THE ADAPTOR PROTEIN SHC IS A CRITICAL REGULATOR OF ANGIOGENIC AND SHEAR STRESS SIGNALING IN ENDOTHELIAL CELLS

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ABSTRACT

DANIEL TIMOTHY SWEET: The Adaptor Protein Shc is a Critical Regulator of Angiogenic and Shear Stress Signaling in Endothelial Cells
(Under the direction of Ellie Tzima)

Endothelial cells (ECs), which form the lining of blood vessels, actively participate in many aspects of cardiovascular development and pathologies such as cancer and atherosclerosis. Vascular ECs are unique in the diverse array of signals that they are capable of sensing from soluble growth factors, immobile extracellular matrix (ECM) proteins and mechanical forces. Studying EC responses to this array of signals will enhance our understanding of the etiology of prevalent diseases such as cancer and atherosclerosis and lead to improved treatments.

Shc is an evolutionarily conserved adaptor protein that mediates signaling cascades downstream of activated receptors and is essential for development of the cardiovascular system. This dissertation focuses on defining the roles that Shc plays in EC responses to angiogenic cues and mechanical force.

Angiogenesis, the growth of new blood vessels from pre-existing vessels, is important during embryonic development as well as in adults for wound healing and tumorigenesis. Using loss-of-function experiments in the mouse and

zebrafish, we found that Shc is required for sprouting angiogenesis *in vivo*. Shc mediates signaling from integrins and VEGF receptors which is required for haptotaxis, survival and sprouting. Interestingly, Shc integrates VEGF and ECM signaling as VEGF-induced survival requires Shc specifically on fibronectin.

Fluid shear stress, the frictional force from blood flowing over ECs, regulates EC function and allows vessels to respond to changes in tissue physiology but also contributes to vessel pathogenesis such as atherosclerosis. We have shown that Shc is required for transducing shear stress signaling directly downstream of the ‘mechanosensory complex’. Shc is required for induction of the inflammatory response that is activated by disturbed shear stress and underlies the development of atherosclerotic plaques. Additionally, Shc is required in mice for shear-induced collateral artery remodeling and arterial specification during arteriogenesis.

Together, Shc plays an important signaling function in ECs and enables ECs to dynamically respond to angiogenic and mechanical stimulation from their environment.

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My family has been incredibly supportive throughout my life no matter what challenge I was facing, and I thank my parents Tim and Sandy Sweet for raising me to always strive be better and also be able to accept failure and persevere through it, which are two useful skills for anyone but particularly for a biological sciences graduate student who encounters failure on a weekly basis. My parents always took an interest in what I was doing even if they didn't fully understand it. I cannot thank my parents enough for all they've done in raising me and supporting me throughout my life.

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LIST OF ABBREVIATIONS AND SYMBOLS

BAEC	Bovine Aortic Endothelial Cell
bFGF	basic Fibroblast Growth Factor
CH1/CH2	Collagen Homology domain 1/2
CL	Collagen
DLAV	Dorsal Longitudinal Anastomotic Vessel
Dll-1, 4	Delta-like 1, Delta-like 4
ECM	Extracellular Matrix
ECs	Endothelial Cells
EGF	Epidermal Growth Factor
eNOS	endothelial Nitric Oxide Synthase
ERK	Extracellular-Related Kinase 1/2
FN	Fibronectin
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-1	Intercellular Adhesion Molecule-1
ISV	Intersegmental Vessel
kDa	kilodaltons
KLF2	Kruppel-like Factor 2
LDI	Laser Doppler Imaging

LM	Laminin
MAPK	Mitogen Activated Protein Kinase
MLEC	Mouse Lung Endothelial Cell
MO	Morpholino
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NICD	Notch Intracellular Domain
NO	Nitric Oxide
PDGF	Platelet Derived Growth Factor
PI3K	Phosphoinositide 3-Kinase
PTB	Phospho-Tyrosine Binding domain
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
SH2	Src Homology-2
Shc	Src homology-2 domain containing
shRNA	short hairpin Ribonucleic Acid
SMC	Smooth Muscle Cell
TNF-α	Tumor Necrosis Factor-alpha
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VEGFR-2	Vascular Endothelial Growth Factor Receptor-2

CHAPTER I.

INTRODUCTION

PREFACE

Parts of this chapter were adapted from a previously published review article [1]. I wrote the original manuscript and Ellie Tzima wrote and edited the final version of the manuscript.

SIGNAL TRANSDUCTION IN ECs

Endothelial cells (ECs) are a specialized cell type that form the inner lining of blood vessels and are actively involved in many aspects of cardiovascular development and pathology. Vascular ECs are quite unique in the diverse array of signals that they are exposed to. The constant flow of blood over the endothelium brings a myriad of soluble growth factors and cytokines that bind to receptors on the EC surface while also imparting mechanical forces that are sensed by the endothelium. Signals from the surrounding ECM are sensed by integrins that form specific cell-ECM adhesions. These signaling cascades are

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[1] **Sweet DT**, Tzima E. Spatial signaling networks converge at the adaptor protein Shc. *Cell Cycle*. 2009 Jan 15; 8(2): 231-5

required for important physiological EC functions, such as wound healing and inflammation, but also pathological EC functions, such as tumor angiogenesis and the chronic inflammation associated with atherosclerosis. This dissertation has aimed to examine signal transduction pathways in ECs that are activated by angiogenic growth factors, mechanical force and the ECM. By studying signal transduction in ECs, we will improve upon our current understanding of the causes of and potential treatments for prevalent diseases such as cancer and atherosclerosis. My research has focused on the adaptor protein Shc, which we have found is an important mediator of EC signaling in response to angiogenic cues and mechanical force.

ADAPTOR PROTEIN SHC

The mammalian *ShcA* gene encodes three Shc isoforms of 46, 52 and 66 kDa – all of which originate from the same mRNA either through alternative RNA splicing or translation initiation sites [1, 2]. The isoforms only differ in the length of their N-terminal CH2 domain and all three include the SH2 and PTB domains important in phospho-tyrosine receptor binding as well as the CH1 domain which houses three important tyrosine phosphorylation sites (see [3] for review). Shc is ubiquitously expressed in adults [4] and has homologues in *Drosophila* and *C. elegans*, indicating an important evolutionarily conserved role for the protein [5, 6]. Shc was initially described as an oncogene due to its key function in activation of Ras and MAPKs [1, 7-10]. Shc binds to phospho-tyrosine residues of activated Receptor Tyrosine Kinasas (RTKs) for growth factors such as Epidermal Growth

Factor (EGF) [11], Platelet-Derived Growth Factor (PDGF) [12, 13], Insulin [14-17], and basic Fibroblast Growth Factor (bFGF) [18, 19]. When Shc binds activated RTKs, Shc itself is phosphorylated and then associates with secondary signaling molecules such as Grb2, which lead to activation of Ras [3]. Not only is Shc important in signaling from a variety of growth factor RTKs, but Shc also associates with and mediates signaling from a subset of integrins. Activation of integrins $\alpha_6\beta_4$ (laminin receptor), $\alpha_1\beta_1$ (collagen/laminin receptor), $\square_5\square_1$ (fibronectin) and $\alpha_v\beta_3$ (vitronectin/fibronectin) induces phosphorylation of Shc and Shc:integrin association. Conversely, ligation of $\alpha_2\beta_1$ (collagen/laminin), $\alpha_3\beta_1$ (promiscuous), $\alpha_6\beta_1$ (laminin) and β_2 integrins does not [20-22]. Shc has an established role in Ras signaling in non-EC tissues, but its role in EC signaling is less well defined.

Shc is critical for cardiovascular development, as Shc knockout mice are embryonic lethal at embryonic day 11.5 [23] due to defects in heart development, as well as cell-cell contacts and mural cell coverage of the blood vessels. Further genetic studies revealed that Shc expression specifically in cardiac myocytes is sufficient for embryonic cardiovascular development and adult heart function [24, 25]. While a role for Shc in heart development and function has been established, relatively little is known about the role of Shc in the function of ECs. Shc has been implicated in signaling downstream of some EC-specific RTKs *in vitro*. For example, Vascular Endothelial Growth Factor (VEGF) stimulation of ECs caused Shc to associate with VEGF Receptor-2 (VEGFR-2) and VE-Cadherin [26, 27]. Similarly, Shc associates with integrins such as $\alpha_5\beta_1$ and $\alpha_v\beta_3$

that are required in ECs for proper angiogenesis [21, 28-30]. Because Shc associates with integrins and RTKs that are critical for angiogenesis and mechanotransduction, we **hypothesized** that Shc may act as a signaling hub in ECs.

ANGIOGENESIS

Blood vessels are formed through two sequential processes: vasculogenesis and angiogenesis. Vasculogenesis is the initial differentiation of endothelial cells from precursors and assembly into vessels whereas angiogenesis is a process of new vessel growth by sprouting from the existing vasculature [31-33]. Angiogenesis is important during embryonic development for proper patterning of the vascular tree as well as in adults for wound healing and during tumorigenesis. Angiogenesis is a highly coordinated sprouting and remodeling process that is controlled by several signaling pathways such as Tie2 and Notch, but the VEGF pathway is the principal master regulator of angiogenesis. VEGF-A (referred to as simply VEGF) is a soluble growth factor whose expression is upregulated in hypoxic or cancer tissue to stimulate angiogenesis. VEGF binds its receptor VEGFR-2 which is expressed specifically on the surface of ECs and activates multiple signaling cascades such as MAPKs, phospho-inositide 3-kinases (PI3Ks), Akt, and small GTPases such as RhoA. As a result, VEGF signaling promotes EC proliferation, survival, migration towards the VEGF gradient, filopodia extension and EC permeability by disrupting adherens junctions and inducing endocytosis of VE-Cadherin [34, 35]. As the nascent vessel sprouts from the parent vessel, the underlying ECM is remodeled and integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ are upregulated in angiogenic ECs. While the role of these integrins is controversial, it is clear that blocking either of these integrins can impede the angiogenic process [29] therefore proper function of integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ is necessary in angiogenic ECs.

MECHANOTRANSDUCTION: ECs RESPOND TO MECHANICAL FORCE

Vascular ECs are constantly exposed to hemodynamic forces due to blood flowing over them. Fluid shear stress, the frictional drag force from blood flow, is crucial in determining the shape, cytoskeletal organization and function of ECs and allowing vessels to respond to changes in tissue physiology but also contributing to vessel pathogenesis [36-38]. Although systemic risk factors such as smoking, diabetes, and high plasma levels of cholesterol and lipoproteins are associated with the development of atherosclerosis, atherosclerosis is a focal disease and forms preferentially at vessel bifurcations and curvatures- sites where blood flow is disturbed or turbulent [39]. In regions of arteries where flow is low (<5 dynes/cm2) and disturbed, atherosclerosis is promoted because disturbed flow increases proliferation and inflammation [40, 41]. These inflammatory processes also lead to a reduction in ‘protective’ functions such as endothelial Nitric Oxide Synthase (eNOS) expression, vasodilation and endothelial repair [36]. In contrast, vessels with steady, laminar flow are protected from atherosclerotic plaque formation [41] and exhibit a phenotype in which the ECs are quiescent and align their cytoskeleton in the direction of flow. Shear stress promotes either an atherogenic or atheroprotective EC phenotype, which underlies the development, or lack, of atherosclerotic plaque formation and therefore studying how ECs sense and respond to shear stress is a critical health care priority.

Shear stress is detected by a variety of molecular force sensors located throughout the EC membrane [39, 42]. Forces from the apical (luminal) surface are transmitted through the cytoskeleton to points of attachment that resist shear stress [41]. In that regard, both cell-cell and cell-ECM adhesions have been implicated in shear stress signal transduction. At cell-cell adhesions, our lab has previously reported a 'mechanosensory complex' comprised of PECAM-1, VE-Cadherin and VEGFR-2 which is necessary and sufficient for a subset of cellular responses to shear stress [43]. Indeed, PECAM-1 is required for cytoskeletal alignment to flow as well as atherosclerosis and flow-mediated vessel remodeling *in vivo* [43-46]. At the basal EC surface, integrins have also been implicated in mechanotransduction. Shear stress stimulates the conversion of integrins to a high affinity state, followed by their binding to the ECM and subsequent activation of multiple signaling pathways downstream of integrin activation [47-49]. Interestingly, the activation of the inflammatory transcription factor NF-κB by flow is dependent upon the ECM composition – as it is activated on fibronectin (FN) but prevented from being activated in cells growing on collagen (CL) [50, 51].

EC responses to shear stress have been well characterized *in vitro* and can be divided into three categories temporally [38]. Rapid responses include production of vasodilator nitric oxide [52, 53] and phosphorylation of PECAM-1 [54], VEGFR-2 [55], and Akt by PI3K [56]. Also, at this early timepoint, integrins undergo a conformational activation and then these high-affinity integrins bind to their respective ligand in the underlying ECM [48]. On a time scale of minutes to hours, shear stress activates Rho family GTPases and initiates the process of cytoskeletal remodeling [48, 57-59], stimulates tyrosine phosphorylation of proteins such as MAPKs [60], release of Reactive Oxygen Species (ROS) [61] and activates transcription factors such as NF-□B which are critical for initiating the inflammatory response to shear stress [62]. Slower responses that occur on a time scale of hours to days include altered expression of shear-responsive genes encoding Kruppel-like factor 2 (KLF2) [63, 64], eNOS, and cell adhesion molecules E-selectin, Intercellular Cell Adhesion Molecule (ICAM)-1 and Vascular Cell Adhesion Molecule (VCAM)-1 [65, 66]. The hallmark of the long-term adaptive EC response to laminar shear stress is the rearrangement of actin microfilaments and microtubules and their elongation in the direction of flow [67-69], while long-term disturbed flow induces chronic inflammation and causes leukocyte adhesion and transmigration into the vessel wall [67, 70].

ARTERIOGENESIS

Shear stress regulates vessel size (caliber) whereas pressure determines vessel wall thickness [71]. Shear force is dependent on vessel diameter, so arteries dynamically respond to changes in shear stress by remodeling inward or outward to compensate for the new shear environment [72, 73]. Following an arterial occlusion, shear-induced vascular remodeling is the driving force for recovery of perfusion to ischemic tissue during a process known as arteriogenesis [74]. Arteriogenesis describes the formation of mature arteries from pre-existent interconnecting arterioles after an arterial occlusion [75]. Artery-to-artery connections, called collateral arteries, arise during development and act as a natural bypass mechanism to circumvent circulation in the case of a large artery occlusion [74, 76]. Following an arterial occlusion, blood is re-routed into pre-existing collaterals, dramatically increasing shear stress and inducing outward remodeling, allowing the vessel to carry more flow and restore circulation to downstream ischemic tissue. The sudden increase in collateral flow is sensed by ECs and activates several signaling pathways that are critical for vessel remodeling including proliferation of ECs and smooth muscle cells, vessel dilation via NO production, and inflammation and leukocyte transmigration into the vessel wall via the NF-κB pathway [74, 77]. Arteriogenesis is critical to prevent tissue death following myocardial infarction or other arterial occlusion, and understanding this process will help in treatment and prevention of cardiovascular disease, the number one cause of death in industrialized nations such as the United States.

RESEARCH PRESENTED IN THIS DISSERTATION

As described in the subsequent chapters, the overarching goals of this dissertation are as follows:

Chapter II.

Determine the role of the adaptor protein Shc in EC signaling during angiogenesis. Previous studies have shown that Shc can bind to activated VEGFR-2 or integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$. Because these transmembrane receptors have been implicated in controlling angiogenesis, ***I hypothesized*** that Shc plays a role in angiogenesis by mediating signaling from one or both of these signaling hubs. Using loss-of-function studies in mouse and zebrafish, I first assessed a possible role for Shc in angiogenesis *in vivo*. Next, I performed a series of functional assays *in vitro* to test which EC functions are regulated through Shc signaling. Finally, I uncovered the molecular mechanism by examining the role of Shc in mediating signaling pathways that are known to be required for angiogenesis.

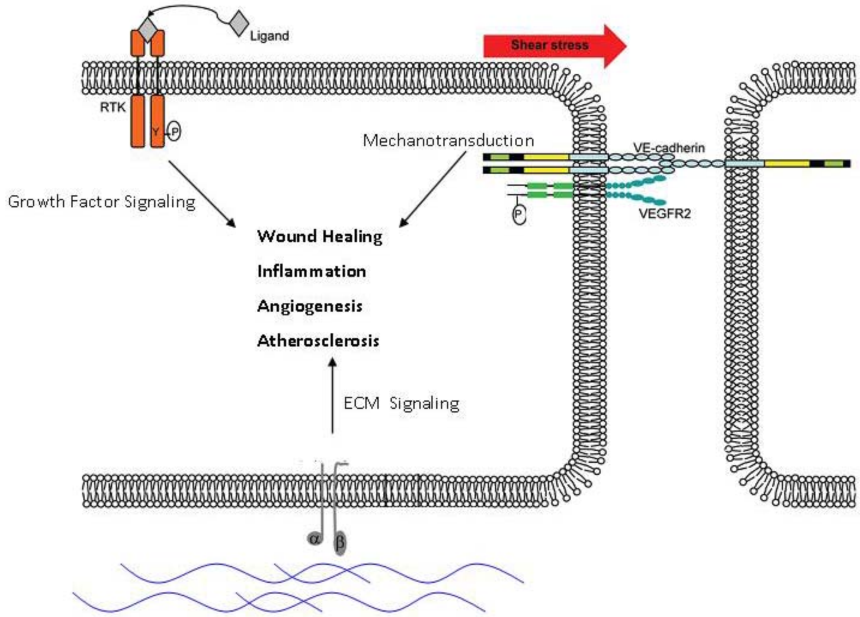
Chapter III.

Determine the role of Shc in EC responses to shear stress. Our lab recently reported a 'mechanosensory complex' in ECs that is necessary and sufficient for conferring cells the ability to respond to shear stress. ***I hypothesized*** that Shc mediates signaling downstream of this 'mechanosensory complex' and therefore is required for mechanotransduction in ECs. First, I performed *in vitro* experiments in ECs to test the role of Shc in mediating signaling from the 'mechanosensory complex'. I then confirmed these findings in mice in which Shc is conditionally removed from ECs. The role of Shc in shear stress-induced arteriogenesis was examined, focusing on inflammation and arterial specification, which underlie collateral vessel remodeling during arteriogenesis.

Chapter IV.

Synthesize the findings of the research chapters and assess the impact of these findings on the field of vascular biology. The findings of this dissertation represent several important contributions to the understanding of angiogenesis, arteriogenesis and EC signaling in general. I briefly outlined the novelty and significance of my research and its implications in the future of cardiovascular research.

Figure 1.1: ECs integrate diverse environmental signals from multiple cell-surface receptors



Endothelial Cells (ECs) use cell-surface receptors to sense a diverse array of signals which direct specific EC responses. Soluble growth factors and cytokines secreted from surrounding tissue are sensed by Receptor Tyrosine Kinases (RTKs). Also, mechanical force such as shear stress from blood flowing over the EC layer is sensed by a 'Mechanosensory Complex' comprised of VE-Cadherin and VEGFR2 located at cell-cell junctions. Third, the composition of the Extracelular Matrix (ECM) is sensed by integrins at the basal surface of the cell.

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CHAPTER II.

THE ADAPTOR PROTEIN SHC INTEGRATES GROWTH FACTOR AND ECM SIGNALING DURING POSTNATAL ANGIOGENESIS

PREFACE

This work was previously published in *Blood* in early 2012 [1]. My role in this project included initiating the study and designing the experiments. I performed all experiments and data analysis, wrote the manuscript and prepared the figures. Zhongming Chen provided technical assistance with tissue staining. David M. Wiley and Victoria L. Bautch provided the protocol, reagents and assistance with the Fibrin Bead assay (Figure 2.3). Ellie Tzima was the principal investigator of the study and designed the experiments, analyzed the data and wrote the manuscript.

OVERVIEW

Angiogenesis requires integration of cues from growth factors, ECM proteins and their receptors in endothelial cells. Here, we show that the adaptor

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[1] **Sweet DT,** Chen Z, Wiley DM, Bautch VL, Tzima E. The adaptor protein Shc integrates growth factor and ECM signaling during postnatal angiogenesis. *Blood*. 2012 Feb 23; 119(8): 1946-55.

protein Shc is required for angiogenesis in zebrafish, mice, and in cell culture models. Shc knockdown embryos show defects in intersegmental vessel sprouting in the zebrafish trunk. *Shc flox/flox; Tie2-Cre* mice display reduced angiogenesis in the retinal neovascularization model and in response to VEGF in the Matrigel plug assay *in vivo*. Functional studies reveal a model whereby Shc is required for integrin-mediated spreading and migration specifically on fibronectin, as well as EC survival in response to VEGF. Mechanistically, Shc is required for activation of the Akt pathway downstream of both integrin and VEGF signaling as well as for integration of signals from these two receptors when cells are grown on fibronectin. Thus, we have identified a unique mechanism in which signals from two critical angiogenic signaling axes, integrins and VEGFR-2, converge at Shc to regulate postnatal angiogenesis.

INTRODUCTION

Angiogenesis, the sprouting and growth of new blood vessels from pre-existing vasculature, is critical for wound healing and in diseases such as rheumatoid arthritis, diabetes and cancer [1]. Angiogenesis is a highly coordinated tissue remodeling process activated by proangiogenic growth factors, such as VEGF, whose expression is upregulated in hypoxic or cancer cells. VEGF receptors expressed on the EC surface become activated when bound to the VEGF ligand, and initiate signaling cascades that lead to EC proliferation, migration, survival and tube formation [2]. Basement membrane deposition and mechanical cues from the extracellular matrix transmitted via integrins also participate to coordinate vessel sprouting and remodeling in conjunction with the VEGF signaling pathway [3]. Given their transmembrane structure, ability to form associations with adaptor molecules and ability to bind to extracellular ligands, VEGF receptors and integrins are well positioned to serve as functional hubs during the angiogenic process [4].

Adaptor proteins, which have no catalytic activity but instead promote protein-protein interactions, are important regulators of signaling pathways downstream of activated cell-surface receptors [5]. The prototypical adaptor protein Shc is an evolutionarily conserved, ubiquitously expressed protein that was originally described as an oncogene because of its participation in the activation of Ras and MAPKinases downstream of a multitude of receptors for various growth factors, cytokines and hormones [6, 7]. Shc is expressed as three isoforms of 46, 52 and 66 kDa, all of which are products of the same gene, *Shc1*

[8, 9]. Global knockout of *Shc1* in mice causes embryonic lethality at E11.5 [10]. These embryos exhibit severe defects in the cardiovascular system, including defective heart development and vessel remodeling. More detailed gene targeting work has shown that expression of the PTB domain of Shc specifically in cardiomyocytes is critical for mid-gestational heart development and embryonic life [11]. Conditional knockout strategies have shown that Shc is also important for the proper development/function of other organs such as skeletal muscle[11], brain [12] , cardiomyocytes [13] and thymocytes [14], as tissue specific deletion of Shc resulted in living, but mis-developed mice. To address the role of Shc in angiogenesis *in vivo*, we studied loss of Shc function using morpholino antisense technology in zebrafish. Additionally, we used the Tie2-Cre transgene to generate mice null for Shc in ECs and some hematopoietic cells [15]. Surprisingly, these mice survive through development, thus enabling us to investigate the role of Shc in postnatal angiogenesis. Here, we show that Shc is required for proper angiogenesis *in vivo* in both the zebrafish and mouse. Mechanistically, Shc is required for transmitting signals downstream of two major angiogenic signaling hubs, VEGFR-2 and integrins.

METHODS

Zebrafish Morpholino Injection

Two splice-blocking morpholinos targeting the zebrafish ortholog of Shc (accession # LOC563639) were designed by GeneTools. The MO sequences are: ShcMO1: 5’- TGAAATGAATTGAATCTTACCCTGA -3’ and ShcMO2: 5’-

ATAAAGAATTGGAAACCTTTCTCCT -3'. ShcMO2 resulted in better Shc knockdown and was used for experiments. Shc or Standard Control morpholinos were injected into one-cell-stage *Tg(kdrl:egfp)* zebrafish embryos at 8 ng (2x) or 16ng (4x) per embryo. Embryos were scored and imaged at 24 hpf stage by embedding in 1% agarose solution with 0.016% tricaine to inhibit movement. Z-stacks were taken using 5x and 20x objectives on a Zeiss Pascal confocal microscope. Control 4x MO n=118; Shc 2x MO n=72; Shc 4x MO n=105 embryos. Numbers indicate all fish counted from 3 independent experiments.

Mice

Shc floxed mice were a kind gift from Dr. Kodi Ravichandran at University of Virginia[16]. Tie2-Cre (B6.Cg-Tg(Tek-cre)12Flv/J) and R26R (B6.129S4-Gt(ROSA)26Sortm1Sor/J) mice were purchased from Jackson Labs. All housing, breeding and experimental procedures using mice were in accordance with national guidelines and regulations and were approved by the Institutional Animal Care and Use Committee at the University of North Carolina- Chapel Hill.

X-gal Stain of R26R Tissue

The Rosa26 Reporter mice (Jackson Labs) were used to monitor expression of Tie2-Cre. Male *Shcflox/flox; Tie2-Cre*+ were mated with female R26R mice to produce *Shcflox/+; R26R+; Tie2-Cre*+ and *Shcflox/+; R26R*+ offspring. Four week old mice were euthanized and tissues were quickly frozen and sectioned into 5um slices. Frozen sections were fixed in 0.2% gluteraldehyde and stained overnight with 1mg/ml X-Gal and then counterstained with Nuclear Fast Red. Slides were imaged using the 4x, 10x and 40x objectives on an Olympus BX61 light microscope. Representative images are shown.

Matrigel Plug Assay

4-6 week old littermates were used to assay angiogenesis from subcutaneous tissue into Growth Factor Reduced Matrigel (BD Biosciences). Cold matrigel was mixed with heparin (50 U/ml; Sigma) and VEGF (250 ng/ml) or vehicle (ddH_2O). Mice were lightly anaesthetized using isofluorane and cold matrigel (0.5 ml) was injected into the abdominal subcutaneous tissue along the peritoneal mid-line. After 7 days, mice were euthanized and matrigel plugs were removed and fixed in 4% paraformaldehyde, embedded in paraffin, sectioned and H&E stained. Blood vessel infiltration into the plug was quantified in 4 sections per plug, each 100μm apart, by counting number of cells per mm^2 using ImageJ software. Images were obtained on an Olympus BX61 light microscope and expressed as mean values of 5-6 mice per condition.

Mouse Lung Endothelial Cell Isolation

Mouse Lung Endothelial Cells (MLECs) were isolated from 6-9 day old *Shcflox/flox and Shcflox/flox; Tie2-Cre+* littermate mice. Lungs were dissected from the mouse and were gently minced, collagenase digested, triturated and strained. The resulting single cell suspension underwent positive cell selection using rat anti mouse PECAM-1 (BD Pharmingen) antibody conjugated magnetic

Dynabeads (Invitrogen). PECAM-positive cells were plated in tissue culture flasks coated with 10μg/mL fibronectin and cultured in EGM-2 (Lonza). ECs were immortalized by transduction with Polyoma Middle T antigen expressing retrovirus, and selected using G418. Four clones per genotype were isolated and validated for expression of endothelial markers VEGFR-2, VE-Cadherin and PECAM-1. Cells were cultured in EGM-2 (10% FBS).

Fibrin Gel Bead assay

Fibrin Gel Bead Assays were performed following the previously published protocol[17]. In short, MLECs were incubated with dextran-coated Cytodex 3 microcarriers (Amersham Pharmacia Biotech) at a concentration of 400 cells per bead in 1.5 ml of EGM-2 medium (Lonza) for 4 h at 37°C. The following day, cell-coated beads were washed with EGM-2 and resuspended in 2 mg/ml Fibrinogen (Sigma) solution plus 0.15 U/ml aprotinin (Sigma) at a concentration of 500 cell-coated beads/ml in 2.5 mg/ml. 500 microliters of fibrinogen/bead solution was added to 0.625 units of thrombin (Sigma) per well of a glass bottom 24-well tissue culture plate. Cultures were grown for 3 days in EGM-2 (10% FBS) and fixed in 4% PFA on day 3. Cells were stained with Phalloidin (FITC) to visualize actin and DRAQ5 to mark EC nuclei and imaged using an Olympus FLV500 inverted confocal microscope using the 10x and 40x objectives. Sprouts were quantitated by counting # sprouts per bead and # nuclei per sprout. Sprouts were defined as protrusions containing 2 or more nuclei and >15 beads were counted per genotype.

Endothelial Cell Culture & Lentivirus Infection

Human umbilical vein endothelial cells were purchased from Lonza and maintained in M199 supplemented with 10% Fetal Bovine Serum, 30ug/mL Endothelial Cell Growth Supplement, 100ug/mL Heparin and 1x Pen/Strep (all purchesd from Sigma). HUVECs were starved in M199 media with 0.5% FBS and 1x Pen/Strep for 4 hours before using in experiments unless noted. HUVEC were used between passage 2 and 9.

shRNAs against Shc or non-specific control were designed by Dharmacon and subcloned into pLentiLox 5.0 vector for production of lentivirus in 293T cells as previously described[18]. shShc target sequence GGGGAGGAGTAACCTGAAA targets all 3 isoforms of the human *Shc1* gene. Control shNS sequence GATCGACTTACGACGTTAT has no match in the human, mouse or rat genomes.

Cell Spreading Assay

HUVECs were detached with trypsin, washed with PBS and kept in suspension for 30 minutes in the starvation medium. An equal number of cells were plated in Starvation medium on glass coverslips coated in 10ug/ml fibronectin, 10ug/ml collagen (Millipore) or PBS. After 25 minutes, cells were fixed with 2% formaldehyde, permeabilized with 0.2% Triton X-100 and stained with Alexa-Fluor-568-conjugated phalloidin to mark actin. Fluorescent images of randomly selected fields were acquired using a Zeiss Axiovert S100 and spread area was measured using the threshold function of ImageJ software (NIH). Values shown are mean +/- SEM (n=2 independent experiments, >100 cells counted per condition per experiment).

Cell Migration Assays

Haptotaxis and chemotaxis to VEGF assays were performed using Boyden Chambers (Transwells– Corning) with 8 μm pores.

Haptotaxis experiments – The underside of the Transwell filter was coated in either 10ug/ml fibronectin or collagen or PBS for 2 hr at 37° C.

Chemotaxis experiments – Both the top and bottom of the filters were coated with 10ug/ml fibronectin for 2 hr at 37 C.Filters were washed in PBS and then blocked in PBS+3% BSA for 1 hr at 37° C and then washed again. HUVECs were FACS sorted to obtain a pure population of GFP-positive cells, and 10,000 cells were loaded into the upper portion of each chamber. After 4 hr incubation, filters were washed 2x in cold PBS and non-migrating cells were removed from top of chamber with a cotton swab. Filters were fixed in 2% formaldehyde and migration was quantitated by blind counting the number of migratory cells on the lower surface of the membrane using an inverted Zeiss Axiovert S100 microscope. Five random fields were imaged per filter. Values shown are mean +/- SEM (n=3 independent experiments, 2 filters per condition per experiment).

EC Survival Assay

Lentivirus-infected HUVEC were seeded on FN or CL coated dishes and cultured in Full HUVEC media for 1 day. ECs were starved for 24 hr in Starvation media supplemented with 100ng/ml VEGF (Millipore) or vehicle control to induce apoptosis. After 24 hr, media was removed and cells were washed 1x with PBS to remove floating cell debris and lysed for western blot. Values shown are mean +/- SEM (n=4 independent experiments)

Retinal EC Proliferation Assays

Proliferating cells were marked using the Click-iT EdU kit (Invitrogen) following manufacturer instructions. 100 mg/g EdU was injected intraperitoneally into each P5 pup. Two hours later, pups were anaesthetized with isofluorane and euthanized by injection of 2% Paraformaldehyde into the left ventricle to fix vessels. Retinas were dissected out and fixed in 4% PFA and then stained with Isolectin B4 (Sigma) to mark ECs, DAPI to mark nuclei and EdU to mark proliferative nuclei. Flatmounted retinas were imaged using a Zeiss LSM 710 confocal microscope – 20x and 60x objectives. Images were analyzed using ImageJ (NIH) to count either number of branchpoints per 100 μm^2 or % of proliferative nuclei by dividing the number of EdU-positive, isolectin-positive nuclei by the total number of isolectin positive nuclei. For branchpoint analysis, *Shcflox/flox* n= 17; *Shcflox/flox; Tie2-Cre*+ n= 15 mice. For proliferation analysis, *Shcflox/flox* n= 5; *Shcflox/flox; Tie2-Cre* n= 8 mice.

EC Adhesion Assay

Lentivirus infected HUVEC were starved for 4 hours and then removed from dish using 0.05% Trypsin-EDTA (Akt activation) or 20mM EDTA (ERK activation), spun down to pellet cells and resuspended in HUVEC starvation media. Equal number of cells were seeded on dishes coated in FN or CL (10μg/mL) and allowed to adhere for 15 min (ERK activation) or 30 min (Akt activation) in incubator. Non-adherent cells were washed away with PBS and adherent cells were lysed and processed for western blot.

VEGF Treatments & Western Blot Analysis

Lentivirus infected HUVEC were grown to confluence on FN coated dishes and starved for 4 hours. Media was removed from all dishes and replaced with fresh starvation media supplemented with 100ng/ml VEGF (Millipore) or vehicle control. Cells were incubated for 5 minutes, then washed 1x in PBS and were lysed in buffer containing 1% Noniodet P-40, 150 mM NaCl, 50 mM Tris-HCL (pH 7.8), 2 mM EDTA, 10 mM NaF, 10 mM Na2P2O7, 2mM Na3VO4 10 μg/mL leupeptin, 4 μg/mL pepstatin and 0.1 U/mL aprotinin. Lysates were cleared by spinning at max speed in a tabletop centrifuge and supernatant was combined with 10x Lamelli Sample Buffer and boiled briefly. Lysate was loaded into a 4-12% NuPage Bis-Tris gel and run according to manufacturer instructions using the Licor Odyssey system.

Zebrafish were lysed in buffer containing 1% Noniodet P-40, 150 mM NaCl, 50 mM Tris-HCL (pH 7.4), 1 mM EGTA, 0.25% Deoxycholate, 10 mM NaF, 2mM Na3VO4, 1mM PMSF, 10 μg/mL leupeptin, and 0.1 U/mL aprotinin. 15 fish per condition were homogenized in a tissue tearor and lysate was cleared as described above.

Antibodies used: α-Shc (BD Transduction), α-GAPDH (Millipore), α-Cleaved Caspase 3 (Cell Signaling). Intensity of bands was quantified using ImageJ and protein intensity was divided by GAPDH to normalize for total protein concentration. Quantification is shown as the mean of >3 experiments.

Quantification & Statistical Analysis

Band intensity of immunoblots was quantified using the ImageJ program. Each experimental group was analyzed using single factor analysis of variance. P-values were obtained by performing two-tailed Student's t test using Excel. Statistical significance was defined as $P < 0.05$.

RESULTS

Angiogenesis in Zebrafish Requires Shc

A role for Shc in patterning of the vascular system and sprouting angiogenesis was assayed by depleting Shc protein from zebrafish embryos. Sprouting of Intersegmental Vessels (ISVs) dorsally from the aorta is a VEGF-driven process that can easily be visualized *in situ* using transgenic zebrafish [19]. Shc protein was depleted from *Tg(kdrl:egfp)* zebrafish embryos at the 1 cell stage using a splice-site blocking morpholino (MO) targeted against Shc (data not shown). Shc-MO did not induce zebrafish death, suggesting that Shc is not required for zebrafish embryonic development during the first 3 days post fertilization. Interestingly, global Shc depletion in the zebrafish embryo caused angiogenesis defects specifically, while all other tissues appeared normal. Shc morphants showed impaired ISV formation at 30 hpf (Figure 2.1A). At high concentration of Shc-MO (16 ng/embryo) as well as low concentration (8ng/embryo), 73% and 55% of Shc-MO fish displayed a cardiovascular defect compared to only 12% of fish injected with high concentration of Standard Control-MO (Figure 2.1B). The predominant vascular phenotype observed was defective growth of ISVs dorsally and improper Dorsal Longitudinal Anastamotic Vessel (DLAV) formation, while other defects ranged from complete loss of ISV sprouts or abnormal overall ISV patterning (termed "Severe CV Defects") to dilated Caudal Ventral Vein and partial connection of ISVs to DLAV ("Mild CV Defects"). In all conditions, a small percentage of zebrafish exhibited non-cardiovascular (CV) defects such as gross defects in head, eye, fin or tail morphology. We believe this is a non-specific effect of accidentally injuring the embryo with the micropipette during morpholino injection. No obvious defects were observed in heartbeat, aorta morphology or overall zebrafish patterning, indicating a specific role for Shc in angiogenesis during zebrafish development. These results are consistent with the phenotype of the global Shc knockout mouse, which exhibited cardiovascular development defects [10]. In contrast to the mouse, Shc-MO zebrafish did not display increased lethality compared to Control-MO injected fish. This apparent discrepancy may be due to the unique ability of zebrafish to survive significantly longer than mice without a functional heart or vascular system [20] and/or the incomplete depletion of Shc protein in the Shc-MO zebrafish. The zebrafish genome is known to have undergone extensive genome duplication, however it appears that the Shc gene has escaped this duplication, and only a single Shc gene exists in zebrafish. The morpholinos used here target unique sequences at the locus LOC563639 and nowhere else in the zebrafish genome. While we cannot rule out the existence of another un-anottated Shc-like gene playing a redundant or unique role in zebrafish development, nobody has reported such a gene.

Endothelial Shc is Required for Proper Angiogenesis *in vivo*

To specifically inactivate the *Shc1* gene in ECs, female mice carrying floxed alleles of Shc exons 1 and 2[16] were intercrossed with male transgenic mice expressing Cre recombinase under the control of the Tie2 promoter which is expressed specifically in ECs and some hematopoietic cells [15]. In this cross, the Tie2-Cre allele was always donated from the father to minimize leakage of Cre expression into other tissues, which can occur when Tie2-Cre is donated by the mother. Surprisingly, *Shcflox/flox; Tie2-Cre+* were born at the expected Mendelian ratio and these animals display no gross anatomic abnormalities or decrease in fertility compared to *Shcflox/flox* controls. To verify tissue specific Cre/loxP recombination in our mice, we crossed *Shcflox/flox; Tie2-Cre+* mice to mice the Rosa26 Lac-Z Reporter allele. X-gal staining of the carotid artery, heart and retina showed staining restricted to the endothelium (data not shown). Cre expression was not mosaic, as X-Gal staining was seen in nearly all ECs. To confirm that Shc protein was reduced in ECs, primary lung ECs were isolated from *Shcflox/flox; Tie2-Cre+* and *Shcflox/flox* littermates. Western blot analysis revealed a complete reduction in all three Shc isoforms in the *Shcflox/flox; Tie2-Cre+* animals (data not shown).

To determine whether angiogenesis is affected in *Shcflox/flox; Tie2-Cre+* animals, we used two *in vivo* models: neonatal retinal neovascularization and the Matrigel plug assay. Vascularization of the murine retina commences after birth, as the vessels originating at the optic nerve spread radially over the inner surface of the retina on the pre-existing template of astrocytes, guided by a gradient of VEGF-A to form a two-dimensional vascular plexus [21]. At postnatal day 5, retinas were isolated and stained with Isolectin B4 to mark ECs. *Shcflox/flox; Tie2-Cre+* mice exhibited a less dense primitive plexus at the vascular front compared to both *Shcflox/flox* and *Shc wt/wt; Tie2-Cre+* controls (Figure 2.2A). Vascular density in the retina was quantified by counting the number of branchpoints per 100 μm^2 area as well as % vascular area, both of which revealed a significant decrease in *Shcflox/flox; Tie2-Cre+* compared to littermate controls (Figure 2.2A, lower). Both genotypes of control mice, *Shcflox/flox* and *Shc wt/wt; Tie2-Cre+*, showed equal retinal vascular density, indicating Tie2-Cre expression itself if not responsible for the phenotype, so only *Shcflox/flox* littermate controls were used in the remaining experiments.

The Matrigel plug assay, in which microvessel growth is induced toward an angiogenic factor source (in this case VEGF), adult mice were injected with two plugs each, one containing vehicle and the other supplemented with VEGF to induce angiogenesis into the plug. VEGF induced neovascularization of Matrigel implants in *Shcflox/flox* controls, whereas neovascularization was impaired in *Shcflox/flox; Tie2-Cre+* littermates (Figure 2.2B). Collectively, these data suggest that endothelial Shc is required for proper postnatal angiogenesis *in vivo*.

Endothelial Shc is Required for Tube Assembly & Sprouting *in vitro*

To further determine the role of Shc in the EC angiogenic response, we performed the Fibrin Gel Bead assay. The Fibrin Gel Bead assay was preferred over other available *in vitro* angiogenesis assays because this assay involves actual sprouting of ECs off of the bead over a period of a few days so EC proliferation, migration and survival are required; whereas standard 'tube formation' assays largely involve assembly of ECs into capillary-like tubes over a timecourse of a few hours, so the processes of proliferation, survival and sprouting are less important. Secondly, fibrin gel is more applicable because it is made of digested fibrinogen, and Shc has previously been shown to bind to fibrinogen-binding integrins such as $\alpha_5\beta_1$ and $\alpha_v\beta_3$ whereas the standard Matrigel is a complex mixture of several ECM proteins such as collagen, laminin and others. ECs isolated from the lungs of *Shcflox/flox; Tie2-Cre+* and *Shcflox/flox* mice (MLECs) were coated on beads and embedded in Fibrin gel. Control *Shcflox/flox* ECs sprouted outward off the bead and lumenized to form capillary-like vessels in the 3D Fibrin matrix, as is typically seen using HUVEC. Interestingly, *Shcflox/flox; Tie2-Cre+* MLECs displayed a striking defect in both number and size of sprouts (Figure 2.3A). While *Shcflox/flox; Tie2-Cre+* MLECs were able to extend filopodia out into the matrix at a normal or even enhanced rate, these filopodia failed to develop into full sprouts and the tip cells remained stuck on the bead. Quantification revealed a significant reduction in number of sprouts per bead as well as number of cells per sprout, indicating an important role for Shc in EC sprouting (Figure 2.3B).

Shc is Required for Integrin-Mediated EC Signaling

To understand the mechanism underlying the role for Shc in angiogenesis, we examined signaling downstream of two major angiogenic receptors: integrins and VEGFR-2 in ECs. Previous work has shown that Shc binds to a subset of activated integrins and mediates signaling. Upon outside-in integrin activation by ligation to its ECM ligand, Shc is phosphorylated and recruited to integrins $\alpha_5\beta_1$ (fibronectin receptor) and $\alpha_v\beta_3$ (fibronectin/vitronectin), but not to $\alpha_2\beta_1$ (collagen) or $\alpha_6\beta_1$ (laminin) [22]. We therefore tested the role of Shc in integrin-dependent angiogenic responses. For the following experiments, Human Umbilical Vein Endothelial Cells (HUVECs) were infected with lentivirus that expresses either Shc (shShc) or non-specific (shNS) shRNA in order to deplete Shc protein (data not shown). The role of Shc in integrin-mediated cell spreading on ECM was tested by seeding equal numbers of ECs on fibronection (FN) or collagen (CL) and measuring the cell area. While there was no difference in spreading on CL in the absence of Shc, Shc-depleted ECs showed impaired spreading on FN compared to control ECs (Figure 2.4A), suggesting that Shc is required specifically for EC spreading on FN. To determine whether migration toward FN also required Shc, we performed haptotaxis assays using Boyden Chambers.

Similar to the spreading experiments, EC migration toward FN was impaired in Shc-depleted ECs, while migration toward CL occurred independent of Shc (Figure 2.4B) These results indicate that Shc is required for integrin-mediated spreading and migration towards FN, therefore suggesting that Shc selectively mediates angiogenic signaling downstream of FN-binding integrins.

Shc is Required for VEGF-Mediated EC Signaling

VEGF induces Shc phosphorylation and its association with VEGFR-2 and VE-Cadherin [23, 24]. However, the role of Shc, if any, in signaling downstream of VEGF remains unexplored. We assayed the role of Shc in VEGF-induced EC survival and migration *in vitro*, as well as proliferation of retinal ECs *in vivo*. EC survival was assayed by inducing apoptosis in ECs in the presence or absence of VEGF. Apoptosis was quantified by measuring the level of cleaved caspase 3 present in the cell lysates. VEGF treatment resulted in a 50% decrease in cleaved caspase 3 in shNS control cells, while shShc ECs showed no significant protection from apoptosis (Figure 2.5A). To determine the role of Shc in VEGF-induced migration, we assayed chemotaxis toward a VEGF gradient. Baseline migration of both shNS and shShc ECs was similar, and interestingly, migration toward VEGF was induced in both cell types, indicating that Shc is not required for EC migration toward a VEGF gradient (Figure 2.5B). In the developing retina, vessel outgrowth occurs by proliferation of ECs and migration of endothelial tip cells in response to the VEGF gradient released from the underlying astrocytes [25]. To investigate if Shc deficiency affects the proliferation rate of retinal ECs, we analyzed EdU (5-ethynyl-2'-deoxyuridine) incorporation into endothelial nuclei 2 hours after injection into the P5 mice (Figure 2.5C). Retinas were stained to mark ECs green (Isolectin), all cell nuclei blue (DAPI) and proliferating cells red (EdU). By comparing the number of cells that stained positive for all three markers divided by the total number of ECs, we found the number of EdU-positive EC nuclei was slightly lower in *Shcflox/flox; Tie2-Cre*+ mice compared to controls (Figure 2.5C), but these differences did not reach statistical significance. Thus, Shc does not play a significant role in EC proliferation. Together, these data are consistent with a model in which Shc function is important for survival signaling in response to VEGF, but not for VEGF-induced migration or proliferation.

Integration of VEGF & integrin signaling via Shc

Our data show a role for Shc in processes downstream of both VEGF and integrins. We hypothesized that Shc mediates crosstalk between these two receptors. To test this hypothesis, we performed survival experiments on ECs plated on FN vs CL. VEGF treatment resulted in a ~50% decrease in apoptosis in shNS control cells grown on either FN or CL, similar to what was seen in Figure 2.5A. Interestingly, shShc ECs grown on CL showed similar VEGF-induced survival as control shNS cells, while shShc ECs grown on FN showed no significant protection from apoptosis (Figure 2.6). These data suggest that Shc integrates VEGF and integrin signals specifically on FN.

Shc Mediates Akt Activation Downstream of VEGF & Integrin Activation

To further delineate the signaling pathways that are mediated by Shc downstream VEGF and integrin signaling, we assayed activation of two key signaling cascades, Akt and ERK 1/2. shShc ECs treated with VEGF failed to activate Akt, whereas VEGF-induced ERK 1/2 activation was similar to shNS control ECs (Figure 2.7A). Interestingly, the requirement for Shc in the activation of Akt was specific to VEGF, as Epidermal Growth Factor (EGF) induced robust activation of Akt in both shNS and shShc ECs (data not shown).

Similarly, Akt activation by adhesion of ECs to FN was impaired in shShc ECs, whereas shShc ECs plated on CL could activate Akt normally (Figure 2.7B), indicating that Shc mediates Akt activation specifically downstream of FN binding integrins. In contrast, ERK 1/2 was activated similarly in both shNS and shShc ECs on both FN and CL, indicating that Shc is not important for ERK 1/2 activation downstream of either FN or CL binding integrins. Together, these data are consistent with a model in which Shc function is important for Akt signaling which promotes survival downstream of VEGF specifically on FN, whereas Shc is dispensable for ERK 1/2 activation and EC proliferation.

DISCUSSION

In this study, we present evidence that the adaptor protein Shc is required for postnatal angiogenesis in zebrafish, mouse and cell culture models. Shc morphant zebrafish embryos show defects in ISV sprouting in the trunk, while *Shcflox/flox; Tie2-Cre+* mice display impaired angiogenesis in the retina and in the Matrigel plug assay *in vivo*. Using an *in vitro* model of angiogenesis, we show that Shc is required for sprouting and tube formation. Mechanistically, Shc integrates signaling downstream of integrins and VEGF. Shc is required for integrin-mediated spreading and migration specifically on FN, as well as survival in response to VEGF. Importantly, Shc integrates VEGF and integrin signaling, as VEGF-induced survival on FN requires Shc, whereas survival in ECs on CL does not. Activation of the Akt, but not ERK1/2, pathway in response to both VEGF and integrin activation depends on Shc. Combined, these processes are critical for angiogenesis and provide a mechanism by which Shc integrates signals from VEGF and integrins to mediate angiogenesis (Figure 2.7C). The observation that Shc is required for VEGF-induced EC survival is reminiscent of the reported role for VE-Cadherin in this process [51]. Indeed, it is likely that Shc is involved in signaling downstream of this VE-Cadherin:VEGFR-2 complex that leads to Akt activation and cell survival because the same lab later showed that VEGF treatment of ECs induces a Shc association with both VE-Cadherin and VEGF-2 [23]. It is unknown whether Shc mediates assembly of the VE-Cadherin: VEGFR-2 complex or merely signals downstream of this complex after it is formed, but this will be an area of further research.

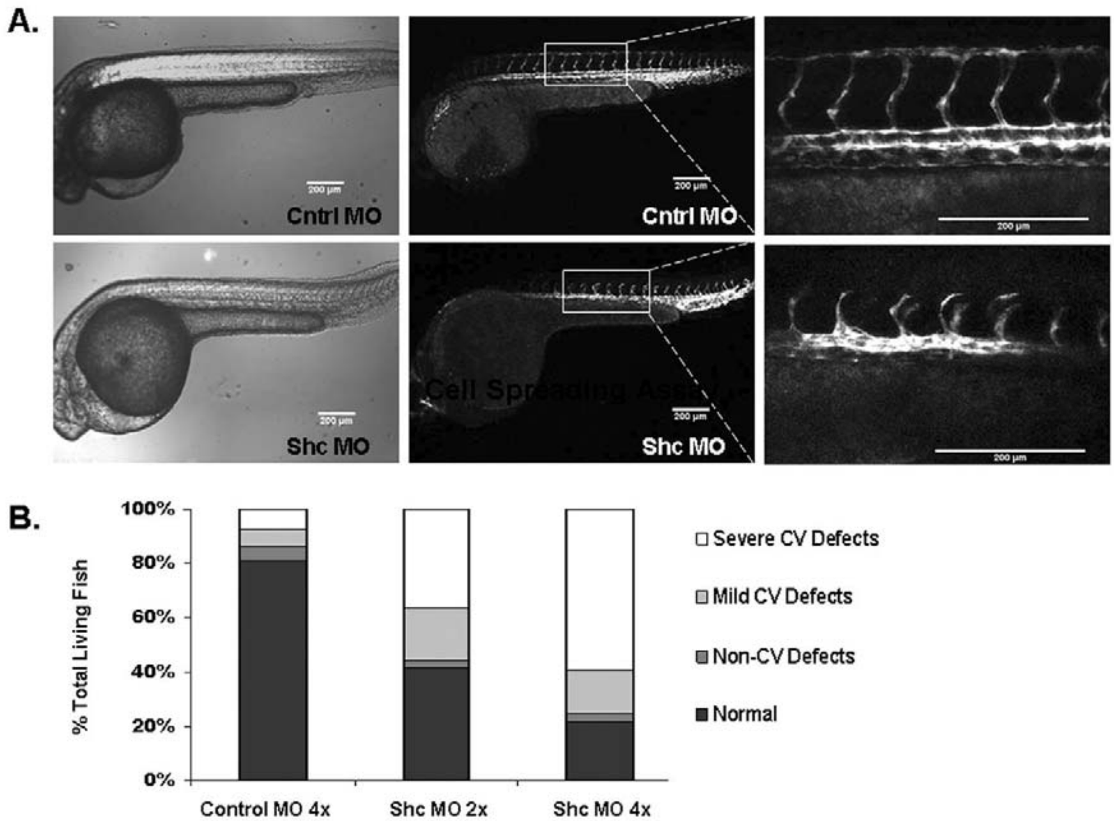
Given the large number of signaling networks that need to be organized and integrated for new vessels to form, signaling hubs may be important during angiogenesis [4]. In this manner, both integrin and VEGF- receptor complexes represent central signaling axes during angiogenesis. Activation of either VEGFR-2 or $\alpha_v\beta_3$ induces physical association of the two receptors, which is important for VEGFR-2 phosphorylation [26]. Function of both receptors is required for proper signaling, as inhibition of $\alpha_v\beta_3$ or VEGFR-2 function decreases VEGFR-2 activation and complex formation[27, 28]. Our work here shows that Shc is required for mediating and integrating angiogenic responses downstream of both integrins (FN-binding integrins specifically) and VEGF, thus coordinating the angiogenic process as a whole.

Shc was originally described as an oncogene, and mutation of Shc attenuates tumor growth in mice [29]. Shc overexpression in fibroblasts causes transformation [9] and Shc is required for cellular transformation in ErbB2-overexpressing breast cancer cells [30], as well as in mammary tumors induced by Polyoma Middle T expression [31]. In humans, clinical studies have associated Shc activation with poor patient prognosis [29]. These data, combined with our current findings, suggest that Shc is critical for many steps of tumorigenesis, including cellular transformation of tumor cells themselves, as well as angiogenesis in ECs that feed the tumor and enable its growth. Therefore, Shc may be an interesting target for cancer treatment at multiple levels.

Expression of the PTB-domain of Shc in cardiomyocytes is essential for embryonic heart development [11]. Interestingly, mice with a conditional deletion of Shc in specific organs such as skeletal muscle [11], thymocytes [14], or brain[12] live to adulthood, exhibiting defects only in the function of the tissue in which Shc was removed. Similarly, we now show that endothelial Shc expression is not required for embryonic development, but it is required postnatally for angiogenesis. Induction of *Tie2-Cre* expression has been reported at E9.5[15], which precedes embryonic lethality of the global Shc knockout at E11.5, so mis-timing of Shc gene excision does not appear to be the reason for *Shcflox/flox; Tie2-Cre*+ mouse survival. Emerging research has set precedence for the idea that conditional gene knockout using the *Tie2-Cre* transgene can result in mice that initially develop a normal vasculature while exhibiting defective angiogenic capacity. Tie2-Cre mediated conditional knockout of genes such as Endothelin-1[32, 33], TFPI[34], ADAM17[35], PPARγ [36] and Dicer [37] yield viable mice with cardiovascular defects, while the corresponding global knockout animal is embryonic lethal. Thus, genes such as Shc and others appear to have differing roles in developmental *vs*. postnatal angiogenesis. This hypothesis is strengthened in light of the literature on proteins that interact with Shc. Our results indicate that Shc is required for signaling downstream of FN-binding integrins such as $\alpha_v\beta_3$ and/or $\alpha_5\beta_1$, which are upregulated during angiogenesis. Surprisingly, endothelial knockout of α_v[38], β_3[39], or α_5[40] results in viable mice, while antagonism of either of these integrins using blocking antibodies results in a defect in angiogenesis[41-43]. We also show that Shc mediates a subset of signaling responses downstream of VEGF. In particular, Shc is required for EC survival but not proliferation in response to VEGF.

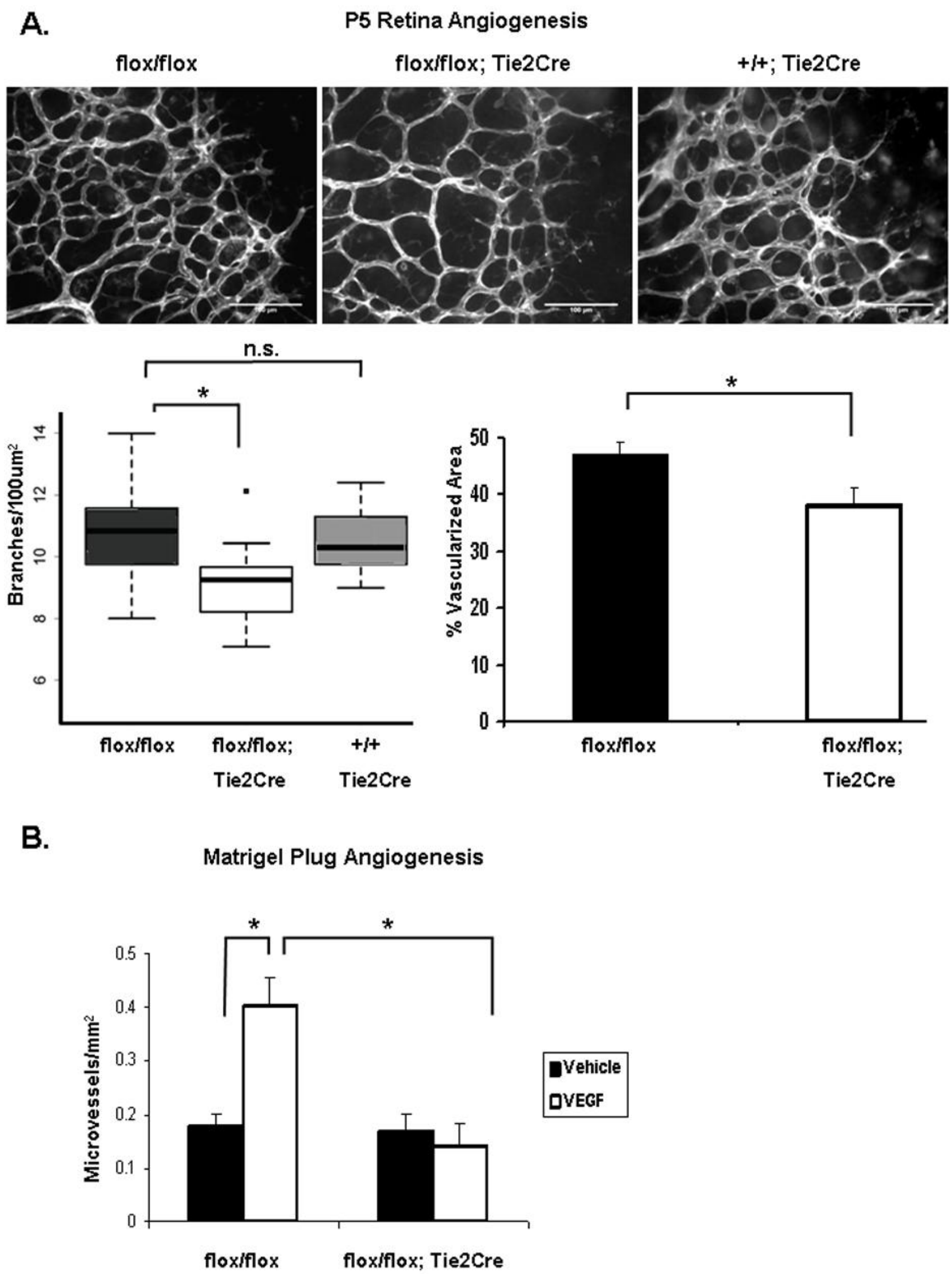
We recently reported a role for Shc in mechanotransduction in response to shear stress [44]. Hemodynamic forces are emerging as an important regulator of angiogenesis in some vascular beds such as aortic arch [45, 46] and yolk sac [47] in both mouse and fish. Flow also promotes hematopoetic cell development [48, 49] *in vivo* and atheroprotective laminar flow inhibits HUVEC tubule formation and migration *in vitro* [50]. Therefore, a role for Shc in flow-driven angiogenesis is an attractive idea. Integration of VEGF- and flow dependant signaling was recently reported during zebrafish vascular remodeling [46], and future experiments are aimed at understanding the role of Shc in these processes. Angiogenesis involves a complex interplay of mechanical forces, ECM remodeling and pro- and anti- angiogenic growth factors, all signaling simultaneously in ECs. Adaptor proteins, such as Shc, are likely responsible for the integration of these signals due to their ability to bind many receptors, and are emerging as signaling nodes critical for many vascular processes.

Figure 2.1: Shc is required for intersegmental vessel sprouting angiogenesis in zebrafish



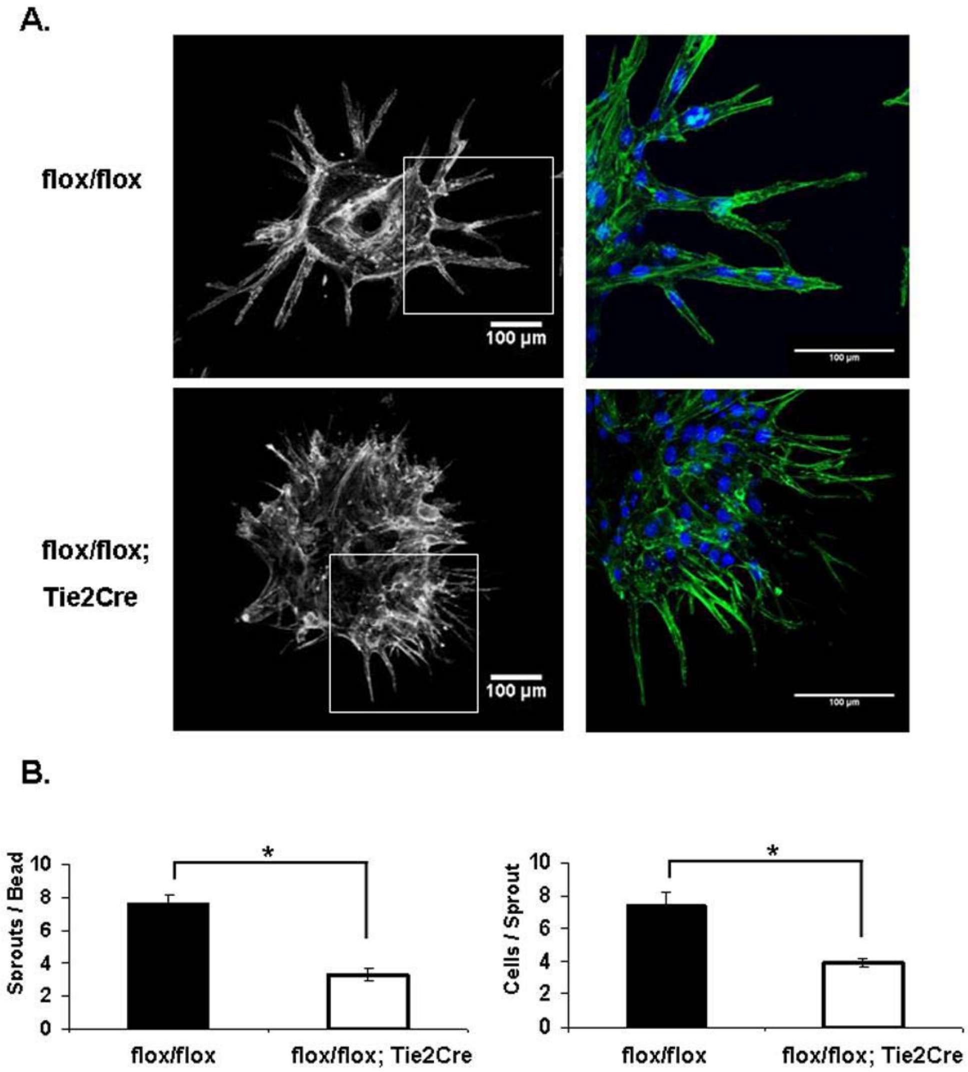
(A) Shc protein depletion in *Tg(kdrl:egfp)* zebrafish embryos results in defective angiogenesis 30 hpf. Representative images of trunk vasculature are shown, with anterior on the left. Shc-MO fish exhibited a range of vascular phenotypes, the most common being delayed or defective intersomitic vessel sprouting and growth dorsally from the aorta. Scale bars = 200 μm. (B) Quantification of phenotypes observed in all living fish at 24 hpf displayed as % of total. Control 4x MO n=118; Shc 2x MO n=72; Shc 4x MO n=105 embryos. Numbers indicate all fish counted from 3 independent experiments.

Figure 2.2: Endothelial Shc knockout causes defective angiogenesis *in vivo*



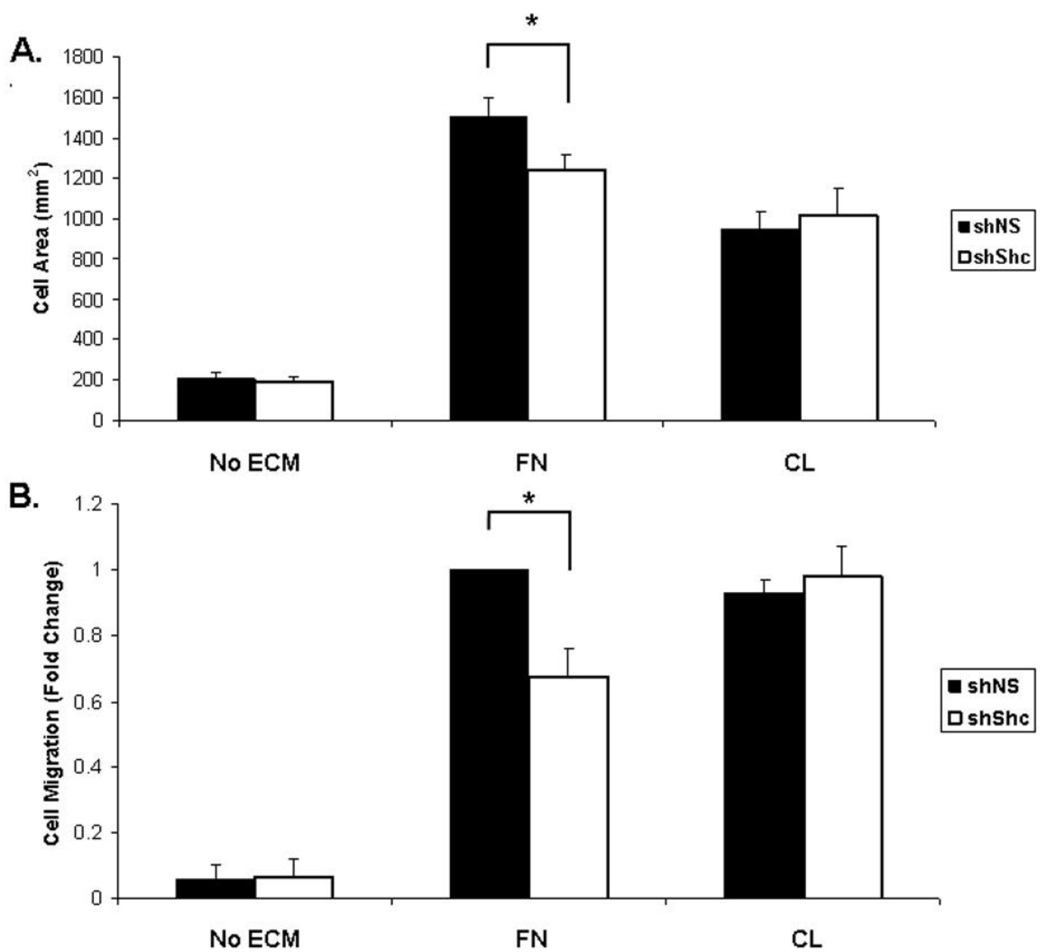
(A) Shc knockout in ECs results in decreased vascular density in the postnatal retina. Retinas from P5 mice were stained with Isolectin-B4 Alexa488 to visualize endothelial cells. Vascular density was quantified by counting branchpoints per 100 μm^2 and % vascular area - performed blind by two different people. *Shcflox/flox* n= 17; *Shcflox/flox; Tie2-Cre+* n= 15 ; *Shc wt/wt;; Tie2-Cre+* n= 9 mice. Scale bars = 100 μm. (B) Matrigel Plug assay in 4-6 week old mice reveals a role for EC Shc in angiogenesis toward VEGF. Matrigel plugs containing 250 ng/ml VEGF and or vehicle alone were implanted into each mouse. After 7 days, plugs were H&E stained and microvessels per mm^2 were counted in serial sections through the plug. Quantification is shown as mean +/- SEM (Student's t-test). *Shcflox/flox* n= 5, *Shcflox/flox; Tie2-Cre+* n=6 mice.

Figure 2.3: Endothelial Shc is required for tube assembly & sprouting *in vitro*



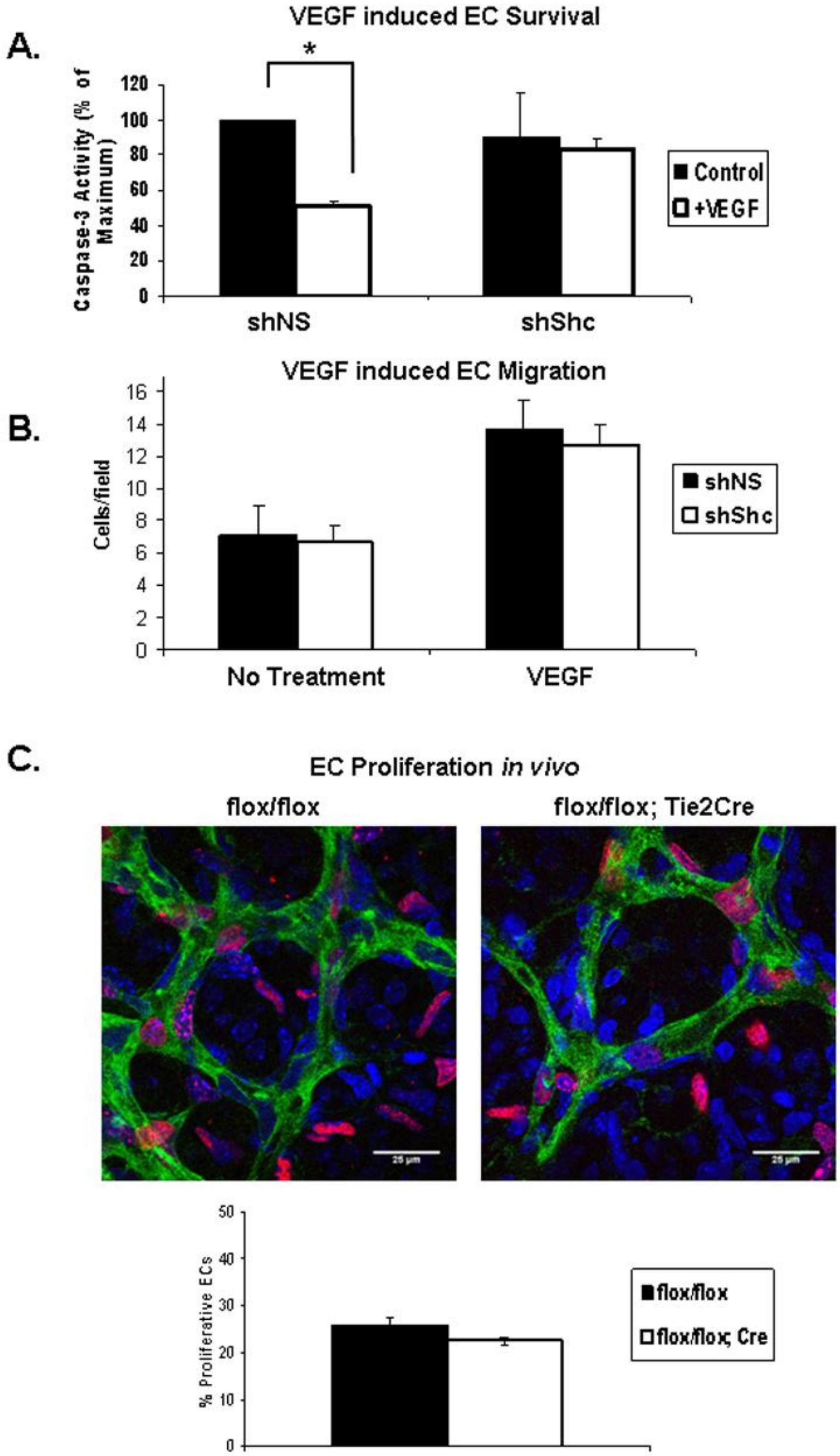
(A) Fibrin Gel Bead Assay was performed using MLEC isolated from mice used in Fig. 2. On Day 3 after seeding cell covered beads in gel, cultures were fixed and stained for phalloidin (green) and DRAQ5 (blue). Quantifications (below) were performed by counting at least 15 beads per genotype and are expressed as mean +/- SEM. n= 2 independent experiments, 4 replicates per experiment. In all graphs, * indicates $p<0.05$

Figure 2.4: Shc is required for integrin-mediated spreading & haptotaxis on fibronectin but not collagen



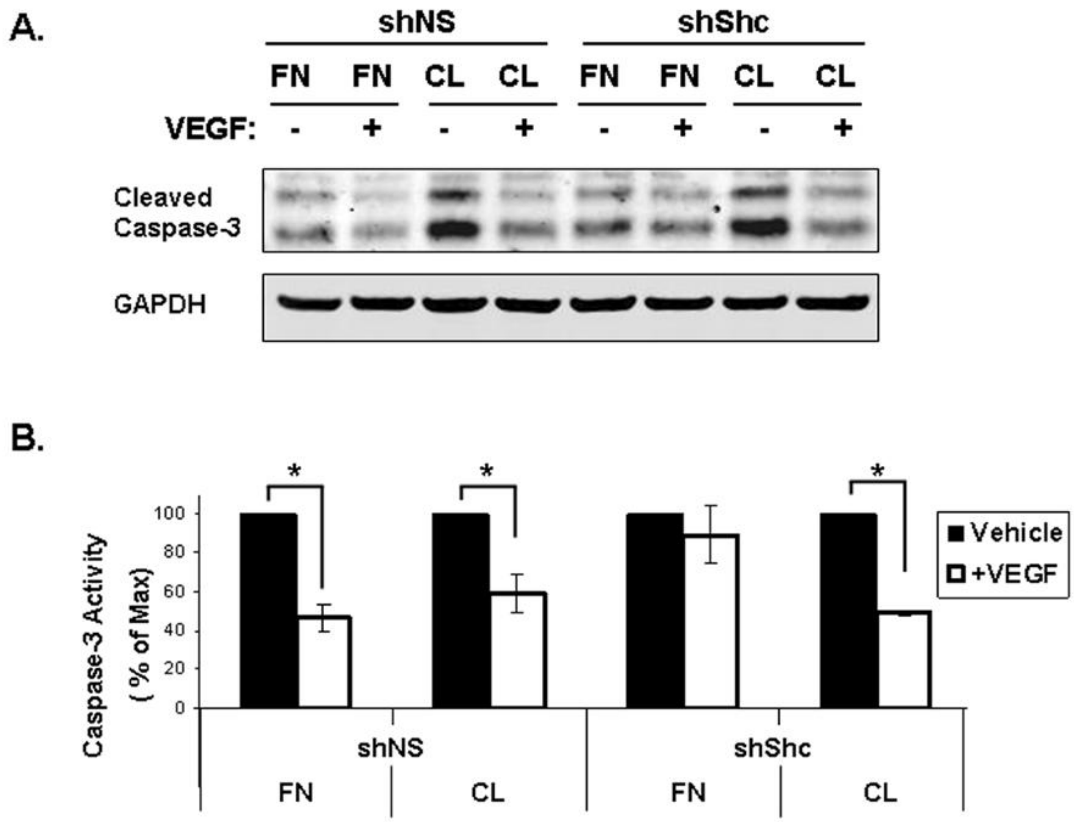
(A) Shc is required for cell spreading on FN. Equal numbers of lentivirus infected HUVECs were seeded on coverslips coated with 10ug/ml FN, 10ug/ml CL or vehicle (PBS) and allowed to spread for 25 minutes. Cell area was measured using ImageJ. Values shown are mean +/- SEM (n=2 independent experiments, >100 cells counted per condition per experiment). (B) Haptotaxis was measured using Boyden Chambers coated on the underside with 10ug/ml FN, 10ug/ml CL or vehicle (PBS) and blocked with 3% BSA. Cells that had migrated to the underside of the chamber were counted using an inverted microscope. Five random fields were imaged per filter. Values shown are mean +/- SEM (n=3 independent experiments, 2 filters per condition per experiment). In all graphs, * indicates $p<0.05$

Figure 2.5: Shc is required for VEGF-induced EC survival but not migration toward VEGF or proliferation



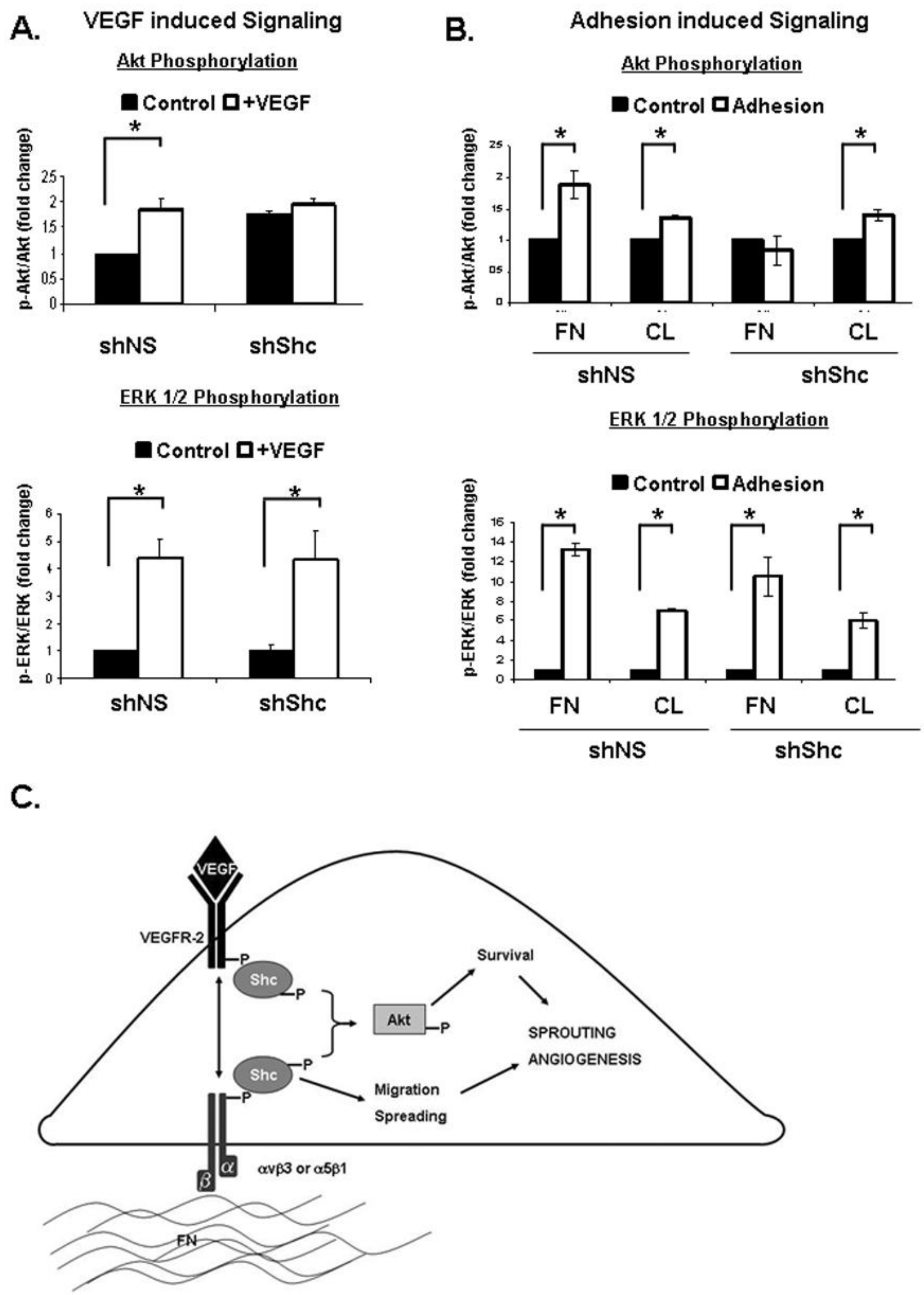
(A) HUVECs were serum starved for 24 hrs with or without 100ng/ml VEGF to induce apoptosis. Lysates were immunoblotted for cleaved caspase 3 and GAPDH as a loading control. Survival was quantified by comparing the amount of cleaved caspase 3 present in lysate. Values shown are mean +/- SEM (n=4 independent experiments). (B) Chemotaxis toward VEGF gradient was measured using Boyden Chambers containing 100ng/ml VEGF or vehicle in the lower well. After 4 hours of migration, cells that had migrated to the underside of the membrane were counted using an inverted microscope. Five random fields were imaged per filter. Values shown are mean +/- SEM (n=3 independent experiments, 2 filters per condition per experiment). In all graphs, * indicates $p<0.05$. (C) EC Proliferation was assayed in the P5 mouse retina. EdU reagent was injected intraperitoneally and 2 hrs later retinas were harvested. Retinas were stained with Isolectin (green) to mark ECs, DAPI (blue) to mark all cell nuclei, and EdU (red) to mark proliferating nuclei. Proliferation of ECs was quantified by counting # isolectin/EdU positive nuclei divided by # isolectin/DAPI positive nuclei. Values shown are mean +/- SEM (*Shcflox/flox* n= 5; *Shcflox/flox; Tie2-Cre* n= 8).

Figure 2.6: Survival requires integration of VEGF & integrin signaling through Shc



(A) HUVECs were seeded on FN or CL coated dishes, then serum starved for 24 hrs with or without 100ng/ml VEGF to induce apoptosis. Lysates were immunoblotted for cleaved caspase 3 and GAPDH as a loading control. Survival was quantified by comparing the amount of cleaved caspase 3 present in lysate. Values shown are mean +/- SEM (n=3 independent experiments).

Figure 2.7: Shc is required for specific signal transduction pathways downstream of integrins & VEGF



(A) The role of Shc in VEGF signaling was assayed in HUVECs. Cells were treated for 5 min with 100ng/ml VEGF or vehicle. Cell lysates were separated by SDS-PAGE and immunoblotted for the indicated proteins. Quantitation values shown are mean +/- SEM (n=4 independent experiments). (B) The role of Shc in integrin signaling was assayed in HUVECs. Cells were allowed to adhere and spread on FN or CL (10ug/ml) for kept as controls. Cell lysates were separated by SDS-PAGE and immunoblotted for the indicated proteins. Quantitation values shown are mean +/- SEM (n=3 independent experiments). In all graphs, * indicates $p<0.05$ (C) Schematic model of how Shc is thought to regulate angiogenesis in endothelial cells. Shc participates in signaling from fibronectin binding integrins such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$ which is required for EC spreading and migration. Simultaneously, Shc is also required for EC survival induced by VEGF. Loss of Shc results in attenuation of Akt activation by the integrin and VEGF pathways in ECs and thus, results in defective angiogenesis, as is seen in the zebrafish and mouse.

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CHAPTER III.

SHC MEDIATES THE ENDOTHELIAL RESPONSE TO SHEAR STRESS *IN VITRO* AND DURING ARTERIOGENESIS *IN VIVO*

PREFACE

This chapter represents results described in two manuscripts. The first manuscript, published in 2008 [1], described a role for the adaptor protein Shc in the endothelial response to shear stress *in vitro*. Some of the data from this paper is included in Figures 3.1-3.3 of this chapter. I was the second author on this paper and my contributions included performing and analyzing the *in vitro* experiments showing Shc phosphorylation and Shc association with the mechanosensory complex in response to flow, and experiments comparing responses to flow on FN and CL. Yunhao Liu performed mouse tissue immunohistochemistry and the Shc siRNA experiments. Mohamad Irani-Tehrani performed initial co-immunoprecipitation experiments and made the discovery that Shc associates with VE-cadherin and integrins in response to shear stress. Nobuyo Maeda provided the ApoE null mice. Ellie Tzima was the principal

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[1] Liu Y, **Sweet DT**, Irani-Tehrani M, Maeda N, Tzima E. Shc coordinates signals from intercellular junctions and integrins to regulate flow-induced inflammation. *J Cell Biol*. 2008 Jul 14; 182(1):185-96

investigator of the study, designed the experiments, analyzed the data and wrote the manuscript.

The second manuscript, which encompasses Figures 3.4-3.7 of this chapter, is currently under review at *Circulation Research* [2]. This paper examines the role of Shc in shear stress-induced arteriogenesis *in vivo.* I am co-first author on this paper and contributed by making the conditional knockout mice, performing the Laser Doppler Imaging, imaging and quantitating tissue sections, performing qPCR experiments and writing the manuscript. Zhongming Chen performed hindlimb ischemia surgeries, harvested mouse tissue and performed immunofluorescence staining and imaging of collateral sections. Ellie Tzima was the principal investigator of the study, designed the experiments, analyzed the data and wrote the manuscript.

OVERVIEW

Shear stress is a potent regulator of the EC phenotype and regulates the inflammatory status of the vessel. Disturbed shear stress can trigger chronic vascular inflammation and cause atherogenic plaque formation, whereas increased shear stress through collaterals can be beneficial following an occlusion, when changes in shear stress can induce vessel remodeling and

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[2] **Sweet DT**, Chen Z, Tzima E. Endothelial Shc regulates arteriogenesis through dual control of arterial specification and inflammation via the Notch and NF-κB pathways. In Review- *Circulation Research*

recovery of perfusion of the ischemic tissue. Shear stress is sensed by several different ‘mechanosensors’ at cell-cell and cell-ECM adhesions, however little is known about signal transduction downstream of these mechanosensors. Here, we show that the adaptor protein Shc is activated by shear stress and associates with cell-cell and cell-matrix adhesions. Shc regulates flow-induced inflammatory signaling by mediating NF-κB activation and subsequent leukocyte adhesion to the endothelium *in vitro*. We confirmed that Shc is required for signaling in response to shear stress i*n vivo*, as conditional knockout mice in which Shc is deleted from ECs exhibited reduced hindlimb perfusion recovery following femoral artery ligation. Reduced perfusion was associated with blunted shear-driven collateral remodeling and reduced capillary density. Mechanistically, Shc deficiency resulted in impaired activation of the NF-κB-dependent inflammatory pathway and reduced CD45+ leukocyte infiltration into the vessel wall. Unexpectedly, Shc is required for arterial specification of the remodeling arteriole by mediating upregulation of the arterial endothelial cell marker ephrinB2 and activation of the Notch pathway. Taken together, these results show that Shc is activated by shear stress and mediates activation of shear stress-induced signaling pathways such as inflammation and arterial specification.

INTRODUCTION

Fluid shear stress, the frictional force from blood flow, acts directly on the endothelium to modulate vessel structure and function [1]. Two different types of shear stress exist due to the natural branched and curved patterning of the vascular tree. In straight vessels such as the descending aorta, laminar flow exerts atheroprotective effects on the ECs by activating anti-inflammatory genes, such as eNOS and Klf-2. Conversely, in curved or bifurcated regions of the vasculature such as the aortic arch, disturbed shear stress occurs and atheroprotective genes are suppressed, while pro-atherogenic genes are upregulated, thereby promoting the atherosclerotic process [2]. Importantly, acute onset of laminar flow *in vitro* stimulates many of the same responses as sustained disturbed shear. However, over longer time periods, the cells adapt to the unidirectional laminar shear forces and downregulate signaling, whereas under prolonged disturbed shear, continual changes in flow magnitude and direction lead to chronic, sustained signaling[3]. Thus, the *in vitro* protocol in which cells under static conditions are exposed to an abrupt increase in flow has been widely used as a model for disturbed flow and is particularly useful in analyzing temporal responses to flow. *In vivo*, onset of flow occurs in pre-existing collateral arteries acutely following occlusion of a large artery upstream, and initiates a shear-stress induced remodeling process called arteriogenesis.

Arteriogenesis, the outward remodeling of pre-existing immature collateral arteries, is critical in recovery and restoration of blood supply to ischemic tissue following a vascular occlusion. Arteriole–arteriole anastomoses, termed collateral arteries, act as a natural bypass mechanism to maintain blood supply to downstream tissue even when major arteries, such as the femoral artery, become blocked [4]. In a healthy animal, blood flow through collateral arteries is negligible, however, after an occlusion, the steep pressure gradient between the pre-and post- occlusive regions of the vessel causes blood to rush into pre-existing collaterals, activating ECs and inducing outward remodeling of the vessel. Outward remodeling of pre-existing collaterals is driven primarily by the sudden spike in hemodynamic forces resulting from the increase in flow through the collaterals[5]. Changes in hemodynamic forces are sensed by ECs[6-8], which initiate signal transduction pathways that ultimately result in outward remodelling of the vessel to increase the lumen diameter and normalize the high shear stress.

EC surfaces are equipped with numerous mechanoreceptors that are capable of detecting and responding to shear stress [10, 11]. After activation of mechanoreceptors, a complex network of several intracellular pathways is triggered, a process known as mechanotransduction. We previously identified a 'mechanosensory complex' located at cell-cell junctions comprised of PECAM-1, VE-Cadherin, and VEGFR-2 that is necessary and sufficient for the EC response to shear stress *in vitro* such as activation of NF-κB and it's pro-inflammatory target genes[12], vascular remodeling[13] and arteriogenesis *in vivo*[14].

The *Shc1* gene encodes an adaptor protein which is a key component of the pathways that activate Ras and MAP kinases downstream of several different growth factors, cytokines, integrins and mechanical forces [15-18]. When activated, Shc associates with phospho-tyrosine residues on activated receptors, inducing Shc phosphorylation at tyrosine residues 239/240 and/or 317. Shc phosphorylation allows association with other signaling molecules and activation of signaling cascades such as Ras/MAPK pathway [17]. In addition, tyrosine-phosphorylated Shc associates with integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ when they are conjugated to appropriate ligands (FN) [19, 20]. Importantly, Shc is expressed primarily in the cardiovascular system of mouse embryos and global knockout of the *Shc1* gene in mice causes embryonic lethality at E11.5 due to defects in embryonic heart development[21, 22]

Here, we show that shear stress induces association of Shc with components of the 'mechanosensory complex' and reveal a surprising role for Shc in flow-induced inflammatory signaling *in vitro*. Additionally, Shc is required specifically in ECs for shear stress-induced arteriogenesis *in vivo*. Our data show impaired plantar perfusion recovery in *Shc flox/flox; Tie2-Cre* conditional knockout mice compared to controls. Furthermore, we demonstrate that Shc is required for ligation-induced inflammation and activation of the NF-κB pathway in collateral arteries. Surprisingly, Shc is also required for shear stress-induced activation of the Notch pathway and downstream expression of the arterial marker ephrinB2, which is important for specifying arterial identity of the remodeling collaterals.

METHODS

BAEC Culture, Transfections & Shear Stress

Bovine aortic endothelial cells (BAECs) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% Fetal Bovine Serum (FBS, Invitrogen), 10 μg/ml penicillin, 0.25 μg/ml streptomycin (Invitrogen). THP-1 leukocytes were maintained in RMPI 1640 medium (Invitrogen) with 10% FBS, 10 μg/ml penicillin, 0.25 μg/ml streptomycin, and 2 mM glutamate (Invitrogen). Control siRNA or Shc siRNA (Dharmacon) were transfected into BAECs as previously described[23]. For shear stress experiments, BAECs were plated on appropriate matrix proteins (10 μg/ml FN or 20 μg/ml Coll I) and allowed to grow for 10 h in medium containing 10% FBS or 4h in 0.5% FBS. Cells were then starved overnight in medium containing 0.5% FBS. Slides were loaded onto a parallel plate flow chamber in 0.5% FBS and 12 dynes/cm^2 of laminar shear stress was applied for indicated times. To perform oscillatory flow, the slides were attached to parallel chambers as with laminar flow. The chambers were subsequently connected to an NE-1050 bi-directional pump purchased from New Era Pump Systems, Inc. (Farmingdale, NY). Cells were sheared at ± 6.5 Dyne/cm^2, 1 Hz in media containing 0.5% FBS.

Immunoprecipitations, Western blotting & Antibodies

Cells were harvested in lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS) supplemented with 1 mM aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF, 1 mM sodium pyrophosphate and 1 mM β-glycerophosphate. Lysates were pre-cleared with 50 μl protein A/G plus sepharose beads (Santa Cruz) for 1 h at 4°C. Supernatants were then incubated with 30 μl protein A/G plus sepharose previously coupled to the primary antibodies for 2 h at 4°C with continuous agitation. The beads were washed three times with lysis buffer supplemented with protease and phosphatase inhibitors and the immune complexes were eluted in 2X SDS sample buffer. Associated proteins were subjected to SDS-PAGE and Western Blotting using the appropriate primary antibodies and HRP-conjugated anti-mouse or anti-rabbit antibodies (Jackson Immunochemicals). Immunoreactive proteins were visualized by enhanced chemiluminescence (GE Health). The phospho-Shc (Tyr239/240 or Tyr317) and phospho-p65 (Ser536) were purchased from Cell Signaling. An anti-Shc phosphoTyr239/240 antibody from Invitrogen BioSource was tested and generated similar results to the Cell Signaling phospho-Shc antibody. Anti-VEGFR-2 and VE-Cadherin antibodies were purchased from Santa Cruz Biotechnology. Anti-Shc and anti-NFκB (p65) were purchased from BD Transduction Laboratories. FITC-conjugated goat anti-mouse IgG and Rhodamine-conjugated goat anti-rabbit IgG were obtained from Jackson ImmunoResearch Inc. and used at 1:200 dilution.

Immunofluorescence Microscopy

To examine the tyrosine phosphorylation of Shc and the nuclear translocation of NFκB, cells were fixed for 20 min in PBS containing 2% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with PBS containing 10% goat serum and 1% BSA for 1h at room temperature. Antibody incubations were performed as previously described[24] and slides mounted in vectashield mounting medium (Vector laboratories, Inc.). Images were obtained using the 60X/1.40 oil objective on a Nikon Eclipse E800 microscope equipped with a Hamamatsu ORCA-ER digital camera and MetaMorph software.

Leukocyte Adhesion Assay

For each adhesion assay, 1×10^6 THP-1 cells were collected by centrifugation. Cells were resuspended in serum-free RMPI 1640 medium containing 1 μM CellTracker Green CMFDA (5-chloromethylfluorescein diacetate, Molecular Probes) and incubated at 37°C for 20 min. Cells were then spun down and resuspended in RMPI 1640 medium containing 10% FBS. After the endothelial cells were sheared for the required times, the pre-labeled THP-1 cells were added onto the monolayers of endothelial cells and incubated at 37°C for 15 min. The unbound cells were rinsed off with PBS and the bound cells fixed with 2% formaldehyde. To quantify the assays, five random fields under the 10X/0.30 objective on a Leica DMIRB inverted microscope were counted for each assay and representative images were acquired using a QImaging RETIGA 1300 camera.

Animals

Shc-flox mice were a kind gift from Dr. Kodi Ravichandran at University of Virginia[25]. Tie2-Cre (B6.Cg-Tg(Tek-cre)12Flv/J) mice were purchased from Jackson Labs. All housing, breeding and experimental procedures using mice were in accordance with national guidelines and regulations and were approved by the Institutional Animal Care and Use Committee at the University of North Carolina- Chapel Hill. Male *Shc flox/flox; Tie2-Cre* and age-matched littermates (*Shc flox/flox*, 10-14 weeks) were used for all experiments. To genotype animals, DNA was isolated from ears at weaning and PCR performed. All analyses were conducted by observers blinded to animal genotype.

Unilateral Hindlimb Ischemia

The surgery procedure was performed on the right hind limb as described previously[14]. Briefly, animals were anesthetized with 1.125% isoflurane supplemented with oxygen, and body temperature was maintained at 37°C. Hair was removed from the hindquarters with a depilating cream. The femoral artery was exposed aseptically through a 2mm incision and isolated from vein and nerve. The femoral artery was ligated with 7-0 ligatures proximal to the bifurcation of the popliteal artery and distal to the lateral caudal femoral artery (LCFA) for the less severe ischemia mode. The incision was closed after the wound was irrigated with sterile saline.

Laser-Doppler Imaging

The animals were placed for 5min at a 37°C chamber before the measurements to avoid vasoconstriction by anesthetic heat loss. A Laser Doppler imager was used to estimate relative blood flow. Ratios of occluded over non-occluded values were compared. Noninvasive measurements of superficial hindlimb perfusion were obtained before ligation, immediately after ligation (acute), 7 and 21 days after ligation [5]. Plantar perfusion was quantified within anatomically defined regions of interest (ROIs). All ROIs were drawn by an investigator blind to animal genotype. Data are reported as perfusion ratios of ligated vs. sham control side.

Morphometry

Collateral arteries were harvested from mice as described previously[14]. Briefly, animals were transcardially perfused at 100 mmHg with PBS containing 10 nmol/l sodium nitroprusside and 10U/ml heparin 3 weeks after hindlimb ischemia. PBS was followed by 2% PFA for 20 min. We harvested the anterior and posterior gracilis muscles which contain three pre-existing collaterals. The midzone of the muscles (i.e., the 5-mm-wide centermost section) was trimmed. A section of the calf (gastrocnemius/soleus) muscle was also harvested for the examination of capillary density (described below). Samples were embedded in paraffin and 5μm thick cross sections were H. & E. stained. Lumen diameter of collateral arteries was measured as previously described[14]. H&E stained cross-sections within 0.5mm from the midzone of the collateral arteries in anterior and posterior gracilis muscles were digitized at 60x magnification. Lumen circumference was measured interactively using NIH Image J package. For each mouse, four arteries were studied, two from the surgery-operated side and two from the sham control side. At least 4 cross-sections from each mouse were measured and the average lumen diameter of collateral vessel was used.

Immunohistochemistry

We used antibodies to NF−κB (1:200, BD Pharmingen, San Diego, CA), VCAM-1 (1:200, Santa Cruz), CD45 (1:100, BD Pharmingen), PCNA (1:1000, Abcam, Cambridge, MA), ephrinB2 (1:200, R & D systems, Minneapolis, MN), Cleaved Notch-1 (1:100, Abcam), Phospho-Shc (1:100, BD Transduction) and Smooth Muscle α-Actin(SMC α-actin, 1: 1000, Sigma-Aldrich). Antigen retrieval was performed for cross sections with Retrogen (BD Pharmingen), except for NFκB and PCNA antibodies. Thyramide signal amplification (TSA, Perkins Elmar Inc, Waltham, MA) was performed for NFκB, VCAM-1, CD45, ephrinB2 and Cleaved Notch-1 staining, per manufacturer's instruction. Primary antibody was incubated at 4^{o}C overnight, followed by 60 minutes for biotinylated secondary antibody (1:500), and 30 minutes for ABC complex (Elite ABC kit, Vector Laboratories, Burlingame, CA). Cy3-thyamide was used to visualize the peroxidase-binding sites. To visualize collateral media, the slides were further incubated with mouse anti-SMC α-actin for 2 hours, followed by the incubation of Cy5-goat anti-mouse Ig G (1:100) for one hour. The slides were counter stained with DAPI to visualize cellular nuclei.

Leukocyte Density

Leukocytes in the adventitia and periadventitia of pre-existing collaterals were detected with CD45 antibody as described above. CD45-positive cells having a blue nucleus surrounded by Cy3 fluorescence (from Cy3-thyramide) on their surface were counted by an observer blinded to the identity of the randomly arranged slides. Average leukocyte density was determined from 4 sections from each animal.

Capillary Density

Capillary density was counted as described previously[14]. Briefly, muscle fibers were harvested from the right and left side, from the *m. adductor* and *m. gastrocnemius*. The plasma membrane of capillary ECs in tissue sections was labeled with Alexa 568-IsolectinB4 (Invitrogen, CA). Micrographs were obtained with the Nikon fluorescence microscope using a 20x objective lens. Digitized images were analyzed with an image analysis package, Image J. The total number of capillaries was counted on 5 random optical fields for each mouse. Results are expressed in capillaries per muscle fiber.

iMLEC Cell Culture & Shear Stress

Shc flox/flox and *Shc flox/flox; Tie2-Cre* MLECs (for isolation protocol see[26]) were subjected to shear stress *in vitro* using a previously described cone and plate viscometer[27]. 100-mm tissue culture dishes were coated in 10□g/ml Fibronectin, then MLECs were seeded and grown to confluence in EGM-2 (Lonza) with 10% FBS. Then, MLECs were serum starved overnight in M-199 (Gibco) with 0.5% FBS and exposed to an arterial level of unidirectional laminar shear stress (15 dyn/cm^2) in the starvation medium for 4 hours or kept static as controls. For inhibitor experiments, ECs were pre-treated for 1 hr with 20μM DAPT (Selleck Chem) or DMSO vehicle control in starvation media, then exposed to shear for 4 hrs in the presence of inhibitor. Immediately after treatment, dishes were washed 1x in cold PBS and frozen. MLECs were lysed in TriZol for subsequent RNA isolation.

Real-Time Quantitative PCR

Total RNA was extracted from MLECs using Trizol reagent (Invitrogen, Inc, Carlsbad, CA) following manufacturer's protocol. Total RNA (5μg) was reverse-transcribed into cDNA with random primers and SuperScript II (Invitrogen) per manufacturer's instruction. The primer pairs were as following ("-F": forward, "-R": reverse): GAPDH-F: 5'-GGC ATT GCT CTC AAT GAC AA -3'; GAPDH-R: 5'-TGT TGC TGT AGC CGT ATT CA -3'; ephrinB2-F: 5'-GCG GGA TCC AGG AGA TCC CCA CTT GGA CT -3'; ephrinB2-R: 5'- GTG CGC AAC CTT CTC CTA AG -3'; HES-F: 5'- TCC TTG GTC CTG GAA TAG TGC TA -3'; HES-R: 5'- ACT GAG CAG TTG AAG GTT TAT TAT GTC T -3'; Deltex-F: 5'- CAC TGG CCC TGT CCA CCC AGC CTT GGC AGG -3'; Deltex-R: 5'- GGG AAG GCG GGC AAC TCA GGC CTC AGG -3'. SYBR Green I based real-time PCR (Absolute SYBR Green ROX Mix, Thermo Fisher Scientific, Surrey, UK) was performed in a Rotor

Gene thermal cycler (Qiagen, Foster City, CA) with the following thermal parameters: 95°C 15 min, followed by 40 cycles of 95°C for 30sec, 57°C for 30sec, 72°C for 30sec. Data were analyzed using relative real-time PCR quantification based on the ΔΔCt method. GAPDH was the endogenous reference gene for ephrinB2, HES and Deltex, and the control was *Shc flox/flox* static MLECs.

Statistical Analysis

Values are presented as means ± SE. Differences was determined by Student t-test (between two groups) and one-way ANOVA (among multiple groups). A value of $P<0.05$ was considered to indicate statistical significance.

RESULTS

Shc is Activated by Shear Stress

Shc signaling function is regulated by three tyrosine residues (Y239, Y240 and Y317) that are phosphorylated when Shc is activated. To test whether Shc can be activated by shear stress, we subjected ECs to different flow patterns *in vitro* and assayed Shc activation using a phospho-Shc specific antibody. ECs stimulated with prolonged disturbed flow (which is pro-inflammatory and atherogenic) showed elevated Shc phosphorylation compared to static ECs whereas ECs stimulated with extended laminar flow (which is considered anti-inflammatory and atheroprotective) showed decreased Shc phosphorylation compared to static ECs (Figure 3.1A). This indicates that Shc is activated by atherogenic disturbed shear but is preferentially de-activated by laminar atheroprotective shear. Acute onset of laminar shear stimulates many of the same responses as chronic disturbed flow[28]; thus, a large number of *in vitro* studies have exploited the acute onset of laminar shear to model atheroprone signaling and obtain a temporal map of signaling cascades activated by shear stress[7]. We therefore used the onset of laminar flow protocol to assay the early responses downstream of Shc. In ECs, Shc phosphorylation was detected upon the onset of flow, as assessed by immunoblotting the cell lysates with a phospho-Shc Tyr239/240 antibody (Figure 3.1B). Notably, all three isoforms of Shc are phosphorylated in response to flow. Immunofluorescence staining showed that a fraction of activated Shc localized to cell-cell junctions (Figure 3.1C). Flow-induced phosphorylation of Shc Tyr317 was not observed in parallel experiments (data not shown), suggesting that the downstream signaling elicited by Shc in response to flow is primarily mediated through its phosphorylation at Tyr 239/240.

Shc Associates with Components of the 'Mechanosensory Complex' in Response to Shear Stress

The distinct spatial activation of Shc in response to the onset of flow suggested that Shc might associate with components of endothelial cell-cell junctions. Recently, our lab identified a minimal complex necessary for a subset of EC shear stress responses, which requires PECAM-1, VE-cadherin and VEGFR-2[12]. To further investigate the role of Shc in shear stress signaling, the association of Shc with crucial components of the VE-cadherin–VEGFR-2 signaling pathway was examined. Onset of flow induced an acute association of Shc with VE-cadherin and VEGFR-2 (Figure 3.2A), suggesting the existence of a multi-protein complex induced by shear. Importantly, the association of Shc with VE-cadherin was sustained under long term disturbed flow (Figure 3.2B), similar to the sustained Shc phosphorylation.

Shear Stress Induced Shc Association with Integrin $\alpha_v\beta_3$ is Dependent on ECM Composition

Binding of integrins to their specific ligand in the ECM induces integrin activation and intracellular recruitment of signaling adaptor proteins to the integrin[29]. Shc has been shown to associate with activated integrin $\alpha_v\beta_3$ when the integrin is bound to its ligand, fibronectin (FN) or vitronectin (VN)[20]. Consistent with previous reports, shear stress induced Shc-integrin association in cells plated on FN and VN (both engage integrin $\alpha_v\beta_3$) but was absent in cells plated on collagen (CL) or laminin (LN) (both engage integrin $\alpha_2\beta_1$) (Figure 3.2C). Importantly, the composition of the subendothelial ECM modulates inflammatory signaling and permeability in response to shear stress[28, 30]. Orr et al. reported that the inflammatory transcription factor NF-κB is activated by shear stress only when ECs are growing on FN whereas shear stress does not activate NF-κB on CL[28]. To determine whether Shc activation is also matrix specific, ECs were plated on either FN or CL and Shc phosphorylation was assayed. Onset of flow triggered an increase in Shc phosphorylation irrespective of the matrix that the cells were plated on (Figure 3.2D), indicating that Shc phosphorylation is not ECM specific. To test whether the flow-induced Shc association with cell-cell junctions is ECM dependent, immunoprecipitation assays were performed with lysates from cells plated on FN or CL. As shown in Figure 3.2E, Shc interaction with VE-cadherin was rapidly enhanced after the onset of flow regardless of the ECM composition. Thus, Shc activation and association with cell-cell junctions correlate closely and occur independently of the matrix composition, whereas the later Shc-integrin association is ECM-dependent.

Shc Mediates the Flow-Induced Inflammatory Response

Shear stress regulates the chronic inflammation associated with atherosclerotic plaque formation[31-33]. The Shc phosphorylation observed in response to atherogenic disturbed flow raises the possibility that Shc may participate in the regulation of the inflammatory response elicited by shear stress. NF-κB is a key regulator of shear stress-induced inflammatory gene expression and contributes to the initiation of atherosclerosis by shear stress. In unstimulated cells, NF-κB is held inactive in the cytoplasm through its interaction with IκB. Degradation of IκB results in NF-κB activation, nuclear targeting and initiation of transcription [34]. To test whether Shc is involved in flow-induced NF-κB activation, we suppressed cellular levels of Shc using small interfering RNAs (siRNAs) (Figure 3.3A). Knockdown of Shc expression prevented nuclear translocation of NF-κB by shear whereas control siRNA had no effect, suggesting that Shc is upstream of NF-κB activation in response to flow (Figure 3.3B).

Nuclear NF-κB binds to a shear stress responsive element found in the promoter of several atherogenic genes, including ICAM-1 and VCAM-1, which regulate monocyte recruitment to the endothelium [35, 36]. To test whether the role of Shc in NF-κB activation by shear stress is functionally relevant for the shear-induced inflammatory response, we tested whether Shc is required for shear stress-induced monocyte adhesion to the EC monolayer. Interestingly, in control siRNA transfected cells, disturbed flow increased monocyte adhesion to the endothelium whereas it had no effect on monocyte adhesion to Shc siRNA transfected ECs (Figure 3.3C). Thus, we conclude that Shc function is important for mediating the initial events in inflammation and atherogenesis induced by shear stress.

Endothelial Shc is Required for Plantar Perfusion Recovery following Femoral Artery Ligation

To examine the role of endothelial Shc in shear stress-induced responses *in vivo*, conditional knockout *Shc flox/flox; Tie2-Cre* and control *Shc flox/flox* littermates (referred to as *flox/flox* and *flox/flox; Cre*, respectively) were subjected to hindlimb ischemia by ligation of the femoral artery, which triggers flow-induced adaptive remodeling of pre-existing collaterals from the deep femoral artery. Blood perfusion of hind paws (plantar) was non-invasively measured using Laser Doppler Imaging before surgery (pre), immediately after surgery (acute), and at various timepoints throughout the 3 week recovery period following surgery (Figure 3.4A). Plantar perfusion was quantified from the Doppler images and normalized to the sham control side of the same animal for the comparisons of different time points. Strikingly, *flox/flox; Cre* mice displayed attenuated perfusion recovery as early as 3 days post-surgery (Figure 3.4B). The defect in perfusion recovery was exacerbated at each timepoint until the final timepoint showed a 40% reduction in plantar perfusion in *flox/flox; Cre* mice compared to *flox/flox* controls. Importantly, in wild-type mice, femoral artery ligation induced rapid Shc phosphorylation in ECs that line pre-existing collateral arteries (Figure 3.4C), indicating that endothelial Shc is activated in this model. These data demonstrate that Shc is activated in ECs during collateral remodeling and that Shc is required for perfusion recovery following hindlimb ischemia.

Shc is Required for Collateral Artery Remodeling & Angiogenesis

Plantar perfusion recovery following femoral artery ligation requires two EC-dependent vascular processes: arteriogenesis and angiogenesis[5, 37]. Ligation of the femoral artery causes a sudden increase in blood flow and hemodynamic force through pre-existing collateral arteries in the *gracilis* muscles, causing flow-induced outward vascular remodeling (arteriogenesis), therefore allowing more blood to be carried by the collateral artery. Simultaneously, ischemia in tissues distal to the ligation, such as the *gastrocnemius* muscle, induces angiogenesis in order to increase vascular density and blood perfusion. In order to test whether Shc is required for arteriogenesis and/or angiogenesis, we examined the *gracilis* and *gastrocnemius* muscles after 3 weeks after ligation (or sham) surgery. While there was no difference in basal *gracilis* collateral size in sham mice, we found that 3 weeks after ligation, the collaterals in *flox/flox; Cre* mice were ~30% smaller than those of control *flox/flox* littermates (Figure 3.5A). Similarly, induction of angiogenesis in the ischemic *gastrocnemius* muscle was defective in *flox/flox; Cre* mice. Capillary density increased by almost 50% in control *flox/flox,* whereas *flox/flox; Cre* mice were refractory to induction of angiogenesis (Figure 3.5B). As arteriogenesis is the largest contributor to perfusion recovery[38] we focused our attention on the role of Shc in collateral growth.

Shc is Required for Flow-Induced NF-κB Activation & Inflammation in Collaterals

The attenuated plantar perfusion recovery and collateral remodeling in *flox/flox; Cre* mice suggests a role for Shc in arteriogenesis. Sharp increases in hemodynamic forces in the collateral induces EC proliferation and inflammation; two EC-dependent processes that underlie arteriogenesis. We assayed EC proliferation in collaterals of *flox/flox; Cre* and *flox/flox* mice by nuclear PCNA staining. EC proliferation in collaterals 3 days after surgery was decreased in *flox/flox; Cre* mice compared to *flox/flox* controls (Figure 3.6A). Similarly, we assayed the role of Shc in activation of inflammation in response to femoral artery ligation. Collaterals were stained for infiltration of CD45-positive leukocytes, an important mediator of collateral remodeling. *flox/flox; Cre* mice exhibited a significant decrease in CD45- positive leukocyte infiltration compared to *flox/flox* controls (Figure 3.6B), indicating a role for Shc in inflammation in response to hindlimb ischemia. CD45-positive cell recruitment following femoral artery ligation requires activation of NF-κB in ECs[52], so we tested the role of Shc in NF-κB activation in this model. While control *flox/flox* mice showed activation of NF-κB as early as 24hrs after ligation, *flox/flox; Cre* mice displayed defects in NF-κB activation (Figure 3.6C). Interestingly, we also observed upregulation of p65 expression in ECs in *flox/flox* mice that was absent in *flox/flox; Cre* mice. This defect in p65 nuclear localization coincided with a decrease in expression of the NF-κB-dependent adhesion molecule Vascular Cell Adhesion Molecule-1 (VCAM-1) (Figure 3.6D). Together, these data indicate that Shc is required for EC proliferation and inflammation during collateral remodeling, both of which are critical for recovery from hindlimb ischemia.

Notch-Dependent Collateral EC Arterial Specification Requires Shc

The EC phenotype is plastic and heterogeneous throughout the vascular tree, and the expression of arterial- and venous-specific genes is the consequence of local hemodynamic cues that may regulate vessel remodeling [39, 40]. Because ECs in collaterals quickly change from a low flow environment to a high shear stress environment following femoral artery ligation[41, 42], we hypothesized that collateral ECs adopt a more 'arterial identity' to suit the new arterial-like blood flow environment. To test this, we stained collateral tissue sections for a marker of arterial (as opposed to venous) ECs, ephrinB2 [43]. EphrinB2 expression was upregulated in *flox/flox* collateral ECs 3 days after ligation, however, ephrinB2 upregulation was absent in *flox/flox; Cre* mice, indicating that Shc is required for arterial specification of ECs in response to changes in the hemodynamic environment in collaterals (Figure 3.7A). Because ephrinB2 is transcriptionally regulated by the Notch transcription factor NICD [44], we next examined activation of Notch in collaterals. In control *flox/flox* mice, femoral artery ligation induced Notch activation (NICD nuclear localization) in collateral ECs, whereas Notch activation was impaired in *flox/flox; Cre* mice (Figure 3.7B). These data indicate that Shc is important for activation of the Notch pathway and arterial specification of the remodeling collateral arterioles.

ECs in collateral arteries experience several mechanical and chemical stimuli simultaneously during arteriogenesis, making it difficult to delineate the exact role of Shc during collateral remodeling. We therefore tested the role of Shc in shear stress-induced Notch activation and ephrinB2 upregulation using an *in vitro* system. Onset of shear stress induced expression of the Notch target genes ephrinB2, HES and Deltex in ECs isolated from *flox/flox* mice. In contrast, shear-induced upregulation of Notch target genes was impaired in the absence of Shc (Figure 3.7C). Shear-induced gene regulation is dependent on Notch, as wild-type cells treated with the γ-secretase inhibitor DAPT failed to activate any of the Notch target genes (data not shown). Together, our data show that Shc is required for shear stress-induced arterial specification by mediating Notch-dependent ephrinB2 upregulation in ECs.

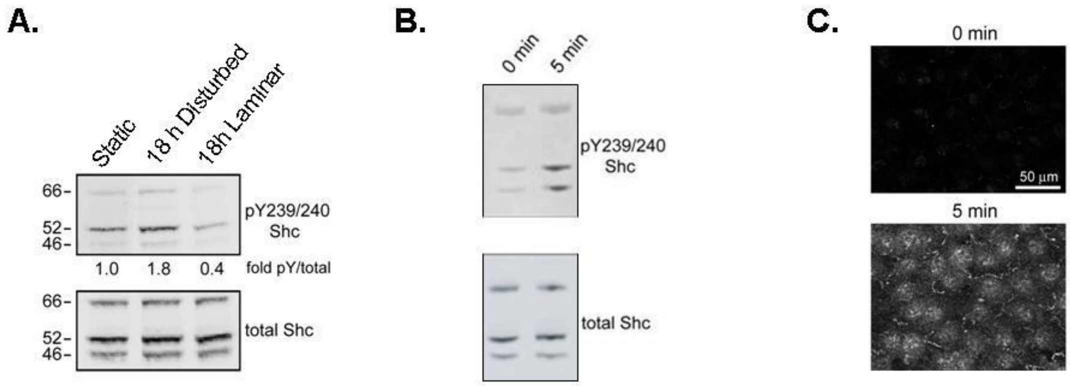
DISCUSSION

In this Chapter, I present evidence that Shc is required for shear stress-induced signaling in ECs both *in vitro* and in the mouse. Shc is phosphorylated in response to acute onset of shear stress and associates with components of the junctional mechanosensory complex VE-cadherin and VEGFR-2 at early times after the onset of flow; and with integrin-ECM adhesions at later times. While Shc phosphorylation and its association with VE-cadherin are ECM-independent, Shc binding to integrins occurs only on FN and not on CL. Depletion of Shc in ECs impairs flow-induced inflammation, including NF-κB activation and leukocyte adhesion to the endothelium. Interestingly, the activation of NF-κB signaling is ECM specific and correlates with the ECM specificity for the Shc-integrin association. In the mouse, Shc is also required for response to shear stress, as *flox/flox; Cre* mice displayed a marked reduction in restoration of blood flow to distal tissue following femoral artery ligation. Onset of flow after ligation induced Shc phosphorylation in ECs of collateral arteries. Histological analyses revealed defects in angiogenesis in the microvasculature in the ischemic tissue, as well as defects in arteriogenesis due to impaired pre-existing collateral remodeling. Mechanistically, Shc mediates vessel inflammation and activation of the transcription factor NF-κB as well as proliferation, both of which are critical for arteriogenesis. Unexpectedly, Shc is also required for arterial specification of the remodeling collateral arterioles by mediating shear-induced Notch activation and expression of the arterial EC marker ephrinB2.

The contribution of Shc to both integrin- and growth factor- signaling is well documented[17, 20, 21], but this is the first report to reveal a role for Shc in inflammatory signaling through NF-κB. It has recently been shown that flow-induced NF-κB activation is ECM-dependent and is only observed in cells plated on FN, but not on CL[28]. In addition, we have shown that the association of Shc with integrins in response to flow is ECM-specific. Interestingly, FN deposition is increased in atheroprone vessels that experience disturbed flow[28] as well as in collateral vessels in animals subjected to femoral artery ligation [45]. Taken collectively, these data suggest a pathway in which FN deposition is upregulated in vessels during atherogenesis and arteriogenesis, which enhances shear stress-induced Shc signaling and leads to inflammation. In curved or branched arteries, disturbed flow is constitutive, leading to a chronic inflammatory response that is pathological whereas in collateral vessels, the increased shear stress is transient leading to a transient inflammatory response that is physiological and beneficial to the animal. Transient Shc phosphorylation occurs in response to acute onset of laminar flow *in vitro* and in collaterals following femoral artery occlusion *in vivo* and this Shc phosphorylation activates transient inflammation that drives physiological remodeling of the collateral arteries. However, sustained Shc phosphorylation occurs in atheroprone curved or branched vessels leading to chronic inflammation that causes plaque formation. Therefore, Shc regulates the switch between chronic, pathological inflammation associated with atherosclerosis and transient, beneficial inflammation associated with arteriogenesis.

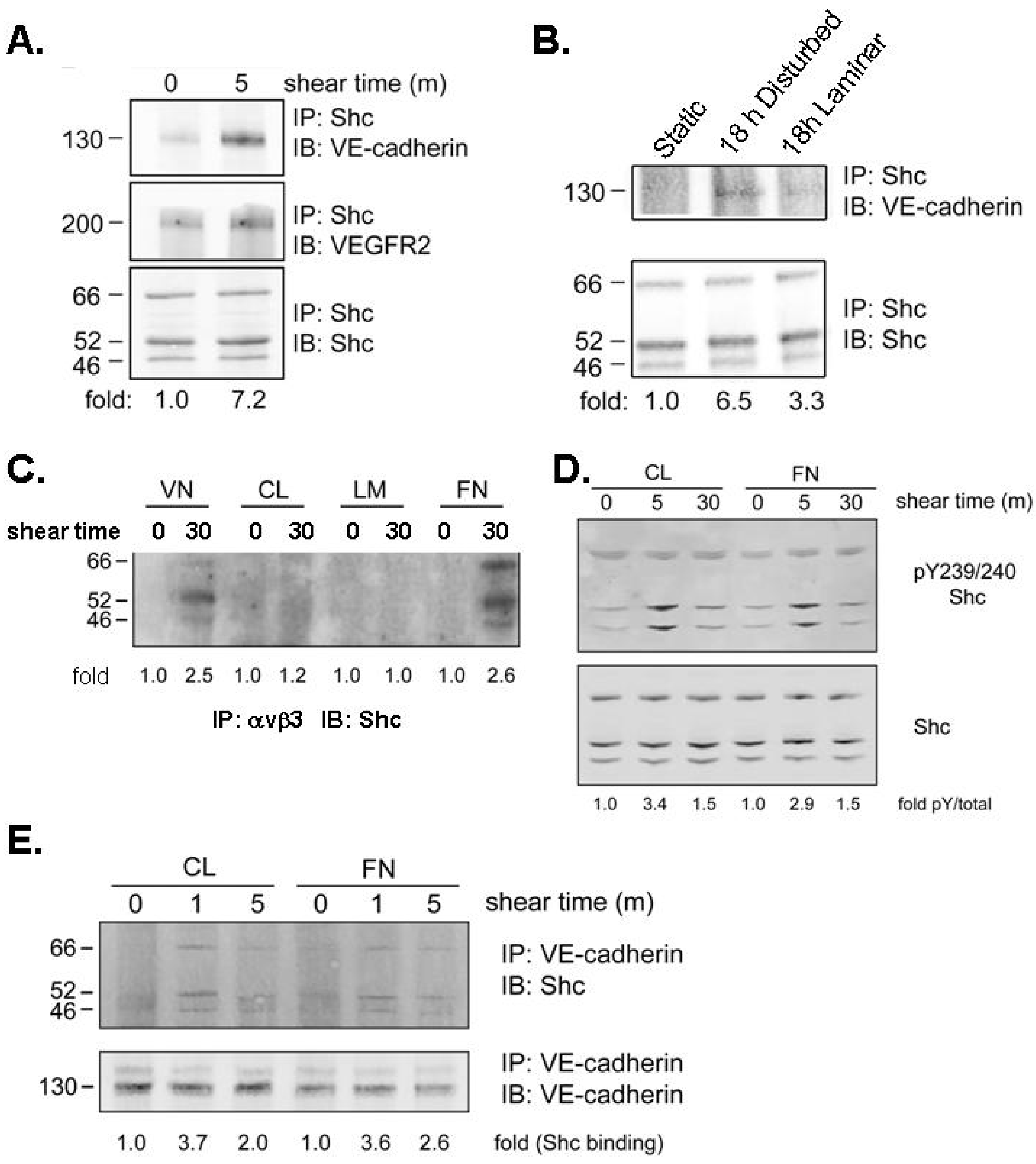
The Notch pathway is critical for embryogenesis [46] and development of the cardiovascular system[47, 48], but its role in adult physiology is less well-defined. Mice heterozygous for Notch-1 or the Notch ligand Dll-1 exhibit reduced plantar perfusion recovery following femoral artery ligation, similar to the phenotype observed in *Shc flox/flox; Tie2-Cre* mice[49, 50]. These studies suggested that the Notch pathway is activated downstream of VEGF, which is produced by ischemic tissue and drives angiogenesis. Here, we introduce an alternative model in which shear stress directly activates the Notch pathway and results in upregulation of Notch target genes such as ephrinB2 in remodeling collateral arteries. We show that activation of the Notch pathway by shear stress requires Shc, which mediates signal transduction downstream of the mechanosensory complex of PECAM-1, VE-Cadherin and VEGFR-2. Shear-stress induced activation of the Notch pathway facilitates arterial specification of collateral ECs as they remodel into high-flow carrying arterioles. The mechanism to explain how Notch is activated by shear stress is currently not well understood, although two hypotheses exist. One possibility is that shear stress induces expression of a Notch ligand which in turn activates Notch in *trans* through the canonical pathway. A second possibility is ligand-independent Notch activation in which intracellular signals activate γ-secretase, which in turn cleaves Notch. Similarly, it remains unclear whether arterial-specification of ECs in remodeling arterioles is required for, or is merely a consequence of, the remodeling process. EC-specific knockout of the arterial EC marker ephrinB2 is embryonic lethal [51], precluding any experiments to address this question in the adult mouse.

Figure 3.1: Shc is activated by shear stress



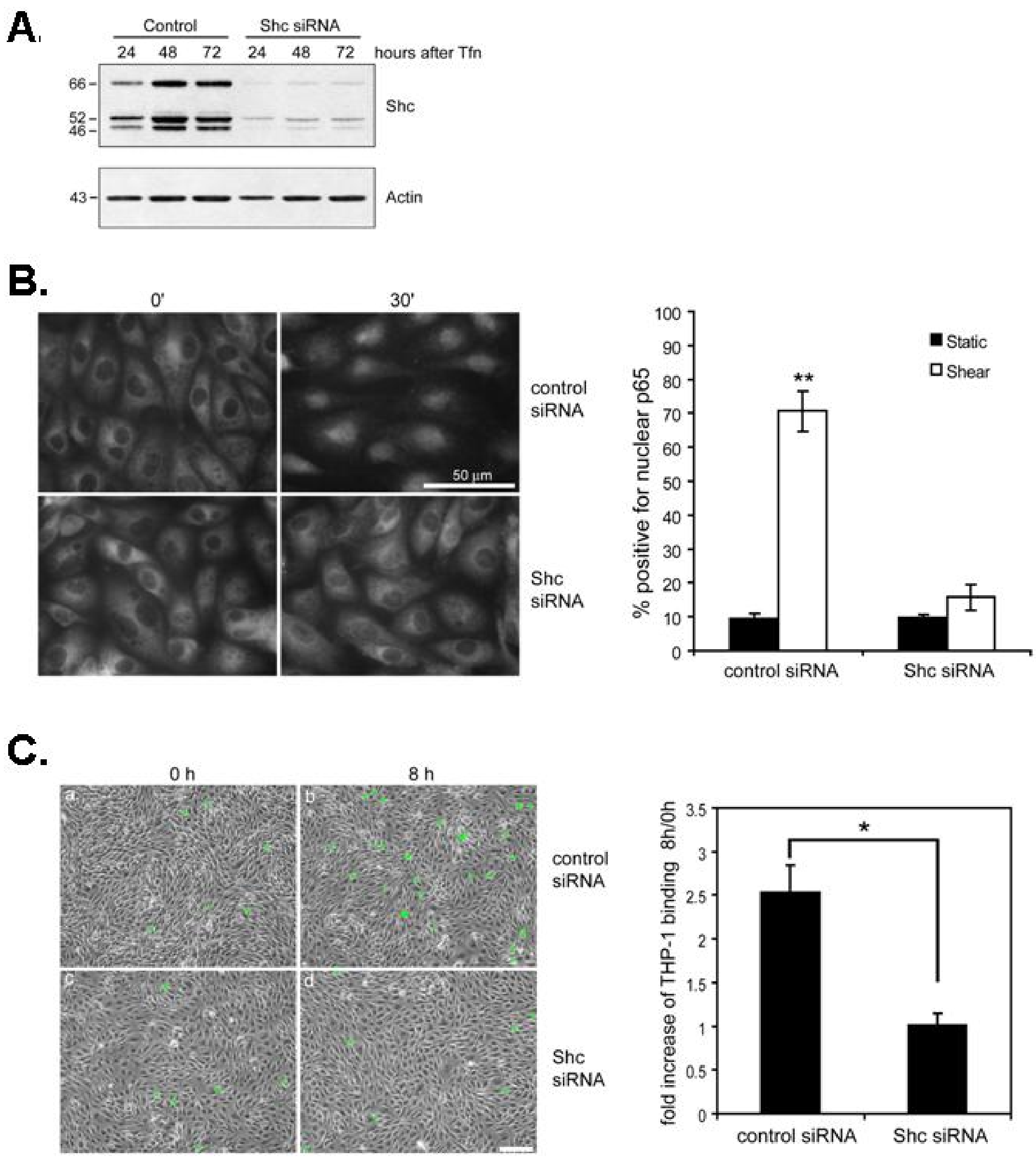
(A) BAECs were plated on FN-coated slides and subjected to disturbed or laminar flow for 18 hrs or kept as static controls. Whole cell lysates were analyzed by immunoblotting with anti-Shc phospho-Tyr239/240 or anti-Shc antibodies. (B) BAECs were plated on FN-coated slides and sheared for 1, 5, 30 min or kept as static controls. Cell lysates were subjected to SDS-PAGE and immunoblotting with anti-Shc phospho-Tyr239/240 or anti-Shc antibodies. (C) BAECs were subjected to laminar flow at 12 dyne/cm^2 for 5 min or kept static as control. Cells were subsequently fixed, permeabilized and immunostained for phospho-Tyr239/240 of Shc.

Figure 3.2: Shear stress induces ECM-independant Shc association with the 'mechanosensory complex' & ECM-dependant Shc association with integrins



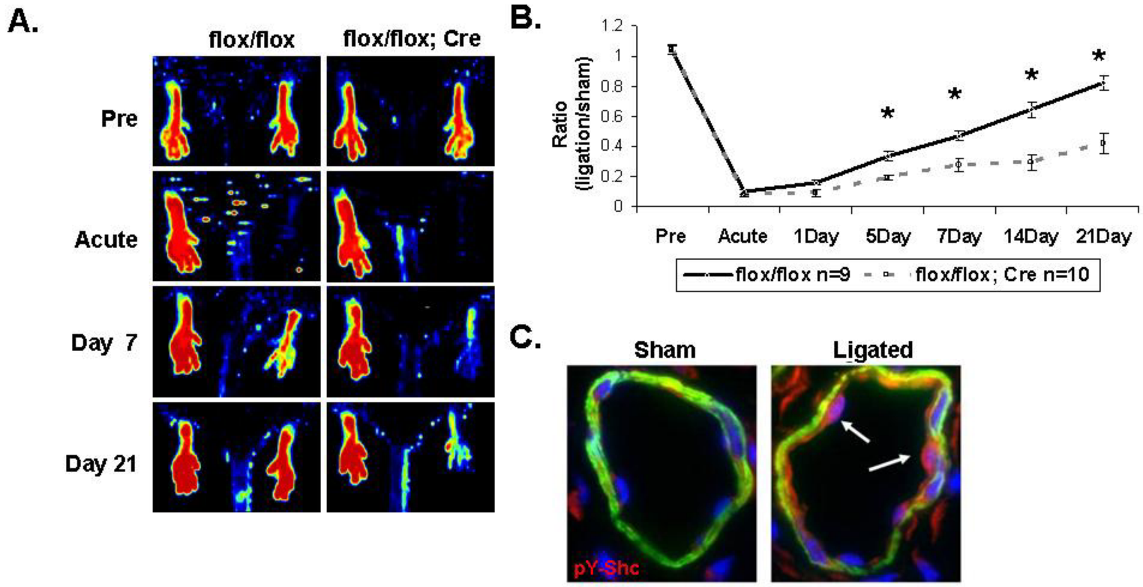
(A) BAECs were plated on FN-coated slides and subjected to laminar flow at 12 dyne/cm^2 for 5 min or kept static as control. Cells were lysed and Shc protein was immunoprecipitated from whole cell lysates using anti-Total Shc antibody. Immunoprecipitated proteins were separated using SDS-PAGE and immunoblotted for Shc, VEGFR-2 and VE-Cadherin. (B) BAECs grown on FN-coated slides were kept static or subjected to laminar or disturbed shear stress for 18 hrs. Cells were lysed and Shc protein was immunoprecipitated from whole cell lysates using anti-Total Shc antibody. Immunoprecipitated proteins were separated using SDS-PAGE and immunoblotted for Shc and VE-Cadherin. (C) Slides were coated with vitronectin (VN), collagen (CL), laminin (LM) or fibronectin (FN). BAECs were sheared for 30 min or kept as static controls. Cell lysates were immunoprecipitated with LM609 anti-$\alpha_v\beta_3$ followed by immunoblotting with anti-Shc antibody. (D) BAECs were plated on CL- or FN-coated slides and sheared for 5, 30 min or kept as static controls. Cell lysates were subjected to SDS-PAGE and immunoblotted with anti-Shc phospho-Tyr239/240 or anti-Shc antibodies. (E) BAECs were plated on CL- or FN-coated slides and sheared for 1, 5 min or kept as static controls. Cell lysates were immunoprecipitated with a VE-cadherin specific antibody and immunoblotted with anti-Shc or anti-VE-cadherin.

Figure 3.3: Shc is required for shear stress-induced NF-κB activation & leukocyte adhesion to the endothelium



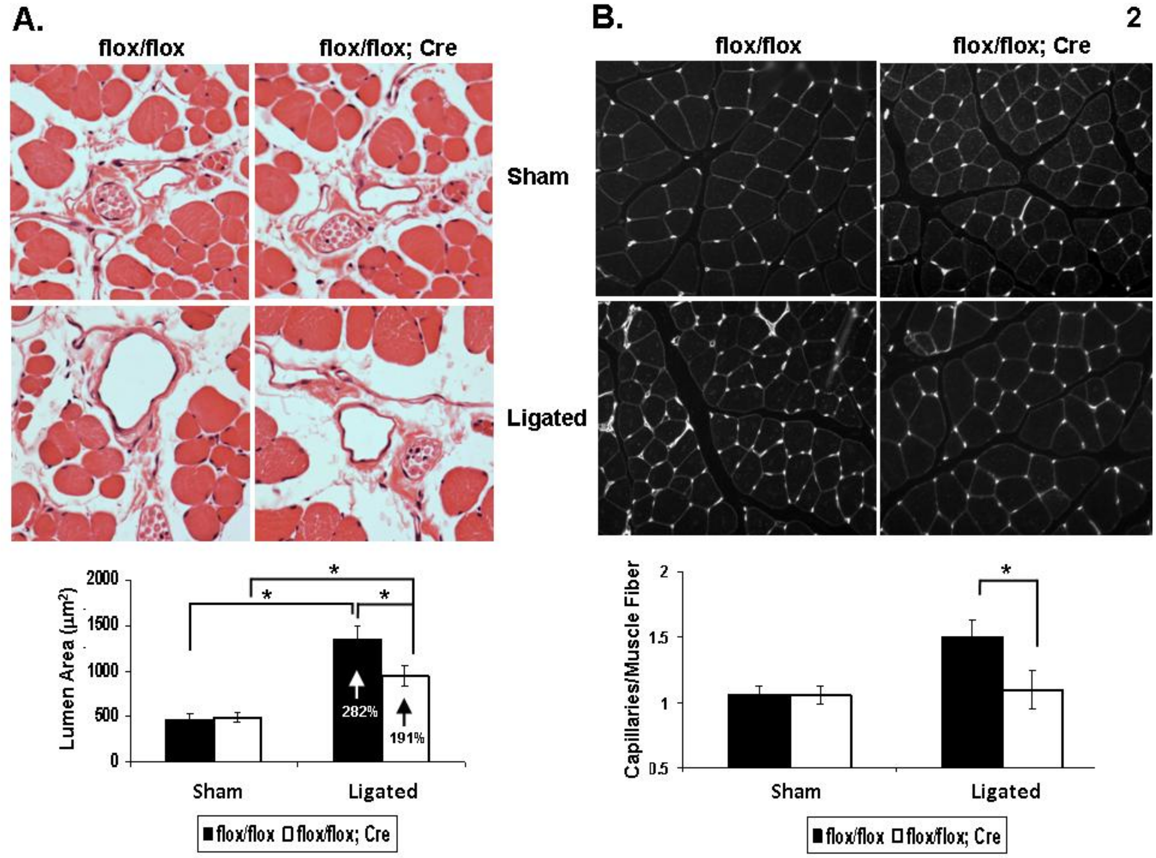
(A) BAECs were transfected with control siRNA or ShcA siRNA as described in the Materials and Methods. Cells were lysed at indicated times after transfection and cell lysates analyzed by immunoblotting with Shc specific antibody to confirm the knockdown effect of the Shc siRNA. Blots were stripped and reprobed with an antibody against Actin as a loading control. (B) BAECs were transfected with control siRNA or Shc-specific siRNA. 48 hours after transfection, cells were exposed to disturbed flow for 30 min or left as static control. Cells were fixed, permeabilized and stained for the p65 subunit of NFκB as described in the Materials and Methods. Three independent experiments were performed and 100 cells were counted for each experiment (**, $p < 0.01$). (C) BAECs were transfected with control siRNA or Shc-specific siRNA. 48 hours after transfection, cells were exposed to oscillatory flow for 8 h or left as static control. THP-1 monocytes pre-labeled with CellTracker™ Green were added to BAECs monolayers and monocyte binding assay was performed as described in the Materials and Methods. Bar, 100μm. (Right) Quantitation of monocyte binding represented as mean ± S.D. for three independent experiments (∗, $p < 0.05$).

Figure 3.4: Shc signaling regulates plantar perfusion recovery following hindlimb ischemia



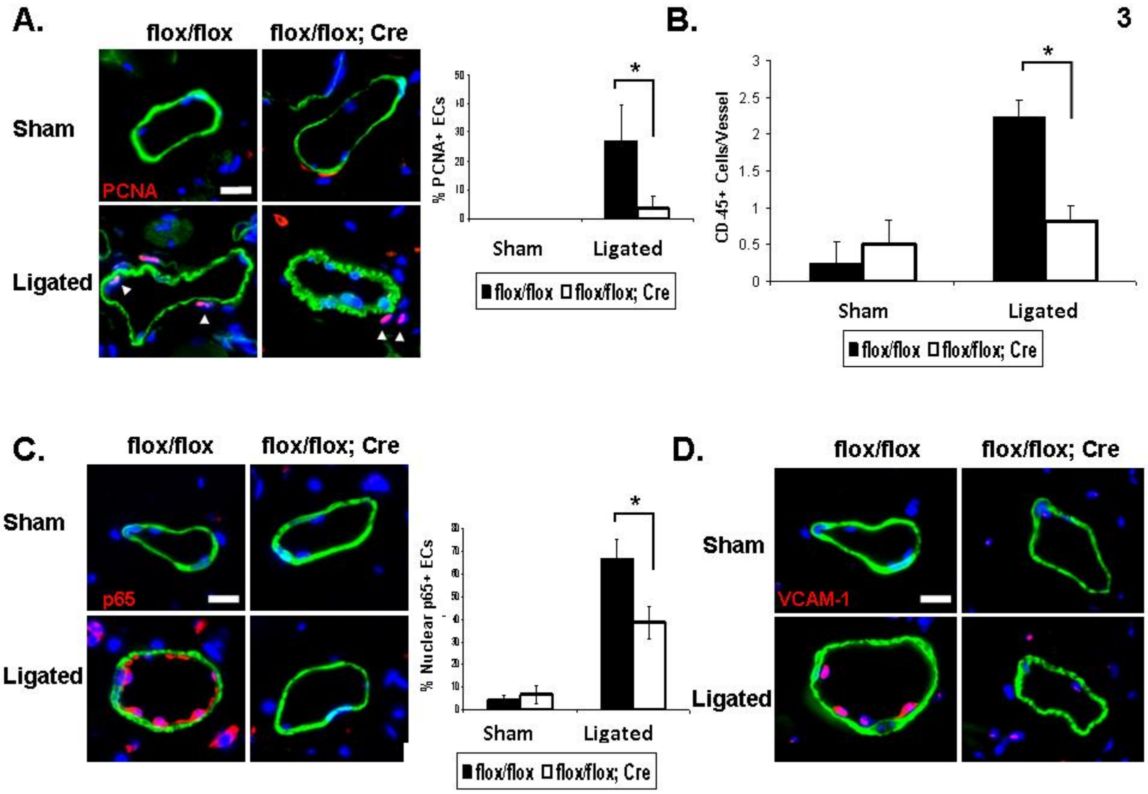
(A) Defective perfusion recovery following femoral artery ligation in conditional knockout *Shc flox/flox; Tie2-Cre* mice compared to *Shc flox/flox* control littermates. Representative LDI scans of plantar perfusion before (Pre), immediately after (Acute), 7 days (7d), and 3 weeks after hindlimb ischemia surgery. Pseudocolor scale (in arbitrary units): black indicates 0; white, 1000. (B) Ratio of plantar perfusion (ligated vs sham control side) quantified from the LDI images. Values are provided as mean +/- SEM. *= $p<0.05$, compared with the respective time point of *Shc flox/flox* controls. n= 9 *flox/flox* and 10 *flox/flox; Tie2-Cre* per timepoint. (C) Cross-section staining of a pre-existing collateral artery 24 hrs after ligation (or sham control). Slides were stained with phospho-Shc Y239, 240 (red), Smooth Muscle α-actin (green) and DAPI (blue).

Figure 3.5: Shc is required for pre-existing collateral remodeling & angiogenesis



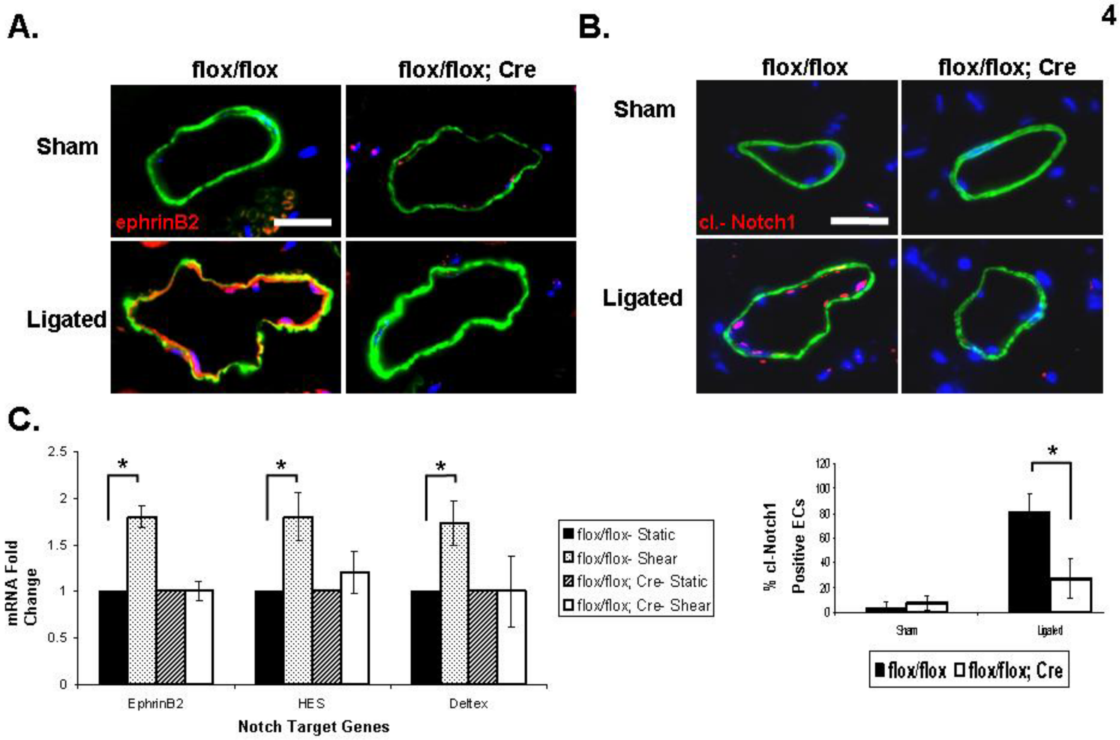
(A) Collateral lumen area in sham and ligated hindlimbs 3 weeks after femoral artery ligation surgery in *Shc flox/flox* and *Shc flox/flox; Tie2-Cre* mice. Cross-sections of the *gracilis* muscle were H&E stained and collateral lumen area was measured. Values are provided as mean +/- SEM. *= p<0.05. (B) Angiogenesis response was measured in sham and ligated hindlimbs 3 weeks after femoral artery ligation surgery in *Shc flox/flox* and *Shc flox/flox; Tie2-Cre* mice. *Gastrocnemius* muscle was cross-sectioned and stained with TRITC-Wheat Germ Agglutinin. Capillary density was quantified as number of capillaries per muscle fiber in 5 random fields per mouse. Values are provided as mean +/- SEM. *= p<0.05. n= 8 *flox/flox* and 8 *flox/flox; Tie2-Cre* for both (A) and (B).

Figure 3.6: Shc is required for collateral EC proliferation & inflammation



Proliferation and inflammation in the vessel wall was assessed by analyzing cross-sections of collateral arteries 3 days after femoral after ligation (or sham) surgery. (A) Sections were stained with PCNA to mark proliferating cells (red), Smooth Muscle α-actin (green) and DAPI (blue). Quantitation (right) is shown as % PCNA positive ECs. (B) Quantitation of CD-45 positive-cell accumulation in the vessel wall 3 days after surgery. (C) Staining of collaterals for NF-κB subunit p65 (red), Smooth Muscle α-actin (green) and DAPI (blue). Quantitation (right) displayed as % ECs with nuclear (active) p65 localization. (D) Staining of collaterals for Vascular Cell Adhesion Molecule (VCAM) (red), Smooth Muscle α-actin (green) and DAPI (blue). Scale bars = 20μm. For all quantitation, values are provided as mean +/- SEM. *= $p<0.05$. n= 8 *flox/flox* and 8 *flox/flox; Tie2-Cre*

Figure 3.7: Shc regulates shear stress-induced collateral EC arterial specification via activation of the Notch pathway



(A) Upregulation of arterial EC marker ephrinB2 during collateral arteriogenesis requires Shc. Staining of collaterals 3 days after femoral ligation (or sham) surgery for ephrinB2 (red), Smooth Muscle α-actin (green) and DAPI (blue). (B) Activation of Notch transcription factor requires Shc. Staining of collaterals for Cleaved Notch-1 (red), Smooth Muscle α-actin (green) and DAPI (blue). Quantitation (right) displayed as % ECs with active Cleaved Notch-1. N= 8 *flox/flox* and 8 *flox/flox;Cre*. (C) Shc is required for shear stress-induced Notch-target gene activation. Relative mRNA expression of Notch target genes ephrin-B2, HES and Deltex in ECs from *flox/flox* vs. *flox/flox; Tie2-Cre* mice. ECs were sheared for 4 hrs at 15 dynes/cm2 or kept static as a control. Values are shown as mean +/- SEM (n=4 independent experiments). *= $p<0.05$.

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CHAPTER IV.

CONCLUSIONS & PERSPECTIVES

OVERVIEW OF FINDINGS

This dissertation identifies the adaptor protein Shc as an important regulator of signaling in ECs. Before this work, Shc was underappreciated as a signaling molecule in ECs, known mostly for its adaptor function in mediating signaling pathways downstream of receptors for EGF [1], PDGF [2, 3], Insulin [4-7] and bFGF [8, 9] in non-endothelial cells, usually leading to activation of Ras and mitogenic signaling through the MAPK pathway. The generation of the Shc knockout mouse revealed a role for Shc in the developing cardiovascular system; however, the embryonic lethality of the global knockout was not conducive to studies on the role of Shc in adult physiology and pathology. This dissertation has outlined an important role for Shc in regulating the EC-specific signaling pathways activated by VEGF and shear stress (Figure 4.1). Additionally, we are the first to implicate Shc in the NF-κB and Notch pathways which are required for vessel inflammation and arterial specification, respectively. While Chapters II and III of this dissertation can be seen as a standalone stories, the findings fit together to form a cohesive picture of Shc signaling in ECs downstream of VEGFR-2 and integrin stimulation. The stimulus that activates Shc signaling defines the EC response, but key players in these distinct responses are conserved: VEGFR-2, integrins $\alpha_v\beta_3$ and/or $\alpha_5\beta_1$ and Shc. This Chapter briefly highlights the key findings from Chapters 2 and 3, explains the significance of the research in terms of the vascular biology field, and proposes future research which may prove beneficial in expanding the understanding of Shc signaling in vascular biology and in the context of human disease.

CHAPTER II: THE ADAPTOR PROTEIN SHC INTEGRATES GROWTH FACTOR AND ECM SIGNALING DURING POSTNATAL ANGIOGENESIS

Angiogenesis, the growth of new blood vessels as they sprout from pre-existing vasculature, has been an active area of research since Dr. Judah Folkman hypothesized that tumor growth could be limited by inhibiting angiogenesis in the early 1970s. This idea persists more than 40 years later, as several angiogenesis inhibitors are currently in use or in clinical trials. The results of the angiogenesis inhibitors currently in use have largely been disappointing, underlying the need for further understanding of the angiogenic process.

Since Dr. Folkman's seminal papers, a wealth of research has uncovered a great deal about how angiogenesis is activated and regulated [10]. Soluble pro-angiogenic growth factors are produced by hypoxic or tumor tissue and bind to receptors expressed on the surface of ECs, activating the previously quiescent EC layer. The activated ECs then loosen cell-cell adherens junctions and express proteases that degrade the surrounding ECM to allow space for the activated ECs to sprout a new vessel off of the parent vessel. The activated EC adopts a 'tip cell' fate, meaning the EC is highly motile and invades the underlying tissue, whereas the neighboring ECs adopt a 'stalk cell' fate, where the ECs maintain connections to the parent vessel, proliferate and lumenize the nascent sprout. Several major signaling pathways are required to coordinate the many steps of the complex angiogenesis process. VEGF is the major 'angiogenic factor' whose receptor, VEGFR-2, is specifically expressed on ECs and induces proliferation (via the MAPK pathway), migration (via Rho small GTPases and p38 MAPK), survival (via the PI3K-Akt pathway) and degradation of the ECM (via MMPs) [11]. VEGF activity can be modulated by expression of the VEGF sink sFlt-1 by stalk cells which sets up a narrow VEGF gradient through which a sprout migrates [12, 13]. Integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ are upregulated in VEGF-activated angiogenic ECs and blockade of these integrins has been shown to decrease angiogenesis by increasing apoptosis of ECs[14]. Together, these pathways orchestrate the complex processes that encompass angiogenesis, and cross-talk between these major pathways is beginning to be uncovered. For example, the Byzova lab has shown that integrin function is required for VEGFR-2 activation in response to VEGF treatment of ECs, and VEGFR-2 binds integrin $\alpha_v\beta_3$ in response to its activation, indicating crosstalk between the two receptors is necessary for proper function[15, 16].

My research has contributed to the field of angiogenesis in two ways. First, I have uncovered a novel function for the adaptor protein Shc in angiogenesis due to its role in mediating a subset of signaling from VEGFR-2 and integrins. These results are important to the angiogenesis field in that they further our understanding of the VEGF and integrin signaling pathways that are critical for angiogenesis. While extensive research has reported that VEGF and integrin signaling are important for angiogenesis due to their roles in activating ERK MAPK, PI3K-Akt and other pathways[17], surprisingly little was known about the proteins that transmit signals from these receptors at the cell surface into a functional response. My research has shown that Shc is required for proper postnatal angiogenesis in several complementary models and the use of pertinent animals models combined with mechanistic studies *in vitro* provide a detailed story of how Shc requlates angiogenesis at the cellular and organismal levels. Until my work, the study of Shc's role in cardiovascular signaling was precluded by embryonic lethality of the global Shc knockout mouse[18]. We circumvented this issue by conditionally removing Shc from ECs in the mouse using Tie2-Cre-LoxP genetics and found that EC-specific Shc knockout mice were born at normal Mendelian ratios, providing an important tool for the future study of Shc in cardiovascular physiology and pathology.

A second, more general contribution to the field of EC signaling and angiogenesis is the observation that Shc mediates cross-talk between VEGF-Receptors and integrins. The critical experiment, Figure 2.6, shows that VEGF-induced EC survival requires Shc only when ECs are grown on FN, whereas ECs on CL do not require Shc for survival in response to VEGF. This experiment indicates that VEGF-induced survival is integrin-dependent and because FN-binding integrins such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$ recruit Shc whereas CL-binding integrins such as $\alpha_2\beta_1$ do not, Shc mediates the crosstalk between VEGFR-2 and integrins when ECs are grown on FN. This idea of crosstalk between multiple receptors via adaptor proteins is largely under-studied, but may become more prevalent as signaling pathways begin to be thought of as interconnected webs rather than linear, binary switches. My research is one example of how an EC functional response depends on coordination of signals from two different receptors by a soluble cytoplasmic adaptor protein such as Shc.

Several further questions have arisen from the above data, and these will be the focus of future research. One major question is mechanistically, how Shc is able to mediate crosstalk between VEGFR-2 and integrins. Previous reports have shown that VEGF treatment of ECs induces physical association of VEGFR-2 with integrin $\alpha_v\beta_3$[15], so perhaps the simplest hypothesis is that Shc mediates the association between VEGFR-2 and integrin $\alpha_v\beta_3$. However, a direct physical association of VEGFR-2 with integrin $\alpha_v\beta_3$ has recently been reported in a test tube in the absence of Shc[45], indicating that Shc does not promote association of VEGFR-2 with integrin $\alpha_v\beta_3$. An alternative hypothesis is that Shc mediates signaling from VEGFR-2 and integrin $\alpha_v\beta_3$ separately and integrates the signal from each of these receptors into one coordinated cellular response. This hypothesis is supported by protein domain structure-function analysis that Shc binds VEGFR-2 via its SH2 domain after VEGF treatment[19], whereas Shc binds integrin β_3 using its PTB domain[20]. This indicates that Shc protein has two distinct binding sites for VEGFR-2 and $\alpha_v\beta_3$ that Shc could use to bind each receptor in succession. Because Shc uses different domains to bind each of these receptors, an important future experiment will be to create mutant Shc constructs in which the SH2 and/or the PTB domains are mutated so they are unable to interact with VEGFR-2 or integrin β_3. We will transfect mutant Shc constructs that can only bind VEGFR-2 or integrin $\alpha_v\beta_3$ but not both into ECs and assay for angiogenesis readouts such as sprouting in Fibrin gel, association of VEGFR-2 with integrin $\alpha_v\beta_3$, haptotaxis on FN, cell survival and Akt activation by VEGF and adhesion to FN. If SH2 and PTB domain-mutant ECs are unable to sprout in Fibrin gel, we will conclude that binding of Shc to both VEGFR-2 *and* integrin $\alpha_v\beta_3$ are required for signaling during angiogenesis. Conversely, if one of the mutants is still able to form productive sprouts, we will conclude that association of Shc with only one receptor is sufficient for sprouting. Similarly, if VEGF-induced signaling occurs normally in the Shc PTB-mutant cells, this would indicate that Shc mediates VEGFR-2 signaling independent of β_3 binding. However, if no signaling occurred and the VEGFR-2:integrin β_3 complex did not form, this would indicate that Shc binds both receptors simultaneously and brings together a ternary complex that is capable of signaling.

Another interesting question related to angiogenesis in general is why do *Shc flox/flox; Tie2-Cre* mice survive embryogenesis and develop into adulthood normally? We know that angiogenesis is critical to embryonic development for expansion and remodeling of the vasculature and we also have shown that Shc is important for angiogenesis, so how do the Shc EC-specific knockout mice survive throughout development? The answer to this question has been elusive, although three hypotheses exist. First, conditional knockout mice could escape embryonic lethality if the Cre driver promoter is turned on after the critical stage of development. This does not appear to be the case for the *Shc flox/flox; Tie2-Cre* mice because several papers have reported that Tie2-Cre is turned on by E9.5 [21] while global Shc knockout mice develop normally until E11.5 [18]. Another possible explanation is that postnatal angiogenesis occurs by a different mechanism than embryonic angiogenesis. Emerging research has set precedent for the idea that conditional gene knockout using the *Tie2-Cre* transgene can result in mice that initially develop a normal vasculature, while exhibiting defective angiogenic capacity. *Tie2-Cre* mediated conditional knockout of genes such as Endothelin-1[22, 23], TFPI[24], ADAM17[25], PPARγ [26] and Dicer [27] yield viable mice with cardiovascular defects, while the corresponding global knockout animal is embryonic lethal. Thus, embryonic angiogenesis may use different adaptor proteins in ECs to transmit signals, or the angiogenic microenvironment (growth factors, ECM, hypoxia conditions) may be different in the embryo than in the adult, activating slightly different signaling pathways that do not require Shc. A third hypothesis to explain how *Shc flox/flox; Tie2-Cre* mice survive embryogenesis is compensation for loss of Shc by some other adaptor protein, which is able to perform enough of Shc's function to permit development. Shc mediates protein-protein interactions of signaling cascades through use of SH2 and PTB phospho-tyrosine binding domains. Approximately 115 SH2 domain containing proteins[28]and 24 PTB domain containing proteins[29] are known in the human genome, so functional compensation by one or more of these proteins is possible, simply because of the high concentration of proteins with similar protein binding domains available in the cytoplasm. ECs express SH2 and PTB-containing adaptor proteins such as Focal Adhesion Kinase (FAK) and Src-Family Kinases Src, Yes and Fyn which have been shown to bind integrin $\alpha_v\beta_3$, while Gab1 is another SH2-containing adaptor protein that has been shown to bind VEGFR-2. More proteins similar in structure such as Grb2, Nck and Cbl exist in ECs and have been shown to mediate signaling downstream of RTKs. We have not yet explored the issue of compensation by other adaptors in *Shc flox/flox; Tie2-Cre* mice or ECs, but it is an interesting possibility to address in the future.

CHAPTER III: SHC MEDIATES THE ENDOTHELIAL RESPONSE TO SHEAR STRESS *IN VITRO* AND DURING ARTERIOGENESIS *IN VIVO*

Atherosclerosis, the buildup of cells, ECM and lipids in the vessel wall causing narrowing of the lumen and reduced perfusion of the downstream tissue is the leading cause of death in industrialized nations. Atherosclerosis was initially thought to be caused by passive deposition of lipid in the vessel wall simply due to high concentrations of circulating low density lipoproteins. However, plaque formation occurs preferentially at sites of vessel curvature and bifurcation while high cholesterol is systemic, indicating some other atherogenic stimulus occurs at these discrete locations. In 1973, Russell Ross proposed the “Response-to-Injury” hypothesis which states that endothelial injury induces chronic inflammation, which is the primary cause of atherosclerotic plaque formation [30]. Subsequent research has shown that shear stress, the frictional drag force of blood flowing over the endothelium, can cause endothelial injury and chronic inflammation that initiates atherogenesis [31].

Disturbed shear stress exists at athero-prone vessel curvatures and bifurcations, and activates a chronic inflammatory response in ECs of these vessels. Shear stress can be sensed by a variety of different mechanoreceptors located at the EC luminal surface (primary cilia and ion channels), basal surface (integrins) and cell-cell adhesions[31]. Tzima et al. reported a mechanosensory complex located at cell-cell junctions comprised of PECAM-1, VE-Cadherin and VEGFR-2 which is necessary and sufficient for the EC response to shear stress[32]. Activation of the mechanosensory complex by shear stress induces several signaling cascades such as activation of Src-family kinases, MAPKs, and the inflammatory transcription factor NF-κB [32, 33]. These pathways activate the normally quiescent EC monolayer and induce EC proliferation, lipid uptake into the vessel wall, and chronic inflammation which promotes leukocyte transmigration into the underlying tissue. Each of these events contributes to the growth of atherosclerotic plaques, and further understanding of the mechanism by which shear stress is sensed and responded to will aid in establishing targets for treatment and prevention of atherosclerosis. PECAM-1, a component of the mechanosensory complex, is required for shear stress-induced inflammation and development of atherosclerotic plaques in regions of disturbed flow [34, 35] in hypercholesterolemic mice, further indicating that the response to disturbed shear is a driving force in atherogenesis.

At the onset of my thesis research, a gap existed between the membrane bound mechanosensory complex and the major signaling nodes that were known to be activated by shear stress (such as NF-κB). My research contributed to the shear stress/mechanotransduction field by identifying an adaptor protein, Shc, which is required for signaling directly downstream of the mechanosensory complex. Shear stress induces a physical association of Shc with VE-Cadherin and VEGFR-2, two components of the mechanosensory complex, as well as Shc phosphorylation at Tyr239,240. Importantly, Shc phosphorylation remained high even after 18 hrs of disturbed flow, whereas Shc was de-phosphorylated (compared to static cells) in response to 18 hrs of atheroprotective laminar flow. These results indicate that Shc is constitutively active in areas of disturbed flow and may contribute to chronic inflammation in these vessels. Shc-depleted ECs showed impaired NF-κB activation and leukocyte adhesion in response to disturbed shear stress. This was the first time that Shc was implicated upstream of NF-κB activation in any cell type. These *in vitro* findings that Shc mediates shear-induced NF-κB activation and is constitutively activated in areas of disturbed flow potentially make Shc a critical player in shear-induced inflammatory signaling.

To confirm that Shc plays a major role in signaling in response to shear stress *in vivo*, Shc was conditionally knocked out of ECs and mice were subjected to femoral artery ligation surgery. Femoral artery ligation reroutes blood from the femoral artery into pre-existing collateral arteries and this sudden increase in blood flow promotes vascular remodeling known as arteriogenesis.

Arteriogenesis is driven by a sudden increase in hemodynamic force, which activates EC and SMC proliferation, NO production and vessel dilation, and inflammation which contribute to outward remodeling and widening of the collateral arteries to allow perfusion of the ischemic tissue[36, 37]. We found that Shc expression in ECs is required for recovery of perfusion to the ischemic hind limb, and *Shc flox/flox; Tie2-Cre* mice exhibited defective arteriogenesis which may explain the decrease in recovery of foot perfusion. Mechanistically, *Shc flox/flox; Tie2-Cre* mice were unable to increase EC proliferation or inflammation in response to femoral artery ligation; two processes that are important for vessel remodeling [38]. These results confirmed our *in vitro* data that Shc is required for NF-κB activation by shear stress and advanced these findings by showing that Shc is required for shear-induced inflammation and vessel remodeling in the mouse.

Vascular ECs are not the only cell type that is capable of responding to shear stress. Epithelial cells lining tubes in the kidney [48] and in the airway [49] can sense fluid or air flow, smooth muscle cells in vessels in which ECs are denuded can respond to flow [47], and interstitial fluid flow in bone regulates differentiation and gene expression in osteoblasts [50]. Shc is ubiquitously expressed in the adult, so one wonders whether the role for Shc in the response to shear stress is conserved in these non-vascular cell types, or if Shc has a unique EC-specific function. Based on the literature and what we know about mechanotransduction in ECs, it is likely that both of these possibilities are true. Shc participates in shear-induced signaling from two important mechanosensors – the PECAM-

1:VE-Cadherin:VEGFR-2 ‘mechanosensory complex’ and integrins. Expression of PECAM-1, VE-Cadherin and VEGFR-2 are restricted to ECs and therefore, the role of Shc in mechanotransduction from this complex is a unique EC-specific function of Shc. However, integrins are expressed by several cell types and recently, Shc has been implicated downstream of integrins in shear-sensing in osteoblast-like cells [51, 52]. Therefore, Shc may have a ubiquitous function in mechanostransduction via integrins in cell types other than vascular ECs.

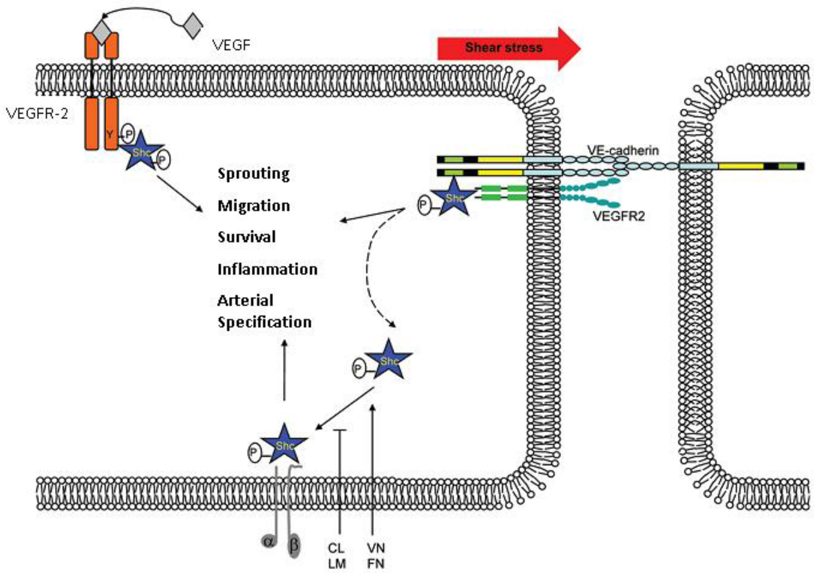
This research has contributed significantly to the fields of atherosclerosis and mechanotransduction by identifying the first adaptor protein that is required for shear stress-induced signal transduction downstream of the ‘mechanosensory complex.’ Shc is activated by atherogenic, disturbed shear stress and mediates vascular inflammation, which has been shown to be a primary cause of atherosclerotic plaque formation. Therefore, it is likely that Shc is required for atherosclerotic plaque formation in areas of disturbed flow and future experiments will aim to examine this question directly. *Shc flox/flox; Tie2-Cre* mice will be crossed to Apolipoprotein E null mice [39] to create hypercholesterolemic *Shc flox/flox; Tie2-Cre; ApoE-/-* mice that lack Shc in ECs. These mice and control *Shc flox/flox; ApoE-/-* mice will be fed a high fat diet to induce atherosclerotic plaque development and aortic plaque burden will be assessed by Oil Red O staining in areas of disturbed vs. laminar flow. We hypothesize that *Shc flox/flox; Tie2-Cre; ApoE-/-* mice will have decreased atherosclerosis in areas of disturbed flow, such as the aortic arch due to reduced inflammation in the vessel wall, which we have shown is regulated through Shc.

We expect less NF-κB activation and less leukocyte accumulation in the vessel wall of *Shc flox/flox; Tie2-Cre; ApoE-/-* mice in areas of disturbed flow due to the inability of these mice to respond to shear stress. Combining the *in vitro* cell biology, the arteriogenesis data and the future atherosclerosis study, we will provide a comprehensive picture of how Shc is required for shear stress-induced vascular inflammation and atherosclerosis. Because Shc is activated in areas of disturbed flow and mediates shear-induced inflammation, phospho-Shc may be a fruitful target for treating and preventing atherosclerosis. However, in light of the results presented in Chapter 3, one important caveat for targeting phospho-Shc as a treatment for atherosclerosis may be the undesirable side-effect that phospho-Shc is also required for arteriogenesis. Therefore, inhibiting phospho-Shc may also prevent beneficial shear-induced collateral remodeling and decrease perfusion recovery in tissues with already-occluded vessels.

Another important future direction will be to assess the importance of Shc phosphorylation in the response to shear stress. We have shown that shear stress induces Shc phosphorylation but is this phosphorylation required for association of Shc with the mechanosensory complex or for the activation of downstream signaling pathways such as NF-κB? To test this we will utilize mutant Shc constructs in which the three tyrosine residues that are sites of phosphorylation (Y239, 240 and 317) are mutated to phenylalanine which cannot be phosphoryled (termed ShcY-F mutants). We will transfect the Shc constructs into ECs and assay the response to shear stress in these mutant ECs. We will assay Shc association with VE-Cadherin, VEGFR-2 and integrin β_3 in response to shear stress as well as NF-κB activation and leukocyte adhesion to the endothelium after disturbed flow. I hypothesize that ECs transfected with ShcY-F mutants will be unable to activate NF-κB in response to shear stress. In conjuction with these proposed *in vitro* experiments to study the importance of Shc phosphorylation, we will perform *in vivo* experiments using transgenic mice that express the ShcY-F mutant downstream of a lox-STOP-lox cassette for conditional expression of the transgene[46]. Currently, I am crossing Tie2-Cre mice to the ShcY-F mice in order to conditionally express the ShcY-F mutant in ECs. These mice will be used in the atherosclerosis studies outlined above as well as in arteriogenesis studies in which the mice are subjected to femoral artery ligation surgery and hindlimb perfusion and collateral remodeling will be assessed. In both models, I expect that the ShcY-F mutant mice will be unable to activate NF-κB and shear stress-induced inflammation; thus rendering the mice defective in atherosclerotic plaque formation and arteriogenesis.

A second contribution that my research has made to the field of mechanotransduction is the novel observation that Shc is required for shear stress-induced activation of the Notch pathway. Notch is a critical pathway in developmental biology as it controls cell-fate determination in many tissue types including the cardiovascular system [40, 41]. However, relatively little is known about the role of the Notch pathway in the adult cardiovascular system. Homozygous deletion of Notch-1 or the Notch ligands Dll-1, Dll-4 or Jag1 results in embryonic lethality due to cardiovascular defects. Adult mice heterozygous for Notch-1 or Dll-1 exhibited reduced plantar perfusion recovery following femoral artery ligation, similar to the phenotype observed in *Shc flox/flox; Tie2-Cre* mice[42, 43]. *In vitro*, shear stress has been shown to activate the Notch pathway in mouse embryonic stem cells in culture [44]. My work is the first to show that Shc is required for shear stress-induced Notch activation *in vivo* and *in vitro* in fully-differentiated mouse ECs, which underlies arterial specification of the remodeling arteriole. Previous studies suggested that the Notch pathway was activated downstream of VEGF, which is produced by ischemic tissue and drives angiogenesis. However, my *in vitro* studies using serum-free normoxic conditions have shown that shear stress itself can activate the Notch pathway and result in upregulation of Notch target genes such as ephrinB2. This begs several new questions regarding the mechanism explaining how Notch is activated by shear stress. Notch is a transmembrane receptor that is activated when it binds to one of five ligands which are also transmembrane proteins that are expressed by a neighboring cell. Binding of Notch to its ligand induces two proteolytic cleavage events by ADAM10 and then by the γ-secretase complex to release Notch Intracellular Domain (NICD) from the cell membrane and allowing it to translocate into the nucleus. Once in the nucleus, NICD associates with the transcription factor CSL and activates transcription of a subset of genes including ephrinB2. The idea that Notch is activated by shear stress is new and therefore it is unknown how mechanical force-induced Notch activation occurs. One possibility is that shear stress upregulates expression of a Notch ligand which in turn activates Notch in *trans* through the canonical pathway. Indeed, my preliminary data suggests that Notch ligands Dll-1 and Dll-4 are upregulated by shear stress *in vitro,* whereas these ligands are not upregulated in *Shc flox/flox; Tie2-Cre* ECs. Future studies will confirm these preliminary results and use siRNAs to knockdown expression of Dll-1 and Dll-4 in ECs and assess Notch activation in response to shear stress to determine if expression of these two ligands drives Notch activation. If Notch activation proceeds normally in Dll-1/Dll-4 knockdown ECs, ligand-independent Notch activation may be occurring. In this scenario, shear stress would activate γ-secretase directly, which in turn cleaves Notch and activates the Notch pathway. Future studies will address the mechanism by which Notch is activated by shear stress and how Shc plays a role in this novel pathway for Notch activation by mechanical force.

Figure 4.1: The adaptor protein Shc is a critical regulator of angiogenic and shear stress signaling in ECs



The adaptor protein Shc regulates signaling from several receptors and is critical for many EC responses to their environment. During angiogenesis, Shc mediates a subset of signaling from VEGFR-2 and fibronectin-binding integrins such as $\alpha_v\beta_3$ and/or $\alpha_5\beta_1$. In the context of mechanotransduction, Shc mediates signaling from the 'Mechanosensory Complex' and fibronectin-binding integrins that is activated by shear stress. Collectively, Shc is required for many EC functions such as sprouting angiogenesis, migration, survival, inflammation and arterial specification.

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