Vegfc is required for vascular development and endoderm morphogenesis in zebrafish

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During embryogenesis, complex morphogenetic events lead endodermal cells to coalesce at the midline and form the primitive gut tube and associated organs. While several genes have recently been implicated in endoderm differentiation, we know little about the genes that regulate endodermal morphogenesis. Here, we show that vascular endothelial growth factor C (Vegfc), an angiogenic as well as a lymphangiogenic factor, is unexpectedly involved in this process in zebrafish. Reducing Vegfc levels using morpholino antisense oligonucleotides, or through overexpression of a soluble form of the VEGFC receptor, VEGFR-3, affects the coalescence of endodermal cells in the anterior midline, leading to the formation of a forked gut tube and the duplication of the liver and pancreatic buds. Further analyses indicate that Vegfc is additionally required for the initial formation of the dorsal endoderm. We also demonstrate that Vegfc is required for vasculogenesis as well as angiogenesis in the zebrafish embryo. These data argue for a requirement of Vegfc in the developing vasculature and, more surprisingly, implicate Vegfc signalling in two distinct steps during endoderm development, first during the initial differentiation of the dorsal

endoderm, and second in the coalescence of the anterior endoderm to the midline.

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INTRODUCTION

Members of the vascular endothelial growth factor (VEGF) family (PlGF, VEGFA, VEGFB, VEGFC, VEGFD) and their receptors (VEGFR-1/Flt1, VEGFR-2/Flk1, VEGFR-3/Flt4 and Neuropilin—a (co)-receptor for some of the isoforms of VEGF family members) are essential components in vasculogenesis and angiogenesis (Carmeliet, 2000). VEGFC (a ligand for VEGFR-3) can, on proteolytic processing, also activate VEGFR-2 and thus promote angiogenesis as well as lymphangiogenesis (Jeltsch *et al*, 1997; Oh *et al*, 1997; Cao *et al*, 1998; Witzensbichler *et al*, 1998). While the effects of these growth factors on endothelial cells have received much attention due to their potential therapeutic applications, for example, in cardiovascular diseases and tumour angiogenesis, little is known about their roles beyond the classical endothelial target cell. Recent studies in *Drosophila* have revealed a requirement for Pdgf/Vegf signalling in two independent processes during early stages of development. In the oocyte, border cell migration is regulated by a Pdgf/Vegf-type receptor (Duchek *et al*, 2001). Furthermore, the same receptor is implicated in blood cell migration at a later stage during *Drosophila* development (Cho *et al*, 2002).

While studies have focused mainly on the function of VEGFA, the roles of VEGFB, VEGFC and VEGFD either in vasculogenesis or beyond are less clear (Carmeliet, 2000; Veikkola *et al*, 2000). We have taken advantage of the zebrafish system to address the role of VEGFC during development. Our findings suggest a requirement for vascular endothelial growth factor C (Vegfc) in the developing vasculature and, more surprisingly, implicate Vegfc signalling in two independent steps during endoderm development. At first, Vegfc is required during the initial differentiation

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of the dorsal endoderm, and in a second phase for the coalescence of the anterior endoderm to the midline.

RESULTS AND DISCUSSION

A full-length zebrafish *vegfc* cDNA was isolated (see supplementary information online for sequence alignment) and expressed in 293-T cells. Like its mammalian homologues, it undergoes proteolytic processing, interacts with human VEGFR-3 and VEGFR-2 (Fig 1), and is able to induce autophosphorylation of human VEGFR-3 (data not shown).

Comparative *in situ* hybridization analyses of *vegfc*, *vegfa* (zebrafish orthologue of mammalian VEGFA; Liang *et al*, 1998) and two zebrafish *vegfr* receptor genes, *flt1* (Fouquet *et al*, 1997; Liao *et al*, 1997; Sumoy *et al*, 1997; Thompson *et al*, 1998) and *flt4* (Thompson *et al*, 1998), show distinct but partially overlapping expression patterns. All four genes are expressed maternally (data not shown). Subsequently, *vegfc* expression becomes prominent in the developing brain, the dorsal aorta and trunk mesoderm (expression patterns from the 10-somite stage to 49 hours post-fertilization (hpf) can be found as supplementary information online). Starting at mid-somitogenesis stages, *vegfc* expression is generally found in areas adjacent to *flt4*-expressing cells. The proximity of expression in combination with the binding studies suggests Flt4 as the potential *in vivo* receptor of Vegfc in zebrafish.

In order to address the role of Vegfc during zebrafish embryogenesis, we knocked down Vegfc levels using antisense morpholino oligonucleotides (Summerton & Weller, 1997). We found that reduced levels of Vegfc led to pronounced pericardial oedema and a failure to establish blood circulation (data not shown), resembling the phenotype observed in *VEGFR-3/Flt4*-deficient mouse embryos (Dumont *et al*, 1998). In the trunk, the dorsal aorta appeared to be incompletely formed, as indicated by the patchy appearance of alkaline phosphatase (AP) staining (Fig 2A–D) and *flt1* expression (data not shown). The posterior cardinal vein (PCV) was most sensitive to reduced Vegfc levels, and in the most severe cases appeared to be completely missing (Fig 2A–D; data not shown). In addition, the intersegmental vessels (Fig 2A–D) and the central arteries (Fig 2G,H, arrowhead), which sprout from the basilar artery in the midbrain/hindbrain region, also appeared to be absent. Two different morpholinos designed to knock down Vegfc levels led to the same phenotype even when injected at relatively low concentrations (1.9–2.5 ng per embryo, see Methods), whereas a number of control morpholinos did not cause this phenotype even when injected at high concentrations (5–10 ng). In addition, we observed a similar phenotype in embryos injected with mRNA encoding a soluble version of human VEGFR-3/Flt4 (sFlt4), which specifically binds and sequesters zebrafish Vegfc but not zebrafish Vegf (Fig 1), further confirming the specificity of the *vegfc* morpholinos (Fig 2E,F). Interestingly, the Vegfc knock-down embryos have a more profound defect than the zebrafish *flt1* mutant (Habeck *et al*, 2002), possibly because Vegfc activates both Flk1 and Flt4. Despite their severe vascular phenotype, the Vegfc knock-down embryos did not exhibit any discernible difference in *gata1* (Detrich *et al*, 1995) expression at mid-somitogenesis stages (data not shown), indicating that Vegfc does not regulate red blood cell differentiation. Altogether these data show that Vegfc has important functions in both vasculogenesis and angiogenesis in zebrafish.

Fig 1 | Zebrafish Vegfc binds human VEGFR-3. The VEGF homology domain of zebrafish Vegfc (zVegfcΔNΔC) (aa 92–212) also interacts with human VEGFR-2. Zebrafish Vegf binds to both human VEGFR-1 and VEGFR-2 but not VEGFR-3. For expression in mammalian cells, the sequences encoding the full-length Vegfc or the VEGF-homology domain (zVegfcΔNΔC) were cloned into the pSecTag vector (Invitrogen), which includes an immunoglobulin κ signal sequence and a C-terminal Myc epitope. 293-T or 293-EBNA cells transfected with the Vegf expression plasmids were metabolically labelled with 100 μCi/ml Pro-mix L-³⁵S (Amersham Pharmacia Biotech) for 6–7 h in serum-free culture medium. VEGFR-Ig fusion proteins were used to precipitate the metabolically labelled VEGFs. An anti-myc antibody was used for the immunoprecipitation shown in lane 1 (IP). The full-length 60 kDa (size corresponds to estimations from reducing SDS-PAGE analysis without the myc tag) zVegfc is proteolytically processed similar to human VEGFC (Joukov *et al*, 1997), generating major polypeptides of approximately 35 and 30 kDa and minor polypeptides of 28 and 18 kDa.

Surprisingly, while analysing the vasculature of embryos injected with *vegfc* morpholinos or sFlt4, we frequently observed a duplication of the presumed liver buds, which also contain endogenous AP activity. Examining the expression of the endodermal marker *foxA1/HNF3α/flk7* (Odenthal & Nüsslein-

Fig 2 | Vegfc is required for vasculogenesis and angiogenesis. Dorsal (A,C,E) and lateral (B,D,F) views of 72 hpf embryos stained for AP activity, anterior to the left. The intersegmental vessels (arrowhead) and dorsal aorta (arrow) are clearly distinct in the control embryos (A,B), but are absent or patchy in the Vegfc knock-down embryos (C,D) and in the embryos overexpressing soluble Flt4 (E,F). (G,H) Lateral views of 56 hpf embryos stained for AP activity, anterior to the left. The central arteries (arrowhead) are missing in the Vegfc knock-down embryos.

Volhard, 1998) at 48 hpf revealed that reducing Vegfc levels led to a unique and previously undescribed phenotype, in which the anterior gut endoderm is split. In the most severe cases, seen in 28% of the injected embryos ($n=509$), the anterior gut tube split just posterior to where the pancreas forms. Consequently, these embryos exhibited a V-shaped anterior gut (Fig 3B,D). In another 45% of the injected embryos, the split terminated more anteriorly (Fig 3C). Interestingly, the split never extended further posterior than the pancreatic domain, indicating that the loss of Vegfc affects only the anteriormost endoderm. Each side of the split anterior endoderm displayed bud-like outgrowths that could be found on the lateral, medial or ventral sides of the V (data not

shown). We used organ-specific markers to determine the identity and analyse the state of differentiation of these endodermal outgrowths: we found that the anterior buds expressed normal levels of liver markers such as *hhx* (Ho et al, 1999), *prox1* (Glasgow & Tomarev, 1998) and *ceruloplasmin* (Korzh et al, 2001), while the posterior buds expressed normal levels of pancreatic markers such as *pdx1* (Milewski et al, 1998). These data indicate that the anteroposterior patterning of the gut endoderm and endodermal organ differentiation is unaffected in the Vegfc knock-down embryos.

To understand the origin of the split-gut phenotype, we examined endodermal morphogenesis at earlier stages. In contrast

Fig 3 | Vegfc is required for endodermal morphogenesis. (A–D) *foxA1* expression at 48 hpf. Dorsal views, anterior to the left. Vegfc knock-down embryos (B,C) and sFlt4-overexpressing embryos (D) show a splitting of the anterior gut tube, in the most severe cases (28%; $n = 509$) accompanied by a duplication of the liver (L; arrows) and pancreatic buds (red arrowheads; pancreatic and swimbladder anlagen marked as points of reference to appreciate the extent of the split). In wild-type embryos (A), the anteriormost endoderm (or pharyngeal endoderm (PE)) consists of a thin sheet of cells across the midline with condensed groups of cells on the edges (black arrowheads point to these lateral edges of the PE). In the Vegfc knock-down and sFlt4-overexpressing embryos, PE cells (black arrowheads) fail to occupy the midline region. In contrast, Vegfc-depleted embryos do not exhibit similar defects in endoderm formation, emphasizing the specificity of the phenotypes observed in Vegfc knock-down embryos (data not shown). MO: morpholino.

to the control embryos, which exhibit a continuous sheet of *foxA1*-expressing cells across the midline at the 15-somite stage, the Vegfc knock-down embryos had distinctly bilateral populations of endodermal cells anteriorly (Fig 4A,B). We also examined the expression of the endodermal marker *sox17* (Alexander & Stainier, 1999) at various stages during gastrulation. By 90% epiboly, the Vegfc knock-down embryos exhibited a clear reduction of endodermal cells on the dorsal side (Fig 4C,D). This phenotype could be due to a local defect in endoderm formation or a defect in endoderm migration to the dorsal side. To distinguish between these possibilities, we analysed endodermal induction by examining the expression of *bonnie and clyde* (*bon*), a mix-type homeobox gene implicated in this process (Alexander et al, 1999; Kikuchi et al, 2000). We found that *bon* expression was also specifically reduced on the dorsal side in 42% of the Vegfc knock-down embryos ($n = 33$; Fig 4E,F). This dorsal reduction of *bon* and *sox17* expression in Vegfc knock-down embryos indicates that lowering Vegfc signalling affects the formation of the dorsal endoderm. This defect appears to be specific to the endoderm as the expression of *no tail* (Schulte-Merker et al, 1992), a mesendodermal gene that is not required for endoderm formation, appeared unaffected in Vegfc knock-down embryos (data not shown). The reduction in *bon* expression in Vegfc knock-down embryos is similar to that seen in embryos lacking zygotic Squint (*Sqt*) function (Alexander & Stainier, 1999), leading us to examine gut tube formation in *sqt* mutant embryos.

Despite a reduction in dorsal endoderm, *sqt* mutant embryos form a continuous gut tube (L. Trinh and D.Y.R. Stainier, unpublished observations; Fig 4G), indicating that a reduction in dorsal endoderm does not necessarily cause a split-gut phenotype; however, reducing Vegfc levels in *sqt* mutant embryos led to a split-gut phenotype in 42% of the cases ($n = 57$; Fig 4H). Altogether, these data indicate that Vegfc regulates two distinct steps during endoderm development: an early step that leads to the formation of the dorsal endoderm, and a later step that leads to the coalescence of the anterior endoderm to the midline. Furthermore, these data also argue that differentiation of the endodermal organs is not dependent on the coalescence of their precursors to the midline.

As we were evaluating receptors that could mediate Vegfc function in the embryo, it was reported that *nrp1*, although expressed mainly in neuronal and vascular tissue, is also expressed in the anterior portion of the endoderm during early somitogenesis (Lee et al, 2002; Shoji et al, 2003). This expression pattern prompted us to look for a possible biochemical interaction between Nrp1 and Vegfc. We found that partially processed human and zebrafish VEGFC (29/31 kDa form) interacted with mouse Nrp1 (see supplementary information online Fig 2). Injection of *nrp1* morpholinos led to a vascular phenotype similar to that shown by Lee et al (2002), but we did not detect any defects in early endoderm formation, or in the coalescence of endodermal cells to the midline. This lack of endodermal

Fig 4 | Vegfc is required for endoderm formation and morphogenesis. (A,B) *foxA1* expression at 15 somites (16.5 hpf). Dorsal views, anterior to the left. *foxA1*-expressing endodermal cells (arrows) stay bilateral in the Vegfc knock-down embryos (B). Arrowheads point to the notochord, which also expresses *foxA1*. (C,D) *sox17* expression at 90% epiboly (9 hpf): dorsal views, animal pole to the top (these embryos were slightly overstained to assess the phenotype fully). Vegfc knock-down embryos exhibit a reduction in the number of *sox17*-expressing endodermal cells specifically on the dorsal side. (The delay in dorsal compaction of forerunner cells (arrowheads) observed in the Vegfc knock-down embryos is sometimes observed in control injected embryos (data not shown).) (E,F) *bon* expression at 50% epiboly (5.3 hpf): animal pole views, dorsal to the right. Vegfc knock-down embryos exhibit a reduction in *bon* expression specifically on the dorsal side, while *no tail* expression is not affected (data not shown). (G,H) *foxA1* expression at 34 hpf. Dorsal views, anterior to the left. Whereas uninjected *sqt* mutant embryos ($n = 31$) show a continuous gut tube, those injected with Vegfc morpholinos exhibit a split gut tube anteriorly (42%; $n = 57$). MO: morpholino.

phenotype in Nrp1-deficient embryos may be due to a functional overlap of Nrp1 with other Neuropilins. For example, it has been shown that in mammals Nrp2 also interacts with Vegfc (Karkkainen *et al*, 2001). Furthermore, knocking down another Vegfc receptor, Flt4, while causing a defect in intersegmental vessel formation, did not affect endoderm formation during late blastula/early gastrula stages or during early somitogenesis stages (E.A. Ober, N. Lawson and D.Y.R. Stainier, unpublished observations), suggesting that other receptors mediate Vegfc function during endoderm formation. Conversely, possible maternal deposits of Nrp1 and Flt4 may also play a role during these early stages of endoderm development (Lee *et al*, 2002; data not shown). In addition, it is likely that there are additional Vegfc receptor genes present in the zebrafish genome, as analysis of the Fugu genome (<http://genome.jgi-psf.org/fugu6/fugu6.home.html>) indicates that there are at least four members of this family in contrast to three in mammals.

Studies in vertebrates have so far identified blood, vascular and neuronal cells as the sole targets of VEGF function. In the *Drosophila* oocyte, border cell migration is regulated by a Vegf/Pdgf-type receptor (Duchek *et al*, 2001). Our data indicate that Vegfc plays an important role in endoderm development in zebrafish. Specifically, we propose that Vegfc signalling modulates endoderm formation during late blastula/early gastrula stages, and the coalescence of the endodermal cells to the midline during early somitogenesis stages. Similarly, in *Xenopus*, VEGF has been shown to direct angioblasts to the midline (Cleaver & Krieg, 1998). Importantly, Vegfc is the first factor implicated in the coalescence of endodermal cells to the midline, and it is likely that many others will be identified.

Vegfc may also be involved in later steps of endoderm morphogenesis. Recent reports have indicated a role for the nascent vasculature in liver (Matsumoto *et al*, 2001) and pancreas (Lammert *et al*, 2001) development, and it will be important to determine whether these effects are directly related to the function of a VEGF family member. Consistent with this idea, it has been shown that activation of the VEGFR-1 pathway in the hepatic endothelium regulates the secretion of mitogenic and survival factors that affect neighbouring hepatocytes (LeCouter *et al*, 2003). While these findings focus on roles of Vegf signalling in later steps of organ formation, our data indicate that Vegfc regulates earlier aspects of endoderm development and, most importantly, argue for a more widespread function of this VEGF family member than previously appreciated.

METHODS

Injections and *in situ* hybridizations. Zebrafish (*Danio rerio*) embryos, wild type or *golden*, were raised at 28 °C under standard laboratory conditions. In some cases, wild-type embryos were grown in the presence of 0.003% 1-phenyl-2-thiourea to reduce pigmentation. The *vegfc* antisense morpholinos 5'-GAAAATC CAAATAAGTGCATTTTAG-3' and 5'-AGACAGAAAATCCAAAT AAGTCAT-3', *vegfc* antisense morpholinos 5'-CTCGTCTTATTT CCGTGACTGTTTT3', *nrp1* antisense morpholinos 5'-GGATCAA CACTAATCCACAATGCAT-3' (Gene Tools, LLC) and control morpholinos were resuspended in 5 mM Hepes (pH 7.6) and injected at the 1–2-cell stage at different concentrations (1.9–7.8 ng/embryo).

Soluble Flt4 consists of the first three extracellular immunoglobulin-homology domains of human VEGFR-3 fused to a human IgG Fc domain (Mäkinen *et al*, 2001). Whole-mount *in situ* hybridizations were performed essentially as described (Alexander & Stainier, 1999) using single-stranded digoxigenin-UTP (Boehringer-Mannheim)-labelled RNA probes. The *vegfc* probe spans bp 537–995 of the cDNA in the 5' coding region (*vegfc* GenBank accession number AF466147). For histology, the stained embryos were dehydrated, embedded in JB-4 (Polysciences) and cut at 5 µm. Endogenous AP activity was detected according to standard techniques. Transfections, immunoprecipitations and soluble receptor binding were performed as described (Achen *et al*, 1998; Mäkinen *et al*, 2001). The zebrafish Vegfc Δ NAC construct encodes amino-acid (aa) residues 92–212.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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