Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature

R. Sathish Srinivasan,1 Miriam E. Dillard,1 Oleg V. Lagutin,1 Fu-Jung Lin,2 Sophia Tsai,2,3 Ming-Jer Tsai,2,3 Igor M. Samokhvalov,4 and Guillermo Oliver1,5

1Department of Genetics and Tumor Cell Biology, St. Jude Children’s Hospital, Memphis, Tennessee 38105, USA; 2Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030, USA; 3Developmental Biology Program, Baylor College of Medicine, Houston, Texas 77030, USA; 4Laboratory for Stem Cell Biology, Center for Developmental Biology, RIKEN Kobe, Kobe 650-0047, Japan

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.

[Keywords: Lymphatic endothelial cells; lymphangiogenesis; Prox1; mouse; lineage tracing; Runx1]

Supplemental material is available at http://www.genesdev.org.

Received June 29, 2007; revised version accepted August 10, 2007.

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,
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-CreERT\textsuperscript{2} 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-expressing cells that contribute to developmental lymphangiogenesis, we genetically labeled Prox1-expressing cells with a TM-inducible Cre-ERT\textsuperscript{2}/LoxP-based tracing system (Danielian et al. 1998; Indra et al. 1999). Gene targeting inserted Cre-ERT\textsuperscript{2} into the mouse Prox1 locus (Supplementary Fig. 1) to generate the Prox1-CreERT\textsuperscript{2} allele. To generate the targeting construct and avoid the haploinsufficiency phenotype observed in Prox1\textsuperscript{1/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-ERT\textsuperscript{2}, and a poly(A) transcription-stop signal (Supplementary Fig. 1). This modified Prox1 allele should express Prox1 and Cre-ERT\textsuperscript{2} from a single bicistronic transcript, thereby recapitulating the normal pattern of Prox1 expression. Targeted embryonic stem cells were used to generate the Prox1-CreERT\textsuperscript{2} 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\textsuperscript{+} 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-CreERT\textsuperscript{2} recapitulates that of endogenous Prox1, we exposed Prox1-CreERT\textsuperscript{2}; 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\textsuperscript{1/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\textsuperscript{+} 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-CreERT\textsuperscript{2} 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\textsuperscript{+} 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-CreERT\textsuperscript{2}. 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\textsuperscript{+} LECs were detected in E10.5 embryos exposed to 3 mg of TM that was administered to pregnant Prox1-CreERT\textsuperscript{2} × R26R females at E9.0 (Supple-
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 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).

**Figure 1.** Venous ECs are the earliest LEC progenitors. Prox1-CreERT2;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]. (D) The number of lacZ+ LECs in the jugular lymph sacs also increased. Bar, 100 µm.

Next, for fate-mapping experiments, we administered 3 mg of TM at E8.5 or E9.5 to Prox1-CreERT2 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].
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-CreERT2;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-CreERT2;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-CreERT2;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
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 cardi-

cnal 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, 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-CreERT2;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-CreERT2;R26R embryos exposed to 3 mg of TM at E10.5. Bars: A–D, 500 µm; E–H, 200 µm.

**Figure 3.** The deeper lymphatic vasculature arises by continuous sprouting, proliferation, and migration of LECs. Prox1-CreERT2;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-CreERT2;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-CreERT2;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-CreERT2;R26R embryos exposed to 3 mg of TM at E10.5. Bars: A–D, 500 µm; E–H, 200 µm.

**Lymphatic vasculature is of venous origin**

Due to the mosaic labeling observed when using the Prox1-CreERT2 mouse strain, we cannot exclude the pos-

sibility that other sources contribute to the formation of the lymph sacs. To conclusively demonstrate that mam-

alian lymph sacs and, therefore, the whole lymphatic 

vasculature are exclusively venous derived, we comple-

mented the studies presented above by performing similar analyses using the available mouse strains Tie2-Cre, 

Prox1flox/+, Coup-TFIIflox/+, and Runx1-MER-Cre-MER, and 

Runx1. Tie2-Cre mice Tie2 is a receptor tyrosine kinase ex-

pressed 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 postna-

tal 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<sup>flox/+</sup> mice** We previously demonstrated that deletion of Prox1 from a conditional mouse strain [Tie2-Cre;Prox1<sup>flox/+</sup>] (Harvey et al. 2005) resulted in conditional-heterozygous mice with lymphatic defects similar to those of standard Prox1<sup>+/LacZ</sup> 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<sup>flox/LacZ</sup>]. 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<sup>+/LacZ</sup> females were bred with Tie2-Cre;Prox1<sup>flox/+</sup> males, and E11.5 Tie2-Cre;Prox1<sup>flox/LacZ</sup>-null embryos were sectioned and analyzed using anti-Prox1 antibody. At this stage, wild-type and Prox1<sup>+/LacZ</sup> embryos showed Prox1+ LECs budding from the anterior cardinal vein [Fig. 5A]; however, the conditional Tie2-Cre;Prox1<sup>flox/LacZ</sup>-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<sup>flox/LacZ</sup>-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<sup>flox/+</sup>-heterozygous mice (Harvey et al. 2005) and the drastic reduction in the number of LECs present in the conditional Tie2-Cre;Prox1<sup>flox/LacZ</sup> 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<sup>flox/+</sup> 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<sup>flox/+</sup>] 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<sup>flox/+</sup> 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.
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-TFIIflox/+ mice. The number of Prox1-expressing LECs is drastically reduced (B) or almost undetected (C) in conditional Tie2-Cre;Coup-TFIIflox/fox mutant littermates. Red staining labels PECAM. 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;Prox1flox/LacZ conditional mutant littermate was severely reduced [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;Prox1flox/LacZ mutant embryo. (E) No lacZ+ LECs were present in the lungs [E] or mesentery [F] of E16.5 conditional Tie2-Cre;Prox1flox/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.
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].

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 arterio–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 rise 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-
onstrasting 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. 

**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-/-; Prox1flox/LacZ, Prox1; Coup-TFIIflox/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-Cre;ER 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'-diaminobenzidine (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).
Lymphatic vasculature is venous derived

Acknowledgments

We thank N. Lenny and J. Downing for Runx1-mutant embryos, M. Yanagisawa for the Tie2-Cre line, T. Sato for the Tie2-GFP; D. Dumont for the Tie2 cDNA; N. Gale, N. Papadopoulos, and G. Yancopoulos for the Lyve-1 fusion protein, M. Self and X. Geng for assistance, N. Johnson for critical reading, and Angela McArthur for editing the manuscript. This work was supported by NIH grant HL 076448 (to S.Y.T.) and R01-HL073402 (to G.O.), Cancer Center Support Grant CA-21765, and the American Lebanese Syrian Associated Charities (ALSAC). R.S.S. is supported by a post-doctoral fellowship grant from the Lymphatic Research Foundation.

References

Abtahian, F., Guerriero, A., Sebzda, E., Lu, M.M., Zhou, R., Banerji, S., Ni, J., Wang, S.X., Clasper, S., Su, J., Tammi, R., Harvey, N.L., Srinivasan, R.S., Dillard, M.E., Johnson, N.C., Sabin, F. 1902. On the origin of the lymphatics system from the somatic translocations in human leukemia. Nature 242: 70–78.

Gale, N.W., Thurston, G., Hackett, S.F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M.H., Jackson, D.G., et al. 2002. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. Dev. Cell 3: 411–423.

Kerjaschki, D., Huttary, N., Raab, I., Regele, H., Bojarski-Nagy, K., Bartel, G., Krober, S.M., Greinix, H., Rosenmaier, A., Karlhofer, F., et al. 2006. Lymphatic endothelial progenitor cells contribute to de novo lymphangiogenesis in human renal transplants. Nat. Med. 12: 230–234.

Kisanuki, Y.Y., Hammer, R.E., Miyazaki, J., Williams, S.C., Richardson, J.A., and Yanagisawa, M. 2001. Tie2-Cre transgenic mice: A new model for endothelial cell-lineage analysis in vivo. Dev. Biol. 230: 230–242.

Maruyama, K., Mi, M., Cursiefen, C., Jackson, D.G., Keino, H., Tomita, M., Van Rooijen, N., Takenaka, H., D’Amore, P.A., Stein-Streilein, J., et al. 2005. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. J. Clin. Invest. 115: 2363–2372.

Morisada, T., Oike, Y., Yamada, Y., Urano, T., Akae, M., Kubota, Y., Maekawa, H., Kimura, Y., Ohmura, M., Miymoto, T., et al. 2005. Angiopoietin-1 promotes LYVE-1-positive lymphatic vessel formation. Blood 105: 4649–4656.

Motoyoshi, S., Loughna, S., Perens, E., Roman, B.L., Liao, W., Chau, T.C., Richardson, C.D., Kawate, T., Kuno, J., Weinstein, B.M., et al. 2000. Universal GFP reporter for the study of vascular development. Genesis 28: 75–81.

Ny, A., Koch, M., Schneider, M., Neven, E., Tong, R.T., Maity, S., Fischer, C., Plaisance, S., Lambrechts, D., Heligon, C., et al. 2005. A genetic Xenopus laevis tadpole model to study lymphangiogenesis. Nat. Med. 11: 998–1004.

Okuda, T., van Deursen, J., Hiebert, S.W., Grosveld, G., and Downing, J.R. 1996. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell 84: 321–330.

Oliver, G. and Alitalo, K. 2005. The lymphatic vasculature: Recent progress and paradigms. Annu. Rev. Cell Dev. Biol. 21: 457–483.

Oliver, G. and Metzger, D. 1999. Temporally-controlled expression of the reporter protein β-gal in mice embryos in utero by a tamoxifen-inducible form of Cre recombinase. Development 125: 2323–2326.

Banerji, S., Ni, J., Wang, S.X., Clasper, S., Su, J., Tammi, R., Jones, M., and Jackson, D.G. 1999. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J. Cell Biol. 144: 789–801.

Buttler, K., KreySing, A., von Kaisenberg, C.S., Schweigerer, L., Gale, N., Papoutsli, M., and Wilting, J. 2006. Mesenchymal cells with leukocyte and lymphendothelial characteristics in murine embryos. Dev. Dyn. 235: 1554–1562.

Dor, Y., Brown, J., Martinez, O.I., and Melton, D.A. 2004. Adult pancreatic β-cells are formed by self-duplication rather than stem-cell differentiation. Nature 429: 41–46.

Mocsai, A., Myers, E.E., Huang, B., Jackson, D.G., Ferrari, V.A., et al. 2003. Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. Science 299: 247–251.
Stadtfeld, M. and Graf, T. 2005. Assessing the role of hematopoietic plasticity for endothelial and hepatocyte development by non-invasive lineage tracing. *Development* **132**: 203–213.

Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S., Sato, T.N., and Yancopoulos, G.D. 1996. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87**: 1171–1180.

Takakura, N., Huang, X.L., Naruse, T., Hamaguchi, I., Dumont, D.J., Yancopoulos, G.D., and Suda, T. 1998. Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. *Immunity* **9**: 677–686.

Takakura, N., Watanabe, T., Suenobu, S., Yamada, Y., Noda, T., Ito, Y., Satake, M., and Suda, T. 2000. A role for hematopoietic stem cells in promoting angiogenesis. *Cell* **102**: 199–209.

Takamoto, N., You, L.R., Moses, K., Chiang, C., Zimmer, W.E., Schwartz, R.J., DeMayo, F.J., Tsai, M.J., and Tsai, S.Y. 2005. COUP-TFII is essential for radial and anteroposterior patterning of the stomach. *Development* **132**: 2179–2189.

Wigle, J.T. and Oliver, G. 1999. Prox1 function is required for the development of the murine lymphatic system. *Cell* **98**: 769–778.

Wigle, J.T., Chowdhury, K., Gruss, P., and Oliver, G. 1999. Prox1 function is crucial for mouse lens-fibre elongation. *Nat. Genet.* **21**: 318–322.

Wigle, J.T., Harvey, N., Detmar, M., Lagutina, I., Grosveld, G., Gunn, M.D., Jackson, D.G., and Oliver, G. 2002. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J.* **21**: 1505–1513.

Wilting, J., Papoutsi, M., Schneider, M., and Christ, B. 2000. The lymphatic endothelium of the avian wing is of somitic origin. *Dev. Dyn.* **217**: 271–278.

Wilting, J., Aref, Y., Huang, R., Tomarev, S.I., Schweigerer, L., Christ, B., Valasek, P., and Papoutsi, M. 2006. Dual origin of avian lymphatics. *Dev. Biol.* **292**: 165–173.

Yaniv, K., Isogai, S., Castranova, D., Dye, L., Hitomi, J., and Weinstein, B.M. 2006. Live imaging of lymphatic development in the zebrafish. *Nat. Med.* **12**: 711–716.

You, L.R., Lin, F.J., Lee, C.T., DeMayo, F.J., Tsai, M.J., and Tsai, S.Y. 2005. Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature* **435**: 98–104.

Zhang, H., Fujitani, Y., Wright, C.V., and Gannon, M. 2005. Efficient recombination in pancreatic islets by a tamoxifen-inducible Cre-recombinase. *Genes & Development* **42**: 210–217.
Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature

R. Sathish Srinivasan, Miriam E. Dillard, Oleg V. Lagutin, et al.

*Genes Dev.* 2007, 21: Access the most recent version at doi:10.1101/gad.1588407