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# Shear Stress–Activated Wnt-Angiopoietin-2 Signaling Recapitulates Vascular Repair in Zebrafish Embryos

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***Objective***—Fluid shear stress intimately regulates vasculogenesis and endothelial homeostasis. The canonical Wnt/β-catenin signaling pathways play an important role in differentiation and proliferation. In this study, we investigated whether shear stress activated angiopoietin-2 (Ang-2) via the canonical Wnt signaling pathway with an implication in vascular endothelial repair.

***Approach and Results***—Oscillatory shear stress upregulated both TOPflash Wnt reporter activities and the expression of Ang-2 mRNA and protein in human aortic endothelial cells accompanied by an increase in nuclear β-catenin intensity. Oscillatory shear stress–induced Ang-2 and Axin-2 mRNA expression was downregulated in the presence of a Wnt inhibitor, IWR-1, but was upregulated in the presence of a Wnt agonist, LiCl. Ang-2 expression was further downregulated in response to a Wnt signaling inhibitor, DKK-1, but was upregulated by Wnt agonist Wnt3a. Both DKK-1 and Ang-2 siRNA inhibited endothelial cell migration and tube formation, which were rescued by human recombinant Ang-2. Both Ang-2 and Axin-2 mRNA downregulation was recapitulated in the heat-shock–inducible transgenic *Tg(hsp70l:dkk1-GFP)* zebrafish embryos at 72 hours post fertilization. Ang-2 morpholino injection of *Tg (kdrl:GFP)* fish impaired subintestinal vessel formation at 72 hours post fertilization, which was rescued by zebrafish Ang-2 mRNA coinjection. Inhibition of Wnt signaling with IWR-1 also downregulated Ang-2 and Axin-2 expression and impaired vascular repair after tail amputation, which was rescued by zebrafish Ang-2 mRNA injection.

***Conclusions***—Shear stress activated Ang-2 via canonical Wnt signaling in vascular endothelial cells, and Wnt-Ang-2 signaling is recapitulated in zebrafish embryos with a translational implication in vascular development and repair. **(*Arterioscler Thromb Vasc Biol*. 2014;34:2268-2275.)**

**Key Words:** angiopoietin-2 ■ DKK-1/dickkopfs-1 ■ endothelial repairs ■ human aortic endothelial cells (HAEC) ■ vasculogenesis ■ Wnt signaling ■ zebrafish

Mechanotransduction is implicated in differentiation of embryonic stem cells to vascular endothelial cells.[1–3] Hemodynamics, namely, fluid shear stress, is intimately involved in stem cell[1,4] and mesenchymal progenitors[5] differentiation to vascular endothelial cells. Although the roles of angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) during vascular development have been extensively investigated, shear stress–mediated Ang-2 in mature vascular endothelium was recently reported to play a role in tubulogenesis[6] and to confer atheroprotection.[7]

Although Ang-1 is constitutively released by the perivascular cells, Ang-2 is released from the Weibel-Palade bodies in endothelial cells.[8,9] Ang-2 binds to endothelial-specific receptor tyrosine kinase 2 (TIE-2) and acts as a negative regulator of Ang-1/TIE-2 signaling during angiogenesis.[10] Earlier studies demonstrated that Ang-2 release from Weibel-Palade bodies is induced by endothelial stretch, which occurs during hypertension.[11] However, the mechanisms underlying reactivation of developmental genes, such as Ang-2 in endothelial cells, remain elusive.

Hemodynamic forces are complex regulators of endothelial homeostasis.[12] Disturbed flow, including oscillatory shear stress (OSS), is a bidirectional flow associated with a net-zero forward flow that develops in the curvatures or branching points of the vasculature.[13–16] OSS-induced Ang-2 promotes tubular formation and migration of cultured endothelial cells.[6] Although stretching isolated arterial endothelial cells further promotes the paracrine effect of Ang-2 release, Ang-1 release

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| Nonstandard Abbreviations and Acronyms | |
|---|---|
| **Ang-2** | angiopoietin-2 |
| **hpf** | hours post fertilization |
| **OSS** | oscillatory shear stress |
| **SIV** | subintestinal vessel |

inhibits these effects.[11] Ang-2 stimulates arteriogenesis in an C57Bl/6J mice with a ligated femoral artery[17] and confers atheroprotection in apoE-null mice. In contrast, overexpression of Ang-1 induces smooth muscle cell migration and monocyte chemotaxis.[7] However, there remains a paucity of literature in shear stress–activated developmental genes, and the mechanisms underlying OSS-induced Ang-2 expression remain to be elucidated.

Canonical Wnt/β-catenin signaling pathway regulates development, cell proliferation, and migration.[18] In this study, we investigated whether shear stress activated Ang-2 via canonical Wnt signaling pathway. Both endothelial Ang-2 expression and Wnt TOPflash reporter activity were upregulated in response to OSS. Although Wnt agonist, Wnt3a, promoted Ang-2 mRNA expression, Dkk-1 treatment or Ang-2 siRNA inhibited endothelial cell migration and tube formation. Wnt-Ang-2 signaling was further recapitulated in the zebrafish embryos, in which mRNA of Ang-2b homolog was downregulated in heat-shock–inducible DKK-1 transgenic *Tg(hsp70l:Dkk1-GFP)* fish (for the zebrafish-related studies, zebrafish Ang-2b homolog is denoted as Ang-2). Ang-2 morpholino microinjection further impaired development of subintestinal vessels (SIV) at 72 hours post fertilization (hpf). Thus, we provide new insights into shear stress–activated Wnt-Ang-2 signaling with a translational implication in vascular development and repair.

## Materials and Methods

Materials and Methods are available in the online-only Supplement.

## Results

### OSS Activated Ang-2 Expression via Wnt Signaling

In a dynamic flow system,[19] OSS upregulated Wnt signaling activity in human aortic endothelial cells. TOPflash reporter assay demonstrated a 2.3-fold increase in Wnt signaling activity in response to OSS, and a 2.8-fold increase in response to LiCl, a positive control ($P<0.05$; n=3; Figure 1A). In parallel, OSS increased nuclear β-catenin content by 1.33-fold when compared with static condition ($P<0.05$; n=4; Figure 1B). Wnt signaling inhibitor ionomycin inhibited nuclear β-catenin translocation (Figure V in the online-only Data Supplement). Furthermore, OSS upregulated Axin-2 mRNA, a well-known Wnt target gene, by 2.3-fold ($P<0.05$; n=4), which was attenuated by a Wnt inhibitor, IWR-1 (Figure 1C). OSS also upregulated Ang-2 mRNA expression by 2-fold ($P<0.05$; n=4), which was attenuated by IWR-1 (Figure 1D). OSS further upregulated Ang-2 mRNA to a greater extent than did pulsatile shear stress, and OSS also upregulated Ang-2 protein expression ($P<0.05$; n=4; Figure 1E and 1F). Thus, OSS induced Ang-2

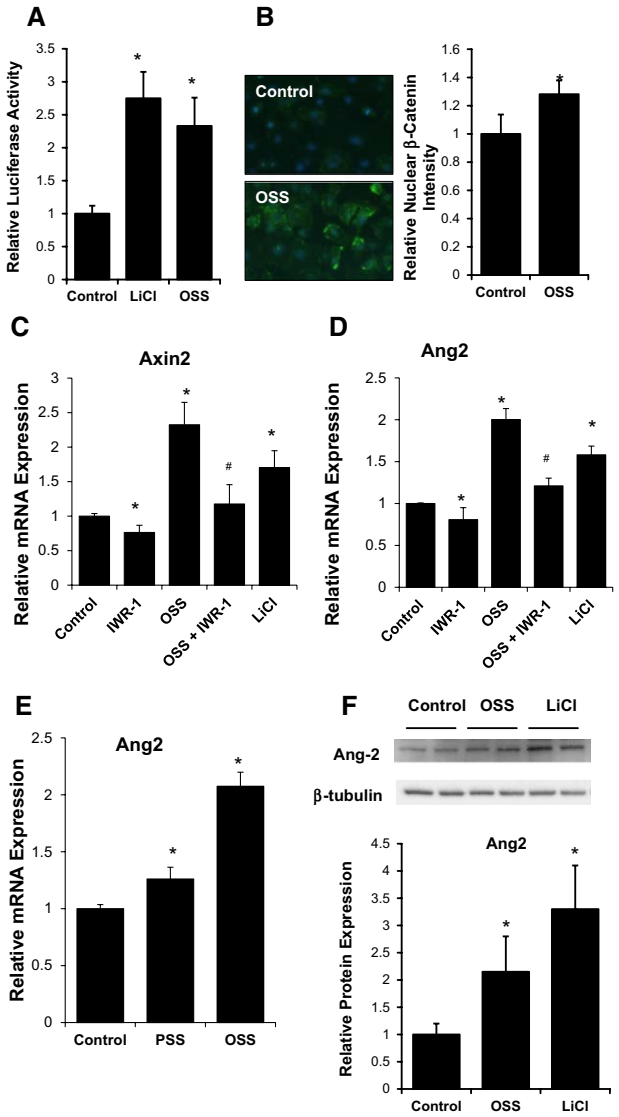


**Figure 1.** Oscillatory shear stress (OSS) promoted Ang-2 expression via Wnt signaling. **A**, Topflash reporter assay revealed that OSS for 8 hours significantly activated Wnt signaling. LiCl, a wnt-signaling inducer, was used as positive control (Control=1.00±0.06; LiCl=2.78±0.42; OSS=2.35±0.46; *$P<0.05$ vs control; n=4). **B**, OSS induced a 1.3-fold increase in nuclear β-catenin fluorescence in canonical Wnt signaling pathway (*$P<0.05$; n=4). **C**, OSS upregulated a well-recognized Wnt target gene, Axin-2, which was attenuated in the presence of IWR-1, a Wnt inhibitor (normalized to GAPDH: control=1.00±0.038; IWR-1=0.76±0.10; LiCl=1.70±0.25; OSS=2.32±0.32; OSS+IWR-1=1.18.±0.28; *$P<0.05$ vs control; #$P<0.05$ vs OSS; n=4). IWR downregulated but LiCl upregulated Axin-2 expression (*$P<0.05$ vs control; n=4). **D**, OSS further upregulated Ang-2 mRNA expression, which was also attenuated in the presence of IWR-1 (normalized to GAPDH: control=1.00±0.01; IWR-1=0.81±0.14; LiCl=1.58.±0.10; OSS=2.00±0.13; OSS+IWR-1=1.21±0.09; *$P<0.05$ vs control; #$P<0.05$ vs OSS; n=4). IWR downregulated but LiCl upregulated Ang-2 expression (*$P<0.05$ vs control; n=4). **E**, Pulsatile shear stress (PSS) upregulated Ang-2 mRNA expression by 1.21.±0.10-fold (*$P<0.05$ vs control; n=4), whereas OSS upregulated Ang-2 expression by 2.08±0.12-fold (normalized to GAPDH: *$P<0.05$ vs control; n=4). **F**, In corollary, both OSS (24 hours) and LiCl significantly upregulated Ang-2 protein expression as quantified by densitometry (normalized to β-tubulin: *$P<0.05$ vs control; n=4).

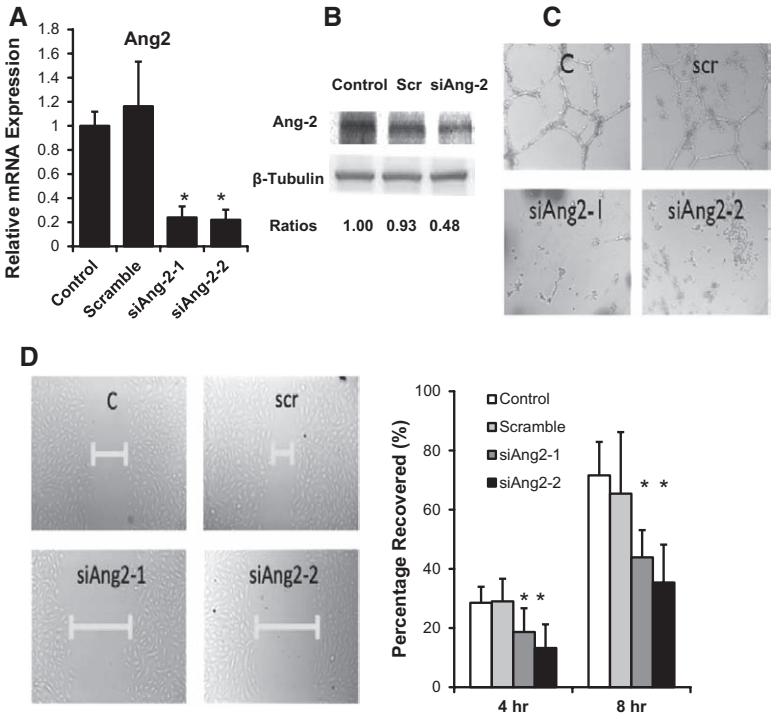


**Figure 2.** Knockdown of angiopoeitin-2 retarded human aortic endothelial cell (HAEC) migration and tube formation. HAEC were transfected with 50 nmol/L scrambled siRNA (Scr), or in-house designed or independently designed Ang-2 siRNA (siAng2-1 and siAng2-2, respectively) for 48 hours. The cells were used for Ang-2 mRNA expression, Matrigel assay for tube formation, and scratch assay for cell migration. **A**, Transfection with siAng-2 significantly reduced Ang-2 mRNA expression (*$P$<0.05; n=4) and (**B**) protein expression by >50%. **C**, HAEC tube formation was inhibited at 8 hours after siAng-2 transfection. **D**, HAEC monolayers were scratched using pipette tips and cultured in the presence of Scr, siAng2-1, and siAng2-2 (50 nmol/L). siAng-2 also inhibited HAEC migration. Bar graphs quantified cell migrations in terms of percentage after scratching at 4 hours (*$P$<0.05; n=4) and 8 hours (*$P$<0.05; n=4). Both the migration studies and the Matrigel assays were representative of 4 independent experiments with reproducible findings.

expression via canonical Wnt signaling in human aortic endothelial cells.[6]

## Ang-2 Is a Wnt Target Gene for Endothelial Repair

Ang-2 knockdown with siRNA (siAng-2) significantly reduced both Ang-2 mRNA and protein expression (Figure 2A and 2B). Transfecting human aortic endothelial cell with siAng-2 impaired tube formation at 8 hours (Figure 2C) and cell migration at both 4 and 8 hours (Figure 2D). siAng-2 studies were further validated with a second set of independently designed Ang-2 siRNA sequences (Figure 2A–2D).

To assess Ang-2 as one of the Wnt target genes, we demonstrated that human recombinant DKK-1 treatment downregulated Ang-2 mRNA expression in a dose- and time-dependent manner (normalized to GAPDH, $P$<0.05 versus control; n=3; Figure 3A), whereas recombinant Wnt3a treatment upregulated

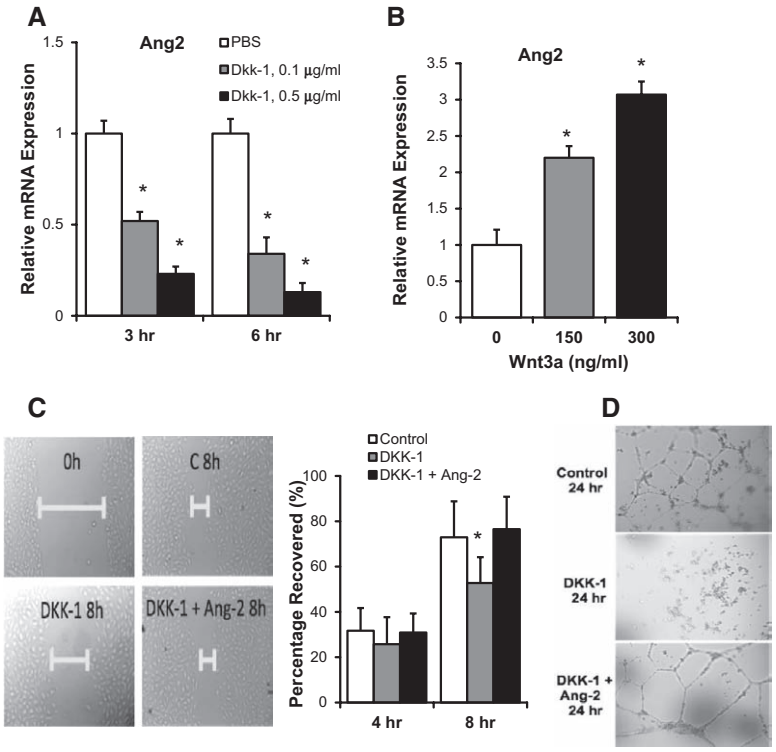


**Figure 3.** Wnt signaling mediated human aortic endothelial cell (HAEC) migration and tube formation is Ang-2-dependent. **A**, HAEC monolayers were treated with 0.1 and 0.5 μg/mL of DKK-1 for 3 and 6 hours, respectively. Quantitative reverse transcriptase-polymerase chain reaction revealed downregulation of Ang-2 mRNA expression in the presence of DKK-1 in a dose- and time-dependent manner (normalized to GAPDH: *$P$<0.05 vs control; n=3). **B**, Ang-2 mRNA expression was upregulated in response to treatment with human recombinant Wnt3a for 3 hours (normalized to GAPDH: *$P$<0.05 vs control; n=3). **C**, HAEC monolayers were scratched using pipette tips and cultured in the presence or absence of 0.5 μg/mL of human recombinant DKK-1. DKK-1 inhibited cell migration, which was rescued by recombinant Ang-2 treatment (0.5 μg/mL). Bar graphs quantified cell migrations in terms of percentage after scratching at 4 and 8 hours (*$P$<0.05; n=4). **D**, HAEC were cultured in the Matrigel in the presence or absence of 0.5 μg/mL of DKK-1. After 8 hours, tube formation was inhibited in the presence of DKK-1 (0.5 μg/mL), which was rescued by Ang-2 treatment (0.5 μg/mL). Both the migration studies and the Matrigel assays were representative of 4 independent experiments with reproducible findings.

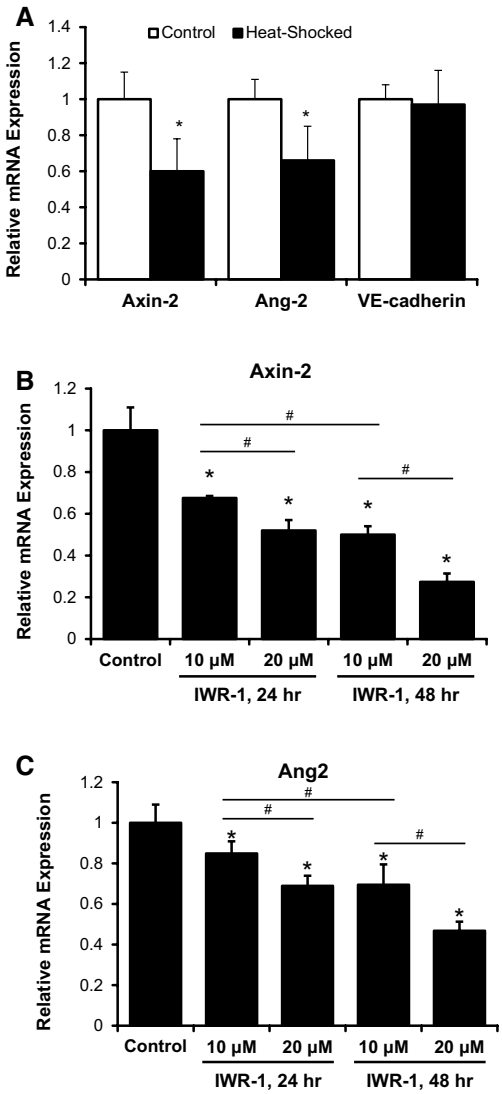


**Figure 4.** Treatment of *Tg(hsp70:DKK-1-GFP)* zebrafish embryos with IWR-1 recapitulated Ang-2 as a Wnt target gene. **A**, *Tg(hsp70l:Dkk1-GFP)* embryos were heat-shocked at 48 hours post fertilization (hpf) at 37°C for 1 hour. Axin-2, a well-recognized Wnt target gene, was used as a reference control. In *Tg(hsp70l:Dkk1-GFP)* embryos, both Axin-2 and Ang-2 mRNA expressions were downregulated in response to heat-shock induction of DKK-1 (*$P$<0.05 vs control; n=4). DKK-1 did not significantly change the expression of VE-Cadherin, suggesting DKK-1–induced downregulation of Axin-2 and Ang-2 was not because of potential vascular toxicity by heat-shock induction of DKK-1. **B** and **C**, IWR-1 also downregulated both Axin-2 and Ang-2 mRNA in a dose-dependent manner in the *Tg (kdrl:GFP)* fish at 72 hpf. Ang-2 expression was downregulated to a greater extent >48 hours treatment (starting at 24 hpf) when compared with 24-hour treatment (starting at 48 hpf; *$P$<0.05 vs control; #$P$<0.05 for pair-wise comparison; n=4).

Ang-2 in a dose-dependent manner ($P$<0.05 versus control; n=3; Figure 3B). DKK-1 treatment also impaired endothelial migration (Figure 3C) and tube formation at 8 hours (Figure 3D), which were rescued by recombinant Ang-2 treatment (Figure 3C and 3D). The downregulation of Ang-2 by DKK-1 was not because of apoptosis because DKK-1 treatment had no effect on cell viability at our time points (Figure II in the online-only Data Supplement). Ionomycin treatment similarly reduced endothelial cell migration and tube formation (Figure VI in the online-only Data Supplement). Taken together, Ang-2 is a Wnt target gene, with an implication in endothelial repair.

## Inhibition of Wnt Signaling Downregulated Ang-2 Expression in Zebrafish Embryos

To recapitulate Ang-2 as a Wnt target gene in zebrafish embryos, we used transgenic *Tg(hsp70l:Dkk1-GFP)* lines (for the zebrafish-related studies, Ang-2b homolog is denoted as Ang-2). Heat-shock induction of DKK-1-GFP resulted in downregulation of both Axin-2 and Ang-2 mRNA expression, whereas VE-cadherin expression remained unchanged (Figure 4A); whereas heat shock of wild-type fish did not have any effect on Axin-2 or Ang-2 expression (Figure III in the online-only Data Supplement). To validate Ang-2 as a Wnt target gene further, we used IWR-1, a small molecule Wnt inhibitor, to interrogate Axin-2 and Ang-2 mRNA expression. Both genes were downregulated in dose- and duration-dependent manners at 72 hpf (Figure 4B and 4C). These findings corroborated Ang-2 as a Wnt target gene in the zebrafish embryos.

## Ang-2 Morpholinos Impaired Vascular Development in Zebrafish Embryos

To elucidate whether Ang-2 was implicated in SIV development further, we used transgenic *Tg(kdrl:GFP)* zebrafish embryos (Figure 5A). Microinjection of 0.5 μmol/L Ang-2 ATG-morpholinos or splicing morpholinos to the 2-cell stage embryos impaired SIV development at 72 hpf (Figure 5B; Figure IV in the online-only Data Supplement). Coinjection of zebrafish Ang-2 (zAng-2) mRNA restored SIV formation (Figure 5B). Quantitatively, SIV length was reduced by 65% in response to ATG-morpholinos injection, which was rescued by zAng-2 mRNA injection ($P$<0.01; n=20; Figure 5C). Furthermore, Wnt inhibitor IWR-1 impaired SIV formation, which was partially rescued by zAng-2 coinjection at 72 hpf (Figure 5D and 5E). A similar effect was observed with ionomycin treatment (Figure VII in the online-only Supplement). Thus, Ang-2 is implicated in SIV development, recapitulating endothelial tube formation (Figure 2).

## IWR-1 Impaired Vascular Repair

We further assessed whether Wnt signaling was implicated in endothelial repair in the *Tg(kdrl:GFP)* zebrafish embryos at 72 hpf. Tail amputation was performed ≈100 μm from the tip (Figure 6A). In the control group, vascular repair led to a closed loop between dorsal longitudinal anastomotic vessels and dorsal aortas at 3 days post amputation (Figure 6A). Treatment with 10 μmol/L IWR-1 inhibited vascular endothelial repair at 3 days post amputation (Figure 6A). Tail amputation performed at 72 hpf to the fish injected with zAng-2 mRNA at 2-cell stage and treated with 10 μmol/L IWR-1 exhibited tail repair at 3 days post amputation (Figure 6A). Both the control and the zAng-2 injection groups exhibited a significantly higher rate of regeneration when compared with IWR-1 treatment alone ($P$<0.05; n=20; Figure 6B) These findings support the implication of Wnt-Ang-2 signaling in vascular repair.

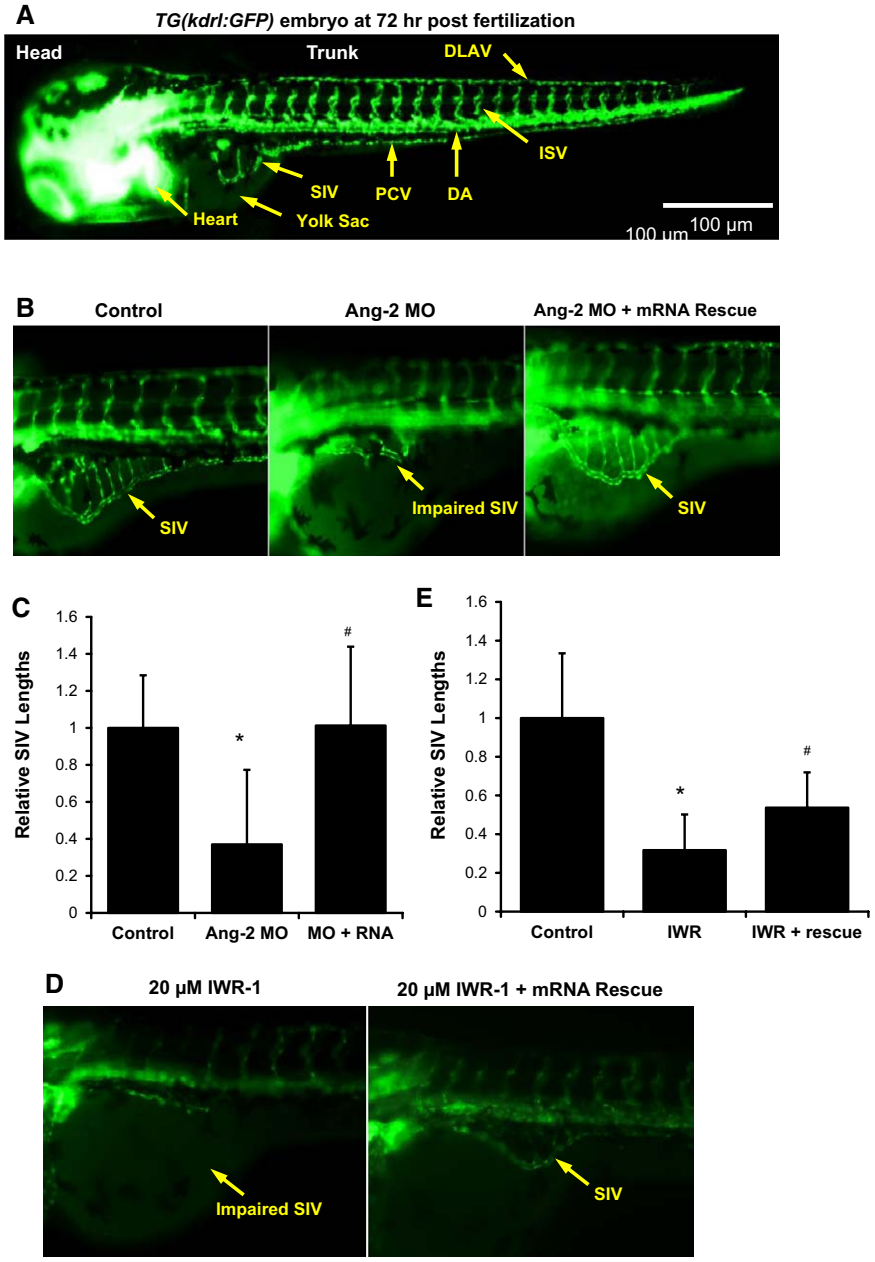


**Figure 5.** Ang-2 morphant injection impaired subintestinal vein (SIV) formation in Zebrafish embryos. **A**, Vasculature of transgenic zebrafish *Tg (kdrl:GFP)* at 72 hours post fertilization (hpf) reveals SIV, intersegmental vessel (ISV), dorsal longitudinal vein (DLAV), dorsal aorta (DA), and posterior cardinal vein (PCV). **B**, Embryos injected with the control MO developed normal SIV at 72 hpf. Ang-2 ATG-MO injection (0.5 µmol/L) impairs SIV formation. Coinjection of zAng-2 mRNA (25 ng) with Ang-2 months rescued SIV formation. **C**, Quantification of SIV length was performed and there was a significant difference between control and injection with morpholino (*$P$<0.001; n=20). zAng-2 mRNA injection rescued the SIV formation (#$P$<0.001; n=20). **D**, Treatment with Wnt signaling inhibitor IWR-1 impaired SIV formation. Injection with zAng-2 mRNA (25 ng) was able to rescue SIV formation partially. **E**, Quantification of SIV length showed significant reduction after IWR-1 treatment (*$P$<0.001; n=20), whereas injection with zAng-2 mRNA significantly increased SIV length (#$P$<0.001; n=20).

## Discussion

In this study, we recapitulate a shear stress–activated Wnt-Ang-2 signaling pathway using the developmental zebrafish model. In our dynamic flow system, canonical Wnt signaling was implicated in OSS-induced Ang-2 expression,[6] which influenced vascular endothelial cell migration and tube formation. In the zebrafish embryos, the mechano-reactivated Wnt-Ang-2 signaling was implicated in both SIV development and tail repair. Thus, shear stress–reactivated Wnt target genes (Table I in the online-only Data Supplement), in this case, Ang-2, confer therapeutic potential in restoring endothelial repair.

The Wnt/β-catenin signaling pathway plays an important role in both development and tissue repair.[20–24] Several molecules negatively regulate canonical Wnt signaling, including Dickkopfs (DKK-1), the secreted frizzled-related proteins (sFRP-1, sFRP-2, sFRP-3, and sFRP-4), and the Wnt inhibitory factor (Wif-1),[25,26] as well as small molecules, such as IWR-1. Treatment with DKK-1 and siAng-2 inhibited endothelial

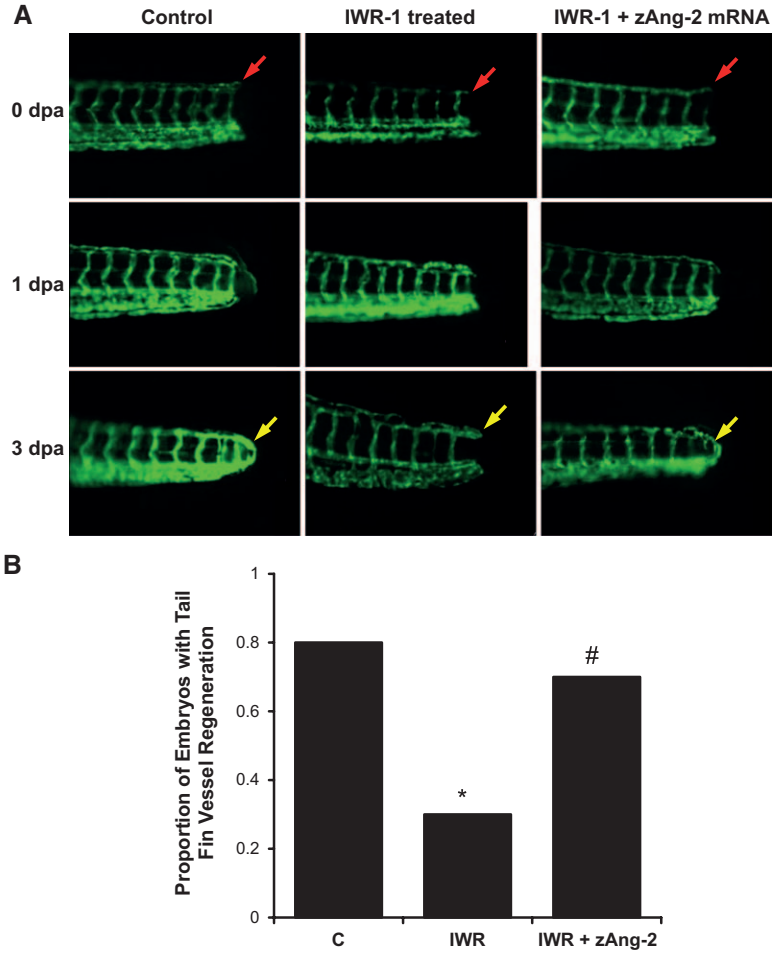


**Figure 6.** Wnt-Ang-2 signaling and vascular endothelial repair. **A**, The tails of transgenic *Tg* (kdrl:*GFP*) zebrafish embryos were amputated at 72 hours post fertilization. At 0 day post amputation (dpa), the red arrow pointed to the site of injury. At 1 dpa, initiation of endothelial repairs was present. At 3 dpa, complete tail repair was observed, as indicated by the yellow arrow. IWR-1 treatment (10 μmol/L) inhibited tail repair at 3 dpa. zAng-2 mRNA injection restored tail repair in IWR-1–treated fishes at 3 dpa. **B**, Quantification of tail repair. These experiments were repeated for n=20 in each treatment group, and each fish was assessed for the presence of tail repair at 3 dpa. The proportion of fish exhibiting tail repair in each group showed a significant difference between the control and IWR-1 treatment conditions (*$P$<0.01; n=20) and between IWR-1 treatment with and without zAng-2 mRNA injection (#$P$<0.05; n=20).

cell migration and tube formation. In corollary, ionomycin, a Calcium ionophore, is well recognized to downregulate β-catenin/T-cell factor (TCF) signaling in Wnt pathway.[27] In the colon cancer cells, ionomycin disrupted β-catenin and TCF binding, nuclear translocation of β-catenin, and suppression of TCF complexes binding to its specific DNA-binding sites.[27] We also demonstrated that ionomycin attenuated nuclear translocation of β-catenin, resulting in (1) downregulation of both Ang-2 mRNA and protein expression (Figure V in the online-only Data Supplement), (2) inhibition of tube formation, (3) endothelial migration, (4) proliferation (Figure VI in the online-only Data Supplement), and (5) inhibition of SIV development in the zebrafish model (Figure VII in the online-only Data Supplement). In this context, the complementary use of Wnt signaling inhibitors; namely, DKK-1, IWR-1, or siAng-2 knockdown, with recombinant or zebrafish Ang-2 mRNA corroborated reactivation of Wnt-Ang-2 signaling in vascular endothelial repair.

Using the angiogenesis PCR SuperArray (PAHS-024), we identified a host of Wnt/β-catenin target genes. Ang-2 was one of the shear stress-responsive angiogenic factors (data not shown). In response to low shear stress (1 dyne/cm$^2$), vascular endothelial growth factor-dependent induction of Ang-2/Tie-2 system is implicated in endothelial homeostasis, proliferation, and differentiation; in response to high shear stress (30 dyne/cm$^2$), FOXO1-dependent downregulation of Ang-2 occurs.[28,29] We demonstrate that OSS upregulated Ang-2 mRNA to a greater extent than did pulsatile shear stress, and OSS regulated Ang-2 protein expression by 2.2-fold (Figure 1E and IF). Furthermore, OSS activated Ang-2 expression via Wnt signaling both in mature endothelial cells and in a developmental zebrafish model. Ang-2 is a secreted glycoprotein that is expressed by endothelial cells and vascular progenitor cells, and the release of Ang-2 from activated endothelial cells antagonizes the binding of Ang-1 to the Tie-2 receptor, thus sensitizing the endothelial cells to proangiogenic and proinflammatory stimuli.[10] Ang-2 promotes endothelial chemotaxis and tube formation by inhibiting Ang-1–mediated phosphorylation of Tie-2.[30] Overexpression of Ang-2 can impart an antiangiogenic effect as an Ang-1/Tie-2 inhibitor by disrupting embryonic blood vessel formation, resulting in a phenotype similar to that of Tie-2 knockout.[31] Ang-2 is further implicated in regulating Wnt target Survivin expression to mitigate oxidized low-density lipoprotein–induced apoptosis in human aortic endothelial cells.[32] Elevated Ang-2 levels promote tumor progression[33] and are associated with obesity.[34] Endothelial-specific Ang-2 overexpression further promotes vascular permeability and hypotension during septic shock, whereas inhibition of the Ang-2/Tie-2 interaction attenuates lipopolysaccharide-induced hypotension and reduces mortality rate.[35] Nevertheless, the precise mechanism whereby OSS modulates Ang-2 expression in maintaining

endothelial homeostasis and in promoting vascular repair warrants further investigation.

The use of transgenic zebrafish model recapitulated shear stress–reactivated Wnt-Ang-2 signaling pathway. Zebrafish Ang-2 orthologs have been recognized to play an important role in zebrafish vascular development, particularly for intersegmental vessel sprouting and SIV formation before 72 hpf.[36] Intersegmental vessel sprouting occurs between 24 and 72 hpf, and SIV formation originates from the duct of Cuvier between 48 and 72 hpf.[37] Both intersegmental vessel and SIV are anatomic milestones for monitoring disrupted angiogenesis.[38] Analogous to the in vitro model of vascular repairs, we demonstrate Ang-2 knockdown with morpholinos resulted in impaired SIV formation in *Tg(kdrl:GFP)* fish (Figure 5). Furthermore, we demonstrate that inhibition of Wnt-signaling pathway disrupted vascular repair in response to tail amputation (Figure 6). Taken together, these findings provide new mechanotransduction insights underlying the reactivation of Wnt target genes with a therapeutic implication for vascular development and repair.

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## Disclosures

None.

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## Significance

The canonical Wnt/β-catenin signaling pathways play an important role in differentiation and proliferation. In this study, we investigated whether the Wnt signaling pathway was implicated in shear stress–activated Ang-2. Both Ang-2 expression and Wnt TOPflash reporter activity were upregulated in response to oscillatory shear stress. Although Wnt agonist Wnt3a promoted Ang-2 mRNA expression, Dkk-1 treatment or Ang-2 siRNA inhibited endothelial cell migration and tube formation. Wnt-Ang-2 signaling was further elucidated in the zebrafish embryos, in which Ang-2 mRNA was downregulated in heat-shock–inducible DKK-1 transgenic *Tg(hsp70l:Dkk1-GFP)* fish. Ang-2 morpholino microinjection further impaired development of subintestinal vessels and blood flow in at 72 hours post fertilization. Thus, we provide new insights in shear stress–activated Wnt-Ang-2 signaling with a translational implication in vascular repair.

## 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 ($\tau_{ave}$), 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 ($\tau_{av}$) = 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 $\tau_{ave}$=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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**Supplemental Materials**

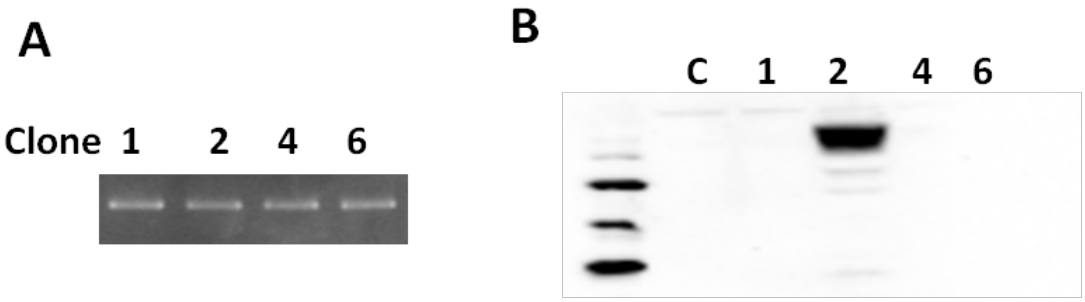
**Supplemental Methods:**

**Cell viability assay**

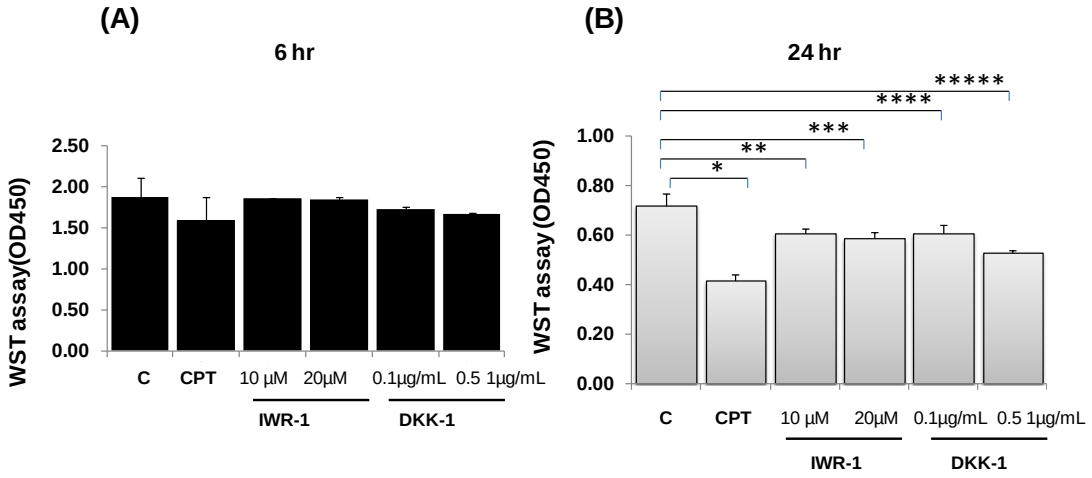
The effects of Wnt inhibitors on endothelial cell apoptosis were assessed with WST-1 cell viability assay (Cayman Chemicals). Briefly, HAEC were seeded in the 96 well plate and grown to sub-confluence. Cells were then treated with or without the Wnt inhibitors, IWR-1 (at 10μM and 20 μM) or Dkk-1 (at 0.1μg/mL and 0.5μg/mL) or a positive control of apoptosis Camptothecin (CPTs at 10 uM) for 6 hours versus 24 hours. 5uL ofWST-1 reagent was then added into cells. After 2 hour incubation, , absorbance at 450nm was measured as an indication of cell viability.

**Supplemental Figures**

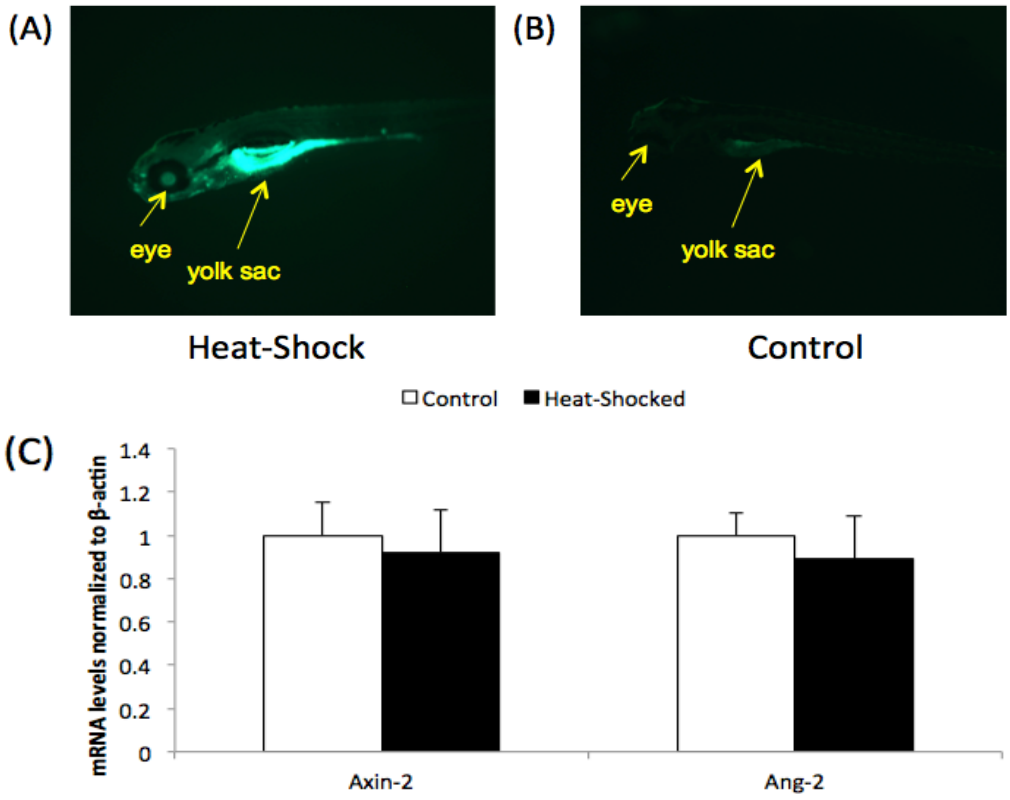
**Supplemental Figure I. Verification of HA-tagged zAng2 expression**. (**A**) pCS2-zAng-HA plasmids were transfected into HEK-293 cells. RNA was isolated to verify zAng2-HA mRNA expression by qRT-PCR. (**B**) zAng2-HA protein expression was verified by Western blots using anti-HA antibody.



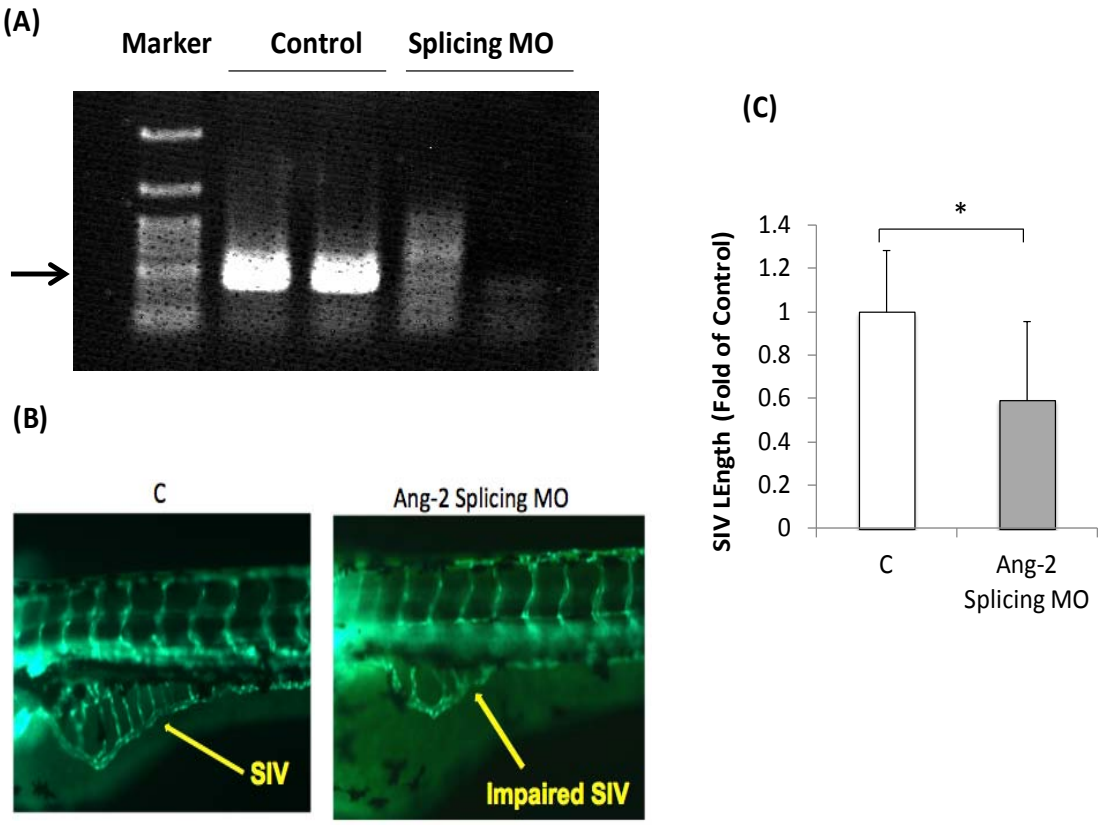
**Supplemental Figure II**. **Inhibition of Wnt signaling and cell viability**. (**A**) After 6 hours of incubation, IWR-1 and DKK did not significantly reduce cell viability ($p > 0.05$ vs. control, n=3). (**B**) After 24 hours of incubation, both IWR-1 (at 10μM and 20 μM) and Dkk-1 (at 0.1μg/mL and 0.5μg/mL) significantly reduced HAEC viability (*, **, ***, ****, *****$p<0.05$ vs. control, n=3). C denotes the control, CPT (Camptothecin) was used as a positive control for apoptosis.



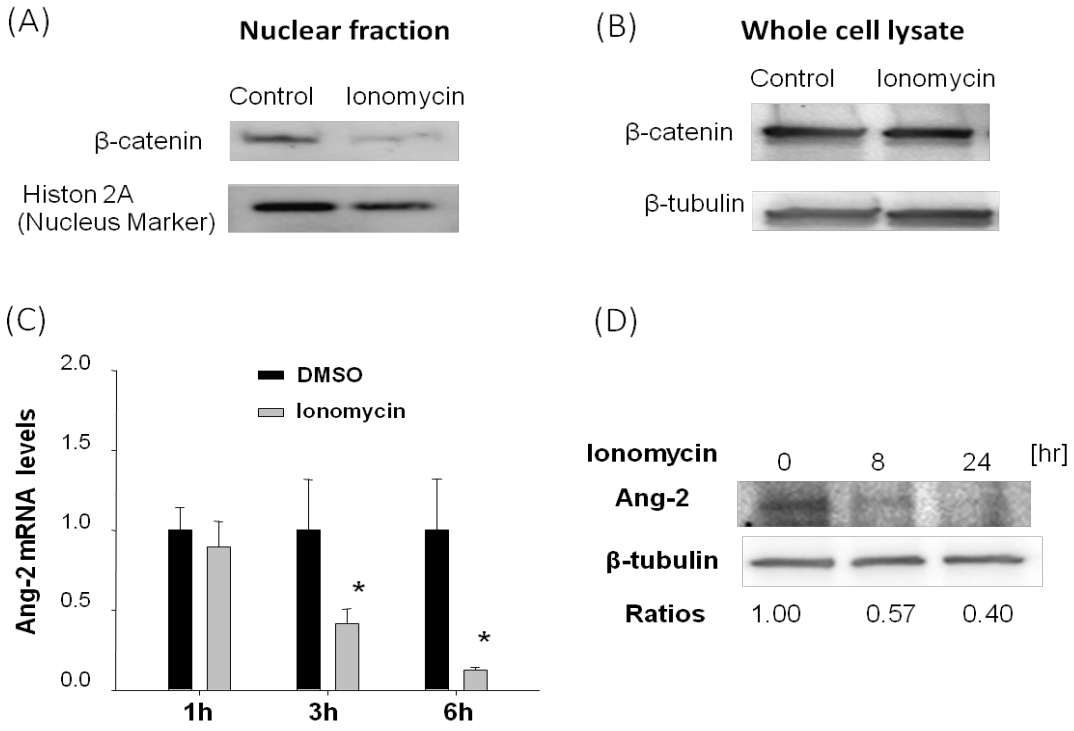
**Supplemental Figure III. The effect of heat shock on non-transgenic fish on Axin2 and Ang-2 mRNA expression. (A)** Heat-shock induction of the *Tg(hsp70l:Dkk1-GFP)* embryos at 48 hpf at 37°C for 1 hour resulted in an increase in DKK-1-GFP expression. **(B)** In the absence of heat-shock induction, DKK-1-GFP was not expressed. **(C)** Heat-shock of non-transgenic (wild-type) fish showed no changes in Axin-2 or Ang-2 expression as compared to control ($p > 0.05$, $n$=3).



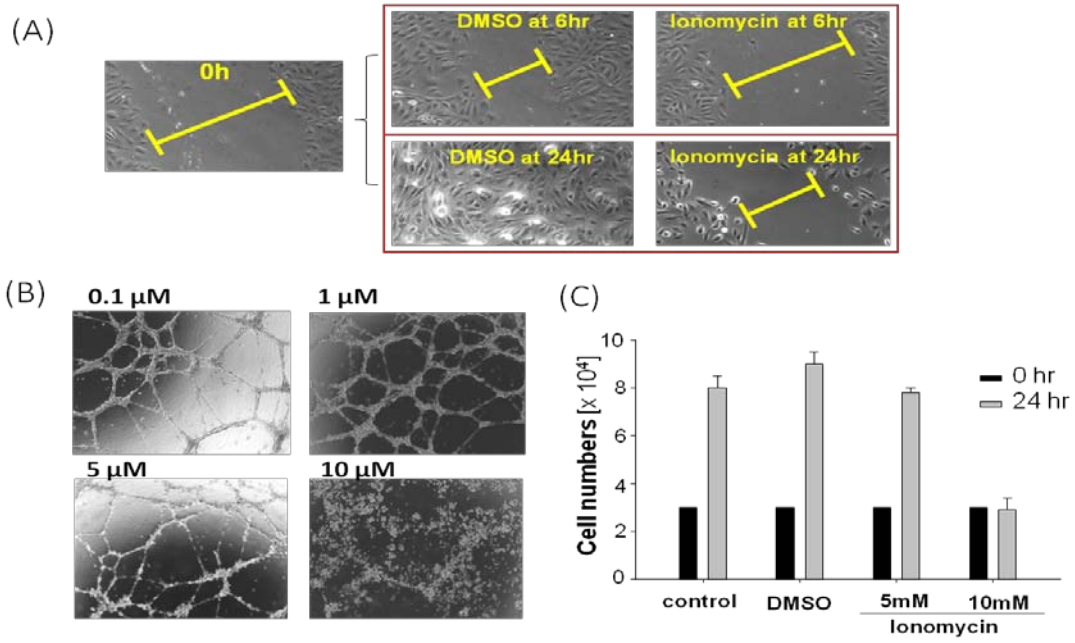
**Supplemental Figure IV. zAng-2 splicing morpholino inhibited SIV formation.** **(A)** RT-PCR was performed to validate the effect of Ang-2 splicing MO using primers that covered the exon 2-intron 2 boundary of Ang-2 (Lamont RE1, Vu W, Carter AD, Serluca FC, MacRae CA, Childs SJ. **Hedgehog signaling via angiopoietin1 is required for developmental vascular stability**. Mech Dev. 2010 Apr;127(3-4):159-68). The splicing morpholino injection resulted in a decrease in the wild type band intensity (arrow) in comparison with the controls. **(B)** The splicing morpholino micro- injection into the 2-egg stage resulted in an impaired SIV formation at 72 hpf (representative photos) . **(C)** Quantification of the SIV length revealed a 41% reduction in SIV length in response to Ang-2 splicing MO (* $p <$ 0.01, $n = 20$).



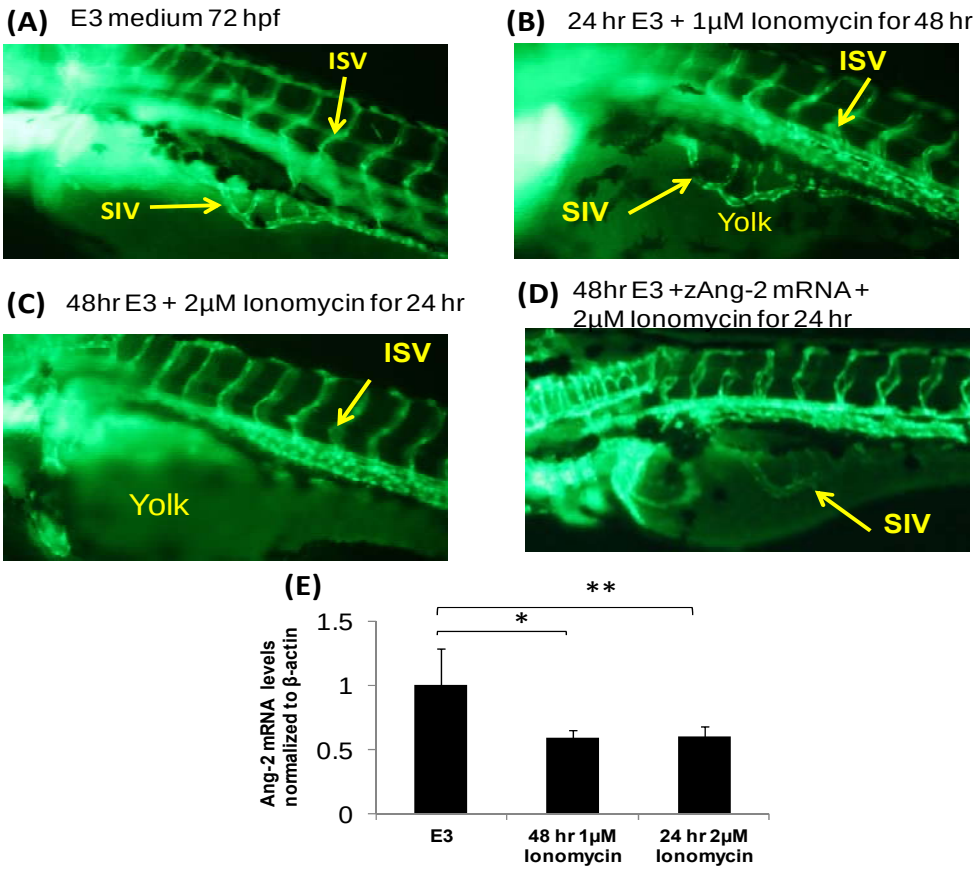
**Supplemental Figure V. Ionomycin reduced Ang-2 expression by attenuating nuclear translocation of β-catenin**. (A) Ionomycin treatment at 5 μmol/L attenuated nuclear fraction of β-catenin in HUVEC. (B) Ionomycin treatment did not alter the overall β-catenin quantity in the whole cell lysate. (C) RNAs were isolated HUVEC that were treated with 5μmol/L of Ionomycin for 1h, 3h, and 6h, respectively. Ionomycin attenuated Ang-2 mRNA expression in HUVEC as normalized to GAPDH (*$p < 0.05$ vs. Control cells with 0.1%DMSO treatment, n = 3). (D) Ionomycin attenuated Ang-2 protein levels. Entire cell lysates were collected by RIPA buffer and 50 μg of entire cell protein was prepared for Ang-2 protein levels. The relative expression was normalized to β-tubulin from density scan data. The blots were representative of two independent experiments with identical results.



**Supplemental Figure VI. Wnt signaling pathway influenced endothelial cell migration and tube formation via Angiopoietin-2.** (A) Ionomycin inhibited cell migration in HUVEC monolayer scratch assay. (B) HUVEC were cultured on the Matrigel in the presence of 0.1 – 10 μmol/L of Ionomycin for 8 hours. Ionomycin inhibited tube formation in a dose-dependent manner. (C) HUVEC were seeded in the 6-well plates and cultured for 24 hours in the presence or absence of Ionomycin at the indicated concentration, and were then trypsinized and counted with hemocytometer. HUVEC treated with the high concentration of Ionomycin ceased to proliferate.



**Supplemental Figure VII. Ionomycin impaired SIV formation that was rescued by Ang-2.** (**A**) Presence of SIV at 72 hpf in E3 medium. (**B**) 1µM Ionomycin starting at 24 hpf disrupted SIV formation, or (**C**) 2µM Ionomycin starting at 48 hpf inhibited SIV formation. (**D**) Micro-injection of 9 ng zAng-2 mRNA at the two-cell stage rescued SIV formation in the presence of 2µM Ionomycin. (**E**) qRT-PCR revealed that treatment at 48 hpf with 2 µM Ionomycin for 24 hr significantly down-regulated Ang-2 expression (**p* < 0.01 vs. E3; ***p* <0.01 vs. E3, n=3).



**Supplemental Table:**

**Supplemental Table I. List of genes in the Stem Cell RT$^2$ Profiler$^{TM}$ PCR Array (SuperArray®)**

■**Stem Cell Specific Markers:**
Cell Cycle Regulators: Apc, Axin1, Ccna2, Ccnd1, Ccnd2, Ccne1, Cdc2a, Cdc42, Ep300, Fgf1, Fgf2, Fgf3, Fgf4, Myc, Notch2, Pard6a, Rb1.
Chromosome and Chromatin Modulators: Gcn5l2, Hdac1, Hdac2, Myst1, Myst2, Rb1, Tert.
Genes Regulating Symmetric/Asymmetric Cell Division: Dhh, Notch1, Notch2, Numb, Pard6a.
Self-Renewal Markers: Hspa9, Myst1, Myst2, Neurog2, Sox1, Sox2.
Cytokines and Growth Factors: Bmp1, Bmp2, Bmp3, Cxcl12, Fgf1, Fgf2, Fgf3, Fgf4, Gdf2, Gdf3, Igf1, Jag1.
Genes Regulating Cell-Cell Communication: Dhh, Dll1, Gja1, Gjb1, Jag1.
Cell Adhesion Molecules: Acan (Agc1), Apc, Bglap1, Cd4, Cd44, Cdh1, Cdh2, Ctnna1, Cxcl12, Ncam1.
Metabolic Markers: Abcg2, Aldh1a1, Aldh2, Fgfr1.
■**Stem Cell Differentiation Markers:**
Embryonic Cell Lineage Markers: Actc1, Ascl2, Foxa2, Pdx1 (Ipf1), Isl1, Krt15, Msx1, Myod1, T.
Hematopoietic Cell Lineage Markers: Cd19, Cd3d, Cd4, Cd8a, Cd8b1, Mme.
Mesenchymal Cell Lineage Markers: Acan (Agc1), Bglap1, Col1a1, Col2a1, Col9a1, Pparg.
Neural Cell Lineage Markers: Cd44, Ncam1, Oprs1, S100b, Tubb3.
■**Signaling Pathways Important for Stem Cell Maintenance:**
Notch Pathway: Dll1, Dll3, Dtx1, Dtx2, Dvl1, Ep300, Gcn5l2, Hdac1, Hdac2, Jag1, Notch1, Notch2, Numb.
Wnt Pathway: Adar, Apc, Axin1, Btrc, Ccnd1, Frat1, Fzd1, Myc, Ppard, Wnt1.

Stem-cell specific markers, stem cell differentiation markers, and genes in signaling pathways important for stem cells maintenance were analyzed using qRT-PCR array. We analyzed expression of a focused panel of genes related to stem cell biology in response to fluid shear stress. Bioinforamtics approaches were based on the above functional gene groupings. Based on the initial grouping of the genes into different categories, we have first identified gene subsets in the Wnt pathway whose gene expression levels were significantly elevated at specific time points in response to shear stress. In the Wnt pathway, genes cooperate with each other to perform certain functions. Thus, the expression levels of genes in the Wnt pathway were highly correlated.

**Supplemental Movies**:

**Supplemental Video I:** Micro-injection with the control MO displayed a normal blood circulation in dorsal aorta (DA), posterior cardinal vein (PCV), and subintestinal vein (SIV) at 72 hpf.

**Supplemental Video II:** Micro-injection with Ang-2 MO (0.5uM) led to absence of circulation despite cardiac contraction at 72 hpf.

**Supplemental Video III:** *Tg(hsp70l:DKK1-GFP)* fish were imaged for blood flow at 72hpf without heat-shock.

**Supplemental Video IV:** *Tg(hsp70l:DKK1-GFP)* fish were imaged for blood flow at 72hpf after one hour heat-shock at 48 hpf to induce DKK1 expression. DKK-1 did not have apparent effect on blood flow.