Supplementary Data

Supplementary Materials and Methods

Reagents

Pharm Lyse, chamber slides, 7AAD, and CD31 antibody for immunohistochemistry, or anti-mouse CD31, VEGFR-2, CD117, Sca-1, CD11b, CXCR-4, CD45R, Ter119, Gr-1, TCR $\gamma\delta$, TCR β, and CD45 antibodies for flow cytometry were obtained from BD Biosciences. Hoechst 33342 was purchased from Molecular Probes. Crystal violet was from Applichem. Mouse HO-1 ELISA was from Enzo. Growth factor-reduced Matrigel was obtained from R&D Systems. BS1-lectin was purchased from Vector Laboratories. Reverse transcriptase was from Fermentas and dNTP was from Finnzymes. DiI-Ac-LDLs were from Invitrogen. EBM-2 medium with supplement was obtained from Lonza. Hemin was from Frontier Scientific. Qiazol was from Qiagen. QCM cell migration assay was obtained from Chemicon. RPMI 1640, DMEM, fetal bovine serum (FBS), and Accutase were from PAA Laboratories. Fenozol was purchased from A&A Biotechnology. All other reagents were purchased from Sigma.

Animals

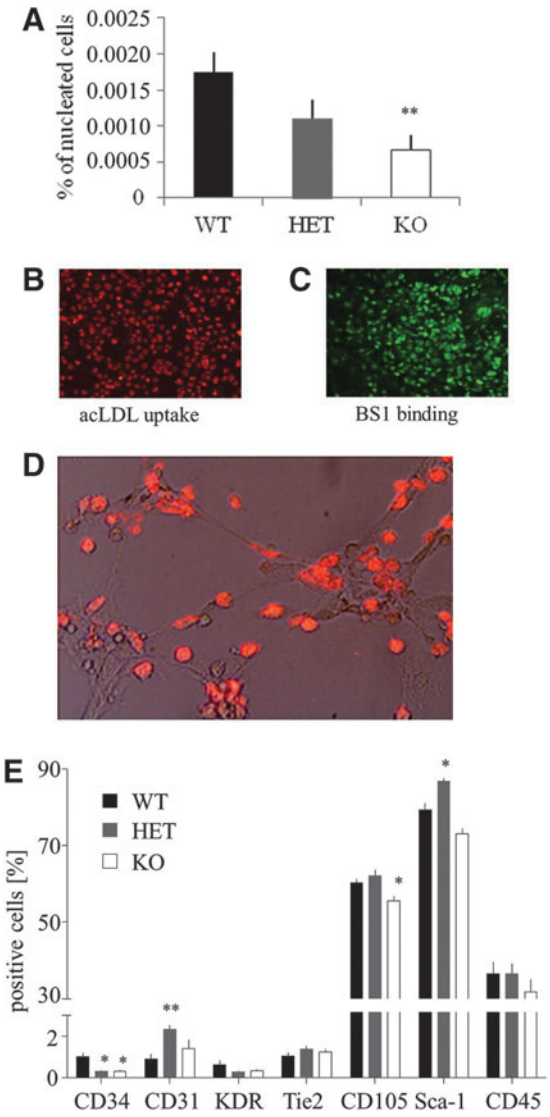
Animals were handled in strict accordance with good animal practice as defined by the relevant national and local animal welfare bodies. All animal work was approved by the local ethics committee for Animal Research at the Jagiellonian University. Breeding heterozygote pairs of HO-1-deficient mice were initially kindly provided by Anupam Agarwal, University of Alabama, (Birmingham, AL).

Flow cytometric detection of endothelial progenitor cell in bone marrow

Bone marrow cells were collected from tibias and femurs of adult mice. Cavities of the bones were flushed with low-glucose DMEM containing 10% FBS. The resulting cell suspension was filtered through a 40 μm strainer, and erythrocytes were lysed by incubation in hypotonic solution. Cells were stained in phosphate-buffered saline (PBS) with 2% FBS for 20 min on ice with antibodies: CD45-FITC (clone 30-F11), VEGFR-2-APC (clone Avas 12α1), CD117-APC-eFluor780 (clone 2B8), and Sca-1-PE-Cy7 (clone D7). For the detection of lineage-committed cells, the following antibodies were used: CD11b-PE (clone M1/70), CD45R-PE (clone RA3-6B2), Ter119-PE (clone TER-119), Gr-1-PE (clone RB6-8C5), TCR $\gamma\delta$-PE (clone GL-3), and TCR β-PE (clone H57-597). In addition, Hoechst 33342 was added at the concentration of 1 μg/ml. Data were collected using an LRS II flow cytometer (BD). Results were analyzed with FACSDiva (BD) and FlowJo (Tree Star) software.

Transcriptome analysis

Quality of isolated RNA was checked using Agilent 2100 Bioanalyzer. Samples with RIN (RNA integrity number) ≥ 9 were used for microarray analysis, according to the vendor's protocols ($n = 3$ per group). In short, RNA (500 ng) was reverse transcribed and cRNA was prepared on the cDNA template using One-Color Low RNA Input Linear Amplification PLUS (Agilent), followed by purification on RNAeasy columns (Qiagen). Resulting cRNA-Cy7 (1500 ng) was analyzed using Agilent Whole Mouse Genome Oligo microarrays with binding sites for 44,000 sequences. Results were analyzed using Feature Extraction Software 9.5.1.1 (Agilent). Values of fluorescence intensity were normalized using CARMAweb.



SUPPLEMENTARY FIG. S1. Characterization of BMDCs. (A) Fraction of endothelial progenitor cell (EPC) ($Hoe^+Lin^-CD45^-cKit^-Sca\text{-}1^+VEGFR\text{-}2^+$) in bone marrow of wild-type (WT, HO-$1^{+/+}$), heterozygous (HET, HO-$1^{+/-}$), and knockout (KO, HO-$1^{-/-}$) mice. Multicolor flow cytometry phenotyping. **(B)** acLDL uptake by bone marrow-derived cells (BMDCs). Representative picture. **(C)** BS1 lectin binding by BMDCs. Representative picture. **(D)** Tube formation by BMDCs seeded on Matrigel. Representative picture. **(E)** Fraction of BMDCs expressing positive endothelial or hematopoietic markers after a 9-day incubation period. Flow cytometry phenotyping. $^*p < 0.05$, $^{**}p < 0.01$ versus WT.

Supplementary Table S1. Venn Analysis Comparing the Effects of Knocking Down HMOX1 on the Transcriptomes of EPCs in Normoxia with Hypoxic Conditions

					Intensities				*HMOX1*$^{-/-}$ vs. *HMOX1*$^{+/+}$				*Hypoxia* vs. *normoxia*			
					Normoxia		*Hypoxia*		*Normoxia*		*Hypoxia*		*HMOX1*$^{-/-}$		*HMOX1*$^{+/+}$	
HMOX1$^{-/-}$ vs. *HMOX1*$^{+/+}$	*Gene symbol*	*Description*	*Accession*	*Mouse entrez ID*	*HMOX1*$^{+/+}$	*HMOX1*$^{-/-}$	*HMOX1*$^{+/+}$	*HMOX1*$^{-/-}$	*Fold*	*FDR (%)*	*Fold*	*FDR (%)*	*Fold*	*FDR (%)*	*Fold*	*FDR (%)*
Altered by HMOX1 knockdown in Normoxia and Hypoxia																
$n=3$ down in HMOX1$^{-/-}$	Hmox1	Heme oxygenase 1	NM_010442	15368	67229	3316	94513	6312	−20.3	0.0	−15.0	0.0	1.9	38.5	1.4	100.0
	Kng1	Kininogen 1	NM_023125	16644	6120	1523	1513	347	−4.0	2.9	−4.4	2.1	−4.4	0.2	−4.0	0.2
$n=3$ up in HMOX1$^{-/-}$	Vsig4	V-set and immunoglobulin domain containing 4	NM_177789	278180	2973	746	2891	808	−4.0	0.4	−3.6	2.1	1.1	100.0	−1.0	100.0
	Aytl1	Acyltransferase like 1	NM_173014	270084	1196	3820	952	3117	3.2	5.3	3.3	8.2	−1.2	100.0	−1.3	100.0
	Wisp1	WNT1 inducible signaling pathway protein 1	NM_018865	22402	761	2446	328	1012	3.2	5.3	3.1	13.5	−2.4	12.3	−2.3	10.8
	Slc40a1	Solute carrier family 40, member 1	NM_016917	53945	5693	18535	5685	16942	3.3	5.2	3.0	16.8	−1.1	100.0	−1.0	100.0
Altered by HMOX1 knockdown in normoxia only																
$n=19$ genes down in HMOX1$^{-/-}$	Cdk5r2	Cyclin-dependent kinase 5, regulatory subunit 2	NM_009872	12570	19590	2627	6875	4569	−7.5	0.0	−1.5	100.0	1.7	100.0	−2.8	4.1
	Synpo	NOD-derived CD11c +ve dendritic cells	AK154958	104027	10916	1734	4553	2548	−6.3	0.4	−1.8	100.0	1.5	100.0	−2.4	34.9
	Agt	Angiotensinogen	NM_007428	11606	9593	1764	1108	510	−5.4	2.0	−2.2	100.0	−3.5	8.3	−8.7	0.0
	Tcfap2e	Transcription factor AP-2, epsilon	NM_198960	332937	53705	12057	34854	22398	−4.5	0.1	−1.6	100.0	1.9	58.7	−1.5	100.0
	Ogn	Osteoglycin	NM_008760	18295	17660	4741	7431	1979	−3.7	18.2	−3.8	20.9	−2.4	45.0	−2.4	33.4
	TC1756779	MUSPTPASE protein tyrosine phosphatase (*Mus musculus*), partial	TC1756779		19428	5524	11349	6191	−3.5	18.7	−1.8	100.0	1.1	100.0	−1.7	100.0
	S3-12	Plasma membrane associated protein, S3-12	NM_020568	57435	5279	1547	7099	2806	−3.4	18.2	−2.5	100.0	1.8	100.0	1.3	100.0
	Rusc1	RUN and SH3 domain containing 1, transcript variant 1	NM_028188	72296	50226	15208	32348	21057	−3.3	5.3	−1.5	100.0	1.4	100.0	−1.6	100.0
	Grin2d	Glutamate receptor, ionotropic, NMDA2D	NM_008172	14814	4345	1389	2450	1708	−3.1	15.8	−1.4	100.0	1.2	100.0	−1.8	75.6
	Rspo2	R-spondin 2 homolog	NM_172815	239405	2062	669	695	445	−3.1	5.0	−1.6	100.0	−1.5	100.0	−3.0	0.6
	AW112010	Bone marrow macrophage	AK153119	107350	7700	2538	13445	5163	−3.0	18.2	−2.6	52.8	2.0	51.0	1.7	78.7
	Cp	Ceruloplasmin, transcript variant 2	NM_007752	12870	17707	6129	18958	13768	−2.9	5.3	−1.4	100.0	2.2	11.8	1.1	100.0
	Lpl	Lipoprotein lipase	NM_008509	16956	21634.5	7825	9272	5768.5	−2.8	2.4	−1.6	100.0	−1.3	100.0	−2.4	2.1

(*continued*)

SUPPLEMENTARY TABLE S1. (CONTINUED)

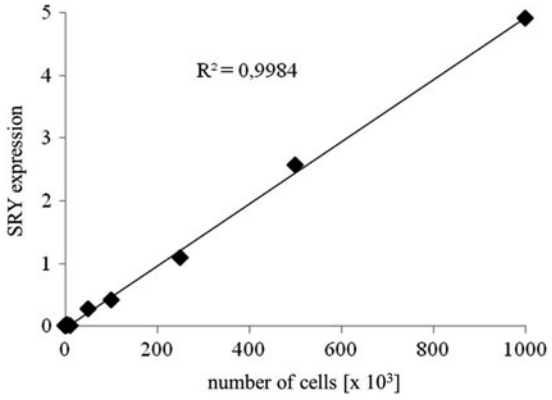
					Intensities				*HMOX1*$^{-/-}$ vs. *HMOX1*$^{+/+}$				*Hypoxia* vs. *normoxia*			
					Normoxia		*Hypoxia*		*Normoxia*		*Hypoxia*		*HMOX1*$^{-/-}$		*HMOX1*$^{+/+}$	
HMOX1$^{-/-}$ vs. *HMOX1*$^{+/+}$	*Gene symbol*	*Description*	*Accession*	*Mouse entrez ID*	*HMOX1*$^{+/+}$	*HMOX1*$^{-/-}$	*HMOX1*$^{+/+}$	*HMOX1*$^{-/-}$	*Fold*	*FDR (%)*	*Fold*	*FDR (%)*	*Fold*	*FDR (%)*	*Fold*	*FDR (%)*
	Ms4a4d	Membrane-spanning 4-domains, subfamily A, member 4D	NM_025658	66607	2109	769	2302	1336	−2.7	13.0	−1.7	100.0	1.7	74.4	1.1	100.0
	Sfrp4	Secreted frizzled-related sequence protein 4	NM_016687	20379	7827	3038	1146	664	−2.6	10.4	−1.7	100.0	−4.6	0.0	−6.8	0.0
	Mmp11	Matrix metallopeptidase 11	NM_008606	17385	4990	2014	4476	3110	−2.5	10.4	−1.4	100.0	1.5	98.9	−1.1	100.0
	Ly6a	Lymphocyte antigen 6 complex, locus A	NM_010738	110454	19984	8894	28404	20668	−2.2	14.8	−1.4	100.0	2.3	1.6	1.4	99.2
	Ly6c	mRNA for Ly-6C variant, complete cds.	D86232	17067	97846	45206	116194	83040	−2.2	15.8	−1.4	100.0	1.8	17.4	1.2	100.0
n=5 genes up in HMOX1$^{-/-}$	Pcolce2	Procollagen C-endopeptidase enhancer 2	NM_029620	76477	470	1651	501	1344	3.5	1.3	2.7	20.9	−1.2	100.0	1.1	100.0
	Ngfb	Nerve growth factor, beta	NM_013609	18049	755	2339	1241	1461	3.1	0.6	1.2	100.0	−1.6	79.5	1.6	51.1
	Serpine1	Serine peptidase inhibitor, clade E, member 1	NM_008871	18787	7291	22710	19872	38063	3.1	2.0	1.9	100.0	1.7	78.6	2.7	0.7
	Hbegf	Heparin-binding EGF-like growth factor	NM_010415	15200	937	2395	1587	3401	2.6	5.3	2.1	40.0	1.4	100.0	1.7	41.1
Altered by HMOX1 knockdown in Hypoxia only																
n=6 genes down in HMOX1$^{-/-}$	Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	NM_010501	15959	4412	1349	15582	1543	−3.3	95.6	−10.1	0.0	1.1	100.0	3.5	6.2
	LOC667370	Similar to interferon-induced protein with tetratricopeptide repeats 3	NM_001005858	667370	306	177	1094	223	−1.7	100.0	−4.9	13.5	1.3	100.0	3.6	6.5
	BC020489	Adult male liver tumor	AK050122	223672	683	282	2265	472	−2.4	100.0	−4.8	3.7	1.7	100.0	3.3	3.5
	Tnfrsf10b	Tumor necrosis factor receptor superfamily, member 10b	NM_020275	21933	564	514	1633	562	−1.1	100.0	−2.9	17.0	1.1	100.0	2.9	1.2
	Mt1	Metallothionein 1, complete cds.	BC027262	17748	538	745	1643	609	1.4	100.0	−2.7	8.2	−1.2	100.0	3.1	0.1
	Lpin1	Lipin 1, transcript variant 2	NM_015763	14245	751	603	2182	897	−1.2	100.0	−2.4	16.8	1.5	100.0	2.9	0.1
n=7 genes up in HMOX1$^{-/-}$	Mcm5	Minichromosome maintenance deficient 5, cell division cycle 46	NM_008566	17218	5024	9556	2347	14072	1.9	100.0	6.0	0.0	1.5	100.0	−2.1	9.4

(*continued*)

SUPPLEMENTARY TABLE S1. (CONTINUED)

					Intensities				$HMOX1^{-/-}$ vs. $HMOX1^{+/+}$				Hypoxia vs. normoxia			
					Normoxia		Hypoxia		Normoxia		Hypoxia		$HMOX1^{-/-}$		$HMOX1^{+/+}$	
$HMOX1^{-/-}$ vs. $HMOX1^{+/+}$	Gene symbol	Description	Accession	Mouse entrez ID	$HMOX1^{+/+}$	$HMOX1^{-/-}$	$HMOX1^{+/+}$	$HMOX1^{-/-}$	Fold	FDR (%)	Fold	FDR (%)	Fold	FDR (%)	Fold	FDR (%)
	Chsy1	Carbohydrate synthase 1	NM_001081163	269941	11879	15851	1956	10176	1.3	100.0	5.2	17.8	−1.6	100.0	−6.1	0.5
	9930013L23Rik	Adult male medulla oblongata	AK018112	80982	4936	6733	4591	14826	1.4	100.0	3.2	13.9	2.3	32.7	−1.1	100.0
	Tk1	Thymidine kinase 1	NM_009387	21877	6284	6670	1563	4653	1.1	100.0	3.0	13.8	−1.4	100.0	−4.0	0.0
	Cyp26b1	Cytochrome P450, family 26, subfamily b, polypeptide 1	NM_175475	232174	953	1483	2457	7310	1.6	100.0	3.0	9.9	4.9	0.0	2.6	2.4
	Col1a1	Procollagen, type I, alpha 1	NM_007742	12842	2577	3896	1748	5017	1.5	100.0	2.9	17.0	1.3	100.0	−1.5	100.0
	Sema7a	Sema domain, immunoglobulin domain, and GPI membrane anchor, 7A	NM_011352	20361	2958	5942	1951	5318	2.0	100.0	2.7	8.6	−1.1	100.0	−1.5	90.8

EPC, endothelial progenitor cell.



SUPPLEMENTARY FIG. S2. Standard curve for quantitation of BMDCs surviving within the skin. The curve was obtained from the *sry* gene expression in the sorted male bone marrow cells.

Detection of proliferation

Bone marrow-derived cells (BMDCs) were cultured in standard conditions until they reached 50% confluence. Then, media were changed to EBM-2 with 0.5% FBS for 24 h. After that, cells were washed with PBS, detached with Accutase, and stained with Hoechst 33342 for 45 min at 37°C and then with pyronin Y for 15 min at 37°C. Data were collected using an LRS II flow cytometer (BD). Results were analyzed with FACSDiva (BD) and FlowJo (Tree Star) software.

Migration assay

Migration was measured using QCM Cell Migration Assay with inserts of diameter of 8 μm (Chemicon). Cultured cells were detached with Accutase, seeded (10,000 cells/well) on transwell filters (untreated or coated on the underside with EGM complete medium), and incubated overnight at 37°C. The migrating cells were fixed in 3% paraformaldehyde for 10 min, washed with PBS, and stained with 0.5% crystal violet for 15 min. Then, the cells localized within the filter or on the underside were counted.

Tube formation on Matrigel

Fifty microliter of growth factor-reduced Matrigel was loaded in a 96-well plate and incubated at 37°C for 15 min. For a paracrine-effect study, BMDCs were incubated in EBM-2 containing 0.5% FBS for 24 h. After this period, conditioned media were collected and used for the incubation of HAEC cells plated on the Matrigel. EBM-2 with 0.5% FBS or EGM-2 with 10% FBS served as negative or positive controls, respectively.

Cell survival in vivo

About 200,000 viable cultured BMDCs isolated from HO-$1^{+/+}$ or HO-$1^{-/-}$ male mice were injected intradermally into female HO-$1^{+/+}$ mice. Skin explants were harvested at 6 h, and at days 3, 7, and 14 after the injection. In another experimental setting, 500,000 viable cultured BMDCs isolated from HO-$1^{+/+}$ male mice were injected intravenously into female HO-$1^{+/+}$ mice, which underwent femoral artery ligation 24 h before the cell injection. Ischemic *caput gastrocnemius* muscle was harvested at 6 h, and at days 3, 7, and 14 after the injection. Genomic DNA was isolated and equal volumes of samples were subjected to real-time PCR (StepOnePlus; Applied Biosystems), with the following primers for male specific *sry* gene: 5′-TTG TCT AGA GAG CAT GGA GGG CCA TGT CAA-3′ and 5′-CCA CTC CTC TGT GAC ACT TTA GCC CTC CGA-3′. The number of cells present in the skin or in ischemic *caput gastrocnemius* muscle was calculated using a standard curve (Supplementary Fig. S2). To prepare a standard curve, the genomic DNA was isolated from 50, 500, 1000, 5000, 10,000, 50,000, 100,000, 250,000, 500,000, and 1,000,000 male BMDCs, sorted using MoFlo XDP sorter (Beckman-Coulter). Then, the quantitative real-time PCR with the *sry*-specific primers was carried out.

Cell therapy of wounds and ischemic limbs with BMDCs

For the wound-healing experiment, BMDCs isolated from HO-$1^{+/+}$ or HO-$1^{-/-}$ mice were stained with PKH67 according to the vendor's protocol. The efficacy of staining was 100% with a cell mortality of 2% as assessed by PI incorporation. HO-$1^{-/-}$ mice were anesthetized with isoflurane and shaved. Then, the skin was disinfected with 70% ethanol, and two full-thickness excisional wounds, both of which were 4 mm in diameter, were generated with a disposable biopsy punch tool (Stiefel) on either side of the dorsal midline of each mouse. The wounds were separated well by >1.5 cm of skin. Immediately after injury, 900,000 viable HO-$1^{+/+}$ or HO-$1^{-/-}$ cells or PBS were injected intradermally into four places around the wound. Each wound was photographed every day using a camera EOS350D (Canon) with an objective EF-S 60 mm f/2.8 Macro USM (Canon). Wound surfaces were measured using the ImageJ program (National Institutes of Health) and expressed as a percentage of the wound area at day 0. Mice were sacrificed at day 14 after injury. The presence of PKH-positive cells within the wounds was assessed in the frozen sections of wounds under the fluorescence microscope (Nicon Eclipse TX-100).

For the hind limb ischemia experiment, HO-$1^{+/+}$ or HO-$1^{-/-}$ mice were subjected to femoral artery ligation according to the procedure described elsewhere (49). The femoral artery was ligated at the proximal end of the femoral artery and at the distal end of the femoral artery proximal to the popliteal artery. PBS or 500,000 BMDCs isolated from HO-$1^{+/+}$ or HO-$1^{-/-}$ individuals were injected intravenously next day after the surgery. Blood flow in ischemic and contralateral limbs was measured before cell injection and at day 14 using a Laser Doppler Perfusion Imager System (PIM II; Perimed). The ischemic-to-nonischemic foot blood flow ratio was calculated as an index of blood flow recovery.

Gene expression analysis in caput gastrocnemius muscle

Caput gastrocnemius muscle was lysed in Qiazol. Total cellular RNA was isolated with a modified guanidinium isothiocyanate method and reversely transcribed. mRNA transcript levels were checked by real-time PCR (StepOnePlus; Applied Biosystems), with the following primers: CXCR-4—5′-AAA CCT CTG AGG CGT TTG GT-3′ and 5′-AGC AGG GTT CCT TGT TGG AG-3′, EF2—5′-GCG GTC AGC ACA ATG GCA TA-3′ and 5′-GAC ATC ACC AAG

GGT GTG CAG-3′, HO-1—5′-CCT CAC TGG CAG GAA ATC ATC-3′ and 5′-CCT CGT GGA GAC GCT TTA CAT A-3′, PlGF—5′-CAA GGG GGA CGA GCA TGG TGA TTG-3′ and 5′-GGC CGA CAG TAG CTG CGA CCC-3′, SDF-1α—5′-CCT TCA GAT TGT TGC ACG GCT GA-3′ and 5′-CCC ACC ACT GCC CTT GCA TC-3′, VEGF—5′-ATG CGG ATC AAA CCT CAC CAA GGC-3′ and 5′-TTA ACT CAA GCT GCC TCG CCT TGC-3′, VEGF-R1—5′-GCA CCT ATG CST GCA GAG C-3′ and 5′-TCT TTC AAT AAA CAG CGT GCT G-3′, VEGF-R2—5′-CCT CAC CTG TTT CCT GTA TGG AG-3′ and 5′-GAK GCC ACA GAC TCC CTG C-3′. The reaction mixture contained cDNA template (20 ng/ml in case of housekeeping EF2 gene or 40 ng/ml in case of the gene of interest), SybrGreen 2$\times$(7.5 μl), primers (1 μl, 10 μM), and RNAse free water for approximately 15 μl.

Measurement of HO-1 protein in *caput gastrocnemius* muscle was done with ELISA (Enzo) according to the vendor's protocol.

Progenitor cell mobilization

Peripheral blood was harvested from *vena cava* superior into heparinized syringe, and erythrocytes were removed with PharmLyse buffer. After washing, cells were incubated with FITC-labeled anti-mouse Sca-1 (clone E13-161.7) and PE-labeled anti-mouse CXCR-4 (clone 247506 IgG_{2b}) antibody for 30 min at 4°C in RPMI 1640 medium containing 2% FBS, according to the manufacturer's recommendation. Data were collected using a cytofluorometer (LSRII; BD) and analyzed using FACSDiva software (BD).

CD31 immunohistochemical staining

Caput gastrocnemius muscles were excised, embedded in OCT compound (Tissue-Tek), and snap frozen in liquid nitrogen. Histological transverse 6 μm-thick cryostat sections were used to assess capillary density. Sections were dried (1 h, room temperature) and fixed for 10 min in cold acetone. Capillaries were stained with rat anti-mouse CD31 antibody (clone 550274) and with the rhodamine-conjugated goat anti-rat antibody (clone 55767). Then, they were blocked in 10% goat serum, 0.05% Tween-20, and 0.1% Triton X-100 (1 h, room temperature); incubated with anti-CD31 antibody (1.5 h, room temperature); washed; incubated with secondary antibody (30 min, room temperature); washed again; and mounted with fluorescent mounting medium. Counting was performed at 200$\times$magnification using a fluorescence microscope (Nikon Eclipse TX100).