

Arteries provide essential guidance cues for lymphatic endothelial cells in the zebrafish trunk

Jeroen Bussmann^{1,*†}, Frank L. Bos^{1,2,*}, Akihiro Urasaki³, Koichi Kawakami^{3,4}, Henricus J. Duckers² and Stefan Schulte-Merker^{1,‡}

SUMMARY

The endothelial cells of the vertebrate lymphatic system assemble into complex networks, but local cues that guide the migration of this distinct set of cells are currently unknown. As a model for lymphatic patterning, we have studied the simple vascular network of the zebrafish trunk consisting of three types of lymphatic vessels that develop in close connection with the blood vasculature. We have generated transgenic lines that allow us to distinguish between arterial, venous and lymphatic endothelial cells (LECs) within a single zebrafish embryo. We found that LECs migrate exclusively along arteries in a manner that suggests that arterial endothelial cells serve as the LEC migratory substrate. In the absence of intersegmental arteries, LEC migration in the trunk is blocked. Our data therefore demonstrate a crucial role for arteries in LEC guidance.

KEY WORDS: Lymphatic system, Cell migration, Endothelium, Pattern formation, Zebrafish

INTRODUCTION

The vertebrate blood vessels are crucial for the exchange of gases, hormones and metabolites, whereas lymphatic vessels are important in the regulation of the immune system and fluid homeostasis. Both types of vessels assemble into complex vascular networks that display a remarkable degree of conservation in pattern formation (Weinstein, 1999), but how lymphatic endothelial cells (LECs) are guided and go on to form the lymphatic vascular system is still unclear. It has been noted that collecting, but not capillary, lymphatic vessels frequently align with blood vessels (Alitalo et al., 2005), but whether this indicates common guidance factors is not known.

The existence of a lymphatic system in zebrafish has recently been documented (Hogan et al., 2009; Kuchler et al., 2006; Yaniv et al., 2006). Here, we have studied the formation of the lymphatic network in the zebrafish trunk in more detail. The lymphatic network arises from cells that directly bud from the posterior cardinal vein (PCV) to the horizontal myoseptum between 1.5 and 2 dpf. Some of these cells reconnect to the primary arterial sprouts and remodel into veins (Isogai et al., 2001). The other cells do not connect, continue to migrate dorsally and constitute so-called parachordal lymphangioblasts (PLs) that align along the horizontal myoseptum (Hogan et al., 2009), before they start migrating dorsally or ventrally (then referred to as LECs) at 3 dpf. These

migratory cells give rise to three distinct types of lymphatic vessels at 4 dpf: the thoracic duct (TD), the intersegmental lymphatic vessels (ISLVs) tightly abutting the intersegmental blood vessels, and the dorsal longitudinal lymphatic vessel (DLLV) (Hogan et al., 2009).

Given the simplicity of this network and the amenability of the zebrafish embryo to *in vivo* imaging, this lymphatic network provides an ideal model in which to study lymphatic endothelial cell migration and patterning in a living embryo. Similar to the collecting lymphatic vessels in mammals (Alitalo et al., 2005), this network forms in close association with the blood vasculature. Using newly established transgenic lines that allow the direct visualization of arteries, veins and lymphatic vessels within a single embryo, we found a striking tendency of ISLVs to align with arterial intersegmental vessels (aISVs), but not venous intersegmental vessels (vISVs). By analyzing embryos with defective arterial patterning, we show that aISVs are required for LEC migration and provide crucial guidance cues for LECs in the zebrafish trunk.

MATERIALS AND METHODS

Zebrafish strains and morpholino injections

All zebrafish strains were maintained under standard husbandry conditions. The *kdr^{rh5088}* allele was identified in a forward genetic screen (Hogan et al., 2009). *Tg(fli1a:gfp)^{yl}* (Lawson and Weinstein, 2002) and a *kdr1* morpholino (Habeck et al., 2002) have been described previously.

Transgenesis

flt1 promoter/enhancer constructs were cloned in the MiniTo2 vector (Balcunas et al., 2006) and injected at 25 ng/μl with tol2 transposase mRNA (25 ng/μl) into eggs. Embryos were selected at 3 dpf for high expression and grown to adults, among which germline founders were identified.

The SAGFF27 line was identified in a large scale gene-trap screen (Asakawa et al., 2008) using the T2KSAGFF construct. The insertion in this line occurred in the first intron of *zgc:171516* (ZFIN) and is located on chromosome 24. Characterization of the homozygous phenotype will be described elsewhere (A.U. and K.K., unpublished). Heterozygous fish were viable and fertile, and did not display obvious defects in blood or lymphatic vascular patterning.

¹Hubrecht Institute-KNAW and University Medical Centre, and Centre for Biomedical Genetics, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands. ²Molecular Cardiology Laboratory, Experimental Cardiology, Thoraxcenter, Erasmus University Medical Centre Rotterdam, 3015 CE Rotterdam, the Netherlands. ³Division of Molecular and Developmental Biology, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan. ⁴Department of Genetics, the Graduate University for Advanced Studies (SOKENDAI), 1111 Yata, Mishima, Shizuoka, 411-8540, Japan.

*These authors contributed equally to this work

[†]Present address: Max Planck Institute for Molecular Medicine, Röntgenstrasse 20, 48149, Münster, Germany

[‡]Author for correspondence (s.schulte@hubrecht.eu)

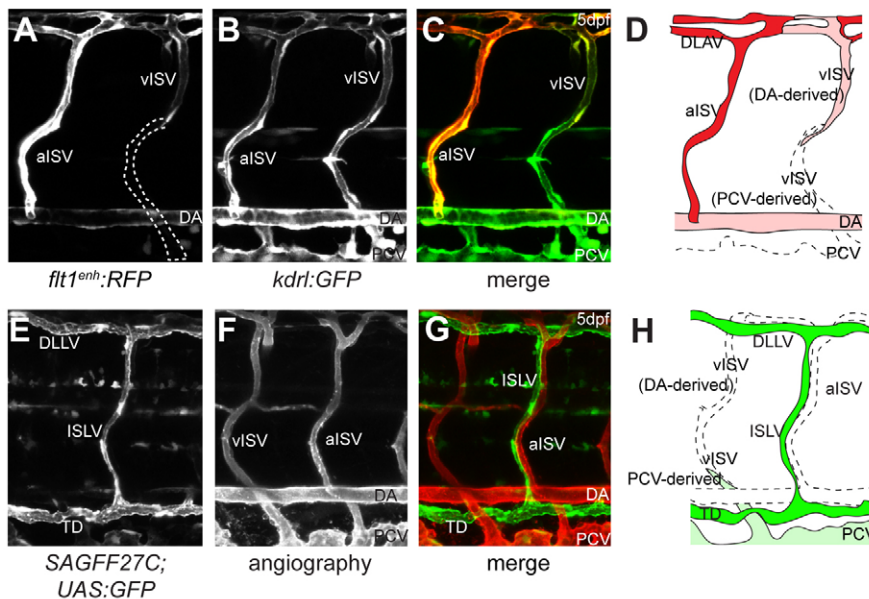


Fig. 1. Generation and visualization of *ftl1^{enh}:RFP* and *SAGFF27C;UAS:GFP* transgenic lines. (A–C) Arterial-restricted reporter expression in the *ftl1^{enh}:RFP* transgenic line at 5 dpf (A), compared with *kdrl:GFP*, which labels all blood endothelial cells (B) allows for discrimination between ISVs connected to the dorsal aorta or PCV. (C) Overlay of A,B. (D) Schematic representation of *ftl1^{enh}:RFP* expression. (E) Lymphatic reporter expression in the *SAGFF27C;UAS:GFP* transgenic line at 5 dpf, compared with an angiography (F) that labels perfused blood vessels (red in G). (G) Overlay of E,F. (H) Schematic representation of *SAGFF27C;UAS:GFP* expression. aISV, arterial ISV; DA, dorsal aorta; DLLV, dorsal longitudinal lymphatic vessel; ISLV, intersegmental lymphatic vessel; PCV, posterior cardinal vein; TD, thoracic duct; vISV, venous ISV.

Imaging

Microangiography was performed as described (Kuchler et al., 2006). Embryos were mounted in 0.5–1.0% low melting point agarose (Invitrogen, Carlsbad, CA, USA) in E3 buffer. Confocal image stacks were collected on SPE, SP2 or SP5 confocal microscopes with 10×, 20×, 40× and 63× objectives (Leica Microsystems, Wetzlar, Germany) and processed using ImageJ (<http://rsbweb.nih.gov/ij>).

RESULTS AND DISCUSSION

Transgenic labelling of arteries, veins and lymphatic vessels

To study lymphatic patterning in relation to the blood vascular network, we aimed to develop a transgenic line in which arteries, veins and lymphatic vessels could be distinguished in a single living embryo. We have previously identified a transgenic line (*ftl1^{BAC}:YFP*) in which arterial endothelial cells are specifically labelled (Hogan et al., 2009). Although *ftl1^{BAC}:YFP* is an early marker for arterial endothelial cells, reporter gene expression becomes upregulated in all blood endothelial cells after the onset of blood flow, making it less useful to visualize arteriovenous differentiation during the stages when lymphatic network formation occurs. In order to obtain a more general marker for zebrafish arteries, we used an in silico approach to identify potential regulatory elements necessary for early *ftl1* expression in arterial endothelial cells. This analysis combined conserved non-coding elements and enhancers within the proximal region of the *ftl1* promoter (for detailed descriptions, see Fig. S1 in the supplementary material). Two conserved elements were found to drive arterial expression and in combination with the *ftl1* proximal promoter, this 0.8 kb promoter-enhancer construct was used to generate the *ftl1^{enh}:RFP* transgenic line (Fig. 1A–D). Within the trunk vascular network, aISVs display high RFP expression (*ftl1^{enh}:RFP^{high}*) whereas vISVs display low RFP expression (*ftl1^{enh}:RFP^{low}*). In lymphatic vessels, RFP expression is undetectable (*ftl1^{enh}:RFP^{neg}*). The expression of *ftl1^{enh}:RFP* was a reliable readout of ISV identity (vISV or aISV), based on the direction of flow (see Fig. S2A–D in the supplementary material). For ISVs without observable blood flow, *ftl1^{enh}:RFP^{high}* ISVs were invariably connected to the dorsal aorta, whereas *ftl1^{enh}:RFP^{low}* ISVs were connected to the PCV (data not shown).

Another line we have previously generated, *stab1^{BAC}:YFP*, labels venous endothelial cells and lymphatic endothelial cells, including parachordal lymphangioblasts (PLs), that are derived from them (Hogan et al., 2009). In this study, by performing a large-scale gene-trap screen using the T2KSAGFF gene trap construct (Asakawa et al., 2008), we identified the *SAGFF27C* transgenic line that, when crossed to UAS:GFP fish, displayed reporter expression at much higher levels than *stab1^{BAC}:YFP*, facilitating high-resolution imaging. This line labels the lymphatic vasculature, i.e. the thoracic duct, DLLV and ISLVs (Fig. 1E–H).

Arteriovenous and lymphatic patterning in the zebrafish trunk

Interestingly, when combining both transgenic lines we observed that in *ftl1^{enh}:RFP; SAGFF27C;UAS:GFP* transgenic embryos, *SAGFF27C;UAS:GFP⁺* ISLVs appeared to be almost exclusively located along *ftl1^{enh}:RFP^{high}* aISVs and only very rarely along *ftl1^{enh}:RFP^{low}* vISVs. To confirm this observation, we quantified lymphatic and blood vascular patterning. To this end, we performed a detailed analysis of lymphatic and blood vascular patterning at 5 dpf, based on the expression of *ftl1^{enh}:RFP* and *SAGFF27C;UAS:GFP* (Fig. 2A). For a total of 30 embryos, the arterial or venous identity of ISVs and the presence of ISLVs in all segments was monitored (Fig. 2B).

We first analyzed the arteriovenous patterning of the trunk vascular network and confirmed the previous observation obtained from six embryos (Isogai et al., 2001) that the first segmental positions are almost invariable and bilaterally symmetrical. We found a vISV at segmental positions 1, 3 and 4, and an aISV at position 2. Between position 5 and 25, the arterial or venous identity becomes essentially random, with 48% (605/1260) aISVs and 52% (653/1260) vISVs (Fig. 2B,C). It has previously been reported that ISVs in neighbouring segments and at the left and right side of the embryo have a tendency to have different identity (Isogai et al., 2001). Of these relationships, we could only find support for a correlation between ISV identities in neighbouring segments at the same side of the embryo. This correlation is strong, but not absolute (which would otherwise result in invariable aISV–vISV–aISV–vISV alternations): in 78% (928/1256) of ISVs between position 5 and 25, the ISV in the neighbouring segment is of

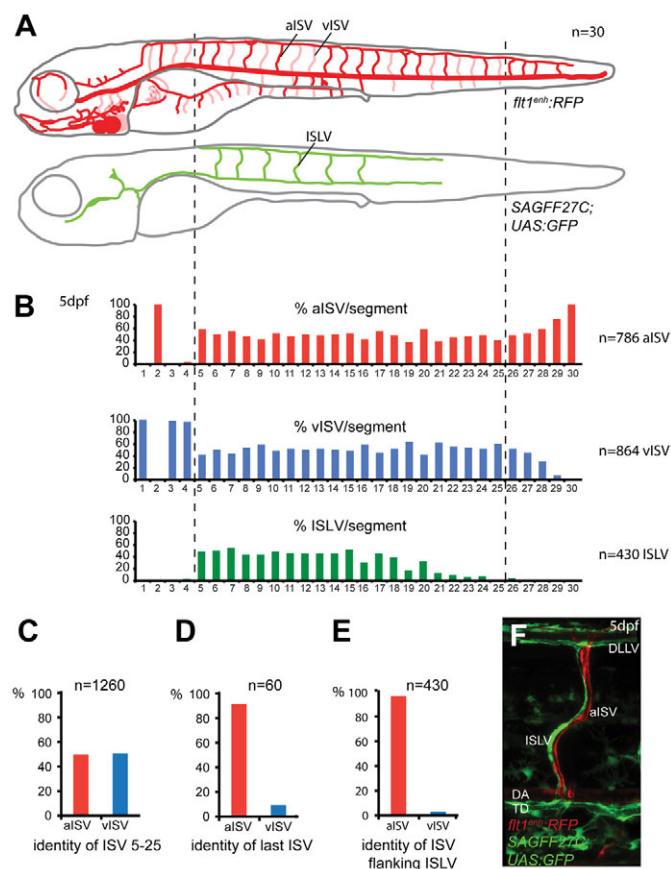


Fig. 2 Arteriovenous and lymphatic patterning in the zebrafish trunk. (A) Schematic representation of arteries, veins and lymphatic vessels at 5 dpf, based on expression of *flt1^{enh}:RFP* and *SAGFF27C;UAS:GFP* over 25–30 segments in 30 embryos (segment numbers vary in zebrafish embryos, with an average of 27.2 segments or 54.4 segmental positions per embryo). (B) Quantification of vessel type distribution per segment, indicating aISV in red bars, vISVs in blue bars and ISLVs in green bars. (C) Quantification of aISV/vISV identity in segments 5–25, $n=1260$ segmental positions scored. (D) Quantification of aISV/vISV identity of the caudal-most ISV/side, $n=60$ indicating a strong preference for aISV over vISV in the last ISV position. (E) Quantification of arterial and venous ISVs abutting an ISLV, $n=430$ intersegmental vessel pairs scored. (F) Overlaid confocal images of *flt1^{enh}:RFP⁺* aISV and *SAGFF27C;UAS:GFP⁺* ISLV. aISV, arterial ISV; DA, dorsal aorta; DLLV, dorsal longitudinal lymphatic vessel; ISLV, intersegmental lymphatic vessel; TD, thoracic duct; vISV, venous ISV.

different identity. The other postulated relationship between vessel identities within the same somite pair we found to be uncorrelated [49% different identity (310/628)]. We additionally found a significantly higher likelihood for arterial identity in the caudal-most ISVs: 92% (55/60) of these are arterial (Fig. 2D). As the number of segmental positions varies slightly (26–29) between embryos, this leads to a statistically significant enrichment of aISVs at, and posterior to, position 26 (Fig. 2B, top panel).

Second, we analyzed the patterning of the lymphatic network. Interestingly, lymphatic ISVs were only found from segment 5, whereas the first four (invariably patterned) ISVs were never flanked by an ISLV. Posterior to this position, on average one ISLV (out of a potential two) was identified per somite pair within a segment. The percentage of ISLVs dropped from segmental

position 18 (Fig. 2B), indicating a rostral-to-caudal succession of lymphatic migration, similar to that observed for primary angiogenic sprouting. Consistently, at 7 dpf, ISLVs were observed at more caudal positions (data not shown). Even though PLs have a choice of either vISVs and aISVs at the time when they migrate dorsally, 97% (418/430) of ISLVs were found neighbouring aISVs, suggesting an important role for arteries in regulating lymphatic patterning (Fig. 2E,F).

Intersegmental arteries are required for LEC migration

The remarkable alignment of ISLVs with aISVs suggests that aISVs act as guides for lymphatic patterning. Alternatively, lymphatic precursors could initially show no preference for arteries over veins, but become attracted to aISVs later. To distinguish between these two possibilities, we performed time-lapse confocal imaging in the *flt1^{enh}:RFP; SAGFF27C;UAS:GFP* transgenic line. We found that PLs migrate away from the horizontal myoseptum (and are now referred to as LECs) almost exclusively along aISVs, both ventrally to form the thoracic duct, as well as dorsally to form the ISLV and DLLV (Fig. 3A; see Movie 1 in the supplementary material). At the stages of development we analyzed, no vascular smooth muscle cells or pericytes have been identified in the intersegmental network of the zebrafish. In addition, we show that migrating LECs use aISVs as a direct substrate for migration: PLs (and LECs) extend protrusions along the external surface of aISVs (Fig. 3B,C; see Movies 2, 3 in the supplementary material) before they migrate along the surface to extend ventrally in the direction of the DA and dorsally in the direction of the DLAV. These findings strongly suggest that the endothelial cells from arterial ISVs are regulators of LEC migration in the zebrafish.

These observations do not rule out the possibility that arteries are simply a preferred substrate for lymphatic precursor migration, and that in the absence of arteries, lymphatic endothelial cells might be able to migrate completely independently or on vISVs. To investigate this possibility, we made use of *plcy¹²⁶⁴⁸⁰* and *kdr^{ihu5088}* mutants that displayed reduced or no primary (arterial) angiogenic sprouting, but retained normal secondary (venous) angiogenic sprouting and PL formation. In *plcy¹²⁶⁴⁸⁰* mutants, we did not observe ISLV or TD formation (data not shown). However, as *plcy¹²⁶⁴⁸⁰* mutants do not establish trunk circulation and display signs of necrosis at the time of our analysis (3–5 dpf), we performed the analysis in zebrafish that are mutant for the vascular endothelial growth factor receptor *kdr-like* (*kdr^{ihu5088}*). These mutants retain circulation in the DA and PCV, and form LECs and a TD. Mutants and morphants have some residual primary angiogenic sprouts. These sprouts were usually found only ventral to the horizontal myoseptum, therefore no DLAV was established. Venous sprouts were occasionally found to connect to these small vessels, establishing a short functional circulatory loop. In *kdr^{ihu5088}* mutants, PLs were found to migrate away from the horizontal myoseptum, but only ventrally along the residual aISVs, but not along vISVs. This led to the establishment of a thoracic duct at the ventral side, but not to the formation of a DLLV at the dorsal side of the embryo (Fig. 4A–F).

This result shows a requirement for aISVs in lymphatic precursor migration. Occasionally, aISVs were formed that extended to the dorsal side of the embryo. When this occurred, lymphatic precursors were also able to migrate dorsally (see Fig. S3 in the supplementary material), thereby demonstrating that arteries are crucial for proper guidance of LEC migration, and that *kdr-like* does not play a role in the process.

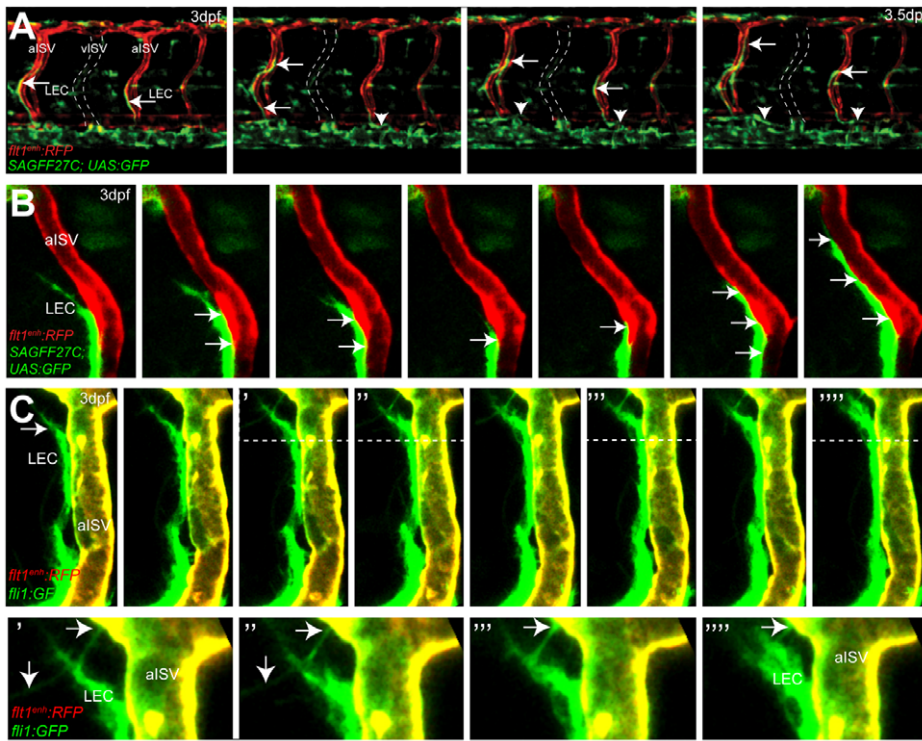


Fig. 3. Migration of lymphatic precursors along intersegmental arteries. (A) Still images of *flt1^{enh}:RFP;SAGFF27C;UAS:GFP* triple transgenic embryos reveal migration of *SAGFF27C;UAS:GFP⁺* LECs (arrows) along *flt1^{enh}:RFP⁺* arteries and establishing the TD (arrowheads) in the trunk. The position of a *viSV* is indicated by broken lines. (B) Still images of migrating *SAGFF27C;UAS:GFP⁺* LECs suggest direct contact (arrows) between the *aLSV* and the LEC. (C) Still images of migrating *fli1a:GFP⁺* LECs and filopodia formation (indicated by arrows) at the leading edge along the surface of a *flt1^{enh}:RFP⁺*; *fli1a:GFP⁺* artery at 3 dpf. Areas above the broken lines are shown at higher magnification in the bottom row.

Our data provide the first *in vivo* demonstration of lymphatic endothelial cells to be dependent on arterial ECs for pathfinding. Time-lapse analysis strongly suggests a physical interaction of these types of endothelial cells. This interaction does not depend on the *kdr-like* VEGF receptor, as in those few cases where arterial

ISV were observed in mutants (see Fig. S3 in the supplementary material), PLs did migrate along arteries. This would predict that deleting arteries by other means would also interfere with this process. Indeed, suppressing the Notch pathway by DAPT treatment of embryos, which results in a shift from arterial to

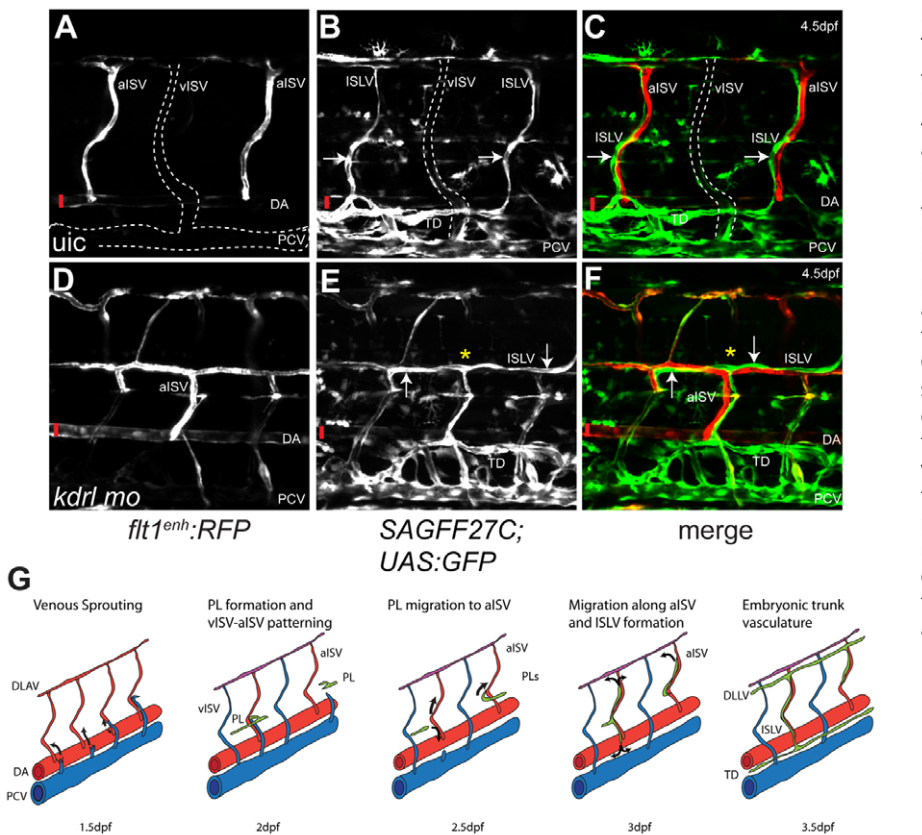


Fig. 4. Intersegmental arteries are required for LEC migration. (A-C) *flt1^{enh}:RFP* (A), *SAGFF27C;UAS:GFP* (B) and merged (C) unilateral confocal images of control embryos at 4.5 dpf showing lymphatic patterning along *aLSVs* in normal blood vasculature. Arrows indicate *ISLVs*. (D-F) *flt1^{enh}:RFP* (D), *SAGFF27C;UAS:GFP* (E) and merged (F) unilateral confocal images of *kdr* morpholino injected embryos at 4.5 dpf showing LEC migration along arteries that cross-connect abnormally at the horizontal myoseptum, and that do not extend into the dorsal half (indicated by yellow asterisk) of the respective somite. Arrows indicate LECs along the surface of an abnormal *aLSV*. Vertical red bars indicate the DA. (G) Model of lymphatic patterning within the zebrafish trunk. Secondary sprouts from the PCV that do not connect to primary intersegmental vessels migrate to the horizontal myoseptum region where they constitute a pool of PLs. PLs will migrate ventrally or dorsally to form the thoracic duct and *ISLVs*, respectively, and use arteries as their substrate.

venous fates (Lawson et al., 2001) effectively blocks migration of PLs dorsally (Geudens et al., 2010), consistent with our model and the situation in *kdr-like* mutants and morphants.

The possible contact-dependent migration of LECs on arterial ECs provides a new paradigm for LEC pathfinding. Several possibilities could be explored in order to obtain more insight into the mechanism regulating LEC migration. The secretion of guidance factors for LECs by arterial ECs, such as VEGF-C, is one possibility, a shared preference of both PLs/LECs and aISVs for the same molecular environment or the same guidance cues (such as semaphorins, integrins or other extracellular matrix molecules) is another. Furthermore, arterial ECs might deposit ECM components that provide crucial substrate components for LECs. Further studies will have to unravel which molecules are central to the interaction between these two types of endothelial cells.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.048207/-/DC1>

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