Materials and Methods

Vascular endothelial cell culture and chemical reagents

Human aortic endothelial cells (HAEC) were purchased from Cell Applications (San Diego, CA, USA). The endothelial cells were cultured in endothelial growth medium (Cell Applications, San Diego, CA) supplemented with 4% Fetal Bovine Serum (FBS). HAEC were propagated for experiments between passages 4 and 7. Human recombinant Dickkopf-1 (DKK-1) and human recombinant Wnt3a were purchased from R&D systems (Minneapolis, MN). Human recombinant Ang-2 (0.5 μM) (ProSpec Inc, East Brunswick, NJ) was used to rescue endothelial cell migration and tube formation.

Mechanotransduction of vascular endothelial cells

A dynamic flow system was used to generate pulsatile shear stress (PSS) and oscillatory shear stress (OSS) as previously described [1, 2]. The flow system was designed to simulate physiologic shear stress occurring at human arterial branching points with well-defined slew rates ($\partial\tau/\partial t$), time-averaged shear stress (τ_{ave}), frequency, and amplitude. The cells were applied to flow in DMEM culture medium supplemented with 1% FBS and maintained at a temperature of 37°C and pH of 7.4. Confluent monolayers of HAEC grown on glass slides were subjected to three flow conditions at 1 Hz for 4 hours: 1) control at no flow state, 2) pulsatile flow with time-average shear stress (τ_{av}) = 23 dyne/cm^2 accompanied by a stress slew rate ($\partial\tau/\partial t$=71 dyne/cm^{-2}/s at 1 Hz), and 3) oscillating flow (0±3 dyne/cm^2) with τ_{ave}=0 dyne/cm^2 at 1 Hz. For oscillating flow, minimal forward flow at a mean shear stress of 0.2 dyne/cm^2 was provided every hour to deliver nutrients and to remove waste products from the cells.

TOPflash Wnt reporter activity assay

Wnt signaling was measured via TOPflash lentivirus reporter (Addgene plasmid 24307). Lentiviruses were prepared as reported [3]. HAEC grown to sub-confluence were infected with TOPflash lentiviruses at 1:1 ratio in the presence of 6μg/ml polybrene for overnight incubation. Next day, HAEC were subjected to OSS or treated with 20mM of LiCl as a positive control for 8 hours. The cells were then collected and lysed in passive lysis buffer (PLB, Promega), and luciferase activities were quantified with Luminometer using Bright-Glow substrate (Promega).

Immunoflurorescence and the quantification of nuclear β-Catenin

HAEC monolayers were subjected to OSS for 4 hours as described above, and were fixed with 4% paraformaldehyde thereafter. The cells were incubated with anti-β-Catenin (Cell Signaling Technologies, MA), stained with Alexa Fluor 488 secondary antibody(Life Techologies, NY), and mounted with Vectashield mounting medium with DAPI (Vector Laboratories, CA). Fluorescent images were acquired using an inverted microscope (Olympus, NJ) and a CCD camera (Jenoptik, FL). Nuclear β-Catenin fluorescent intensities were quantified via Matlab (Mathworks, MA). Fluorescent β-Catenin signals would be considered positive if co-localized with DAPI fluorescent, and would be compared with the control as fold-change of control.

Ang-2 knock-down

Scrambled control siRNA, and Ang-2 siRNA were obtained from Qiagen (Valencia, CA). siRNA (60nmol/L) was transfected to HAEC with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) as described previously [4]. Cells were used for confirmation of gene knockdown or function assay 48 hours after transfection. There was no observable damage due to the transfection procedure.

Endothelial cell migration and tube formation assays with Wnt inhibitors and Ang-2 siRNA

For the migration assay, confluent HAEC monolayers were scratched by using the 1000 µL pipette tips. The monolayers were washed once, and the medium was replaced with the endothelial cell medium in the presence or absence of Wnt inhibitor DDK-1 or DMSO (vehicle control). After 6 hours of incubation at 37°C, the original scratch lines were photographed and compared with the control.

For the tube formation assay, HAEC were suspended in DMEM (Invitrogen, Carlsbad, CA) with 25ng/ml of VEGF and 5% FBS. HAEC were added to 96-well plate coated with growth factor-reduced Matrigel (BD Biosciences, San Jose, CA) at 20,000 cells/well. The cells were incubated for 8 hours in the presence or absence of DDK-1. Tube formation was compared between the treatment and control using a phase contrast microscope (Olympus IX70). To determine cell viability, we incubated cells in Matrigel with Calcein AM dye (Molecular Probes) at 5 µM for 15 minutes. In the live cells, this dye was converted to a green-fluorescent Calcein after acetoxymethyl ester hydrolysis by intracellular esterases [5].

Construction of HA-tagged zebrafish Ang-2 (zAng-2) and preparation of zAng-2 mRNA

To facilitate the detection of zAng2 protein, we constructed zAng2 with HA tag. zAng2 cDNA (in plasmid pDONR221) was provided by Dr. Sara Childs at the University of Calgary. The zAng2 cDNA was amplified from the donor plasmid and cloned into the plasmid pCS2+ at the BamH I and EcoR I sites with HA tag sequence at the C-terminal end. Clones with the z-Ang-2 cDNA insert were selected by PCR screening. Four clones with z-Ang2 insert were verified by transfecting the plasmids into HEK-293 cells. RNA was extracted to verify mRNA expression by RT-PCR and zAng2 protein expression was verified by Western blot with anti-HA-tagged antibody. Clone #2 was confirmed to express both zAng-2 mRNA and the HA-tagged z-Ang2 protein (**Supplemental Figure I**). zAng2 mRNA was made from the clone 2 plasmid using the mMessage SP6 kit (Invitrogen, CA) following the manufacturer's instruction.

Quantitative real-time PCR analysis

Angiopoietin-2 (Ang-2) and Axin2 mRNA expressions were measured by quantitative RT-PCR. Total RNA was isolated using Bio-Rad Total RNA kit (Bio-Rad, Hercules, CA). RNA was reverse-transcribed using iScript™ cDNA synthesis kit (BioRad), followed by PCR amplification with qPCR Master Mix (Applied Biological Materials Inc. Richmond, BC, Canada). Ang-2 mRNA expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequence of Ang-2, Axin2 and GAPDH

were provided in **Table 2.** The differences in C_T values for various intervals versus control were used to determine the relative difference in the levels of Ang-2 mRNA expression.

Western blot analysis

Cells were washed with phosphate-buffered saline, harvested, and lysed with RIPA buffer. The lysate was centrifuged at 12,000g for 10 minutes, and the resulting supernatants were used as the whole cell lysate. Protein concentration was determined using DCP assay (Bio-Rad, Hercules, CA). Proteins were separated by 4–20% polyacrylamide gel with SDS and electroblotted onto the polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK) and were blocked overnight at 4°C in Tris-buffered saline-Tween20 (TBS-T) containing 5% non-fat dry milk (Bio-Rad, Hercules, CA). Ang-2 protein expression was detected with anti-Ang-2 (SantaCruz), and equal loading was verified by blotting with anti-β-tubulin (Millipore Inc). After treatment with horse radish peroxidase-conjugated anti-goat (Santa Cruz) or anti-mouse IgG antibody (Jackson ImmunoResearch, PA) for 1 hour at room temperature, chemilluminescence signals were developed with Supersignal Western Pico (Pierce) and recorded with FluorChem FC2 (Alpha Inotech Inc, San Leandro, CA). Densitometry scans of western blots were performed by using the software installed in FluorChem FC2.

Vasculogenesis assay using *Tg(kdrl:gfp)* transgenic zebrafish embryos

Transgenic *Tg(kdrl:GFP*) fish, were provided by both Ellen C. Lien at Children's Hospital Los Angles and Jau-Nian Chen at UCLA. Kdrl, also known as flk-1, a VEGF receptor 1, is tissue-specific for vascular endothelial cells. Fish were collected at 0 hour-post-fertilization (hpf). Ang-2 inhibition was performed via micro-injection of anti-sense morpholino oligomer (MO) (GeneTools, LLC, Philomath, OR). The MO sequences used were provided in **Table 2**. Control and Ang-2 MOs were dissolved in water to make 0.3mM stock solution with addition of 0.1mM p53 MO. Immediately after collection at 0 hpf, approximately 30-40 embryos were randomly chosen for morpholino micro-injections with 2nL MO stock for the control, Ang-2 Splicing MO, and Ang-2 ATG MO solutions. To rescue, we co-injected 25ng zAng-2 mRNA with Ang-2 MO. All of the embryos were maintained in E3 medium at 28°C. After 72 hpf, all of the embryos were examined under fluorescence microscope (Olympus IX70, Olympus, Japan) for vasculature phenotypes. Embryos from each treatment condition were then collected for Ang-2 mRNA expression by quantitative RT-PCR.

Subintestinal vein (SIV) Quantification

Fluorescent Tg(*flk1:GFP*) zebrafish SIV lengths were quantified using a custom Matlab script. Briefly, SIV areas were cropped out from embryo image and fluorescent intensities standardized between samples. SIV structures were extracted by thresholding and skeletonizing the image. The result was summed to determine total SIV lengths in pixels and compared to controls to determine fold changes in lengths.

Heat-shock induction of DKK-1 in transgenic *Tg(hsp70l:dkk1-GFP)* embryos to inhibit Wnt signaling and Wnt target genes

Heat-shock inducible transgenic *Tg(hsp70l:dkk1-GFP)* embryos, provided by Neil C. Chi at University of California, San Diego, were used to inhibit canonical Wnt signaling. DKK-1 acts as a potent inhibitor by binding to Wnt receptors LPR5/6. We heat-shocked the embryos at 48 hpf to show a robust GFP expression. Heat shock-induction of DKK-1 was performed in a 38°C water bath for an hour for twenty embryos (*n*=20). Heat shocked embryos exhibited DKK-1-GFP expression (Olympic IX70 Fluorescence microscope). Quantitative RT-PCR using previously reported Wnt target gene, Axin-2 [6], was performed as a positive control for the effect of DKK-1 induction. Individual heat-shock treatments and subsequent assays were performed in four independent experiments. For each experiment, 4 embryos were collected from heat-shock and control groups and lysed for RNA isolation using Bio-Rad Total RNA kit (Bio-Rad, Hercules, CA). The primer sequences for zebrafish Ang-2, Axin-2 and the reference gene β-actin were presented in **Table 2.**

Inhibition of Wnt signaling via IWR-1 in *Tg(kdrl:GFP)* transgenic embryos

Transgenic *Tg(kdrl:gfp)* zebrafish embryos were used to assess vasculogenesis in response to inhibition of Wnt signaling. IWR-1(Sigma-Aldrich) acts as an inhibitor of the canonical Wnt signaling pathway by affecting the gene Porcupine (porcn), which adds a palmitoyl group to Wnt proteins essential to their signaling ability, and is required for Wnt secretion. To assess the time- and dose-dependent effects on the Wnt signaling pathway, we introduced IWR-1 to the growth medium at two different time frames (24 and 48 hpf) and at two different concentrations (10 and 20 μM) (**Table 1**). DMSO (0.1%) was also introduced to the growth medium at which IWR-1 was added to nullify any unknown effects of DMSO at the gene expression levels. IWR-1 was administered in a solution mixed with 0.1% DMSO (as a solvent). At 72 hpf, quantitative RT-PCR was performed to assess Ang-2 and Axin-2 mRNA expression.

Tail injury study using *Tg(kdrl:GFP)* zebrafish embryos

Transgenic *Tg(kdrl:GFP)* embryos were used to assess vascular repair in response to tail injury. Fish larvae were grown to 72 hpf in standard E3 medium. The larvae were first anaesthetized in 0.02% tricaine solution to allow for precise tail placement. The posterior tail segment was then amputated by approximately 100 μm from the tip of the tail using a surgical scalpel under a stereo microscope (MEIJI Techno EMZ series, MEIJI, Japan). After amputation, fish were isolated and placed into E3 medium, E3 medium with 20μM IWR-1. zAng-2 mRNA injection at 2-cell stage plus IWR-1 was also performed. Fish tail sections were imaged under a fluorescent microscope (Olympus IX71, Olympus, Japan) to visualize the blood vessels immediately after amputation and every 24 hours thereafter over the next 3 days. Images were compared to show the differences in regrowth of blood vessels between the different treatment groups at 0 day post amputation (dpa), 1 dpa, and 3 dpa.

Statistical analysis

Data were expressed as mean ± SD and compared among separate experiments. Comparisons of multiple values were made by one-way analysis of variance (ANOVA), and statistical significance for pairwise comparison was determined by using the Turkey test. *P*-values of < 0.05 were considered statistically significant.

Table 1. IWR-1 treatment scheme for *Tg* (*kdrl:GFP*）zebrafish embryos

Condition	IWR-1 Concentration	Treatment Start time	Treatment duration
E3 medium + 0.1% DMSO	0	24 hpf	48 hr
E3 medium + 0.1% DMSO	10 μM	24 hpf	48 hr
E3 medium + 0.1% DMSO	20 μM	24 hpf	48 hr
E3 medium + 0.1% DMSO	10 μM	48 hpf	24 hr
E3 medium + 0.1% DMSO	20 μM	48 hpf	24 hr

Table 2. Sequencing Information of qRT-PCR primers and Mopholinos

Primer/MO name	**Sequence**
Human Ang-2 forward	5´- GAC CAC GAG ACT TGA ACT TCA G-3´
Human Ang-2 reverse	5´- GGA TGA TGT GCT TGT CTT CCA TAG -3´
Human GAPDH forward	5'- CCT CAA GAT CAT CAG CAA TGC CTC CT -3'
Human GAPDH reverse	5'- GGT CAT GAG TCC TTC CAC GAT ACC AA -3'
Zebrafish Ang-2 forward	5'- CCA ATC TT CTA AGC CAA TCA GCG GAA -3'
Zebrafish Ang-2 reverse	5'- CCA CAT CTG TCA GTT TGC GCG TGT TT -3'
Zebrafish Axin2 forward	5'- GGA CAC TTC AAG GAA CAA CTA C -3'
Zebrafish Axin2 reverse	5'- CCT CAT ACA TTG GCA GAA CTG -3'
Zebrafish β-Actin forward	5'- TGG ATC AGC AAG CAG GAG TAC G -3'
Zebrafish β-Actin reverse	5'- AGG AGG GCA AAG TGG TAA ACG C -3'
Zebrafish Standard Control MO	5'- CCT CTT ACC TCA GTT ACA ATT TAT A-3'
Zebrafish Ang-2 Splicing MO	5'- TCA TTT GAT CAG CCT CAC CTG CGT C -3'
Zebrafish Ang-2 ATG MO	5'- GGC AGG CTG TCC ATC CCA GGA AAC C -3'
Zebrafish p53 MO	5'- GCG CCA TTG CTT TGC AAG AAT TG -3'
Zebrafish Ang-2 Splicing MO PCR primer forward	5'- AGGAAAGGAAGCTGGAGACC-3'
Zebrafish Ang-2 Splicing MO PCR primer reverse	5'- TGTTACGAGTGGAGCTGGCC-3'

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