# HEMODYNAMIC MECHANISMS REGULATING INFLAMMATORY VASCULAR REMODELING

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**ABSTRACT**

Christopher Stephen Givens: Hemodynamic Mechanisms Regulating Inflammatory Vascular Remodeling
(Under the direction of Ellie Tzima)

Hemodynamic forces are critical regulators of vascular health and disease. Shear stress, the frictional force of blood flowing over the endothelium, is a major hemodynamic input into vascular function. For example, inflammatory blood flow patterns induce programs of intercellular signaling and gene expression that cause local vascular inflammation. Endothelial cells mediate the interactions between blood flow and blood vessels, and as such are exquisitely sensitive to forces. Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1), a well-known endothelial mechanosensor, is required for flow-induced endothelial inflammation, yet the signaling pathways connecting hemodynamics, PECAM-1, and vascular inflammation remain to be elucidated. This dissertation provides mechanistic insight into two PECAM-1 dependent inflammatory pathways: assembly of fibronectin (FN) into fibrils and the role of the adaptor protein Shc in inflammatory vascular remodeling.

FN is an extracellular matrix protein that plays major roles in vascular development and disease. Required for embryonic development of the vascular plexus and heart, FN is also heavily deposited in atherosclerotic plaques and chronically inflamed vessels. As FN is known to sustain endothelial inflammation, knowledge of its assembly is critical to understanding vascular disease. In Chapter 2, I demonstrate that FN assembly is controlled by altered hemodynamics via PECAM-1. Additionally, I delineate a mechanism by which the small GTPase RhoA and β1 integrins regulate FN assembly in a PECAM-1 dependent manner. This is the first time that FN assembly has been mechanistically linked to hemodynamics, and suggests a route by which chronic inflammation may take hold in the endothelium.

Vascular inflammation may result in remodeling of blood vessels, and depending on the physical cues at work, this remodeling may result in widening or narrowing of the blood vessels. The adaptor protein Shc is a known regulator of endothelial inflammation and outward vascular remodeling, and is phosphorylated upon the onset of shear. In Chapter 3, I demonstrate that Shc tyrosine phosphorylation is required for outward collateral remodeling in response to femoral artery ligation. I also demonstrate that Shc is required for atherogenesis, which is pathological inward remodeling of arteries in response to inflammatory flow. Critically, I show the involvement of Shc in atherogenesis to be responsive to changes in hemodynamics. These data suggest that Shc is a major regulator of vascular remodeling, as well as suggest potential targets for downstream vascular therapeutics.

**ACKNOWLEDGMENTS**

The old adage is true: it certainly took a village to raise this scientist, and I would like to start by giving heartfelt thanks to my advisor, Ellie Tzima. My first rotation of graduate school was in the Tzima lab, and I found the research so fascinating that I had to join. Since then, Ellie has had a tremendous impact on my development as a scientist and a person. She is passionate about science and unafraid of new ideas or strange twists in the path of a research project, qualities that have made a lasting impression on me as a scientist. She also deeply cares about her trainees and has supported me well throughout my journey. Even from across the Atlantic, she has continued to be a steadfast and insightful mentor, for which she has my deepest gratitude.

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## PREFACE

The work in Chapter 1 entitled “Endothelial Mechanosensing: Does One Sensor Fit All?” was published in *Antioxidants and Redox Signaling* in September 2016. I was the principal author of the review article, and Ellie Tzima provided guidance on the article’s structure, edited the manuscript, and saw it through to submission. The citation for the review is as follows: Givens C and Tzima E. Endothelial Mechanosignaling: Does One Sensor Fit All? *Antioxidants and Redox Signaling*. 2016 Sep 1;25(7):373-88. Additionally, the work in Chapter 1 entitled, “Stiffness and Vascular Phenotypes” is in preparation for publication as a separate review.

The work presented in Chapter 2 has been published at *Scientific Reports*. My roles in this project included repeating experiments for the original submission, data analysis, and writing the manuscript. Ellie Tzima was the principal investigator, who helped design experiments. Dr. Tzima and John Reader both provided critical input on and helped edit the manuscript. The citation is as follows: Chen, Z; Givens, C; and Tzima E. PECAM-1 regulates fibronectin assembly in endothelial cells. Scientific Reports. 2017 January 25. The overall work presented in this thesis was funded by NIH HL117256 and a Wellcome Trust Senior Fellowship, both to Ellie Tzima, as well as NIH T32 HL069768, to Chris Givens.

Several manuscripts not included in this dissertation were published over the course of my graduate work. These include two commentaries co-written with Ellie Tzima in *Arteriosclerosis, Thrombosis, and Vascular Biology* and *Developmental Cell*. Their citations are Givens, C and Tzima E. Vessels With Cingulin Are Leakproof. Arteriosclerosis, Thrombosis, and Vascular Biology. 2016 April; 36:584-585 and Givens, C and Tzima, E. $S1P_1$ Bridges Mechanotransduction and angiogenesis during vascular development. Developmental Cell. 2012 Sep 11; 23(3):451-2, respectively.

I also co-authored two manuscripts. The first was published with Daniel T. Sweet as the lead author and Ellie Tzima as the senior author. I assisted with experiments and data analysis. The

citation is as follows: Sweet, DT; Chen, Z; Givens, C; Owens, AP; Mackman, N; and Tzima, E. Endothelial Shc regulates arteriogenesis through dual control of arterial specification and inflammation via the Notch and NF-κB pathways. Circulation Research. 2013 June 21; 113(1):32-39.

The second paper I co-authored was led by Leigh Ann Samsa, with Jiandong Liu as the senior author. I assisted with shear stress experiments and experimental design. The citation is as follows: Samsa, LA; Givens, C; Tzima, E; Stainier, D; Qian, L; Liu, J. Cardiac contraction activates endocardial notch signaling to modulate chamber maturation. Development. 2015 Dec 1; 142(23):4080-4091.

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**ApoE** = Apolipoprotein E

**BAEC** = Bovine aortic endothelial cell

**CAL** = partial carotid artery ligation

**Cdh5** = Cadherin 5

**CH1** = Collagen homology 1 domain

**CL** = Collagen I

**E10.5** = Embryonic day 10.5

**ECM** = Extracellular Matrix

**EC** = Endothelial Cell

**EDA** = Extra Domain A

**EDB** = Extra Domain B

**eNOS** = Endothelial nitric oxide synthase

**ER** = Endoplasmic Reticulum

**ERK** = Extracellular signal related kinase

**FAK** = Focal adhesion kinase

**FAL** = Femoral artery ligation

**FITC** = Fluorescein isothiocyanate

**FN** = Fibronectin

**GAPDH** = Glyceraldehyde 3-phosphate dehydrogenase

**GFND** = Glomerulopathy with fibronectin deposits

**Grb2** = Growth Factor Receptor Bound Protein 2

**H&E** = Hematoxylin and Eosin

**HFD** = High fat diet

**ICAM-1** = Intercellular Adhesion Molecule 1

**DOC** = Deoxycholate

**KO** = PECAM Knockout endothelial cells

**LCA** = Left common carotid artery

**LDL** = Low density lipoprotein

**LM** = Laminin

**LPA** = Lysophosphatidic acid

**MEK** = Mitogen activated protein kinase kinase

**MCP-1** = Monocyte Chemoattractant Protein 1

**NF-κB** = Nuclear Factor Kappa B Subunit 1

**NO** = Nitric Oxide

**ORO** = Oil Red O

**oxLDL** = Oxidized low density lipoprotein

**PAK** = p21-activated kinase

**PBS** = Phosphate Buffered Saline

**PECAM** = Platelet endothelial cell adhesion molecule 1

**PECAM$^{-/-}$** = PECAM Knockout

**PTB** = Phosphotyrosine binding domain

**RC** = PECAM reconstituted endothelial cells

**ROI** = Region of interest

**ROS** = Reactive Oxygen Species

**RTK** = Receptor Tyrosine Kinase

**SH2** = Src homology 2 domain

**Shc** = Src homology 2 containing transferring protein

**Shc3F** = Shc phosphorylation-null mutant

**Shc$^{p66}$** = p66 isoform of ShcA

**Shc fl/fl** = Shc flox/flox genotype

**SMC** = Smooth Muscle Cell

**SNO** = s-Nitrosylation

**SOS** = Son-of-Sevenless

**tTG** = Tissue transglutaminase

**TNFα** = Tumor necrosis factor alpha

**V** = Variable region

**VCAM-1** = Vascular Cell Adhesion Molecule 1

**VLDL** = Very low density lipoprotein

**WT** = Wild Type

## Chapter 1 Introduction

### ENDOTHELIAL MECHANOSIGNALING: DOES ONE SENSOR FIT ALL?

**Introduction**

Mechanical forces influence every area of biology, from early development to adult physiology and pathology. During development, left-right asymmetry of the growing embryo; pruning of the immature vascular plexus; and renal morphogenesis are all regulated by mechanical forces (Lucitti et al., 2007; Orr et al., 2006b). Similarly, in the adult organism, several physiological processes are dependent on mechanical force sensing, including the senses of touch and hearing as well as pulmonary surfactant production resulting from breathing. There is also a dark side to force sensing, as tumor metastasis and atherosclerosis are regulated by pathological forces and resultant mechanosignaling (Orr et al., 2006b).

In the cardiovascular system, forces are critical determinants of vascular homeostasis and pathological processes. Vascular smooth muscle cells increase collagen production in response to stretch, which contributes to normal collagen synthesis and turnover, but can also lead to the development of atherosclerosis (Leung et al., 1976; Rodriguez-Feo et al., 2005). Additionally, increases in cardiac load due to exercise or hypertension lead to extensive cardiac remodeling known as cardiac hypertrophy (Dorn, 2007). Devastating conditions, such as aortic dissection, are fundamentally problems of mechanobiology, and result when wall stress exceeds the strength of arterial walls (Humphrey et al., 2015). Also, inflammatory flow patterns, including disturbed and low flow conditions, contribute to focal atherosclerotic plaque formation, which is the pathology behind debilitating cardiovascular events like stroke and myocardial infarction (Hahn and Schwartz, 2009). The past twenty-five years have yielded many insights into the mechanisms behind shear sensing in endothelial cells (ECs), including the identification of many putative endothelial shear stress sensors.

This review focuses on the shear stress mechanosensors that have been identified in ECs, and categorizes them based on their subcellular localization: luminal, junctional, or basal.

**Shear Stress and EC Responses**

The vasculature is constantly subjected to two main forces: circumferential stretch and fluid shear stress. The force of stretch, which results from the natural pulsatility of blood flow, is normal to the vessel wall (Chien, 2007; Tarbell et al., 2014). Stretch can also arise as the result of chronic hypertension, causing thickening of arterial walls and decreasing responsiveness to vasodilatory stimuli (Lu and Kassab, 2011). Stretch induces specific signaling pathways in the endothelium and vascular smooth muscle cells, leading to a complex suite of phenotypes and communication between the two cell types (Ando and Yamamoto, 2011; Birukov, 2009; Lu and Kassab, 2011). Fluid shear stress is the frictional force felt by ECs as a result of blood flow parallel to the vessel wall (Hahn and Schwartz, 2009). Shear stress is represented as a force per unit area; the most common units are dynes or Newtons. ECs throughout the vasculature experience a wide range of shear stresses and magnitudes (Figure 1.1). Arterial shear stresses range from approximately 10dyn/cm$^2$ in the aorta to 50dyn/cm$^2$ in smaller arterioles (Papaioannou and Stefanadis, 2005). Shear stress in the venous circulation is lower, ranging from 20dyn/cm$^2$ in venules to 1dyn/cm$^2$ in the vena cava (Lipowsky et al., 1978; Papaioannou and Stefanadis, 2005).

*In vivo*, blood flow exhibits differential patterns, which fall into two broad categories: laminar and disturbed flow, also known as atheroprotective and atheroprone flow, respectively (Figure 1.2) (Hahn and Schwartz, 2009). These flow patterns are determined by vessel geometry, and give rise to vastly different gene expression profiles. The difference in flow patterns is sensed by endothelial mechanosensors, which transfer differential physical information into divergent biochemical signals. Laminar flow is characterized by high, uniform flow, and occurs mostly in straight areas of the vasculature, such as the descending aorta (Chatzizisis et al., 2007). ECs in areas of laminar flow align in the direction of flow, forming large stress fibers, and exhibit low cellular turnover. Anti-inflammatory genes are highly expressed, including the transcription factor KLF2 (Chatzizisis et al., 2007; Parmar et al., 2006). Conversely, disturbed flow is characterized by low and oscillatory flow

patterns, and is the main flow regime in areas of the vasculature that are highly curved or bifurcated. ECs that experience disturbed flow do not align in the direction of flow and proliferate more rapidly (Buchanan et al., 1999; Chatzizisis et al., 2007). Importantly, inflammatory gene expression is increased in ECs experiencing disturbed flow. Inflammatory adhesion molecules such as VCAM-1 are highly expressed, which can lead to recruitment of monocytes and formation of atherosclerotic plaques (Malek et al., 1999). In this manner, areas of disturbed flow are highly correlated with atherogenesis. In addition to systemic factors such as smoking or high blood cholesterol, EC inflammation caused by disturbed shear stress "primes" the endothelium for atherosclerotic plaque growth. A similar mechanism underlies the growth of collateral vessels after stenosis of the nutritive artery. Found throughout the body, collateral vessels are connectors between different arterial beds (Faber et al., 2014). Collaterals normally do not conduct blood, but upon stenosis of a major artery, blood flow markedly increases through the collaterals, increasing the shear stress experienced by ECs (Cai and Schaper, 2008; Schaper, 2009; Schaper and Scholz, 2003). This increase in shear stress leads to mechanotransduction and activation of signaling pathways that ultimately result in transient inflammation of the collateral ECs (Schaper, 2009). Collateral ECs increase their proliferation and and promote recruitment of immune cells (Z. Chen et al., 2010; Schaper and Scholz, 2003; Sweet et al., 2013). These processes guide collateral remodeling, and the collateral vessels eventually increase their caliber such that they conduct the amount of blood normally carried by the nutritive artery (Schaper, 2009).

Endothelial responses to flow have been studied extensively *in vitro.* Early responses include K+ and $Ca^{2+}$ influx, NO production, and ROS production (Hsieh et al., 1998; Nauli et al., 2008; Olesen et al., 1988; Yamamoto et al., 2000). Soon thereafter, activation of MAP kinases, eNOS and Akt also occur. The discovery that shear stress activates integrins in ECs (Jalali et al., 2001; Tzima et al., 2002; 2001) opened up a new avenue of research on the importance of the identity of the extracellular matrix (ECM) in shear stress signaling (please see Review Forum article by Yurdagul and Orr). The current view is that the matrix, and as a result the specific integrins that are binding to it, serve as a "check-point" that determines which intracellular signaling responses will be activated and which will be inhibited. Examples include the activation of JNK when cells are plated on

fibronectin and the activation of p38 MAP kinase when cells are on collagen (Hahn et al., 2011; Liu et al., 2008; Orr et al., 2005). Similarly, the activation of p21-activated kinase and the inflammatory transcription factor NF-kB is also matrix specific, occurring specifically on fibronectin (Orr et al., 2005; 2007)

Another important set of signaling molecules is also regulated by shear stress: members of the Rho family of GTPases are highly sensitive to both spatial and temporal regulation by shear stress (Tzima, 2006). RhoA is transiently downregulated within 5 minutes of onset of shear stress, allowing breakdown and subsequent re-formation of actin stress fibers, while Rac is transiently activated starting at 5 minutes and peaking at 30 minutes after onset of shear stress (Tzima et al., 2002; 2001). Interestingly, activation of Rho family members is subject to integrin control (Tzima, 2006). Shear stress activates endothelial integrins, which form new connections with the ECM (Tzima et al., 2001). This activity activates RhoA, which directs rearrangement of focal adhesions (Collins and Tzima, 2013). In unstimulated ECs, the focal adhesion protein vinculin is evenly distributed in small puncta throughout the cell. However, after application of shear stress, vinculin aggregates into large focal adhesions at the upstream edge of ECs (Girard and Nerem, 1995). Proper spatiotemporal activation of both Rho and Rac is required for alignment of ECs in the direction of flow (Tzima et al., 2002; 2001)as well as establishment of polarity in ECs (Liu et al., 2013). Cdc42 is also activated in response to shear (Wojciak-Stothard and Ridley, 2003) and its activation is required for polarization of the microtubule organizing center (MTOC) in ECs (Tzima et al., 2003). Furthermore, EC migration speed increases when ECs are exposed to shear, an effect mediated by RhoA (Shiu et al., 2004).

Intermediate responses to shear include transcriptional activation of NF-kB target genes, such as ICAM-1 (Orr et al., 2005). In areas of laminar flow, shear signaling cascades culminate with downregulation of NF-kB, followed by atheroprotective transcription resulting from ERK5-dependent KLF2 expression (Hahn and Schwartz, 2009; Parmar et al., 2006). At this time, ECs also align parallel to the direction of flow (Tzima et al., 2001; 2005). In areas of disturbed flow, however, NF-kB and other inflammatory signaling is sustained. Long term EC inflammation leads to continuation of ICAM-1 and VCAM-1 expression, causing recruitment of monocytes to inflamed endothelium, beginning atherogenesis (Chatzizisis et al., 2007). Additionally, increased fibronectin deposition occurs in areas

of inflamed endothelium, enhancing and sustaining inflammation and atherogenesis in those areas (Feaver et al., 2010; Green et al., 2014; Orr et al., 2005). Extensive endothelial protein S-nitrosylation also occurs in response to shear stress (Hoffmann et al., 2003). These proteins include heat shock proteins, as well as cytoskeletal elements like tropomyosin and vimentin (Huang et al., 2009). The diverse suite of shear-induced endothelial signaling and phenotypes implies that ECs possess exquisite mechanosensitivity, which is indeed the case. ECs express many putative mechanosensors, the major groups of which are discussed below (Figure 1.3).

**Apical Mechanosensors**

***Primary Cilia*** Primary cilia are 3-5μm long cellular protrusions provided structure by microtubular bundles. Embryonic evidence suggests a requirement for cilia in developing vasculature, where flow regimes are low. Developing zebrafish present with cranial hemorrhage after disruption of intraflagellar transport protein Ift81, while Pkd2 EC-specific knockout causes hemorrhage in the cranium and back of developing mice (Garcia-Gonzalez et al., 2010; Hamik et al., 2007; Kallakuri et al., 2015; Parmar et al., 2006; Sen-Banerjee et al., 2005).

EC primary cilia are primarily found in areas of the vasculature that experience low or disturbed flow. Their presence has been demonstrated in chicken embryonic endocardial and venous ECs (Van der Heiden et al., 2006). Cilia exist in embryonic mouse aorta and the inner curvature of adult mouse aorta, both low flow areas of the vasculature (Nauli et al., 2008; Van der Heiden et al., 2008). Additionally, zebrafish arteries and veins exhibit cilia (Goetz et al., 2014). The presence of cilia is known in primary chicken ECs (Hierck et al., 2008), human umbilical vein ECs (HUVECs) (Iomini et al., 2004), and embryonic mouse aortic ECs (Nauli et al., 2008). Laminar shear causes rapid disassembly of cilia in ECs, and as such the contribution of cilia to long-term shear stress signaling remains unclear (Iomini et al., 2004).

A body of research investigating cilia-dependent shear responses shows a role for cilia in early calcium signaling and nitric oxide (NO) production. To mediate shear-induced calcium and NO signaling, primary cilia partner with polycystin 1 and 2 (PKD1 and PKD2, expressed from the Pkd1 and Pkd2 genes). PKD1, a G-protein coupled receptor (GPCR), constitutively activates Gi/o proteins.

Physical association with PKD2, a $Ca^{2+}$ channel, represses this constitutive activation (Delmas et al., 2002). Using microfluorimetry, early shear-dependent calcium influx and NO production were examined *in vitro* using aortic ECs from $Pkd1^{null/null}$ mice. Twenty seconds after the application of shear, high levels of calcium influx and NO production were observed in wild type cells (Nauli et al., 2008). However, early calcium influx and NO production were blocked in $Pkd1^{null/null}$ ECs. Additionally, Pkd2 is required for shear-induced calcium influx and NO production. These early events are blocked by Pkd2 knockdown in ECs (AbouAlaiwi et al., 2009). Interestingly, ECs isolated from an autosomal dominant polycystic kidney disease (ADPKD) patient show heterogeneous expression of PKD2. PKD2-null ECs from these patients display aberrant localization of eNOS after the application of shear stress (AbouAlaiwi et al., 2009).

Recent *in vivo* studies of primary cilia complement previous *in vitro* studies of cilia. In zebrafish embryos, a morpholino against Pkd2 blocks calcium influx in the arterial endothelium (Goetz et al., 2014). *Tnnt2* and *gata1* morpholinos are also used, blocking heartbeat and reducing hematocrit, respectively. *Tnnt2* morphants display no ciliary deflection, while cilia in *gata1* morphants only deflect moderately. Importantly, endothelial calcium influx correlates with degree of ciliary deflection, as tnnt2 morphants show a severe reduction in EC calcium influx and gata1 morphants display only a moderate reduction (Goetz et al., 2014; Nauli et al., 2008).

Despite the published work on the involvement of cilia in $Ca^{2+}$ and NO signaling, the status of primary cilia as shear sensors remains unclear. Cilia reside in low flow areas of the vasculature (Y.-S. J. Li et al., 2005; Liu et al., 2008; Tzima et al., 2001; Van der Heiden et al., 2008), and are mostly absent in areas of high flow. This suggests the intriguing possibility that cilia formation itself is a process that depends on blood flow, since cilia form as a response to low or disturbed flow. Although this does not exclude the possibility of cilia functioning as mechanosensors, their role as amplifiers of shear signals (Cuhlmann et al., 2011; Hierck et al., 2008; Lan et al., 1994) is certainly compatible with their presence in areas of low flow. Cilia may act as low flow sensors in the endothelium, but disentangling ciliary functions in development and overall EC shear signaling remains a challenge.

***Glycocalyx*** The endothelial glycocalyx consists of a mixture of glycoproteins, hyaluronin, and proteoglycans such as syndecan-1 and -4. The glycocalyx has a net negative charge due to the presence of glycoprotein-bound sialic acid moieties and proteoglycan-bound glycosaminoglycans (GAGs). As a result, an extended endothelial surface layer (ESL) is formed as a result of glycocalyx interaction with components of blood. The ESL is comprised of plasma proteins, growth factors, and cytokines (Chatzizisis et al., 2007; Parmar et al., 2006; Weinbaum et al., 2007).

Early work on the endothelial glycocalyx revealed a hydrodynamically relevant surface layer capable of regulating capillary hematocrit (Desjardins and Duling, 1990; Malek et al., 1999; Pries et al., 1997). Using *in vivo* solute exclusion assays, the height of the glycocalyx was determined to be approximately 500nm. High molecular weight dextran was excluded from this 500nm-thick later, and the thickness of the layer was reduced by degrading the glycocalyx with an infusion of hyaluroninidase (Henry and Duling, 1999; Vink and Duling, 2000; S. Yuan and Z. Sun, 2013). Additionally, vascular TNFα infusion reduces glycocalyx height, suggesting that endothelial inflammation negatively regulates glycocalyx density (Essner et al., 2005; Henry and Duling, 2000; Murcia et al., 2000). Degradation of hyaluronin or inhibition of its synthesis decreases glycocalyx thickness in collateral vessels, reducing collateral remodeling after hindlimb ischemia. Shear dependent EC proliferation and inflammatory gene expression are also reduced (Grundmann et al., 2009; Huangfu et al., 2003; Potter et al., 2015).

The glycocalyx is thought to dampen the force of shear stress that that reaches the EC cell surface (Weinbaum et al., 2007). Thus, cells with an intact glycocalyx do not experience appreciable shear forces at their plasma membranes. Instead, syndecans link to the cytoskeleton through their cytoplasmic termini, providing a possible mechanism for force transfer from shear stress to the cytoskeleton (Tkachenko et al., 2005; Van der Heiden et al., 2006; Weinbaum et al., 2003). Indeed, ablation of syndecan function through enzymatic or genetic means leads to blockade of shear stress responses in ECs. Degradation of heparin sulfate GAGs, the major GAGs associated with syndecans, leads to misregulated actin dynamics in ECs. As a result, ECs do not break down their dense peripheral actin band and fail to form new stress fibers after application of shear (Nauli et al., 2008; Thi et al., 2004; Van der Heiden et al., 2008; Weinbaum et al., 2007). Syndecan-1 knockout causes

misregulation of RhoA and Akt, as well as upregulation of EC inflammatory gene expression after the application of shear stress (Goetz et al., 2014; Voyvodic et al., 2014). Correlating with the syndecan-1 studies, syndecan-4 knockout ECs do not align in the direction of flow and display an increased inflammatory phenotype. This leads to a higher atherosclerotic burden *in vivo*. Importantly, in Syndecan-4 KD ECs, VEGFR2 phosphorylation is not altered immediately after application of shear. This indicates that syndecan-4 dependent signaling may act independently, or downstream, of junctional shear stress signaling (Baeyens et al., 2014; Hierck et al., 2008).

Enzymatic and genetic studies indicate the importance of glycocalyx components in shear-related signaling. However, syndecans act as co-receptors for integrins during adhesion, complicating the determination of specific function in shear stress signaling (Hassan et al., 2013; Iomini et al., 2004; McQuade et al., 2006; Stepp et al., 2010; Tkachenko et al., 2005). Many EC responses that are misregulated in response to syndecan ablation, including RhoA activation and ICAM-1 and VCAM-1 exression, are downstream of integrin activation after the application of shear (Liu et al., 2008; Nauli et al., 2008; Tzima et al., 2001). Thus, syndecan function in shear signaling may be required for integrin function, and not direct shear sensing. Further studies are required to completely disentangle specific glycocalyx signaling from integrin-associated signaling in ECs. The glycocalyx as an EC mechanosensor remains attractive, but clarity about its function remains elusive.

***Heterotrimeric G-proteins and G-protein coupled receptors*** Heterotrimeric G-proteins participate in shear stress signaling and may also form mechanosensitive complexes with known mechanosensors. Work done using vesicles loaded with G-proteins and $^{32}P$ suggests that increasing membrane fluidity or applying shear stress leads to GTPase activation (Gudi et al., 1998; Iomini et al., 2004). The heterotrimeric G-protein Gαq/11 associates with PECAM-1 in unstimulated ECs, rapidly dissociating upon application of shear. *In vivo,* Gαq/11 colocalizes with PECAM-1 in areas of laminar, but not disturbed, flow (Delmas et al., 2002; Otte et al., 2009). Interestingly, the association of Gαq/11 with PECAM-1 is mediated by heparan sulfate, suggesting a link between three candidate mechanosensors: PECAM-1, Gαq/11, and the glycocalyx (Murcia et al., 2000; Paz et al., 2014). Gαq/11 is also required for activation of Ras by flow, implicating G-proteins in shear-induced MAPK

signaling (Gudi et al., 2003; Nauli et al., 2008). Also, siRNA knockdown of Gαq/11 increases the time required for calcium influx after the onset of retrograde shear stress (AbouAlaiwi et al., 2009; Melchior and Frangos, 2012).

GPCRs are also implicated as mechanosensitive molecules. The bradykinin B2 receptor undergoes a ligand-independent conformational change in response to shear stress or membrane fluidizing agents (AbouAlaiwi et al., 2009; Chachisvilis et al., 2006). Furthermore, sphingosine-1 phosphate receptor 1 ($S1P_1$) is required for signal transduction of many well-characterized shear-responsive pathways, such as ERK, AKT, and eNOS activation. In this study, small-molecule induction of $S1P_1$ proteasomal degradation blocked phosphorylation of ERK and EC alignment after application of shear stress. Interestingly, blockage of ERK activation and EC alignment was rescued using endocytosis- and ligand binding-deficient mutants of $S1P_1$, indicating that the involvement of $S1P_1$ in shear signaling is ligand-independent (B. Jung et al., 2012). Endothelial $S1P_1$ is also required for angiogenesis in the mouse retina (Goetz et al., 2014; B. Jung et al., 2012). These studies suggest that GPCRs and G-proteins are heavily involved in shear-responsive mechanotransduction and may be mechanosensors in their own right.

***Ion Channels*** Hyperpolarization of ECs was one of the first identified EC responses to shear, and is among the earliest EC responses to shear (Nakache and Gaub, 1988; Olesen et al., 1988). $K^+$ ion channel activity is regulated by shear stress induced deformation of ECs, rather than high flow rate over ECs (Ando et al., 1993). After initial hyperpolarization of ECs in response to shear, $Ca^{2+}$ channels open and depolarize the EC (Barakat et al., 1999). Shear-induced $Ca^{2+}$ influx increases with increasing magnitude of shear stress (Yamamoto et al., 2000). Interestingly, the activity of one channel, transient receptor potential channel 5 (TRPC5), is enhanced by S-Nitrosylation (Yoshida et al., 2006). Though it is not known how shear stress might regulate S-Nitrosylation of TRPC5, the the S-Nitrosylation is dependent on eNOS, a shear-responsive protein (Yoshida et al., 2006).

Several ion channels are putative shear stress sensors in ECs. The endothelial Kir2.1 $K^+$ channel has been shown to be mechanosensitive, and when expressed in *Xenopus laevis* oocytes, the oocytes become sensitive to shear (Hoger et al., 2002). Additionally, transient receptor potential

vanilloid 4 (TRPV4) has been proposed as a mechanosensor. TRPV4 is sensitive to multiple stimuli, including heat, phorbol esters, arachidonic acid, hypotonic shock, and shear stress (Gao et al., 2003; Watanabe et al., 2002a; 2003; 2002b). Interestingly, TRPV4 also has demonstrated importance in the vasculature, where it regulates vasodilation in carotid arteries, rat mesenteric arteries, and arterioles (Earley et al., 2009; Hartmannsgruber et al., 2007; Köhler et al., 2006; Mendoza et al., 2010) Regulation of vasodilation by TRPV4 may be achieved through TRPV4-dependent NO production from ECs (Cabral and Garvin, 2014). TRPV4 also forms a heterotrimeric, flow-sensitive complex with TRPP2 and TRPC1(Du et al., 2014). TRPV4, however, is activated by arachidonic acid, which is synthesized by phospholipase A2 (PLA2) in response to shear stress(Pearce et al., 1996). If PLA2 activity is inhibited, intercellular calcium and vasodilation responses are blocked (Köhler et al., 2006; Marrelli et al., 2007). Thus, TRPV4 activation is downstream of enzymatic activity and may not be sensitive to the force of flow. However, arachidonic acid synthesis takes place on a slower time scale (Pearce et al., 1996) than TRPV4-dependent calcium flux (Du et al., 2014), suggesting that there may be a yet undiscovered mechanism behind activation of TRPV4 by shear stress.

Recent work has identified the mechanically activated cation channels Piezo1 and Piezo2 in neurons (Coste et al., 2010). Piezo1 and Piezo2 are implicated in touch sensation in mice and red blood cell volume (Cahalan et al., 2015; Ranade et al., 2014b)Transfection into non-mechanically activated HEK293T cells confers sensitivity to mechanical stimulation as well as shear stress (Coste et al., 2010; 2012; J. Li et al., 2014). HEK293T cells transfected with Piezo1 exhibit increasing calcium influx with increasing shear stress. Piezo1 does not share structural features with known channels and may represent an entirely new class of mechanically activated ion channel (Coste et al., 2012). Piezo1 knockout mice are embryonic lethal as a result of major vascular defects manifesting as early as E8.5 (Ranade et al., 2014a). The vascular tree fails to prune correctly in both whole-body and EC-specific Piezo1 knockouts, and ECs in haploinsufficient arteries do not align in the direction of flow (J. Li et al., 2014). Treatment of ECs with Piezo1 siRNA also largely blocks flow-induced EC alignment (Ranade et al., 2014a). Though little is known about these channels at the moment, they hold tremendous potential for the study of EC shear stress signaling.

***Caveolae*** Caveolae are flask-shaped invaginations present in the plasma membrane of various cell types. Caveolae are mostly found in cell types that receive mechanical stress, such as fibroblasts, adipocytes, and ECs. Caveolae are given their structure by proteins of the caveolin and cavin family (CAV1-3 and cavin1-3, respectively) (Echarri and Del Pozo, 2015). Importantly, caveolae rapidly disassemble after hypotonic shock, suggesting a role in buffering the cell membrane against mechanical stress (Sinha et al., 2011). Caveolae are also important in murine development, as Cav1 knockout mice display cardiac hypertrophy and thickened coronary vessel walls, along with bronchiolar hyperplasia (Murata et al., 2007).

Evidence for caveolae in endothelial mechanosensing was first described in rat lungs, where increased perfusion of the rat pulmonary vasculature led to enhanced protein tyrosine phosphorylation, especially in caveolar compartments (Rizzo et al., 1998b). Additionally, eNOS was enriched in rat lung EC caveolae, which served as the main location of eNOS activation after shear (Rizzo et al., 1998a). The cholesterol binding antibiotic filipin and cholesterol sequestering compound methyl-β-cyclodextrin reduced shear-induced ERK activation in BAECs, suggesting that cholesterol-rich regions of the plasma membrane are required for shear signaling. However, a limitation of these pharmacological treatments is that both caveolae and lipid raft function are disrupted, complicating interpretation of results (Park et al., 1998). Transmission electron microscopy images revealed a luminal surface increase in caveolae density in ECs subjected to shear stress (Boyd et al., 2003). Additionally, shear stress induced Cav-1 translocation to the upstream edge of ECs, where hydrostatic pressure is highest on ECs (R. J. Sun et al., 2002). This correlates with the hypothesis that caveolae buffer cell membranes against high mechanical stress (Sinha et al., 2011).

Genetic evidence for caveolae as mechanosensors has revealed a role for caveolae in transduction of shear stress signals but not directly in mechanosensing. In one study, Cav1 knockout mice were subjected to partial carotid artery ligation. Whereas WT mice showed a decrease in lumen diameter, the Cav1 knockout mice displayed no such change. However, the arterial walls of the Cav1 knockout carotids did thicken significantly, a result of significant increases in endothelial, medial, and adventitial proliferation (Yu et al., 2006). Furthermore, Cav1 knockout carotid arteries were deficient in vasodilation as a result of impaired eNOS activation (Yu et al., 2006). In a model of atherosclerosis,

Cav1 whole body knockout mice were crossed to ApoE knockout mice, leading to a reduction in aortic plaque burden. The reduction in plaque burden caused by Cav1 knockout was accompanied by reductions in ICAM-1 and VCAM-1 expression (FernAndez-Hernando et al., 2009). Interestingly, reconstitution of only endothelial Cav1 increased the aortic plaque burden and ICAM-1 and VCAM-1 expression back to control levels, suggesting that caveolin proteins mediate endothelial inflammation leading to atherosclerosis. Caveolae have also been linked to the actin cytoskeleton. Knockdown of Cav1 increases basal RhoA activity, blocking stress fiber formation in ECs (B. Yang et al., 2011). Additionally, Cav1 is required for luminal β1 integrin activation after the onset of shear stress. This activation of luminal integrins does not require the actin cytoskeleton. When blocked, luminal β1 integrin activation reduces shear-induced Src family kinase, Akt, and eNOS signaling in ECs (B. Yang and Rizzo, 2013). Whether caveolae are mechanosensors is unclear, and will take more integrative research to fully determine. Given that caveolae number increases as a response to shear stress, it is likely that they do not act as sensors but are rather shear-regulated, but more research could uncover a role for these structures in endothelial force sensing.

***Tie Receptors*** Members of the Tie family of receptor tyrosine kinases have also been implicated in shear stress sensing (Chen-Konak et al., 2003; Porat et al., 2004). Tie1, a tyrosine kinase with immunoglobulin-like and EGF-like domains 1, is expressed in ECs and its expression maps distinctively to regions exposed to disturbed shear stress (Woo et al., 2011). In vitro, its expression is downregulated by laminar shear stress, while disturbed flow increases Tie1 promoter activity. Deletion of Tie1 increased activation of eNOS and resulted in decreased inflammatory signals in response to laminar flow in vitro. The same group demonstrated a dose-dependent reduction in atherosclerosis in Tie1-attenuated ApoE-/- mice, giving credence to the idea that Tie1 is a critical regulator of the endothelial response to disturbed shear stress. Although it is mechanoresponsive insofar as it alters its expression and modulates downstream signaling, it is unclear if it is a direct sensor of shear stress.

**Junctional Mechanosensors**

***PECAM-1*** PECAM-1 is an adhesion molecule of the immunoglobulin superfamily. The extracellular domain of PECAM-1, which mediates homophilic binding to neighboring ECs, consists of 6 Ig-like subunits (Figure 5). The intracellular and extracellular domains of PECAM-1 are linked by a short transmembrane domain. The major features of the intracellular domain are 2 ITIM domains. These domains contain two tyrosines, 663 and 686, that are phosphorylated rapidly after the onset of shear stress. Along with $Ca^{2+}$ influx and $K^{+}$ channel activation, PECAM-1 phosphorylation is one of the earliest known responses to shear stress. Importantly, it is thought that PECAM-1 phosphorylation is independent of calcium influx, as $Ca^{2+}$ agonists do not increase PECAM-1 phosphorylation on their own.

*In vitro* studies of the role of PECAM-1 in shear stress signaling are numerous. Early evidence implicating PECAM-1 in EC force sensing showed that PECAM-1 phosphorylation, Shp2 and Gab1 recruitment, and ERK phosphorylation all increase in response to hypo-osmotic shock and fluid shear stress (Osawa et al., 2002). Additionally, Shp2 phosphatase activity is required for PECAM-1 dependent ERK activation. Using an extracted EC model, Chiu et al identified Fyn as the kinase that mediates stretch- and shear stress-induced PECAM-1 phosphorylation (Chiu et al., 2008). Later evidence demonstrated that PECAM-1 acts in concert with VE-Cadherin and VEGFR2 to mediate a large number of shear stress responses. These responses include EC alignment, NF-kB activation, and Akt phosphorylation following application of shear stress (Fleming et al., 2005; Tzima et al., 2005). Importantly, PECAM-1 is required for both anti-inflammatory and inflammatory signaling in ECs (Tzima et al., 2005). PECAM-1 is required for establishment of polarity in ECs after onset of shear stress via spatiotemporal activation of Rac GTPase (Liu et al., 2013). In this model, PECAM-1 is required for Rac activation downstream of Src and Vav2 phosphorylation, while VE-Cadherin acts as a scaffold for localized Rac activation on the downstream edge of ECs. PECAM-1 knockout also leads to misregulation of eNOS localization and resultant increased NO production in unstimulated ECs (McCormick et al., 2011). Additionally, in PECAM-1 knockout ECs, eNOS activation is decreased in response to shear stress (Fleming et al., 2005).

Force application directly on PECAM-1 using ferromagnetic beads has allowed the study of specific PECAM-1-dependent signaling pathways (Figure 1.4). Using magnetic beads coated with PECAM-1 antibody, early studies demonstrated that direct force on PECAM-1 elicits both PECAM-1 and ERK phosphorylation (Osawa et al., 2002). PECAM-1 engagement is required for these responses, highlighting the importance of confluency in studies of EC shear stress signaling. Further studies of direct force application on PECAM-1 have shown that localized force on PECAM-1 can activate global cellular stiffening pathways in ECs. Application of force on PECAM-1 using magnetic beads manipulates only a small area of the EC; however, after 5 minutes of force, global focal adhesion growth and RhoA activation are observed(Collins et al., 2012). The observed focal adhesion growth and RhoA signaling culminate in cellular stiffening in response to force application. Interestingly, cellular stiffening, related cytoskeletal dynamics, and focal adhesion growth are all ECM-specific events. Plating ECs on collagen blocks force-induced focal adhesion growth and cytoskeletal dynamics that are normally observed in ECs growing on fibronectin (Collins et al., 2014). This suggests that PECAM-1 and integrins act cooperatively in order to mediate downstream signaling to force-based events. Since pharmacological inhibition of PI3-Kinase blocks force-induced focal adhesion formation and integrin activation (Collins et al., 2012), PECAM-1 may communicate with integrins via biochemical means, rather than direct force-transfer through the cytoskeleton.

PECAM-1 null mice are healthy and fertile (Duncan et al., 1999), which is surprising given the role of shear stress signaling in the development of the vascular plexus. However, starting at 4 weeks of age, PECAM-1 knockout mice display cardiac defects including increased left ventricle diameter, reduced fractional shortening, and reduced ejection fraction (McCormick et al., 2014). Flow-dependent dilation is blocked in *ex vivo* arteries from PECAM KO mice due to failure to activate eNOS (Bagi et al., 2005). Additionally, PECAM-1 KO mice subjected to partial carotid artery ligation display defects in flow-mediated vascular remodeling and intima-media thickening, due to defects in the NF-kB pathway (Z. Chen and Tzima, 2009). PECAM-1 KO mice also show reduced collateral remodeling after femoral artery ligation, once again due to reduced NF-kB-dependent transcriptional activity (Z. Chen et al., 2010). Results from studies of atherosclerosis using PECAM-1 knockout mice have been difficult to interpret, possibly owing to differences in atherogenic genetic background and

feeding time. However, across several studies, PECAM-1 knockout mice show reduced atherosclerotic plaque formation in the lesser curvature of the aortic arch (Goel et al., 2008; Harrison et al., 2013a; Harry et al., 2008; Stevens et al., 2008). This area is constantly exposed to disturbed flow, suggesting that PECAM-1 knockout dampens EC inflammatory signaling arising from disturbed flow.

***VE-Cadherin*** We have previously shown that PECAM-1, VE-Cadherin and VEGFR2 are sufficient and required for shear stress signaling in ECs (Tzima et al., 2005). When VE-Cadherin-expressing cells are mixed with VE-Cadherin knockout cells and exposed to flow, the VE-Cadherin expressing cells align in the direction of flow, whereas VE-Cadherin knockout cells do not (Tzima et al., 2005). Thus, even in the absence of homophilic VE-cadherin adhesion, WT ECs align correctly. This suggests that VE-Cadherin acts as an adaptor in shear stress signaling. VE-cadherin knockout additionally blocks integrin activation after application of shear stress, but not Src activation (Tzima et al., 2005). Src activation is one of the earliest events in shear stress signaling, taking place just after PECAM-1 phosphorylation. This suggests that VE-cadherin acts downstream of PECAM-1 in shear stress signaling, which allows transactivation of VEGFR2 and induction of downstream signaling pathways (Liu et al., 2008; Tzima et al., 2005). The VE-Cadherin transmembrane domain is also required for recruitment of VEGFR2 to the mechanosensory complex, further implicating VE-Cadherin as an adaptor in this system (Figure 1.5) (Coon et al., 2015).

Recent evidence suggests that VE-cadherin may also be a mechanosensitive molecule. Previous studies suggest that force application on VE-Cadherin using antibody-coated magnetic beads does not activate known force-sensitive signaling, however recent evidence shows that this phenomenon may be epitope-specific (Barry et al., 2015; Tzima et al., 2005). Application of force to VE-Cadherin using the VE-Cadherin FC antibody elicited responses similar to those elicited by force on PECAM-1; namely, cell stiffness and actin reorganization (Barry et al., 2015). Additionally, FRET studies using PECAM-1 and VE-Cadherin tension sensors suggest that VE-cadherin carries a high-tension load in unstimulated ECs. This load is reduced once shear stress is applied. Interestingly, tension on PECAM-1 is very low before application of shear stress. After the onset of shear, tension

increases on PECAM-1 as a result of association with vimentin (Conway et al., 2013). The main role of VE-Cadherin in shear stress signaling is likely as an adaptor. However, the recent studies mentioned above imply that VE-Cadherin may have other roles in EC shear stress signaling. More research is needed to determine whether the force-sensing properties of VE-Cadherin are relevant in physiological signaling.

***VEGF Receptors*** VEGF receptors 2 and 3 (VEGFR2 and VEGFR3) are also involved in shear stress sensing. VEGFR2, the better characterized of the two, is rapidly phosphorylated at the onset of shear (Liu et al., 2008; Shay-Salit et al., 2002; Tzima et al., 2005). Shear stress-induced Akt activation is blocked when tyrosines 801 and 1175 of VEGFR2 are mutated, implying that VEGFR2 is required for shear-induced PI3 kinase activation and resultant downstream signals (Figure 5). Thus, while there is no evidence to suggest VEGFR2 is directly mechanosensitive, its activity is required for intact shear stress signaling.

Recently, VEGFR3 was shown to be as a constituent of the mechanosensory complex containing PECAM-1, VE-Cadherin, and VEGFR2. This evidence also implicates VEGFR3 in sensing different magnitudes of shear, adding to our understanding of how different shear patterns are sensed. At the onset of shear, VEGFR2 and VEGFR3 are recruited to the mechanosensory complex by the transmembrane domain of VE-Cadherin, followed by phosphorylation of both VEGFR2 and VEGFR3 (Coon et al., 2015). Evidence also suggests that VEGFR2 and VEGFR3 signal redundantly in response to shear stress. Knockdown of both proteins, but not VEGFR2 or VEGFR3 individually, blocks shear-induced Akt activation (Coon et al., 2015). Interestingly, VEGFR3 has been implicated in determining the sensitivity of different types of ECs to shear stress (Baeyens et al., 2015). Lymphatic ECs (HDLECs) and HUVECs, when exposed to a range of shear stress levels, show peak alignment at different shear magnitudes. HDLECs align most efficiently around 5 dyn/cm$^2$, while HUVECs align most efficiently around 10 dyn/cm$^2$. Additionally, HDLECs express higher levels of VEGFR3 than HUVECs do. HDLECs phenocopy HUVECs upon knockdown of VEGFR3, aligning at higher levels of shear than normal HDLECs. Taken together, these results suggest that shear stress sensing may be more variable across the phenotypic spectrum of ECs than previously thought. Endothelial

mechanosensing may not simply be a matter of efficient force transmission and biochemical information transfer by one or more specialized EC proteins, the mechanisms of which are uniform across ECs. Mechanisms of mechanosensing may vary with endothelial niche bestowed upon them by differential expression of mechanosensory constituents.

**Basal sensors: Integrins**

Integrins act as connections between the extracellular matrix and the actin cytoskeleton, making them attractive candidate mechanosensors in ECs. However, evidence that integrins are direct shear stress sensors in ECs is limited. After application of shear stress or force on PECAM-1, integrins are activated globally in ECs (Collins et al., 2012; Tzima et al., 2005; 2001). This global activation requires PI3 kinase, suggesting that shear-induced activation of integrins is mediated by a biochemical stimulus, such as $PIP_3$, rather force transfer through the cytoskeleton (Collins et al., 2012; Tzima et al., 2005). Additionally, the amount of force that integrins feel from shear stress is 1000-5000 times lower than traction forces on integrins (Katsumi et al., 2004). This further suggests that integrins are not activated by the direct force of shear stress. However, it has been hypothesized that mechanosensitive cells have evolved specialized mechanosensors capable of sensing extremely small forces unique to their niche (Orr et al., 2006b). Thus, it is possible that the integrins act as specialized endothelial mechanosensors. Integrins are mechanosensitive proteins, and as such, could be responding to minute shear stress forces or changes in cytoskeletal tension (Choquet et al., 1997; Guilluy et al., 2011; Matthews et al., 2006; N. Wang et al., 1993) (For further information about the interaction between hemodynamics, integrins, and the ECM, please see Yurdagul and Orr later in this issue).

Integrins serve as the integration site for shear-dependent signaling cascades (Liu et al., 2008; Tzima et al., 2002; 2001). After the application of shear on ECs, integrins are activated and form new connections with the underlying ECM. These new connections with the ECM are required for downstream activation of RhoGTPases , and NF-κB (Tzima et al., 2003; 2002; 2001). Integrins also influence the endothelial phenotype through integration of upstream signals with signals from the ECM. The adaptor protein Shc, which binds with the mechanosensory complex at the onset of shear,

binds integrins around 30 minutes after the onset of shear (Jalali et al., 2001; Liu et al., 2008). As a result of interaction with two separate mechanosensors, Shc integrates signals of junctional and subendothelial origin in both shear and non-shear contexts (Liu et al., 2008; Sweet et al., 2012; Sweet and Tzima, 2009) Also, focal adhesion growth resulting from force on PECAM-1 requires fibronectin; no growth is observed when cells are plated on collagen (Collins et al., 2014). Collagen binding also regulates cytoskeletal dynamics in ECs. When force was applied directly to PECAM-1 in ECs grown on collagen, protein kinase A (PKA) was activated, leading to repression of RhoA activity and reduction of cell stiffness, while the opposite was true of ECs grown on fibronectin (Collins et al., 2014). Passive rheology experiments performed on isolated mouse aortas confirmed these observations. EC stiffness was high in the aortic arch, where fibronectin density is high, while EC stiffness was lower in the descending aorta, where collagen makes up most of the ECM. Interestingly, *in vivo* inhibition of PKA reversed this effect, causing higher EC stiffness in the descending aorta as a result of removal of RhoA repression (Collins et al., 2014). Additionally, ECs plated on collagen align less effectively than cells plated on fibronectin (Hahn et al., 2011). Other shear-induced signaling pathways are also matrix specific. Shear-induced phosphorylation of p21-activated kinase (PAK) requires fibronectin connections, while shear-induced phosphorylation of p38 MAP kinase occurs only on collagen (Orr et al., 2007; 2005). Furthermore, disturbed flow regimes induce fibronectin expression and deposition, which sustains endothelial inflammation through NF-kB-dependent gene expression (Feaver et al., 2010). Though integrins likely do not directly sense shear stress, their function as integrators of shear stress signaling pathways demonstrates that they are an integral part of the EC shear sensing milieu.

## Conclusions

### *What is a mechanosensor? Do they work alone?*

A mechanosensor is the primary cellular structure or protein that senses a physical force, setting off downstream biochemical signaling pathways. ECs may have one, but their number is likely many. PECAM-1, the best studied of the putative EC mechanosensors, is capable of initiating global biochemical/signaling cascades in the cell when force is applied on it. A mechanotransducer is a cell

structure or protein that is required for conductance of signals generated by force, but not for sensing the originating force itself. In ECs under shear stress, integrins likely fall into this category as their activation seems to originate from biochemical, and not force-based, signals in ECs. The status of the other putative mechanosensors mentioned in this article remains unclear, however. Further experiments must be done to eliminate the caveats inherent in the knowledge of each mechanosensor. Only then can we definitely assign force-sensing properties to putative EC mechanosensors, and begin untangling the signaling relationships they share.

An unanswered question is the extent to which the putative mechanosensors work together to sense flow and maintain EC mechanosensitivity. There may be only one ultimate mechanosensor in ECs, but this seems unlikely given the known cooperation between sensors. Perhaps the best example of this is the cooperation between PECAM-1 and integrins, but evidence is starting to accumulate that other mechanosensors cooperate as well. For example, it has been shown that ligand-independent $S1P_1$ activity is required for maintenance of the glycocalyx, a possible example of mechanosensor cooperation as well as a possible mechanism for specific localization of the glycocalyx in areas of laminar flow (Zeng et al., 2014). Additionally, the heterotrimeric G-protein Gαq/11 complexes with PECAM-1, suggesting that more than one mechanosensor may be working together to sense flow (Paz et al., 2014). Interestingly, the Gαq/11 – PECAM-1 interaction is mediated by heparin sulfate, a component of the glycocalyx, further suggesting mechanosensor cooperation. Lastly, one of the roles of primary cilia is thought to be amplification of signals through the cytoskeleton. This could be an instance where cilia act alongside other major mechanosensors to help sense the smaller forces from low flow and influence EC phenotype. Related questions center around the relationship between shear stress and cyclic stretch. Though some *in vitro* studies (Zheng et al., 2012) suggest that stretch and shear signaling are interrelated processes, little is known about any overlap in these two signaling cascades. In order to understand the full scope of vascular mechanosignaling, knowledge of the mechanisms of cyclic stretch signaling and shear signaling must be incorporated.

### *Developmental questions and endothelial heterogeneity*

Mice with deleted PECAM-1, Syndecan-1, and Syndecan-4 are healthy and fertile, although PECAM-1 knockouts suffer from dilated cardiomyopathy and systolic dysfunction (McCormick et al., 2014). In knockout mice for other mechanosensory genes, like Pkd1 or Pkd2, defects in development haven't been disentangled from any possible defects in shear stress signaling (Alexander et al., 2000; Baeyens et al., 2014; Garcia-Gonzalez et al., 2010). A major issue for the shear stress field moving forward is understanding the importance of shear sensing *in utero*, and the signaling pathways involved. Similarly, another challenge for the field is understanding the phenotypes of ECs from different vascular beds and how those phenotypes influence shear signaling. Already, we have evidence that a shear sensing set point may be regulated by VEGFR3 expression in lymphatic ECs (Baeyens et al., 2015). This suggests the tantalizing possibility that specialized vascular beds, like lymphatic, brain, or renal ECs may be specialized in their function and methods of shear sensing. Perhaps, for different vascular beds, one mechanosensor does not fit all.

### *Clinical Perspectives*

Shear stress, and therefore endothelial mechanosignaling, are central to many vascular pathologies. Atherogenesis, for example, preferentially occurs in areas of the vasculature that are inflamed due to experiencing disturbed flow (Chatzizisis et al., 2007). Furthermore, shear stress may influence the rupture of unstable plaques. The fibrous cap of a plaque is exposed to the highest strain, and shear stress, at its upstream edge (Gijsen et al., 2008). Previous work has shown that macrophage invasion is higher in the upstream edge of plaques (Cicha et al., 2011; Dirksen et al., 1998), and that resident macrophages in the upstream edge increase expression of proteolytic enzymes (Cicha et al., 2011), thereby weakening the plaque. Additionally, the fibrous cap on the upstream edge of plaques tends to be more ulcerated and thinner, and when combined with high shear stresses may lead to destabilization and rupture of the plaque (de Weert et al., 2009; Gijsen et al., 2008). Another common vascular pathology, intimal stiffening, occurs with age. Intimal stiffening is associated with increased RhoA activity and vascular permeability, leading to increased leukocyte transmigration (Huynh et al., 2011). Additionally, conditions that affect vasodilation, such as aging

and hypertension, modify the hemodynamic environment. In tests of femoral artery shear rates in old and young men, old men showed lower shear rates (Trinity et al., 2014), predisposing the older men to endothelial inflammation. Flow patterns are also altered in older men: anterograde flow is reduced, while retrograde flow is increased. These changes combine to increase the overall oscillatory nature of blood flow in the femoral artery, once again predisposing older men to increased endothelial inflammation (Young et al., 2010). While exercise transiently reduces age-related increases in oscillatory shear index (Padilla et al., 2011), much work remains before the full involvement of endothelial mechanosignaling in a range of vascular pathologies can be determined.

***Future directions***

How should new mechanosensors be discovered? How should putative mechanosensors be validated? There are many *in vitro* and *in vivo* models for determining function of cellular structures or proteins in shear sensing (for more in depth information on these models, please see Bowden, et al, later in this issue). Moving forward, every effort should be made to mix reductionist and integrative approaches as much as possible (Figure 1.6). For example, magnetic bead pulling experiments are an excellent way to assign force-sensing function to a protein. However, these experiments do little to illuminate how that protein may behave in the context of a monolayer or an adult animal. Likewise, simply studying mouse knockouts or *in vitro* models of shear makes assignment of direct force-sending function problematic. By mixing reductionist and integrative approaches, we can better understand known putative mechanosensors and more easily find new candidate mechanosensors. When the mechanosensory infrastructure of ECs is more fully understood, a more full knowledge of intracellular signaling pathways will follow. By studying these pathways using a mixture of *in vitro* and *in vivo* approaches, the aim is that one or more signaling pathways that regulate inflammatory, but not atheroprotective, signaling are identified. Discovery of such a pathway could allow rational design of treatments that block endothelial inflammation, and thus development of atherosclerosis and other shear-induced vascular pathologies. This could lead to novel therapeutics of enormous potential.

## STIFFNESS AND VASCULAR PHENOTYPES

### Introduction

Mechanical forces are potent biological stimuli, especially in the vasculature where forces act as critical determinants of vascular physiology and pathology (Hahn and Schwartz, 2009; Orr et al., 2006b). Vascular pruning and tone are regulated by hemodynamics, and pathologies like atherosclerosis are potentiated by inflammatory blood flow patterns (Chatzizisis et al., 2007; Davies, 1995; Hahn and Schwartz, 2009; Lucitti et al., 2007; Orr et al., 2006b). The endothelium acts as a major mechanosensor in the vasculature, receiving major mechanical input from shear stress, the force exerted on the endothelium by blood flow. ECs also experience mechanical input in the form of stiffness, which manifests in two ways: vascular stiffness and endothelial stiffness. Vascular stiffness increases as a result of age and other factors(Kohn et al., 2015a) and causes upregulation of RhoA signaling(Huynh et al., 2011). Conversely, endothelial stiffness requires RhoA signaling, and increases as a result of direct force applied to endothelial mechanosensors (Collins et al., 2014; 2012). Importantly, along with its involvement in stiffness-related signaling, the RhoA pathway is regulated by shear stress(Tzima et al., 2001) Though substrate stiffness, endothelial stiffness, and hemodynamics may be interrelated processes, the exact mechanisms underlying these processes remain little understood. This review will focus on the common mechanisms shared between shear stress, vascular stiffness, and endothelial stiffness, in an attempt to define what is known and what must be determined about the interrelation of these mechanical pathways in the vasculature.

### Shear stress is a critical determinant of endothelial phenotype

As a result of their luminal position, ECs are constantly subjected to fluid shear stress, the frictional force felt by ECs as a result of blood flow parallel to the vessel wall (Hahn and Schwartz, 2009). Depending largely on vessel geometry, blood flow patterns fall into two broad categories: laminar and disturbed flow. Laminar flow, also known as atheroprotective flow, is characterized by high, unidirectional flow, and occurs mostly in straight areas of the vasculature (Chatzizisis et al., 2007). Laminar flow induces a unique endothelial phenotype, where ECs form large stress fibers, align in the direction of flow, express anti-inflammatory genes such as KLF2, and exhibit low turnover

(Chatzizisis et al., 2007; Parmar et al., 2006). Areas of disturbed flow, also known as atheroprone flow, exhibit low and oscillatory flow patterns, and mainly occur in areas of the vasculature that are highly curved or bifurcated. ECs that experience disturbed flow do not align in the direction of flow, exhibit increased proliferation, and increase their expression of inflammatory adhesion molecules like ICAM-1 and VCAM-1 (Buchanan et al., 1999; Chatzizisis et al., 2007; Malek et al., 1999). Increased inflammation "primes" the endothelium for atherosclerotic plaque growth, and when combined with pathologic systemic factors, like smoking of high cholesterol, induce focal plaque growth in areas of disturbed flow.

In order to sense and respond to flow, ECs express numerous mechanosensory proteins, including a junctional mechanosensory complex comprised of PECAM-1, VE-Cadherin, and VEGFR2 (Tzima et al., 2005). PECAM-1 is required for many shear-induced endothelial processes. *In vivo* manipulation of shear stress by partial carotid artery ligation uncovered a requirement for PECAM-1 in vascular remodeling (Z. Chen and Tzima, 2009). In PECAM-1 knockout mice, ECs in partially ligated carotid arteries are unable to activate NF-kB, blocking ICAM-1 and VCAM-1 expression and consequent recruitment of leukocytes. Similar results are observed after femoral artery ligation, where collateral vessel remodeling is reduced in PECAM-1 knockout mice as a result of blunted endothelial inflammation (Z. Chen et al., 2010). *In vitro* studies confirm that PECAM-1 is required for endothelial inflammatory signaling, as well as ERK and Akt activation (Osawa et al., 2002; Tzima et al., 2005). In addition to its role in regulation of shear-induced endothelial inflammation and kinase activation, PECAM-1 is an important regulator of the actin cytoskeleton in response to shear. Studies have shown that PECAM-1 is required for shear-induced endothelial alignment, as well as force-induced integrin activation downstream of PI3K (Tzima et al., 2005). Furthermore, PECAM-1 is required for Rac1 activation, which leads to the establishment of polarity after the induction of shear stress (Liu et al., 2013).

**Rho GTPases regulate cytoskeletal dynamics and mechanics**

A major subset of endothelial responses to shear is based in regulation of the actin cytoskeleton. These include alignment in the direction of flow, stress fiber formation, focal adhesion

maturation, and increased endothelial contractility. The Rho family of GTPases, including RhoA, Rac1, and Cdc42, work together dynamically to regulate these processes (Nobes and Hall, 1995; Tzima, 2006; Wojciak-Stothard and Ridley, 2003). These proteins are differentially regulated in response to shear and other stimuli (Tzima et al., 2002; 2001; Wojciak-Stothard and Ridley, 2003; Yamaguchi et al., 2001). Additionally, RhoA and Rac1 actively repress one another, adding to the complex interplay between this family of GTPases (Nimnual et al., 2003; Rottner et al., 1999; Yamaguchi et al., 2001).

Alignment in the direction of flow is a hallmark of endothelial shear stress responses(Tzima et al., 2005; 2001). Essentially a process of polarity determination followed by stress fiber formation, alignment relies on dynamic regulation of Rac1 and RhoA (Liu et al., 2013; Tzima et al., 2002; 2001). At the onset of flow, Rac1 is activated at the downstream edge of ECs, while RhoA is transiently downregulated (Wojciak-Stothard and Ridley, 2003). This allows for the simultaneous creation of a polarity complex and destruction of stress fibers. If downregulation of RhoA does not occur at this step, stress fibers are not abolished and ECs do not align in the direction of flow (Tzima et al., 2001). After polarity is established, Rac1 is downregulated simultaneous with an upregulation of RhoA activity, as stress fibers are formed (Tzima et al., 2002; 2001).

Endothelial contractility is derived from RhoA-regulated myosin contractility. Contractility has implications for barrier formation, immune cell transmigration, and migration of ECs (Dudek and Garcia, 2001; Lessey-Morillon et al., 2014; Shiu et al., 2004). The main source of contractility in ECs is activation of myosin light chain (MLC) through phosphorylation (Dudek and Garcia, 2001). The phosphorylation state of MLC is a delicate balance, and is maintained through oppositional MLC kinase (MLCK) and MLC phosphatase (PP1) activity (Kimura et al., 1996; Tinsley et al., 2000). While MLCK is regulated through cytokine-dependent $Ca^{2+}$/Calmodulin mechanisms (Dudek and Garcia, 2001), PP1 activity is regulated by RhoA-Rho associated kinase (ROCK) signaling pathways (Amano et al., 1997; Essler et al., 1998; Kimura et al., 1996). When ECs are activated with thrombin, cell contractility increases dramatically (Essler et al., 1998). This effect is negated by inhibition of RhoA or cellular injection of PP1, indicating that RhoA-dependent inhibition of PP1 is necessary for an increase in cellular contraction. Regulation of cellular tension is also critical for focal adhesion

maturation. While Rac1 and Cdc42 are required for the initial formation of focal complexes, RhoA, through ROCK-MLC-dependent contractility, causes focal adhesion maturation through an increase in tensional forces on the focal complexes (Rottner et al., 1999; Wolfenson et al., 2013).

Integrins also play a central role in many cellular mechanical processes. Critically, shear-dependent RhoA and Rac1 activation require new integrin ligations (Tzima et al., 2002; 2001). When new integrin ligation is blocked, ECs cannot polarize and do not align in the direction of flow. Traction forces are also generated by integrin-based focal adhesions (S. Li et al., 2005). These centripetal forces are required for cell motility, and increase in response to shear stress (Shiu et al., 2004). Traction forces are also generated by cell adhesion. Over the course of endothelial adhesion, traction forces are the highest early in the spreading process, before being confined mostly to the edges of cells later in adhesion (Reinhart-King et al., 2005). Traction forces also increase with ligand density during the spreading process, further implicating integrins in force generation and substrate sensing.

**Vessel stiffness regulates endothelial contractility**

Vessel stiffness is strongly correlated with age (Benetos et al., 2002; Huveneers et al., 2015; Kohn et al., 2015a; Lakatta, 2003). Vessels derive their mechanical characteristics from elastin and collagen (CL), the two main matrix proteins in vessel walls (Burton, 1954). The ratio of elastin to CL in the vessel wall is regulated by balancing matrix protein and matrix metalloproteinase expression (Kohn et al., 2015a). Elastin is more distensible than CL, while CL confers the majority of stiffness to blood vessel walls (Burton, 1954). Young vessels exhibit a higher elastin to CL ratio, and as such are more compliant than older vessels, which express a lower elastin to CL ratio (Burton, 1954; Schlatmann and Becker, 1977). As vessels age, the elastin to CL ratio is changed through the fragmentation of elastin fibers and gradual accumulation of CL. During this process, CL both replaces smooth muscle cells (SMCs) and contributes to intima-media thickening (Lakatta, 2003; Schlatmann and Becker, 1977). Thus, when an organism ages, its vessels progressively stiffen over time due to natural processes. Increased vessel stiffness also strongly correlates with adverse cardiovascular events, like atherosclerosis, hypertension, and heart failure (Benetos et al., 2002; Kohn et al., 2015a). Understanding vessel stiffness is necessary for designing therapeutics for vascular diseases. In

particular, understanding the mechanisms by which vessel stiffness regulates endothelial phenotype is critical for integrating the fields of shear stress and extracellular matrix biology, which comprise the two major mechanical inputs that regulate endothelial phenotype.

When taken together, studies into the effects of substrate stiffness on endothelial phenotype begin to tell a cohesive story of increased endothelial contractility and sensitization to stimuli. Early work from Peng et al demonstrated the effects of differing substrate stiffness on shear-induced eNOS phosphorylation(Peng et al., 2003). ECs were coated on silastic tubing of differing stiffness, followed by pulse perfusion with culture media. eNOS phosphorylation increased 2.5-fold in ECs from the softer tubes, while eNOS phosphorylation was blocked in ECs cultured on stiff tubes. These results suggest that stiffness may counteract the effects of laminar flow on ECs, conditions normally conducive for eNOS activation. Additional studies suggest that increasing substratum stiffness sensitizes ECs to cytokine treatment. After treatment with LPS, ICAM-1 and VCAM-1 expression was higher in ECs plated on a 40 kPa substratum than in ECs plated on a 1.5 kPa substratum, suggesting an intersection between increased stiffness and inflammatory signaling in ECs (Mambetsariev et al., 2014). Also, when ECs are plated on 11 kPa and 1.2 kPa substrata, the difference in stiffness leads to a difference in cellular responses to thrombin treatment. The ECs plated on stiffer substratum exhibit higher permeability and ERM phosphorylation after thrombin treatment, both suggestive of increased RhoA activity as a result of stiffer substrate (Krishnan et al., 2011). In addition, ECs grown on stiff substrates and treated with LPS show a significant increase in GEF-H1 expression when compared to cells grown on soft substrates (Mambetsariev et al., 2014). Since GEF-H1 is an activator of RhoA, this suggests that ECs grown on stiff substrates possess an intrinsically greater capacity for RhoA activation than ECs grown on soft substrates.

Increased RhoA-dependent contractility is a hallmark of ECs growing on stiffer substrates. ECs cultured on stiffer substrates exhibit increased junctional width and permeability to FITC-dextran(Huynh et al., 2011). Interestingly, permeability and junction width were normalized by inhibition of ROCK, implicating RhoA signaling (Huynh et al., 2011). Further *in vitro* experiments show that substrate stiffness influences endothelial responses to shear stress. Compared to soft substrates, stiffer substrates induce higher shear-dependent RhoA activation (Kohn et al., 2015b).

ERK and eNOS signaling are also reduced on stiffer substrates, suggesting that stiffness may induce endothelial dysfunction through mechanisms outside of RhoA activation. *In vivo*, atomic force microscopy (AFM) measurements showed that the aortas of aged mice were found to be stiffer than aortas from young mice. This age-dependent increase in stiffness lead to an increase in permeability to Evans blue dye and junction width in the aortic endothelium of older mice, both of which were normalized by ROCK inhibition (Huynh et al., 2011). Several other studies also demonstrate barrier integrity decreases induced by stiffness. Kohn et al showed that while shear stress normalizes barrier integrity on stiff substrates, junction width remains increased(Kohn et al., 2015b). Also, ECs plated on stiff substrates exert increased baseline traction forces, which can be normalized by ROCK inhibition (Huynh et al., 2011; Krishnan et al., 2011). This further supports the idea that RhoA-dependent contractility increases with substrate stiffness.

Arterial stiffness positively correlates with cardiovascular risk (Mitchell et al., 2010). While the mechanistic details of stiffness-induced pathologies are little understood, the relation of stiffness to the progression of atherosclerosis is becoming clearer. Interestingly, blood vessels in $ApoE^{-/-}$ mice are stiffer as a result of increased CL and fibronectin (FN) expression and deposition (Kothapalli et al., 2012). The observed increase in VSMC expression of FN and CL is reduced by treatment with either HDL or ApoE3, two factors that are known to be anti-atherogenic. ApoE3 treatment also reduces RhoA activation and cellular stiffness in VSMCs (Hsu et al., 2015). Importantly, when $ApoE^{-/-}$ mice are treated with BAPN, an inhibitor of CL crosslinking, aortic stiffness is reduced. Additionally, chronic treatment with BAPN reduces atherosclerotic burden in $ApoE^{-/-}$ aortas, providing mechanistic evidence that vessel stiffness plays an important role in the development of atherosclerosis. Increased substrate stiffness also increases immune cell accumulation *in vivo* and *in vitro* (Huynh et al., 2011; Kothapalli et al., 2012). Furthermore, shear stress is also known to regulate ECM expression by ECs. Oscillatory flow patterns have been shown to induce FN expression in ECs in a PECAM-1 dependent manner. The increase in FN expression leads to sustenance of endothelial inflammation, stimulating atherosclerotic development (Feaver et al., 2010; Gelfand et al., 2011). What is not known, but is tantalizing to speculate about, is whether increased FN deposition in areas of the vasculature that experience oscillatory flow translates into a focal increase in stiffness. Such a

focal increase in stiffness would provide a second atherogenic input into local plaque formation, but it is unknown whether this is the case. The mechanisms by which arterial stiffness regulates the development of atherosclerosis are just beginning to be understood. When combined with our knowledge of endothelial shear stress signaling, a greater understanding of stiffness signaling mechanisms will help create a fully integrated picture of endothelial mechanosensing. Additionally, such a full understanding will help open new avenues for vascular therapeutics.

**Direct force on ECs induces cellular stiffening**

While vessel stiffness derives from increased amounts of ECM proteins deposited into the vessel wall in response to age, hemodynamics, and other stimuli, endothelial stiffness derives from contractility of the actin cytoskeleton in response to force. The stiffness in ECs is RhoA-dependent, and selectively occurs based on the type of ECM the ECs are growing on. At this early stage, the relationship between endothelial stiffness and shear stress is unclear, but recent data suggest that atheroprotective flow may lead to more compliant ECs, while atheroprone flow may induce a stiffer phenotype.

Recently, the effects of force applied directly on the endothelial mechanosensor PECAM-1 have been determined(Collins et al., 2014; 2012; Tzima et al., 2005). Force is directly applied to ECs using either permanent magnets or a magnetic tweezers system (Tim O'Brien et al., 2008). Combined use of these systems allows sustained and pulsatile force application, respectively. By pulling specifically on PECAM-1 using magnetic beads, Collins et al determined that pulsatile force on PECAM-1 induced an increase in cellular stiffness on the order of minutes (Collins et al., 2012). The observed increase in stiffness occurred downstream of integrin activation, where RhoA was activated by GEF-H1 and LARG. This activation of RhoA resulted in both adaptive stiffening and focal adhesion formation. Importantly, the observed integrin ligation, RhoA activation, and downstream cytoskeletal stiffening all occurred on a global scale in force-stimulated ECs. Global activation of these pathways occurred even though force on PECAM-1 is delivered to only one small area of each stimulated cell. This, along with the timescale of the stiffening response, suggests a biochemical method of signal

transduction, as opposed to direct mechanotransduction through cytoskeletal elements (Ingber, 1993; Na et al., 2008).

In another study from our group, Collins et al determined that endothelial stiffening is not a universal phenomenon; ECs selectively stiffened based on the composition of the underlying ECM (Collins et al., 2014). When force was applied directly to ECs growing on FN, cellular stiffening and focal adhesion growth occurred. However, if ECs were grown on CL, force-dependent stiffening and focal adhesion growth did not occur, suggesting differential activation of RhoA. Indeed, reduced force-dependent activation of RhoA occurred on ECs cultured on CL. This was a result of inhibitory phosphorylation of RhoA by PKA (Collins et al., 2014). Notably, when ECs grown on CL were treated with a PKA inhibitor, they phenocopied ECs grown on FN by increasing stiffness, stress fiber formation, and focal adhesion formation in response to force. Since hemodynamics are known to regulate ECM compotision (Feaver et al., 2010), *ex vivo* passive microbead rheology (Hoffman et al., 2006) was used to determine whether local differences in ECM composition affected the the stiffness of aortic ECs. The stiffness of ECs in the aortic arch, an area that experiences disturbed flow, and the descending aorta, which experiences laminar flow, was assayed. Interestingly, ECs in the aortic arch were stiffer than ECs in the descending aorta. These results correlated with the known high levels of FN in the aortic arch and high levels of CL in the descending aorta, respectively, and suggest that hemodynamics may play a direct and indirect role in endothelial stiffening. Notably, when the mice were treated with a PKA inhibitor, aortic ECs in the descending aorta once again phenocopied ECs in the aortic arch, stiffening in spite of the high proportion of CL in the ECM.

The physiological significance of endothelial stiffening remains unknown. It has been shown that neutrophil adherence on TNFα treated ECs increases endothelial stiffness and F-actin content (Q. Wang et al., 2001; Q. Wang and Doerschuk, 2000). Additionally, using magnetic tweezers, force applied directly to ICAM-1 induces a rapid stiffening of ECs. Notably, endothelial stiffening downstream of force on ICAM-1 is much more rapid than stiffening downstream of PECAM-1. Also, stiffening downstream of ICAM-1 is not ECM-selective, suggesting that integrin ligation is not required for this process (Lessey-Morillon et al., 2014).

## Conclusion

The interplay between hemodynamics, vessel stiffness, and endothelial stiffness is a new and exciting avenue of study. While early work shows promise, many aspects of the relationship between hemodynamics and stiffness remain unclear. For example, endothelial contractility and stiffening are both RhoA dependent processes. However, whether these two processes are separate, or are two measures of the same phenotype, is unknown. Additionally, it is tantalizing to speculate about a link between atherogenic flow patterns and focal vessel stiffness. Does increased FN deposition in areas of the vasculature that experience oscillatory flow translate into a focal increase in arterial stiffness? Along with oscillatory flow, such a focal increase would provide a second atherogenic input into local plaque formation, but it is unknown whether this is the case. The mechanisms by which arterial stiffness regulates the development of atherosclerosis are just beginning to be understood.

Additionally, the physiological significance of endothelial stiffness remains unknown. Taken together, the studies of neutrophil-induced stiffening suggest that ECs stiffen in response to immune cell binding, possibly to aid transmigration. Additionally, the studies conducted by applying force on PECAM-1 suggest the possibility that hemodynamics may also influence endothelial stiffness *in vivo*. Importantly, this focal stiffness could be neutrophil-independent, yet still aid transmigration, therefore enhancing atherosclerotic plaque formation. Perhaps this focal stiffness could aid the cessation of leukocyte rolling along the endothelium, which must occur before transmigration. However, further study is required to delineate the signaling relationships that underlie endothelial stiffening, as well as the full physiological significance of endothelial stiffness.

When combined with our knowledge of endothelial shear stress signaling, a greater understanding of stiffness signaling mechanisms will help create a more fully integrated picture of endothelial mechanosensing. Such a picture may present novel mechanisms underlying the progression of atherosclerosis. An integrative knowledge of new mechanisms and how they work together will open up new avenues for vascular therapeutics.

## BIOLOGY OF THE ADAPTOR PROTEIN SHC

The Src homology 2 containing transferring protein (Shc) proteins are a family of adaptor proteins that are specialized for mediating receptor tyrosine kinase (RTK) and other phosphotyrosine signaling. Perhaps the best known member of the family, ShcA (hereafter referred to as Shc), consists of three ubiquitously expressed isoforms: p46, p52, and p66(Ravichandran, 2001; Wills and Jones, 2012). These three isoforms are all structurally similar, sharing a common layout of three domains: an amino-terminal phosphotyrosine binding (PTB) domain, a carboxy-terminal Src homology 2 (SH2) domain, and a Collagen homology 1 (CH1) domain which connects the two(Wills and Jones, 2012)(Figure 1.7). Additionally, the three isoforms' difference in size owes to an amino-terminal Collagen homology 2 (CH2) domain, which is longest in p66Shc and absent in p46Shc. Shc is a multifunctional protein whose variety of functions arises from combinatorial functions of each individual domain, resulting in profound consequences for cell signaling and development(Hardy et al., 2007). The PTB domain is known to mediate epidermal growth factor receptor (EGFR) signaling through binding to autophosphorylation sites on the intracellular domain of EGFR(Sakaguchi et al., 1998). Furthermore, PTB domain interaction with phospholipids and phosphatidylinositol 3-kinase (PI3K) is required for Shc signaling in response to insulin, but not epidermal growth factor (EGF) or platelet derived growth factor (PDGF)(Ugi et al., 2002). The SH2 domain also mediates signaling pathways, interacting with T-cell receptor (TCR) in response to TCR and CD4 stimulation(Ravichandran et al., 1993). The SH2 domain additionally interacts with PDGF receptor (PDGFR) at intracellular autophosphorylation sites in response to PDGF treatment(Yokote et al., 1994). In mice, the SH2 domain is required for nerve and skeletal muscle development(Hardy et al., 2007). Expressing Shc with a nonfunctional SH2 domain causes disruption of nerve growth; when expressing the mutant SH2 protein in skeletal muscle, the mice develop but have poor motor function resulting from reduced spindle and intrafusal fiber generation(Hardy et al., 2007).

The CH1 domain of Shc contains three tyrosines that are subject to phosphorylation by RTKs and Src family kinases: Tyr239/240 and Tyr317. These tyrosines are phosphorylated in response to cellular stimulation by EGF, PDGF, and insulin(Ravichandran et al., 1993; Sakaguchi et al., 1998; Ugi et al., 2002; Yokote et al., 1994). In addition to regulating the conformation of Shc, phosphorylation of

these tyrosines mediates interaction with downstream signaling partners, notably the adaptor protein Grb2(R. George et al., 2008; Suenaga et al., 2004; Wills and Jones, 2012). Association with Grb2 is a well-understood pathway, in which Tyr230/240 and Try317 of the CH1 domain are phosphorylated, which leads to recruitment of Grb2. The association of Shc with Grb2 increases the affinity of Grb2 for SOS, a Ras-activating GTPase that activates Ras after association with Grb2(Ravichandran et al., 1995; Salcini et al., 1994; Walk et al., 1998).

Shc is also a major player in cardiovascular biology. The ShcA knockout mouse is embryonic lethal due to massive cardiovascular defects(K. M. Lai and Pawson, 2000). These include cardiac defects, such as poor trabeculation and cardiac cushion formation, and angiogenic defects that lead to a less complex, dilated vascular phenotype(K. M. Lai and Pawson, 2000). Interestingly, fibroblasts from these mice are hypercontractile and do not spread in culture, suggesting inappropriate activation of RhoA. The PTB domain also plays a specific role in cardiac development, as impaired trabeculation occurs in embryonic mice expressing a nonfunctional PTB domain(Hardy et al., 2007). Shc also regulates endothelial responses to shear stress. After application of shear, Shc associates with VE-Cadherin and VEGFR2, where it is phosphorylated. This phosphorylation is dependent on Src and VEGFR2 tyrosine kinase activity(Liu et al., 2008). Additionally, Shc is required for shear-dependent NF-κB nuclear localization, attendant inflammatory gene expression, and ERK activation(Liu et al., 2008). A similar phenotype was seen in an endothelial-specific Shc knockout mouse, when collateral arteries show impaired remodeling after femoral artery ligation(Sweet et al., 2013). The lack of remodeling is due to impaired endothelial inflammatory signaling, Notch activation, and arterial specification(Sweet et al., 2013).These findings establish Shc as a critical player in endothelial mechanosignaling.

## BIOLOGY OF FIBRONECTIN, A CRITICAL EXTRACELLULAR MATRIX PROTEIN

Fibronectin (FN) is a major constituent of the ECM, where it is actively assembled into fibrils that abet cellular adhesion in a variety of tissues(Schwarzbauer and DeSimone, 2011). In pre-chordate species, some homologs of FN domains have been found, but no protein homologous to FN itself exists(Hynes, 2012). Thus, it is thought that FN arose along with the blood vasculature, a hypothesis

supported by the critical importance of FN in vascular development and function(Astrof and Hynes, 2009; Hynes and Zhao, 2000). Developmental studies of genetically ablated FN in mice show a wide variety of vascular defects, including thickened cardiac walls, lack of cardiac jelly, and poor red blood cell development(E. L. George et al., 1993). Furthermore, while FN null embryos display normal specification of primitive heart cells, heart morphology in FN null embryos is abnormal, suggesting a role for FN in mechanical processes, even early in development(E. L. George et al., 1997). Additionally, when FN was ablated in cardiac progenitor cells, the resulting embryos displayed malformed aortas and septal defects between the ventricles of the heart(D. Chen et al., 2015)

FN achieves a broad diversity of function through the use of many functional domains. FN is composed of three types of domains: FNI, FNII, and FNIII domains (Figure 1.8). FNI and FNII domains are highly ordered, containing disulfide bonds to give the protein three-dimensional structure(Schwarzbauer and DeSimone, 2011). Conversely, FNIII domains consist of 7-stranded β-barrels and no disulfide bonds, allowing the conformational changes necessary to achieve fibrillogenesis(Schwarzbauer and DeSimone, 2011). The domains of FN associate with one another in various combinations, producing higher order structures like detergent-insoluble fibrils. One major assembly domain is a 70 kDa stretch of FN, including $FNI_{1-5}$, which mediates FN-FN binding(Schwarzbauer and DeSimone, 2011). Other domains, such as $FNIII_{1-2}$, are also required for FN assembly, though denaturation or other structural breakdown is required to uncover this cryptic site and increase binding to $I_{1-5}$(Hocking et al., 1994; Sechler et al., 2001). The Arg-Gly-Asp (RGD) amino acid sequence, which is present in $III_{10}$, is required for integrin α5β1binding to FN, and without this domain, efficient fibrillogenesis does not occur(Sechler et al., 1997; Singh et al., 2010). Mutation of the RGD sequence to a nonfunctional Arg-Gly-Glu (RGE) sequence induces a shortened anterior-posterior axis in embros, as well as increased apoptosis at E9.5(Takahashi et al., 2007). In agreement with these data, genetic ablation of α5 integrin produces cardiovascular defects, such as abnormal vessel formation and primitive blood cell leakage at E9.5(J. T. Yang et al., 1993). The HepII domain ($FNIII_{12-14}$) also participates in adhesion by acting as a co-receptor for Syndecans, where Syndecan-2 is notably required for FN assembly in transformed fibroblasts(Galante and Schwarzbauer, 2007; Singh et al., 2010).

Alternative splicing is also an important facet of FN function. Alternative spliceforms of FN may contain Extra Domain A (EDA), Extra Domain B (EDB), or a part of the Variable (V) region, which can be 0, 64, 89, 95, or 120 amino acids long in humans (V0-V120, respectively)(Schwarzbauer and DeSimone, 2011). The diversity of spliceforms imparts further variety of function on FN. The EDA and EDB regions, for example, are absent in plasma FN but present in cellular FN, and are dispensable for development when individually genetically ablated(Schwarzbauer and DeSimone, 2011). Additionally, the EDA and EDB regions are highly expressed during blood vessel development, and downregulated postnatally(Ffrench-Constant and Hynes, 1989; Ffrench-Constant et al., 1989). These domains are then upregulated during wound healing, suggesting they might function in cell migration(Ffrench-Constant et al., 1989). Further studies show that EDA-null mice exhibit blunted wound healing and reduced lifespan(Muro et al., 2003). Additionally, EDA-null mice show reduced atherosclerotic burden and lower cholesterol in VLDL particles(Tan et al., 2004). EDA is dispensable for ECM formation(Muro et al., 2003), so taken together, these results suggest a signaling, rather than structural, role for the EDA domain of FN. Interestingly, when both the EDA and EDB domains are removed from FN, the result is embryonic lethal by E10.5(Astrof et al., 2007). Embryonic death comes as a result of massive cardiovascular defects, including posterior and cranial hemorrhage, defective embryonic angiogenesis, and poor heart cushion formation(Astrof et al., 2007). Again, these mutations did not affect the deposition of FN matrix, suggesting a signaling role for EDA and EDB during development. The V region is also intimately involved with cardiovascular biology, as FN dimers missing the V region in both subunits are not secreted, and are degraded in the ER(Schwarzbauer and DeSimone, 2011). V0-V+ FN dimers, however, are efficiently secreted, and previous work suggests that these dimers are easily incorporated into fibrin clots in the bloodstream(Wilson and Schwarzbauer, 1992). FN and its alternative spliceforms are critical to vascular biology, participating in a wide range of functions during development, physiology, and disease. Though many mechanisms of FN function are understood, more work is needed to determine the precise functions of FN spliceforms, as well as the exact function of its complex domain structure.

**Figure 1.1 Shear stress levels are variable throughout the vasculature**

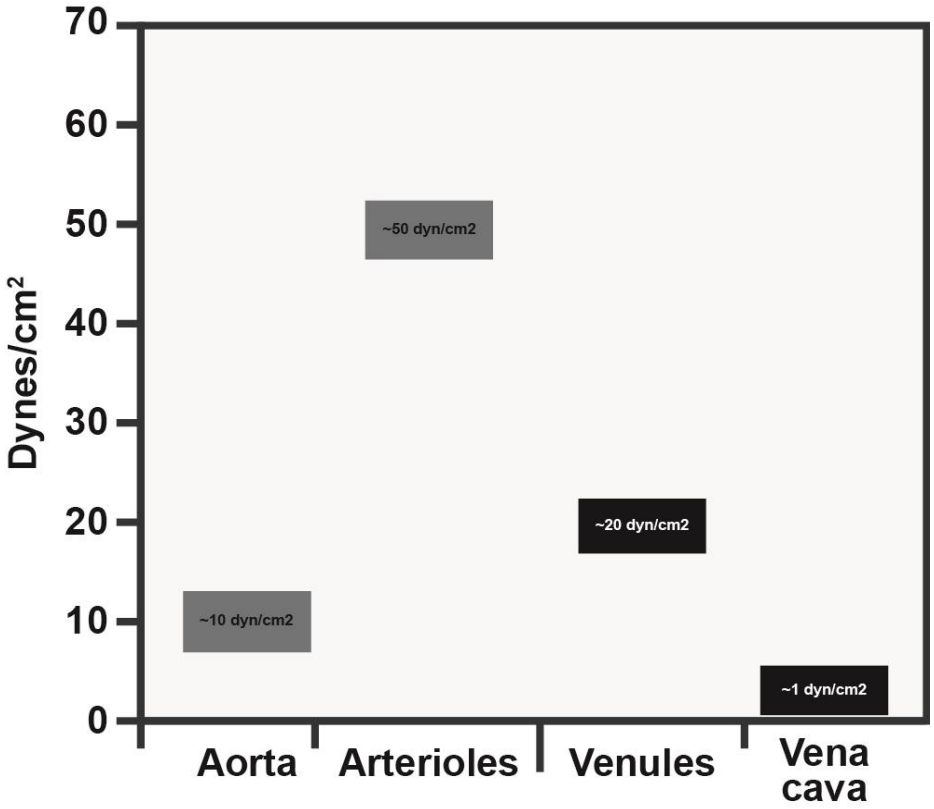
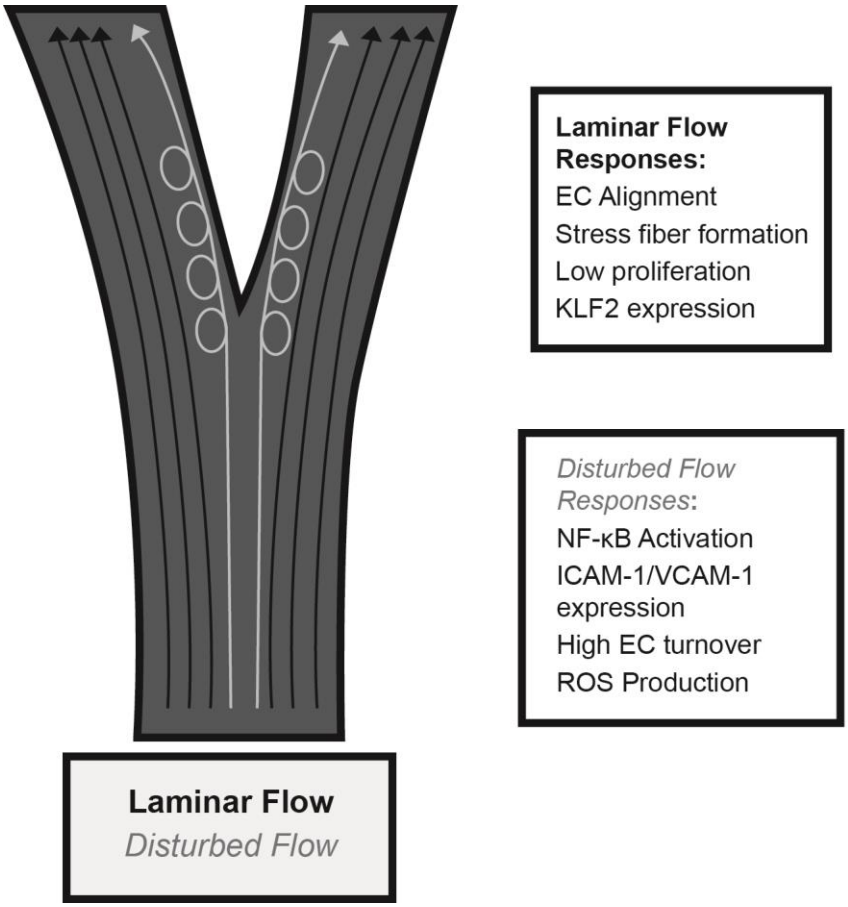


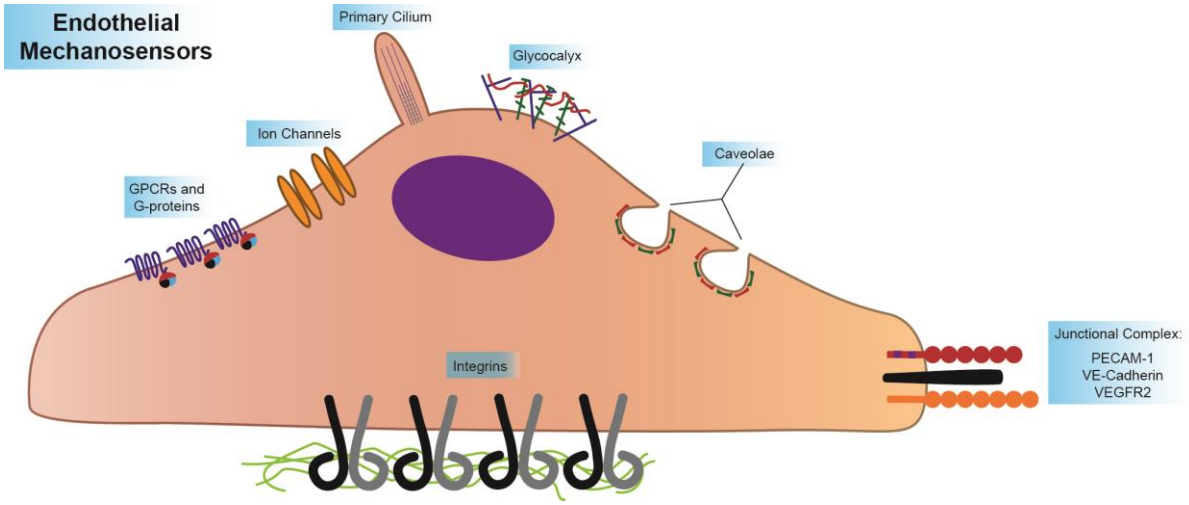

Illustration demonstrating relative levels of shear stress throughout the vasculature. Arterial shear stress levels are higher than venous levels, and larger vessels have lower shear than smaller vessels.

**Figure 1.2 Two main types of flow exist in the vasculature: laminar and disturbed flow**



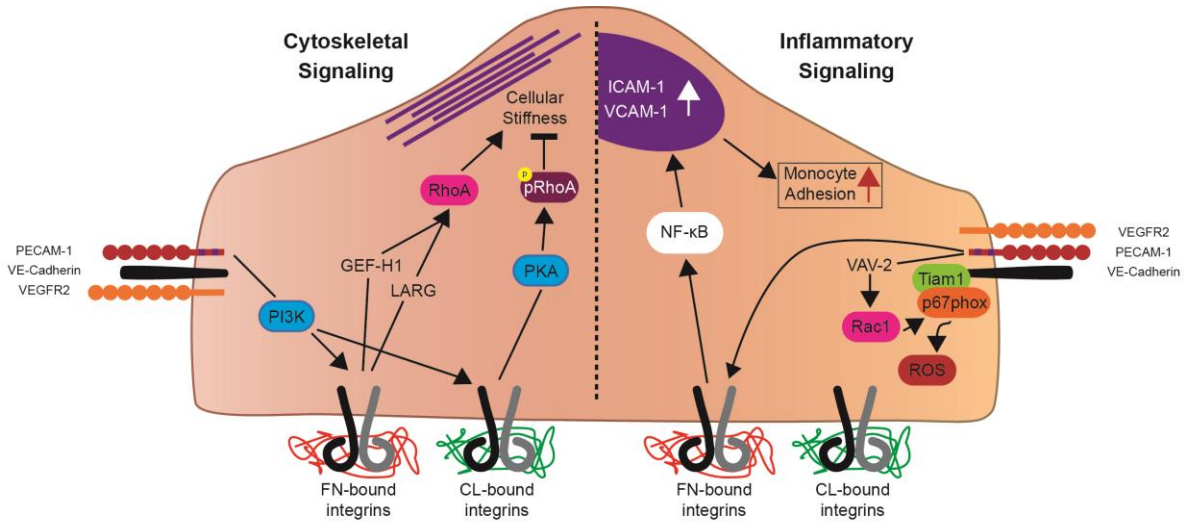

Laminar flow occurs where vessel gemoetry is straight and uniform, whereas disturbed flow occurs where vessels bifurcate or curve highly. The different types of flow elicit different endothelial responses. Responses to laminar flow, termed “atheroprotective” include EC alignment in the direction of flow, stress fiber formation, and KLF2 expression, which leads to anti-inflammatory gene expression. Disturbed flow responses, termed “atheroprone” are more inflammatory and include NF-κB activation and associated transcription. Additionally, ECs in areas of disturbed flow are more proliferative and produce more ROS than ECs in areas of laminar flow.

**Figure 1.3 ECs express many mechanosensors**



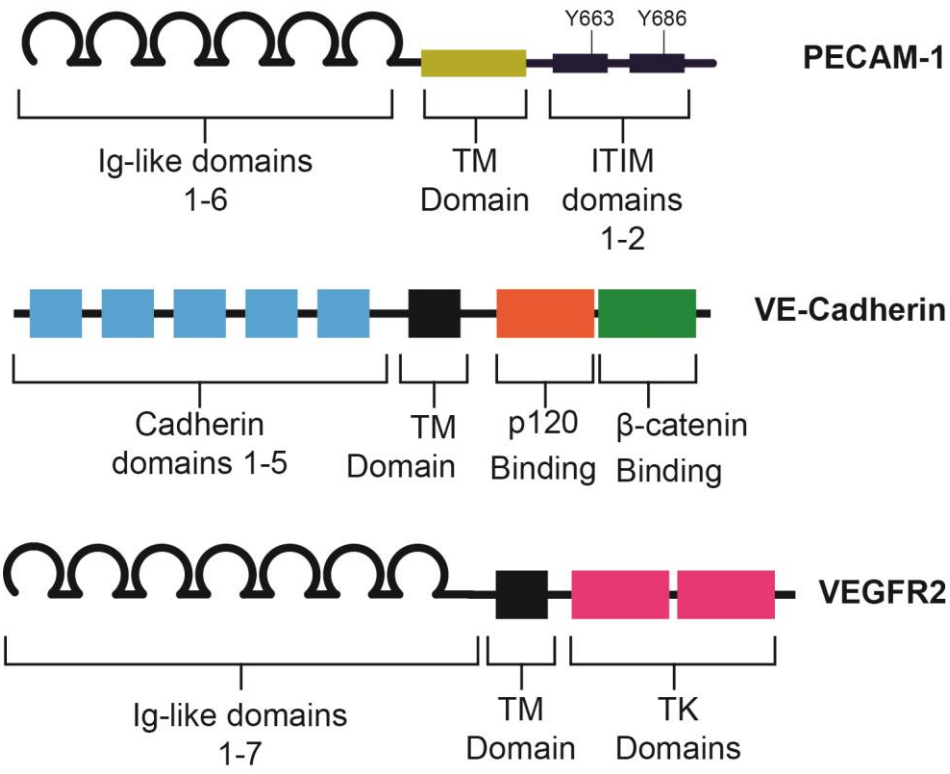

These sensors can be divided into luminal, junctional, and basal mechanosensors. Luminal mechanosensors include G-protein coupled receptors (GPCRs, including the sphingosine 1-phosphate receptor 1, $S1P_1$, and the bradykinin B2 receptor) and heterotrimeric G-proteins (namely, Gαq/11), ion channels (including TRPV4, TRPP2, TRPC1, Piezo1 and Piezo2), microtubule-based primary cilia (which associated with the ion channels PKD1 and PKD1), the glycocalyx (where Syndecan-1 and -4, as well as heparin sulfate glycosaminoglycans are involved in shear signaling), and protein-coated membrane pits called caveolae (given structure by Caveolin 1-3 and Cavin 1-3). The known junctional mechanosensors, PECAM-1, VE-Cadherin, and VEGFR2, form a mechanosensory complex that elicits many signaling pathways as a response to shear. The basal mechanosensors consist of the integrins, which sense ECM type and substrate stiffness, all while integrating signaling pathways originating from other mechanosensors.

**Figure 1.4 The junctional mechanosensory complex regulates cytoskeletal stiffening and inflammatory signaling in response to force**



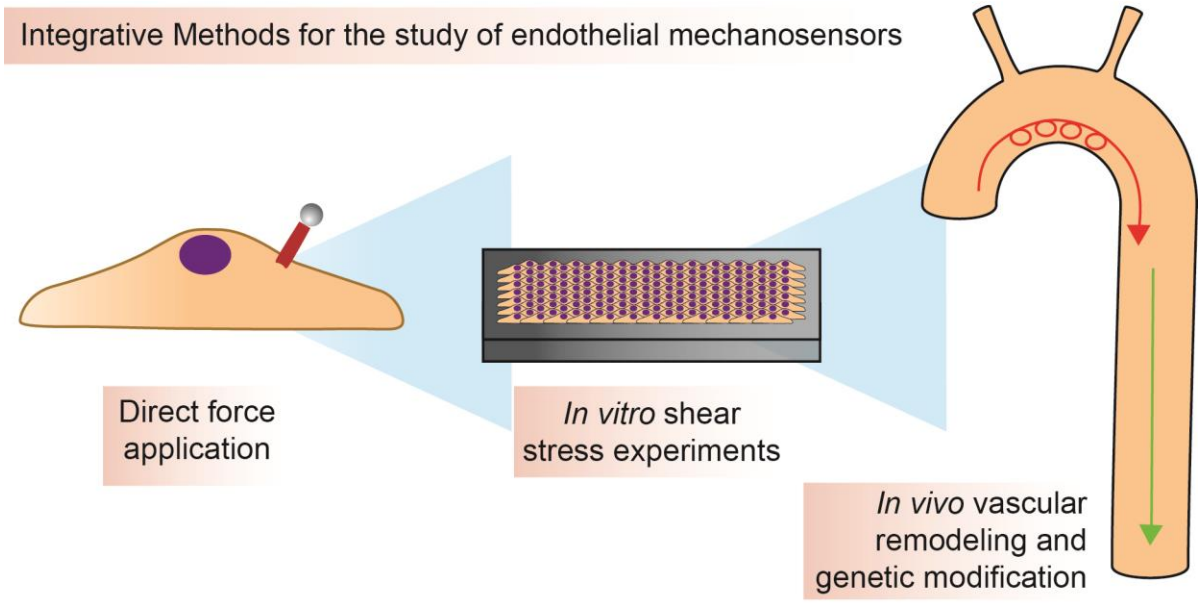

Force on PECAM-1 activates phosphoinositide 3-kinase (PI3K), which is required for downstream activation of integrins. ECM-dependent integrin activation occurs, with fibronectin (FN) bound integrins positively regulating cellular stiffness through GEF-H1/LARG-RhoA signaling. Collagen (CL) bound integrins have the opposite affect; cellular stiffness is negatively regulated downstream of CL-bound integrins through a protein kinase A (PKA)-phospho-RhoA pathway. Additionally, Rac1 is activated downstream of force on PECAM-1, which leads to increased inflamatory ROS production. Additonally, shear stress activates FN-bound integrins, leading to NF-κB activation, ICAM-1 and VCAM-1 expression, and an increase in moncyte adhesion to “activated” ECs.

**Figure 1.5 Domain structure of the junctional mechanosensors**



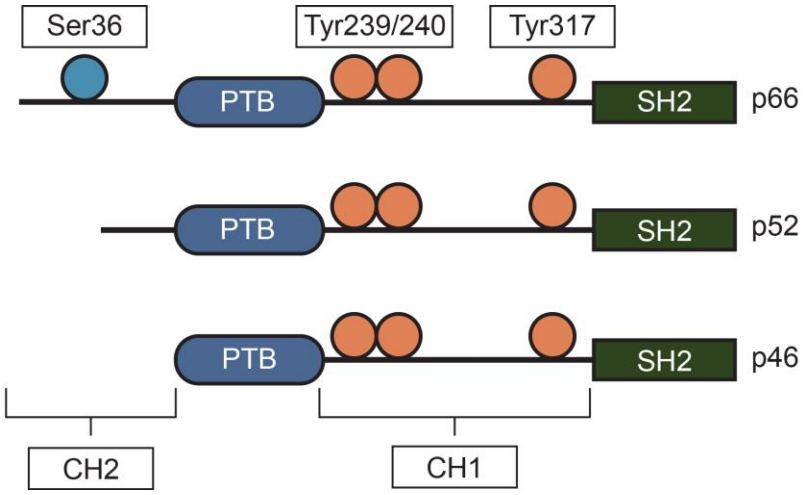

The extracellular domain of PECAM-1 is comprised of 6 immunoglobulin (Ig)-like repeats, which participate in homophilic binding with other PECAM-1 molecules on neighboring ECs. These Ig-like repeats are linked by a transmembrane (TM) domain to the intracellular domain. The intracellular domain contains two immunoreceptor tyrosine-based inhibitory motif (ITIM) domains, which contain critical tyrosines that are rapidly phosphorylated at the onset of shear. VE-Cadherin, a classical cadherin, has an extracellular domain comprised of 5 cadherin domains, which mediate adhesion, and features p120 and β-catenin binding sites on its intracellular domain. These intracellular domains interact with p120 and β-catenin, mediating VE-Cadherin binding to the cytoskeleton. VEGFR2, a receptor tyrosine kinase has 7 Ig-like repeats in its extracellular domain. The intracellular domain contains two tyrosine kinase (TK) domains that interact with downstream effectors. Critically, tyrosines 801 and 1175 bind PI3K upon stimulation of the receptor. Mutation of these tyrosines blunts the cellular response to shear.

**Figure 1.6 Integration of approaches is necessary for determining the precise function of various mechanosensors**



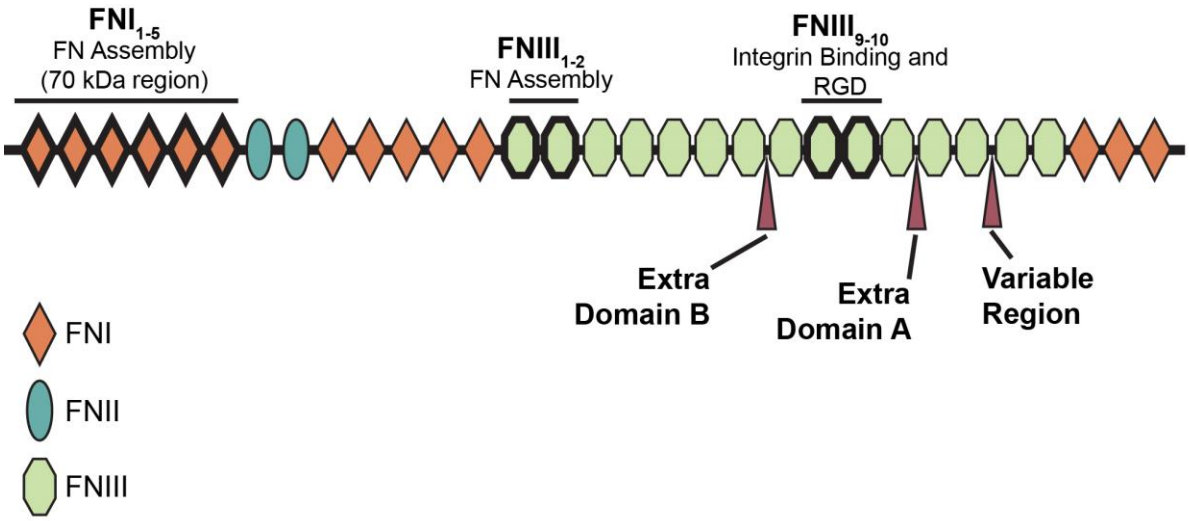

Methods available for the study of mechanosensing range from reductionist biophysical experiments to in vivo hemodynamic modification. Magnetic beads are used to apply force directly to putative mechanosensors, and can be useful for directly determing mechanosensitivity of a protein. In vitro shear stress experiments are useful for determining mechanoresponsiveness of proteins in the context of an endothelial monolayer, while in vivo experiments determine whether information gained using the aforementioned reductionist approaches is physiologically relevant.

**Figure 1.7 Domain structure of the ShcA family of proteins**




The ShcA family of proteins consists of three members: p66Shc, p52Shc, and p46Shc. These proteins all share a similar structure, composed of a PTB domain and an SH2 domain connected by a CH1 domain. The size difference between the proteins is determined by the N-terminal CH2 domain, which is longest in p66Shc. The PTB an SH2 domains mediate interactions with phosphotyrosines on RTKs and other proteins. Several amino acids are also phosphorylated. These are Tyr239/240 and Tyr317 in the CH1 domain, and Ser36 in the CH2 domain of p66Shc. Importantly, the tyrosines of the CH1 domain are phosphorylated by acute application of shear stress in a process that requires VEGFR2 and Src kinase activities. The Ser36 of p66Shc is phosphorylated in response to oxidant stress, and is involved with apoptosis pathways (see Chapter 4 for more information).

**Figure 1.8 Domain structure of Fibronectin**




Fibronectin is a large glycoprotein that mainly consists of three types of domain: FNI, FNII, and FNIII. These domains combine to confer many activities on FN. A 70 kDa fragment composed of FNI1-5 resides at the N-terminus of the protein, and participates in FN assembly. FNIII1-2 are also important for FN assembly, while FNIII9-10 provide a binding site for integrins known as the RGD domain. Alternative spliceforms of FN can be made by adding or omitting Extra Domain A, Extra Domain B, and varying lengths of the Variable Region.

## Chapter 2 Haemodynamics Regulate Fibronectin Assembly via PECAM

### OVERVIEW

Fibronectin (FN) assembly and fibrillogenesis are critically important in both development and the adult organism, but their importance in vascular functions is not fully understood. Here we identify a novel pathway by which haemodynamic forces regulate FN assembly and fibrillogenesis during vascular remodelling. Induction of disturbed shear stress *in vivo* and *in vitro* resulted in complex FN fibril assembly that was dependent on the mechanosensor PECAM. Loss of PECAM also inhibited the cell-intrinsic ability to remodel FN. Gain- and loss-of-function experiments revealed that PECAM-dependent RhoA activation is required for FN assembly. Furthermore, PECAM$^{-/-}$ mice exhibited reduced levels of active β1 integrin that were responsible for reduced RhoA activation and downstream FN assembly. These data identify a new pathway by which endothelial mechanotransduction regulates FN assembly and flow-mediated vascular remodelling.

### INTRODUCTION

The extracellular matrix (ECM) is critical for a host of physiological processes. Not simply a structural scaffold, the ECM is critical for a number of cellular functions, including proliferation and survival(Chiang et al., 2009a; Choi and Hynes, 1979; McKeown-Longo and Mosher, 1983). Specifically in the vasculature, the ECM plays an important role in capillary morphogenesis and vascular tube stabilization(Ambesi et al., 2005; Davis and Senger, 2005; Zhou et al., 2008). A major constituent of the ECM in many tissues is fibronectin (FN), which exists in both plasma and cellular forms(Schwarzbauer and DeSimone, 2011). FN is a glycoprotein consisting of multiple binding domains for a plethora of proteins, including integrins, fibrin, collagen, heparin and other FN molecules(Schwarzbauer and DeSimone, 2011). FN is indispensable for development and,

interestingly, mice that lack FN expression are embryonic lethal due to cardiovascular defects(E. L. George et al., 1997; 1993). FN also plays an important role during pathological processes: during early atherogenesis, FN deposition is promoted in areas of disturbed flow and is associated with endothelial dysfunction(Collins et al., 2014; Orr et al., 2007; 2006a; 2008).

FN monomers are soluble, and when assembled, form highly adhesive, insoluble fibrils. Polymerisation of FN into the ECM is important for both development and adult physiology. FN fibrillogenesis is essential for cleft formation during epithelial branching, the process by which nascent salivary glands begin developing(Sakai et al., 2003). FN fibrils also provide a mesodermal guidance cue during gastrulation(Darribère and Schwarzbauer, 2000; D. L. Shi et al., 1989). Injection of an RGD peptide blocks FN fibrillogenesis in the amphibian blastocoel cell roof, leading to a loss of directionality in mesodermal migration(Winklbauer and Nagel, 1991; Winklbauer and Stoltz, 1995). In adult physiology, mutations in the Hep-II and Hep-III domains of FN are present in carriers of glomerulopathy with FN deposits (GFND)(Castelletti et al., 2008). These mutations occur in regions of FN that are critical for fibrillogenesis, implicating impaired FN assembly in the progression of GFND. Additionally, FN fibrillogenesis regulates the remodeling and organization of other ECM molecules, as blocking FN polymerization also blocks collagen (CL) polymerization(F. Shi et al., 2010). FN assembly is a complex process that involves FN binding with integrins, an increase in FN-FN interaction, and conversion to deoxycholate (DOC) insoluble FN fibrils(Choi and Hynes, 1979; Schwarzbauer and DeSimone, 2011). The integrin binding step is primarily mediated by the FN binding integrin α5β1, which binds the RGD sites on FN molecules and increases clustering after binding occurs(Huveneers et al., 2008; Schwarzbauer and DeSimone, 2011). The small GTPase RhoA is also required for interaction of FN proteins. RhoA activity, and downstream activation of ROCK, increases cell contractility which acts upon FN proteins through α5β1 integrins, opening up cryptic binding sites in FN(Zhong et al., 1998). FN dimers assemble into fibrils that are eventually augmented to become large, DOC-insoluble FN polymers that make up the ECM. Although a great deal is known about the process of FN assembly, its importance in cardiovascular biology is incompletely understood. FN assembly is required for some endothelial functions, like network formation and proliferation(Ambesi et al., 2005; Davis and Senger, 2005; Zhou et al., 2008).

Assembled FN also regulates phenotypic plasticity in smooth muscle cells(Chiang et al., 2009a; F. Shi et al., 2014). Early studies suggested that ECs are involved in remodeling FN in the basal lamina and that FN organization, similar to actin stress fiber organization, shows flow dependency(AR et al., 1985; Jinguji and Fujiwara, 1994). In the descending aorta, an area of the vasculature that experiences laminar flow, FN fibrils are thick and align in the direction of flow. However, at the renal artery bifurcation, an area of disturbed flow, FN fibrils are randomly distributed(Jinguji and Fujiwara, 1994). The role of FN assembly in vessel function was more definitively tested using a peptide that inhibits FN polymerization: when this peptide was utilised in a mouse model of partial carotid artery ligation (CAL), it inhibited intima-media thickening (IMT) and flow-mediated vascular remodeling(Chiang et al., 2009a).

Vascular remodeling resulting from CAL is driven by haemodynamic forces, as CAL induces chronic disturbed flow in the ligated carotid artery(Z. Chen and Tzima, 2009; Chiang et al., 2009a; Nam et al., 2009). Although it is appreciated that shear stress sensing by ECs is a major driving force for vascular remodeling, the molecular mechanisms by which endothelial mechanosignaling leads to vascular remodeling are unknown. We have previously shown that the cell adhesion molecule, PECAM, is a member of an endothelial mechanosensory complex. This complex is necessary and sufficient for endothelial mechanosignalling, including alignment of stress fibers in the direction of flow(Tzima et al., 2005). *In vivo*, PECAM is necessary for vascular remodeling after CAL(Z. Chen and Tzima, 2009), as well as collateral remodelling in response to hindlimb ischaemia(Z. Chen et al., 2010). Using a magnetic tweezers system, we have shown that local force application on PECAM results in a global mechanotransduction response that involves remodeling of the actin cytoskeleton and activation of mechanical stiffening(Collins et al., 2012). However, very little is known about the specific mechanisms underlying shear induced FN assembly. Here, we show that haemodynamics, and shear stress in particular, regulate FN assembly *in vitro* and *in vivo* in a PECAM-dependent manner. Mechanistically, PECAM-dependent FN assembly requires RhoA and β1 integrin activation. These data identify a new pathway by which endothelial mechanotransduction regulates FN assembly and flow-mediated vascular remodeling.

## RESULTS

### *In vivo* flow-mediated FN remodeling is dependent on PECAM

Previous work from our group has shown that PECAM$^{-/-}$ mice exhibit impaired flow-mediated vascular remodeling and reduced IMT after partial CAL(Z. Chen and Tzima, 2009). Since FN assembly is required for IMT(Chiang et al., 2009a), we hypothesized that flow-mediated vascular remodeling, FN assembly and PECAM mechanotransduction are connected. To test this, we first examined if FN assembly is sensitive to changes in haemodynamics. We subjected WT mice to partial CAL or sham surgery, and after 3 weeks, left common carotid arteries (LCA) were mounted *en face* and immunostained with an anti-FN antibody. Sham LCAs, which experience laminar shear stress, exhibited long FN fibrils that were aligned in the direction of flow (Figure 2.1A and C). In contrast, exposure of LCAs to low and disturbed flow due to ligation resulted in formation of thicker and randomly-oriented FN fibrils (Figure 2.1A and C). Interestingly, LCAs from PECAM$^{-/-}$ mice displayed differences in FN organization compared to WT LCAs in both sham and ligated conditions (Figure 2.1A and D). Under laminar flow conditions present in sham LCAs, PECAM$^{-/-}$ animals had few and randomly oriented FN fibrils; even after changing flow in response to ligation, PECAM$^{-/-}$ LCAs failed to show the complex FN fibrils evident in the WT ligated LCAs, suggesting that absence of PECAM results in impaired flow-mediated FN assembly and remodeling (Figure 2.1B). Consistent with the results in WT and PECAM$^{-/-}$ carotid arteries, the WT descending aorta displays a high degree of FN assembly, whereas PECAM$^{-/-}$ descending aortas lack complex FN assembly (Figure 2.7). Given that the FN matrix serves as a template for deposition of other ECM proteins(Kubow et al., 2015), we also examined the distribution of collagen I (CL) in sham and ligated LCAs. Similar to the observations made for FN fibrils, PECAM$^{-/-}$ aortas were characterized by wispy and fewer CL fibrils (Figure 2.8).

### Disturbed shear stress induces FN assembly in a PECAM-dependent manner.

To determine whether the observed deficit in FN assembly in KO mice is an endothelial-specific phenotype, we exposed PECAM knockout (KO) and PECAM reconstituted (RC) ECs to oscillatory shear stress, followed by visualization of FN fibrils (Figure 2.2A and B). Striking differences in FN assembly were observed between genotypes. RC cells showed a high degree of FN fibril formation in static conditions and responded to shear stress by increasing fibril formation 3-fold. In

contrast, KO cells did not form any detectable FN fibrils in either static or sheared conditions. The few FN deposits that did form resembled the punctate nature of FN deposits observed in PECAM$^{-/-}$ mice (Figure 2.1). Similar results were obtained when ECs were immunostained for CL (Figure 2.8). Interestingly, KO ECs also exhibited reduced FN mRNA levels, suggesting that PECAM is also important for FN expression (Figure 2.9).

FN assembly renders fibrils insoluble in DOC-containing lysis buffer, making DOC insolubility an appropriate biochemical method for assaying the degree of FN assembly. To complement our immunofluorescence data, we performed DOC-solubility experiments. RC and KO lysates were fractionated using a DOC-containing lysis buffer after application of shear stress, followed by immunoblotting for FN (Figure 2.2C). In static conditions, RC cells showed 5-fold more assembled FN than KO cells. Application of shear increased the amount of assembled FN 2-fold in RC cells, while no increase in assembled FN was observed in sheared KO cells. These results are consistent with the immunofluorescence data and collectively show that PECAM is required for both basal and shear-dependent FN assembly and remodeling in ECs.

**PECAM is required for the intrinsic ability to assemble FN**

To determine whether PECAM is required for the cell-intrinsic ability to assemble FN, we utilized a fibrillogenesis assay whereby RC and KO cells were supplied with FITC-FN as ECM, soluble media supplement, or both, followed by fixation and fluorescence microscopy (Figure 2.3). Under all three conditions, RC cells displayed increased accumulation of FITC-FN into fibrils compared to KO cells. Whether FITC-FN was provided as ECM glass coating or media supplement, RC cells assembled more extensive networks of FN fibrils. KO cells organized the FN into punctate deposits, but failed to form FN fibrils. When FN was provided as both a glass coating and a media supplement, RC cells demonstrated augmented assembly of fibrillar FN matrix. Under these same conditions, KO cells assembled significantly less FITC-FN-containing fibrillar matrix that was characterized by primarily punctate deposits of FN. Together, these data suggest that PECAM is required for EC fibrillar FN matrix assembly independent of the expression of endogenous FN.

**Disturbed shear stress regulates PECAM-dependent FN assembly via a β1-integrin-RhoA pathway**

FN assembly in fibroblasts requires actomyosin signaling via the Rho-ROCK pathway(Schwarzbauer and DeSimone, 2011; Singh et al., 2010; Zhong et al., 1998). Notably, RhoA-dependent contractility is required for exposure of a cryptic self-assembly site in the FN protein(Zhong et al., 1998). Additionally, knockdown of ROCK isoforms I or II leads to reduced FN matrix assembly and altered MLC localization, further suggesting a role for RhoA-dependent contractility in FN assembly(Yoneda et al., 2007). Since KO cells lack the ability to properly assemble FN into fibrils, we examined the role of the Rho pathway in our system. We assayed RhoA activation levels in RC and KO cells by RBD pulldown, which uses the Rho binding domain from the Rhotekin protein to bind and isolate GTP bound RhoA. As shown in Figure 4a and 4b, KO ECs have reduced levels of active RhoA compared to RC cells. The observed reduction in Rho-GTP levels correlates with lower DOC-soluble FN in the KO cells. To determine whether RhoA activation levels were causative for FN assembly, we performed gain-and loss-of-function experiments. Firstly, to determine if low FN assembly levels could be rescued by stimulation of RhoA activity, we treated KO cells with lysophosphatidic acid (LPA) to activate RhoA(Anliker and Chun, 2004), followed by RBD pulldown and DOC insolubility assays (Figure 2.4C and D). LPA stimulation increased Rho-GTP levels approximately 140%, as expected, which was also accompanied by a concomitant and comparable increase in FN assembly compared to unstimulated cells. RhoA knockdown experiments were also performed in RC cells to determine whether decreased expression of RhoA reduces levels of assembled FN (Figure 2.4E and F). Indeed, Rho-GTP levels were significantly reduced after RhoA knockdown, leading to a 60% reduction in DOC-soluble FN levels. Overall, these gain-of-function and loss-of-function studies show that PECAM-dependent RhoA activation is necessary for FN assembly in ECs.

β1 integrin is a major FN binding integrin, and is reported to be the master integrin involved in stretching and assembling individual FN monomers into FN fibrils(Huveneers et al., 2008). Given the deficit in FN assembly in the absence of PECAM, we examined the activation status of β1 integrins in the absence of PECAM. We performed immunofluorescence staining using an antibody specific for active β1 integrins in WT and PECAM$^{-/-}$ carotid arteries. Imaging revealed a stark difference between

the two genotypes (Figure 2.5A). WT carotids displayed high levels of β1 integrin activation, concentrated in the endothelial layer, compared to PECAM$^{-/-}$ carotids. These results raise the intriguing possibility that levels of active integrins are connected to both activation of RhoA and downstream FN assembly. To test this, we performed β1 integrin knockdown experiments (Figure 2.5B). In RC cells treated with β1 integrin siRNA, Rho-GTP levels were reduced by 70% compared to control siRNA transfected ECs. This was accompanied by a comparable reduction in levels of DOC-insoluble FN, indicative of reduced FN assembly. In complementary experiments, when using a β1 integrin function-blocking antibody we also saw reduced levels of DOC-insoluble FN (Figure 2.5C). Overall, these data show that PECAM-dependent β1 integrin activation is required for RhoA activation and downstream FN assembly in ECs.

## DISCUSSION

We have previously shown that PECAM knockout mice exhibit impaired flow-mediated vascular remodeling and IMT in response to CAL(Z. Chen and Tzima, 2009). Similarly, mice treated with a FN polymerization inhibitor are also characterized by reduced IMT and vascular remodeling(Chiang et al., 2009a). Although endothelial responses to shear stress are known to regulate expression and deposition of ECM components(Feaver et al., 2010; Gelfand et al., 2011; Green et al., 2014; Orr et al., 2006a), virtually nothing is known about the role of shear stress in the regulation of FN assembly. We hypothesized that PECAM, a known endothelial mechanosensor, is important for regulation of the organization of the subendothelial ECM, especially in response to shear stress. Our results here provide a mechanistic link between mechanosensing and FN organization during flow-mediated vascular remodeling. Using both PECAM knockout ECs and mice, we show that PECAM regulates FN fibrillogenesis in a RhoA- and integrin β1-dependent pathway (Figure 2.6).

CAL is a method for modification of the carotid artery hemodynamic environment. Proximal to the carotid bifurcation, the left carotid artery normally experiences laminar flow. After CAL, blood flow in the left carotid artery becomes disturbed, leading to IMT and vascular remodeling in order to normalize shear stress. A main result from the present study is the failure of PECAM knockout mice to assemble FN after CAL. Other studies have focused on the role of haemodynamics in the regulation of expression of ECM components. Atheroprone areas of the vasculature exhibit increased deposition of FN and ECs exposed to disturbed flow exhibit increased FN expression(Feaver et al., 2010; Gelfand et al., 2011; Green et al., 2014; Orr et al., 2006a; 2005). Not surprisingly, the role of FN in inflammation and atherosclerosis is rather complex: while FN promotes increased plaque area, it has also been shown to promote formation of the protective fibrous cap, which in humans prevents plaque rupture(Rohwedder et al., 2012). Our results are in agreement and extend these findings to show increased FN deposition in areas of disturbed flow in response to CAL. Importantly, PECAM knockout mice, which lack much of their endothelial shear sensing capability, deposit very little FN and exhibit almost no fibril assembly. The observed deficiency in FN fibrillogenesis in the absence of PECAM could be the result of two causes: a reduction in FN expression or a lack of endothelial

capacity to physically assemble the fibrils. Our results show that both are the case in PECAM KO ECs. We observe that PECAM KO cells exhibit deficient basal FN mRNA expression, consistent with previous work showing that atheroprone shear stress turns on transcription of FN via a PECAM/ NFκB-dependent pathway in ApoE KO mice(Feaver et al., 2010). We also show that when provided exogenous FN, KO cells are unable to assemble the FN into fibrils, demonstrating an intrinsic inability of KO cells to remodel FN, independent of the expression of FN.

RhoA-mediated contractility is thought to be the physical force that stretches FN proteins and assembles them into fibrils(Fernandez-Sauze et al., 2009; Singh et al., 2010; Zhong et al., 1998). We show here that PECAM is required for RhoA activity in ECs, as PECAM KO ECs display reduced levels of active, GTP-bound RhoA. Importantly, gain-of-function studies show that stimulation of RhoA activity in KO ECs leads to increased FN assembly. Reciprocally, loss-of-function studies using siRNA-mediated knockdown of RhoA in PECAM-expressing ECs show that abrogation of Rho signaling results in significant attenuation of FN assembly. Since β1 integrins are the main integrins that bind to the RGD domain of FN and shear stress leads to increased integrin activation and increased binding to the ECM(Tzima et al., 2001), we examined the activation of β1 integrins in $PECAM^{-/-}$ carotid arteries. We found that PECAM is required for increased levels of active β1 integrins. A causative role for β1 integrins in FN assembly was shown using two complementary approaches: use of siRNA-mediated knockdown and function blocking antibodies, both of which resulted in reduced RhoA activation and downstream FN assembly. This demonstrates a requirement for β1 integrin in PECAM-mediated FN assembly.

Taken together with previous studies, our results here provide a mechanistic link between endothelial mechanosensing, FN assembly, and flow-mediated vascular remodeling. Although the observation that FN fiber organization shows an apparent flow dependency was made more than 20 years ago, the molecular mechanisms of this process remained a mystery. We now show that shear stress serves as a potent activator of β1 integrins and RhoA signaling through a PECAM-dependent mechanism that, in turn, drives FN (and CL) assembly. Our results show that increased FN assembly resulting from chronic disturbed flow *in vitro* and *in vivo* requires PECAM, indicating that FN assembly is a mechanoresponsive event. Previous work from our lab described a requirement for PECAM in

flow-induced inflammation, IMT and vascular remodeling in a model of partial carotid ligation(Z. Chen and Tzima, 2009). Using two dfferent models of atherosclerosis (ApoE KO and LDLR KO), previous studies have shown that the functions of PECAM depend on the type of shear stress that vessels experience, such that PECAM promotes atherosclerosis in areas of disturbed shear stress while it prevents atherosclerosis in areas of laminar shear stress(Goel et al., 2008; Harrison et al., 2013b; Harry et al., 2008; Stevens et al., 2008). The present study shows that under disturbed flow regimes, PECAM promotes FN assembly. Given that FN assembly is required for vascular remodeling and attendant inflammation(Chiang et al., 2009a), and that haemodynamics regulate FN deposition(Feaver et al., 2010), increased FN assembly may be a hallmark of vessel inflammation caused by PECAM signaling in response to disturbed flow. It is therefore tempting to hypothesize that PECAM- dependent FN assembly underlies inflammatory remodeling, such as IMT and atherosclerosis, in the vasculature. However, further studies are needed to delineate if FN assembly begets endothelial inflammation, or whether the converse is true and endothelial inflammation begets FN assembly. Further research in this area could lead to targeted therapies to atherosclerotic areas, where plaque progression could be slowed or fibrous caps could be stabilized through mechanisms that block or induce FN assembly, respectively.

## MATERIALS AND METHODS

**Cell culture, shear stress assays** PECAM$^{-/-}$ (KO) cells and cells reconstituted (RC) with full-length PECAM were prepared as described(Graesser et al., 2002). Levels of PECAM in reconstituted cells are similar to wild-type levels. Primary bovine aortic ECs (BAEC) were obtained from VEC Technologies (Rensselaer, NY). Nearly confluent cells were incubated in starvation media (DMEM with 0.5% fetal bovine serum and 1% penicillin/streptomycin) overnight before shear stress experiments. Cells on fibronectin-coated glass slides were mounted in a parallel plate flow chamber connected to a NE-1050 bi-directional syringe pump by rigid wall tubing (New Era Pump Systems, Inc., Farmingdale, NY). Cells were exposed to oscillatory flow for 24 hours at ± 6.5 Dynes/cm$^2$ with a frequency of 1 Hz. Flowed slides were washed with cold phosphate buffered saline (PBS) for Rho-

GTPase pulldown, deoxycholate (DOC) solubility assays or fixed with 1% formaldehyde for 10min for immunostaining as described below. Cells not subjected to shear stress were used as static controls.

**DOC solubility assay** Cells were washed with cold PBS for 3 times, and lysed in cold 2% DOC buffer(Wierzbicka-Patynowski et al., 2004). Cell lysates were processed with a bead homogenizer for 30s and aliquoted. To separate DOC-insoluble fraction, an aliquot was centrifuged at 16000rpm in 4ºC for 15m. The pellet was dissolved in SDS loading buffer and DOC-insoluble proteins were examined with Western blot assay. BCA assays were performed to ensure equal protein loading, and DOC-insoluble vimentin is used as a loading control protein.

**Western Blotting** Samples were loaded on to pre-cast 4-15% polyacrylamide gradient gels (Bio-Rad, Hercules, CA). Using MOPS running buffer, electrophoresis was carried out at 200V for 30 minutes, followed by transfer to a nitrocellulose membrane. Membranes were blocked for 1 hour in 50% Li-Cor blocking buffer in 1X PBS (Li-Cor, Lincoln, NE). Membranes were then probed with primary antibodies overnight at 4°C in 50% Li-Cor blocking buffer in 1X PBS. Primary Antibodies: Goat anti-β1 integrin (1:200; Santa Cruz Biotechnology, Dallas, TX; SC-6622), Rabbit anti-Fibronectin (1:1000; Sigma-Aldrich Inc., St. Louis, MO; F3648), Mouse anti-RhoA (1:500; Santa Cruz Biotechnology, Dallas, TX; SC-418), Mouse anti-GAPDH (1:1000; Millipore, Billerica, MA; MAB374), Goat anti-vimentin (1:200; Santa Cruz Biotechnology, Dallas, TX; SC-7559). Membranes were then probed using fluorescent secondary antibodies for 1 hour at room temperature, followed by scanning on a Li-Cor Odyssey scanner. Fluorescent secondary antibodies were all used at a 1:2000 dilution.

**Quantitative PCR** Quantitative PCR was performed using SYBR Green 2X master mix as per manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Primer Sequences: Fibronectin- F: TGGTTTGGTCTGGGATCAAT; R: ACAGTGCTGCAGGTCAGATG. GAPDH- F: CATGTTTGTGATGGGTGTGA, R: CTAAGCAGTTGGTGGTGCAG. Cycler Conditions: 5 minutes at 95°C, followed by 45 cycles of: 20 seconds at 94°C, 20 seconds at 60°C, and 20 seconds at 72°C. The last step of the cycle was a melt curve to test primer efficiency. qPCR peaks were quantified using the delta-delta Ct method.

**FITC-Fibronectin assay** 1mg/ml fibronectin stock was conjugated to fluorescein isothiocyanate isomer I (Sigma-Aldrich Inc., St. Louis, MO) as per manufacturer instructions. After dialysis, protein

concentration was determined using a BCA assay. Slides were then coated with 10µg/ml FITC-FN or 0.1% gelatin for 1 hour at room temperature. PECAM RC or PECAM KO cells were plated and allowed to grow to confluence, after which they were serum starved for 16h. After serum starvation, reduced serum media was either replaced with fresh media or fresh media with 10µg/ml FITC-FN added. Cells were incubated with FITC-FN for 24h, followed by fixation and mounting with DAPI-containing media.

**Rho-GTPase assay** Cells were plated on fibronectin-coated glass slides and allowed to grow overnight to 95% confluency. Rho-GTPase pulldown was performed with RBD-conjugated beads per manufacturer's instructions (Cytoskeleton Inc., Denver CO). To stimulate Rho-GTP, lysophosphatidic acid (LPA) (Sigma-Aldrich Inc., St. Louis, MO) was dissolved in deionized water and added to cell media at 20µM final concentration. Deionized water was added to control cells. To inactivate Rho-GTP, cells were transfected with RhoA siRNA as described below.

**siRNA transfection** The following siRNA duplexes were synthesized (Dharmacon Inc., Lafayette, CO) : bovine β1 integrin CUUAAUAUGUGGAGGAAAUUU; bovine RhoA: CUAUGUGGCAGAUAUUGAdTdT BAECs were plated in 100mm dishes to grow to 30-40% confluence in full media (DMEM with 10% FBS and 1% penicillin/streptomycin). 25µl 20µM siRNA was mixed with 500µl 2x HBS pH 7.05, followed by mixing with 30µl 2.5M $CaCl_2$. The mixture was incubated at room temperature for 20min to form siRNA-calcium phosphate complex. The siRNA complex was added to the cell media dropwise. The cells were incubated with siRNA complex overnight, trypsinized and re-plated to a fibronectin-coated dish, and culture in full media. Total β1 integrin protein was monitored before, 24, 48 and 72 hours after transfection. Cells were harvested for Rho-GTPase assays or DOC solubility assay 48 hours after the initial transfection.

**Immunofluorescence** Cells subjected to shear stress were washed 3 times with cold PBS, followed by fixation with 1% formaldehyde for 10min. Cells were permeabilized with 0.3% Trition X-100 in tris-buffered saline (TBS) for 3m, and blocked with 0.5% non-fat dry milk, 1% BSA and 0.3% Triton X-100 in TBS for 60m. A primary antibody, fibronectin (1:200; Sigma-Aldrich Inc., St. Louis, MO; F3648), active β1-integrin (clone 9EG7, 1:100, BD, 553715; or clone HUTS4 1:100; Millipore, Billerica, MA; MAB2079Z) or beta-catenin (1:100, Sigma-Aldrich Inc., St. Louis, MO; C7082), was incubated with

the cells overnight in 4ºC, followed by the incubation with Alexa 568-labeled goat anti-rabbit or mouse antibodies (1:200, Life Technologies, Grand Island, NY). Images were taken with a Carl-Zeiss 700 laser confocal microscope.

**Carotid artery ligation model** Wild-type C57/BL6 (PECAM$^{+/+}$) mice were purchased from Jackson Laboratories. PECAM$^{-/-}$ C57BL/6 mice were kindly provided by Dr. P. Newman (Blood Research Institute, Blood Center of Wisconsin, Milwaukee), bred in house and used in accordance with the guideline of the National Institute of Health and for the care and use of laboratory animals (approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill). Male PECAM $^{-/-}$ and age-matched littermates (WT, 10-14 weeks) were used for all experiments. Blood flow reduction in the left common carotid artery (LCA) and blood flow measurements were performed as previously described(Z. Chen and Tzima, 2009; Korshunov and Berk, 2003). The ligation operation closed internal, external and occipital carotid arteries, and left the thyroid artery open. Partial blood flow (5-10% within 3 weeks after surgery) remained in common carotid artery through the opening thyroid branch. The sham procedure consisted of vessel isolation and ligature placement without ligation.

***En face* preparation of carotid arteries** The common carotid arteries were perfusion-fixed with 1% paraformaldehyde for 10m and harvested 5 days or 3 weeks after carotid artery ligation. The adventitia was removed using surgical forceps. The remaining intimal-medial tissue of the carotid arteries was cut longitudinally and mounted flat on glass slides for immunofluorescence and imaging.

***En face* tissue immunofluorescence** *En face* mounted tissue samples were rehydrated with water for 10 minutes. The tissue was then permeabilized using Tris-Triton buffer (50mM Tris, 500mM NaCl, 0.3% Triton X-100) for 1 hour, followed by 3 washes with water. Permeabilized tissue was then blocked using 0.5% non-fat dry milk and 1% BSA in TBST for 60 minutes at room temperature. Primary antibodies (Rabbit anti-FN, 1:100, Sigma F3648, St. Louis, MO; rat anti-VE-Cadherin, 1:50, BD Pharmingen 550548, Franklin Lakes, NJ, ; or rabbit anti-Collagen α1, 1:100, Abcam Ab292, Cambridge, UK) were then diluted in blocking buffer and incubated with the tissue overnight at 4°C. After primary antibody incubation, slides were washed 3 times with TBST. Secondary antibodies (Goat anti-rat Alexafluor 488, 1:100, A11006 Life Technologies; Goat anti-rabbit Alexafluor 568,

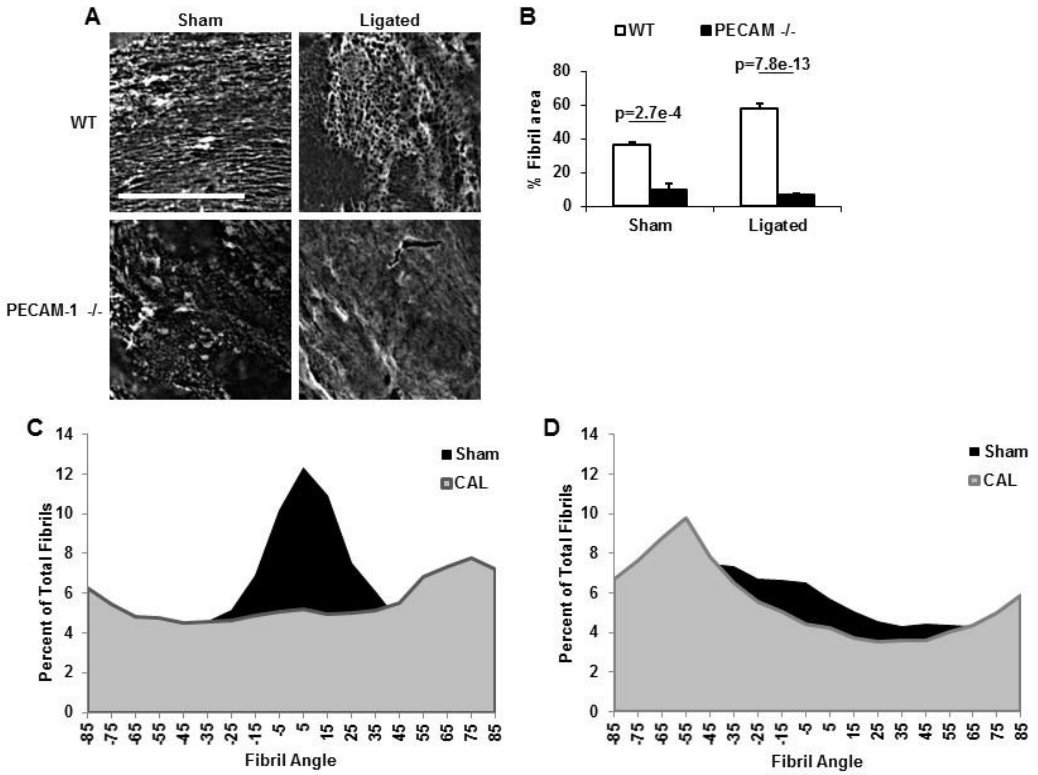
1:100, A11011 Life Technologies) were diluted in blocking buffer and incubated with the tissue for 60 minutes at room temperature. The tissue was washed 3 times in TBST, and then mounted with VectaShield plus DAPI (Vector Labs, Burlingame, CA) and sealed with clear nail polish. Slides were imaged on a Zeiss 700 or Zeiss 710 confocal microscope.

**Percent Fibril Area Calculations and Fibril Angle Morphometry** Grayscale images of fluorescently labeled FN were opened in Fiji image analysis software. The images were then adjusted using the threshold command with the "dark background" option selected. The threshold values were recorded and used across all images for each experiment. After thresholding, the analyze particles command was used, which produces the area of each fibril in an image. These fibril areas were added together to obtain the total fibril area. This area was then divided by the total image area, giving the percent fibril area. To obtain fibril angles, thresholding was performed, followed by analysis using the Directionality plugin in Fiji. Angles from 90 to -90 were analyzed, and the resulting particle frequencies were grouped together in 10-degree bins, followed by plotting in Microsoft Excel.

**β1 integrin function blocking studies** BAECs were grown to 95% confluence, followed by 4 hours of serum starvation in 0.5% FBS. BAECs were then treated with either 1μg/ml β1 integrin function blocking antibody AIIB2 (University of Iowa Hybridoma Bank) or 1μg/ml ChromPure mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA; 015-000-003) for 24 hours at 37° and 5% $CO_2$.

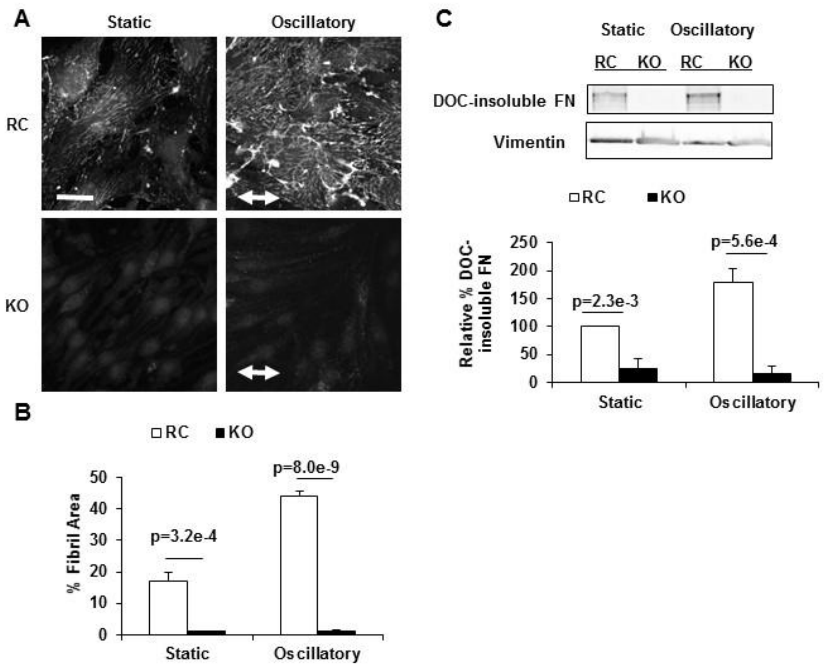
**Statistical Analysis** Results are described as mean ± SEM. Statistical tests were performed with Microsoft Excel analysis package, using Student's t-test for 2 groups or one-way ANOVA followed by multiple comparisons with Tukey's Honest Significance Difference test. Level of $p<0.05$ was considered significant.

**Figure 2.1 PECAM is required for FN assembly in response to CAL**



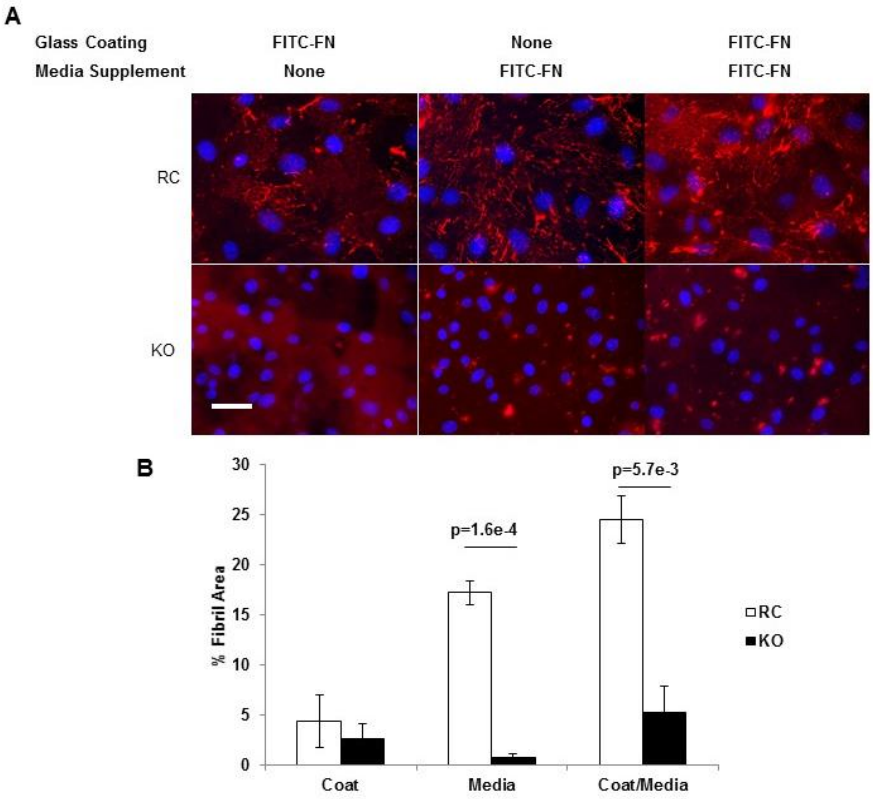

WT and PECAM$^{-/-}$ mice were subjected to CAL. (A) 3 weeks after CAL, sham and ligated carotid arteries were harvested, mounted *en face* and stained for FN. (B) FN fibril area was quantified. Scale Bar = 20μm; n=16 WT and 10 PECAM$^{-/-}$ mice. (C) WT FN fibril angle was analyzed in sham and 3-week ligated LCAs n=3 sham and n=5 CAL. (D) KO FN fibril angle was analyzed in sham and 3-week ligated LCAs n=2 sham and n=3 CAL.

**Figure 2.2 Disturbed shear stress induces PECAM-dependent FN assembly**



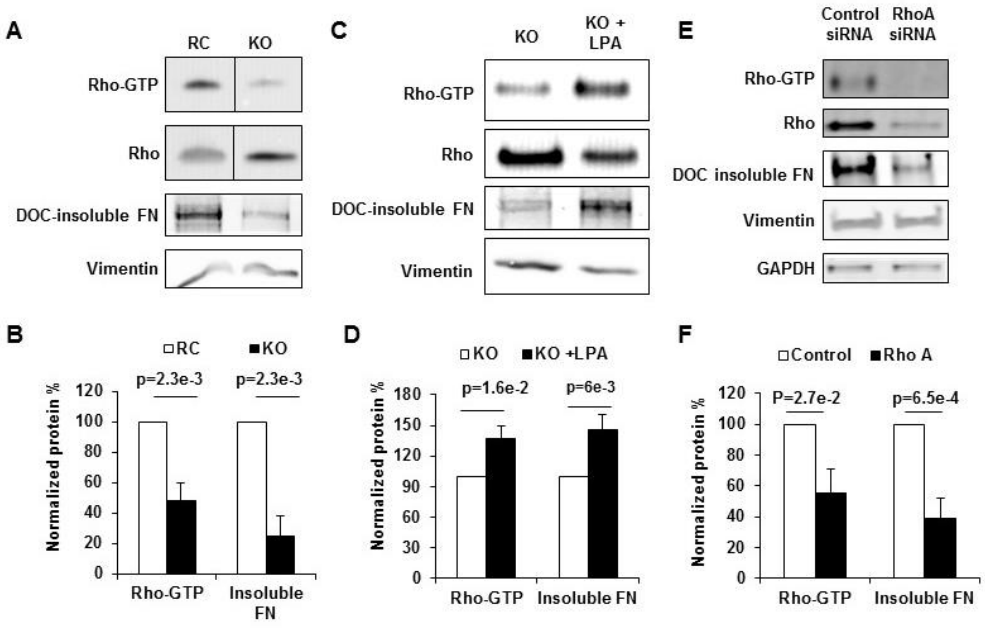

RC and KO cells were subjected to flow or kept as static controls. (A) After flow, RC and KO cells were fixed and stained for FN, followed by (B) quantification of FN fibril area. (C) Representative western blots showing DOC-insoluble FN in RC and KO ECs before or after oscillatory shear stress. Quantification of DOC-insoluble FN relative to static RC lysates is shown, and was performed after normalization to vimentin. Scale bar in (A) is 20um. (A) and (B) n=5 independent experiments; (C) n=4 independent experiments.

**Figure 2.3 PECAM is required for the intrinsic ability of ECs to remodel FN**



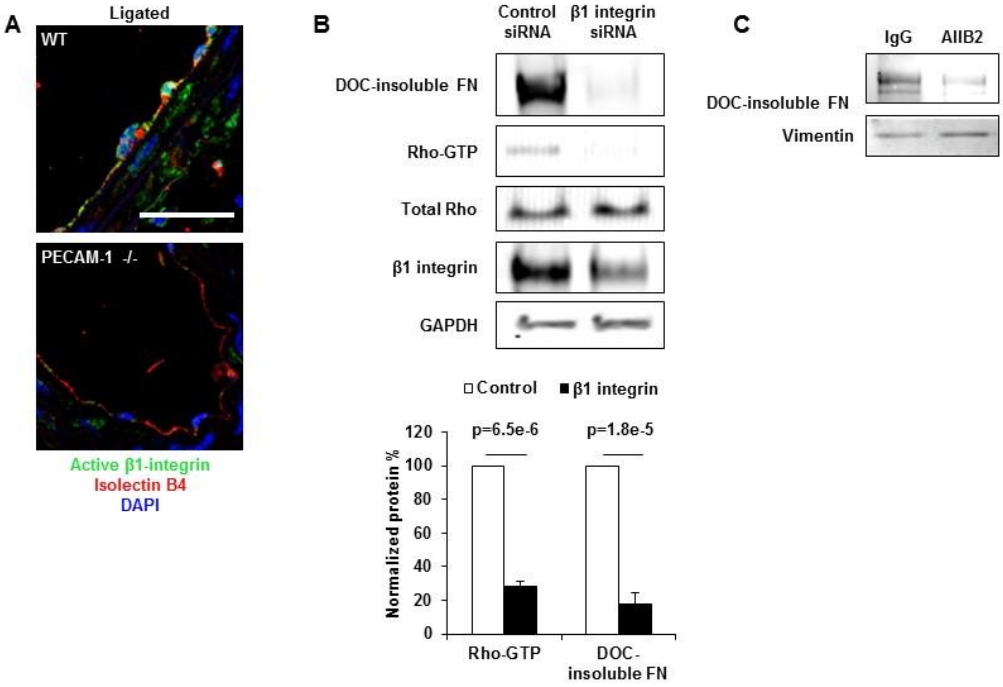

(A) FITC-conjugated FN was provided to static RC and KO cells as a tissue culture glass coat, media supplement, or both. Representative images are shown (FN was pseudocolored in red for clarity). Representative images are shown (FN was pseudocolored in red for clarity). Scale Bar=40μm (B) Percent fibril area was also quantified. (A) and (B) n=3 independent experiments.

**Figure 2.4 PECAM-dependent RhoA activity is required for FN assembly**



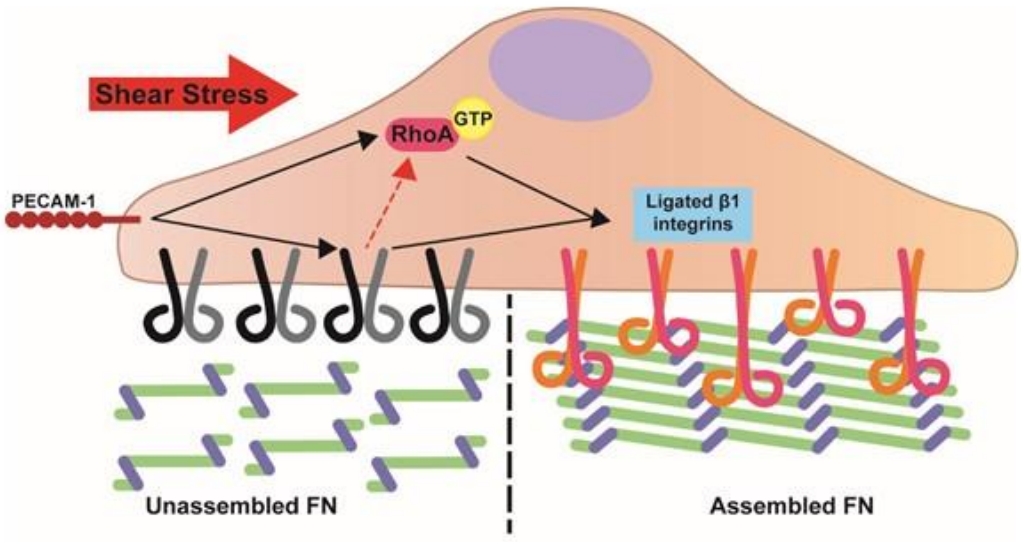

(A) RhoA-GTP levels in RC and KO cells were determined by RBD pulldown. FN assembly was assayed by DOC-insolubility. (B) Quantification of RhoA-GTP and DOC-insoluble FN is below. (C) KO cells were stimulated with LPA, a RhoA agonist. RhoA-GTP was determined by RBD pulldown and FN assembly was assayed by DOC-insolubility. (D) Quantification of RhoA-GTP and DOC-insoluble FN is below. (E) ECs were transfected with control or RhoA siRNA and Rho-GTP levels and DOC-insuble FN were assayed. (F) Quantification of RhoA-GTP and DOC-insoluble FN is below. Experiments were performed in static cells. (A) and (B) n=4 independent experiments; (C) and (D) n=6 independent experiments; (E) and (F) n=4 independent experiments.

**Figure 2.5 Role of Beta1 integrin activation in FN assembly**



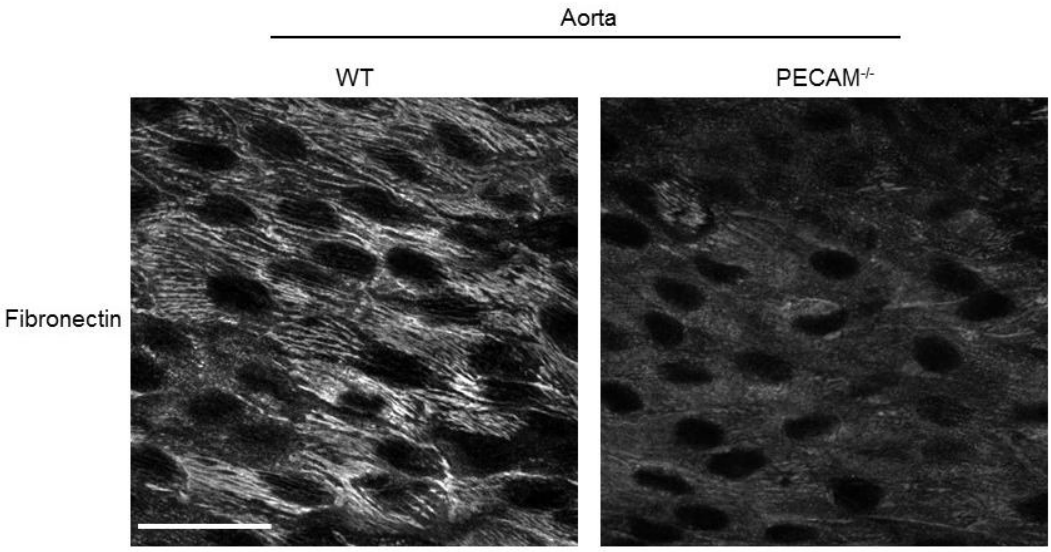

(A) Active β1-integrin staining in cross sections of WT and PECAM$^{-/-}$ carotid arteries harvested 5 days post-CAL. Scale Bar=20μm; n=3 independent experiments. (B) ECs were transfected with control or β1 integrin siRNA and levels of GTP-RhoA and DOC-insoluble FN were assayed and quantified. (C) ECs were incubated with control IgG or the β1 integrin function blocking antibody AIIB2 before determination of FN assembly by DOC-insolubility. (B) n=at least 3 independent experiments. (C) n=4 independent experiments.

**Figure 2.6 Disturbed flow regulates PECAM-dependent FN assembly through a Beta1 integrin-RhoA pathway**



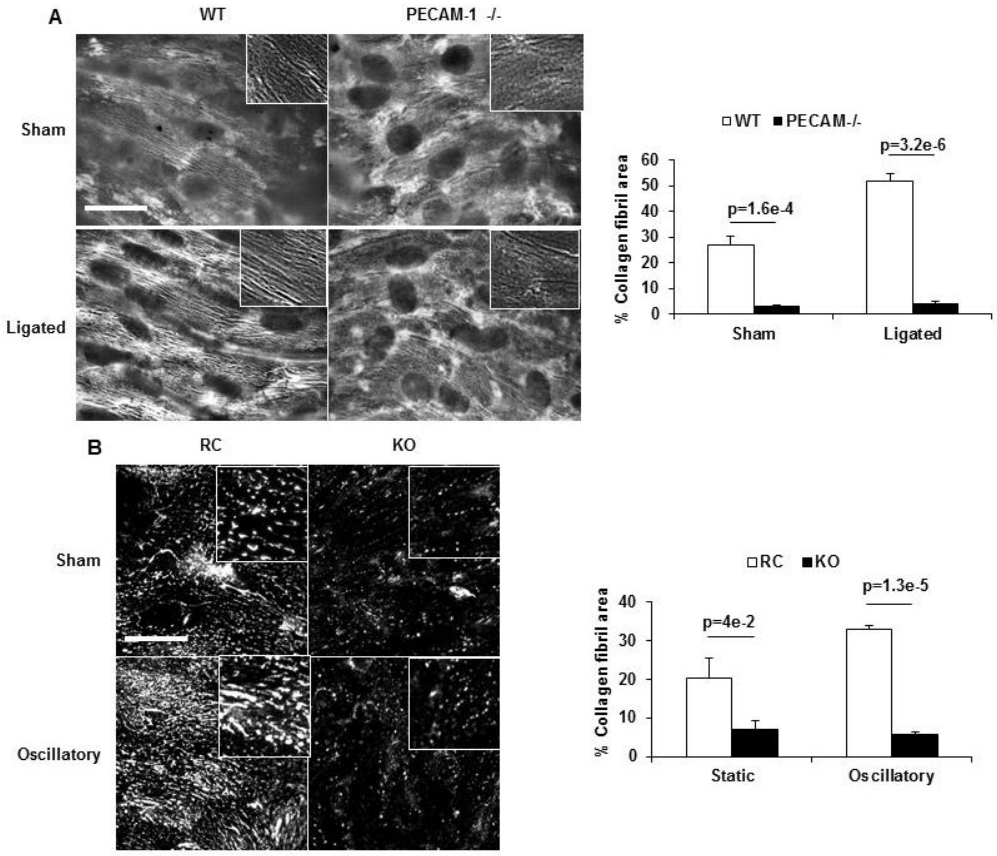

Distrubed flow regulates FN fibrillogenesis; mechanistically, this is dependent on PECAM-dependent activation of β1 integrins and RhoA.

**Figure 2.7 PECAM-1 is required for FN assembly in the descending aorta**



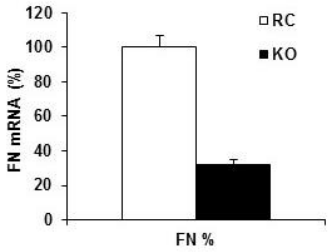

WT and PECAM$^{-/-}$ aortas were mounted *en face* and stained for fibronectin. Scale Bar = 20μm, n=1 mouse per genotype

**Figure 2.8 PECAM-1 is required for shear-induced CL assembly**




(A) WT and PECAM$^{-/-}$ mice were subjected to CAL. 5 days after CAL, sham and ligated carotid arteries were harvested, mounted *en face* and stained for CL. CL fibril area was quantified. Scale bar = 30μm, Sham n = 5 WT and 4 PECAM-1$^{-/-}$ , 5d CAL n = 4 WT and 4 KO. (B) RC and KO cells were subjected to flow, followed by immunostaining for CL. CL fibril area was quantified. Scale bar = 10μm, n= 3 independent experiments.

**Figure 2.9 PECAM-1 is required for FN expression**




mRNA was harvested from RC and KO cells, and qPCR was performed using primers specific to FN. n = 3 biological replicates for each genotype.

## Chapter 3 The adaptor protein Shc is a major regulator of endothelial inflammation

### OVERVIEW

Forces are critical regulators of vascular physiology and pathology. Shear stress, the frictional force of blood flowing over the endothelium, regulates both inflammatory and anti-inflammatory signaling in ECs. The adaptor protein Shc is a central regulator of endothelial inflammation, and is phosphorylated upon the application of shear stress to ECs. The data in this chapter describe a role for Shc tyrosine phosphorylation in arteriogenesis. In mice expressing a phosphorylation-null Shc construct, arteriogenesis is blunted due to a reduction in collateral vessel growth. Additionally, this chapter demonstrates that Shc is required for atherogenesis, the chronic inflammatory condition by which atherosclerotic plaques form and develop. Critically, Shc is shown to be involved in atherogenesis in response to altered hemodynamics. Taken together, these results suggest that Shc may play a major role in vascular inflammation, and may present a target for future vascular therapeutics.

### INTRODUCTION

Shear stress, which is the frictional force of blood flowing over the endothelium, is a critical regulator of cardiovascular physiology and pathology, influencing vascular tone, pruning of the vascular tree, and formation of atherosclerotic plaques(Boo and Jo, 2003; Davies, 1995; Hahn and Schwartz, 2009; Lucitti et al., 2007). Shear patterns fall into two broad categories: laminar and disturbed. Laminar, or atheroprotective, shear stress, is anti-inflammatory and occurs in uniform, straight areas of the vasculature. Laminar flow induces eNOS activation and NO production(Pan, 2009), endothelial expression of KLF2(Parmar et al., 2006), and alignment of ECs in the direction of flow(Tzima et al.,

2005; 2001). Conversely, disturbed shear stress is highly inflammatory(Hahn and Schwartz, 2009). ECs do not align in the direction of flow, and instead retain a polygonal shape. Additionally, ROS production and NF-κB activation occur, leading to expression of inflammatory adhesion markers like ICAM-1 and VCAM-1(Chatzizisis et al., 2007).

Endothelial inflammation underlies many physiological and pathological conditions in the vasculature. For example, inflammation directs the process of arteriogenesis, in which small collateral arterioles remodel and increase their size to resemble conductance arteries (Schaper and Scholz, 2003; Sweet et al., 2013). Collateral vessels, which are arteriole-arteriole anastomoses, normally experience no net blood flow as a result of the flat pressure gradient between healthy arterial beds. Upon major vessel occlusion, the gradient tips and shear stress dramatically increases. This increase in shear stress induces endothelial inflammation, including NF-κB activation, endothelial proliferation, and expression of ICAM-1, VCAM-1, and MCP-1(Z. Chen et al., 2010; Schaper, 2009; Sweet et al., 2013). In turn, leukocyte recruitment and SMC proliferation increase, leading to an increase in collateral size. Importantly, shear stress, not ischemia, is a major driver of arteriogenesis. The point at which shear stress becomes normalized determines the final size of remodeling collaterals(Schaper, 2009). If shear is artificially increased, the collaterals grow larger as a result of higher shear forces(Eitenmüller et al., 2006).

Another result of endothelial inflammation is atherosclerosis, a decades-long inflammatory disease characterized by focal accumulation of cholesterol and immune cells under the endothelium(Chatzizisis et al., 2007; Hahn and Schwartz, 2009). The progression of atherosclerosis is associated with genetic and environmental factors, including family history, elevated levels of LDL and VLDL, smoking and diabetes(Green et al., 2014; Lusis, 2000). However, though many contributing factors are systemic, atherosclerotic plaque distribution is focal, which is thought to be due to the localization of low and disturbed flow to branch points and areas of high curvature in the vasculature(Chatzizisis et al., 2007; Hahn and Schwartz, 2009). Chronic disturbed shear contributes to the growth of atherosclerosis in a number of ways. Disturbed shear activates ECs through induction of NF-κB(Hahn and Schwartz, 2009), sustaining this activation through deposition of fibronectin(Feaver et al., 2010). Moreover, disturbed shear represses eNOS expression in ECs,

leading to a reduction in NO production(Chatzizisis et al., 2007; Ziegler et al., 1998). Endothelial mechanosensing plays a central role in the regulation of atherosclerosis by disturbed flow. An endothelial mechanosensor, PECAM-1, has been implicated in the formation of atherosclerosis in the lesser curvature of the aortic arch, an area of the aorta exposed to chronic disturbed flow(Goel et al., 2008; Harrison et al., 2013a; Harry et al., 2008; Stevens et al., 2008). Furthermore, acute induction of disturbed flow is sufficient to induce plaque formation in the carotid arteries of mice(Nam et al., 2009).

Playing a central role in the endothelial mechanosensory response is the adaptor protein ShcA, henceforth referred to as Shc. Shc is a ubiquitously expressed adaptor protein that has three isoforms, all differentiated by the length of their N-terminal tails: p46, p52, and p66 Shc(Wills and Jones, 2012). All isoforms of Shc share PTB-, CH1-, and SH2-domains. The PTB and SH2 domains mediate interaction with RTKs, while the CH1 contains critical phosphorylation sites at tyrosines 239, 240, and 317 that mediate interactions with downstream effectors(Wills and Jones, 2012; L. Zhang et al., 2002). Shc is critical for cardiovascular development, as Shc knockout mice lack cardiovascular function due to malformed hearts(K. M. Lai and Pawson, 2000). ECs from these mice also fail to spread in culture due to improper RhoA function. Other studies have linked defects in heart development specifically to PTB domain function, while the phosphorylation sites of the CH1 domain are dispensable(Hardy et al., 2007). Shc also participates in shear signaling, and has a known role in inflammatory shear stress pathways, including activation of NF-κB and expression of ICAM-1 and VCAM-1(Liu et al., 2008). Shc also associates with VE-Cadherin after treatment with VEGF or induction of shear stress(Liu et al., 2008; Zanetti, 2002). Crucially, the onset of shear stress induces transient Shc phosphorylation, which remains high in areas of the vasculature that experience disturbed flow(Liu et al., 2008). Shc is also known to participate in angiogenesis, where it is instrumental in growth factor and survival signaling(Sweet et al., 2012). Furthermore, endothelial Shc is required for arteriogenesis(Sweet et al., 2013). After FAL, endothelial Shc knockout display blunted arteriogenesis due to an impaired inflammatory response, characterized by a failure to activate NF-κB, recruit leukocytes, or activate Notch signaling in collateral endothelium(Sweet et al., 2013).

This chapter details studies undertaken to understand the role of Shc in vascular inflammation culminating in arteriogenesis or atherosclerosis. First, FAL studies are performed with

Shc phosphorylation-null mice to test the role of Shc tyrosine phosphorylation in arteriogenesis. Second, atherogenic endothelial Shc knockout mice are used to determine the whether Shc is required for shear-induced atherosclerotic plaque formation. High fat diet and CAL studies are used to determine whether Shc is required for plaque formation.

## RESULTS

### Endothelial Shc phosphorylation is required for perfusion recovery after hindlimb ischemia

Mice containing a mutant p52Shc allele carrying tyrosine to phenylalanine (Y-F) mutations at amino acids 239, 240, and 317 were generated previously (L. Zhang et al., 2002) (Figure 3.1A). When expressed, this construct represses endogenous Shc signaling through a dominant negative mechanism(Plyte et al., 2000; Pratt et al., 1999; Ravichandran, 2001). These animals were crossed to *Tie2-Cre* mice, resulting in endothelial-specific expression of the *Shc3F* construct. Expression of the *Shc3F* construct was detectable by flag staining in the endothelium of *Shc3F;Tie2-Cre*, but not *Shc3F*, mice (Fig 3.1B). To determine whether Shc phosphorylation is required for reperfusion after femoral artery ligation (FAL), we subjected *Shc3F* and *Shc3F;Tie2-Cre* mice to femoral artery ligation, followed by measurement of plantar perfusion by Laser Doppler scanning over the course of 3 weeks post-surgery (Figure 3.2A). Recovery in *Shc3F* mice was maximal at 21d post-injury, reaching an average of 80% reperfusion in the ligated paw, as compared to the unligated paw. In contrast, recovery was impaired in *Shc3F;Tie2-Cre* mice, plateauing at 50% reperfusion in the ligated paw 3 weeks post-surgery (Figure 3.2B). Importantly, immediately post-surgery, perfusion values in the ligated paws were similar between *Shc3F* and *Shc3F;Tie2-Cre* mice, suggesting that the observed reperfusion derives entirely from collateral growth after induction of ischemia, and not a basal difference in collateral size or number. These results indicate that endothelial Shc phosphorylation is required for reperfusion after FAL.

### Endothelial, not hematopoietic, Shc phosphorylation is required for reperfusion after FAL

When used as a promoter for Cre expression, *Tie2* drives excision in ECs and a large portion of hematopoietic lineages(Constien et al., 2001; Kisanuki et al., 2001; Tang et al., 2010). To disentangle

the role of endothelial versus hematopoietic Shc phosphorylation, *Shc3F* mice were crossed with *Cadherin5 (Cdh5)-CreERT2* mice, generating a tamoxifen-inducible, endothelial specific Shc3F expressing mouse line. Use of this mouse removes any possibility of hematopoietic Cre-mediated excision, because Cdh5 promoter activity only occurs in ECs in adult mice. Thus, the Shc3F construct is expressed solely in ECs.

After corn oil or tamoxifen treatment, Shc 3F;Cdh5-CreERT2 mice were subjected to FAL. Similar to Shc3F and Shc3F;Tie2-Cre mice, Shc3F;Cdh5-CreERT2 mice experience almost complete loss of plantar perfusion in the ligated paw post-surgery (Figure 3.3A). By 7d post-surgery, both corn oil and tamoxifen treated mice displayed 25% recovery. However, at 14d and 21d post-surgery, the control mice exhibit significantly increased plantar perfusion in the ligated paw. Reperfusion reaches maximal levels in control mice at 55%, compared to 35% for tamoxifen treated mice (Figure 3.3B). These results demonstrate that endothelial, not hematopoietic, Shc phosphorylation is required for reperfusion after hindlimb ischemia.

**Shc phosphorylation is required for collateral remodeling after FAL**

Hindpaw reperfusion post FAL is mediated by outward remodeling of collateral vessels in the gracilis muscle of the leg(Schaper and Scholz, 2003). To determine whether growth of these vessels was impaired in Shc3F;Tie2-Cre mice, morphometry was performed in Hematoxylin and Eosin (H&E) stained collateral vessels from Shc3f and Shc3F;Tie2-Cre mice after 21d of recovery from FAL. The lumen area before surgery is similar in both genotypes and increases approximately 2.5-fold in control mice after FAL (Figure 3.4 A and B). In contrast, collateral growth in Shc3F;Tie2-Cre mice is blunted, increasing by 1.5-fold. These results demonstrate that endothelial Shc phosphorylation is required for collateral growth after FAL, providing a mechanistic link between blockade of Shc phosphorylation and reduction in perfusion recovery.

**Shc is required for atherosclerotic plaque formation**

Given the requirement for Shc in physiological vessel growth(Sweet et al., 2012), remodeling(Sweet et al., 2013), and shear-induced endothelial inflammation(Liu et al., 2008), we

hypothesized that Shc is required for the development of atherosclerosis in mice. To test this hypothesis, Shc fl/fl;ApoE-/-;Tie2-Cre mice were generated, resulting in an atheroprone endothelial-specific Shc knockout. To determine whether Shc is required for atherogenesis, Shc fl/fl;ApoE-/- and Shc fl/fl;ApoE-/-;Tie2-Cre mice were fed high fat diet (HFD) for 8 weeks, followed by perfusion fixation, removal of the aorta, and plaque visualization using Oil Red O (ORO) staining. ORO staining in control aortas revealed a heavy plaque burden in the aortic arch (Figure 3.5A). The largest concentrations of plaque occurred at branch points in the greater curvature of the arch, and throughout the endothelium of the lesser curvature of the arch. Sporadic plaque formation was seen in the descending aorta. In the Shcfl/fl;ApoE-/-;Tie2-Cre mice, overall plaque burden was reduced in the arch, while very little reduction was observed in the descending aorta. Plaque area was then calculated and quantified as part of the whole aorta, aortic arch, or descending aorta (Figure 3.5B). Overall plaque burden was significantly higher in control mice than in Shcfl/fl;ApoE-/-;Tie2-Cre mice, with approximately 4% plaque coverage in the whole aorta, versus 1.5% coverage in the Shcfl/fl;ApoE-/-;Tie2-Cre mice. The largest difference was observed in the aortic arch, where control mice exhibited 10.5% plaque coverage, versus 4% coverage in Shcfl/fl;ApoE-/-;Tie2-Cre mice. No difference was observed in the descending aorta. Overall, these results demonstrate a requirement for Shc in atheroma formation, suggesting a further role for Shc in shear-induced EC signaling.

**Endothelial Shc is required for acute plaque formation after partical carotid artery ligation**

The aorta studies establish a role for Shc in atheroma formation. However, no distinction is made between a requirement for Shc in atherogenic shear stress responses and other EC inflammatory signaling pathways. To test the requirement for endothelial Shc in atherogenic endothelial mechanosignaling, partial carotid artery ligation (CAL), a model of acutely induced disturbed flow, was utilized. Control and Tie2-Cre mice were subjected to CAL, followed by HFD feeding for 4 weeks. After the feeding period, the carotid arteries were removed and the plaques burden was visualized by ORO staining. ORO staining revealed large, complex plaques in control mice, while plaques in Tie2-Cre mice were smaller and less complex (Figure 3.6A). Additionally, plaque burden in control mice was 77% of lumen area, while plaques covered 48% of lumen area in

Tie2-Cre mice (Figure 3.6B). These results suggest that endothelial Shc is required for shear-induced plaque formation in carotid arteries.

## DISCUSSION

Inflammatory signaling is critical for the progression of physiological and pathological vascular remodeling. Physiologically, collateral arterioles increase in size in response to major vessel occlusion(Schaper, 2009). This increase results from shear-induced endothelial inflammation, which begins a program of leukocyte recruitment, smooth muscle proliferation, and size increase of collateral vessels(Z. Chen et al., 2010; Schaper, 2009; Sweet et al., 2013). In a pathological situation such as in atherosclerosis, shear-induced inflammation primes the endothelium for plaque formation by promoting lipid uptake, oxidative stress, and inflammatory gene expression(Chatzizisis et al., 2007; Hahn and Schwartz, 2009).

Previous work demonstrates the importance of Shc in endothelial inflammation(Liu et al., 2008; Sweet et al., 2013) and collateral remodeling(Sweet et al., 2013). Using two models of inflammatory vascular remodeling, this study further defines the role of Shc in vascular remodeling and disease. By blunting endothelial Shc phosphorylation using *Shc3F;Tie2-Cre* mice in a model of hindlimb ischemia, we demonstrate a requirement for Shc phosphorylation in collateral remodeling. Second, using *Shcflox/flox;ApoE-/-;Tie2-Cre* mice, which are an atheroprone endothelial Shc knockout, we demonstrate a role for Shc in atherosclerosis in the aorta and in ligated carotid arteries.

Collateral remodeling is a shear-directed process. Collateral vessels normally experience no net blood flow, but after nutritive vessel occlusion, a major increase in blood flow and shear stress occurs(Eitenmuller, 2006; Schaper and Scholz, 2003). This acute change in shear causes an increase in endothelial proliferation and NF-κB activation, both of which require Shc(Sweet et al., 2013). Crucially, Shc is highly phosphorylated in collateral vessels within 24 hours of nutritive vessel occlusion(Sweet et al., 2013). Thus we hypothesized that phosphorylated Shc plays a crucial role in the endothelial shear stress response, and therefore collateral remodeling. To this end, we used Shc phosphorylation-null mice in a model of hindlimb ischemia to determine whether Shc phosphorylation is required for arteriogenesis. Our results show that 21 days after FAL, our phosphorylation-null mice

exhibited significantly lower plantar perfusion in the ligated paw, compared to control mice. Additionally, we observed smaller collaterals in *Shc3F;Tie2-Cre* mice after 21 days of FAL. Though the mechanisms at work remain unclear, these data suggest a role for Shc tyrosine phosphorylation in recovery from FAL. Outstanding questions remain about the role of Shc phosphorylation in arteriogenesis. Specifically, is Shc phosphorylation required for the host of signaling that leads to vessel growth, such as endothelial inflammation, endothelial proliferation, and leukocyte recruitment? These processes are all abrogated when endothelial Shc is knocked out(Sweet et al., 2013), but whether they specifically require Shc phosphorylation remains unknown.

This study also addresses the role of Shc in the progression of atherosclerosis. While Shc is known to be required for endothelial inflammation *in vitro*(Liu et al., 2008) and arteriogenesis *in vivo*(Sweet et al., 2013), its involvement in a chronic inflammatory condition such as atherosclerosis has not been tested. Using *Shc fl/fl;ApoE-/-;Tie2-Cre* mice, this study establishes a requirement for Shc in atherosclerosis. Specifically, we show that the incidence of atherosclerosis in the aortic arch, an area of the aorta that experiences disturbed flow, sharply decreases in *Shc fl/fl;ApoE-/-;Tie2-Cre* mice. Additionally, we demonstrate that atherogenesis is blunted in *Shc fl/fl;ApoE-/-;Tie2-Cre* mice after acute application of disturbed shear stress using CAL. Taken together, these data demonstrate that endothelial Shc is required atherogenesis, and that Shc is a major component of the disturbed shear-responsive signaling pathway that gives rise to atherosclerosis.

While these data suggest a role for Shc in plaque formation, the underlying mechanisms remain unknown. Atherosclerosis occurs as cholesterol and immune cells accumulate under the endothelium, processes which both rely on high endothelial inflammation and permeability(Chatzizisis et al., 2007). Given its known role in endothelial inflammation(Liu et al., 2008), the observed reduction in plaque size may be a result of reduced endothelial inflammation. Additionally, cell spreading and focal adhesion arrangement are aberrant in Shc knockout ECs(K. M. Lai and Pawson, 2000), raising the possibility that Shc may also regulate endothelial permeability through regulation of the actin cytoskeleton. Furthermore, p66Shc is a known regulator of reactive oxygen species (ROS) generation(Migliaccio et al., 1999) and is phosphorylated in response to oxidized low density lipoprotein (oxLDL) treatment(Y. Shi et al., 2014). p66Shc also promotes atherosclerosis(Martin-

Padura et al., 2008), raising the possibility that knockout of all ShcA isoforms may repress mechanical and biochemical signaling pathways which promote atherosclerosis.

## MATERIALS AND METHODS

**Mouse Lines** Shc floxed and Shc3F mice were a kind gift from Kodi Ravichandran of the University of Virginia (L. Zhang et al., 2002). ApoE-/- mice were a kind gift from Nobuyo Maeda of the Univeristy of North Carolina- Chapel Hill(S. H. Zhang et al., 1992). Cdh5-CreERT2 mice were a kind gift from Ralph Adams of The Max Planck Institute for Biomedicine (formerly of Cancer Research UK). Tie2-Cre mice were purchased from Jackson Labs. All housing, breeding, and experimental procedures were done in accordance with national guidelines, and were approved by the Institutional Animal Care and Use Committee at the University of North Carolina- Chapel Hill. For FAL experiments, Shc3F, Shc3F;Tie2-Cre, and Shc3F;Cdh5-CreERT2 age-matched littermates were used between the ages of 10 and 14 weeks. For long-term HFD studies, Shcfl/fl;ApoE-/- and Shcfl/fl;ApoE-/-;Tie2-Cre age-matched littermates were used at 8 weeks of age. For CAL experiments, Shcfl/fl;ApoE-/- and Shcfl/fl;ApoE-/-;Tie2-Cre age-matched littermates were used at 8 weeks of age. Genotyping was performed by toe clip on neonatal pups. Analysis was carried out by observers blind to genotype.

**Femoral Artery Ligation Surgery** The surgical procedure was described previously (REF). Animals were anesthetized with 1.125% isofluorane mixed with oxygen. While under anesthesia, body temperature was maintained by a 37° heating pad. Before the incision, hair was removed from the hindquarters with a depilating cream. Using aseptic technique, the femoral artery was then exposed through a 2mm incision. After isolation from the vein and nerve, the femoral artery was ligated with 7-0 sutures proximal to the bifurcation of the popliteal artery and distal to the lateral caudal femoral artery. The artery was then severed between the sutures to further prevent blood flow. After irrigation of the wound with sterile saline, the incision was closed and the mouse was allowed to recover in a large, warmed chamber.

**Laser-Doppler Imaging** Mice were anesthetized using 1.5% isoflurane, and maintained at this level for the duration of scanning. After initial anesthesia, the mouse was transferred to a 37° heat block to maintain body temperature. 1.5% isoflurane was continuously supplied via a nosecone. The core temperature of the mouse was maintained between 36.5° and 37.5° to ensure consistent vascular tone between mice. The paws of the mouse were gently taped flat to the heating block to ensure identical positioning, and a Laser-Doppler imager (Perimed PeriScan PIM3) was used to measure relative blood flow. Values from ligated and unligated paws were obtained before and immediately after surgery, 24h, 3d, 5d, 7d, 14d, and 21d post-surgery. Plantar perfusion was determined within anatomically defined regions of interest (ROIs), which were drawn by an investigator blind to genotype.

**Collateral Vessel Morphometry** To harvest collateral vessels, animals were transcardially perfused with PBS containing 10nmol/L sodium nitroprusside and 10U/ml heparin after 3 weeks of hindlimb ischemia. After PBS perfusion, 2% paraformaldehyde was perfused for 20 minutes. The anterior and posterior gracilis muscles were then harvested and fixed for 16 hours in 2% PFA. A 5mm section was trimmed from the center of the gracilis muscle and embedded in paraffin. This was then sectioned into 5μm sections and stained with H&E. These H&E stained cross-sections were then imaged at 60x magnification on an Olympus IX-81 microscope. The circumference of the lumen was then traced using NIH ImageJ. At least 3 collaterals were measured from the ligated and unligated muscles, and then an average lumen area was computed for each animal. Morphometry was performed by an investigator blinded to mouse genotype.

**Tamoxifen Administration** Sterile 20mg/ml Tamoxifen (Sigma Aldrich, T5648-1G) and corn oil were prepared and stored at -20 until use. Mice received 2mg/kg tamoxifen or an equivalent amount of corn oil via intraperitoneal injection once daily for three days. Mice were used one week after the cessation of tamoxifen administration to allow for induction of Cre-mediated recombination.

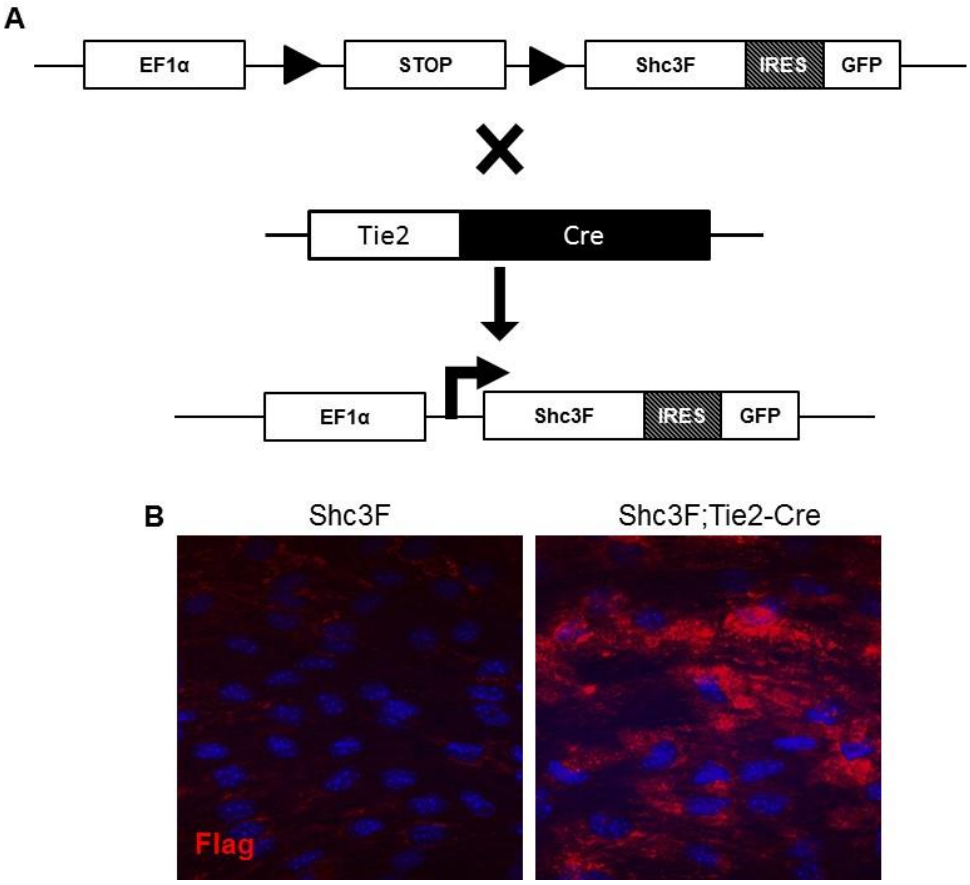
**Partial Carotid Artery Ligation** Partial carotid artery ligation was performed as described previously(Z. Chen and Tzima, 2009; Nam et al., 2009). Briefly, Shcfl/fl;ApoE-/- or Shcfl/fl;ApoE-/-

;Tie2-Cre mice were anesthetized using 1.5% isoflurane. Hair around surgery area was removed using a depilating cream. Using sterile technique, a 2mm incision was made into the neck and the left external, occipital, and internal carotid arteries were isolated and ligated using 7-0 sutures. The thyroid artery was left unligated, which maintained approximately 10% blood flow through the ligated carotid artery. The wound was then closed, and buprenorphine was administered as an analgesic.

**High Fat Diet Studies** For long term high fat diet studies, 8-week old Shc fl/fl;ApoE-/- and Shc fl/fl;ApoE-/-;Tie2-Cre mice were fed high fat diet (Harlan Teklad TD.88137, 42% of calories from fat, 0.2% cholesterol) ad libidum for 8 weeks, following dissection of the aorta and collection of blood for lipid profiling. For CAL studies, 8-week old Shc fl/fl;ApoE-/- and Shc fl/fl;ApoE-/-;Tie2-Cre mice were fed HFD ad libidum for 4 weeks, before fixation and removal of carotid arteries.

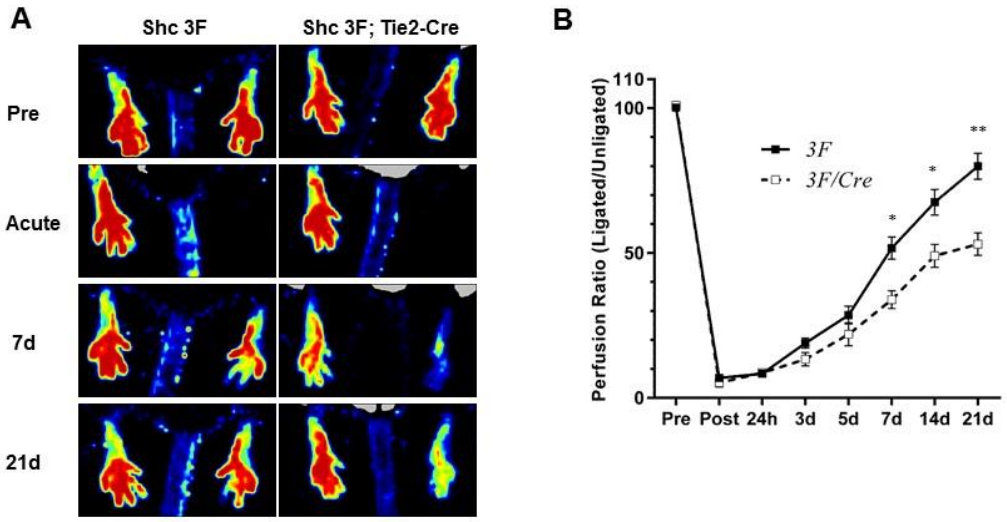
**Plaque Area Quantification** *Aortic measurements:* After high fat diet feeding, aortas were removed from mice, stained with ORO, and mounted en face in black wax for imaging. Images were obtained using a handheld digital camera. Aortic plaque burden was determined by tracing plaques and measuring their surface area using NIH ImageJ software. Total aortic area was determined in the same manner. To measure total plaque burden, the total plaque area was divided by total aortic area. For site-specific plaque burden measurements, the aorta was divided into the arch region and descending regions. The first intercostal branch was used as the beginning of the descending region. *Carotid artery measurements*: Measurements were performed as previously reported(Nam et al., 2009). Briefly, after surgery and high fat diet feeding, carotid arteries were perfusion fixed and removed from mice, followed by cryopreservation on 30% sucrose for 16h. After cryopreservation, the carotids were embedded in OCT and sectioned. Sections from within 2mm of the apex of the carotid ligation were taken and stained with Oil Red O and Hematoxylin. Using NIH ImageJ software, the circumferences of the internal elastic lamina (IEL) and lumen were measured. Values were expressed as percent of total lumen area filled with plaque. Plaque area was calculated by subtraction of lumen area from the area within the IEL, and percent plaque area was calculated by dividing plaque area by the total area inside the IEL.

**Figure 3.1 The Shc phospho-null mutant construct**



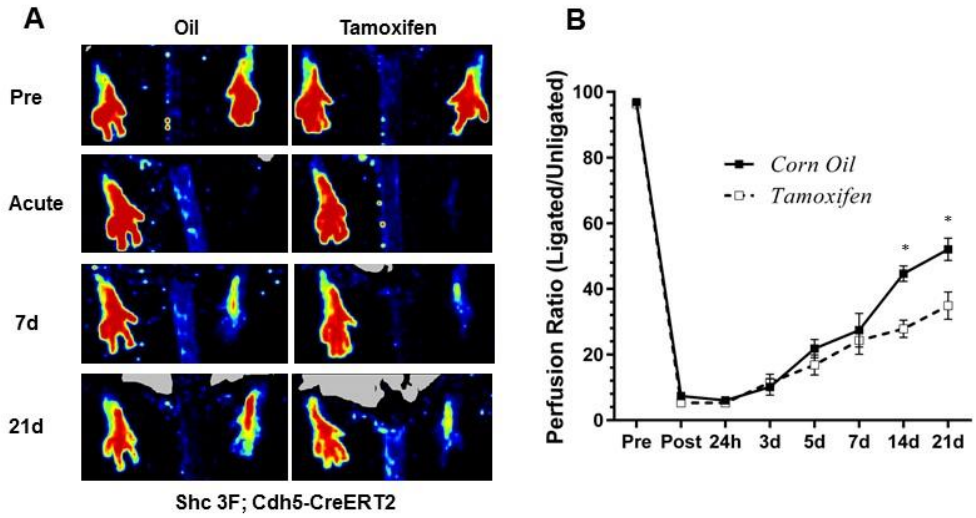

(A) Depiction of the Shc phospho-null mutant construct. The central aspect of the construct is a FLAG-tagged ShcA allele with Tyr239/240 and 317 mutated to Phe, rendering the end product phospho-null. The construct co-expresses GFP via an internal ribosomal entry site, and is driven by the human EF1α promoter. A floxed stop sequence intervenes between the promoter and coding region. When Shc3F mice express Tie2-Cre, the stop sequence is excised and the Shc3F construct is expressed. (B) Immunofluorescence for FLAG shows robust staining in the endothelium of Shc3F;Tie2-Cre mice, but not Shc3F mice.

**Figure 3.2 Shc tyrosine phosphorylation is required for plantar reperfusion after FAL**



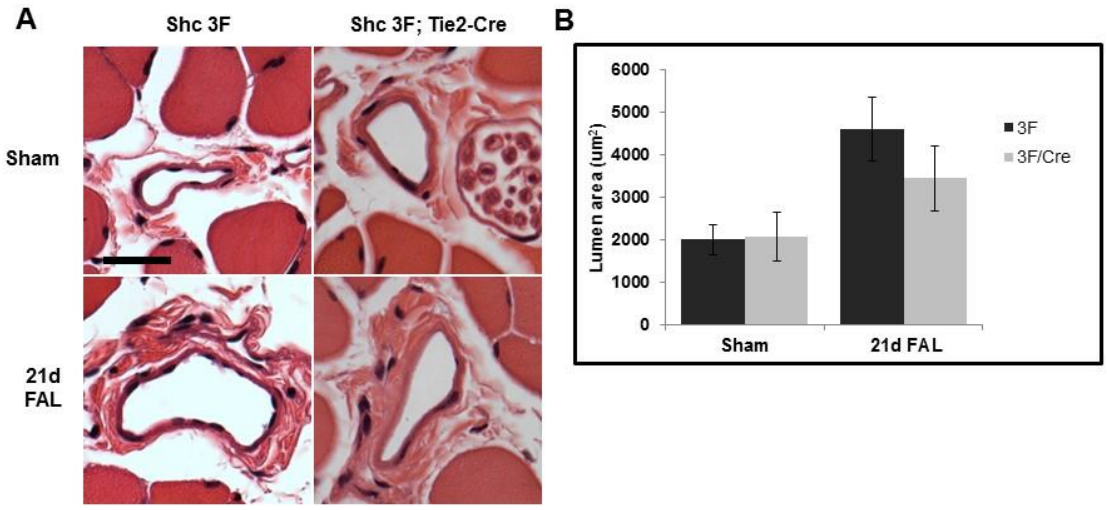

(A) Plantar reperfusion is blunted in Shc3F;Tie2-Cre mice as compared to Shc3F littermates. Representative Laser Doppler images from Shc3F and Shc3F;Tie2-Cre mice before (Pre), immediately after (Acute), 7 days after, and 3 weeks after femoral artery ligation. The images are pseudocolored: Black = 0 and bright red = 1000 arbitrary units. (B) Ratio of plantar perfusion (ligated versus sham paw), as quantified from the Laser Doppler image. The graph represents the mean reperfusion ± SEM. * = $p<0.05$; ** = $p<0.01$. n=11 Shc 3F and 10 Shc 3F/Tie2-Cre mice.

**Figure 3.3 Endothelial, not hematopoietic, Shc is required for plantar reperfusion after FAL**



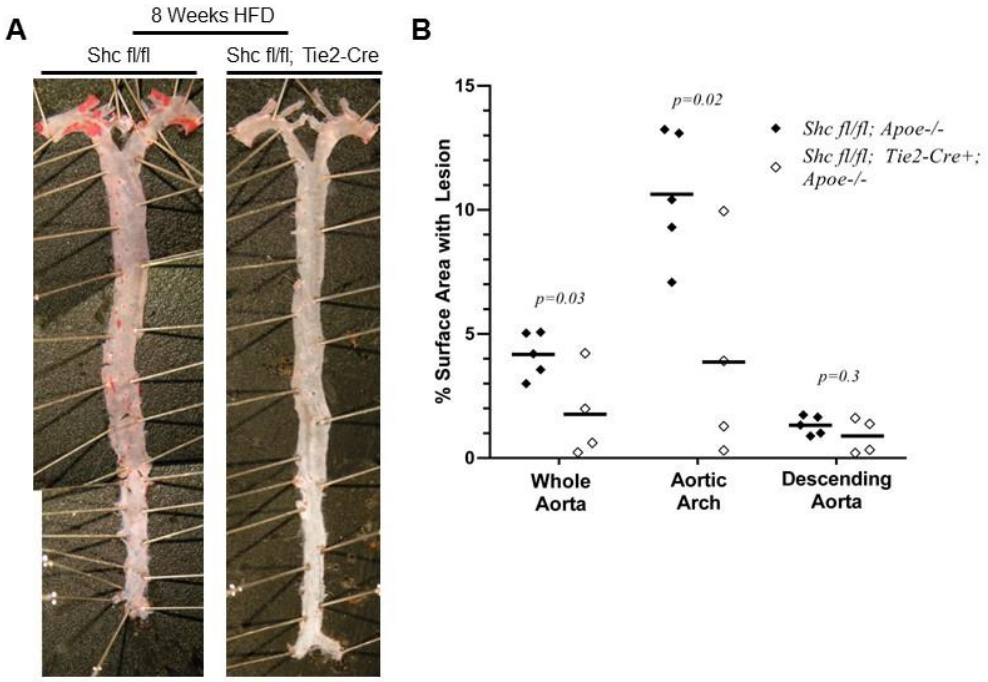

(A) Plantar reperfusion is blunted in Shc3F;Cdh5-CreERT2 mice treated with Tamoxifen as compared to Corn Oil-treated littermates. Representative Laser Doppler images from Corn Oil- and Tamoxifen-treated Shc3F;Cdh5-CreERT2 mice before (Pre), immediately after (Acute), 7 days after, and 3 weeks after femoral artery ligation. The images are pseudocolored: Black = 0 and bright red = 1000 arbitrary units. (B) Ratio of plantar perfusion (ligated versus sham paw), as quantified from the Laser Doppler image. The graph represents the mean reperfusion ± SEM. * = $p<0.05$. n= 8 Corn Oil and 9 tamoxifen treated mice.

**Figure 3.4 Shc tyrosine phosphorylation is required for collateral remodeling in response to FAL**



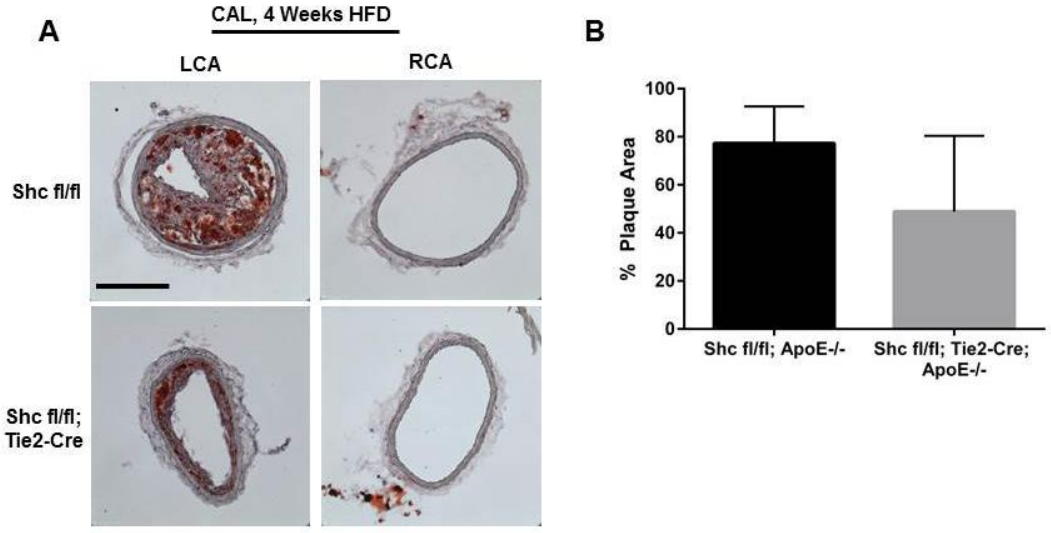

(A) Blunted arteriogenesis in Shc3F;Tie2-Cre mice. The gracilis muscle was harvested from Shc3F and Shc3F;Tie2-Cre mice 21d after FAL surgery. The muscle was sectioned and stained with H&E, and collateral circumference was measured. 2 collaterals per condition per mouse were measured. (B) Lumen area was calculated from the collateral measurements. Sham: n= 6 Shc 3F and 5 Shc 3F/Tie2-Cre mice; Ligated: n= 5 Shc 3F and 4 Shc 3F/Tie2-Cre mice.

**Figure 3.5 Endothelial Shc is required for atherogenesis**




(A) Shc fl/fl and Shc fl/fl;Tie2-Cre mice were fed high fat diet for 8 weeks, followed by isolation of the aorta and ORO staining. Aortas were mounted whole and imaged with a digital camera. (B) Plaque area was quantified. All plaques were measured, and then subdivided based on location in the aorta. The perimeter of the whole aorta was used for % surface area measurements. Horizonal black bars represent the mean. n = 5 Shc fl/fl and 4 Shc fl/fl;Tie2-Cre mice.

**Figure 3.6 Endothelial Shc is required for atherogenesis in response to altered hemodynamics**




(A) Shc fl/fl and Shc fl/fl;Tie2-Cre mice were subjected to CAL surgery, followed by high fat diet feeding for 4 weeks. After 4 weeks of recovery, the carotid arteries were harvested, sectioned, and stained with ORO. (B) Plaque area was quantified. n= 3 Shc 3F and 6 Shc 3F/Tie2-Cre mice.

# Chapter 4 Discussion and Future Perspectives

## OVERVIEW

It is well established that PECAM-1 is an endothelial mechanosensor that is required for inflammatory and anti-inflammatory endothelial phenotypes(Tzima et al., 2005). PECAM-1-dependent mechanosignaling presents a quandary for vascular biologists. It is at once beneficial and detrimental to vascular health(Goel et al., 2008; Harrison et al., 2013b; Harry et al., 2008; Stevens et al., 2008); a critical player in endothelial mechanosignaling that is seemingly dispensable for development, as PECAM-1 null mice are born healthy and at normal Mendelian ratios(Duncan et al., 1999; Tzima et al., 2005). However, PECAM-1 plays a critical role in processes such as vascular remodeling(Z. Chen and Tzima, 2009), arteriogenesis(Z. Chen et al., 2010), and atherosclerosis(Harry et al., 2008). Therefore, understanding the signaling pathways downstream of PECAM-1 activation is critically important for understanding the biology of PECAM-1 and designing vascular therapeutics based in endothelial mechanosignaling. This thesis provides mechanistic insights into how initiation of PECAM-1 signaling can lead to regulation of vascular phenotypes. Specifically, Chapter II of this thesis describes a mechanism by which PECAM-1 regulates FN assembly through EC-generated tension and integrin binding. As FN is known to be a pro-inflammatory stimulus(Feaver et al., 2010; Orr et al., 2005) for ECs and a scaffold for assembly of other ECM molecules, Chapter II identifies a mechanism by which shear stress may regulate endothelial inflammation through assembly of FN fibrils. Additionally, Chapter III presents data which describe a role for the adaptor protein Shc, a mechanotransducer activated downstream of PECAM-1/VE-Cadherin/VEGFR2 mechanosignaling(Liu et al., 2008), in vascular inflammation. My data suggest that Shc tyrosine phosphorylation is required for arteriogenesis, and that Shc is required for the formation of atherosclerotic plaques. These results

suggest that Shc may regulate inflammation in ECs, providing a potent target for vascular therapeutics for pathologies arising from aberrant endothelial mechanosignaling. Taken as a whole, these data provide novel insights into the mechanisms of endothelial mechanosignaling, but also open up many unanswered questions. These questions are discussed in further detail below.

## CHAPTER 2: HAEMODYNAMICS REGULATE FIBRONECTIN ASSEMBLY VIA PECAM

The aim of this chapter was to determine whether PECAM-1 regulates FN assembly in response to hemodynamics. To that end, we subjected PECAM-1 null mice to CAL surgery and utilized PECAM-1 KO cells to determine how PECAM-1 might regulate FN assembly. This study found that PECAM-1 is required for FN assembly in areas of laminar and disturbed flow, and that FN assembly requires cell contractility regulated by integrin binding and RhoA. Additionally, we found that expression of FN is dramatically reduced in KO cells and that when exogenous FN was provided to KO cells, the cells were unable to assemble FN into fibrils. This suggests that PECAM-1 is required for cellular contractility that induces FN fibrillogenesis.

### Hemodynamic Regulation of FN Assembly

A novel insight in this body of work is that PECAM-1 is required for FN fibrillogenesis. This is the first time that the specific mechanism linking hemodynamics and FN assembly has been delineated, though previous studies suggested a link between hemodynamics and FN. Evolutionarily, FN is thought to have arisen with the vasculature(Hynes, 2007), which suggests close involvement of FN with vascular function, including the possibility of FN interacting with the physical forces that impinge on the endothelium. Additionally, early work examining ECM assembly in the abdominal aorta revealed that in areas of laminar flow, FN fibrils align in the direction of flow, while in areas of disturbed flow, these fibrils exhibit a random pattern of alignment(Jinguji and Fujiwara, 1994). Furthermore, it has been shown PECAM-1, a well-known mechanosensor(Tzima et al., 2005), regulates FN expression(Feaver et al., 2010).

Neointima formation in PECAM-$1^{-/-}$ mice is blunted when these mice are subjected to CAL. This reduction in neointima formation is a result of impaired NF-κB signaling, a hallmark of vascular

inflammation(Z. Chen and Tzima, 2009). FN is required for both short term(Liu et al., 2008; Orr et al., 2005) and long term(Chiang et al., 2009b) inflammatory phenotypes in the vasculature, and assembled FN is required for shear-induced endothelial inflammation(Chiang et al., 2009a). Additionally, deposition of FN induces a positive feedback loop that sustains endothelial inflammation(Feaver et al., 2010). Therefore it is tempting to speculate that regulation of FN assembly and expression by PECAM-1 modulates endothelial phenotype via control of initiation and continuation of endothelial inflammation. If FN assembly is blocked, endothelial inflammation and attendant FN expression do not occur, and therefore cannot sustain chronic inflammation in the presence of altered hemodynamics. This potentially has profound consequences for vascular physiology, including alterations in endothelial permeability and inflammation, and pathologies such as atherosclerosis.

**FN Assembly and EC Permeability**

In ECs, FN induces several matrix-specific signaling events that are associated with vascular pathologies like atherosclerosis. ECs exhibit specific inflammatory and anti-inflammatory phenotypes; FN may be part of the switch between anti-inflammation and inflammation. Though ECs normally reside on basement membrane consisting of Collagen (CL) and Laminin (LM), FN is deposited in response to inflammatory insults like disturbed flow(Feaver et al., 2010; Orr et al., 2005). The deposition of FN helps initiate and sustain inflammatory EC phenotypes(Feaver et al., 2010). FN deposition also increases endothelial permeability, a process that is regulated by p21-activated kinase (PAK)(Orr et al., 2007). Importantly, the increase in endothelial permeability is specific to FN matrix and does not occur on CL(Orr et al., 2007). Matrix-specific regulation of PAK activity by protein kinase A (PKA) also occurs. Basement membrane promotes PKA activation and concomitant PAK repression, while FN matrix promotes PAK activation through repression of PKA(Funk et al., 2010). Taken together, these results introduce a major feature of FN deposition and assembly—FN is necessary for induction of inflammatory endothelial phenotypes, and its expression can have major consequences for vessel health. For example, increases in endothelial permeability are associated with atherogenic phenotypes and with areas of disturbed flow in the vasculature(Himburg et al.,

2004). Sites of disturbed flow are also associated with increased LDL accumulation(HERRMANN et al., 1994; Sakellarios et al., 2013). Therefore, it is tempting to hypothesize that reduced FN expression and assembly in PECAM-$1^{-/-}$ mice serves as an atheroprotective mechanism, and works through modulation of junctional integrity in the endothelium.

**FN Assembly and Vessel Stiffness**

PECAM-1 is also known to regulate eNOS signaling; in PECAM-$1^{-/-}$ cells, eNOS activation is misregulated(McCormick et al., 2011). This may have consequences for FN assembly and matrix stiffness, as eNOS activity represses the activity of tissue transglutaminase (tTG), a critical crosslinker of ECM proteins. Repression is achieved through production of NO species, which lead to S-nitrosylation (SNO) of tTG, thus preventing its extracellular crosslinking function(T. S. Lai et al., 2001). Critically, tTG activity enhances, but is not sufficient for, FN assembly which is completely blocked through inhibition of α5β1 integrins(Akimov and Belkin, 2001; L. Yuan et al., 2007). Overactivation of tTG has also been implicated in the vascular processes of vascular remodeling and age-related vascular stiffening. In porcine carotid arteries cultured in vitro, tTG activity was required for the lumen narrowing that is characteristic of vascular remodeling(Bakker et al., 2005). Importantly, stimulation of tTG expression increased the narrowing of the artery lumen, while inhibiting tTG function using cadaverine completely blocked lumen narrowing(Bakker et al., 2005). Similar results were found in a study examining age-related stiffening in rat arteries. tTG-null mice show impaired matrix crosslinking, and do not stiffen their arteries in response to eNOS inhibition, which removes blockade of tTG activation in mice(Santhanam et al., 2010). Matrix crosslinking also increased with age in WT mice, while tTG expression did not. A reduction in age-related matrix crosslinking was observed when tTG was inhibited(Santhanam et al., 2010). Additionally, tTG activity increases in eNOS null mice, leading to increased matrix crosslinks and stiffer vessels in the absence of a major repressor of tTG(S. M. Jung et al., 2013). These data suggest that the endothelial dysfunction that attends age may remove barriers to vessel stiffening.

Given that PECAM-1 knockout leads to abberant eNOS activation, it is tempting to hypothesize that PECAM-1-/- mice inappropriately repress tTG function, therefore leading to further

deficits in FN assembly. Data supporting this hypothesis are slim, however I performed immunofluorescence studies for SNO levels in PECAM-$1^{-/-}$ and WT carotid arteries after the mice were subjected to carotid artery ligation. 24h after ligation, WT mice show an approximately 1.5-fold increase in SNO in ECs, which returns to baseline by 5 days post-ligation. The PECAM-$1^{-/-}$ mice, however, show an approximately 2.5-fold increase in SNO through 3 weeks of recovery post-ligation. These data suggest that a defect in matrix crosslinking may augment the observed defect in FN assembly. However, more work is needed to delineate the mechanism.

Does PECAM-1 regulate age-related vascular stiffening? If the relationship between PECAM-1 and tTG is supported by more data, it may provide a robust link between hemodynamics and age-related vascular stiffening. This would be an important discovery, as vascular stiffness is a potent contributor to devastating vascular pathologies such as atherosclerosis, hypertension, and aortic dissection. It has been shown that PECAM-1 regulates endothelial stiffness in a matrix-specific manner, as ECs stiffen in response to direct force on PECAM-1 on FN but not CL(Collins et al., 2014; 2012). Additionally, matrix selectivity in EC stiffness translates *in vivo*. Using passive microbead rheology, it was shown that ECs are stiffer in areas of higher FN content, namely the aortic arch, versus areas of higher CL content like the descending aorta. (Collins et al., 2014). A similar technique could be used on aortas from PECAM-$1^{-/-}$ mice in order to determine the relative stiffness of ECs in areas of higher FN. Though the relationship between endothelial stiffness and matrix stiffness is unclear, it is reasonable to speculate that endothelial stiffness may reflect matrix stiffness. Endothelial stiffness arises as a result of highly activated RhoA, the same pathway that is highly activated in ECs when cultured on stiff substrates(Collins et al., 2012; Huynh et al., 2011). Understanding the relationship between hemodynamics, endothelial mechanosensing, and vascular stiffness is an important challenge moving forward. As the mechanisms of shear sensing become more well understood, it is important that findings from studies of shear stress be integrated with studies of other mechanical functions of vessels. This will allow a more holistic view of the vessel to emerge, where mechanical and biological factors are studied together in order to understand physiology and better design therapeutics for vascular disease.

## CHAPTER 3: THE ADAPTOR PROTEIN SHC IS A MAJOR REGULATOR OF ENDOTHELIAL INFLAMMATION

The aim of this chapter was to determine the specific role of endothelial Shc in acute and chronic vascular inflammatory remodeling. I tested the role of Shc tyrosine phosphorylation in arteriogenesis, which is the outward remodeling of collateral arteries, and found blunted arteriogenesis in Shc phosphorylation-null mice. Additionally, I tested the role of Shc protein in the formation of atherosclerotic plaques, finding a significant reduction in plaque burden in endothelial Shc-null mice. These results demonstrate a central role for Shc, and specifically Shc tyrosine phosphorylation, in inflammatory remodeling in the vasculature. However, the present study raises many important questions, which are discussed below.

### The role of Shc tyrosine phosphorylation in endothelial inflammation and atherogenesis

The work presented in Chapter III utilize a knock-in mouse that expresses a Shc phosphorylation-null mutant (Shc3F) protein after Cre recombinase-mediated excision of a stop cassette. In Chapter III, these mice were used to test the requirement of Shc phosphorylation in arteriogenesis. My data show a requirement for Shc tyrosine phosphorylation in arteriogenesis, and acute inflammatory condition of the vasculature(Schaper, 2009).

In murine arteriogenesis, vessel inflammation subsides after flow is normalized, a period of time lasting two to three weeks(Sweet et al., 2013). Shc phosphorylation occurs briefly at the outset of arteriogenesis, in agreement with *in vitro* studies of ECs exposed to laminar shear stress showing transient Shc phosphorylation at early shear time points(Liu et al., 2008; Sweet et al., 2013). Interestingly, Shc phosphorylation is not always transient. In areas of the vasculature that normally experience disturbed flow, such as the lesser curvature of the aortic arch or the fibrous cap of an atherosclerotic plaque, Shc phosphorylation remains high(Liu et al., 2008). This difference in Shc phosphorylation between areas of different flow regimes raises the possibility that Shc phosphorylation acts as a switch between inflammatory and anti-inflammatory signaling pathways in ECs. In such a system, Shc would induce inflammatory flow patterns downstream of disturbed flow, ceasing such induction after the normalization of inflammatory flow. Thus, if disturbed flow is chronic,

then Shc would continue to be activated and endothelial inflammation would persist. If Shc phosphorylation is blocked, endothelial inflammation would also be blocked.

My results support this model, while underscoring the need for further studies. In the HLI model, loss of Shc phosphorylation blunted arteriogenesis. My immunofluorescence data suggest that this blunted arteriogenesis was attended by a reduction in NF-κB signaling, demonstrating a role for Shc phosphorylation in endothelial inflammation. To extend these findings, the role of Shc phosphorylation in arteriogenesis must be delineated further through *in vitro* and *in vivo* methods. A hallmark of arteriogenesis, macrophage accumulation(Schaper, 2009; Sweet et al., 2013), will be assessed around growing collaterals to determine whether loss of Shc phosphorylation leads to loss of immune cell recruitment. Additionally, ICAM-1 and VCAM-1, the cell surface proteins intimately involved in recruitment of immune cells, expression should be assayed in post-HLI collaterals. Should deficits be found in all of these areas in Shc3F;Tie2-Cre mice, it would demonstrate a profound defect in the endothelial inflammatory response to shear stress, placing Shc at the center of inflammatory signaling. Another *in vivo* aim is to test the role of Shc phosphorylation in atherogenesis, a chronic inflammatory condition where endothelial Shc phosphorylation is also chronically high. Using Shc3F;Cdh5-CreERT2;ApoE-/- mice, this line of experimentation is reasonable and would provide detailed mechanistic insight into a central regulator of endothelial shear signaling. In this model, if Shc phosphorylation is central to the endothelial inflammatory response, atherogenesis will be either reduced or completely blocked. These studies are necessary to delineate whether Shc phosphorylation is a cause endothelial inflammation, or is simply secondary to chronic disturbed flow. While my data and previous studies suggest that Shc phosphorylation is required for atherogenesis, the involvement of Shc phosphorylation in atherogenesis must be fully determined. If Shc phosphorylation is required for atherogenesis, such a result would provide more evidence that Shc is a central regulator of endothelial inflammation. It may also provide a target for future therapeutics. By blocking chronic endothelial inflammation, while allowing anti-inflammatory responses to persist in the endothelium, shear signaling might be used to combat atherogenesis and other pathological vessel remodeling. This also underscores the importance of understanding whether Shc is required for anti-

inflammatory shear signaling in ECs. If Shc does not regulate anti-inflammatory signaling, it may truly represent a switch between inflammatory and anti-inflammatory signaling in ECs.

Lastly, the role of p66Shc must be taken into account in vascular dysfunction. p66Shc is the largest isoform of ShcA, and is notably different because of the presence of its long N-terminal tail that contains a phosphorylation site, Serine 36(Wills and Jones, 2012). In response to oxidant stress, Serine 36 is phosphorylated by Protein Kinase C β (PKCβ), and localizes to the mitochondrial membrane under the guidance of Prolyl Isomerase 1 (Pin1)(Pinton et al., 2007). Once recruited to the mitochondria, p66Shc then stimulates apoptosis via interaction with cytochrome c(Wills and Jones, 2012). Importantly, p66Shc has a role in vascular disease, where it promotes age-related vascular dysfunction and advanced atherosclerosis(Francia et al., 2004; Martin-Padura et al., 2008). In aged mice, increased levels of superoxide production and nitrotyrosine species are present in the vasculature of WT mice as compared to p66Shc-null mice(Francia et al., 2004). Additionally, atherosclerotic plaque growth is reduced in p66Shc-null mice(Martin-Padura et al., 2008). This may be partly a result of oxidative stress induced by oxLDL avtivating p66Shc, which leads to uncoupling of eNOS signaling(Y. Shi et al., 2014). However, regulation of p66Shc by shear stress signaling remains poorly understood. As oxidative stress plays a major role in shear signaling and pathologies emanating from shear signaling, p66Shc is a worthwhile avenue of study, and could yield important insight into the regulation of endothelial inflammation by Shc.

**The role of downstream effectors on Shc signaling**

Shc phosphorylation is important because of its ability to effect multiple downstream signaling cascades. In the case of Shc, the best known effector is the adaptor protein Growth Factor Receptor Bound Protein 2 (Grb2). When bound to Shc, Grb2 leads to activation of the RasGEF Son-of-Sevenless (SOS), which potentiates downstream Ras signaling, including mitogen-activated protein kinase kinase (MEK) and extracellular signal related kinase (ERK) signaling(Ravichandran, 2001). The interaction between Shc and Grb2 has been shown to occur very early, approximately 5 minutes, after the onset of shear stress(K. D. Chen et al., 1999), but its overall function in shear signaling has not been fully explored. Additionally, Shc associates with integrins after the onset of shear stress, but

this association occurs later in shear signaling. Association with $\alpha_v\beta_3$ integrins occurs at 30 minutes after the onset of shear, while association with $\beta_1$ and $\beta_5$ integrins occurs after 1 hour of shear(K. D. Chen et al., 1999; Liu et al., 2008). In the context of shear, Shc phosphorylation occurs upstream of integrins and is VE-Cadherin dependent, as is Shc association with $\alpha_v\beta_3$ integrins(Liu et al., 2008). Shc phosphorylation, however, is independent of matrix composition, further suggesting that Shc is phosphorylated upstream of its interaction with integrins(Liu et al., 2008).

The consequences of the association of Shc with integrins remain to be explored in the context of shear signaling, and could be key in understanding the role of Shc phosphorylation in endothelial inflammation. EC adhesion to FN induces Shc association with $\alpha_5\beta_1$ integrins(Wary et al., 1996). This association leads to Shc phosphorylation, recruitment of Grb2, and cell cycle regulation. New integrin ligation is a shared attribute of cellular adhesion and shear stress signaling. Many shear responses are downstream of integrins, including regulation of RhoA and endothelial alignment in the direction of flow, both of which require new integrin ligations to FN(Tzima et al., 2001). Major inflammatory signaling, notably NF-κB-dependent transcription, also occurs downstream of integrins. This is evidenced by the matrix selectivity of NF-κB activation, which peaks at about 60 minutes after the onset of shear—a timescale similar to that of Shc binding to integrins(Liu et al., 2008; Orr et al., 2005). NF-κB activation is dependent on the small GTPase Rac1, the activation of which is dependent on new integrin binding to FN(Tzima et al., 2002). Another modulator of NF-κB activation, PAK, is also activated in a matrix-specific manner(Orr et al., 2008). Though shear-induced ROS production is matrix independent, PAK modulates this signaling and promotes NF-κB activation specifically FN and not CL(Orr et al., 2008). These data, taken together with evidence supporting a central role for Shc in regulating endothelial inflammation *in vitro* and *in vivo*(Liu et al., 2008; Sweet et al., 2013), suggest that Shc may play a major role in inflammatory signaling downstream of integrins. Perhaps phosphorylated Shc translocates and binds to integrins, altering the conformation of integrin-based signaling platforms and allowing NF-κB activation to occur in the process? In this case, Shc phosphorylation may not bind partners directly required for signaling downstream of integrins, but rather might be required for translocation. It is also possible that Shc may bind as yet undefined

partners in response to shear stress; mass spectrometry experiments may help in elucidating novel interactors with Shc.

Lastly, whether Shc regulates the actin cytoskeleton remains an open question. The regulation of cytoskeletal function by Shc is an important question, given that important endothelial phenotypes, such alignment in the direction of shear and permeability, are chiefly regulated by the actin cytoskeleton(Huynh et al., 2011; Tzima et al., 2001). To date, not enough work has been done to mechanisticly link Shc function to regulation of cytoskeletal phenotypes, especially in the context of shear stress. However, the data that do exist, mostly obtained in systems outside of the endothelium, suggest that Shc is involved in force-dependent regulation of the cytoskeleton. Fibroblasts isolated from ShcA-null mice display a hypercontractile phenotype, staying round and declining to spread when grown in tissue culture(K. M. Lai and Pawson, 2000). Also, since Shc associates with integrins after the onset of shear stress, it is possible that it participates in the regulation of cytoskeletal elements. In felines, Shc forms an insoluble complex on integrins with FAK after right ventricle pressure overload(Laser et al., 2000). A similar result was seen in cardiomyocytes in the presence of RGD peptide; Shc, FAK, and other proteins associate in focal complexes at integrins in response to stimulation with RGD(Laser et al., 2000). More recently, a study done in HUVECs demonstrated that FAK is required for the recruitment of Shc to integrins after the application of tension on integrins with FN-coated magnetic beads(Wu et al., 2016). Furthermore, p66Shc was shown to be required for activation of p115-RhoGEF and GEF-H1, as well as RhoA activation downstream of force on integrins(Wu et al., 2016). These data represent an excellent use of a reductionist system to delineate signaling relationships; however, the role of Shc in shear-induced cytoskeletal regulation remains to be uncovered.

The data presented in Chapter III underscore the physiological significance of Shc signaling in vascular inflammation. These data demonstrate specific requirements for Shc tyrosine phosphorylation in arteriogenesis, and for Shc in atherosclerosis. They also suggest a mechanism by which Shc may regulate shear responses based on the stimulus perceived, whether it be laminar or disturbed flow. Shear-induced inflammation and vascular remodeling occur as a result of altered hemodynamics, whether the insult is acute or chronic. The purpose of inflammatory remodeling is to

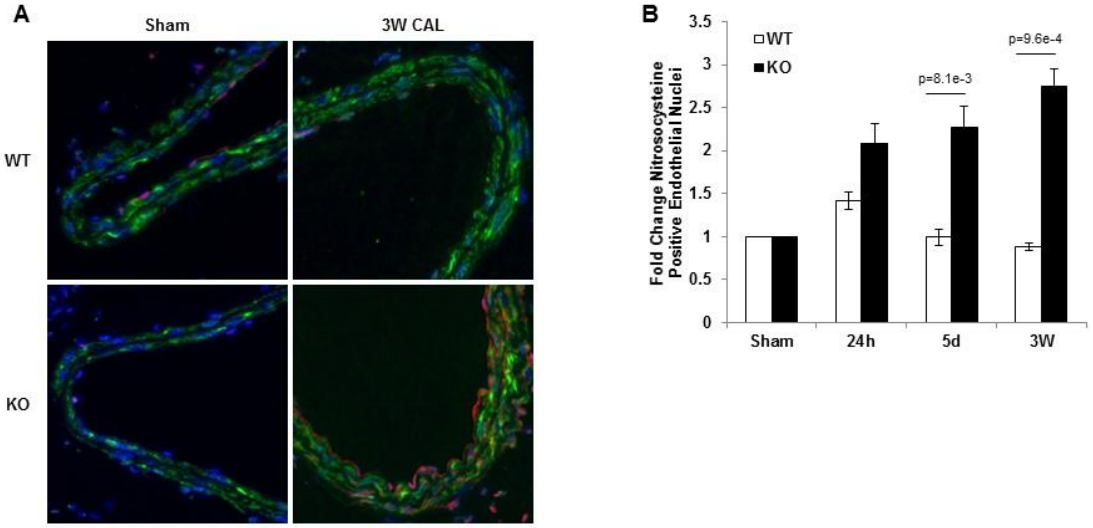
normalize flow conditions—in the case of arteriogenesis, reduction of shear stress to physiological levels is the end result(Schaper, 2009). Arteriogenesis is an example of outward vascular remodeling in response to high shear stress(Schaper and Scholz, 2003), where the extent of collateral remodeling is governed by the magnitude of shear forces experienced by collateral vessels(Eitenmüller et al., 2006; Pipp et al., 2004). Conversely, atherosclerosis is an example of pathological inward remodeling, which occurs in response to low and disturbed flow(Dajnowiec and Langille, 2007). Chronic disturbed flow initially causes outward remodeling of the vessel, but when plaques grow large enough, they eventually begin to block the vessel lumen, and lead to pathologies like heart attack, stroke or angina pectoralis(Dajnowiec and Langille, 2007; Hahn and Schwartz, 2009). Though arteriogenesis and atherosclerosis arise from different conditions in the vasculature, they share several basic mechanisms. Through endothelial inflammation, endothelial proliferation and leukocyte recruitment take place(Hahn and Schwartz, 2009; Schaper, 2009), leading to outward remodeling in arteriogenesis and inward remodeling in atherosclerosis. Taken together, the data in Chapter III and from previous studies suggest that Shc is a central regulator of endothelial inflammation, that acts as a critical endothelial mechanotransducer in response to altered hemodynamics. It is likely that *in vivo*, Shc is necessary for transduction of signals generated by inflammatory hemodynamics, and for the initiation and continuation of endothelial inflammation. Under physiological hemodynamic regimes, Shc signaling is reduced, which coincides with a reduction in endothelial inflammation.

## FINAL PERSPECTIVES

The study of vascular inflammation is of major importance for the treatment and curing of vascular diseases. The data in this thesis, along with demonstrating the role of PECAM-1 in regulation of FN assembly and the role of Shc in inflammatory vascular remodeling, demonstrate that vascular inflammation cannot be fully understood without taking endothelial mechanosignaling into account. Shear stress causes two broad phenotypes in ECs: inflammatory and anti-inflammatory. If an overarching goal exists in the field of shear stress research, it is to understand the signaling

pathways and gene expression signatures of both endothelial phenotypes, and to exploit those differences for use in the design and delivery of vascular therapeutics. This thesis provides substantial insight into two known regulators of vascular inflammation: FN matrix, and the adaptor protein Shc. Considering both subjects together presents an interesting question about the origin of endothelial inflammation. Flow-induced FN expression is NF-κB-dependent(Feaver et al., 2010), and endothelial Shc is required for NF-κB activation in response to shear stress(Liu et al., 2008; Sweet et al., 2013), so it follows that inflammation may originate with Shc signaling, downstream of PECAM-1 sensing altered hemodynamics. However, integrin ligation to FN is required for shear signaling downstream of integrins(Tzima et al., 2001), part of which includes Shc-dependent NF-κB activation, hence the uncertainty regarding the origins of endothelial inflammation. Regardless of the origins of inflammation, this thesis delves into signaling that sustains endothelial inflammation. This cascade is a positive feedback loop of inflammatory signals emanating from PECAM-1 and FN-bound integrins, integrated and processed by the adaptor protein Shc. Hopefully, the information contained herein, combined with many other studies, may provide a way to stop the inflammatory feedback that sustains the devastating vascular pathologies that plague human health.

**Figure 4.1 Altered nitrosocysteine production in PECAM-1 KO mice**




WT and PECAM-/- mice were subjected to CAL and harvested at 24 hours, 5 days, or 3 weeks post-CAL. (A) Cross sections of carotid arteries from WT and PECAM-$1^{-/-}$ mice were immunostained for S-nitrosocysteine, followed by quantification of nuclear S-nitrosocysteine. (B) Representative images from WT and PECAM-$1^{-/-}$ mice from the sham and 3 week timepoints. (A) and (B) Sham: n=5 WT and 4 PECAM-$1^{-/-}$ mice; 24 hours: n=5 WT and 4 PECAM-$1^{-/-}$ mice; 5 days: n=3 WT and 4 PECAM-$1^{-/-}$ mice; 3 weeks: n=4 WT and 3 PECAM-$1^{-/-}$ mice.

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