**lyve1 expression reveals novel lymphatic vessels and new mechanisms for lymphatic vessel development in zebrafish**

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**SUMMARY**
We have generated novel transgenic lines that brightly mark the lymphatic system of zebrafish using the *lyve1* promoter. Facilitated by these new transgenic lines, we generated a map of zebrafish lymphatic development up to 15 days post-fertilisation and discovered three previously uncharacterised lymphatic vessel networks: the facial lymphatics, the lateral lymphatics and the intestinal lymphatics. We show that a facial lymphatic vessel, termed the lateral facial lymphatic, develops through a novel developmental mechanism, which initially involves vessel growth through a single vascular sprout followed by the recruitment of lymphangioblasts to the vascular tip. Unlike the lymphangioblasts that form the thoracic duct, the lymphangioblasts that contribute to the lateral facial lymphatic vessel originate from a number of different blood vessels. Our work highlights the additional complexity of lymphatic vessel development in the zebrafish that may increase its versatility as a model of lymphangiogenesis.

**KEY WORDS:** Zebrafish, Lyve1, Lymphatic, Lymphangioblast

**INTRODUCTION**

Lymphatic vessels are important for the regulation of tissue fluid homeostasis, immune surveillance and dietary fat absorption (Tammela and Alitalo, 2010). Aberrant lymphatic growth is associated with pathological conditions, including cancer metastasis and chronic inflammation, while a malfunctioning lymphatic system results in lymphoedema (Alitalo, 2011). Fuelled by the identification of lymphatic specific markers and growth factors, extensive lymphatic research is being conducted with the hope of identifying therapeutic targets for these lymphatic abnormalities (Normen et al., 2011).

Florence Sabin proposed the centrifugal growth of lymphatic vessels from the lymph sac, which originates from the venous vasculature system (Sabin, 1902). The venous origin of the lymph sacs has since been validated in mice and zebrafish (Kuchler et al., 2006; Yaniv et al., 2006; Srinivasan et al., 2007). Although subsequent lymphatic vessel formation is proposed to occur through both sprouting from the lymph sacs and from the direct delamination of lymphatic endothelial cells from the cardinal vein (Francois et al., 2012), little is known about how the lymph sacs remodel to form a complete lymphatic network. The optically transparent zebrafish embryo provides an ideal platform to investigate this.

The formation of the thoracic duct, the major trunk lymphatic vessel in the zebrafish embryo, has been established as a model of lymphangiogenesis (Kuchler et al., 2006; Yaniv et al., 2006; Hogan et al., 2009b; Coffindaffer-Wilson et al., 2011). However, the remaining lymphatic network in the zebrafish embryo is poorly characterised and little is known about the development of lymphatic vessels outside the trunk.

In this study, we generated transgenic lines using the zebrafish lymphatic vessel endothelial hyaluronan receptor 1 (*lyve1*) promoter. LYVE1 is one of the most specific and widely used mammalian lymphatic endothelial markers and its expression in a subpopulation of venous endothelial cells located in the cardinal vein provides the first indication of lymphatic endothelial commitment (Oliver, 2004; Tammela and Alitalo, 2010). Recently, we identified the zebrafish orthologue of *LYVE1*; zebrafish Lyve1 displays 34% amino acid similarity to human LYVE1 and is also expressed in the lymphatic vessels (Flores et al., 2010). These novel *lyve1* transgenics enabled us to identify previously uncharacterised lymphatic vessels in the head, intestine and the superficial area of the trunk. The identification of these new lymphatic networks increases the versatility of the zebrafish as a tool with which to investigate lymphatic development. Using live-imaging approaches, we show that a facial lymphatic vessel, termed the lateral facial lymphatic (LFL), develops in a manner distinct to that previously described for the thoracic duct (TD). The LFL initially develops by the migration of a vascular sprout at the tip of the developing vessel; lymphangioblasts are recruited to this vascular tip to form a lymphatic vessel. Unlike the TD, we have shown that the lymphangioblasts that contribute to the LFL do not derive from a single source, showing that lymphatic vessel formation in zebrafish is more complex than previously thought.

**MATERIALS AND METHODS**

**Zebrafish**

All zebrafish strains were maintained under standard husbandry conditions. The published transgenic lines used were *Tg(kdrl:egfp)146* (Jin et al., 2005), *Tg(kdrl:rfp)122* (Huang et al., 2005), *Tg(fgata1:DsRed)hu462* (Traver et al., 2003), *Tg(flt1:Yfp)hu24a* (Bussmann et al., 2010), *Tg(kdrl:nlsmCherry)hu24a* (Lam et al., 2010) and *Tg(sagf27c:usagfp)hu24a* (Bussmann et al., 2010). Lines generated in this study are *Tg(lyve1:egfp)42* and *Tg(lyve1:DsRed2)42*.

**Transgenesis**

A promoter insert, which contained 5.2 kb upstream of the start codon and the first exon of *lyve1*, was cloned into the pT2KXGΔamin and pT2KXIRΔamin vectors (Kawakami et al., 2004). Stable transgenic lines were generated as described previously (Hall et al., 2007).
Morpholino injections and whole-mount in situ hybridisation
Morpholino injections and whole-mount in situ hybridisations were performed as described previously (Nasevicius and Ekker, 2000; Flores et al., 2010). The cce1 and flt4 morpholinos have been described previously (Hogan et al., 2009b; Hogan et al., 2009a).

Confocal live imaging
Microangiography was performed as described previously (Kuchler et al., 2006). Dye-uptake assays were performed by subcutaneous injection of 500 kDa fluorescein dextran (Inviogen, Carlsbad, CA, USA) resuspended in distilled water, to the dorsal area of the facial lymphatic. Embryos were imaged as described (Hall et al., 2009) with a Nikon D-Eclipse C1 confocal microscope or with an Olympus FV1000 confocal microscope for time-lapse microscopy. Still images were taken using z stacks 5 μm apart. For time-lapse microscopy, z stacks 5 μm apart were taken at 10-minute intervals. Images in this paper are maximum projections of z series stacks. Images were processed using ImageJ (NIH, Bethesda, MD, USA), Photoshop CS5 (Adobe, San Jose, CA, USA) and Velocity 5.4 image analysis software (Improvision/PerkinElmer Life and Analytical Sciences, Shelton, CT, USA). The blood vascular nomenclature used in this paper is as described previously (Isogai et al., 2001).

Image analysis and statistics
Lymphatic vessel length was measured using Velocity 5.4 image analysis software. The middle of the developing lateral facial lymphatic was traced from its origin at the common cardinal vein to the vascular tip. The total length of this trace was used to determine the total length. For the ventral aorta lymphangiblast (VA-L), measurements were taken from a ventral view at 2 dpf and only the length of the VA-L, which was perpendicular to the LFL, was considered. Intestinal lymphatic branches were counted as any lyve1-expressing vessels branching from the left intestinal lymphatics anterior to the boundary of the 6th to 7th somite. Statistical analysis was performed using Prism 5.0 software (GraphPad Software). Significance was determined by Mann-Whitney tests.

RESULTS
lyve1 expression marks zebrafish lymphatic vessels
We generated novel transgenic lines using the promoter for lyve1, a gene previously described as being expressed in zebrafish lymphatics (Flores et al., 2010), to drive the expression of either enhanced green fluorescent protein (EGFP) or Discosoma red fluorescent protein (DsRed2) in the developing lymphatic vessels. The spatiotemporal expression of lyve1:EGFP is generally consistent with the endogenous expression of lyve1. However, lyve1:EGFP expression has a slightly wider expression pattern than endogenous lyve1, as gfp mRNA is observed in the primordial hindbrain channel at 1 dpf and in secondary sprouts at 2 dpf (see supplementary material Fig. S1A-L). The wider expression of lyve1:EGFP could be due to the stronger gfp signal, which allows detection of gfp in tissues where lyve1 is normally difficult to visualize.

To confirm the lymphatic expression in our lyve1 transgenic lines, we crossed the TG(lyve1:DsRed2)zm101 line with a known marker of zebrafish lymphatics, SAGFF27C;UAS:GFP (Bussmann et al., 2010), and observed co-expression in the trunk lymphatic network (supplementary material Fig. SIM-M'). Fluorescent protein expression in the TG(lyve1:egfp)zm12 (referred to as lyve1:EGFP) and TG(lyve1:DsRed2)zm101 (referred to as lyve1:DsRed2) is completely overlapping and they both highlight the previously described developmental steps in the formation of the trunk lymphatic vessel network (Geudens et al., 2010) (supplementary material Fig. SIN-R'). In addition to expression in the lymphatic vessels, we also observed lyve1 expression in the major venous vasculature such as the common cardinal vein (CCV), posterior cardinal vein (PCV) and the caudal vein (CV). In addition, veins developing after blood circulation also display lyve1 expression, but in general this is not maintained and expression is lost following vessel development; e.g. the intersegmental veins only display lyve1:EGFP expression until 6 dpf (supplementary material Fig. S1O-R'). An exception to this is the primary head sinus (PHS), which maintains lyve1:EGFP expression until at least 15 dpf (supplementary material Fig. S2).

Taking advantage of the lack of lymphatic expression in the kinase insert domain receptor like (kdrl) transgenic line (Hogan et al., 2009b), compound lyve1:DsRed2;kdrl:EGFP and lyve1:EGFP;kdrl:RFP transgenic lines were generated to differentiate the lymphatic and blood vasculature. As lyve1-positive venous endothelium is marked yellow owing to the co-expression of red and green fluorescent proteins, the bona fide lymphatic vessels in these compound transgenics are specifically marked by red and green fluorescent protein respectively. Montage diagrams of the entire lyve1:DsRed2;kdrl:EGFP and lyve1:EGFP;kdrl:RFP transgens at various time points were generated (supplementary material Fig. S2) and were the basis of subsequent studies.

Zebrafish contain a facial lymphatic network that sprouts from the common cardinal vein
Analysis of both the lyve1:EGFP;kdrl:RFP and lyve1:DsRed2;kdrl:EGFP transgenics identified a previously uncharacterised lymphatic network that extends through the jaw, otolith and branchial arch region of the zebrafish head in a bilateral manner (Fig. 1A-E'). We have termed this group of lymphatic vessels the facial lymphatics (FL), which include the lateral facial lymphatic (LFL), the medial facial lymphatic (MFL), the otolithic lymphatic vessel (OLV), and the first, second, third and fourth lymphatic brachial arches (LAA1-4). Although a facial lymphatic vessel has previously been described using lymphangiography in 5-week-old zebrafish (Yaniv et al., 2006), we are not sure whether this previously described vessel is connected to, or derived from, the FL network that we have identified. The FL network originates from the CCV from a large vascular sprout visible at around 1.5-2 days post fertilisation (dpf) (n=62/62 embryos) (Fig. 1F'). This vascular sprout was termed the facial lymphatic sprout (FLS) and it extends along the primary head sinus (PHS) (Fig. 1A,A'). The FLS develops into the LFL that diverts towards the mandibular arch (AA1) and tracks around the eye towards the jaw (Fig. 1A-E'). By 3 dpf, the OLV sprouts from the LFL and develops parallel to the posterior cerebral vein over the otolith, but does not follow any blood vessels. By 4 dpf, the MFL forms from the LFL and develops ventromedially towards the ventral aorta (VA) (Fig. 1C-E'). Additionally, LAA1-4 sprout from the LFL and develop along the first, second, third and fourth brachial arches, respectively (supplementary material Fig. S3). By 5 dpf, the FL network connects to the thoracic duct (TD) through the jugular lymphatic vessel (JLV), which sprouts from the LFL and migrates along the lateral dorsal aorta (LDA) and joins to the anterior end of the TD near its connection to the CCV (Yaniv et al., 2006) (Fig. 1G,G'). The connection between the CCV and the LFL becomes thinner at around 4 dpf and eventually disappears at around 5 dpf leaving a remnant lyve1-expressing structure attached to the CCV (Fig. 1C-D'). Interestingly, the OLV branches out towards this structure at around 6 dpf and re-establishes a connection by 15 dpf (observed in 12/16 embryos) (Fig. 1H,I).

We hypothesise that these blind-ended facial vessels are bona fide lymphatic vessels. Supporting this, high molecular weight fluorescein dextran injected dorsal to the FL network was taken up
by the FL (supplementary material Fig. S4A-A’). As no
erthrocytes were observed in the FL of the
lyve1:EGFP;gata1:DsRed transgenic, we concluded that the FL
did not have blood flow (Long et al., 1997) (supplementary
material Fig. S4B-C). Thus the facial lymphatics have functional
characteristics that are consistent with them being a network of
lymphatic vessels.

Development of the intestinal lymphatics
A supraintestinal lymphatic vessel (SIL) has been previously
identified in the zebrafish (Coffindaffer-Wilson et al., 2011).
Further investigation of the SIL using the
lyve1:DsRed2;kdr:EGFP lymphatic vessel revealed that the SIL
is actually two paired vessels that we termed the right and left
supraintestinal lymphatic vessels (R-SIL and L-SIL). Both SILs
are located either side of the PCV in the posterior region of the
intestinal (see Movie 1 in the supplementary material). We also
observed a previously uncharacterised lymphatic vessel network
that extends over the entire intestine. The first indication of this
network is at 4 dpf, when we observe lyve1-positive vessels that
run alongside the anterior mesenteric artery (AMA),
supraintestinal artery (SIA) and the pancreatic anlage (Fig.
2A,A’). By 5 dpf, these vessels extend to follow the SIA and the
right subintestinal vein (R-SIV); the branch following the SIA
we termed the upper right intestinal lymphatic (UR-IL) and the
branch following the R-SIV we termed the lower right intestinal
lymphatic (LR-IL) (Fig. 2B,B’,D). Together, we term these
vessels the right intestinal lymphatics (R-IL). In addition to the
R-IL we also observe two lymphatic vessels on the left side of
the gut; one vessel extends along the left subintestinal vein (L-
SIV), which we termed the lower left intestinal lymphatic (LL-
IL), whereas the other vessel runs parallel to the UR-IL but does
not appear to follow any blood vessels (Fig. 2B-D). Although we
believe that both the R-IL and the left intestinal lymphatics (L-
IL), which include the LL-IL and the UL-IL, derive from the
same initial lyve1-positive vessels, the exact way in which they
connect to each other has been difficult to determine owing to
their location. Interestingly, several lymphatic vessels that
connect the TD and the left and right SIL were observed at 5 dpf
(Fig. 2E, E’; supplementary material Movie 1). At 6 dpf, the LR-
IL appears to bifurcate and form two separate vessels that both
run in close proximity to one another along the R-SIV. As it is
difficult to distinguish between these two branches, and as they
both follow the R-SIV, we have termed both of these vessels the
LR-IL (Fig. 2F,F’). Both the R-IL and the L-IL extend caudally
at 7 dpf (Fig. 2G-H’). However, the L-IL do not extend as far as
the R-IL; in the case of the LL-IL, this is possibly due to the
degeneration of the caudal region of the L-SIV (Isogai et al.,
1997).
Fig. 2. The intestinal lymphatics span the entire intestine and its development is closely associated with the intestinal blood vasculature. All the images used in this figure are generated from the lyve1:DsRed2;kdr:EGFP transgenic. (A,A') Lateral image of the right-hand side of the embryo showing the initial lyve1-positive vessel (indicated by asterisks) forming along the AMA, SIA and the PA at 4 dpf (A), with schematic diagram of arteries (red), veins (blue) and lymphatics (green) (A'). (B,B') Dorso-lateral image of the right side of the embryo showing the UR-IL and the LR-IL developing along the SIA and the R-SIV, respectively (B), with schematic diagram (B'). (C,C') Lateral image of the left side of the embryo showing the UL-IL and the LL-IL that develops along the L-SIV (C), with schematic diagram (C'). Asterisks indicate the connection between the L-IL and the R-IL. The posterior regions of the SIA and the UR-IL are also visible. (D) Dorsal view of the intestinal vessels at 5 dpf. Dotted green lines represent proposed vessel connections between the L-IL and the R-IL. (E,E') Lateral image showing the connections (indicated by asterisks) between the SILs and the TD at 5 dpf. Blue and black asterisks represent connections between the R-SIL and L-SIL with the TD, respectively. (F,F') Ventral image showing a pair of LR-IL that forms in close proximity along the R-SIV at 6 dpf (F), with schematic diagram (F'). (G-H') Lateral images of the right (G), and the left (H) side of the embryo showing the intestinal vessels at 7 dpf, with schematic diagrams (G', H'). The L-IL branch is labelled with an asterisk in H and H'. (I,I') Lateral image of the left side of the embryo showing the L-IL branches (indicated by asterisks) and branches extending from the R-IL to the left side of the intestine (indicated by pink arrows) at 15 dpf (I), with schematic diagram (I'). (J) Quantification of the number of L-IL branches at 7 dpf (average 1.3, n=22) and 15 dpf (average 4.4, n=10). Error bar represents 95% confidence interval. ***P<0.001. (K,K') Lateral image of the right side of the embryo showing the intestinal vessels at 15 dpf (K), with schematic diagram (K'). Lymphatic vessels whose positions are variable between individual embryos are in pink. Connection between the L-IL and the R-IL is indicated with an asterisk. (L,L') Lateral image showing the SIL branches (indicated by asterisks) at 15 dpf (L), with schematic diagram (L'). Only the lyve1:DsRed2 expression was shown for clarity. B,E,H,I,K,L are montage images. B,E,G,H,I used two z series stacks. K used six z series stacks. L used four z series stacks. AMA, anterior mesenteric artery; CCV, common cardinal vein; DA, dorsal aorta; DLLV, dorsal longitudinal lymphatic vessel; ISLV, intersegmental lymphatic vessel; L-IL, left intestinal lymphatics; LL-IL, lower-left intestinal lymphatic; LR-IL, lower-right intestinal lymphatic; L-SIL, left supraintestinal lymphatic vessel; L-SIV, left subintestinal vein; PA, pancreatic anlage; PCV, posterior cardinal vein; R-IL, right intestinal lymphatics; R-SIV, right subintestinal vein; SIA, supraintestinal artery; TD, thoracic duct; UL-IL, upper-left intestinal lymphatic; UR-IL, upper-right intestinal lymphatic. Scale bars: 100 μm in A-H; 200 μm in I-L.

2001) (Fig. 2F-H'). At 15 dpf, several lymphatic branches develop along both the R-IL and L-IL (Fig. 2I,I'). These branches were visible at 7 dpf (Fig. 2H,H'), but the number of the L-IL branches was significantly higher at 15 dpf (Fig. 2J). We hypothesize that these branches serve as a connection between and within the L-IL and the R-IL. Consistent with this,
branches extending from the R-IL to the left side of the intestine were visible at 15 dpf (9/10 embryos) (Fig. 2I). Furthermore, connections between the R-IL and L-IL were observed (4/10 embryos) (Fig. 2K). Unlike the R and L-IL that develop in a consistent pattern up to 15 dpf, the location of these branches varies between individual embryos (Fig. 2K). Similar to the R-IL and L-IL, the SIL also form branches that extend ventrally toward the intestine at 15 dpf (Fig. 2L). Although we hypothesise that the SIL forms a connection with the R-IL and the L-IL at later stages, we rarely observed any connection by 15 dpf (not connected in 9/10 embryos).

Development of the lateral lymphatics

Analysis of the lyve1:DsRed2;kdr:EGFP transgenic at 5-15 dpf revealed a lateral lymphatic (LL) network, which includes the parachordal lymphatic vessel (PLV) and the intercostal lymphatic vessels (ICLVs). We believe the PLV to be analogous to the previously described lateral lymphatic vessel in teleosts (Kampmeier, 1969; Yaniv et al., 2006; Deguchi et al., 2012). The PLV sprouts from the ISLVs and migrates along the parachordal vessel (PAV) (Fig. 3A-C). The sprouts from neighbouring ISLVs fuse to form vessel fragments; a mechanism of vessel development similar to that observed during TD formation (Fig. 3C) (Yaniv et al., 2006). The PLV develop superficial to the intersegmental vessels (Fig. 3D) and, by 15 dpf, extend along the PAV both anteriorly and posteriorly (Fig. 3E). We propose that these vessel fragments will fuse to become a single PLV later in development. In addition, the intercostal lymphatic vessels (ICLVs) sprout ventrally from the PLV and develop along the intercostal vessel (Fig. 3D,E).

Two distinct lymphangioblast populations contribute to the development of lateral facial lymphatic

We noted that the FL appeared to develop from one lymphatic sprout originating from the CCV (Fig. 1A-F). This unidirectional lymphatic vessel growth contrasted with the previously described development of the TD, which forms through bidirectional migration and fusion of lymphatic vessel fragments (Yaniv et al., 2006). To further investigate the mechanism of FL development, we analysed the initial FLS development from the CCV at 1.5 to 2.5 dpf. At around 1.5 dpf, the FLS was first observed sprouting from the CCV (Fig. 4A). At the same time-point, we identified a number of lyve1-expressing structures that were distinct from the venous endothelium that could be potential lymphangioblasts (Fig. 4A). At the same time-point, we identified a number of lyve1-expressing structures that were distinct from the venous endothelium that could be potential lymphangioblasts (Fig. 4A). These included lymphangioblasts that appeared to originate from the PHS (PHS-L) and another lymphangioblast that appeared to originate from near the ventral aorta, termed the ventral aorta lymphangioblast (VA-L). All lymphangioblasts, including those described in the trunk, co-express kdr, which is indicative of their vascular origin (Fig. 4A-D; supplementary material Fig. S2A). Further investigation revealed that although the VA-L co-expressed kdr and lyve1, it did not express fli1e:YFP – a marker of arterial endothelium (Bussmann et al., 2010) – suggesting it has a venous origin (supplementary material Fig. S5). We believe the VA-L originates from an uncharacterised vein near the VA. At 1.75 dpf, the VA-L migrates dorsally along the VA and AA1, and the FLS elaborates near the PHS (Fig. 4B). Between 2 and 2.5 dpf we observed the joining of the VA-L to the FLS to form the LFL (n=50/50 embryos) (Fig. 4C-D).
proceeds anteriorly near the PHS and contains both filopodia and lamellapodia-like structures at the growing tip (Fig. 5A). Cells within the FLS are continually migrating, and we consistently observed a cell division during the time-course (n=3/5 movies) (Fig. 5A’, see supplementary material Movie 2). We also observed migratory behaviour in the PHS-Ls as they appear to bud off and migrate from the PHS (supplementary material Movie 2). Between 1.8 and 2 dpf, we observed recruitment of these PHS-L to the growing FLS (n=3/3 movies) (Fig. 5A,A’). We consistently observed an initial recruitment of one PHS-L cell at around 1.8-1.95 dpf, followed by the recruitment of 6-10 PHS-L cells at ~1.9-2 dpf (n=3 movies) (Fig. 5A,A’). Interestingly, once these PHS-Ls were added to the FLS, they become part of the vascular tip and drive further migration (Fig. 5). Further imaging from 2 to 2.5 dpf showed that the final group of nine or ten PHS-L cells fuse with the FLS at ~2-2.15 dpf (n=4 movies), whereas ~8-10 VA-L cells fused with the FLS at ~2.1-2.2 dpf (n=4 movies) to create the LFL (Fig. 5B,B’; supplementary material Movie 3). Once the VA-L has fused, cells that were previously at the tip of the FLS stop migrating.

**flt4 and ccbe1 are required for FL, IL and LL development**

**flt4/vegfr3** and **ccbe1** (collagen and calcium binding EGF domain 1) are required for the development of the thoracic duct in the zebrafish and both genes are essential for mammalian lymphatic development (Karkkainen et al., 2000; Alders et al., 2009; Hogan et al., 2009b; Hogan et al., 2009a). flt4 is expressed in the PCV, and complementary expression of its ligand, **vegfc**, in the trunk mesoderm is known to cause migration of flt4-expressing lymphatic endothelial cells from the PCV to form the secondary sprouts (Kuchler et al., 2006; Hogan et al., 2009a). ccbe1 is also required for secondary sprout formation and it is thought to be involved in the flt4 signalling pathway (Boš et al., 2011). We observed a similar complementary pattern of flt4 expression in vessels in the head region and of vegfc in the non-vascular region surrounding these vessels at 2 dpf (supplementary material Fig. S6A-D). These data suggested that, similar to the trunk lymphatics, flt4, and therefore ccbe1, could also be required for the development of the FL network.

In support of this hypothesis, antisense morpholinos against **flt4** and **ccbe1** prevented the initial FLS forming from the CCV at 2 dpf (Fig. 6A-D; supplementary material Movie 4). This was not due to a morpholino-induced developmental delay as blood vessel development proceeded normally in the morphant embryos. Quantification of the length of the FL sprouting from the CCV from 2 to 4 dpf revealed that ccbe1 morphants completely failed to form a FLS (Fig. 6C,D). However, flt4 morphants did display some FL development, as from 3 dpf the FLS does form from the CCV, but this still fails to develop into a complete facial lymphatic network (Fig. 6B,D). By 5 dpf, flt4 morphants develop both the MLV and the OLV (16/24 embryos), but the LAA rarely formed (1/24 embryos). Consistent with this, the VA-L also failed to migrate in both flt4 and ccbe1 morphant embryos (supplementary material Fig. S6). In addition, both ccbe1 and flt4 morphants lacked the IL network at 6 dpf and likewise the initial LL network (PLV) failed to form owing to the absence of trunk lymphatics (Fig. 6E-G’) (IL and LL network were absent in 19/19 flt4 morphants and 20/20 ccbe1 morphants). Thus, ccbe1 and flt4 are required for the development of all lymphatic vessels in the zebrafish embryo.

**Fig. 4. Distinct lymphangioblast populations contribute to the development of the LFL.** (A–D*) Lateral images of lyve1:EGFP expression (A–D) and both lyve1:EGFP and kdrl:RFP expression (A’–D’) of the lyve1:EGFP,kdrl:RFP transgenic at 1.5 dpf (A), 1.75 dpf (B), 2 dpf (C) and 2.5 dpf (D), with schematic diagrams of arteries (red), veins (blue) and lymphatic (green) at each stage (A’–D’). Lymphangioblast populations are indicated by asterisks. The PHS-L is in pink; the VA-L is in grey. AA1, mandibular arch; CCV, common cardinal vein; FLS, facial lymphatic sprout; LFL, lateral facial lymphatic; PHS, primary head sinus; PHS-L, primary head sinus lymphangioblast; VA, ventral aorta; VA-L, ventral aorta lymphangioblast. Scale bars: 100 μm.

To determine how the FLS develops, and to establish the contribution of the PHS-L to the formation of the LFL, we used confocal time-lapse imaging to follow the development of the FLS from ~1.6 dpf to 2 dpf. To be able to observe the behaviour of single cells within the FLS, we took advantage of residual kdrl expression in developing lymphatics and imaged a lyve1:EGFP,kdrl:nlsCherry compound transgenic. We found that the FLS resembles a dynamic vascular sprout. This sprout
DISCUSSION
The zebrafish is increasingly gaining importance as a model for lymphatic research. However, the majority of this research has focused on the development of the trunk lymphatics. In part, this is due to the lack of specific zebrafish lymphatic markers; initial studies of zebrafish lymphatic development used a pan-endothelial marker (\textit{fli1a:EGFP}) (Kuchler et al., 2006; Yaniv et al., 2006). When \textit{fli1a:EGFP} was crossed with the blood vessel specific marker, \textit{kdrl:RFP}, the trunk lymphatic vasculature could be identified (Hogan et al., 2009b); however, owing to strong expression of \textit{fli1a:EGFP} in the head, it is difficult to identify vessels in this area. Subsequent studies used either the \textit{stab1l:YFP} or the \textit{SAGFF27C;UAS:GFP} lines, which are expressed in the lymphatic vasculature, but the \textit{stab1l:YFP} is

**Fig. 5.** The LFL develops from a vascular sprout with migration, duplication, and recruitment of lymphangioblast populations to the vascular tip. (\textbf{A},\textbf{A'}) Still images from supplementary material Movie 2 showing the developing LFL in the \textit{lyve1:EGFP;kdrl:nlsmCherry} transgenic from 1.63 to 2 dpf (39 to 48 hpf). (\textbf{A}) \textit{lyve1} expression shows the vascular tip of the FLS, which resembles a dynamic vascular sprout with filopodia (red arrow) and lamellipodia (yellow arrow), and the migration and the recruitment of PHS lymphangioblasts (indicated by a white asterisk) to the vascular tip of the FLS. (\textbf{A'}) Nuclear \textit{kdrl} expression shows duplication of a lymphangioblast cell (yellow and red) within the FLS (green outline), the migration of lymphangioblast cells (yellow, red, blue, and beige) and the recruitment of PHS-Ls (purple outline). (\textbf{B},\textbf{B'}) Still images from supplementary material Movie 3 showing the developing LFL in the same \textit{lyve1:EGFP;kdrl:nlsmCherry} transgenic embryo as in \textbf{A} from 2 to 2.5 dpf (48 to 60 hpf). (\textbf{B}) \textit{lyve1} expression shows the migration and recruitment of another PHS-L (white asterisk) and the VA lymphangioblast (yellow asterisk) to the growing tip of the FLS to form the LFL. (\textbf{B'}) Nuclear \textit{kdrl} expression shows duplication of lymphangioblast cells (green and light blue) within the FLS (green outline), and the migration of lymphangioblast cells from the PHS-L that have been recruited to the FLS (orange, green, and light blue) to another PHS lymphangioblast (pink outline) and the VA lymphangioblast (red outline). CCV, common cardinal vein; FLS, facial lymphatic sprout; LFL, lateral facial lymphatic; PHS, primary head sinus; PHS-L, primary head sinus lymphangioblast. Scale bars: 50 µm.
only weakly expressed whereas the SAGFF27C;UAS:GFP has non-specific expression, particularly in the intestine (supplementary material Fig. S1) (Hogan et al., 2009b; Bussmann et al., 2010). The lyve1 transgenic developed in this study shows strong expression in the lymphatic vessels and a subset of veins. When co-expressed with the blood vessel-specific marker kdrl, lymphatic vessels are easily identified. Importantly, the low level of non-vascular expression enabled the identification of lymphatic vessels that were not visible in previous transgenic lines.

We have established a map of zebrafish lymphatic development up to 15 dpf and have identified three new lymphatic networks; the FL, the IL and the LL (Fig. 7A). The FL network displays bilateral symmetry, develops from the CCV and extends through the jaw, otolith and branchial arch region of the zebrafish head (Fig. 1A-E'). They connect to the TD by 5 dpf near its connection to the CCV (Fig. 1G). The IL network includes the SIL, previously described (Coffindaffer-Wilson et al., 2011), but also include a network of lymphatic vessels that develop in close proximity to the intestinal blood vasculature. The LL network is an extension of the previously described trunk lymphatic network, forming from the ISLVs following TD formation.

Arterial guidance cues have been shown to be essential for the development of the TD (Bussmann et al., 2010). Consistent with this we observed that a number of our newly characterised lymphatic vessels extend along arteries: the JLV, LAA1-4, UR-IL develop along the LDA, AA1-4 and SIA, respectively. However, during the construction of the lymphatic atlas we noticed that not all lymphatic vessels appeared to develop along arteries. For example, the LL-IL and the LR-IL vessels extend along the L-SIV
and R-SIV, respectively (Fig. 2F-H’) whereas the SIL develops along the PCV (supplementary material Movie 1). Thus, both arterial and venous guidance cues are used during lymphatic vessel development. We also noted that the development of the OLV, MFL, LFL (Fig. 1B-E) and the UL-IL (Fig. 2D) are partly or fully independent of the blood vasculature. This data suggests that other guidance cues are important for lymphatic development. One candidate non-vascular cue could be motoneurons, as these are implicated in development of the zebrafish trunk lymphatic network (Lim et al., 2011).

The identification of lymphatic vessels associated with the zebrafish intestine raised the possibility that the zebrafish lymphatic network could play a similar role in lipid uptake as is observed in mammals (Coffindaffer-Wilson et al., 2011). In mammals, lipids that have been absorbed in the lacteals in the small intestine are transported to the thoracic duct (Alexander et al., 2010). In the zebrafish, we observed several lymphatic vessels that connect the TD and the SIL at 5 dpf and persist to 15 dpf (Fig. 2E,E’; supplementary material Movie 1). These lymphatic vessels may serve as a link for lipid transportation in the zebrafish between the intestine and the TD. The development and function of the IL, in particular its similarities to the mammalian lacteal capillaries, require further investigation.

To date, only the development of the zebrafish trunk lymphatic network has been characterised in detail (Kuchler et al., 2006; Yaniv et al., 2006; Hogan et al., 2009b; Coffindaffer-Wilson et al., 2011). The trunk lymphatics develop from secondary lymphatic sprouts that originate from the PCV. These sprouts migrate dorsally towards the myoseptum, becoming parachordal lymphangioblasts. The parachordal lymphangioblasts migrate both dorsally and ventrally along the arterial ISVs to form the ISLVs, DLLV and the TD (Yaniv et al., 2006; Hogan et al., 2009b; Coffindaffer-Wilson et al., 2011). Thus, the trunk lymphatics are derived entirely from lymphangioblasts originating from the PCV. These sprouts migrate dorsally towards the myoseptum, becoming parachordal lymphangioblasts. The parachordal lymphangioblasts migrate both dorsally and ventrally along the arterial ISVs to form the ISLVs, DLLV and the TD (Yaniv et al., 2006; Hogan et al., 2009b; Coffindaffer-Wilson et al., 2011). Thus, the trunk lymphatics develop from secondary lymphatic sprouts that originate from the PCV. These sprouts migrate dorsally towards the myoseptum, becoming parachordal lymphangioblasts. The parachordal lymphangioblasts migrate both dorsally and ventrally along the arterial ISVs to form the ISLVs, DLLV and the TD (Yaniv et al., 2006; Hogan et al., 2009b; Coffindaffer-Wilson et al., 2011).
derived from an undefined vein near the VA fuses with the FLS to form the LFL. These results show that the development of the FL involves dynamic and co-ordinated communication between different cell types from various locations in the zebrafish head, which is in contrast to the formation of the TD that is derived entirely from lymphangioblasts originating from the PCV (Fig. 7B) (Yaniv et al., 2006). The multi-vessel origin of the FL network is perhaps not surprising given that there is no single vein that aligns along the course of FL development. It will be interesting to elucidate the molecular mechanisms involved in FL development; in particular, the developmental cues that coordinate lymphangioblast budding and migration from different vessels.

Time-lapse imaging revealed that the FLS was highly dynamic, containing filopodia and lamellipodia structures. We also observed cell proliferation within the migrating FLS. Cells at the tip of the FLS were migratory whereas cells further back were less so. This structure has many features in common with the angiogenic sprout observed in various angiogenic models, including the zebrafish (Isogai et al., 2003; Blum et al., 2008; Geudens and Gerhardt, 2011). Angiogenic sprouts, such as the FLS, contain a migratory leading tip cell, whereas cells adjacent to the tip, termed stalk cells, are less polarised, can undergo cell proliferation and contribute to the formation of the vascular lumen (Geudens and Gerhardt, 2011). Angiogenic tip cells also contain filopodia, which help to guide the developing vessel (Isogai et al., 2003). Recently, it has been shown that angiogenic tip and stalk cell fate is dynamic and cells can interchange their fate as either a tip or a stalk cell (Jakobsson et al., 2010). Interestingly, we noted that the leading cell within the FLS often changed, although one key difference from an angiogenic sprout is that this was often due to the addition of a new lymphangioblast cell(s) at the tip.

Many genes have been implicated in lymphatic development, particularly the vegfr3/flt4 receptor and ccbe1, which when mutated in humans result in Milroys disease and Hennekam syndrome, respectively – both of which are forms of primary lymphoedema (Karkkainen et al., 2000; Alders et al., 2009; Hogan et al., 2009b). FLT4-expressing lympho endothelial cells migrate towards the FLT4 ligand VEGF-C, which is expressed in the surrounding mesenchyme (Tammela and Alitalo, 2010). Previous work has shown that silencing flt4 or ccbe1 prevents TD development in zebrafish owing to a reduction in secondary sprout formation (Hogan et al., 2009b; Hogan et al., 2009a). Consistent with this, we find that both flt4 and ccbe1 morphants do not form a FLS at 1.5-2 dpf and fail to develop a complete FL network (Fig. 6). The lack of FLS was not due to a morpholin-induced developmental delay, as blood vessel development proceeded normally (supplementary material Movie 4). We did note that flt4 morphants formed an FLS later in development (3 dpf) but it did not extend as normal. flt4 and ccbe1 morphants also failed to develop the IL network, and as the ISLV did not form in the trunk, the LL network was also absent. Thus, both vegfr3/flt4 and ccbe1 are required for the formation of the entire lymphatic network in the zebrafish embryo. Interestingly, a ccbe1 mutant, full of fluid (jopu3653), was reported to display oedema around the eye and the lower intestine (Hogan et al., 2009b), which supports our hypothesis that ccbe1 is required for both FL and IL development.

In summary, we have established a map of lymphatic development in the zebrafish up to 15 dpf. In the process we have identified novel lymphatic networks. We believe the characterisation of these new lymphatic vessels will increase the versatility of the zebrafish as a model for lymphatic vessel development and function. Our atlas now enables investigators to test the role of genes in lymphatic vessel development outside of the trunk lymphatic network, as we have done for both flt4 and ccbe1. In addition, we have shown that the facial lymphatic network develops initially from a single lymphatic sprout that migrates from the CCV; lymphangioblasts are added to the tip of this lymphatic sprout and drive further migration of the developing vessel. Future work will focus on the molecular signals that enable the coordinated budding and migration of these lymphangioblasts from different veins in the head.

Acknowledgements

The kdrtt, crr/pp, gata1:DsRed, kdrtt/EGFP and the fli1:YP transgenic lines were provided by Graham Lieschke, David Traver and the Zebrafish International Resource Center (ZIRC, supported by grant P40 RR012546 from the NIH-NCRC), respectively. The SAGGF27C/US:GFP transgenic line, the fli1 in situ probe, and the flt4 and ccbe1 morpholinos were kindly provided by Ben Hogan. We thank Alhad Mahagaonkar for managing the fish facility, Alisha Malik for technical support and the University of Auckland Biomedical Imaging Research Unit for imaging. We also thank Stefan Dehler, Leslie Sandenon, Chris Hall and the rest of Crosier laboratory for comments on the manuscript.

Funding

J.W.A., J.P.M. and M.V.F. were supported by the Ministry of Science and Innovation grant [UOA08013] awarded to K.E.C and P.S.C. (New Zealand). K.S.O. was supported by the Ministry of Science, Technology and Innovation scholarship (Malaysia).

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.077701/-/DC1

References


