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Los Angeles

Dynamic Blood Flow Modulates Endothelial Mitochondrial
Redox States and Vascular Repair

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Bioengineering

by

Nelson Jen

2015

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ABSTRACT OF DISSERTATION

Dynamic Blood Flow Modulates Endothelial Mitochondrial Redox States and Vascular Repair

By

Nelson Jen

Doctor of Philosophy in Bioengineering

University of California, Los Angeles 2015

Professor Tzung K. Hsiai, Chair

Temporal and spatial variations in shear stress are intimately linked with vascular metabolic effects. Fluid shear stress plays a major role in regulating endothelial homeostasis, dysfunction, and vasculogenesis. The following studies characterized the mechanisms whereby shear stress modulates these effects.

In the first study we demonstrated atherogenic oscillatory shear stress (OSS) induced mitochondrial superoxide ($\text{mtO}_2^{\cdot-}$) production via NADPH oxidase and c-Jun NH₂-terminal kinase (JNK-1 and JNK-2) signaling. OSS ($\pm 3 \text{ dyn}\cdot\text{cm}^{-2}$) induced JNK peak activation at 1 h, accompanied by increased MitoSOX Red ($\text{mtO}_2^{\cdot-}$ indicator) and dihydroethidium ($\text{O}_2^{\cdot-}$ indicator) intensities in bovine aortic endothelial cells which were all attenuated by either pretreatment with apocynin (NADPH oxidase inhibitor), N-acetyl cysteine (antioxidant), or JNK knockdown by siRNA (siJNK) and pretreatment with

SP600125 (JNK inhibitor). Immunohistochemistry of explanted human coronary arteries further revealed prominent phosphorylated JNK (active JNK) staining in OSS-exposed regions. These results demonstrate that OSS-induced $\text{mtO}_2^{\bullet-}$ production via NADPH oxidase and JNK activation is relevant for vascular oxidative stress and the initiation of atherosclerosis.

Next we revealed that OSS-mediated oxidative stress and JNK activation induced autophagy but impaired autophagic flux to promote $\text{mtO}_2^{\bullet-}$ production, mtDNA damage, and mitochondrial dysfunction in the disturbed flow-exposed regions. OSS significantly increased (1) microtubule-associated protein light chain 3 (LC3) II to I ratios in human aortic endothelial cells (HAEC), (2) autophagosome formation as quantified by GFP-LC3 dots per cell, and (3) p62 protein levels, a reverse marker of autophagic flux. MnSOD over-expression by recombinant adenovirus, N-acetyl cysteine (NAC) treatment, or c-Jun N-terminal Kinase (JNK) inhibition reduced OSS-mediated LC3-II/LC3-I ratios and mitochondrial DNA damage. Inhibition of autophagy completion by bafilomycin incrementally increased both LC3-II/ LC3-I ratios and p62 levels under OSS conditions, implicating impaired autophagic flux. Immunohistochemical analysis revealed prominent staining of p62 in the oscillatory shear stress (OSS) -exposed aortic arch as compared with that in the pulsatile shear stress (PSS) -exposed descending aorta. Knockdown of ATG5 with siRNA further enhanced OSS-mediated $\text{mtO}_2^{\bullet-}$ production whereas starvation or rapamycin-induced autophagy reduced OSS-mediated $\text{mtO}_2^{\bullet-}$, mitochondrial respiration, and Complex II activity. These findings reveal OSS-mediated oxidative stress and JNK activation induced autophagy but impaired

autophagic flux to promote $\text{mtO}_2^{\cdot-}$ production, mtDNA damage, and mitochondrial dysfunction in the disturbed flow-exposed regions.

In the next study we demonstrated rapid and irregular pacing, to mimic atrial fibrillation (AF), reduced intravascular shear stress (ISS) with implications to endothelial response modulation. We paced the left atrial appendage of New Zealand White (NZW) rabbits at rapid and irregular intervals, then recorded surface electrocardiograms (ECG) for atrial and ventricular rhythm, and calculated ISS from measured intravascular convective heat transfer by micro thermal sensors. Rapid and irregular pacing decreased arterial systolic and diastolic pressures, and reduced ISS. We recapitulated the altered ISS in an *in vitro* system and further demonstrated that rapid pulse rates at 150 bpm down-regulated endothelial levels of nitric oxide (NO), promoted superoxide ($\text{O}_2^{\cdot-}$) production, and increased monocyte binding to endothelial cells. These findings suggest that rapid pacing reduces ISS and $\partial\tau/\partial t$, and rapid pulse rates modulate endothelial responses.

In the last study, we investigated whether shear stress activated angiopoietin-2 (Ang-2) via the canonical Wnt signaling pathway with implications in vascular endothelial repair. OSS upregulated both TOPflash Wnt reporter activities and Ang-2 mRNA and protein expressions in HAEC. OSS-induced Ang-2 mRNA expression was upregulated by Wnt agonists LiCl and Wnt3a and downregulated by Wnt inhibitor IWR-1 and Wnt signaling inhibitor DKK-1. Inhibition of Ang-2 by DKK-1 and Ang-2 siRNA attenuated endothelial cell migration and tube formation with implications to vascular repair. Inhibition of Wnt signaling with IWR-1 in *Tg(kdr1:GFP)* zebrafish impaired vascular repair after tail amputation and downregulated Ang-2 expression, both of which

were rescued by zebrafish Ang-2 mRNA injection. Furthermore Ang-2 morpholino injection impaired subintestinal vessel formation at 72 hours post fertilization, which was rescued by zebrafish Ang-2 mRNA coinjection. These data demonstrated shear stress activated Ang-2 via canonical Wnt signaling as an essential signaling component in vascular development and repair.

The dissertation of Nelson Chiang Jen is approved.

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2015

DEDICATION

This work is dedicated to my parents, Chin Ping Jen and Chun Lien Jen.

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Chapter One: Introduction

1.1 Shear stress and Endothelium

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the world, accounting for a third of all deaths in developed countries [1]. The primary cause of CVD is atherosclerosis, which results from a maladaptive inflammatory response initiated by the accumulation of fatty streaks which develop into a lesion and result in vessel obstruction or lesion rupture [1-2]. The latter can abruptly restrict blood flow to the heart by giving rise to a blood clot, thereby leading to a heart attack or stroke.

While atherosclerosis is a systemic disease, its manifestations tend to be focal and eccentric [3-4]. Atherosclerotic lesions develop preferentially at the inner curvature of arteries or at the lateral wall of arterial bifurcations [5]. These predisposed areas are exposed to bidirectional, low wall shear stress and flow separation, a departure from the pulsatile, unidirectional flow that exists in straight portions of the arteries [6-8]. Hemodynamic flow imparts both shear stress, cyclic stretch, and hydrostatic pressure on the endothelium due to the pulsatile nature of blood pressure and flow [9-10]. While cyclic stretch plays an important role maintaining endothelial function [10], shear stress exerts a significant impact in directing developmental, physiological, and pathophysiological endothelial responses [5, 7, 10-14].

Shear stress is the parallel friction drag force that blood flow imparts on the endothelium. The rheological properties of blood are complex due to its heterogeneous composition including erythrocytes, leukocytes, and organic compounds such as fibrinogen and albumin [15]. Under high shear rates, such as during peak systole, blood behaves as a Newtonian fluid near the vessel wall. However the non-Newtonian

properties of blood dominate during diastole and rheological characteristics require complex constitutive equations [16-17]. In our investigations we used culture media which was treated as a Newtonian fluid. Under steady flow across a surface, shear stress can be defined by the equation:

$$\tau = \mu \cdot \dot{\gamma} = \mu \cdot \frac{\partial u}{\partial y}$$

where μ is the dynamic viscosity and $\dot{\gamma}$ the shear rate (sec^{-1}), which could be further defined with u the fluid velocity, and y the distance from the wall [18]. For blood flow within a vessel (inelastic, cylindrical, straight), shear stress can be defined by the Haagen- Poiseuille equation:

$$\tau = 32 \cdot \mu \cdot \frac{Q}{\pi \cdot d^3}$$

where Q is the volumetric flow rate and d the diameter of the vessel [18-19]. This equation indicates that shear stress is directly proportional to blood flow rate and inversely proportional to vessel diameter.

The contractile nature of the heart imparts cyclic variations in shear stress. The local spatial ($\partial\tau/\partial x$) and temporal ($\partial\tau/\partial t$) components of shear stress largely determine phenotypic responses of endothelial cells [3, 20-22]. At the lateral wall of bifurcations, oscillatory shear stress (OSS), bidirectional with net zero forward flow, has been implicated in inducing oxidative stress and inflammation, initiating atherosclerosis, and is considered to be “pro-atherogenic” [21, 23-25]. Conversely, pulsatile shear stress (PSS), unidirectional with high shear flow, which develops in the straight portions of arteries, promotes anti- thrombotic, anti-inflammatory, anti-oxidant responses, and is considered “anti-atherogenic” [10, 26-28].

Endothelial cells are continually subjected to shear stress due to blood flow. These mechanical stimuli modulate many endothelial functions, such as vessel permeability, proliferation, endothelial activation, and other homeostatic responses, through complex mechanotransduction signaling pathways [9-10, 12]. Endothelial cells rely on multiple different mechanosensor systems to transduce mechanical signals. Integrins, transmembrane protein receptors that bind cells to the extracellular matrix (ECM), are responsible for transducing bulk deformations in cell cytoskeleton through focal adhesions – integrin contacts, leading to the activation of focal adhesion kinase (FAK) and c-Src to initiate signaling [9, 29]. Deformations in the cellular cytoskeleton in response to shear stress can also activate the cell adhesion protein PECAM1, which in turn transduces intracellular signaling with PKC [30-31]. Shear stress can also activate mechanically sensitive K^+ and Ca^{2+} ion channels [32]. Additionally, transmembrane proteins such as G protein-coupled receptors and membrane bound structures such as the glycocalyx play an important role in transducing mechanical signals [33]. The activation of these cellular shear stress sensors in turn lead to the activation of multiple signaling molecules including protein kinase C (PKC), FAK, c-Src, Rho family GTPases, PI3K, and MAPKs, for the initiation and propagation of multiple signaling pathways [9, 31, 33].

1.2 Oxidative stress and endothelium

Reactive oxygen species (ROS) are key mediators in signaling pathways that underlie inflammation and the initiation of atherosclerosis. There are numerous sources of cellular superoxide ($O_2^{\cdot-}$) production which contribute to vascular oxidative stress, including NADPH oxidase systems, mitochondria respiratory chain, xanthine oxidase,

eNOS uncoupling, p450 isoenzymes, and peroxisomes [3, 34-35]. NADPH oxidase is considered the major source of ROS generation in vascular endothelial cells [34] and OSS is implicated in the production of reactive oxygen species (ROS) via NADPH oxidase systems [24, 36]. While a low level of ROS is associated with normal cellular functions, such as cell survival [37-38], differentiation [39-40], post-translational protein modifications [21], and host defense [41], a high level of ROS production induces oxidative stress, tissue damage [25, 42], and contributes to endothelial dysfunction relevant for the initiation of atherosclerosis [34].

Mitochondria are an important source of ROS in the endothelium. Normally 1% of mitochondrial oxygen is incompletely reduced, leading to the generation of mitochondrial superoxide ($mtO_2^{\bullet-}$) at complex I and III of the electron transport chain by transfer of a single free electron to molecular oxygen [43]. This radical is then scavenged by manganese-dependent superoxide dismutase (MnSOD) to form less reactive H_2O_2 . The protective role of MnSOD is essential for homeostasis; a homozygous knockout of MnSOD in mice ($MnSOD^{-/-}$) leads to lethality a few days after birth [44]. Increase in cellular superoxide has been associated with reduced mitochondrial membrane potential ($\Delta\Psi_m$), leading to greatly increased mitochondrial superoxide production [27, 45]. Increased mitochondria ROS can lead to MnSOD deficiency and increased mitochondrial DNA damage by oxidation, both of which have been linked to atherosclerosis [46-47].

Endothelial cells have endogenous anti-oxidant protective mechanisms to respond to oxidative injury. Intense exercise has been demonstrated to increase cellular enzymatic anti-oxidants, increase nitric oxide (NO) production, and reduce inflammation

as a protective mechanism against oxidative stress [48-49]. Exercise-induced increases in blood flow and shear stress are attributed to improved vascular function. These salutary effects are primarily due to activation of the PI3K-kinase-Akt-eNOS pathway to increase bioavailable NO, inactivation of inflammatory stress-responsive kinase JNK, increases in antioxidant CuZn-SOD, and improved mitochondrial respiration [24, 27, 50]. Collectively these effects impart a protective phenotype by down-regulating pro-inflammatory gene expression and maintaining endothelial redox states.

1.3 Shear stress, oxidative stress, and autophagy

Autophagy is a highly regulated process associated with the activation of autophagy-related (ATG) genes [51] whereby cellular components, including soluble and aggregated proteins as well as organelles, are sequestered in autophagosomes and degraded in lysosomes to adapt to nutrient restriction or to eliminate modified macromolecules and damaged organelles [52-56]. The initiation of autophagy in mammalian cells begins at the formation of the isolation membrane which is influenced by ULK kinase complex, which is formed by UNC-51-like autophagy kinase 1 (ULK1), ULK2, and ATG13. Under basal conditions the mechanistic target of rapamycin complex 1 (MTORC1) binds directly to ULK1 and inhibits the ULK1/2 and ATG13 by phosphorylation [57-58]. Upon stress-induced activation, MTORC disassociates from the ULK kinase complex and allows ATG13 dephosphorylation and activation of ULK1/2 to induce autophagy [58]. The nucleation of the phagophore is catalyzed by the class III phosphatidylinositol 3-kinase (PtdIns3K) where binding to ATG14 and autophagy/beclin-1 regulator 1 (AMBRA1) directs and induces autophagy [58-59]. The ATG8/Microtubule-associated protein light chain 3 (LC3) family and ATG5 play key roles in

autophagosome biogenesis by building protein scaffolds [60-62] and mediating expansion of the lipid membrane of the autophagosome [51]. Activation of LC3-I (ATG8) requires ATG3 conjugation to mediate binding to phosphatidylethanolamine (PE) for conversion to LC3-II (ATG8-PE) [51, 58]. This allows the anchorage of ATG8/LC3 to both the inner and outer membrane of the autophagosome and is essential for phagophore expansion [51]. Autophagy substrates are targeted for degradation by associating with p62/SQSTM1, a multi-domain protein that functions as a selective autophagy receptor, which physically links autophagic cargo to ATG8/MAP1-LC3/GABARAP family members located on the forming autophagic membranes [63]. Mature autophagosomes then fuse with lysosomes for cargo degradation by a RAB7-mediated process where resulting macromolecules are transported back to the cytoplasm for recycling [58, 63].

While autophagy is essential for cell survival, differentiation, and development, dysfunctional autophagy is associated with a number of pathological conditions, including cardiovascular and neurodegenerative diseases, muscular dystrophies, and cancer [57, 64-65]. Increasing evidence supports reactive oxygen species (ROS), oxidized lipoproteins, and endoplasmic reticulum (ER) stress as autophagy inducers [56, 66-67]. Furthermore, under excess oxidative stress, autophagic flux can be disabled, leading to an intracellular accumulation of p62, a marker for an incomplete autophagy process known as impaired autophagic flux [68]. Impaired autophagy results in an inability to respond to stress and promotes vascular inflammatory responses, atherogenesis [69], myocardial contractile dysfunction, and heart failure [64, 70-71].

1.4 Rapid, irregular pulsatile shear stress and endothelium

Endothelial responses are closely linked with cardiac function [72]. The rhythmicity of the heart drives the temporal ($\partial\tau/\partial t$) components of shear stress. The unsteady nature of pulsatile flow can be characterized by the Womersley number (α), which is the ratio of the transient inertial force versus the shear force [18]:

$$\text{Transient inertial force: } \rho\omega V$$

$$\text{Shear force: } \mu V/L^2$$

where μ is the viscosity of the fluid, ρ the density of the fluid, ω the heart rate, V the velocity of the fluid, and L the diameter of the vessel. The ratio of the two terms gives [18]:

$$\frac{\text{Transient inertial force}}{\text{Shear force}} = \frac{\rho\omega L^2}{\mu} = \text{Stokes number} = (\text{Womersley Number})^2$$

For large Womersley numbers (α) such as in vessels with larger diameters like the aorta, oscillatory inertial force dominates, and for small α , viscous force dominates. The healthy human aorta gives a α that ranges from 12.0 to 16.1 [73], which typically experiences transient turbulent flow [74]. In the case of elevated heart rates, α is further increased, leading to greater instabilities in flow and disturbed flow.

Patients with arrhythmia, or irregular cardiac rhythms, experience irregular ventricular responses and pulsatile blood flow [75]. Atrial fibrillation (AF) is the most common type of cardiac arrhythmia, affecting up to 5 million people in the United States and 5% of the population over 65 years old [76-78]. AF promotes rapid and irregular atrial contraction rates exceeding 400–600 times per minute (bpm) [79], resulting in rapid and irregular ventricular contraction rates above 100 bpm [80], and leading to a decrease in both atrial pressure and cardiac output [81-82]. This response leads to rapid, irregular complex hemodynamic changes which are sensed by the endothelium

[33, 83]. Increasing evidence has supported a link between chronic AF and vascular endothelial dysfunction [84]. Patients with AF have been reported with elevated circulating von Willebrand factor (vWF) and increased E-selectin [85-86]. AF is also implicated in decrease in endothelial dependent vasodilation in forearm vessels [85, 87], serum nitrates [88-89], and coronary flow reserve (CFR) [90].

1.5 Shear stress, angiogenesis, and wound repair

Shear stress plays a crucial role in regulating angiogenesis, vasculogenesis and wound repair [14, 91]. While high shear stress induces a quiescent state by suppressing proliferation and apoptosis [92], low shear stress induces angiogenesis-related gene expressions, including VEGFA, Ang-2, and MMP-9 [93]. Additionally, shear stress also plays a role in vasculogenesis through the differentiation of embryonic stem cells to vascular endothelial cells [94-96]. These endothelial progenitor cells are involved in vascular repair and are sensitive to shear stress [14, 97]. Increased blood flow has been linked to vessel sprouting and wound healing [98-99]. During wound healing, angiogenic sprouts invade the wound clot, leading to the formation of a microvascular network [100].

Shear stress was previously shown to activate TIE-2 receptors and upregulate Ang-2 in endothelial cells, both of which are requirements for the formation of blood vessels [93, 101]. Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are key growth factors that mediate blood vessel formation [102-103]. 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 [104]. Furthermore, Ang-2 promotes endothelial

migration and tubular formation [91]. However, the mechanism whereby shear-induced Ang2 promotes angiogenesis remains unclear.

Chapter Two: Oscillatory Shear Stress Induces Mitochondrial Superoxide Production: Implication of NADPH Oxidase and c-Jun NH₂-Terminal Kinase Signaling

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Takabe W, Jen N, Ai L, Hamilton R, Wang S, Holmes K, Dharbandi F, Khalsa B, Bressler S, Barr ML, Li R, Hsiai TK. "Oscillatory shear stress induces mitochondrial superoxide production: implication of NADPH oxidase and c-Jun NH₂-terminal kinase signaling." *Antioxid Redox Signal*. 2011 Sep 1;15(5):1379-88. Copyright © 2011 by Mary Ann Liebert, Inc.

2.1 Introduction

Atherosclerosis is a systemic disease; however, its manifestations tend to be focal and eccentric [3, 21-22]. The spatial ($\partial\tau/\partial x$) and temporal ($\partial\tau/\partial t$) components of shear stress largely determine the focal characteristics of vascular oxidative stress, leading to proinflammatory states [21, 23-25]. In the arterial regions exposed to atheroprotective hemodynamics, pulsatile flow develops, whereas at the lateral wall of arterial bifurcations, disturbed flow, including atherogenic oscillatory shear stress (OSS), prevails [24-25]. The latter flow characteristics, defined as bidirectional net zero forward flow, are implicated in the production of reactive oxygen species (ROS) via NADPH oxidase systems [24, 36], and an elevated level of ROS production contributes to endothelial dysfunction relevant for the initiation of atherosclerosis [34].

Numerous sources of cellular superoxide ($O_2^{\cdot-}$) production contribute to vascular oxidative stress, including NADPH oxidase systems, mitochondria, xanthine oxidase, eNOS uncoupling, p450 isoenzymes, and peroxisomes [3, 34-35]. NADPH oxidase is considered the major source of ROS generation in vascular endothelial cells (ECs) [34]. A low level of ROS is implicated in normal cellular functions, such as cell survival [37-38], differentiation [39-40], post-translational protein modifications [21], and host defense [41], whereas a high level of ROS production engenders oxidative stress and tissue damage [25, 42].

Both biomechanical and biochemical stimuli mediate mitochondrial ROS generation. Atheroprotective pulsatile shear stress induces endothelial mitochondrial membrane potential ($\Delta\Psi_m$) and a reduction in mitochondrial superoxide ($mtO_2^{\cdot-}$) production via an increase in manganese superoxide dismutase (Mn-SOD) activities

[27]. In contrast, oxidized low-density lipoprotein (oxLDL) induces $\text{mtO}_2^{\bullet-}$ production, leading to apoptosis via JNK-mediated Mn-SOD ubiquitination and protein degradation [105]. However, the mechanisms whereby atherogenic OSS regulates $\text{mtO}_2^{\bullet-}$ remain unknown.

Mounting evidence supports the notion that shear stress activates c-Jun NH₂-terminal kinase (JNK-1 and JNK-2) in cultured vascular ECs [106-108]. JNK is one of the signaling molecules in the mitogen-activated protein kinase super family, and is implicated in stress responses to inflammatory cytokines, growth factors [109] and ROS [108]. Li et al. reported that laminar shear stress (LSS) at 12 $\text{dyn}\cdot\text{cm}^{-2}$ induced a transient and rapid activation of JNK-1 and JNK-2 via extracellular signal-regulated kinases (ERK-1 and ERK-2) [107]. Moreover, Li et al. showed that LSS inhibited tumor necrosis factor-mediated JNK activation via MEK5-BMK1 in vascular ECs [110-111]. In this context, we proposed that atherogenic OSS induced $\text{mtO}_2^{\bullet-}$ production via NADPH oxidase and JNK activation.

In this study, we demonstrated in bovine aortic ECs (BAECs) that OSS ($\pm 3 \text{ dyn}\cdot\text{cm}^{-2}$) induced JNK activation peaked at 1 h, accompanied by an increase in $\text{mtO}_2^{\bullet-}$ production. Pretreatment with apocynin (an inhibitor to NADPH oxidase assembly) or N-acetyl cysteine (NAC) (an antioxidant) resulted in a significant attenuation of OSS-induced JNK activation. Apocynin further reduced OSS-mediated cytosolic $\text{O}_2^{\bullet-}$ production. As a corollary, transfecting BAECs with JNK siRNA (siJNK), pretreating with SP600125 (JNK inhibitor), or with apocynin significantly reduced OSS-mediated $\text{mtO}_2^{\bullet-}$ production. Immunohistochemistry on explants of human coronary arteries also revealed prominent phosphorylated JNK staining in the arterial regions prone to

atherogenic hemodynamics. Hence, our findings support the notion that OSS induced $\text{mtO}_2^{\cdot-}$ production via NADPH oxidase and JNK activation is relevant for vascular oxidative stress.

2.2 Materials and Methods

2.2.1 EC culture and inhibitor study

Confluent BAECs between passages 4 and 7 were seeded on Collagen Type I (BD Biosciences)-coated glass slides (5 cm^2) at 1.5×10^5 cells per slide and grown to confluent monolayers in high glucose (4.5 g/l) Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone) and 100 U/ml L-glutamine–penicillin–streptomycin (Sigma) for 48 h in 5% CO_2 at 37°C . Before shear stress exposure, the cells were starved in Dulbecco's modified Eagle's medium with 0.5% FBS overnight to reduce phosphorylative background. For inhibitor studies, the cells were pretreated with either JNK inhibitor SP600125 ($10 \mu\text{M}$) for 30 min, NADPH oxidase inhibitor apocynin (1 mM) for 2 h, or antioxidant (5 mM) before shear stress exposure.

2.2.2 Dynamic flow system to simulate OSS in the arterial bifurcation

A dynamic flow channel was used to implement OSS by simulating hemodynamics in human carotid arterial bifurcations. The flow system was designed to generate well-defined flow profiles across the width of the parallel flow chamber at various temporal gradients ($\partial\tau/\partial t$), frequencies, and amplitudes [112]. BAECs were exposed to two conditions: (i) control at static conditions, and (ii) OSS at $\tau_{\text{avg}} = 0.02$ and $\partial\tau/\partial t$ at $\pm 3 \text{ dyn}\cdot\text{cm}^{-2}$. At the lateral wall of arterial bifurcations, flow separation and

migrating stagnation points create low and oscillating shear stress (OSS: bidirectional net zero forward flow), which is commonly considered as an inducer of vascular oxidative stress [113-114].

2.2.3 Detection of intracellular superoxide

The intracellular superoxide ($O_2^{\cdot-}$) production was measured using dihydroethidium (DHE). BAEC were exposed to OSS for 1 h and cells were washed with the culture medium without phenol red. Cells were then incubated with DHE (5 μ M) for 20 min followed by five times of washing. Images were acquired from three chosen fields using an inverted epifluorescence microscope and using a ProgRes C3 digital microscope camera.

2.2.4 siRNA transfection

The siRNA target sequence for Bovine JNK-1 was 5'-CATGGAGCTCATGGATGCAAATCTT-3° and Bovine JNK-2 was 5'-CATGAAAGAATGTCCTACCTTCTTT-3°. siRNA (each 30 nM) was transfected to BAEC with Lipofectamine RNAiMAX (Invitrogen) as described previously [27]. Cells were used for confirmation of gene knockdown or function assay 48 h after transfection. Negative control siRNA (Qiagen) was used as the scramble siRNA. There was no observable damage due to the transfection procedure.

2.2.5 Western blots analysis

After OSS exposure, BAECs were assessed for JNK phosphorylation. The cells were rinsed with phosphate-buffered saline (PBS) and lysed using RIPA buffer supplemented with protease and phosphatase inhibitors. Protein concentration was measured using the Bio-Rad DC assay and 50 μ g of protein was loaded for Western

blot. Activated JNK (p-JNK) was measured using an anti-phosphor-JNK antibody (Upstate Cell Signaling Solutions). Parallel blots were performed with anti-total JNK (Upstate Cell Signaling Solutions) and/or anti- β -actin (Millipore Corp.) to standardize protein abundance. Densitometry was performed using NIH Scion Image Software (Scion Corp.).

2.2.6 Flow cytometry analysis to quantify $\text{mtO}_2^{\cdot -}$ production

MitoSOX Red superoxide indicator (Invitrogen) is a fluorogenic dye that is selective for $\text{mtO}_2^{\cdot -}$ in live cells [115]. It localizes into cellular mitochondria and is readily oxidized by superoxide, but not other sources of ROS or nitrogen species. The oxidation of the probe is prevented by superoxide dismutase and exhibits a bright red fluorescence upon binding to nucleic acids (excitation/emission maxima = 510/580 nm). After OSS exposure, BAECs were incubated with MitoSOX Red (3 μM) for 10 min at 37°C. The cells were collected by trypsinization and washed in PBS supplemented with 2% FBS. Cells were fixed in 2% paraformaldehyde and suspended in PBS. Measurements were performed in duplicates using the BD LSR II flow cytometer (BD Biosciences) at the USC Center for Stem Cell and Regenerative Medicine FACS Core. MitoSOX Red was excited at 488 nm, and the data were collected by a 575/26 nm (FL2) channel. The data were presented by histograms in terms of the mean intensity of MitoSOX fluorescence normalized to those of the static controls.

2.2.7 Immunohistochemistry analyses of phosphorylated JNK in explants of human coronary arteries

Three explants of human coronary arteries were isolated from the transplant patients with ischemic cardiomyopathy. The protocol was approved by the USC

Institutional Review Board for identifier-stripped specimens. Cross sections of the left and right coronary arteries with and without atherosclerotic lesions were stained for phosphor-JNK, cytochrome c, and succinyl dehydrogenase. The latter two antibodies were specific mitochondrial inner membrane proteins. Immunostaining was performed in frozen sections using anti-p-JNK antibody (Santa Cruz Biotech.), anti-cytochrome c (Abcam), biotinylated secondary antibodies, and horseradish peroxidase-conjugated streptavidin (Sigma-Aldrich Corp.). Diaminobenzidine (DAB) was used as a chromogen and the sections were counterstained with hematoxylin for observation of intima, media, and adventitia. Counterstaining with α -smooth muscle actin antibody (ab5694 at 1:100 dilution; Abcam) allowed for distinguishing smooth muscle cells (SMCs) in the media and intima. EC were stained with monoclonal antibodies (Dakocytomation) for EC-specific von Willebrand factor at a dilution of 1:25. Negative controls were performed by omitting the primary antibody. Positive controls were established by using the brain and kidney tissues.

2.2.8 Statistical analysis

Data are expressed as mean $\pm$ standard deviation and compared among separate experiments. Comparisons of multiple values were made by one-way analysis of variance, and statistical significance for pairwise comparison was determined using the Tukey test. p -values of < 0.05 are considered statistically significant.

2.3 Results

2.3.1 OSS transiently induced JNK activation

The characteristics of shear stress regulate JNK activation in BAECs. OSS induced both JNK-1 and -2 activations (**Fig. 1**). The intensities of individual JNK isoforms were normalized to total JNK, and they peaked by 3.06-fold and 3.89-fold, respectively, as compared to the static condition at 1 h. Confocal fluorescence microscopy also supported OSS-induced JNK activation (**Fig. 2**). Activated JNK was stained with fluorescein isothiocyanate-conjugated anti-phosphorylated JNK (green), the mitochondria with MitoTracker Red (red), and the nuclei with DAPI (blue). Under static conditions, BAECs did not exhibit visible JNK green fluorescence. In response to OSS, BAECs displayed a significant increase in JNK intensity after 30 min. The merged images showed orange/yellow signals, implicating a potential role of activated JNK in the mitochondrial redox status.

2.3.2 NADPH oxidase-mediated cytosolic $O_2^{\cdot-}$ production induced JNK activation

OSS induced NADPH oxidase, which, in turn, generated cytosolic $O_2^{\cdot-}$ production [24-25]. In the presence of NADPH oxidase inhibitor, apocynin (1 mM), OSS-induced JNK-1 and -2 activations were significantly attenuated from 3.35- to 2.30-fold and 4.57- to 3.27-fold, respectively, as compared to the static condition (**Fig. 3a**). As a corollary, pretreatment with antioxidant, N-acetylcysteine (NAC; 5 mM), further reduced OSS-mediated JNK-1 and -2 activations from 2.95- to 2.11-fold and 3.02- to 2.0-fold, respectively, as compared to the static condition (**Fig. 3b**). Apocynin also reduced OSS-mediated cytosolic $O_2^{\cdot-}$ production as illustrated by a reduction in DHE staining (**Fig. 4**). These findings suggest that NADPH oxidase-mediated cytosolic $O_2^{\cdot-}$ production was implicated in OSS-induced JNK phosphorylation.

2.3.3 OSS induced mt $O_2^{\cdot-}$ production via NADPH oxidase and JNK activation

To assess whether NADPH oxidase and JNK activation were implicated in the mitochondrial redox status, we tested the effects of Apocynin, JNK inhibitor (SP600125), and JNK knock-down (with JNK siRNA, siJNK) on $\text{mtO}_2^{\cdot-}$ production. Flow cytometry was employed to quantify MitoSOX Red intensities specific for $\text{mtO}_2^{\cdot-}$. OSS induced a 2.57-fold increase in MitoSOX Red intensity as compared to the static condition (**Fig. 5**). This induction was inhibited by SP600125 (10 μM) by 62%. Next, we further demonstrated the effect of JNK on $\text{mtO}_2^{\cdot-}$ production with siJNK. The effect of siRNA was confirmed by Western blot analysis (**Fig. 6a**). JNK-1 protein level was decreased by 71% and JNK-2 by 74% following siJNK transfection. JNK knockdown with siJNK completely inhibited OSS-induced MitoSOX Red intensity as compared to the static condition (**Fig. 6b**). Pretreatment with Apocynin (1 mM) further reduced OSS-mediated MitoSOX Red intensity from 1.80- to 0.89-fold as compared to the static conditions (**Fig. 7**). Taken together, these findings demonstrated the notion that OSS mediated $\text{mtO}_2^{\cdot-}$ production via NADPH oxidase and JNK activation.

2.3.4 Activated JNK was present in the OSS-exposed regions of human coronary arteries

Explants of human coronary arteries isolated from heart transplant patients with ischemic cardiomyopathy were analyzed for JNK activation (**Fig. 8**). Cytochrome c staining identified mitochondria, and von Willebrand factor staining identified ECs [21]. JNK activation was present in the left main bifurcation (**Fig. 8a, b**). Also prominent was activated JNK staining in the vaso vasorum (brown) (**Fig. 8c**). Cross sections from the greater curvature of the right coronary artery further revealed prominent phosphorylated JNK staining, accompanied by cytochrome c staining in the ECs (**Fig. 8d–g**). These

immunohistochemistry findings supported the aforementioned in vitro findings of OSS-induced JNK activation in explants of human coronary arteries.

2.4 Discussion

In this study, we demonstrated that OSS induced $\text{mtO}_2^{\bullet-}$ production via NADPH oxidase and JNK activation. We showed that (i) siJNK and SP600125 (inhibitor of JNK) reduced OSS-induced $\text{mtO}_2^{\bullet-}$ generation ($\text{mtO}_2^{\bullet-}$), (ii) antioxidant, NAC, attenuated OSS-induced JNK activation, and (iii) Apocynin (inhibitor of NADPH oxidase assembly) reduced OSS-induced cytosolic $\text{O}_2^{\bullet-}$ production, JNK activation, and $\text{mtO}_2^{\bullet-}$ generation. Ex vivo analyses of human coronary arteries further supported the notion that JNK activation was present in the OSS-exposed ECs. Collectively, the novelty of our observations is that OSS-activated JNK plays an important role in modulating $\text{mtO}_2^{\bullet-}$ generation relevant for vascular oxidative stress.

JNK, a stress-activated protein kinase, plays a role in EC activation and macrophage recruitment. JNK activation was also linked with atherosclerosis. Marked decrease of macrophage and foam cell infiltration was observed in the arterial wall of $\text{ApoE}^{-/-}\text{JNK-2}^{-/-}$ double knockout mice as compared to $\text{ApoE}^{-/-}$ mice [116], whereas oxLDL activated JNK in macrophages in $\text{ApoE}^{-/-}$ mice [116]. Osto et al. further showed that $\text{JNK-2}^{-/-}$ knockout mice displayed an elevated level of Mn-SOD [117], supporting the notion that oxLDL-activated JNK mediated Mn-SOD ubiquitination/protein degradation with relevance to atherogenesis [105].

Mitochondrial function is intimately linked with endothelial metabolic homeostasis [118]. Oxidative phosphorylation through complexes I to II, III, and IV drives the proton

translocation across the inner membrane to mitochondrial intermembrane space. While mitochondrial electron transport chain drives the synthesis of ATP, ~1.5%–2% of electrons leak out to form superoxide anion ($O_2^{\bullet-}$) [119]. Activated JNK colocalizes with mitochondria [120], inhibiting electron transport through mitochondrial complexes II, III, and IV, and promoting EC apoptosis [121] in response to ischemia-reperfusion injury [109]. Mitochondrial dysfunction as a result of increased $mtO_2^{\bullet-}$ production has been well implicated in Diabetes Mellitus and neurodegenerative diseases [122]. Knocking out JNK-1 and -2 protected mice (*JNK1^{-/-}JNK2^{-/-}*) from apoptosis [123]. Hence, activated JNK plays an important role in modulating mitochondrial redox status.

NADPH oxidase is an important source of vascular endothelial superoxide production, and has been implicated in the development of atherosclerosis. OSS induces cytosolic superoxide production through the upregulation of NADPH oxidase expression, which, in turn, induces monocyte chemoattractant protein 1 [24-25]. In this study, inhibition of NADPH oxidase and its superoxide production reduced OSS-induced JNK activation, suggesting the role of NADPH oxidase in JNK activation.

The use of Apocynin to inhibit NADPH oxidase remains controversial. Apocynin is commonly used to inhibit p47phox translocation and assembly of NADPH oxidase complexes [124]. In the phagocytic cells, the inhibitor activity of apocynin requires oxidation by myeloperoxidase (MPO) and H_2O_2 to form an apocynin radical, which, in turn, oxidizes thiols in NADPH oxidase [125]. In HEK293 cells that overexpressed Nox1, Nox2, or Nox4, apocynin was reported to act as an antioxidant, and it does not inhibit NADPH oxidase in the absence of MPO [126]. In vascular ECs, specific NADPH oxidase inhibitor gp91ds-tat attenuated superoxide production in response to shear

stress [24-25, 127], and oxidation of apocynin was mediated by other peroxidases (rather than MPO) [128]. In in vivo systems, apocynin has been used to reduce NADPH oxidase-mediated superoxide production in *ApoE*^{-/-} mice [129-130]. Taken together, the use of apocynin is cell type dependent and a viable inhibitor to link OSS-mediated superoxide production with JNK activation.

Previous work by Berk and colleagues [110] and Jo and colleagues [106] suggest that the time-dependent JNK phosphorylation was activated by different signal pathways. Berk's group reported that H₂O₂ induced JNK activation in ECs [110]. Our current study also supports the notion that OSS induced JNK activation via NADPH-mediated cytosolic O₂^{•-} production. Go et al. and Jo and colleagues previously demonstrated that LSS-mediated ·NO production was required for JNK activation after 60 min. By transfecting BAEC with Akt mutant, Go et al. and Jo and colleagues showed that LSS at 10 dyn·cm⁻² stimulated JNK by activating the cascade of PI3K-Akt- eNOS and ·NO production. However, the precise mechanisms whereby pulsatile versus OSS modulates JNK activation remain to be defined.

JNK activation is prominent in the atherosclerotic lesions [131]. Our ex vivo findings revealed prominent activated JNK staining in OSS-exposed regions of human coronary arteries. In the left main bifurcation, activated JNK staining was prominent in ECs, SMCs, and macrophages/foam cells. JNK staining was also prominent in the vasa vasorum in the adventitia (**Fig. 8c**). Unlike our in vitro cultured BAEC model, human coronary arteries were constantly exposed to the circulating cytokines and growth factors as well as the paracrine effects of SMCs, all of which may contribute to JNK activation [132-133].

In summary, our data support the hypothesis that OSS increased cytosolic superoxide production via NADPH oxidase. Cytosolic superoxide subsequently activated JNK, which in turn induced the production of $mtO_2^{\bullet-}$ (**Fig. 9**). Hence, atherogenic OSS induced $mtO_2^{\bullet-}$ production via NADPH oxidase-JNK signaling pathway is relevant for initiation of atherosclerosis.

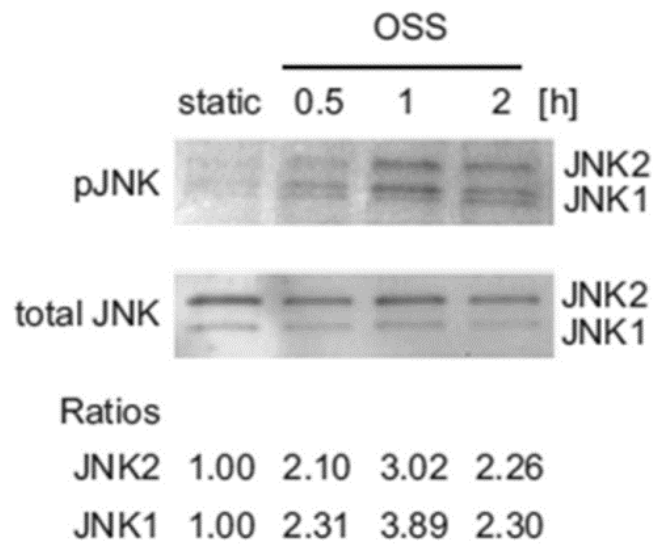


Figure 1. Oscillatory shear stress (OSS) induced transient c-Jun NH₂-terminal kinase (JNK) activation. Bovine aortic endothelial cells (BAEC) monolayers were exposed to static condition or OSS for 30 min, 1 h, or 2 h. Phosphorylated JNK was then analyzed by Western blot analysis, quantified by densitometry, and expressed as fold ratios relative to total JNK and static conditions. OSS induced a peaked JNK activation at 1 h (both JNK isoforms) ($p < 0.01$ vs. static condition). The experiments were performed in triplicates.

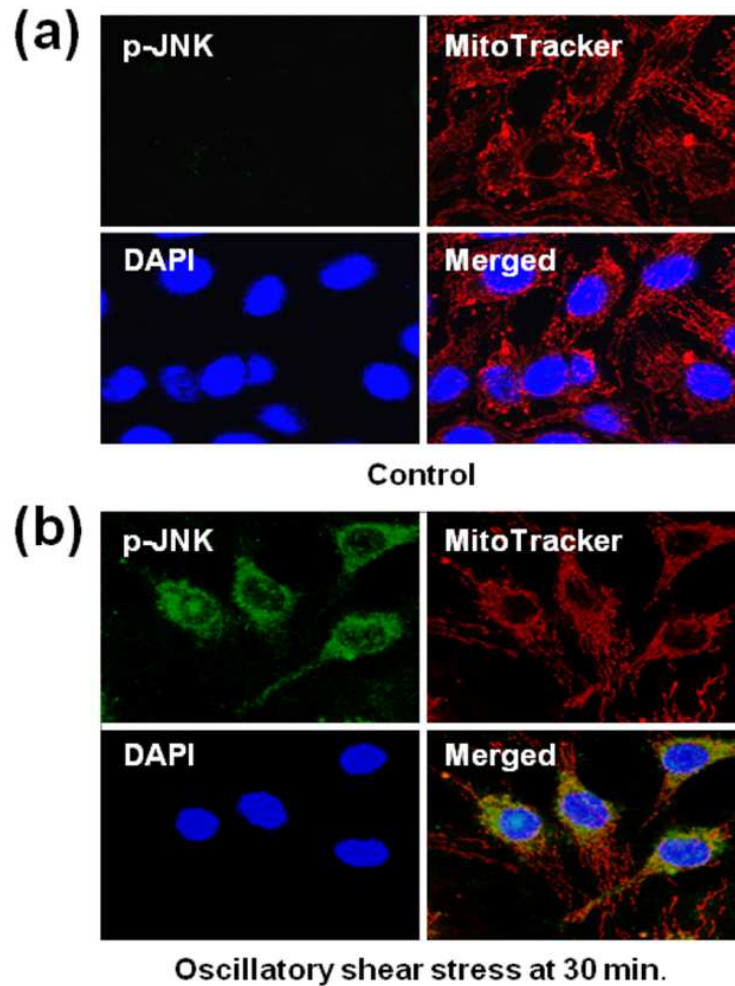


Figure 2. JNK activation in response to OSS. p-JNK was stained with fluorescein isothiocyanate-anti-p-JNK (green). Active cellular mitochondria were localized using MitoTracker Red (red). Nuclei were stained with DAPI (blue). **(a)** Under static conditions, JNK green fluorescence was hardly visible. **(b)** In response to OSS, a significant JNK green fluorescence developed after 30 min, accompanied by yellowish/orange signals as a result of merged spectra between fluorescein isothiocyanate and MitoTracker Red.

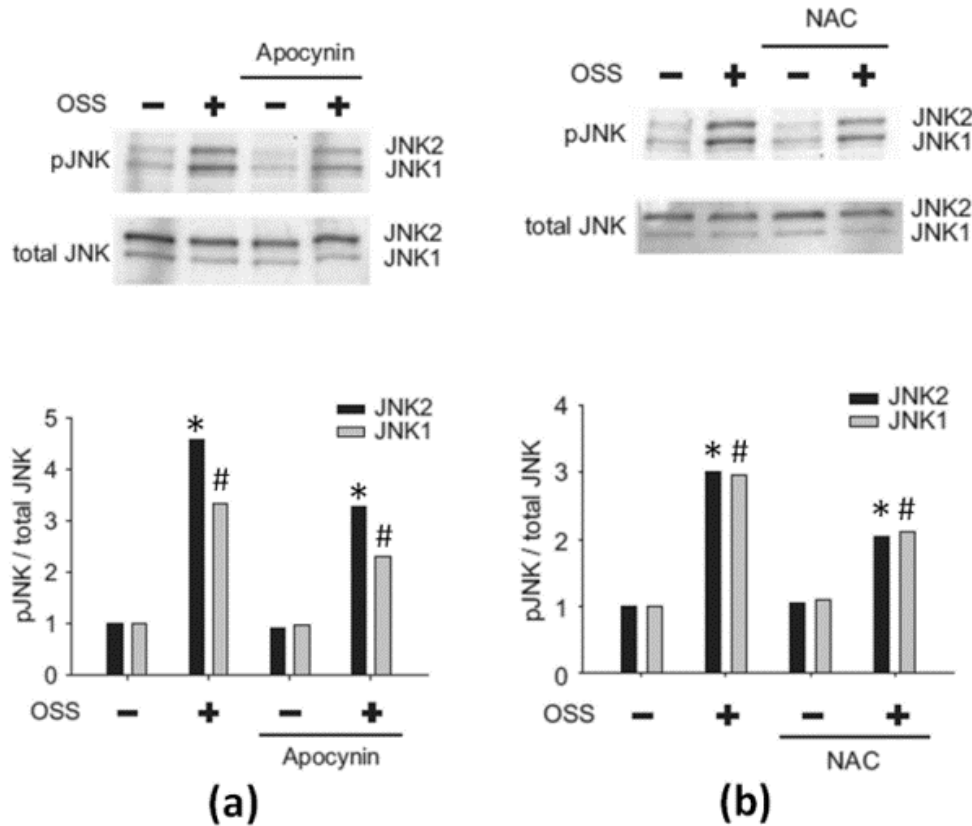


Figure 3. Apocynin or NAC attenuated OSS-induced JNK phosphorylation. (a) BAECs were pretreated with apocynin (1 mM) for 2 h before OSS exposure. Apocynin significantly reduced OSS-induced JNK phosphorylation expressed as fold change relative to total JNK in comparison with the untreated condition ($*p < 0.01$ vs. static conditions. $\#p < 0.01$ vs. OSS, $n = 3$). **(b)** Pretreatment with 5 mM of N-acetyl cysteine (NAC) also significantly reduced OSS-induced JNK phosphorylation ($*p < 0.01$ vs. static conditions. $\#p < 0.01$ vs. OSS, $n = 3$). All studies were performed in duplicates.

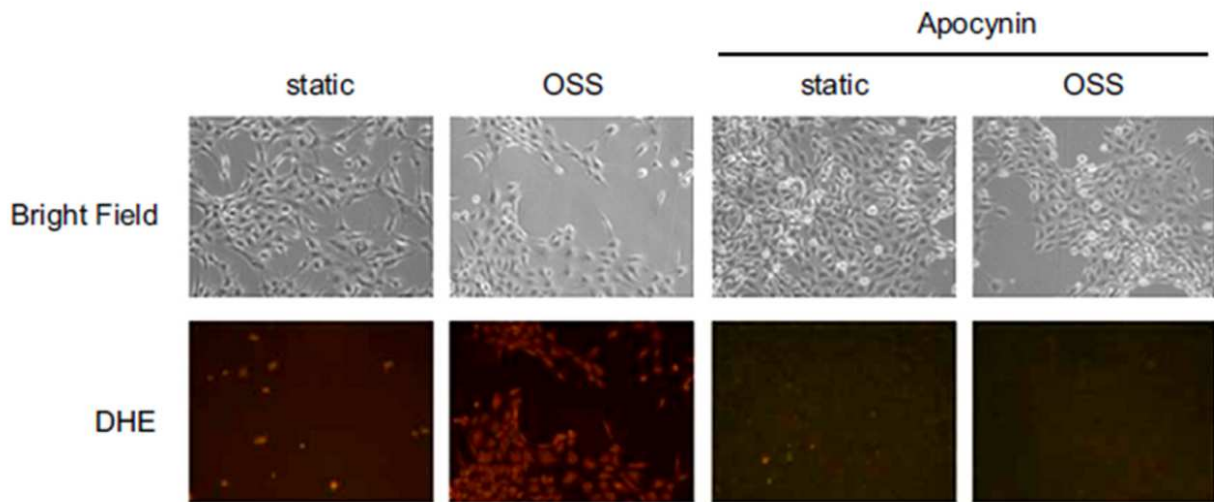


Figure 4. Apocynin attenuated oscillatory shear-induced dihydroethidium (DHE) intensities. OSS exposure increased DHE staining compared to the static conditions. This increase in DHE intensity was attenuated in response to Apocynin treatment (1 mM).

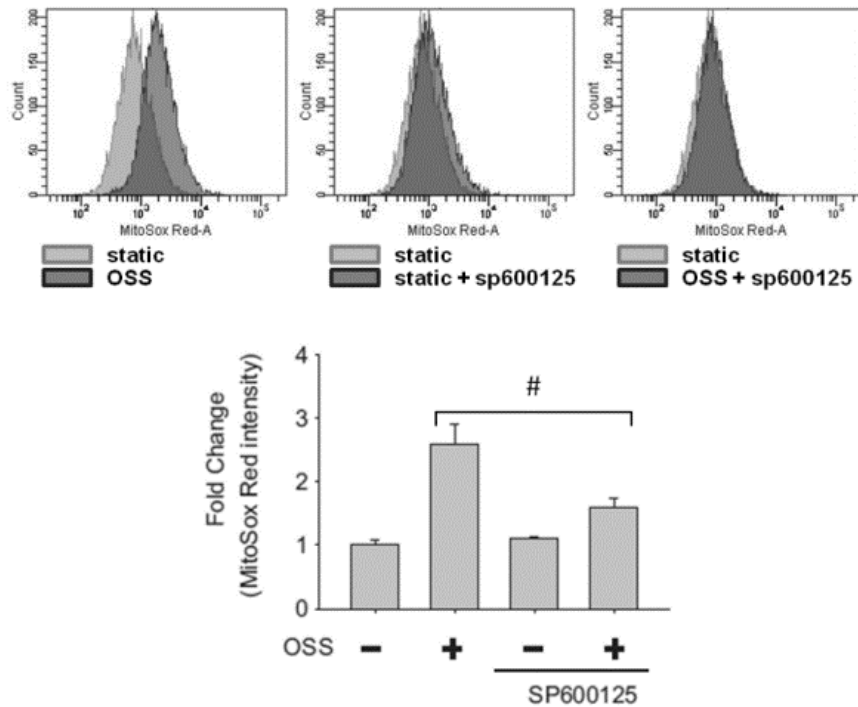


Figure 5. Inhibition of JNK attenuated MitoSOX Red intensities. BAEC monolayers were pretreated with JNK inhibitor, SP600125, and mitochondrial superoxide (mtO₂^{•-})-specific dye, MitoSOX Red, before flow exposure. Measurements were performed using BD LSR II flow cytometer. (Top) The data were presented by histograms in terms of the mean intensity of MitoSOX fluorescence normalized to those of the static conditions. (Bottom) OSS-induced MitoSOX intensity was significantly attenuated in response to SP600125 ($p < 0.01$ vs. static condition. # $p < 0.01$ vs. OSS, $n = 3$).

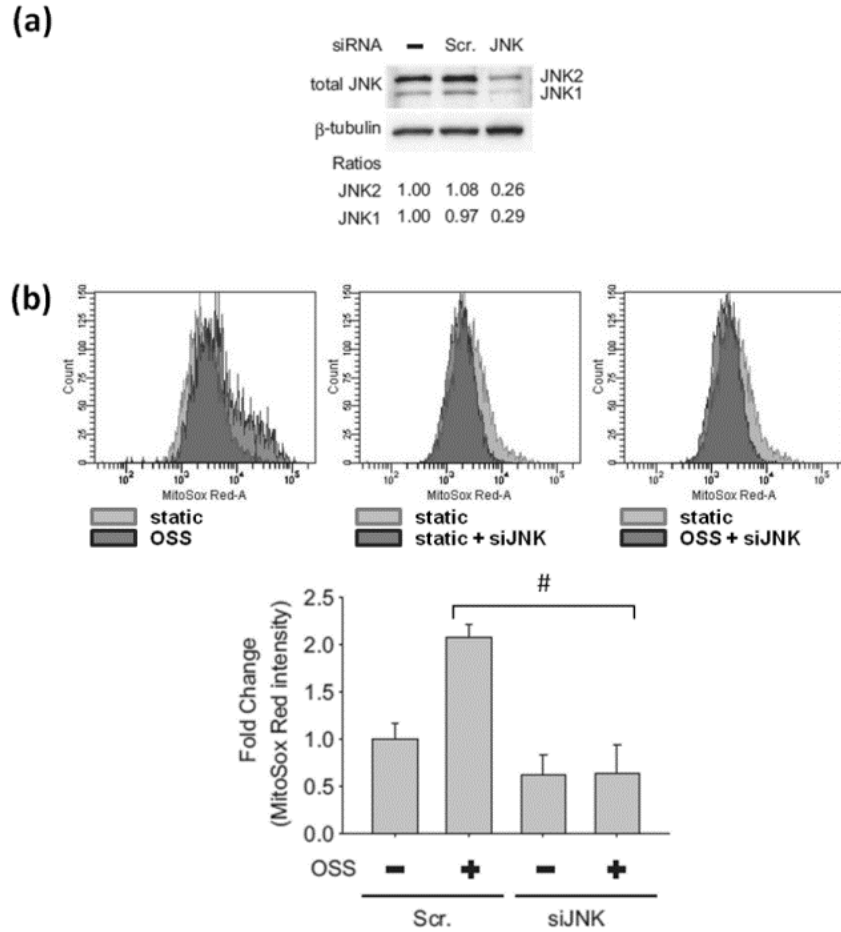


Figure 6. Knockdown of JNK reduced OSS-mediated MitoSOX Red intensities. (a) BAEC were transfected with siJNK or scramble (scr) siRNA for 48 h. Cell lysate was used to verify the efficiency of siJNK on the protein level of JNK. The blots were representative of two independent experiments with similar results. **(b, top)** The data were presented by histograms in terms of the mean intensity of MitoSOX fluorescence normalized to those of the static controls. **(b, bottom)** While scrambled JNK did not affect OSS-mediated MitoSOX intensity ($p < 0.01$ vs. static with scr siRNA, $n = 3$), transfecting BAECs with siJNK significantly reduced OSS-mediated MitoSOX intensity ($\#p < 0.01$ vs. OSS with scr siRNA, $n = 3$).

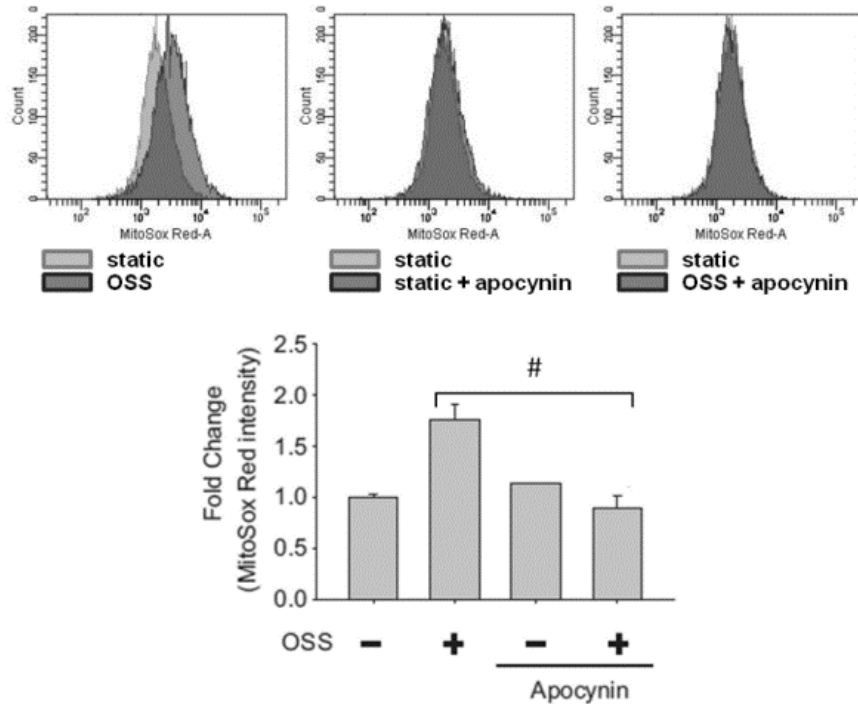


Figure 7. Inhibition of NADPH oxidase attenuated OSS-induced MitoSOX Red intensities. Measurements were performed using BD LSR II flow cytometer. (*Top*) The data were presented by histograms in terms of the mean intensity of MitoSOX Red fluorescence normalized to those of the static conditions. (*Bottom*) While OSS induced an increase in MitoSOX Red fluorescence by 1.75 ± 0.2 ($p < 0.01$ vs. static conditions, $n = 3$), pretreatment with apocynin significantly attenuated OSS-induced MitoSOX Red intensity ($^{\#}p < 0.01$ vs. OSS, $n = 3$).

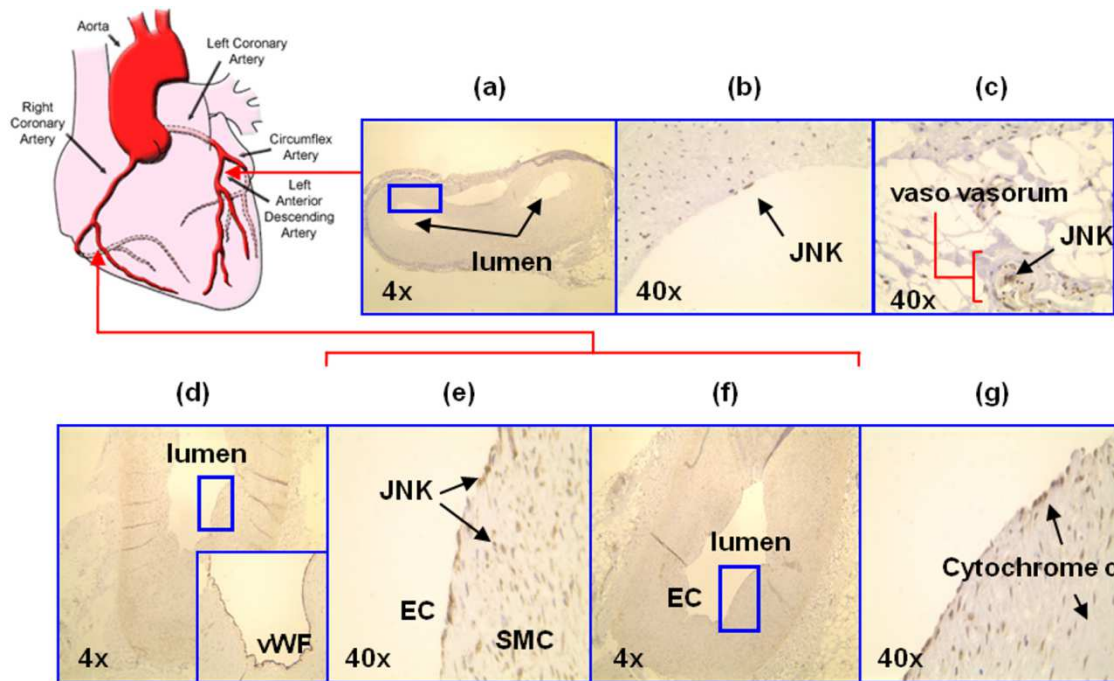


Figure 8. Immunohistochemistry of explants of human coronary arteries. The blue boxes indicate areas of interest that are subsequently magnified in successive panels. **(a–c)** In the OSS-exposed regions such as the left main coronary bifurcation, endothelial cells were stained positive for activated JNK. **(c)** Vaso vasorum from the same cross section also revealed prominent activated JNK staining (brown). **(d–g)** Cross section from the greater curvature of the right coronary artery revealed prominent activated JNK and cytochrome c staining in the endothelial cells. Positive von Willebrand factor (vWF) staining indicated presence of endothelial cells, while cytochrome c revealed presence of mitochondria.

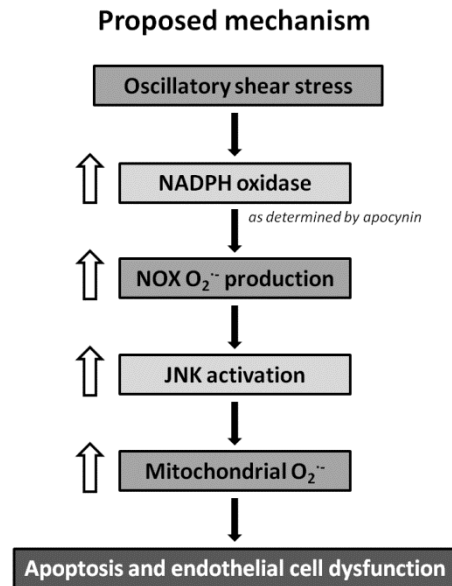


Figure 9. Proposed mechanism of OSS-mediated $mtO_2^{\cdot -}$ production. OSS increased cytosolic superoxide production via NADPH oxidase. Cytosolic superoxide subsequently activated JNK, which in turn induced the production of $mtO_2^{\cdot -}$.

Chapter Three: Disturbed Flow Induces Autophagy But Impairs Autophagic Flux to Perturb Mitochondrial Homeostasis

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Li R, Jen N, Wu L, Lee J, Fang K, Quigley K, Lee K, Wang S, Zhou B, Vergnes L, Chen YR, Li Z, Reue K, Ann DK, Hsiai TK. "Disturbed Flow Induces Autophagy, but Impairs Autophagic Flux to Perturb Mitochondrial Homeostasis." *Antioxid Redox Signal*. 2015 Nov 20;23(15):1207-19. Copyright © 2015 by Mary Ann Liebert, Inc.

3.1 Introduction

Autophagy is an evolutionarily conserved process. Cellular components, including soluble and aggregated proteins as well as organelles, are sequestered in autophagosomes and degraded in lysosomes to adapt to nutrient restriction or to eliminate modified macromolecules and damaged organelles [52-56]. While autophagy is essential for cell survival, differentiation, and development, dysfunctional autophagy is associated with a number of pathological conditions, including cardiovascular and neurodegenerative diseases, muscular dystrophies, and cancer [57, 64-65]. Impaired autophagy promotes vascular inflammatory responses and atherogenesis [69], myocardial contractile dysfunction and heart failure [64, 70-71]. Increasing evidence supports reactive oxygen species (ROS), oxidized lipoproteins, and endoplasmic reticulum (ER) stress as autophagy inducers [56, 66-67]; however, the mechanotransduction mechanisms underlying hemodynamic shear stress and autophagy remain elusive.

Autophagy is a highly regulated process associated with the activation of autophagy-related (ATG) genes [51]. The initiation of autophagy is influenced by the UNC-51-like kinase (ULK)-Atg17 complex, which is inhibited by mechanistic target of rapamycin (mTOR) [134]. Inhibition of mTOR by rapamycin results in the activation of autophagy [51]. The ATG8/Microtubule-associated protein light chain 3 (LC3) family and ATG5 play key roles in autophagosome biogenesis by building protein scaffolds [60-62, 135] and by mediating expansion of the lipid membrane of the autophagosome [51]. Conversion of LC3-I (ATG8) to LC3-II allows the anchorage of ATG8/LC3 to the phagophore and is essential for its expansion to form autophagosomes [51]. Autophagy

substrates are targeted for degradation by associating with p62/SQSTM1, a multi-domain protein that functions as a selective autophagy receptor, which physically link autophagic cargo to ATG8/MAP1-LC3/GABARAP family members located on the forming autophagic membranes [63]. Deficiency in autophagy leads to intracellular accumulation of p62, a marker for an incomplete autophagy process known as impaired autophagic flux [136].

Shear stress imparts both metabolic and mechanical effects on vascular endothelial cells [137], with a pathophysiological relevance in the focal and eccentric nature of atherosclerotic lesions [6, 21, 24-25, 132, 138-141]. A complex flow profile develops at arterial bifurcations; namely, flow separation and migrating stagnation points create low and oscillating shear stress (OSS). At the lateral walls of bifurcations, disturbed flow, including oscillatory flow (bidirectional and axially misaligned flow), preferentially induces oxidative stress to initiate atherosclerosis; whereas in the medial walls of bifurcations, pulsatile flow (unidirectional and axially aligned flow) down-regulates inflammatory responses and oxidative stress [3]. Specifically, pulsatile shear stress (PSS) increased endothelial mitochondrial membrane potential ($\Delta\Psi_m$), accompanied by a decrease in mitochondrial superoxide production ($mtO_2^{\cdot-}$) [27], whereas OSS and oxidized low-density lipoprotein (LDL) increased $mtO_2^{\cdot-}$ production and apoptosis [105, 142]. In this context, spatial ($\partial\tau/\partial x$) and temporal variations ($\partial\tau/\partial t$) in shear stress largely determine the focal nature of vascular oxidative stress and pro-inflammatory state.

Oxidative stress is considered to be a major inducer of autophagy [54, 66]. Oxidized Low Density Lipoprotein (ox-LDL) induces autophagy accompanied by

mitochondrial DNA (mtDNA) damage [143]. Unlike nuclear DNA, the lack of protective histones in mitochondria renders mtDNA vulnerable to oxidative stress [144]. A significantly higher incidence of mtDNA with 4977 base pair deletion mutation is present in circulating blood cells and atherosclerotic lesions [145]. ApoE-null mice deficient in protein kinase ATM (ataxia telangiectasia mutated) also exhibit an increase in frequency of mtDNA damage and a decrease in oxidative phosphorylation, leading to defects in cellular proliferation and initiation of atherosclerosis [146]. OSS activates NADPH oxidase to increase cytosolic superoxide ($O_2^{\bullet-}$) production, which in turn activates c-Jun NH2-terminal Kinase (JNK), leading to $mtO_2^{\bullet-}$ production [105, 113, 142]. Increased $mtO_2^{\bullet-}$ production impairs electron transport chain and promotes mtDNA damage [147]. While biomechanical forces have been reported to induce autophagy [55], whether spatial and temporal variations in shear stress modulate autophagy to influence $mtO_2^{\bullet-}$ production remains undefined. We demonstrated prominent staining for p62, a reverse marker of autophagic flux, in the disturbed flow-exposed aortic arch, but attenuated p62 in the PSS-exposed descending aorta. OSS significantly increased the LC3-II/LC3-I ratios and p62 levels, whereas PSS minimally increased LC3 ratios and decreased P62 expression. Both anti-phospho-JNK and anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) staining for DNA damage were prominent in the OSS-exposed aortic arch, whereas the staining was nearly absent in the PSS-exposed descending aorta. Our results indicate that OSS-mediated oxidative stress and JNK activation induced autophagy but impaired autophagic flux to promote $mtO_2^{\bullet-}$ production, mtDNA damage, and mitochondrial dysfunction in the disturbed flow-exposed regions.

3.2 Materials and Methods

3.2.1 Immunohistochemistry staining

p62, also known as sequestosome (SQSTM1), is an ubiquitin-binding scaffold protein. p62 accumulates when autophagy is inhibited, and decreases when autophagy is induced (44,47). Cross sections of aortic arch and descending aorta from New Zealand White rabbits were stained with anti-p62 antibody (Boster Biological Technologies) to assess changes in autophagy in vivo. Staining of the aortic arch and descending aorta was performed with anti-8-OHdG (Cayman Chemicals, MI) and anti-phospho-JNK (Cell Signaling, MA) to assess DNA damage by oxidation.

3.2.2 Three-Dimensional Computational Fluid Dynamic (CFD) simulation

Aortic arch geometry was extracted from an angiogram video of the aorta. Diameters were measured every 5mm along the length of the aorta and cross-sections were assumed to be circular at every measured site. The aorta was reconstructed in Solidworks (Concord, Massachusetts, USA). The pulsatile arterial profiles were captured from the MRI images and the video frames were extracted by ImageJ (National Institute of Health, Bethesda, MD, USA). Arterial velocity profiles were used as inlet boundary condition and the outlet was defined at 0 Pa, static pressure. CFD simulation was performed with Solidworks Flow Simulation (Concord, Massachusetts, USA) with 3176 fluid mesh cells and 6082 partial mesh cells. The simulation was run until defining 2s physical time with a time step size of 0.01. Solidworks Flow Simulation automatically sets convergence conditions with changing iteration numbers. The governing equations were solved by laminar incompressible blood with non-slip unsteady flow condition.

3.2.3 Endothelial cell culture

Human aortic endothelial cells (HAEC) were cultured in endothelial cell growth medium (Cell Applications) and used between passages 5 and 9. For autophagy study in response to shear stress, cells were seeded on gelatin coated glass slides (25x75 mm) and grown to confluent monolayers for 48 hrs in 5% CO₂ at 37°C before flow was applied. For inhibitor and stimulator studies, the cells were pretreated with either c-Jun NH₂-terminal Kinase (JNK) inhibitor SP600125 (2-10 μ M), antioxidant NAC (5 mM), or rapamycin (1 μ M) prior to shear stress exposure.

3.2.4 Dynamic flow system to simulate pulsatile (PSS) versus oscillatory shear stress (OSS) profiles

A dynamic flow channel was used to recapitulate hemodynamics in human carotid arterial bifurcations [23, 148]. The flow system was designed to generate well-defined flow profiles across the width of the parallel flow chamber at various temporal gradients ($\partial\tau/\partial t$), frequencies, and amplitudes. HAEC were exposed to three conditions in DMEM/1%FBS unless otherwise stated: (1) Control at static conditions, (2) PSS at $\tau_{avg} = 23 \text{ dyn}\cdot\text{cm}^{-2}$ and $\partial\tau/\partial t$ at $\pm 8 \text{ dyn}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ at 1 Hz and (3) OSS at $\tau_{avg} = 0.02 \text{ dyn}\cdot\text{cm}^{-2}$ and $\partial\tau/\partial t$ at $\pm 3 \text{ dyn}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ at 1 Hz.

3.2.5 Western blot analyses

After flow exposure, HAEC were rinsed with phosphate-buffered saline (PBS) and lysed using RIPA buffer supplemented with protease and phosphatase inhibitors. Protein concentration was measured using the Bio-Rad DC assay and 50 μ g of protein was loaded for Western blotting essentially as previously described [149]. Antibodies against autophagy associated genes were purchased from Abcam for microtubule-associated

protein light chain 3 (LC-3) and from Boster Biological Technologies for p62. Parallel blots were performed with anti- β -tubulin (Millipore Inc, MA) for loading normalization. Densitometry was performed to quantify blot bands as previously described [149] .

3.2.6 Autophagosome visualization with GFP-LC3

HAEC seeded onto glass slides were infected with recombinant GFP-LC3 adenoviruses, kindly provided by Dr. Junichi Sadoshima from Rutgers New Jersey Medical School, at multiple of infection (MOI) 1:20. The cells were then applied to EBSS, static condition, or shear stress for 4 hours. Fluorescent images were acquired using an inverted microscope (Olympus, PA) and a DSLR camera (Canon, NY). The punctate autophagosome structures were assessed by quantifying the dots inside cells. Dots were averaged from 50 cells for each condition as indication of autophagosome formation.

3.2.6 Mitochondrial respiration Assay

HAECs treated with dynamic shear stress were collected by trypsinization and seeded on XF24 V7 microplates (North Billerica, MA) at 25,000 cells/well. The cells were allowed to adhere to the plate for 2 hours then analyzed for mitochondrial respiration using the Seahorse XF24 analyzer (North Billerica, MA) as previously described [150]. Mitochondrial respiration was measured and normalized to protein levels using the Bio-Rad protein assay (Hercules, CA).

3.2.7 Complex II activity assay

Mitochondrial complex II activity was measured with an assay kit from Abcam. Briefly, HAEC were collected after flow and re-suspended at 5.5 mg/mL. The cells were lysed with the lysis buffer provided in the kit. After 30 min incubation, the lysates were

centrifuged at 25,000 g for 20 minutes at 4°C. Supernatants were collected for the assay following the manufacturer's instructions. Relative complex II activities were normalized to protein levels.

3.2.8 Mitochondrial DNA damage assay

Mitochondrial DNA (mtDNA) damage was assessed by quantifying a large base pair deletion along the major arch of the mitochondrial genome by quantitative PCR. A common 4977 bp deletion in mtDNA was quantified by quantitative PCR (8) using primers flanking the deletion and normalized to mitochondrial COX1 gene. The pair of primers for detection of the 4977 bp deletion was: forward: CCTTACACTATTCCTCATCACC; reverse: TGTGGTCTTTGGAGTAGAAACC); and the pair of primers for mitochondrial COX1 was: forward: TTCGCCGACCGTTGACTATTCTCT, reverse: AAGATTATTACAAATGCATGGGC.

3.2.9 siRNA transfection

Validated siRNA for ATG5 was purchased from Qiagen Inc. Negative control siRNA or ATG5 siRNA (30 nM) was transfected to HAEC with Lipofectamine RNAiMAX (Life Technologies, CA) following the manufacturer's instructions. Real-time PCR was used for confirmation of gene knockdown at 48 hrs after transfection.

3.2.10 Over-expression of MnSOD

Over-expression of the mitochondrial antioxidant enzyme manganese superoxide dismutase (MnSOD) was done by transfecting HAEC with MnSOD adenovirus at MOI 1:100 for one hour. HAECs were allowed to recover for 24 hours before flow exposure, as previously described [142].

3.2.11 Immunofluorescence staining

Following flow exposure, HAEC were fixed with 4% PFA and stained with antibody against 8-Hydroxy-2'-Deoxyguanosine (8-OHdG) for DNA oxidation and anti-cytochrome C for mitochondria. Fluorescent images were acquired using an inverted microscope (Olympus, PA) and a DSLR camera (Canon, NY).

3.2.12 Flow cytometry analysis to quantify mtO₂^{•-} production

Mitochondrial superoxide (mtO₂^{•-}) production was assessed by staining with MitoSOX Red as previously described [105, 142], and was quantified using flow cytometry as previously described [151].

3.2.13 Nuclear DNA damage assay (Comet assay)

Nuclear DNA damage was assessed by quantifying nuclear DNA fragmentation using the Comet Assay kit (Trevigen, MD) according to the manufacturer's suggested procedures. Briefly, HAEC were trypsinized and immobilized on a glass slide with low-melting point agarose. The cells were lysed, treated with alkaline buffer, and stained with a SYBR green nucleic acid stain. The sample was then run at 28V for 30m, "comet" tails were visualized by fluorescence microscopy, and the tail moments were quantified using CaspLab Comet Assay Software.

3.2.14 Statistical analysis

Data were expressed as mean ± standard deviation, and compared among separate experiments. Comparisons of multiple values were made by one-way analysis of variance (ANOVA), and statistical significance for pair-wise comparisons was determined post-hoc using Tukey's method. *p*-values of <0.05 were considered statistically significant.

3.3 Results

3.3.1 Spatial variations in shear stress modulates p62/SQSTM1 immunostaining in rabbit aorta

Atherosclerosis preferentially develops in the branch points and curvature of arterial trees where disturbed flow, including OSS, occurs [6, 12, 24, 141, 152]. To examine whether there is a relationship between spatial variations in shear stress and autophagy, we stained cross-sections of the rabbit aortic arch and descending aorta with an antibody against autophagy-related gene p62, an ubiquitin-binding scaffold protein to promote autophagosome formation. Computational fluid dynamic (CFD) was employed to illustrate both spatial ($\partial\tau/\partial x$) and temporal ($\partial\tau/\partial t$) variations in wall shear stress (WSS) at an instantaneous moment in systole and diastole (**Fig. 1A**). Immunohistochemistry revealed prominent endothelial p62 staining in the OSS-exposed aortic arch (**Fig. 1B**), but p62 staining was nearly absent in the PSS-exposed descending aorta (**Fig. 1C**) [153-154]. This observation suggests a link between spatial variations in shear stress and p62 staining, and provides a basis to determine whether temporal variations in shear stress ($\partial\tau/\partial t$); namely OSS versus PSS, differentially induces autophagy.

3.3.2 Temporal variations (PSS vs. OSS) in shear stress modulate LC3-II to LC3-I ratios and autophagosome formation

Human aortic endothelial cells (HAEC) were exposed to OSS versus PSS for 4 hours, and autophagy was assessed by normalizing the LC3-II to LC3-I ratios to the static condition. The LC3-II to LC3-I ratios significantly increased in response to OSS by

120% ($p < 0.001$ vs. static control, $n=5$) and 77% over PSS ($p < 0.001$, $n=5$), whereas PSS minimally increased LC3 ratios by 23% ($p < 0.05$, $n=5$) (**Fig. 2A**). To further assess the completion of autophagy process, we examined the p62 levels, a reverse marker for autophagy flux. OSS increased p62 by 37% ($p < 0.01$, $n=4$), whereas PSS did not significantly change the p62 (**Fig. 2A**). In parallel, OSS significantly increased autophagosome formation, as illustrated by GFP-LC3 puncta or dots/cell, but not PSS (**Fig. 2B**, arrows). Earle's Balanced Salt Solution (EBSS) to induce autophagy by starvation also increased GFP-LC3 puncta (**Fig. 2B**). Our data indicate that OSS significantly induced autophagy by nearly 2-fold, and thus, provided a basis to focus on OSS modulation of autophagy.

3.3.3 Oscillatory Shear Stress Impairs autophagic Flux

To further determine OSS modulation of autophagic flux, we compared the LC3 ratios and p62 levels in the presence of autolysosome inhibitor bafilomycin. EBSS-induced autophagy increased LC3-II to LC3-I ratios by 28% ($p < 0.05$, $n=6$), but decreased p62 levels by 10% ($p < 0.05$, $n=6$), co-treatment of EBSS and bafilomycin increased LC3-II to LC3-I ratios by 55% ($p < 0.05$ vs. EBSS, $n=6$) and p62 levels by 120% ($p < 0.01$ vs. EBSS, $n=4$) (**Fig. 2C**). Co-treatment of OSS and bafilomycin increased LC3-II to LC3-I ratios by 47% ($p < 0.05$ vs. OSS, $n=6$), and p62 levels by 41% ($p < 0.01$ vs. OSS, $n=6$) (**Fig. 2C**). The increase in p62 levels in response to introducing bafilomycin to EBSS or OSS indicates an incomplete autophagy process, supporting the notion that OSS induces autophagy by an increase in LC3-II to LC3-I ratios, but impaired autophagic flux by a concomitant increase in p62 levels.

3.3.4 OSS-induced oxidative stress and JNK signaling increase LC3-II to LC3-I ratios

Previous studies demonstrated that OSS induces endothelial $O_2^{\bullet-}$ production via activation of NADPH oxidase enzyme system and c-Jun NH2-terminal kinase (JNK-1 and JNK-2) signaling [24-25, 105, 119, 142]. Here, we transfected HAEC with control (Adv-LacZ) or recombinant MnSOD adenovirus (Adv-MnSOD) to reduce mitochondrial superoxide production ($O_2^{\bullet-}$) (74). Over-expression of MnSOD significantly attenuated OSS-induced LC3-II to LC3-I ratios ($p < 0.05$ vs. static condition, $n=4$) (Fig. 3A). Similarly, treatment with anti-oxidant NAC and JNK inhibitor SP600125 significantly mitigated OSS-induced LC3-II to LC3-I ratios (NAC: $p < 0.01$, $n=4$; SP600125: $p < 0.05$, $n=4$) (**Figs. 3B & C**). Thus, OSS-mediated $O_2^{\bullet-}$ production and JNK phosphorylation induce autophagy.

3.3.5 EBSS or rapamycin-induced autophagy mitigates OSS-induced mitochondrial superoxide ($mtO_2^{\bullet-}$) production

OSS significantly increased $mtO_2^{\bullet-}$ production in HAEC as measured via MitoSOX Red fluorescent intensity quantification by flow cytometry ($p < 0.05$, $n=4$) (**Fig. 4A**). EBSS attenuated OSS-induced inhibition of autophagy flux ($p < 0.05$, $n=6$) (**Supplementary Fig. S1**) and $mtO_2^{\bullet-}$ production ($p < 0.05$, $n=4$) (**Fig. 4A**). Treatment of HAEC with rapamycin resulted in a similar finding (**Supplementary Fig. S2**), whereas ATG5 knockdown with siRNA (siATG5) accentuated MitoSOX Red intensities ($p < 0.05$, $n=4$) (**Fig. 4B**). Thus, induction of autophagy attenuated OSS-mediated $mtO_2^{\bullet-}$ production.

3.3.6 EBSS or rapamycin-induced autophagy restores OSS-mediated reduction in mitochondrial respiration

OSS significantly reduced mitochondrial respiration by 46% ($p < 0.01$, $n=4$). EBSS-induced autophagy completely restored OSS-mediated reduction in mitochondrial respiration ($p < 0.01$, $n=4$) (**Fig. 5A**). Mitochondrial complex II generates reactive oxygen species at high rates during oxidative phosphorylation [150]. OSS reduced complex II activity in HAEC by 43% ($p < 0.05$, $n=3$), which was restored by EBSS ($p < 0.05$, $n=3$) (**Fig. 5B**). rapamycin-induced autophagy also completely restored OSS-mediated decrease in complex II activity ($p < 0.05$, $n=4$) (**Supplementary Fig. S3**). Collectively, induction of autophagy restores mitochondrial respiration.

3.3.7 OSS-mediated JNK signaling and $mtO_2^{\cdot-}$ production induce mitochondrial DNA damage

Unlike nuclear DNA, mtDNA is not bound directly to the histones family in the mitochondrial matrix [155-156], and is susceptible to oxidative DNA damage (76). OSS-induced mtDNA damage was assessed by accumulation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) [156], and was predominantly co-localized with mitochondria (anti-Cytochrome c, red fluorescence) (**Fig. 6A**). OSS induced a 4.5-fold increase in mtDNA damage ($p < 0.001$ vs. control, $n=4$) (**Fig. 6B**). OSS-induced nuclear DNA damage was statistically insignificant by the Comet Assay ($p = 0.9$, $n=3$) (**Fig. 6C**). Furthermore, OSS-induced mtDNA damage was significantly mitigated by over-expression of MnSOD with recombinant adenoviruses ($p < 0.05$, $n=4$) (**Fig. 7A**), treatment with antioxidant NAC ($p < 0.05$, $n=4$) (**Fig. 7B**), and the JNK inhibitor

SP600125 ($p < 0.01$, $n=4$) (**Fig. 7C**). These observations demonstrate that OSS-induced $\text{mtO}_2^{\cdot-}$ production and JNK signaling promote mtDNA damage.

3.3.8 DNA damage and JNK activation develop in the disturbed flow-exposed aortic arch

To recapitulate spatial variations in OSS-induced JNK activation and mtDNA damage in vivo, we performed immunohistochemistry staining in both the aortic arch and descending aorta of New Zealand White rabbits fed on normal chow diet. Both anti-8-OHdG antibody staining for oxidative DNA damage and anti-phospho-JNK staining were prominent in the OSS-exposed aortic arch, but were nearly absent in the PSS-exposed descending aorta (**Fig. 8**). Taken together, spatial (lesser vs. greater curvature vs. descending aorta) and temporal variations (OSS vs. PSS) in shear stress differentially induce autophagy and autophagic flux (**Fig. 1**) to modulate mitochondrial homeostasis (**Fig. 6**).

3.4 Discussion

Spatial and temporal variations in hemodynamic shear stress modulate endothelial metabolic states to support the focal and eccentric nature of atherosclerotic lesions. While the mechanotransduction mechanisms underlying fluid shear stress and oxidative stress have been well-elucidated [11-12, 83, 157-158], we hereby provide new metabolic insights into the disturbed flow-mediated (1) LC3-II to LC3-I ratios to promote autophagosome biogenesis, and (2) p62 accumulation to impair autophagic flux. We further elucidate OSS-mediated oxidative stress and JNK signaling underlying activation of autophagy to modulate mitochondrial homeostasis (Fig. 9).

Organisms experience frequent changes in their biophysical and biochemical environment. Induction of basal autophagy preserves cellular function and adapts to stress responses via delivery of proteins, macromolecules, organelles, and microbes to the lysosome for digestion [155, 159-165]. Macrophage-specific ATG5-null mice develop an increase in atherosclerotic lesions accompanied by elevated inflammatory markers, supporting the impairment of autophagy as an underlying mechanism in the initiation of atherosclerosis [69]. In our dynamic flow system, ATG5 knockdown accentuated OSS-induced $\text{mtO}_2^{\bullet-}$ production, whereas EBSS or rapamycin activated autophagy to attenuate OSS-induced $\text{mtO}_2^{\bullet-}$ production (**Fig. 4A** and **Supplementary Fig. S2**).

In the disturbed flow-exposed regions of the adult porcine aorta, both pro-inflammatory and anti-oxidative endothelial transcription profiles coexist [166]. While disturbed flow promotes inflammatory responses, the anti-oxidative transcript profiles, including MnSOD expression, prevent initiation of atherosclerosis [166]. Shear stress-responsive MnSOD is located in the mitochondrial matrix to dismutate the conversion of superoxide anion ($\text{O}_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) [23, 167]. MnSOD^{+/-} mice exhibit impaired endothelium-dependent vasodilation [168]. ApoE^{-/-} MnSOD^{+/-} mice display earlier mtDNA damage and accelerated atherosclerosis compared with ApoE^{-/-} MnSOD^{+/+} mice [169]. In our dynamic HAEC model, induction of autophagy mitigates OSS-induced $\text{mtO}_2^{\bullet-}$ production in the absence of hyperlipidemia. EBSS or rapamycin-induced autophagy mitigates OSS-mediated JNK activation and $\text{mtO}_2^{\bullet-}$ production to reduce mtDNA damage, which initiates atherogenesis [143, 146, 170]. Over-expression of MnSOD, treatment with NAC or JNK inhibitor significantly attenuates OSS-induced

mtDNA damage (**Fig. 7**). In corollary, over-expression of MnSOD attenuates JNK-mediated MnSOD protein degradation and ubiquitination, leading to an increase in cleaved Caspase-3 [105]. Preferential accumulation of p62 levels in the OSS-exposed aortic arch rather than PSS-exposed descending aorta further supports an incomplete autophagy process known as impaired autophagic flux (**Fig. 1**) [68, 136, 171]. Thus, the combination of impaired autophagic flux and mtDNA damage in the disturbed flow-exposed regions is conducive to initiate endothelial dysfunction (Fig. 8).

It is well-recognized that reactive oxygen species (ROS) activate antibacterial autophagy and autophagic cell death through distinct mechanisms, depending on the specific cell types in the micro-environment [54, 66]. Induction of autophagy by pharmacological intervention, over-expression of ATG genes, and exercise-mediated shear stress augmentation confer cellular protection against the aggregate-prone proteins associated with neurodegeneration [172-173]. Here, we demonstrate that spatial ($\partial\tau/\partial x$) and temporal variations ($\partial\tau/\partial t$) in shear stress induce cytosolic $O_2^{\cdot-}$ production, JNK activation, mitochondrial metabolic changes to increase LC3-II to LC3-I ratios.

NADPH oxidase-cytosolic $O_2^{\cdot-}$ -JNK phosphorylation signaling was implicated in OSS-mediated $mtO_2^{\cdot-}$ production [142]. Immunostaining of aorta from NZW rabbits fed on normal diet supports the notion that DNA damage and JNK activation co-exist in the OSS-exposed region or lesser curvature of the aortic arch (**Fig. 8**). In our dynamic HAEC model, OSS increased LC3-II/LC3-I ratios and GFP-LC3 dots/cell to a greater extent than did PSS, and OSS-mediated cytosolic $O_2^{\cdot-}$ -JNK phosphorylation signaling induces autophagy (**Fig. 3**). Conversely, EBSS and rapamycin-induced autophagy

mitigate OSS-induced $\text{mtO}_2^{\bullet-}$ production, whereas ATG5 siRNA-inhibited autophagy increased OSS-induced $\text{mtO}_2^{\bullet-}$ production. We further observed that autophagy restored OSS-mediated mitochondrial respiration (**Fig. 5A**).

Due to the lack of protective histones, mtDNA is vulnerable to oxidative damage [144]. Oxidative phosphorylation is coupled with respiratory chain complexes I to II, III and IV [174-175]. While the transfer of electrons from ubiquinone through the mitochondrial respiratory chain is more than 98% efficient, 1.5 to 2 % of electrons leak out to form $\text{O}_2^{\bullet-}$ [176]. For these reasons, autophagy modulates OSS-mediated changes in mitochondrial respiration and complex activity to mitigate $\text{mtO}_2^{\bullet-}$ production and mtDNA damage (**Fig. 4A** and **Supplementary Fig. S1, S2**).

mtDNA damage is frequently observed in blood cells and the vascular wall. Despite its low abundance, a specific 4977-bp “common” deletion (mtDNA[4977]) is associated with mitochondrial dysfunction [145]. mtDNA damage primes the initiation of atherosclerosis [146]. Our lab and others have demonstrated that OSS activates NADPH oxidase, which, in turn, induces an increase in cytosolic $\text{O}_2^{\bullet-}$ production to activate JNK and to increase $\text{mtO}_2^{\bullet-}$ production [105, 113, 142]. Thus, OSS induces JNK signaling pathway to initiate $\text{mtO}_2^{\bullet-}$ production and mtDNA damage.

Overall, disturbed flow modulates the cross-talk between autophagy and mitochondrial function [66, 143, 177-179]. In the athero-prone or OSS-exposed arterial regions, basal endothelial homeostasis may be dependent on the balance between OSS-induced mtDNA damage and autophagy. In the presence of cardiovascular risk factors, disturbed flow impairs autophagic flux to initiate inflammatory responses and

atherosclerosis. We hereby provide a dynamic model with translational implications to modulate mechano-sensitive tissues to maintain cellular homeostasis.

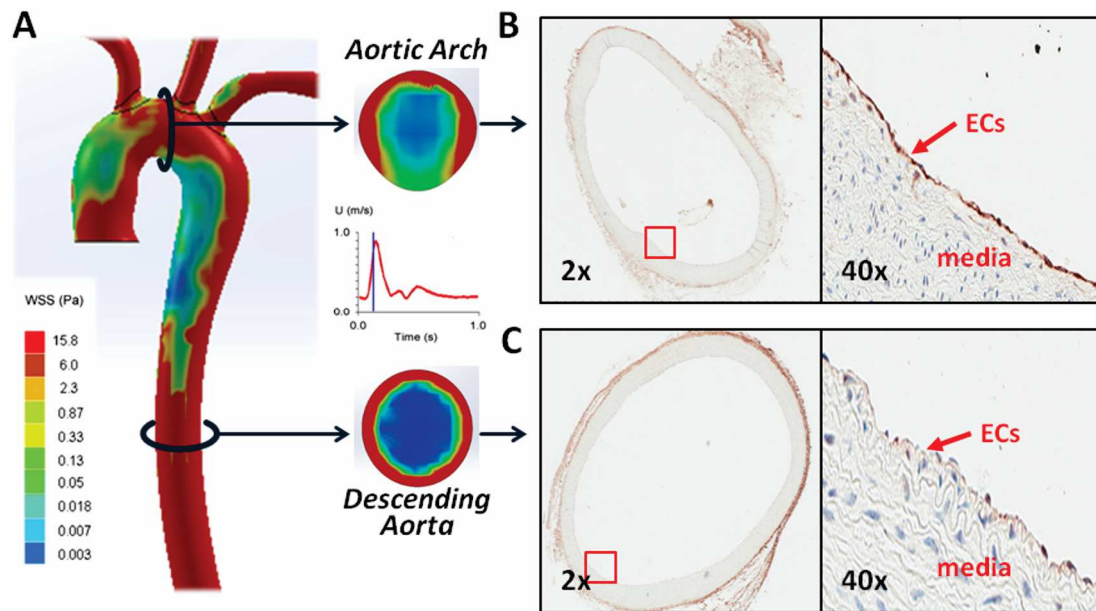


Figure 1. Spatial variations in shear stress differentially increased p62 accumulation in rabbit aorta. (A) Cross-section in Rabbit aorta highlights instantaneous spatial variations in wall shear stress (WSS) in the aortic arch versus descending aorta during systole. The instantaneous WSS is low in the lesser curvature of aortic arch, but is circumferentially high in the descending aorta. Immunohistochemistry reveals more prominent p62 staining lesser curvature of aortic arch (B) where endothelial cells (ECs) were exposed to disturbed flow, including oscillatory shear stress (OSS), when compared to the descending aorta (C) where ECs were exposed to pulsatile shear stress (PSS).

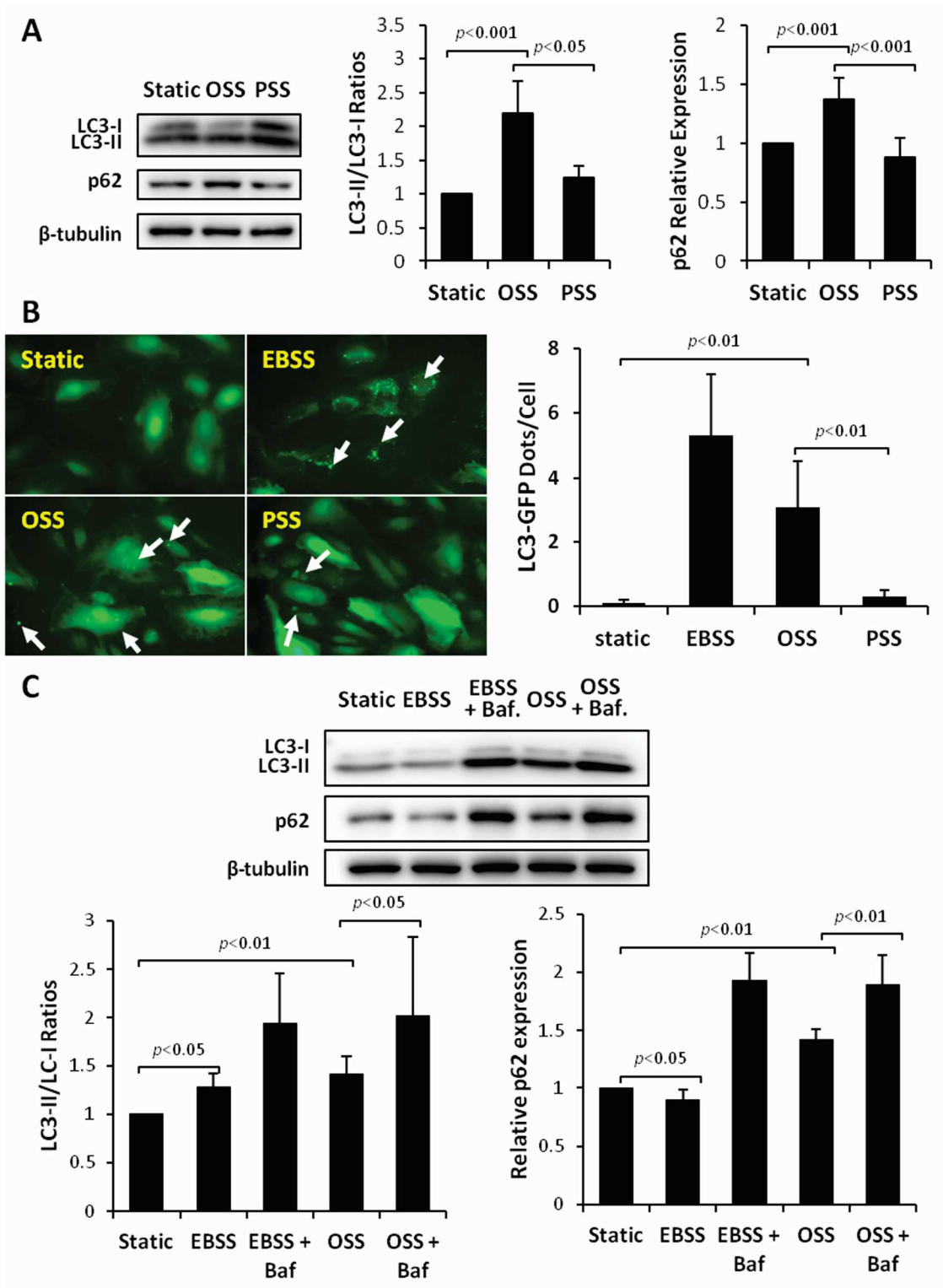


Figure 2. Temporal variations in shear stress (oscillatory shear stress vs. pulsatile shear stress) differentially induced autophagy in HAEC. (A) HAEC were

exposed to oscillatory shear stress (OSS) versus pulsatile shear stress (PSS) for 4 hours. Autophagy was assessed by normalizing the ratios of LC3-II to LC3-I with the static condition and comparing p62 levels by western blot. OSS significantly increased the LC3-II/LC3-I ratios ($p < 0.001$, $n=5$) when compared to static condition and PSS, whereas PSS minimally increased LC3 ratios ($p < 0.05$, $n=5$) in comparison with static condition. OSS also increased protein levels of p62 against static condition and PSS ($p < 0.01$, $n=4$), whereas there was no significant change between static condition and PSS. **(B)** HAEC were infected with recombinant LC3-GFP adenovirus (1:20) overnight, and then were exposed to OSS versus PSS for 4 hours. LC3-GFP puncta or dots/cell (arrows) indicated that OSS significantly increased the number of autophagosomes in comparison to static control ($p < 0.01$, $n=3$) similarly to Earle's Balanced Salt Solution (EBSS)-induced autophagy ($p < 0.01$, $n=3$). PSS only minimally increased LC3-GFP puncta versus static condition ($p < 0.05$, $n=3$). **(C)** HAEC were treated with either OSS or EBSS in the presence or absence of 1nM of bafilomycin (Baf). While EBSS increased LC3 ratios ($p < 0.05$, $n=6$) and decreased p62 levels ($p < 0.05$, $n=6$) over static condition, bafilomycin treatment significantly increased LC3 ratios and p62 to a lesser extent in OSS exposed cells (LC3: $p < 0.05$, $n=6$; p62: $p < 0.01$, $n=6$) than EBSS treated cells (LC3: $p < 0.001$, $n=6$; p62: $p < 0.001$, $n=6$).

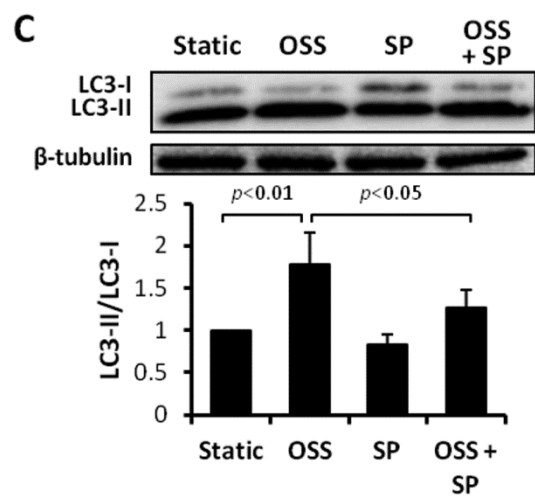
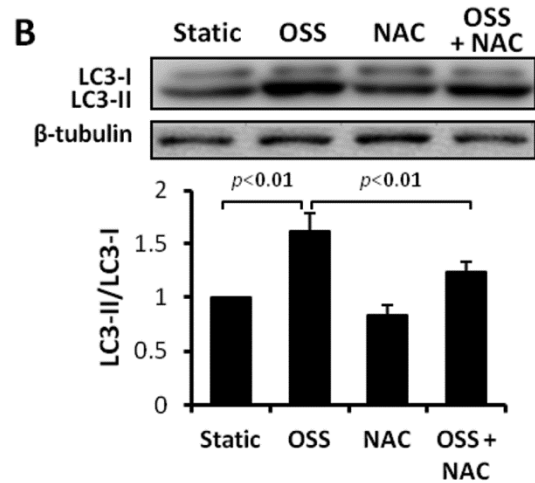
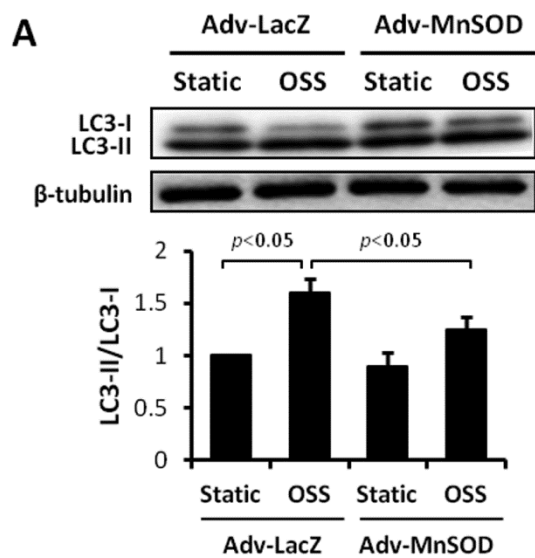


Figure 3. OSS induced oxidative stress and JNK activation to activate autophagy.

(A) HAEC were infected with control or recombinant MnSOD adenovirus (MOI 1:100) overnight, and then were exposed to static condition or OSS for 4 hours. Over-expression of MnSOD significantly attenuated OSS-induced LC3-II/LC3-I ratios (OSS: $p < 0.05$, $n=4$; MnSOD: $p < 0.05$, $n=4$). **(B)** HAEC were exposed to static condition or OSS for 4 hours in the presence or absence of NAC at 5mM or **(C)** a JNK inhibitor SP600125 (SP) at 2 μ M. Both anti-oxidant NAC and JNK inhibitor significantly attenuated OSS-induced LC3-II/LC3-I ratios (NAC: $p < 0.01$; SP: $p < 0.05$, $n=4$).

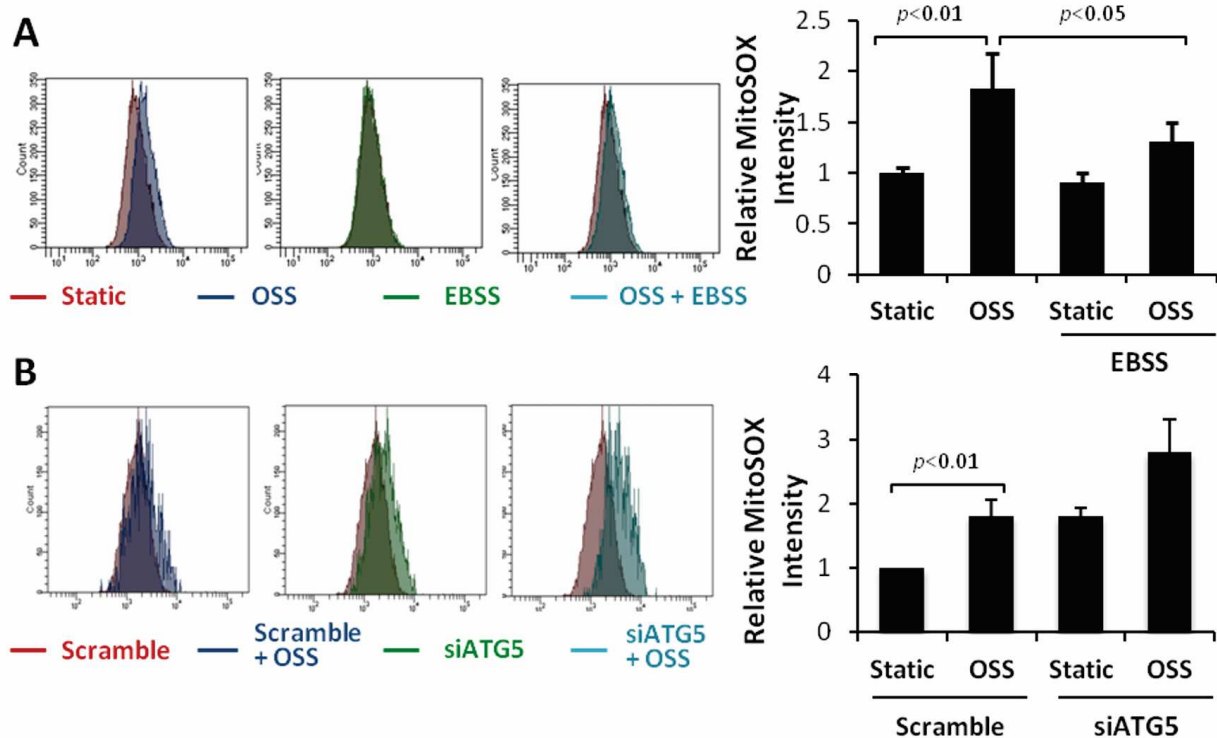


Figure 4. EBSS-activated autophagy mitigated OSS-induced mitochondrial $O_2^{\cdot-}$ production. (A) HAEC were exposed to OSS in DMEM/1%FBS (control) or in Earle's Balanced Salt Solution (EBSS) for 4 hours. $MtO_2^{\cdot-}$ production was measured by the MitoSox Red fluorescent intensity. EBSS attenuated OSS-induced MitoSox Red intensity ($p < 0.05$, $n=4$). **(B)** HAEC were transfected with scrambled siRNA or ATG5 siRNA for 48 hours, and then were exposed to static condition or OSS. Knockdown of ATG5 augmented OSS-induced MitoSox Red intensity ($p = 0.059$, $n=4$).

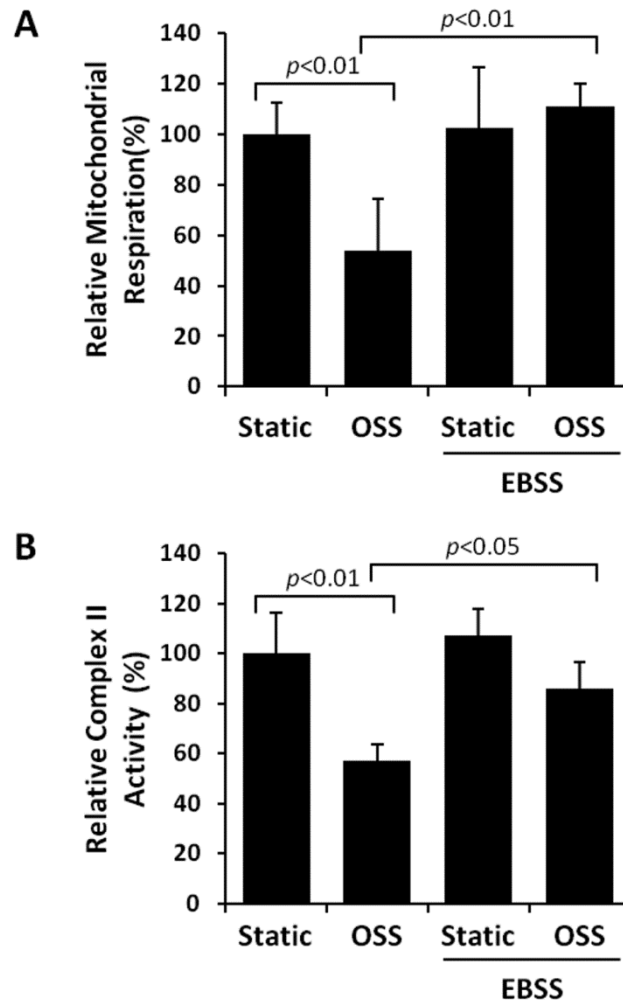


Figure 5. EBSS-activated autophagy restored OSS-mediated reduction in mitochondrial respiration. HAEC were exposed to OSS in DMEM/1%FBS (control) or in EBSS for 4 hours. **(A)** Mitochondrial respiration was assessed by Seahorse Flux Analyzer. EBSS restored OSS-mediated reduction in mitochondrial respiration (OSS: $p < 0.01$, $n=4$; EBSS: $p < 0.01$, $n=4$). **(B)** EBSS restored OSS-mediated reduction in mitochondrial complex II activity ($p < 0.05$, $n=4$).

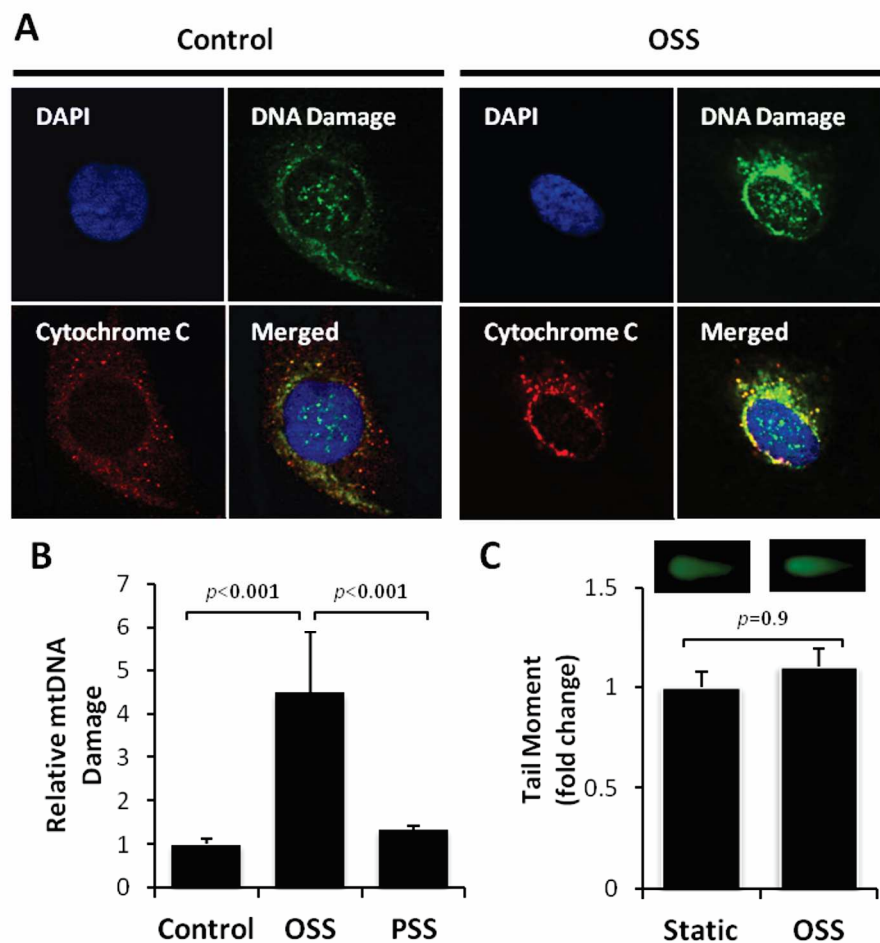


Figure 6. OSS induced mitochondrial DNA damage. (A) Immunofluorescent staining revealed mtDNA damage. HAECs exposed to OSS (4 hr) developed a prominent intensity for anti 8-OHdG (8-Hydroxy-2'-Deoxyguanosine) (Green) as an indicator for DNA damage, which co-localized with anti-Cytochrome C (Red) for mitochondria. DAPI (blue) stained for nuclei. (B) HAECs were exposed to OSS vs. PSS for 4 hours, and mitochondrial DNA (mtDNA) damage was measured by quantitative PCR. OSS induced a 4.5-fold increase in mtDNA damage ($p < 0.001$, $n=4$), and PSS induced an insignificant increase ($p > 0.05$, $n=3$). (C) OSS induces negligible nuclear DNA damage. Tail moment analysis of Comet Assay (CometScore software) revealed insignificant difference in nuclear DNA damage between static and OSS conditions ($p=0.9$, $n=3$).

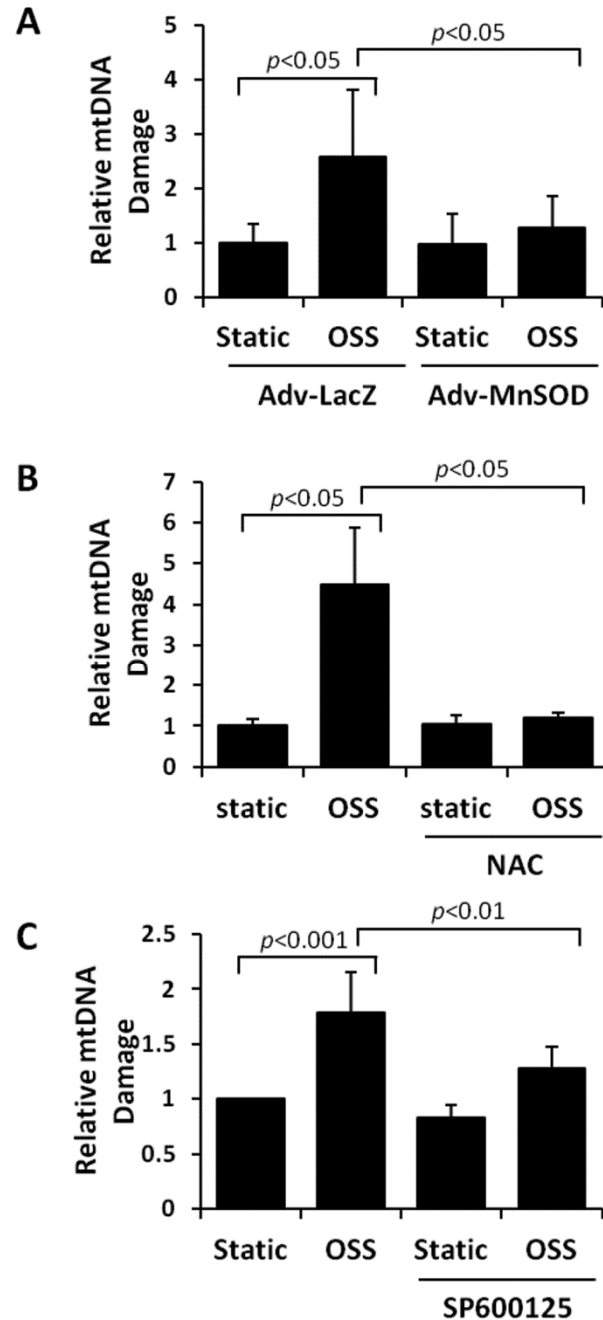


Figure 7. Antioxidants and JNK inhibitor reversed OSS-induced mtDNA damage.

(A) HAEC were infected with control or recombinant MnSOD adenovirus (MOI 1:100) overnight, and then were exposed to OSS for 4 hours. MnSOD over-expression significantly reversed OSS-induced mtDNA damage ($p < 0.05$, $n=4$). **(B)** HAEC were

exposed to static condition or OSS for 4 hours in the presence or absence of 5mM of antioxidant (NAC) or (C) 10μM of JNK inhibitor SP600125 (SP). Both NAC and SP significantly reversed OSS-induced mtDNA damage (NAC: $p < 0.05$; SP: $p < 0.01$, $n=4$).

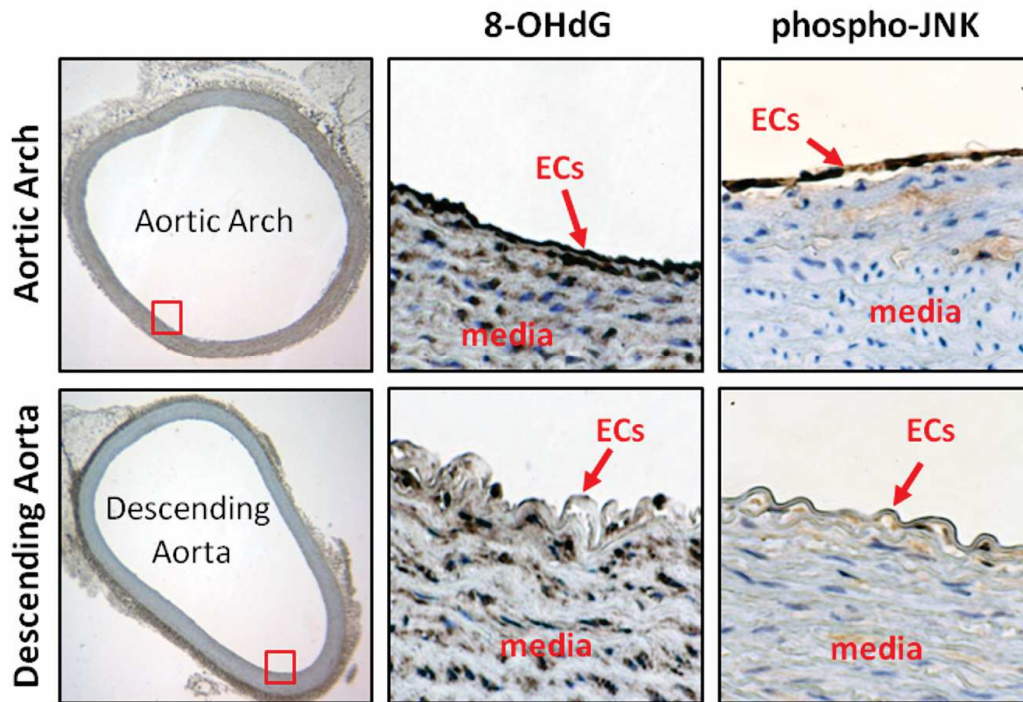


Figure 8. Spatial variations in shear stress differentially promoted DNA damage and JNK activation in New Zealand White rabbits. Cross-sections of aortic arch versus descending aorta were stained with anti-8-OHdG (8-Hydroxy-2'-Deoxyguanosine) for DNA damage and anti-phospho-JNK for JNK activation. Prominent anti-8-OHdG staining was observed in aortic arch but nearly absent in the descending aorta. Phospho-JNK staining was prominent in the aortic arch but nearly absent in the descending aorta. The red squares denote enlarged areas (100x magnification).

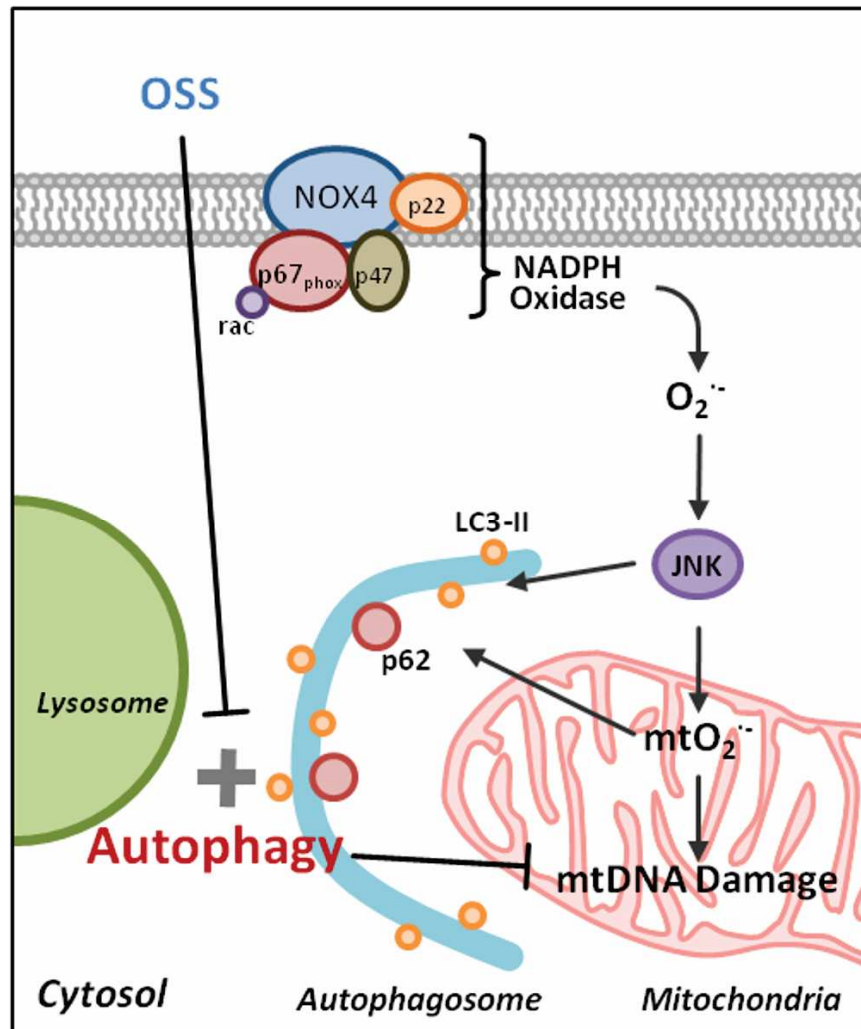
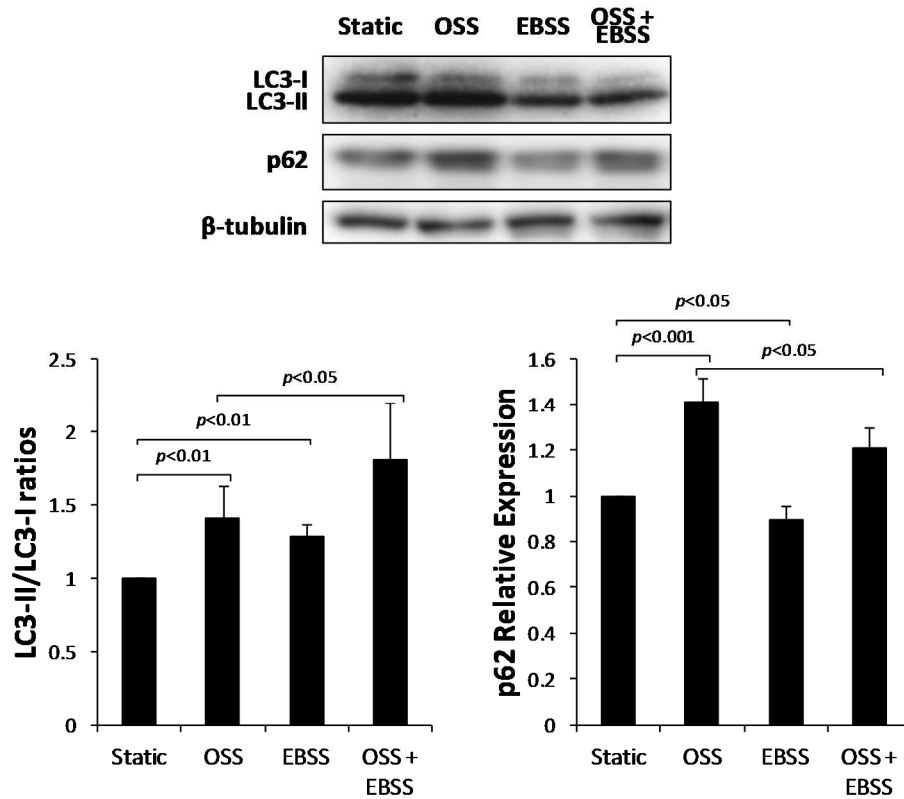
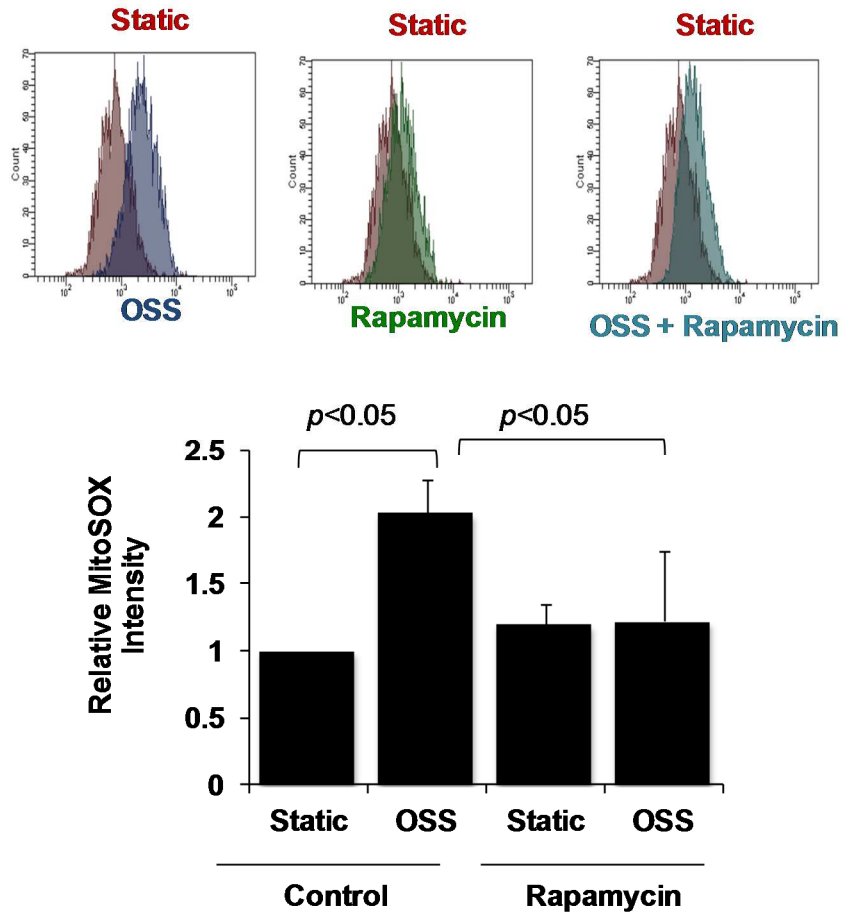


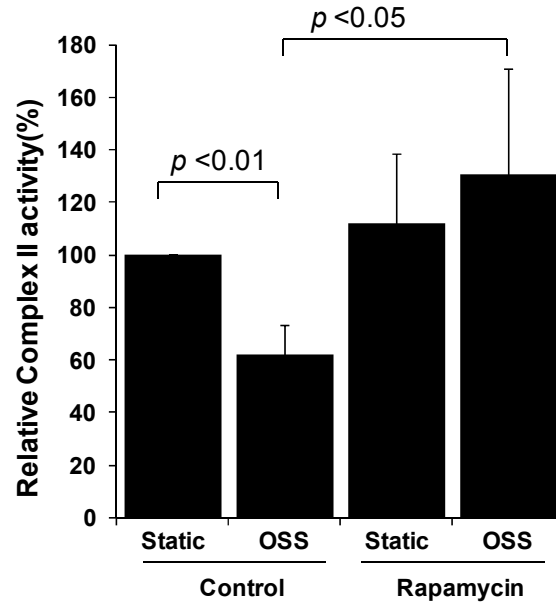
Figure 9. Scheme of OSS modulation of autophagy. OSS-induced oxidative stress activates JNK which plays a dual role in inducing mitochondrial dysfunction and initiating autophagy. However, sustained OSS impairs autophagic flux, leading to accumulation of p62, increased $mtO_2^{\bullet-}$ production, and mtDNA damage.



Supplemental Figure. S1. EBSS attenuated OSS-inhibited autophagy flux. HAEC were treated with OSS or EBSS for 4 hours. OSS and EBSS both increased LC3-II/LC3-I ratios ($p < 0.01$, $n=6$). Adding EBSS to OSS further increased LC3 ratios ($p < 0.01$, $n=6$). EBSS reduced OSS-inhibited p62 levels (OSS: $p < 0.001$, $n=6$; EBSS: $p < 0.05$, $n=6$).



Supplemental Figure. S2. Rapamycin mitigated OSS-induced mitochondrial superoxide production (mtO₂^{•-}). HAEC were treated with Rapamycin at 1μM for 1 hour to activates autophagy, and then were exposed to static condition or OSS for 4 hours. mtO₂^{•-} was measured. OSS-induced mitochondrial O₂^{•-} production was reversed with Rapamycin ($p < 0.05$, n=4)



Supplemental Figure. S3: Rapamycin restored OSS-induced reduction in mitochondrial complex II activity. Mitochondrial complex II activity was reduced after OSS exposure for 4 hours ($p < 0.01$, $n=4$), whereas treatment with Rapamycin at $1\mu\text{M}$ restored mitochondrial complex II activity ($p < 0.05$, $n=4$).

Chapter Four: Atrial Fibrillation Pacing Decreases Intravascular Shear Stress in a New Zealand White Rabbit Model: Implications in Endothelial Function

This manuscript is adapted with permission and excerpted from:

Jen N, Yu F, Lee J, Wasmund S, Dai X, Chen C, Chawareeyawong P, Yang Y, Li R, Hamdan MH, Hsiai TK. "Atrial fibrillation pacing decreases intravascular shear stress in a New Zealand white rabbit model: implications in endothelial function." Biomech Model Mechanobiol. 2013 Aug;12(4):735-45. Copyright © 2013 by Springer

4.1 Introduction

Cardiac arrhythmia is a leading cause of syncope and sudden cardiac death in the United States [79, 90, 180-182]. Atrial fibrillation (AF) is the most common type of cardiac arrhythmia, affecting up to 5 million people in the United States and 5% of the population over 65 years old [76-78]. While the effects of AF-mediated atrial remodeling have been widely characterized [78, 87], the hemodynamic effects of AF on endothelial function has remained poorly understood.

Mounting evidence suggests that AF is implicated in increased serum oxidative stress markers and impaired acetylcholine-mediated vasodilation [183-186]. AF promotes rapid and irregular atrial contraction rates exceeding 400–600 times per minute (bpm) [79], resulting in rapid and irregular ventricular contraction rates above 100 bpm [80], and leading to a decrease in both atrial pressure and cardiac output [81-82]. While a plethora of literature supports the mechanisms linking wall shear stress with the regulation of endothelial function [9, 187], there is a paucity of data to support AF-mediated changes in hemodynamics on endothelial responses.

In this context, we sought to determine whether AF-mediated changes in temporal gradients ($\partial\tau/\partial t$) and intravascular shear stress (ISS) modulated endothelial responses. Microelectromechanical systems (MEMS) thermal sensors have allowed for measurement of convective heat transfer in the aorta of New Zealand White (NZW) rabbits, from which intravascular shear stress (ISS) was inferred as an approximation to wall shear stress [23-24, 188-191]. Surface electrical cardiogram (ECG) recordings were synchronized with ISS in the thoracic aorta in response to rapid and irregular atrial pacing. In an in vitro flow system in which the time-averaged shear stress was

maintained at a constant value, we further assessed the effects of rapid pulse rates on the production of endothelial nitric oxide (NO), reactive oxygen species (ROS), and on monocyte binding to human aortic endothelial cells (HAEC). Our findings suggest that rapid atrial pacing reduces ISS and $\partial\tau/\partial t$, and rapid pulse rate is implicated in modulating endothelial function.

4.2 Materials and methods

4.2.1 Catheter-based flexible MEMS sensor to measure intravascular convective heat transfer

The sensor was fabricated using surface micromachining with biocompatible materials including Parylene C, Ti, and Pt as previously described [188] (**Supplementary Figure 1**). Based on the heat transfer principle, the output voltage of the MEMS sensors under the constant current circuits was sensitive to the fluctuations of ambient temperature. The temperature overhear ratio (α_T) is expressed as temperature variations of the sensor over the ambient temperature (T_0):

$$\alpha_T = \frac{(T - T_0)}{T_0} \quad (1)$$

where T denotes the temperature of the sensor. The relation between resistance and temperature overhear ratios is expressed as:

$$\alpha_R = \frac{(R - R_0)}{R} = \alpha(T - T_0) \quad (2)$$

where α is the temperature coefficient of resistance (TCR). The operating resistance of the sensing element was approximately 1.189 k Ω 37.8°C with a temperature overhear ratio (α_T) of 0.046 and a TCR (α) of 0.84 $\times 10^{-3}/^\circ\text{C}$ (**Supplementary Figure 2a**). The sensor provided a maximum frequency response at 5 kHz accompanied by a gain of

8.72 (**Supplementary Figure 2b**). Calibration was performed in a platinum-cured silicone tube for individual sensors to establish a relationship between heat exchange (from the heated sensing element to the blood flow) and shear stress over a range of steady flow rates (Q_n). The theoretical shear stress value corresponding to each flow rate was calculated as:

$$\tau_w = \frac{4Q_n\mu}{\pi r^3} \quad (3)$$

where τ_w is the wall shear stress, μ is the blood viscosity, and r is the radius of the circular tube. The viscosity of the blood as a function of flow rate was measured using a viscometer (Brookfield Engineering, Newhall, CA). The calibrated sensors were then deployed into the NZW rabbit's aorta for convective heat transfer on the MEMS thermal sensors, from which intravascular shear stress (ISS) was inferred as previously described [192-193]:

$$\tau_w^{1/3} \propto Q_{conv} \cong \frac{V_0^2}{R_s} \quad (4)$$

where Q_{conv} denotes the convective heat transfer, V the changes in voltage to the MEMS thermal sensors as flow past the resistively heated sensor, and R the resistors of the sensor.

4.2.2 Assessment of intravascular shear stress

In light of experimental constraints to directly measure wall shear stress in an in vivo model, we acquired ISS to approximate wall shear stress in NZW rabbit aortas [191]. Flexible MEMS sensors deployment into rabbit aortas was performed in compliance with the Institutional Animal Care and Use Committee in the Heart Institute of the Good Samaritan Hospital (Los Angeles, CA), which is accredited by the American Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC).

Four male NZW rabbits (10 weeks old; mean body weight, $2,442 \pm 210$ g) were acquired from a local breeder (Irish Farms, Norco, CA) and maintained in the Good Samaritan Hospital Vivarium. After a seven-day quarantine period, the rabbits were anesthetized for percutaneous access, and anesthesia was induced through an intramuscular injection of 50mg/kg ketamine (JHP Pharmaceuticals, LLC) combined with 10 mg/kg xylazine (IVX Animal Health, Inc.). A 23-gauge hypodermic needle and 26-gauge guide wire were introduced via cut-down of the left femoral artery. A rabbit femoral catheter (0.023 in. ID $\times$ 0.038 in. OD) was passed through the left femoral artery. The circulatory system of the individual animal was anti-coagulated with heparin (100 units/kg) prior to the sensor deployment, and catheters and needles were rinsed with heparin at 1,000 units/ml prior to the procedures.

An ultrasound transducer (Philips SONOS 5500 at 12 MHz) was positioned over the abdomen to interrogate arterial blood flow. Periodic blood pressure measurements were recorded with an automated tail cuff (IITC/Life Science Instruments). Using the fluoroscope in the animal angiographic laboratory (Phillips BV-22HQ C-arm), we were able to visualize and steer the catheter-based MEMS sensors (**Fig. 1a**). Contrast dye was injected to delineate the position of the MEMS sensors in relation to the inner aortic diameter. The voltage recordings were synchronized with the rabbit's cardiac cycle via ECG.

4.2.3 Data acquisition

The constant temperature circuit was used for real-time voltage signal acquisition in the aorta [192]. When the current passed through the sensor, sensing element was heated up at an overheat ratio of $\sim 3\%$. The voltage across the sensing element was

monitored by a LabVIEW-based data acquisition system, which included a data acquisition board (USB-6216 DAQ device, National Instruments, Austin, TX) and a laptop computer (ThinkPad T61, Lenovo) loaded with LabVIEW. The acquisition was sampled at 1,000 Hz. Wavelet decomposition and low-pass filters were applied to remove background noise, resulting in a signal-to-noise ratio of 4.8 as previously described [194].

4.2.4 Rapid atrial fibrillation pacing

Rapid atrial fibrillation (AF) was performed via a standard pacemaker lead (St. Jude Medical, Minneapolis, MN, USA) that was advanced through the jugular vein into the right atrium. The lead was connected directly to a Grass SD9 stimulator (Grass Technologies, West Warwick, RI, USA). The stimulator was triggered using a custom software program (LabVIEW, National Instruments, Austin, TX). The pacing protocols were as follows: (1) baseline sinus rhythm at 121.8 ± 1.6 , (2) rapid regular and tachycardia atrial pacing at 168.3 ± 0.5 , and (3) rapid irregular pacing at 167.1 ± 57.4 (Table 1).

4.2.5 ECG recordings and signal processing

Two 29-gauge stainless steel microelectrodes (AD Instrument, Colorado Springs, CO) were positioned at 90° to the animal's ventral epidermis. The recording electrode was positioned directly above the ventricle, while the reference electrode was positioned distal to the heart. Both electrodes were secured onto the skin to approximately 1mm in depth. The ECG signals were amplified by 1,000-fold (A-M 1700 Differential Amplifier, A-M Systems Inc., Carlsborg, WA) and band-pass filtered between 0.1 and 500Hz as well as at 60Hz (notch) [194]. The signals were acquired and digitized

at a sampling rate of 1,000Hz (National Instruments USB-6216 DAQ device, and LabVIEW 8.2). To enhance signal-to-noise ratios (SNR), we digitally processed the signals using the wavelet transform and thresholding MATLAB algorithm (MATLAB 7.1 Mathworks Inc, Natick, MA) developed in our laboratory [195]. The parameters used in the algorithm allowed for systematic recordings for QRS and QTc intervals regardless of electrode placements or the cardiac vector orientations.

4.2.6 Computational fluid dynamics (CFD) simulation

CFD code was developed to compare between analytical and experimental data. Three-dimensional modeling of healthy rabbit aortic geometries (aortic arch, thoracic, abdominal, renal aorta) was reconstructed by SolidWorks (Concord, Massachusetts, USA). To exclude contributions from lumen elasticity, the aorta was assumed to be a rigid tube. Aorta diameters were taken from angiography. The inlet pulsatile blood velocity profiles were obtained from the pulsed-wave Doppler velocity measurements. The velocity values were filtered by a low-pass filter using MATLAB (Natick, MA, USA). To compensate for the model's flat velocity inlet boundary layer, we allowed the fluid to flow along the rigid tube for 20 cm to develop fully parabolic flow before the flow stream passes down to the sensor. The outlet boundary condition was determined from the mean arterial pressure and defined as 80 mmHg. Geometries were meshed by 9424 fluid/12484 partial hexahedron cells after defining the inlet and outlet boundary conditions. Meshed models were solved by SolidWorks Flow simulation until it met the defining time which is 2 s, with a time step size of 0.005 s. SolidWorks Flow simulation automatically set up to meet the convergence condition with changing iterating numbers.

The governing equations were solved by assuming laminar, incompressible, and unsteady flow under the non-slip condition.

4.2.7 A flow system to assess cultured endothelial responses to rapid pulse rates

A train of pulsatile shear stress (PSS: unidirectional flow) was generated to deliver distinct pulse rates (50, 100 vs. 150 bpm), slew rates ($\partial\tau/\partial t = 31, 57, \text{ vs. } 89 \text{ dyn}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$), and minimum versus maximal shear stress (12 vs. $36 \text{ dyn}\cdot\text{cm}^{-2}$) at a constant time-averaged shear stress ($\tau_{avg} = 23\pm4 \text{ dyn}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$) (Supplemental Figure 3). Oscillatory shear stress (OSS at $0.1\pm3.0 \text{ dyn}\cdot\text{cm}^{-2}$) was also generated to compare with PSS. Human aortic endothelial cells (HAEC) (Cell Applications, San Diego, CA) were cultured on gelatin-coated dish in endothelial growth medium (Cell Applications) supplemented with 5% heat-inactivated fetal bovine serum (FBS, GIBCO, Carlsbad, CA) at 37°C in a 5% CO_2 atmosphere. The cells were exposed to five flow conditions for 4 h: (1) control at static conditions as a reference point, (2) PSS at 50 bpm, (3) PSS at 100 bpm, (3) PSS at 150 bpm, and (5) OSS at 60 bpm (Supplementary Figure 3). Subsequently, the cells were used to assess NO production and monocyte-binding assay.

4.2.8 Analysis of no production: nitrite (NO_2^-) and Nitrate (NO_3^-)

Quantitative measurements of NO_2^- and NO_3^- were performed as an index of global nitric oxide (.NO) production following methods described previously [196]. NO is metabolized or decomposed via various reactions to the metabolites as NO_2^- and NO_3^- , which serve as useful measurements of overall NO production and metabolism [197].

4.2.9 Measurement of reactive oxygen species (ROS)

The intracellular superoxide ($O_2^{\cdot-}$) production was measured using dihydroethidium (DHE). HAEC were exposed to PSS at 50, 100, 150 bpm, and OSS at 1 Hz for 4 h, followed by incubation with DHE (5 μ M). Images were acquired from three chosen fields (Nikon Inverted Epifluorescence Microscope and a ProgRes C3 digital microscope camera, Japan). Image intensities were quantified by using a custom MATLAB script.

4.2.10 Monocyte-binding assay

THP-1 cells, grown in suspension, were centrifuged, rinsed with phosphate-buffered saline (PBS) twice, and labeled with 1.0 μ M Calcein AM (Invitrogen). Following shear stress exposures as described above, HAEC were incubated with calcein-labeled THP-1 cells for 30 min in a humidified incubator with 5% CO₂ at 37°C. Unbound THP-1 cells were washed away three times with PBS. 1ml of PBS/2%PFA was added and incubated for 30 min at room temperature. The cells were rinsed with PBS, and 1ml of PBS was added onto each slide. THP-1 cells attached to endothelial cells were counted under fluorescence microscope. Bound THP-1 cells were counted in five fields for each slide, averaged, and normalized to static control.

4.2.11 Statistical analyses

Data were expressed as mean $\pm$ SD. For comparisons between two groups, Student's t test was used for significance analysis. For comparisons among multiple values, one-way analysis of variance (ANOVA) was used. A *p* value < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Assessment of intravascular shear stress via convective heat transfer

The sensor was deployed into NZW rabbit aortas via femoral cut-down, and navigated to the specific regions under fluoroscopic guidance (**Fig. 1a, b**). Real-time voltage signals were measured and filtered using a Hilbert transform, from which convective heat transfer was converted to intravascular shear stress (**Fig. 1c**). The sensor was positioned in the aortic arch, thoracic aorta, supra renal artery, and abdominal aorta for intravascular thermal profiles (**Supplementary Figure 4**).

4.3.2 Rapid irregular pacing in NZW rabbits

Surface ECG recordings were synchronized with ISS in the rabbit thoracic aorta at the baseline sinus rhythm (**Fig. 2a**), revealing distinct P waves, QRS complexes, and T waves. The P waves were indistinct in response to rapid irregular pacing (**Fig. 2b**). Increasing regular pacing rate at 178.3 ± 10.0 bpm resulted in a decrease in systolic pressure by 7.3 ± 1.8 mmHg, a decrease in mean pressure by 3.8 ± 1.8 mmHg, and a decrease in time-averaged shear stress by 3.8 ± 8.4 dyn·cm⁻². Rapid and irregular pacing at 178.1 ± 38.1 bpm further decreased diastolic pressure by 9.1 ± 4.3 mmHg, accompanied by a decrease in mean arterial pressure by 6.3 ± 5.5 mmHg, and a similar reduction in decrease in time-averaged shear stress by 3.8 ± 6.9 dyn·cm⁻² (**Table 1**).

Next, we analyzed the effects of rapid irregular pacing in terms of ISS and temporal gradients ($\partial\tau/\partial t$) (**Fig. 3**). There were positive correlations between ISS and cycle lengths ($r = 0.58$), and between temporal gradients and cycle lengths ($r = 0.86$). There was also correlation between ISS and temporal gradients ($r = 0.76$). We further compared ISS with computed wall shear stress (WSS) in the thoracic aorta. ISS overlapped with computed WSS in terms of the magnitude and frequency (**Fig. 3d**). The

minimum to maximum values of ISS ranged from 4 to 39 dyn·cm⁻², which was in a close approximation to the range of computed WSS from 5 to 38 dyn·cm⁻². Despite the experimental constraint to directly measure shear stress acting on the arterial wall, ISS approach provided a close approximation to the theoretical values in the thoracic aorta in an in vivo model. Taken together, rapid irregular pacing promoted a reduction in mean arterial pressure, ISS and $(\partial\tau/\partial t)$ in our in vivo model.

4.3.3 Implications of rapid pulse rates on cultured human aortic endothelial cells (HAEC)

To assess the effects of rapid pulse rates on endothelial function, we exposed HAEC to shear stress in a well defined flow system in which we were able to vary the pulse rates at a constant time-averaged shear stress (τ_{avg}) (Supplementary Figure 3). PSS-induced eNOS mRNA up-regulation was significantly attenuated in the setting of increasing pulse rates (PSS at 50bpm [PSS₅₀]: 1.62±0.13 -fold; PSS at 100bpm [PSS₁₀₀]: 1.47±0.1-fold; PSS at 150bpm [PSS₁₅₀]: 1.38±0.12 -fold vs. static condition, * p < 0.05, n = 6) (**Fig. 4a**), whereas OSS did not significantly alter eNOS expression. In corollary, endothelial NO production was quantified in terms of its metabolites; namely nitrate (NO₂⁻) and nitrite NO₃⁻) [21]. We demonstrated a decrease in NO production in response to rapid pulse rates (PSS at 50bpm [PSS₅₀]: 3.66±0.54-fold increase compared to static condition; PSS at 100bpm [PSS₁₀₀]: 2.45±0.12-fold; PSS at 150bpm [PSS₁₅₀]: 1.87±0.47-fold, * p < 0.05, n = 4) (**Fig. 4b**). OSS further reduced NO production as reported previously [21] when compared to PSS condition. Hence, rapid pulse rates attenuated PSS-mediated eNOS expression and subsequent NO production.

In parallel, we assessed reactive oxygen species (ROS) production and monocytes binding. PSS is well recognized to down-regulate ($O_2^{\bullet-}$) formation [21, 24], whereas OSS promotes ROS production via the NADPH oxidase system [105]. Reduced pulse rates attenuated PSS-mediated reduction in DHE intensities (PSS₅₀: $26 \pm 5\%$, $*p < 0.05$, $n=3$), whereas rapid pulse rates did not significantly alter DHE intensities (PSS₁₀₀: $8 \pm 5\%$ vs. static conditions, PSS₁₅₀: $4 \pm 7\%$ vs. static conditions). OSS remained a potent inducer of ROS production. Thus, rapid pulse at (PSS₁₅₀) reversed the salutary effect of PSS₅₀ in terms of DHE-indicated ROS production (**Fig. 4c**).

We further quantified monocyte binding to HAEC. PSS₅₀ attenuated monocyte binding to a greater extent compared to PSS₁₅₀ (PSS₅₀: $37 \pm 6\%$ reduction; PSS₁₀₀: $20 \pm 8\%$; PSS₁₅₀: $12 \pm 6\%$ vs. static condition, $*p < 0.05$, $n = 4$) (**Fig. 4d**). As a positive control [24], bidirectional OSS significantly increased monocyte binding (OSS: $96 \pm 40\%$ vs. static condition, $*p < 0.01$, $n=4$). Taken together, rapid pulse rates (PSS₁₅₀) attenuated the salutary effects of PSS₅₀ in terms of eNOS mRNA expression, NO production, reduction in ROS, and monocytes binding.

4.4 Discussion

In our New Zealand White rabbit model, we elucidated how decreased cycle lengths and pulse pressure during rapid and irregular atrial pacing resulted in a reduction in both mean arterial pressure and shear stress. In our dynamic flow system, we recapitulated these in vivo findings by demonstrating how decreased cycle lengths and slew rates modulated endothelial function. Hence, both in vivo and in vitro models

were complementary to provide new insights into the interplay between increased pulse rates (during sinus tachycardia or atrial fibrillation) and endothelial dysfunction.

Atrial fibrillation (AF) induces atrial and ventricular electrical remodeling [198-199]. Adverse hemodynamic effects in response to rapid and irregular sequence of ventricular cycle lengths were notable for decreased cardiac output, increased pulmonary capillary wedge pressure, and increased right atrial pressure [82]. AF reduced cardiac output through increased mean atrial pressure relative to left ventricular end diastolic pressure [200], thereby decreasing stroke volume [200-201] and cardiac output in a canine model [75]. Furthermore, increasing heart rates and decreasing cycle lengths reduced the Reynolds numbers, but increased the Womersley numbers [202]. In our study, deployment of flexible MEMS thermal sensors allowed for real-time measurement of convective heat transfer with high spatial and temporal resolution in the aorta of ZNW rabbits, from which we could infer a decrease in ISS and slew rates ($\partial\tau/\partial t$). Our experimental data indicated that rapid regular pacing led to a decrease in both mean blood pressure and shear stress in comparison with those of baseline measurements (Table 1). Rapid irregular pacing further reduced mean blood pressure. However, time-averaged shear stress remained similar to that of rapid regular pacing.

Experimental constraint has hampered accurate wall shear stress (WSS) measurement in vivo. WSS vectors were estimated from time-resolved three-dimensional phase-contrast MRI measurements of the velocity field [203]. However, MRI is unable to detect high WSS values along the divider wall of carotid artery, and the precise measurement of velocity field near the wall and in the regions of disturbed flow remains a challenge [204]. For this reason, we have addressed how to optimize ISS

measurement to approximate WSS in the region of thoracic aorta [191]. The experimental parameters that govern the deployment of the catheter-based MEMS sensors into the aorta of NZW rabbits include the dimension of coaxial wires, position of sensors in the vessel, and entrance length (L_e) to minimize flow disturbance to the sensors. In our NZW rabbit model, ISS values were influenced by the (1) presence or absence of guide wire in the aorta, (2) ratios of aorta to guide wire diameters ($n = D_{aorta}/D_{guidewire} = 1.5 - 9.5$), and (3) range of Reynolds numbers (116–1,550).

Our computational results indicated that the entrance length in response to the maximal inlet Reynolds numbers in the rabbits ($Re_{max_rabbit} = 459$) and human abdominal aorta ($Re_{max_human} = 1, 150$) was $L_{e_rabbit} = 1.244$ cm and $L_{e_human} = 1.985$ cm, respectively. For a Womersley number >1 , the above L_e values could be overestimated. Sufficient time was allowed for the flow to develop toward a parabolic velocity profile during individual cardiac cycles and the pulsatile flow behaved in a quasi-steady manner [18]. WSS in the presence of coaxial wire, τ_{w_wire} , can be expressed as [191]:

$$\tau_{w_wire} = -\mu \left. \frac{du}{dr} \right|_{r=R} = E_{wss} \cdot \tau_w \quad (5)$$

where τ_w is the WSS in the absence of coaxial wire and E_{wss} is the WSS elevation factor (WEF), u denotes the velocity in the circular pipe, and R and is the radius of the pipe.

$$E_{wss} = \frac{n^2(2n^2 \ln n - n^2 + 1)}{2[(n^4 - 1) \ln n - (n^2 + 1)^2]} \quad (6)$$

Similarly, the shear stress on the MEMS sensor, τ_{i_wire} , can be evaluated at $r = R_i$ (radius of the catheter/coaxial wire):

$$\tau_{i_wire} = -\mu \left. \frac{dU}{dr} \right|_{r=R_i} = E_{iss} \cdot \tau_w \quad (7)$$

where E_{iss} is defined as the ISS elevation factor (IEF):

$$E_{iss} = \frac{n^3(2 \ln n - n^2 + 1)}{2[(n^4 - 1) \ln n - (n^2 + 1)^2]} \quad (8)$$

To optimize real-time ISS in NZW rabbit, CFD analyses revealed that an entrance length of 2.9 mm and the diameter ratio (n) of 4.5 would minimize the pressure and shear stress elevation in the rabbit aorta. When the catheter was positioned off the center of vessel [205], reported that an eccentricity of the velocity profiles developed, and shear stress on the catheter and the vessel wall varied circumferentially [190]. In humans, the diameter ratio could approach to 100 for an aortic inner diameter of 2.5 cm with a catheter outer diameter of 0.254 mm. Hence, ISS assessment could be further optimized by positioning the catheter near the vessel wall using a steerable catheter with a large diameter ratio in a large animal model [191]. In NZW rabbits, convective heat transfer-inferred time-averaged ISS was within the range of computed WSS by 18.5% in the presence of guide wire [191, 193]. Thus, the application of ISS afforded a reasonable approximation to computed WSS in the thoracic aorta.

Increasing evidence has supported a link between chronic AF and vascular endothelial dysfunction [84]. Patients with AF have been reported to harbor elevated circulating VonWillebrand factor (vWF) and increased E-selectin [85-86]. AF is also implicated in decrease in endothelial-dependent vasodilation in forearm vessels [85, 206], serum nitrates [88-89], and coronary flow reserve (CFR) [90]. Our in vitro flow system allows for delivering a constant time-averaged shear stress (τ_{avg}) while varying pulse rates to recapitulate rapid pacing-mediated reduction in temporal gradients ($\partial\tau/\partial t$) and pulse pressure (ΔP) in rabbits. Our current study demonstrated that rapid pulse rates modulated endothelial NO and ROS production and interaction with monocytes

binding, thus supporting the rapid and irregular pacing model to further elucidate the molecular mechanisms underlying AF-mediated endothelial dysfunction in an in vitro flow system.

Mounting evidence supports that fluid shear stress is intimately involved in vascular oxidative stress [119], inflammatory responses, and atherosclerosis [50, 138, 207-208]. In the medial wall of arterial bifurcations or relatively straight segments of carotid arteries, unidirectional pulsatile flow (net positive forward flow) develops, whereas in the lateral wall or atheroprone regions, flow separation and secondary flow develop. In response to cardiac contraction, the migrating stagnation points generate low and oscillating flow (bidirectional zero net forward flow), otherwise known as disturbed or secondary flow [18]. Pulsatile flow down-regulates adhesion molecules and reactive oxygen species, whereas oscillatory flow increases oxidative stress, promoting the initiation of atherosclerosis [22, 113, 209-211]. PSS attenuates vascular O₂·⁻ production, in part, via down-regulation of NADPH oxidase system [24]. In the present study, we found that pulse rates ranging from 50 to 150 bpm influenced eNOS mRNA expression and subsequent NO production. While the precise molecular mechanisms remain to be defined, we provided a flow model system to isolate the effects of rapid pulse rates on endothelial responses with clinical relevance

Analysis of sensor performance in terms of temperature overheat ratio and thermal coefficient of resistance further supported feasibility of flexible MEMS sensors for realtime physiological recording with high sensitivity and frequency responses. We previously reported changes in heat transfer in atherosclerotic lesions in fat-fed NZW rabbits [189]. Here, we further assessed rapid and irregular pacing-mediated changes in

heat transfer-inferred ISS and $\partial\tau/\partial t$. Changes in temporal gradient or known as slew rates ($\partial\tau/\partial t$) in shear stress have been well recognized in nitric oxide-dependent dilation of arterioles, endothelial cell proliferation, and monocytes-adhesion to active lipid-induced endothelial cells [20, 28, 112, 212]. Thus, heat transfer-inferred ISS and $\partial\tau/\partial t$ offered a potential entry point to link rapid and irregular pulse rates with vascular endothelial function.

In conclusion, the current study provides an in vivo model to link AF-mediated changes in convective heat transfer with a decrease in ISS in a NZW rabbit model. We established a link between rapid pulse rates and endothelial responses in a well-defined flow system. Our intravascular heat transfer strategy ($\Delta\tau$, $\partial\tau/\partial t$, and ΔP) will likely provide a basis to further investigate whether AF cardioversion to sinus rhythm improves endothelial function [84].

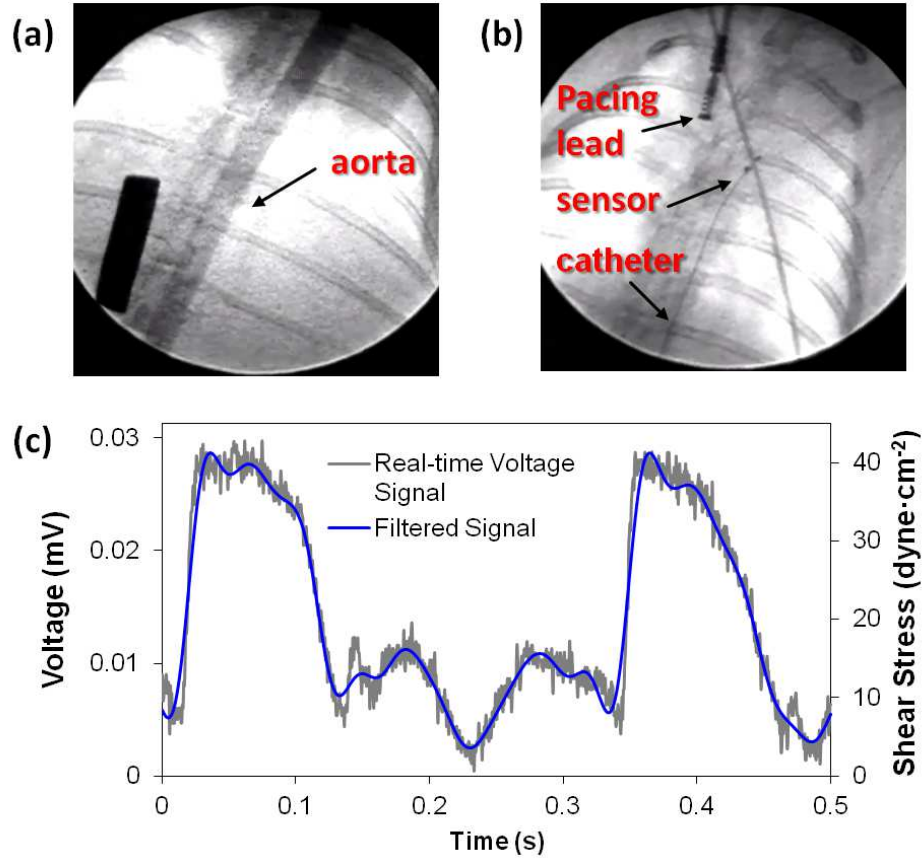


Figure 1. Assessment of intravascular shear stress via convective heat transfer.

(a) Rabbit aorta was visualized by fluoroscopic angiography with contrast for measurements of aortic diameter. **(b)** Fluoroscopy further guided deployment of flexible intravascular sensor to the ascending aorta and the pacing lead to the right atrium. **(c)** Real-time intravascular measurements (**grey line**) captured the pulsatile voltage profiles. The signal was filtered by Hilbert wavelet transform (**blue line**). Voltage measurements were then converted to the corresponding shear stress values as described in the methods.

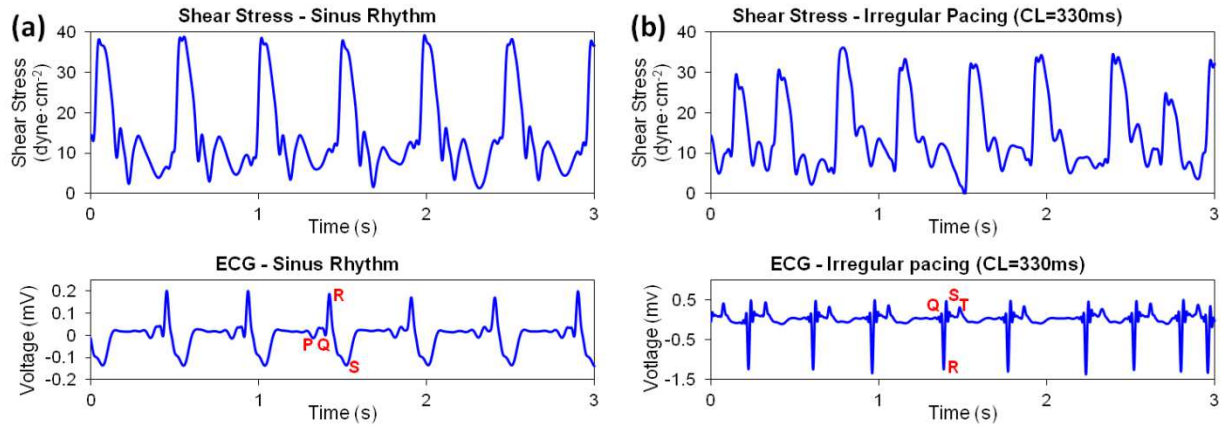


Figure 2. Intravascular shear stress in synchrony with surface ECG recording. (a) ISS and surface ECG were synchronized in the setting of normal sinus rhythm. **(b)** Atrial pacing at an irregular rhythm resulted in indistinct P waves and irregular R-R intervals, as well as negative QRS amplitude in response to atrial pacing.

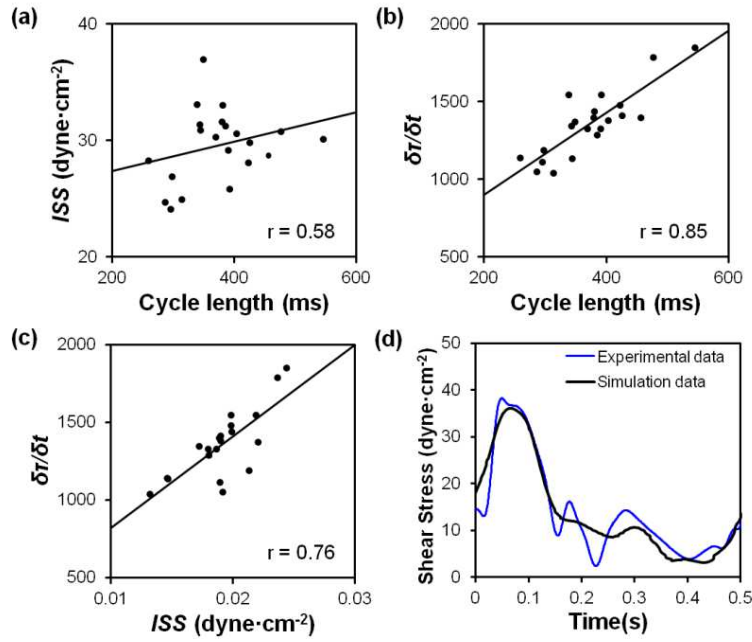


Figure 3. Rapid and irregular pacing altered ISS and temporal gradients. We analyzed the relation between changes in intravascular shear stresses (ISS) and cycle lengths, between temporal gradients ($\partial\tau/\partial t$) and cycle lengths, and between slew rates and shear stress in response to irregular and tachycardia pacing protocol. **(a)** Changes in shear stress correlated with the cycle lengths ($r = 0.58$). **(b)** Changes in $\partial\tau/\partial t$ also correlated with the cycle lengths ($r = 0.86$). **(c)** Changes in $\partial\tau/\partial t$ further correlated with changes in ISS ($r = 0.76$). **(d)** Experimental ISS and simulated wall shear stress (WSS) overlapped in terms of magnitude and frequency in a resting rabbit. ISS ranged between 4 to 39 dyne/cm², corresponding to a simulated range of 5 to 38 dyne/cm².

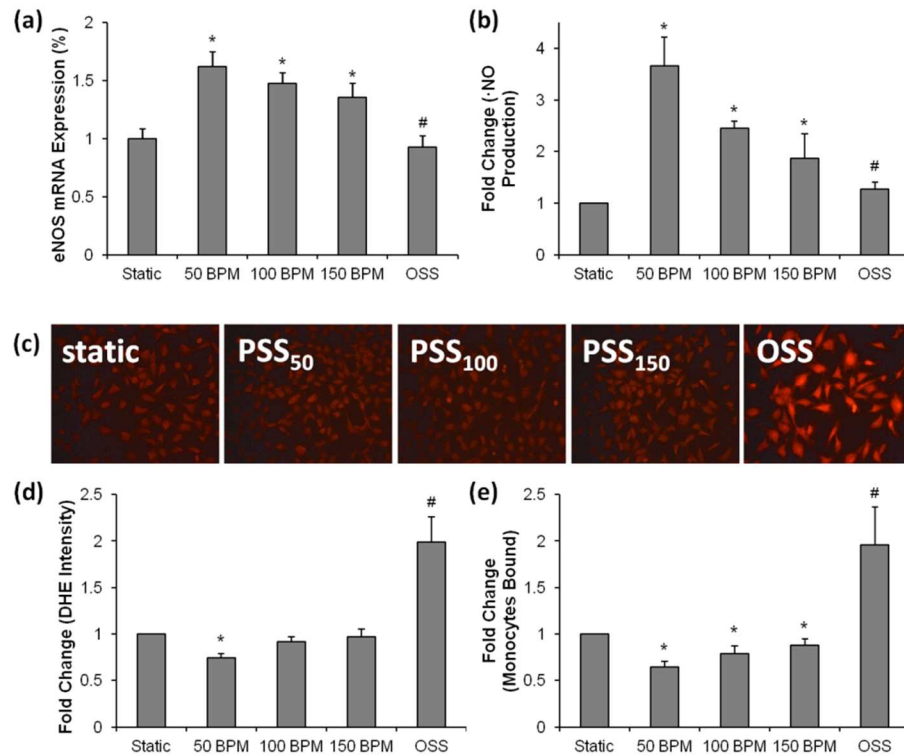


Figure 4. Rapid pulse rates modulated endothelial responses. (a) Decreases in eNOS mRNA expression in response to increases in pulse rates. At an identical time-averaged shear stress (τ_{avg}), eNOS mRNA expression in HAECs was significantly up-regulated by 1.62 ± 0.13 -fold in response to PSS at 50 BPM (PSS₅₀) and by 1.38 ± 0.12 -fold in response to PSS at 150 bpm (PSS₁₅₀) (* $P < 0.05$ vs. static condition, $n = 6$). eNOS mRNA expression remained unchanged statistically in response to oscillatory shear stress at 60 bpm (# $P > 0.05$ vs. static condition, $n = 6$). **(b) Decreases in NO metabolites production in response to increases in pulse rates.** NO metabolites, nitrate (NO_2^-) and nitrite (NO_3^-), were increased by 3.66 ± 0.54 -fold in response to PSS₅₀ and by 1.87 ± 0.47 -fold in response to PSS₁₅₀ (* $P < 0.05$ vs. static

condition, n=4). OSS metabolite production in response to OSS was also elevated ($\#P < 0.05$ vs. static condition, n=6). **(c,d) The effects of pulsatile and oscillatory shear stress on DHE intensities.** Dihydroethidium fluorescent intensities. PSS₅₀ significantly decreased fluorescent intensities as indicated by DHE staining in HAEC ($*p < 0.05$, n=3), whereas OSS significantly increased DHE intensities ($\#p < 0.05$, n=3). PSS₁₀₀ and PSS₁₅₀ did not significantly reduce DHE intensities. DHE intensities were quantified from fluorescent images in the graph. **(e) Incremental monocyte binding in response to increases in pulse rates.** PSS at 50 BPM (PSS₅₀) attenuated monocyte binding by $37 \pm 6\%$, whereas PSS at 150 bpm (PSS₁₅₀) attenuated monocyte binding by $12 \pm 6\%$ ($*p < 0.05$ vs. static condition, n=4). OSS at 60 bpm significantly increased monocyte binding by 96% ($\#p < 0.01$ vs. static condition, n=4).

	Baseline Rhythm	Regular Cardiac Pacing	Irregular Cardiac Pacing
Heart Rate (bpm)	122.1 $\pm$ 5.4	178.1 $\pm$ 10.0	178.1 $\pm$ 38.1
Systolic Pressure (mmHg)	99.4 $\pm$ 0.9	92.1 $\pm$ 1.6	90.3 $\pm$ 4.2
Diastolic Pressure (mmHg)	74.9 $\pm$ 1.2	73.2 $\pm$ 1.9	68.4 $\pm$ 5.3
Mean Pressure (mmHg)	83.0 $\pm$ 1.0	79.1 $\pm$ 1.5	75.9 $\pm$ 3.6

Table 1. Changes in arterial pressure in response to atrial pacing. Systolic, diastolic, and mean arterial blood pressure in response to baseline sinus rhythm at 121.8 $\pm$ 1.6, regular and tachycardia atrial pacing at 168.3 $\pm$ 0.5, and irregular pacing at 167.1 $\pm$ 57.4. Increasing atrial pacing rates resulted in a reduction in systolic, diastolic and mean blood pressures. Irregular pacing led to a further reduction in blood pressures.

Chapter Five: Shear Stress–Activated Wnt-Angiopoietin-2 Signaling Recapitulates Vascular Repair in Zebrafish Embryos

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Li R, Beebe T, Jen N, Yu F, Takabe W, Harrison M, Cao H, Lee J, Yang H, Han P, Wang K, Shimizu H, Chen J, Lien CL, Chi NC, Hsiai TK. “Shear stress-activated Wnt-angiopoietin-2 signaling recapitulates vascular repair in zebrafish embryos.” *Arterioscler Thromb Vasc Biol.* 2014 Oct;34(10):2268-75. Copyright © 2014 by American Heart Association

5.1 Introduction

Mechanotransduction is implicated in differentiation of embryonic stem cells to vascular endothelial cells [94-96]. Hemodynamics, namely, fluid shear stress, is intimately involved in stem cell [94, 213] and mesenchymal progenitors [13] 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 [91] and to confer atheroprotection [214].

Although Ang-1 is constitutively released by the perivascular cells, Ang-2 is released from the Weibel-Palade bodies in endothelial cells [215-216]. 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 [104]. Earlier studies demonstrated that Ang-2 release from Weibel-Palade bodies is induced by endothelial stretch, which occurs during hypertension [217]. 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 [218]. 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 [24-25, 219-220]. OSS-induced Ang-2 promotes tubular formation and migration of cultured endothelial cells [91]. Although stretching isolated arterial endothelial cells further promotes the paracrine effect of Ang-2 release, Ang-1 release inhibits these effects [217]. Ang-2 stimulates arteriogenesis in an C57Bl/6J mice with a ligated femoral artery [221] and confers atheroprotection in apoE-null mice. In

contrast, overexpression of Ang-1 induces smooth muscle cell migration and monocyte chemotaxis [214]. 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 [222]. 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.

5.2 Materials and Methods

5.2.1 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 μ M) (ProSpec Inc, East Brunswick, NJ) was used to rescue endothelial cell migration and tube formation.

5.2.2 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 [112, 148]. 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_{avg}), 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_{avg}) = 23 dyne/cm² accompanied by a stress slew rate ($\partial\tau/\partial t$) = 71 dyn·cm⁻²·s⁻¹ at 1 Hz), and 3) oscillating flow (0 ± 3 dyne/cm²) with τ_{avg} = 0 dyn·cm⁻² at 1 Hz. For oscillating flow, minimal forward flow at a mean shear stress of 0.2 dyne/cm² was provided every hour to deliver nutrients and to remove waste products from the cells.

5.2.3 TOPflash Wnt reporter activity assay

Wnt signaling was measured via TOPflash lentivirus reporter (Addgene plasmid 24307). Lentiviruses were prepared as reported [223]. HAEC grown to sub-confluence were infected with TOPflash lentiviruses at 1:1 ratio in the presence of 6 μ g/ml polybrene for overnight incubation. Next day, HAEC were subjected to OSS or treated with 20 mM 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).

5.2.4 Immunofluorescence 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 Technologies, 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.

5.2.5 Ang-2 knock-down

Scrambled control siRNA, and Ang-2 siRNA were obtained from Qiagen (Valencia, CA). siRNA (60 nmol/L) was transfected to HAEC with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) as described previously [149]. 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.

5.2.6 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 μ L 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 25 ng/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 μ M for 15 minutes. In the live cells, this dye was converted to a green-fluorescent Calcein after acetoxymethyl ester hydrolysis by intracellular esterases [224].

5.2.7 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.

5.2.8 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 CT values for various intervals versus control were used to determine the relative difference in the levels of Ang-2 mRNA expression.

5.2.9 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.

5.2.10 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 Angeles 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 0.1 mM p53 MO. Immediately after collection at 0 hpf, approximately 30-40 embryos were randomly chosen for morpholino microinjections with 2 nL 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.

5.2.11 Subintestinal vein (SIV) Quantification

Fluorescent *Tg(flkl1: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.

5.2.12 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 [225], was performed as a positive control for the effect of DKK-1 induction. Individual heatshock 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**.

5.2.12 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 μ M) (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.

5.2.13 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 μ m 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 20 μ M 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

5.2.14 Statistical analysis

Data were expressed as mean $\pm$ 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.

5.3 Results

5.3.1 OSS Activated Ang-2 Expression via Wnt Signaling

In a dynamic flow system [148], 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 (**Supplemental Figure V**). 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 expression via canonical Wnt signaling in human aortic endothelial cells.

5.3.2 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 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 (**Supplemental Figure II**). Ionomycin treatment similarly reduced endothelial cell migration and tube formation (**Supplemental Figure VI**). Taken together, Ang-2 is a Wnt target gene, with an implication in endothelial repair.

5.3.3 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 (**Supplemental Figure III**). 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.

5.3.4 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 $\mu\text{mol/L}$ Ang-2 ATG-morpholinos or splicing morpholinos to the 2-cell stage embryos impaired SIV development at 72 hpf (**Figure 5B**; **Supplemental Figure IV**). 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 (**Supplemental Figure VII**). Thus, Ang-2 is implicated in SIV development, recapitulating endothelial tube formation (Figure 2).

5.3.5 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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ 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.

5.4 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 [91], 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 (**Supplemental Table I**), 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 [226-230]. 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) [231-232], as well as small molecules, such as IWR-1. Treatment with DKK-1 and siAng-2 inhibited endothelial 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 [233]. 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 [233]. We also demonstrated that ionomycin attenuated nuclear translocation of β -catenin, resulting in (1) downregulation of both Ang-2 mRNA and protein expression (**Supplemental Figure V**) [95], (2) inhibition of tube formation, (3) endothelial migration, (4) proliferation (**Supplemental Figure VI**), and (5) inhibition of SIV development in the zebrafish model (**Supplemental Figure VII**). 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 ($1 \text{ dyn}\cdot\text{cm}^{-2}$), 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 [234-235]. 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 **1F**). 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 [236]. 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 [237]. Ang-2 is further implicated in regulating Wnt target Survivin expression to mitigate oxidized low-density lipoprotein–induced apoptosis in human aortic endothelial cells [238]. Elevated Ang-2 levels promote tumor progression [239] and are associated with obesity [240]. 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 [241]. 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 [242]. Intersegmental vessel sprouting occurs between 24 and 72 hpf, and SIV formation originates from the duct of Cuvier between 48 and 72 hpf [243]. Both intersegmental vessel and SIV are anatomic milestones for monitoring disrupted angiogenesis [244]. 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.

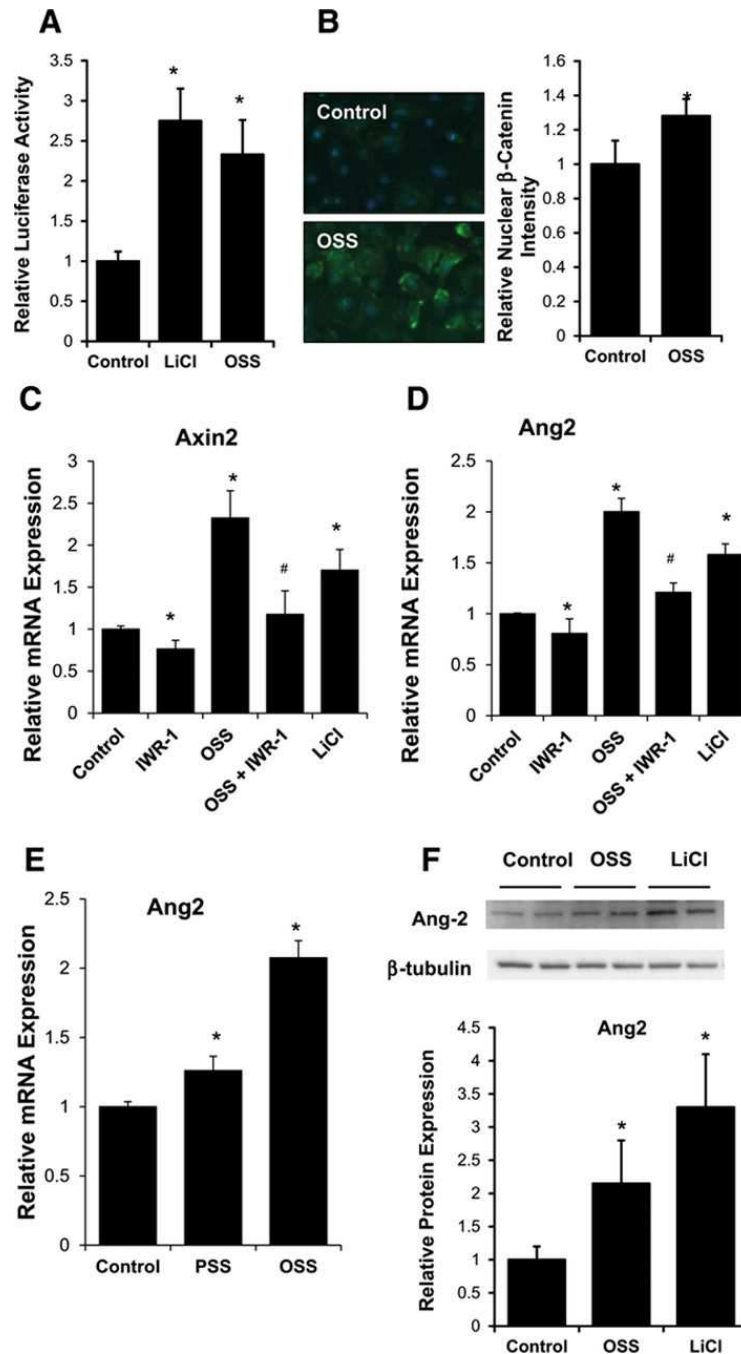


Figure 1. Oscillatory shear stress (OSS) promoted Ang-2 expression via Wnt signaling. (A) Topflash reporter assay revealed that OSS for 8 hours significantly activated Wnt signaling. LiCl, a wnt-signaling inducer, was used as positive control (Control=1.00±0.06; LiCl=2.78±0.42; OSS=2.35±0.46; **p*<0.05 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; $n = 4$).

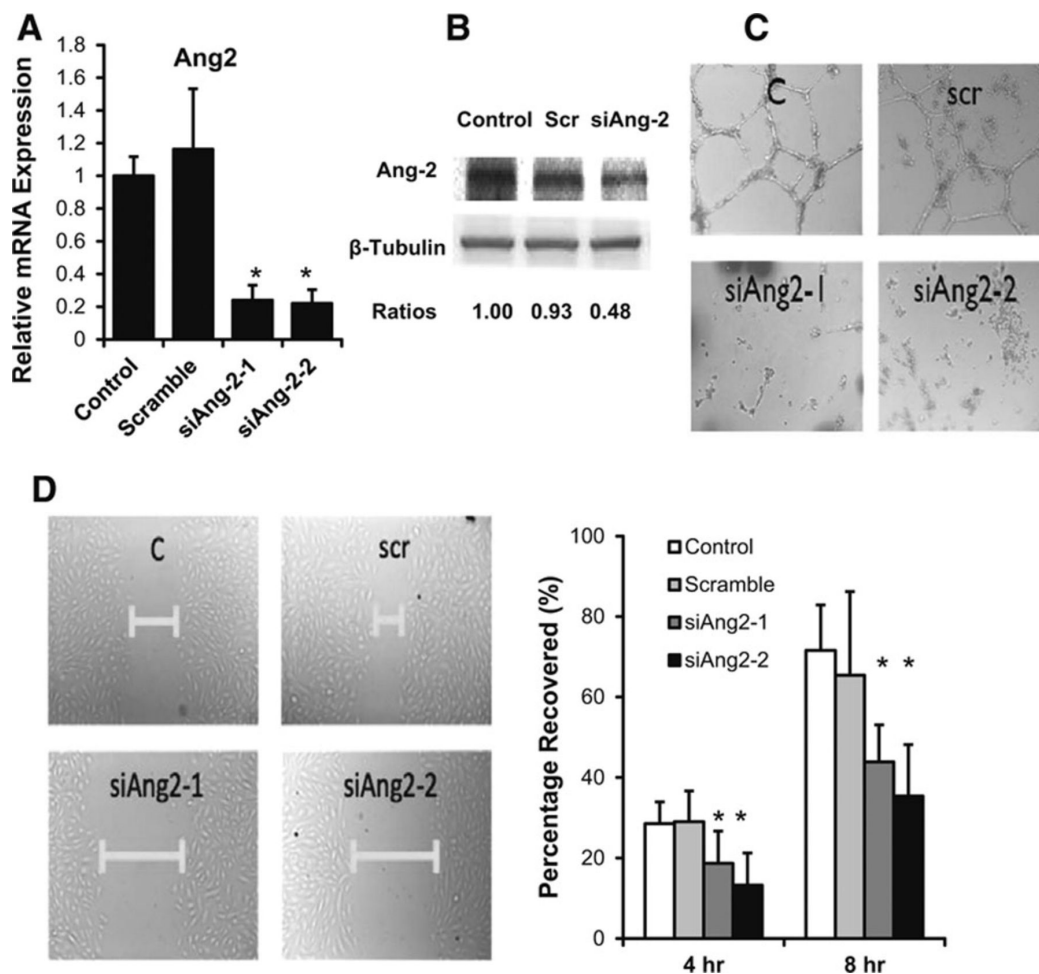


Figure 2. Knockdown of angiopoietin-2 retarded human aortic endothelial cell (HAEC) migration and tube formation. HAEC were transfected with 50 nmol/L scrambled siRNA (Scr), or in-house designed or independently designed Ang-2 siRNA (siAng2-1 and siAng2-2, respectively) for 48 hours. The cells were used 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.

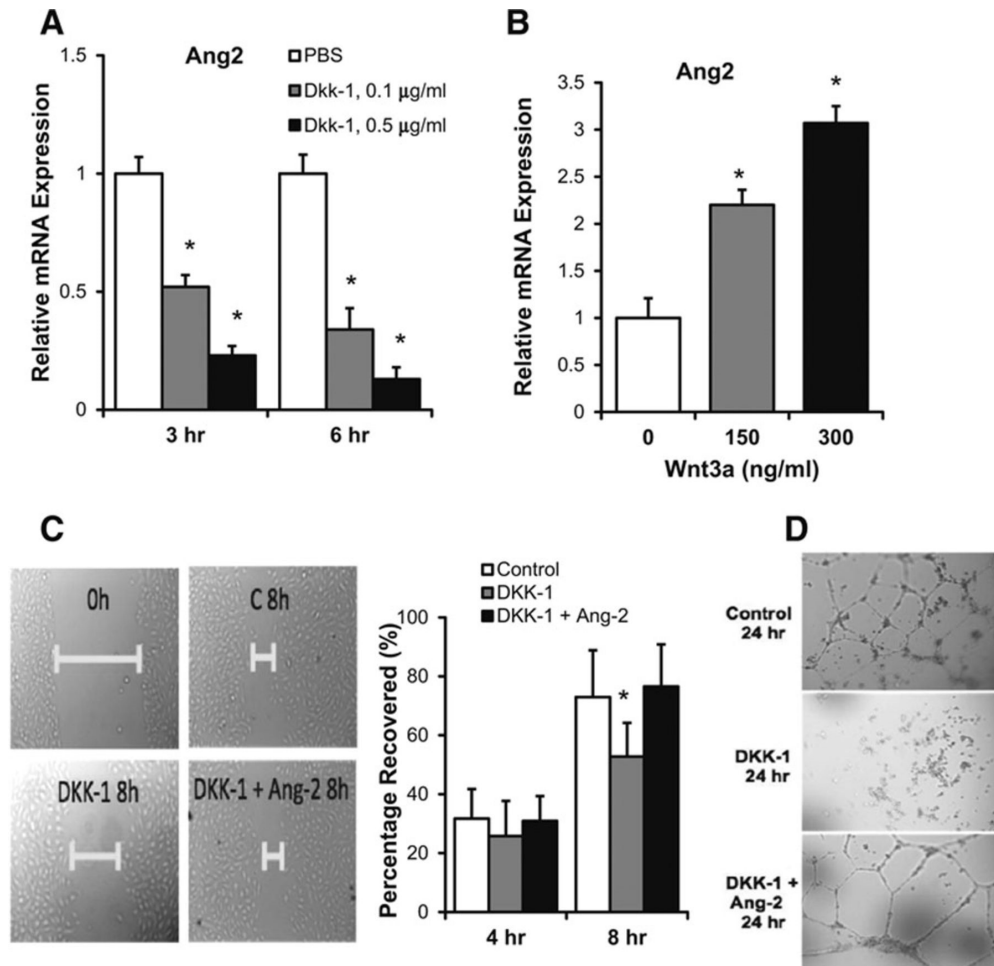


Figure 3. Wnt signaling mediated human aortic endothelial cell (HAEC) migration and tube formation is Ang-2-dependent. (A) HAEC monolayers were treated with 0.1 and 0.5 µg/mL of DKK-1 for 3 and 6 hours, respectively. Quantitative reverse transcriptase-polymerase chain reaction revealed downregulation 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 $\mu\text{g/mL}$). 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 $\mu\text{g/mL}$ of DKK-1. After 8 hours, tube formation was inhibited in the presence of DKK-1 (0.5 $\mu\text{g/mL}$), which was rescued by Ang-2 treatment (0.5 $\mu\text{g/mL}$). Both the migration studies and the Matrigel assays were representative of 4 independent experiments with reproducible findings.

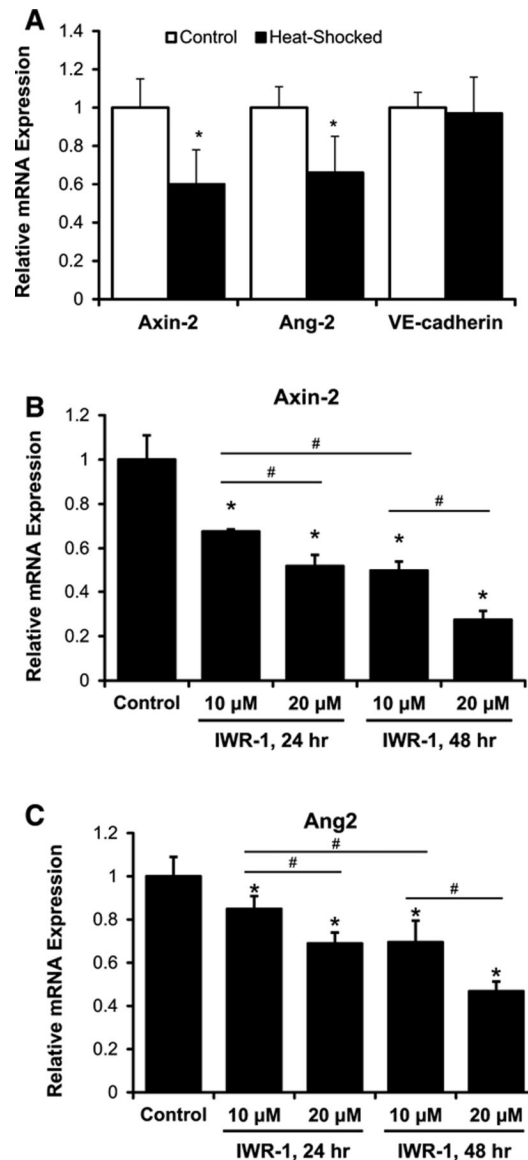


Figure 4. Treatment of *Tg(hsp70:Dkk-1-GFP)* zebrafish embryos with IWR-1 recapitulated Ang-2 as a Wnt target gene. (A) *Tg(hsp70l:Dkk1-GFP)* embryos were heat-shocked at 48 hours post fertilization (hpf) at 37°C for 1 hour. Axin-2, a well-recognized Wnt target gene, was used as a reference control. 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).

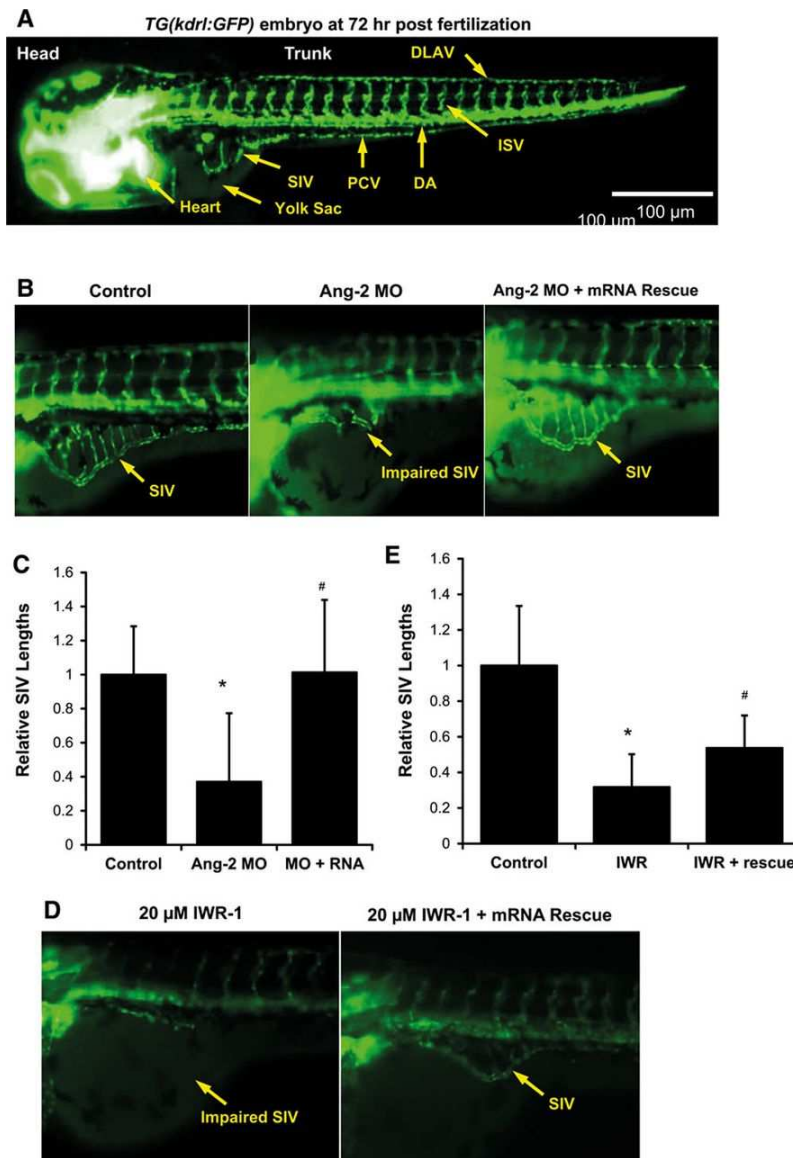


Figure 5. Ang-2 morphant injection impaired subintestinal vein (SIV) formation in Zebrafish embryos. (A) Vasculature of transgenic zebrafish *Tg(kdrl:GFP)* at 72 hours post fertilization (hpf) reveals SIV, intersegmental vessel (ISV), dorsal longitudinal vein (DLAV), dorsal aorta (DA), and posterior 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 ($\#p < 0.001$; $n = 20$).

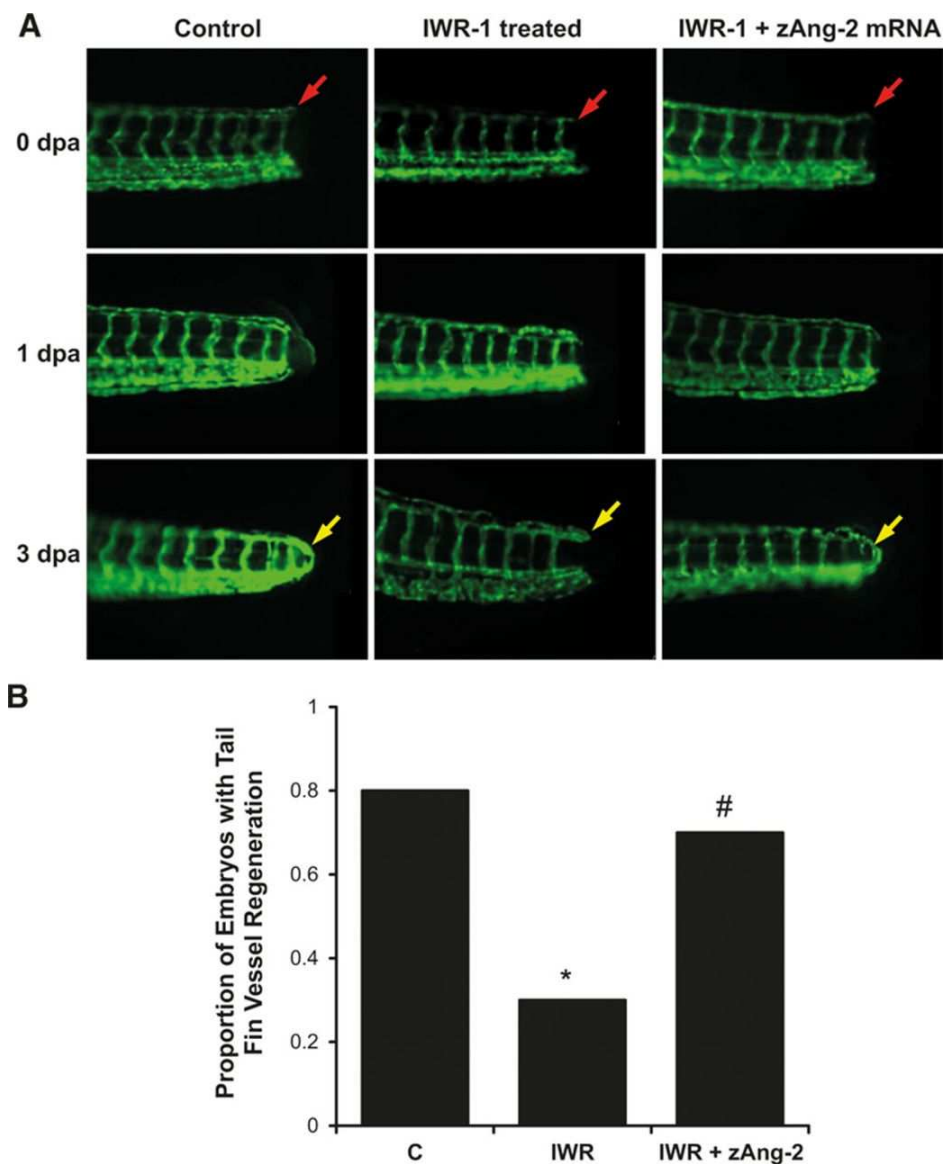
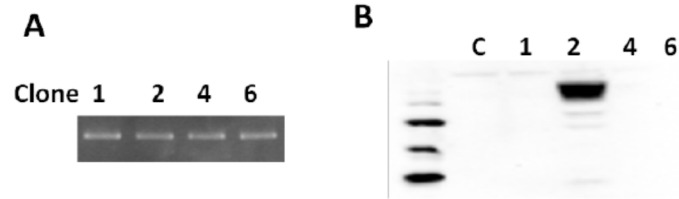
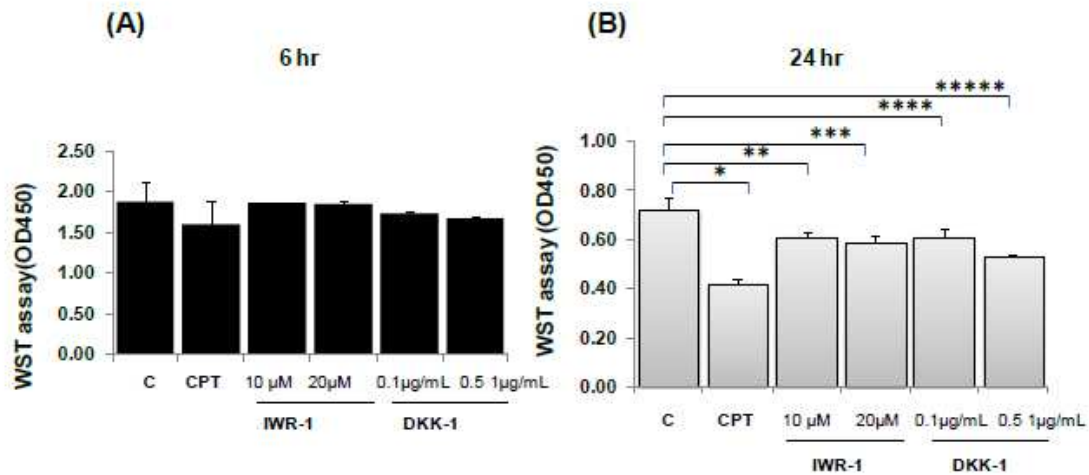


Figure 6. Wnt-Ang-2 signaling and vascular endothelial repair. (A) The tails of transgenic *Tg(kdrl:GFP)* zebrafish embryos were amputated at 72 hours post fertilization. At 0 day post amputation (dpa), the red arrow pointed to the site of injury. At 1 dpa, initiation of endothelial repairs was present. At 3 dpa, complete tail repair was observed, as indicated by the yellow arrow. IWR-1 treatment (10 $\mu\text{mol/L}$) 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

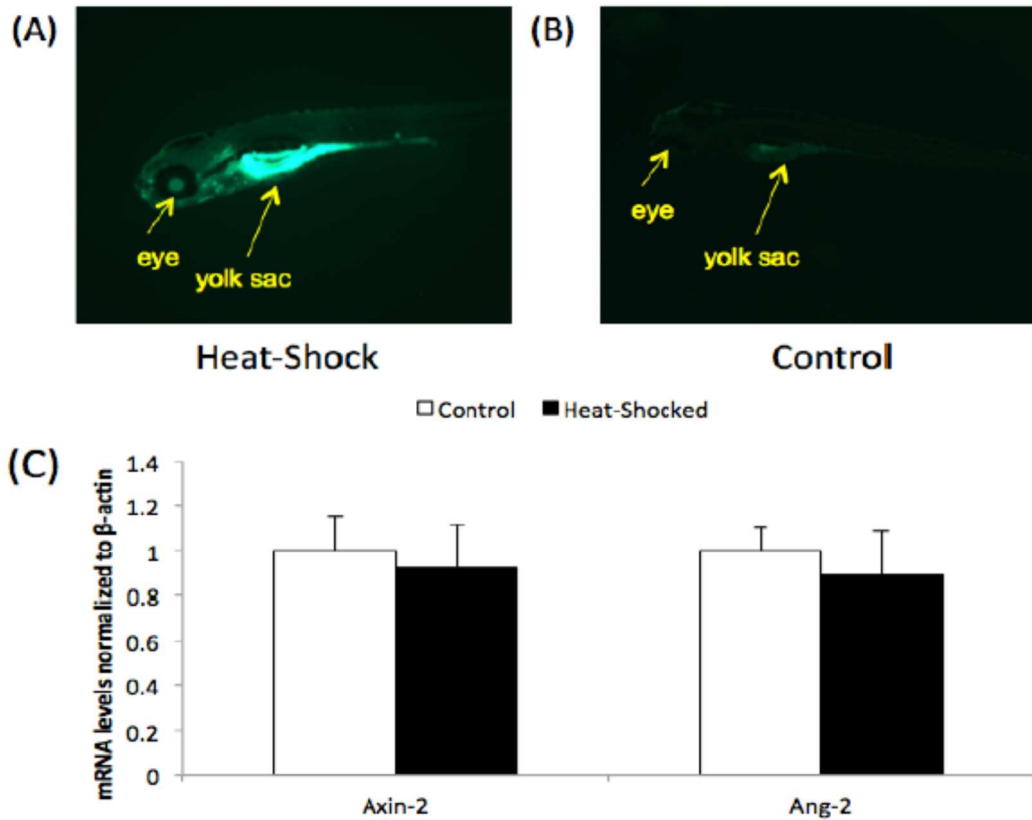
treatment group, and each fish was assessed for the presence of tail repair at 3 dpa. The proportion of fish exhibiting tail repair in each group showed a significant difference between the control and IWR-1 treatment conditions (* $p < 0.01$; $n = 20$) and between IWR-1 treatment with and without zAng-2 mRNA injection (# $p < 0.05$; $n = 20$).



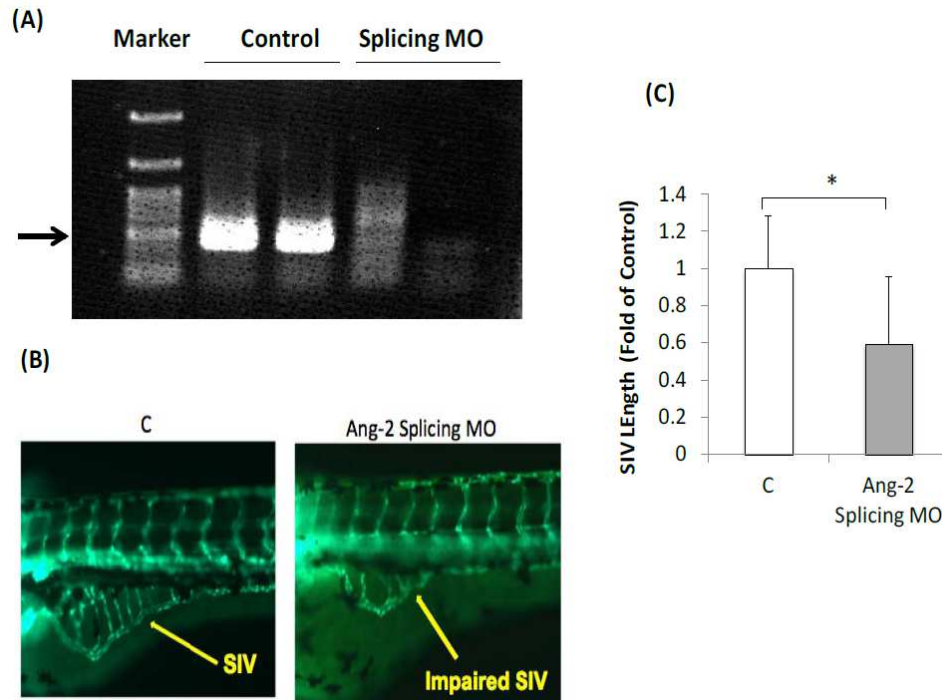
Supplemental Figure I. Verification of HA-tagged zAng2 expression. (A) pCS2-zAng-HA plasmids were transfected into HEK-293 cells. RNA was isolated to verify zAng2-HA mRNA expression by qRT-PCR. (B) zAng2-HA protein expression was verified by Western blots using anti-HA antibody.



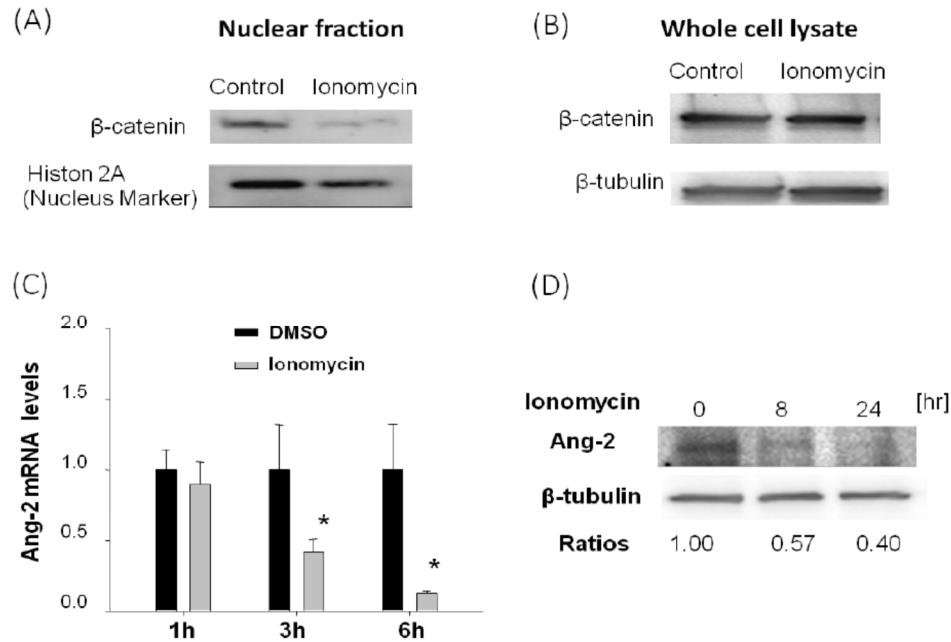
Supplemental Figure II. Inhibition of Wnt signaling and cell viability. (A) After 6 hours of incubation, IWR-1 and DKK did not significantly reduce cell viability ($p > 0.05$ vs. control, $n=3$). (B) After 24 hours of incubation, both IWR-1 (at 10 μ M and 20 μ M) and Dkk-1 (at 0.1 μ g/mL and 0.5 μ g/mL) 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.



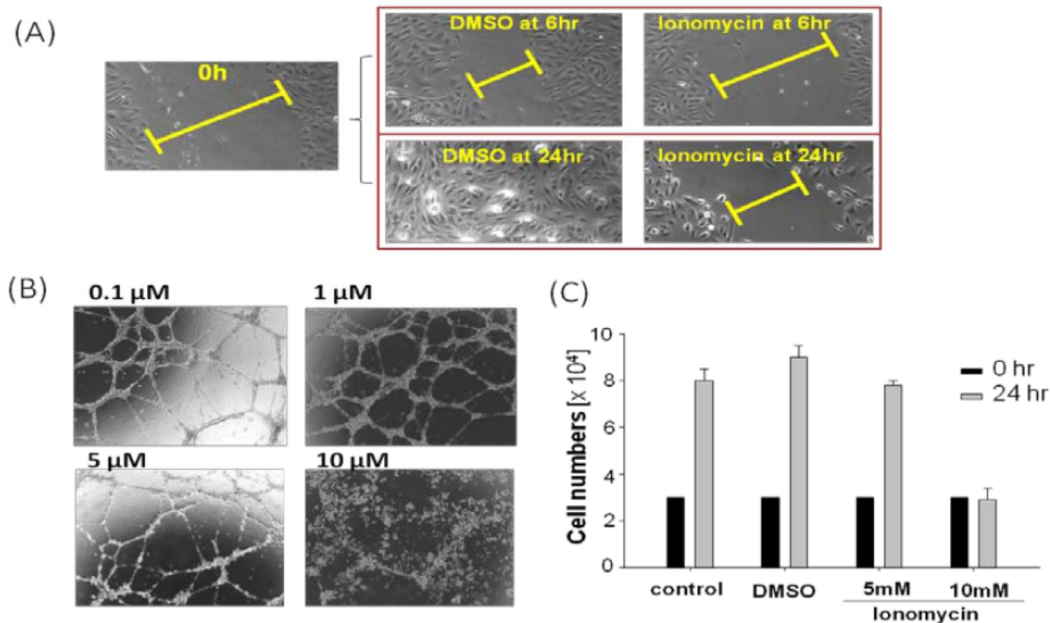
Supplemental Figure III. The effect of heat shock on non-transgenic fish on Axin2 and Ang-2 mRNA expression. (A) Heat-shock induction of the *Tg(hsp70l:Dkk1-GFP)* embryos at 48 hpf at 37°C for 1 hour resulted in an increase in DKK-1-GFP expression. (B) In the absence of heat-shock induction, DKK-1-GFP 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 ($p > 0.05$, $n=3$).



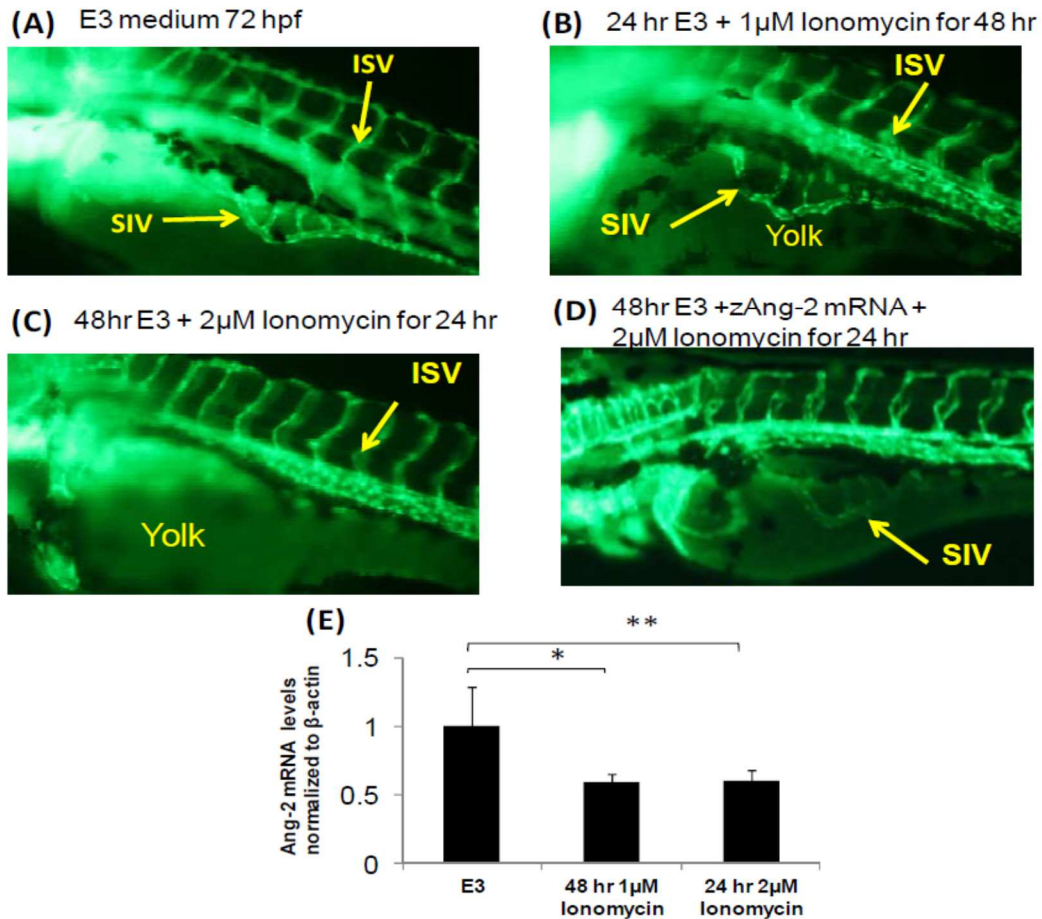
Supplemental Figure IV. zAng-2 splicing morpholino inhibited SIV formation. **(A)** RT-PCR was performed to validate the effect of Ang-2 splicing MO using primers that covered the exon 2-intron 2 boundary of Ang-2 (Lamont RE1, Vu W, Carter AD, Serluca FC, MacRae CA, Childs SJ. **Hedgehog signaling via 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 μ mol/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) 1 μ M Ionomycin starting at 24 hpf disrupted SIV formation, or (C) 2 μ M 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 2 μ M Ionomycin. (E) qRT-PCR revealed that treatment at 48 hpf with 2 μ M Ionomycin for 24 hr significantly down-regulated Ang-2 expression (* p < 0.01 vs. E3; ** p < 0.01 vs. E3, n=3).

■ **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.

Supplemental Table I. List of genes in the Stem Cell RT² Profiler™ PCR Array (SuperArray®). 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. Bioinformatics 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.

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