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Report

Development of the Zebrafish Lymphatic System Requires Vegfc Signaling

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Summary

Lymphangiogenesis results in the formation of a vascular network distinct from arteries and veins that serves to drain interstitial fluid from surrounding tissues and plays a pivotal role in the immune defense of vertebrates as well as in the progression of cancer and other diseases [1, 2]. In mammals, lymph vessels are lined by endothelial cells possibly sprouting from embryonic veins, and their development appears to be critically dependent on the function of PROX1 [3] and VEGFC signaling [4]. The existence of a lymphatic system in teleosts has been a matter of debate for decades. Here we show on the morphological, molecular, and functional levels that zebrafish embryos develop a lymphatic vasculature that serves to retrieve components of the interstitium to the lymph system. We demonstrate the existence of vessels that are molecularly and functionally distinct from blood vessels and show that the development of these vessels depends on Vegfc and VEGFR-3/Flt4 signaling. These findings imply that the molecular components controlling lymphangiogenesis in zebrafish and mammals are conserved and that the zebrafish lymphatic system develops early enough to allow in vivo observations, lineage tracing, and genetic as well as pharmacological screens.

Results and Discussion

In order to investigate the existence of lymph vessels in the zebrafish, we examined both embryonic and adult stages for morphological and molecular evidence of lymphatics. In embryos at 5 days postfertilization (dpf) that were transgenic for a largely endothelial-specific fli:GFP transgene [5], we found a previously undescribed vessel in close proximity to the dorsal aorta (DA) in the trunk (Figure 1A). This vessel was usually considerably thinner than the DA and not as straight as the DA. We noticed that, in life embryos, we were never able to observe blood flow in this vessel. To investigate whether this vessel was indeed a part of the vasculature distinct from blood vessels, we injected rhodamine dextran into the sinus venosus of anesthetized embryos. The dye was distributed within all blood-perfused vessels. We observed that the DA, the posterior cardinal vein (PCV), and other vessels readily filled with the dye, but not the vessel adjacent to the aorta (Figure 1B). This was an indication that the vessel in question might indeed be part of a lymphatic system, as in mammals and other vertebrates blood can not be found in the lymphatics either.

Using the fli:GFP transgenic line, we examined the first appearance of this vessel in early embryos. The vessel became apparent at around day 3.5 dpf, when we noticed, along the length of the DA, independent appearances of cells just ventral to the aorta. An example of this is shown in Figure 1C, where at least four independent aggregations of endothelial cells within the same embryo can be observed. There are distinct gaps (highlighted by arrowheads) between these four sets of cells. Figures 1D–1G represent still frames from time-lapse cinematography (see Supplemental Data available with this article online) confirming that the patent vessel is formed by initially independent cells that spread anteriorly and posteriorly in order to connect with their rostral and caudal counterparts.

Ang2 Is Expressed in Cells Characteristic of Lymphatic Endothelial Cells

Subsequently, at later stages of development (see below, Figure 4), we have observed sprouts derived from this vessel, indicating that a more complex system will derive from this primary vessel. We have therefore addressed the question of whether there is morphological and molecular evidence for a possible lymphatic system in adult zebrafish.

In mice, Ang2 has been shown to play an essential role in the development and the function of lymphatics [6]. In order to examine the zebrafish adult lymphatic system, we made use of an antibody [7] that was raised against one of the zebrafish angiopoietins [7, 8], ang2. Using this antibody, we could, in adult zebrafish, distinguish two sets of vessels (Figures 2A and 2B), i.e., vessels that contained blood cells and were immunonegative, and vessels that were blood cell-free and were immunopositive. The Ang2 antibody specifically stained vessels that appeared more similar to veins rather than arteries in that these vessels are not surrounded by muscle cells (in contrast to arteries) and appear irregular in shape (Figures 2A and 2B). We wanted to examine whether these blood-free vessels were indeed lined by endothelial cells, and we confirmed this by electron microscopy (Figures 2C–2F). Both vessel types (ones with and ones without

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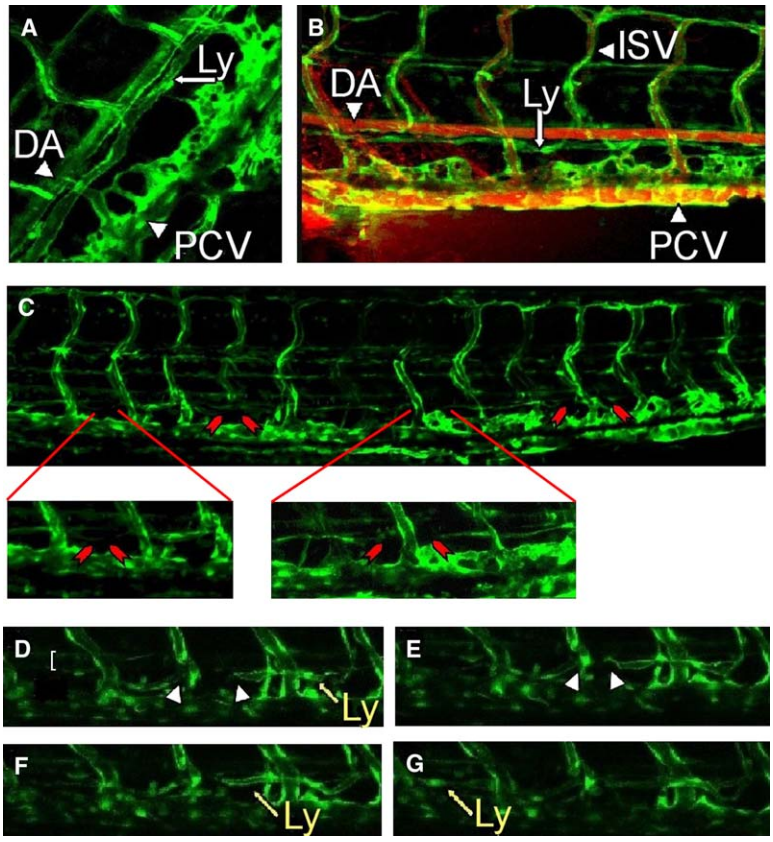

Figure 1. Confocal Microscopy Analysis of Vessel Formation in the Trunk of Zebrafish Embryos

(A) Lateral view of a 5 dpf embryo transgenic for fli:GFP. There is a previously undescribed vessel just underneath the DA, which was found to be devoid of circulating blood cells. Arrowheads point to the DA and the PCV; arrow points to the newly described vessel.

(B) Conventional angiography was performed using rhodamine dextran injection into the sinus venosus of a 5 dpf transgenic embryo. While the blood-filled vessels such as the DA, the PCV, and the intersegmental vessels (ISV) readily stained with the dye, the vessel ventral to the DA was never dye filled. Labeling as in (A).

(C) Lateral view of a transgenic embryo at 3.5 dpf. At this stage, the vessel described above is not fully formed, but there are independent clusters of cells along the length of the trunk in the position where the respective vessel will form eventually. Arrowheads point to gaps between the clusters, demonstrating that at this point there is not a fully patent vessel. Two of the areas containing such a gap are enlarged below the overview panel.

(D–G) Transgenic embryos such as in (C) were subjected to time-lapse confocal microscopy, which revealed that the individual cell clusters will extend anteriorly and posteriorly and join their posterior and anterior neighbors, respectively. A movie demonstrating this process can be viewed in the Supplemental Data. Four individual frames from this movie can be seen in (D)–(G), with the arrowheads pointing toward a gap between individual cells. In (F) and (G), the gap has been closed and the cells have joined.

blood cells within them) were lined by endothelial cells that were connected by tight junctions. The morphological difference to arteries, the absence of blood cells, and the difference in immunoreactivity strongly suggest that this newly discovered vessel type is distinct from the vascular vessel system and rather constitutes the adult lymphatic system. Angiopoietin 2, therefore, appears to be a specific marker for endothelial cells of the lymphatics in the adult zebrafish. Clearly, more work is required to understand the specific role of Ang2 in the zebrafish. However, in respect to the present study and the lymphatic system, our data support an evolutionary conserved role for Ang2 within lymphatic endothelial cells and demonstrate that there is a previously undescribed part of the adult vasculature that is molecularly and morphologically distinct from arteries and veins.

Dependence on Vegfc Signaling

In mice, lymph vessels do not develop in homozygous mutants for VEGFC [4], and the specific responsiveness of lymphatic endothelial cells to VEGFC and VEGFR-3/Flt-4 signaling is one of the molecular hallmarks of this vessel type [2]. We wanted to examine whether the development of zebrafish lymphatic vessels would be sensitive to VEGFC/Flt4 signaling. In comparison to injection control cases (Figure 3A), which were either left uninjected (n = 60) or were injected with 4 ng per embryo (n = 15) of an antisense morpholino directed against zebrafish flt1/VEGFR1, the injection of morpholinos directed against zebrafish Vegfc (Figure 3B) led to a dose-dependent variety of phenotypes, including embryos lacking circulation, as described previously [9]. We have analyzed only embryos that appeared phenotypically normal at 4 dpf and that exhibited normal circulation, and we found that the vessel adjacent to the aorta did not form in 100% of cases (n = 10). Identical results were obtained when injecting mRNA encoding a soluble, dominant-negative form of the human VEGFR-3, which has been shown previously to bind zebrafish Vegfc [9]. Again, we analyzed only embryos that established normal circulation and showed no effects in the formation of the DA and PCV (n = 129, from four independent injection experiments) and found a complete absence of this specific vessel (Figure 3C). No other vessels were affected. About 25% (17 of 74) of these embryos develop severe edema at 5 dpf.

We also attempted to use two different morpholinos directed against zebrafish Prox1 [10, 11], but formation of a severe heart defect forbid further analysis of the affected embryos. However, the extreme sensitivity of the newly discovered vessel to levels of Vegfc and VEGFR3/Flt4 signaling, as well as the observation that angiographies fail to stain this vessel type, strongly suggests that there is a lymphatic vessel system in zebrafish and that the vessel adjacent to the DA constitutes the primary lymph vessel in fish.

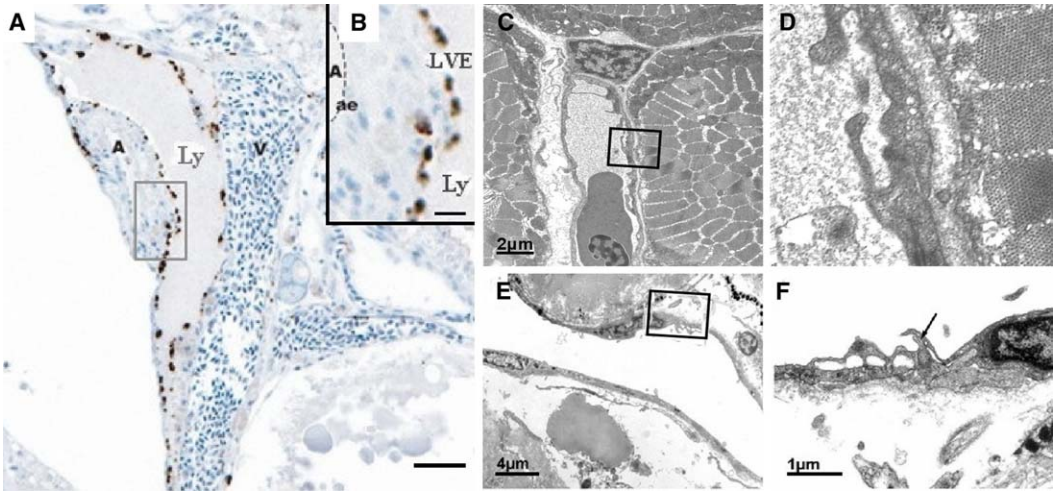

Figure 2. Zebrafish Ang2 Is Expressed in Adult Lymphatic Vessels

(A) Staining of sectioned adult zebrafish by using a peptide antibody directed against zebrafish Ang2 reveals the existence of lymphatics in zebrafish adults. Endothelial cells of the inner lining in blood cell-free vessels are stained positive for Ang2, while blood vessels do not show specific staining. The scale bar represents 50 μm. Controls including the peptide used for immunization were negative.

(B) Enlargement of the region boxed in (A). The scale bar represents 10 μm.

(C–F) Transmission electron microscopy shows endothelial lining of blood vessels (C and D) and blood cell-free vessels (E and F) in adult zebrafish. Arrow in (F) points to junctions between two endothelial cells. Boxes in (C) and (E) are enlarged in (D) and (F). Tissue was taken from the dorsal trunk region (distal to the neural tube) and processed using standard techniques. A, artery; ae, arterial endothelium; Ly, lymphatic vessel; LVE, lymphatic vessel endothelium; V, vein.

Removal of Substances from the Interstitium Functionally Defines the Zebrafish Lymphatic System

Ever since the classical study of Sabin [12], the physiological role of the lymphatic system has been considered to retrieve substances from the interstitium. We determined whether lymph vessels in the zebrafish embryo are functional in that respect by injecting fluorescent dextran subcutaneously into larvae older then 7 days. Fluorescin dextran injected between day 7 and day 12 into the muscle mass of the posterior trunk (Figure 4B) was significantly enriched in the lymphatic vessels after 3 hr (Figure 4C). Upon injection of the dye, we observed bright fluorescence around the site of injection over a period of a few hours, which became considerably weaker and was usually not detectable after 3 hr. Instead, the vessel ventral to the aorta, but not the DA itself or the PCV, accumulated the fluorescent dye. Over time, we observed distribution of the fluorescent dye from this vessel to the venous system, particularly the PCV (Figure 4D). This pattern of subcutaneously injected dye being taken up first by the vessel next to the DA and becoming redistributed to the PCV was highly reproducible. In a set of 32 scored embryos, we observed, after 6 hr, accumulation of the dye in the dorsal vessel in 12 cases, while in nine embryos the dye was distributed roughly equally between the vessel and the PCV, and in 11 cases there was more dye in the PCV than in the dorsal vessel. In control cases in which we injected dye into embryos that had been injected with sFlt4 mRNA at the one-cell stage and that were sorted for not having the dorsal vessel next to the DA, we never (n = 12) observed accumulation of the dye in the region of the DA (Figure 4E) or in the PCV (Figure 4F). This constitutes a strong line of evidence that lymphatic function is completely abolished in embryos lacking the vessel

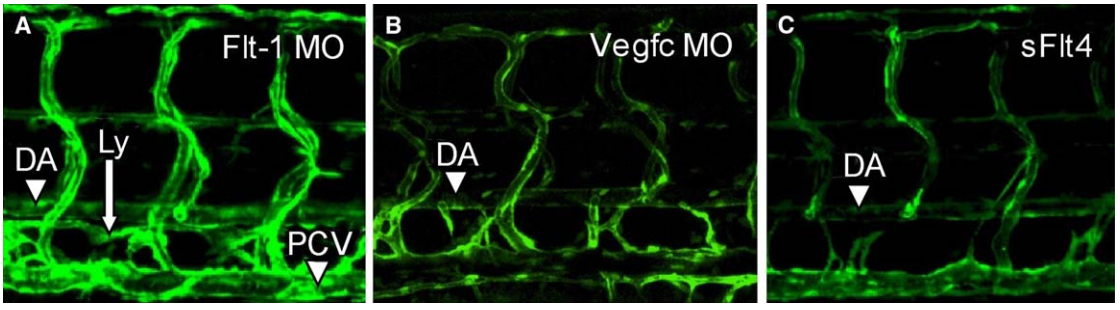

Figure 3. Development of Lymphatic Vessels Is Sensitive to Levels of Vegfc and VEGFR-3/Flt4 Signaling

(A–C) Embryos transgenic for fli:GFP were injected at the one-cell stage with a control morpholino directed against zebrafish VEGFR1/flt1 (A), a morpholino against zebrafish Vegfc (B), or capped mRNA of human sFlt4 (C). Embryos with intact circulation were selected for confocal microscopy analysis at 4.5 dpf. In uninjected controls (data not shown) and control-injected cases (A), the vessel just ventral to the DA was visible in all cases. In contrast, embryos that had been injected with the Vegfc morpholino or the dominant-negative human FLT4 receptor never developed this vessel.

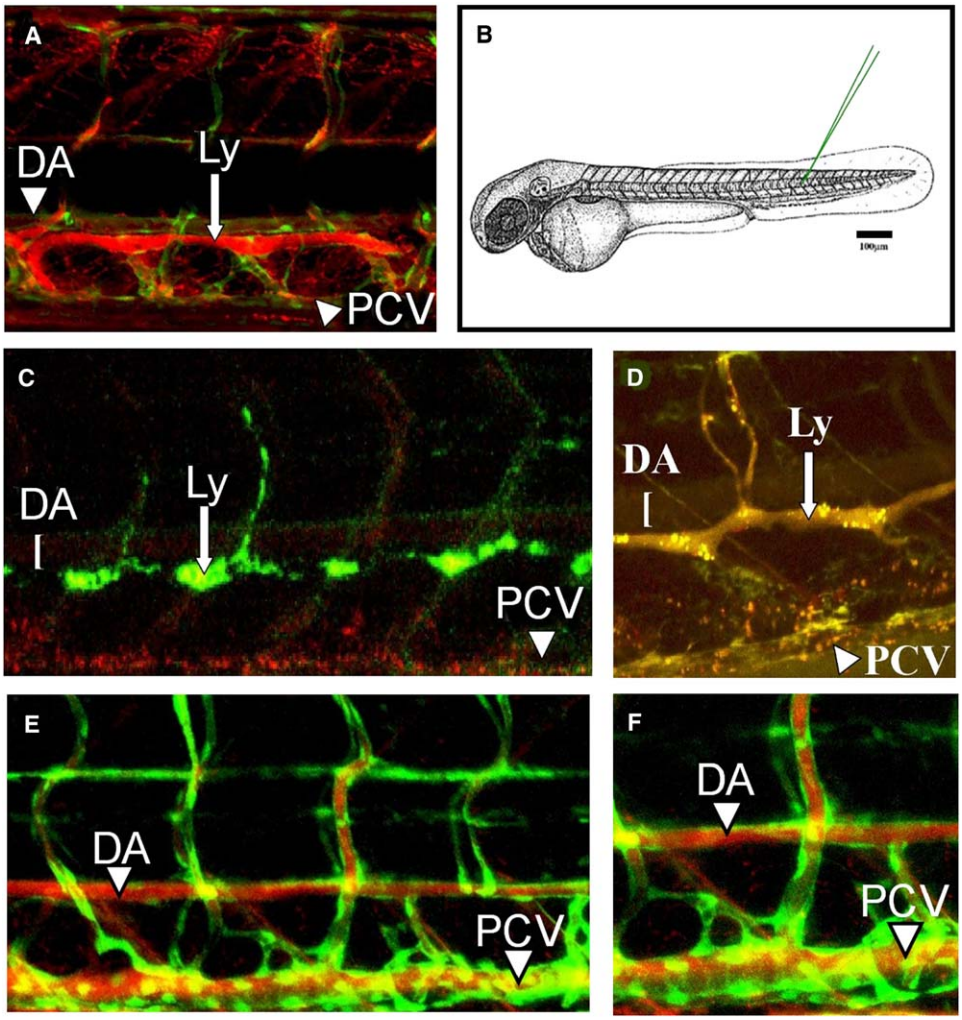

Figure 4. Interstitial Components Are Selectively Taken Up by the Lymphatic System, which Is Connected to the Common Cardinal Vein

(A) Fli:GFP transgenic embryos (5 dpf) were injected with rhodamine dextran into the common cardinal vein (CCV) in the region where the CCV branches into the anterior cardinal vein and connects to the pectoral fin vessel. This results in a preferential filling of the blood cell-free vessel adjacent to the DA, while the DA, the PCV, and all other blood vessels are not highlighted.

(B) Schematic representation of the site of subcutaneous injection in a day 7 and day 8 larva.

(C) Fluorescin dextran was subcutaneously injected into fli:GFP transgenic embryos. After roughly 3 hr, an uptake of the dye into the lymphatics was clearly visible. Brackets indicate the extent of the DA.

(D) A few hours later, part of the dye becomes apparent in the PCV. Please also note dorsal sprouts emanating from the primary lymphatic vessel in (C) and (D).

(E and F) Larvae that miss the lymphatic vessel do not accumulate injected dye. Embryos were injected at the zygote stage with mRNA encoding sFlt4 and sorted for lack of lymphatics while having normal circulation. Subcutanous injection of fluorescin dextran at 7 dpf as described above resulted in a failure to accumulate the dye at a specific location in the larvae. In (C)–(F), rhodamin dextran was used for angiographies to highlight the vascular system, and color intensities were calibrated either according to the levels of fluorescin dextran in the lymphatic vessel (C and D) or according to eGFP expression in endothelial cells (E and F).

next to the DA. We conclude that this vessel constitutes the primary lymphatic vessel in the zebrafish embryo.

The redistribution of the dye from the lymph system to venous vessels suggested that the lymph is eventually channeled into the blood via a connection to the venous system, which is similar to the situation in mammals. Here, the connection between the lymphatic system with the venous system is through anastomoses in the jugular area [13]. We injected rhodamine dextran into the venous blood stream of day 8 embryos at a position where the common cardinal vein branches into the anterior cardinal vein (equivalent to the vena jugularis in mammals) and the pectoral fin vessel (equivalent to the subclavian vein in mammals). In a number of cases (8 out of 12), this has enabled us to preferentially fill the lymphatic vessel without staining the main blood vessels at the same time (Figure 4A). We take this as a strong indication that the lymphatic system connects to the venous vessels in this area of the zebrafish larva and that the wiring of the lymphaticovenous system is evolutionary conserved among all vertebrates.

Conclusions

While lymphatic vessels had been shown to exist in other vertebrate classes [14, 15], and recently also have been demonstrated in *Xenopus tropicalis* [16], there has

been no conclusive evidence of a teleost lymph system (for review, see [17]) up to now. Vogel [18] has described a vessel system in adult trout (*Oncorhynchus mykiss*) with a low hematocrit; however, this vessel system is connected to arteries rather than veins, and there are no embryological or larval data available [19]. Our data demonstrate the existence of a lymphatic system in zebrafish and resolve this long-standing issue conclusively. Morphological, molecular, and functional data show that the initial formation of lymphatics in the zebrafish occurs during late embryogenesis and establishes a vessel that is connected to the venous system in an anatomical position equivalent to the mammalian situation. Further studies will have to show where the precursors of the initial cell clusters come from and how the initial lymphatic trunk vessel branches to form the patent adult lymph system.

Zebrafish are highly amenable to forward and reverse genetic screens [20], as well as compound testings [21], and have already contributed greatly to our understanding of vertebrate vasculogenesis and angiogenesis. Simultaneously studying blood vessels and lymphatics in this transparent model system holds immense potential for identifying genes and pathways that play a crucial role in development and function of the vascular system.

Experimental Procedures

Angiographies and confocal microscopy were performed as described in [22]. For functional studies, anesthetized larvae were subcutaneously injected with 1 nl of fluorescent dextran or rhodamine dextran (2.5 mg/ml) into the muscle mass of the posterior trunk of anesthetized larvae by using glass capillaries and a conventional microinjection setup.

Supplemental Data

Supplemental Data include one movie and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/12/1244/DC1/.

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