SUPPLEMENTAL MATERIAL

Supplementary Methods

Mouse model of revascularization without or with exercise

Studies of wild-type (WT, MMP-$2^{+/+}$) and MMP-2–deficient (KO, MMP-$2^{-/-}$) mice in a C57/BL6 background were approved by the Institutional Animal Care and Use Committee at Nagoya University. Male young (6 months) and aged (18 and 24 months) mice of both genotypes were subjected to unilateral hindlimb ischemic surgery under sodium pentobarbital anesthesia (50 mg/kg intraperitoneally).[1] Exercise training, which consisted of swimming for 1 hour per day for 2 weeks, began on postoperative day 1. The mice swam in a pool (35 × 20 × 20 cm) filled with warm water (36˚ ± 1˚C) to a depth of 10 cm. Swimming is thought to be a natural behavior of mice, and it is less stressful than, and avoids the foot injuries associated with, other forced exercise protocols. In specific experiments, the HIF-1α stabilizer deferoxamine (DFO, 10 mg/kg), the PI3K inhibitor LY2940029 (LY, 20 mg/kg; both from Calbiochem and dissolved in dimethyl sulfoxide [DMSO]), or vehicle (DMSO) was intraperitoneally injected into the abdomen of aged mice (without or with training) 1 d before surgery and every other day until sacrifice.

Analysis of hindlimb blood flow

Hindlimb blood flow was determined by using a laser Doppler blood flow (LDBF) analyzer (PIM II LDI, Linkoping, Sweden). LDBF analyses were performed on legs and feet before surgery and on the indicated postoperative days. Blood flow is shown as changes in the laser frequency using different color pixels. Quantitative analysis of blood flow was expressed as the ratio of left (ischemic) to right (nonischemic) LDBF to avoid data variations because of ambient light and temperature.[1]

Western Blot Analysis

Total extracted protein was separated with 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and blocked with 5% milk/Tris-buffered saline with Tween. Primary antibodies against the following proteins were used for Western blotting: HIF-1α, prolyl hydroxylase (PHD)1, and PHD3 (Novus Biologicals); VEGF-A, PHD2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology); Shc (BD Transduction Laboratories); β-actin (Sigma-Aldrich); and total Akt and phosphor-Akt (Cell Signaling).

Quantitative real-time gene expression assay

RNA was harvested from tissue with an RNeasy Fibrous Tissue Mini-Kit and from cultured cells with an RNeasy Micro Kit (Qiagen) in accordance with the manufacturer's instructions. The mRNA was reverse transcribed to cDNA with an RNA PCR Core kit (Applied Biosystems). Quantitative gene expression was studied by using the ABI 7300 Real-Time PCR System with TaqMan Universal PCR

Master Mix (Applied Biosystems). All experiments were performed in triplicate. Sequences of primers specific to mouse matrix metalloproteinase-2 (MMP-2), vascular endothelial growth factor (VEGF), VEGF receptor-1 (Flt-1), and stroma cell-derived factor-1 (SDF-1) were described previously.[2] Transcription of targeted genes was normalized to GAPDH.

Gelatin zymography

Gelatinolytic activity in extracts from the tissues and cell-conditioned media was detected as previously described.[7]

Measurement of capillary density

Capillary density in cross-sections of adductor muscle (6-μm cryosections) was assayed at postoperative day 14 or 28 with anti–*Griffonia simplicifolia* lectin-I (GSL-I, Vector Laboratories). Capillary endothelial cells (ECs) were quantified by measuring the number of GSL-I–positive cells per high-power (400×) field. The number of capillaries per muscle fiber was measured in 15 randomly chosen microscopic fields from 3 different sections in each tissue block.[1]

Endothelial progenitor cell (EPC) mobilization assay

On postoperative day 7, bone marrow (BM) and peripheral blood (PB) were obtained from young and aged mice, and erythrocytes were lysed with ammonium chloride and separated into pellets. The cells were washed with phosphate-buffered saline (PBS)/EDTA and sorted by flow cytometry using fluorescein isothiocyanate (FITC)-labeled PECAM-1 and phycoerythrin-labeled CD117 (BD Pharmingen).[1]

Analysis of collateral vessel formation and pathological vessel formation

On postoperative day 15, the collateral vessels were evaluated by iliac microangiography. Vessel density was expressed as the number of vessels in a fixed quantification area in each diameter range, as described previously.[1] Pathological vessels were evaluated by intravascular vital staining with a fluorescently labeled *Lycopersicon esculentum* lectin (Vector Laboratories) that binds the luminal surface of all blood vessels, as previously described.[3] In brief, on the operative day 15, mice were anesthetized and FITC-labeled lectin was injected through a $24_{\times 3/4}$-gauge soft-tip catheter into the abdominal aorta; 2 minutes later the tissues were fixed by perfusion of 1% paraformaldehyde in PBS. The skeletal muscle was embedded and frozen in Tissue-Tek OCT medium. Quantitative analysis of the cryosections (8-μm) was performed with the use of a confocal laser scanning microscope (WinRF version 5.0 image-processing software, Mitani, Tokyo, Japan). The number of pathological vessels per high-power (400×) field was measured in 8 randomly chosen microscopic fields from 3 different sections in each tissue block.[1]

Histological analysis

On day 4, macrophages that had infiltrated the ischemic muscles of the aged mice (24 months) were detected immunohistochemically in cross-sections (6 μm) by using a mouse anti–Mac-3 mAb (1:40, BD Pharmingen). Macrophages were counted in four random microscopic fields from three independent sections of each animal (n = 6 per group), and the infiltration was expressed as the number of Mac-3–labeled cells per high-power (400×) field.

Mouse BM transplantation model

BM transplantations were performed in 6 groups of mice (n = 5 per group): MMP-$2^{+/+}$ donor/MMP-$2^{+/+}$ recipient, MMP-$2^{+/+}$ donor/MMP-$2^{+/+}$ recipient with training, MMP-$2^{+/+}$ donor/ MMP-$2^{-/-}$ recipient, MMP-$2^{+/+}$ donor/ MMP-$2^{-/-}$ recipient with training, MMP-$2^{-/-}$ donor/ MMP-$2^{-/-}$ recipient, and MMP-$2^{-/-}$ donor/ MMP-$2^{-/-}$ recipient with training. In each case, the donor was 18 months old and the recipient was 24 months old. BM was harvested from femurs and tibias of aged male mice, and c-Kit^{+}/$lineage^{-}$ (including CD5, CD11b, CD45R, and Gr-1) cells were isolated by Magnetic Cell Sorting (MACS) using a Lineage Cell Depletion Kit (Miltenyli Biotec Inc, GmbH, Bergisch Gladbach, Germany).[1] BM-derived MCs (2×10^6/mouse) were injected through the tail vein into lethally irradiated (10 Gy) aged MMP-$2^{+/+}$ mice. After 40 days had been allowed for reconstitution of the BM, mice were subjected to hindlimb ischemia without or with exercise.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA) and cultured in EBM-2 plus 10% fetal bovine serum (FBS) and EGM-2 SingleQuotes (Clonetics) in a humidified atmosphere of 5% CO_2 and 95% air. Population doubling (PD) was determined at each passage.[4] In the experiments, cells at 5–7 PD and 15–17 PD were used as young and aged HUVECs, respectively. The senescent phenotype was confirmed with β-galactosidase (β-gal) staining solution (Supplemental Figure VIII). HUVECs were seeded into 6-well plates (5×10^5 cells/well) and transferred to a modular incubator chamber (Billups-Rothenberg),

which was flushed with 1% O_2, 5% CO_2, and 94% N_2; sealed; and placed at 37˚C. BM-derived EPC-like c-Kit$^+$ cells were isolated by using CD117 MicroBeads and MACS (Miltenyli Biotec GmbH)[1] and separated into pellets. Cells were then washed with PBS and subjected to cellular assays.

Cell adhesion and migration assay

HUVECs and BM-derived EPC-like c-Kit$^+$ cells were isolated from aged mice without or with training, and cell adhesion and migration assays were performed as previously described.[1]

EPC tube formation assay

EPCs (2.5×10^5 cells/well) were seeded onto Matrigel (BD Biosciences, Le Pont de Claix, France) in a 12-well plate and incubated overnight in EBM-2 containing VEGF (20 ng/mL) to induce tube formation. Matrigel wells were observed under an Olympus IIX 70 microscope (Tokyo Japan), and WinRF version 5.0 image-processing software (Mitani, Tokyo, Japan) was used to count the number of sprouts in 5 fields (100×) of each well.

siRNA Transfection Protocol

Specific siRNA duplexes (ON-TARGETplus siRNA) for HIF-1α (J-004018-07 and J-004018-09), VEGF (J-40812-06), lamin A/C (D0010500105, as a positive control), and a nontargeting control siRNA (ON-TARGETplus non-targeting siRNA, D-001810-01-20) were purchased from Dharmacon (Brébières, France). Briefly, cells were grown on 6-well plates until 80% confluent. The siRNA

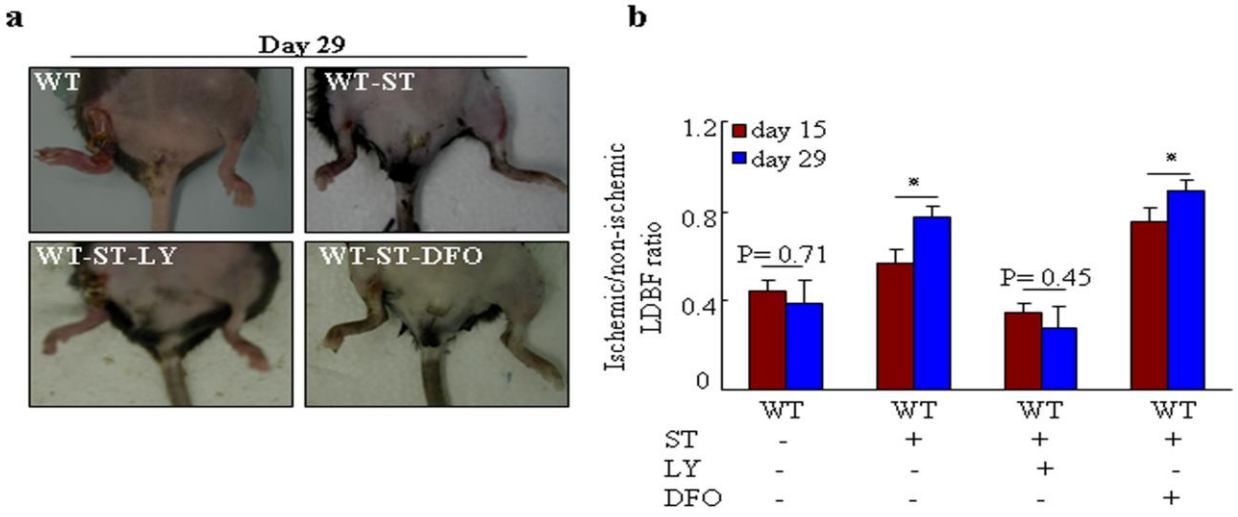
solution was mixed with serum-free and antibiotic-free EBM-2 medium containing DharmaFECT2 siRNA Transfection reagent. The culture medium was removed and replaced with 800 μL of antibiotic-free EBM-2 medium containing 8% FCS, and 200 μL of the transfection mix was added to each well to achieve a final siRNA concentration of 20 nmol/L. Transfected cells were incubated under hypoxic conditions (1% O_2, 5% CO_2, and 94% N_2) for 24 h, and expression of MMP-2 mRNA and protein were analyzed with PCR assays and Western blotting, respectively. In addition, maxGFP was also used to examine the effect of GFP gene transfection on MMP-2 mRNA expression in mouse endothelial cells.

Statistical analysis

Data are expressed as means ± SE. Student's *t* tests (for comparison between 2 groups) or one-way ANOVA (for comparison of 3 ≥ groups) followed by Tukey post hoc tests were used for statistical analyses. The non-parametric Kruskal-Wallis test (Tukey type multiple comparison) was used analysis of variance for the gene expression data. Blood flow data were subjected to two-way repeated measures ANOVA and Bonferroni post hoc tests. SPSS software version 17.0 (SPSS Inc, Chicago, III) was used. A value of $P < 0.05$ was considered statistically significant. Collateral vessel diameter, pathological vessel, capillary density, foot amputation, and length and number of endothelial sprouts were evaluated by two observers in a blind manner, and the values they obtained were averaged

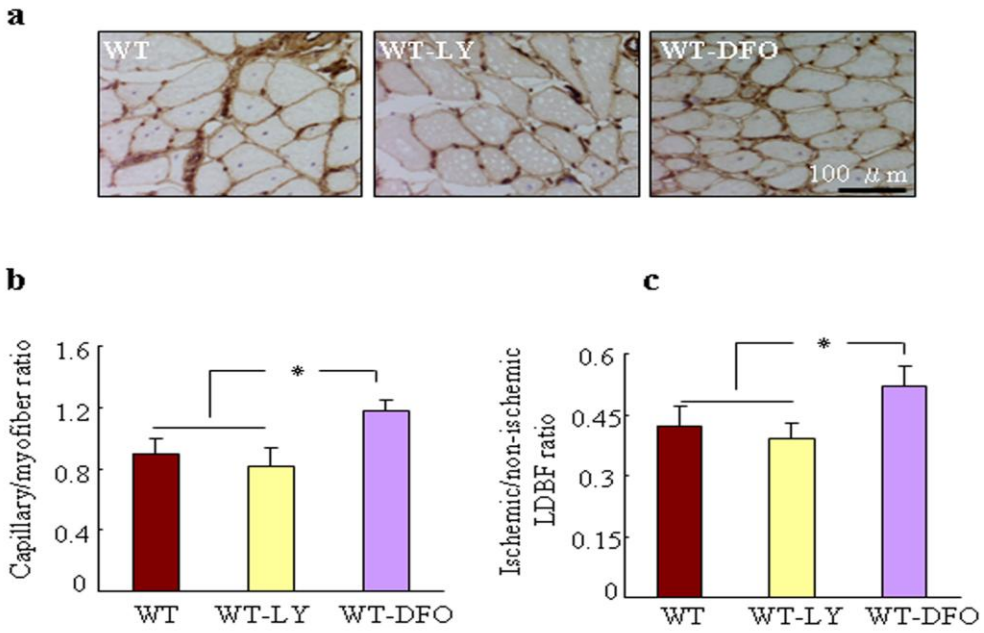
The authors had full access to and talk full responsibility for the data. All authors have read and agree to the manuscript as written.

Supplemental Figure I



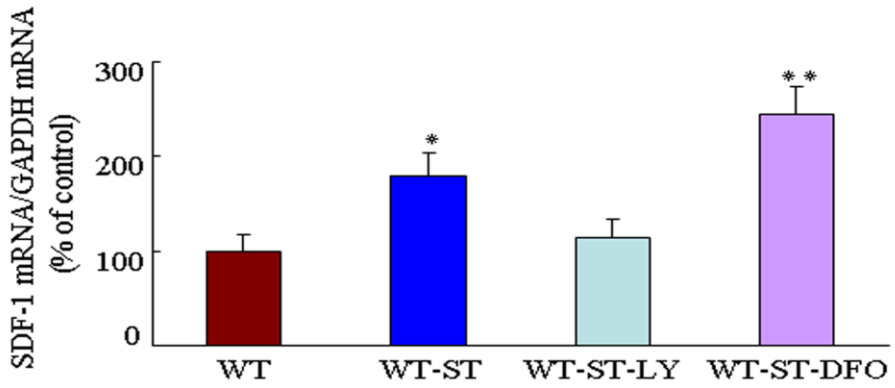
Supplemental Figure I. Long-term effect of ST on blood perfusion in ischemic tissues of aged MMP-$2^{+/+}$ mice (24 months old). (**a**) Legs from mice in the 4 experimental groups (WT, WT-ST, WT-ST-LY, and WT-ST-DFO) on postoperative day 29. (**b**) The ratios of ischemic-to-normal LDBF were higher in MMP-$2^{+/+}$ mice on day 29 than on day 15 ($n = 6$ per group, * $P < 0.05$, two-way repeated measures ANOVA followed Bonferroni post hoc tests).

Supplemental Figure II



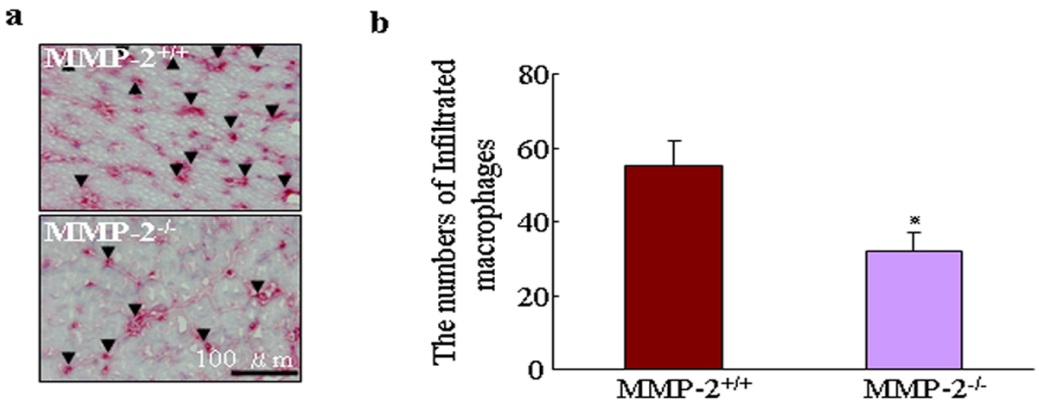
Supplemental Figure II. Effects of LY or DFO alone on capillary formation and blood perfusion in the ischemic tissues of aged MMP-$2^{+/+}$ mice (24 months old). (**a**) Immunohistostaining was performed using an anti-CD31 monoclonal antibody to determine the capillary density of ischemic muscle on postoperative day 15. Scale bar = 100 μ m. (**b**) DFO alone improved the capillary density ($CD13^+$ signals/muscle fiber) in aged MMP-$2^{+/+}$ mice, whereas LY had no effect on ischemic revascularization in MMP-$2^{-/-}$ mice (n = 6 per group, * $P < 0.05$; Tukey post hoc tests).

Supplemental Figure III



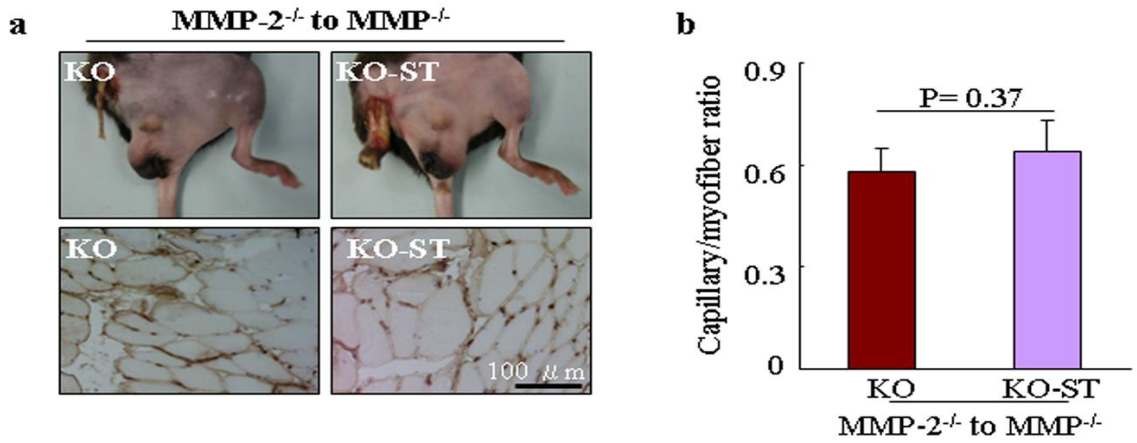
Supplemental Figure III. Expression of SDF-1 mRNA in 4 experimental groups (WT, WT-ST, WT-ST-LY, and WT-ST-DFO). ST enhanced the levels of SDF-1 mRNA in the ischemic muscles of aged MMP-$2^{+/+}$ mice on postoperative day 8; this effect was diminished by LY and enhanced by DFO. ($n = 4$ per group, * $P < 0.05$ versus WT mice; Tukey post hoc tests).

Supplemental Figure IV



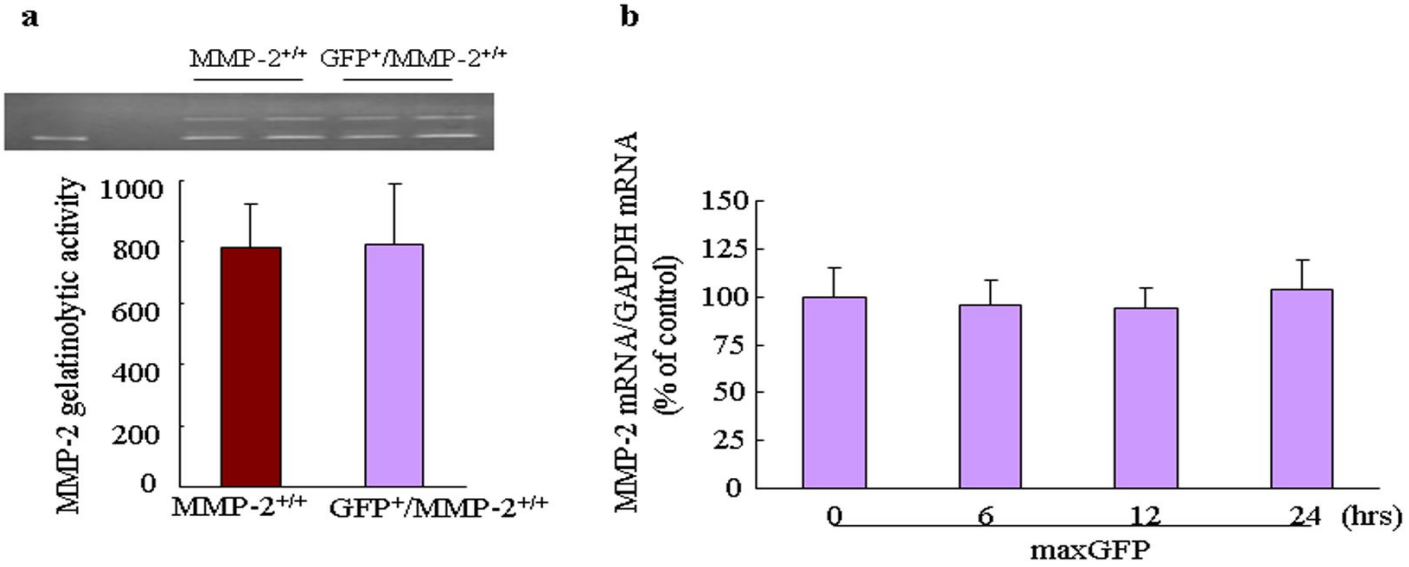
Supplemental Figure IV. Infiltration of macrophage into the ischemic muscles of aged MMP-$2^{+/+}$ and MMP-$2^{-/-}$ mice. (**a**) Representative microscopy of macrophage infiltration in mice of both genotypes. Arrowheads indicate infiltrated macrophages. (**b**) Macrophage infiltration into the ischemic muscles of aged MMP-$2^{-/-}$ mice is reduced ($n = 6$ per group, * $P < 0.05$ versus aged WT mice; Student's t test).

Supplemental Figure V



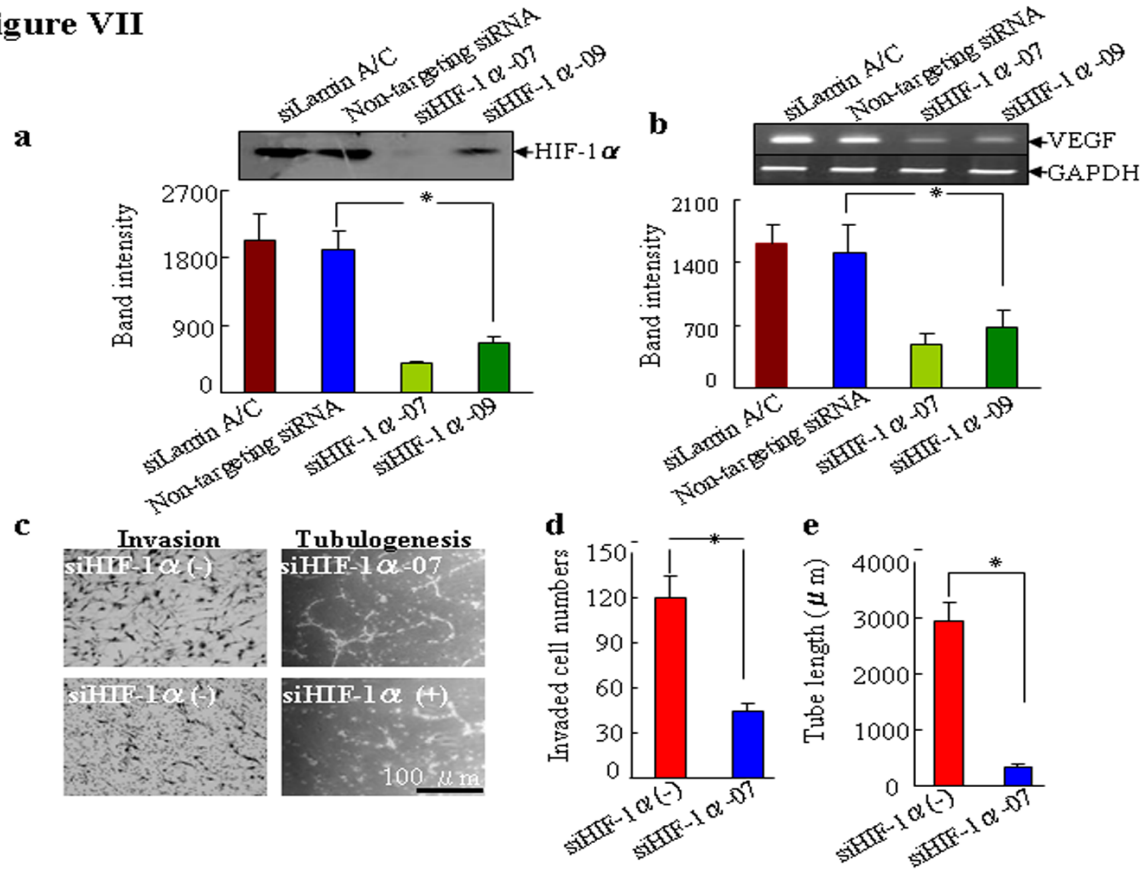
Supplemental Figure V. Effect of cell therapy with the BM of aged MMP-2$^{-/-}$ mice (18 months old) on neovascularization in aged MMP-2$^{-/-}$ mice (24 months old) treated with or without ST. (**a**) Representative photos (upper panels) and immunostaining with an anti-CD31 antibody (lower panels) in the ischemic muscles of aged mice. (**b**) Cell therapy with aged MMP-2$^{-/-}$ BM abolished ST-mediated capillary formation (* $P > 0.05$ versus KO mice, Student's t test).

Supplemental Figure VI



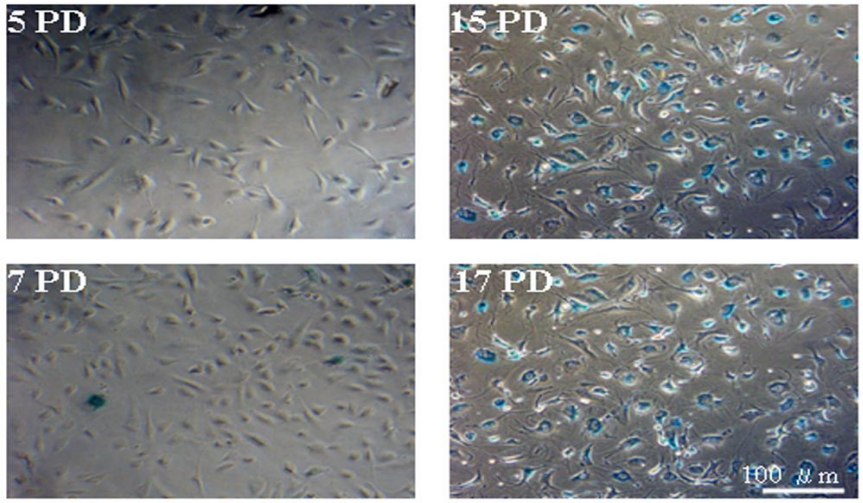
Supplemental Figure VI. Effects of transgenic GFP expression on MMP-2 mRNA expression and protein activity in vivo and in vitro. (**a**) Representative zymography (upper panel) and quantitative analysis of the bands showed that there was no significant difference in MMP-2 activity between mice of these genotypes ($n = 4$, * $P > 0.05$ versus corresponding controls; Student's t test). (**b**) Quantitative real-time PCR showed that maxGFP had no effect on MMP-2 mRNA expression at the indicated time points ($n = 8$, * $P > 0.05$ versus 0 hour; Tukey post hoc tests).

Supplemental Figure VII



Supplemental Figure VII. Effects of HIF-1 α knockdown on invasion and tubulogenesis in HUVECs (passages 5 to 7). (**a, b**) Representative Western and PCR blots (upper panels) showing siRNA-mediated HIF-1 α knockdown and reduction of VEGF mRNA expression in HUVECs. Following transfection with siLamin A/C, a non-targeting siRNA, siHIF-1 α -07, or siHIF-1 α -09, the HUVECs were exposed to hypoxia for 24 h, and the cell lysates were subjected to Western blotting and PCR assays. Levels of HIF-1 α protein and VEGF mRNA were reduced by both siHIF-1 α s ($n = 4$, * $P < 0.05$; Tukey post hoc tests). (**c**) Micrographs showing siHIF-1 α –mediated impairment of invasive and tubulogenic responses in HUVECs. (**d** and **e**) HUVEC invasion and tubulogenesis were impaired by siHIF-1 α ($n = 4$, * $P < 0.05$; Tukey post hoc tests).

Supplemental Figure VIII



Supplemental Figure VIII. Cellular senescence was studied with senescence-associated β -gal staining solution. The relative levels of β -gal–positive staining were higher in old HUVECs (15–17 PD) than in young HUVECs (5–7 PD), suggesting that old HUVECs were recognized as senescent.

Supplemental References

1. Cheng XW, Kuzuya M, Nakamura K, Maeda K, Tsuzuki M, Kim W, Sasaki T, Liu Z, Inoue N, Kondo T, Jin H, Numaguchi Y, Okumura K, Yokota M, Iguchi A, Murohara T. Mechanisms underlying the impairment of ischemia-induced neovascularization in matrix metalloproteinase 2-deficient mice. *Circ Res.* 2007;100:904-913.

2. Cheng XW, Kuzuya M, Nakamura K, Liu Z, Di Q, Hasegawa J, Iwata M, Murohara T, Yokota M, Iguchi A. Mechanisms of the inhibitory effect of epigallocatechin-3-gallate on cultured human vascular smooth muscle cell invasion. *Arterioscler Thromb Vasc Biol.* 2005;25:1864-1870.

3. Kobayashi K, Kondo T, Inoue N, Aoki M, Mizuno M, Komori K, Yoshida J, Murohara T. Combination of in vivo angiopoietin-1 gene transfer and autologous bone marrow cell implantation for functional therapeutic angiogenesis. *Arterioscler Thromb Vasc Biol.* 2006;26:1465-1472.

4. Hayashi T, Matsui-Hirai H, Miyazaki-Akita A, Fukatsu A, Funami J, Ding QF, Kamalanathan S, Hattori Y, Ignarro LJ, Iguchi A. Endothelial cellular senescence is inhibited by nitric oxide: implications in atherosclerosis associated with menopause and

diabetes. *Proc Natl Acad Sci U S A.* 2006;103:17018-17023.