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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 the 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 *ccbe1* 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 set of bloodless 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 (LECs) 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). Heterozygote inactivation of *Vegfr3* in mice causes severe lymphatic defects (Karkkainen et al., 2001), and *Vegfr3*$^{-/-}$ mice die during early embryogenesis (E9.5) owing to failure in the development of the cardiovasculature (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 progenitors 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 *Vegfd* has confirmed that it is not essential for mammalian lymphatic vessel development (Baldwin et al., 2005); however, *Vegfd* 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 they 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).

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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 (LAAs) 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 *ccbe1* 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 (*flt4*^{hu4602}) (Hogan et al., 2009b); or injection of an mRNA encoding the Ig domain of human FLT4 (*sFLT4*) 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 *flt4*^{hu4602} 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. 1F,I,L) due to a defect in secondary sprouting from the PCV (supplementary material Fig. S2). A similar result was observed in embryos injected with a MO against *ccbe1*, with an inhibition of both LFL and TD development (Fig. 1E,J,K,L; supplementary material Fig. S2). There are a number of *lyve1*-positive vessels that remain in the head following knockdown of either *flt4* or *ccbe1* in 3 dpf embryos, but these were confirmed to be veins (PHS, CCV and PCeV) as they retain *kdrl* expression and have blood flow (supplementary material Figs S1, S3).

We used two different MOs designed to prevent the splicing of *vegfc*: *vegfc* MO-1 was designed to bind to the intron 1/exon 2 boundary, whereas *vegfc* MO-2 was designed against the exon 3/intron 3 boundary. We confirmed by RT-PCR that both *vegfc* MO-1 and *vegfc* MO-2 were able to prevent the correct expression of *vegfc* mRNA at 1 dpf (supplementary material Fig. S4). We also found that embryos injected with either of these *vegfc* MOs did not form the TD (Fig. 1G,L) due to a reduction in the number of secondary sprouts from the PCV (supplementary material Fig. S2), confirming that *vegfc* is essential for trunk lymphatic development. However, *vegfc* morphant embryos displayed normal LFL formation, with the length of the LFL being the same as in controls (Fig. 1B,K). Similar results were observed in *vegfc*^{hu5055} embryos that contain a missense mutation, that removes a highly conserved cysteine residue in the C-terminal propeptide of Vegfc (C365R), and that has been previously shown to have a defect in the formation of the TD (Fig. 1C,H,K,L) (Le Guen et al., 2014).

Next, we examined the formation of the FLS by live imaging *lyve1:egfp* embryos from 1.5 dpf when the FLS forms from the CCV. In three embryos injected with a control MO we found that the FLS consistently formed by 1.6 dpf and had migrated towards the PHS by 1.9 dpf (Fig. 2A-E; supplementary material Movie 1). Time-lapse imaging of three *flt4* morphant embryos showed that no embryos developed an FLS by 1.9 dpf, despite all three embryos displaying normal blood vessel development, providing evidence that *flt4* is essential for FLS formation and explaining the inhibition of LFL vessel length at 3 dpf in *flt4* morphant embryos (Fig. 2F-J; supplementary material Movie 2). However, in three embryos injected with *vegfc* MO-1 (the MO with the best efficacy at silencing *vegfc*), we observed normal FLS formation between 1.6 and 1.9 dpf, despite the absence of lymphatic development in the trunk in the same morphant embryos (Fig. 2K-O; supplementary material Movie 3). Taken together, our results show that whereas *flt4* and *ccbe1* are required for development of the facial lymphatics, *vegfc* is not essential for the formation of the FLS and embryos with impaired Vegfc are still able to develop an LFL vessel at 3 dpf.

Next, we examined the effect of the Flt4/Vegfc/Ccbe1 pathway on development of the facial lymphatic network after 3 dpf, when the OLV, MFL and LAA begin to form from the developing LFL (supplementary material Fig. S1). Imaging of 5 dpf *lyve1* embryos showed that injection of either *ccbe1* 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 *flt4*^{hu4602} 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 *vegfc*^{hu5055} embryos and to a lesser extent in *vegfc* morphant embryos (Fig. 1M-R; supplementary material Fig. S3). In addition, although the LFL had extended anteroventrally from the level of the PHS in the majority of *vegfc*^{hu5055} 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 *ccbe1* 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).

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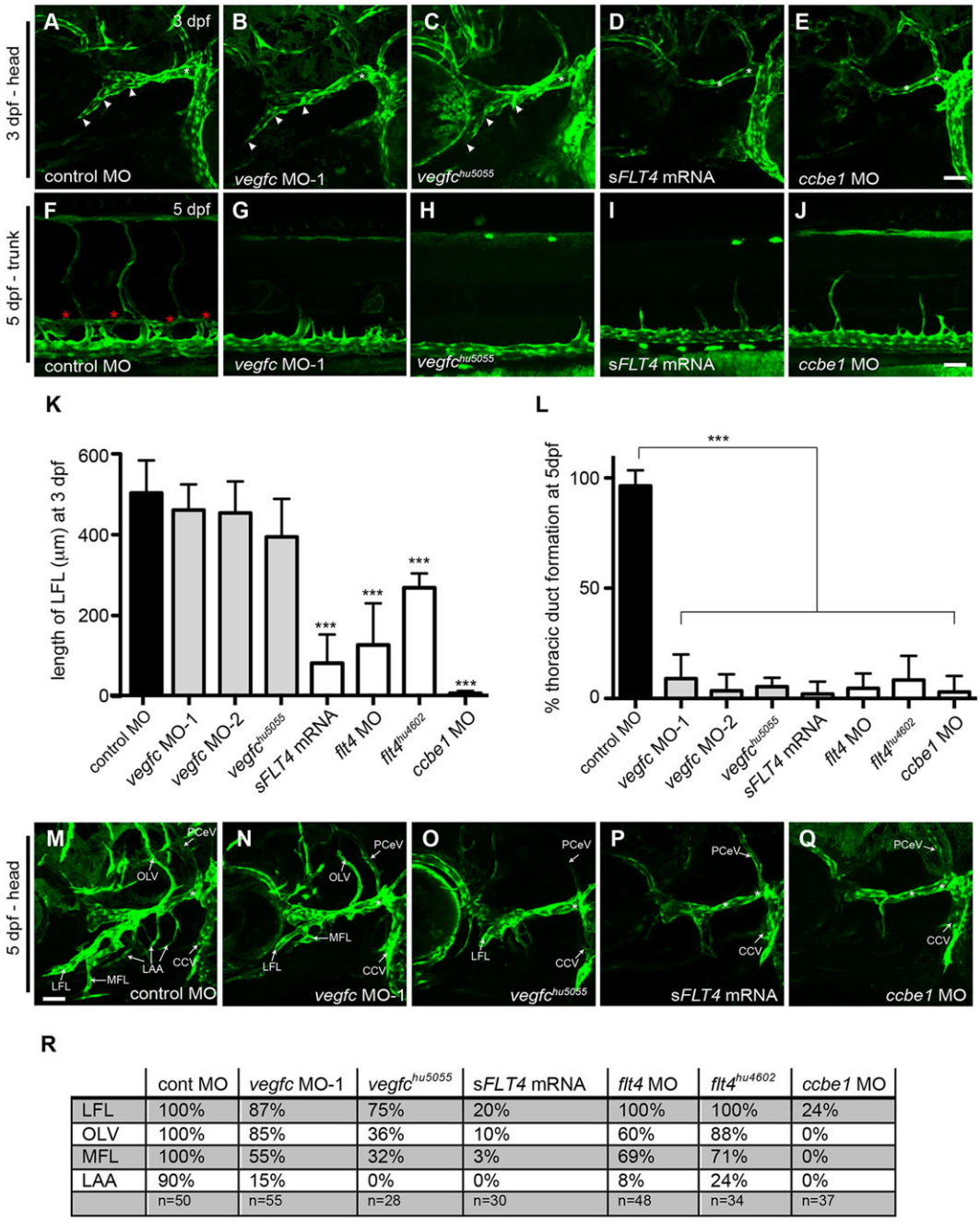

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	cont MO	*vegfc* MO-1	*vegfc*^{hu5055}	s*FLT4* mRNA	*flt4* MO	*flt4*^{hu4602}	*ccbe1* MO
LFL	100%	87%	75%	20%	100%	100%	24%
OLV	100%	85%	36%	10%	60%	88%	0%
MFL	100%	55%	32%	3%	69%	71%	0%
LAA	90%	15%	0%	0%	8%	24%	0%
	n=50	n=55	n=28	n=30	n=48	n=34	n=37

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), *vegfc*^{hu5055} (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 *vegfc*^{hu5055} mutant embryos. (K) Quantitation of LFL length at 3 dpf. (L) Quantitation of TD formation at 5 dpf. (M-Q) Confocal images of the facial lymphatics in *lyve1* embryos at 5 dpf in control MO (M), *vegfc* MO-1 (N), *vegfc*^{hu5055} (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 *flt4*^{hu4602} and *vegfc*^{hu5055} 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.

cxcr4a and *cxcl12a* 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 *cxcrl12a* were expressed in a spatiotemporal manner consistent with a possible role in facial lymphatic development, MO knockdown of *cxcr4a* or *cxcrl12a* 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 *cxcrl12a* morphant embryos. Taken together, our results suggest that, with

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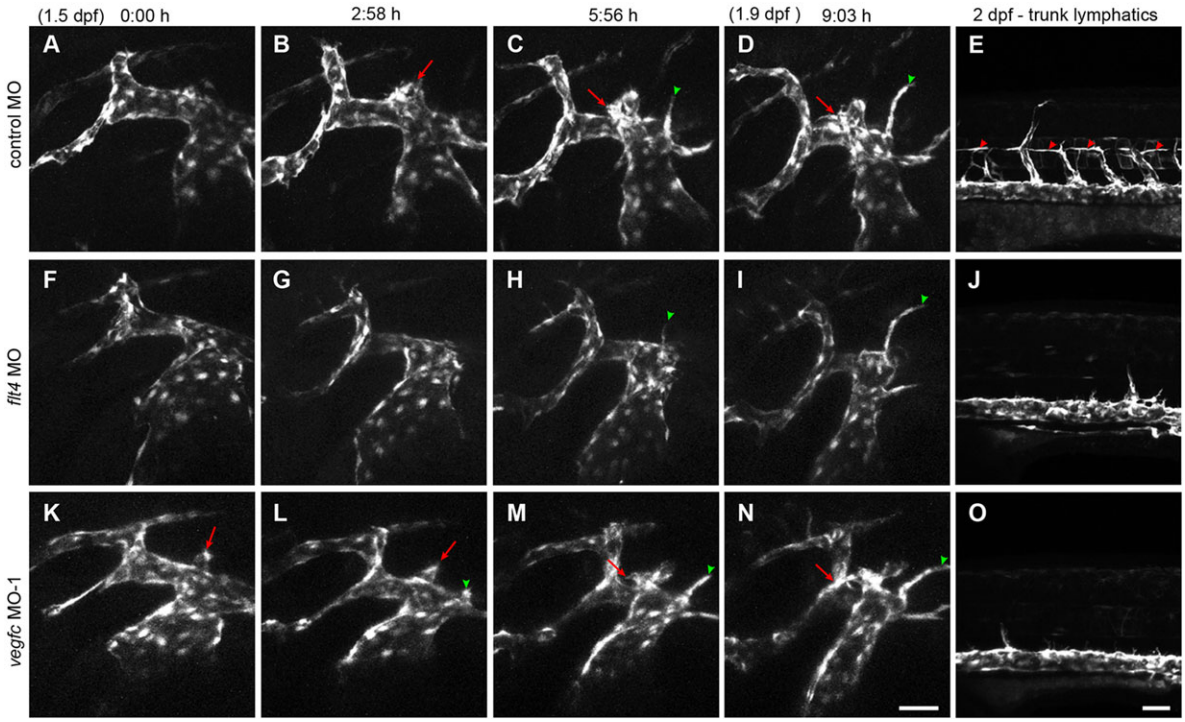

Fig. 2. The facial lymphatic sprout forms normally in *vegfc* morphant embryos. (A-D,F-I,K-N) Stills from confocal time-lapse imaging of the facial lymphatic sprout in *lyve1:egfp* embryos from 1.5 dpf to 1.9 dpf (9:03 h) with (E,J,O) a confocal image of the developing trunk lymphatics in the same embryo 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.

regard to lymphatic development, *cxcr4a* and *cxcrl12a* 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 *ccbe1* 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) and we observed expression of *vegfd* in the vicinity of the developing FLS at 1.5 dpf (Fig. 3F′). Taken together, these data suggest that *vegfd* is expressed in a spatiotemporal manner consistent with a role in the development of facial lymphatics. Quantitative PCR (qPCR) analysis of *vegfd* and *vegfc* mRNA levels in isolated heads of 1.5, 3 and 5 dpf embryos showed that mRNA levels of both genes are maintained from 1.5 to 5 dpf (Fig. 3K).

We were also able to show that *flt4*, *vegfc* and *ccbe1* are all expressed in the trunk, matching the spatiotemporal patterns observed in previous studies and consistent with the essential role of these genes in trunk lymphatic development (Hogan et al., 2009a,b; Kuchler et al., 2006; Villefranc et al., 2013) (Fig. 3; supplementary material Fig. S7). We did not observe expression of *vegfd* in regions of trunk lymphatic development, suggesting that it might not be involved in this process (Fig. 3I), but did observe expression in the pectoral fin buds, which, along with previous reports of *vegfd* expression in the tail bud (Hogan et al., 2009a), suggests a possible role for *vegfd* in fin development.

Previous studies have shown that zebrafish Vegfd, like Vegfc, has angiogenic properties (Hogan et al., 2009b; Song et al., 2007). We were able to confirm this by injecting either *vegfc* or *vegfd* mRNA into embryos expressing GFP in the blood vasculature (*kdrl:egfp*), and we observed that both genes, when expressed ectopically, can induce misguidance of the arterial intersegmental blood vessels (ISVs) along the horizontal myoseptum (Fig. 4A-C). Recently, it has been shown that *ccbe1* can genetically interact with *vegfc*; silencing *ccbe1* can inhibit the ability of *vegfc* to induce branching of the ISVs (Le Guen et al., 2014). We were able to confirm this observation and further showed that *vegfd* and *ccbe1* also interact genetically, as injection of *vegfd* mRNA into *ccbe1* morphants had reduced levels of ISV branching compared to embryos injected with *vegfd* mRNA and control MO (Fig. 4D-G). To determine whether zebrafish *vegfd* can compensate for *vegfc*, we injected *vegfd* mRNA into either *vegfc* morphant embryos or progeny from an incross of *vegfc*^{hu5055} heterozygous carriers and observed that *vegfd* mRNA was able to partially rescue TD development in these embryos with a similar efficacy to that of rescue by *vegfc* mRNA (Fig. 4H-L).

To determine the ability of Vegfd to induce sprouting of *lyve1*-positive vessels, we utilised a xenograft model in which human breast cancer cell lines, transfected with expression vectors containing zebrafish *vegfc* or *vegfd*, were implanted into the perivitelline space of 2 dpf *lyve1:dsred* embryos and then examined at 5 dpf for the formation of ectopic *lyve1*-positive vessels that form from the CCV around the xenograft. We called these ectopic vessels, '*lyve1*-positive vessels' as they also express the blood vessel marker *kdrl* (data not

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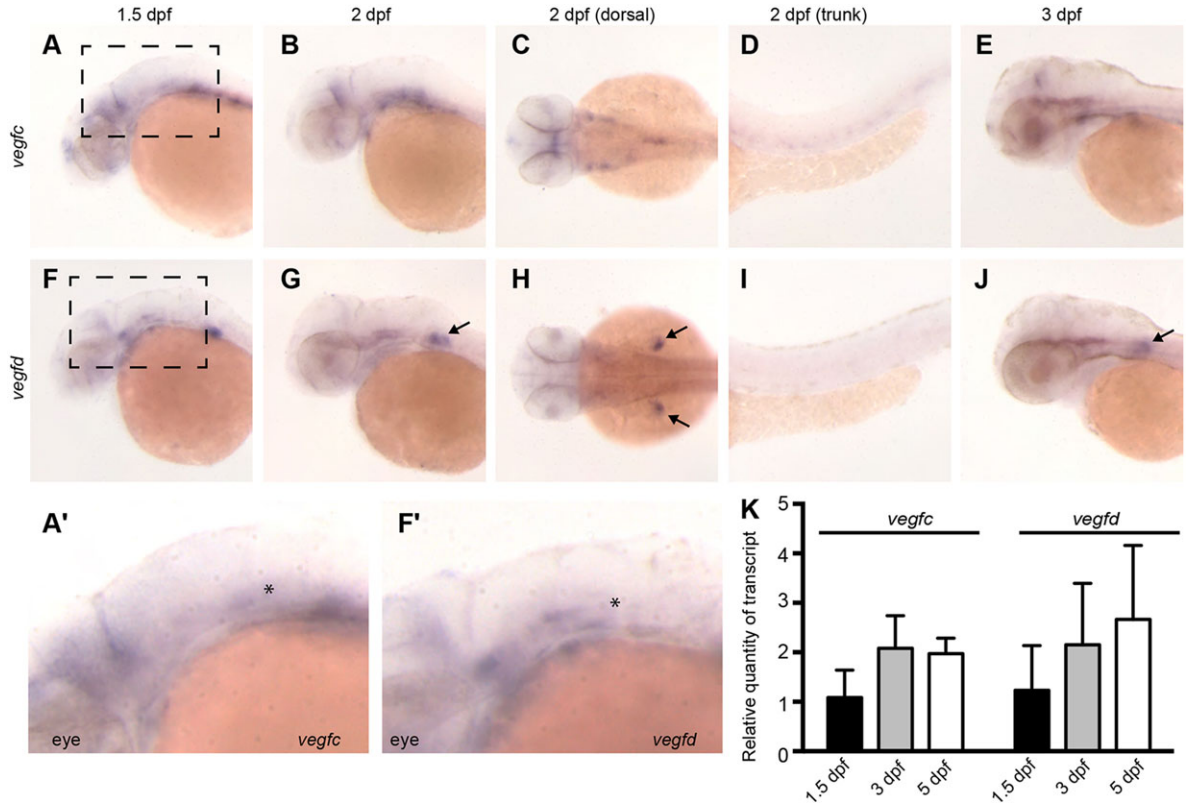

Fig. 3. *vegfd* is expressed in the head and pectoral fin buds. Representative images of whole-mount *in situ* hybridisation showing the expression of *vegfc* (A-E) and of *vegfd* (F-J), in 1.5 dpf (A,F), 2 dpf (B-D,G-I) and 3 dpf embryos (E,J). (C,H) The dorsal aspect of the head region at 2 dpf and (D,I) lateral aspect of the trunk region at 2 dpf. *vegfd* expression in the pectoral fin buds is marked with black arrows in G,H,J. (A′,F′) Magnified images of the boxed regions in A and F; the black asterisks show the approximate position of the FLS. (K) qPCR analysis of *vegfc* and *vegfd* mRNA levels from isolated heads of 1.5, 3 and 5 dpf embryos. Data are shown relative to mRNA levels in 1.5 dpf embryos.

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 *vegfc*^{hu5055} embryos (Fig. 6L,P,Q). Examination of OLV, MFL and LAA vessel formation at 5 dpf revealed that both *vegfc/vegfd* double morphants and *vegfc*^{hu5055} 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 Ccbe1 in development of the intestinal and the facial lymphatics and have found that they are all essential for development of the intestinal

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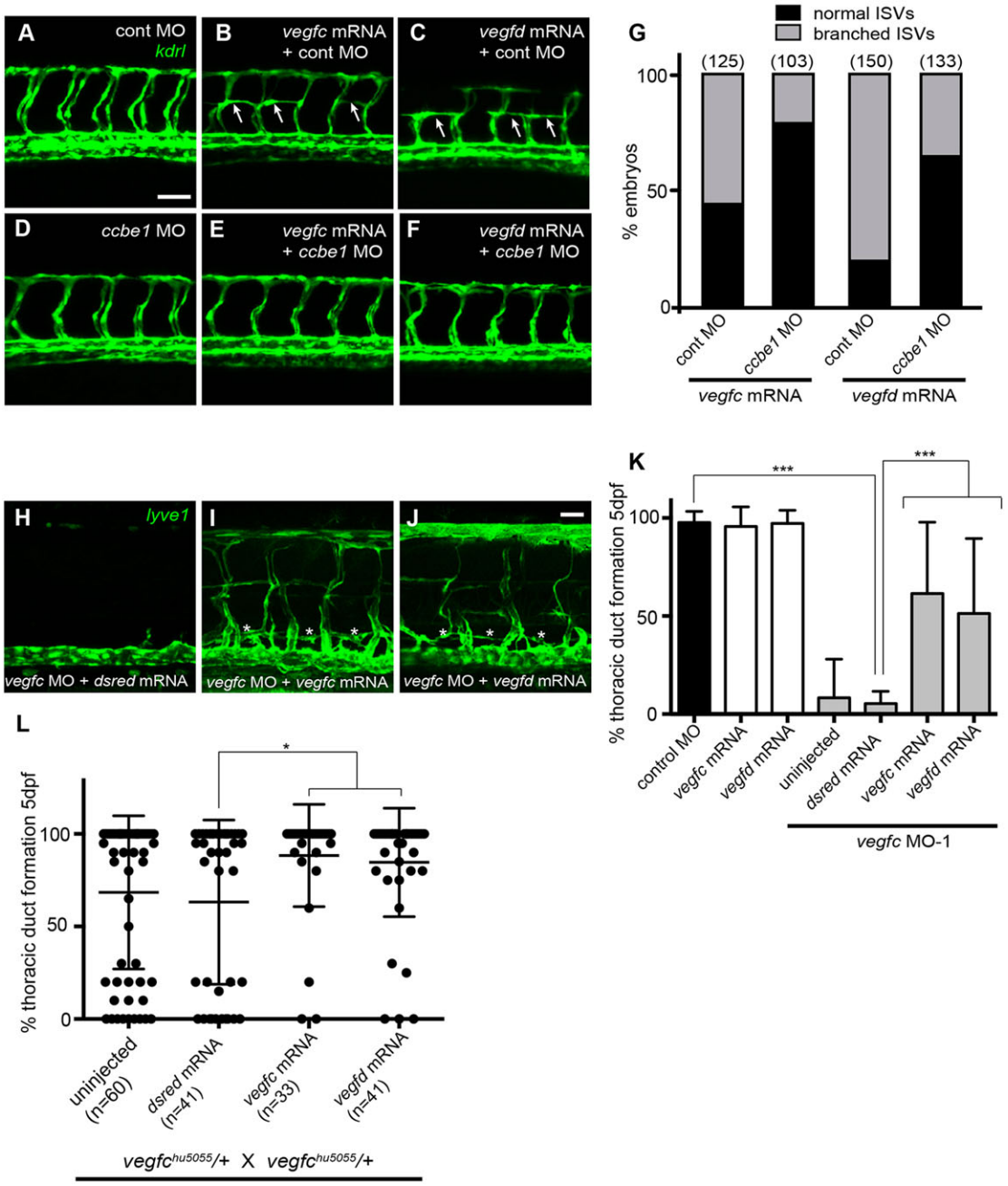

Fig. 4. *vegfd* genetically interacts with *ccbe1* and can compensate for loss of *vegfc*. (A-F) Confocal images of the trunk blood vasculature in 30 hpf *kdrl:egfp* embryos injected with control MO (A-C), *ccbe1* MO (D-F), 400 pg *vegfc* mRNA (B,E) and 200 pg *vegfd* mRNA (C,F). Injection of either *vegfc* or *vegfd* mRNA induced misguidance of the intersegmental blood vessels along the horizontal myoseptum (arrows). (G) The percentage of embryos with branched ISVs; numbers in brackets denote number of embryos scored. Silencing *ccbe1* reduces the number of embryos with branched ISVs induced by injection of either *vegfc* or *vegfd* mRNA. (H-J) Confocal images of the trunk lymphatics in 5 dpf *lyve1:egfp* embryos injected with *vegfc* MO-1+*dsred* mRNA (H), *vegfc* MO-1+400 pg *vegfc* mRNA (I) and *vegfc* MO-1+200 pg *vegfd* mRNA (J). Injection of either *vegfc* or *vegfd* mRNA can rescue the formation of the TD (asterisks) in *vegfc* morphant embryos. (K) Quantitation of TD formation at 5 dpf. (L) Quantitation of TD formation at 5 dpf in F1 progeny from an incross of *vegfc*^{hu5055} heterozygous carriers. ***P<0.001, *P<0.05 by a Mann–Whitney test; error bars indicate s.d. Scale bars: 50 μm.

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 *cxcr4a* or *cxcl12a* 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 *ccbe1* 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

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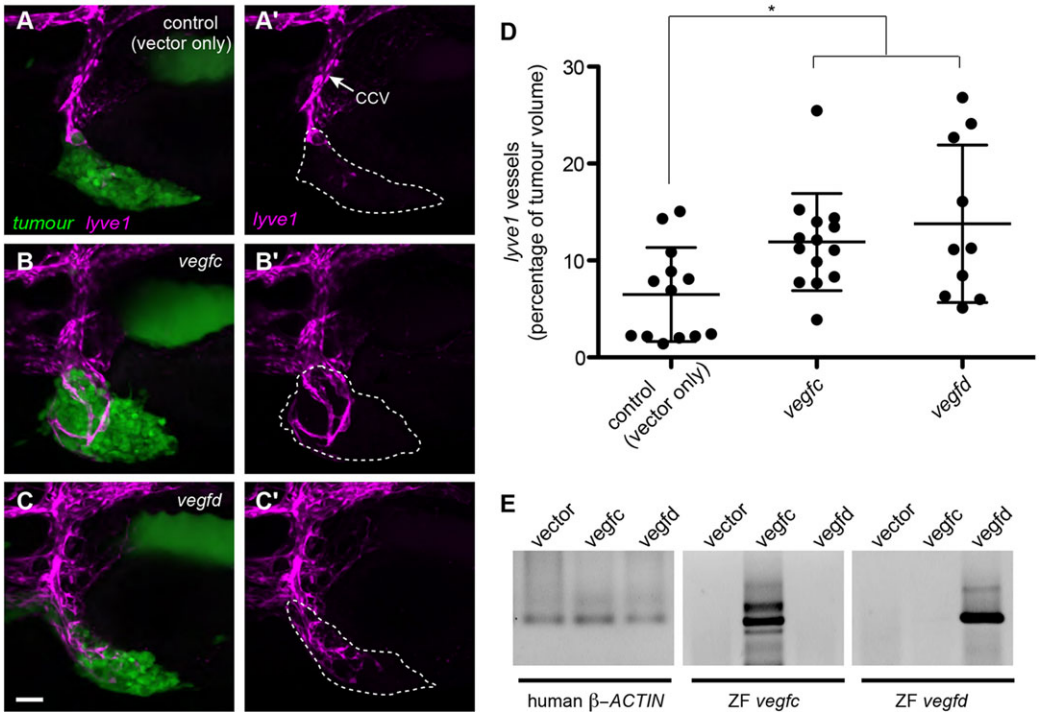

Fig. 5. Expression of *vegfd* can induce ectopic *lyve1*-positive vessels. (A-C) Confocal images of human breast cancer cells labelled with Cell Tracker Green (MDA-MD-231) xenografted into *lyve1:dsred* embryos and imaged at 5 dpf. (A) MDA-MD-231 cells transfected with vector only. (B) MDA-MD-231 cells 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-MB-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*^{hu5055}) 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*^{hu5055} 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*^{hu5055} 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*^{hu5055} 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; Kataru et al., 2009). It has also been shown to compensate for loss of VEGFC (Haiko et al., 2008; Karkkainen 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,

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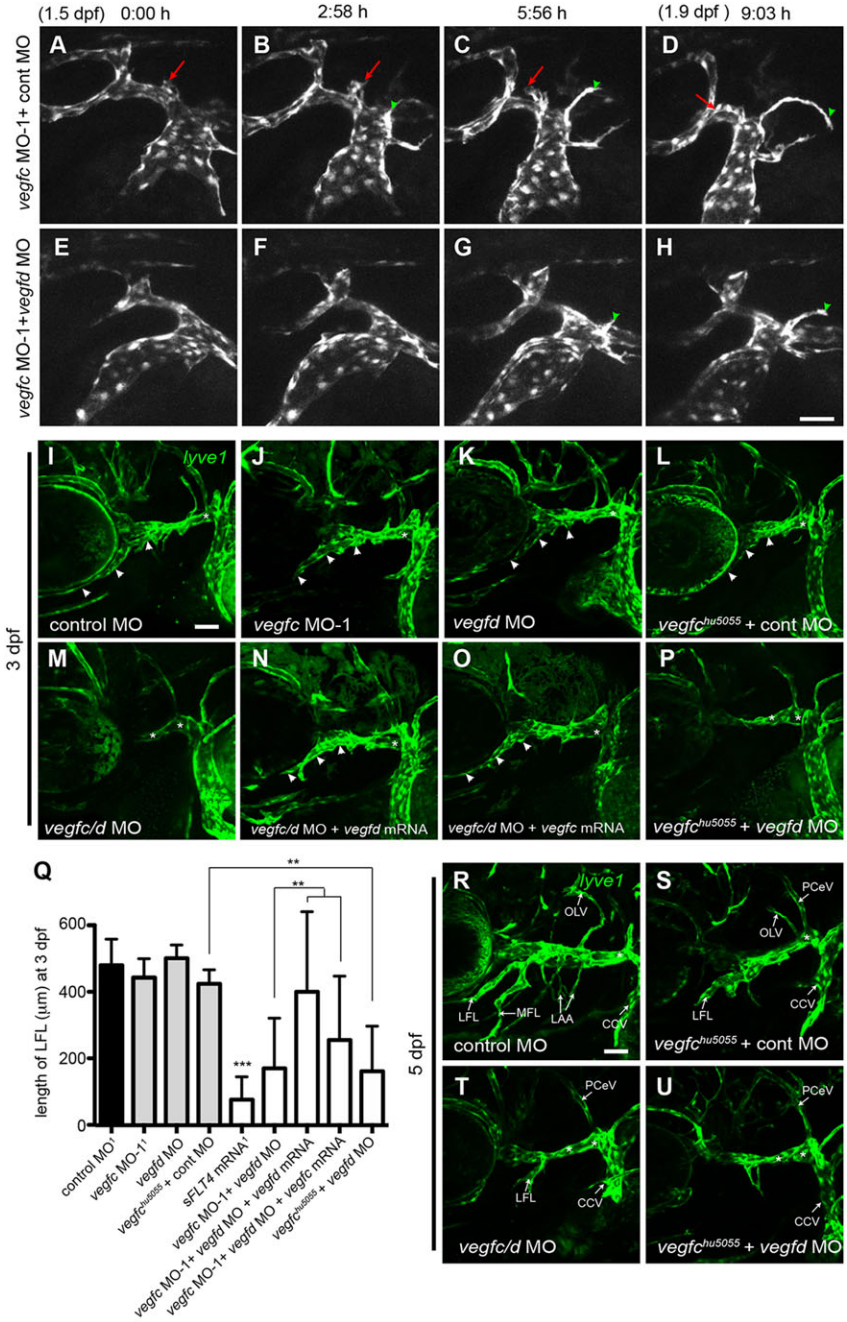

V

	cont MO[1]	*vegfc*^{hu5055} cont MO	*vegfc* MO-1 *vegfd* MO	*vegfc*^{hu5055} *vegfd* MO	s*FLT4* mRNA[1]
LFL	100%	69%	27%	45%	20%
OLV	100%	36%	10%	13%	10%
MFL	100%	28%	0%	0%	3%
LAA	90%	0%	0%	0%	0%
	n=50	n=32	n=45	n=40	n=30

Fig. 6. Knockdown of both *vegfc* and *vegfd* is required to prevent facial lymphatic development. (A-H) 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) injected with either *vegfc* MO-1 and control MO (A-D, supplementary material Movie 4) or *vegfc* MO-1 and *vegfd* MO (E-H, supplementary material Movie 5). Data are representative of three independent time-lapse experiments. The facial lymphatic sprout (red arrows) forms in *vegfc* MO-1 and control MO morphants but is not present in double-knockdown morphant embryos. The formation of the pectoral vein (green arrowheads) serves as a control to show that the embryos are developing normally. (I-P) Confocal images of the facial lymphatics in *lyve1* embryos at 3 dpf in control MO (I), *vegfc* MO-1 (J), *vegfd* MO (K), *vegfc*^{hu5055}+control MO (L), *vegfc* MO-1 +*vegfd* MO (M), *vegfc* MO-1+*vegfd* MO+200 pg *vegfd* mRNA (N), *vegfc* MO-1+*vegfd* MO+400 pg *vegfc* mRNA (O) and *vegfc*^{hu5055}+*vegfd* MO (P). Silencing either *vegfc* or *vegfd* has no effect on early facial lymphatic development but double knockdown of both genes prevents the formation of the LFL at 3 dpf (arrowheads). (Q) Quantitation of the length of the LFL vessel in 3 dpf embryos ([1]data reproduced from Fig. 1 for comparison). (R-U) Confocal images of the facial lymphatics in *lyve1* embryos at 5 dpf in control MO (R), *vegfc*^{hu5055}+control MO (S), *vegfc* MO-1+*vegfd* MO (T) and *vegfc*^{hu5055}+*vegfd* MO (U). Asterisks indicates the PHS. (V) The percentage formation of different facial lymphatic vessels at 5 dpf. ([1]data reproduced from Fig. 1 for comparison). For *vegfc*^{hu5055} embryos, *n*=number of mutant embryos. Knockdown of both *vegfc* and *vegfd* results in a more severe defect in the development of the facial lymphatic network than knockdown of *vegfc* alone. 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, **P<0.01 by a Mann–Whitney test, unless indicated, significance was determined to control MO; error bars indicate s.d. Scale bars: 50 μm.

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 *ccbe1* or embryos with impaired Flt4 signalling. It has been shown that Ccbe1 is required for the activity of Vegfc (Jeltsch et al., 2014; Le Guen et al., 2014) and our data suggest that Ccbe1 is also required for Vegfd activity. In support of this we found that *ccbe1* and *vegfd* genetically interact, as silencing *ccbe1* 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

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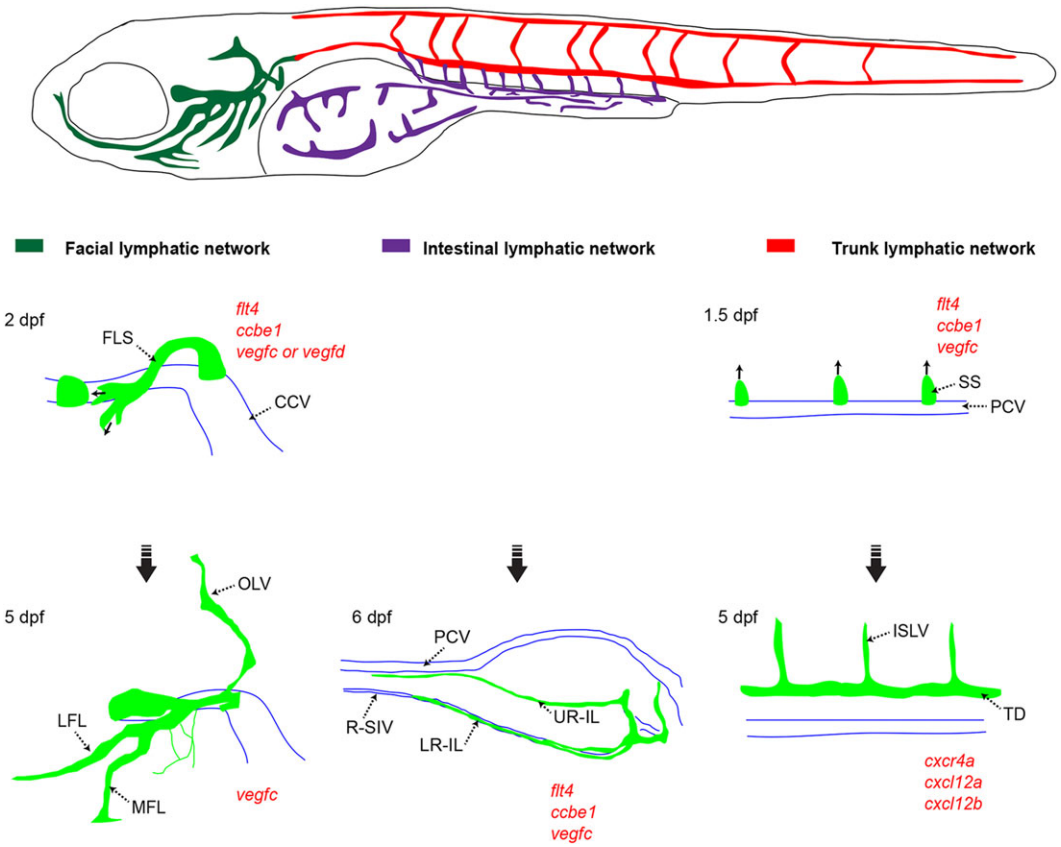

Fig. 7. Lymphatic networks have distinct genetic requirements. Genes required for the development of the facial, intestinal or trunk lymphatic networks during early (1.5-2 dpf) and late (5-6 dpf) lymphatic development are given in red. Whereas *flt4* and *ccbe1* are required for the development of all lymphatic networks, *vegfc* is not essential for early facial lymphatic development due to the expression of *vegfd* in the head. *cxcr4a* and *cxcl12a* are required for the formation of the trunk lymphatics but are not necessary for normal facial or intestinal lymphatic development. CCV, common cardinal vein; FLS, facial lymphatic sprout; ISLV, intersegmental lymphatic vessel; LFL, lateral facial lymphatic; MFL, medial facial lymphatic; OLV, otolithic lymphatic vessel; LR-IL, lower right intestinal lymphatic; UR-IL, upper right intestinal lymphatic; R-SIV, right subintestinal vein; PCV, posterior cardinal vein; SS, secondary sprout; TD, thoracic duct.

head region. However, why *vegfd* is expressed in this region and not in other areas of lymphatic vessel development, and whether *vegfd* plays an active role in facial lymphatic development, are not clear. In mammalian embryos, *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 *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 *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 *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 *vegfd*. However, we do show that *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 have 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), *TG(lyve1:egfp)*^{nz150}, *TG(lyve1:dsred)*^{nz101} (Okuda et al., 2012), *TG(kdrl:egfp)*^{s843} (Jin et al., 2005), *TG(kdrl:rfp)*^{la4} (Huang et al., 2005), *flt4*^{hu4602} (Hogan et al., 2009b) and *vegfc*^{hu5055} (Le Guen et al., 2014).

Whole-mount *in situ* hybridisation

In situ hybridisation was performed as described previously (Thisse and Thisse, 2008). Antisense RNA probes for *ccbe1* (Hogan et al., 2009a), *flt4*, *vegfc* (Hogan et al., 2009b), *cxcr4a*, *cxcl12a* and *cxcl12b* (Cha et al., 2012)

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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 *in vitro* transcription kit (Ambion) from an Sp6 primer and 200 pg (*sFLT4*, *vegfd*) or 400 pg (*vegfc*) mRNA 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 (Improvision/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 anteroventrally 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 *lyve1:dsred*-expressing vessels around and in the tumour was determined by Volocity 5.4 image analysis software and expressed as a percentage of 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 (Oehlers 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

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.106591/-/DC1

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