

Ocular vessel patterning in zebrafish is indirectly regulated by Hedgehog signaling

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ABSTRACT The superficial ocular vasculature of the embryonic zebrafish develops in a highly stereotypic manner and hence provides a convenient model for studying molecular mechanisms that regulate vascular patterning. We have used transgenic zebrafish embryos in which all endothelial cells express enhanced Green Fluorescent Protein and small molecule inhibitors to examine the contribution of two signaling pathways, vascular endothelial growth factor (VEGF) and Hedgehog (Hh) pathways, to the development of the superficial system. We find that most, but not all vessels of the superficial system depend on VEGF signaling for their growth. Hh signaling appears to limit superficial vessel growth over the dorsal eye and is required to promote superficial vessel growth over the ventral eye. These effects of Hh signaling are indirect. Our initial analyses of factors that regulate growth and patterning of superficial ocular vessels suggest that early patterning events in the embryo during organogenesis stages could influence vascular patterning later on. By studying development of specific vascular systems it should be possible to identify new roles for signaling pathways in regulating vascular development.

KEY WORDS: *zebrafish, ocular vasculature, Hedgehog, VEGF*

Introduction

The embryonic vertebrate eye receives blood supply from two networks of blood vessels: the hyaloid system inside the eye and the choroidal vasculature on the outer surface (Saint-Geniez and D'Amore, 2004). In zebrafish, the first vessels that supply blood to the outer surface of the eye form a highly stereotypic and simple pattern, which we have named the superficial ocular vasculature (Kaufman *et al.*, 2015). The initial pattern of superficial ocular vasculature is completed by approximately 54 hours post fertilization (hpf) and includes one vessel that serves as an artery, the nasal radial vessel (NRV), which receives blood through the cranial division of the carotid artery (CrDI). Blood is drained from the system via two veins, the dorsal radial vessel (DRV) and the ventral radial vessel (VRV), the latter also draining the hyaloid system. NRV, DRV and VRV are interconnected by the superficial annular vessel (SAV) (see Fig. 1) (Kaufman *et al.*, 2015; Kitambi *et al.*, 2009). The superficial ocular system begins to develop at 22-23 hpf and is entirely derived from a single venous source, the primordial midbrain channel (PMBC) (Kaufman *et al.*, 2015).

The highly stereotypic pattern in which superficial ocular vessels of zebrafish develop begs the question of how formation of this pattern is directed. Two main processes underlie the formation of new blood vessels, namely vasculogenesis and angiogenesis. In vasculogenesis, angioblasts, the precursors of endothelial cells, coalesce at a specific region where they differentiate into endothelial cells and rearrange to form new blood vessels. In angiogenesis, new blood vessels arise from an existing vessel, either by sprouting of new vessels (sprouting angiogenesis) or by splitting of the vessel (intussusceptive angiogenesis) (Ellertsdottir *et al.*, 2010; Heinke *et al.*, 2012). All ocular vessels in zebrafish form by angiogenesis (Hartsock *et al.*, 2014; Kaufman *et al.*, 2015), whereas the PMBC, which gives rise to superficial vessels forms by vasculogenesis (Proulx *et al.*, 2010).

Abbreviations used in this paper: CrDI, cranial division of the carotid artery; Dpf, days post fertilization; DRV, dorsal radial vessel; eGFP, enhanced green fluorescent protein; Hh, Hedgehog; Hpf, hours post fertilization; NRV, nasal radial vessel; PMBC, primordial midbrain channel; VEGF, vascular endothelial growth factor; VRV, ventral radial vessel.

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Angiogenesis is a complex process, requiring formation of sprouts at correct locations, endothelial cell proliferation, guided migration of the growing vessel and differentiation and maturation of the new vessel as artery or vein (Carmeliet, 2003). Vascular endothelial growth factor (VEGF) family of ligands and receptors (VEGFRs) is the central regulator of angiogenesis, and promotes endothelial cell proliferation, migration and arterial fate. VEGF-A signaling through VEGFR-2 (Kdr, Flk-1) is the main pro-angiogenic mechanism (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Shalaby *et al.*, 1995), but other family members, e.g. VEGF-B, VEGF-C and VEGFR-3 (Flt-4) have also been shown to promote angiogenesis in specific contexts (Covassin *et al.*, 2006; Jensen *et al.*, 2015; Ober *et al.*, 2004).

In addition to VEGF, other signaling pathways can also promote angiogenesis. The Hedgehog (Hh) signaling pathway has been shown to promote angiogenesis upstream of VEGF expression in several contexts (Nagase *et al.*, 2008; Pola *et al.*, 2001), and also during arterial differentiation (Lawson *et al.*, 2002). Hh signaling can also promote angiogenesis by acting directly on endothelial cells (Chinchilla *et al.*, 2010; Renault *et al.*, 2010). However, whether Hh signaling affects, either directly or indirectly, vascular patterning is currently unknown.

In this work we begin to address the question of what are the molecular signals that help to shape the superficial ocular vasculature in zebrafish. We first examine the implications of blocking VEGF signaling and find that almost all ocular vessels require VEGF signaling for their growth and early maintenance. Surprisingly, growth of vessels arising from the ventral PMBC appears independent of VEGF signaling. We then examine whether Hh signaling contributes to promoting angiogenesis upstream of VEGF. Surprisingly, we find that reduced Hh pathway activity leads to excess angiogenesis over the dorsal eye and failure of blood vessel formation over the ventral eye. The effect of loss of Hh signaling on superficial vessel angiogenesis is indirect and is only partially dependent on VEGF signaling.

Together, the results described in this work begin to identify signaling pathways that are required for proper formation of ocular vessels in zebrafish and raise the idea that early patterning events during organogenesis influence later-developing patterns of blood vessels.

Results

VEGF signaling is required for development and early survival of most ocular vessels

To begin to identify the mechanisms that govern patterning of superficial ocular vessels in zebrafish, we asked what signals drove growth of these vessels. VEGF signaling is known for pro-

moting angiogenesis in multiple contexts; however, recent work has shown that in the trunk of zebrafish embryos, Bone Morphogenetic Signaling (BMP) signaling drove venous angiogenesis whereas VEGF signaling drove arterial angiogenesis (Wiley *et al.*, 2011). Given that all superficial vessels are derived from a vein, the PMBC, we asked which signaling pathway drove their growth. To examine the contribution of these signaling pathways to ocular angiogenesis we used small molecule inhibitors to block VEGF or BMP signaling and analyzed the development of ocular vessels in Tg(*kdrl:EGFP*) embryos, in which all endothelial cells express EGFP under regulation of *vegfr-2* promoter/enhancer elements, thus affording visualization of the vasculature in live embryos.

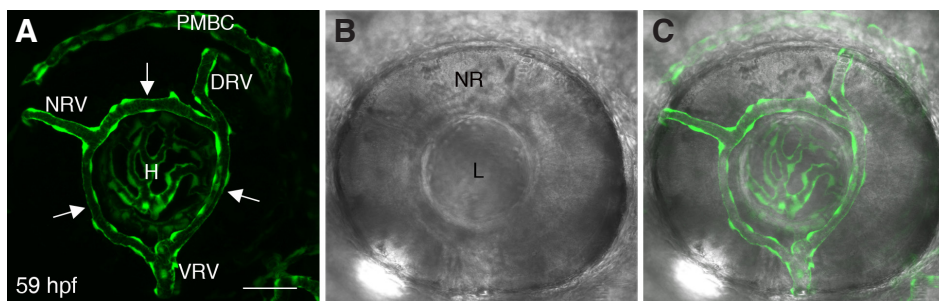
Ocular vessels in zebrafish begin growing around 18 hpf, starting with the hyaloid artery that later grows to form the arterial components of the hyaloid system and following with growth from the PMBC around 20 hpf (Kaufman *et al.*, 2015). The PMBC itself becomes apparent in Tg(*kdrl:EGFP*) embryos only around 18 hpf. To block VEGF signaling we treated embryos with the VEGFR inhibitor SU5416, which has been shown to efficiently block angiogenesis (Fong *et al.*, 1999; Serbedzija *et al.*, 1999), starting at 17 hpf (16 somite-stage) and examined vessel development at 30 hpf and 50 hpf. Whereas in control embryos superficial vessels appeared normal at 30 hpf (n=6/6), in treated embryos superficial vessels completely failed to develop (n=15/15) (Fig. 2 A,B). Moreover, CrDI and hyaloid vessels also did not form. By contrast, the PMBC did form and surprisingly, a rudimentary VRV was observed in the treated embryos, suggesting that PMBC and VRV formation were VEGF-independent (Fig. 2B). By 50 hpf, in SU5416-treated embryos there was no appreciable growth of dorsal superficial vessels, and the PMBC appeared to be disintegrating. In most of the embryos the VRV and a rudimentary hyaloid vein were present (n= 12/14) (Fig. 2D).

In contrast to inhibition of VEGF signaling, a similar experiment in which BMP signaling was blocked using the specific BMP inhibitor DMH1 (Hao *et al.*, 2010) showed that blocking BMP signaling results in abnormal superficial vessel patterning, but does not inhibit the growth of these vessels or of hyaloid vessels (n=13/16) (Fig. 2 E,F).

These results suggest that ocular vessel development largely depends on VEGF signaling, however formation of vessels originating in the ventral PMBC appears to be VEGF-independent.

Next, we examined whether components of the VEGF pathway were expressed in the developing eye and ocular vessels. Expression of VEGF receptors *kdr1* (*vegfr-2*) and *flt-4* (*vegfr-3*) in the PMBC, which gives rise to all superficial vessels, has been described (Thisse *et al.*, 2001). We examined expression of VEGF ligands *vegfaa* and *vegfab*. *vegfaa* was expressed in the developing eye during stages of ocular vessel development (24 hpf and 30 hpf), with expression mostly localized in the periphery of the lens

Fig. 1. Superficial ocular vasculature in zebrafish embryos. (A) Confocal maximum projection image of ocular vessels from a live 59 hpf Tg(*kdr1:EGFP*) embryo. (B) Bright field image of the eye from the embryo imaged in (A). (C) Overlay of (A,B) to show localization of vessels relative to eye tissues. Arrows in (A) point at the superficial annular vessel (SAV). DRV, dorsal radial vessel; H, hyaloid vessels; L, lens; NR, neural retina; NRV, nasal radial vessel; PMBC, primordial midbrain channel; VRV, ventral radial vessel. Lateral view, anterior to the left. Scale bar, 50 μ m.



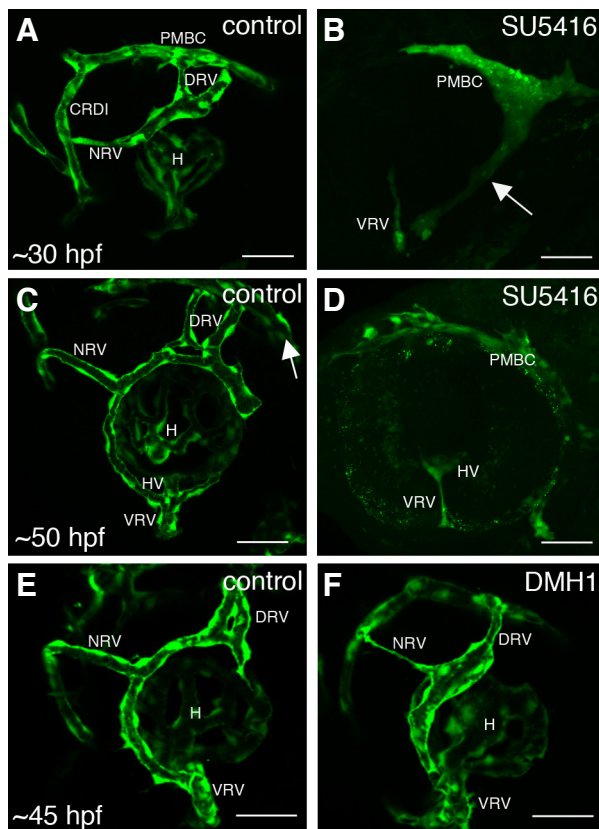


Fig. 2. Vascular endothelial growth factor (VEGF) signaling is required for superficial vasculature development. (A-D) Confocal maximum projection images of ocular vasculature in live *Tg(kdr):EGFP* embryos treated with $1 \mu\text{M}$ SU5416 from 18 hpf (B,D) and control embryos (A,C), at 13 (A,B) and (C,D) 33 h after initiation of treatment and control embryo (E) or embryo treated with $10 \mu\text{M}$ DMH1 (F) from 18 hpf. Arrow in (B) points at the optic vein that grew from the ventral PMBC in a treated embryo. All panels are lateral views, anterior to the left. CrDI, cranial division of internal carotid artery; DRV, dorsal radial vessel; H, hyaloid vessels; HV, hyaloid vein; NRV, nasal radial vessel; PMBC, primordial midbrain channel; VRV, ventral radial vessel. Scale bars, $50 \mu\text{m}$.

(Fig. 3 A,B). *vegfab* was weakly expressed in the optic (choroid) fissure region (Fig. 3 C,D). *vegfc* and *vegfd* do not appear to be expressed in or near the developing eye during the time ocular vessels develop (Astin *et al.*, 2014; Ober *et al.*, 2004) (our unpublished observations).

VEGF signaling is required for survival of ocular vessels only at early developmental stages

VEGF signaling is required not only to promote angiogenesis but also for survival of endothelial cells (Alon *et al.*, 1995). To find whether superficial ocular vessels required VEGF signaling for their survival we treated embryos with the VEGFR-2 inhibitor 1-[4-(6,7-Dimethoxy-quinolin-4-yloxy)-3-fluoro-phenyl]-3-(2-fluoro-phenyl)-urea (Ilovich *et al.*, 2008), hereafter referred to as U7, at different time windows and examined the effect on ocular blood vessels. This VEGFR-2 inhibitor was used in this work interchangeably with SU5416 and gave the same results. Ocular vasculature was documented just prior to treatment and imaged again at the end of the treatment. The first time window of treatment was between 18 and 25 hpf. At 18 hpf, ocular vessels have not formed yet and the PMBC was not detected. Only the hyaloid artery was observed beginning to grow inside the eye (Fig. 4 A,B). After blocking VEGF signaling from 18 hpf until 25 hpf, consistent with the data described above when using SU5416, the PMBC formed but no sprouting of DRV was observed and only a rudimentary VRV could be seen ($n=7/7$), whereas in control embryos the DRV has begun growing ($n=6/7$) (Fig. 4 A',B'). Next we blocked VEGF signaling from 27 hpf, when the DRV has already sprouted (Fig. 4 C,D), until 47 hpf, a time point when in control embryos most superficial ocular vessels have already formed, including DRV, NRV, VRV and anterior SAV ($n=11/11$) (Fig. 4C'). In treated embryos, whereas at 27 hpf a well-developed DRV was observed (Fig. 4D), at 47 hpf almost none of the vessels could be detected, and only a remnant of the VRV was present ($n=9/9$) (Fig. 4D'), indicating that VEGF signaling was also required for the survival of newly formed superficial vessels. Finally, we blocked VEGF signaling from 45 hpf, when much of the superficial system has formed except the posterior SAV (Fig. 4 E,F) until 62 hpf, when the superficial system has fully formed. Whereas in control embryos superficial vessels have generated

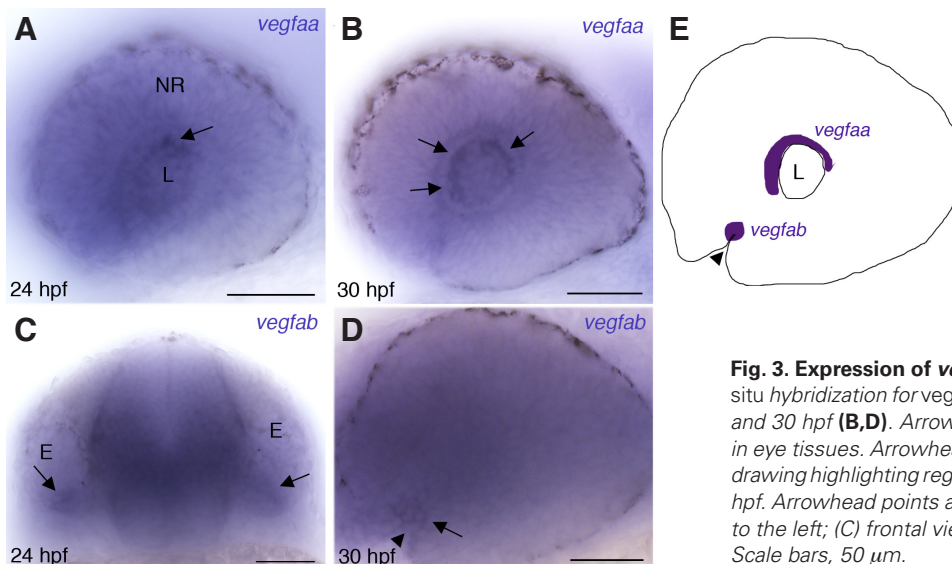


Fig. 3. Expression of *vegfa* ligands in developing eye tissues. (A-D) In situ hybridization for *vegfaa* (A,B) and *vegfab* (C,D) mRNAs at 24 hpf (A,C) and 30 hpf (B,D). Arrows point at regions where expression is observed in eye tissues. Arrowhead in (D) points at the optic fissure. (E) Schematic drawing highlighting regions of *vegfaa* and *vegfab* expression (purple) at 30 hpf. Arrowhead points at the optic fissure. (A,B,D) Lateral views, anterior to the left; (C) frontal view, dorsal is up. E, eye; L, lens; NR, neural retina. Scale bars, $50 \mu\text{m}$.

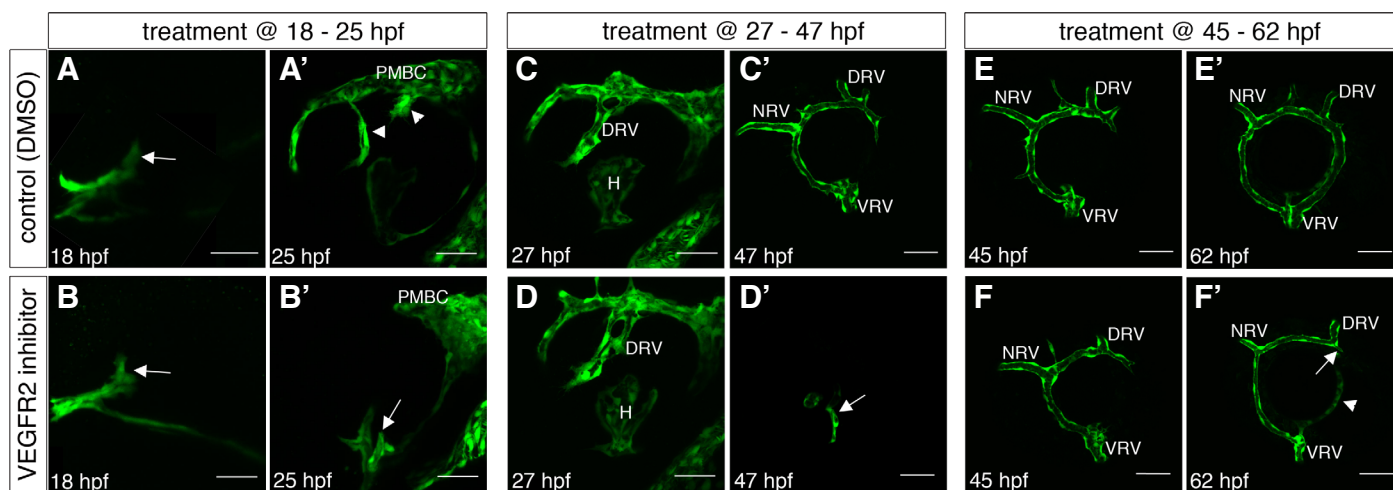


Fig. 4. Vascular endothelial growth factor (VEGF) is required for survival of newly formed superficial vessels. (A-F, A'-F') Confocal maximum projection images of superficial ocular vasculature in live *Tg(kdrl:EGFP)* control embryos (upper panel) and embryos treated with 500 nM VEGFR-2 inhibitor U7 (lower panel) at different time windows. Time window is depicted above each panel group. In each time window group, left images are just before treatment and right images are at the end, in the same embryos and eyes. Arrows in (A,B) point at the hyaloid artery that begins growing into the eye. Arrowheads in (A') point at sprouts from the PMBC and arrow in (B') points at the developing VRV and hyaloid artery. Arrow in (D') points at a remnant of the VRV. Arrow in (F') points at the point where growth of superficial annular vessel (SAV) was arrested and arrowhead points at a hyaloid vessel. In (C; E, E'; F, F') hyaloid vessels are not shown to help focus on superficial vasculature. All panels are lateral views, anterior to the left. DRV, dorsal radial vessel; H, hyaloid vessels; NRV, nasal radial vessel; PMBC, primordial midbrain channel; VRV, ventral radial vessel. Scale bars, 50 μm.

the final pattern (n=7/7), in treated embryos development of the superficial system was arrested and remained as it was at 45 hpf (n=7/7) (Fig. 4 E',F,F'). Importantly, vessels did not degrade, suggesting that from 45 hpf and possibly even earlier, superficial vessels no longer require VEGF signaling for their survival.

Together, the results show that consistent with known functions of VEGF signaling, it is required for the formation of most superficial ocular vasculature and for the survival of these vessels only during the stages shortly after they have formed.

Loss of Hedgehog signaling results in excess sprouting from the PMBC and abnormal superficial vessel patterning

We next asked which additional signaling pathways might influence superficial vessel development. We chose to examine

the effect of Hh signaling because it has been shown to positively regulate VEGF signaling in several contexts and it also influences eye development. To find whether and how Hh signaling affected superficial vessel development we used *smoothened* (*smo*) mutant embryos, in which activity of Smoothened, the obligatory mediator of Hh signaling, is missing (Varga et al., 2001). Surprisingly, we found that in *smo* mutants, sprouting from the PMBC was increased. At 27 hpf, in normal embryos 1-2 sprouts from the PMBC give rise to 1-2 DRVs (Kaufman et al., 2015) (Fig. 5A). By contrast, in mutant embryos we observed up to six sprouts, which gave rise to an ectopic network of vessels over the dorsal aspect of the developing eye (n=19/19) (Fig. 5 B,C,F). By 54 hpf, when development of the superficial system is complete (Fig. 5D), in *smo* mutants the abnormally patterned ectopic network of vessels persisted over

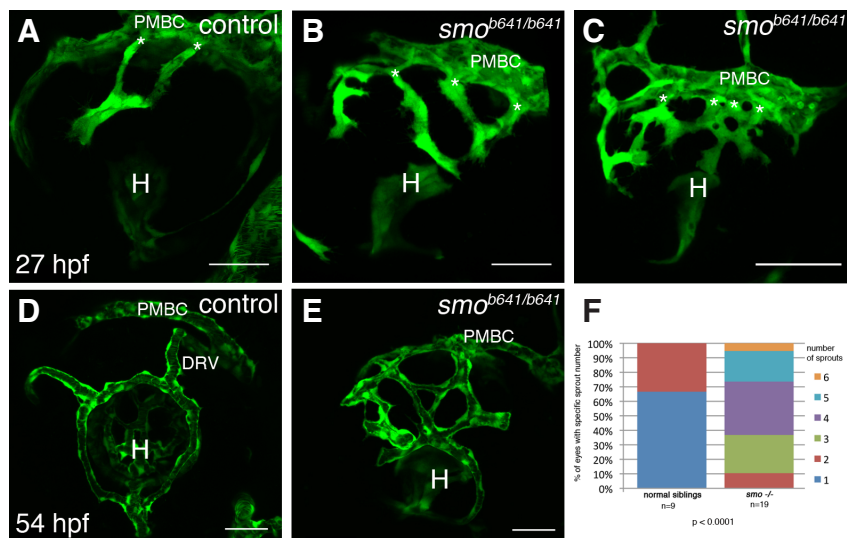
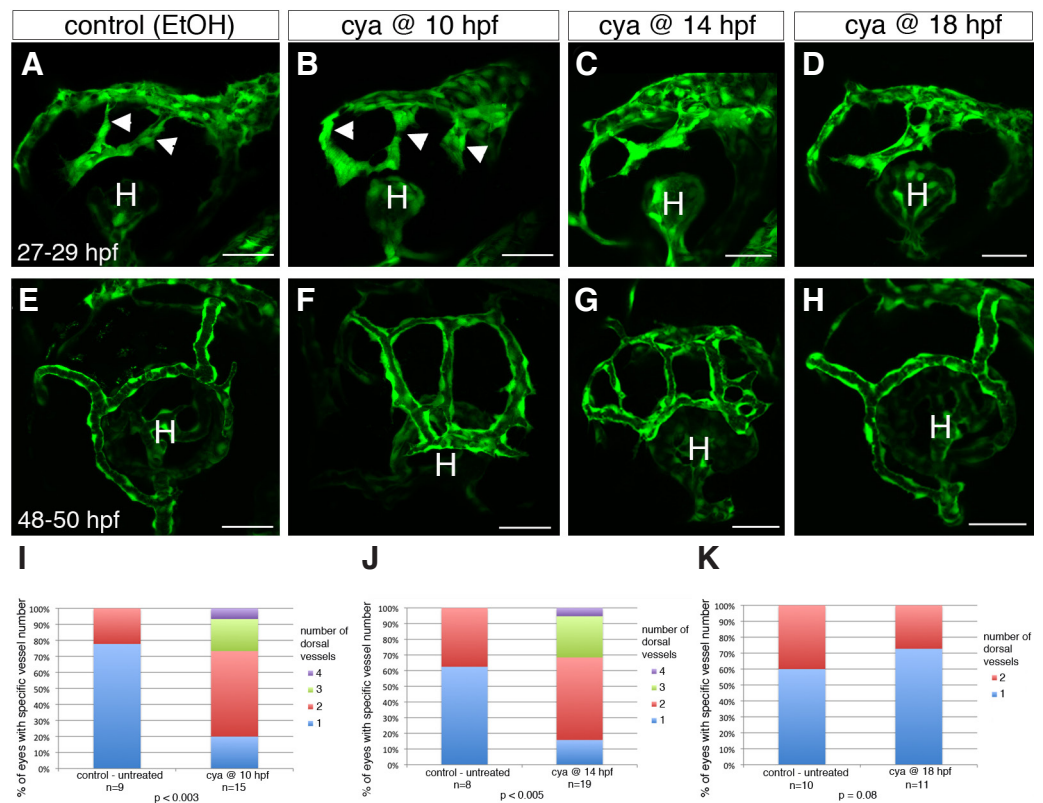


Fig. 5. Excess superficial vessel sprouting in *smo* mutant embryos. (A-E) Confocal maximum projection images of superficial ocular vasculature in live normal or *smo* mutant embryos carrying *kdrl:EGFP* transgene, at 27 hpf (A-C) or 54 hpf (D,E). Asterisks in (A-C) mark exit points of sprouts from the PMBC. (F) Quantification of the number of sprouts from dorsal PMBC in *smo* mutants versus normal siblings at 27 hpf. The difference in the number of sprouts is statistically significant (Student's *t*-test). In all images, anterior is left and dorsal up. DRV, dorsal radial vessel; H, hyaloid vessels; PMBC, primordial midbrain channel. Scale bars, 50 μm.

Fig. 6. Early cyclopamine treatment phenocopies *smo* superficial vessel phenotype. (A-H) Confocal maximum projection images of superficial ocular vasculature in live *Tg(kdrl:EGFP)* control embryos (A,E) and embryos treated with 10 μ M cyclopamine (*cya*) from different time points (B-D, F-H). Treatment start time is depicted above each pair of images from the same embryo, showing phenotypes at 27-29 hpf (top panel) and 47 hpf (bottom panel). Arrowheads in (A,B) point at sprouts from the dorsal PMBC over the eye. (I-K) Quantification of the number of dorsal vessels connected to the PMBC in control embryos and embryos treated with cyclopamine at 48 hpf. Separate comparisons are shown for each time point of cyclopamine addition (I: 10 hpf; J: 14 hpf; K: 18 hpf). P values for each comparison are depicted (Student's *t*-test). H, hyaloid vasculature. All images are lateral views, anterior to the left. Scale bars, 50 μ m.



the dorsal eye whereas vessels failed to grow over the ventral eye ($n=15/15$) (Fig. 5E). Hence, in the absence of Hh activity superficial vessel patterning is abnormal, suggesting a role for Hh signaling in limiting angiogenesis from the dorsal PMBC and in promoting vascular development over the ventral eye.

Hedgehog functions indirectly to influence ocular vessel patterning

Because Hh ligands are not expressed near the dorsal PMBC during the time of sprouting (Egger *et al.*, 1995; Krauss *et al.*, 1993), we hypothesized that the effect of Hh on ocular angiogenesis was indirect. To test this hypothesis we interfered with Hh signaling during different time windows and assayed development of superficial ocular vessels. The rationale of this experiment is that if Hh signaling directly affected vessel growth, then blocking Hh signaling around the time of sprouting from the PMBC would lead to a similar phenotype to *smo* mutants. Alternatively, if Hh signaling acted indirectly, then in order to phenocopy the *smo* mutant phenotype one would need to interfere with Hh signaling before vessel sprouting. To block Hh signaling we used the small molecule cyclopamine, which inhibits Smo function (Chen *et al.*, 2002). For all time points of control and treatment, results represent at least 10 embryos. When embryos were treated with cyclopamine from gastrulation stages (6-10 hpf) and assayed at 27-29 hpf and at 48-50 hpf, superficial vessels showed similar phenotypes to those of *smo* mutants at both time points (Fig. 6 B,F,I). When embryos were treated from 14 hpf (12 somite stage), their superficial vasculature appeared relatively normal at 27-29 hpf, but phenocopied *smo* mutants at 48-50 hpf (Fig. 6 C,G,J). When treatment was initiated at 18 hpf, superficial vessels developed normally at both time points (Fig. 6 D,H,K). Since sprouting from

the PMBC starts only at 22-23 hpf, these data are consistent with the idea that Hh signaling influences sprouting and patterning of superficial vessels indirectly.

Excess sprouting in *smo* mutants is only partially VEGF-dependent

The excessive sprouting from the PMBC in *smo* mutant embryos is surprising given that, to date, Hh signaling was found to be pro-angiogenic. As Hh pathway activity can regulate VEGF expression and ocular vessel growth is largely dependent on VEGF signaling, we next asked whether and how expression of VEGF ligands was affected in *smo* mutants. *In situ* hybridization for *vegfaa* showed clear reduction of expression in the trunk region compared to normal (Fig. 7 B,D), as previously described (Lawson *et al.*, 2002), however, expression levels in the head and eye region appeared much less affected (Fig. 7 A,C). *vegfab* expression also appeared largely unaffected in the head and eye regions (Fig. 7 E,G), whereas in the trunk region it appeared somewhat elevated (Fig. 7 F,H). Hence, Hh activity does not appear to play a major role in regulating relevant VEGF pathway ligand expression in the head region.

Next, we blocked VEGF signaling in *smo* mutant embryos by treating them with SU5416 from 18 hpf and assayed ocular vessel development at 30 hpf and 50 hpf. Surprisingly, treated *smo* mutant embryos showed at 30 hpf some sprouting from the PMBC over the dorsal eye, whereas their treated normal siblings, similar to treated wild-type embryos, had no sprouting (Fig. 8 B,C,G). Although sprouts were present in *smo* mutant embryos, they were underdeveloped compared to untreated embryos (Fig. 8B). At 50 hpf, sprouts of treated *smo* mutant embryos continued to develop, while treated siblings showed degeneration of vasculature. At both time points, treated *smo* mutant embryos had significantly

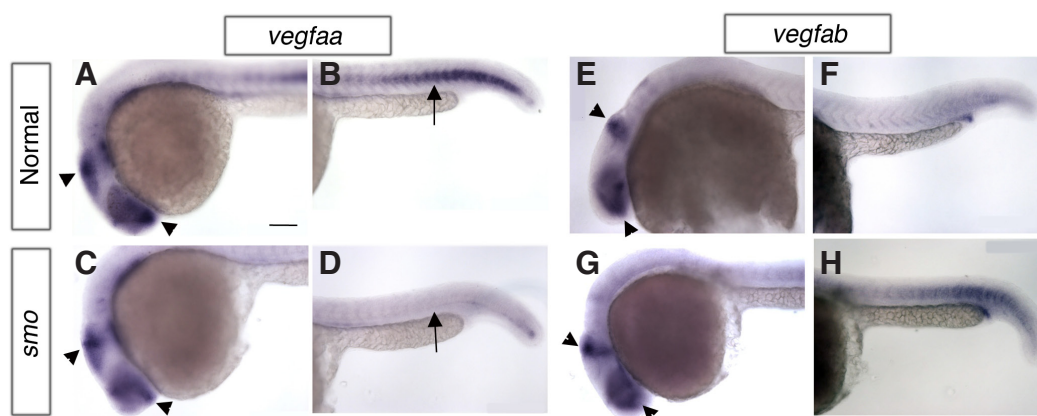


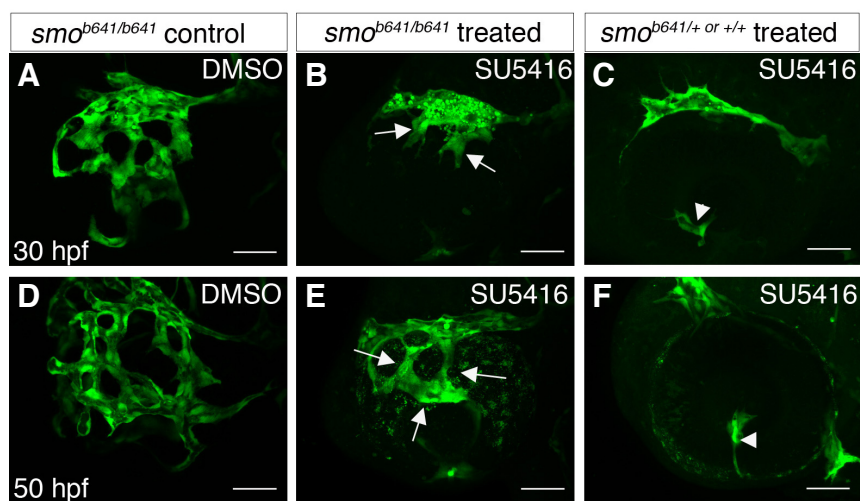
Fig. 7. *vegfaa* and *vegfab* expression in *smo* mutant embryos. (A-H) In situ hybridization for *vegfaa* (A-D) and *vegfab* (E-H) in normal (A, B, E, F) and *smo* mutant (C, D, G, H) embryos. (A, C, E, G) show expression in the head region, (B, D, F, H) show expression in the trunk region. Arrowheads point at major sites of expression in the head, and arrows in (B, D) point at somite region that expresses *vegfaa* in normal but not in *smo* mutant embryos. All images are lateral views, anterior to the left. Scale bar, 100 μ m.

more sprouts than treated normal siblings, but significantly less than untreated *smo* mutant embryos (Fig. 8G). This result suggests that excessive sprouting in embryos lacking Hh signaling is only partially dependent on VEGF activity. The mechanism that enables angiogenesis when VEGF activity is blocked remains to be determined.

Discussion

In this work we examine the contributions of VEGF and Hh signaling pathways to formation and patterning of superficial ocular vessels in zebrafish. We find that VEGF signaling is required to promote growth and survival of newly formed ocular vessels, with exception of vessels that originate in the ventral PMBC. Hh signaling is not required for superficial vessels growth but is required for their correct patterning.

Although VEGF signaling is the pathway most commonly implicated in promoting angiogenesis, other signaling pathways have been shown to promote vascular growth independently of VEGF. For example, Bone morphogenetic protein (BMP) signaling has been shown to promote venous sprouting angiogenesis in the zebrafish trunk, whereas VEGF signaling promoted arterial sprouting angiogenesis (Wiley *et al.*, 2011). However, we find that even though the entire superficial vasculature is derived from a venous source, the PMBC, it is largely dependent on VEGF for its development by angiogenesis. Only vessels derived from the ventral PMBC, i.e. the VRV and hyaloid venous vessels appear to develop independently of VEGF signaling. It is interesting to note that the growth of these vessels from the ventral PMBC appears somewhat different from angiogenesis from the dorsal PMBC as endothelial cells originating from the ventral PMBC form a thin chain of cells that reaches the ventral aspect of the eye, near the optic fissure (Kaufman *et al.*, 2015). This appears different from the sprouts arising from the dorsal PMBC that have a prominent tip cell, as also described in other contexts. It is possible that the different appearance correlates with a different molecular mechanism underlying the angiogenic process from the ventral PMBC. What is the molecular mechanism that promotes angiogenesis from the ventral PMBC remains to be determined. We have ruled out Hh, Fibroblast growth factor (FGF) and BMP signaling (Weiss, Kaufman and Inbal, unpublished observations). Still, several other pro-angiogenic molecules can



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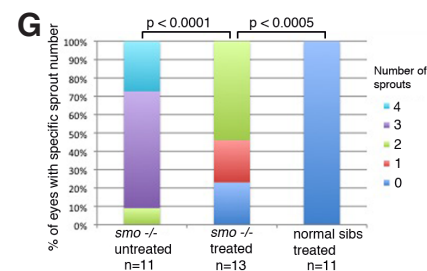


Fig. 8. Excess sprouting in *smo* mutants is only partially dependent on vascular endothelial growth factor (VEGF) signaling. (A-F) Confocal maximum projection images of superficial ocular vasculature in live normal (C, F) or *smo* mutant (A, B, D, E) embryos carrying *kdr1:EGFP* transgene, at 30 hpf (A-C) or 50 hpf (D-F). (A, D) Control *smo* mutant embryo showing the typical superficial vessel phenotype of *smo* mutant embryos. (B, E) A *smo* mutant embryo treated with 1 μ M SU5416 from 18 hpf to inhibit VEGF signaling, showing reduced, but not complete lack of superficial vessel growth (arrows, compare to C, F). (C, F) A normal sibling treated with 1 μ M SU5416 from 18 hpf showing no superficial vessel growth. (G) Quantification of the number of sprouts from dorsal PMBC in *smo* mutants, treated or untreated with SU5416 and normal siblings treated with SU5416 at 30 hpf. Comparing the number of sprouts between treated and untreated mutants or treated mutants and sibs shows that the difference in the number of sprouts is statistically significant in both cases (Student's *t*-test). Arrowheads point at residual hyaloid vein and VRV. All images are lateral views, anterior to the left. Scale bars, 50 μ m.

account for this growth, e.g. *apelin/elabela* (Helker *et al.*, 2015) and their potential role needs to be examined.

The lack of Hh signaling resulted in a surprising effect on ocular vessels. Given that Hh is known to function as a pro-angiogenic factor, we expected that if it had a role in ocular vessel development, its loss of function would lead to reduced angiogenesis. However, we observed the opposite effect over the dorsal eye, namely increased sprouting from the dorsal PMBC. Over the ventral eye vessels failed to grow, which is consistent with a pro-angiogenic role of Hh. These opposite effects are indirect, hence, they are mediated by another process that takes place by mid-late segmentation stages. One possible candidate process is eye patterning, which is largely completed by the time superficial vessels begin to form at approximately 22–23 hpf. Both dorsoventral and nasotemporal patterning of the eye are influenced by Hh signaling (Ekker *et al.*, 1995; Hernandez-Bejarano *et al.*, 2015; Lupo *et al.*, 2005; Takeuchi *et al.*, 2003). We hypothesize that the stereotypic growth of superficial ocular vessels, which takes place over the surface of the eye, is at least in part directed by the molecular landscape of the eye. If this hypothesis is correct, abnormal eye patterning could lead to abnormalities in superficial vessel patterning. Hence, it is possible that eyes of Hh-deficient embryos, which have been shown to be abnormally patterned, mediate the effect of Hh signaling on superficial vessel development. Alternatively, abnormalities of other adjacent structures due to lack of Hh activity, such as brain tissues or the surface ectoderm in the eye region, could also influence ocular vessel development. At the molecular level, it is possible that factors that restrict angiogenesis such as, for example, PlexinD1 (Childs *et al.*, 2002; Torres-Vazquez *et al.*, 2004) are reduced in the dorsal PMBC or eye region in Hh-deficient embryos. The exact mechanism through which Hh influences ocular vessel patterning will likely remain unclear until better understanding of the direct signals that underlie ocular vessel patterning is achieved.

Materials and Methods

Fish lines and husbandry

Zebrafish (*Danio rerio*) were maintained according to standard procedures on a 14 hour light/10 hour dark cycle at 28.5°C and embryos were staged as previously described (Kimmel *et al.*, 1995). The following published lines were used in this work: Tg(*kdr1:EGFP*)s843 (Jin *et al.*, 2005), *smoothened* null allele *smob641* (Varga *et al.*, 2001). AB and TL lines were used as wild type. All experiments were approved by the Hebrew University Authority for Biological and Biomedical Models.

In situ hybridization

Whole-mount *in situ* hybridization (WISH) using riboprobes was performed according to standard protocols (Thisse and Thisse, 2008). BM Purple (Roche) was used as color substrate.

Probes used in this work were *vegfaa* (Liang *et al.*, 2001); *vegfab*: *vegfab* coding sequence was PCR amplified from wild-type total cDNA, cloned into pGEM-T Easy vector (Promega) and confirmed by sequencing. The plasmid was linearized using NcoI and RNA was transcribed using SP6 RNA polymerase.

Small molecule treatments

To block VEGF signaling we used interchangeably two VEGFR-2 inhibitors: SU5416 (Sigma-Aldrich #S8442) was dissolved in DMSO and was used at 1 μM and 1-[4-(6,7-Dimethoxy-quinolin-4-yloxy)-3-fluoro-phenyl]-3-(2-fluoro-phenyl)-urea (Ilovich *et al.*, 2008) was dissolved in DMSO and was used at 500 nM. DMH1 (Sigma-Aldrich #D8946) was dissolved in

DMSO and was used at 10 μM. Cyclopamine (Sigma-Aldrich #C4116) was dissolved in EtOH and used at 10 μM.

Imaging

To block pigmentation when imaging embryos older than 24 hpf, embryos were raised from 22 hpf in the presence of 0.003% N-Phenylthiourea (PTU; Sigma-Aldrich #P7629). Live embryos were anaesthetized using tricaine and mounted in 0.5% low-melting-point agarose (SeaPlaque, Lonza).

Images were acquired using Zeiss LSM700 confocal microscope and Axio Imager.M2 compound microscope or with Discovery.V8 stereoscope and AxioCam MRc digital camera (Zeiss). Microscope objectives used were 40x 1.0 NA water objective or 25x 0.8 NA or 10x 0.3 NA. Images were exported as JPEG or TIFF files using ZEN 2009 LE software (Zeiss) and figures were assembled using Adobe Photoshop CS4 software.

Quantification and statistical analysis

Quantification of dorsal sprouts of superficial vessels was performed by manually counting exit points of sprouts from the PMBC in 3D reconstructions of confocal image data. Statistical analysis was done using Student's t-test.

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References

- ALON, T., HEMO, I., ITIN, A., PE'ER, J., STONE, J. and KESHET, E. (1995). Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med* 1: 1024–1028.
- ASTIN, J. W., HAGGERTY, M. J., OKUDA, K. S., LE GUEN, L., MISA, J. P., TROMP, A., HOGAN, B. M., CROSIER, K. E. and CROSIER, P. S. (2014). Vegfd can compensate for loss of Vegfc in zebrafish facial lymphatic sprouting. *Development* 141: 2680–2690.
- CARMELIET, P. (2003). Angiogenesis in health and disease. *Nat Med* 9: 653–660.
- CARMELIET, P., FERREIRA, V., BREIER, G., POLLEFEY, S., KIECKENS, L., GERTSENSTEIN, M., FAHRIG, M., VANDENHOECK, A., HARPAL, K., EBERHARDT, C. *et al.*, (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380: 435–439.
- CHEN, J. K., TAIPALE, J., COOPER, M. K. and BEACHY, P. A. (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev* 16: 2743–2748.
- CHILDS, S., CHEN, J. N., GARRITY, D. M. and FISHMAN, M. C. (2002). Patterning of angiogenesis in the zebrafish embryo. *Development* 129: 973–982.
- CHINCHILLA, P., XIAO, L., KAZANIETZ, M. G. and RIOBO, N. A. (2010). Hedgehog proteins activate pro-angiogenic responses in endothelial cells through non-canonical signaling pathways. *Cell Cycle* 9: 570–579.
- COVASSIN, L. D., VILLEFRANC, J. A., KACERGIS, M. C., WEINSTEIN, B. M. and LAWSON, N. D. (2006). Distinct genetic interactions between multiple Vegf receptors are required for development of different blood vessel types in zebrafish. *Proc Natl Acad Sci USA* 103: 6554–6559.
- EKKER, S. C., UNGAR, A. R., GREENSTEIN, P., VON KESSLER, D. P., PORTER, J. A., MOON, R. T. and BEACHY, P. A. (1995). Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr Biol* 5: 944–955.
- ELLERTSDOTTIR, E., LENARD, A., BLUM, Y., KRUEDEWIG, A., HERWIG, L., AFFOLTER, M. and BELTING, H. G. (2010). Vascular morphogenesis in the zebrafish embryo. *Dev Biol* 341: 56–65.
- FERRARA, N., CARVER-MOORE, K., CHEN, H., DOWD, M., LU, L., O'SHEA, K. S., POWELL-BRAXTON, L., HILLAN, K. J. and MOORE, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380: 439–442.
- FONG, T. A., SHAWVER, L. K., SUN, L., TANG, C., APP, H., POWELL, T. J., KIM, Y. H., SCHRECK, R., WANG, X., RISAU, W. *et al.*, (1999). SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. *Cancer Res* 59: 99–106.

- HAO, J., HO, J. N., LEWIS, J. A., KARIM, K. A., DANIELS, R. N., GENTRY, P. R., HOPKINS, C. R., LINDSLEY, C. W. and HONG, C. C. (2010). *In vivo* structure-activity relationship study of dorsomorphin analogues identifies selective VEGF and BMP inhibitors. *ACS Chem Biol* 5: 245-253.
- HARTSOCK, A., LEE, C., ARNOLD, V. and GROSS, J. M. (2014). *In vivo* analysis of hyaloid vasculature morphogenesis in zebrafish: A role for the lens in maturation and maintenance of the hyaloid. *Dev Biol* 394: 327-339.
- HEINKE, J., PATTERSON, C. and MOSER, M. (2012). Life is a pattern: vascular assembly within the embryo. *Front Biosci (Elite Ed)* 4: 2269-2288.
- HELKER, C. S., SCHUERMAN, A., POLLMANN, C., CHNG, S. C., KIEFER, F., REVERSADE, B. and HERZOG, W. (2015). The hormonal peptide Elabela guides angioblasts to the midline during vasculogenesis. *Elife* 4.
- HERNANDEZ-BEJARANO, M., GESTRI, G., SPAWLS, L., NIETO-LOPEZ, F., PICKER, A., TADA, M., BRAND, M., BOVOLENTA, P., WILSON, S. W. and CAVODEASSI, F. (2015). Opposing Shh and Fgf signals initiate nasotemporal patterning of the zebrafish retina. *Development* 142: 3933-3942.
- ILOVICH, O., JACOBSON, O., AVIV, Y., LITCHI, A., CHISIN, R. and MISHANI, E. (2008). Formation of fluorine-18 labeled diaryl ureas--labeled VEGFR-2/PDGFR dual inhibitors as molecular imaging agents for angiogenesis. *Bioorg Med Chem* 16: 4242-4251.
- JENSEN, L. D., NAKAMURA, M., BRAUTIGAM, L., LI, X., LIU, Y., SAMANI, N. J. and CAO, Y. (2015). VEGF-B-Neuropilin-1 signaling is spatiotemporally indispensable for vascular and neuronal development in zebrafish. *Proc Natl Acad Sci U S A* 112: E5944-5953.
- JIN, S. W., BEIS, D., MITCHELL, T., CHEN, J. N. and STAINIER, D. Y. (2005). Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development* 132: 5199-5209.
- KAUFMAN, R., WEISS, O., SEBBAGH, M., RAVID, R., GIBBS-BAR, L., YANIV, K. and INBAL, A. (2015). Development and origins of zebrafish ocular vasculature. *BMC Dev Biol* 15: 18.
- KIMMEL, C. B., BALLARD, W. W., KIMMEL, S. R., ULLMANN, B. and SCHILLING, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn* 203: 253-310.
- KITAMBI, S. S., MCCULLOCH, K. J., PETERSON, R. T. and MALICKI, J. J. (2009). Small molecule screen for compounds that affect vascular development in the zebrafish retina. *Mech Dev* 126: 464-477.
- KRAUSS, S., CONCORDET, J. P. and INGHAM, P. W. (1993). A functionally conserved homolog of the *Drosophila* segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* 75: 1431-1444.
- LAWSON, N. D., VOGEL, A. M. and WEINSTEIN, B. M. (2002). sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev Cell* 3: 127-136.
- LIANG, D., CHANG, J. R., CHIN, A. J., SMITH, A., KELLY, C., WEINBERG, E. S. and GE, R. (2001). The role of vascular endothelial growth factor (VEGF) in vasculogenesis, angiogenesis, and hematopoiesis in zebrafish development. *Mech Dev* 108: 29-43.
- LUPO, G., LIU, Y., QIU, R., CHANDRARATNA, R. A., BARSACCHI, G., HE, R. Q. and HARRIS, W. A. (2005). Dorsal-ventral patterning of the *Xenopus* eye: a collaboration of Retinoid, Hedgehog and FGF receptor signaling. *Development* 132: 1737-1748.
- NAGASE, T., NAGASE, M., MACHIDA, M. and FUJITA, T. (2008). Hedgehog signaling in vascular development. *Angiogenesis* 11: 71-77.
- OBER, E. A., OLOFSSON, B., MAKINEN, T., JIN, S. W., SHOJI, W., KOH, G. Y., ALITALO, K. and STAINIER, D. Y. (2004). Vegfc is required for vascular development and endoderm morphogenesis in zebrafish. *EMBO Rep* 5: 78-84.
- POLA, R., LING, L. E., SILVER, M., CORBLEY, M. J., KEARNEY, M., BLAKE PEPINSKY, R., SHAPIRO, R., TAYLOR, F. R., BAKER, D. P., ASAHARA, T. et al., (2001). The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat Med* 7: 706-711.
- PROULX, K., LU, A. and SUMANAS, S. (2010). Cranial vasculature in zebrafish forms by angioblast cluster-derived angiogenesis. *Dev Biol* 348: 34-46.
- RENAULT, M. A., RONCALLI, J., TONGERS, J., THORNE, T., KLYACHKO, E., MISENER, S., VOLPERT, O. V., MEHTA, S., BURG, A., LUEDEMANN, C. et al., (2010). Sonic hedgehog induces angiogenesis via Rho kinase-dependent signaling in endothelial cells. *J Mol Cell Cardiol* 49: 490-498.
- SAINT-GENIEZ, M. and D'AMORE, P. A. (2004). Development and pathology of the hyaloid, choroidal and retinal vasculature. *Int J Dev Biol* 48: 1045-1058.
- SERBEDZIJA, G. N., FLYNN, E. and WILLETT, C. E. (1999). Zebrafish angiogenesis: a new model for drug screening. *Angiogenesis* 3: 353-359.
- SHALABY, F., ROSSANT, J., YAMAGUCHI, T. P., GERTSENSTEIN, M., WU, X. F., BREITMAN, M. L. and SCHUH, A. C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376: 62-66.
- TAKE-UCHI, M., CLARKE, J. D. and WILSON, S. W. (2003). Hedgehog signalling maintains the optic stalk-retinal interface through the regulation of Vax gene activity. *Development* 130: 955-968.
- THISSE, B., PLUMIO, S., FÜRTHAUER, M., LOPPIN, B., HEYER, V., DEGRAVE, A., WOEHL, R., LUX, A., STEFFAN, T., CHARBONNIER, X. Q. et al., (2001). Expression of the zebrafish genome during embryogenesis. *ZFIN Direct Data Submission* (<http://zfin.org>).
- THISSE, C. and THISSE, B. (2008). High-resolution *in situ* hybridization to whole-mount zebrafish embryos. *Nat Protoc* 3: 59-69.
- TORRES-VAZQUEZ, J., GITLER, A. D., FRASER, S. D., BERK, J. D., VAN, N. P., FISHMAN, M. C., CHILDS, S., EPSTEIN, J. A. and WEINSTEIN, B. M. (2004). Semaphorin-plexin signaling guides patterning of the developing vasculature. *Dev Cell* 7: 117-123.
- VARGA, Z. M., AMORES, A., LEWIS, K. E., YAN, Y. L., POSTLETHWAIT, J. H., EISEN, J. S. and WESTERFIELD, M. (2001). Zebrafish smoothed functions in ventral neural tube specification and axon tract formation. *Development* 128: 3497-3509.
- WILEY, D. M., KIM, J. D., HAO, J., HONG, C. C., BAUTCH, V. L. and JIN, S. W. (2011). Distinct signalling pathways regulate sprouting angiogenesis from the dorsal aorta and the axial vein. *Nat Cell Biol* 13: 686-692.

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