-expressing ECs are the earliest source of LEC progenitors but also eliminated the possibility that other hematopoietic- or mesenchyme-derived Prox1$^+$ lymphatic progenitors are present before Prox1 expression in the cardinal vein. Furthermore, *Runx1$^{-/-}$* embryos defective in definitive hematopoiesis had morphologically normal lymph sacs, and lineage tracing using the *Runx1-MER-Cre-MER* strain demonstrated that descendents of *Runx1*$^+$ cells do not contribute to the developing lymph sacs. These results conclusively demonstrate that hematopoietic cells do not significantly contribute to the forming lymph sacs or lymphatic vasculature. Therefore, if mesenchymal cells with leukocyte and lymphoendothelial characteristics or hematopoietic-derived CEPs exist in the mouse embryo, they are so rare they probably cannot directly contribute to the developing murine lymphatic network. We must emphasize that additional LEC sources such as transdifferentiating macrophages (Maruyama et al. 2005) and bone marrow (Salven et al. 2003; Religa et al. 2005; Kerjaschki et al. 2006) probably contribute to postnatal lymphangiogenesis that occurs in normal and pathological conditions.

In summary, our work validates Sabin's model, which almost 100 years ago proposed that from venous-derived primary lymph sacs, the peripheral lymphatic system originates and spreads by endothelial sprouting into the surrounding tissues and organs, where capillaries form (Sabin 1902, 1904). We conclusively determined that during mammalian embryonic lymphangiogenesis, Prox1-expressing LEC progenitors in early veins are the main, and most likely unique source of LECs required for the formation of the lymphatic vasculature.

Materials and methods

Mice

Tie2-Cre mice were provided by Dr. M. Yanagisawa (University of Texas Southwestern Medical Center, Dallas, TX); *Tie2-GFP* mice were supplied by Dr. T. Sato (Cornell University, New York); *R26R* mice were provided by Dr. G. Grosveld (St. Jude Children's Research Hospital, Memphis, TN); and *Runx1*-mutant embryos were supplied by Dr. J. Downing (St. Jude Children's Research Hospital, Memphis, TN). The methods for generating *Prox1$^{+/LacZ}$*, *Prox1$^{flox/flox}$*, *Coup-TFII$^{flox/flox}$*, and *Runx1-MER-Cre-MER* mice have been reported previously (Wigle et al. 1999; Harvey et al. 2005; Takamoto et al. 2005; Samokhvalov et al. 2007). To generate the *Prox1-CreER^{T2}* embryonic stem cell line, we fused a synthetic splice acceptor site, *Prox1* exons 3 and 4, an internal ribosome entry site, and *Cre-ER^{T2}* and Poly(A) tail. This fusion was targeted for insertion into intron 2 of the mouse *Prox1* locus by electroporation into the W9.5 embryonic stem cell line. Following selection and standard screening, the correctly targeted cells were used to generate chimeric mice. The developmental stage of mouse embryos was determined by considering 9 a.m. of the day the vaginal plug was detected in the pregnant dam as E0.5. All of the mouse experiments were approved by the St. Jude Children's Research Hospital Animal Care and Use Committee.

Immunohistochemistry

Fluorescent or horseradish peroxidase staining using 3,3′-diaminobenzidene (DAB) as a substrate was performed on frozen or paraffin-embedded sections (10 µm) as described previously (Harvey et al. 2005). Primary antibodies were rabbit anti-β-gal (MP Biomedicals), rabbit (AngioBio), and guinea pig (G. Oliver, unpubl.); anti-mouse Prox1; rat anti-mouse PECAM (BD Pharmingen); guinea pig anti-Lyve-1 (G. Oliver, unpubl.); and rabbit anti-GFP (Molecular Probes). Secondary antibodies were Alexa 488-conjugated donkey anti-rabbit (Molecular Probes), Cy3-conjugated donkey anti-guinea pig (Jackson ImmunoResearch Laboratories), and Cy3-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories).

TM injection

For lineage tracing using the *Prox1-CreER^{T2}* line, TM (20 mg/mL; Sigma) was dissolved in corn oil. Pregnant mice were injected intraperitoneally with either 3 or 5 mg per 40 g of body weight at the indicated time points. Lineage tracing using the *Runx1-MER-Cre-MER* line has been described previously (Samokhvalov et al. 2007).

Detection of β-galactosidase activity in embryos and tissues

To detect β-gal activity in embryos, we performed X-gal staining as described previously (Harvey et al. 2005). Embryos were post-fixed in 4% paraformaldehyde overnight at 4°C and then cleared by soaking in sequentially increasing concentrations of glycerol dissolved in a solution of PBS and 0.1% Tween 20. Alternatively, embryos were embedded in paraffin and sectioned (10 µm).

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