Endothelial Shc Regulates Arteriogenesis Through Dual Control of Arterial Specification and Inflammation via the Notch and Nuclear Factor-κ–Light-Chain-Enhancer of Activated B-Cell Pathways

Daniel T. Sweet,* Zhongming Chen,* Christopher S. Givens, A. Phillip Owens III, Mauricio Rojas, Ellie Tzima

**Rationale:** Arteriogenesis, the shear stress–driven remodeling of collateral arteries, is critical in restoring blood flow to ischemic tissue after 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 with 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 because loss of Shc in bone marrow–derived hematopoietic cells had no effect on recovery. Mechanistically, Shc deficiency resulted in impaired activation of the nuclear factor κ–light-chain-enhancer of activated B-cell–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.

**Conclusions:** Shc mediates activation of 2 key signaling pathways that are critical for inflammation and arterial specification; collectively, these pathways contribute to arteriogenesis and the recovery of blood perfusion. (Circ Res. 2013;113:32-39.)

**Key Words:** arteriogenesis ■ NF-κB ■ Notch ■ Shc ■ shear stress

Arteriogenesis, the outward remodeling of preexisting collateral arteries, is critical for recovery and restoration of blood supply to ischemic tissue after 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. Blood flow through collateral arteries in a healthy animal is negligible; however, after an occlusion, the steep pressure gradient between the preocclusive and postocclusive regions of the vessel causes blood to rush into preexisting 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. Changes in hemodynamic forces are sensed by endothelial cells (ECs), which initiate signal transduction pathways that induce cell proliferation and inflammation, including the upregulation of cell adhesion molecules on ECs and leukocyte accumulation in the vessel wall.

ECs are equipped with numerous mechanoreceptors capable of responding to shear stress, including caveolae, ion channels, integrins, receptor tyrosine kinases, the apical glycolcayx, primary cilia, heterotrimeric G proteins, and intercellular

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junctures. We previously identified a mechanosensory complex located at cell–cell junctions comprising platelet endothelial cell adhesion molecule-1, vascular endothelial (VE)-cadherin, and VE growth factor receptor-2 that is necessary and sufficient for EC response to shear stress in vitro, vascular remodeling, and arteriogenesis in vivo.

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. Global knockout of the Shc1 gene in mice causes embryonic lethality at embryonic day 11.5 as a result of defects in embryonic heart development. Conditional knockout mice in which Shc was deleted from ECs displayed a defect in postnatal angiogenesis. Using an in vitro shear stress system that models the sudden increase in hemodynamic forces in collaterals immediately after femoral artery ligation, we previously demonstrated that the onset of shear stress activates Shc and induces its association with components of the mechanosensory complex (VE-cadherin and VE growth factor receptor-2), as well as integrins. 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 nuclear factor-κ-light-chain-enhancer of activated B-cell (NF-κB) pathway and downstream inflammation in collateral arteries. Surprisingly, Shc also is 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 delta-like 1 (Dll-1) and Dll-4.

Methods
The detailed materials and methods are described in the online-only Data Supplement.

Results
She Is Required for Plantar Perfusion Recovery After Hindlimb Ischemia
To determine the role of endothelial Shc in perfusion recovery after 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 noninvasively measured using laser Doppler imaging before surgery (pre), immediately after surgery (acute), and at various time points throughout the 3-week recovery period after 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 percentage 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 postsurgery (Figure 1B). The defect in perfusion recovery was exacerbated at each time point until day 21, when Shc flox/flox; Tie2-Cre mice showed a 40% reduction in plantar perfusion when compared with Shc flox/flox controls. Importantly, in wild-type mice, femoral artery ligation induced rapid Shc phosphorylation in ECs that line preexisting 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 after hindlimb ischemia.

Shc Is Required for Collateral Remodeling
Plantar perfusion recovery after femoral artery ligation requires 2 EC-dependent vascular processes: arteriogenesis and angiogenesis. Ligation of the femoral artery causes a sudden increase in blood flow and hemodynamic force through preexisting 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 to increase vascular density and blood perfusion. To test whether Shc is required for arteriogenesis and 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 2 genotypes in basal gracilis collateral size or vascular architecture of the gracilis muscle in unligated 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 50% in control Shc flox/flox mice, whereas Shc flox/flox; Tie2-Cre mice were refractory to induction of angiogenesis (Online Figure III). Because arteriogenesis and collateral remodeling are the largest contributors to perfusion recovery, 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 suggest a role for Shc in arteriogenesis. The sharp increase in hemodynamic forces in the collateral induces EC proliferation and inflammation. 2 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 proliferating cell nuclear antigen (PCNA) staining. Proliferation in

Nonstandard Abbreviations and Acronyms

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<tr>
<td>BM</td>
<td>bone marrow</td>
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<td>EC</td>
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<td>Dll-1</td>
<td>delta-like 1</td>
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<td>Dll-4</td>
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<td>NF-κB</td>
<td>nuclear factor-κ-light-chain-enhancer of activated B cells</td>
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collaterals 3 days after surgery was decreased in \textit{Shc flox/flox; Tie2-Cre} mice compared with \textit{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. \textit{Shc flox/flox; Tie2-Cre} mice exhibited a significant decrease in CD45-positive leukocyte infiltration compared with \textit{Shc flox/flox} controls (Figure 2B), indicating a role for Shc in inflammation in response to hindlimb ischemia. CD45-positive cell recruitment after femoral artery ligation requires activation of NF-κB in ECs, so we tested the role of Shc in NF-κB activation in this model. Although \textit{Shc flox/flox} mice showed significant activation of NF-κB as early as 24 hours after ligation, \textit{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 \textit{Shc flox/flox} mice that was absent in \textit{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, 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.

\textbf{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 and in a significant portion of bone marrow (BM)–derived hematopoietic cells. To determine whether Shc in ECs or hematopoietic cells determines perfusion recovery, we performed BM transplants. Four groups of mice (\textit{Shc flox/flox} recipients [flox] reconstituted with \textit{Shc flox/flox} [flox] or \textit{Shc flox/flox; Tie2-Cre} [knockout] BM and \textit{Shc flox/flox; Tie2-Cre} recipients reconstituted with \textit{Shc flox/flox} or \textit{Shc flox/flox; Tie2-Cre} BM) were subjected to femoral artery ligation surgery, and plantar perfusion recovery was assessed by laser Doppler imaging (Figure 3A and 3B). The recovery of plantar perfusion was significantly decreased in \textit{Shc flox/flox; Tie2-Cre} mice reconstituted with either \textit{Shc flox/flox} or \textit{Shc flox/flox; Tie2-Cre} BM, whereas the recovery of the \textit{Shc flox/flox}
mice reconstituted with either Shc flox/flox or Shc flox/flox; Tie2-Cre BM was normal. Similarly, remodeling of collaterals after femoral artery ligation was defective in both the Shc flox/flox; Tie2-Cre mice reconstituted with Shc flox/flox BM and the Shc flox/flox; Tie2-Cre mice reconstituted with Shc flox/flox; Tie2-Cre BM (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 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 or a nonspecific target 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 because adhesion to the endothelium was blocked when performed in the presence of compound A, a specific inhibitor of IκB kinase-β (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-specific and venous-specific genes is the consequence of local hemodynamic cues that may regulate vessel remodeling. Because ECs in collaterals quickly change from a low-flow environment to a high-shear-stress environment after femoral artery ligation, 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. EphrinB2 expression was upregulated in Shc flox/flox collateral ECs within 1 to 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, we next examined activation of Notch in collaterals. In control
HES-1, and Deltex-1 in ECs isolated from Shc flox/flox induced expression of the Notch target genes ephrinB2, regulation using our in vitro system. The onset of shear stress in shear stress–induced Notch activation and ephrinB2 up-collateral remodeling. Therefore, we tested the role of Shc making it difficult to delineate the exact role of Shc during arteriogenesis, the remodeling collateral arterioles.

Importantly, shear stress–induced expression of Notch target genes was impaired in Shc knockout cells (Figure 4D). 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. Therefore, we tested the role of Shc in shear stress–induced Notch activation and ephrinB2 up-regulation using our in vitro system. The 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 stress–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 stress–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 the expression of Notch ligands. Notch ligands DII-1 and DII-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 DII-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 DII-4 and possibly DII-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 postischemic neovascularization after 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 after femoral artery ligation. Histological analyses revealed defects in arteriogenesis because of 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 stress–induced upregulation of Notch ligands and downstream activation of the Notch pathway.

Figure 3. Endothelial cell (EC), 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 (BM) transplantation 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 BM. Simultaneously, irradiated Shc flox/flox; Tie2-Cre mice were repopulated with either Shc flox/flox; Tie2-Cre or Shc flox/flox BM. 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×floxflox BM, n=6 flox/flox×floxflox; Tie2-Cre BM, n=6 flox/flox; Tie2-Cre×floxflox BM, and n=6 flox/flox; Tie2-Cre×floxflox; Tie2-Cre BM per time point. C, Collateral lumen area in ligated hindlimbs 3 weeks after femoral artery ligation surgery. Cross-sections of the gracilis muscle were hematoxylin and eosin-stained, and collateral lumen area was measured. Quantitation provided as mean±SEM. *P<0.05. n=5 flox/flox×floxflox BM, n=4 flox/flox×floxflox; Tie2-Cre BM, n=4 flox/flox; Tie2-Cre×floxflox BM, and n=4 flox/flox; Tie2-Cre×floxflox; Tie2-Cre BM. D, Shc flox/flox or Shc flox/flox; Tie2-Cre murine lung ECs 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. KO indicates, knockout; shNS, shRNA against nonspecific target; shShc, shRNA against Shc.
including upregulation of the arterial EC marker ephrinB2 (Figure 4G).

The Notch pathway is critical for embryogenesis and development of the cardiovascular system, 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 after femoral artery ligation because of attenuated collateral remodeling, similar to the phenotype observed in Shc flox/flox; Tie2-Cre mice. Similarly, the inhibition of Dll-4 delayed plantar perfusion recovery after femoral artery ligation because of disorganized, low-perfused angiogenic sprouting in the ischemic capillary network. Although the deletion of Shc does not affect basal collateral vessel organization, it is required for Dll-4 expression during collateral remodeling and in response to the onset of shear stress in vitro. Previous studies suggested that the Notch pathway is activated downstream of vascular endothelial growth factor, 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 preexisting 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 (vascular endothelial cadherin [VE-Cad] and VE growth factor receptor-2 [VEGFR2]) and integrins. Shc is required for shear stress–induced nuclear factor-κB activation, downstream cell adhesion molecule upregulation (vascular cell adhesion molecule-1 [VCAM-1]), and arterial specification. MCP-1 indicates monocyte chemoattractant protein-1; NICD, Notch intracellular domain; PECAM-1, platelet endothelial cell adhesion molecule-1.

Figure 4. Shc regulates shear-stress-induced collateral endothelial cell (EC) arterial specification via activation of the Notch pathway. A, Upregulation of Notch-dependent arterial EC marker ephrinB2 during collateral arteriogenic remodeling requires Shc. Staining of collaterals 3 days after femoral artery ligation (or sham) surgery for ephrinB2 (red), smooth muscle α-actin (green), and 4′,6-diamidino-2-phenylindole (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 (cl-Notch1; red), smooth muscle α-actin (green), and DAPI (blue). C, Quantitation of (B) displayed as % ECs with nuclear (active) cl-Notch1 localization. Values are provided as mean±SEM. *P<0.05. n=8 flox/flox and n=8 flox/flox; Cre. D, Shc is required for shear stress–induced Notch target gene (D) and Notch ligand (E) expression. Relative mRNA expression of Notch target genes ephrinB2, hairy and enhancer of split 1 (HES-1), and Deltex or Notch ligands delta-like (Dll) 1, Dll-4, Jagged (Jag)-1, and Jag-2 in ECs isolated from flox/flox vs flox/flox; Tie2-Cre mice. ECs were sheared for 4 hours at 15 dynes/cm² or kept static as a control. Values are shown as means±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 representation 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 (vascular endothelial cadherin [VE-Cad] and VE growth factor receptor-2 [VEGFR2]) and integrins. Shc is required for shear stress–induced nuclear factor-κB (NF-κB) activation, downstream cell adhesion molecule upregulation (vascular cell adhesion molecule-1 [VCAM-1]), and inflammation (blue arrows).
and work of others suggest that shear stress induces the expression of Notch ligands, Dll-1 and Dll-4, which in turn activate Notch in trans it 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 embryonically lethal, precluding any experiments to address this question in the adult mouse.

Hemodynamic forces, such as fluid shear stress, are the primary morphogenetic cue that induces collateral remodeling after 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 mechano-sensor complex composed of EC-specific proteins platelet endothelial cell adhesion molecule-1, VE-cadherin, and vascular endothelial growth factor receptor-2, are required for activation of inflammation in response to flow and, thus, regulation of perfusion recovery in response to ischemic insult. We previously have shown that Shc binds to both integrins and the mechano-sensor complex after the onset of flow 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 the recovery of blood flow to ischemic tissues after an occlusion (Figure 4G).

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Disclosures
None.

References


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**Novelty and Significance**

**What Is Known?**
- Collateral arteries reroute 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, preexisting collaterals remodel to larger, mature vessels.
- Collateral remodeling or arteriogenesis is driven, in part, by shear stress imposed on endothelial cells by blood flow.

**What New Information Does This Article Contribute?**
- Endothelial, but not hematopoietic, Shc is required for collateral remodeling and perfusion recovery after occlusion of the femoral artery.
- Shc regulates inflammatory signaling in collateral endothelial cells by mediating nuclear factor-κ–light-chain-enhancer of activated B-cell activation.
- Shc regulates activation of the Notch pathway and downstream arterial specification of remodeling collaterals.

**Arteriogenesis** is the outward remodeling of collateral arteries in response to an increase in blood flow, is essential for maintaining tissue perfusion after occlusion of a large artery. Arteriogenesis is driven by increased shear stress, but the molecular mechanism by which endothelial cells sense and respond to shear stress is incompletely understood. We conditionally deleted Shc from endothelial cells 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 the required nuclear factor-κ–light-chain-enhancer of activated B-cell activation in remodeling collaterals and for mediating shear stress–induced Notch activation via upregulation of Notch ligands δ-like 1 and δ-like 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 after vascular occlusions.
Activated B-Cell Pathways

Endothelial Shc Regulates Arteriogenesis Through Dual Control of Arterial Specification and Inflammation via the Notch and Nuclear Factor-κ–Light-Chain-Enhancer of Activated B-Cell Pathways

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS:

Mice
Shc-flox mice were a kind gift from Dr. Kodi Ravichandran at University of Virginia. 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.

Femoral artery ligation surgery
The surgery procedure was performed on the right side as described previously. 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. 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. 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 two preexisting 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. 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 (C) 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°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-thyramide 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 preexisting 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. 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.

**Bone marrow transplants**

Bone marrow transplants were performed as previously described. Recipient *Shc flox/flox* and *Shc flox/flox; Tie2-Cre* mice (6-8 weeks old) were irradiated with a total of 11 Gy (2 doses of 550 rads 4 hours apart) using a Cs137 irradiator. Irradiated mice were repopulated with bone marrow (approx. 2 x 10⁶ cells per animal) harvested from *Shc flox/flox* and *Shc flox/flox; Tie2-Cre* donor mice via retroorbital injection. Mice were allowed to recover for 4 weeks and then subjected to femoral artery ligation surgery as described above.

**Monocyte adhesion experiments in vitro**

THP-1 cells were transduced with a lentivirus as previously described. Successfully transduced cells express GFP and were collected by flow cytometry and resuspended in serum-free RPMI 1640 medium. Confluent dishes of serum starved MLECs were either kept static or exposed to 8hrs oscillatory shear stress (+/- 5 dynes/cm²) using a cone and plate viscometer. Then 1 million THP-1 cells per dish were added onto the monolayers of ECs and incubated at 37°C for 25 min. The unbound cells were rinsed off
with PBS and the bound cells were fixed with 2% formaldehyde. To quantify the assays, five random fields under the 5x objective on an inverted microscope (DMIRB; Leica) were counted for each assay, and representative images were acquired using a RETIGA 1300 camera.

For assays using Compound A to inhibit the NF-κB pathway, the above protocol was used with the following modifications: Uninfected THP-1 cells were labeled with 1 µM CellTracker green 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) and for 20 min at 37°C, then washed and resuspended in serum-free RPMI 1640. Confluent dishes of MLECs were pre-treated with 5 µM Compound A or equal volume DMSO (vehicle) for 1 hr in serum-free RPMI and then exposed to shear stress in the presence of the inhibitor.

**Cell culture and shear stress assays**

*Shc flox/flox* and *Shc flox/flox; Tie2-Cre* MLECs (isolation protocol in 5) were subjected to shear stress in *vitro* using a previously described cone and plate viscometer 6. 100-mm tissue culture dishes were coated in 10 µg/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/cm²) in the starvation medium for 4 hours or kept static as controls. For inhibitor experiments, MLECs were pre-treated for 1 hr with 20 µM 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 reverse transcription 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 GTG ATT CA -3'; ephrinB2-F: 5'-GCC CAA TCC AGG AGA CCA CCA ATT GGA C -3'; ephrinB2-R: 5'- GTG CCG AAC CTT CTC CTA AG -3'; HES-F: 5'- TCT TTG GTC CTG GAA TAG TGC TA -3'; HES-R: 5'- ACT GAG CAG TTG AAG GTG TAT TAT GTC T -3'; Deltex-F: 5'- CAC TGG CCC TGT CCA CCC AGC CTT GGC -3'; Deltex-R: 5'- GGG AAG GCG GGC AAC TCA GGC CTC AGG -3'; Delta-like 1-F: 5'- GAC AC CAA GTA CCA GTC GGT GTA TG -3'; Delta-like 1-R: 5'- AAC CTG CTA GTC AGC AGC AGT C -3'; Delta-like 4-F: 5'- CGG GTC ATC TGC AGT GAC AAC -3'; Delta-like 4-R: 5'- AGT TGA GAT CTT GGT CAC AAA ACA G -3'; Jagged-1-F: 5'- CCA GCG GTC CTA ATG GTG ATG -3'; Jagged-1-R: 5'- GCT GTG GTT CTC AGG TGC AAA G -3'; Jagged-2-F: 5'- GAT GGC CAC CGG GTG TAT AG -3'; Jagged-2-R: 5'- TCT CAC AGG GTG GCT GCA AG -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, Deltex, Delta-like 1, Delta-like 4, Jagged-1 and Jagged-2 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.
Online Figure I: Shc is transiently activated 1-3 days after femoral artery ligation
(A) Staining of gracilis collaterals 3 and 21 days after femoral artery ligation surgery, or in unligated sham mice. Sections were stained phospho-Shc Y239,240 (red) and Smooth Muscle α-actin (green) and DAPI (blue).
Online Figure II: Loss of endothelial Shc does not alter baseline collateral lumen area or gracilis muscle vascular architecture

(A) Baseline collateral lumen area of gracilis collaterals was measured in H&E stained sections from unligated mice. Values are provided as mean +/- SEM. *= p<0.05 from 2 mice per genotype. (B) Vascular architecture in the gracilis muscle was assessed by staining sections with Smooth Muscle α-Actin to mark blood vessels and counting the number of large arteries, large veins, small arterioles and small venules per gracilis muscle in unligated mice. Values are provided as mean +/- SEM. from 2 mice per genotype.
Online Figure III: Shc is required for ischemia induced angiogenesis following femoral artery ligation

(A) 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. Quantitation is shown in (B). Values are provided as mean +/- SEM. * = p<0.05. n = 8 Shc flox/flox and 8 Shc flox/flox; Tie2-Cre.
Online Figure IV: Time course of signaling after femoral artery ligation

Staining of gracilis collaterals 1, 3 and 21 days after femoral artery ligation surgery, or in unligated sham mice. Sections were stained for (A) NF-κB subunit p65 (red), (B) VCAM-1 (red), (C) ephrinB2 (red) or (D) cleaved Notch-1 (red) and Smooth Muscle α-actin (green) and DAPI (blue).
Online Figure V: Activation of Akt and MCP-1 by femoral artery ligation require Shc
Staining of gracilis collaterals 3 and 21 days after femoral artery ligation surgery, or in unligated sham mice. Sections were stained for (A) Akt (red) or (B) Monocyte Chemoattractant Protein-1 (red) and Smooth Muscle α-actin (green) and DAPI (blue).
Online Figure VI: The NF-κB pathway is required for shear stress induced monocyte adhesion to the endothelium.
Control Shc flox/flox MLEC s were pre-treated with DMSO (Vehicle) or Compound A and exposed to shear stress or left as a static controls in the presence of lentivirus-infected THP-1 cells.
Online Figure VII: Shear stress-induced Notch target gene expression is Notch-dependent

Relative mRNA expression of Notch target genes Ephrin-B2, HES-1 and Deltex-1 in wild-type ECs. ECs were treated with 20µM DAPT (γ-secretase inhibitor) or DMSO (vehicle) and exposed to shear stress or kept static as a control. Values are mean +/- SEM (n=3 independent experiments). * = p<0.05.
SUPPLEMENTAL REFERENCES


