RESEARCH ARTICLE

Vegfd can compensate for loss of Vegfc in zebrafish facial lymphatic sprouting

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ABSTRACT

Lymphangiogenesis is a dynamic process that involves the sprouting of lymphatic endothelial cells (LECs) from veins to form lymphatic vessels. Vegfr3 signalling, through its ligand Vegfc and the extracellular protein Ccbe1, is essential for the sprouting of LECs to form the trunk lymphatic network. In this study we determined whether Vegfr3, Vegfc and Ccbe1 are also required for development of the facial and intestinal lymphatic networks in the zebrafish embryo. Whereas Vegfr3 and Ccbe1 are required for development of all lymphatic vessels, Vegfc is dispensable for facial lymphatic sprouting but not for the complete development of the facial lymphatic network. We show that zebrafish vegfd is expressed in the head, genetically interacts with ccb1 and can rescue the lymphatic defects observed following the loss of vegfc. Finally, whereas knockdown of vegfd has no phenotype, double knockdown of both vegfc and vegfd is required to prevent facial lymphatic sprouting, suggesting that Vegfc is not essential for all lymphatic sprouting and that Vegfd can compensate for loss of Vegfc during lymphatic development in the zebrafish head.

KEY WORDS: Vegfd, Lymphatic, Vegfc, Zebrafish, Lymphangiogenesis

INTRODUCTION

The lymphatic vasculature is a network of extracellular vessels crucial for maintaining fluid homeostasis in the body, with additional important roles in immune cell trafficking and lipid absorption (Tammela and Alitalo, 2010). The growth of new lymphatic vessels, which is termed lymphangiogenesis, can occur aberrantly in tumours and in areas of chronic inflammation (Stacker et al., 2014).

It is now well established from experiments in mice and in zebrafish that the lymphatic vasculature arises from the veins. In mice the sprouting of lymphatic endothelial cells (LEC) from the cardinal vein requires vascular endothelial growth factor receptor 3 (VEGFR3; FLT4 – Mouse Genome Informatics) signalling through its ligand VEGFC (Karkkainen et al., 2004; Veikkola et al., 2001). Vegfr3 is expressed in blood endothelial cells but becomes upregulated in LECs during their specification from the venous endothelial cells (Kaipainen et al., 1995). However, genetic inactivation of Vegfr3 in mice causes severe lymphatic defects (Karkkainen et al., 2004), and Vegfr3−/− mice die during early embryogenesis (E9.5) owing to failure in the development of the cardiovascular system (Dumont et al., 1998). Vegfr3-expressing LECs respond to VEGFC secreted in the mesenchyme, causing LECs to migrate from the cardinal vein during the early steps of lymphatic vessel development. Accordingly, Vegfc−/− mice lack a lymphatic vasculature because LEC progenitor cells fail to sprout from the cardinal vein (Karkkainen et al., 2004), whereas overexpression of Vegfc causes lymphatic hyperplasia (Jeltsch et al., 1997). The importance of the VEGFR3/VEGFC signalling pathway in human lymphatic vessel development is highlighted by the observation that patients with mutations in either of these genes develop lymphedema (Gordon et al., 2013; Irrthum et al., 2000; Karkkainen et al., 2000). The other ligand for VEGFR3 is Vegfd (FIGF – HUGO Gene Nomenclature Committee; Mouse Genome Informatics). A mouse knockout of Vegfr3 is Vegfd−/− (Baldwin et al., 2005); however, Vegfr3 can rescue the lymphatic hypoplasia in Vegfc−/− mice (Haiko et al., 2008) and is able to induce both lymphangiogenesis and angiogenesis in a variety of assays (Achen and Stacker, 2012).

Recently, zebrafish (Danio rerio) have emerged as an excellent model for the study of lymphangiogenesis; they have the advantage that developing lymphatic vessels are easily observed in transparent embryos (Yaniv et al., 2006). The majority of zebrafish studies have focused on development of the trunk lymphatic network, which by 5 days post fertilisation (dpf) consists of the thoracic duct (TD) that runs between the dorsal aorta and the posterior cardinal vein (PCV), the dorsal longitudinal lymphatic vessel (DLLV) and the intersegmental lymphatic vessels (ISLVs) that connect the TD to the dorsal longitudinal lymphatic vessel (Koltowska et al., 2013). The trunk lymphatic network is derived from lymphatic precursors that sprout from the PCV at around 1.5 dpf and migrate to the horizontal myoseptum, where the are termed parachordal lymphangioblasts. These lymphangioblasts then migrate both dorsally and ventrally using arterial intersegmental blood vessels as guides to remodel into the TD, ISLVs and the DLLV (Bussmann et al., 2010). Importantly, the formation of this network requires Vegfr3 (known as Flt4 in zebrafish) signalling through its ligand Vegfc, as mutation or morpholino (MO)-mediated knockdown of these genes prevents the sprouting of lymphatic cells from the cardinal vein (Hogan et al., 2009b; Kuchler et al., 2006; Villefranc et al., 2013). In addition, the chemokine receptor Cxcr4a and its ligands Cxcl12a and Cxcl12b have been shown to be required for the correct migration and remodelling of lymphangioblasts to form the TD (Cha et al., 2012). Studies using zebrafish have identified the collagen and calcium binding EGF domains 1 (ccbe1) gene as also being required for both zebrafish and mammalian lymphatic development (Alders et al., 2009; Bos et al., 2011; Hogan et al., 2009a). Recently, Ccbe1 has been identified as a crucial component of the Vegfc/Vegfr3 pathway; it has been shown to genetically interact with vegfc and flt4 in zebrafish (Le Guen et al., 2014) and also to indirectly promote the proteolytic cleavage of mammalian VEGFC into its active form (Jeltsch et al., 2014).
We recently generated a map of lymphatic development in zebrafish and characterised the development of two lymphatic networks, namely the facial lymphatics and the intestinal lymphatics, that are distinct from the previously characterised trunk lymphatic network (Okuda et al., 2012). We were able to show that the facial lymphatics develop initially from a single facial lymphatic sprout (FLS) that forms from the common cardinal vein (CCV) near the primary head sinus (PHS) at 1.5 dpf. Following initial migration, lymphangioblasts from the PHS, as well as other veins, are recruited to the tip of the FLS to drive the migration and development of the lateral facial lymphatic vessel (LFL) at 3 dpf, and, by 5 dpf, the otolithic lymphatic vessel (OLV), the medial facial lymphatic (MFL) and the lymphatic branchial arches (LAA)s have formed.

In this study, we investigated whether formation of the intestinal and facial lymphatic networks requires the same signalling pathways as those required for formation of the trunk lymphatic network. We find that flt4 and ccbel are required for the development of all three lymphatic networks; however, vegfc is only essential for the formation of the trunk and intestinal lymphatics and for the complete development of the facial lymphatic network, but is not required for initial facial lymphatic sprouting. We go on to show that expression of the other Flt4 ligand, Vegfd, in the head allows the FLS to form in the absence of Vegfc. In addition, we show that chemokine signalling is only required for the formation of the trunk lymphatics. These findings show that although there are common signalling pathways that drive lymphatic vessel development, there are also differences between the growth factors required to form different lymphatic networks.

RESULTS

vegfc is not essential for initial development of the facial lymphatic network

We investigated whether Flt4, Vegfc and Ccbe1 are required for development of the facial lymphatic network. Facial lymphatic vessels were identified by the expression of the lymphatic and venous marker lyve1 and by the absence of the blood vessel-specific marker kdrl (Okuda et al., 2012) (supplementary material Fig. S1). We confirmed that flt4 is essential for both facial and trunk lymphatic formation by: injecting a MO against flt4 into lyve1:dsred or lyve1:egfp embryos; examining lymphatic vessel formation in a previously characterised hypomorphic mutant of Flt4 in which signalling is impaired (flt4hu4602) (Hogan et al., 2009b); or injection of an mRNA encoding the Ig domain of human FLT4 (flt4hu4602) that has been shown to act as a dominant inhibitor of zebrafish Flt4 signalling (Hogan et al., 2009b; Ober et al., 2004).

Following injection with either flt4 MO or sFLT4 mRNA and in flt4hu4602 mutant embryos, we observed a decrease in the length of the LFL at 3 dpf (Fig. 1A,D,K) and also a failure in the formation of the TD (Fig. 1C,H,K,L) (Le Guen et al., 2014). Analysis of 5 dpf flt4 morphant embryos showed that injection of either ccbel MO or sFLT4 mRNA inhibited the formation of these facial lymphatic vessels (Fig. 1M-R); however, the phenotype was less severe in flt4 morphants or in flt4hu4602 mutant embryos (Fig. 1R). Despite vegfc not being essential for the formation of the FLS and early LFL development, it is required for the formation of the OLV, MFL and LAA, as the development of all of these lymphatic vessels was severely impaired in vegfcas5055 embryos and to a lesser extent in vegfc morphant embryos (Fig. 1M-R; supplementary material Fig. S3). In addition, although the LFL had extended anteroverally from the level of the PHS in the majority of vegfcas5055 embryos, it was still shorter than the LFL in control embryos that had extended below the eye, suggesting that vegfc is required for the later development of the LFL.

vegfc is required for development of the intestinal lymphatic network

The intestinal lymphatic network in zebrafish has been shown to be dependent on both Flt4 and Ccbe1 signalling (Okuda et al., 2012). By 6 dpf the intestinal lymphatics consist of the upper right intestinal lymphatic (UR-IL) that runs alongside the subintestinal artery and the of lower right intestinal lymphatic (LR-IL) that runs alongside the subintestinal vein. Analysis of flt4 and ccbel morphants confirmed that these genes are essential for the formation of the intestinal lymphatics. We also show that vegfc is required, as vegfc morphants consistently displayed either complete loss or fragmentation of the intestinal lymphatic network at 6 dpf (supplementary material Fig. S5).
cxcr4a and cxcr112a are only necessary for development of the trunk lymphatic network

Recently, the chemokine receptor Cxcr4a and its ligands Cxcl12a and Cxcl12b were identified as being required for early lymphatic vessel development in the zebrafish trunk (Cha et al., 2012). We therefore examined the role of chemokine signalling in facial and intestinal lymphatic development. Although both cxcr4a and cxcr112a were expressed in a spatiotemporal manner consistent with a possible role in facial lymphatic development, MO knockdown of cxcr4a or cxcr112a did not result in any defect in facial lymphatic development at either 3 or 5 dpf, despite causing a modest inhibition of formation of the TD (supplementary material Fig. S6). Formation of the intestinal lymphatics also appeared normal in cxcr4a or cxcr112a morphant embryos. Taken together, our results suggest that, with

Fig. 1. vegfc is not essential for zebrafish early facial lymphatic development. (A–J) Confocal images of the facial lymphatics in lyve1 embryos at 3 dpf (A–E) or trunk lymphatics at 5 dpf (F–J) in control MO (A,F), vegfc MO-1 (B,G), vegfcMO-1 (C,H), sFLT4 mRNA (D,I), and ccbe1 MO (E,J). Loss of Vegfc prevents formation of the TD (red asterisks), but the LFL forms normally at 3 dpf (white arrowheads) in vegfc morphant or vegfcMO-1 mutant embryos. (K) Quantitation of LFL length at 3 dpf. (L) Quantitation of TD formation at 5 dpf. (M-O) Confocal images of the facial lymphatics in lyve1 embryos at 5 dpf in control MO (M), vegfc MO-1 (N), vegfcMO-1 (O), sFLT4 mRNA (P), and ccbe1 MO (Q). Knockdown of Flt4, Ccbe1 or Vegfc inhibits the correct development of the facial lymphatic network. Asterisk indicates the PHS. (R) The percentage formation of different facial lymphatic vessels at 5 dpf. For flt4hu4602 and vegfcMO-1 embryos, n=number of mutant embryos. CCV, common cardinal vein; PHS, primary head sinus; PCeV, posterior cerebral vein; LFL, lateral facial lymphatic; OLV, otolithic lymphatic vessel; MFL, medial facial lymphatic; LAA, lymphatic branchial arches. ***P<0.001, by a Mann–Whitney test versus control MO; error bars indicate s.d. Scale bars: 50 μm.
regard to lymphatic development, ccr4a and ccr112a are only required for the growth of trunk vessels.

**vegfd is expressed in the head and can compensate for loss of vegfc**

Given that Flt4 signalling is required for the development of the FLS but its ligand Vegfc appears to be dispensable, we examined whether the other Flt4 ligand, Vegfd, has a role in the development of the facial lymphatics. Previous studies have shown that zebrafish vegfd is expressed in the tail bud but the expression of vegfd in the head was not determined (Hogan et al., 2009a). Using whole-mount in situ hybridization, we examined the expression of flt4, vegfc, vegfd and ccbel1 in the head and trunk during early lymphatic vessel development. We found that mRNA of all four genes was present in the head region at 1.5-2 dpf (Fig. 3; supplementary material Fig. S7) but is present in injected with either control MO (A-E; supplementary material Movie 1), flt4 MO (F-J; supplementary material Movie 2), or vegfc MO-1 (K-O; supplementary material Movie 3). Data are representative of three independent time-lapse experiments. The facial lymphatic sprout (red arrows) does not form in morphant embryos that do not have parachordal lymphangioblasts (red arrowheads) in the trunk. The formation of the pectoral vein (green arrowheads) at approximately 6 h into the experiment serves as a control to show that the embryos are developing normally. Scale bars: 50 μm.

Fig. 2. The facial lymphatic sprout forms normally in vegfc morphant embryos. (A-D,F-I,K-N) Stills from confocal time-lapse imaging of the facial lymphatic sprout in lyve1:egfp embryos from 1.5 dpf to 1.9 dpf (9:03 h) with (E,J,O) a confocal image of the developing trunk lymphatics in the same embryo at 2 dpf injected with either control MO (A-E; supplementary material Movie 1), flt4 MO (F-J; supplementary material Movie 2), or vegfc MO-1 (K-O; supplementary material Movie 3). Data are representative of three independent time-lapse experiments. The facial lymphatic sprout (red arrows) does not form in flt4 morphants but is present in vegfc morphant embryos that do not have parachordal lymphangioblasts (red arrowheads) in the trunk. The formation of the pectoral vein (green arrowheads) at approximately 6 h into the experiment serves as a control to show that the embryos are developing normally. Scale bars: 50 μm.
shown) and thus we were unable to distinguish whether these vessels are veins or immature lymphatic vessels that still retain 

kdrl expression. Both Vegfc- and Vegfd-expressing cancer cells were able to induce more 

lyve1-positive vessels than cancer cells expressing the vector alone, suggesting that both Vegfc and Vegfd have the potential to induce 

lyve1 vessel growth in zebrafish (Fig. 5A-E).

Double knockdown of both 

vegfc and vegfd is required to prevent initial development of the facial lymphatics

Given that vegfd can compensate for vegfc and can induce similar phenotypes to vegfc when expressed ectopically, we examined whether vegfd had a function in lymphatic vessel development. We used an MO targeting the exon 4/intron 4 boundary of vegfd, and injection of 1 pmol of this MO resulted in a 65 bp deletion of the 819 bp vegfd transcript (supplementary material Fig. S8). Full-length vegfd mRNA encodes a 273 amino acid protein. Sequencing the morphant vegfd mRNA showed that the deletion causes a frameshift from position 186 and a premature stop at position 200, resulting in loss of the C-terminal end of the VEGF homology domain that is essential for the function of VEGF ligands. From these data we predict that the vegfd MO is disrupting any potential Vegfd signalling in morphant embryos. Injection of vegfd MO into either lyve1:egfp or lyve1:dsred embryos showed no lymphatic defects in either the trunk, intestinal or facial lymphatic networks, suggesting that vegfd is dispensable for zebrafish lymphatic vessel development (Fig. 6K; supplementary material Fig. S8). However, given that vegfd is expressed in the head during the formation of the facial lymphatics, we examined whether it could compensate for disrupted vegfc levels.

To test this hypothesis, we injected a mixture of vegfc MO-1 and vegfd MO and examined the formation of the FLS by live imaging lyve1:egfp embryos from 1.5 dpf. In three embryos injected with control MO and vegfc MO-1 we found that the FLS consistently formed by 1.9 dpf (45 hpf) (Fig. 6A-D; supplementary material Movie 4). In three embryos injected with vegfc MO-1 and vegfd MO we found that the FLS failed to form by 1.9 dpf (45 hpf) (Fig. 6E-H; supplementary material Movie 5). We also observed a reduction in LFL length in vegfc/vegfd double morphants at 3 dpf that could be rescued by injection of either vegfd or vegfc mRNA (Fig. 6I,M-O, Q). A reduction in LFL length was also observed when vegfd MO was injected into vegfchu5055 embryos (Fig. 6L,P,Q). Examination of OLV, MFL and LAA vessel formation at 5 dpf revealed that both vegfc/vegfd double morphants and vegfchu5055 mutants injected with vegfd MO lacked all facial lymphatic vessels and phenocopied embryos injected with sFLT4 mRNA (Fig. 6R-V). These results show that although vegfd is not essential for lymphatic vessel development, it can compensate for loss of vegfc in the formation of the facial lymphatics. Furthermore, knockdown of both vegfc and vegfd is required to prevent the formation of the FLS and the initial development of the LFL in zebrafish embryos.

**DISCUSSION**

Our results show that different lymphatic networks in the zebrafish have distinct requirements for lymphatic growth factors (Fig. 7). We have analysed the requirement for Flt4, Vegfc and Ccebl in development of the intestinal and the facial lymphatics and have found that they are all essential for development of the intestinal
lymphatics and also for maturation of the facial lymphatics. The LAA, MFL and OLV did not form in embryos with knockdowns or mutations in these genes. Recently, it has been shown that chemokine signalling is required for the development of the trunk lymphatic network (Cha et al., 2012). Although we were able to reproduce the requirement for Cxcr4a and Cxcl12a in the development of the trunk lymphatics, MO knockdown of ccr4a or ccl12a had no effect on the development of the facial or the intestinal lymphatics, despite being highly expressed in the head. Thus, chemokine signalling appears to be a specific requirement for the trunk lymphatic network and highlights how different lymphatic networks may utilise different growth factors (Fig. 7).

The facial lymphatic network is derived from a single sprout, the FLS, which forms from the CCV. Surprisingly, whereas flt4 and ccb1 are essential for the formation of the FLS and the early LFL, we found that knockdown of vegfc by MO injection did not inhibit LFL formation in 3 dpf embryos. Further analysis showed that the
Fig. 5. Expression of vegfd can induce ectopic lyve1-positive vessels. (A–C) Confocal images of human breast cancer cells labelled with Cell Tracker Green (MDA-MD-231) xenografted into lyve1:dsred embryos and imaged at 5 dpf. (A) MDA-MD-231 cells transfected with vector only. (B) MDA-MD-231 cells transfected with zebrafish vegfc. (C) MDA-MD-231 cells transfected with zebrafish vegfd. (A–C) Confocal images of the lyve1:dsred vessels only. Cancer cells in green, lyve1 in magenta. Dashed lines indicate the boundary of the xenograft. The xenografts expressing either vegfc or vegfd are able to induce more ectopic lyve1-positive sprouts from the CCV than the control xenografts. (D) Quantitation of the tumour-induced lyve1-positive vessels, expressed as a percentage of tumour volume. (E) RT-PCR using RNA isolated from MDA-MD-231 cells transfected with vector only, vegfc or vegfd, with primers designed against human β-actin (ACTB), zebrafish vegfc or zebrafish vegfd. *P<0.05 by a Mann–Whitney test; error bars indicate s.d. Scale bar: 50 μm.

FLS formed normally in vegfc morphants despite these embryos showing a defect in secondary sprouting from the PCV. One possible explanation for this result is that MO knockdown of vegfc might be less efficacious in the zebrafish head compared with the trunk, possibly due to the higher levels of vegfc expression in the head. To account for this we also examined lymphatic vessel formation in a vegfc mutant (vegfc<sup>hu5055</sup>) that has lost a conserved cysteine residue in the C-terminal propeptide (C365R) (Le Guen et al., 2014). It is possible that this mutant allele of vegfc is not completely null, as other mutations in vegfc have been shown to have more penetrant defects in trunk lymphatic development (Le Guen et al., 2014). Nevertheless, we have shown that the vegfc<sup>hu5055</sup> embryos have a significant defect in TD development but, like vegfc morphant embryos, these mutant embryos display normal facial lymphatic development up to 3 dpf. Taken together, these observations suggest that the formation of the FLS does not require vegfc or at least is more resistant to loss of vegfc than the formation of lymphatic sprouts in the trunk. As far as we are aware, this is the first report showing that Vegfc is not essential for all lymphatic sprouting. Whereas early facial lymphatic development (up to 3 dpf) was normal in embryos with impaired vegfc, we noted that the OLV, MLV and LAA did not form in the majority of vegfc<sup>hu5055</sup> embryos at 5 dpf, suggesting that vegfc is essential for the development of these vessels. We also noted that the later development of the LFL under the eye also required vegfc. The presence of the OLV and MFL in a small percentage of 5 dpf vegfc<sup>hu5055</sup> embryos might be due to the incomplete penetrance of this allele. Therefore, there appears to be a differential requirement for Vegfc in early versus late lymphatic vessel development in the zebrafish head.

There are two possible explanations for the observation that Vegfr3/Flt4 is required for FLS formation but its ligand Vegfc is not essential: Flt4 is signalling through ligand-independent mechanisms, or another Flt4 ligand is involved in the formation of the FLS. Whereas VEGFR3 is known to have ligand-independent roles, to date the lymphangiogenic function of VEGFR3 has primarily been demonstrated to be via ligand signalling (Haiko et al., 2008; Veikkola et al., 2001). In support of this, we were able to robustly inhibit all facial lymphatic development by the injection of sFLT4 mRNA, which functions as a dominant inhibitor by binding endogenous Flt4 ligands (Hogan et al., 2009b; Ober et al., 2004). We therefore examined the role of the other Flt4 ligand, Vegfd, in facial lymphatic development.

In mammals, it has been shown that VEGFD is closely related in structure to VEGFC (Leppanen et al., 2011) and, like VEGFC, can activate both VEGFR3/Flt4 and VEGFR2, thereby promoting both angiogenesis and lymphangiogenesis (Achen and Stacker, 2012). Unlike VEGFC, VEGFD does not appear to be essential for mammalian lymphatic vessel development (Baldwin et al., 2005), but it has been shown in Xenopus embryos to subtly modify lymphangioblast migration (Ny et al., 2008). Mammalian VEGFD is known to be involved in the growth of lymphatic vessels in tumours and also in areas of chronic inflammation (Huggenberger et al., 2010; Katafu et al., 2009). It has also been shown to compensate for loss of VEGFC (Haiko et al., 2008; Karkkanen et al., 2004). In this study, we show that, like mammalian VEGFD, zebrafish Vegfd has both angiogenic and lymphangiogenic potential and can compensate for loss of Vegfc. Furthermore, we show that at 1.5 dpf vegfd is expressed in a region near the intersection of the CCV and the PHS, where the FLS develops,
consistent with vegfd having a role in early facial lymphatic development.

We show that, as in mammals, vegfd is not essential for lymphatic development, as the trunk, intestinal and facial lymphatics form normally in vegfd morphant embryos. However, we found that double knockdown of both vegfc and vegfd inhibits FLS formation and the subsequent formation of the facial lymphatics, which phenocopies knockdown of either ccbel or embryos with impaired Flt4 signalling. It has been shown that Ccbel is required for the activity of Vegfc (Jeltsch et al., 2014; Le Guen et al., 2014) and our data suggest that Ccbel is also required for Vegfd activity. In support of this we found that ccbel and vegfd genetically interact, as silencing ccbel reduced the levels of ISV branching induced by ectopic expression of vegfd.

We believe that Vegfd can compensate for Vegfc in early facial lymphatic formation due to the restricted expression of vegfd in the...
head region. However, why \textit{vegfd} is expressed in this region and not in other areas of lymphatic vessel development, and whether \textit{vegfd} plays an active role in facial lymphatic development, are not clear. In mammalian embryos, \textit{Vegfd} is expressed in many tissues, including the lung, skin, kidney, limb buds and the mandibular arches (Avantaggiato et al., 1998; Stacker et al., 1999). We observed expression of zebrafish \textit{vegfd} in some of the tissue equivalents to counterparts in mice, such as the pectoral fin buds and the mandibular arches, suggesting some conservation of Vegfd function between teleosts and mammals. It is possible that \textit{vegfd} has other developmental roles that necessitate its expression in the head region and it would therefore be serendipitously available for facial lymphatic development when Vegfc levels are impaired. It is intriguing that endogenous \textit{vegfd} can rescue the formation of the FLS and the early development of the LFL in embryos depleted of Vegfc levels are impaired. It is intriguing that endogenous \textit{vegfd} can rescue the formation of the FLS and the early development of the LFL in embryos depleted of Vegfc, but not the formation of lymphatic vessels after 3 dpf (OLV, MLV and LAA). We were unable to determine if this is due to differences in the spatial expression of \textit{vegfd}. However, we do show that \textit{vegfd} expression is maintained in the head throughout lymphatic development, so it is possible that the OLV, MLV and the LAA have an essential requirement for Vegfc signalling compared with the FLS and the early LFL.

To date, no characterisation of mouse ‘facial lymphatics’ has been conducted and it is unclear if equivalent lymphatic structures exist in mammals. This might explain why our results appear to contradict mouse studies, which have shown that double mutants of Vegfc and Vegfd had similar lymphatic defects to the single Vegfc mutant mouse (Haiko et al., 2008; Karkkainen et al., 2004). In addition, these studies would not have been able to evaluate possible delays in lymphatic sprouting due to technical limitations in the imaging of early lymphatic vessel development in the mouse.

Our findings have three important implications for lymphatic vessel development. First, we show that vegfc is not essential for all lymphatic sprouting in the zebrafish. Second, we demonstrate that although vegfd may not be required for lymphatic vessel development, it nevertheless can play a role in lymphatic development by compensating for loss of vegfc. Third, and more importantly, this study provides evidence that the development of different lymphatic networks can involve different sets of lymphatic growth factors.

**MATERIALS AND METHODS**

**Zebrafish**

All zebrafish strains were maintained under standard husbandry conditions and animal studies were approved by the University of Auckland Animal Ethics Committee. The lines used in this study were: wild type (AB), \textit{tg(lyve1:egfp)1250}, \textit{tg(lyve1:dsred)101} (Okuda et al., 2012), \textit{tg(kdrl:egfp)843} (Jin et al., 2005), \textit{tg(kdrl:rfp)la4} (Huang et al., 2005), \textit{flt4hu4602} (Hogan et al., 2009b) and \textit{vegfchu5055} (Le Guen et al., 2014).

**Whole-mount in situ hybridisation**

\textit{In situ} hybridisation was performed as described previously (Thiess and Thiess, 2008). Antisense RNA probes for \textit{ccbe1} (Hogan et al., 2009a), \textit{flt4}, \textit{vegf} (Hogan et al., 2009b), \textit{ccr4a}, \textit{ccel2a} and \textit{ccel2b} (Cha et al., 2012)
were designed as described previously. The antisense probe for vegfd was based on the full-length CDNA. Primers used to generate the vegfd probe are given in supplementary material Table S1.

MO and RNA injections
MO sequences and doses are given in supplementary material Table S2. Full-length vegfc and vegfd cDNAs were cloned (primers given in supplementary material Table S1) and ligated into the pcS2′ vector. The sFLT4 construct was described previously (Hogan et al., 2009b; Ober et al., 2004). mRNA was synthesised using the mMessage mMachine (Ambion) from an Sp6 primer and 200 pg (construct was described previously (Hogan et al., 2009b; Ober et al., 2004)). RNA was injected into 1-cell embryos.

Confocal live imaging of zebrafish
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 min intervals. Confocal images in this study are maximum projections of z-series stacks. Images were processed using ImageJ (NIH), Photoshop CS5 (Adobe) and Volocity 5.4 image analysis software (Improvement/PerkinElmer Life and Analytical Sciences). Microangiography was performed as described previously (Isogai et al., 2001). Lymphangiography was performed by subcutaneous injection of 2.5 mg/ml 500 kDa fluorescein dextran (Invitrogen) diluted in water as described (Okuda et al., 2012).

Image analysis and statistics
TD formation at 5 dpf was scored by counting the presence of a TD in the first 15 somites using either lyve1:egfp or lyve1:dsred and was represented as percentage. The length of the LFL was measured at 3 dpf as described previously (Okuda et al., 2012). For 5 dpf embryos the LFL was considered as present if it had developed anterogradely from the PHS (towards the eye). The LAA, MFL and OLV were scored as present at 5 dpf if they could be distinguished from the LFL. Secondary sprout formation was scored using the lyve1:egfp transgenic at 36 hpf. Only the numbers of secondary sprouts from the anterior portion of the PCV (from the first somite to the cloaca) were counted. Statistical analysis was performed using Prism 5.0 software (GraphPad Software). Significance was determined by Mann–Whitney tests.

Zebrafish xenografts
Full-length zebrafish vegfc and vegfd cDNAs were cloned into the mammalian expression vector pIRES-P (Hobbs et al., 1998) that was transfected into the human breast cancer line MDA-MB-231 using Lipofectamine 2000 reagent (Invitrogen). Transfected cells were selected and maintained in 2 μg/ml puromycin, grown in MEM-Alpha medium supplemented with 10% FBS. Cells were trypsinised, labelled with 2 μM Cell Tracker Green (Invitrogen) and injected into the ventral part of the perivitelline space of 2 dpf lyve1:dsred embryos as described previously (Nicoli and Presta, 2007). Xenografted embryos were imaged at 5 dpf by confocal imaging through the tumour volume. To normalise for differences in xenograft size, the volume of ectopic lymphatic vessels was calculated from confocal imaging through the tumour volume. To normalise for differences in xenograft size, the volume of ectopic lymphatic vessels was calculated from confocal imaging through the tumour volume. To normalise for differences in xenograft size, the volume of ectopic lymphatic vessels was calculated from confocal imaging through the tumour volume. To normalise for differences in xenograft size, the volume of ectopic lymphatic vessels was calculated from confocal imaging through the tumour volume.

RNA extraction and RT-PCR
RNA was extracted from zebrafish embryos and human cells using Trizol reagent (Ambion) as per the manufacturer’s instructions. Quantitative PCR was conducted as described previously (Oehlerls et al., 2011). The primers used for RT-PCR and qPCR analysis are given in supplementary material Table S1.

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Competing interests
The authors declare no competing financial interests.

Author contributions
J.W.A., B.M.H., K.E.C. and P.S.C. conceived and planned the experiments and analysed the data. J.W.A., M.J.L.H., K.S.O., L.L., J.P.M. and A.T. performed the experiments. The manuscript was written by J.W.A., with assistance from L.L., B.M.H., K.E.C. and P.S.C.

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Supplementary material
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