HHS Public Access

Author manuscript

Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2023 March 24.

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2023 January ; 43(1): 92–108. doi:10.1161/ATVBAHA.122.318205.

Repair of Limb Ischemia Is Dependent on Hematopoietic Stem Cell Specific-SHP-1 Regulation of TGF-β1

Chen Wang[*],
Center for Precision Medicine, University of Missouri School of Medicine, Columbia.

Ravi Nistala,
Center for Precision Medicine, Division of Nephrology, University of Missouri School of Medicine, Columbia.

Min Cao,
Center for Precision Medicine, University of Missouri School of Medicine, Columbia.

De-Pei Li,
Center for Precision Medicine, University of Missouri School of Medicine, Columbia.

Yi Pan,
Center for Precision Medicine, University of Missouri School of Medicine, Columbia.

Mojgan Golzy,
University of Missouri School of Medicine, Columbia. Department of Family and Community Medicine – Biostatistics Unit, School of Medicine, University of Missouri, Columbia.

Yuqi Cui,
Center for Precision Medicine and Division of Cardiovascular Medicine, Department of Medicine, University of Missouri School of Medicine, Columbia.

Zhenguo Liu,
Center for Precision Medicine and Division of Cardiovascular Medicine, Department of Medicine, University of Missouri School of Medicine, Columbia.

XunLei Kang
Center for Precision Medicine, University of Missouri School of Medicine, Columbia.

Abstract

BACKGROUND: Hematopoietic stem cell (HSC) therapy has shown promise for tissue regeneration after ischemia. Therefore, there is a need to understand mechanisms underlying endogenous HSCs activation in response to ischemic stress and coordination of angiogenesis and repair. SHP-1 plays important roles in HSC quiescence and differentiation by regulation of

Correspondence to: Xunlei Kang, One Hospital Dr, M754, Laboratory # 573-884-0863, Columbia, MO 65212-1000. kangxu@health.missouri.edu.
[*]C. Wang and R. Nistala contributed equally.

Disclosures
None.

Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/ATVBAHA.122.318205.

TGF-β1 signaling. TGF-β1 promotes angiogenesis by stimulating stem cells to secrete growth factors to initiate the formation of blood vessels and later aid in their maturation. We propose that SHP-1 responds to ischemia stress in HSC and progenitor cells (HSPC) via regulation of TGF-β1.

METHODS: A mouse hind limb ischemia model was used. Local blood perfusion in the limbs was determined using laser doppler perfusion imaging. The number of positive blood vessels per square millimeter, as well as blood vessel diameter (μm) and area (μm^2), were calculated. Hematopoietic cells were analyzed using flow cytometry. The bone marrow transplantation assay was performed to measure HSC reconstitution.

RESULTS: After femoral artery ligation, TGF-β1 was initially decreased in the bone marrow by day 3 of ischemia, followed by an increase on day 7. This pattern was opposite to that in the peripheral blood, which is concordant with the response of HSC to ischemic stress. In contrast, SHP-1 deficiency in HSC is associated with irreversible activation of HSPCs in the bone marrow and increased circulating HSPCs in peripheral blood following limb ischemia. In addition, there was augmented autoinduction of TGF-β1 and sustained inactivation of SHP-1-Smad2 signaling, which impacted TGF-β1 expression in HSPCs in circulation. Importantly, restoration of normal TGF-β1 oscillations helped in the recovery of limb repair and function.

CONCLUSIONS: HSPC-SHP-1-mediated regulation of TGF-β1 in both bone marrow and peripheral blood is required for a normal response to ischemic stress.

Keywords

bone marrow; flow cytometry; ischemic stress; limb ischemia; perfusion

Vascular remodeling that manifests as inflammation and fibrosis is a major consequence of ischemic stress seen in both physiological injuries and cardiovascular diseases, including myocardial infarction, cardiomyopathy, and peripheral vascular disease. Hind limb ischemia (HLI) models of varying severity and animal background are frequently used to mimic the physiological and pathological mechanisms for vascular remodelling under conditions of ischemic stress. The healing in both physiological and pathological processes is often times challenging due to poor cell/tissue regeneration and maladaptive repair. There are data supporting a major role for endogenous stem cells in the response to ischemic stress due to their rapid activation, mobilization, and secretion of angiogenic factors.[1] Specifically, studies show that bone marrow (BM)-derived stem cells, such as hematopoietic stem cells (HSCs), play obligatory roles in vascular repair.[1-5] Therefore, there is keen interest in utilizing and enhancing the potential of HSCs in ischemic repair. HSCs switch from a quiescent status during homeostasis and become activated to proliferate and differentiate in response to different stressors, such as ischemia, infection, chemotherapy, or radiation.[6,7] For example, FGF1 (fibroblast growth factor 1) signaling has been shown to stimulate HSC expansion under a chemotherapeutic challenge.[8] However, the mechanisms that lead to the switch in HSC status during ischemia stress are not well understood.[9]

Src homology region 2 domain-containing phosphatase 1(SHP-1) homology region 2 domain-containing phosphatase 1(SHP-1), a Src homology 2 domain–containing protein tyrosine phosphatase, is primarily expressed in hematopoietic cells, with low expression in muscle, bone, and connective tissue. SHP-1 is a negative regulator in the signaling

pathways of many growth factors and cytokine receptors.[10-12] Previously, we identified SHP-1 as a novel intrinsic regulator of HSC quiescence in the BM that functions via binding to the immunoreceptor tyrosine-based inhibitory motif (ITIM) of Transforming growth factor beta-1 proprotein (TGF-β1) receptor (TβR1) and facilitates TGF-β1 signaling.[13] Intriguingly, the role of SHP-1 is context-dependent. On the one hand, deficiency of SHP-1 has been associated with several human inflammatory diseases, including psoriatic inflammatory skin disease,[14] multiple sclerosis,[15] and human allergies and asthmatic disease,[16,17] and overexpression of SHP-1 leads to potent suppression of hepatocellular carcinoma and metastasis.[18] On the other hand, SHP-1 is an oncogene in the context of acute myeloid leukemia.[19] However, the role of SHP-1 in the regulation of HSCs response to ischemic stress is unknown.

Cytokines are known to regulate HSC function through autocrine or paracrine effects. Several cytokines, such as TGF-β1, CXCL12, GM-CSF, and G-CSF that are differentially expressed under ischemia stress, have been shown to regulate HSC function.[19-22] TGF-β1 is believed to play a crucial role in mediating the function of HSC in inflammation, vessel formation, and fibrosis in injured tissues.[23,24] TGF-β1 is a pleiotropic cytokine that plays an essential role in angiogenesis during ischemic stress,[25] as well as a pivotal role in BM homeostasis.[26] We and others have previously shown that TGF-β1 in concert with SHP-1 signaling is required for the maintenance of HSCs quiescence in the BM as well as angiogenesis.[13,27] Because TGF-β1 has other functions beyond vascular biology, its effects are complex and pathways are not always well defined.[25,28] Therefore, the precise regulation of TGF-β1 under conditions of ischemic stress needs further understanding, which can facilitate the targeting of TGF-β1 in cardiovascular diseases.

To understand the role of SHP-1 and its interaction with TGF-β1 in regulating the function of HSC under ischemia stress, we used a 1-step HLI model. We performed HLI in C57BL/6J mice because the blood flow recovery after femoral artery ligation (FAL) is fast and reliable as this strain has an extensive pre-existing collateral artery network, and a greater capacity to recruit collateral arteries.[29] We examined the dependence of HLI repair on SHP-1 in endogenous HSC and progenitors cells (HSPC) by utilizing a HSPC-specific deletion of the SHP-1 mouse model and the mechanisms underlying the dependence. We found that TGF-β1 dysregulation in both BM and peripheral blood (PB) in SHP-1-deficient mice leads to a defective response of HSPCs to ischemic stress and repair.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Mice

C57BL/6J, CD45.2, CD45.1 mice were purchased from Charles River, Inc. To obtain an HSPC-specific deletion of *Shp-1,* C57BL/6J mice carrying the loxP-flanked *Shp-1* gene (B6.129P2-Ptpn6tm1Rsky/J [Shp-$1^{fl/fl}$], Jackson laboratory)[30] were crossed with transgenic C57BL/6J mice expressing the tamoxifen-inducible Cre recombinase under the control of stem cell leukemia (Scl) HSC enhancer (Scl-Cre ER mice were provided by J.R. Gothert).[31]

Shp-1 genotyping PCR primers were the following: (1) 5'-ACC CTC CAG CTC CTC TTC-3', (2) 5'-TGA GGT CCC GGT GAA ACC-3', and (3) 5'-TGT TAT GCA TGT GTG TAT CG-3'. The Scl-Cre-ER genotyping PCR primers were the following: (1) 5'-GAACCTGAAGATGTTCGCGAT-3' and (2) 5'-ACCGTCAGTACGTGAGATATC-3'. To induce expression of Cre-ER recombinase, mice received tamoxifen via intraperitoneal injection (Sigma, 1 mg/0.1 ml of corn oil) as described.[31]

All mouse strains used in this study had a C57BL/6J genetic background. Animals were randomly assigned to the experiments after genotyping with the same sex, similar age group (6–8 weeks old) and approximately same weight. For HLI model, only male mice were used because of the higher blood flow recovery after ischemic stress.[32] The figure legends detail the number of animals used per study. The investigators were not blinded to the allocation of animals during the experiments and outcome assessment. The major content of the standard laboratory diet is formulated with 20% protein diet and 4.5% fat. The source and diet number can be found (https://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/mdrf/mdi4/~edisp/ducm04_028436.pdf) All procedures were approved in advance by the Institutional Animal Care and Use Committee of the University of Missouri (MU) and performed in the animal facility of MU.

Creation of Hind Limb Ischemia Model, Blood Flow, and Functional Measurement of Ischemic Hind Limb

FAL was performed to affect hind limb ischemia in the mice as described.[33,34] Briefly, mice were anesthetized with air anesthesia (1.25% isoflurane/O2), and the animal's body temperature was maintained at 37±0.5 °C. The left femoral artery was exposed through a 3-mm incision with minimal retraction and tissue disturbance. A 7.0-ligature was placed distal to the origin of the lateral caudal femoral and superficial epigastric arteries (the latter was also ligated) and proximal to the genu artery. The femoral artery was transected and separated by 1 to 2 mm between the sutures. The incision was cleaned and treated with saline before being closed. Local blood perfusion in the limbs was determined using laser Doppler perfusion imaging (LDPI, Moor Instruments, Devon, United Kingdom) pre-operatively, 30 minutes post-operatively and at day 3, 7, 14, and 21 post-operatively. Before imaging, excess hair was shaved from the limbs of mice, and the animals were placed on a heating pad set to 37 °C to reduce temperature variance. Blood flow in the right limb was also monitored as a baseline control. Blood flow recovery was measured using the ratio of left ischemic limb blood flow/right normal limb blood flow. Treadmill test was performed as described[33] to evaluate functional recovery of the ischemic limb. Functional recovery was reflected by running time for each mouse on a rodent treadmill equipped with an electric grid at the rear. Mice were made to run continuously until fatigued (indicated by falling on the electric grid twice) at days 3, 7, and 21 after limb ischemia and the running time was recorded. Two different experiments were carried out, each with 8 *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice.

Immunofluorescence Staining and Quantification

Femurs or tibias were perfused with PBS before removal and fixed with 4% paraformaldehyde. Frozen sections were retrieved using 1 μg/ml proteinase K in TE buffer

(100 mM Tris-HCl, pH 8.0, and 50 mM EDTA) at 37 °C for 30 minutes. Blocking was done with Universal Blocking Reagent (HK112-5K; BioGenex). Sections were incubated overnight with α - Smooth Muscle Actin Antibody (R&D system) at 4 °C. Secondary staining was done with donkey anti–mouse AF555 (Invitrogen; 1:500) at room temperature for 1 hour. DAPI stock solution was diluted to 300 nM in PBS, and 300 mL was added to the coverslip preparation for 1 minute. Sections were rinsed 3× in PBS, excess buffer drained from the coverslip, and mounted with Thermo Scientific Shandon ImmuMount medium. Image stitching was done to capture the entire specimen at high magnification and seamlessly create a single high-resolution image. For high-resolution 3-dimensional images, images were captured with Keyence BZ-8000 fluorescence microscope with a 20× objective (resulting in ≈ 200× magnification; the zoom function of the camera was also employed). The number of positive blood vessels per square millimeter, as well as blood vessel diameter (μm) and area (μm^2), were calculated from 5 tissue sections (8 μm) in each mouse (from the low frontal muscles).

Flow Cytometry

We used BD Accuri C6 Plus Flow Cytometer or Cytek Aurora flow cytometry machines to analysis of mouse PB, BM, hematopoietic cells were labeled with antibodies from Biolegend (San Diego, CA) unless specifically indicated. The intracellular staining was performed using the Foxp3/Transcription factor staining set (eBioscience, Grand Island, NY) and Fixation/Methanol protocol (eBioscience, Grand Island, NY) provided by the manufacturers. After harvesting 1 million mononuclear cells and analyzing them using Flowjoo software, HSPCs are sequentially gated through single-cell gate, nucleated cell gate, and lineage-negative cell gate, and then selected as Sca-1+cKit+CD150+CD48−, or Sca-1+cKit+CD34− FLK2−. The antibodies used have been listed in Major Resources Table. Please see the Major Resources Table in the Supplemental Material.

Mouse Competitive Reconstitution Analyses

Five hundred Sca-1^+cKit$^+$CD150$^+$CD48$^-$ BM HSC (CD45.2) isolated by flow cytometry sorting machine (Beckman Coulter MoFlo XDP) from *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice 3 days after FAL surgery were mixed with 2×10^5 CD45.1 competitor BM cells, and the mixture was injected intravenously into 6 to 8-week-old CD45.1 mice irradiated with a total dose of 10 Gy. To measure reconstitution, PB was collected at the indicated time points after transplantation, and CD45.2$^+$ cells were measured as described before.[13]

Cytokine Analyses

Cytokine concentrations in plasma and BM extracellular fluid were determined using the LEGENDplex Multi-Analyte Flow Assay Kit (BioLegend, San Diego, CA), a bead-based immunoassay that quantifies multiple soluble analytes in biological samples simultaneously by flow cytometry. Briefly, a custom mouse cytokine and chemokine panel was employed to measure the concentrations of the designated cytokines/chemokines. For measurements with the LEGENDplex Multi-Analyte Flow Assay Kit, FACSVerse (BD Biosciences, San Jose CA) was employed, and data were evaluated with the LEGENDplex Data Analysis software. Specific to TGF-β1, measurements were further analyzed using the mouse TGF-β1 ELISA kit (LEGEND MAXTM free active TGF-β1 ELISA Kit No. 437707, BioLegend). The

assays were performed in 96-well plates, following the manufacturer's instructions and were recorded using a microplate photometer (Fisherbrand, Pittsburgh, PA).

TGF-β1 Luciferase Assay

We used Poly J (SignaGen Laboratories) to transiently transfect 293T cells with a TGF-β1-responsive luciferase reporter construct[8] and a Renilla luciferase (pRL-SV40; Promega) vector. Twenty-four hours after transfection, cells were serum starved in 1% FBS Opti-MEM and then incubated for 16 hours with bone marrow supernatant (harvested as BM extracellular fluid in PBS) from *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice. PBS or 5 ng/mL TGF-β1 (BioLegend) were used as negative and positive controls, respectively. Experiments were performed in triplicate. We used the Dual Reporter Assay (Promega) to quantify luciferase reporter activity and calculated relative luciferase activity as the ratio of luciferase units to Renilla units, as described before.[35]

Colony Assays

Mouse BM cells were diluted to the indicated concentration in IMDM with 2% FBS and were then seeded into methylcellulose medium M3434 (2×10^4/ml; STEMCELL Technologies, Cambridge, MA) for colony formation analysis, as previously described.[36]

Western Blotting

Cells were lysed in Laemmli sample buffer (Sigma-Aldrich, St. Louis, MO) supplemented with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Samples were separated on SDS–PAGE gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) for protein detection as described. The antibodies used have been listed in Major Resources Table in the Supplemental Material.

Quantitative RT-PCR

RT-PCR was performed on 5 ng total RNA with gene-specific primers (as shown below) and a QIAGEN One Step RT-PCR kit (210210; Qiagen, Germantown, MD) according to the manufacturer's protocol. β-actin was used as the reference gene to normalize the relative expression for quantitative RT-PCR analysis. RT-qPCR primers used in this study were as follows: *shp-1*: forward (5′- ACT ACC AGA GAG GTG GAG AAA-3′) and reverse (5′-GGT CAC AGA GTA GAG ACC ATA GAT-3′), *tgfb1*: forward (5′-AAG ACC TGG GTT GGA AGT G-3′) and reverse (5′-CTT CTC CGT TTC TCT GTC ACC CTA T-3′), β-actin: forward (5′-GCT CTT TTC CAG CCT TCC TT-3′), and reverse (5′- CTT CTG CAT CCT GTC AGC AA-3′).

Chromatin Immunoprecipitation Assay

A ChIP assay was performed as described previously.[37] In brief, formaldehyde-crosslinked chromatin was prepared from PB Lin$^-$ cells and immunoprecipitations were performed using the Chip Assay Kit according to the manufacturer's recommended protocol (Upstate Biotechnology). DNA was recovered with the PCR Purification Kit (QIAGEN) and then subjected to real-time PCR analysis. Pairs of real-time PCR primers were used for amplification of promoter segments of the

mouse *tgfb1* gene (forward: 5'-GCGGATCCTCCAGACAGCCA-3' and Reverse 5'-ACTCCGAGTGCGGCTCAGAG-3').[38]

TGF-β1 Blocking Assay

Shp-1$^{+/+}$ and *Shp-1*$^{-/-}$ mice were given one of the following antibodies, intravenously for 4 days, after the hind limb ischemia model was established as described[39,40]: anti-TGF-β1 (19D8, Biolegend, 150 μg/shot) or IgG (RTK2758, Biolegend, 150 μg/shot). All mice participated in treadmill test, blood flow and blood vessel measurements on day 21 to assess for recovery.

Quantification and Statistical Analysis

Data are expressed as mean±SEM. Median and IQR are given in Appendix A. Statistical analyses were performed using GraphPad Prism Version 9.0 (Graph Pad Prism Software, Inc, San Diego, CA) and SAS version 9.4. Specific details on how many independent biological samples or mice were included in an experiment or how many times experiments were repeated independently are given in the corresponding figure legends. Two-group comparisons were performed using Mann-Whitney Rank-sum test, and multigroup comparisons were performed using the Kruskal-Wallis 1-way ANOVA on ranks test with Dunn post hoc test. $P<0.05$ was considered statistically significant. Test for Median score was performed to compare median outcomes between 2 groups overall or at fixed time point (see Appendix A). Boxplots were used for visualization of the quantiles (see Appendix B). To compare outcomes between 2 groups, for the repeated data (repeated measure on same subjects at different days), Friedman's 2-way nonparametric ANOVA was performed by computing the ranks for the outcome variables by "Day" variable and then using the ANOVA procedure on ranks (see Appendix C). The R-squared measure was used to assess the goodness of model fit.

RESULTS

Loss of *Shp-1* in HSCs Impairs Recovery of Blood Flow and Function After Limb Ischemia

To examine the role of SHP-1 in HSPC response to ischemic injuries, we generated tamoxifen-inducible deletion of *shp-1* in HSCs (*Scl-cre*$^{+}$*ER; Shp1*$^{flox/flox}$ mice, *Shp-1*$^{-/-}$ knockout [KO] mice)[31,41] and utilized a classic HLI model. We chose to create the ischemia model in C57BL/6J mice over other strains, such as nude mice, to allow fast recovery of circulation in the ischemic leg and to prevent possible critical limb ischemia, fluctuations in blood pressure and heart rate, and to prevent ongoing immune complications more common in strains other than C57BL/6J. *Shp-1*$^{-/-}$ mice and control *Scl-cre*$^{-}$*ER; Shp1*$^{flox/flox}$(*Shp-1*$^{+/+}$) mice were subjected to limb ischemia by FAL of the left hindlimb (Figure 1A). *Shp-1* deficiency in HSC significantly increased the incidence of defective repair compared with wild type (WT, *Shp-1*$^{+/+}$ mice) control (*P* corresponding to Fisher exact test are 0.0014, 0.0256 for limb salvage, and toe necrosis, respectively). *Shp-1*$^{-/-}$ HLI mice underwent limb loss (12.5%, 1 of 8) or toe necrosis (62.5%, 5 of 8) with only 1 case of limb salvage. On the other hand, all the *Shp-1*$^{+/+}$ HLI mice exhibited limb salvage (Figure 1B). The ratio of blood flow (left ischemic limb blood flow/right normal limb blood flow) and treadmill running time were measured at different time points to evaluate recovery

of blood flow and muscle function in mice after acute HLI. Post-FAL, there was gradual recovery of blood flow in *Shp-1*$^{+/+}$ control mice, with 25.3%, 32.5%, and 71.5% of blood flow recovered on day 3, day 7, and day 21 after ischemia, respectively. The recovery of blood flow correlated with functional recovery of the ischemic limb as determined by an increase in treadmill running time from 56±11 minutes at day 7 to 216±35 at day 21. In contrast, following an initial recovery of blood flow on day 3 (17.6%), blood flow in *Shp-1*$^{-/-}$ mice did not recover further. In fact, it dropped lower at days 7 (14.3%), and 21 (9.6%) post-FAL (Figure 1C and 1D), indicating a lack of recovery. Consistent with a lack of blood flow recovery, functional recovery of the ischemic limb of *Shp-1*$^{-/-}$ mice was absent (Figure 1E). Visual assessment of capillary density in limb tissue revealed that on day 3, both *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice possessed an equal number of capillaries. However, further increase in capillary density was mitigated in *Shp-1*$^{-/-}$ mice, while the *Shp-1*$^{+/+}$ control mice exhibited continuous increase through Day 21. Upon quantitation, capillary density levels in *Shp-1*$^{+/+}$ mice increased after FAL (by 2.4-fold at day 21 versus day 3) in *Shp-1*$^{+/+}$ mice, whereas they decreased drastically in *Shp-1*$^{-/-}$ mice (0.4-fold less at day 21 versus day 3; Figure 1F and 1G). In addition, both the density and vessel diameter size of arterioles in the ischemic area of *Shp-1*$^{-/-}$ mice significantly decreased on day 21 compared with day 3 (*P* were *P*=0.0122 and 0.0027 corresponding to MWU test and Median test, respectively), while *Shp-1*$^{+/+}$ mice exhibited an increase, which is consistent with the capillary density analysis above (Figure 1H through 1J). Taken together, these results indicate that *Shp-1* deficiency in HSCs impairs the angiogenic response to ischemic injury.

SHP-1 Deficiency Is Associated With Excessive and Irreversible Activation of HSPCs in the BM of the HLI Model

To investigate how SHP-1 deletion affects HSC after ischemic injury, we examined the phenotype of HSPCs in the BM of *Shp-1*$^{-/-}$ and compared with *Shp-1*$^{+/+}$ control mice. Ischemic stress leads to the activation of quiescent HSC in *Shp-1*$^{+/+}$ control mice, followed by the sequential expansion of HSPCs from day 3 to day 7, which we consider to be the critical period of HSC regeneration. We observed small but significant increases in the frequency and absolute number of long-term HSCs (LT-HSCs; CD34^{-}FLK2^{-}Lin^{-}Sca1^{+}c-KIT^{+} (LSK); 41%- and 26%-increase at day 3, *P* were <0.001 for both MWU and Median tests), short-term HSCs (ST-HSCs; CD34^{+}FLK2^{-} LSK; 38%- and 21%-increase at day 3, *P* were <0.001 for both MWU and Median tests), and multipotent progenitor cells (MPPs; CD34^{+}FLK2^{+} LSK; 27%- and 33%-increase at day 3; 35%- and 42%-increase at day 7, *P* were <0.001 for both MWU and Median tests) at different time points (Figure 2A through 2D). Twenty-one days after FAL, these changes reverted to normal levels in *Shp-1*$^{+/+}$ control mice. In contrast, *Shp-1*$^{-/-}$ mice exhibited a larger increase in HSC expansion under ischemic stress 3 days after FAL, the frequency, and the numbers of LT-HSC (78%- and 82%-increase), ST-HSC (46%- and 48%-increase), and MPP (56%- and 54%-increase) intensely increased (Figure 2A through 2D). At 21 days after FAL, the increase in total HSPC number remained persistent in *Shp-1*$^{-/-}$ mice compared with *Shp-1*$^{+/+}$ control mice, which is consistent with the changes of downstream progenitors of different lineages, including granulocyte-macrophage progenitor, common myeloid progenitor, megakaryocyte-erythroid progenitor, and common lymphoid progenitor (Figure 2E). Moreover, further downstream lineage changes, such as the dynamic shift in macrophage polarization toward

M1 in *Shp-1*$^{-/-}$ mice at day 21 (Figure S1A and S1B), corroborates with the opinion that macrophage plasticity plays an important role in coordinating muscle repair.[42] To identify whether SHP-1 deficiency persistently increased HSC activation during ischemic stress, we analyzed the cell cycle profile of HSCs at different time points after FAL. Consistent with increases in the number of HSPCs, we observed small but significant changes in the cell cycle phases of HSC in *Shp-1*$^{+/+}$ control mice (G0 phase 10.1% decrease, G1 phase 13.0% increase, and S/G2/M phase 21.2% increase) on day 3 after FAL, while HSC activation in *Shp-1*$^{-/-}$ mice was greater in magnitude and persistently elevated at day 3 (G0 phase 18.6% decrease, G1 phase 18.3% increase, and S/G2/M phase 66.7% increase) and beyond, suggesting that SHP-1 controls the degree and duration of HSC activation in response to ischemic stress (Figure 2F through 2H; Figure S2). In summary, HSCs in the BM undergo a SHP-1-dependent transition from activation to quiescence in response to ischemic stress.

HSC Activation in Response to Ischemia Stress Is Controlled by SHP-1 In Vivo

To assess the direct ischemia healing effect of SHP-1-deficient HSCs, we first performed a competitive repopulation assay by using *Shp-1*$^{+/+}$ or *Shp-1*$^{-/-}$ HSC and then evaluated the blood flow recovery in these experimental mice (Figure 3A). We transplanted 500 LT-HSCs from the BM of *Shp-1*$^{+/+}$ or *Shp-1*$^{-/-}$ mice 3 days after FAL, together with competitor cells, into lethally irradiated CD45.1 recipient mice. Consistent with the in vitro finding that ischemic stress enhances HSC activation, BM cells from both *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice showed a high level of donor cell reconstitution at an earlier time point (54.6% versus 60.4%; P=0.083; Figure 3B). However, the engraftment of *Shp-1*$^{+/+}$ HSCs decreased after 4 weeks (48.2% at 8 weeks, 45.4% at 12 weeks, 43.7% at 16 weeks, and 44.3% at 24 weeks), while the engraftment of *Shp-1*$^{-/-}$ HSCs continued to maintain at a high level (61.2% at 8 week, 59.9% at 12 weeks, 63.4% at 16 weeks, and 61.9% at 24 weeks). We performed FAL surgery on these hematopoietically reconstituted mice 8 months following HSC grafting. Consistent with the findings in Figure 1, there was increase in toe necrosis (66.7%, 4 of 6) in SHP-1 deficient HSCs reconstituted mice, when compared with WT HSCs reconstituted mice (Figure 3C). Furthermore, both the blood flow ratio (Figure 3D and 3E) and treadmill running time (Figure 3F) measured at 21 days after FAL showed significant reductions in SHP-1-deficient HSCs reconstituted mice. Consistent with the functional measurements, the angiogenic response to ischemic injury evaluated by the density of both capillaries (Figure 3G) and arterioles (Figure 3H and 3I) decreased greatly in the ischemic area of SHP-1-deficient HSCs reconstituted mice compared with that of WT HSCs reconstituted mice. Overall, these results directly support the premise that SHP-1 modulates the level of HSC activation in vascular repair and functional recovery in response to ischemic stress.

TGF-β1 Regulation of HSC Activation in Ischemic Stress Is Impaired by SHP-1 Deficiency

TGF-β1 is the dominant signal in the BM that maintains HSC quiescence by suppressing HSC proliferation.[8,43] We previously demonstrated that SHP-1 interacts with TβR1 and is required for TGF-β1 signaling in HSC for maintenance of HSC quiescence.[13] However, it is not known if the TGF-β1 signal responds to ischemic stress and whether SHP-1 modulates this response. First, we analyzed the alterations in concentration of several cytokines known to be involved in the regulation of HSC upon stress, including TGF-β1, CXCL12, G-CSF, GM-CSF in BM extracellular fluid at different time points after surgery.[19-22] Ischemic

stress led to a sharp decrease in free-active TGF-β1 in the BM of both *Shp-1*$^{+/+}$ mice and *Shp-1*$^{-/-}$ mice. In *Shp-1*$^{+/+}$ mice, the concentration of TGF-β1 in the BM started to return to baseline by the seventh day after FAL and was fully back to baseline level after 21 days, while in *Shp-1*$^{-/-}$ mice, the TGF-β1 levels remained low in the BM at both time points (Figure 4A, Figure S3A). When the other cytokines were screened, they were all low at Day 0 and increased to their peak by Day 7 or Day 21. Moreover, this pattern was not significantly different between the *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice (Figure S3B through S3D). Next, we measured the change in biological activity of TGF-β1 by stimulating a luciferase reporter with BM extracellular fluid from *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice that underwent FAL. We observed that TGF-β1 activity mimicked the in vivo cytokine levels at Days 0, 3, 7, and 21 (Figure 4B). The concentration changes and the biological activity of TGF-β1 in the BM correlated well with the activation of HSPCs we observed in Figure 2, which suggests that TGF-β1-SHP-1 signaling is an important intrinsic regulatory mechanism for HSC quiescence and activation in response to ischemia stress. Next, to assess how SHP-1 mediated TGF-β1 signaling regulates HSC activation in ischemia stress, we performed the colony-forming unit assay. TGF-β1 treatment significantly suppressed the colony-forming unit activity of BM HSCs obtained from *Shp-1*$^{+/+}$ mice 3 days after FAL. In contrast, there was no discernible difference in the colony forming ability of corresponding HSCs from *Shp-1*$^{-/-}$ mice after TGF-β1 treatment (Figure 4C). Furthermore, the *Shp-1*$^{+/+}$ BM HSCs exhibited an increased G0 phase fraction (from 52% to 58.3%) and concomitantly a reduced G1 phase fraction (from 42.3% to 39.1%) and a G2/M phase fraction (from 4.3% to 2.5%) after TGF-β1 treatment. In contrast, the cell cycle profile of *Shp-1*$^{-/-}$ BM HSCs did not change significantly after TGF-β1 treatment (Figure 4D). Taken together, these results indicate that SHP-1 is necessary for the shift of the HSC state back from activation to quiescence in the BM during resolution of ischemia stress.

SHP-1-Deficient HSPCs Accumulate in the PB After FAL

HSPCs that are mobilized from the BM to the peripheral circulation by ischemic stress, play a key role in the ischemic angiogenesis process.[9,44,45] To assess the response of HSPCs to ischemic stress from FAL, we measured the circulating HSPCs in the PB of *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice. In *Shp-1*$^{+/+}$ mice, the number of HSPCs (LSK cells) in the PB rapidly increased by day 3, remained high at day 7 (30.7- and 19.7-fold, respectively) and then reverted toward baseline numbers at day 21 (2.8-fold). In contrast, the increase of HSPCs in the PB of *Shp-1*$^{-/-}$ mice was similarly rapid but sustained at high levels (30.1-, 50.3-, and 68.9-fold at day 3, 7, and 21, respectively; Figure 5A and 5B), which parallels the accumulation of SHP-1-deficient HSPCs in the BM after FAL (Figure 2). Consistent with *Shp-1* deletion only in HSPCs (Figure 5C) and not other hematopoietic cells in PB, the assessment of cell frequency of other hematopoietic cells in the PB, such as dendritic cells, natural killer cells, T cells, B cells, monocytes, and megakaryocytes/platelets, did not show significant differences between *Shp-1*$^{+/+}$ mice and *Shp-1*$^{-/-}$ mice upon ischemic stress (Figure S4A through S4G), although they were differentially regulated in response to stress. Furthermore, cytokines involved in HSPC trafficking, such as CXCL12, G-CSF, and GM-CSF, reacted similarly to the ischemic stress in the BM and PB of *Shp-1*$^{+/+}$ mice and *Shp-1*$^{-/-}$ mice (Figure S5A through S5C). Together, these results suggest that overactivation

of HSPCs in the BM in response to ischemic stress is likely responsible for the accumulation of *Shp-1*$^{-/-}$ HSPCs in the circulation.

SHP-1-Smad2 Signaling Suppresses Autoinduction of TGF-β1 in HSPCs in the Circulation in Response to Ischemic Stress

SHP-1 is considered as a negative regulator of inflammation and fibrosis that functions via inhibiting the production of proinflammatory cytokines, including TGF-β1.[46-49] To explore how accumulated *Shp-1*$^{-/-}$ PB HSPCs affect angiogenesis in ischemic limb, we first measured the free/active TGF-β1 in PB at different time points after surgery (Figure 6A; Figure S6A). Surprisingly, the change in TGF-β1 levels in PB, is polar opposite of the trend in BM after FAL. TGF-β1 levels in the PB of both *Shp-1*$^{-/-}$ and *Shp-1*$^{+/+}$ mice increased 3 days after FAL, but they remained high in *Shp-1*$^{-/-}$ mice even at 21 days after surgery. In comparison, the TGF-β1 levels returned to baseline in *Shp-1*$^{+/+}$ mice. To determine the source of the TGF-β1 level shift, we measured the LAP (latency-associate peptide, latent TGF-β1) in hematopoietic cells, in circulation at different time points after FAL, by flow cytometry analysis. Interestingly, LAP levels in HSPCs changed in the same direction that HSPC concentrations did, in *Shp-1*$^{+/+}$ mice and *Shp-1*$^{-/-}$ mice after ischemic stress, that is, LAP increased dramatically in HSPCs after ischemic stress (Day 3) and returned to baseline levels in *Shp-1*$^{+/+}$ mice but remained high in *Shp-1*$^{-/-}$ mice (Figure 6B). To test whether LAP level change in HSPCs is dependent on SHP-1, we measured the LAP levels in other lineage cells in both *Shp-1*$^{-/-}$ and *Shp-1*$^{+/+}$ mice at 21 days after FAL. Indeed, there was no significant difference in LAP levels of natural killer cells, dendritic cells, and MKs/platelets, which are known to secrete TGF-β1 (Figure S6B). Furthermore, we isolated PB HSPCs from *Shp-1*$^{-/-}$ and *Shp-1*$^{+/+}$ mice at 21 days after FAL and cultured them for 6 hours and measured the concentration of TGF-β1 secreted into the culture medium by ELISA. We found that HSPCs of *Shp-1*$^{-/-}$ mice produce 43.2× more active TGF-β1 than that of *Shp-1*$^{+/+}$ mice (Figure 6C). Together, these findings suggest that HSPCs are the major sources of TGF-β1 over-production in the PB of *Shp-1*$^{-/-}$ mice after FAL.

Next, we explored the mechanisms for dysregulated TGF-β1 secretion by SHP-1-deficient HSPCs that accumulated in the PB after FAL. TGF-β1 transcription is induced by ischemia stress in both *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ HSPCs. While the transcripts declined to normal levels in *Shp-1*$^{+/+}$ mice, they remained at very high levels in *Shp-1*$^{-/-}$ HSPCs at day 21 (Figure 6D), which suggests that autocrine induction (autoinduction) of TGF-β1 might underlie activation of HSPCs in PB in the absence of SHP-1.[38] To investigate this possibility, we added TGF-β1 (5 ng/ml) to HSPCs and discovered that exogenous TGF-β1 induced TGF-β1 mRNA level in both *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ HSPCs. Interestingly, *Shp-1*$^{-/-}$ HSPCs are more sensitive to this autoinduction (Figure 6E), which is consistent with endogenous TGF-β1 upregulation in *Shp-1*$^{-/-}$ HSPCs and with the reports that SHP-1 negatively regulates TGF-β1 transcription level.[13,18] Because Smad2 is a well-known transcriptional factor of TGF-β1 signaling in various cells[50,51] and Smad2 negatively regulates TGF-β signaling in inflammatory cells,[38] we further investigated Smad2 activity after FAL. We observed that ischemia stress can suppress Smad2 activity in Lin^{-} cells in PB (Figure 6F and 6G; Figure S7). Importantly, the activity of SHP-1 and Smad2 changed synchronously in cells from *Shp-1*$^{+/+}$ mice (Figure 6F), while SHP-1 deficiency led to persistently low levels of Smad2

activity, even at 21 days after FAL (Figure 6G). Notably, the total protein level of Smad2 remained unchanged. Smad2 has been reported to be bound to the Smad-binding element (SBE)-rich region around the transcription initiation site of the *Tgfb1* gene.[38] To investigate the mechanism by which Smad2 activation regulates transcription of *Tgfb1* in ischemic stress (Figure 6H), we quantitated the interaction between Smad2 and the promoter of *Tgfb1* via a chromatin immunoprecipitation (ChIP) assay. Consistent with the notion of Smad2 inactivation and *Tgfb1* transcription induction, Smad2 binding was quickly reduced after ischemic stress, and returned to the baseline levels 7 days later in *Shp-1*$^{+/+}$ HSPCs, while this binding was similarly reduced but remained at the low levels after FAL in *Shp-1*$^{-/-}$ HSPCs (Figure 6I). Thus, we conclude that SHP-1-Smad2 signaling negatively regulates TGF-β1 production in HSPCs accumulated in PB after ischemic stress, and this negative regulation is absent in SHP-1-deficient HSPCs leading to persistence of TGF-β1 at high levels.

Restoration of TGF-β1 Oscillations and Signaling in PB Leads to Repair of the Ischemic Limb

To confirm whether the sustained increase and lack of oscillation in TGF-β1 levels in the PB of *Shp-1*$^{-/-}$ mice critically affects angiogenesis after ischemic stress, we treated mice with a TGF-β1 blocking antibody (19D8) and control IgG between day 3 and day 7 after FAL and checked for angiogenesis on day 21 (Figure 7A).[40] TGF-β1 antibody treatment of *Shp-1*$^{-/-}$ HLI mice, reduced limb loss and limb necrosis, and increased the ratio of limb salvage (Figure 7B). Moreover, in vivo blockade of TGF-β1 in *Shp-1*$^{-/-}$ HLI mice, led to blood flow recovery (Figure 7C and 7D), significant functional restoration of the ischemic limb (Figure 7E), as well as increased collateral vessel density and vessel diameter in the ischemic area (Figure 7F through 7H). Thus, our in vivo data demonstrates that SHP-1 controls TGF-β1 signaling enforced regulation of angiogenesis under ischemic stress.

DISCUSSION

Ischemic injury in cardiovascular, musculoskeletal, and dermatological systems leads to induction of a stress response, whereby several different cell types and signaling events work in coordination to repair injured tissue.[52-54] Depending on the context, the repair may lead to restoration of normal structure and function or lead to defective repair/fibrosis with loss of normal structure and function. Endogenous HSPCs are understood to contribute majorly to the repair process both via differentiation into cell types important in repair and the secretion of cytokines/chemokines that aid in repair.[55,56] Herein, we demonstrate the dependence of endogenous HSPCs on SHP-1 for normal repair in the HLI model. Furthermore, it seems that SHP-1 regulation of TGF-β1 oscillations is important in the postischemic period as loss of this pattern in the PB and BM likely led to derangement of repair. Blockade of TGF-β1 on Day 3 after ischemia and restoration of normal TGF-β1 oscillation assisted in the recovery of limb function, highlighting the importance of SHP-1 control of TGF-β1 oscillation as central to normal repair.

There is a keen interest in the use of stem cells in post-ischemic regeneration and repair. Exogenous stem cell therapy with endothelial progenitor cells, mesenchymal stem cells, and

BM-derived myeloid cells has been tested for their repair potential in various conditions of ischemia stress.[54,57,58] However, these therapies have not lived up to their promise due to various factors, such as graft versus host disease, need for immunosuppression and resultant toxicity from antirejection medications, transformation of stem cells to a proinflammatory phenotype, and unsuccessful/inadequate homing and engraftment. In contrast, endogenous stem cells overcome many of these issues, although in some contexts, such as diabetes or other vascular disease, endogenous stem cell–mediated repair is not adequate. Therefore, there is a need to understand the mechanisms by which stem cell–mediated repair can be optimized.[1] HSCs are capable of striking a balance between activation and quiescence in response to a broad variety of stresses.[7,59] Our findings indicate that BM HSC are capable of a rapid response to ischemic stress and return to a stable quiescent state in 7 to 21 days post-ischemia. This “normal response” was disrupted in HSC-specific SHP-1-deficient mice, implicating SHP-1 as an important regulator of both HSC activation and quiescence. We previously demonstrated that SHP-1 interacts with TβRI and coordinates TGF-β signaling to regulate HSC quiescence in the BM during homeostasis,[13] and in support of these findings, we found that ischemic injury could overactivate and persistently activate BM HSC in the presence of SHP-1 deficiency, which slowed down angiogenesis, blood flow, and recovery of limb function. In addition to its role in TGF-β1 regulation, SHP-1 is believed to function as a negative signal regulator associated with the phosphorylated ITIM of cell surface receptors in other cells, such as myeloid,[60] T cells,[61,62] and B cells,[63] which suppresses activation of these immune cells and dependent immune diseases.[62] However, further studies are needed to understand the long-term effects of SHP-1 deficiency in differentiated HSPCs. In the short term, our experiment was designed to establish ischemia rapidly after the introduction of tamoxifen, to confine the effect of SHP-1 to HSPCs while minimizing the influence on other hematopoietic cells due to differentiation.

In comparison to our study in nondiabetic conditions, studies in diabetic conditions showed that specific deletion of SHP-1 in smooth muscle cells partially restores blood flow reperfusion in the diabetic ischemic limb[11,64] and deletion of angiotensin II type 2 receptor suppresses SHP-1 activation mediated suppression of VEGF. These results seem contrary to our findings, but actually may be in agreement with SHP-1’s role in the suppression of TGF-β1 secretion. Under conditions of diabetes, SHP-1 upregulation in smooth muscle cells may lead to suppression of TGF-β1 secretion (important for initiation of repair), which in turn leads to suppression of VEGF and PDGF secretion, thereby impairing ischemic repair. Therefore, the secretion of TGF-β1 found in different ischemic models and tissue-specific knockout systems likely underscores the context-dependence of SHP-1 function. Other studies using siRNA targeting of *Shp-1* observed that SHP-1 deficiency accelerates angiogenesis via increased VEGF signaling, likely through suppression of SHP-1 in different cells that are not HSCs.[10] Furthermore, SHP-1 may be important in maintaining the quiescence of HSCs in the BM and preventing the differentiation of HSCs peripherally.[65] When SHP-1 is not present, it may lead to activation of HSCs and differentiation into hematopoietic cells and secretion of TGF-β1.[65] In our study, in addition to HSPCs, endothelial cells may have played a minor role in ischemic repair, as SCl-Cre is weakly expressed in endothelial progenitor cells,[31] thereby affecting VEGF/PDGF secretion, factors that are important for ischemic repair.[25]

A number of pro- and anti-inflammatory mediators have been implicated in the pathophysiology of cardiovascular diseases and in ischemia repair.[23] Cytokines and chemokines are known to orchestrate the various steps of repair that either lead to successful repair or failure to heal. HSPCs and other cells involved in repair secrete and respond to these signals (autocrine and paracrine). Prominent among the signals are TGF-β1, CXCL12, G-CSF, and GM-CSF. TGF-β1 is known to be activated in the ischemic zone, and free/active TGF-β1 (C-terminal peptide) is a major player in the maturation of blood vessels in angiogenesis after it is cleaved from LAP.[66] In the BM, we found high levels of TGF-β1 prior to ischemia at baseline, which plummeted to very low levels upon FAL, and these levels recovered gradually from Day 3 to Day 21, which is consistent with its role in maintaining homeostatic quiescence and promoting post-injury regeneration of HSCs.[8,51] We previously demonstrated that inhibition of TGF-β1 signaling significantly promoted HSC proliferation in homeostasis.[13] Consistent with a role in maintaining quiescence, in this study, we discovered that in vitro TGF-β1 treatment of HSCs derived from animals at Day 3 post-ischemia inhibited proliferation and colony formation. Furthermore, the switch between HSC activity and quiescence, regulated by TGF-β1, is SHP-1 dependent in both homeostasis and ischemic stress.

In contrast to the change in BM, the levels of active TGF-β1 in the circulation changed in the opposite direction following ischemia stress in HSPC-SHP-1 replete animals. There was an acute increase of TGF-β1 by Day 3 in the PB, which was followed by a steep decline to baseline by Day 21. This pattern was somewhat unexpected as ischemic tissues are believed to continuously secrete TGF-β1 to induce angiogenesis and repair. In the SHP-1-deficient mice, TGF-β1 levels increased in PB as expected, in response to ischemia. However, TGF-β1 levels never dropped to baseline by Day 21. The persistently high levels and loss of normal oscillations of TGF-β1 in the plasma of SHP-1-deficient mice correlated with high LAP protein and TGF-β1 mRNA expression in HSPCs and increased sensitivity to stimulus by TGF-β1. TGF-β1 has a pro- or antiangiogenic effect on blood vessel formation depending on the context.[25,67] Studies have shown that latent TGF-β1 overexpression in keratinocytes may result in a severe psoriasis-like skin disorder, similar to SHP-1 deficiency.[68,69] Another study found that at Day 1 post-ischemia, wound macrophages secrete more TNF-alpha and IL-6, and less TGF-β1 than at Day 7.[70] Yet another study found that expression of TGF-β1 is persistent in defective repair compared to normal wound healing, where receptor expression decreases during the remodeling phase. Investigators in this study suggest that the persistence of TGF-β receptor expression may trigger a feedback loop, resulting in a fibrotic phenotype.[71] In agreement with these studies, we observed that the ischemic limb of *Shp-1*$^{-/-}$ mice had much greater fibrosis surrounding the surgery area 21 days after FAL, which could be linked to the persistently high level of TGF-β1 in PB (Figure S8). Surprisingly, the number of HSPCs in circulation followed a similar pattern of fluctuation to TGF-β1 levels. We found that HSPCs and not MKs/platelets, natural killer cells, or DCs were mainly responsible for the fluctuation in TGF-β1 levels. We did not measure tissue TGF-β1 levels, which may also contribute to the TGF-β1 fluctuation.[72] When we looked at why TGF-β1 levels remained high, PB-HSPCs had a reduction in p-SHP-1 levels following ischemia, parallel to reduction in p-Smad2 levels, which were never restored to baseline levels. Autocrine induction has been suggested as

another important mechanism by which cells sense and respond to their surroundings.[73] Autoinduction of TGF-β1 is known in tumor cells[74,75] and other hematopoietic cells[38] and is an essential mechanism for TGF-β1 interaction with the environment. Our study supports the contribution of autoinduction to the persistently high TGF-β1 levels that we observed in the PB of SHP-1-deficient mice.

Previously, ischemic stress has been reported to trigger the mobilization of HSPCs from BM into PB,[9] but the role of HSPCs in repair is unclear. Studies have shown that platelet infusion promotes bone marrow–derived cells mobilization into the circulation, bone marrow–derived cell recruitment into growing neovasculature, tumor vascularization, and blood flow restoration in ischemic limbs, whereas platelet depletion inhibits these effects. Thus, platelets are required for bone marrow–derived cell recruitment into ischemia-induced vasculature.[76] Our data indicate that HSPCs play an important role in revascularization and function recovery likely by appropriately secreting TGF-β1, which is regulated by SHP-1. Although several other hematopoietic lineage cells including MKs/platelets,[77] dendritic cells,[38,78] and natural killer cells[79] produce high amounts of TGF-β1 in response to ischemic stress, we posit that HSPCs-derived TGF-β1 in PB is responsible for the fluctuation of TGF-β1 levels in our *Shp-1*$^{-/-}$ FAL model due to several lines of evidence we gathered about the response of HSPCs to TGF-β1. Furthermore, SHP-1 deficiency in HSC did not result in differences in the mobilization of cytokines such as, CXCL12, G-CSF, and GM-CSF (Figure S3B and SBC), as they all increased in response to ischemia and stayed high regardless of SHP-1 status. The mechanism for the increased number of HSPCs in circulation after SHP-1 deletion in the ischemic model needs further characterization.

Our study revealed the mechanism for suppression of TGF-β1 production in HSPCs by SHP-1 and Smad2 in the circulation after ischemia stress. We found that in SHP-1 replete conditions, the activity of SHP-1 and Smad2 decreased and was gradually restored after FAL, while SHP-1 deficiency maintained Smad2 activity at a low level, which is consistent with the change in transcriptional level of TGF-β1. SHP-1 has been shown to negatively regulate a variety of cytokines, and its absence lets cytokine signaling proceed unchecked, resulting in aberrant or pathogenic responses.[12,80] Smad transcription factors play a pivotal role in relaying signals from the plasma membrane to the nucleus to activate or repress TGF-β1 signaling in the cardiovascular system.[25] In addition, SHP-1 is a negative regulator of TGF-β1 signaling through both the Smad-dependent and Smad-independent mechanism.[18,81,82] The role of Smad2 protein on *Tgfb1* expression has been reported in stem cells,[83] and DCs,[38] and a Smad2-mediated *Tgfb1* transcriptional suppression region on the *Tgfb1* promoter has been identified. However, the signaling upstream of this regulation is unknown. Indeed, we observed that Smad2 binding at this location is associated with its own activity, which is completely congruent with SHP-1 activation. Because SHP-1 activation causes downstream substrates to dephosphorylate, it is unlikely that Smad2 will be activated directly by SHP-1. More studies are needed to understand how Smad2 is regulated by SHP-1.

Results from our study suggest that wound healing may be potentially improved by increasing SHP-1 levels in the HSPCs/other cells in the early stages of ischemia (3–7 days post-FAL). Conditions where SHP-1 levels are reduced in HSPCs at baseline and

under ischemic stress and remain low through wound healing, such as in SHP-1 mutations, polymorphisms, and medications leading to such a decrease, may be potentially improved by giving SHP-1 enhancers/stimulators such as SC-43, a sorafenib derivative. In contrast, in other conditions such as diabetes, where SHP-1 levels are abnormally high at baseline in tissues, may result in an inappropriate response to ischemic stress by inadequately increasing TGF-β1 in the initial stage of wound healing. These conditions may benefit from SHP-1 suppression in the PB by providing SHP-1 inhibitors, such as sodium stilbogluconate, during the initial phase of ischemic injury.

In summary, SHP-1 regulation of TGF-β1 signaling is different in BM versus PB after FAL. In the BM, TGF-β1 levels fall sharply post-ischemia and recover gradually to baseline, whereas in the PB, TGF-β1 levels rise sharply post-ischemia and return gradually to baseline. SHP-1 deficiency leads to dysregulation of normal TGF-β1 oscillations in response to ischemic stress, that is, TGF-β1 levels do not return to their normal levels both in BM and PB due to lack of suppression from SHP-1 and autoinduction by persistently high TGF-β1 levels. The derangement of TGF-β1 levels is likely responsible for the defective repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

R. Nistala and X. Kang designed the study and wrote the article. C. Wang, R. Nistala, D.-P. Li, Y. Cui, Z. Liu, and X. Kang contributed to the experimental plan and data interpretation. C. Wang and Y. Pan performed animal experiments. C. Wang and M. Cao performed western blotting, immunofluorescence staining, cytokine analyses, Transforming growth factor beta-1 proprotein (TGF-β1) luciferase assay, real-time polymerase chain reaction colony assays, Chip assay. C. Wang and X. Kang performed flow cytometry assays. M. Golzy, C. Wang, and R. Nistala performed statistical analysis. X. Kang conceived, coordinated, and supervised the project.

Sources of Funding

We thank the American Heart Association (19CDA34770036 to X. Kang), the National Cancer Institute (R37CA241603 to X. Kang), the National Institute of Diabetes and Digestive and Kidney Diseases (K08DK115886 to R. Nistala), the Center for Precision Medicine/Department of Medicine (Start-up funds to both X. Kang and R. Nistala) for their support.

Nonstandard Abbreviations and Acronyms

BM	bone marrow
FAL	femoral artery ligation
FGF1	fibroblast growth factor 1
HLI	hind limb ischemia
HSC	hematopoietic stem cell
HSPC	HSC and progenitor cell
LAP	latency-associate peptide

PB	peripheral blood

REFERENCES

1. Al-Sharea A, Lee MKS, Purton LE, Hawkins ED, Murphy AJ. The haematopoietic stem cell niche: a new player in cardiovascular disease? Cardiovasc Res. 2019;1 15:277–291. doi: 10.1093/cvr/cvy308
2. Tanaka R, Masuda H, Kato S, Imagawa K, Kanabuchi K, Nakashioya C, Yoshiba F, Fukui T, Ito R, Kobori M, et al. Autologous G-CSF-mobilized peripheral blood CD34+ cell therapy for diabetic patients with chronic nonhealing ulcer. Cell Transplant. 2014;23:167–179. doi: 10.3727/096368912X658007 [PubMed: 23107450]
3. Lara-Hernandez R, Lozano-Vilardell P, Blanes P, Torreguitart-Mirada N, Galmés A, Besalduch J. Safety and efficacy of therapeutic angiogenesis as a novel treatment in patients with critical limb ischemia. Ann Vasc Surg. 2010;24:287–294. doi: 10.1016/j.avsg.2009.10.012 [PubMed: 20142004]
4. Kwon SM, Lee YK, Yokoyama A, Jung SY, Masuda H, Kawamoto A, Lee YM, Asahara T. Differential activity of bone marrow hematopoietic stem cell subpopulations for EPC development and ischemic neovascularization. J Mol Cell Cardiol. 2011;51:308–317. doi: 10.1016/j.yjmcc.2011.04.007 [PubMed: 21557947]
5. Li DW, Liu ZQ, Wei J, Liu Y, Hu LS. Contribution of endothelial progenitor cells to neovascularization (Review). Int J Mol Med. 2012;30:1000–1006. doi: 10.3892/ijmm.2012.1108 [PubMed: 22922670]
6. Zhang CC, Sadek HA. Hypoxia and metabolic properties of hematopoietic stem cells. Antioxid Redox Signal. 2014;20:1891–1901. doi: 10.1089/ars.2012.5019 [PubMed: 23621582]
7. Raaijmakers MH, Scadden DT. Divided within: heterogeneity within adult stem cell pools. Cell. 2008;135:1006–1008. doi: 10.1016/j.cell.2008.11.034 [PubMed: 19070570]
8. Zhao M, Perry JM, Marshall H, Venkatraman A, Qian P, He XC, Ahamed J, Li L. Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. Nat Med. 2014;20:1321–1326. doi: 10.1038/nm.3706 [PubMed: 25326798]
9. Paczkowska E, Larysz B, Rzeuski R, Karbicka A, Jałowiński R, Kornacewicz-Jach Z, Ratajczak MZ, Machaliński B. Human hematopoietic stem/progenitor-enriched CD34(+) cells are mobilized into peripheral blood during stress related to ischemic stroke or acute myocardial infarction. Eur J Haematol. 2005;75:461–467. doi: 10.1111/j.1600-0609.2005.00536.x [PubMed: 16313257]
10. Sugano M, Tsuchida K, Maeda T, Makino N. SiRNA targeting SHP-1 accelerates angiogenesis in a rat model of hindlimb ischemia. Atherosclerosis. 2007;191:33–39. doi: 10.1016/j.atherosclerosis.2006.04.021 [PubMed: 16725144]
11. Paquin-Veillette J, Lizotte F, Robillard S, Béland R, Breton MA, Guay A, Despatis MA, Geraldes P. Deletion of AT2 receptor prevents SHP-1-Induced VEGF inhibition and improves blood flow reperfusion in diabetic ischemic hindlimb. Arterioscler Thromb Vasc Biol. 2017;37:2291–2300. doi: 10.1161/ATVBAHA.117.309977 [PubMed: 29074590]
12. Oh SY Zheng T, Kim YK, Cohn L, Homer RJ, McKenzie AN, Zhu Z. A critical role of SHP-1 in regulation of type 2 inflammation in the lung. Am J Respir Cell Mol Biol. 2009;40:568–574. doi: 10.1165/rcmb.2008-0225OC [PubMed: 18952567]
13. Jiang L, Han X, Wang J, Wang C, Sun X, Xie J, Wu G, Phan H, Liu Z, Zhang C, et al. SHP-1 regulates hematopoietic stem cell quiescence by coordinating TGF-beta signaling. J Exp Med. 2018. doi: 10.1084/jem.20171477 Vol. 215 Issue 5 Pages 1337–134 [PubMed: 29669741]
14. Wakefield LM, Hill CS. Beyond TGFbeta: roles of other TGFbeta superfamily members in cancer. Nat Rev Cancer. 2013;13:328–341. doi: 10.1038/nrc3500 [PubMed: 23612460]
15. Christophi GP, Panos M, Hudson CA, Christophi RL, Gruber RC, Mersich AT, Blystone SD, Jubelt B, Massa PT. Macrophages of multiple sclerosis patients display deficient SHP-1 expression and enhanced inflammatory phenotype. Lab Invest. 2009;89:742–759. doi: 10.1038/labinvest.2009.32 [PubMed: 19398961]
16. Xiao W, Kashiwakura J, Hong H, Yasudo H, Ando T, Maeda-Yamamoto M, Wu D, Kawakami Y, Kawakami T. Phospholipase C-β3 regulates FcεRI-mediated mast cell activation by recruiting

the protein phosphatase SHP-1. Immunity. 2011;34:893–904. doi: 10.1016/j.immuni.2011.04.010 [PubMed: 21683628]

17. Zhou L, Oh SY, Zhou Y, Yuan B, Wu F, Oh MH, Wang Y, Takemoto C, Van Rooijen N, Zheng T, et al. SHP-1 regulation of mast cell function in allergic inflammation and anaphylaxis. PLoS One. 2013;8:e55763. doi: 10.1371/journal.pone.0055763 [PubMed: 23390550]
18. Fan LC, Shiau CW, Tai WT, Hung MH, Chu PY, Hsieh FS, Lin H, Yu HC, Chen KF. SHP-1 is a negative regulator of epithelial-mesenchymal transition in hepatocellular carcinoma. Oncogene. 2015;34:5252–5263. doi: 10.1038/onc.2014.445 [PubMed: 25619838]
19. Kang X, Lu Z, Cui C, Deng M, Fan Y, Dong B, Han X, Xie F, Tyner JW, Coligan JE, et al. The ITIM-containing receptor LAIR1 is essential for acute myeloid leukaemia development. Nat Cell Biol. 2015;17:665–677. doi: 10.1038/ncb3158 [PubMed: 25915125]
20. Ings SJ, Balsa C, Leverett D, Mackinnon S, Linch DC, Watts MJ. Peripheral blood stem cell yield in 400 normal donors mobilised with granulocyte colony-stimulating factor (G-CSF): impact of age, sex, donor weight and type of G-CSF used. Br J Haematol. 2006;134:517–525. doi: 10.1111/j.1365-2141.2006.06223.x [PubMed: 17018030]
21. Brenner C, Galluzzi L, Kepp O, Kroemer G. Decoding cell death signals in liver inflammation. J Hepatol. 2013;59:583–594. doi: 10.1016/j.jhep.2013.03.033 [PubMed: 23567086]
22. Gao X, Zhang D, Xu C, Li H, Caron KM, Frenette PS. Nociceptive nerves regulate haematopoietic stem cell mobilization. Nature. 2021;589:591–596. doi: 10.1038/s41586-020-03057-y [PubMed: 33361809]
23. Borthwick LA, Wynn TA, Fisher AJ. Cytokine mediated tissue fibrosis. Biochim Biophys Acta. 2013;1832:1049–1060. doi: 10.1016/j.bbadis.2012.09.014 [PubMed: 23046809]
24. Kuhlmann MT, Klocke R, Nikol S. Therapeutic angiogenesis for peripheral artery disease: cytokine therapy. Vasa. 2007;36:253–260. doi: 10.1024/0301-1526.36.4.253 [PubMed: 18357917]
25. Goumans MJ, Ten Dijke P. TGF-β Signaling in control of cardiovascular function. Cold Spring Harb Perspect Biol. 2018;10:a022210. doi: 10.1101/cshperspect.a022210 [PubMed: 28348036]
26. Vaidya A, Kale VP. TGF-β signaling and its role in the regulation of hematopoietic stem cells. Systems Synthetic Biol. 2015;9:1–10. doi: 10.1007/s11693-015-9161-2
27. Zawada AM, Rogacev KS, Rotter B, Winter P, Marell RR, Fliser D, Heine GH. SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. Blood. 2011;118:e50–e61. doi: 10.1182/blood-2011-01-326827 [PubMed: 21803849]
28. van Meeteren LA, Goumans MJ, ten Dijke P. TGF-β receptor signaling pathways in angiogenesis; emerging targets for anti-angiogenesis therapy. Curr Pharm Biotechnol. 2011;12:2108–2120. doi: 10.2174/138920111798808338 [PubMed: 21619534]
29. Lotfi S, Patel AS, Mattock K, Egginton S, Smith A, Modarai B. Towards a more relevant hind limb model of muscle ischaemia. Atherosclerosis. 2013;227:1–8. doi: 10.1016/j.atherosclerosis.2012.10.060 [PubMed: 23177969]
30. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell. 2007;131:324–336. doi: 10.1016/j.cell.2007.08.025 [PubMed: 17956733]
31. Gothert JR, Gustin SE, Hall MA, Green AR, Gottgens B, Izon DJ, Begley CG. In vivo fate-tracing studies using the Scl stem cell enhancer: embryonic hematopoietic stem cells significantly contribute to adult hematopoiesis. Blood. 2005;105:2724–2732. doi: 10.1182/blood-2004-08-3037 [PubMed: 15598809]
32. Peng X, Wang J, Lassance-Soares RM, Najafi AH, Sood S, Aghili N, Alderman LO, Panza JA, Faber JE, Wang S, et al. Gender differences affect blood flow recovery in a mouse model of hindlimb ischemia. Am J Physiol Heart Circ Physiol. 2011;300:H2027–H2034. doi: 10.1152/ajpheart.00004.2011 [PubMed: 21398592]
33. Liu L, Cui Y, Li X, Que X, Xiao Y, Yang C, Zhang J, Xie X, Cowan PJ, Tian J, et al. Concomitant overexpression of triple antioxidant enzymes selectively increases circulating endothelial progenitor cells in mice with limb ischaemia. J Cell Mol Med. 2019;23:4019–4029. doi: 10.1111/jcmm.14287 [PubMed: 30973215]

34. Zhu Q, Hao H, Xu H, Fichman Y, Cui Y, Yang C, Wang M, Mittler R, Hill MA, Cowan PJ, et al. Combination of antioxidant enzyme overexpression and N-acetylcysteine treatment enhances the survival of bone marrow mesenchymal stromal cells in ischemic limb in mice with type 2 diabetes. J Am Heart Assoc. 2021;10:e023491. doi: 10.1161/JAHA.121.023491 [PubMed: 34569277]
35. Hao F, Sholy C, Wang C, Cao M, Kang X. The role of T cell immunotherapy in acute myeloid Leukemia. Cells. 2021;10:3376. doi: 10.3390/cells10123376 [PubMed: 34943884]
36. Kang X, Cui C, Wang C, Wu G, Chen H, Lu Z, Chen X, Wang L, Huang J, Geng H, et al. CAMKs support development of acute myeloid leukemia. J Hematol Oncol. 2018;11:30. doi: 10.1186/s13045-018-0574-8 [PubMed: 29482582]
37. Kang X, Qi Y, Zuo Y, Wang Q, Zou Y, Schwartz RJ, Cheng J, Yeh ET. SUMO-specific protease 2 is essential for suppression of polycomb group protein-mediated gene silencing during embryonic development. Mol Cell. 2010;38:191–201. doi: 10.1016/j.molcel.2010.03.005 [PubMed: 20417598]
38. Kashiwagi I, Morita R, Schichita T, Komai K, Saeki K, Matsumoto M, Takeda K, Nomura M, Hayashi A, Kanai T, et al. Smad2 and Smad3 inversely regulate TGF-β autoinduction in clostridium butyricum-activated dendritic cells. Immunity. 2015;43:65–79. doi: 10.1016/j.immuni.2015.06.010 [PubMed: 26141582]
39. de Streel G, Bertrand C, Chalon N, Liénart S, Bricard O, Lecomte S, Devreux J, Gaignage M, De Boeck G, Mariën L, et al. Selective inhibition of TGF-β1 produced by GARP-expressing Tregs overcomes resistance to PD-1/PD-L1 blockade in cancer. Nat Commun. 2020;11:4545. doi: 10.1038/s41467-020-17811-3 [PubMed: 32917858]
40. Mansouri S, Katikaneni DS, Gogoi H, Pipkin M, Machuca TN, Emtiazjoo AM, Jin L. Lung IFNAR1(hi) TNFR2(+) cDC2 promotes lung regulatory T cells induction and maintains lung mucosal tolerance at steady state. Mucosal Immunol. 2020;13:595–608. doi: 10.1038/s41385-020-0254-1 [PubMed: 31959883]
41. Pao LI, Lam KP, Henderson JM, Kutok JL, Alimzhanov M, Nitschke L, Thomas ML, Neel BG, Rajewsky K. B cell-specific deletion of protein-tyrosine phosphatase Shp1 promotes B-1a cell development and causes systemic autoimmunity. Immunity. 2007;27:35–48. doi: 10.1016/j.immuni.2007.04.016 [PubMed: 17600736]
42. Hsieh PL, Rybalko V, Baker AB, Suggs LJ, Farrar RP. Recruitment and therapeutic application of macrophages in skeletal muscles after hind limb ischemia. J Vasc Surg. 2018;67:1908–1920.e1. doi: 10.1016/j.jvs.2017.04.070 [PubMed: 29273298]
43. Yamazaki S, Iwama A, Takayanagi S, Eto K, Ema H, Nakauchi H. TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. Blood. 2009;113:1250–1256. doi: 10.1182/blood-2008-04-146480 [PubMed: 18945958]
44. Machaliński B, Paczkowska E, Koziarska D, Ratajczak MZ. Mobilization of human hematopoietic stem/progenitor-enriched CD34+ cells into peripheral blood during stress related to ischemic stroke. Folia Histochem Cytobiol. 2006;44:97–101. [PubMed: 16805134]
45. Gójska-Grymajło A, Zieliński M, Gąsecki D, Kowalczyk K, Kwarciany M, Seroczyńska B, Nyka WM. CD271+, CXCR7+, CXCR4+, and CD133+ stem/progenitor cells and clinical characteristics of acute ischemic stroke patients. Neuromolecular Med. 2018;20:301–311. doi: 10.1007/s12017-018-8494-x [PubMed: 29744773]
46. Lin L, Jian J, Song CY, Chen F, Ding K, Xie WF, Hu PF. SHP-1 ameliorates nonalcoholic steatohepatitis by inhibiting proinflammatory cytokine production. FEBS Lett. 2020;594:2965–2974. doi: 10.1002/1873-3468.13879 [PubMed: 32619269]
47. Qian H, Deng X, Huang ZW, Wei J, Ding CH, Feng RX, Zeng X, Chen YX, Ding J, Qiu L, et al. An HNF1α-regulated feedback circuit modulates hepatic fibrogenesis via the crosstalk between hepatocytes and hepatic stellate cells. Cell Res. 2015;25:930–945. doi: 10.1038/cr.2015.84 [PubMed: 26169608]
48. Khalil H, Kanisicak O, Prasad V, Correll RN, Fu X, Schips T, Vagnozzi RJ, Liu R, Huynh T, Lee SJ, et al. Fibroblast-specific TGF-beta-Smad2/3 signaling underlies cardiac fibrosis. J Clin Invest. 2017;127:3770–3783. doi: 10.1172/JCI94753 [PubMed: 28891814]
49. Giani JF, Munoz MC, Mayer MA, Veiras LC, Arranz C, Taira CA, Turyn D, Toblli JE, Dominici FP. Angiotensin-(1-7) improves cardiac remodeling and inhibits growth-promoting pathways in

the heart of fructose-fed rats. Am J Physiol Heart Circ Physiol. 2010;298:H1003–H1013. doi: 10.1152/ajpheart.00803.2009 [PubMed: 20061544]

50. Guo W, Dong Z, Guo Y, Kuang G, Yang Z, Shan B. Concordant repression and aberrant methylation of transforming growth factor-beta signaling pathway genes occurs early in gastric cardia adenocarcinoma. Mol Biol Rep. 2012;39:9453–9462. doi: 10.1007/s11033-012-1810-x [PubMed: 22722999]
51. Vaidya A, Kale VP. TGF-beta signaling and its role in the regulation of hematopoietic stem cells. Syst Synthetic Biol. 2015;9:1–10. doi: 10.1007/s11693-015-9161-2
52. Wu JM, Hsueh YC, Ch'ang HJ, Luo CY, Wu LW, Nakauchi H, Hsieh PC. Circulating cells contribute to cardiomyocyte regeneration after injury. Circ Res. 2015;116:633–641. doi: 10.1161/CIRCRESAHA.116.304564 [PubMed: 25398235]
53. Swirski FK, Nahrendorf M. Leukocyte behavior in atherosclerosis, myocardial infarction, and heart failure. Science. 2013;339:161–166. doi: 10.1126/science.1230719 [PubMed: 23307733]
54. Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, Chandraker A, Yuan X, Pu WT, Roberts AB, et al. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med. 2007;13:952–961. doi: 10.1038/nm1613 [PubMed: 17660828]
55. Felfly H, Haddad GG. Hematopoietic stem cells: potential new applications for translational medicine. J Stem Cells. 2014;9:163–197. doi: jsc.2014.9.3.163 [PubMed: 25157450]
56. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature. 2004;428:664–668. doi: 10.1038/nature02446 [PubMed: 15034593]
57. Terry T, Chen Z, Dixon RA, Vanderslice P, Zoldhelyi P, Willerson JT, Liu Q. CD34(+)/M-cadherin(+) bone marrow progenitor cells promote arterio-genesis in ischemic hindlimbs of ApoE(−)/(−) mice. PLoS One. 2011;6:e20673. doi: 10.1371/journal.pone.0020673 [PubMed: 21677770]
58. Teraa M, Sprengers RW, Westerweel PE, Gremmels H, Goumans MJ, Teerlink T, Moll FL, Verhaar MC; JUVENTAS study group. Bone marrow alterations and lower endothelial progenitor cell numbers in critical limb ischemia patients. PLoS One. 2013;8:e55592. doi: 10.1371/journal.pone.0055592 [PubMed: 23383236]
59. Mendelson A, Frenette PS. Hematopoietic stem cell niche maintenance during homeostasis and regeneration. Nat Med. 2014;20:833–846. doi: 10.1038/nm.3647 [PubMed: 25100529]
60. Ganesan LP, Fang H, Marsh CB, Tridandapani S. The protein-tyrosine phosphatase SHP-1 associates with the phosphorylated immunoreceptor tyrosine-based activation motif of Fc gamma RIIa to modulate signaling events in myeloid cells. J Biol Chem. 2003;278:35710–35717. doi: 10.1074/jbc.M305078200 [PubMed: 12832410]
61. Kaneko T, Saito Y, Kotani T, Okazawa H, Iwamura H, Sato-Hashimoto M, Kanazawa Y, Takahashi S, Hiromura K, Kusakari S, et al. Dendritic cell-specific ablation of the protein tyrosine phosphatase Shp1 promotes Th1 cell differentiation and induces autoimmunity. J Immunol. 2012;188:5397–5407. doi: 10.4049/jimmunol.1103210 [PubMed: 22539788]
62. Zhang Q, Raghunath PN, Vonderheid E, Odum N, Wasik MA. Lack of phosphotyrosine phosphatase SHP-1 expression in malignant T-cell lymphoma cells results from methylation of the SHP-1 promoter. Am J Pathol. 2000;157:1137–1146. doi: 10.1016/S0002-9440(10)64629-9 [PubMed: 11021818]
63. Khalil AM, Cambier JC, Shlomchik MJ. B cell receptor signal transduction in the GC is short-circuited by high phosphatase activity. Science. 2012;336:1178–1181. doi: 10.1126/science.1213368 [PubMed: 22555432]
64. Mercier C, Brazeau T, Lamoureux J, Boisvert E, Robillard S, Breton V, Paré M, Guay A, Lizotte F, Despatis MA, et al. Diabetes impaired ischemia-induced PDGF (Platelet-Derived Growth Factor) signaling actions and vessel formation through the activation of scr homology 2-containing phosphatase-1. Arterioscler Thromb Vasc Biol. 2021;41:2469–2482. doi: 10.1161/ATVBAHA.121.316638 [PubMed: 34320834]
65. Xiao W, Hong H, Kawakami Y, Kato Y, Wu D, Yasudo H, Kimura A, Kubagawa H, Bertoli LF, Davis RS, et al. Tumor suppression by phospholipase C-beta3 via SHP-1-mediated

dephosphorylation of Stat5. Cancer Cell. 2009;16:161–171. doi: 10.1016/j.ccr.2009.05.018 [PubMed: 19647226]

66. Kim SY, Senatorov VV Jr, Morrissey CS, Lippmann K, Vazquez O, Milikovsky DZ, Gu F, Parada I, Prince DA, Becker AJ, et al. TGFβ signaling is associated with changes in inflammatory gene expression and perineuronal net degradation around inhibitory neurons following various neurological insults. Sci Rep. 2017;7:7711. doi: 10.1038/s41598-017-07394-3 [PubMed: 28794441]
67. Seghers L, de Vries MR, Pardali E, Hoefer IE, Hierck BP, ten Dijke P, Goumans MJ, Quax PH. Shear induced collateral artery growth modulated by endoglin but not by ALK1. J Cell Mol Med. 2012;16:2440–2450. doi: 10.1111/j.1582-4934.2012.01561.x [PubMed: 22436015]
68. Li AG, Wang D, Feng XH, Wang XJ. Latent TGFbeta1 overexpression in keratinocytes results in a severe psoriasis-like skin disorder. EMBO J. 2004;23:1770–1781. doi: 10.1038/sj.emboj.7600183 [PubMed: 15057277]
69. Wang XJ, Liefer KM, Tsai S, O'Malley BW, Roop DR. Development of gene-switch transgenic mice that inducibly express transforming growth factor beta1 in the epidermis. Proc Natl Acad Sci USA. 1999;96:8483–8488. doi: 10.1073/pnas.96.15.8483 [PubMed: 10411901]
70. Daley JM, Brancato SK, Thomay AA, Reichner JS, Albina JE. The phenotype of murine wound macrophages. J Leukoc Biol. 2010;87:59–67. doi: 10.1189/jlb.0409236 [PubMed: 20052800]
71. Penn JW, Grobbelaar AO, Rolfe KJ. The role of the TGF-β family in wound healing, burns and scarring: a review. Int J Burns Trauma. 2012;2:18–28. [PubMed: 22928164]
72. Pakyari M, Farrokhi A, Maharlooei MK, Ghahary A. Critical role of transforming growth factor beta in different phases of wound healing. Adv Wound Care (New Rochelle). 2013;2:215–224. doi: 10.1089/wound.2012.0406 [PubMed: 24527344]
73. Scheel C, Eaton EN, Li SH, Chaffer CL, Reinhardt F, Kah KJ, Bell G, Guo W, Rubin J, Richardson AL, et al. Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. Cell. 2011;145:926–940. doi: 10.1016/j.cell.2011.04.029 [PubMed: 21663795]
74. Ungefroren H, Christl J, Eiden C, Wellner UF, Lehnert H, Marquardt JU. Autocrine TGFβ1 Opposes Exogenous TGFβ1-Induced Cell Migration and Growth Arrest through Sustainment of a Feed-Forward Loop Involving MEK-ERK Signaling. Cancers (Basel). 2021 Mar 17;13(6):1357. doi: 10.3390/cancers13061357 [PubMed: 33802809]
75. Chen J, Zhu H, Liu Q, Ning D, Zhang Z, Zhang L, Mo J, Du P, Liu X, Song S, et al. DEPTOR induces a partial epithelial-to-mesenchymal transition and metastasis via autocrine TGFβ1 signaling and is associated with poor prognosis in hepatocellular carcinoma. J Exp Clin Cancer Res. 2019;38:273. doi: 10.1186/s13046-019-1220-1 [PubMed: 31228948]
76. Feng W, Madajka M, Kerr BA, Mahabeleshwar GH, Whiteheart SW, Byzova TV. A novel role for platelet secretion in angiogenesis: mediating bone marrow–derived cell mobilization and homing. Blood. 2011;117:3893–3902. doi: 10.1182/blood-2010-08-304808 [PubMed: 21224474]
77. Alpuche J, Quírino L, Sánchez-Vega JT, Yap J, Pérez-Campos E, Cabrera-Fuentes HA. The role of platelets in ischemic conditioning. Cond Med. 2018;1:313–318. [PubMed: 30556056]
78. Nakano R, Tran LM, Geller DA, Macedo C, Metes DM, Thomson AW. Dendritic cell-mediated regulation of liver ischemia-reperfusion injury and liver transplant rejection. Front Immunol. 2021;12:705465. doi: 10.3389/fimmu.2021.705465 [PubMed: 34262574]
79. Viel S, Marçais A, Guimaraes FS, Loftus R, Rabilloud J, Grau M, Degouve S, Djebali S, Sanlaville A, Charrier E, et al. TGF-β inhibits the activation and functions of NK cells by repressing the mTOR pathway. Sci Signaling. 2016;9:ra19. doi: 10.1126/scisignal.aad1884
80. Wu C, Sun M, Liu L, Zhou GW. The function of the protein tyrosine phosphatase SHP-1 in cancer. Gene 2003;306:1–12. doi: 10.1016/s0378-1119(03)00400-1 [PubMed: 12657462]
81. Park IK, Shultz LD, Letterio JJ, Gorham JD. TGF-beta1 inhibits T-bet induction by IFN-gamma in murine CD4+ T cells through the protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1. J Immunol. 2005;175:5666–5674. doi: 10.4049/jimmunol.175.9.5666 [PubMed: 16237056]
82. Jiang M, Zheng C, Shou P, Li N, Cao G, Chen Q, Xu C, Du L, Yang Q, Cao J, et al. SHP1 regulates bone mass by directing mesenchymal stem cell differentiation. Cell Rep. 2016;16:769–780. doi: 10.1016/j.celrep.2016.06.035 [PubMed: 27396328]

83. Liu L, Liu X, Ren X, Tian Y, Chen Z, Xu X, Du Y, Jiang C, Fang Y, Liu Z, et al. Smad2 and Smad3 have differential sensitivity in relaying TGFβ signaling and inversely regulate early lineage specification. Sci Rep. 2016;6:21602. doi: 10.1038/srep21602 [PubMed: 26905010]

Highlights

- In a mouse model that recapitulates human angiogenesis under ischemic stress, Shp-1 deficiency in hematopoietic stem cell and progenitor cells impaired recovery of blood flow and function after limb ischemia.
- SHP-1 regulation of TGF-β1 oscillations is important in the post-ischemic period as loss of this pattern in the BM and PB resulted in repair disarray.
- SHP-1-Smad2 signaling suppressed the autoinduction of TGF-β1 in hematopoietic stem cell and progenitor cells in circulation in response to ischemic stress.

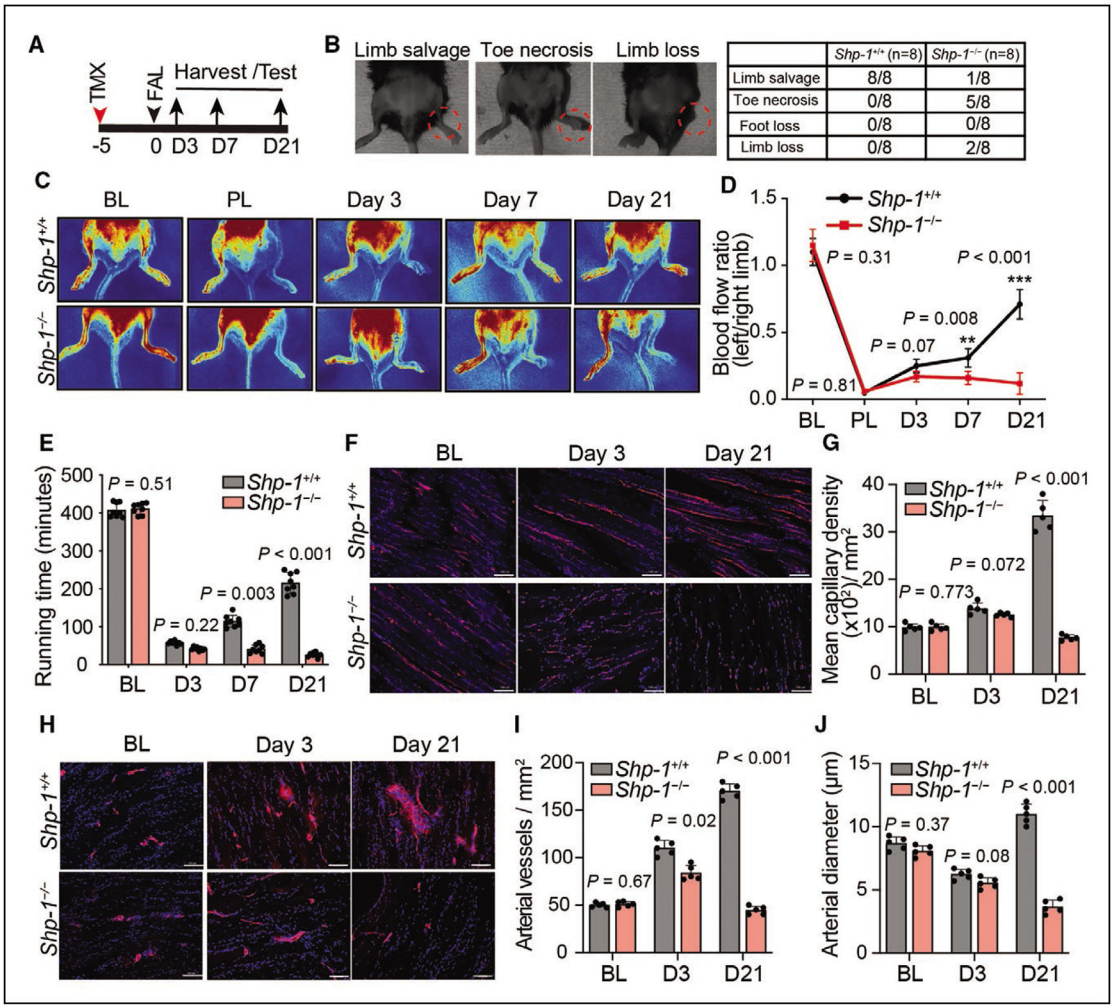


Figure 1. Loss of *Shp-1* in hematopoietic stem cells impairs recovery of blood flow and function after limb ischemia.

A, Schema for tamoxifen treatment, femoral artery ligation (FAL, left limb) surgery, and animal/sample analysis time points. TMX, tamoxifen; D, day (n=8 mice/group). **B**, Representative photographs and ratio quantification of ischemic limbs physiological status 3 weeks after hind limb ischemia. **C**, Representative images of mouse hind limb blood flow measured with laser doppler perfusion imaging (LDPI) before (BL), immediately after ligation (PL) and at day 3, 7, and 21. **D**, Graphical representation of blood flow recovery showing significant reduction in *Shp-1*$^{-/-}$ mice. **E**, The limb function was measured by treadmill test (n=8 mice/group). **F** and **G**, CD31 staining to measure the capillary density on ischemic limb muscle (n=5 mice/group), Scale bar: 100 μM. **H–J**, Immunofluorescent images were taken to measure vascular density and diameter size of arteriole by using anti-mouse smooth muscle α-actin (red) and DAPI (blue) before FAL, at day 3 and day 21, Scale bar: 100 μM (n=5 mice/group). Error bars, SEM.

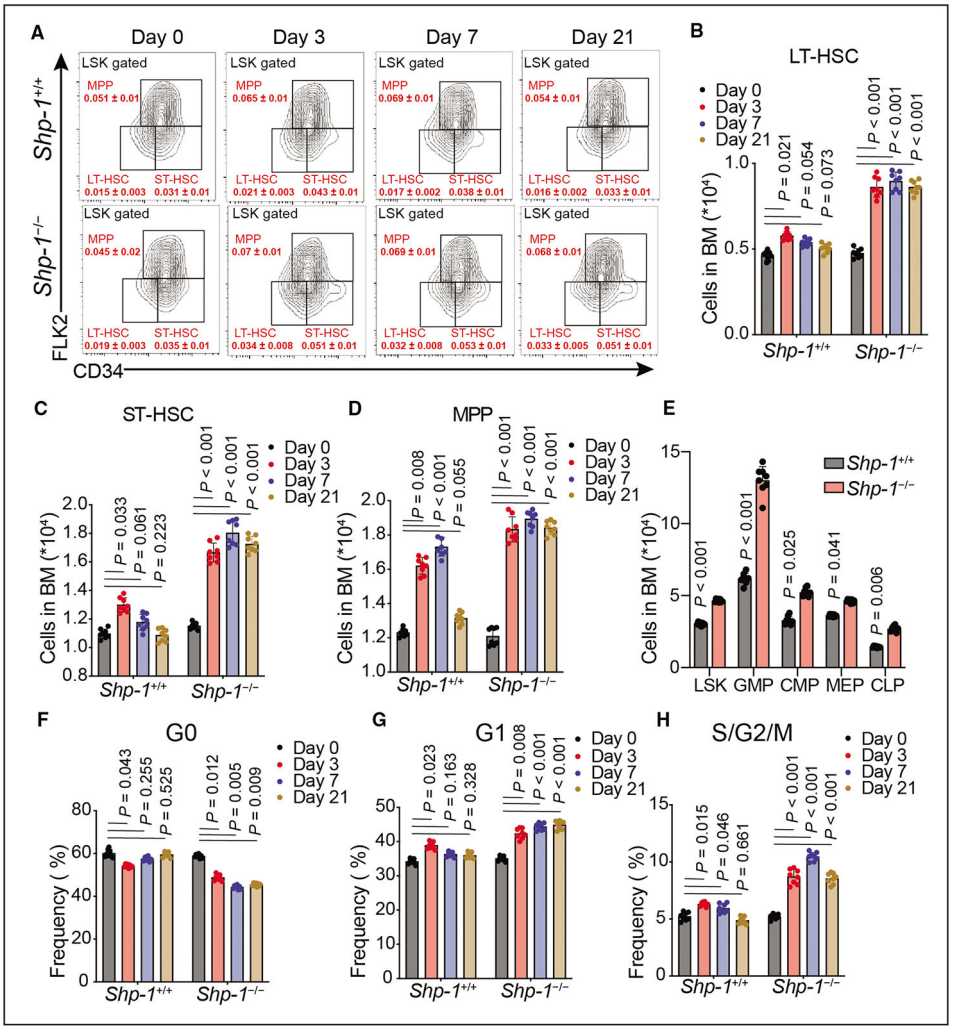


Figure 2. Shp-1 deficiency is associated with excessive and irreversible activation of hematopoietic stem cell (HSC) and progenitor cell (HSPCs) in the bone marrow (BM) of the hind limb ischemia model.

A–E, Flow cytometric analyses to determine the frequency (of total nucleated cells [TNCs]; **A**) and absolute numbers of HSPCs (LT-HSCs, ST-HSCs, and MPPs) in the BM of *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice (**B–D**) at indicated time points and the population of progenitors (granulocyte-macrophage progenitor, common myeloid progenitor, megakaryocyte-erythroid progenitor, common lymphoid progenitor) at day 21 after femoral artery ligation (**E**; n=8 mice/group). **F–H**, Cell cycle analysis of HSC in *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice (n=8 mice per group) at indicated time points by flow cytometry. Error bars, SEM.

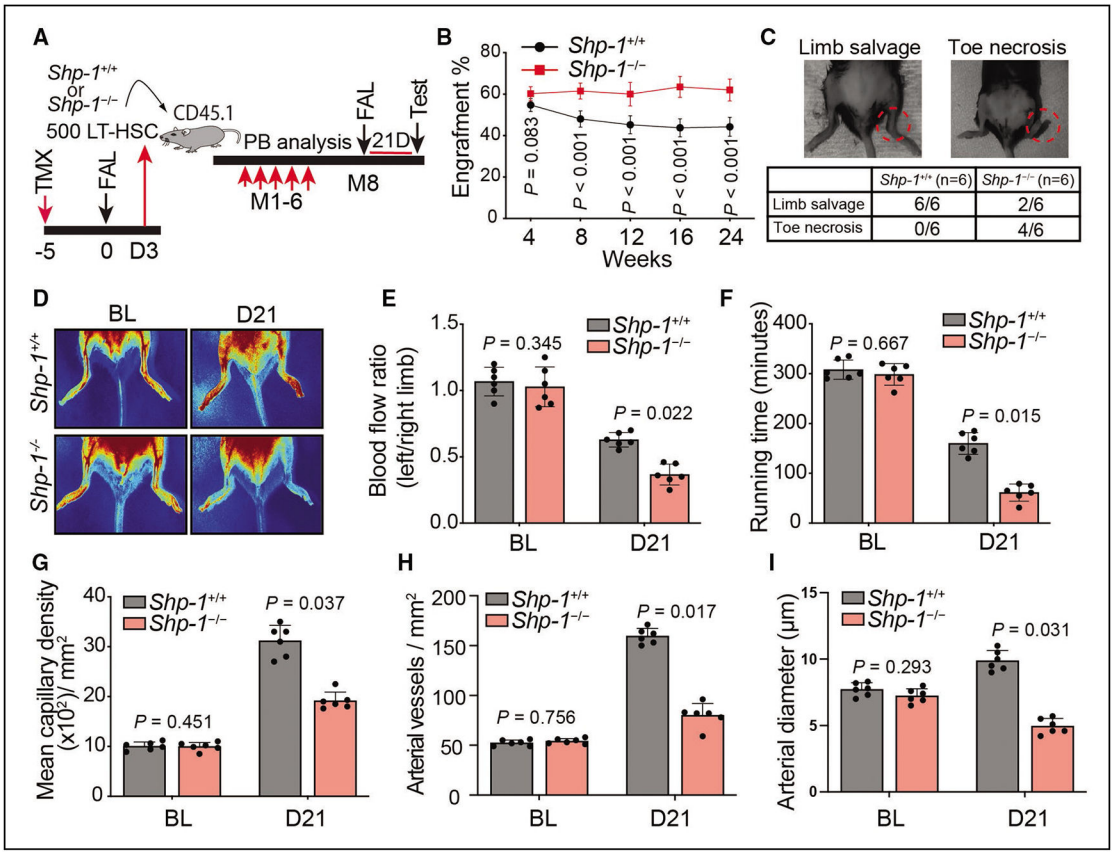


Figure 3. Hematopoietic stem cell (HSC) activation in response to ischemia stress is controlled by SHP-1 in vivo.

A, Schema for competitive reconstitution analysis using 500 HSCs from tamoxifen-treated *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice 3 days after femoral artery ligation (FAL) together with wild-type competitive cells, 8 months after bone marrow transplantation, FAL surgery was performed on the reconstituted receipt mice, and animal/sample analysis at day 21 time point. TMX, tamoxifen; D, day; M, month (n=6 mice/group). **B**, Peripheral blood analysis for total engrafted donor cells at the indicated number of weeks after transplantation (n=6 mice/group). **C**, Representative photographs and ratio quantification of ischemic limbs physiological status 3 weeks after hind limb ischemia. **D** and **E**, Representative images and conclusion of mouse hind limb blood flow measured with laser doppler perfusion imaging (LDPI) before ligation (BL), and 21 days after ligation. **F**, The limb function was measured by treadmill test (n=6 mice/group). **G**, CD31 staining to measure the capillary density on ischemic limb muscle BL and day 21 after ligation (n=6 mice/group). **H** and **I**, Measurement of vascular density and diameter size of arteriole BL and day 21 after ligation (n=6 mice/group).

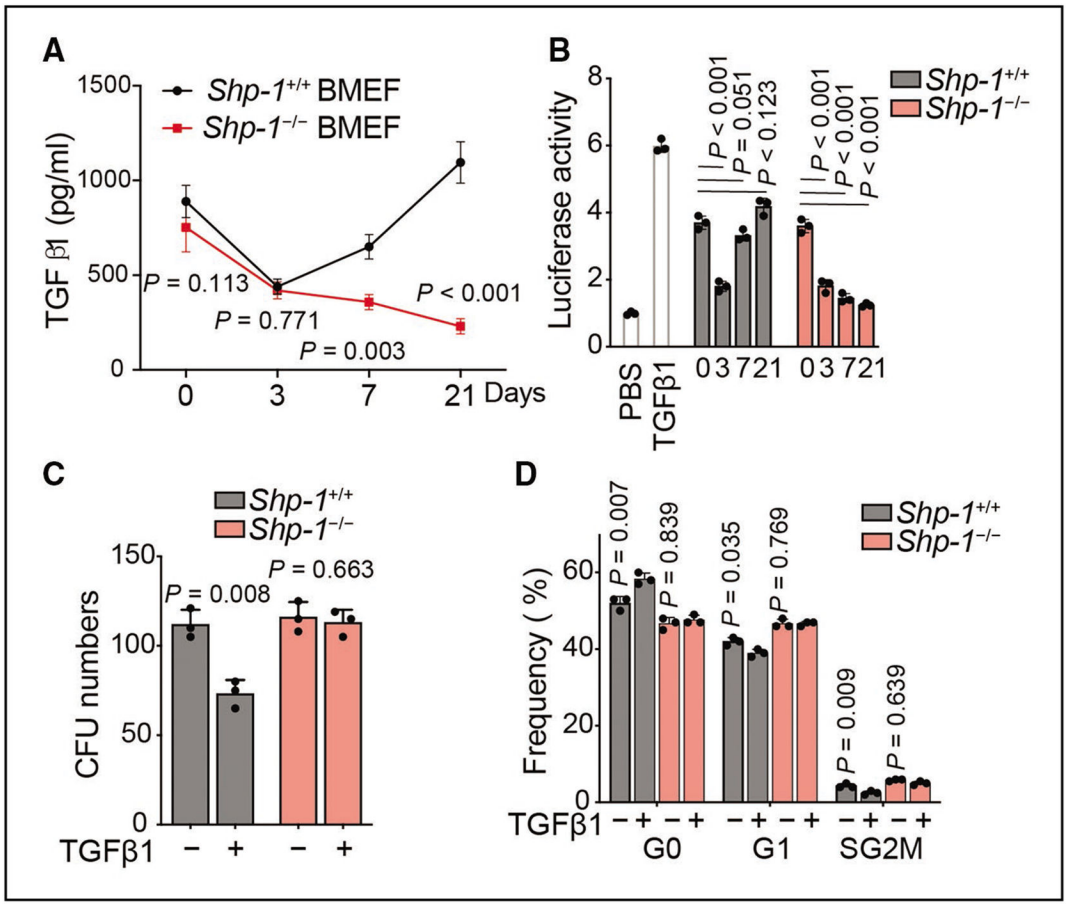


Figure 4. TGF-β1 regulation of hematopoietic stem cell (HSC) activation in ischemic stress is impaired by SHP-1 deficiency.

A, Free active TGF-β1 protein levels were measured in bone marrow (BM) extracellular fluid (BMEF) of *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice by ELISA (n=5 mice per group) at indicated time points. Repeated measure ANOVA is used to analysis the data overall time points. **B**, Bioactive TGF-β1 was determined by a luciferase reporter assay in BM from *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice at indicated time points after femoral artery ligation (FAL). PBS and TGF-β1 treatment were used as negative and positive controls, respectively. **C** and **D**, *Shp-1*-deficient HSC and progenitor cells (HSPCs) are resistant to the inhibitory effect of TGF-β1 in vitro after FAL. HSPCs (LSK cells) isolated from the BM of *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice 3 days after FAL were treated with or without 5 ng/ml TGF-β1 in CFU medium. Seven days later, the total CFU numbers were calculated (**C**; n=3 wells). These cells were also used for HSC cell cycle analysis 10 hours after TGF-β1 treatment (**D**; n=3 wells). Error bars, SEM.

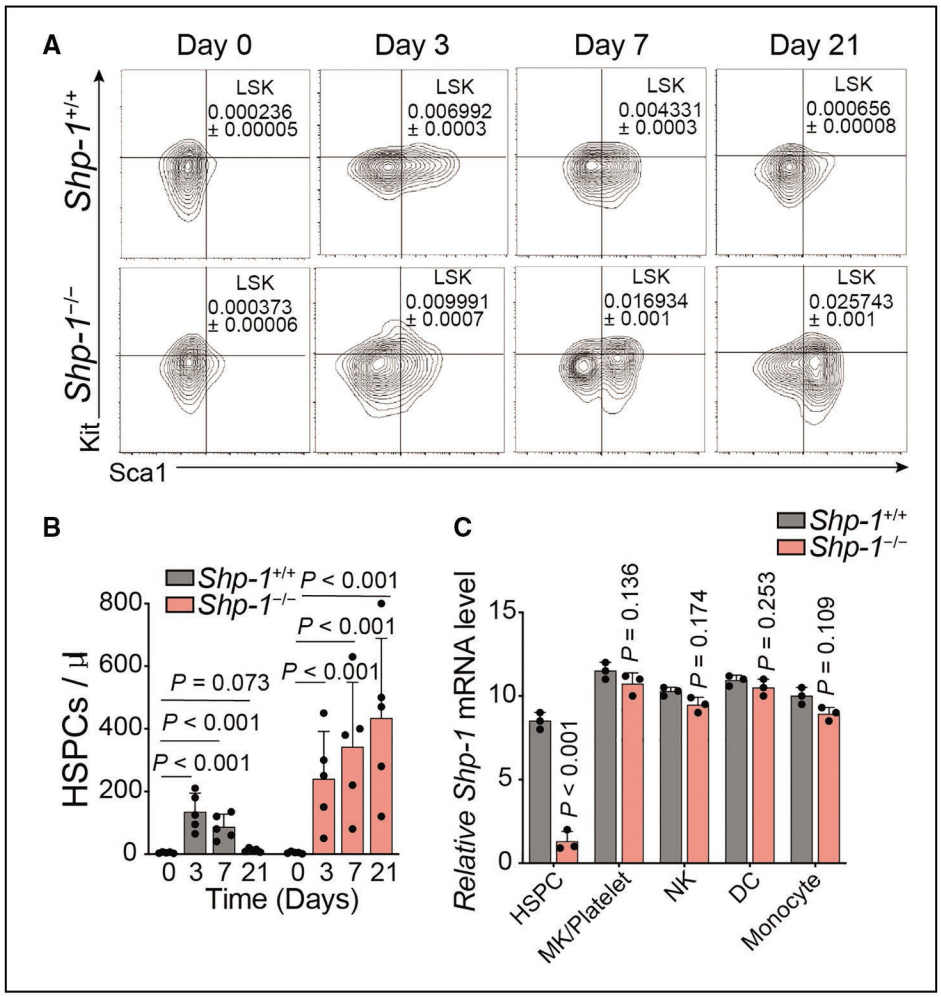


Figure 5. SHP-1-deficient hematopoietic stem cell and progenitor cells (HSPCs) accumulate in the peripheral blood (PB) after femoral artery ligation (FAL).

A and **B**, Representative flow cytometry measurements of the frequency (**A**) and concentration (**B**, cells/μl) of HSPCs (LSK, $Lin^{-}Sca\text{-}1^{+}Kit^{+}$) in PB of mice after FAL at indicated time points (n=5 mice per group). **C**, *Shp-1* was specifically deleted in HSPCs in PB measured at 21 days after FAL. HSPC, MK/platelet, natural killer, dendritic cells, and monocytes were collected by cell sorters and analyzed by RT-PCR. β-actin was used as the reference gene to normalize the relative expression for quantitative RT-PCR analysis. Cells were pooled from 3 mice. Error bars, SEM.

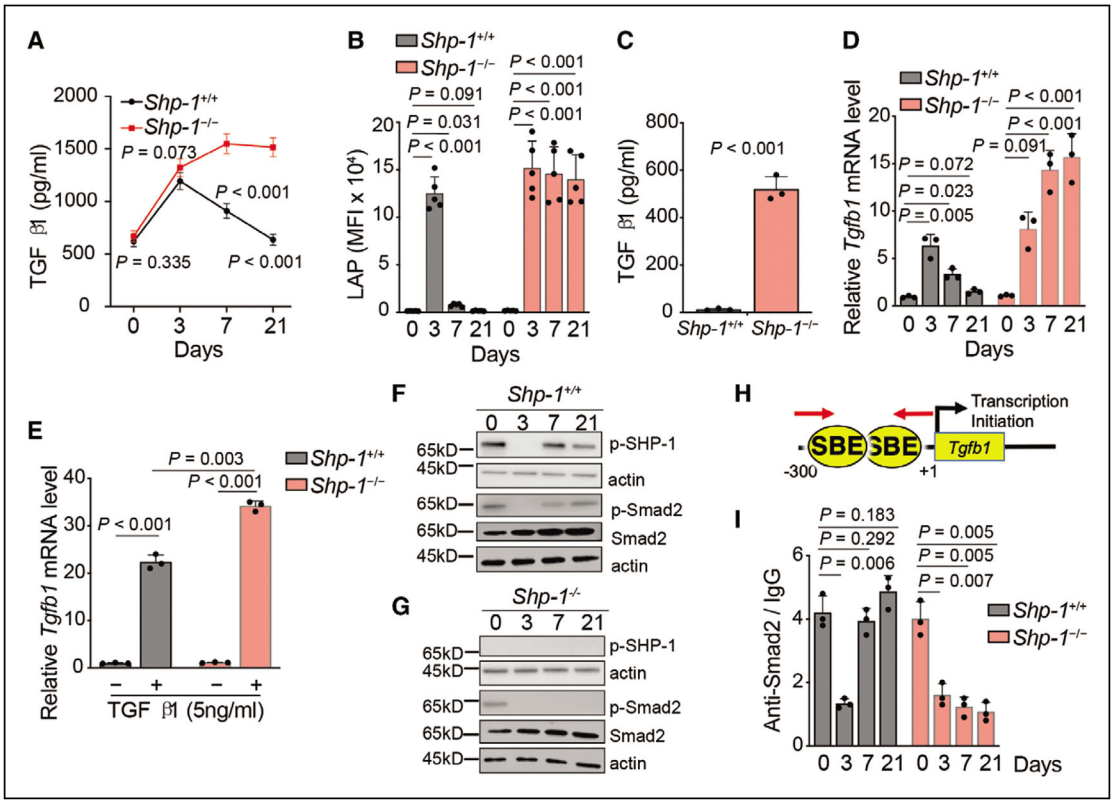
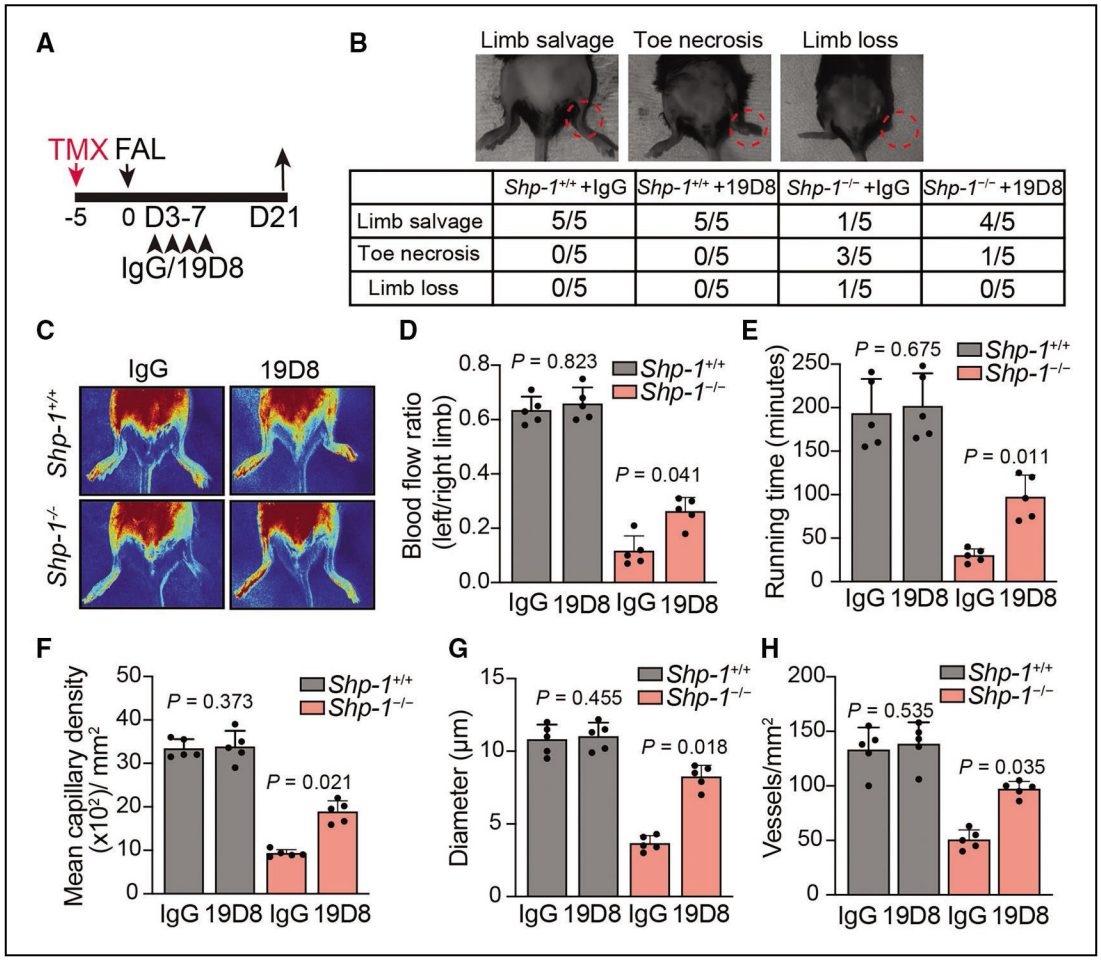


Figure 6. SHP-1-Smad2 signaling suppresses the autoinduction of TGF-β1 in the HSC and progenitor cells (HSPCs) in circulation in response to ischemic stress.

A, Free active TGF-β1 protein levels were measured in plasma of *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice by ELISA (n=5 mice) at indicated time points. Repeated measure ANOVA is used to analysis the data overall time points. **B**, HSPCs were stained with an anti-mouse LAP (latency-associated peptide; TGF-β1) antibody and were assessed by flow cytometry (n=5 mice per group; **C**) TGF-β1 protein levels as determined by ELISA after isolated HSPCs were cultured for 6 hours. **D**, Quantitative RT-PCR (q-PCR) analysis of *Tgfb1* mRNA amounts in HSPCs isolated from PB of *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice on indicated days after femoral artery ligation (FAL). Expression is normalized to 18S control. Each sample was obtained by pooling cells from 3 mice, and the PCR was repeated 3 times. **E**, HSPCs isolated from *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice were stimulated with TGF-β1 (5 ng/ml) for 8 hours. *Tgfb1* mRNA amounts were measured using qRT-PCR. Cells were pooled from 5 mice and PCR repeated 3 times. **F** and **G**, Lin^{-} PBMCs from *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice were collected on indicated days after FAL for immunoblotting of denoted proteins. Actin was used as a loading control. Each sample was obtained by pooling cells from 3 mice. **H**, Schema for Smad2 regulated regions on the Tgfb1 promoter and the primers to amply the −196 to −113 region used for the ChIP assay. SBE, Smad responsive region. **I**, Lin^{-} PBMCs from *Shp-1*$^{+/+}$ and *Shp-1*$^{-/-}$ mice after FAL were fixed, and the ChIP assay was performed with Smad2 antibody. IgG antibodies were used as a control. Each sample was obtained by pooling cells from 3 mice, and experiment was repeated twice. Error bars, SEM.



	Shp-1+/+ +IgG	Shp-1+/+ +19D8	Shp-1−/− +IgG	Shp-1−/− +19D8
Limb salvage	5/5	5/5	1/5	4/5
Toe necrosis	0/5	0/5	3/5	1/5
Limb loss	0/5	0/5	1/5	0/5

Figure 7. Restoration of TGF-β1 oscillations and signaling in peripheral blood (PB) leads to repair of the ischemic limb.

A, Schema for tamoxifen treatment, femoral artery ligation (FAL) surgery, control (IgG) or TGF-β1 blocking antibody (19D8) treatment and animal/sample analysis time points. TMX, tamoxifen; D, day (n=5 mice/group). **B**, Physiological status of ischemic limbs 3 weeks after surgery. TGF-β1 blocking antibody treatment improved the salvage of ischemic limbs of *Shp-1*$^{-/-}$ mice compared with IgG control (n=5 mice/group). **C**, Representative images of mouse hind limb blood flow measured with laser Doppler perfusion imaging after blocking antibody treatment at day 21 post-FAL. **D**, The blood flow ratio (left to right limb) was measured at day 21 post-FAL (n=5 mice/group). **E**, The limb function was measured by a treadmill test (n=5 mice/group). **F**, CD31 staining to measure the capillary density on ischemic limb muscle in the 4 indicated groups (n=5 mice/group). **G** and **H**, The number of vessels and diameter size were measured in the 4 indicated groups (n=5 mice/group). Error bars, SEM.