# The anti-microbial peptide SR-0379 stimulates human endothelial progenitor cell-mediated repair of peripheral artery diseases

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Ischemia is a serious disease, characterized by an inadequate blood supply to an organ or part of the body. In the present study, we evaluated the effects of the anti-microbial peptide SR-0379 on the stem cell-mediated therapy of ischemic diseases. The migratory and tube-forming abilities of human endothelial progenitor cells (EPCs) were enhanced by treatment with SR-0379 *in vitro*. Intramuscular administration of SR-0379 into a murine ischemic hindlimb significantly enhanced blood perfusion, decreased tissue necrosis, and increased the number of blood vessels in the ischemic muscle. Moreover, co-administration of SR-0379 with EPCs stimulated blood perfusion in an ischemic hindlimb more than intramuscular injection with either SR-0379 or EPCs alone. This enhanced blood perfusion was accompanied by a significant increase in the number of CD31- and α-SMA-positive blood vessels in ischemic hindlimb. These results suggest that SR-0379 is a potential drug candidate for potentiating EPC-mediated therapy of ischemic diseases. [BMB Reports 2017; 50(10): 504-509]

## INTRODUCTION

Peripheral artery disease is a circulatory condition, in which blood flow to the limbs, most often the lower limbs, is reduced by the narrowing and hardening of the peripheral arteries. The disease has several stages and symptoms, including painful cramping, leg weakness, toe sores, color changes in the legs, and either no pulse or a weak pulse in the legs or feet (1). Risk factors for peripheral artery disease include age, smoking, diabetes, being overweight or obese, a sedentary lifestyle, high cholesterol, high blood pressure, and a family history of vascular disease (2-5). Various therapeutic agents such as angiogenic cytokines, growth factors, and stem cells have been developed for the treatment of peripheral artery disease, but they remain clinically unsatisfactory (6). Accordingly, novel therapeutic agents for the treatment of peripheral artery disease are required.

Angiogenesis is critical to restore blood supply to ischemic tissues, and the recovery of organ function (7-9). Angiogenesis is initiated by endothelial cells of pre-existing blood vessels forming new capillaries through migration, proliferation, and morphologic differentiation (10, 11). Vasculogenesis is the process of new blood vessel formation by *de novo* production through migration, proliferation and differentiation of endothelial progenitor cells (EPCs), which undergo tubulogenesis into capillaries, arteries and veins (11, 12). Mobilized EPCs from the bone marrow are immature endothelial cells that are released into the bloodstream. These cells are essential in vasculogenesis and angiogenesis. EPCs are necessary to repair the injured blood vessels and to enable neovascularization after ischemic injuries (13-15). The EPCs are released into the blood in response to ischemic insult and are transported to the injury site, where they contribute to neovascularization and tissue repair (16, 17). EPCs directly stimulate neovascularization by incorporating into newly-forming vessels; and indirectly via the release of various paracrine factors (18, 19).

Although research has established the safety and efficacy of EPC-based therapies in animal models, including critical limb ischemia and myocardial infarction, success in clinical trials is limited due to low engraftment rates and poor cell survival after transplantation. For these reasons, drugs that regulate the mobilization and survival of EPCs are of great interest (18, 20). AG30, an antimicrobial peptide containing 30 amino acids, possesses both angiogenic and antibacterial properties (21, 22). A more stable and shorter peptide, SR-0379, contains

twenty amino acids, and can be generated from AG30 (20). SR-0379 is an angiogenic peptide with potent, broad spectrum, antibacterial activity (20). The angiogenic activity of SR-0379 suggested to us that it could affect the therapeutic efficacy and angiogenic properties of EPCs.

In this study, we explored the effects of SR-0379 on angiogenic activities of EPCs *in vitro*; and the effects of its administration on EPC-mediated tissue repair and angiogenesis in a murine hindlimb ischemia, an animal model of peripheral artery disease.

## RESULTS

### Pro-angiogenic effects of SR-0379 on EPCs *in vitro*

Endothelial progenitor cell migration is required for both angiogenesis and tissue repair (13). To assess whether SR-0379 can affect the angiogenic activities of EPCs, the effects of SR-0379 on the migration and tube formation of human EPCs were evaluated *in vitro*. In a transwell migration assay, SR-0379 treatment significantly increased the migration of EPCs in a dose-dependent manner, with maximal stimulation at 1 μg/ml concentration (Figs. 1A and C). The SR-0379-stimulated cell migration was as potent as 1% FBS-induced cell migration. Moreover, the SR-0379 treatment resulted in a significant increase in tube formation of EPCs compared with control with
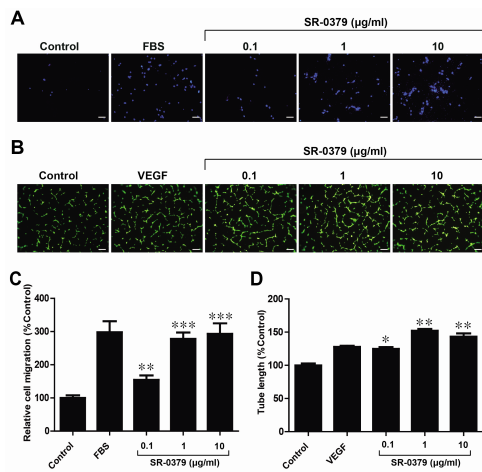
a maximal stimulation at 1 μg/ml concentration (Fig. 1B and D). SR-0379 has been reported to stimulate the angiogenic activities of normal human dermal fibroblast cells *via* the FAK and the PI-3-kinase-AKT-mTOR pathways (20). In this study, we investigated the effects of SR-0379 on the activation of FAK and PI-3-kinase-AKT pathways in EPCs. SR-0379 treatment increased phosphorylation levels of AKT and FAK in EPCs (Supplementary Fig. 1), suggesting that SR-0379 activates the FAK- and the PI-3-kinase-Akt-depenent pathways in EPCs. These results indicate that SR-0379 has pro-angiogenic properties in promoting migration and tube formation of EPCs *in vitro*.

### Repair of hindlimb ischemia by SR-0379 administration *in vivo*

To determine whether SR-0379 can induce an angiogenic effect *in vivo*, we evaluated the effects of SR-0379 on blood perfusion and tissue repair in the mouse hindlimb ischemia model. After surgical removal of the femoral artery of the mouse hindlimb, ischemic hindlimbs were administered with increasing doses of SR-0379. Blood perfusion was measured by Laser Doppler Perfusion Imaging (LDPI), on day 0, 7, 14, and 21 after induction of ischemia. Fig. 2A depicts representative LDPI images of a hindlimb at 0 and 21 days after inducing ischemia. On days 14 and 21, intramuscular
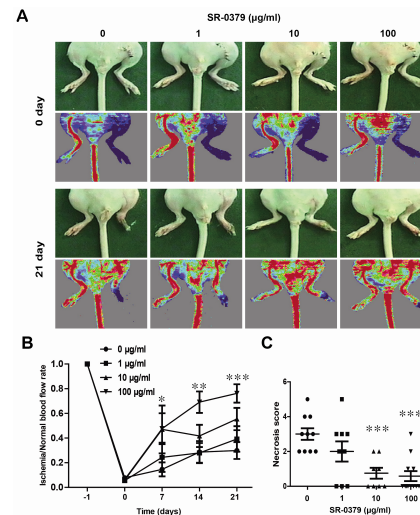


**Fig. 1.** Effects of SR-0379 on the migration and tube forming ability of EPCs. (A) SR-0379-induced migration of EPCs. Chemotactic migration of EPCs was measured by a transwell migration assay in response to treatment with increasing concentrations of SR-0379 or 1% FBS as a positive control. Scale bar = 100 μm. (B) Effects of SR-0379 on tube formation in EPCs. Shown are tube formations induced by the increasing concentrations of SR-0379 or 10 ng/ml VEGF as a positive control. Scale bar = 200 μm. (C) The effects of SR-0379 and FBS on EPC migration were quantified. Data represent mean ± S.D. (n = 8). **P < 0.01; ***P < 0.001 vs. control. (D) Effects of SR-0379 and VEGF on tube formation of EPCs. Data represent mean ± S.D. (n = 4). *P < 0.05; **P < 0.01 vs. control.



**Fig. 2.** Effects of SR-0379 on blood perfusion and tissue necrosis in a mouse hindlimb ischemia model. Ischemic hindlimbs of Balb/c mice were administered by intramuscular injection with the indicated concentrations of SR-0379. (A) Representative images of hindlimbs and blood flow measured by LDPI on post-operative days 0 and 21. (B) Quantitative evaluation of blood flow measured by LDPI. The LDPI ratio was calculated as the ratio of ischemic to contralateral hindlimb blood perfusion over the observation period. (C) Statistical analysis of the necrosis score on day 21. Data represent mean ± S.D. (n = 10 per each group). *P < 0.05; **P < 0.01; ***P < 0.001 vs. HBSS.

injection of 10 μg/ml or 100 μg/ml SR-0379 significantly increased blood flow in the ischemic hindlimb compared with the control group, which was injected with saline buffer (Fig. 2A, B).

To confirm these results, the effect of SR-0379 on hindlimb necrosis in the ischemic mouse model was measured. Intramuscular administration of SR-0379 significantly reduced tissue necrosis and limb loss at concentrations of 10 μg/ml or 100 μg/ml, whereas the saline-injected control group exhibited severe necrosis and loss of the ischemic hindlimb (Fig. 2C). This suggests that SR-0379 improves blood perfusion and alleviates tissue necrosis in ischemic hindlimbs. SR-0379 treatment was not found to significantly affect cell viability of EPCs *in vitro* (Supplementary Fig. 2), alleviating any safety concerns that might otherwise deter its pharmacological application.

## SR-0379-induced angiogenesis in ischemic hindlimbs

To establish whether SR-0379 enhanced angiogenesis in ischemic hindlimbs, the densities of CD31-positive capillaries and α-SMA-positive vessels were measured by the immuno-staining of ischemia tissues on day 21 after initiation of ischemia. Intramuscular injection of SR-0379 significantly increased the numbers of CD31-positive capillaries and α-SMA-positive blood vessels in the ischemic limb (Fig. 3A-D). The SR-0379-induced increase of angiogenesis in ischemic limbs was dependent on the dose of SR-0379, with maximal
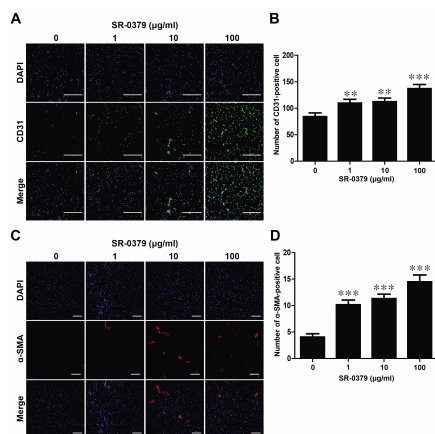
stimulation at 100 μg/ml concentration. These results show that treatment with SR-0379 promotes angiogenesis in ischemic hindlimbs *in vivo*.

## SR-0379-induced stimulation of the EPC-mediated ischemic tissue repair

To determine whether SR-0379 can potentiate the stimulatory effect of EPCs on the repair of ischemic hindlimb, SR-0379 and EPCs were administered into the ischemic hindlimb of nude mice individually or in combination. Nude mice underwent unilateral hindlimb ischemia surgery and received an intra-muscular injection of SR-0379, EPCs, or the co-injection of SR-0379 and EPCs at the ischemic limbs. LDPI analysis showed that the blood flow ratio of the ischemic/non-ischemic
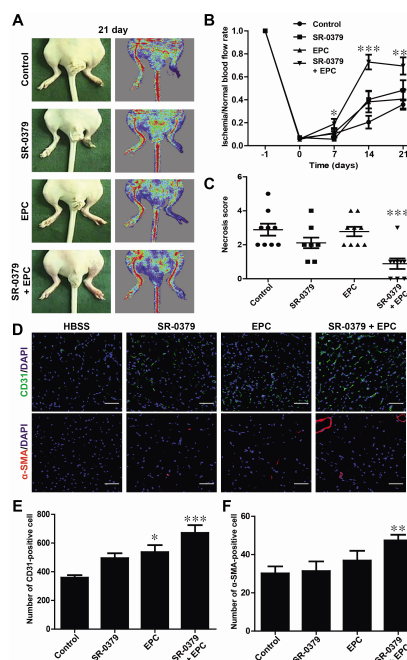


**Fig. 4.** Effects of SR-0379 and EPCs on angiogenesis and limb salvage in an ischemic hindlimb animal model. (A) Effects of SR-0379 and EPCs on blood perfusion in ischemic hindlimbs. Ischemic limbs of nude mice were administered with either SR-0379 (100 μg/ml) or EPCs alone, or combined injections with SR-0379 (100 μg/ml) together with EPCs in the ischemic hindlimb. Representative images of hindlimbs and blood flow measured by LDPI on day 21 are shown. (B) Quantitative analysis of blood flow measured by LDPI. The LDPI value was expressed as a ratio of ischemia to contralateral hindlimb blood perfusion at day 0, 7, 14 and 21. (C) Statistical analysis of the necrosis score on day 21. (D) Immunostaining of CD31-positive capillaries (green) or α-SMA-positive blood vessels (red) in ischemic limbs. Nuclei (blue) were counterstained with DAPI and overlaid images are shown. Scale bar = 100 μm. Quantitative analysis of CD31-positive capillaries (E) and α-SMA-positive blood vessels (F) in the ischemic hindlimb. Data represent mean ± S.D. (n = 10 for each group). *P < 0.05; **P < 0.01; ***P < 0.001 vs. control.



**Fig. 3.** Effects of SR-0379 on neovascularization in the ischemic hindlimb. (A) Immunostaining images for CD31-positive capillaries (green) in ischemic hindlimbs on day 21 after intramuscular injection of the indicated concentrations of SR-0379. Nuclei (blue) were counterstained with or DAPI, and overlaid images are shown. Scale bar = 100 μm. (B) Quantitative analysis of CD31-positive capillaries per high power field in the immuno-stained tissues. (C) Immunostaining images of α-SMA-positive arterioles (red) or DAPI (blue) in ischemic limbs on day 21. Scale bar = 100 μm. (D) Quantitative analysis of α-SMA-positive arterioles per HPF in the ischemic hindlimb. Data represent mean ± S.D. (n = 10 mice per group). **P < 0.01; ***P < 0.001 vs. control.

hindlimbs was significantly higher in the co-injected group (SR-0379 plus EPCs) compared with both the SR-0379 or EPC individually injected group, and the mock-treated group (Fig. 4A and B). When the necrosis score was measured at day 21, injection of SR-0379 slightly decreased the necrosis score in comparison with saline-injected control (P value = 0.0567) whereas individual injection of EPCs did not show a significant reduction (P value = 0.1907). However, the co-injection of SR-0379 and EPCs significantly reduced the hindlimb necrosis score in comparison with individual injection of SR-0379 and EPCs (Fig. 4C), suggesting a synergistic activation of ischemic tissue repair by co-administration of both SR-0379 and EPCs to have taken place.

To determine whether intramuscular co-injection of EPCs and SR-0379 can stimulate angiogenesis in the ischemic hindlimb, the numbers of CD31-positive cells and α-SMA-positive cells in the ischemic hindlimb were examined. Intramuscular co-injection of EPCs and SR-0379 resulted in a significant increase in the number of CD31-positive capillaries and α-SMA-positive blood vessels in the ischemic limb, compared with the saline-injected control group (Fig. 4D-F).

These results suggest a synergistic effect of SR-0379 and EPCs in the repair of hindlimbs from ischemic injury and in the promotion of neovascularization during the repair process, which may inspire the development of a novel therapeutic protocol to treat patients with ischemic conditions.

## DISCUSSION

SR-0379 is a peptide derived from AG-30, an anti-microbial peptide that promotes angiogenesis in murine and porcine wound-healing models (20, 22). Herein, we demonstrated that SR-0379 stimulates angiogenic activities of EPCs *in vitro*. It has been reported that AG-30 and SR-0379 increased angiogenic activity in various cell types, including human umbilical vein endothelial cells, human aortic endothelial cells, and human aortic smooth muscle cells (20, 21). In human aortic endothelial cells, AG-30 also increased the expression of various angiogenic factors including angiopoietin-2, interleukin-8, jagged 1, VEGF, and insulin-like growth factor (21), all of which play an important role in neovascularization (23, 24). SR-0379 has angiogenic effects including proliferation, tube formation, and migration of human umbilical vein endothelial cells (20). In the present study, we consistently demonstrated that SR-0379 promoted the migration and tube forming ability of human EPCs. SR-0379 enhanced not only the angiogenic activities of human endothelial cells, but also the proliferation of human dermal fibroblasts *in vitro* (20). These results suggest that SR-0379 had pro-angiogenic activity stimulating migration and tube forming ability of both endothelial cells and EPCs.

We also demonstrated that intramuscular injection of SR-0379 accelerated blood perfusion in ischemic hindlimbs, and prevented tissue necrosis. Intramuscular administration of SR-0379 increased the numbers of CD31- and α-SMA-positive

blood vessels in ischemic hindlimbs. SR-0379 has been reported to stimulate secretion of angiogenic factors and promote angiogenesis through activation of the FAK- and PI-3-kinase-AKT-mTOR-dependent pathways in endothelial cells (20). Activation of the Akt-mTOR pathway plays an important role in the recovery of hindlimb ischemia by accelerating angiogenesis (25). We also found that SR-0379 stimulated phosphorylation of FAK and AKT in EPCs. Co-administration of SR-0379 with EPCs was found to be the most effective means of reducing the necrosis score, promoting neovascularization, and restoring blood flow, in comparison with administration of SR-0379 or EPCs alone. Accordingly, it is likely that SR-0379 treatment stimulates ischemic tissue repair by promoting angiogenic activities of not only endogenous endothelial cells, but also exogenously co-administered EPCs. Together with the pro-angiogenic activities of SR-0379 on EPCs *in vitro*, these results prove that SR-0379 promotes angiogenic activity and enhances the therapeutic effect of transplanted EPCs in peripheral artery disease.

The present study also demonstrates the potentiating effect of SR-0379 on the EPC-mediated repair of ischemic hindlimbs. Despite the beneficial effects of SR-0379 on EPC-mediated tissue repair, it is still unknown whether SR-0379 or related peptides are endogenously produced within injured or ischemic tissues. Although the physiological relevance of SR-0379 stimulated angiogenesis of EPCs and ischemic tissue repair is still unclear, the present study suggests that SR-0379 is a strong candidate for the development of drugs to treat tissue injury and enhance the therapeutic efficacy of EPCs in patients with ischemic diseases.

## MATERIALS AND METHODS

### Materials
Recombinant human vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were purchased from R&D Systems (Minneapolis, MN, www.rndsystems.com). Anti-CD31 antibody (MEC 13.3) and growth factor-reduced Matrigel were purchased from BD Biosciences (Bedford, MA, www.bdbiosciences.com). Anti-α-smooth muscle actin (α-SMA) antibody (ab5694) and GAPDH antibody (MAB374) were purchased from Abcam PLC (Cambridge, U.K., www.abcam.com). The synthetic peptide SR-0379 was synthesized by Synpeptide (Shanghai, China, www.synpeptide.com). The purity of the synthesized SR-0379 was > 95%. Antibodies against Akt (9272s), phosphorylated Akt (Ser473) (3787s), FAK (3285s) and phosphorylated FAK (Tyr397) (3283s) were purchased from Cell Signaling Technology (Danvers, MA, www.cellsignal.com). Disposable, sterile, pyrogen-free bags were purchased from Green Cross (Yongin, Korea, www.greencross.com). Endothelial cell basal medium-2 (EBM-2) was purchased from Clonetics (San Diego, CA, www.lonza.com). A transwell migration chamber was pur-

chased from Neuro Probe (Inc., Gaithersburg, MD, www. neuroprobe.com). An LDPI analyzer was purchased from Moor instruments (Devon, U.K., www.moor.co.uk). Alexa Fluor 488 goat anti-mouse, Alexa Fluor 488 goat anti-rat and Alexa Fluor 568 goat anti-rabbit secondary antibodies were purchased from Life Technologies (Carlsbad, CA, www. lifetechnologies.com). Vectashield medium was purchased from Vector Laboratories (Burlingame, CA, www.vectorlabs. com). Laser scanning confocal microscopy was purchased from Olympus Corp (Tokyo, Japan, www.olympus-global. com).

### Cell culture

Human EPCs were isolated from human umbilical cord blood, which was collected in disposable, sterile, pyrogen-free bags containing anticoagulant. Written informed consent was obtained from blood donors, and the protocol for this study was approved by the Institutional Review Board of Pusan National University Hospital (Permit Number: H-1302-005-015). EPCs were seeded on culture dishes and maintained in EBM-2 supplemented with EGM-2 MV SingleQuots containing 5% fetal bovine serum (FBS), human VEGF-1, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, and ascorbic acid. The medium was exchanged 24 h after the initial plating to remove non-adherent cells and was exchanged every day for the first week. EPCs were used at passages 5-8 for all experiments, although the cell morphologies were healthy and consistent to proliferate over passage 9. The expression profiles of cell surface markers in EPCs were examined by immunofluorescence staining and a fluorescence activated cell sorter (FACS) analysis. EPCs expressed several endothelial lineage-specific phenotypic markers including CD31, Flk1, and von Willebrand factor, as well as functional markers, including endothelial nitric oxide synthase (eNOS) and phospho-eNOS. Furthermore, expression of EPC-specific cell surface markers CD34, CD31, CD133, c-kit, Flk1, CXCR4, and CD144, was confirmed by flow cytometric analysis. However, the hematopoietic lineage markers CD11b, CD14, and CD45, were not expressed in EPCs.

### Cell migration assay

Chemotactic migration of EPCs were assayed using a disposable 96-well chemotaxis chamber. Transwell migration assays were performed according to the manufacturer's instructions. Cells ($5 \times 10^3$) in 50 µl of serum-free medium were seeded onto the top of each membrane well. 320 µl of serum-free medium containing SR-0379 peptide was put in the lower chamber well. Following incubation for 12 h at 37°C, non-invasive cells were removed from the membrane. Cells that invaded the membrane were fixed with 4% paraformaldehyde, stained with 0.1% Hoechst, and counted under a florescence microscope.

### Tube formation assay

For tube formation assay of EPCs, aliquots (50 µl) of growth factor-reduced Matrigel (10 mg protein/ml) were added to 96-well culture dishes and polymerized for 30 min at 37°C. EPCs were trypsinized, resuspended in EBM-2 basal medium supplanted with 1% FBS, and plated onto a layer of Matrigel at a density of $1 \times 10^5$ cells/well. The cells were then exposed to EBM containing 0.1% FBS, or supplemented with VEGF or SR-0379. After incubation of the Matrigel cultures at 37°C for 12 h, the cultures were photographed using an inverted microscope with a digital camera, and tube networks were quantified by measuring the tube length by using Image J software (https://imagej.nih.gov/ij/).

### Hindlimb ischemia and blood flow measurement

Animal experiments were performed using protocols approved by the Pusan National University Institutional Animal Use and Care Committee. BALB/C or BALB/CA-nu/nu (male, age 6-9 weeks, weighing 22-24 g) were anesthetized with an intraperitoneal injection of 400 mg/kg 2,2,2-tribromoethanol (Avertin; Sigma-Aldrich, St. Louis, MO) for operative resection of one femoral artery and laser Doppler perfusion imaging (LDPI). The femoral artery was excised from its proximal origin as a branch of the external iliac artery to the distal point where it bifurcates into the saphenous and popliteal arteries. After arterial ligation, ischemic hindlimbs received injections with SR-0379 or HBSS buffer into five sites (20 µl at each site) of the muscle in the medial thigh three times per week. The extent of necrosis in the ischemic hindlimb was recorded on day 21 after surgery. Scores for necrosis were assessed as follows: 0 = no necrosis, 1 = toes necrosis, 2 = foot necrosis, 3 = knee necrosis, 4 = femoral necrosis, 5 = whole leg necrosis. Blood flow of the ischemic and normal limb was measured using a LDPI analyzer on days 0, 7, 14 and 21 after surgery of hindlimb ischemia. Perfusion of the ischemic and non-ischemic limb was calculated based on colored histogram pixels. Red colors indicate high perfusion, while blue indicates low. Blood perfusion is expressed as the LDPI index representing the ratio of ischemic versus non-ischemic limb blood flow. A ratio of 1 before surgery indicates equal blood perfusion of both legs.

### Immunofluorescence analyses

For immunofluorescence staining of the tissue samples, hindlimb muscles were harvested, fixed with acetone, and embedded in paraffin. Endothelial cells and smooth muscle cells were stained with mouse anti-CD31 and rabbit anti-α-SMA antibodies. The samples were incubated with Alexa Fluor 488 goat anti-mouse, Alexa Fluor 488 goat anti-rat, or Alexa Fluor 568 goat anti-rabbit secondary antibodies followed by washing and mounting in Vectashield medium with 4[prime],6-diamidino-2-phenylindole for staining of nuclei. The stained sections were visualized using laser scanning confocal microscopy. Capillary density and the number of

arterioles/arteries were assessed by counting the number of CD31-positive and α-SMA-positive cells. Six randomly selected microscopic fields from section in each tissue block were examined for the numbers of capillary density and α-SMA-positive arteries cells for each mouse.

## Statistical analysis

Results of multiple observations are presented as mean $\pm$ SD. For analysis of multivariate data, group differences were assessed using one-way or two-way ANOVA, followed by Scheffe's post hoc test.

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## CONFLICTS OF INTEREST

The authors have no conflicting interests.

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