microRNA-mediated integration of haemodynamics and Vegf signaling during angiogenesis

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Abstract

Within the circulatory system, blood flow regulates vascular remodeling1, stimulates blood stem cell formation2, and plays a role in the pathology of vascular disease3. During vertebrate embryogenesis, vascular patterning is initially guided by conserved genetic pathways that act prior to circulation4. Subsequently, endothelial cells must incorporate the mechanosensory stimulus of blood flow with these early signals to shape the embryonic vascular system4. However, few details are known about how these signals are integrated during development. To investigate this process, we focused on the aortic arch (AA) blood vessels, which are known to remodel in response to blood flow1. By using 2-photon imaging of live zebrafish embryos, we observe that flow is essential for angiogenesis during AA development. We further find that angiogenic sprouting of AA vessels requires a flow-induced genetic pathway in which the mechano-sensitive zinc finger transcription factor klf2α5-7 induces expression of an endothelial-specific microRNA, mir-126, to activate Vegf signaling. Taken together, our work describes a novel genetic mechanism in which a microRNA facilitates integration of a physiological stimulus with growth factor signaling in endothelial cells to guide angiogenesis.

Within a blood vessel, flow exerts tangential and perpendicular forces upon endothelial cells, leading to cytoskeletal rearrangements and changes in gene expression4. While initial embryonic vascular patterning is largely independent of these hemodynamic forces, the onset of circulation drives subsequent remodeling of the circulatory system4. For example,
flow plays an important role in the unilateral regression of the sixth AA during mouse development. In zebrafish, the fifth and sixth AA arise after flow begins and form a persistent connection to the lateral dorsal aortae (LDA) that provides circulation to the trunk. These vessels continue to undergo angiogenesis throughout larval stages to comprise the gill vasculature. To investigate how flow affects angiogenesis, we observed development of AA5 and 6 in zebrafish embryos by 2-photon time-lapse imaging. We performed microangiography on Tg(kdrl:egfp)la116 embryos, which display fluorescent green endothelial cells, using unconjugated Quantum dots (QDots). At 46 hpf, we observed AA perfusion, but no connection between the fifth and sixth AA and the LDA (data not shown and Supplementary Fig. 1a-c). Several hours later, the AA 5/6 connecting vessel (referred to as AA5x according to reference 8) sprouted from the left and right AAs (Supplementary Movie 1). At this point, the sprouts were sufficiently lumenized to allow perfusion with Qdots (Supplementary Fig. 1d, 53.75 magnified; Supplementary Movies 1 and 2). However, blood cells entering from the ventral aorta (VA) became trapped in AA5 and 6 (Supplementary Movie 3). AA5x sprouts then fused with the LDA to form a patent circulatory connection (Supplementary Fig. 1d, 59.75h, Supplementary Movie 1). Subsequently, the AA5x fully lumenized and blood flow through AA5 and 6 commenced (Supplementary Movie 4). These observations indicated that the AA5x develops via concomitant angiogenesis and lumenization in the presence of flow.

To determine if flow was required for this process, we performed unilateral laser microsurgery on Tg(kdrl:egfp)la116 embryos to sever the connection between the VA and AA5 and 6 prior to AA5x sprouting (Supplementary Figs. 1e, 2a). Following microsurgery at 46 hpf, we observed normal AA perfusion on the unoperated side by microangiography at 72 hpf (Fig. 1a). By contrast, on the operated side (right) AA5 and 6 failed to bear flow (Fig. 1b), although cranial blood vessels and the AAs appeared morphologically normal (Fig. 1b; Supplementary Figure 2b). A dorsal view of the same embryo revealed that the AA5x formed on the left side of the embryo, but not on the right side where flow was blocked (Fig. 1c, Supplementary Table 1). To support these results, we treated Tg(kdrl:egfp)la116 embryos beginning at 46 hpf with the myosin ATPase inhibitor 2,3-butanedione 2-monoxime (BDM) or the anesthetic Tricaine methanesulfonate to arrest the heart and block circulation. In both treatments, embryos failed to form the AA5x (Fig. 1d, e; Supplementary Table 1), although vascular morphogenesis in other anatomical locations appeared normal (Supplementary Figure 3). 2-photon time-lapse microscopy of embryos without flow suggested that a failure to initiate sprouting, rather than vessel regression, was responsible for loss of AA5x (Supplementary Movies 5 and 6). Time lapse analysis using Tg(fli1a:negfp)y7 embryos, in which endothelial cell nuclei are labeled with Egfp, revealed decreased migratory activity of cells within the aortic arches in the absence of flow when compared to wild type (Supplementary Movies 7 and 8). Interestingly, embryos injected with a gata1 Morpholino displayed normal AA5x development (Fig 1f, Supplementary Table 1), suggesting that shear stress from blood cells was dispensable for AA5x angiogenesis. Together, these results indicate that the AA5x forms via angiogenesis and that this process is dependent on flow.
Vascular endothelial growth factor (Vegf) signaling has been implicated in flow-mediated AA remodeling in mouse embryos. Accordingly, we observed AA expression of the zebrafish Vegf receptor-2 ortholog, kdrl, including expression in the developing AA5x at 48 hpf (Supplementary Fig. 4a). We also observed vegfa expression in the developing glomerulus (Supplementary Fig. 4b, c), which is located near the branch point of the dorsal aorta and towards which the AA5x sprouts (Supplementary Fig. 4d), and in cells surrounding the AA blood vessels (Supplementary Fig. 4e). Consistent with a role for Vegf signaling during AA5x angiogenesis, embryos bearing a kinase-dead mutation in Kdrl (referred to as kdrl<sup>y17</sup>; ref 11) failed to form a patent AA5x (Fig. 1g; Supplementary Table 1). Furthermore, treatment with the Vegf receptor inhibitor SU5416 from 46 to 65 hpf resulted in a block in AA5x formation, while DMSO had no effect (Fig. 1h, i; Supplementary Table 1). Similarly, partial reduction of Vegfa using a low Morpholino dose (3 ng; see reference 12) blocked AA5x development (Fig. 1j, k). Overall vascular morphology and circulatory function, including initial perfusion of the aortic arches, were normal following these manipulations (Supplementary Fig. 3). These observations demonstrate that AA5x formation requires Vegf signaling. In other developmental settings, Notch signaling coordinates Vegf-stimulated angiogenesis<sup>13, 14</sup>. However, we did not detect expression of Notch signaling molecules or Notch activation in the AA5x (Supplementary Fig. 5a-c) and AA5x was not affected by loss of the Notch ligand dll4 (Supplementary Fig 5d). These results suggest that a Notch-independent mechanism is responsible for Vegf-stimulated AA5x angiogenesis.

A possible candidate gene responsible for integrating flow and Vegf signaling during AA5x formation was the zinc finger transcription factor, klf2, which is induced by flow in endothelial cells<sup>6, 7</sup>. We observed that zebrafish klf2a was expressed in the AA in a pattern similar to the endothelial marker, vascular-endothelial cadherin (cdh5; Fig. 2a) and was expressed in the developing AA5x (Supplementary Fig. 4f). Furthermore, AA expression of klf2a, but not cdh5, was reduced in cardiac troponin T2 (tnnt2)-deficient embryos, which lack circulation (Fig. 2a; Supplementary Table 2; Supplementary Fig. 6a, ref 15) and in embryos treated with Tricaine (Supplementary Fig. 6b; Supplementary Table 2). To determine if klf2a was required for AA5x angiogenesis, we utilized Morpholinos targeting either the klf2a exon 3 splice acceptor site (Supplementary Fig. 7a, b) or the klf2a start codon. Embryos injected with either Morpholino displayed normal morphology and grossly normal circulatory patterns, including perfusion of the aortic arches following angiography (Supplementary Fig. 7c, d and data not shown), consistent with recent work demonstrating relatively normal flow patterns and heart rate in klf2a-deficient zebrafish embryos at 48 hpf<sup>16</sup>. However, the normal transient AA circulatory block persisted in klf2a-deficient embryos (compare Supplementary Movies 3, 4, and 9), suggesting a defect in AA5x formation. Indeed, while embryos injected with control Morpholino appeared normal, klf2a-deficient siblings failed to develop the AA5x (Fig. 2b; Supplementary Fig. 7e, f; Supplementary Table 3). Thus, despite the presence of flow, loss of klf2a mimics the AA5x defect observed in embryos lacking flow or Vegf signaling.

In Xenopus laevis embryos, klf2 is important for Vegf receptor-2 expression<sup>17</sup>. However, kdrl expression appeared normal in klf2a-deficient zebrafish embryos (Supplementary Fig
Similarly, neither kdr nor vegfa were altered in embryos lacking circulation (Supplementary Fig. 6a, c) and we did not observe consistent reduction in other known klf2 responsive genes5-7 in the absence of flow or klf2a (Supplementary Fig. 6a). These results raised the possibility that a post-transcriptional mechanism linked flow, klf2a, and Vegf signaling. A candidate for this role was the endothelial-restricted microRNA, miR-12618, which can enhance Vegf signaling19, 20. While miR-126 expression was apparent in the embryonic vasculature prior to circulation (Supplementary Fig. 8a), at later stages its expression appeared much higher in the AAs (Fig 3a, Supplementary Fig. 8a). Strikingly, we found that AA miR-126 expression was dependent on both flow and klf2a expression. While control embryos expressed high levels of miR-126 within the AAs, tntt2- or klf2a-deficient embryos did not (Fig. 3a-c; Supplementary Figs. 6a and 8b, Supplementary Table 2). By contrast, expression of cdh5 and let-7a was unchanged in the absence of flow or klf2a (Fig. 3d-f, Supplementary Fig. 8b), ruling out a general defect in endothelial gene expression or microRNA processing, respectively. Tricaine treatment to block flow similarly reduced miR-126 AA expression (Supplementary Fig. 6b; Supplementary Table 2). Embryos injected with a Morpholino to prevent miR-126 processing (Supplemental Fig. 8c) displayed blocked AA circulation (Supplementary Movie 10) and hemorrhage in this region by 60 hpf (Supplementary Fig. 8d). Similar to loss of klf2a, the AA5x did not form in miR-126-deficient embryos (Fig. 3g; Supplementary Table 3; Supplementary Movie 11). We also observed ectopic branching of segmental vessels and abnormal patterning of cranial blood vessels in miR-126-deficient embryos (Supplementary Fig. 8e). These results demonstrate that AA expression of miR-126 requires flow and klf2a and that miR-126 itself is required for AA5x angiogenesis.

Our results suggested that klf2a acted upstream of miR-126 to induce flow-stimulated angiogenesis. Consistent with this possibility, exogenous klf2a in embryos lacking blood flow restored AA miR-126 expression (Supplementary Fig. 9a-c). To further test their genetic interaction, we co-injected klf2a and miR-126 Morpholinos at suboptimal doses that individually caused no, or mild low penetrant aortic arch defects (Fig. 3h, i; Supplementary Fig. 9d,e; Supplementary Table 3). Co-injection of both Morpholinos in this case caused a drastic increase in the penetrance of AA5x defects, suggesting that miR-126 and klf2a act in a common pathway (Fig. 3j, Supplementary Fig. 9e, Supplementary Table 3). Interestingly, other vascular defects observed in miR-126-deficient embryos were not apparent in co-injected embryos (Supplementary Fig. 8e, data not shown), suggesting a specific genetic interaction between miR-126 and klf2a during AA5x development. To further confirm that miR-126 functioned downstream of klf2a, we drove mosaic endothelial expression of a miR-126/monomeric cherry (mcherry) transgene in klf2a-deficient embryos using the fliepl promoter fragment (Supplementary Fig. 10a; ref 21). This construct drove flow-independent endothelial expression of mature miR-126 (Supplementary Fig. 10b, c and data not shown) and led to an increased proportion of klf2a-deficient embryos with AA5x formation as compared to injection of klf2a Morpholino alone (Supplementary Table 3). Rescued embryos displayed miR-126/mcherry transgene expression in AA5x endothelial cells, including cases of bi- and uni-lateral rescue (Fig. 3k, l), while the control fliepl:mcherry transgene failed to rescue (Fig. 3m). These results indicate that miR-126 acts downstream of klf2a to drive flow-stimulated angiogenesis.
miR-126 promotes angiogenesis by repressing spred1 and pik3r2, which normally inhibit Vegf signaling\textsuperscript{19, 20}. Our observations suggested that in the absence of flow and klf2a, reduced miR-126 expression allows upregulation of these molecules thereby preventing Vegf-induced AA5x angiogenesis. While miR-126 can repress the zebrafish spred1 3'UTR, it had no effect on pik3r2 in whole embryo miRNA sensor assays (Supplementary Fig. 11a). Using an endothelial autonomous miRNA sensor assay (Supplementary Fig. 11b), we further found that the spred1 3' UTR prevented expression of a mcherry transcript in blood vessels, while egfp fused to a control 3'UTR was expressed (Fig. 4a, b; Supplementary Fig. 11c). By contrast, the mcherry-spred1-3'UTR transgene was robustly expressed in embryos lacking miR-126, blood flow, or klf2a (Fig. 4c-h, Supplementary Fig. 11c). These results support a genetic pathway in which spred1 repression is mediated by klf2a and miR-126 in response to flow. Accordingly, over-expression of mRNA encoding Spred1 blocked AA5x formation (Fig. 4i, j, Supplementary Table 3), while reducing Spred1 in miR-126-deficient embryos rescued AA5x development (Fig. 4k, l, Supplementary Table 3). Taken together, our findings support the existence of a genetic pathway in which flow induces klf2a and miR-126 (Fig. 4m). While our data suggest that the interaction between these genes occurs in AA endothelial cells, we cannot rule out the possibility of an indirect role for klf2a upstream of miR-126. Nevertheless, flow-stimulated miR-126 subsequently inhibits spred1 in endothelial cells to allow angiogenesis to proceed in response to Vegf (Fig 4m). In the absence of flow, klf2a and miR-126 are reduced allowing spred1 to repress Vegf-stimulated angiogenesis. Thus, miR-126 provides a crucial link between flow and Vegf signaling to promote angiogenesis. Importantly, flow, klf2a, and miR-126 were similarly required for angiogenesis in the zebrafish-xenograft model\textsuperscript{22} (Supplementary Fig 12), suggesting that this pathway may represent a general mechanism for flow-stimulated angiogenesis in the zebrafish.

The stereotyped pattern of the vertebrate circulatory system is initially established by conserved genetic pathways that act before circulation to drive endothelial differentiation and provide guidance cues. How haemodynamic forces subsequently modulate these pathways \textit{in vivo} is largely unknown. Our current work provides new insights into how an endothelial cell's response to flow can be integrated with early developmental signals to drive angiogenesis in the presence of flow.

**Methods summary**

Zebrafish and their embryos were handled according to standard protocols\textsuperscript{23} and in accordance with University of Massachusetts Medical School IACUC guidelines. For laser-assisted microsurgery, embryos at 46 hpf were anesthetized and immobilized in 0.5% of low-melt agarose (Biorad). The connection between AA5 and AA6 and the ventral aorta was ablated using a Micropoint laser (Photonic Instrument, Inc) mounted on a Zeiss AX10 Imager M1. SU5416 (Calbiochem) was prepared and used as described previously\textsuperscript{11}. Control embryos were treated with 0.1% dimethyl sulfoxide (DMSO). To arrest heartbeat, embryos were treated with 15 mM of 2,3-butanedione 2-monoxime (BDM; Sigma-Aldrich) or with buffered Tricaine methanesulfonate (Sigma-Aldrich) at 0.66 mg/ml in egg water for the indicated times. Two-photon time-lapse imaging, confocal microscopy and microangiography was performed as previously\textsuperscript{13, 24}, with additional modifications as

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noted in Supplementary Methods. Antisense riboprobes against dll4, vegfa, kdrl, fli1a, and cdh5 were generated and used for whole mount in situ hybridization as described elsewhere25. A klf2a fragment was PCR amplified and cloned by Gateway recombination. The resulting clone was linearized with BglII and a DIG-labeled riboprobe was synthesized using T7 polymerase. Digoxigenin (DIG)-labeled locked nucleic acid (LNA) probes (Exiqon, Copenhagen) were used to detect mature miR-126 and let-7 using in situ hybridization or Northern analysis as described elsewhere18. Morpholinos, mRNA and Tol2-based plasmids were prepared and injected as previously11,21. In cases of co-injection with Morpholinos, Tol2-plasmids and transposase, a DNA/transposase mRNA mixture was initially injected, followed by Morpholino. Plasmid construction details are provided in the full methods section. Morpholinos against vegfa, mnt2 and gata1 have been described elsewhere15, 26, 25; all other Morpholino and oligonucleotide sequences are provided in the full methods section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Methods

Zebrfish lines

The Tg(kdrl:egfp)la116 and kdrЂ17 lines have been described elsewhere 8,11. The Notch sensor line, Tg(tp1bglob:egfp)um14, has been described and validated as Notch-responsive in previous studies 27. A description of the Tg(fli1ep:dsRedEx)um13 line can be found in Covassin et al 28 while the Tg(fli1a:negfp)y7 line is described in Roman et al 29.

Two-photon time lapse imaging and confocal microscopy

Embryos were treated with 0.003% mM 1-phenyl-2-thiourea to prevent pigmentation and immobilized using 0.1% Tricaine as elsewhere 13. Microangiography was performed using Qtracker 655 non-targeted quantum dots (Invitrogen) or Rhodamine-labeled Dextran as described elsewhere 13. Embryos were mounted in low-melt agarose containing PTU and Tricaine as previously13. Two-photon imaging was performed as previously 24, except that GFP and unconjugated Qdots (655 nm emission) were simultaneously excited at 830 nm and the emitted fluorescence separated using a two-photon specific filter cube with 510/50 nm and 645/60 nm emission bandpasses. Both the sample and the objective were kept at 28°C during the experiment. Laser scanning confocal microscopy was performed as described.
elsewhere 11. To generate time-lapse movies and vertical projections, we utilized Imaris (Bitplane). Flash Professional 8 (Macromedia) was used to label movies.

**Transgenic miRNA expression construct**

To create a vector capable of expressing a zebrafish mature miRNA in a cell type-specific manner, we relied on multisite Gateway cloning. For the miRNA cassette, we constructed a middle entry (pME) clone containing a 753bp fragment of the zebrafish EF1alpha gene (accession number NM_131263) encompassing the TATA box, first non-coding exon 1, intron 1 and partial exon 2, 5' of the ATG. This fragment was PCR-amplified from genomic zebrafish DNA using high fidelity DNA polymerase (forward 5'-CGCTCGGTCTCCCTCTCGAGTATAAATTCTCTC-3' and reverse 5'-CTTTCCATGTGACTAAGTTTCTGCGGACC-3'), cloned into a TOPO TA cloning vector (Invitrogen) and validated by sequencing. A multicloning site was inserted between the StuI and ClaI restriction sites of intron 1 by annealing the primers 5'-CCTCTGTGGTACCATTCTACATGTGTTGATTTTCTGTATTTTAGTGATGATTTCTG

GAT-3' and 5'-

CGATCACAGAATTGCACTAAAATACAGAAAATCAACACATGTAGAATGGTACC

ACAGAGG-3' to allow insertion of a pri-miRNA with KpnI and EcoRI restriction sites. Finally, the recombinant fragment was shuttled into pDONR 221 (Invitrogen) by first amplifying the insert using the primers 5'-

GGGACAAGTTTGTACAAAAAAGCAGGCTCTCGAGTATAAATTCTCCAACCAA

AGC-3' (forward) and 5'-

GGGGACCACTTTGTACAAGAAAGCTGGGTCGATACCGTCGACTAAGTTTCTG

CGGACC-3' (reverse). The resulting PCR fragment was used in a BP reaction with pDONR221 to generate a miRNA middle entry vector (pME-miR) and confirmed by sequencing.

To generate a miR-126 middle entry cassette, 600 bp of genomic sequence flanking the mature miR-126 sequence on chromosome 8 (miRBase accession number MI0001979,Zv7) was amplified by PCR (see Table below for primer sequences). The resulting fragment was cloned by Zero Blunt TOPO PCR Cloning (Invitrogen) and validated by sequencing. The miR-126 fragment was subcloned using flanking EcoRI sites into pME-miR to give pME-miR126 (see above). To generate an endothelial autonomous miRNA expression construct, we utilized multisite Gateway LR cloning. The following plasmids were included in the multisite reaction: p5E-fli1ep28, pME-miR-126, p3E-mcherry 21, 30, and pDestTol2pA 30. A control vector was constructed by using the empty pME-miR in a parallel LR multisite reaction. The reaction was performed using LR clonase II plus (Invitrogen) as previously 21.

**miRNA sensor assays**

We amplified the 3’UTR of zebrafish spred1 or pik3r2 by PCR and cloned them via BP Gateway recombination with pDONR P2-P3 (Invitrogen) to generate 3’ entry plasmids (p3E-spred1-3’utr, p3E-pik3r2-3’utr, see Table below for primer sequences). For whole embryo assays, we generated a pCS2-based construct by performing multisite Gateway LR reactions with pCS2Dest2 21, pENTR-egfp 21 and the p3E-spred1-3’utr or p3E-
pik3r2-3’utr entry clones. The resulting plasmids were referred to as pCS-egfp-spred1-3’utr or pCS-egfp-pik3r2-3’utr. These plasmids were linearized by digestion with NotI and used as templates to synthesize capped mRNA using SP6 polymerase (mMessage Machine, Ambion). As a control, we synthesized mRNA encoding the monomeric red fluorescent protein, mcherry containing the SV40 late polyadenylation sequence found in pCS2. 60 pg of mcherry mRNA was co-injected with 60 pg of egfp sensor mRNA with or without 60 fmol of miR-126 duplex (see below for sequences) into 1-cell stage zebrafish embryos. Embryos were observed at 24 hpf for MCherry and Egfp expression using a MZFLIII microscope equipped with epifluorescence. Digital images were captured using a Zeiss mRC digital camera and Axiovision software.

To generate an endothelial autonomous miRNA sensor construct, we cloned the basal promoter-EGFP-polyA cassette from plasmid pENTRbasEGFP21 into the PspOMI site of pDest Tol2pA 30, in an opposite orientation to the attR4-attR3 Gateway cassette in the parent vector (see Supplementary Figure 11b); this plasmid is referred to as pTolbasPegfprev-R4R3. To generate the injection construct, we performed a multisite Gateway reaction using pTol-basPegfprev-R4R3, a 5’ entry clone containing the fli1ep fragment (p5e-fli1ep28), a middle entry clone encoding mcherry (pME-mcherry), and p3E-spred1-3’utr.

For endothelial autonomous assays, we co-injected 25 pg of Tol2 transposase mRNA, 25 pg of Tol2-reporter plasmid, with or without miR-126, tntt2, or klf2a Morpholino, as indicated. Embryos were observed for mCherry and Egfp expression using a MZFLIII microscope equipped with epifluorescence. Subsequently, confocal stacks were acquired as previously 28 and voxel intensity of red and green fluorescence was quantified in 3-dimensional reconstructions using Imaris.

**klf2a mRNA expression construct**

We amplified the open reading frame of zebrafish klf2a (NM_131856) by PCR and cloned it via BP Gateway recombination with pDONR 221 to generate a middle entry (referred to as pME-klf2a; see Table below for primer sequences). In parallel, we constructed a Destination vector in which mCherry was placed upstream of the viral 2A sequence and an attB1/attB2 Gateway cassette (pCSmCherry2ADest). Transfer of an in-frame entry clone into this vector generates a cassette in which two proteins are expressed from a single transcript. To generate an injection construct, we performed a LR Gateway reaction with pCSmCherry2ADest and pME-klf2a. The resulting plasmid is referred to as pCS-mcherry-2A-klf2a. This plasmid was linearized by digestion with NotI and used as template to synthesize capped mRNA using SP6 polymerase (mMessage Machine, Ambion). As a control, we synthesized mRNA encoding the monomeric red fluorescent protein, mcherry. Wild type embryos were injected with 400 pg of mRNA into one cell stage embryos with or without tntt2 MO (3 ng) or klf2a MO splice site (2.5 ng) and control MO (3 ng). We assayed for expression and presence of the injected mRNA at 48 hpf by visualization of mCherry fluorescence and RT-PCR, respectively (see Supplementary Fig. 9a, b).
Xenografts

Implantation of Ras-transformed NIH3T3 cells into zebrafish embryos was performed as previously described\textsuperscript{22}. Cells were labeled with PKH26 red fluorescent linker (Sigma) and subsequently grafted into 48 hpf \textit{Tg(kdrl:egfp)la116} embryos that had been injected with control (20 ng), \textit{tnnt2} (3 ng), \textit{klf2a} splice blocking MO (2.5 ng) or \textit{miR-126} (20 ng) morpholino. Microangiography was performed on embryos 24 hours following the graft followed by confocal microscopy. Imaris (Biplane) software was use to measure and compare vascular tumor volume in 3-dimensional reconstructions of confocal stacks.

Quantitative RT-PCR

Total RNA was extracted from 20 to 40 embryos per group in Trizol (Invitrogen) according to the manufacturer’s instructions. Products were amplified in a real-time PCR reaction with StepOne Plus Real-Time PCR System (Applied Biosystems) using a Power SYBR Green mix (Applied Biosystems) according to the manufacturer’s instructions. Mature \textit{miR-126} was amplified using the miScript Reverse Transcription Kit and miScript SYBR Green PCR Kit (Qiagen) with primers indicated below.

| Morpholino/MiR-126 primers | Primers.
|---------------------------|-------------------
| \textit{klf2a} ATG MO     | GGACCTGTCCAGTTCATCTCCAC |
| \textit{klf2a} splice blocking MO | CTCGCCATGAAAGAGAGGATT |
| \textit{miR-126} MO       | TGCAATTACTCACGTACAGTTTGAGTC |
| \textit{spred1} MO        | AAACCTGAGGAGAGAAACC |
| \textit{control} MO       | Oligo-31N |
| \textit{tnnt2} MO         | CATGGTTCGCTATCTGCACAGCA |

### Primers

- **Klf2a attb1**
  - gggg aca agt tgt ttaaaaaagcaggttacacgtgc acatgcagccacggagtc
- **Klf2a attb2**
  - GGGGACACTTTGTACAAGAAAGCTGGGTTCATCCTGACAGCTGC
- **Spred 3’UTR attb2**
  - GGGG ACA GCT TTC TTG TAC AAA GTG G
  - GCAGACGTTGCCACGGTC
- **PIK3r2 3’UTR attb2**
  - GGGG ACA GCT TTC TTG TAC AAA GTG G
  - CAAACGACTCCTGCAATGT
- **PIK3r2 3’UTR attb3**
  - GGGG AC AAC TTA TTA TAA TAA AGT TG
  - TCCCAAGCTGGGATCATGT
- **Spred ORF attb1**
  - GGGGACAATGTtacacaaaugcaggttacacgtgacagccagtaa cacc aac
- **Spred ORF attb2**
  - GGGGACACTTTGTACAGGAGGGGACACAGCAGGTACATCGCAGCAGGG
- **miR126 chm8 F**
  - TGCAATTACTCACGTACAGTTTGAGTC
- **miR126 chm8 R**
  - GTGATTTTAATGTACATCGC
- **qPCR Bact F**
  - AGCTTGAAACTCGCAAGTG
- **qPCR Bact R**
  - CAGCTTTATAGCCGCACTG
- **qPCR vegf F**
  - GCAAAAGCAGAAGTCAAG

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qpcr vegf R    TGCAGGAGCATTTACAGGTG
qpcr Kdrl F    TCTACTGGGCTTTTCCCTCTC
qpcr Kdrl R    AGGGTTACTATGGTGACGTTGC
qpcr Zelastin F GCTCGTCTCCATACAAAGCA
qpcr Zelastin R CAGAACTCTCCTGGTGATGCC
qpcr Z Tie2 F    GCCTCAAGGAGGATGAAAGA
qpcr Z Tie2 R    GCTGCTGTGAGGAGAGTGTG
qpcr Znos1 F    TTAAATACGCCACCAACAAAA
qpcr Znos1 R    GAGAAGAAGGGGCAAAACATC
qpcr ZItgb5 F    TGGGAAGGATGGACAAAGAG
qpcr ZItgb5 R    CGGGTGAATGAGGAGACACT
qpcr ZEdg1 F    GCCGTCAAGAGGATGAAAGA
qpcr ZEdg1 R    GCTGCTGTGAGGAGAGTGTG
qpcr ZEdn 1 F    TTAAATACGCCACCAACAAAA
qpcr ZEdn 1 R    GAGAAGAAGGGGCAAAACATC
F-qpcr klf2a    CCAACGCCAACCAAAGAGTA
R-qpcr klf2a    CTCGCCTGTGTTGTTTCTGT
Mir 126 qpcr F for miRscript CATTGCAGTGAGTAAATGC
R-RT-PCR klf2a    ACGTGGTACCCGCCACGGCGAACTCACACTTG
F-RT-PCR klf2a    ACGTGGTACCacgcacggcgactcacacttg
miRNA Duplex
miR-126 sense    ucuuaacgagaguanacua
miR-126 antisense gcauauauacuacguacguuga
miR-126 scramble antisense gcacacacucacguacguuga

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Figure 1. AA5x angiogenesis requires flow and Vegf signaling

a-k, Tg(kdrl:egfp)la116 embryos at 72 hpf (a-c), 60 hpf (d-f) or 65 hpf (g-k). a-c, g-k
Embryos subjected to microangiography. Endothelial cells are green, flow is red. a, b,
Lateral views, anterior to left (a), or right (b), dorsal is up. c-k, dorsal view, anterior is up. a,
b, aortic arches (AA, numbered, indicated by arrows) after severing right AA5 and 6 from
ventral aorta; opa – opercular artery. c, dorsal view of embryo in a, b, d, e, Stills from
Supplementary Movies 5 and 6. Embryos treated beginning at 46 hpf with BDM (d),
Tricaine (e), or injected with gata1 MO (f). g, kdrl<sup>y17</sup> mutant embryo at 65 hpf. h-k,
Embryos treated with 2.5 μM SU5416 (h) or 0.1% DMSO (i) beginning at 46 hpf or injected
with 3 ng of scrambled MO (j) or 3 ng of Vegfa MO (k). c-k, Arrows: lateral dorsal aortae
(LDA), arrowheads: AA5x.
Figure 2. AA5x angiogenesis requires klf2a

a. Embryos subjected to whole mount in situ hybridization at 65 hpf using indicated riboprobes (klf2a, cdh5). Lateral view, anterior to the left, dorsal is up. Embryos were injected with 2 ng of scrambled control (left) or tnnt2 (right) MO. Aortic arch region is denoted by black boxes; aortic arches 4-6 are indicated by numbered arrows in cases where staining is present. b. Microangiogram of 72 hpf Tg(kdrl:egfp)la116 embryo injected with 11 ng-embryo scrambled control MO (top) or klf2a ATG MO (bottom); arrowheads indicate position of normal AA5x formation, arrows denote lateral dorsal aortae. Dorsal views, anterior is up.
Figure 3. *miR-126* acts downstream of *klf2a* during AA5x development

**a-f**, Ventral view, anterior is up. Bracket: AA3-6. Expression of *miR-126* (a-c) or *cdh5* (d-f).

Embryos injected with 11 ng control MO (a, d), 2 ng *tnnt2* MO (b, e), 11 ng *klf2a* ATG MO (c, f).

**g-j**, *Tg(kdrl:egfp)la116* embryos at 65 hpf, dorsal view, anterior is up. Endothelial cells are green, blood flow is red. Embryos injected with 20 ng of control or *miR-126* MO (g), 2 ng *klf2a* ATG MO (h), 7 ng *miR-126* MO (i), or co-injected with 2 ng *klf2a* ATG MO and 7 ng *miR-126* MO (j).

**k-m**, *Tg(kdrl:egfp)la116* embryos co-injected with 11 ng *klf2a* ATG MO and pTol-fli1ep:*miR-126/mcherry* (k, l) or 11 ng *klf2a* ATG MO and pTol-fli1ep:*mcherry*.
(m). **k-m**, Yellow indicates *egfp* and *mcherry* co-expression; dorsal views, anterior is up. **g-m**, Arrows: lateral dorsal aortae (LDA), arrowheads: AA5x.
Figure 4. Flow-mediated repression of Spred1 is required for AA5x angiogenesis

a-h, Cranial vessel expression of a miR-126 sensor at 65 hpf; lateral views, anterior to the left, dorsal is up. a, c, e, g, Expression of Egfp fused to control 3’UTR (green) and mCherry-spred1-3’UTR (red), coexpression is yellow. b, d, f, h, Expression of mCherry-spred1-3’UTR (red). Embryos co-injected with miR-126 sensor construct and 20 ng control MO (a, b), 20 ng miR-126 MO (c, d), 2 ng tnt2 MO (e, f), or 11 ng klf2a ATG MO (g, h).

i-l, Tg(kdrl:egfp)la116 embryos at 65 hpf, dorsal view, anterior is up. Endothelial cells in green, circulation in red. Arrow - Lateral dorsal aortae; AA5x - arrowheads. Embryos left uninjected (i), injected with 100 pg spred1 mRNA (j), 20 ng control MO (k), or 1 ng spred1 MO and 20 ng miR-126 MO (l). m, Pathway responsible for flow-stimulated angiogenesis.