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Endothelial Shc Regulates Arteriogenesis Through Dual Control of Arterial Specification and Inflammation via the Notch and NF-κB Pathways

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Abstract

Rationale—Arteriogenesis, the shear stress-driven remodeling of collateral arteries, is critical in restoring blood flow to ischemic tissue following a vascular occlusion. Our previous work has shown that the adaptor protein Shc mediates endothelial responses to shear stress in vitro.

Objective—To examine the role of the adaptor protein Shc in arteriogenesis and endothelial-dependent responses to shear stress in vivo.

Methods and Results—Conditional knockout mice in which Shc is deleted from endothelial cells were subjected to femoral artery ligation. Hindlimb perfusion recovery was attenuated in Shc conditional knockout mice compared to littermate controls. Reduced perfusion was associated with blunted collateral remodeling and reduced capillary density. Bone marrow transplantation experiments revealed that endothelial Shc is required for perfusion recovery as loss of Shc in bone marrow-derived hematopoietic cells had no effect on recovery. Mechanistically, Shc deficiency resulted in impaired activation of the NF-κB-dependent inflammatory pathway and reduced $CD45^+$ cell infiltration. Unexpectedly, Shc was required for arterial specification of the remodeling arteriole by mediating upregulation of the arterial endothelial cell marker ephrinB2 and activation of the Notch pathway. In vitro experiments confirmed that Shc was required for shear stress-induced activation of the Notch pathway and downstream arterial specification through a mechanism that involves upregulation of Notch ligands Delta-like 1 and Delta-like 4.

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DISCLOSURES
None.

Conclusions—Shc mediates activation of two key signaling pathways that are critical for inflammation and arterial specification; collectively, these pathways contribute to arteriogenesis and recovery of blood perfusion.

Keywords

Arteriogenesis; Shc; shear stress; NF-κB; Notch

INTRODUCTION

Arteriogenesis, the outward remodeling of pre-existing collateral arteries, is critical in recovery and restoration of blood supply to ischemic tissue following occlusion of a vessel. Arteriole–arteriole anastomoses, termed collaterals, act as a natural bypass mechanism to maintain blood supply to downstream tissue even when major arteries, such as the femoral artery, become blocked[1]. Blood flow through collateral arteries in a healthy animal 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 and induces outward remodeling of the vessel. Outward remodeling of preexisting collaterals is driven primarily by the sudden spike in hemodynamic forces, especially shear stress, resulting from the increase in flow through collaterals[2]. Changes in hemodynamic forces are sensed by endothelial cells (ECs)[3-5], which initiate signal transduction pathways that induce cell proliferation and inflammation, including upregulation of cell adhesion molecules on ECs and leukocyte accumulation in the vessel wall[6].

ECs are equipped with numerous mechanoreceptors capable of responding to shear stress, including caveolae, ion channels, integrins, Receptor Tyrosine Kinases, the apical glycocalyx, primary cilia, heterotrimeric G proteins, and intercellular junctions[4]. We previously identified a mechanosensory complex located at cell-cell junctions comprised of Platelet Endothelial Cell Adhesion Molecule (PECAM)-1, Vascular Endothelial (VE)-Cadherin, and Vascular Endothelial Growth Factor Receptor (VEGFR)2 that is necessary and sufficient for EC response to shear stress in vitro [7]; vascular remodeling[8]; and arteriogenesis in vivo[9].

The adaptor protein Shc mediates signaling cascades downstream of a multitude of Receptor Tyrosine Kinases for various growth factors, cytokines, hormones and recently, mechanical force[10, 11]. Global knockout of the *Shc1* gene in mice causes embryonic lethality at E11.5 due to defects in embryonic heart development[12, 13]. Conditional knockout mice in which Shc was deleted from ECs displayed a defect in post-natal angiogenesis [14]. Using an in vitro shear stress system that models the sudden increase in hemodynamic forces in collaterals immediately following femoral artery ligation, we previously demonstrated that onset of shear stress activates Shc and induces its association with components of the mechanosensory complex (VE-cadherin and VEGFR2), as well as integrins[15]. Here, we hypothesize that endothelial Shc is required for shear stress-driven arteriogenesis and blood flow recovery in response to femoral artery ligation. Our data show impaired plantar perfusion recovery in *Shc flox/flox; Tie2-Cre* mice, which was dependent on Shc expression in ECs but not hematopoietic cells. Furthermore, we demonstrate that Shc is required for activation of the NF-κB pathway and downstream inflammation in collateral arteries. Surprisingly, Shc is also required for shear stress-induced activation of the Notch pathway and downstream expression of the arterial EC marker ephrinB2 through a mechanism that involves upregulation of Notch ligands Dll-1 and Dll-4.

METHODS

The materials and methods used are detailed in the Online Supplement.

RESULTS

Shc is required for plantar perfusion recovery following hindlimb ischemia

To determine the role of endothelial Shc in perfusion recovery following ischemia, *Shc flox/flox; Tie2-Cre* and *Shc flox/flox* littermates were subjected to hindlimb ischemia by ligation of the femoral artery, which triggers shear stress-mediated adaptive remodeling of preexisting 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 1A). Plantar perfusion was quantified from the Doppler images and normalized to the sham control side of the same animal to determine the percent of perfusion of the ligated hindlimb compared with the unligated sham hindlimb. Strikingly, *Shc flox/flox; Tie2-Cre* mice displayed attenuated perfusion recovery as early as 5 days post-surgery (Figure 1B). The defect in perfusion recovery was exacerbated at each timepoint until day 21, when *Shc flox/flox; Tie2-Cre* mice showed a 40% reduction in plantar perfusion in compared to *Shc flox/flox* controls. Importantly, in wild-type mice, femoral artery ligation induced rapid Shc phosphorylation in ECs that line pre-existing collateral arteries (Figure 1C, Online Figure I), 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 remodeling

Plantar perfusion recovery following femoral artery ligation requires two EC-dependent vascular processes: arteriogenesis and angiogenesis [2, 16]. 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, 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 before and 3 weeks after ligation. At day 21 after ligation, the collaterals in *Shc flox/flox; Tie2-Cre* mice were ~30% smaller than those of control *Shc flox/flox* littermates, indicating defective arteriogenesis (Figure 1D). Importantly, no difference existed between the two genotypes in basal *gracilis* collateral size or vascular architecture of the *gracilis* muscle in un-ligated animals (Online Figure II). Similarly, induction of angiogenesis in the ischemic *gastrocnemius* muscle was defective in *Shc flox/flox; Tie2-Cre* mice at day 21. Capillary density increased by almost 50% in control *Shc flox/flox* mice, whereas *Shc flox/flox; Tie2-Cre* mice were refractory to induction of angiogenesis (Online Figure III). As arteriogenesis and collateral remodeling are the largest contributor to perfusion recovery[17] we focused our attention on the role of Shc in collateral growth.

Shc is required for shear stress-induced NF-κB dependent inflammation in collaterals

The attenuated plantar perfusion recovery and collateral remodeling in *Shc flox/flox; Tie2-Cre* mice suggests a role for Shc in arteriogenesis. The sharp increase 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 *Shc flox/flox; Tie2-Cre* and *Shc flox/flox* mice by nuclear PCNA staining. Proliferation in collaterals 3 days after surgery was decreased in *Shc flox/flox; Tie2-Cre* mice compared to *Shc flox/flox*

controls (Figure 2A). 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. *Shc flox/flox; Tie2-Cre* mice exhibited a significant decrease in CD45-positive leukocyte infiltration compared to *Shc flox/flox* controls (Figure 2B), 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 [18], so we tested the role of Shc in NF-κB activation in this model. While *Shc flox/flox* mice showed significant activation of NF-κB as early as 24hrs after ligation, *Shc flox/flox; Tie2-Cre* mice displayed defects in NF-κB activation in ECs (Figure 2C, Online Figure IV). Interestingly, we also observed upregulation of p65 expression in ECs in *Shc flox/flox* mice that was absent in *Shc flox/flox; Tie2-Cre* mice. This defect in p65 nuclear localization coincided with a decrease in upregulation of the NF-κB-dependent adhesion molecule Vascular Cell Adhesion Molecule-1 (VCAM-1) and Monocyte Chemoattractant Protein-1 (Figure 2D, Online Figure V). 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.

Shc expression in ECs, but not hematopoietic cells, is required for plantar perfusion recovery, arteriogenesis and leukocyte adhesion

The Tie2 promoter used to drive Cre expression in these experiments is expressed primarily in ECs as well as in a significant portion of bone marrow-derived hematopoietic cells [19, 20]. To determine whether Shc in ECs or hematopoietic cells determines perfusion recovery, we performed bone marrow transplants. Four groups of mice (*Shc flox/flox* recipients (flox) reconstituted with *Shc flox/flox* (flox) or *Shc flox/flox; Tie2-Cre* (KO) bone marrow and *Shc flox/flox; Tie2-Cre* (KO) recipients reconstituted with *Shc flox/flox* or *Shc flox/flox; Tie2-Cre* bone marrow*)* were subjected to femoral artery ligation surgery, and plantar perfusion recovery was assessed by Laser Doppler Imaging (Figure 3A&B). Recovery of plantar perfusion was significantly decreased in *Shc flox/flox; Tie2-Cre* mice reconstituted with either *Shc flox/flox* or *Shc flox/flox; Tie2-Cre* bone marrow, whereas the recovery of the *Shc flox/flox* mice reconstituted with either *Shc flox/flox* or *Shc flox/flox; Tie2-Cre* bone marrow was normal. Similarly, remodeling of collaterals following femoral artery ligation was defective in both the *Shc flox/flox; Tie2-Cre* mice reconstituted with *Shc flox/flox* bone marrow and *Shc flox/flox; Tie2-Cre* mice reconstituted with *Shc flox/flox; Tie2-Cre bone marrow* (Figure 3C). These results suggest that Shc expressed in ECs, but not hematopoietic cells, determines arteriogenesis and plantar perfusion recovery.

To specifically test whether Shc expression in ECs and/or leukocytes is required for shear-stress induced leukocyte adhesion to the endothelium, we performed in vitro leukocyte adhesion assays. ECs isolated from *Shc flox/flox* or *Shc flox/flox; Tie2-Cre* mice were subjected to inflammatory shear stress to induce cell adhesion molecule expression and adhesion of monocytic cells (THP-1 cells) that express shRNA against Shc (shShc) or a non-specific target (shNS) was assayed (Figure 3D). As expected, shear stress induced a 3-fold increase in shNS monocytic cell adhesion to *Shc flox/flox* ECs. Interestingly, *Shc flox/flox* ECs incubated with shShc monocytic cells exhibited a similar 3-fold induction, whereas no induction of adhesion was observed in *Shc flox/flox; Tie2-Cre* ECs incubated with either shNS or shShc THP-1 cells (Figure 3D). Together, these in vitro data show that Shc expressed in ECs, but not hematopoietic cells, is required to support shear stress-induced monocytic cell adhesion. Importantly, activation of the NF-κB pathway is required for this response, as adhesion to the endothelium was blocked when performed in the presence of Compound A, a specific inhibitor of Inhibitor of Kappa B Kinase-beta (IKK-β) [21] (Online Figure VI).

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 [22, 23]. Because ECs in collaterals quickly change from a low flow environment to a high shear stress environment following femoral artery ligation[24, 25], we hypothesized that collateral ECs adopt an arterial identity to suit the new arterial-like blood flow environment. To test this, we stained collateral tissue sections for a marker of arterial ECs, ephrinB2 [26]. EphrinB2 expression was upregulated in *Shc flox/flox* collateral ECs within 1-3 days after ligation, however, ephrinB2 upregulation was attenuated in *Shc flox/flox; Tie2-Cre* mice, suggesting that Shc is required for arterial specification of ECs in response to changes in the hemodynamic environment in collaterals (Figure 4A, Online Figure IV). Because ephrinB2 is transcriptionally regulated by the Notch transcription factor NICD [27], we next examined activation of Notch in collaterals. In control *Shc flox/flox* mice, femoral artery ligation induced Notch activation (NICD nuclear localization) in collateral ECs, whereas Notch activation was impaired in *Shc flox/flox; Tie2-Cre* mice (Figure 4B-C, Online Figure IV). 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 our in vitro system. Onset of shear stress induced expression of the Notch target genes ephrinB2, HES-1 and Deltex-1 in ECs isolated from *Shc flox/flox* mice. In contrast, shear-induced upregulation of Notch target genes was impaired in Shc knockout cells (Figure 4D). Importantly, shear stress-induced expression of Notch target genes was impaired in wild-type ECs treated with the γ-secretase inhibitor DAPT (Online Figure VII), indicating that shear-induced expression of these genes is Notch-dependent. To gain mechanistic insights into the role of Shc in shear stress-induced Notch activation, we assessed expression of Notch ligands. Notch ligands Delta-like 1 (Dll-1) and Delta-like 4 (Dll-4) were upregulated by shear stress in vitro in *Shc flox/flox* but not in *Shc flox/flox; Tie2-Cre* ECs (Figure 4E). Shc-dependent upregulation of Dll-4 was confirmed in collateral ECs 3 days after femoral ligation (Figure 4F), indicating that Shc controls activation of the Notch pathway by mediating shear stress-induced upregulation of canonical Notch ligands Dll-4 and possibly Dll-1.

DISCUSSION

Restoration of blood flow to ischemic tissue downstream of an occlusion is a highly complex process requiring activation of several signaling pathways to induce simultaneous angiogenesis and arteriogenesis. Here, we show that the adaptor protein Shc is instrumental in orchestrating EC signaling during post-ischemic neovascularization following ligation of the femoral artery. *Shc flox/flox; Tie2-Cre* mice in which Shc was removed from ECs displayed a marked reduction in restoration of blood flow following femoral artery ligation. Histological analyses revealed defects in arteriogenesis due to impaired 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 upregulation of Notch ligands and downstream activation of the Notch pathway, including upregulation of the arterial EC marker ephrinB2 (Figure 4G).

The Notch pathway is critical for embryogenesis [28] and development of the cardiovascular system[29, 30], but its role in adult physiology is less well-defined. Mice heterozygous for Notch-1 or the Notch ligand Delta-like 1 exhibit reduced plantar perfusion recovery following femoral artery ligation due to attenuated collateral remodelling, similar to the phenotype observed in *Shc flox/flox; Tie2-Cre* mice[31, 32]. Similarly, inhibition of Delta-like 4 delayed plantar perfusion recovery following femoral artery ligation due to disorganized, low perfused angiogenic sprouting in the ischemic capillary network[34]. Although deletion of Shc does not affect basal collateral vessel organization, it is required for Delta-like 4 expression during collateral remodeling and in response to onset of shear stress in vitro. Previous 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 in pre-existing collateral arterioles and results in upregulation of Notch target genes during arteriogenic remodeling and ECs in culture. 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 VEGFR2 and integrins [15]. 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 our work and work of others suggests that shear stress induces expression of Notch ligands Delta-like 1 and/or Delta-like 4, which in turn activate Notch *in trans* through the canonical pathway. 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 [33], precluding any experiments to address this question in the adult mouse.

Hemodynamic forces, such as fluid shear stress, are the primary morphogenic cue that induces collateral remodeling following femoral artery ligation. ECs are responsible for sensing shear stress, and thus are equipped with numerous ‘mechanoreceptors’ capable of sensing and responding to changes in shear stress. Two such mechanoreceptors: integrins and a ‘mechanosensory complex’ comprised of EC-specific proteins PECAM-1, VE-Cadherin and VEGFR2, are required for activation of inflammation in response to flow and, thus, regulation of perfusion recovery in response to ischemic insult. We have previously shown that Shc binds both integrins and the mechanosensory complex following onset of flow[15], and mediates signaling from these mechanoreceptors. Here, we show that Shc is required for the EC response to changes in shear stress in vivo. Endothelial Shc relays signals from these mechanoreceptors into whole-vessel responses, such as inflammation and arterial specification, both of which are critical for arteriogenesis and recovery of blood flow to ischemic tissues following an occlusion (Figure 4G).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

EC	Endothelial cell
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
VE-Cadherin	Vascular Endothelial Cadherin
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B Cells
HES-1	Hairy and Enhancer of Split 1
BMT	Bone Marrow Transplant

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Novelty and Significance

What Is Known?

- Collateral arteries re-route blood flow around an occluded artery to maintain perfusion of downstream tissue.
- Collateral arteries form during embryonic development but are unused in healthy animals unless there is an occlusion. After an occlusive event, pre-existing collaterals remodel to larger, mature vessels.
- Collateral remodeling or arteriogenesis is driven in part by sheer stress imposed on endothelial cells by blood flow.
- The adaptor protein Shc is regulates shear stress signaling pathways in ECs in vitro.

What New Information Does This Article Contribute?

- Endothelial, but not hematopoietic Shc, is required for collateral remodeling and perfusion recovery following occlusion of the femoral artery.
- Shc regulates inflammatory signaling in collateral ECs by mediating NF-κB activation.
- Shc regulates activation of the Notch pathway and downstream arterial specification of remodeling collaterals.

Arteriogenesis, the outward remodeling of collateral arteries in response to an increase in blood flow, is essential for maintaining tissue perfusion following occlusion of a large artery. Arteriogenesis is driven by increased shear stress, , but the molecular mechanism by which ECs sense and respond to shear stress is incompletely understood. We conditionally deleted Shc from ECs and hematopoietic cells and examined the response to femoral artery occlusion. We found that mice lacking Shc exhibited impaired perfusion recovery and collateral remodeling after femoral occlusion. In bone marrow transplantation experiments, we found that endothelial, but not hematopoietic, Shc is required for perfusion recovery. We found that, Shc is required NF-κB activation in remodeling collaterals and for mediating shear stress-induced Notch activation via upregulation of Notch ligands Dll-1 and Dll-4. This leads to expression of the arterial marker ephrinB2 and arterial specification of the remodeling arteriole. These findings contribute to our understanding of arteriogenesis, and how it could be enhanced to prevent tissue death following vascular occlusions.

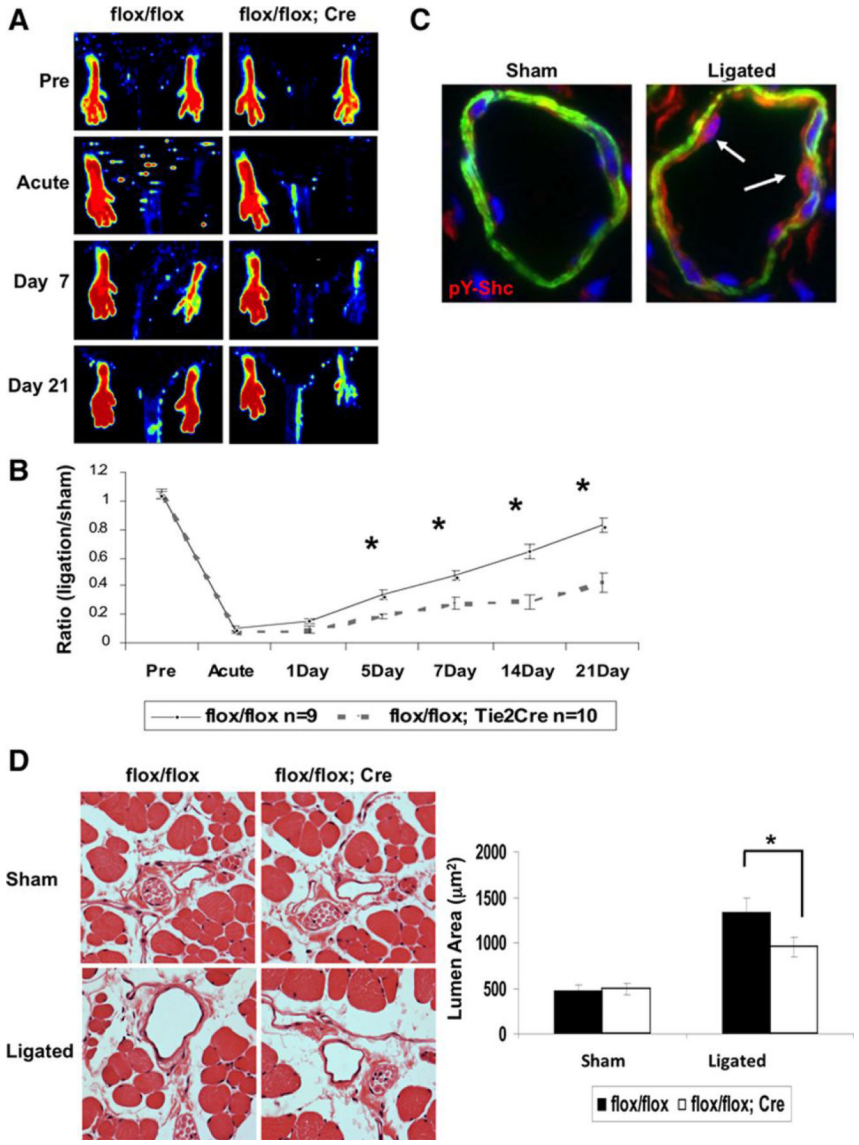

Figure 1. Shc signaling regulates plantar perfusion recovery and arteriogenesis following hindlimb ischemia surgery

(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 Laser Doppler Imaging 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 Doppler 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) Representative 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). (D) 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. *= $p<0.05$.

Values are provided as mean +/− SEM. *= p<0.05. n=8 *Shc flox/flox* and 8 *Shc flox/flox; Tie2-Cre.*

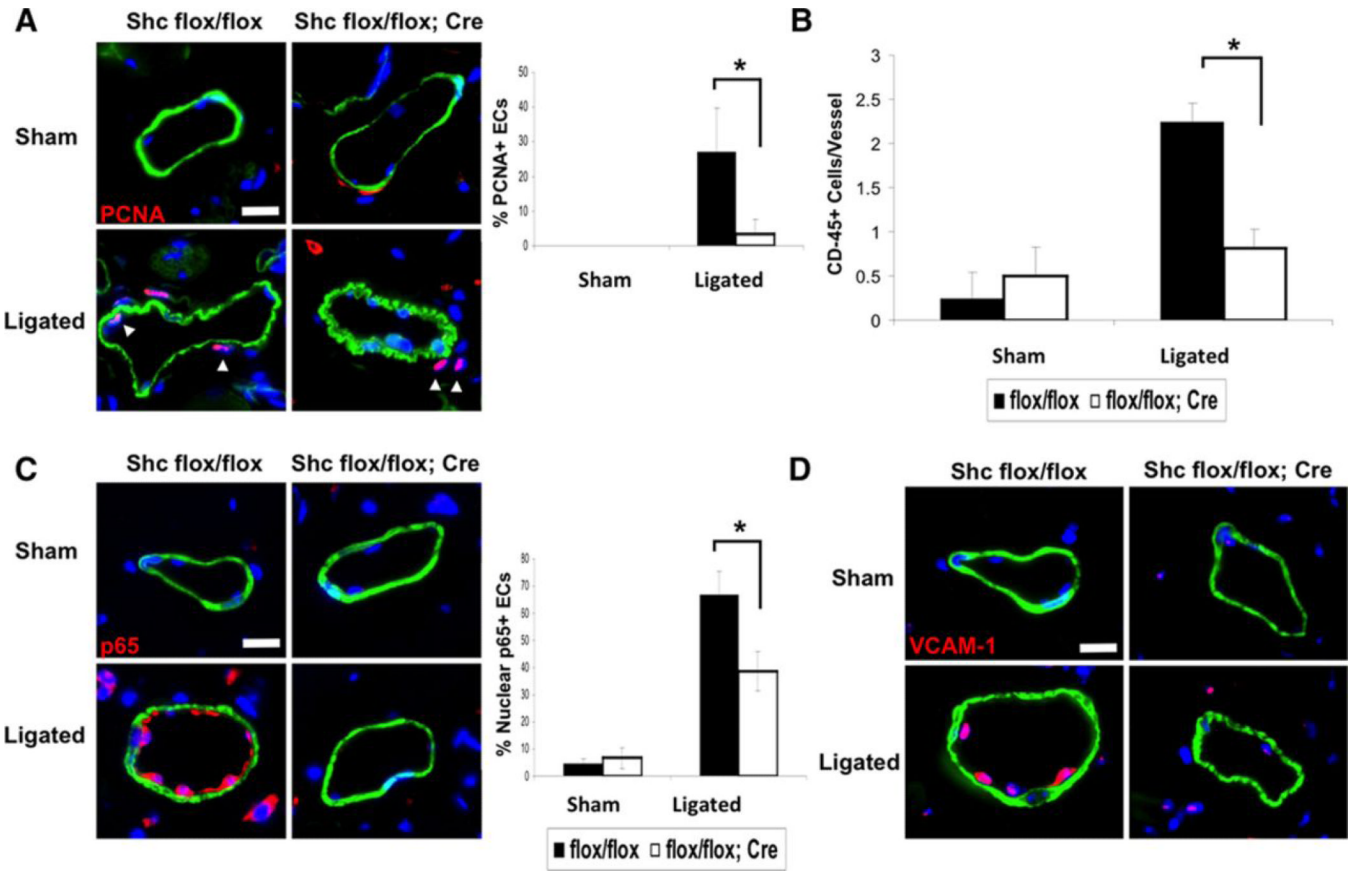

Figure 2. Shc is required for collateral EC proliferation and inflammation

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 VCAM-1 (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 *Shc flox/flox* and 8 *Shc flox/flox; Tie2-Cre*

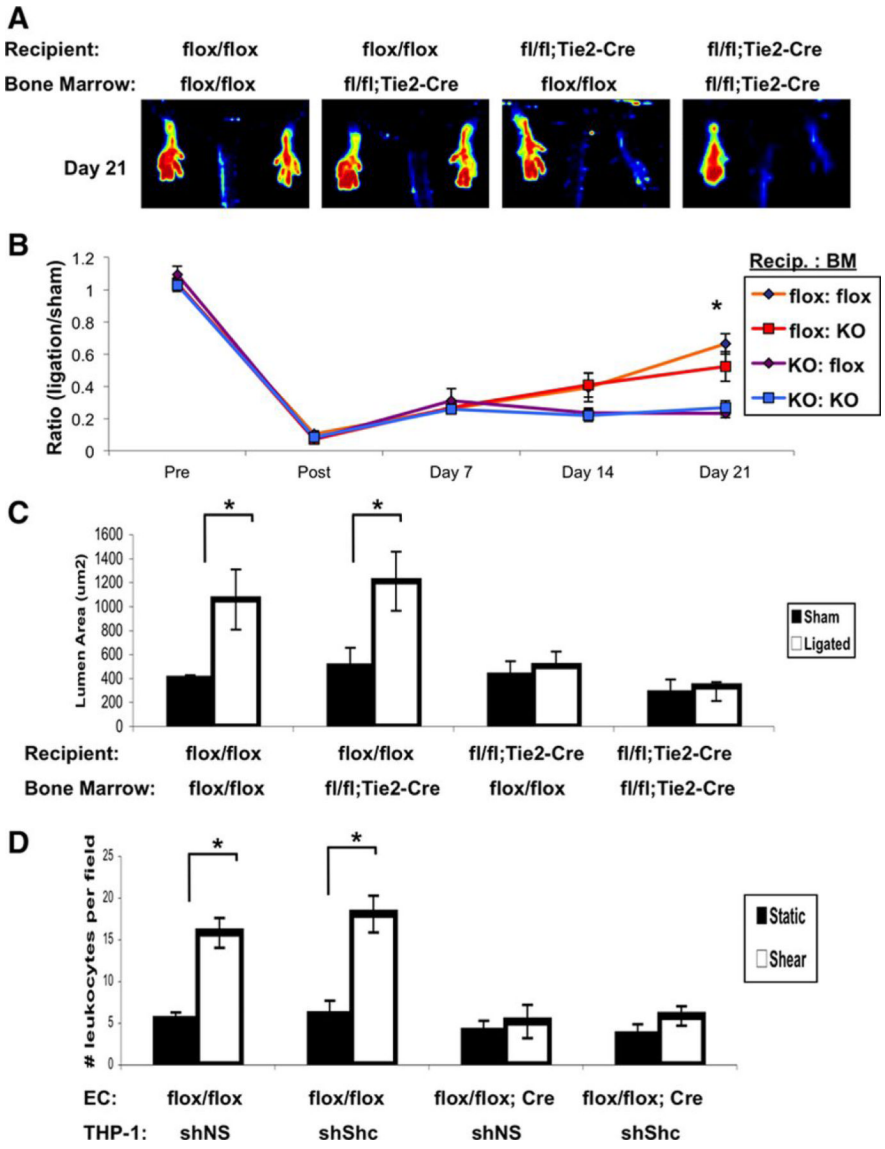

Figure 3. Endothelial, but not hematopoietic cell Shc is required for plantar perfusion recovery, arteriogenesis and monocyte adhesion to the endothelial monolayer

The contribution of Shc in endothelial vs. hematopoietic cells was examined by performing bone marrow transplant and assessing recovery after femoral artery ligation. Irradiated *Shc flox/flox* mice were repopulated with either *Shc flox/flox; Tie2-Cre* or *Shc flox/flox* bone marrow. Simultaneously, irradiated *Shc flox/flox; Tie2-Cre* mice were repopulated with either *Shc flox/flox; Tie2-Cre* or *Shc flox/flox* bone marrow. (A) Plantar perfusion recovery was monitored by Laser Doppler Imaging as in Figure 1. Representative images from day 21 are shown for each condition. (B) Ratio of plantar perfusion (ligated vs sham control side) quantified from the Doppler images. Values are provided as mean +/− SEM. *= $p<0.05$. n= 7 *flox/flox+flox/flox* BM, 6 *flox/flox+flox/flox;Tie2-Cre* BM, 6 *flox/flox;Tie2-Cre+flox/flox* BM and 6 *flox/flox;Tie2-Cre+flox/flox;Tie2-Cre* BM per timepoint. (C) Collateral lumen area in ligated hindlimbs 3 weeks after femoral artery ligation surgery. Cross-sections of the *gracilis* muscle were H&E stained and collateral lumen area was measured. Quantitation provided as mean +/− SEM. *= $p<0.05$. n= 5 *flox/flox+flox/flox* BM, 4 *flox/flox+flox/flox;Tie2-Cre* BM, 4 *flox/flox;Tie2-Cre+flox/flox* BM and 4 *flox/flox;Tie2-Cre+flox/*

flox;Tie2-Cre BM (D) *Shc flox/flox* or *Shc flox/flox; Tie2-Cre* MLECs were exposed to shear stress or left as static controls in the presence of lentivirus-infected THP-1 cells. Monocyte adhesion to the endothelial monolayer was quantified by counting the number of adherent monocytes in 5 random fields. These data are presented as mean +/− SEM. *= $p<0.05$.

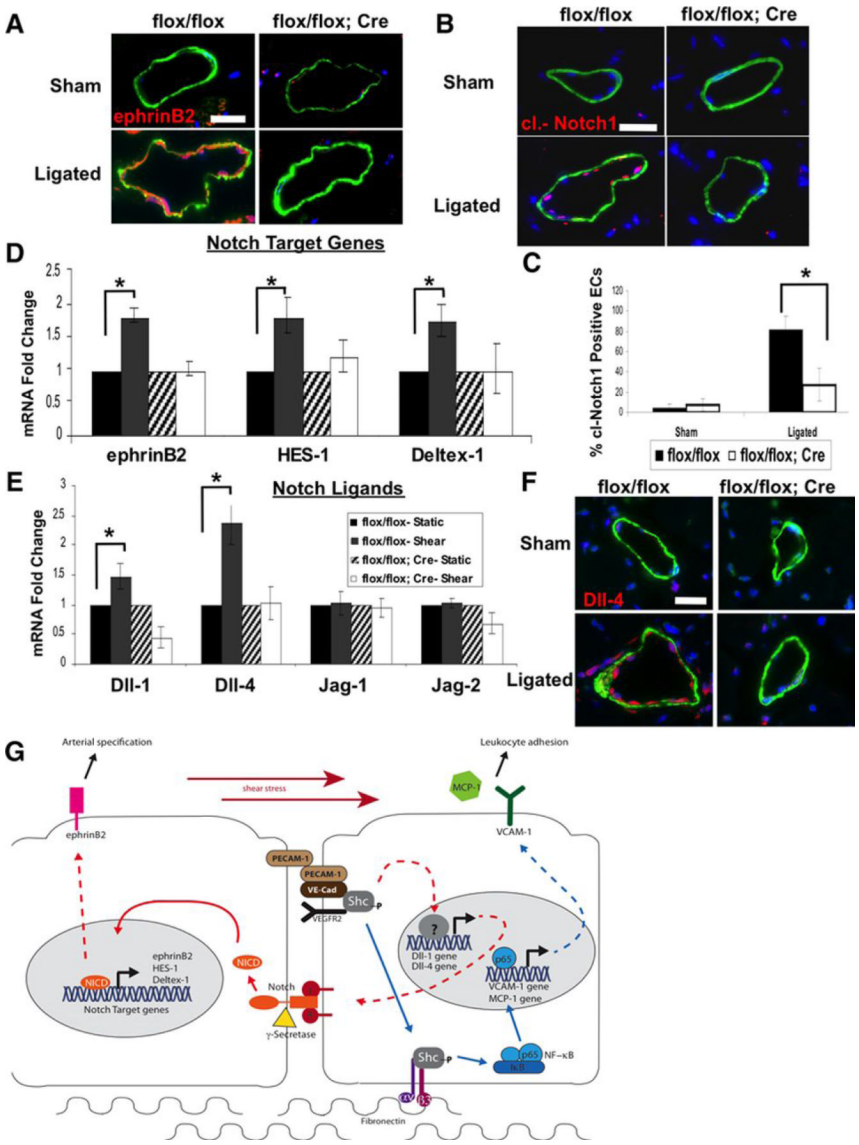

Figure 4. Shc regulates shear stress-induced collateral EC arterial specification via activation of the Notch pathway

(A) Upregulation of Notch-dependent arterial EC marker ephrinB2 during collateral arteriole remodeling requires Shc. Staining of collaterals 3 days after femoral artery 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 3 days after femoral artery ligation (or sham) surgery for Cleaved Notch-1 (red), Smooth Muscle α-actin (green) and DAPI (blue). (C) Quantitation of (B) displayed as % ECs with nuclear (active) Cleaved Notch-1 localization. Values are provided as mean +/− SEM. *= $p<0.05$. n= 8 *flox/flox* and 8 *flox/flox;Cre*. (D&E) Shc is required for shear stress-induced Notch target gene (D) and Notch ligand (E) expression. Relative mRNA expression of Notch target genes Ephrin-B2, HES-1 and Deltex or Notch ligands Delta like-1, Delta like-4, Jagged-1 and Jagged-2 in ECs isolated from *flox/flox* vs. *flox/flox; Tie2-Cre* mice. ECs were sheared for 4 hrs at 15 dynes/cm^2 or kept static as a control. Values are shown as mean +/− SEM (n=4 independent experiments). *= $p<0.05$. (F) Upregulation of Notch ligand Dll-4 requires Shc.

Staining of collaterals 3 days after femoral artery ligation (or sham) surgery for Dll-4 (red), Smooth Muscle α-actin (green) and DAPI (blue).
(G) Schematic showing the role of Shc during collateral remodeling: onset of shear stress induces Shc phosphorylation and the association of Shc with components of the mechanosensory complex (VE-cadherin and VEGFR2) as well as integrins. Shc is required for shear stress-induced NF-κB activation, downstream cell adhesion molecule upregulation (VCAM-1) and inflammation (blue arrows). Additionally, Shc is required for shear stress-induced notch ligand upregulation (Dll-1 and Dll-4), downstream canonical Notch activation and Notch-target gene expression (ephrin B2, HES-1 and Deltex-1) and arterial specification (red arrows).