ROLE OF SHEAR STRESS IN ANGIOPOIETIN-2-DEPENDENT NEOVASCULARIZATION: IMPLICATIONS IN OCCLUSIVE VASCULAR DISEASE AND ATHEROSCLEROSIS

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ROLE OF SHEAR STRESS IN ANGIOPOIETIN-2-DEPENDENT NEOVASCULARIZATION: IMPLICATIONS IN OCCLUSIVE VASCULAR DISEASE AND ATHEROSCLEROSIS

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For my family, especially my grandmother
Maria Aline Fitzpatrick (1930-2007), my angel

"*joaninha, boa, boa*"

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LIST OF SYMBOLS AND ABBREVIATIONS

A	Aortic
Ang II	Angiotensin II
Ang1	Angiopoietin-1
Ang2	Angiopoietin-2
ANOVA	Analysis of Variance
ApoE	Apolipoprotein E
BAEC	Bovine Aortic Endothelial Cells
bFGF	basic Fibroblast Growth Factor
BrDU	Bromodioxyuridine
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CM	Conditioned Media
Cox-2	Cyclooxygenase 2
CT	Computed Tomography
Cy3	Cyanine 3
Cy5	Cyanine 5
D	Diameter
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid

EC	Endothelial Cells
eNOS	Endothelial Nitric Oxide Synthase
EPC	Endothelial Progenitor Cells
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothyiocyanate
G_0	Quiescent phase of the cell cycle
G_1	Phase of the cell cycle
GC	Greater Curvature
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HCL	Hydrochloric Acid
HDL	High Density Lipoprotein
HIF-1	Hypoxia Inducible Factor-1 alpha
HMEC	Human Microvascular Endothelial Cells
HMG-CoA	3-hydroxyl-3-methyl-glutaryl-Coenzyme A
HRP	Horse Radish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intercellular Adhesion Molecule - 1
kDa	kilodalton (unit of weight)
LC	Lesser Curvature
LDL	Low Density Lipoprotein
LDPI	Laser Doppler Perfusion Imaging
LS	Laminar Shear Stress
mmHg	Milimeters of Mercury (unit of pressure)

MMPs	Matrix Metalloproteinases
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NIH	National Institutes of Health
NO	Nitric Oxide
NS	Non-silencing siRNA
OS	Oscillatory Shear Stress
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PECAM-1	Platelet/Endothelial Cell Adhesion Molecule-1
PI3K	Phosphatidyl Inositol-3 kinase
PlGF	Placenta Growth Factor
pTyr	Phosphorylated Tyrosine
PVDF	Polyvinyldifluoride
Q	Flow
r	Radius
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
S	Synthesis phase of the cell cycle
SDS	Sodium Dodecyl Sulfate

SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
Si	Silencing siRNA
siRNA	Small Interfering Ribonucleic Acid
SMA	Smooth Muscle Alpha Actin
ST	Static
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TNF-α	Tumor Necrosis Factor - alpha
TSP-1	Thrombospondin-1
τ_w	Shear stress
Tyr	Tyrosine
V	Ventricularis
VCAM-1	Vascular Cell Adhension Molecule-1
VE	Vascular Endothelial
VEGF	Vascular Endothelial Growth Factor
VEGFR-2	Vascular Endothelial Growth Factor Receptor -2
VLDL	Very Low Density Lipoprotein
VSMC	Vascular Smooth Muscle Cells
vWF	von Willebrand Factor
WT	Wildtype
α	Cone Angle
μ	Viscosity

ν	Kinematic Viscosity
τ	Shear Stress
υ	Velocity
ω	Angular Velocity

SUMMARY

The formation of blood vessels is important in both normal physiological processes as well as pathophysiological processes. The main players in blood vessel formation are endothelial cells. Endothelial cells line the blood vessel wall and act as a barrier by inhibiting platelet aggregation and coagulation and allow for the selective transport of molecules from the blood into the tissue. In addition, the endothelium is highly influenced by the effects of hemodynamic flow and this may play an important role in blood vessel formation. Fluid flow causes shear stress which is sensed by the endothelium leading to the activation of many signaling pathways. Two typical types of shear stress found in the vascular tree are a unidirectional laminar shear stress (LS) found in straight regions and a disturbed or oscillatory shear stress (OS) found at branches or curves. Both types of shear stress lead to the activation of different signaling pathways. At the cellular and molecular level, LS is thought to promote endothelial cell quiescence; laminar sheared endothelial cells are anti-proliferative, anti-apoptotic, and anti-thrombotic. OS is thought to promote endothelial cell dysfunction; oscillatory sheared endothelial cells are pro-proliferative, pro-migratory, pro-thrombotic, and secrete growth factors that stimulate smooth muscle cell proliferation and migration. The secretion of growth factors from dysfunctional endothelial cells could also play a role in neovascularization. The detailed mechanisms resulting in neovascularization remain uncertain.

As such, this project focuses on understanding how shear stress regulates neovascularization and the physiological implications *in vivo*. There are several human

diseases that involve both disturbed shear stress and neovascularization, such as atherosclerosis, aortic valve disease, and occlusive vascular disease. In these pathophysiological scenarios fluid shear stress may provide a driving force for neovascularization. **The *objective* of this project is to investigate how shear stress mediates endothelial cell functions such as neovascularization and to identify how shear-induced factors, in particular Angiopoietin-2 (Ang2), play a role physiologically and pathophysiologically. The *overall hypothesis* of this work is that oscillatory shear stress promotes greater neovascularization compared to unidirectional laminar shear stress through the secretion of angiogenic factors, which play a physiological role in neovascularization *in vivo*.** This hypothesis was tested using *in vitro* and *in vivo* approaches.

During neovessel formation, endothelial cells must migrate and form tubules through which blood can flow. Therefore, we examined the effect of shear stress on endothelial cell migration and tubule formation. For the first time, we were able to show that OS promotes greater tubule formation and migration of endothelial cells as compared to LS and this was mediated through secreted factors. Taken together, OS promotes greater proliferation, migration, and tubule formation than LS, suggesting that OS may play an important role in promoting neovascularization.

To identify a molecular mechanism for shear-mediated migration and tubule formation, we first examined vascular endothelial growth factor receptor-2 (VEGFR-2) because it has been shown to be activated by shear stress independent of ligand binding. We found that inhibiting VEGFR-2 blunted OS-mediated tubule formation as well as inhibited migration. OS also stimulated the phosphorylation of VEGFR-2 at tyrosine

1175, a site that is known to initiate the ERK1/2 pathway and proliferation. This data suggests that OS may promote tubule formation and migration through the phosphorylation of VEGFR-2 at tyrosine 1175 leading to the subsequent ERK1/2 signaling pathway and proliferation. However, the VEGFR-2 pathway has been highly studied and we wanted to determine a more novel pathway involved in shear-mediated neovessel formation. In addition, VEGFR-2 inhibition did not completely block tubule formation and migration, suggesting that other factors may be playing a role.

Gene and protein array analysis was performed to determine a novel mediator of shear induced neovessel formation. From gene and protein array analysis, we identified several angiogenic genes that were shear-sensitive. In particular, Ang2 was upregulated by OS compared to LS on both gene and protein arrays. We found that inhibiting Ang2 blocked OS-mediated tubule formation and migration and that LS-inhibited tubule formation could be rescued by addition of Ang2. In addition, Ang2 was found to be upregulated at sites of disturbed flow *in vivo*, implicating a physiological role for Ang2.

To further investigate the physiological role of Ang2 in neovascularization, we examined the effects of inhibiting Ang2 in a mouse model of hindlimb ischemia. In the hindlimb ischemia model, the femoral artery is ligated blocking flow into the mouse hindlimb. In the adductor muscle, increased flow is directed through collaterals bypassing the occlusion, resulting in decreased and retrograde flow in the post-occlusive site with minimal ischemia as compared to the lower hindlimb. Therefore this is a good model involving both disturbed flow and neovascularization. We found that Ang2 is upregulated in the adductor muscle of the ischemic hindlimb suggesting that it plays a role in recovery from femoral artery ligation. In addition, we found that inhibiting Ang2

decreased reperfusion or blood flow recovery. To determine the mechanism by which Ang2 inhibition impairs recovery, we examined neovascularization in the ischemic adductor muscle. Ang2 inhibition resulted in decreased smooth muscle cell coverage of vessels as well as decreased macrophage infiltration. These findings suggest that Ang2 promotes blood flow recovery through the recruitment of smooth muscle cells and formation of collaterals, as well as the recruitment of macrophages that secrete important growth factors and help degrade the extracellular matrix in order for neovascularization to occur.

In this dissertation, we have identified an important mediator of shear stress-induced neovessel formation that plays an important physiological role *in vivo.* Identifying factors that play an important role in neovascularization at sites of disturbed flow could help provide localized therapies at sites of disease. This work illustrates the shear stress regulation of neovessel formation through the expression of angiogenic factors, in particular Ang2, and the role of Ang2 in neovascularization *in vivo*. This work has implications toward several cardiovascular diseases which are the number one killers in the United States. By understanding how angiogenic factors are regulated and what role they play *in vivo*, we can better understand human disease and develop important therapeutic targets.

CHAPTER 1

INTRODUCTION

This review will focus on the role of shear stress in blood vessel formation and its implications in human diseases, such as atherosclerosis, occlusive vascular disease, and aortic valve disease, as well as possible molecular mediators of shear stress induced neovascularization.

Shear Stress

Fluid shear stress is the tangential force created by fluid moving over a surface. In the cardiovascular system this corresponds to the dragging force created by blood as it moves over the endothelium[1]. For Newtonian fluids, or fluids that exhibit a linear relation between stress and strain rate, fluid flow is governed by a set of equations called the Navier-Stokes equations[1]. In larger diameter vessels, blood can be considered a Newtonian fluid because the size of the cells and proteins within the blood are so small compared to the diameter of the vessel[2]. The shear stress (τ) may be computed from an equation showing that the viscosity (μ) and velocity gradient ($\partial u/\partial r$) are proportional to the shear stress:

$$\tau = \mu \, \partial u/\partial r$$

For steady laminar flow in a straight tube the shear stress at the wall (τ_{wall}) can be described by:

$$\tau_{wall} = 32\mu Q/\pi D^3$$

Where μ is the viscosity, Q is the flow and D is the diameter of the vessel[2].

At the cellular level, the endothelium is what senses the mechanical forces from blood shear stress. Endothelial cells (EC) are known to align with the direction of

unidirectional laminar flow, however in oscillatory flow this alignment is eliminated and the cells are oriented randomly. The mechanical force from shear stress is sensed by "mechanoreceptors" on the cell's surface which either transmit the force/signal through the intracellular cytoskeletal filaments that connect to organelles, nucleus, as well as junctional complexes, integrins and focal adhesions, or activate a biochemical pathway that can lead to altered gene expression[3]. This distribution of luminal signal throughout the entire cell leads to activation of receptors and cell signaling molecules at sites distant from the source of the stimulus, particularly at sites of mechanical attachment such as cell-to-cell junctions or cell-matrix junctions which lead to activation of many signaling pathways.

There are several mechanoreceptors or the molecules implicated in mechanotransduction of fluid shear stress into a biochemical signal. Mechanosensitive ion channels are thought to be mechanoreceptors which are regulated by changes in membrane tension[4]. As mentioned previously, focal adhesions and integrins can also serve as mechanoreceptors. They are the sites responsible for cell attachment and are involved in many cell signaling pathways. When shear stress is applied force is transmitted through cytoskeletal elements that connect to focal adhesions and integrins resulting in activation of signaling pathways. Other mechanoreceptors include G-Protein linked receptors and tyrosine kinase receptors[3].

Neovascularization: Angiogenesis and Arteriogenesis

Blood vessels form through three different mechanisms: vasculogenesis, angiogenesis and arteriogenesis. Blood vessels in the embryo form through vasculogenesis which is the *in situ* differentiation of undifferentiated precursor cells

(angioblasts) to endothelial cells. New vessels in the adult occur mainly by angiogenesis, which is the sprouting of endothelial cells from pre-existing postcapillary venules. There are three mechanisms of angiogenesis: sprouting, intussusception (venule becomes divided by pillars of periendothelial cells) or transendothelial bridges which then split into individual capillaries[5]. There are two different definitions of arteriogenesis. Some define arteriogenesis as the maturation of new endothelium-lined channels via the recruitment of smooth muscle cells[5]. Others define it as the enlargement of preexisting collateral vessels, and not the formation of new arteries[6]. The former may be more applicable in normal growth, whereas the latter may be more applicable to pathological conditions.

The exact molecular mechanisms that trigger neovascularization remain unclear. Molecules such as vascular endothelial growth factor (VEGF) and its receptor (VEGFR2), Angiopoietin 1 (Ang1), Angiopoietin 2 (Ang2), Tie2, and hypoxia inducible factor-1α (HIF-1α) are all thought to play a role[5]. Once initiated, angiogenesis begins with vasodilation involving nitric oxide (NO). Vascular permeability increases in response to VEGF which allows the extravasation of plasma proteins that can lay down a provisional scaffold for endothelial cells to migrate on. Ang1, a Tie2 activating ligand, is an inhibitor of vascular permeability and thought to prevent excessive leakage of vessels[7]. Endothelial cells then need to loosen interendothelial cell contacts or become destabilized in order to migrate. Ang2, a Tie2 inactivating ligand, is thought to play a role in this process and in promoting endothelial cells to migrate[8]. Proteinases such as matrix metalloproteinases (MMPs), degrade the matrix and liberate growth factors that may be sequestered in the extracellular matrix[9].

Once the endothelial cells have been activated and the matrix degraded endothelial cells proliferate and migrate. Endothelial cells first form as a solid cord and then acquire a lumen. Once assembled into new vessels the endothelial cells enter a stabilization phase where endothelial cell proliferation is arrested and the endothelial cells become quiescent, there is reconstruction of the basement membrane around the neovessel, and lastly, the new capillary is covered with pericytes. Hemodynamics are essential for endothelial cell survival, as normal shear stress reduces endothelial cell turnover and inhibits TNF-α which causes apoptosis[5].

Neovascularization and Shear Stress

Fluid shear stress is thought to play a role in angiogenesis and arteriogenesis. Under healthy conditions, normal levels of shear stress promote endothelial cell quiescence. However, changes in the normal shear pattern and the integrity of the endothelium, such as higher than normal shear stress, low and oscillatory shear stress or wounding, may disrupt endothelial quiescence and promote neovascularization.

Shear stress at approximately 3 dyn/cm^2 has been found to promote microvascular endothelial cell microvessel formation in collagen gels[10] and enhance human umbilical vein endothelial cell and human coronary artery endothelial cell wound closure in vitro[11]. In addition, higher levels of shear stress are thought to promote capillary angiogenesis in exercised trained muscle[12, 13]. At the microvessel level, unidirectional laminar shear stress, depending on the level of shear stress, appears to promote angiogenesis.

The majority of research investigating the effects of disturbed flow on angiogenesis focuses on migration and proliferation, and little research has been

conducted on the effects of disturbed flow on endothelial tubule formation. Tardy et al. studied the effects of unidirectional laminar flow and disturbed flow on endothelial cell migration and proliferation through time-lapse video microscopy over a 48 hour period[14]. They observed in a uniform laminar flow field that cells continually rearranged their relative position with no net migration. However, in a disturbed flow field there was a net migration directed away from the region of high shear gradient, a two-fold increase in cell motility, and an increase in cell division. However, Hsu et al. using a similar flow system found that laminar shear stress enhanced EC migration into a wounded area whereas disturbed flow had less effect on wound healing[15]. Such discrepancies could be due to the effects of wounding or differences in experimental methods.

It is well established that laminar shear stress causes a reduction in the rate of EC proliferation and reduces the number of cells entering the cell cycle, with the majority of cells arrested in the G_0 or G_1 phase[16-18]. Contrastingly, disturbed flow causes an increase in the rate of EC proliferation[16, 19]. Thus, laminar shear stress protects the endothelium by reducing EC turnover and promoting endothelial quiescence.

Shear stress is also known to play a role in arteriogenesis. Arterial occlusion causes increased flow through collateral vessels and is known to cause the enlargement of these collaterals. The increased shear stress leads to the upregulation of adhesion molecules in the endothelium[20], the activation of monocytes which secrete TNF-α and VEGF and stimulate the endothelium to produce bFGF and PDGF[21]. However, these findings are somewhat contradictory because TNF-α is anti-mitogenic and increased shear stress increases the expression of NO synthase which leads to the increase expression of NO, which is known to downregulate VEGF and its receptor[21]. These

conflicting results could be due to the magnitude of shear stress and the length of shear stress, as chronic shear stress has different effects on the endothelium than short-term shear stress experiments.

In vivo experiments have also shown that shear stress plays a role in angiogenesis. Sho et al. created abdominal aortic aneurysm in mice that were exposed to low flow, high flow or left unchanged. They found that abdominal aortic aneurysms exposed to low flow had more adventitial capillaries and increased expression of VEGF, PDGF and GM-CSF as compared to aneurysms exposed to high flow[22]. Ichioka et al. found that increased blood flow enhanced wound-healing angiogenesis in the rabbit ear chamber[23].

There are several human diseases that involve both disturbed shear stress and neovascularization, such as atherosclerosis, aortic valve disease, and occlusive vascular disease. In these pathophysiological scenarios fluid shear stress may provide a driving force for neovascularization.

Atherosclerosis

Atherosclerotic cardiovascular disease is one of the leading causes of death in the western world[24]. In the United States alone, nearly 2600 people die of cardiovascular disease each day, an average of 1 death per 34 seconds[24]. The increased prevalence of cardiovascular disease suggests a need for further research of the entire atherosclerotic process and therapies that address early detection and prevention.

Atherosclerosis is a focal disease that occurs in areas of the arterial system exposed to disturbed flow causing unstable and oscillatory wall shear stress. This disturbed flow occurs at branched, bifurcated and curved arteries such as the carotid

bifurcation, the lesser curvature of the ascending aorta, and the coronary arteries. At these sites, the initiation of atherosclerosis is thought to be a "response-to-injury" that leads to endothelial dysfunction[25]. Possible causes of endothelial dysfunction include hypercholesterolemia, especially oxidized LDL, smoking, hypertension, diabetes mellitus, genetic diseases, and elevated plasma homocysteine levels[25]. In response to one or a combination of these risk factors or "injuries", the endothelium becomes dysfunctional and increases the expression of inflammatory adhesion molecules, secretes cytokines and growth factors. The progression of atherosclerosis is marked by the accumulation of lipid-laden macrophages, also called foam cells, within the intimal layer of the artery wall, the proliferation and migration of smooth muscle cells, and the appearance of neovessels within the developing plaque, also called plaque angiogenesis. Continued inflammatory cell infiltration can cause the plaque to become unstable and rupture, leading to thrombosis and embolism causing heart attack or stroke.

Atherosclerosis and Shear Stress

As mentioned previously, atherosclerosis preferentially occurs in regions of the arterial system exposed to low and oscillatory shear stress, whereas straight arterial regions exposed to unidirectional laminar shear stress are well protected from atherosclerosis development. These two different shear waveforms, oscillatory shear stress and laminar shear stress have been termed "atheroprone" and "atheroprotective", respectively. Caro et al. in 1969 was the first to identify the correlation between low shear stress and atherosclerosis development[26]. Zarins et al. further investigated this

concept by using a scale human carotid bifurcation model that showed disturbed flow in areas that correlated to atherosclerosis found at autopsy[27].

Atherosclerosis and Neovascularization

Vulnerable plaques are characterized by a large lipid core, thin fibrous cap, inflammation at the shoulders of the cap, decreased smooth muscle cells within the cap, and increased angiogenesis within the intima and media[28]. Neovessels within the plaque make it unstable, as they are prone to rupture and cause hemorrhage. Therefore, preemptive intervention strategies to stop the progression of these high risk lesions, such as anti-angiogenic therapies, could help prevent heart attack or stroke.

The discovery that angiogenesis is found within atherosclerotic plaques occurred over a century ago, when it was noted that atheromas contained new blood vessels[29]. However, only in the past 20 years have researchers been investigating the link between neovascularization and atherosclerotic disease progression. In the 1980s, Silverman and colleagues conducted imaging studies in which silicone polymers were injected into fixed human hearts[30, 31]. Histological examination of these specimens found that neovessels were located in the media and thickened intima of atheromas and the extent of neovascularization correlated with the severity of disease[31]. Casts of the network of coronary arteries in human hearts have shown the presence of "extensive mesh-like vascular networks" near the lumen of atheromatous vessels[32].

Since these first initial studies, the majority of research on human plaque neovascularization has been conducted using histological sections of atherosclerotic plaques and staining for markers of endothelial cells, such as von Willebrand factor,

CD31 and CD34, to identify new blood vessels within the plaques[33]. Newly formed intimal blood vessels can originate from the adventitial vasa vasorum or the arterial lumen, although the adventitial blood vessels are much more common than the luminal blood vessels[34]. Kumamoto et al. found that the number of adventitial blood vessels increased with the severity of stenosis, whereas the number of luminal vessels was most frequent when the severity of stenosis reached 40% to 50% and were more frequently associated with intimal hemorrhage than blood vessels from the adventitia[34]. In addition, plaque neovascularization is most frequently found asymmetrically in the shoulder and fibrous cap region of atherosclerotic plaques and a small proportion of the vessels in the shoulder region originate from the arterial lumen[33]. The local expression of matrix degrading metalloproteinases has also been found in the shoulder region of atheromas[35], which could cause local proteolytic damage to micro-vessel walls, and together with the pulsatile forces from the arterial lumen would facilitate intraplaque hemorrhages. Hemorrhages in the plaque shoulder may contribute to further inflammation, plaque destabilization, rupture, and thrombosis leading to myocardial infarction or stroke.

The molecular and cellular mechanisms involved in plaque neovascularization remain poorly understood, although hypoxia, inflammation, and other cell types may be responsible for the expression of angiogenic factors. Hypoxia-driven plaque neovascularization would suggest that neovessels would be distributed at the lesion base arising from the adventitial vasa vasorum. Instead, neovascularization is localized asymmetrically in the shoulder and fibrous cap of atheromas which are supplied oxygen from the arterial lumen, suggesting that other factors may be driving plaque neovascularization. Inflammation may also be a driving force for plaque

neovascularization. Regions of marked neovascularization are also associated with accumulation of macrophages, mast cells, and T-cells[36]. However, whether this is a cause or a result of plaque angiogenesis is unclear because inflammatory infiltrates are common in initial atherosclerotic plaques where plaque angiogenesis is usually not found. However, the endothelium of intimal capillaries shows increased expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) which may allow entry of inflammatory cells into the intima[37]. Other cell types may also contribute to the expression of angiogenic factors, such as smooth muscle cells and possibly the endothelium overlying the atherosclerotic plaque. Most likely, it is a combination of all these factors that drives plaque neovascularization.

Interestingly, many factors involved in promoting atherosclerosis development are also thought to induce angiogenesis. Reactive oxygen species (ROS) generated by NADPH oxidase are known to be upregulated by oscillatory shear stress and are known to play an important role in promoting atherogenesis[38]. ROS are also thought to promote angiogenesis[39, 40]. In addition, HMG-CoA reductase inhibitors, also called statins, inhibit cholesterol formation and are a very common drug therapy for patients suffering from cardiovascular disease. Statins have also been found to be anti-angiogenic suggesting that one mechanism of action of statins is to inhibit plaque neovascularization and cause the regression of vulnerable plaques[41, 42].

Occlusive Vascular Disease

Atherosclerosis often leads to occlusive vascular diseases, such as myocardial infarction, stroke, and peripheral artery disease[43]. When unstable atherosclerotic plaques

rupture it can lead to thrombosis and blockage of the vessel[25]. The surrounding tissue then becomes ischemic from the lack of blood flow carrying vital oxygen[43]. The blockage of blood flow often leads to the formation of collaterals or 'natural bypasses' that circumvent the occlusion site[6, 43]. During occlusive vascular disease both angiogenesis and arteriogenesis occur. Whereas angiogenesis is believed to be driven by both hypoxia and fluid shear stress, arteriogenesis is believed to be driven only by fluid shear stress[6].

Occlusive Vascular Disease and Shear Stress

During occlusive vascular disease, fluid shear stresses in the vasculature change drastically. Whereas fluid flow increases in the collaterals bypassing the occlusion, fluid flow is low and reversed in the post-occlusive site[6]. When a vessel is occluded, blood pressure drops in the post-occlusive site. This creates a steep pressure gradient and increases the flow in pre-existent arterioles towards the area of low pressure. In some areas of the post-occlusive site the flow becomes reversed[6].

During arterial occlusion, the surrounding vessels are exposed to changes in several mechanical forces, such as fluid shear stress and wall stresses as a result of blood pressure. An increase in blood pressure resulting in an increase in circumferential wall stress is known to trigger the proliferation of smooth muscle cells and this may contribute to the remodeling process[6]. However, Pipp et al. found that arteriogenesis occurring after femoral artery occlusion was driven mainly by changes in fluid shear stress and not by changes in blood pressure, implicating fluid shear stress as an important mediator of blood vessel remodeling during occlusive vascular disease[44].

Occlusive Vascular Disease and Neovascularization

Both angiogenesis and arteriogenesis are important during occlusive vascular disease to relieve the subsequent ischemia. Arteriogenesis occurs in pre-existing vessels to circumvent the occlusion and angiogenesis occurs in the post-occlusive site. Arteriogenesis occurs independently of the presence of hypoxia and is mainly driven by shear stress[43]. In contrast, angiogenesis is known to be driven by both shear stress and hypoxia[43]. During hypoxia, hypoxia inducible factor-1 (HIF-1) is expressed leading to the transcription of the potent angiogenic factor VEGF-A[43]. Changes in shear stress may lead to the expression of other factors and the combination of hypoxia and locally secreted factors may initiate capillary tube formation.

The changes in fluid shear stress during occlusive vascular disease lead to activation of the endothelium. Genes are upregulated that code for cytokines, growth factors, and adhesion molecules[45-47]. Monocytes adhere and invade the collateral wall where they produce growth factors and proteases[48]. Invading monocytes play a critical role in arteriogenesis by stimulating the proliferation of smooth muscle cells and degrading extracellular matrix to allow for migration of cells.

During arteriogenesis collateral arteries do not develop *de novo* but develop from pre-existing vessels[45]. The collateral vessels are much more efficient at rescuing fluid flow than the smaller capillaries. The contribution of angiogenesis to rescuing fluid flow is almost negligible when compared to arteriogenesis[45]. However, angiogenesis still plays an important role in rescuing tissue from ischemia in localized regions.

Aortic Valve Disease

The aortic valve experiences very complex fluid flow and shear stresses. The fibrosa side of the leaflet, or the side facing the aorta, experiences a low and disturbed shear stress and the ventricularis side of the leaflet, or the side facing the ventricle of the heart, experiences a high unidirectional shear stress[49]. Aortic valve disease often results in calcification of the valve and this calcification is often associated with neovascularization[50]. The aortic valve is normally avascular, however when diseased, neovessels can form and are thought to promote the progression of aortic valve disease leading to valve stenosis[50]. However, whether there is a correlation between shear stress and neovascularization in the valve leaflet is not known.

Model Systems

In vitro Shear Stress Systems

The two most common systems to simulate arterial fluid shear stress on endothelial cells are the cone and plate viscometer and the parallel plate flow chamber.

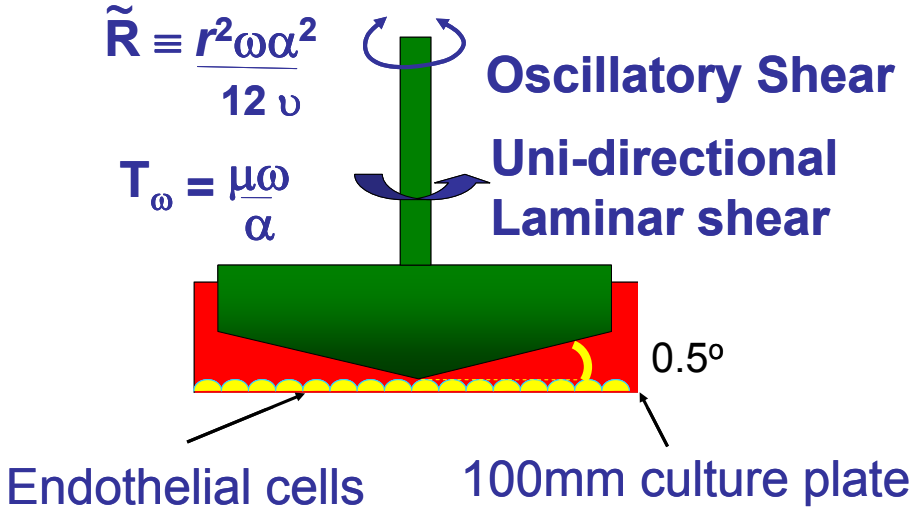

Figure 1.1. Cone and plate shear apparatus.

With the cone and plate viscometer, endothelial cells are grown in a monolayer on a circular tissue culture dish and a cone is placed into the medium (Figure 1.1). The cone is rotated at a speed to obtain uniform arterial levels of shear stress[1]. The angle of the cone is very small and rotation of the cone forces flow between the cone and plate to flow azimuthally which produces a shear stress on the stationary endothelial cell monolayer[1]. Governing equations have been derived from the Navier-Stokes equations for this system and have determined the limits to which this system can produce laminar arterial flow profiles[1]. Secondary flow can occur at larger rotational speeds, but are neglible at the speeds used in our lab[51].

In the parallel plate flow chamber, cells are grown on glass slides which are placed in a chamber. Medium is then pumped through the chamber to provide uniform levels of shear stress[52]. A comparison between the cone and plate viscometer and the parallel plate flow chamber reveals many advantages and disadvantages of each system. The cone and plate viscometer can shear a larger number of cells in each plate than can be sheared on the glass coverslips. This is very useful when collecting RNA and protein for analysis. The cone and plate viscometer can also be used to simulate reversal of flow by rotating the cone back and forth. However, the cone and plate viscometer uses much less media than the parallel plate chamber, leaving questions about whether secreted growth factors and cytokines are more concentrated and have a non-physiological effect. Also, in the cone and plate viscometer the cells in the middle of the dish experience a lower shear stress than the rest of the dish. Our lab has chosen to use the cone and plate viscometer because of its ability to produce flow reversal.

***In vitro* Neovascularization Systems**

There are several *in vitro* models of neovascularization that examine functions important in angiogenesis such as proliferation, migration and the formation of tubules. Proliferation assays often examine the incorporation of a reagent into the DNA of proliferating cells, such as bromodeoxyuridine (BrDU), or use antibodies against antigens expressed during proliferation, such as Ki67 and proliferating cell nuclear antigen (PCNA)[53]. BrDU is a thymidine analog that is incorporated into the DNA during the synthesis phase[53]. Antibodies against BrDU then detect its presence in the DNA[53]. Ki67 is a nuclear antigen expressed during all stages of the cell cycle except G_0 and early G_1[53]. Both Ki67 and PCNA can be detected in cells using antibodies. Flow cytometry is also used to determine what phase of the cycle a cell is in such as G_0 quiescent phase or S phase[54].

Migration assays include scratch wound healing assays where endothelial cells are removed in a distinct area such as by a scratch, and then the movement of endothelial cells into the denuded area is examined[55]. Transwell filters or modified Boyden Chambers are also used for migration assays where cells are added to the top well and the migration through a filter into the bottom well is examined[56]. The latter assay is often used to examine the chemotaxis of cells toward a certain reagent or factor. Other migration assays include time-lapse video microscopy and colloidal gold cell tracking.

Tubule formation assays examine the ability of endothelial cells to form tubes similar to the formation of capillaries *in vivo*. In tubule formation assays, endothelial cells are placed either within or on top of a substrate that is similar to the extracellular matrix found *in vivo*, such as a collagen gel, fibrin gel, or a substance called Matrigel[57].

Matrigel is an extracellular matrix derived from a mouse tumor and contains a multitude of growth factors. Matrigel can also be purchased with reduced growth factor content. The limitations of *in vitro* angiogenesis assays are that they do not fully simulate the formation of functional blood vessels as a whole; they only simulate individual parts of the angiogenesis process. Therefore, *in vivo* models are important to fully examine the neovascularization process.

In vivo Shear Stress and Neovascularization Systems

There are many models to examine fluid shear stress *in vivo*, and in some of these models the effect of shear stress on neovascularization can be examined. The natural geometry of the vascular system can be examined to look at different flow regions such as at bifurcations in larger arteries including the carotid artery and branches in the coronary arteries. The aortic arch is a good region to examine the effects of different types of shear stress. The lesser curvature of the arch is exposed to a disturbed shear stress, whereas the greater curvature and the descending aorta are exposed to a unidirectional laminar shear stress[58]. Using histological sectioning or *en face* immunostaining, the different flow regions can be examined[58]. In addition, in the presence of advanced atherosclerosis, neovascularization can also be examined in the adventitia[59]. Other models look at the effect of altering fluid flow such as by completely blocking fluid flow or by partially blocking fluid flow.

Flow Cessation Models

Flow cessation models look at the effect of blocking blood flow on vessel remodeling. In the carotid ligation model, the commom carotid artery is ligated and the subsequent vascular remodeling is examined[60]. This model causes severe inflammation with macrophage infiltration and intimal hyperplasia. Neovessels may also be present in the vascular wall.

In the hindlimb ischemia model, the superficial femoral artery is ligated proximal to the branching of the deep femoral artery and proximal to the branching of the tibial arteries and is then removed[61]. In this model, collateral vessels form to bypass the occlusion and angiogenesis occurs in the post-occlusive site. Therefore this is a good model to examine the effects of altered shear stress on two different types of blood vessel formation: arteriogenesis and angiogenesis. Unfortunately, at this time shear stress cannot be quantified in small pre-existing collaterals due to technical limitations so it is not feasible to determine the exact shear stresses occurring during femoral artery ligation[43]. Therefore, there is not direct evidence that shear stress is playing a role in neovascularization only inferential evidence.

Low Flow Models

Low flow models examine the effect of reducing blood flow on vascular remodeling. In the partial carotid ligation model, three of the branches off the common carotid artery are ligated and only one is left open[62]. This causes reduced flow through one carotid and increased flow in the other carotid, and the effects of low flow and increased flow can be examined. The non-ligated carotid experiences an increase in

lumen diameter whereas the low flow side experiences an increase in adventitia, medial and intimal thickness[62]. Neovascularization can be examined in the vascular wall.

Another model similar to the partial carotid ligation model is the carotid cuff model where a cuff is placed over the carotid artery to create reduced flow. In this model there is reduced shear stress upstream of the cuff, increased shear stress within the cuff and an oscillatory, disturbed shear stress downstream of the cuff[63]. This model also examines several different types of shear stress. Neovascularization can also be examined in the vascular wall. The disadvantage of these models is that it is difficult to determine if shear stress has a direct effect on neovascularization or an indirect effect possibly mediated through ischemia or inflammation.

Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2)

The vascular endothelial growth factor (VEGF) family has been implicated in vascular development and neovascularization in physiological and pathological conditions, including cancer, rheumatoid arthritis, ocular neovascular disorders and cardiovascular disease[64]. The VEGF family consists of five members, VEGF- A, B, C, D, E and placenta growth factor (PlGF)[65]. VEGFs bind to three different receptors: VEGFR-1 (also known as Flt-1), VEGFR-2 (also known as KDR in humans, Flk-1 in mice), and VEGFR-3. VEGF-B and PlGF bind only to VEGFR-1 and VEGF-A can also bind VEGFR-1; VEGF-A, VEGF-C, VEGF-D, and VEGF-E all bind VEGFR-2; VEGF-C and VEGF-D primarily bind to VEGFR-3[65]. However, we are primarily interested in VEGFR-2 because it has been implicated in diseases involving deregulated angiogenesis[66] and has been shown to be shear stress sensitive[67].

VEGFR-2 is a 200-230 kDa high affinity receptor for VEGF-A. The receptor is crucial for vascular development as VEGFR-2 null mice die at embryonic day 8.5-9.5 due to defects in the development of endothelial and hematopoietic precursor cells and vasculogenesis[68]. VEGFR-2 can be autophosphorylated through a ligand-dependent and ligand-independent manner. Currently, the following sites have been identified as autophosphorylation sites: Tyr801, Tyr951 and Tyr996 present in the kinase insert domain, Tyr1054 and Tyr1059 in the kinase domain, and Tyr1175 and Tyr1241 in the C-terminal tail[65].

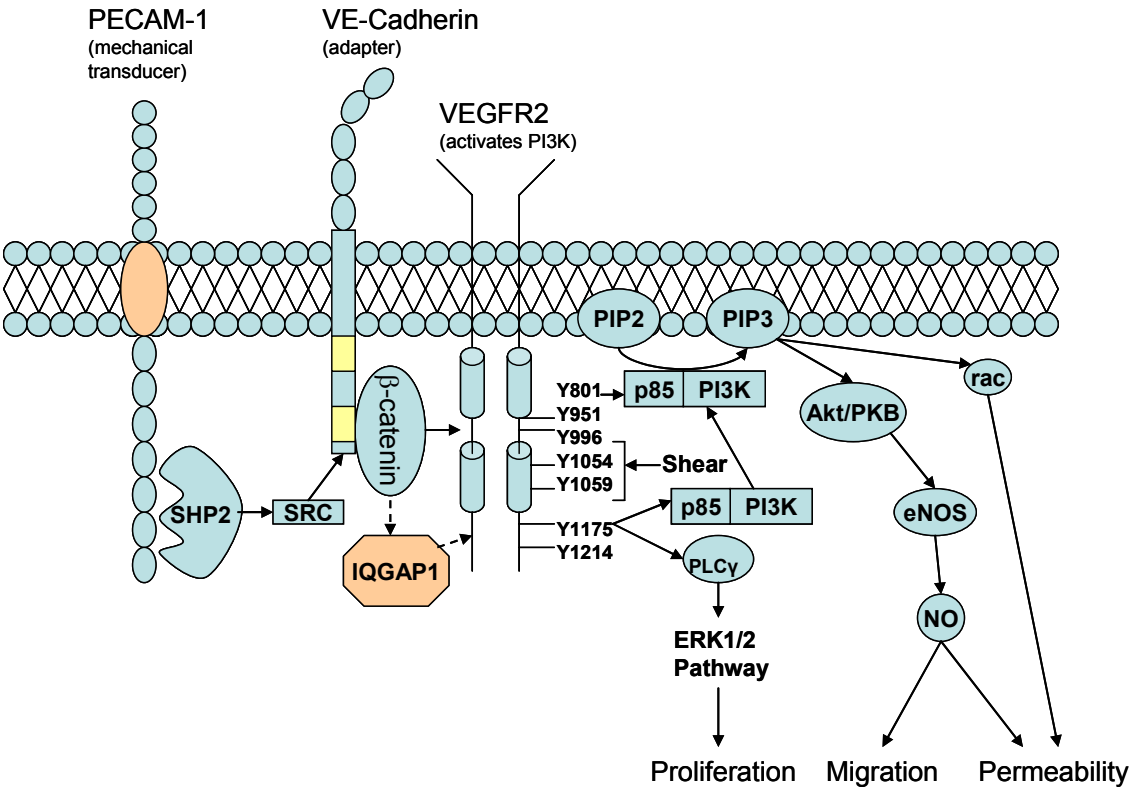

Figure 1.2. VEGFR-2 shear-mediated signaling pathways[67,69].

VEGFR-2 mediates several physiological effects including proliferation, survival, migration, and vascular permeability. VEGFR-2 can initiate proliferation through the classical ERK1/2 pathway leading to gene transcription[65]. This is most likely mediated through Tyr1175 which when phosphorylated can bind PLCγ leading to the ERK1/2 pathway[65]. VEGFR-2 also activates the Akt/PKB pathway and Rac pathway through binding of Tyr1175 or Tyr801 sites to the p85 sunbunit of PI3K[69] which then converts phosphatidylinositol (4,5)P_2 (PIP_2) to phosphatidylinositol(3,4,5)P_3 (PIP_3)[70]. The Akt/PKB pathway is important in inhibiting apoptosis and it also activates endothelial nitric oxide synthase (eNOS) which generates NO to increase vascular permeability and migration[71].

VEGFR-2 is part of a mechanosensory complex that can sense fluid shear stress leading to ligand-independent activation (Figure 1.2)[67, 69]. Vascular endothelial cadherin (VE-cadherin) is known to form a complex with VEGFR-2 and includes β-catenin which is phosphorylated and translocates to the nucleus in VEGF-A stimulated cells[72, 73]. Tzima et al. identified that PECAM-1 also forms a complex with VEGFR-2 and VE-cadherin, and acts as the "mechanical transducer" that senses the force of shear stress and transmits the signal to VE-cadherin, "the adapter", which activates VEGFR-2 leading to the subsequent signaling pathways[69]. Unidirectional laminar shear stress transiently activates VEGFR-2 through phosphorylation of Tyr1054/1059 and after 30 minutes of shear the receptor is de-phosphorylated[67, 69, 74]. The role of oscillatory shear stress in VEGFR-2 activation has not been studied. Laminar shear stress often rapidly and transiently activates signaling pathways, whereas oscillatory shear stress often chronically activates

these signaling pathways. Therefore, oscillatory shear stress may chronically activate VEGFR-2 through specific phosphorylation sites leading to proliferation and migration.

IQGAP1 has been identified as a novel binding partner of VEGFR-2. Yamaoka-Tojo et al. identified IQGAP1 through a yeast two-hybrid system screening for proteins that bind the cytoplasmic tail of VEGFR-2[75]. IQGAP1 is a scaffolding protein that is involved in migration and interacts directly with cytoskeletal elements[75]. IQGAP1 is also involved in VEGF mediated reactive oxygen species dependent migration and proliferation[75]. IQGAP1 may also play a role in shear mediated VEGFR-2 activation.

Angiopoietins

The Angiopoietins are a family of growth factors known to be essential in blood vessel formation. There are four known Angiopoietins, Angiopoietin-1 (Ang1), Angiopoietin-2 (Ang2), Angiopoietin-3 (Ang3), and Angiopoietin-4 (Ang4). Ang1 and Ang2 are the most well studied, Ang3 is found only in mice and Ang4 is its homolog in humans[76]. Ang2 is an antagonist of Ang1 and competes with Ang1 for binding to their shared receptor Tie2. Ang1 has been shown to clearly activate Tie2 autophosphorylation whereas Ang2 binding can both antagonize and agonize Tie2 receptor phosphorylation under varying conditions. Maisonpierre et al. identified Ang2 by homology screening and found that it is 496 amino acids long with six potential N-glycosylation sites and has a secretion signal peptide[77, 78]. Kim et al. identified an alternative splice variant of Ang2 called Ang2(443) because it lacked 53 amino acids found in the full length protein[78]. Ang2(443) is also secreted and retains 4 of the 6 N-glycosylation sites. Western blot analysis revealed that Ang2 and Ang2(443) are expressed as 68- and 61- kDa proteins

that after deglycosylation are 57 and 51- kDa, respectively[78]. Ang2 and Ang2(443) exist in a homodimeric form and under non-reducing conditions produce bands at ~ 80 and ~160 kDa for Ang2 and ~70 and ~140 kDa for Ang2(443)[78].

Overexpression of Ang2 in mice disrupted blood vessel formation in the mouse embryo and in adult mice Ang2 was only expressed at sites of vascular remodeling[77]. Ang2 knockout mice display early postnatal vascular remodeling defects in the eye[79] and show that Ang2 is not required for embryonic angiogenesis but is requisite for postnatal angiogenesis[80].

Ang2 has been found to stimulate the migration of endothelial progenitor cells (EPCs)[81]. Circulating EPCs are recruited to sites of injury and tumorigenesis and contribute to neovascularization. Hildbrand et al. found that CD34+CD11b+ EPCs are capable of autonomously producing and binding angiopoietins and that Ang2 enhances the expansion of this endothelial cell progeny[82].

Recently, it has been found that Ang2 is downregulated by laminar shear stress in an Akt and FOXO1 dependent manner[83]. Laminar shear stress results in the phosphorylation of Akt leading to increased Akt activity[84]. The transcription factor FOXO1 is then phosphorylated in an Akt-dependent manner leading to nuclear exclusion of FOXO1[83]. FOXO1 is a transcription factor for Ang2 and therefore the nuclear exclusion of FOXO1 causes decreased expression of Ang2[83]. Conversely, a decrease in Akt leads to an increase in FOXO1 and an increase in Ang2 expression. Interestingly, Ang2 expression activates the Akt pathway which then downregulates FOXO1, thus resulting in a negative feedback loop[85] (Figure 1.3). This negative feedback loop could

allow for the transient expression of Ang2. Tie2 is known to be upregulated by laminar shear stress; however, Ang1 is not affected by shear stress.

Ang2 is thought to be involved in several disease pathologies. Significant research has been conducted on Ang2's role in tumor angiogenesis. Local production of Ang2 is thought to induce glioma- and glioblastoma neovascularization[86]. Ang2 has been implicated in human gastric cancers[87], human prostate cancer[88], and human breast cancer[89]. Ang2 has also been implicated in macular degeneration, rheumatoid arthritis, osteoarthritis, and psoriasis[90-94]. Ang2 has also been found to be expressed in neovessels from advanced atherosclerotic plaques, but not early plaques, from human coronary and carotid arteries[95].

Inhibiting Ang2 may prove to be an effective treatment for these diseases. White et al. created a nuclease-resistant RNA aptamer that binds and inhibits Ang2 but not Ang1. Delivery of this aptamer inhibited bFGF-mediated neovascularization in the rat

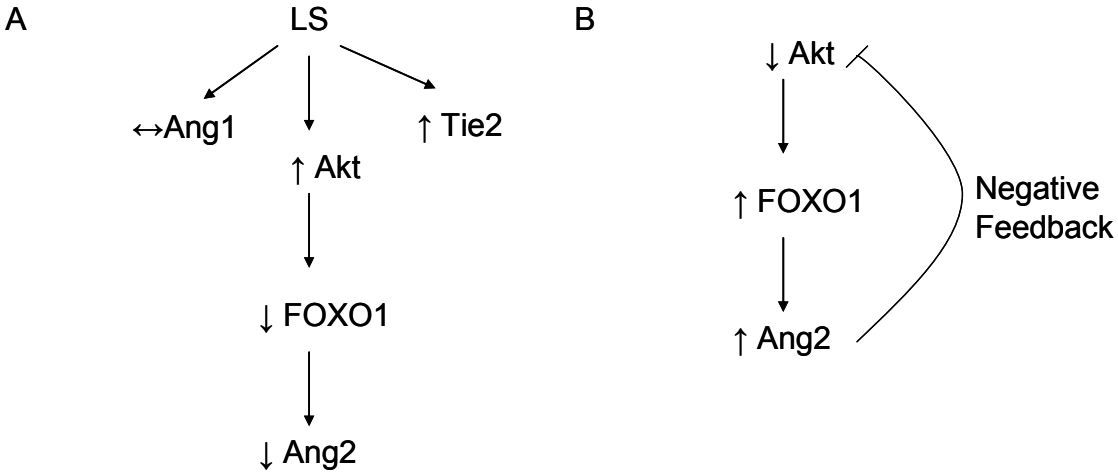

Figure 1.3. Ang2 signaling pathways. A, Mechanism of laminar shear stress (LS) regulation of Ang2. B, Negative feedback mechanism involved in Ang2 signaling [83-85].

corneal micropocket angiogenesis assay[96]. Oliner et al. generated an antibody and peptide-Fc fusion protein that potently and specifically inhibited the interaction between Ang2 and Tie2[97]. The Ang2-blocking agents were found to inhibit VEGF-stimulated neovascularization in the rat corneal micropocket assay[97]. In addition, systemic treatment of the Ang2-blocking agents to mice with tumors resulted in tumor stasis, followed by elimination of all measurable tumors[97]. These results indicate that a specific inhibitor of Ang2 can act as an anti-angiogenic agent and could be used as an effective anti-angiogenic strategy for patients with diseases involving impaired angiogenesis, such as atherosclerosis and occlusive vascular disease.

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CHAPTER 2

SPECIFIC AIMS

Introduction

Neovascularization, or blood vessel formation, has been implicated in many disease pathologies including cancer, atherosclerosis, and occlusive vascular disease. Shear stress, or the dragging force created by blood, is sensed by the endothelium and thought to play a role in the two main types of neovascularization: angiogenesis and arteriogenesis. Two typical types of shear stress found in the vascular tree are a unidirectional laminar shear stress (LS) which is found in straight arterial regions, or a disturbed oscillatory shear stress (OS) which is found at branches or curved regions. At the cellular and molecular level, laminar shear stress is thought to promote endothelial cell quiescence; laminar sheared endothelial cells are anti-proliferative, anti-apoptotic, and anti-thrombotic. Oscillatory shear stress is thought to promote endothelial cell dysfunction; oscillatory sheared endothelial cells are pro-proliferative, pro-migratory, pro-thrombotic, and secrete growth factors that stimulate smooth muscle cell proliferation and migration. The secretion of growth factors from dysfunctional endothelial cells could also play a role in neovascularization. The detailed mechanisms resulting in neovascularization remain uncertain. There are several physiological scenarios in which shear stress may play a role in blood vessel growth, such as in atherosclerosis, occlusive vascular disease, and aortic valve disease. Here, we will test the effects of shear stress, particularly oscillatory shear stress, on endothelial cell neovessel formation *in vitro* and investigate the possible role of shear stress on *in vivo* neovascularization.

Overall Hypothesis

Oscillatory shear stress promotes greater neovascularization compared to unidirectional laminar shear stress through the secretion of angiogenic factors, which play a physiological role in neovascularization *in vivo*. This hypothesis was tested using *in vitro* and *in vivo* approaches according to the following Specific Aims:

Specific Aim 1

Investigate the functional effects of shear stress on endothelial cell migration and neovessel formation

Hypothesis: Oscillatory shear stress promotes endothelial cell tubule formation and migration compared to laminar shear stress.

To determine the effects of shear stress on endothelial cell neovessel formation, we examined the direct effects of shear stress on endothelial cell migration and tubule formation and the indirect effects of shear conditioned media from aortic endothelial cells on microvascular endothelial cell migration and tubule formation. To do this, shear conditioned media or sheared endothelial cells (EC) were used to test whether tubule formation or migration is promoted or inhibited *in vitro* through Matrigel tubule formation and scratch migration assays.

Specific Aim 2

Identify a molecular mechanism involved in shear-induced neovessel formation

Hypothesis: Oscillatory shear stress promotes neovessel formation through a secreted factor.

To identify a molecular mechanism for shear-induced neovessel formation, we first examined the canonical vascular endothelial growth factor (VEGF) pathway. However, to identify a novel mechanism, we performed a high throughput DNA array specific to human angiogenic genes to evaluate the relative angiogenic gene expression between oscillatory- and laminar-sheared endothelial cells. We also performed a protein array with OS or LS conditioned media to determine secreted angiogenic proteins. The selected proteins were then blocked by an inhibitor or siRNA and tested in shear mediated migration and tubule formation assays.

Specific Aim 3

Determine the physiological relevance of Angiopoietin-2 in shear-mediated neovascularization, *in vivo*

Hypothesis: Inhibiting Ang2 impairs blood flow recovery during hindlimb ischemia by inhibiting angiogenesis and arteriogenesis.

From Specific Aim 2, Angiopoietin-2 (Ang2) was identified as a mediator of OS-induced tubule formation and migration. To establish the physiological importance of Ang2 in neovascularization, we examined the role of Ang2 in the hindlimb ischemia model, which is a model similar to occlusive vascular disease. In the hindlimb ischemia model, the femoral artery is ligated resulting in increased flow in collaterals and low and reversed flow in the post-occlusive site. In this model, Ang2 was inhibited and the effects on blood flow recovery and neovessel formation were examined.

The goal of this project is to investigate how shear stress mediates endothelial cell functions such as neovascularization and to identify how shear mediated proteins, in particular Angiopoietin-2, play a role physiologically. By understanding how Angiopoietin-2 is regulated and what role it plays *in vivo*, we can better understand human disease and develop important therapeutic targets.

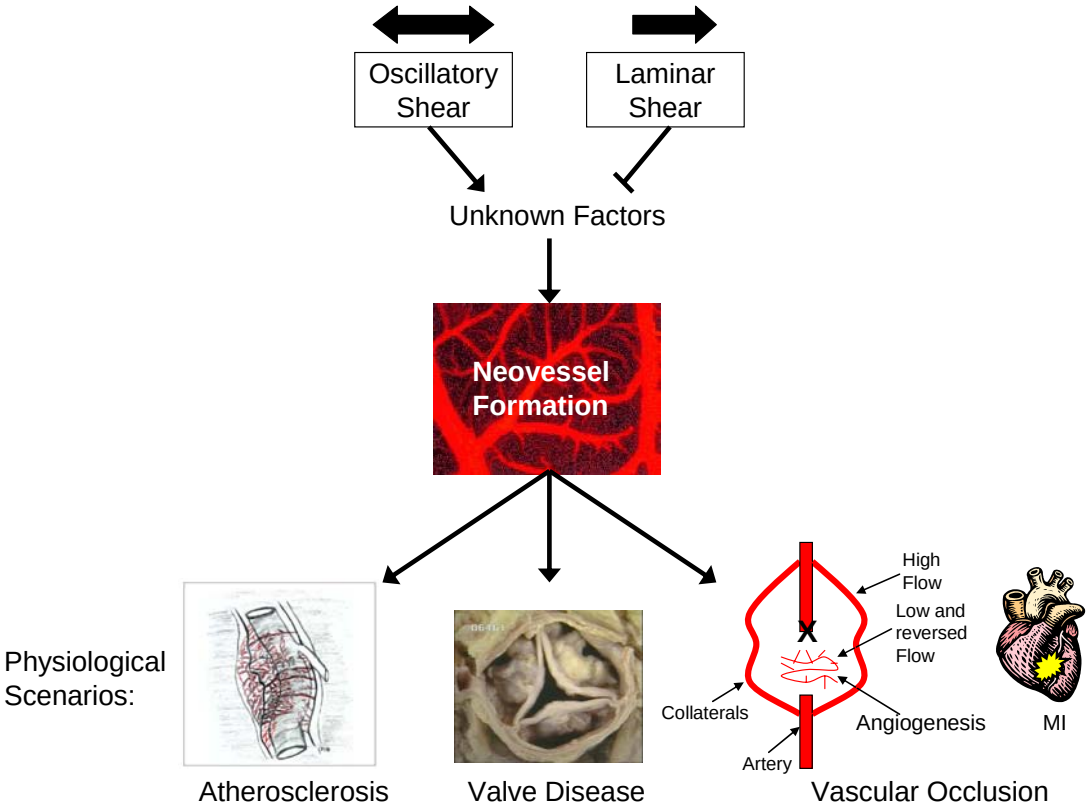

Figure 2.1. Overall Hypothesis.

CHAPTER 3

ROLE OF SHEAR STRESS IN TUBULE FORMATION, MIGRATION, AND PROLIFERATION

The goal of specific aim 1 was to investigate the functional effects of shear stress on endothelial cell migration and neovessel formation. Understanding the role of shear stress on endothelial cell neovascularization may further elucidate the role of shear stress in endothelial cell functions. To achieve this aim, shear conditioned media or sheared endothelial cells (EC) were used to test whether tubule formation or migration is promoted or inhibited *in vitro* through Matrigel tubule formation and scratch migration assays.

Introduction

Neovascularization plays an important role in normal physiological responses as well as pathophysiological responses. Endothelial cells play an important role in neovascularization and are greatly influenced by fluid shear stress. Two typical types of shear stress found in the vascular system are a unidirectional laminar shear stress (LS), found in straight arterial regions, and an oscillatory shear stress (OS) found in branched or curved regions[1]. At the cellular level, LS is known to promote endothelial quiescence, whereas OS is known to promote endothelial dysfunction[1]. Under OS, the endothelium is pro-thrombotic, pro-migratory, and secretes growth factors that stimulate the proliferation of smooth muscle cells[1]. The secretion of growth factors could also play a role in neovascularization.

The three main functions important in neovascularization are migration, proliferation, and the formation of tubules, functions that may also be regulated by fluid

shear stress. Tardy et al. observed in a uniform laminar flow field that cells continually rearranged their relative position with no net migration[2]. However, in a disturbed flow field there was a net migration directed away from the region of high shear gradient, a two-fold increase in cell motility, and an increase in cell division[2]. In addition, it has been found that LS causes a reduction in the rate of EC proliferation and reduces the number of cells entering the cell cycle, with the majority of cells arrested in the G_0 or G_1 phase[3-5]. Contrastingly, disturbed flow causes an increase in the rate of EC proliferation[3, 6]. Thus, LS protects the endothelium by reducing EC proliferation and promoting endothelial quiescence whereas OS activates the endothelium by increasing EC proliferation and migration. However, the underlying mechanisms by which OS and LS differentially regulate the angiogenic responses are not clear.

Tubule formation requires a combination of both migration and proliferation of endothelial cells. Tubule formation is similar to the formation of capillaries *in vivo*. Here, we investigated the effects of shear stress on endothelial cell migration, proliferation and tubule formation. We hypothesize that OS will promote greater migration, proliferation, and tubule formation compared to LS through the secretion of angiogenic factors.

Methods

Cell Culture and Shear Studies

Human umbilical vein endothelial cells (HUVEC) were obtained from the Department of Dermatology, Emory University, cultured in M199 media (Cellgro) with 20% heat inactivated fetal bovine serum (FBS, Atlanta Biologicals), and used between

passage 4 and 5. Bovine aortic endothelial cells (BAEC) were purchased from Cell Applications, cultured in 10% FBS DMEM and used between passages 7-9. The human microvascular endothelial cell line (HMEC-1) was obtained from the Center for Disease Control and cultured in MCDB131 media (Cellgro) with 10% heat inactivated FBS. EC one day post-confluence were exposed to unidirectional LS (5 or 15 dyn/cm^2), OS (± 5 or ± 15 dyn/cm^2 at 1 Hz frequency), or static control (ST) for 24 hours using a cone-and-plate device as described by us[7].

Matrigel Tubule Formation Assay

Following shear exposure, HUVEC, HMEC-1, or BAEC were trypsinized and re-suspended in reduced serum media (HUVEC: M199-2% FBS; HMEC-1: MCDB131-1% FBS; BAEC: DMEM-10% FBS for 1 hr then DMEM-0.5% FBS). Re-suspended cells (HUVEC & HMEC-1: 20,000 cells/well; BAEC 40,000 cells/well) were added to a growth factor reduced Matrigel (BD Bioscience) coated 96-well plate and incubated for 6 hours (HUVEC, HMEC) or 22 hours (BAEC) at 37°C. To test the effects of conditioned media (CM) on tubule formation, CM were collected from HUVEC or BAEC sheared in reduced serum media (M199-2% FBS). Non-sheared HUVEC were then resuspended in the CM and 20,000 cells/well were added to a Matrigel coated 96-well plate. Tubule formation was observed using a phase contrast microscope at 5x and 10x magnification. Tubule length was quantified using NIH ImageJ and the total length of tubules was summed over 4 high powered fields (5x magnification).

Scratch Migration Assay

After shear, cell monolayers were scratched with a 200 µL pipette tip. The monolayer was washed once and the medium was replaced with M199-2% FBS (HUVEC) or DMEM-0.5% FBS (BAEC). The scratch line was photographed. After 6 hours of incubation at 37°C, the same scratch line was photographed and the amount of cells migrated into the scratch area were counted using NIH ImageJ. To examine the effects of CM on migration, non-sheared monolayers were scratched with a 200 µL pipette tip. The monolayer was washed once and the medium was replaced with the shear CM.

Bromodeoxyuridine (BrDU) Incorporation Proliferation Assay

After shear, the media was replaced with M199 (2% FBS) media and BrDU (25µM) was added. The cells were incubated for 6 hours at 37°C. The cell monolayer was then washed three times with PBS and fixed for 5 minutes with ice cold 90% Methanol, 10% MeS buffer. The monolayer was blocked with 3% BSA for 1 hour and the DNA was denatured with HCL-Triton X 100 for 10 minutes. The monolayer was incubated with anti-BrDU antibody (1:200) overnight, and then incubated for 1 hour with FITC-tagged anti-rat secondary (1:200). The monolayer was imaged using a fluorescent microscope (Zeiss) and the number of positive nuclei was counted per high powered field.

Statistical Analysis

Data are reported as average ± SEM obtained from at least 3 independent studies. Statistical significance ($p<0.05$) was assessed by Student's t-test using a Microcal Origin statistical package.

Results

OS promotes tubule formation compared to LS in EC

To determine whether shear stress regulates the tubule forming capability of EC, we performed a Matrigel tubule formation assay. Static culture conditions (ST), cells cultured under no shear stress, were used as a control for our shear system. However, the majority of arterial endothelial cells *in vivo* are continuously exposed to shear stress, and static effects are not physiologically relevant. LS is a more appropriate control, representing a healthy "normal" state, which we will compare to OS, our disease state.

HUVEC that were preconditioned under static or OS (±5 dyn/cm^2) formed more, longer tubules than those preconditioned under LS (15 dyn/cm^2) (Figure 3.1A). The low level of OS (± 5 dyn/cm^2), which is typically found in atheroprone areas of human arteries[8], also promoted greater tubule formation compared to that of static cells (Figure 3.1A). HUVEC that were exposed to two different levels of LS (5 and 15 dyn/cm^2) and OS (±5 and ±15 dyn/cm^2) were compared to each other to investigate the magnitude-dependence and directional-dependence of the shear stress effect. LS exposure at both 5 and 15 dyn/cm^2 significantly inhibited tubule formation of HUVEC compared to both low and high levels of OS (±5 and ±15 dyn/cm^2) and ST (Figure 3.1A). This suggests that the unidirectional component of the shear stress plays a greater role than the

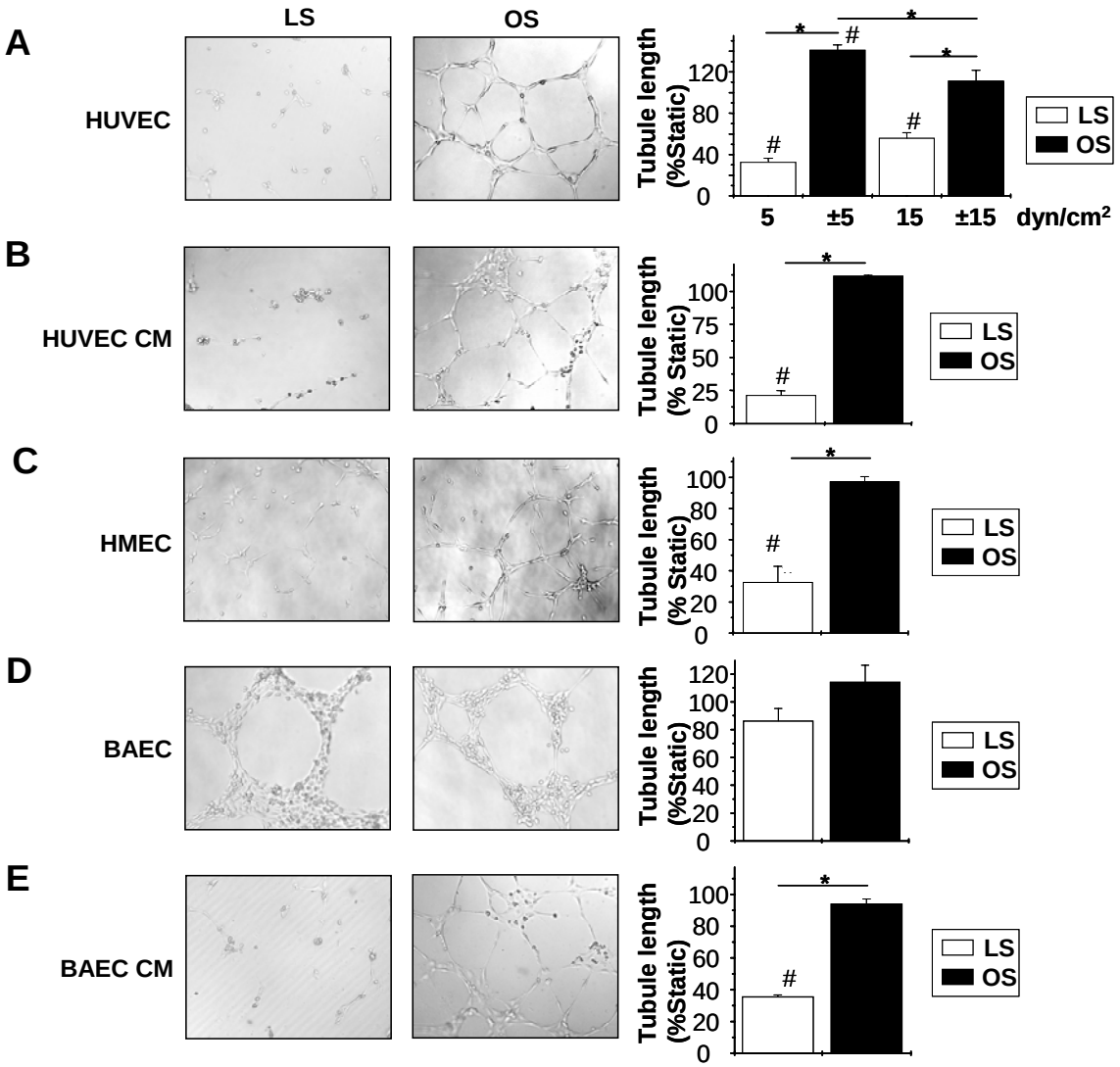

Figure 3.1. OS promotes tubule formation compared to LS in HUVEC and HMEC but not BAEC. A, HUVEC were sheared at 5 and 15 dyn/cm^2 unidirectional LS or ±5 and ±15 dyn/cm^2 OS for 24 hours with static condition (ST) as a control and then used in a Matrigel tubule formation assay. B, Conditioned media (CM) collected from HUVEC that were sheared at 15 (LS), ±5 (OS) dyn/cm^2 or ST for 24 hours were added to static HUVEC in a Matrigel tubule formation assay. HMEC-1 (C) or BAEC (D) were sheared at 15 (LS) or ±5 dyn/cm^2 (OS) for 24 hours and then used in the Matrigel tubule formation assay. E, CM collected from sheared BAEC as in D were added to static HUVEC in a Matrigel tubule formation assay. Shown are representative images (10x magnification) and tubule length was quantified over 4 high powered fields at 5x magnification and normalized to percent static. (mean ±SEM, n=3-9; * P<0.05 LS vs. OS; # P<0.05 compared to ST).

magnitude of the shear stress in inhibiting tubule formation. Based on this finding, we used two typical arterial levels of shear conditions: atheroprotective 15 dyn/cm^2 LS and pro-atherogenic ±5 dyn/cm^2 OS for the rest of the studies[8].

The inhibitory effect of LS on tubule formation was also observed in EC obtained from microvascular origins (HMEC-1, Figure 3.1C). However, EC obtained from aortas (BAEC), which are less likely to participate in angiogenesis *in vivo*, showed no significant differences in tubule formation between LS and OS (Figure 3.1D), suggesting that shear stress does not play a role in tubule formation by aortic EC.

We next examined whether EC would produce a secreted factor(s) which regulates shear-mediated tubule formation. We added conditioned media (CM) collected from sheared cells (LS, OS and ST for 1 day) to static HUVEC in the Matrigel tubule formation assay. LS CM obtained from HUVEC significantly inhibited tubule formation of static HUVEC whereas OS and ST CM did not (Figure 3.1B). Similarly, LS CM from BAEC inhibited tubule formation of static HUVEC compared to OS and ST CM (Figure 3.1E). These results suggest that aortic EC may not form capillary-like structures by themselves, but they still could produce factors promoting tubule formation in response to OS.

OS promotes migration compared to LS

To investigate whether shear stress would regulate EC migration we performed the scratch migration assay. We found that HUVEC and BAEC preconditioned with LS had inhibited migration into the denuded zone compared to ST whereas OS promoted migration with similar results as ST (Figure 3.2A and 3.2B).

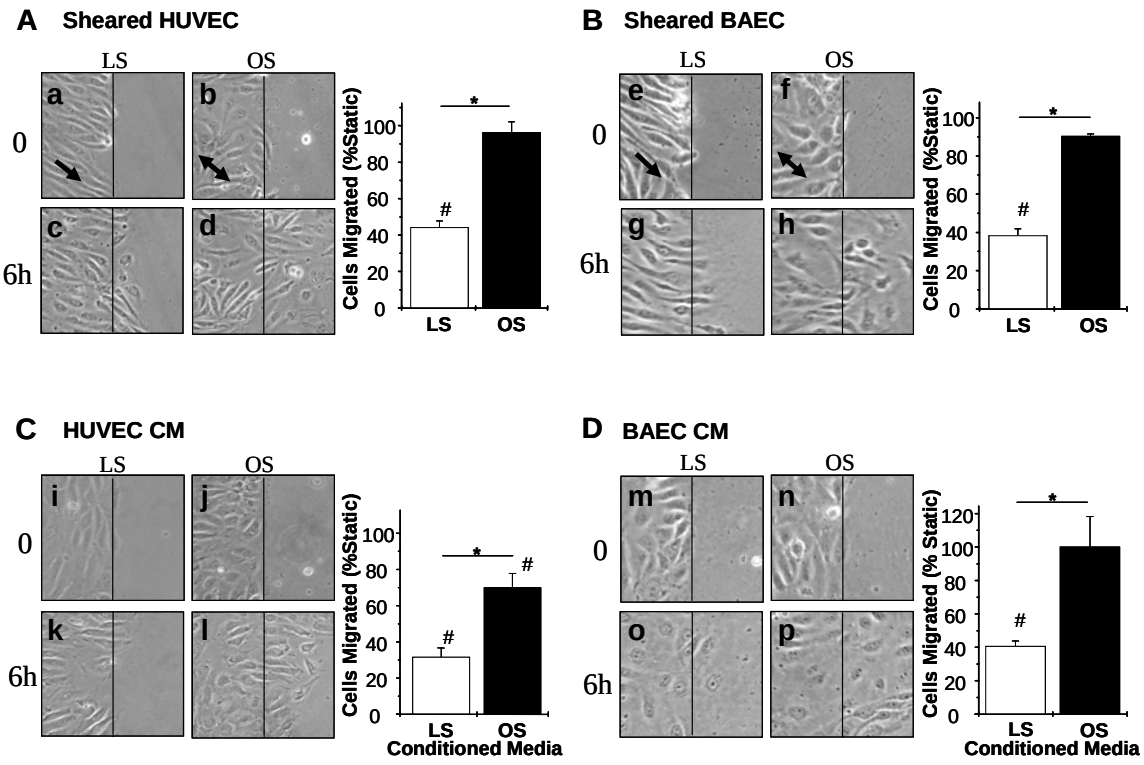

Figure 3.2. OS promotes migration compared to LS in HUVEC and BAEC. HUVEC (A) or BAEC (B) were sheared at 15 (LS) and ±5 (OS) dyn/cm^2 for 24 hours. The monolayers were scratched, and photographed immediately (0 h) (HUVEC:a, b; BAEC: e,f) and again after 6 hours incubation in fresh reduced serum medium (HUVEC: c,d; BAEC: g,h). For C and D, confluent static cultured HUVEC were scratched and the media were replaced with CM obtained from sheared HUVEC or BAEC as in A and B. Cells were photographed at 0 and 6 hours incubation. The center lines indicate the original scratch margins and the arrows indicate flow directions. Graphs represent number of cells migrated into the scratched area as a percent of static. (mean ±SEM, n=3; * P<0.05 LS vs OS; # P<0.05 compared to ST).

Next, we investigated whether a secreted factor(s) in response to shear stress regulates migration. For this study, we added shear CM to static HUVEC or BAEC in a scratch migration assay. LS CM significantly inhibited migration compared to those of both ST and OS (Figure 3.2C and 3.2D). This suggests that EC produce a secreted factor(s) which regulates shear-mediated migration.

OS promotes proliferation compared to LS

Although, it has been previously published[2], we also wanted to confirm that LS inhibits proliferation compared to OS and ST in our system. To do this, we performed a Bromodeoxyuridine (BrDU) incorporation assay to examine the effect of shear stress on the proliferation of EC. BrDU is incorporated into the DNA of proliferating cells and its presence can be detected by immunocytochemistry (Figure 3.3A). We found that HUVEC exposed to LS had inhibited proliferation compared to OS and ST (Figure 3.3B) corroborating previous published data and validating our system.

Discussion

In this study we found that: 1) LS inhibits tubule formation in HUVEC and HMEC-1, but not BAEC, however CM from laminar sheared BAEC can inhibit tubule formation of HUVEC, 2) Pre-conditioning with LS inhibits migration in HUVEC and BAEC, whereas OS does not inhibit migration and this is mediated through secreted protein, and 3) Proliferation is downregulated by LS compared to OS and ST. Collectively, these findings suggest that OS promotes functions important in

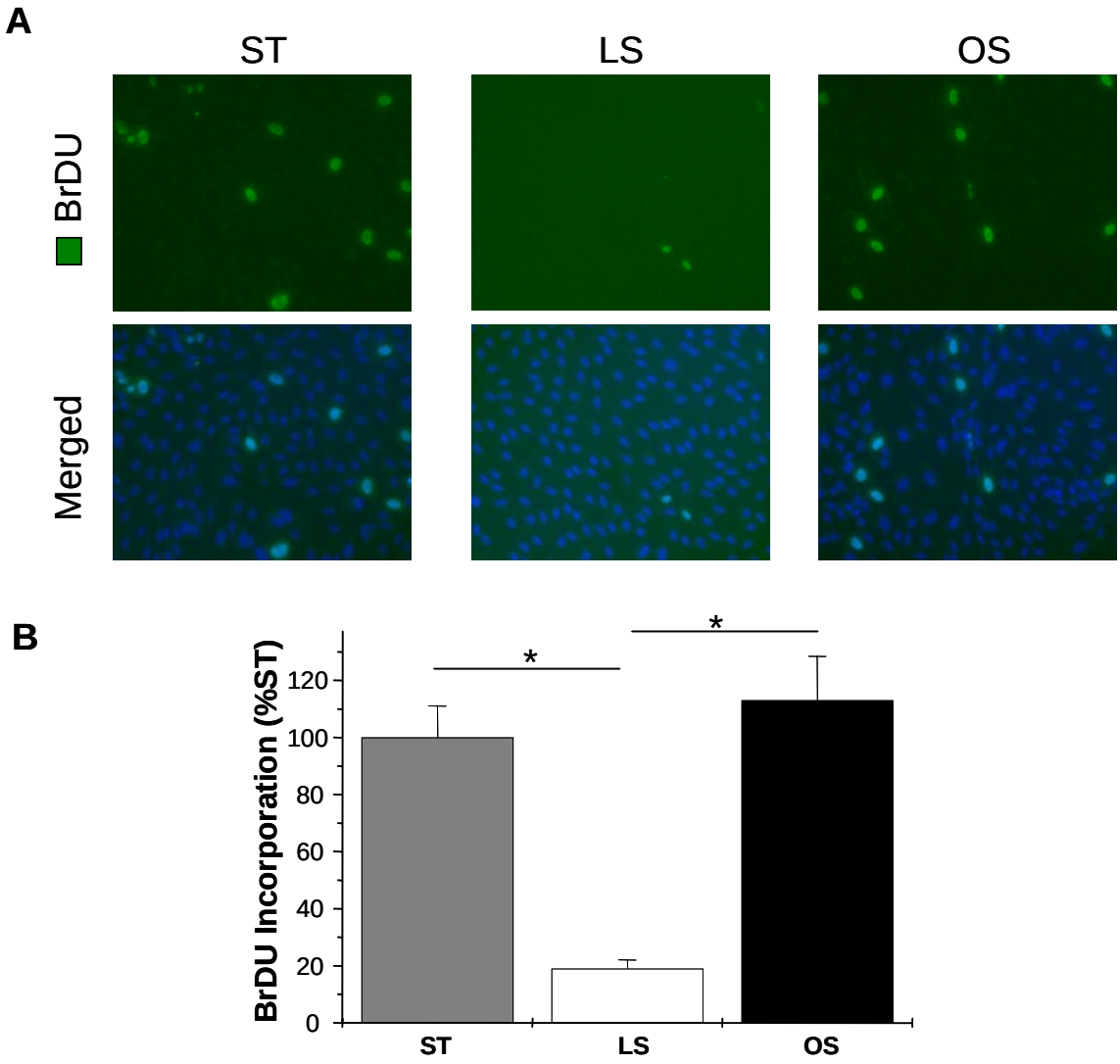

Figure 3.3. OS promotes proliferation of EC compared to LS in HUVEC. HUVEC were sheared at 15 (LS) and ±5 (OS) dyn/cm^2 for 24 hours. After which, BrDU was added and the cells were incubated at 37°C for 6 hours. Immunocytochemistry was performed to detect BrDU incorporation (green) (A). The number of positive nuclei were quantified per 4 hpf using a fluorescence microscope and normalized to a percent of ST. Nuclei were stained with Hoechst (blue) (mean ±SEM, n=7-8; * P<0.05).

neovascularization whereas LS does not, and this could be important in understanding diseases involving disturbed shear stress and neovascularization

Fluid shear stress is thought to play a role in angiogenesis and arteriogenesis. In these processes, endothelial proliferation and migration are two important responses. It is well established that LS causes a reduction in the rate of EC proliferation[2]. Contrastingly, disturbed flow causes an increase in the rate of EC proliferation[2]. The effect of shear stress on migration appears to be conflicting at first glance. Consistent with our finding, Tardy et al. have also shown that EC exposed to 48 hours of LS continually rearranged their relative position with no net migration, whereas EC exposed to 48 hours of OS had a two-fold increase in cell motility[2]. However, Hsu et al. reported that LS increases migration of EC into a scratched area as compared to cells exposed to static and disturbed flow[9]. These discrepancies are likely due to differences in experimental conditions: our cells were pre-conditioned with shear stress for 1 day, then scratched to see what the cell was 'programmed' to do, whereas in Hsu et al.'s experiments static cultured cells were scratched and then sheared. Our unpublished results indeed confirm Hsu et al's result, suggesting that endothelial migration responses are different depending upon when shear stress is applied.

Thus far, we have shown that LS inhibited tubule formation and migration of sheared HUVEC themselves. In addition, LS also stimulated or inhibited production of a secreted factor(s) that inhibited tubule formation and migration of non-sheared EC. These findings suggest that EC differentially respond to OS and LS to produce pro- and anti-angiogenic protein(s) or cytokines. We next went on to study the molecular mechanisms by which shear stress regulates tubule formation and migration by examining the

canonical Vascular Endothelial Growth Factor (VEGF) pathway as well as identifying secreted factors that may be mediating these responses.

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CHAPTER 4

OSCILLATORY SHEAR STRESS PROMOTES TUBULE FORMATION OF ENDOTHELIAL CELLS IN A VEGFR-2 DEPENDENT MANNER

The goal of specific aim 2 was to identify molecular mechanisms involved in shear-induced neovessel formation. Identifying the molecular mediators involved in shear-induced neovascularization could provide useful therapeutic targets for diseases that involve disturbed shear stress. To achieve this aim, we first examined the canonical vascular endothelial cell growth factor (VEGF) pathway.

Introduction

The classical mediators of neovascularization are the VEGF family. The majority of VEGF signaling is through the VEGF receptor, VEGFR-2, which has also been shown to be an important mediator of shear stress responses[1, 2]. Therefore, VEGFR-2 may play an important role in shear mediated neovascularization.

VEGFR-2 is essential for vascular development as VEGFR-2 knock out mice are embryonic lethal due to defects in the development of endothelial cells and vasculogenesis[3]. VEGFR-2 can be autophosphorylated through a ligand-dependent and ligand-independent mechanism. Currently, the following sites have been identified as autophosphorylation sites: Tyr801, Tyr951 and Tyr996 present in the kinase insert domain, Tyr1054 and Tyr1059 in the kinase domain, and Tyr1175 and Tyr1241 in the C-terminal tail[4]. VEGFR-2 can initiate proliferation through the classical ERK1/2 pathway leading to gene transcription. This is most likely mediated through Tyr1175 which when phosphorylated can bind PLCγ leading to the ERK1/2 pathway[4].

VEGFR2 is part of a mechanosensory complex that can sense fluid shear stress leading to ligand-independent activation [1, 2]. Vascular endothelial cadherin (VE-cadherin) is known to form a complex with VEGFR-2 and includes β-catenin which is phosphorylated and translocates to the nucleus in VEGF-A stimulated cells [5, 6]. Tzima et al. identified that PECAM-1 also forms a complex with VEGFR-2 and VE-cadherin, and acts as the "mechanical transducer" that senses the force of shear stress and transmits the signal to VE-cadherin, "the adapter", which activates VEGFR-2 leading to the subsequent signaling pathways [2]. Unidirectional laminar shear stress transiently activates VEGFR-2 through phosphorylation of Tyr1054/1059 and after 30 minutes of shear the receptor is de-phosphorylated [1, 2, 7]. Laminar shear stress often rapidly and transiently activates signaling pathways, whereas oscillatory shear stress often chronically activates these signaling pathways. Therefore, oscillatory shear stress may chronically activate VEGFR-2 through specific phosphorylation sites leading to proliferation and migration.

Here, we investigated the role of VEGFR-2 in shear-mediated migration and tubule formation, as well as investigated the effects of shear stress on the phosphorylation of VEGFR-2. We hypothesize that inhibiting VEGFR-2 will inhibit OS-mediated tubule formation and migration and this is mediated through the phosphorylation of VEGFR-2 at Tyr1175.

Methods

Cell Culture and Shear Studies

Human umbilical vein endothelial cells (HUVEC) were obtained from the Department of Dermatology, Emory University, cultured in M199 media (Cellgro) with

20% heat inactivated fetal bovine serum (FBS, Atlanta Biologicals), and used between passage 4 and 5. Endothelial cells (EC) one day post-confluence were exposed to unidirectional LS (15 dyn/cm^2), OS (± 5 dyn/cm^2 at 1 Hz frequency), or static control (ST) for 24 hours using a cone-and-plate device as described by us[8].

Matrigel Tubule Formation Assay

Following shear exposure, HUVEC were trypsinized and re-suspended in reduced serum media (M199 with 2% FBS). Re-suspended cells (20,000 cells/well) were added to a growth factor reduced Matrigel (BD Bioscience) coated 96-well plate and incubated for 6 hours at 37°C. To test the effects of conditioned media (CM) on tubule formation, CM were collected from HUVEC sheared in reduced serum media (M199 with 2% FBS). Non-sheared HUVEC were then resuspended in the CM and 20,000 cells/well were added to a Matrigel coated 96-well plate. Tubule formation was observed using a phase contrast microscope at 5x and 10x magnification. Tubule length was quantified using NIH ImageJ and the total length of tubules was summed over 4 high powered fields (5x magnification).

Scratch Migration Assay

After shear, cell monolayers were scratched with a 200 μL pipette tip. The monolayer was washed once and the medium was replaced with M199-2% FBS (HUVEC). The scratch line was photographed. After 6 hours of incubation at 37°C, the same scratch line was photographed and the amount of cells migrated into the scratch area were counted using NIH ImageJ. To examine the effects of CM on migration, non-

sheared monolayers were scratched with a 200 µL pipette tip. The monolayer was washed once and the medium was replaced with the shear CM.

VEGFR-2 inhibitor experiments

For VEGFR-2 inhibitor experiments, the VEGFR-2 inhibitor, SU1498 (30µM, Calbiochem) or 0.1% DMSO vehicle control was added during shear and cells were used in Matrigel tubule formation and scratch migration assays.

Preparation of Cell lysates and Immunoblotting

Cells were lysed in RIPA buffer and further homogenized by sonication. Protein content of each sample was measured using a Bio-Rad DC assay. Protein (25 µg) was resolved on 8% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were probed with the following primary antibodies: rabbit anti-phospho-VEGF Receptor 2 (Tyr1175) (Cell Signaling), rabbit anti-VEGFR-2 phospho specific (Tyr1054/1059) (Calbiochem), anti-Flk-1 (VEGFR-2) (Santa Cruz) and goat-anti-actin (Santa Cruz). They then were incubated with secondary antibody conjugated to alkaline phosphatase, and then detected using chemiluminescence. The intensities of immunoreactive bands were analyzed using Scion Image.

Statistical Analysis

Data are reported as average ± SEM obtained from at least 3 independent studies. Statistical significance ($p<0.05$) was assessed by Student's t-test using a Microcal Origin statistical package.

Results

VEGFR-2 inhibition blunts OS-mediated EC tubule formation

To investigate the role of VEGFR-2 in shear-mediated tubule formation, we examined the effect of inhibiting VEGFR-2 on shear induced tubule formation through the use of SU1498, a potent and selective inhibitor of VEGFR-2. As seen in Chapter 3, LS significantly inhibited tubule formation compared to ST and OS (Figure 4.1A and 4.1B). Treatment with SU1498 significantly inhibited OS-mediated tubule formation but not LS or ST tubule formation suggesting that VEGFR-2 plays a specific role in OS-mediated tubule formation (Figure 4.1A and 4.1B).

VEGFR-2 inhibition blunts EC migration

Similarly, to examine the role of VEGFR-2 in shear mediated migration, we again inhibited VEGFR-2 using SU1498 during shear induced migration assays. As seen in Chapter 3, LS significantly inhibited migration compared to ST and OS (Figure 4.2A and 4.2B). SU1498 treatment significantly inhibited migration of ST, LS and OS cells compared to non-treated ST, LS, and OS cells, respectively (Figure 4.2A and 4.2B), suggesting that VEGFR-2 plays a role in migration but not necessarily OS-mediated migration.

OS phosphorylates VEGFR-2 at Tyrosine 1175 and Tyrosine 1054/1059 whereas LS phosphorylates VEGFR-2 only at Tyrosine 1054/1059

To determine how shear is mediating tubule formation and migration through VEGFR-2, we examined the effects of shear stress on the phosphorylation of VEGFR-2.

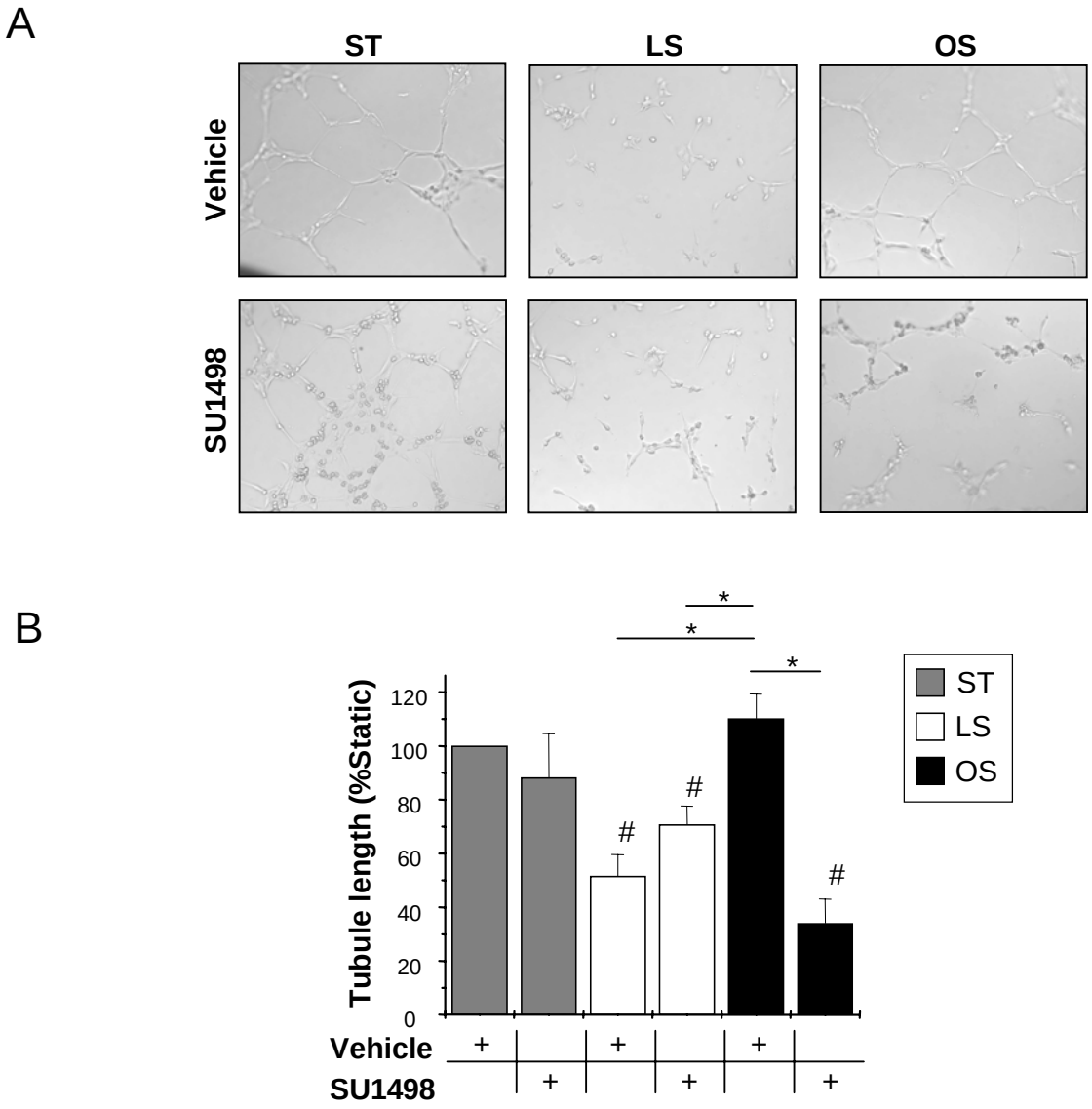

Figure 4.1. VEGFR-2 inhibition blunts OS-mediated EC tubule formation. A, HUVEC were treated with 30 μM SU1498 or vehicle and exposed to LS (15 dyn/cm^2), OS (±5 dyn/cm^2), or ST for 24 hours. Cells were then used in a Matrigel tubule formation assay. B, tubule length was quantified using NIH-Image J. (mean ±SEM, n=7-8; * P<0.05; $^{\#}$ P<0.05 compared to ST-Vehicle)

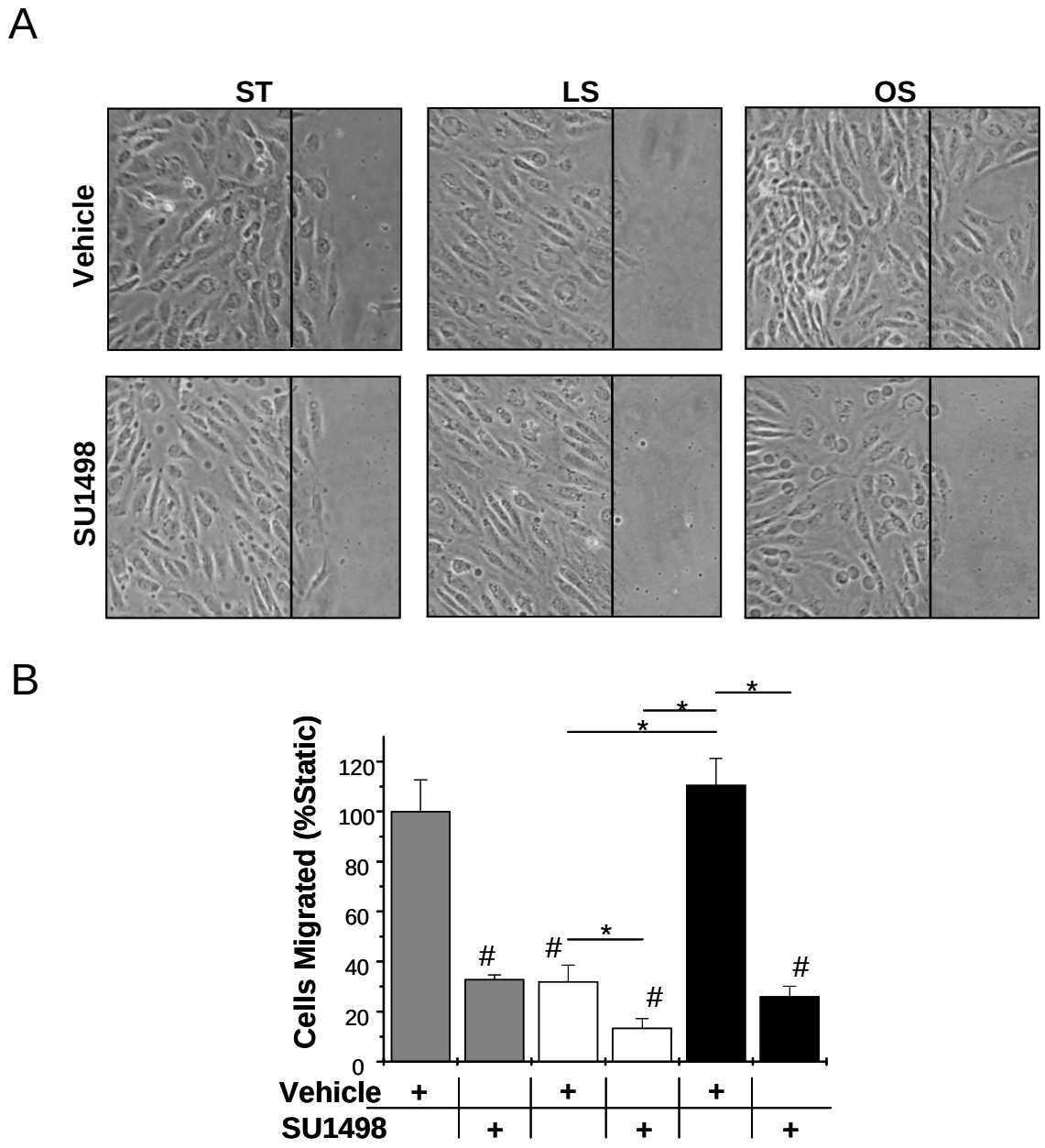

Figure 4.2. VEGFR-2 inhibition blunts EC migration. A, HUVEC were treated with 30 μM SU1498 or vehicle and exposed to LS (15 dyn/cm^2), OS (±5 dyn/cm^2), or ST for 24 hours. Cells were then used in a scratch migration assay. B, The number of cells migrated was counted and normalized to a percent of ST. (mean ±SEM, n=3-4; * $P<0.05$; $^{\#}$ $P<0.05$ compared to ST-Vehicle)

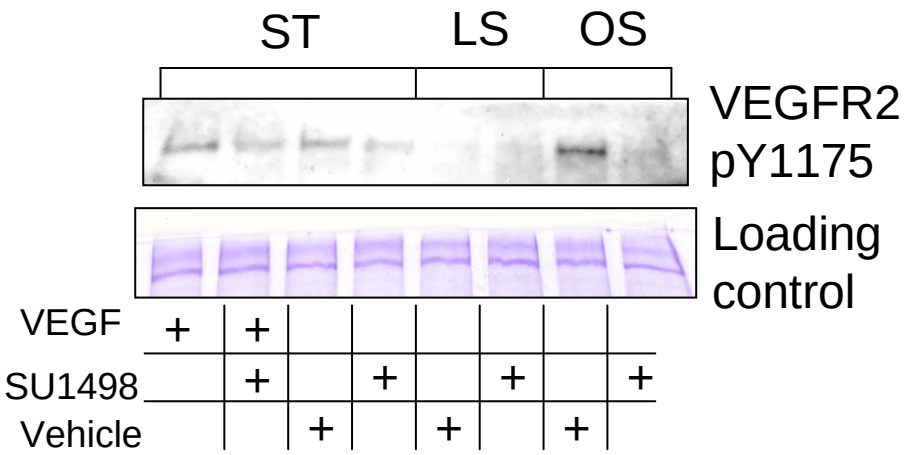

Figure 4.3. OS phosphorylates VEGFR-2 at Tyr1175 which can be blocked by SU1498. HUVEC were treated with 30 μM SU1498, VEGF, or vehicle and exposed to LS (15 dyn/cm^2), OS (±5 dyn/cm^2), or ST for 24 hours. Cell lysates used for Western blots with primary antibody to VEGFR-2 phospho-Tyr1175. Coomossie blue staining of the gel was used as a loading control. (n=3)

VEGFR-2 has several phosphorylation sites that mediate different responses[4]. Two important phosphorylation sites are at Tyrosine 1054/1059 (Tyr1054/1059) and at Tyrosine 1175 (Tyr1175)[4]. Using western blot analysis, we found that after 24 hours of shear, OS phosphorylated VEGFR-2 at Tyr1175 whereas LS did not, and this could be blocked by SU1498 treatment (Figure 4.3).

We next examined phosphorylation of VEGFR-2 by shear at several timepoints. Both LS and OS phosphorylate VEGFR-2 at Tyr1054/1059 by 6 hours, however by 24 hours LS still phosphorylates VEGFR-2 at Tyr1054/1059 but OS phosphorylation has declined (Figure 4.4A). OS also phosphorylates VEGFR2 at Tyr1175 after 6 hours of shear whereas LS does not (Figure 4.4B). Shear had no effect on VEGFR-2 phosphorylation at time points less than 30 minutes (Figure 4.5).

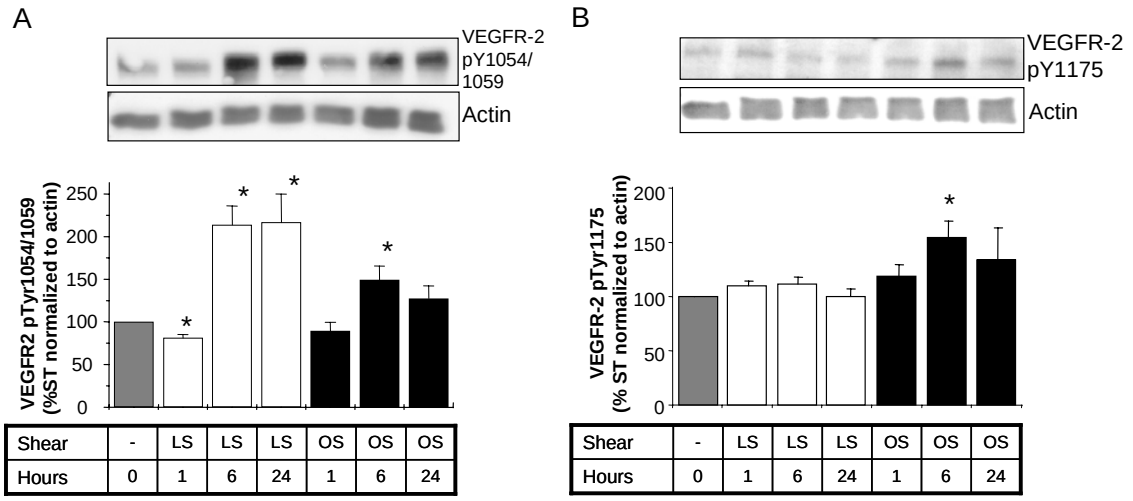

Figure 4.4. OS phosphorylates VEGFR-2 at Tyr1175 and Tyr1054/1059 whereas LS phosphorylates VEGFR-2 at Tyr1054/1059 only. HUVEC were exposed to LS (15 dyn/cm^2), OS (±5 dyn/cm^2), or ST for 1, 6, and 24 hours. Cell lysates were used for Western blots with antibodies to VEGFR-2 phospho-Tyr1054/1059 (A) and VEGFR-2 phospho-Tyr1175 (B). As controls, cell lysates were blotted for β-actin. Protein levels were quantified by densitometry and normalized to actin. (mean ±SEM, n=3; * P<0.05 compared to ST)

Discussion

In this study we found: 1) VEGFR-2 inhibition blunts OS-mediated tubule formation, 2) VEGFR-2 inhibition blunts EC migration, 3) OS phosphorylates VEGFR-2 at Tyr1054/1059, and 4) LS phosphorylates VEGFR-2 at Tyr1054/1059 only. These results suggest that VEGFR-2 plays an important role in OS-mediated tubule formation and migration and this is possibly mediated through OS phosphorylating VEGFR-2 at Tyr1175.

It is known that VEGF stimulates EC migration and proliferation through its receptor VEGFR-2[9]. VEGFR-2 is one of the most important VEGF receptors since it has been shown that most physiological VEGF signaling in EC is mediated through VEGFR-2 and its deletion in mice is lethal[3, 10, 11]. Therefore, it is not surprising that inhibiting

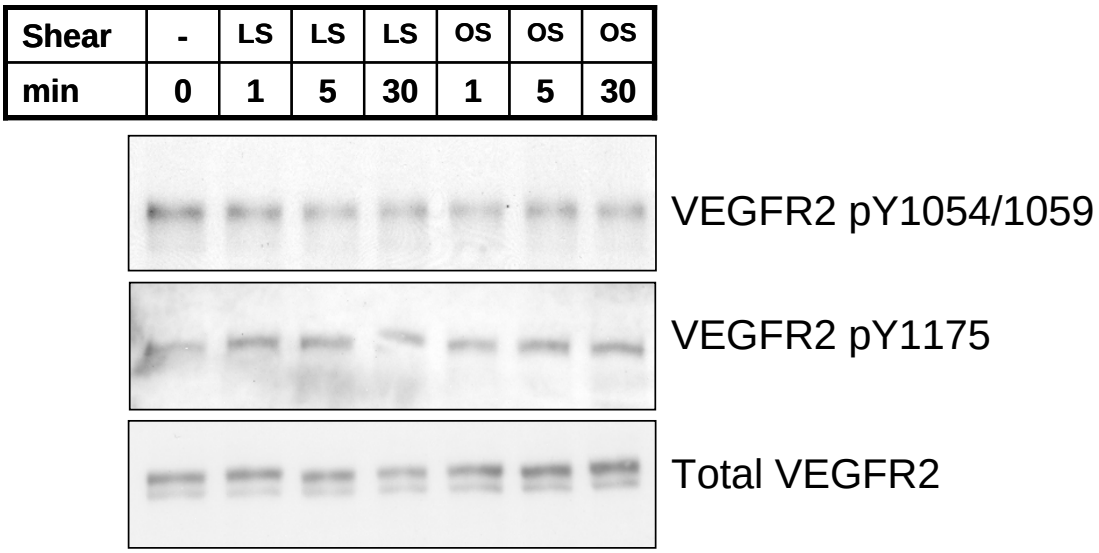

Figure 4.5. Shear has no effect on VEGFR-2 phosphorylation at short timepoints. HUVEC were exposed to LS (15 dyn/cm^2), OS (±5 dyn/cm^2), or ST for 1, 5, and 30 minutes. Cell lysates were used for Western blots with antibodies to VEGFR-2 phospho-Tyr1054/1059, VEGFR-2 phospho-Tyr1175. As controls, cell lysates were blotted for total VEGFR-2. (n=3)

VEGFR-2 inhibited OS-mediated tubule formation. Interestingly, VEGFR-2 inhibition did not inhibit ST or LS mediated tubule formation in our system, suggesting that VEGFR-2 specifically mediates OS-induced tubule formation. It has been published that VEGFR-2 can be activated by shear stress independent of ligand binding[1]. Therefore, OS and LS could have differing effects on VEGFR-2 activation. OS may activate VEGFR-2 independent of ligand which leads to downstream signaling pathways stimulating tubule formation. However, VEGFR-2 inhibition blunted migration in all cases, suggesting that VEGFR-2 mediates migration independent of shear stress. VEGFR-2 inhibition did not completely block OS-mediated tubule formation, so we suggest that other mechanisms may also be involved.

VEGFR-2 is activated through receptor dimerization and autophosphorylation of tyrosine residues in the cytoplasmic kinase domain[4]. Six phosphorylation sites have been

identified[4], each of which leads to different signaling pathways. Of interest, Tyr1175 is known to bind PLCγ which mediates activation of ERK1/2 signaling, proliferation, and expression of adhesion molecules leading to inflammation[12]. We found that OS phosphorylated VEGFR-2 at Tyr1175 whereas LS did not. The phosphorylation of VEGFR-2 at Tyr1175 may lead to PLCγ binding and activation of the Erk1/2 proliferation pathway and subsequent tubule formation, as well as stimulate inflammation, a well known result of OS[8]. We also found that both LS and OS led to the phosphorylation of VEGFR-2 at Tyr1054/1059, with LS maximally stimulating phosphorylation. Others have published that LS leads to the phosphorylation of VEGFR-2, however, no one has examined the role of OS in Tyr1054/1059 phosphorylation[2].

In summary, OS partially mediates tubule formation in a VEGFR-2 dependent manner and possibly through the phosphorylation of VEGFR-2 at Tyr1175. Understanding the role of VEGFR-2 in shear mediated diseases could be of great therapeutic importance. However, to identify more novel mechanisms by which shear mediates neovascularization, we next went on to perform DNA and protein array analysis.

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CHAPTER 5

OSCILLATORY SHEAR STRESS PROMOTES TUBULE FORMATION AND MIGRATION OF ENDOTHELIAL CELLS BY AN ANGIOPOIETIN-2-DEPENDENT MECHANISM

The goal of specific aim 2 was to identify molecular mechanisms involved in shear-induced neovessel formation. In Chapter 4, we identified VEGFR-2 as a possible mediator of OS-induced tubule formation and migration. However, the VEGFR-2 pathway has been highly studied and is already known to be shear-mediated. In addition, our data from Chapter 4 suggested that VEGFR-2 is not the only factor involved and that other factors may also be playing a role. Therefore, to identify a novel mechanism of shear-mediated neovessel formation, we performed gene and protein array analysis.

Introduction

Fluid shear stress, the dragging force created by flow through blood vessels, is sensed by the endothelium and plays an important role in normal physiological responses as well as disease pathologies. In particular, shear stress is thought to play a role in angiogenesis, or the formation of new blood vessels from pre-existing blood vessels[1-3]. At the cellular and molecular level, unidirectional laminar shear stress (LS) is thought to promote endothelial cell (EC) quiescence; laminar sheared EC are anti-proliferative, anti-apoptotic, and anti-thrombotic [4]. However, oscillatory shear stress (OS), a type of disturbed shear stress implicated in diseases such as atherosclerosis, is thought to promote EC dysfunction; oscillatory sheared EC are pro-proliferative, pro-migratory, pro-thrombotic, and secrete growth factors that stimulate smooth muscle cell proliferation

and migration [4]. The secretion of growth factors from dysfunctional EC exposed to OS could also play a role in blood vessel remodeling and angiogenesis.

There are several diseases associated with both angiogenesis and disturbed flow, such as atherosclerosis, aortic valve calcification, and arterial occlusion. In atherosclerosis, atherosclerotic plaques preferentially occur in areas of the arterial system exposed to disturbed flow and angiogenesis in the plaque is thought to promote the progression of atherosclerosis[5, 6]. Disturbed flow is found in the aortic valve sinus and angiogenesis in the valve leaflet is associated with calcification[7, 8]. In arterial occlusion, disturbed flow is found in the post-occlusive site and angiogenesis is important for the subsequent ischemia [9, 10]. The detailed mechanisms resulting in pathological angiogenesis and neovessel formation remain uncertain. In these physiological and pathophysiological scenarios, fluid shear stress may provide a driving force for angiogenesis.

Here, we hypothesized that LS inhibits angiogenesis by downregulation of the secretion of pro-angiogenic factors or cytokines. To examine this hypothesis, we carried out gene array and protein array studies specific for angiogenic factors and cytokines, respectively. From these and subsequent functional studies, we demonstrate that LS inhibits angiogenesis through the downregulation of Angiopoietin-2 (Ang2), a secreted protein that is required for postnatal angiogenesis[11].

Methods

Cell Culture and Shear Studies

Human umbilical vein endothelial cells (HUVEC) were obtained from the Department of Dermatology, Emory University, cultured in M199 media (Cellgro) with 20% heat inactivated fetal bovine serum (FBS, Atlanta Biologicals), and used between passage 4 and 5. Bovine aortic endothelial cells (BAEC) were purchased from Cell Applications, cultured in 10% FBS DMEM and used between passages 7-9. The human microvascular endothelial cell line (HMEC-1) was obtained from the Center for Disease Control and cultured in MCDB131 media (Cellgro) with 10% heat inactivated FBS. EC one day post-confluence were exposed to unidirectional LS (15 dyn/cm^2), OS (± 5 dyn/cm^2 at 1 Hz frequency), or static control (ST) for 24 hours using a cone-and-plate device as described by us[12].

Gene and Protein Array

For the gene array study, total RNA was prepared from HUVEC exposed to LS (15 dyn/cm^2) and OS (± 5 dyn/cm^2 at 1 Hz) for 1 day, using Qiagen RNAeasy kit as we previously described[12, 13]. Total RNA (3μg) was reverse transcribed into cDNA and then transcribed into biotinylated cRNA containing Biotin-16-UTP using TrueLabeling-AMP™ Linear RNA Amplification kit (Superarray) according to the manufacturer's instructions. Prepared cRNA was hybridized to the Human Angiogenesis oligo GEArray (Superarray) containing 128 angiogenesis related genes according to the manufacturer's instructions. Bound biotinylated cRNA was detected using alkaline phosphatase-conjugated streptavidin and CDP-star chemiluminescence. Images of the arrays were

developed on X-ray film and digitally recorded using a scanner. Data analysis was performed using GEArray Expression Analysis Suite (www.superarray.com), and background was corrected by subtraction of the minimum value and loading was corrected by normalizing to actin on each individual array. The protein array study was carried out with cytokine antibody arrays (containing 68 cytokine antibodies) as described previously[14, 15]. Conditioned media (10 ml each) collected after shear exposure (LS or OS for 1 day as above) were concentrated ~5-fold and equal amounts of protein were incubated with the blocked membrane. After extensive washing to remove unbounded materials, the membranes were then incubated with a cocktail of biotin-labeled antibodies against different individual cytokines. The membranes were then washed and incubated with horse radish peroxidase (HRP)-conjugated streptavidin (2.5 pg/ml) for 1 hr at room temperature. Finally the signals were detected by ECL system (Amersham Pharmacia Biotech). The intensities of signals were densitometrically quantified and normalized to positive and negative controls included in each membrane as previously described[15]. Statistical analyses for gene and protein arrays (n=3 each) were carried out using Microcal Origin software. The functions for each gene/protein listed in Appendix A & B, Table A.1 and B.1 were found from NCBI Entrez Gene database.

Preparation of Cell lysates and Immunoblotting

Cells were lysed in RIPA buffer and further homogenized by sonication. To detect secreted protein in conditioned media, HUVEC were sheared in M199-2% FBS media for 24 hours. The conditioned media were then centrifuged at 2,000 rpm for 10

minutes. Aliquots of the media (2 mL) were then precipitated in 10 mL acetone at -20°C overnight. Protein content of each sample was measured using a Bio-Rad DC assay. Protein (25 µg) was resolved on 8% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were probed with the following primary antibodies: goat anti-Ang-2 (F-18), goat anti-Ang-1 (N-18) and goat-anti-actin (Santa Cruz), and anti-TSP-1 (clone D4.6) (Lab Vision) and then with secondary antibody conjugated to alkaline phosphatase, which was then detected using chemiluminescence. The intensities of immunoreactive bands were analyzed using Scion Image.

Real time quantitative polymerase chain reaction (PCR)

Four µg of total RNA was reverse transcribed by using random primers and a Superscript-II kit (Life Technology) to synthesize first-strand cDNA. The cDNA was assayed by real time PCR using a LightCycler (Roche), recombinant Taq polymerase (Life Technology), Taq start antibody (Clontech) and SYBR green detection of products. Copy numbers were determined based on standard curves generated with human standards for Ang2, TSP-1 and 18S templates. The following primers were used to amplify human Ang2 (5' to 3')- forward: CGACGTGAGGATGGCAGCGTTGA and reverse: ATCAAACCACCAGCCTCCTGTTAGCA, and for human TSP-1- forward: AACATGCCACGGCCAACAAA and reverse: TGCACTTGGCGTTCTTGTTGC. Real-time PCR for Ang2 and TSP-1 was carried out with the annealing temperature at 60ºC and 35 cycles. DNA gels were run to confirm specificity of PCR products.

Immunocytochemistry

Following shear, HUVEC were washed with PBS and fixed with 90% Methanol, 10% MeS buffer for 10 minutes. Cells were blocked with 3% BSA for 1 hour and then incubated with primary antibody (Ang2, Santa Cruz) overnight. Cells were then incubated with FITC-labeled secondary antibody for 1 hour and nuclei stained with Hoechst. Cells were imaged with a fluorescent microscope at 63x magnification.

Aorta en face immunostaining

Male C57BL/6J (wildtype) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice (8 weeks of age, n=5) were sacrificed by CO_2 inhalation and pressure perfused with saline (0.9% NaCl) and pressure fixed with 10% formalin. The aortic arch and thoracic aorta were isolated and cleaned of adventitia. The tissue was then placed in a 24 well plate and permeabolized for 20 minutes with 0.2% Triton-X 100, washed and then blocked with 10% donkey serum for 1.5hrs. The tissue was incubated overnight at 4°C with primary antibody at a dilution of 1:25. The tissue was then washed and incubated for 2 hours with secondary antibody at a dilution of 1:50 followed by washing. The greater curvature (GC) of the arch was separated from the lesser curvature (LC) using a scalpel and both pieces were splayed open and laid flat on a microscope slide with endothelium facing up. The tissue was mounted with Vectashield with DAPI and imaged using a Zeiss LSM 510 confocal microscope. Primary antibodies used were goat anti-Ang2 (Santa Cruz) and goat anti-Ang1 (Santa Cruz).

Matrigel Tubule Formation Assay

Following shear exposure, HUVEC were trypsinized and re-suspended in reduced serum media (M199 with 2% FBS). Re-suspended cells (20,000 cells/well) were added to a growth factor reduced Matrigel (BD Bioscience) coated 96-well plate and incubated for 6 hours at 37°C. To test the effects of conditioned media (CM) on tubule formation, CM were collected from HUVEC sheared in reduced serum media (M199-2% FBS). Non-sheared HUVEC were then resuspended in the CM and 20,000 cells/well were added to a Matrigel coated 96-well plate. Tubule formation was observed using a phase contrast microscope at 5x and 10x magnification. Tubule length was quantified using NIH ImageJ and the total length of tubules was summed over 4 high powered fields (5x magnification).

Scratch Migration Assay

After shear, cell monolayers were scratched with a 200 μL pipette tip. The monolayer was washed once and the medium was replaced with M199-2% FBS (HUVEC) or DMEM-0.5% FBS (BAEC). The scratch line was photographed. After 6 hours of incubation at 37°C, the same scratch line was photographed and the amount of cells migrated into the scratch area were counted using NIH ImageJ. To examine the effects of CM on migration, non-sheared monolayers were scratched with a 200 μL pipette tip. The monolayer was washed once and the medium was replaced with the shear CM.

Ang2 knockdown by siRNA and Ang2 addition experiments

Sub-confluent (50%) HUVEC were transfected with 50 nM of annealed siRNA duplexes for Ang2 [sense: 5' –AGAACCAGACGGCUGUGAUGAUAGAAA-3', antisense 5' –UUUCUAUCAUCACAGCCGUCUGGUUCU-3'] (MWG) or non-silencing duplexes [sense: 5'-UUCUCCGAACGUGUCACGUtt-3', antisense: 5'-ACGUGACACGUUCGGAGAAtt-3'] (Qiagen) using Oligofectamine (Invitrogen) in Opti-MEM media (Gibco). After 4 hours, the medium was supplemented with FBS (20% final concentration). Following transfection (48 hours), the media was changed to M199-2% FBS and the cells were sheared for 24 hours. The shear conditioned media was then collected and used in Matrigel tubule formation assays. Alternately, following transfection, the cells were sheared for 24 hours, and were then used in a scratch migration assay. For Ang2 addition experiments, HUVEC shear conditioned media (M199-2% FBS) were collected and recombinant human Ang2[16] was added to the CM at concentrations of 50, 200, 500, and 800 ng/mL during Matrigel tubule formation.

Statistical Analysis

Data are reported as average ± SEM obtained from at least 3 independent studies. Statistical significance ($p<0.05$) was assessed by Student's t-test using a Microcal Origin statistical package.

Results

Shear-sensitive angiogenic genes and proteins

To identify the genes and the secreted proteins that are responsible for shear-mediated tubule formation and migration, we performed a gene array containing 128

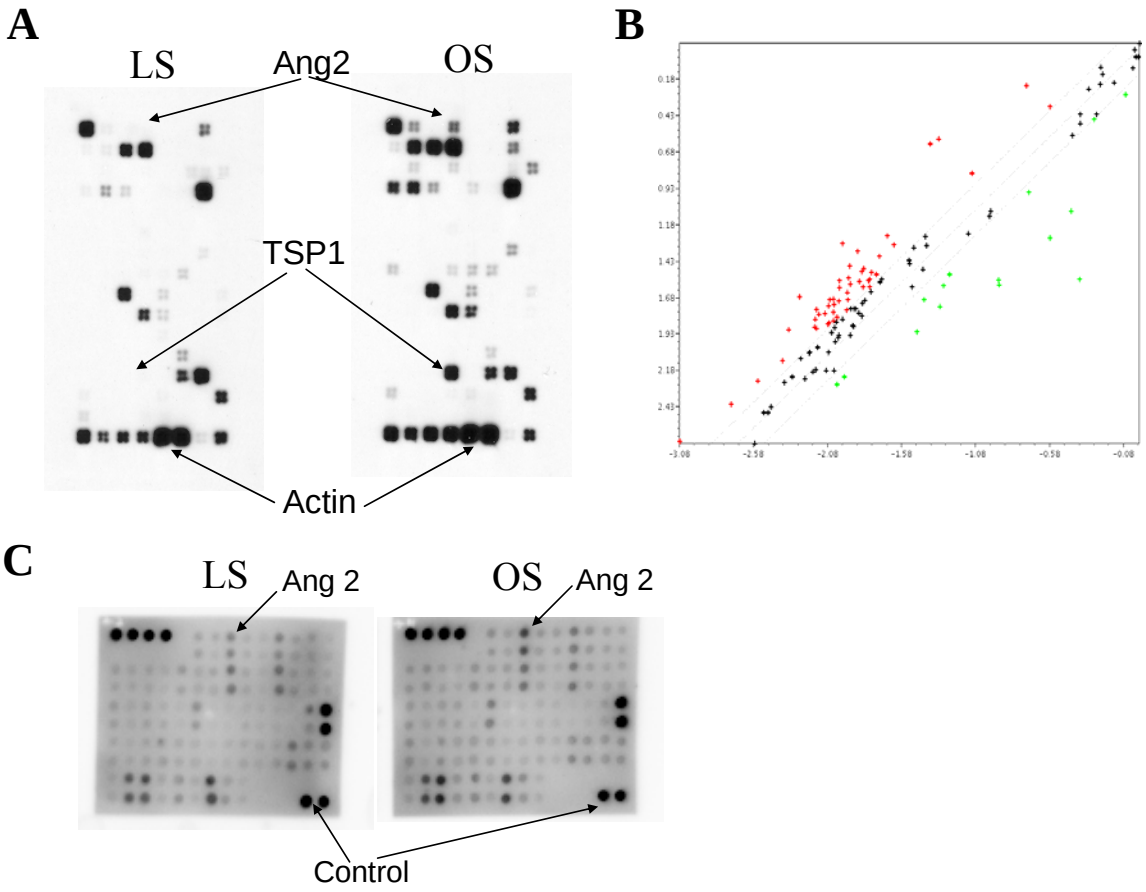

Figure 5.1. Gene and protein arrays show shear-mediated angiogenic genes and cytokines. A, Oligo GEArray human angiogenesis microarrays were hybridized with RNA from HUVEC exposed to LS (15 dyn/cm^2) or OS (±5 dyn/cm^2) for 24 hours. Actin was used as a loading control. A scatterplot of the 128 genes are shown in (B). Genes upregulated by oscillatory shear stress are in green and genes upregulated by laminar shear stress are red. Black genes did not change greater than 1.5-fold. C, Conditioned media from laminar- or oscillatory- sheared HUVEC was collected and incubated with a cytokine antibody array. Bound protein was detected using secondary antibody and chemiluminescence. Representative blots are shown.

human angiogenic genes and a protein array of 68 human cytokines (Figure 5.1 and Appendix A & B). Figure 5.2 shows genes significantly different between OS and LS. Of interest, *thrombospondin 1* (*TSP-1*) was upregulated 14.6-fold by OS over LS (Figure 5.2). From the protein array, Ang2 was upregulated 3.4-fold by OS compared to LS (Figure 5.1).

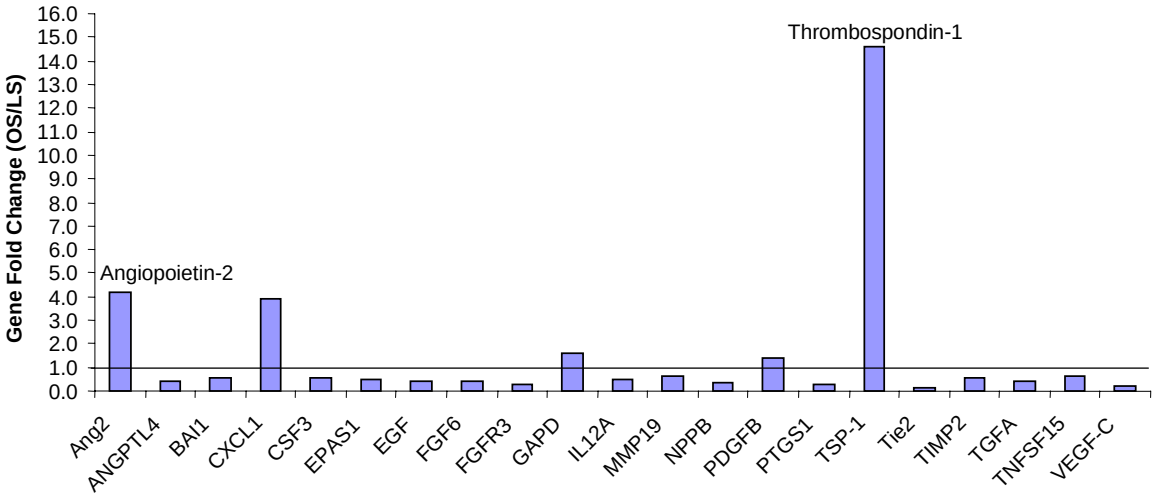

Figure 5.2. Shear-sensitive Angiogenic genes. Oligo GEArray human angiogenesis microarrays were hybridized with RNA from HUVEC exposed to LS (15 dyn/cm^2) or OS (±5 dyn/cm^2) for 24 hours. Actin was used as a loading control. Shown are fold changes of OS/LS for genes that changed significantly. (mean; n=3; P<0.05 with the exception of Ang2 P<0.06)

The gene and protein array results for Ang2 were confirmed using real time PCR and Western blot, while TSP-1 was used as an additional control. The CM and cell lysates of HUVEC exposed to LS expressed 2 to 3-fold less Ang2 protein than that of OS (Figure 5.3A). The molecular mass of Ang2 is known to be 68 kDa (the upper band), while the lower band (~61 kDa) seems to correspond to Ang2(443), an alternative splice variant of Ang2 [17]. Real time PCR showed that LS exposure significantly downregulated

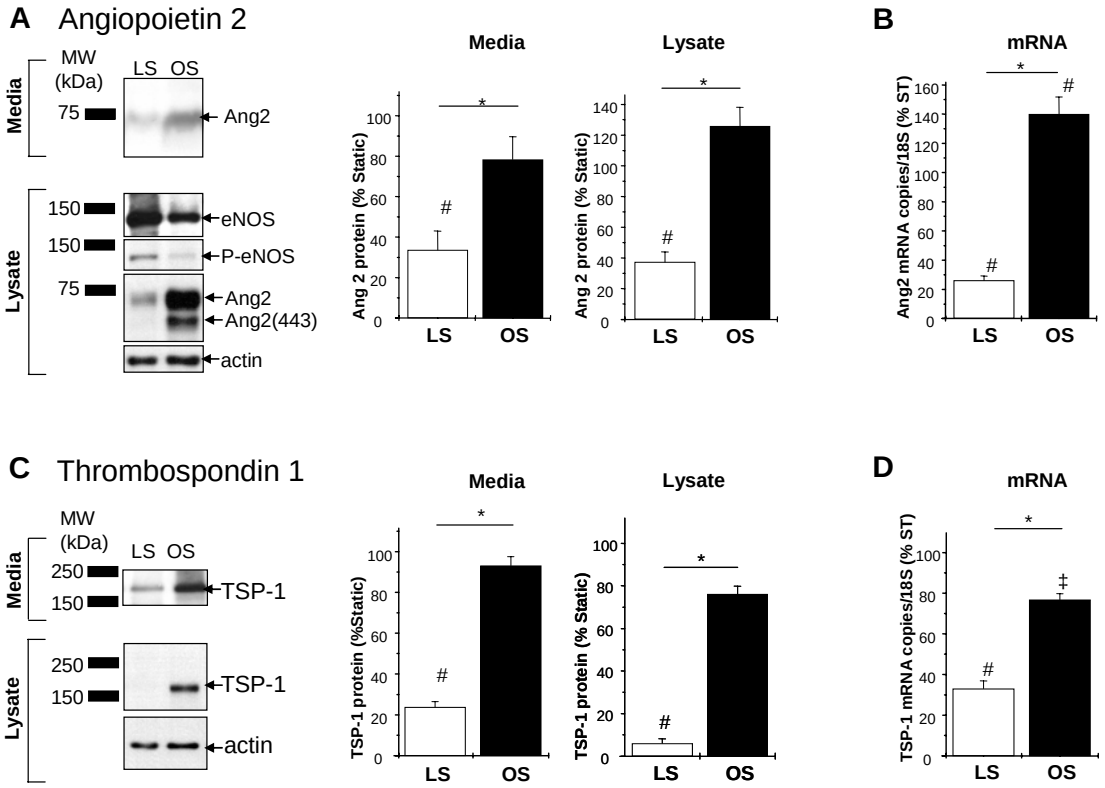

Figure 5.3. OS upregulates angiopoietin-2 (Ang2) and thrombospondin-1 (TSP-1) compared to LS in HUVEC. Confluent HUVEC were exposed to LS (15 dyn/cm^2), OS (±5 dyn/cm^2) and ST for 24 hours. Cell lysates and CM were used for Western blots with antibodies to Ang2 (A) and TSP-1 (C) and total RNA was used for real-time PCR analyses (Ang2: B, TSP-1: D). As controls, cell lysates were blotted with total eNOS and phosphorylated eNOS (p-eNOS) as well as β-actin. Ang2 and TSP-1 protein levels were quantified by densitometry and normalized to actin. mRNA copy numbers were normalized to 18S and represented as a percent of static. (mean ±SEM, n=3; * P<0.05 LS vs. OS; # P<0.01 compared to ST)

Ang2 mRNA level in HUVEC by almost 7-fold below that of OS (Figure 5.3B). While OS significantly increased Ang2 mRNA levels above that of static, Ang2 protein levels in OS and static cells were similar to each other (Figure 5.3A and B). In this study, the well-known effect of LS on increased endothelial nitric oxide synthase (eNOS) protein level and its phosphorylation at Ser-1177 (P-eNOS)[18] was used as a control for shear experiments (Figure 5.3A).

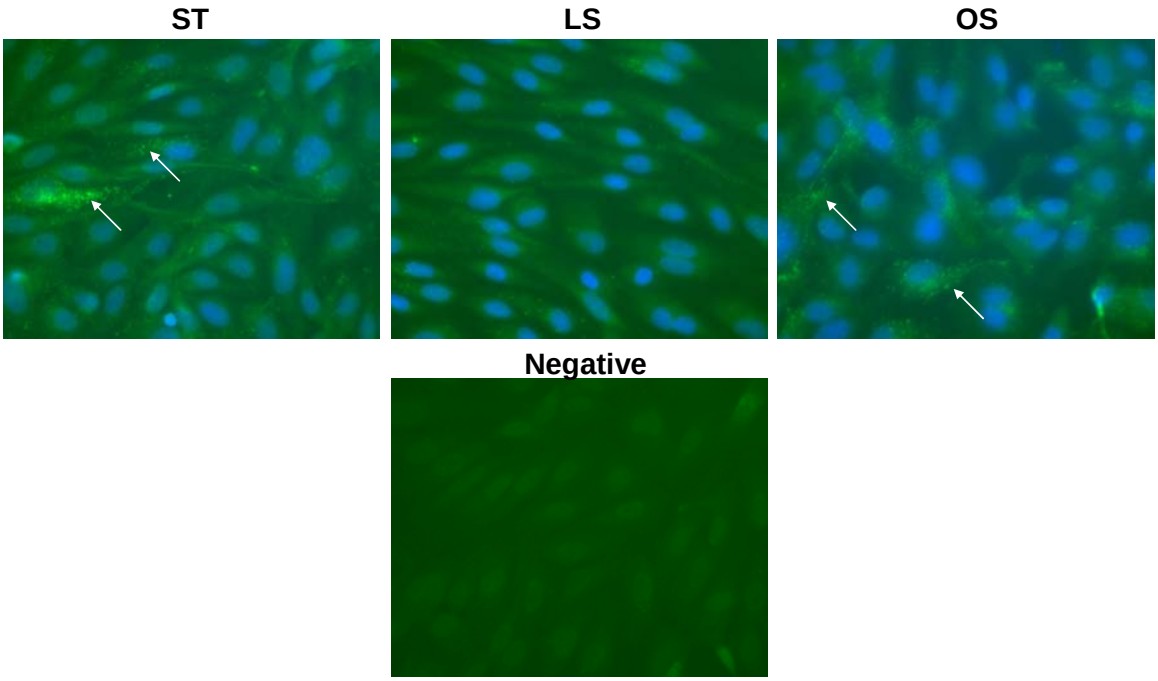

Figure 5.4. Ang2 localization in sheared EC. HUVEC were sheared as in Figure 5.1 and then immunocytochemistry was performed with primary antibody against Ang2 (green). Nuclei were stained with Hoechst (blue). Shown are representative images (63x) (n=3)

TSP-1 protein was downregulated in the CM and cell lysate of HUVEC exposed to LS (Figure 5.3C). Static cells and OS exposed cells expressed similar amounts of TSP-1 protein. Real time PCR data showed that TSP-1 mRNA level was ~2.5-fold higher in OS exposed HUVEC than that of LS (Figure 5.3D), which was not as high as

the 14.6 fold change found on the array, which may reflect differences between the array and PCR assays.

Ang2 localization in sheared EC

To investigate the localization of Ang2 in sheared EC, we performed immunocytochemisty analysis. HUVEC were sheared (LS, OS, or ST) for 24 hours and then immunostained for Ang2. In ST and OS cells, Ang2 is localized in the cytosol with a punctate staining (white arrows) (Figure 5.4). However, in LS cells the punctate staining ofAng2 is almost undetectable in the cytosol (Figure 5.4). Ang2 is known to be stored in Weibel-palade bodies which exhibit a similar staining pattern as shown here suggesting that under ST and OS Ang2 is stored in Weibel-palade bodies[19].

Ang2 is upregulated at sites of disturbed flow *in vivo*

The aortic arch has been shown to be exposed to both unidirectional laminar shear stress in the greater curvature and disturbed, oscillatory shear stress in the lesser curvature[20]. Using *en face* immunostaining of the aortic arch in mice, we found that Ang2 was downregulated in the atheroprotected greater curvature compared to the atheroprone lesser curvature[20] (Figure 5.5A & B), suggesting that Ang2 is downregulated by laminar shear stress and upregulated by disturbed, oscillatory shear stress *in vivo*. Ang1 which is known to be unchanged by shear stress[21] was used as a control and found to be unchanged by shear stress *in vivo* (Figure 5.5D & E). Both Ang1 and Ang2 were found only in the endothelium as depicted in orthogonal images of Z-stacks showing

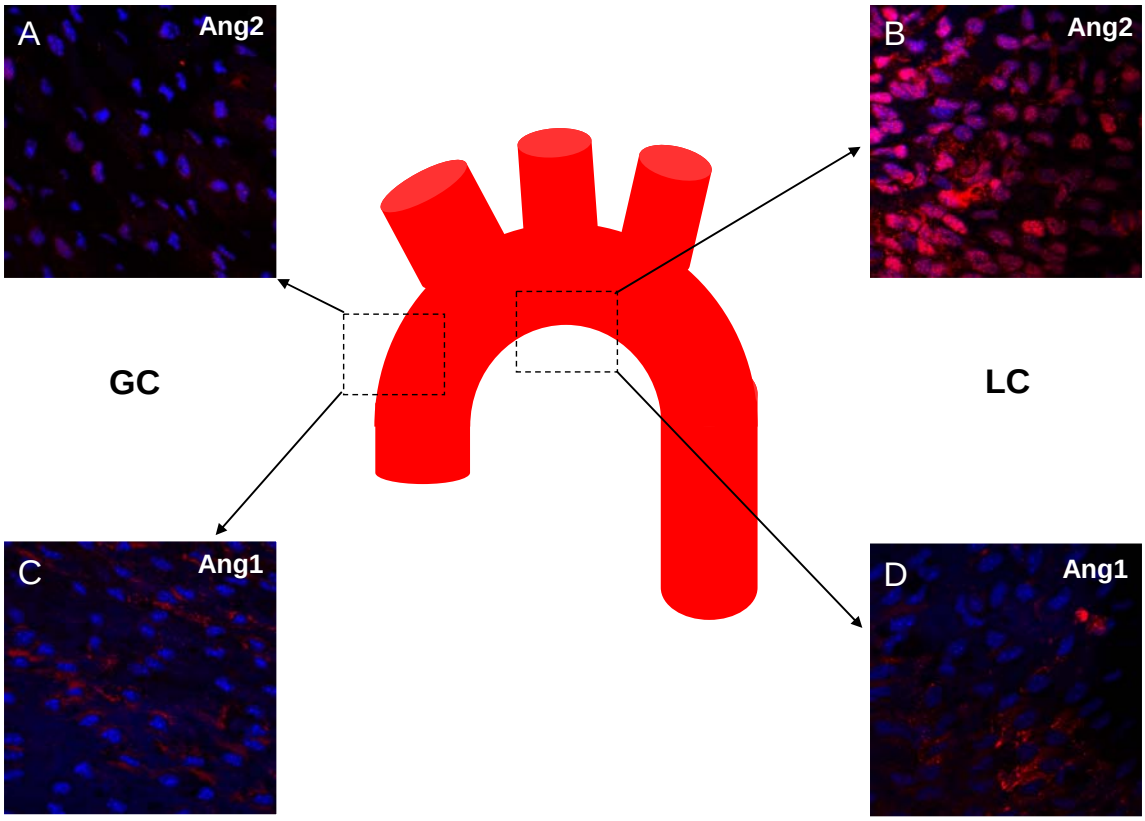

Figure 5.5. Ang2 is upregulated at sites of disturbed flow *in vivo*. C57BL/6J mice were pressure fixed with 10% formalin and the aortic arch and thoracic aorta were isolated. The tissue was incubated with primary antibodies against Ang2 (A & B), Ang1 (D & E) or secondary antibody only (rhodamine-red conjugated secondary) (C & F). The greater curvature (GC) and lesser curvature (LC) were isolated and splayed open on a microscope slide, endothelium facing up. Blue indicates nuclei stained with DAPI. Shown are representative confocal images at 63x magnification (n=5).

stained endothelial cells on top of the internal elastic lamina, with negative smooth muscle cells underneath (Figure 5.6)

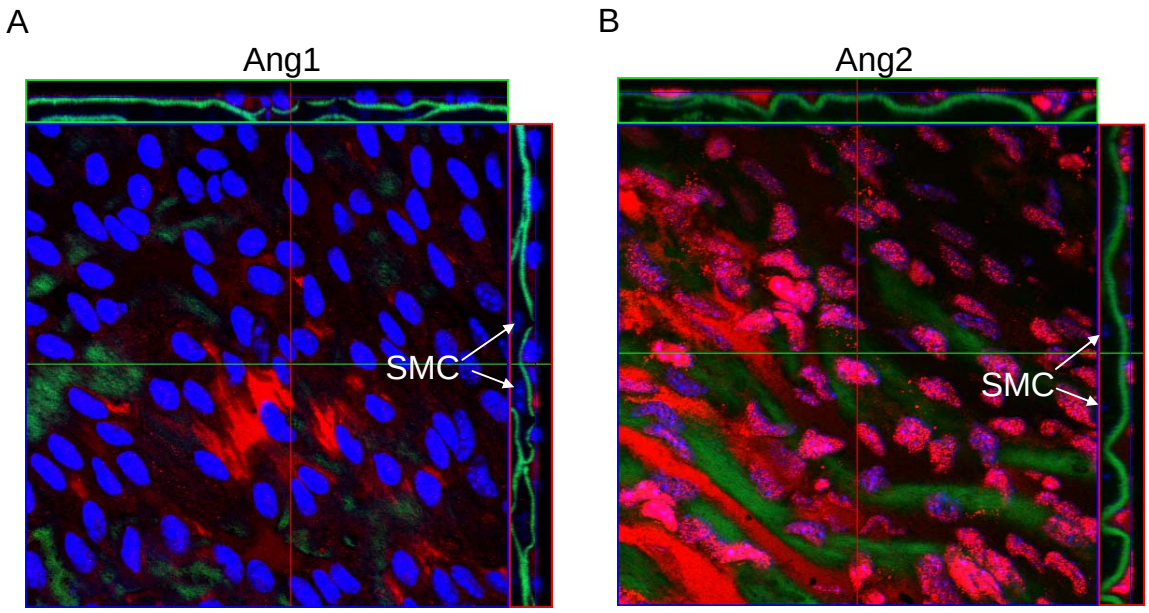

Figure 5.6. Ang2 is localized in the endothelial layer of mouse aortas. C57BL/6J mice were pressure fixed with 10% formalin and the lesser curvature (LC) of the aortic arch was isolated. The tissue was incubated with primary antibodies against Ang1 (A) and Ang2 (B). Blue indicates nuclei stained with DAPI. Green indicates elastic lamina. Shown are representative confocal images at 63x magnification (n=5).

Knocking down Ang2 inhibits OS-mediated migration

We next examined whether Ang2 was responsible for shear-mediated migration of HUVEC using Ang2 siRNA. Ang2 protein level was significantly and specifically reduced in HUVEC by siAng2 but not by non-silencing siRNA as determined by Western blot (Figure 5.7A-C). siAng2 did not knock down Ang1 (Figure 5.7A), showing its specificity for Ang2. Silencing Ang2 significantly inhibited OS-mediated migration

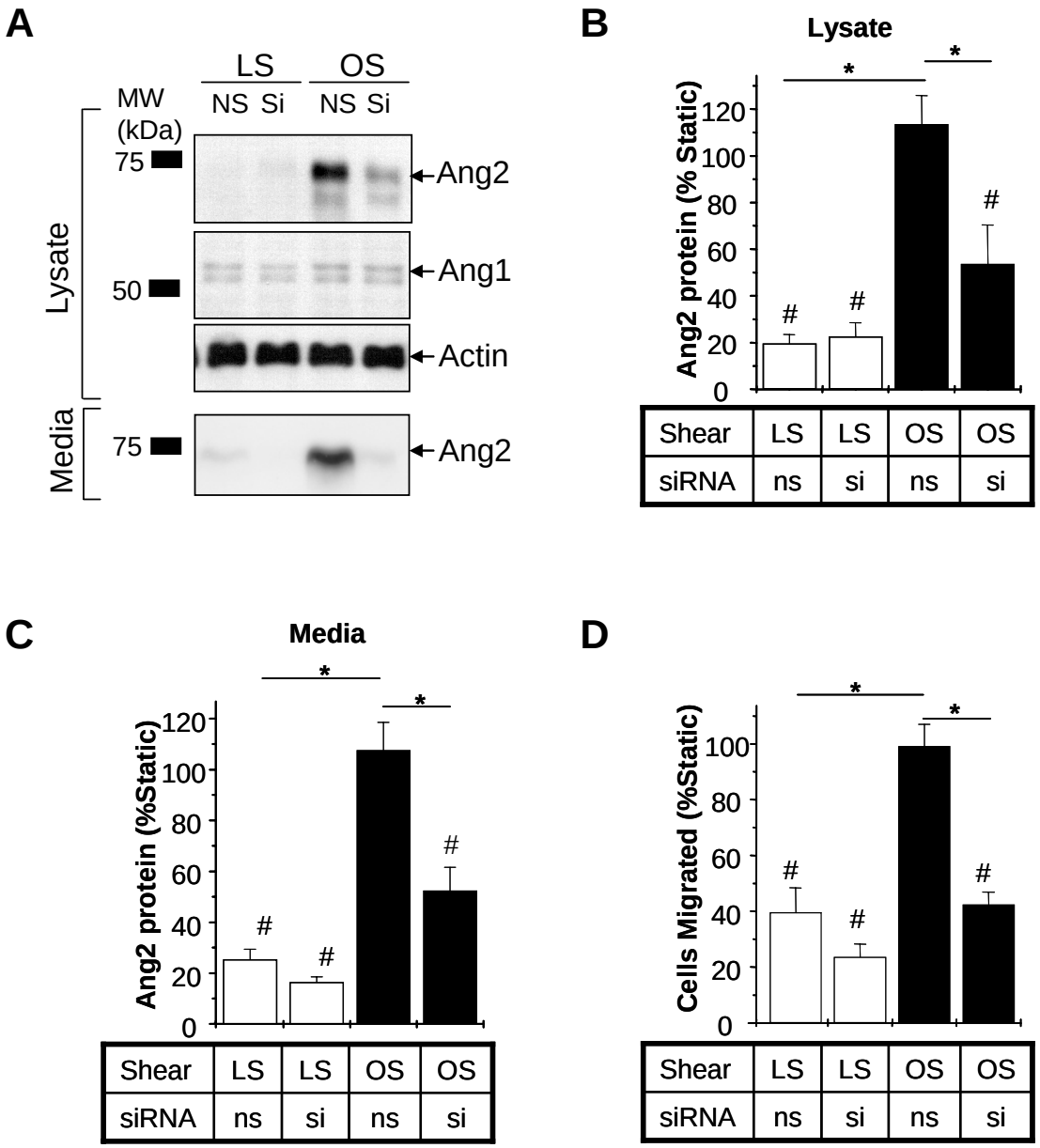

Figure 5.7. Ang2 knockdown by siRNA inhibits endothelial cell migration. A, HUVEC were treated with 50 nM of non-silencing siRNA (ns) or Ang2 siRNA (Si, siAng2) for 2 days. Cells were then sheared (LS, OS and ST) for 24 hours as in Fig. 2. Cell lysates and CM were Western blotted with an Ang2 antibody, while Ang1 and β-actin antibodies were used as controls (A). Ang2 bands were quantified by densitometry (B, C). D, HUVEC transfected with non-silencing siRNA or siAng2 were sheared for 24 hours and sheared cells were used in a scratch migration assay. (mean ±SEM, n=3-10; * $P<0.05$; # $P<0.05$ compared to ST)

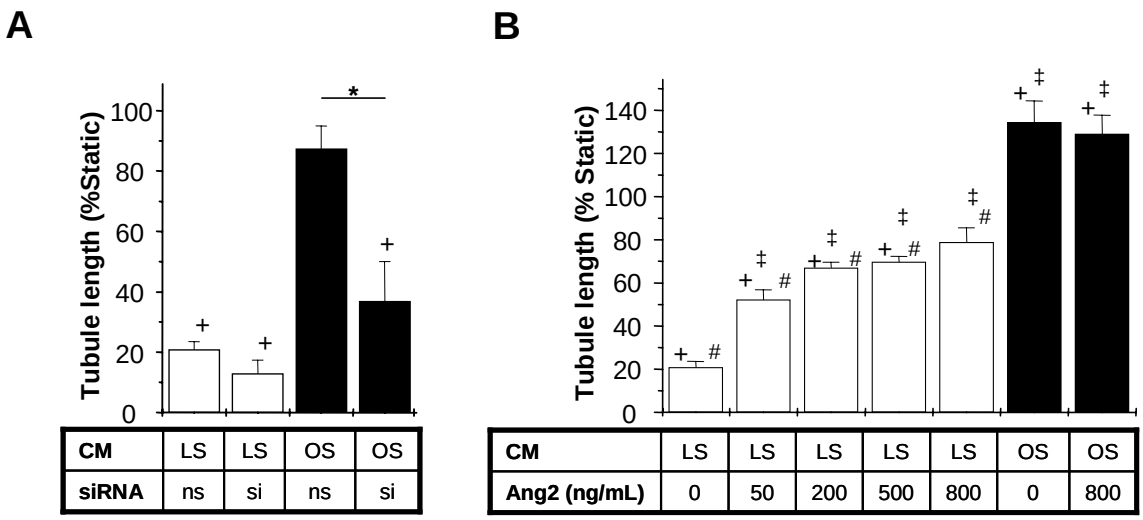

Figure 5.8. Ang2 knockdown inhibits OS-mediated tubule formation and LS-inhibited tubule formation can be rescued by exogenous addition of Ang2. A, HUVEC treated with non-silencing siRNA (NS) or siAng2 (si) as in Fig. 5 were sheared (LS, OS and ST for 1 day), and CM were collected. CM were then added to static HUVEC in a tubule formation assay. B. CM collected from sheared HUVEC (LS, OS and ST for 1 day) were added to static HUVEC for tubule formation assay and Ang2 was added at the concentrations indicated. (mean ±SEM, n=3-6; * $P<0.05$; $^{+}$ $P<0.05$ compared to ST; ‡ $P<0.05$ compared to LS-0; $^{\#}$ $P<0.05$ compared to OS-0)

(Figure 5.7D), suggesting that Ang2 plays an important role in migration. Ang2 knockdown had no significant effect on LS-mediated migration (Figure 5.7D).

Knocking down Ang2 inhibits OS-mediated tubule formation, and LS-inhibited tubule formation can be partially rescued by recombinant Ang2

Next, we examined whether secreted Ang2 in response to OS was responsible for OS-induced tubule formation. Silencing Ang2 inhibited OS-mediated tubule formation but not LS-mediated tubule formation (Figure 5.8A), suggesting the specific role of Ang2 in OS-mediated tubule formation. We next tested whether the inhibitory effect of LS on tubule formation is due to a lack of Ang2 secreted into the CM. To test this hypothesis,

recombinant Ang2 protein was added to LS CM in the Matrigel assay. As shown in Figure 5.8B, recombinant Ang2 partially rescued the inhibitory effect of LS on tubule formation in a dose-dependent manner. In contrast, recombinant Ang2 protein added to OS shear CM did not have an effect on tubule formation (Figure 5.8B), suggesting that Ang2 level in the OS CM already reached a maximum effective level. Ang2 protein is found physiologically at a concentration of ≤300 ng/mL[22] and therefore physiologically relevant levels of Ang2 can partially rescue LS-inhibited tubule formation. The fact that Ang2 only partially rescues tubule formation suggests that other factors may also be playing a role.

Discussion

In this study we found that: 1) Ang2 is upregulated by OS both at the gene and protein level *in vitro,* 2) Ang2 is downregulated at sites of LS and upregulated at sites of OS *in vivo*, 3) Knockdown of Ang2 inhibits OS-mediated tubule formation and migration, and 4) Addition of recombinant Ang2 partially rescues LS-inhibited tubule formation. Collectively, these findings suggest that Ang2 secreted by EC in response to OS, acts as a pro-migratory and pro-angiogenic molecule that could play an important role in diseases with altered shear stress.

To identify a molecular mechanism for the shear-mediated migration and tubule formation, we performed a gene and protein array from which we identified Ang2 and TSP-1 as being downregulated by LS compared to OS. Both Ang2 and TSP-1 have been previously identified as shear-sensitive *in vitro*[21, 23], however it has not been studied how shear stress affects tubule formation and migration through expression of Ang2 and TSP-

1. The role of TSP-1 in angiogenesis however is controversial, with most groups claiming it is anti-angiogenic and some claiming that it is pro-angiogenic in certain settings[24, 25]. In our hands, a TSP-1 inhibitory peptide did not show any significant effect on tubule formation induced by OS (data not shown). The downregulation of TSP-1 by LS may be more important in regulating inflammation as TSP-1 has been shown to bind CD36 and promote monocyte binding[23].

Angiopoietin-1 (Ang1) and Tie 2, the shared receptor of Ang1 and 2, were both on the gene array. However, Ang1 was not significantly changed by shear stress. Interestingly, Tie 2 was significantly upregulated by LS compared to OS (Appendix A, Table A.1). This could be due to a compensatory mechanism where either lack of the ligand causes increased expression of the receptor or conversely, too much of the ligand causes receptor de-sensitization which decreases the expression of the receptor. In fact, it has recently been shown that Tie 2 is internalized upon binding with Ang2 or Ang1, and this internalization could act as feedback to downregulate mRNA expression of Tie 2 [26].

Our gene array found that VEGF was unchanged by shear (Appendix A, Table A.2). VEGF has been shown to be important for the angiogenic potential of Ang2[11], however, Bureau et al. found that Ang2 can induce angiogenesis in a VEGF-independent manner[27]. VEGF-C was significantly upregulated by LS compared to OS on our gene array. VEGF-C has been shown to be important in lymphangiogenesis[28]. In Chapter 4, we found that VEGFR-2 was regulated by shear stress; however, VEGFR-2 inhibition did not completely abolish OS-mediated tubule formation suggesting that another mechanism could also be involved such as Ang2.

We identified Ang2 as being downregulated by LS compared to OS, both *in vitro* and *in vivo*. Ang2 is a 496 amino acid long protein with 6 potential N-glycosylation sites and a secretion signal peptide[17]. Ang2 knockout mice show that Ang2 is not required for embryonic angiogenesis but is requisite for postnatal angiogenesis [11]. Recently, it has been published that after inhibition of the PI3K/Akt pathway Ang2 expression is rapidly induced in EC by the transcription factor FOXO1 [29]. LS is known to stimulate the phosphorylation of Akt [18, 30] which inhibits Ang2 expression by phosphorylating and inhibiting FOXO1 [29], suggesting a possible mechanism for the downregulation of Ang2 in response to LS.

Ang2 is thought to be involved in several disease pathologies including cancer[31] and Ang2 has been found in neovessels of advanced human atherosclerotic plaques[32]. Inhibiting Ang2 may prove to be an effective treatment for these diseases. We were able to show that knocking down Ang2 using siRNA, blocked OS-mediated migration and tubule formation, and this effect was partially recaptured by addition of Ang2 to LS shear CM. These results indicate that regulating Ang2 could be used as an effective strategy for patients with diseases, such as atherosclerosis or ischemia that involve pathological angiogenesis.

In summary, we showed that LS inhibited pro-angiogenic responses while OS did not and this was through an Ang2-dependent manner. Elucidating the role of shear stress in regulating angiogenic signaling molecules such as Ang2 is important for understanding how these forces play a role in blood vessel remodeling and angiogenesis.

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CHAPTER 6

ANGIOPOIETIN-2 INHIBITION IMPAIRS BLOOD FLOW RECOVERY DURING HINDLIMB ISCHEMIA THROUGH THE INHIBITION OF ARTERIOGENESIS

After having identified Angiopoietin-2 (Ang2) as a mediator of shear-induced neovessel formation in specific aim 2, it is the goal of specific aim 3 to perform *in vivo* experiments to determine the physiological relevance of Ang2 in shear-mediated neovascularization. To achieve this aim, the role of Ang2 was examined in a mouse model of hindlimb ischemia. This model was selected because the femoral artery is ligated and this leads to disturbed flow in the post-occlusive site.

Introduction

Blood vessel formation plays an important role in normal physiology as well as in pathophysiology. In particular, the formation of new blood vessels is involved in diseases involving ischemia, including tumors, atherosclerotic plaques, and ischemic heart disease[1]. Therefore, understanding the mechanisms of blood vessel formation and the ability to control blood vessel formation could be important in developing therapies for these diseases. The two main forms of blood vessel formation in the adult are angiogenesis and arteriogenesis[1]. Angiogenesis is the formation of new blood vessels from pre-existing blood vessels whereas arteriogenesis is the enlarging of existing blood vessels to form collaterals and the recruitment of smooth muscle cells[1, 2]. The exact molecular mechanisms that drive blood vessel formation remain unclear.

Numerous factors are thought to be involved in blood vessel formation, including hypoxia, inflammation, and the secretion of growth factors[1]. Proteins such as vascular

endothelial cell growth factor (VEGF), angiopoietin-1 (Ang1), angiopoietin-2 (Ang2), Tie2, and hypoxia inducible factor-1α (HIF-1α) are all thought to play a role[1]. We have recently identified Ang2 as a mechano-sensitive protein involved in shear stress-mediated tubule formation and migration *in vitro* using cultured endothelial cells[3]. However, the role of Ang2 in blood vessel formation *in vivo* remains controversial and needs to be clarified[4, 5].

In human diseases, Ang2 has been found to be upregulated within neovessels of advanced atherosclerotic lesions as compared to early lesions[6]. In addition, Ang2 was upregulated in an animal model of myocardial ischemia[7]. In both advanced atherosclerosis and myocardial ischemia, hypoxia and inflammation are present[8, 9]. Therefore, we hypothesized that Ang2 plays an important role in blood flow recovery during ischemia through the modulation of angiogenesis and arteriogenesis. To test this hypothesis, we used a mouse model of hindlimb ischemia to examine the role of Ang2 in blood flow recovery as well as angiogenesis and arteriogenesis. From this, we demonstrate that Ang2 plays an important role in blood flow recovery during ischemia through the formation of collaterals and the recruitment of inflammatory cells, implicating Ang2 as an important factor in regulating ischemic diseases.

Methods

Hindlimb ischemia model

Male C57Bl/6J mice were purchased from the Jackson Laboratory and used at 6-8 weeks of age. All protocols were approved by the Institutional Animal Care and Use Committee and done in accordance with the federal guidelines on the principles for the care and use of animals in research. Animals were anesthetized by intraperitoneal injection of Xylazine (10 mg/kg) and Ketamine (80 mg/kg). The left superficial femoral artery and vein were isolated from the femoral nerve and ligated proximal to the caudally branching deep femoral artery. A second ligation was then made proximal to the branching of the tibial arteries and the artery and vein were removed between the two ligation points, leaving the femoral nerve intact. The skin was closed by interrupted sutures and the mouse was allowed to recover and ambulate freely[10]. Where indicated, L1-10 (4mg/kg), an FC-fusion protein Ang2 specific inhibitor (Amgen), vehicle (PBS), or IgG_1-FC control (R&D systems) was injected subcutaneously every other day starting with 1 day before surgery.

Real time quantitative polymerase chain reaction (PCR)

Animals were sacrificed by CO_2 inhalation and pressure perfused with 0.9% NaCl solution. The adductor muscle was isolated and immediately flash frozen in liquid nitrogen. The frozen tissue was disrupted using a mortar and pestle and homogenized using QIAshredders (Qiagen). Total RNA was isolated and purified using RNeasy Mini Kit (Qiagen). Four µg of total RNA was reverse transcribed by using random primers and a Superscript-II kit (Life Technology) to synthesize first-strand cDNA. The cDNA

was assayed by real time PCR using a LightCycler (Roche), recombinant Taq polymerase (Life Technology), Taq start antibody (Clontech) and SYBR green detection of products. Copy numbers were determined based on standard curves generated with mouse standards for Ang2, Ang1, c-fms, and 18S templates. The following primers were used to amplify mouse Ang2 (5' to 3')- forward: AGATCCAACAGAATGTGGTGC and reverse: TGTTGACGGTCTCCATTAGG[11], for mouse Ang1- forward: CACGAAGGATGCTGATAACG and reverse: AAGTGGCGATTCTGTTGACC[11], for mouse c-fms- forward: CACAGGAGGTGACAGTGGTT and reverse: TGGTCTTGCACACGTAGGTA[12]. Real-time PCR for Ang2, Ang1, and c-fms was carried out with the annealing temperature at 60ºC and 40 cycles. DNA gels were run to confirm specificity of PCR products.

Whole mount immunohistochemistry

On day 2, 5, 7 or 10 after surgery as indicated, the animal was sacrificed by CO_2 inhalation and pressure perfused with 0.9% NaCl solution then pressure fixed with 10% formalin. The adductor muscle was isolated, removed and fixed for 1 hour with 1% paraformaldehyde. The tissue was blocked for 1 hour with 5% goat or donkey serum in Tris buffered saline containing 0.3% Triton-X 100 (TBST) and incubated overnight at 4°C with primary antibody diluted 1:1000 in TBST. After several washes with TBST, the tissue was then incubated with fluorescently tagged secondary antibody diluted in TBST for 4 hours. The tissue was washed and post-fixed with 4% paraformaldehyde for 10 minutes. The whole tissue was placed on a coverslip and imaged using a LSM 510 Meta confocal microscope (Zeiss). Images were constructed into 3D projections of Z-

stacks. Antibodies used were: hamster anti-mouse PECAM-1 (Chemicon), goat anti-mouse Ang2 (Santa Cruz), anti-mouse smooth muscle alpha actin-FITC (Sigma) and rat anti-mouse CD11b (BD).

Preparation of cell lysates and immunoblotting

Human umbilical vein endothelial cells (HUVEC) were serum starved for 1 hour and then treated with Ang1 (800 ng/mL, R&D Systems), Ang2 (800 ng/mL), and/or L1-10 (6.5μg/mL which is equivalent to ~10 times the molar concentration of Ang1 and Ang2) for 15 minutes. Cells were lysed in RIPA buffer and further homogenized by sonication. Protein content of each sample was measured using a Bio-Rad DC assay. Protein (50 μg) was resolved on 8% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore)[3]. The membranes were probed with the following primary antibodies: anti-pTie2 (Tyr992) (Cell Signaling), anti-Tie2 (Santa Cruz) and anti-actin (Santa Cruz) and then with secondary antibody conjugated to alkaline phosphatase, which was then detected using chemiluminescence. The intensities of immunoreactive bands were analyzed using Scion Image and normalized to β-actin.

Shear studies and Matrigel tubule formation assay

Human umbilical vein endothelial cells (HUVEC) were obtained from the Department of Dermatology, Emory University, cultured in M199 media (Cellgro) with 20% heat inactivated fetal bovine serum (FBS, Atlanta Biologicals), and used between passage 4 and 5. HUVEC were grown to confluency and exposed to laminar shear stress (LS, 15 dyn/cm^2), oscillatory shear stress (OS, ± 5 dyn/cm^2 at 1 Hz frequency) or static

no shear control (ST) for 24 hours using a cone-and-plate device as described by us[3]. Following shear, conditioned media (CM) were collected from HUVEC sheared in reduced serum media (M199-2% FBS). Non-sheared HUVEC were then resuspended in the CM and 20,000 cells/well were added to a growth factor reduced Matrigel coated 96-well plate. The Ang2 specific inhibitor, L1-10, or vehicle was then added at 10 µg/mL and the cells were incubated at 37°C for 6 hours. Tubule formation was observed using a phase contrast microscope at 5x and 10x magnification. Tubule length was quantified using NIH ImageJ and the total length of tubules was summed over 4 high powered fields (5x magnification).

Laser Doppler perfusion imaging (LDPI)

LDPI was performed 1 day before hindlimb ischemia surgery (Pre-Op) to establish a baseline and on days 2, 5, 7, and 10 after surgery. Mice were anesthetized with Xylazine (10mg/kg) and Ketamine (80 mg/kg) and allowed to warm on a 37°C heating pad for 5 minutes. The footpad was then scanned with a PIM II Laser Doppler Perfusion Imager. Average perfusion in the ischemic paw was then normalized to the contralateral control for each animal.

Statistical analysis

Data are reported as average ± SEM obtained from at least 3 independent studies. Statistical significance ($p<0.05$) was assessed by Student's t-test using a Microcal Origin statistical package as well as one-way ANOVA followed by Bonferroni test for comparisons over a timecourse within the same group.

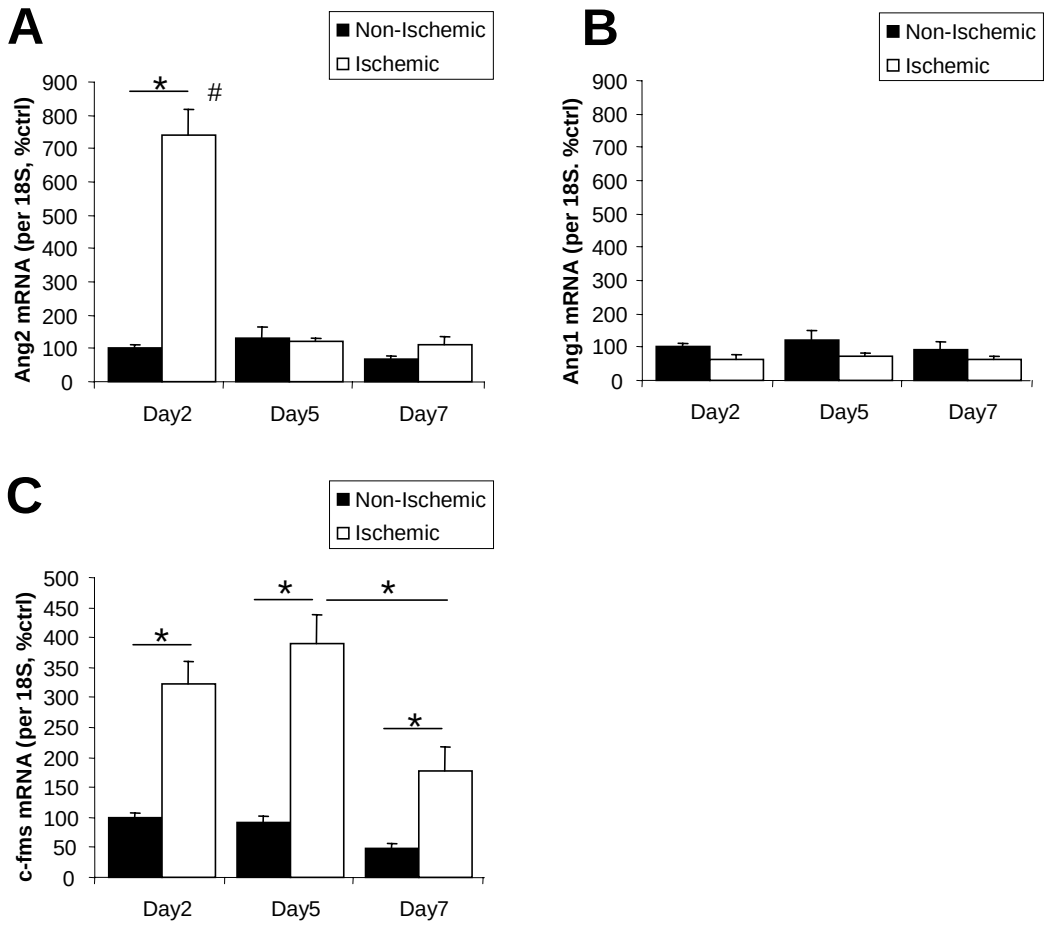

Figure 6.1. Ang2 and c-fms mRNA are upregulated in the ischemic hindlimb whereas Ang1 mRNA is not. C57Bl/6J mice underwent hindlimb ischemia surgery. RNA was collected after 2, 5, or 7 days from the ischemic and non-ischemic adductor muscle. Real-time PCR was performed for Ang2 (A), Ang1 (B), and c-fms (C). (mean ±SEM, n=4-6; * $P<0.05$; # $P<0.05$ compared to day 5 and day 7, ANOVA followed by Bonferroni test).

Results

Ang2 is Transiently Upregulated in the Ischemic Hindlimb Whereas Ang1 is Not

To investigate the role of angiopoietins in neovascularization, we examined the expression of Ang1 and Ang2 in the mouse hindlimb after femoral artery ligation. Male C57Bl/6J mice underwent femoral artery ligation surgery to induce hindlimb ischemia. After 2, 5, or 7 days the adductor muscle was collected for mRNA. Real time

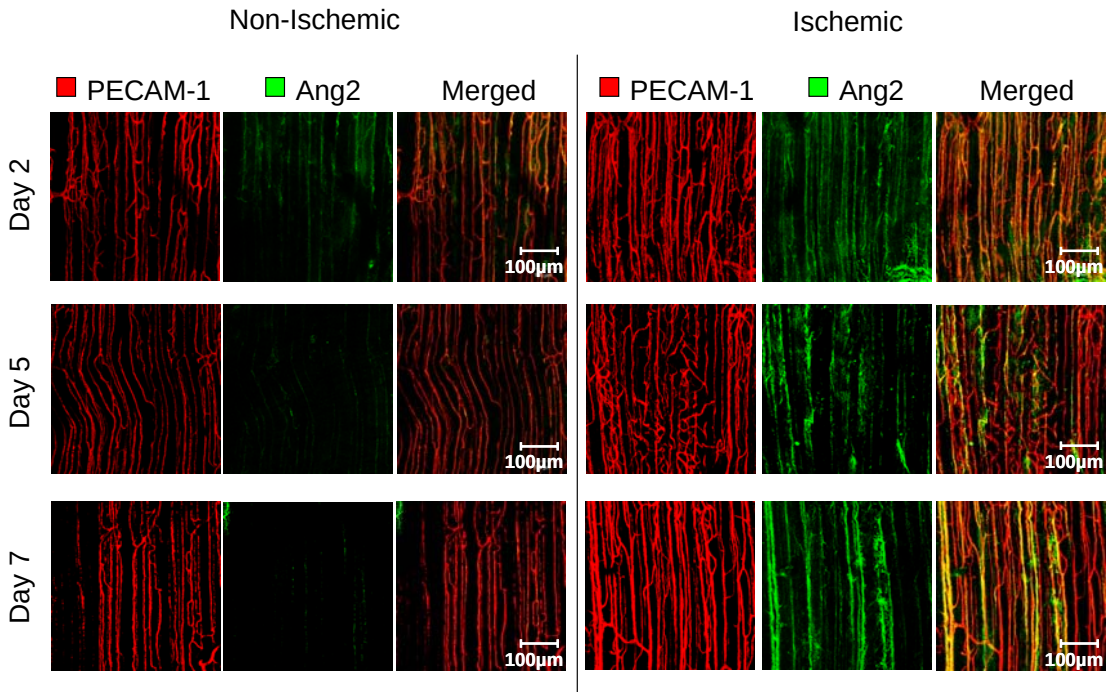

Figure 6.2. Ang2 protein expression is upregulated in blood vessels of the ischemic hindlimb. C57Bl/6J mice underwent hindlimb ischemia surgery. Whole mount immunohistochemistry was performed after 2, 5, or 7 days on the adductor muscle for Ang2 (green) and PECAM-1 (red). Shown are projection images from Z-stacks of the proximal region of the adductor muscle. (mean ±SEM, n=4-5).

quantitative PCR revealed that Ang2 mRNA was upregulated 7-fold in the ischemic adductor muscle compared to the contralateral non-ischemic control adductor on day 2, but returned to equivalent values on day 5 and day 7 (Figure 6.1A), suggesting an early role for Ang2 in hindlimb ischemia. In contrast, Ang1 mRNA levels were not significantly different between the ischemic and non-ischemic adductor muscle (Figure 6.1B). C-fms mRNA, a macrophage marker, was upregulated in the ischemic adductor on day 2, 5 and 7, with day 7 starting to decline back to normal levels (Figure 6.1C) suggesting a role for macrophages in hindlimb ischemia recovery.

Whole mount immunostaining of the mouse adductor muscle was performed for PECAM-1 and Ang2 protein expression (Figure 6.2). PECAM-1 staining for endothelial

cells revealed blood vessels in the non-ischemic control that align parallel to the muscle fibers (Figure 6.2). The ischemic adductor muscle had increased PECAM-1 staining intensity and a slight increase in blood vessel density compared to the non-ischemic control (Figure 6.2). By day 7, blood vessels in the ischemic adductor muscle are larger in diameter (Figure 6.2). Ang2 protein expression was upregulated in the ischemic adductor muscle on day 2, 5, and 7, suggesting that Ang2 protein expression lags behind mRNA expression (Figure 6.2).

L1-10 Inhibits Ang2 and Shear-Mediated Tubule Formation

To investigate the specific role of Ang2 in neovascularization, we used an FC-fusion peptide called L1-10, which is a specific inhibitor of Ang2 binding to its receptor, Tie2. L1-10 is a related compound to L1-7 which was used in previous publications and shown to be a specific inhibitor of Ang2[13, 14]. To confirm the inhibitory effects of L1-10 on Ang2, we examined the phosphorylation of Tie2 by Ang2 binding. HUVEC were treated with control, Ang1, Ang2, Ang1 + L1-10, or Ang2 + L1-10 and the phosphorylation of Tie2 was examined. Ang2 treatment was found to phosphorylate Tie2 at concentrations of 800 ng/mL which could be blocked by addition of L1-10 (Figure 6.3A). However, L1-10 did not block Ang1 induced phosphorylation of Tie2 (Figure 6.3A). Total Tie2 levels were not affected. In addition, to examine the functional role of L1-10, we examined its effect on shear-mediated tubule formation. Previously, we showed that fluid shear stress, specifically OS, mediated tubule formation through a mechanism dependent on Ang2, but not Ang1, in endothelial cells[3]. We found

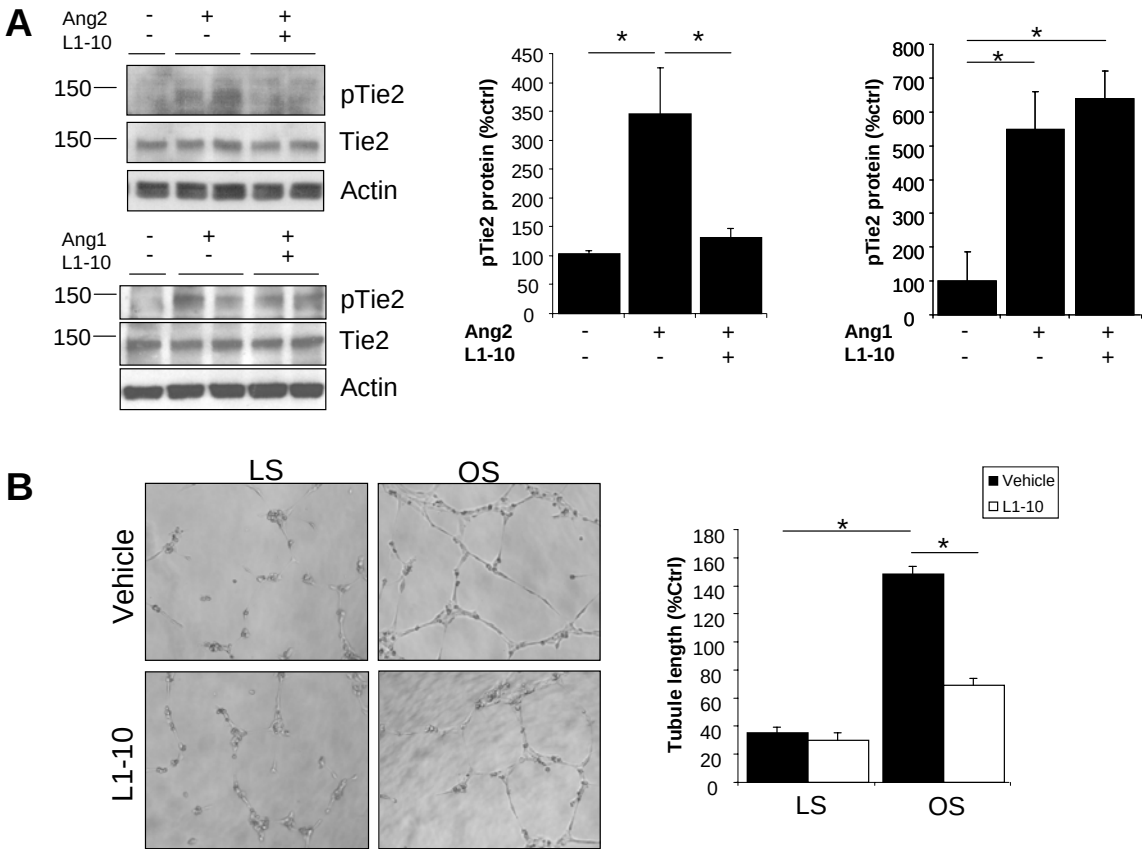

Figure 6.3. L1-10 specifically inhibits Ang2 and inhibits *in vitro* shear-mediated tubule formation. A, HUVEC were treated with Ang1 (800 ng/mL), Ang2 (800 ng/mL) ± L1-10 for 15 minutes. Cell lysates were used for Western blots with antibodies to phospho-Tie2, Tie2, and actin. Blot densitometry was quantified using Scion Image and normalized to actin as a loading control. B, Conditioned media collected from HUVEC that were sheared at 15 dyn/cm^2 laminar shear stress (LS), ±5 dyn/cm^2 oscillatory shear stress (OS) or no shear static control (ST) for 24 hours were added to HUVEC in a Matrigel tubule formation assay. L1-10 was added at 10µg/mL where indicated. Shown are representative images and tubule length was quantified over 4 high powered fields (5x magnification) and normalized to percent ST. (mean ±SEM, n=3-6; * P<0.05).

that L1-10 inhibited OS-mediated tubule formation of HUVEC (Figure 6.3B), suggesting that L1-10 can inhibit the functional role of Ang2 *in vitro*.

Ang2 Inhibition Impairs Blood Flow Recovery During Hindlimb Ischemia

To investigate the *in vivo* role of Ang2 in neovascularization during ischemia, we investigated the effect of inhibiting Ang2 on blood flow recovery and vascular remodeling after femoral artery ligation. We found that inhibiting Ang2 via the Ang2 specific FC-fusion peptide, L1-10, impaired blood flow recovery compared to vehicle and FC control as evidenced by LDPI measurement (Figure 6.4). Ang2 inhibition significantly impaired blood flow recovery in the ischemic hindlimb on days 5 and 7 post-surgery (Figure 6.4). FC control had no effect compared to vehicle control. Therefore, vehicle was used as the control for all subsequent experiments and FC was only examined at the 10 day time point.

Ang2 Inhibition Blocks Arteriogenesis

To examine how Ang2 inhibition was impairing blood flow recovery, we examined blood vessel formation in the ischemic adductor muscle. Briefly, the adductor muscle was isolated and whole mount immunostained for PECAM-1, an endothelial cell specific marker, and smooth muscle α-actin (SMA), a VSMC marker. Since arteriogenesis is the formation of collaterals requiring the participation of VSMC, we denoted PECAM-1 positive vessels covered with VSMC as a marker of arteriogenesis and PECAM-1 positive vessels lacking VSMC as a marker of angiogenesis. We found that on day 7 and day 10, there was no significant difference in angiogenesis, or the

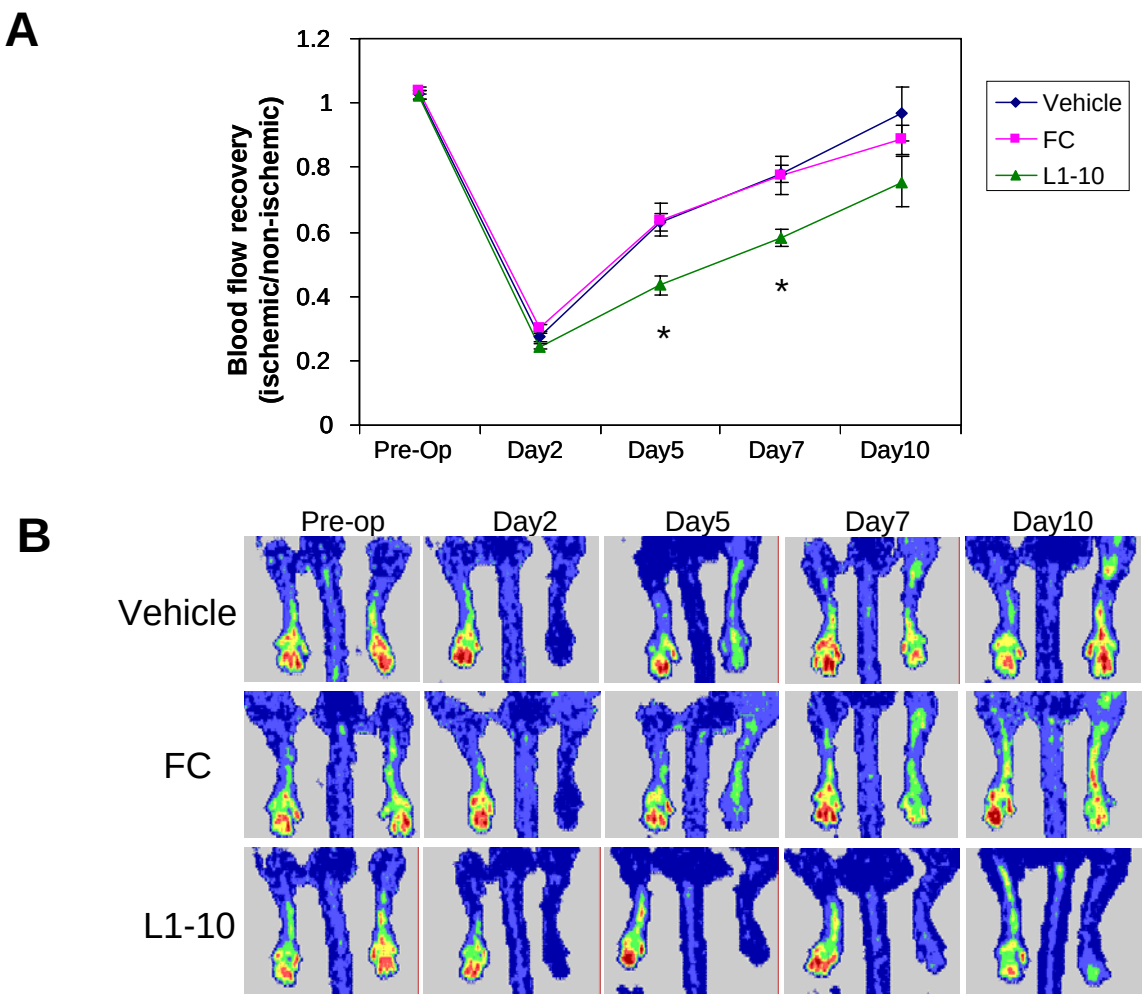

Figure 6.4. Ang2 inhibition impairs blood flow recovery during hindlimb ischemia. C57Bl/6J mice underwent hindlimb ischemia surgery and were treated with Vehicle, L1-10, or FC control. LDPI was measured before surgery (pre-op), and 2, 5, 7, and 10 days after surgery. A, Mean perfusion was expressed as a ratio of the ischemic paw to the contralateral non-ischemic paw. B, Shown are representative LDPI images at the indicated timepoints. (mean±SEM, n=6-12; * P<0.05 compared to vehicle and FC control).

VSMC-free blood vessels (capillaries), between the Ang2 inhibitor treated and vehicle treated ischemic adductor muscles (Figure 6.5). Although the morphology looked slightly different, with L1-10 treated vessels being less tortuous, the overall capillary density did not change. However, on day 7 and day 10, the Ang2 inhibitor treated ischemic adductor muscle showed a significant decrease in arteriogenesis, or VSMC-covered blood vessels (arterioles), as compared to the vehicle treated ischemic adductor muscle (Figure 6.5). On day 10, FC control treated ischemic adductor muscle showed no significant difference compared to vehicle control (Figure 6.5B). Ang2 inhibitor treated ischemic adductor muscle showed a significant decrease in arteriogenesis as compared to FC control on day 10, similar to vehicle control results (Figure 6.5B). These results suggest that Ang2 improves blood flow recovery via arteriogenesis or collateral formation.

Ang2 Inhibition Reduces Inflammatory Cell Infiltration

Since macrophages are known to play an important role in hindlimb ischemia and have been shown to be recruited by Ang2[15], we investigated the effects of Ang2 inhibition on macrophage infiltration during hindlimb ischemia. The ischemic adductor muscle was isolated and whole mount immunostained for PECAM-1 and CD11b, a marker of macrophages and neutrophils. On day 7 and day 10 after hindlimb ischemia surgery, Ang2 inhibition significantly decreased the number of macrophages/neutrophils in the ischemic adductor muscle as compared to vehicle control (Figure 6.6), suggesting that Ang2 inhibition reduces inflammatory cell infiltration. FC control treated was not significantly different from vehicle control (Figure 6.6B).

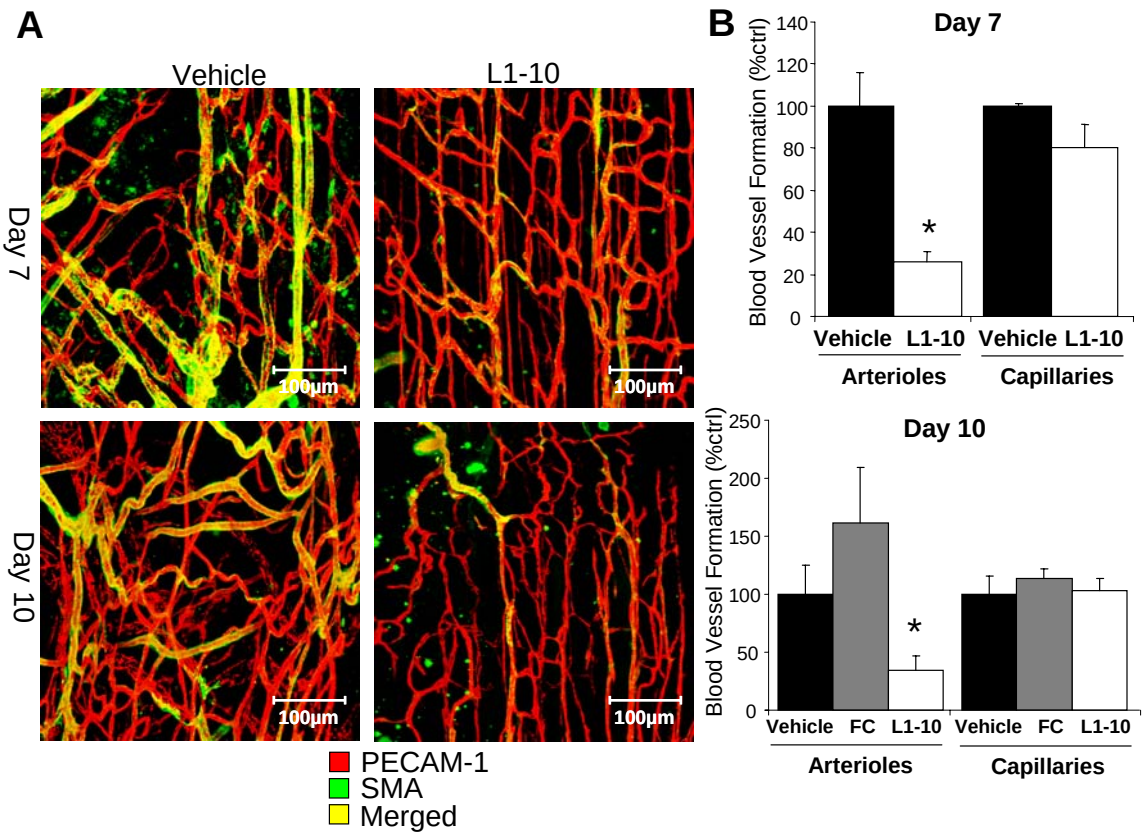

Figure 6.5. Ang2 inhibition blocks arteriogenesis and the formation of collaterals. C57Bl/6J mice underwent hindlimb ischemia surgery and were treated with vehicle or L1-10. Whole mount immunohistochemistry was performed after 7 and 10 days on the ischemic adductor muscle for PECAM-1 (red) and smooth muscle α-actin (SMA) (green). Shown are representative merged projection images of Z-stacks from the distal region of the ischemic adductor muscle (A). B, Each channel was quantified using ImageJ with green representing arterioles and red only covered vessels representing capillaries and expressed as a percent of vehicle control. (mean±SEM, n=4-5; * $P<0.05$ compared to vehicle and FC control).

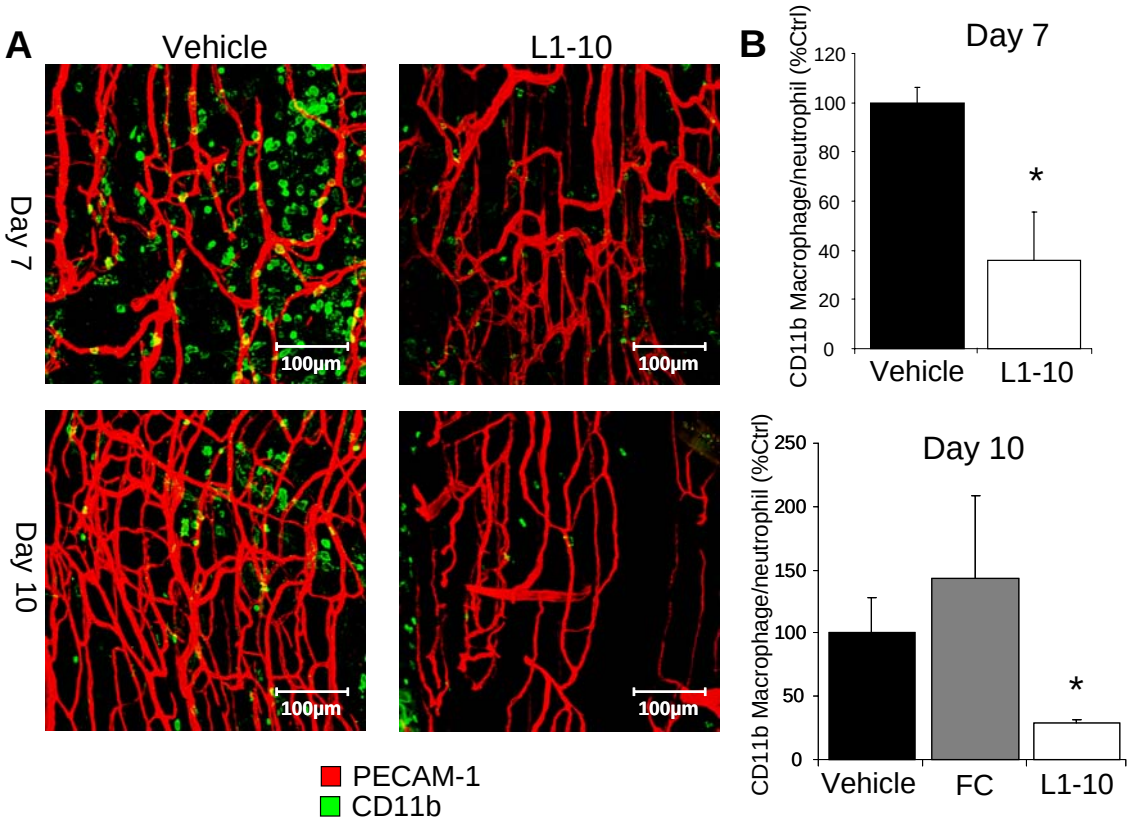

Figure 6.6. Ang2 inhibition reduces inflammatory cell infiltration. C57Bl/6J mice underwent hindlimb ischemia surgery and were treated with vehicle or L1-10. Whole mount immunohistochemistry was performed after 7 and 10 days on the ischemic adductor muscle for PECAM-1 (red) and CD11b (green). Shown are representative merged projection images of Z-stacks from the distal region of the ischemic adductor muscle (A). B, Macrophage/neutrophils were quantified using ImageJ and expressed as a percent of vehicle control. (mean±SEM, n=4-6; * $P<0.05$ compared to vehicle and FC control).

Discussion

The novel findings of this study are: 1) Ang2 is upregulated at early time points in the mouse ischemic hindlimb both at the mRNA level and protein level, 2) Ang2 induced phosphorylation of the Tie2 receptor can be inhibited by the FC-fusion peptide L1-10 and L1-10 inhibits shear-mediated tubule formation *in vitro*, 3) Inhibition of Ang2 impairs blood flow recovery in the mouse ischemic hindlimb, 4) Inhibition of Ang2 inhibits VSMC coverage of neovessels and collateral formation in the ischemic adductor muscle, 5) L1-10 treatment inhibits macrophage/neutrophil infiltration into the ischemic adductor muscle. Collectively, these findings suggest that Ang2 plays an important role in the early response to ischemia, by the recruitment of inflammatory cells as well as the accumulation of VSMC.

There are four known angiopoietins, however, Ang1 and Ang2 are the most well characterized[16]. Ang1 appears to be involved in vessel stabilization whereas Ang2 appears to be involved in vascular remodeling and vessel destabilization[17]. However, the complex role of Ang2 in vascular remodeling still remains unclear. We previously found that Ang2 was upregulated by a disturbed shear stress termed OS when compared to a normal shear stress termed LS[3]. In addition, we found that OS-induced tubule formation was mediated through Ang2[3]. In the current study, we examined the role of Ang2 in hindlimb ischemia which is produced by the cessation of flow in the femoral artery, altering flow conditions including disturbance and/or reversal of shear stress in collaterals[2].

During hindlimb ischemia, fluid shear stresses in the vasculature change drastically. Whereas fluid flow increases in the collaterals bypassing the occlusion, fluid

flow is low and reversed in the post-occlusive site[2]. When a vessel is occluded, blood pressure drops in the post-occlusive site. This creates a steep pressure gradient and increases the flow in pre-existing arterioles towards the area of low pressure. In some areas of the post-occlusive site the flow becomes reversed[2]. The mouse hindlimb ischemia model is similar to occlusive vascular disease seen in humans, such as myocardial infarction, where both ischemia and disturbed flow are present.

Here, we found that Ang2 expression was upregulated in the ischemic hindlimb in a time-dependent manner. Ang2 mRNA expression in the ischemic hindlimb peaked at day 2 and then declined back to similar levels with the non-ischemic control by day 5. Ang2 protein expression lagged behind mRNA expression with a peak increase in the ischemic hindlimb on day 2 and day 5 and slightly declining on day 7. Similarly, Matsunaga et al. found that Ang2 expression increased during ischemia and peaked at day 3 in a canine model of myocardial ischemia[7]. In a similar mouse model of hindlimb ischemia, Silvestre et al. found no significant differences in Ang2 protein expression between the ischemic and non-ischemic hindlimb, however, this was likely because the protein expression was only examined after 28 days and earlier timepoints were not studied[18].

Ang2 has been previously found to be induced by hypoxia in endothelial cells *in vitro* [19-21], as well as *in vivo* in various tissues[11, 21, 22], suggesting that Ang2 plays an important role in hypoxia- and ischemia-induced vascular remodeling. Pichiule et al. found that Ang2 expression was increased by hypoxia independent of the canonical hypoxia inducible factor (HIF) pathway, but through a COX-2-dependent pathway[23].

The presence of ischemia coupled with disturbed fluid shear stress could be possible mechanisms by which Ang2 is upregulated during hindlimb ischemia.

We found that inhibiting Ang2 impaired blood flow recovery in the ischemic hindlimb, suggesting that the upregulation of Ang2 is necessary in the early phase of collateral formation after arterial occlusion. However, the role of Ang2 in hindlimb ischemia is somewhat conflicting. Recently, Reiss et al. found that transgenic overexpression of Ang2 in endothelial cells impaired revascularization during hindlimb ischemia[4]. However, the same authors previously reported that Ang2 promotes neovessel formation during cerebral ischemia[5] suggesting a complex role of Ang2 during ischemia. Reiss et al. concluded that the dose of Ang2 is a critical determinant of whether there is vessel growth or regression[4]. We speculate that perhaps a physiological increase in Ang2 can stimulate revascularization, as seen in our hindlimb ischemia model, whereas Ang2 expression well above physiological levels and for longer durations reaching beyond 7 days could promote vessel regression. Supraphysiological amounts or excessive duration of Ang2 exposure could cause chronic inflammation or continued vessel destabilization. Consistent to this notion, Ang2 has been shown to recruit monocytes and promote vascular leakage[15, 17, 24] In contrast to the findings of Reiss et al., Shyu et al. found that intramuscular injection of plasmid DNA encoding Ang2 showed no further increase in neovascularization in the rabbit ischemic hindlimb[25]. Our finding that Ang2 is only transiently upregulated further supports the initial role of Ang2 in neovascularization and that prolonged expression of Ang2 could have a negative impact on vessel growth.

A possible explanation for the reduced reperfusion in mice treated with Ang2 inhibitor is through inhibited recruitment and proliferation of VSMC. Mice deficient in

Ang2 tend to be lethal within two weeks after birth due to postnatal defects in vascular growth[26]. However, the few that survive show defects in pericyte and smooth muscle cell recruitment in lymphatic vessels[27], implicating Ang2 as an important regulator of smooth muscle cell recruitment. Here, we found that L1-10 treatment inhibited VSMC coverage of blood vessels and this may be attributed to the drug blocking VSMC recruitment to vessels or VSMC proliferation. Interestingly, we did not see a difference in capillary density between the L1-10 treated and vehicle treated ischemic adductor muscles, suggesting that Ang2 mediates blood flow recovery mainly through the formation of collaterals and not through the formation of smaller capillaries. It has been reported that the majority of blood flow recovery during hindlimb ischemia is achieved by arteriogenesis and not angiogenesis and the contribution of angiogenesis is almost negligible[28]. Therefore, the decrease in blood flow recovery in mice treated with L1-10 is most likely mediated by decreased arteriogenesis and this is evidenced by the decrease in VSMC covered vessels. Ang2 may promote arteriogenesis by the recruitment and proliferation of VSMC.

Surprisingly, we found that Ang2 inhibition reduced macrophage/neutrophil infiltration in the ischemic hindlimb. Recently, a subset of monocytes has been found to express the Tie2 receptor, which was originally thought to be restricted to endothelial cells[29]. Murdoch et al. found that monocytes and macrophages upregulate Tie2 when exposed to hypoxia and that Ang2 acts as a chemoattractant for Tie2-positive monocytes and macrophages[15]. In addition, Ang2 is known to promote vascular leakage *in vivo* which also may increase macrophage infiltration[24]. Macrophages may further promote

neovascularization by the secretion of growth factors suggesting another mechanism by which Ang2 promotes blood flow recovery during hindlimb ischemia[30].

Ang2 has been shown to be involved in several diseases including cancer, atherosclerosis, and myocardial ischemia[6, 7, 31]. In tumors and atherosclerotic plaques, an ischemic core develops and is thought to promote neovascularization[1, 32]. In myocardial infarction, coronary blood flow is blocked resulting in myocardial ischemia and the formation of collaterals is important to relieve the ischemia[33]. Understanding the role of Ang2 in blood vessel formation during ischemia and the ability to control the expression of Ang2 could be of great therapeutic importance.

In conclusion, we found that Ang2 plays an important role in blood flow recovery and reperfusion during ischemia and this was mediated through arteriogenesis and the recruitment of inflammatory cells. Elucidating the role of Ang2 in neovascularization could be important in understanding ischemic diseases. The spatial and temporal controlled expression of Ang2 could have novel therapeutic applications for diseases involving impaired blood vessel formation.

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CHAPTER 7

ROLE OF NEOVASCULARIZATION AND ANGIOPOIETIN-2 IN ATHEROSCLEROSIS

To further examine the role of Ang2 in *in vivo* models of neovascularization, as outlined in specific aim 3, we also examined the role of shear stress and Ang2 in atherosclerosis neovascularization. This was achieved by using mouse models of atherosclerosis.

Introduction

Increased neovascularization has been found in the atherosclerotic plaque suggesting a role for neovascularization in atherosclerosis development[1, 2]. Atherosclerosis is an inflammatory disease that preferentially occurs in regions of the arterial system exposed to disturbed flow, such as at branch points or curves, which results in low and oscillatory shear stress (OS)[3, 4]. Contrastingly, straight regions of the arterial system exposed to unidirectional laminar shear straight (LS) are well protected from atherosclerosis[3, 4]. At the cellular and molecular level, laminar shear stress is thought to promote endothelial cell quiescence; laminar sheared endothelial cells are anti-proliferative, anti-apoptotic, and anti-thrombotic[4]. Oscillatory shear stress is thought to promote endothelial cell dysfunction; oscillatory sheared endothelial cells are pro-proliferative, pro-migratory, pro-thrombotic, and secrete growth factors that stimulate smooth muscle cell proliferation and migration[4]. The secretion of growth factors from dysfunctional endothelial cells could also play a role in plaque neovascularization.

We have recently identified Angiopoietin-2 (Ang2) as a shear-sensitive angiogenic factor secreted by EC exposed to OS[5]. In addition, we found that Ang2

mediates OS-induced tubule formation and migration[5]. Ang2 has been found to be expressed in the neovessels of advanced atherosclerotic plaques suggesting a role for Ang2 in plaque neovascularization[6].

The detailed mechanisms resulting in plaque angiogenesis and neovessel recruitment remain uncertain. Here we will examine the role of Ang2 in atherosclerosis development and plaque neovascularization using a mouse model of atherosclerosis. We hypothesize that inhibiting Ang2 will slow the progression of atherosclerosis by inhibiting plaque neovascularization.

Methods

Models of Advanced Atherosclerosis

To develop advanced atherosclerosis, male apolipoprotein E deficient mice ($ApoE^{-/-}$, Jackson Laboratory) were purchased at 5 weeks of age and fed a high fat diet for 5 months. C57Bl/6J wildtype mice were fed a normal chow diet and used as controls. To develop advanced atherosclerosis in a shorter period of time, $ApoE^{-/-}$ mice were fed a high fat diet and subcutaneously implanted with an osmotic mini-pump containing Angiotensin II (AngII) (0.7mg/kg/day) or vehicle control for 4 weeks. All experimental protocols were approved by the institutional Animal Care and Use Committee at Emory University.

Whole Mount Immunohistochemistry

Mice were sacrificed by CO_2 inhalation and pressure perfused with 0.9% NaCl solution then pressure fixed with 10% formalin. The aorta was isolated from the aortic

arch to past the renal arteries, cleaned of adventitial fat and fixed for 1 hour with 1% paraformaldehyde. The tissue was blocked for 1 hour with 5% goat or donkey serum in Tris buffered saline containing 0.3% Triton-X 100 (TBST) and incubated overnight at 4°C with primary antibody diluted 1:1000 in TBST. After several washes with TBST, the tissue was then incubated with fluorescently tagged secondary antibody diluted in TBST for 4 hours. The tissue was washed and post-fixed with 4% paraformaldehyde for 10 minutes. The aorta was cut open lengthwise and mounted on a coverslide with adventitial side facing up and imaged using a LSM 510 Meta confocal microscope (Zeiss). Images were constructed into 3D projections of Z-stacks. Antibodies used were: hamster anti-mouse PECAM-1 (Chemicon) and guinea pig anti-mouse perilipin (Fitzgerald).

Ang2 Inhibition in an Animal Model of Atherosclerosis

Male ApoE$^{-/-}$ mice were purchased at 5 weeks of age (Jackson Laboratory). Mice starting at 6 weeks of age were injected subcutaneously twice a week with 4 mg/kg L1-10 (Ang2 specific inhibitor, Amgen)[7], or Vehicle control (PBS) for 8 weeks. Mice were fed a high fat atherogenic diet throughout the study[8]. The animals were humanely sacrificed by CO_2 inhalation at the end of the 8 week period.

Blood Pressure Measurements

Systolic blood pressure was measured before the treatment period (baseline) and after 1, 4 and 8 weeks by a computerized tail cuff plethysmograph (Model BP-2000 Visitech Systems). Each mouse was accustomed to the apparatus for 4 days before

measurements were recorded. Ten measurements were obtained and averaged for each mouse.

Lipid Profile Analysis

After 8 weeks of treatment, mice were sacrificed by CO_2 inhalation and blood was collected through the inferior vena cava for lipid profiles. Plasma lipid analyses were performed by Cardiovascular Specialty Labs (Atlanta, GA).

***En face* Aorta Lesion Analysis**

Aortas were pressure perfused with 0.9% NaCl solution and then pressure fixed with 10% formalin solution *in situ*. The aorta was isolated from the area of the ascending aorta to the branches of the femoral artery and dissected free. The aorta was then opened lengthwise and pinned open. Digital images were taken for subsequent lesion area analysis using Image Pro.

Statistical Analysis

Data are reported as average ± SEM obtained from at least 3 independent studies. Statistical significance ($p<0.05$) was assessed by Student's t-test using a Microcal Origin statistical package as well as general linear model ANOVA followed by Bonferroni test for comparisons over a timecourse.

Results

Adventitial Neovascularization in ApoE$^{-/-}$ mice

We first sought to characterize the extent of neovascularization in a mouse model of atherosclerosis by performing whole mount immunohistochemistry of the adventitia of ApoE$^{-/-}$ mice with advanced atherosclerosis. ApoE$^{-/-}$ mice were fed a high fat diet for 5 months in order to develop extensive neovascularization. Mice aortas normally do not have a vasa vasorum or adventitial vascular network. Therefore, it was necessary to allow the mice to develop severe plaque development in order to see if neovascularization would occur. Blood vessels were immunostained with a PECAM-1 antibody which is specific for endothelial cells. Wildtype control fed a normal chow diet had no adventitial blood vessels (Figure 7.1). On the contrary, ApoE$^{-/-}$ mice had extensive vascular formation in the adventitia (Figure 7.1). The greater curvature, the lesser curvature, the thoracic region and the abdominal region were all examined. Interestingly, the lesser curvature had greater neovascularization than the greater curvature. The lesser curvature is known to be exposed to a disturbed shear stress and develops more atherosclerosis than the greater curvature which is exposed to a unidirectional laminar shear stress. This suggests that the disturbed flow region develops more neovascularization than the laminar flow region. In the thoracic region, neovascularization tended to occur around branches to the intercostal arteries, which is also a region that experiences disturbed flow and extensive atherosclerosis (Figure 7.1). The abdominal aorta was the region that developed the most neovascularization (Figure 7.1).

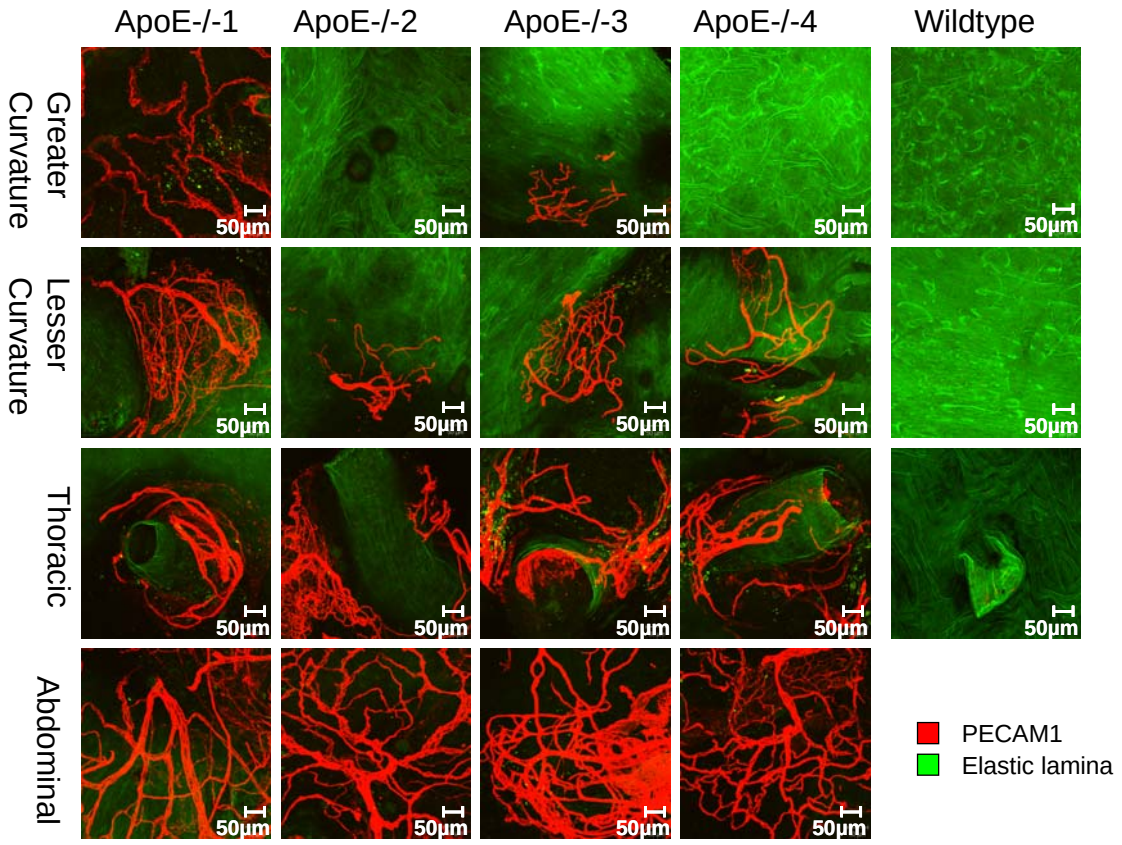

Figure 7.1. Adventitial neovascularization in ApoE$^{-/-}$ mice. Male ApoE$^{-/-}$ mice were fed a high fat diet for 5 months. After which mice were sacrificed and pressure fixed with 10% formalin. The aorta was isolated and incubated with PECAM-1 antibody and then Cy3 conjugated secondary antibody (red). The aorta was cut lengthwise and mounted with adventitia facing up. Shown are representative images from 4 different ApoE$^{-/-}$ of confocal projection images of Z-stacks. Wildtype C57Bl/6J was used as control. Green represents autofluorescence from elastic lamina. (N=4)

We next wanted to examine adventitial neovascularization in a more accelerated model of atherosclerosis, as well as determine if the neovascularization is associated with adventitial fat deposits. To do this, ApoE$^{-/-}$ mice were treated for 4 weeks with Angiotensin II (Ang II) which increases blood pressure and causes advanced atherosclerosis development. The adventitial aorta was again immunostained for endothelial cells as well as perilipin which is a marker of adipocytes. In this model we found that ApoE$^{-/-}$ mice treated with Ang II had greater neovascularization in the abdominal and thoracic aorta than ApoE$^{-/-}$ mice treated with vehicle and this neovascularization was not associated with fat (Figure 7.2A). However, at this timepoint there was no neovascularization in the lesser and greater curvature of the aorta in both treatment groups. The majority of the neovascularization was not associated with adventitial fat, although there was some fat found on the aorta that was missed during cleaning and is shown here as a positive control to show that the antibody is working (Figure 7.2B). Adventitial fat is highly vascularized as shown in Figure 7.2B and therefore we wanted to determine that the neovascularization we see is not from fat deposits but part of the adventitia itself.

Inhibition of Ang2 had no effect on blood pressure or lipid profiles

To investigate the role of Ang2 in cardiovascular disease, we first examined the effect of inhibiting Ang2 on blood pressure. ApoE$^{-/-}$ mice were treated with L1-10, an Ang2 specific inhibitor, and fed a high fat diet for 8 weeks. Blood pressure analysis revealed that there was no significant difference in systolic blood pressure between L1-10 treated and vehicle control over the 8 week treatment period (Figure 7.3). Systolic blood

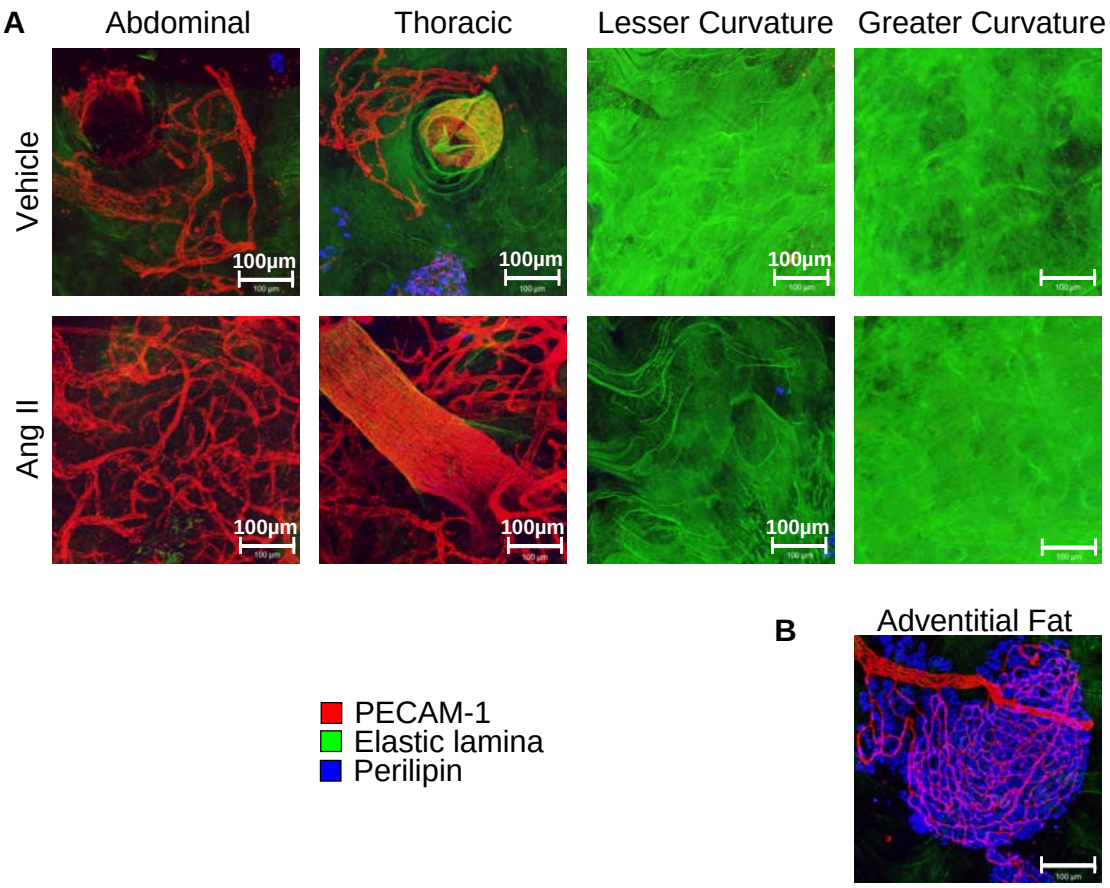

Figure 7.2. Adventitial neovascularization in ApoE$^{-/-}$ mice treated with AngII. ApoE$^{-/-}$ mice were fed a high fat diet and treated with AngII (0.7mg/kg) for 4 weeks. After which, the mice were sacrificed and pressure fixed with 10% formalin. The aorta was isolated and incubated with antibody against PECAM-1 (red) and Perilipin (blue). The aorta was cut lengthwise and mounted with adventitia facing up. Shown are representative projection images of Z stacks from 4 regions of the aorta. Green represents autofluorescence from elastic lamina. B shows an example of vascularized adventitial fat. (N=4)

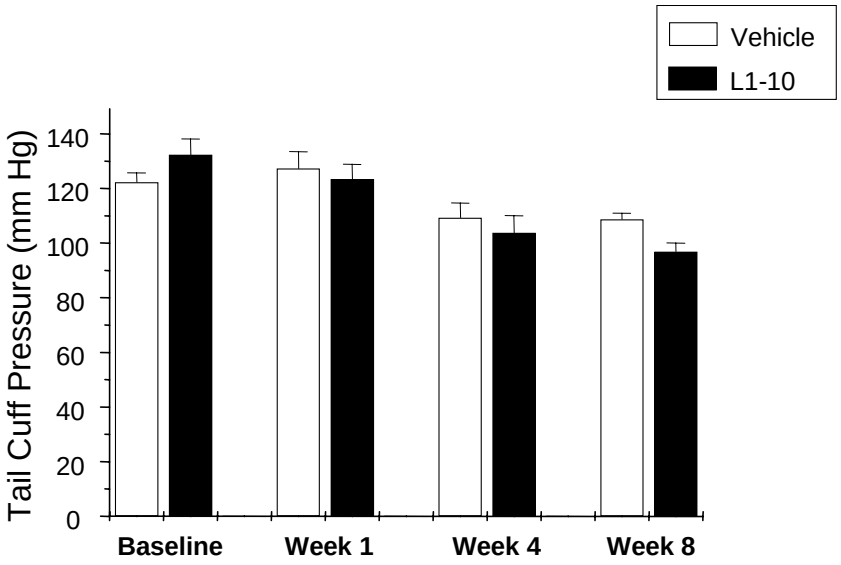

Figure 7.3. Inhibition of Ang2 has no effect on blood pressure. ApoE$^{-/-}$ mice were fed a high fat diet and treated with the Ang2 inhibitor L1-10 or vehicle for 8 weeks. Systolic blood pressure was measured using a computerized tail cuff plethysmograph before treatment (baseline) and at week 1, week 4 and week 8. Mice were accustomed to the device 4 days prior and shown are the average of 10 measurements per mouse. (N=5)

pressure did slightly decrease over time in both groups, but this was most likely due to the mice being more acclimated to the blood pressure machine.

We also examined lipid profiles of ApoE$^{-/-}$ mice on high fat diet treated with L1-10 or vehicle after 8 weeks. Lipid analysis showed no significant difference between L1-10 and vehicle treated mice in plasma cholesterol, triglycerides, HDL, LDL, and VLDL (Figure 7.4), suggesting that Ang2 does not play a role in regulating cholesterol and triglycerides.

Ang2 inhibition had no effect on atherosclerotic lesion area

To investigate the role of Ang2 in atherosclerosis development, we examined the effects of inhibiting Ang2 on atherosclerotic lesion area in a mouse model of

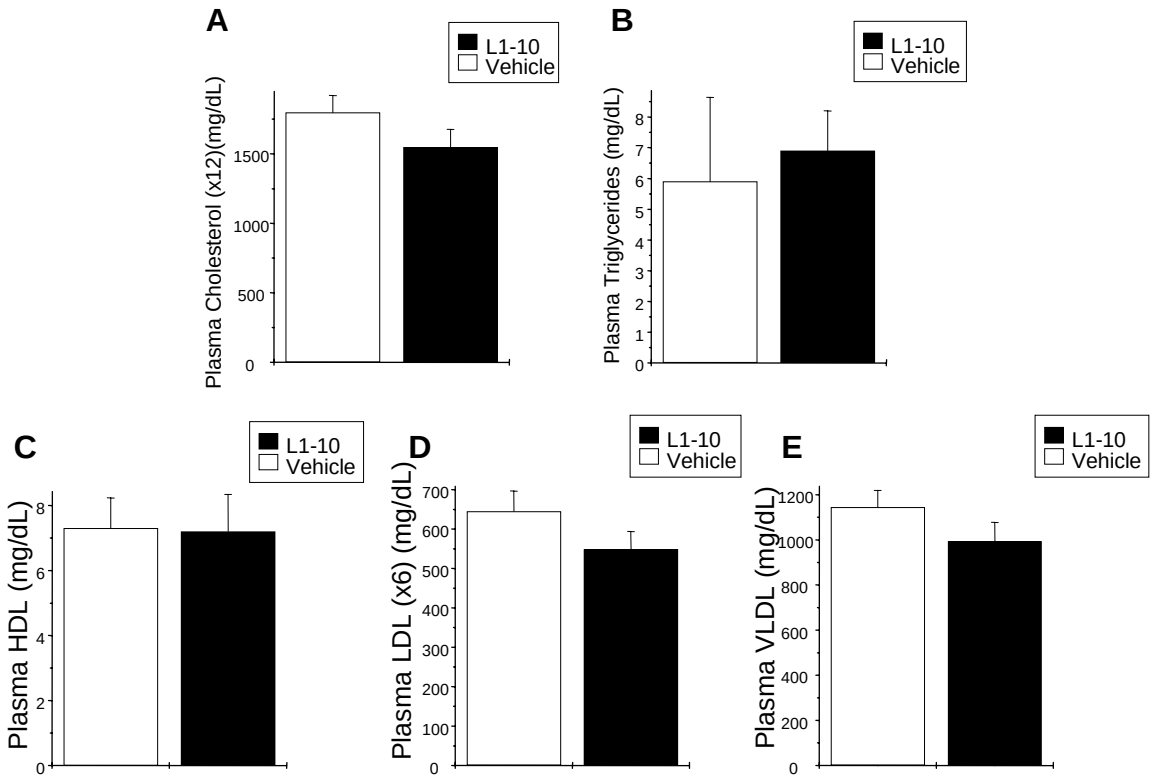

Figure 7.4. Inhibition of Ang2 had no effect on lipid profiles. ApoE$^{-/-}$ mice were fed a high fat diet and treated with the Ang2 inhibitor L1-10 or vehicle for 8 weeks. After which, blood plasma was collected and lipid analysis was performed for cholesterol (A), triglycerides (B), high density lipoprotein (HDL) cholesterol (C), low density lipoprotein (LDL) cholesterol (D), and very low density lipoprotein (VLDL) cholesterol (E). (N=5)

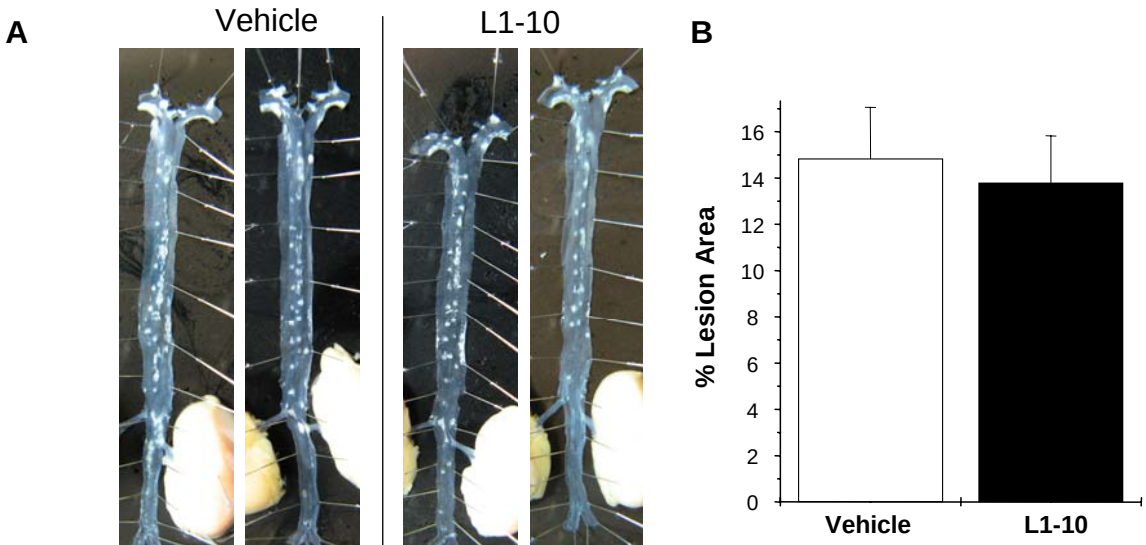

Figure 7.5. Inhibition of Ang2 has no effect on atherosclerotic lesion size. ApoE$^{-/-}$ mice were fed a high fat diet and treated with L1-10 or vehicle for 8 weeks as in Figure 7.4. After which, the mice were sacrificed and pressure fixed with 10% formalin. The aorta was isolated, cleaned of adventitial fat, cut lengthwise and pinned open. White color represents atherosclerotic lesion. Shown are two representative images. Percent lesion area was calculated using ImagePro (B). (N=5)

atherosclerosis. ApoE$^{-/-}$ mice were fed a high fat diet and injected with L1-10 or vehicle for 8 weeks. At the conclusion of the 8 week treatment period mouse aortas were isolated to examine atherosclerotic lesion area. At this timepoint, Ang2 inhibition did not affect total lesion area significantly, although there was a slight decrease in total lesion area in the L1-10 treated group (Figure 7.5). Longer timepoints may be necessary to develop more advanced lesions where neovascularization plays more of a role. Interestingly, L1-10 treatment may have decreased lesion area in the aortic arch region, a region known to experience disturbed shear stress (Figure 7.5).

Discussion

From this study we found that: 1) C57BL/6J wildtype mice have no adventitial aorta neovascularization, 2) on the contrary, ApoE$^{-/-}$ mice fed a high fat diet for 5 months develop extensive aortic adventitial neovascularization, 3) neovascularization is highest in disturbed flow regions of the aorta, 4) neovascularization is not associated with fat deposits but is part of the adventitia, 5) Ang2 inhibition does not effect blood pressure or lipid profiles, and 6) Ang2 inhibition has no effect on atherosclerotic lesion area. These results suggest that longer timepoints are required to develop adventitial neovascularization, and that Ang2 does not play a role in early atherosclerosis development but may play a role in later atherosclerosis when neovascularization is present.

We found that wildtype mice fed a normal diet did not develop adventitial neovascularization whereas mice with advanced atherosclerosis developed extensive adventitial neovascularization. Moulton et al. was first to demonstrate that ApoE$^{-/-}$ mice fed a high fat diet for 9 months develop intimal vessels in advanced lesions of atherosclerotic plaque and that using an angiogenesis inhibitor reduces intimal neovascularization and plaque growth[9].

Here we demonstrate that adventitial neovascularization can occur as early as 5 months in the ApoE$^{-/-}$ mouse fed a high fat diet and that adventitial neovascularization is associated with regions of the vasculature exposed to disturbed shear stress. This suggests that the disturbed flow region develops more neovascularization than the laminar flow region.

Interetingly, we previously identified Ang2 to be upregulated in the lesser curvature as compared to the greater curvature in wildtype mice[5]. The region corresponding to increased Ang2 expression is also the region corresponding to greater neovascularization. Therefore, we hypothesized that increased Ang2 in the disturbed flow regions was contributing to plaque neovascularization. However, when we inhibited Ang2 using an Ang2 specific inhibitor, we were unable to see any difference in atherosclerotic lesion area. We speculate that a longer timepoint is needed to develop more advanced atherosclerosis with extensive neovascularization before we can see the impact of inhibiting neovascularization on plaque development. In fact, Moulton et al. first fed ApoE$^{-/-}$ mice high fat diet for 20 weeks in order to develop advanced atherosclerosis and then treated mice for 16 weeks with angiogenesis inhibitors[9]. A similar timeline as this may also reveal the role of Ang2 in advanced atherosclerosis and plaque neovascularization.

If Ang2 does play a role in advanced plaque neovascularization, then inhibiting Ang2 could be of great therapeutic importance because it could be a way to inhibit the progression of advanced atherosclerosis. Most atherosclerosis doesn't present itself until it is in advanced stages, and therefore the ability to inhibit plaque development at late stages could be an important therapy.

In summary, we found that adventitial neovascularization occurs in advanced models of atherosclerosis in the ApoE$^{-/-}$ mouse and that neovascularization is associated with regions of disturbed flow. However, Ang2 inhibition did not reduce atherosclerotic plaque development in early stages of atherosclerosis development. We therefore conclude that plaque neovascularization occurs at later stages and although Ang2 does

not play a role in early atherosclerosis development, it may play a role in later stages of atherosclerosis when neovascularization is present.

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CHAPTER 8

DISCUSSION

Summary and Conclusions

Neovascularization is a necessary and important process in normal physiology. However, in disease, neovascularization can play both a positive and negative role in disease progression and has often been termed the 'double-edged sword'. For example, in cancerous tumors and atherosclerotic plaques neovascularization is thought to promote the progression. However, in occlusive vascular diseases such as myocardial infarction, neovascularization is necessary to relieve the subsequent ischemia. Therefore, understanding neovascularization and the forces that drive it, such as shear stress, is of great therapeutic importance.

The *objective* of this dissertation was to investigate how shear stress mediates neovessel formation of endothelial cells and to identify how shear-mediated proteins play a role in physiological neovascularization. By understanding how these factors are regulated and what role they play *in vivo*, we can better understand human disease and develop important therapeutic targets. Our *overall hypothesis* was that an oscillatory shear stress promotes greater neovascularization compared to a unidirectional laminar shear stress through the secretion of angiogenic factors, which play a physiological role in neovascularization *in vivo*. This hypothesis was tested using *in vitro* and *in vivo* approaches according to three specific aims:

Specific Aim 1: Investigate the functional effects of shear stress on endothelial cell migration and neovessel formation

Specific Aim 2: Identify a molecular mechanism involved in shear-induced neovessel formation

Specific Aim 3: Determine the physiological relevance of Angiopoietin-2 in shear-mediated neovascularization, *in vivo*

To achieve these specific aims, we first examined the *in vitro* role of shear stress on endothelial cell neovessel formation. During neovessel formation, endothelial cells must first proliferate, then migrate, and lastly form tubules through which blood can flow. Therefore, we examined the effect of shear stress on endothelial cell proliferation, migration and tubule formation. Several groups have examined the role of shear stress on proliferation, citing that a unidirectional laminar shear stress (LS) is known to inhibit proliferation whereas an oscillatory shear stress (OS) is known to promote proliferation[1-3]. We were also able to corroborate these results in our system. For the first time, we were able to show that OS promotes greater tubule formation and migration of endothelial cells as compared to LS. Taken together, OS promotes greater proliferation, migration, and tubule formation than LS, suggesting that OS may play an important role in promoting neovascularization.

To identify a molecular mechanism for shear-mediated migration and tubule formation, we first examined the canonical VEGF pathway. We examined VEGF first because it is one of the most important mediators of neovascularization[4-7]. In addition, VEGFR-2, the main receptor for VEGF in endothelial cells, has been shown to be

activated by shear stress independent of ligand binding[8]. Therefore, we examined the role of VEGFR-2 in shear-mediated tubule formation and migration. We found that inhibiting VEGFR-2 blunted OS-mediated tubule formation as well as inhibited migration. OS also stimulated the phosphorylation of VEGFR-2 at tyrosine 1175, a site that is known to initiate the ERK1/2 pathway and proliferation. This data suggests that OS may promote tubule formation and migration through the phosphorylation of VEGFR-2 at tyrosine 1175 leading to the subsequent ERK1/2 signaling pathway and proliferation. However, the VEGFR-2 pathway has been highly studied and we wanted to determine a more novel pathway involved in shear-mediated neovessel formation. In addition, VEGFR-2 inhibition did not completely block tubule formation and migration, suggesting that other factors may be playing a role.

Gene and protein array analysis was performed to determine a novel mediator of shear induced neovessel formation. From gene and protein array analysis, we identified several angiogenic genes that were shear-sensitive. Thrombospondin-1 (TSP-1) was greatly upregulated by OS compared to LS. However, subsequent inhibitor studies examining TSP-1's role in shear mediated tubule formation revealed that it did not have an effect. Ang2 was also upregulated by OS compared to LS on both our gene and protein arrays. We found that inhibiting Ang2 blocked OS-mediated tubule formation and migration and that LS-inhibited tubule formation could be rescued by addition of Ang2. In addition, Ang2 was found to be upregulated at sites of disturbed flow *in vivo*, implicating a physiological role for Ang2.

To investigate the physiological role of Ang2 in neovascularization, we examined the effects of inhibiting Ang2 in a mouse model of hindlimb ischemia. In the hindlimb

ischemia model, the femoral artery is ligated blocking flow into the mouse hindlimb. This model is both a flow cessation model as well as an ischemia model. Increased flow is directed through collaterals bypassing the occlusion, resulting in decreased and retrograde flow in the post-occlusive site, therefore this is a good model involving both disturbed flow and neovascularization. We found that Ang2 is upregulated in the adductor muscle of the ischemic hindlimb suggesting that it plays a role in recovery from femoral artery ligation. Interestingly, in the hindlimb ischemia model, the adductor muscle does not experience much ischemia, most ischemia is found in the lower leg such as within the gastrocnemius muscle, suggesting that Ang2 is upregulated independent of ischemia and disturbed shear stress may be the driving force for increased Ang2 [9-11]. In addition, we found that inhibiting Ang2 decreased reperfusion or blood flow recovery. To determine the mechanism by which Ang2 inhibition impairs recovery, we examined neovascularization in the ischemic adductor muscle. Ang2 inhibition resulted in decreased smooth muscle cell coverage of vessels as well as decreased macrophage infiltration. These findings suggest that Ang2 promotes blood flow recovery through the recruitment of smooth muscle cells and formation of collaterals, as well as the recruitment of macrophages that secrete important growth factors and help degrade the extracellular matrix in order for neovascularization to occur.

By achieving the three specific aims listed, we have identified an important mediator of shear stress induced neovessel formation that plays an important physiological role *in vivo.* Identifying factors that play an important role in neovascularization at sites of disturbed flow could help provide localized therapies at sites of disease. This work illustrates the shear stress regulation of neovessel formation

through the expression of angiogenic factors, in particular Ang2, and the role of Ang2 in neovascularization *in vivo* (Figure 8.1). This work has implications toward several cardiovascular diseases which are the number one killers in the United States. By understanding how angiogenic factors are regulated and what role they play *in vivo*, we can better understand human disease and develop important therapeutic targets.

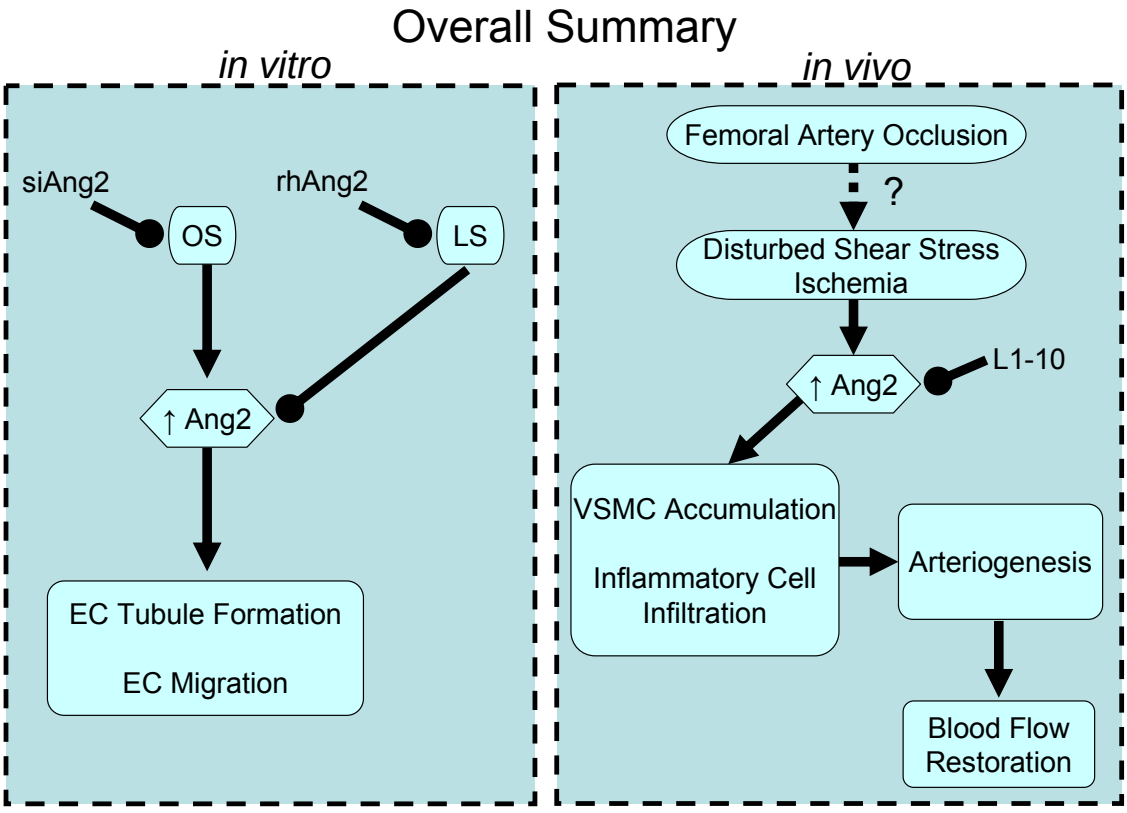

Figure 8.1. Overall summary and conclusions.

Limitations

There are several limitations to this study that will be outlined here. First, there are always limitations when working with cultured cells in an *in vitro* setting, as this setting does not fully simulate cells *in vivo*. We used a cone and plate shear apparatus to simulate fluid flow; however, the fluid flow *in vivo* is much more complex and is pulsatile. Several groups have shown that *in vitro* shear systems induce the same responses in endothelial cells as seen *in vivo*[12, 13]. Indeed, we were able to show that Ang2 is also upregulated at sites of disturbed flow *in vivo*. However, *in vitro* shear systems can only simulate *in vivo* conditions and some responses may be different.

To simulate neovascularization *in vitro*, we examined proliferation, migration, and tubule formation. However, *in vitro* neovascularization assays do not fully simulate the formation of functional blood vessels as a whole; they only simulate individual parts of the angiogenesis process. Therefore, *in vivo* models of neovascularization are important to fully examine the neovascularization process. In addition, we were not able to examine shear stress and neovascularization assays at the same time. Instead we pre-conditioned cells with 24 hours of shear stress, and then examined what the sheared cells were 'programmed' to do in migration and tubule formation assays. However, some of this concern was addressed by using the shear conditioned media and examining its effects on endothelial cell migration and tubule formation.

The biggest limitation of this study is the inability to determine shear stresses in our *in vivo* model of hindlimb ischemia. At this time, shear stress cannot be quantified in small pre-existing collaterals due to technical limitations so it is not feasible to determine the exact shear stresses occurring during femoral artery ligation[14]. Although, it is thought

that there is increased flow in the collaterals and low and reversed flow in the post-occlusive site[9]. Therefore, it is difficult to determine if the increase in Ang2 in the ischemic hindlimb is due to disturbed shear stresses or other factors such as ischemia. Although we have shown that Ang2 is upregulated at sites of disturbed shear stress in the aortic arch and we have shown that Ang2 is upregulated in vessels in the ischemic hindlimb, but we cannot show that vessels in the ischemic hindlimb are exposed to disturbed shear stress.

In our atherosclerosis model, we cannot conclude that shear stress directly promotes neovascularization; it could also promote neovascularization indirectly. It has been published that a disturbed shear stress promotes the expression of adhesion molecules leading to increased inflammation[13, 15]. This inflammation may in turn promote neovascularization. Therefore, we cannot conclude that shear stress promotes neovascularization directly but maybe indirectly through increased inflammation. In addition, we were able to determine that the neovascularization was in the adventitia and localized at sites of plaque, but we were not able to determine if it entered the medial and intimal layers. Further microscopic analysis would need to be done in order to determine if the neovascularization infiltrates the plaque.

Moreover, this project only examines two pathways, the VEGFR-2 pathway and Angiopoietin pathway. The detailed mechanisms on how shear stress regulates these proteins were not examined. Other than looking at receptor binding and phosphorylation, the downstream signaling pathways were not examined, only functional outcomes were examined. Future work should address some of these limitations.

Future Directions

The work set forth in this dissertation has created the foundation for future work examining neovascularization in diseases involving disturbed shear stress. To advance these findings further work should be done in our *in vitro* model as well as our *in vivo* model. As mentioned in the Limitations section, the mechanisms by which shear stress regulates Ang2 and VEGFR-2 should be examined. Also, the downstream signaling pathways should also be investigated. For VEGFR-2 studies, we determined that OS induces the phosphorylation of VEGFR-2 at tyrosine 1175. However, we did not examine whether the phosphorylation at tyrosine 1175 plays a role in OS-mediated tubule formation. To determine this, a dominant negative mutant of VEGFR-2, mutated at tyrosine 1175 should be created as well as examine the downstream signaling pathway of tyrosine 1175, the ERK1/2 pathway. In addition, we have preliminary data examining the role of IQGAP1, a novel binding partner of VEGFR-2, in OS-mediated tubule formation and migration. We have found that inhibiting IQGAP1 by siRNA inhibits OS-mediated tubule formation and migration, similar to results seen when inhibiting VEGFR-2 (Figure 8.2 and 8.3). Further studies, should be performed to examine the role of IQGAP1 in shear mediated neovascularization.

In Chapter 6, we examined the role of Ang2 in hindlimb ischemia and found that inhibiting Ang2 impaired arteriogenesis or the formation of smooth muscle cell covered collaterals. Future work could also be done using micro-Computed Tomography (microCT), to examine the effect of Ang2 inhibition of vessel size. With microCT, you can discriminate between different vessel diameters in the entire hindlimb which may give an overall picture of how Ang2 inhibition is changing vessel size. In addition,

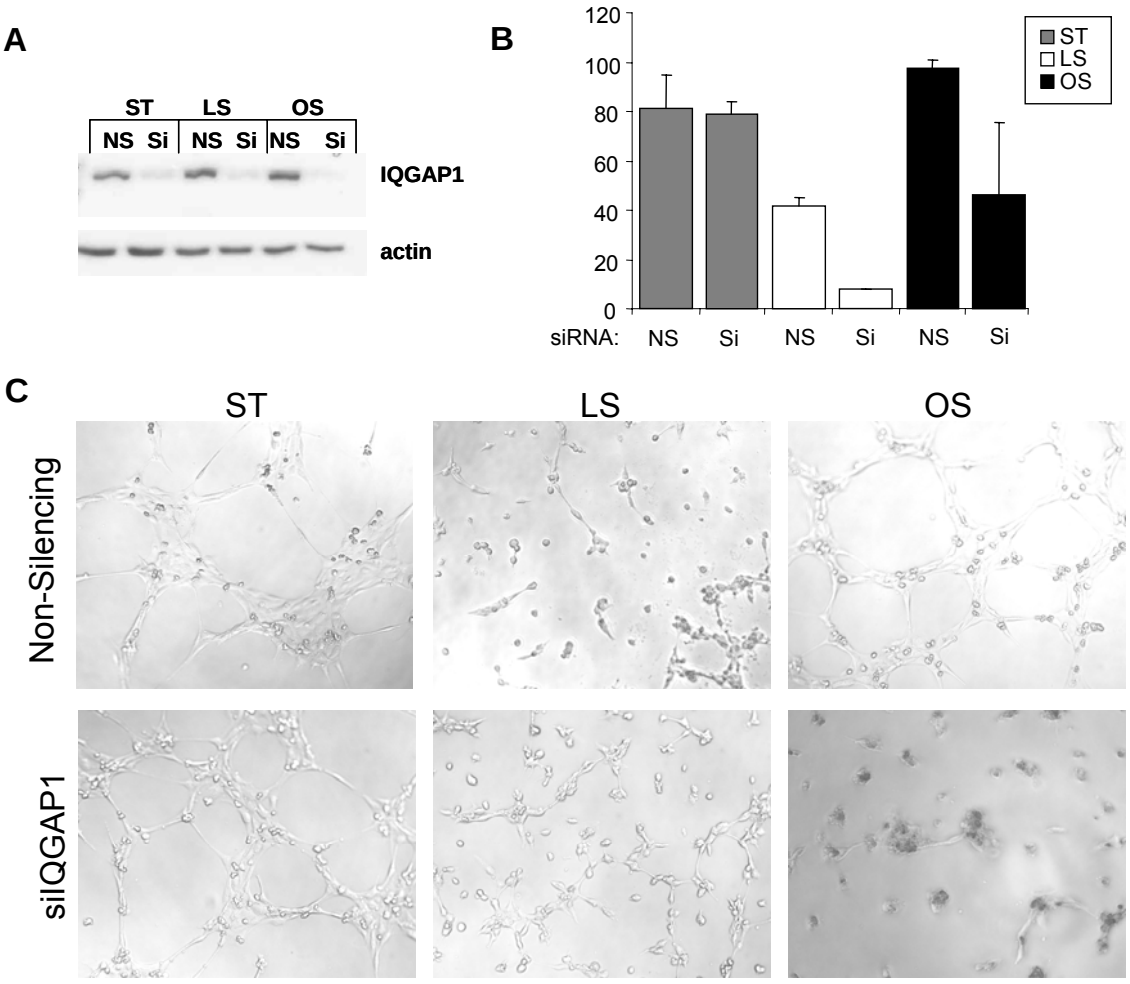

Figure 8.2. IQGAP1 knockdown inhibits shear-mediated tubule formation. A, HUVEC were transfected with 50nM IQGAP1 siRNA (Si, siIQGAP1) or non-silencing siRNA (NS). Cells were then sheared (LS, OS or ST) for 24 hours. Cell lysates were western blotted with IQGAP1 antibody and actin antibody was used as controls. HUVEC were transfected with siIQGAP1 or nonsilencing siRNA and then used in tubule formation assays (B & C). Tubule length was quantified using ImageJ (B). (mean ±SEM; N=2-3)

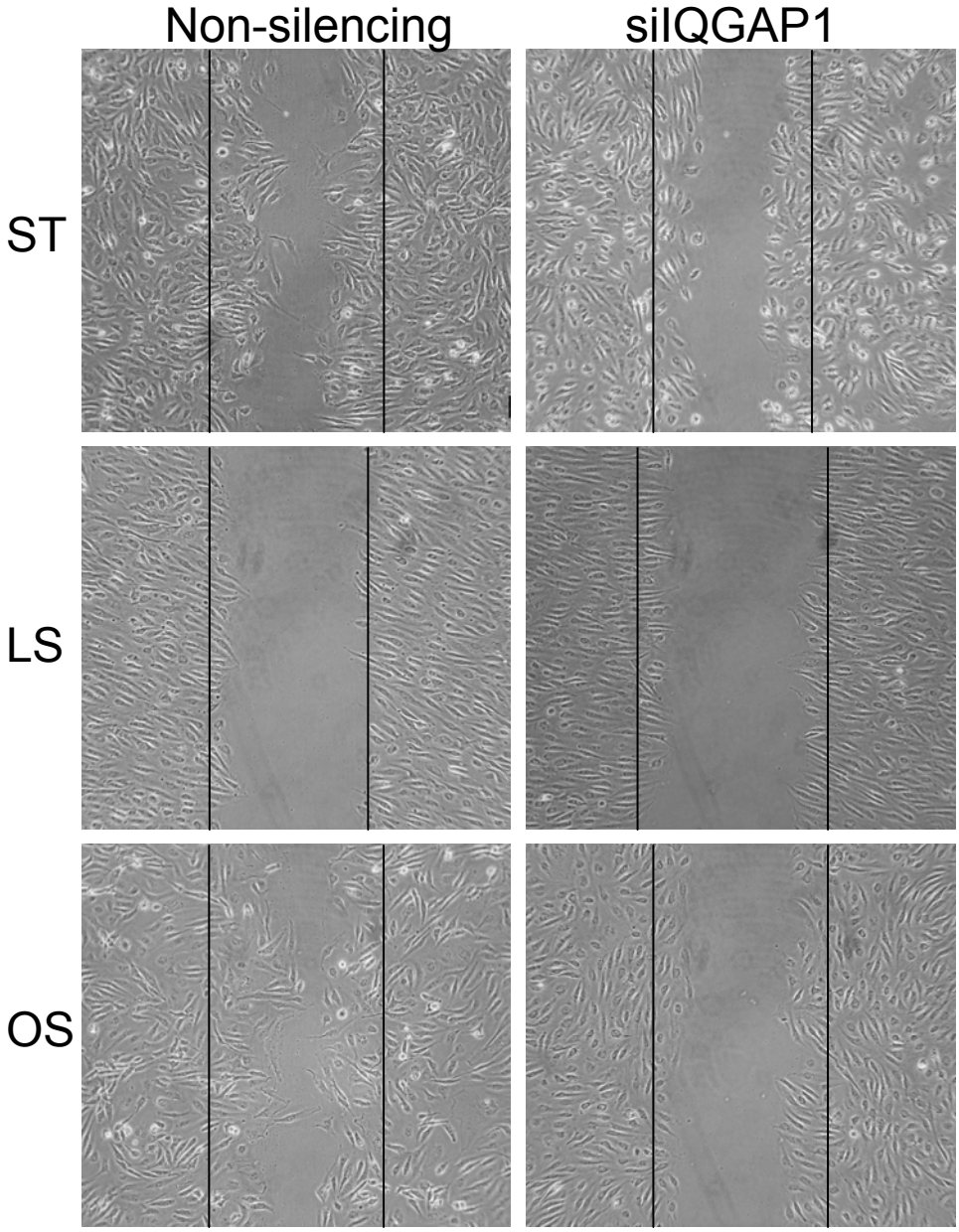

Figure 8.3. IQGAP1 knockdown inhibits shear-mediated migration. HUVEC were transfected with 50nM IQGAP1 siRNA (Si, siIQGAP1) or non-silencing siRNA (NS). Cells were then sheared (LS, OS or ST) for 24 hours. HUVEC were then used in scratch migration assays.

further work could be performed to examine the upstream and downstream signaling pathways involved. We found that Ang2 was transiently upregulated in the ischemic hindlimb, and the regulation of Ang2 expression could also be examined. Daly et al. found that inhibition of the PI3K/Akt pathway leads to FOXO1 expression, which is a transcription factor for Ang2 expression[16]. The resultant increase in Ang2 expression then results in activation of the PI3K/Akt pathway which acts as a negative feedback mechanism to inhibit FOXO1. This pathway could be one mechanism by which Ang2 is expressed only transiently in the ischemic hindlimb. Further studies could be performed to examine how our findings fit into the current knowledge in the field (Figure 8.4).

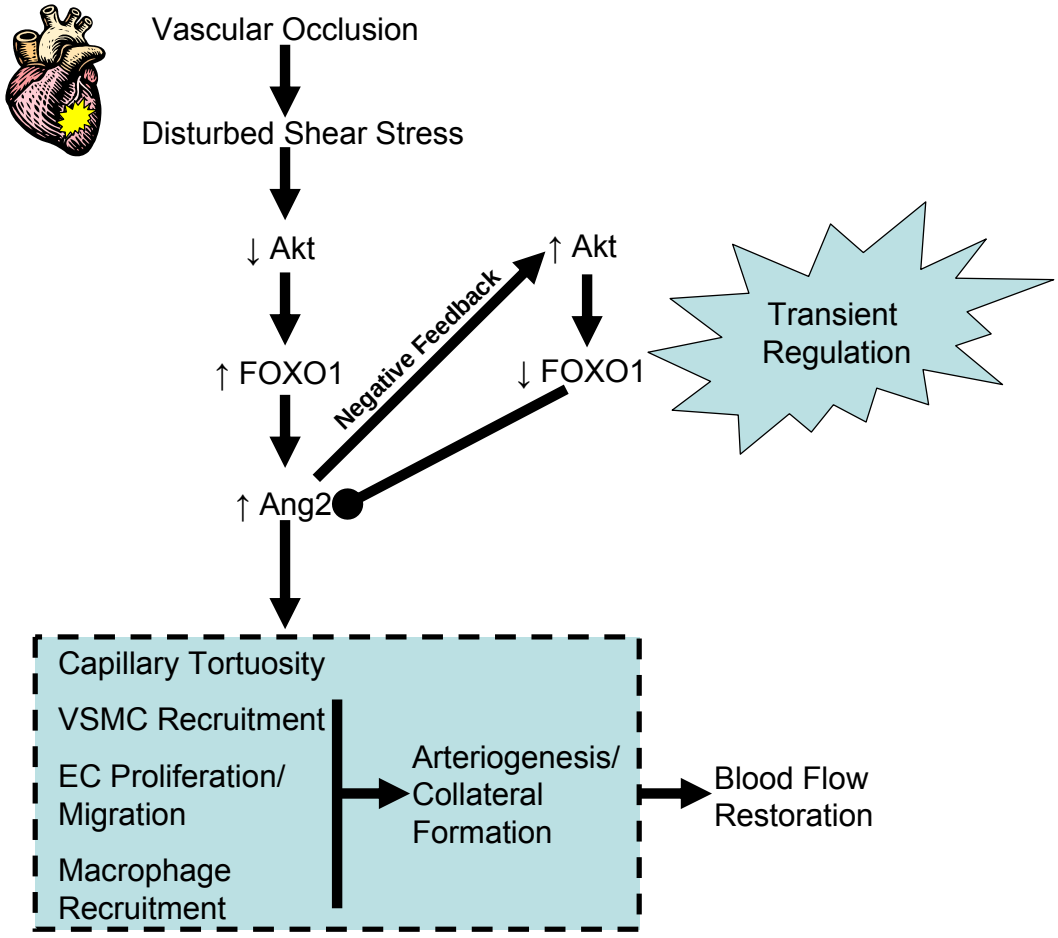

Figure 8.4. How our findings fit into the current knowledge in the field[16].

We have begun studies examining the role of Ang2 in human aortic valve neovascularization. Healthy aortic valve leaflets are avascular, however when they become diseased neovascularization is often seen. Preliminary immunohistochemistry data has revealed that neovessels in a diseased aortic leaflet, identified by von Willebrand Factor (vWF) staining for endothelial cells, express Ang2 (Figure 8.5-8.6). Ang2 could also play a role in aortic valve neovascularization. Further studies would need to be completed to examine Ang2 expression in both healthy and diseased aortic valve leaflets. However, it is difficult to develop a good model of aortic valve calcification in mice. Aortic valve stenosis can occur in ApoE$^{-/-}$ mice fed a high fat diet for over 6 months but it would be difficult to identify neovascularization in the mouse aortic valve leaflet because it is so small. Therefore, this study would have to be conducted on human valve leaflets and possibly cultured valve cells *in vitro*.

In Chapter 7, we examined the role of neovascularization in a mouse model of atherosclerosis as well as the role of Ang2 in atherosclerosis. Further studies should be completed to examine the role of shear stress in neovascularization. The partial carotid ligation model is a good model to look at the effect of reducing shear stress on adventitial neovascularization. In the partial carotid ligation model, three of the branches of the carotid artery are ligated and one is left open. This reduces the flow through the ligated carotid artery and increases the flow through the contralateral carotid artery. On the low flow side there is rapid vessel remodeling and intimal thickening. In this model, neovascularization in the vessel wall could be examined. In addition, the effect of inhibiting Ang2 on this process could also be examined. In our 8 week model of atherosclerosis, we were not able to see any effect of inhibiting Ang2 on atherosclerosis

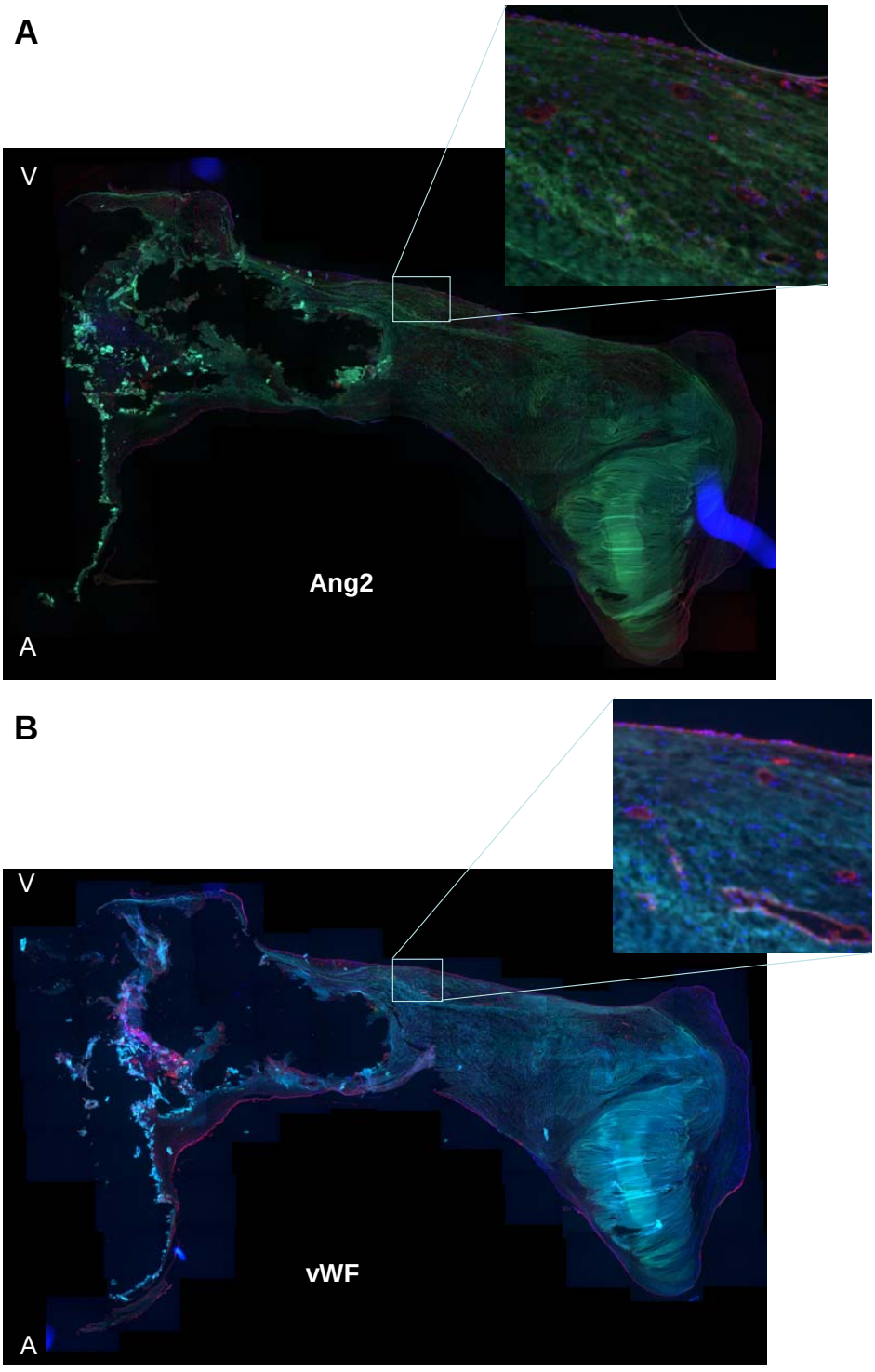

Figure 8.5. Ang2 expression in diseased human aortic valve leaflets. Human aortic valve leaflets were frozen in liquid nitrogen and then sectioned with the ventricularis side (white V) at the top and the aortic side (white A) at the bottom. Tissue sections were incubated with antibodies against Ang2 (A) and vWF (B) as a marker of endothelial cells. Sections were then incubated with secondary antibody conjugated to rhodamine red X. Nuclei were stained blue with Hoechst. Green represents autofluorescence.

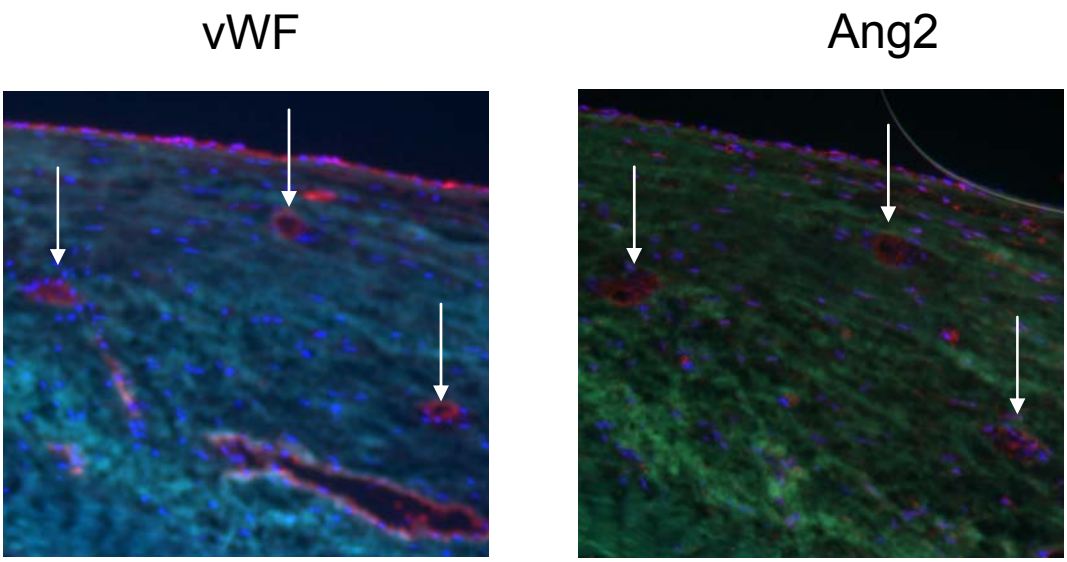

Figure 8.6. Ang2 expression co-localizes with neovessels in the human aortic valve leaflet. Human aortic valve leaflets were sectioned as in Figure 8.5 and incubated with antibodies to vWF and Ang2, then secondary antibodies conjugated with rhodamine red-X. Nuclei were stained blue with Hoechst and green represents autofluorescence. Shown are consecutive sections with positive staining in neovessels (white arrows).

development. This time period may not have been enough time to develop advanced atherosclerosis with neovascularization. Therefore, Ang2 should be examined in more advanced models of atherosclerosis, such as the partial carotid ligation model or through treatment with Angiotensin II.

Overall, the future directions of this project should examine the *in vivo* implications of shear stress in neovascularization and what factors play a role. By understanding what drives neovascularization in disease, important therapies can be developed. Cardiovascular disease is the number one killer in the United States and elucidating the role of factors such as Ang2 in these diseases could help develop molecular targets for pharmaceutical intervention and be of great therapeutic importance.

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APPENDIX A

GENE ARRAY STUDY

Table A.1 Shear-sensitive angiogenic genes (Gene array study)

GenBank no.	Gene name	OS / LS ratio	*P* value	Function
NM_003246	Thrombospondin 1	14.6	0.003	Glycoprotein that mediates cell-to-cell and cell-to-matrix interactions
NM_001147	Angiopoietin 2	4.2	0.060	Expressed at sites of vascular remodeling; vessel destabilization
NM_001511	Chemokine (C-X-C motif) ligand 1	3.9	0.035	Chemokine
NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	1.6	0.047	Carbohydrate metabolism
NM_002608	Platelet-derived growth factor beta polypeptide	1.4	0.047	Mitogenic for cells of mesenchymal origin
NM_002429	Matrix metalloproteinase 19	0.6	0.007	Breakdown of ECM, migration and adhesion
NM_005118	Tumor necrosis factor (ligand) superfamily, member 15	0.6	0.039	Induces apoptosis, inhibits EC proliferation
NM_000759	Colony stimulating factor 3 (granulocyte)	0.6	0.019	Cytokine that controls production, differentiation, & function of granulocytes
NM_001702	Brain-specific angiogenesis inhibitor 1	0.6	0.008	Brain specific angiogenesis inhibitor regulated by p53
NM_003255	Tissue inhibitor of metalloproteinase 2	0.5	0.050	Inhibitor of metalloproteinases; suppresses proliferationo of endothelial cells
NM_000882	Interleukin 12A	0.5	0.043	Cytokine that acts on T and natural killer cells
NM_001430	Endothelial PAS domain protein 1	0.5	0.002	Differentiation; Transcription; response to hypoxia
NM_001963	Epidermal growth factor	0.5	0.029	Differentiation and potent mitogenic factor
NM_016109	Angiopoietin-like 4	0.4	0.013	Glucose homeostasis, lipid metabolism, insulin sensitivity, apoptosis survival factor
NM_003236	Transforming growth factor, alpha	0.4	0.042	Competes with EGF for binding to EGF receptor producing a mitogenic response
NM_020996	Fibroblast growth factor 6	0.4	0.016	Mitogenic and cell survival, oncogenic

Table A.1 (continued)

NM_002521	Natriuretic peptide precursor B	0.4	0.016	Cardiac hormone, diuresis, vasorelaxation, inhibition of renin and aldosterone secretion
NM_000962	Prostaglandin-endoperoxide synthase 1	0.3	0.008	Prostaglandin synthesis; Regulates angiogenesis; involved in cell-cell signaling and tissue homeostasis
NM_000142	Fibroblast growth factor receptor 3	0.3	0.014	Mitogenesis and differentiation. Bone development and maitenance
NM_005429	Vascular endothelial growth factor C	0.2	0.0004	Lymphangiogenesis
NM_000459	Tie2	0.2	0.019	Receptor for Angiopoietin 1 and 2

Table A.2 Shear-insensitive angiogenic genes (Gene array study)

GenBank no.	Gene Name	*OS/LS ratio*	*Pvalue*
NM_182685	Ephrin-A1	4.2	0.240
NM_001147	Angiopoietin 2	4.2	0.067
NM_002982	Chemokine (C-C motif) ligand 2	3.8	0.275
NM_000584	Interleukin 8	2.4	0.400
NM_000362	Tissue inhibitor of metalloproteinase 3	2.3	0.157
NM_003873	Neuropilin 1	2.1	0.223
NM_003872	Neuropilin 2	2.0	0.500
NM_000600	Interleukin 6 (interferon, beta 2)	2.0	0.504
NM_002210	Integrin, alpha V	1.8	0.220
NM_002089	Chemokine (C-X-C motif) ligand 2	1.7	0.084
NM_000442	Platelet/endothelial cell adhesion molecule (CD31 antigen)	1.6	0.163
NM_000301	Plasminogen	1.6	0.580
N/A	Biotinylated Artificial Sequence 2 Complementary sequence	1.5	0.360
NM_007083	Nudix (nucleoside diphosphate linked moiety X)-type motif 6	1.5	0.420
NM_001405	Homo sapiens ephrin-A2	1.3	0.647
NM_004557	Notch homolog 4 (Drosophila)	1.2	0.445
NM_003377	Vascular endothelial growth factor B	1.2	0.589
NM_004093	Ephrin-B2	1.2	0.552
NM_004952	Ephrin-A3	1.2	0.295
NM_002954	Ribosomal protein S27a	1.2	0.248
NM_000576	Interleukin 1, beta	1.2	0.371
NM_021973	Heart and neural crest derivatives expressed 2	1.2	0.623
NM_004530	Matrix metalloproteinase 2	1.2	0.490
NM_005163	V-akt murine thymoma viral oncogene homolog 1	1.1	0.768
NM_030582	Collagen, type XVIII, alpha 1	1.1	0.550
NM_001101	Actin, beta	1.1	0.717
NM_004048	Beta-2-microglobulin	1.1	0.877
NM_000619	Interferon, gamma	1.1	0.935
NM_002986	Chemokine (C-C motif) ligand 11	1.0	0.881
NM_005409	Chemokine (C-X-C motif) ligand 11	1.0	0.947
NM_000618	Insulin-like growth factor 1 (somatomedin C)	0.9	0.907
NM_001101	Actin, beta	0.9	0.653
NM_007355	Heat shock 90kDa protein 1, beta	0.9	0.830
NM_002019	Fms-related tyrosine kinase 1	0.9	0.804
NM_003975	SH2 domain protein 2A	0.9	0.883
NM_000230	Leptin	0.9	0.792
NM_001432	Epiregulin	0.9	0.615
NM_000118	Endoglin	0.9	0.616
NM_001145	Ribonuclease, RNase A family, 4	0.9	0.793
NM_000212	Integrin, beta 3	0.9	0.727
NM_002391	Midkine (neurite growth-promoting factor 2)	0.9	0.635
NM_000572	Interleukin 10	0.9	0.758
NM_000506	Coagulation factor II (thrombin)	0.8	0.578
NM_003247	Thrombospondin 2	0.8	0.599

Table A.2 (continued)

NM_003239	Transforming growth factor, beta 3	0.8	0.773
NM_001795	Cadherin 5, type 2, VE-cadherin	0.8	0.059
NM_007355	Heat shock 90kDa protein 1, beta	0.8	0.547
NM_002167	Inhibitor of DNA binding 3	0.7	0.523
NM_000660	Transforming growth factor, beta 1	0.7	0.496
NM_000214	Jagged 1	0.7	0.651
NM_003254	Tissue inhibitor of metalloproteinase 1	0.7	0.404
NM_021935	Prokineticin 2	0.7	0.417
NM_002176	Interferon, beta 1, fibroblast	0.7	0.375
NM_002994	Chemokine (C-X-C motif) ligand 5	0.7	0.280
NM_004994	Matrix metalloproteinase 9	0.7	0.142
NM_004612	Transforming growth factor, beta receptor I	0.7	0.532
NM_002090	Chemokine (C-X-C motif) ligand 3	0.7	0.095
NM_000906	Natriuretic peptide receptor A/guanylate cyclase A	0.7	0.307
NM_014495	Angiopoietin-like 3	0.7	0.352
NM_004444	EphB4	0.7	0.279
NM_015136	Stabilin 1	0.7	0.626
NM_001146	Angiopoietin 1	0.7	0.061
NM_000594	Tumor necrosis factor (TNF superfamily, member 2)	0.7	0.209
NM_016639	Tumor necrosis factor receptor superfamily, member 12A	0.6	0.359
NM_002607	Platelet-derived growth factor alpha polypeptide	0.6	0.175
NM_018046	Angiogenic factor VG5Q	0.6	0.161
NM_002253	Kinase insert domain receptor	0.6	0.399
NM_001962	Ephrin-A5	0.6	0.082
NM_000601	Hepatocyte growth factor (hepapoietin A)	0.6	0.303
NM_002006	Fibroblast growth factor 2 (basic)	0.6	0.108
NM_003376	Vascular endothelial growth factor	0.6	0.293
NM_005424	Tyrosine kinase	0.6	0.138
NM_002416	Chemokine (C-X-C motif) ligand 9	0.6	0.105
NM_001565	Chemokine (C-X-C motif) ligand 10	0.6	0.086
NM_024013	Interferon, alpha 1	0.6	0.192
NM_000963	Prostaglandin-endoperoxide synthase 2	0.5	0.279
NM_003283	Troponin T1, skeletal, slow	0.5	0.294
NM_020405	Plexin domain containing 1	0.5	0.154
NM_006665	Heparanase	0.5	0.064
NM_000800	Fibroblast growth factor 1 (acidic)	0.5	0.389
NM_006291	Tumor necrosis factor, alpha-induced protein 2	0.5	0.254
NM_001562	Interleukin 18 (interferon-gamma-inducing factor)	0.5	0.069
NM_004469	C-fos induced growth factor	0.5	0.077
NM_017564	Stabilin 2	0.5	0.219
NM_001530	Hypoxia-inducible factor 1, alpha subunit	0.5	0.412
NM_021972	Sphingosine kinase 1	0.5	0.243
NM_007015	Leukocyte cell derived chemotaxin 1	0.5	0.083
NM_002632	Placental growth factor	0.5	0.208
NM_001400	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 1	0.5	0.268
NM_002825	Pleiotrophin	0.4	0.094
NM_002993	Chemokine (C-X-C motif) ligand 6	0.4	0.197

Table A.2 (continued)

NM_003238	Transforming growth factor, beta 2	0.4	0.414
NM_002619	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)	0.4	0.145
NM_000091	Collagen, type IV, alpha 3 (Goodpasture antigen)	0.4	0.186
NM_002165	Inhibitor of DNA binding 1	0.4	0.125
NM_002615	Serine (or cysteine) proteinase inhibitor, clade F	0.4	0.401
NM_000314	Phosphatase and tensin homolog	0.3	0.165
NM_002658	Plasminogen activator, urokinase	0.3	0.144
NM_001150	Alanyl (membrane) aminopeptidase	0.3	0.069
NM_005560	Laminin, alpha 5	0.2	0.067

APPENDIX B

PROTEIN ARRAY STUDY

Table B.1: Shear-sensitive proteins (Protein array study)

Protein name	OS / LS ratio	*P* value	Function
Angiopoietin-2	3.4	0.01	Expressed only at the sites of vascular remodeling; Vessel destabilization
Interleukin 7 (IL-7)	2.7	0.02	Cytokine important for B and T cell development
Neurotrophin 3 (NT-3)	2.1	0.00	Survival and differentiation of mammalian neurons
Insulin-like growth factor binding protein 6 (IGF-BP-6)	2.0	0.05	Modulates Insulin growth factor activity
dtk (tyro3)	1.8	0.02	Growth factor receptor tyrosine kinase
Cutaneous T-cell-attracting chemokine (CTACK) (CCL27)	1.8	0.01	Chemotactic for T-cells
Macrophage Inflammatory Protein 1 delta (MIP-1-delta) (CCL15)	1.7	0.01	Chemotactic for T-cells and monocytes
Insulin-like growth factor binding protein 3 (IGF-BP-3)	1.6	0.03	Modulates Insulin-like growth factor activity
Hepatocyte growth factor (HGF)	0.4	0.02	Regulates cell growth, cell motility, and morphogenesis
TNFreceptor superfamily member 1A (sTNF-RI)	0.4	0.04	Mediates apoptosis, activates NF-kappa B signaling, regulates inflammation

Table B.2: Shear-insensitive proteins (Protein array study)

Protein name	OS / LS ratio	*P* value
AgRP	3.04	0.28
IL-10	2.73	0.09
ICAM-3	2.45	0.36
MIP-3-alpha	2.31	0.16
TECK	1.86	0.19
IGFBP-4	1.84	0.10
EGF-R	1.81	0.08
NAP-2	1.81	0.08
BLC	1.77	0.09
IGF-I SR	1.76	0.19
VEGF-D	1.75	0.44
ENA-78	1.57	0.52
Eotaxin-3	1.53	0.19
Lymphotactin	1.51	0.40
FGF-4	1.50	0.11
I-TAC	1.48	0.13
MCP-2	1.45	0.31
IL-16	1.44	0.15
FGF-9	1.43	0.13
SDF-1	1.41	0.29
MSP-a	1.40	0.60
IL-15	1.40	0.22
Fas/TNFRSF6	1.40	0.28
IL-13	1.36	0.49
I-309	1.32	0.25
Leptin	1.32	0.26
MCP-3	1.28	0.46
GITR ligand	1.27	0.16
MIP-1-alpha	1.27	0.18
TRAIL-R3	1.24	0.49
IGFBP-1	1.23	0.32
MIF	1.23	0.45
IL-1 R4/ST2	1.22	0.59
Beta-NGF	1.21	0.77
IL-2	1.20	0.68
TARC	1.19	0.74
IL-1 RI	1.19	0.82
Osteoprotegerin	1.19	0.63
Amphiregulin	1.19	0.78
IL-4	1.18	0.69
IL-3	1.18	0.62
GITR	1.17	0.69
IFN-gamma	1.17	0.68
BMP-6	1.16	0.06

Table B.2 (continued)

FGF-7	1.14	0.62
LIGHT	1.13	0.63
Oncostatin M	1.13	0.62
MIP-3-beta	1.13	0.68
RANTES	1.13	0.61
TPO	1.13	0.53
Eotaxin	1.12	0.59
FGF-6	1.12	0.41
IL12-p40	1.11	0.83
IL-1beta	1.11	0.78
IGF-I	1.11	0.53
MIG	1.10	0.59
IL-6	1.10	0.89
Flt-3 Ligand	1.09	0.66
VEGF	1.06	0.79
G-CSF	1.05	0.90
MIP-1-beta	1.03	0.94
TRAIL-R4	1.02	0.88
Acrp30	1.02	0.92
IL8	1.00	1.00
axl	1.00	1.00
MCP-4	0.99	0.96
CNTF	0.98	0.93
SCF	0.97	0.90
IL11	0.97	0.97
PDGF-BB	0.96	0.93
NT-4	0.95	0.92
GM-CSF	0.95	0.85
TIMP-2	0.94	0.89
TNF-beta	0.93	0.72
TGF-beta 3	0.92	0.74
TGF-beta 1	0.92	0.73
IL-1alpha	0.90	0.74
BDNF	0.90	0.36
MCP-1	0.89	0.66
EGF	0.87	0.38
bFGF	0.85	0.76
TIMP-1	0.83	0.41
ICAM-1	0.81	0.75
IL-1ra	0.79	0.43
Angiogenin	0.79	0.23
PlGF	0.75	0.70
GRO	0.74	0.71
BMP-4	0.73	0.08
IL-6 R	0.73	0.53
IGFBP-2	0.72	0.38
GRO-alpha	0.68	0.50

Table B.2 (continued)

TNF-alpha	0.66	0.47
BTC	0.66	0.36
Fractalkine	0.65	0.15
sTNF RII	0.60	0.15
IL-5	0.57	0.48
GDNF	0.57	0.19
sgp130	0.54	0.23
IL12-p70	0.54	0.51
CCL28	0.45	0.08
uPAR	0.45	0.51
HCC-4	0.43	0.06
GCP-2	0.34	0.09
Blank	0.33	0.11
IL-2 Ra	0.27	0.41
IL17	0.00	0.37

VITA

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Sarah was born on May 15, 1981 in Whitman, Massachusetts. At 8 years old, she moved to East Bridgewater, Massachusetts where she attended middle school and high school. In 2003, Sarah graduated with highest honors from Worcester Polytechnic Institute with a Bachelor of Science degree in Biomedical Engineering. She then enrolled in the Wallace H. Coulter Department of Biomedical Engineering at Georgia Tech and Emory University. While at Georgia Tech and Emory, she obtained a Master of Science in Mechanical Engineering and completed her doctoral degree in Bioengineering. In her free time she likes to play the piano, develop black and white photography and go shopping with her mom and sister. Sarah especially enjoys traveling and her favorite places are Bangkok, Thailand; Cinque Terre, Italy; and Grindelwald, Switzerland.