Cellular and molecular analyses of vascular tube and lumen formation in zebrafish

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Summary

Tube and lumen formation are essential steps in forming a functional vasculature. Despite their significance, our understanding of these processes remains limited, especially at the cellular and molecular levels. In this study, we analyze mechanisms of angioblast coalescence in the zebrafish embryonic midline and subsequent vascular tube formation. To facilitate these studies, we generated a transgenic line where EGFP expression is controlled by the zebrafish *flk1* promoter. We find that angioblasts migrate as individual cells to form a vascular cord at the midline. This transient structure is stabilized by endothelial cell-cell junctions, and subsequently undergoes lumen formation to form a fully patent vessel. Downregulating the VEGF signaling pathway, while affecting the number of angioblasts, does not appear to affect their migratory behavior. Our studies also indicate that the endoderm, a tissue previously implicated in vascular development, provides a substratum for endothelial cell migration and is involved in regulating the timing of this process, but that it is not essential for the direction of migration. In addition, the endothelial cells in endodermless embryos form properly lumenized vessels, contrary to what has been previously reported in *Xenopus* and avian embryos. These studies provide the tools and a cellular framework for the investigation of mutations affecting vasculogenesis in zebrafish.

Key words: Endothelial cell, Migration, Endoderm, VEGF, Angioblast, Zebrafish

Introduction

The formation of a functional vascular system is essential for the proper development of vertebrate embryos, as well as for the survival of adults. The vascular system provides oxygen, carries away metabolic waste products, serves as the conduit for hormones and provides space for the immune response. Owing to its clinical importance, physiological aspects of the vascular system have been extensively studied. However, the development of the vascular system in vertebrate embryos remains relatively unexplored. Studies thus far have shown a high degree of conservation of the molecular mechanisms that govern vascular development as well as in the anatomical structures that comprise the vascular system.

Several signaling molecules and transcription factor genes have been implicated in the development of the vertebrate vasculature: *vegf*, *vegfr1*, *vegfr2*, *vegfr3*, *tie1*, *tie2*, *angiopoietin1*, *angiopoietin2*, *ephrinB2*, *ephB4*, *scl*, *fli1*, *ets1*, *runx*, semaphorins and plexins have been analyzed in zebrafish, amphibians, birds and mammals, and found to display similar temporal and spatial expression patterns (Fouquet et al., 1997; Liao et al., 1997; Gering et al., 1998; Liang et al., 1998; Liao et al., 1998; Lyons et al., 1998; Kataoka et al., 2000; Lawson et al., 2001; Pham et al., 2001; Habeck et al., 2002; Torres-Vazquez et al., 2004). For example, *flk1/vegfr2* is strongly expressed in developing angioblasts/endothelial precursors in zebrafish (Liao et al., 1997), and its level of expression significantly decreases as vessels mature, as observed in *Xenopus*, chick and mouse (Quinn et al., 1993; Cleaver et al., 1997; Eichmann et al., 1998).

In addition to exhibiting similar expression patterns, the function of these genes appears to be conserved in all vertebrates. For example, Vegf and Hedgehog (Hh) signaling have been shown to be crucial for vascular development in several vertebrate model systems. Vegf regulates the migration and survival of endothelial cells. Mice lacking a functional Vegf signal show various developmental defects, including a reduced number of endothelial cells and a failure to form a functional vasculature (Carmeliet et al., 1996; Ferrara et al., 1996) (reviewed by Carmeliet and Storkebaum, 2002). Similar phenotypes have been reported in *Xenopus* and chick embryos with compromised Vegf signaling (Cleaver and Krieg, 1998). In zebrafish, Vegf appears to be crucial for angioblast formation as well as for the subsequent differentiation into arterial endothelial cells (Nasevicius and Ekker, 2000; Lawson et al., 2002). Another example of functional conservation in vascular development is Hh. In zebrafish embryos, Shh in the

notochord appears to regulate *vegf* expression in the somites, which in turn regulates vascular development (Lawson et al., 2002). In chick and mice, Hh signaling also appears to be important in vascular development (Vokes et al., 2004).

Anatomically, vascular development in zebrafish proceeds in a fashion analogous to what is observed in other vertebrates. Studies using microangiography (Isogai et al., 2001) or transgenic lines that specifically express green fluorescent protein (GFP) in endothelial cells (Motoike et al., 2000; Lawson et al., 2002) have delineated vascular development in zebrafish. The major axial vessels in zebrafish, the dorsal aorta (DA) and posterior cardinal vein (PCV), form as the result of angioblast migration from the lateral plate mesoderm (LPM) and subsequent coalescence in the midline (Torres-Vazquez et al., 2003). Two waves of angioblast migration have been observed and it has been hypothesized that the endothelial precursors that migrate during the initial wave contribute to the DA, while those that migrate during the second wave contribute to the PCV (Torres-Vazquez et al., 2003). This pattern of angioblast migration to the midline resembles the previously reported process of vasculogenesis in quail embryos documented with the QH1 antibody (Pardanaud et al., 1987). Secondary vessel formation by angiogenesis follows shortly after the coalescence of angioblasts at the midline. As previous studies have primarily focused on vascular development after the onset of circulation (24 hpf), the mechanisms of angioblast migration and coalescence, as well as subsequent vascular tube formation, remain largely unexplored.

During early development, angioblasts migrate to the midline in response to an unidentified attractive signal. In *Xenopus* embryos, the endoderm appears to be required for proper vascular tube formation (Vokes and Krieg, 2002), as surgical removal of this tissue severely affected vascular tube formation. In quail and mouse embryos, migrating angioblasts are in close contact with the endoderm, indicating that the endoderm is required for vascular development (Vokes and Krieg, 2002; Vokes et al., 2004). However, it is not clear from these studies how the endoderm affects vascular tube or lumen formation and whether it has a direct or indirect role in these processes.

In this report, we identify distinct steps of early vascular development at single cell resolution. We examine how angioblasts migrate from the LPM to the midline, how these cells coalesce to form a vascular cord, and how this transient structure is stabilized by cell-cell junctions and later lumenized. In addition, we analyze the role of Vegf signaling in these processes. We find that downregulation of Vegf signaling, while affecting the number of angioblasts does not appear to affect their migratory behavior. In order to further understand the function of the endoderm in vascular development, we analyzed the migratory path of angioblasts relative to the endoderm in wild-type embryos. Furthermore, we delineated vascular development in *casanova* (*cas*) and *bonnie and clyde* (*bon*) mutant embryos, which show either a complete absence (*cas*) or strong reduction (*bon*) of the endoderm (Alexander et al., 1999; Kikuchi et al., 2000). To our surprise, these mutant embryos formed wild-type like vascular tubes, suggesting that the endoderm plays a more limited role in vascular tube and lumen formation than previously thought.

Materials and methods

Zebrafish husbandry and the generation of *Tg(flk1:EGFP)* lines

Zebrafish (*Danio rerio*) embryos were obtained from the mixed wild-type strain in the laboratory and raised at 28°C as previously described (Westerfield, 1993).

Approximately 6.5 kb of upstream sequence of the zebrafish *flk1* gene (Liao et al., 1997; Thompson et al., 1998) was amplified from wild-type genomic DNA based on the sequence information previously deposited (from –6410 to –3 of the transcriptional start site, GenBank Accession Number AY045466) (Chan et al., 2002), and subcloned into the pCRII TOPO vector (Invitrogen). This fragment has been shown to drive endothelial-specific expression of the green-red coral fluorescent protein (G-RCFP) (Cross et al., 2003). The *Eco*RI cleaved fragment from this construct was placed 5′ upstream of the enhanced green fluorescent protein (EGFP) vector (Clontech) to drive endothelial specific expression of EGFP (Fig. 1A). Approximately 300 pg of this linearized construct was microinjected into one-cell stage embryos to generate transgenic lines as previously described (Motoike et al., 2000). Embryos were screened for transient expression of EGFP under a Leica epifluorescence microscope. Embryos showing specific expression of EGFP in the vasculature were raised to adulthood and crossed to identify founder fish with germline integration, from which stable transgenic lines were established.

Heterozygous carriers of the *cas* (Alexander and Stainier, 1999) or *bon* mutations (Stainier et al., 1996) were crossed to *Tg(flk1:EGFP)*^{s843} zebrafish to generate *Tg(flk1:EGFP)*^{s843}/+; *cas*/+ double heterozygotes and *Tg(flk1:EGFP)*^{s843}/+; *bon*/+ double heterozygotes, respectively. Homozygous embryos were obtained by incrossing these fish. Heterozygous carriers of *Tg(her5:GFP)*^{ne2067} zebrafish (Tallafuss and Bally-Cuif, 2003) were crossed to *Tg(flk1:EGFP)*^{s843} zebrafish to generate *Tg(flk1:EGFP)*^{s843}; *Tg(her5:GFP)*^{ne2067} embryos.

Microinjections and chemical treatment

Microinjections of Morpholinos (MO) were performed as previously described (Nasevicius and Ekker, 2000; Horne-Badovinac et al., 2001). The sequence of the *vegf* MO used is 5′-CTCGTCT-TATTTCCGTGACTGTTTT3′ (Ober et al., 2004; Parker et al., 2004), and the sequence of the *plcg1* MO is 5′-ATTAGCA-TAGGGAACTTACTTTCG-3′ (Lawson et al., 2003). One-cell or two-cell stage embryos were injected with 4 ng of either MO and raised at 28°C until harvested.

Embryos were treated with 1.5 μM of the Vegfr antagonist SU5416 (Calbiochem) as previously described (Fong et al., 1999; Serbedzija et al., 1999). As a control, embryos from the same batch were treated with 1% DMSO. Embryos were treated from 6 hpf until they were harvested for fixation.

Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed as previously described (Trinh and Stainier, 2004). Briefly, embryos were fixed overnight with 2% paraformaldehyde and embedded in 4% NuSieve GTG low melting agarose. Embedded embryos were cut with a VT1000S vibratome (Leica) into 250 μm sections. Sections were processed in PBDT [1% BSA, 1% DMSO, and 0.1% Triton X-100 in PBS (pH 7.3)]. The following antibodies were used at the following dilutions: rabbit polyclonal anti-Fibronectin (Sigma) at 1:200 (Trinh and Stainier, 2004), mouse IgG anti-β-catenin (Sigma) at 1:100 (Horne-Badovinac et al., 2003), mouse IgG anti-Zona Occludin-1 (BD Transduction Laboratory) (Horne-Badovinac et al., 2003) at 1:200, goat anti-EphrinB2 (R&D System) at 1:100, and mouse IgG anti-Claudin5 (Zymed) at 1:100. Filamentous actin was visualized with rhodamine phalloidin (Molecular Probes) at 1:100 (Trinh and Stainier, 2004). Nuclei were visualized with TOPRO (Molecular Probes) at 1:10000 (Oomman et al., 2004). Processed samples were mounted in

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Vectashield (Vector Laboratories) and the images were acquired using a Zeiss LSM5 Pascal confocal microscope.

Whole-mount in situ hybridization was performed as previously described (Alexander and Stainier, 1999). Riboprobes for *flk1* (Liao et al., 1997), *ephrinB2a* (Chan et al., 2001; Lawson et al., 2001), *VE-cadherin* (Larson et al., 2004), and *flt4* (Thompson et al., 1998) were prepared with Ambion mMessage Machine. Embryos were mounted in benzylbenzoate:benzyl alcohol and documented with a Zeiss Axiocam.

Results

Generation of *Tg(flk1:EGFP)* line

In order to visualize endothelial precursor migration and early vascular tubulogenesis, we generated transgenic lines that express GFP under the control of the *flk1/vegfr2* promoter (Fig. 1A), one of the earliest acting promoters in the endothelial lineage. Approximately 300 embryos were injected, and those showing transient GFP expression were raised to generate stable transgenic lines. From those embryos, six founders were identified, and they produced GFP positive progeny with percentages ranging from 5 to 37.5%. All but one out of six founder fish seemed to have a single insertion. Two founders chosen because of their higher percentage of GFP-positive embryos were propagated and named according to the nomenclature *Tg(flk1:EGFP)^{s843}* and *Tg(flk1:EGFP)^{s844}*. For this study, we used *Tg(flk1::EGFP)^{s843}* which carries the transgene on linkage group 16 (Fig. 1B,C).

The GFP expression pattern in *Tg(flk1:EGFP)^{s843}* appears to be specific to the vasculature and to faithfully recapitulate the endogenous expression of *flk1* (Liao et al., 1997). Furthermore, analyzing GFP expression in these embryos allows higher resolution analyses than previously used methods, such as detecting alkaline phosphatase activity (Childs et al., 2002; Parker et al., 2004) (Fig. 1D-K). The expression of GFP in the endothelial precursors can be detected as early as 12 hpf (data not shown). At this stage, GFP-positive cells are scattered within the LPM. At 14 hpf, GFP expression, like *flk1* expression, is detected in bilateral stripes along the anteroposterior axis of the embryos, and these stripes merge at the midline at approximately 17 hpf (see Fig. S1 in the supplementary material). In addition, similar to the endogenous expression of *flk1* (Lawson et al., 2001), the intensity of GFP expression in the arterial endothelial cells appears stronger than that in the venous endothelial cells. The expression of GFP persists in the vasculature, even in adult fish, similar to what is observed in *Tg(fli1:EGFP)^{y1}* (Lawson and Weinstein, 2002) transgenic and *Tg(Tie2:GFP)^{s849}* (Motoike et al., 2000) lines. It appears that all endothelial cells express GFP as the expression pattern of *Tg(flk1:EGFP)^{s843}* is indistinguishable from that of *VE-cadherin*, a known pan-endothelial cell marker in zebrafish (see Fig. S2 in the supplementary material) (Larson et al., 2004).

In addition to this vascular expression, the hindbrain of the transgenic embryos also shows consistent GFP expression (see Fig. S3A in the supplementary material), consistent with *flk1* expression reported in zebrafish (Liao et al., 1997) and in chick embryos (Hashimoto et al., 2003) (data not shown). Interestingly, transient, but consistent, expression of GFP is also detected in the pharyngeal region. It appears that this expression is endodermal, not endothelial in origin: *Tg(flk1:EGFP)^{s843};cloche* mutant embryos, which lack endothelial precursors completely in the head and anterior trunk regions (Stainier et al., 1995), exhibit this expression; *Tg (flk1:EGFP)^{s843};cas* mutant embryos, which lack endodermal cells, and *Tg(flk1:EGFP)^{s843};bon* mutant embryos, which lack most endodermal cells, show a total absence or dramatic reduction of GFP expression in this region (see Fig. S3B-F in the supplementary material). As all the lines derived from all the different founders show the same pharyngeal expression pattern, it is unlikely that this non-endothelial GFP expression is the result of the integration site, but instead reflects a low level endogenous *flk1* expression in the pharyngeal region as previously reported (Liao et al., 1997).

Formation of the vascular cord by angioblast migration to the midline

Using the *Tg(flk1::EGFP)^{s843}* line, we analyzed the distinct phases of early vascular development in zebrafish, focusing on two crucial events: the coalescence of angioblasts to the midline and the lumenization of the vascular tube. Angioblasts first appear within the LPM around 12 hpf (data not shown). These cells then migrate as individual cells to the midline

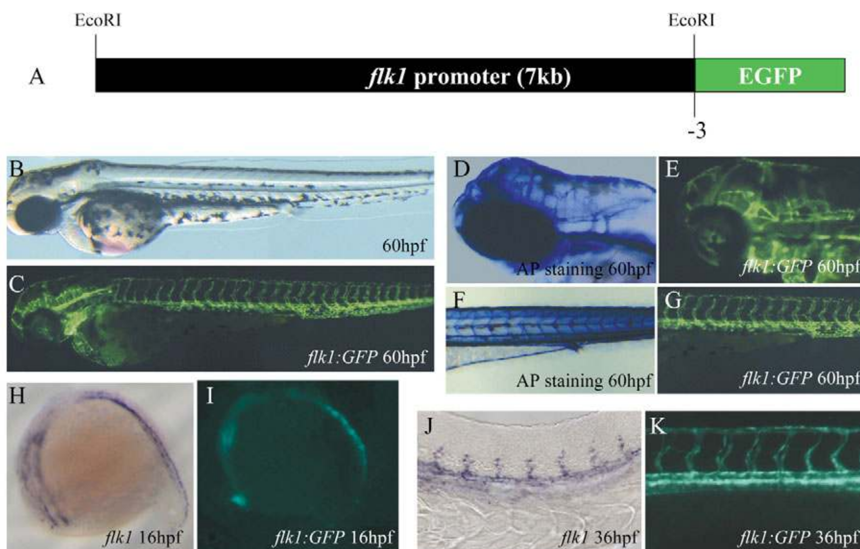

Fig. 1. Generation of the *Tg(flk1:EGFP)^{s843}* line. (A) A 6.5 kb upstream sequence of zebrafish *flk1* was used to generate the *Tg(flk1:EGFP)^{s843}* line. (B) Bright-field micrograph of a 60 hpf *Tg(flk1:EGFP)^{s843}* embryo. (C) Epifluorescence micrograph of the same embryo. (D) Head vasculature of a 60 hpf embryo visualized by endogenous alkaline phosphatase (AP) activity. (E) Similar area in a *Tg(flk1:EGFP)^{s843}* embryo. (F) Trunk vasculature of a 60 hpf embryo visualized by endogenous AP activity. (G) Similar area in a *Tg(flk1:EGFP)^{s843}* embryo. (H) *flk1* expression at 16 hpf in a 16 hpf embryo. (I) Expression of GFP at the same stage. (J) *flk1* expression at 36 hpf in a 36 hpf embryo. (K) Expression of GFP at the same stage. The *flk1:EGFP* transgene recapitulates *flk1* expression and allows higher resolution analyses than staining for *flk1* expression or AP activity.

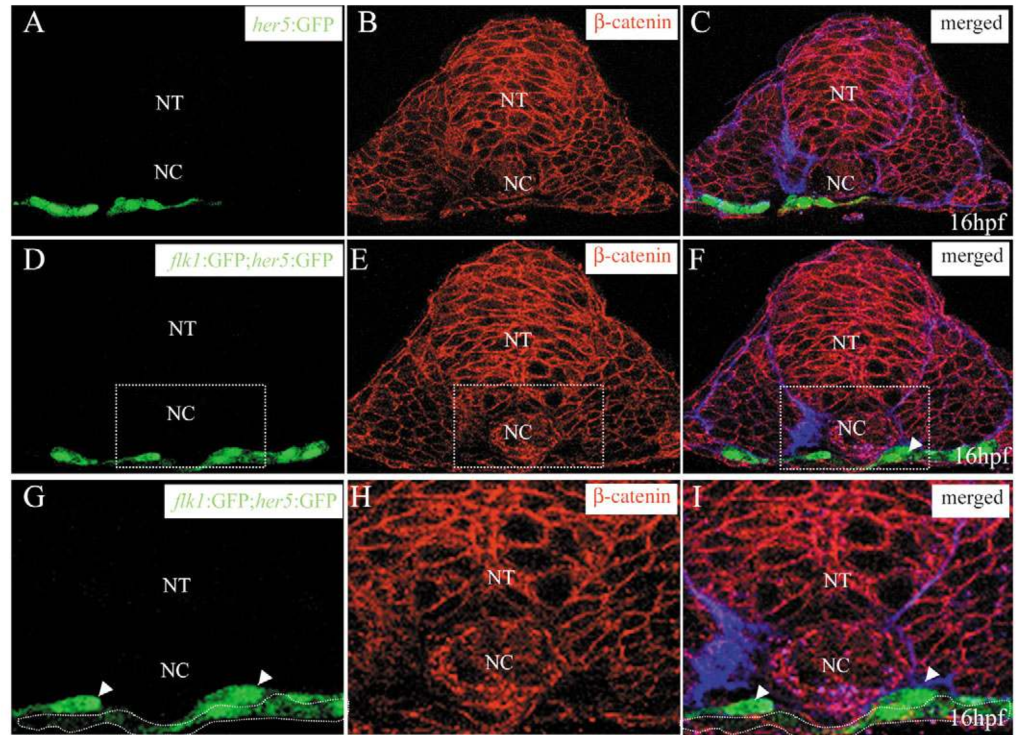

Fig. 2. Angioblasts migrate directly on top of the endodermal layer. Transverse sections of *Tg(her5:EGFP)*^{ne2067} embryo (A-C) and *Tg(flk1:EGFP)*^{s843}; *Tg(her5:EGFP)*^{ne2067} embryo (D-I) visualized for GFP (green) (A,D,G), β-catenin (red) (B,E,H) and fibronectin (blue) (C,F,I). The sections shown are at the level of the 6th somite. White arrowheads mark migrating angioblasts localized right on top of the endodermal layer (outlined by a broken white line). β-Catenin staining outlines the endodermal layer. NT, neural tube; NC, notochord.

directly on top of the endodermal layer (Fig. 2). It appears that there are at least two distinct waves of angioblast migration during vascular development. The initial wave of migration begins at ~14 hpf, while the second wave begins at ~16 hpf (Fig. 3A,B). Upon completion of the initial wave of migration, angioblasts aggregate at the midline to form a cord-like structure, which is situated directly dorsal to the endoderm and ventral to the hypochord (Fig. 3C). We name this cellular aggregate the vascular cord. Although cells in the vascular cord are located in close proximity to one another, they do not appear to form any detectable cell-cell junctions, suggesting that the vascular cord is initially a simple cluster of angioblasts, rather than an organized structure. Within this aggregate, fibronectin is deposited between the angioblasts (Fig. 3C),

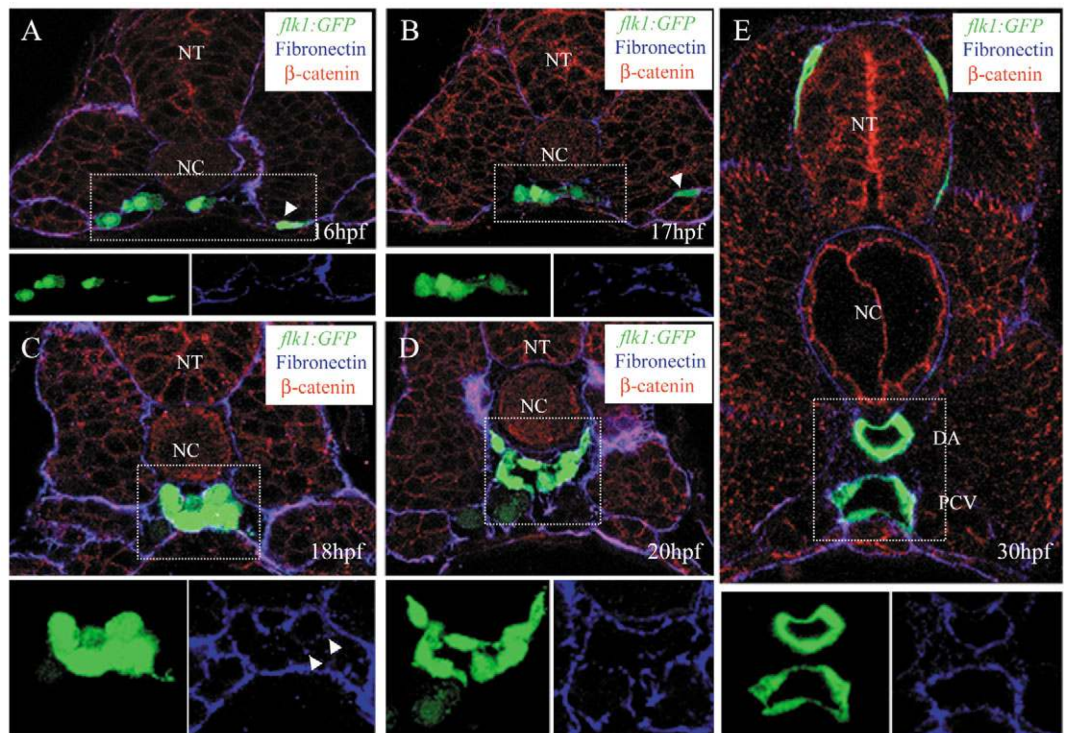

Fig. 3. Angioblast migration to the midline. (A-E) Transverse sections visualized for GFP (green), fibronectin (blue) and β-catenin (red). The GFP (green) and fibronectin (blue) signals of the outlined areas are also shown separately. The sections shown are at the level of the 6th (A,B), 10th (C) and 14th (D,E) somite. White arrowheads in A and B mark angioblasts that are still residing within the LPM and that will migrate during a second wave. White arrowheads in C show fibronectin deposition around a single endothelial cell within the vascular cord. Over the span of 14 hours, the angioblasts migrate to the midline where they aggregate to form a vascular cord that subsequently lumenizes. NT, neural tube; NC, notochord; DA, dorsal aorta; PCV, posterior cardinal vein.

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further supporting the idea that these cells have not yet formed a cohesive structure at this stage. However, within the next 2 hours, cell-cell junctions appear (see below) and, subsequently, a lumen is formed.

Lumenization of the vascular cord leads to the formation of patent vessels, the basal side of which is coated with fibronectin (Fig. 3D,E). The vascular cord starts to lumenize to form the dorsal aorta at ~18 hpf. It appears that the 'hollow out' mode of lumen formation is used to generate the vascular tube, which initially contains 4 to 6 angioblasts around its circumference. Concomitant with an increase in size of the vascular lumen, these cells undergo morphological changes; their cuboidal shape rapidly changes to an elongated shape (Fig. 3D,E), the prevalent shape of endothelial cells in the mature vasculature.

Vascular tube formation and angioblast differentiation

Cell-cell junctions between angioblasts first form at ~17 hpf. Immunostaining with anti-zona occluding 1 (ZO1) antibody clearly shows junctions formed between adjacent angioblasts (Fig. 4A,B). At the same time, adherens junctions are present, as indicated by the localized deposition of β-catenin, which is heightened at the focal point of cell-cell contacts (see Fig. S4 in the supplementary material). At 30 hpf, when vascular development is complete and active circulation is established, ZO1 deposition can be detected in both the DA and PCV (Fig. 4C). Although the expression of ZO1 is pan-endothelial, that of another junctional protein, claudin 5, appears to be more restricted. The expression of claudin 5 can be detected ~1 hour later than that of ZO1 (Fig. 4D). Whereas ZO1 appears to mark cell-cell junctions in both arterial and venous endothelial cells, claudin 5 seems to mark cell-cell junctions only in arterial endothelial cells (Fig. 4D-F).

Differentiation of the angioblasts into arterial and venous endothelial cells first becomes evident at 17 hpf (see Fig. S5 in the supplementary material). Endothelial expression of *ephrinB2a*, a marker of differentiated arterial endothelial cells (Lawson et al., 2001; Zhong et al., 2001), and expression of *flt4*, a marker of differentiated venous endothelial cells (Thompson et al., 1998) can first be detected by in situ hybridization at ~17 hpf (see Fig. S5 in the supplementary material). Although both arterial and venous markers are expressed at this time in angioblasts located in the midline, it is not clear how these cells are spatially organized within the vascular cord. In order to analyze the relative position of the different endothelial cells within the vascular cord, we used a commercially available antibody against zebrafish ephrin B2. Consistent with the in situ hybridization data, ephrin B2a is expressed in the angioblasts located in the dorsal region of the vascular cord as early starting at ~17 hpf (Fig. 5A,F). These ephrin B2a positive cells give rise to the first sprouts of intersegmental vessels by 18 hpf (Fig. 5G-I). Ventrally located angioblasts do not appear to express ephrin B2a, suggesting that some of the angioblasts that migrated during the first wave differentiate into venous endothelial cells. This differentiation may depend on the relative position of the angioblasts within the vascular cord. The ephrin B2a-negative cells, along with the angioblasts in the second wave of migration, appear to contribute to the PCV, which forms later than the DA. By 24 hpf, the vasculature in the zebrafish embryo contains two defined axial vessels, the DA and the PCV, and several intersegmental vessels. In the mature vasculature, ephrin B2a is evenly distributed on the DA arterial endothelial cells (Fig. 5J-L).

The number of angioblasts is not critical for their migration to the midline

In order to test the role of Vegf signaling in the migration of

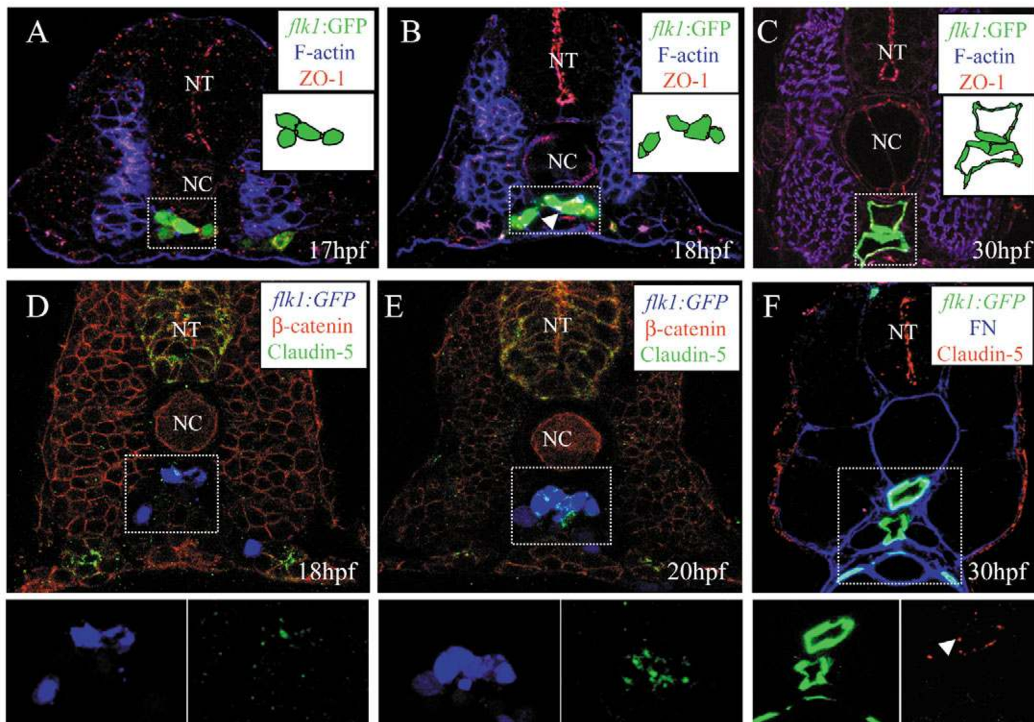

Fig. 4. Junction formation between endothelial cells in the vascular cord. Transverse sections visualized for: (A-C) GFP (green), filamentous actin (blue) and ZO1 (red); (D,E) GFP (blue), claudin 5 (green) and β-catenin (red); (F) GFP (green), fibronectin (blue) and claudin 5 (red). The sections shown are at the level of the 7th (A), 10th (B,D,E), and 14th (C,F) somites. Schematic drawings of the outlined areas are shown as insets (A-C). The GFP and claudin 5 signals of the outlined areas are shown separately as insets as blue and green channels, respectively (D,E) and green and red channels, respectively (F). Junctions form between endothelial cells after they reach the midline. NT, neural tube; NC, notochord. Arrowheads in B,F indicate ZO1 and claudin 5 localization, respectively.

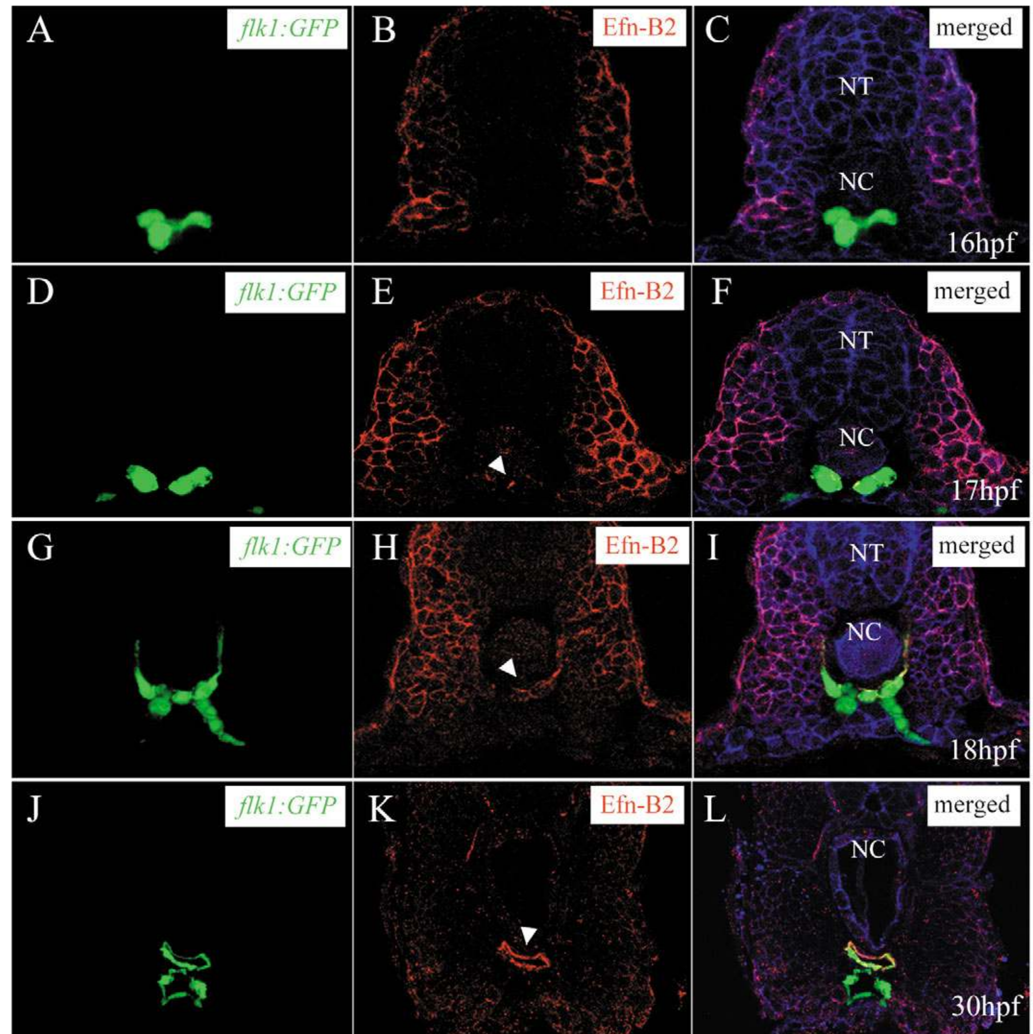

Fig. 5. Differentiation of arterial endothelial cells within the vascular cord. Transverse sections visualized for GFP (A,D,G,J) (green), ephrin B2 (B,E,H,K) (red) and β-catenin (C,F,I,L) (blue). Sections shown are at the level of the 7th (A to C), 10th (D to F), and 14th somite (G to L). Arrowheads mark the ephrinB2 expression within the vascular cord. Subsets of angioblasts within the vascular cord start to express ephrin B2 at 17 hpf, and this expression later becomes restricted to the DA. NT, neural tube; NC, notochord.

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angioblasts to the midline, we investigated this process in embryos with compromised Vegf signaling. We first blocked the activity of Vegf by injecting morpholino antisense oligonucleotides (MO) targeting *vegf* (Nasevicius et al., 2000) or its downstream effector *plcg1* (phospholipase C γ) (Lawson et al., 2003).

Both *vegf* MO- and *plcg1* MO-injected embryos showed a significantly reduced number of angioblasts compared with the uninjected control embryos (Fig. 6). Whereas the uninjected control embryos contained an average of 6.7 angioblasts per focal plane (n=25), *vegf* MO-injected embryos contained an average of 4.3 angioblasts per focal plane (n=33) and *plcg1* MO-injected embryos contained an average of 3.5 angioblasts per focal plane (n=41).

Despite this reduction in their number, the angioblasts appeared to migrate to the midline at approximately the same time as their counterparts in uninjected embryos, and formed a wild-type like vascular cord. Analyses of both transverse sections (Fig. 6A-F) and whole-mount in situ hybridization with the pan-endothelial marker *cdh5* (VE-cadherin) (Fig. 6G-I) indicated that angioblasts in *vegf* MO- or *plcg1* MO-injected embryos behave normally.

To verify the results from the MO experiments, we employed the pharmacological reagent SU5416, a widely used antagonist of VEGF signaling (Fong et al., 1999). When embryos were treated from 6 hpf onwards, SU5416 caused a dramatic reduction in the number of angioblasts, as was observed in *vegf* MO- or *plcg1* MO-injected embryos (data not shown). However, despite this reduction, the angioblasts in SU5416-treated embryos migrated to the midline, suggesting that the Vegf signal is dispensable for this process (data not shown).

The endoderm is required for proper temporal regulation of angioblast migration, but not for angioblast differentiation

Several studies have suggested a pivotal role for the endoderm in vascular development. Surgical removal of the endoderm in *Xenopus* and avian embryos resulted in the failure to form functional vascular tubes (Vokes and Krieg, 2002). In order to further investigate how the endoderm regulates vascular tube formation, we analyzed angioblast migration in a previously described zebrafish mutant, *cas*, which is completely devoid of endoderm (Alexander and Stainier, 1999; Alexander et al., 1999).

Angioblasts in *Tg(flk1:EGFP)*^{s843}*;cas* mutant embryos appear to differentiate normally (Fig. 7). *Tg(flk1:EGFP)*^{s843}*;cas* mutant embryos and their wild-type siblings are morphologically indistinguishable until 16 hpf.

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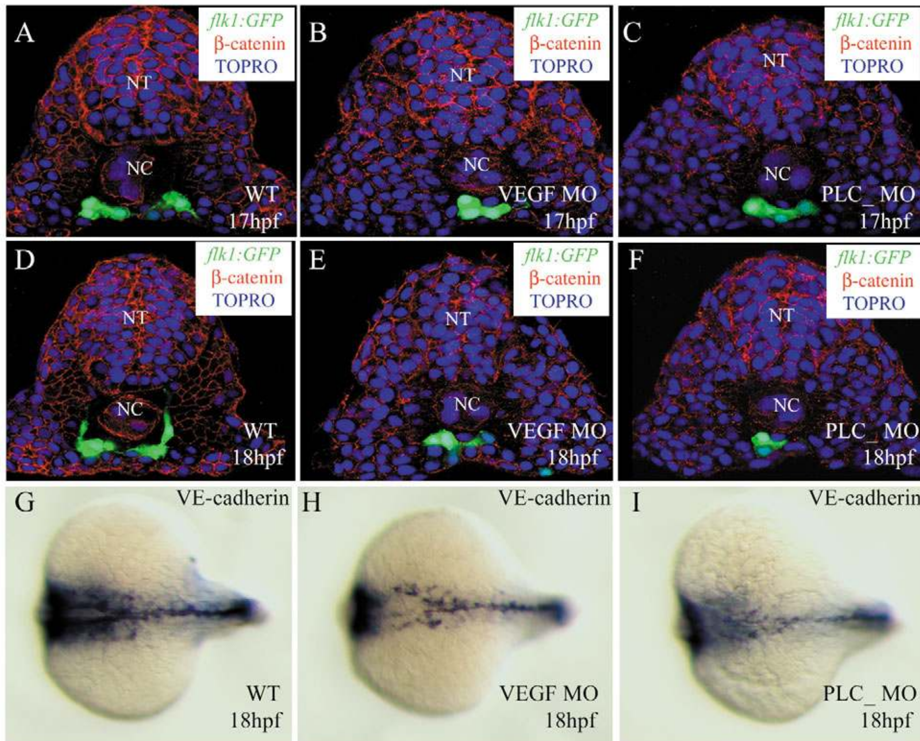

Fig. 6. Cell number is not crucial for angioblast migration. Transverse sections of embryos with compromised VEGF signaling visualized for GFP (green), β-catenin (red) and TOPRO (blue) (A-F), and in situ hybridization with VE-cadherin (*cdh5*) (G-I). The sections shown are at the level of the 6th somite (A-F). Control embryos (A,D,G), *vegf* MO-injected embryos (B,E,H), *plcg1* MO-injected embryos (C,F,I). The angioblasts in embryos with compromised VEGF signaling are in a similar position to those in control embryos, while the numbers of angioblasts are significantly reduced. NT, neural tube; NC, notochord.

However, angioblasts in these embryos show severe migration defects. At this stage, the angioblasts in *cas* mutant embryos and their wild-type siblings begin to migrate to the midline (Fig. 7A,B, compare with Fig. 3A,B). Angioblasts in *cas* mutant embryos eventually reach the midline at 22 hpf, which is significantly later than their wild-type counterparts (Fig. 7C-D, compare with Fig. 3C,D). Despite this initial delay in migration, angioblasts in *cas* mutant embryos eventually form two distinct axial vessels that are properly lumenized and coated with fibronectin (Fig. 7E). Although

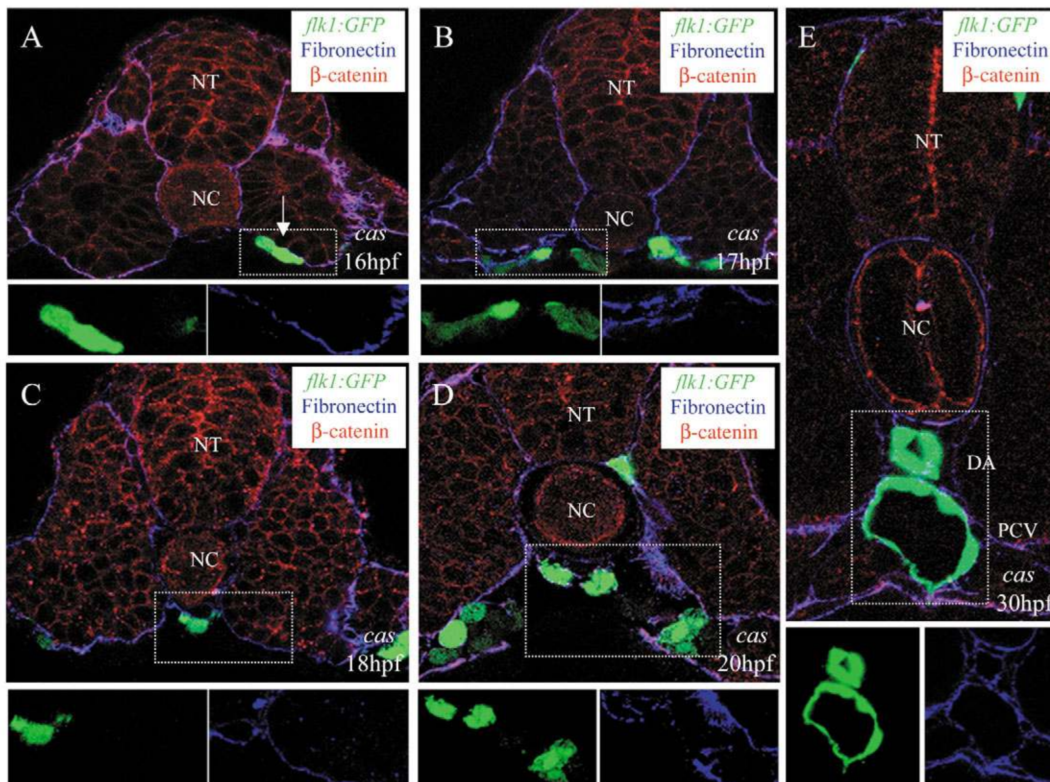

Fig. 7. Angioblast migration in endodermless embryos. Transverse sections of endodermless embryos *Tg(flk1:EGFP)^{s843};cas* mutants visualized for GFP (green), fibronectin (blue), and β-catenin (red). The GFP (green) and fibronectin (blue) signals of the outlined areas are shown separately (A-E). The sections shown are at the level of the 6th (A,B), 10th (C), 14th (D) and 18th (E) somites. White arrow in A shows a cluster of angioblasts exiting the LPM. Despite the absence of endoderm, angioblasts migrate to the midline. NT, neural tube; NC, notochord; DA, dorsal aorta; PCV, posterior cardinal vein.

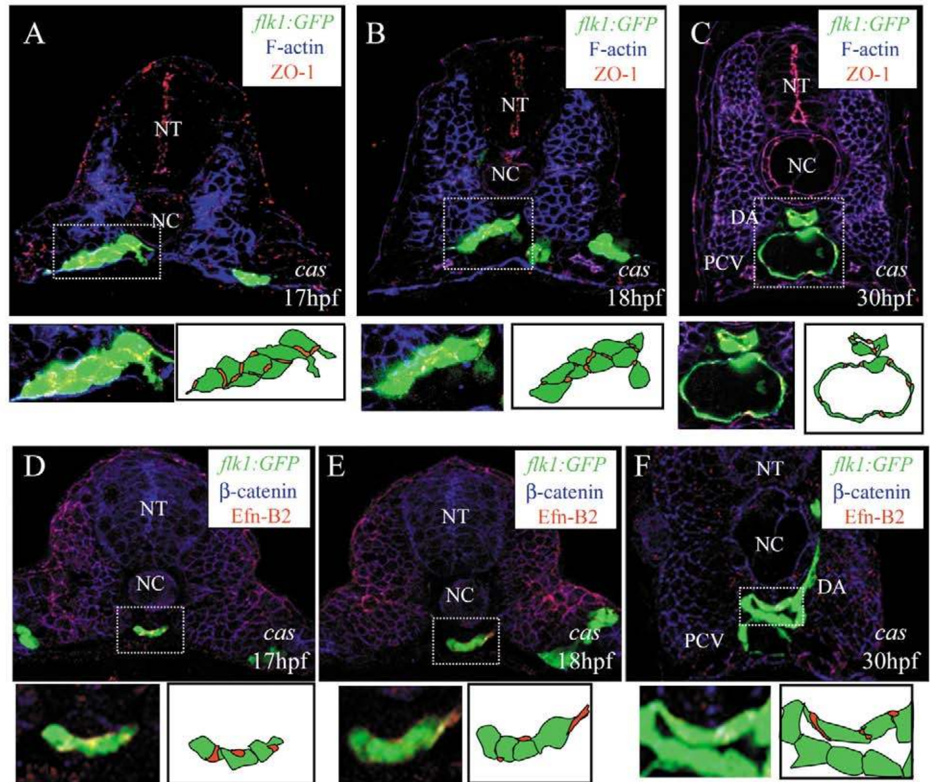

Fig. 8. Junction formation and arterial endothelial cell differentiation in endodermless embryos. Transverse sections of endodermless embryos. *Tg(flk1:EGFP)^{s843};cas* mutants visualized for: (A-C) GFP (green), filamentous actin (blue) and ZO1 (red); and (D-F) GFP (green) ephrin B2 (red) and β-catenin (blue). The sections shown are at the level of the 6th (A,D), 10th (B,E), and 14th somites (C,F). The outlined areas are magnified and shown with schematic drawings. Despite the absence of endoderm, differentiation of angioblasts into arterial and venous endothelial cells occurs as in wild-type embryos. NT, neural tube; NC, notochord; DA, dorsal aorta; PCV, posterior cardinal vein.

the DA in these mutant embryos is similar to that in wild-type siblings, the PCV appears to be dilated to various degrees as observed in many mutants that lack circulation (Sehnert et al., 2002).

Despite their relatively normal migratory behavior, the angioblasts in *Tg(flk1:EGFP)^{s843};cas* mutant embryos aggregate to form aberrant clusters along the mediolateral axis of the embryo (Fig. 8A,B). Angioblasts inside these clusters appear to form cell-cell junctions, as detected by the anti-ZO-1 antibody (Fig. 8A,B, compare with Fig. 4A,B). During their migration, it appears that the angioblasts in *cas* mutant embryos maintain cell-cell junctions, thereby migrating as a group rather than as individual cells (Fig. 8A,B). At 30 hpf, ZO1 expression is localized between endothelial cells within the DA and PCV in *Tg(flk1:EGFP)^{s843};cas* mutant embryos as in wild-type embryos (Fig. 8C).

Angioblasts in *Tg(flk1:EGFP)^{s843};cas* mutant embryos undergo proper differentiation: the arterial specific marker, ephrin B2a, is restricted to the dorsal aorta, as determined by in situ hybridization and immunostaining (Fig. 8D-F), while in situ hybridization with the venous specific marker *flt4* detects this marker only in the cardinal vein (data not shown). Thus, it appears that the differentiation of angioblasts into arterial or venous endothelial fates does not require the endoderm. Similar phenotypes were observed in *cas* MO-injected embryos, as well as in *bon* mutant embryos, which lack most endodermal cells (Alexander and Stainier, 1999; Kikuchi et al., 2000) (data not shown). These results suggest that the endothelial phenotype in *cas* mutant embryos is not allele specific or *cas* specific, but rather reflects the role of the endoderm in vascular development, as analyzed genetically.

Discussion

In this study, we report the generation of the *Tg(flk1:EGFP)^{s843}* line and use it to analyze angioblast migration and subsequent vascular tube formation. The expression of GFP persists throughout development into adulthood, and appears to be regulated like the endogenous *flk1* gene (Liao et al., 1997). Several transgenic lines that visualize the vasculature have been established and they have facilitated the understanding of vascular development (Motoike et al., 2000; Lawson and Weinstein, 2002; Cross et al., 2003). However, the *Tg(Tie2:GFP)^{s849}* line, which uses the mouse *Tie2* promoter, shows relatively weak fluorescence in the vasculature except for the endocardium (Motoike et al., 2000; Walsh and Stainier, 2001), which limits its usefulness. The *Tg(fli1:EGFP)^{y1}* line, despite showing strong vascular GFP expression, also shows non-endothelial expression in mesenchyme and cartilage (Lawson et al., 2001). This non-vascular GFP expression makes it difficult to analyze the vasculature in the head and aortic arch region. By contrast, *Tg(flk1:EGFP)^{s843}* fish exhibits vascular specific GFP expression with strong intensity, allowing observation of endothelial cells during development under an epifluorescence microscope, which will facilitate future screens for vascular specific phenotypes.

In addition to the vasculature, the hindbrain and the pharyngeal region of the transgenic embryos express GFP. The GFP expression in the pharyngeal region might be related to the role of Vegfc in endoderm migration and morphogenesis. It has been reported that embryos with compromised Vegfc function show a split gut and duplicated liver primodia owing to defects in endoderm migration (Ober et al., 2004). As Vegfc can also interact with Vegfr2 (Joukov et al., 1996; Ober et al., 2004), and the major receptor for Vegfc, Vegfr3/Flt4, is not

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expressed in the pharyngeal endoderm, it is plausible that the pharyngeal expression of Flk1 is crucial for transducing Vegfc signaling during endoderm migration.

Angioblast migration and the factors regulating this process

Angioblasts migrate extensively during development. The absolute number of angioblasts does not seem to be crucial for this process. Embryos injected with *vegf* or *plcg1* MOs do not display any obvious defects in angioblast migration despite a significant reduction in their number. Similar results were observed in embryos treated with SU5416, a chemical antagonist of Vegf signaling. These data suggest that Vegf signaling is required for angioblast formation, but is not necessary to direct angioblasts during their migration to the midline. Other signals, such as Fgf or Pdgf, might be responsible for the directionality of migration (Yang and Moses, 1990; Sa et al., 1995; Thommen et al., 1997). Alternatively, repulsive signals such as Semaphorins (Shoji et al., 2003; Torres-Vazquez et al., 2004) might play a more significant role in angioblast migration than previously thought.

Unlike the precardiac mesoderm, which migrates as a polarized pseudo-epithelium, angioblasts appear to migrate as individual cells. Throughout this migration process, fibronectin deposition is observed on the ventral side of the angioblasts (data now shown), although it is not clear whether the fibronectin provides a directional cue or is merely a substratum for migration. Two fibronectin genes are present in the zebrafish genome (Trinh and Stainier, 2004; Sun et al., 2005), and they may have partially overlapping function in angioblast migration.

Role of endoderm during early vascular development

Our study shows that the endoderm is required for the temporal regulation of angioblast migration. Although angioblasts in endodermless embryos start their migration to the midline at the same time as those in wild-type embryos, they reach the midline much later than their wild-type counterparts. Interestingly, the angioblasts in the endodermless mutant embryos display a distinct behavior that is not observed in wild-type embryos. They appear to aggregate during their migration to the midline and form ectopic cell-cell junctions with each other. These angioblasts then migrate as groups and maintain their cell-cell junctions during this process. It is possible that the endoderm somehow prevents cell-cell contact between migrating angioblasts, and thereby promotes their migration as individual cells. Once the angioblasts in endodermless embryos reach the midline, they form a single aggregate as observed in wild-type embryos. It is not clear how the cell-cell junctions between endothelial precursors are redistributed once they reach the midline.

The mechanism by which the endoderm regulates angioblast migration is also unclear. It is possible that the endoderm provides an unidentified attractant for angioblasts. However, it appears to be an undifferentiated homogenous cell layer at this stage. A more likely scenario may be that the endoderm functions as a substratum for the angioblasts to migrate upon, and prohibits them from aggregating.

Several studies have indicated that the endoderm is required for proper vascular tubulogenesis (Palis et al., 1995; Bielinska et al., 1996; Vokes and Krieg, 2002). However, we found no evidence for a pivotal role of the endoderm in vascular tube formation or angioblast differentiation in zebrafish. Although the angioblasts in endodermless embryos show a distinct phenotype and a delay during migration, they form comparatively normal vascular tubes. Two distinct vascular tubes with proper junctional complexes are formed. Furthermore, the expression patterns of the arterial endothelial-specific marker ephrin B2a and the venous endothelial specific marker *flt4* (Lawson et al., 2001) appear to be unaltered in endodermless embryos, suggesting that the endoderm is not required for angioblast differentiation into arterial and venous endothelial cells.

The PCV in endodermless embryos appears to be dilated at later stages. We observed a severe accumulation of blood cells in these dilated vessels that might in fact be responsible for the dilation as many other zebrafish mutations that affect circulation exhibit a similar phenotype (Sehnert et al., 2002; Lawson et al., 2003). We believe that the defects in *cas* and *bon* mutant embryos reflect the function of the endoderm during vascular development, rather than previously uncharacterized functions of these genes in the lateral plate mesoderm or angioblasts. Previous studies have indeed shown that the *cas* mutation affects the endoderm specific HMG transcription factor Sox32 (Kikuchi et al., 2001).

Why is the endoderm dispensable for vascular tube formation in zebrafish, unlike what appears to happen in other organisms? It is possible that the fast development of zebrafish embryos allows us to monitor later stages of development than previously observed in *Xenopus* or avian embryos. For example, we analyzed vascular tube formation in endodermless embryos until 72 hpf. By contrast, previous studies (Vokes and Krieg, 2002; Vokes et al., 2004) analyzed vascular tube formation until stage 34 in *Xenopus* embryos, and the eight-somite stage in quail embryos, which is approximately equivalent to 24 hpf for zebrafish embryos. A similar temporal delay in endothelial precursor migration and vascular tube formation as we observed in endodermless zebrafish embryos might occur in *Xenopus* and avian embryos, but was misinterpreted because later stage embryos were not examined. Alternatively, the temporal and spatial expression patterns of molecular cues regulating angioblast migration might have evolved differently.

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Supplementary material

Supplementary material for this article is available at
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