Data Supplement

Murine hind limb ischemia model

All surgical procedures and laser speckle blood flow analysis (see below) were performed under anesthesia with intraperitoneal pentobarbital sodium salt injection (80 mg/kg). To induce unilateral hind limb ischemia, skin incision was performed at the mid-portion of the right hind limb overlying the femoral vessels. The femoral vessels were then gently isolated without damaging the femoral nerve. The proximal portion of the femoral artery and the distal portion of the saphenous artery were ligated with 7-0 silk ligatures (Natsume Seisakusyo Co., Ltd., Tokyo, Japan). The remaining branches between these two sites as well as veins were all dissected free and then excised. The overlying skin was closed using a 3-0 silk suture (Akiyama Seisakusyo, Tokyo, Japan). After surgery, mice were kept on a heating plate at 37°C to monitor the operated animals until they recovered completely from anesthesia [1, 2]. All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee, The University of Tokushima Graduate School.

Macroscopic evaluation of ischemic severity

After the operation, clinical outcomes of all mice (n=17 for each male mice group, n=12 for each female mice group) were observed and recorded at 4 time points (at days 3, 7, 14 and 21 after surgery). The ischemic limb was macroscopically evaluated, and diagnosis of hindl imb autoamputation was determined as extension of necrosis above the crus (knee loss and total hind limb loss).

Laser speckle blood flow analysis

We measured hind limb blood flow using a laser speckle blood flow (LSBF) imager (OMEGAZONE OZ-1, OMEGAWAVE, Inc., Tokyo, Japan) at 6 time points (before surgery, and at days 1, 3, 7, 14 and 21 after surgery). In the LSBF imager, low or no perfusion is displayed as dark blue, whereas the highest perfusion interval is displayed as yellow to red. The stored perfusion values behind the color-coded pixels remain available for data analysis. Previous studies [1, 3] established that laser Doppler flow velocity correlates with capillary density in ischemic limbs of animal models, and Briers et al. demonstrated that the two techniques of laser Doppler blood flow analysis and LSBF analysis were essential for measuring line-of-sight velocities [4]. Excess hair was removed from the hind limb using a depilatory cream, and before initiating scanning, mice were placed on a heating plate at 37°C to minimize variations in temperature. For each time point, we obtained two consecutive LSBF images over the same region of interest (legs and feet) (n=17 for each male mice group, n=12 for each female mice group). Accordingly, the averaged flows of the ischemic and nonischemic limbs were calculated on the basis of colored histogram pixels. To avoid the influence of ambient light and temperature, LSBF data were expressed as the ratio of ischemic (right) to normal (left) hindlimb perfusion. At day 21, the male mice were sacrificed, and the thigh adductor muscles of bilateral limbs were harvested for capillary density analysis (see below).

TUNEL staining

< Immunofluorescence > At day 1 after hind limb ischemic surgery, the male mice (n=8 for each

male mice group) were euthanized. After the thigh adductor muscles of ischemic limbs had been removed, they were embedded in OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and snap-frozen in liquid N_2. Multiple cross cryosections of 6 μm in thickness were prepared, and apoptotic cells were analyzed by TUNEL staining using an *In situ* Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany). Briefly, the samples were fixed in 4% paraformaldehyde for 20 min at room temperature and then washed with PBS for 30 min. After incubating in permeabilization solution for 2 min and washing with PBS two times, the samples were incubated with TUNEL reaction mixture at 37°C in a humid condition for 1 hr and then encapsulated by mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA). We evaluated the number of apoptotic cells using fluorescence microscopy (Nikon Corporation, Tokyo, Japan). TUNEL-positive cells were counted in five randomly selected microscopic fields in each plate (magnification, 100×), and the percentages of apoptotic cells were expressed as TUNEL-positive cells per total number of DAPI-positive cells.

<Enzyme antibody technique> The ischemic adductor muscles at day 1 were fixed in 4% paraformaldehyde overnight. After fixation, the muscles were embedded in paraffin and were serially cut into 3-μm-thick slices. After being deparaffinized and hydrated, the sections were washed in deionized water, and endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. Next, the sections were treated with 20 μg/ml proteinase K for 10 min at room temperature and rinsed in PBS. Then the sections were incubated with a reaction reagent (ApopTag Peroxidase *In Situ* Apoptosis Detection Kit, CHEMICON International, Inc., Temecula, CA) for 60 min at 37°C and with 1:500-diluted peroxidase-conjugated streptavidin

for 30 min at room temperature, rinsed in PBS, incubated with 3.3'-diaminobenzidine tetrahydrochloride for 10 min at room temperature, washed in water, and finally counterstained with methyl green for 30 min. The sections were examined under a light microscope (Olympus Corporation, Tokyo, Japan, magnification, 400×).

CD31 and αSMA immunostaining

As described above, fresh-frozen sections of hindlimb adductor muscles at day 21 after ischemic surgery (n=8 in each male mice group) were prepared. The sections were histochemically stained with CD31 antibody (BD Pharmingen, Franklin Lakes, NJ) to identify capillary endothelial cells and smooth muscle actin (SMA) antibody (Dako North America, Inc. Carpinteria, CA) to identify vascular pericytes. Immunostains were visualized by using an amino acid-polymer system (Nichirei Bio, Tokyo, Japan), DAB (for CD31) and Permanent Red (for SMA). Capillaries and arterioles in both ischemic and nonischemic limbs were analyzed for specific evidence of neovascularity. Endothelial cells positively stained with CD31 antibody, vascular pericytes positively stained with SMA antibody and muscle fibers were counted under a light microscope (Olympus Corporation, Tokyo, Japan, magnification, 200×). Five random microscopic fields from 2 different sections (50 μm apart) in each mouse were counted. Capillary density was expressed as number of CD31-positive cells per muscle fiber or per square millimeter. Arteriolar (pericyte) numbers were expressed as number of SMA-positive cells per square millimeter [2, 5].

Aortic ring assay

Descending thoracic aortas from male WT and *AR*KO mice were excised (*n*=6 in each male mice group), and fibro-adipose tissue around the aorta was carefully removed. Under a dissecting microscope, five 1-mm-thick aortic rings were prepared from each aorta. The aortic rings were embedded in 3-dimensional gels of Matrigel Basement Membrane Matrix (BD Biosciences, Bedford, MA). Matrigel Basement Membrane Matrix solution (200 μl) was transferred to each well of 48-well plates and gelatinized to make a base layer at 37°C for 30 min. Each aortic ring was placed on the basal layer of the Matrigel in the center of the well, covered with an additional 100-μl aliquot of Matrigel solution and allowed to gel for 30 min. Five hundred μl of EGM-2 medium (Lonza, Walkersville, MD) was then added to each well and cultured for 7 days. At day 7, the total number and total length of sprouting microvessels were determined under a light microscope (Olympus Corporation, Tokyo, Japan, magnification, 40×) and using ImageJ version 1.29 [6, 7].

***In vivo* angiogenesis assay**

Quantitative *in vivo* angiogenesis assay using DIVVA (Trevigen, Inc. Gaithersburg, MD) was performed in male WT and *AR*KO mice. According to the manufacturer's instructions, implant-grade silicone cylinders were each filled with 20 μl of basement membrane extract (BME) premixed with PBS as negative control or 30 ng FGF-2 with 10 ng VEGF. These cylinders were implanted subcutaneously in the dorsal flanks of male WT and *AR*KO mice (*n*=10 for each male mice group, 2 cylinders implanted in each mouse). At 15 days after implantation, vascular endothelial cells had migrated into and proliferated in the BME with the angiogenic factor to form vessels in the silicone cylinders. The cylinders were removed from

each mouse and BME/vessel complex was extracted and collected as cell pellets. The cell pellets were labeled with DIVVA FITC-Lectin and fluorescence was measured.

Bone marrow transplantation (BMT)

Nineteen-week-old male WT and *AR*KO mice (n=10 in each recipient group) were irradiated with a dose of 9.5 Gy from an X-ray source at a dose rate of approximately 0.90 Gy/min. Bone marrow cells (BMCs) from age-matched donor male WT and *AR*KO mice were harvested by flushing the femurs and tibias, and red cells were removed by lysis using ammonium chloride. BMCs in suspension were counted, and 1×10^7 cells/mouse were injected through the orbital vein of the recipient immediately after irradiation [8]. In order to determine whether BMT was successfully completed, we created chimeric mice transplanted with BMCs from CD45.1 mice (B6.SJL-PtprcaPep3b/BoyJ). The chimeric ratio of BMCs was more than 95% as determined by fluorescence-activated cell sorting analysis of the chimeric mice at 6 weeks after BMT [8] (data not shown). Six weeks after BMT, the recipient male WT and *AR*KO mice were subjected to hindlimb ischemia as described above. At indicated time points, we also examined limb survival rate and blood flow reperfusion.

Western blot analysis

For Western blotting analysis and real-time PCR analysis (see below), adductor muscles before ischemic surgery and ischemic muscles at day 1 after surgery were excised (n=8 in each male mice group). Phosphorylation of Akt, eNOS and Erk1/2 and expression of Bcl-2 and BAX were evaluated by Western blot analysis. Protein extraction from muscle and Western blot

analysis were performed as described previously [9]. In brief, 50-μg protein extracts from the muscles of WT and *AR*KO mice were boiled for 5 min in Laemmli sample buffer and then run on SDS-PAGE. The protein extracts were then transferred to a PVDF membrane (Millipore Corporation, Bedford, MA). The membrane was blocked for 20 min at room temperature with SuperBlock T20 TBS Blocking Buffer (Thermo Scientific, Rockford, IL). The blots were incubated overnight at 4°C with each primary antibody, followed by incubation for 1 hr with anti-rabbit secondary antibody (horseradish peroxidase-conjugate). Immunoreactive bands were visualized using enhanced chemiluminescence with ECL-PLUS reagents (GE Healthcare, Buckinghamshire, UK) and exposure to a lumino image analyzer (LAS-3000mini) (Fujifilm Corporation, Tokyo, Japan). The signals were quantified by densitometry using ImageJ version 1.29. We analyzed 8 independent samples for each time point in each group and detected the phosphor-specific antibody first, then the total antibody, and finally the internal control antibody. Phospho-specific proteins were normalized by total protein, and total protein was corrected by GAPDH as an internal control. We used primary antibodies against phosphorylated Akt (Ser473), total Akt, phosphorylated ERK1/2 (Thr202/Tyr204), total ERK1/2, phosphorylated eNOS (Ser1177), GAPDH (Cell Signaling Technology, Beverly, MA), total eNOS (BD Bioscience, San Jose, CA), Bcl-2 and BAX (Santa Cruz Biotechnology, Inc., CA).

Quantitative real-time PCR

RNA extraction and reverse transcriptase-polymerase chain reaction were performed as described previously [9, 10]. In brief, thigh adductor muscles were homogenized in TRIzol (Invitrogen, Carlsbad, CA) and total RNA was extracted. Total RNA of 1 μg was used for

cDNA synthesis with a QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The PCR mixture contained cDNA, generated from 2.5 ng of total RNA, 0.1 nmol/l forward and reverse primer mix, and SYBR Green (Platinum SYBR Green qPCR SuperMi-UDG, Invitrogen Carlsbad, CA). Assays were performed with a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Amplification included one stage of 2 min at 50°C and one stage of 2 min at 95°C followed by 40 cycles of a 2-step loop: 15 seconds at 95°C and 30 seconds at 60°C. Commercially available PCR primers were purchased from Perfect real-time primer (TAKARA BIO INC. Ohtsu, Japan) for *Hif1a, Vegfa, Fgf2, Kdr, Bcl2, Bax* and *Gapdh*. Results were analyzed with the 7300 System software, and transcript levels were adjusted relative to the expression of *Gapdh* as an internal control. The eight independent samples for each time point in each group were used, and duplicate results per sample were averaged.

Cell preparation

Male HUVECs were purchased from Cell Applications Inc. (San Diego, CA) and were maintained in EGM-2 medium (Lonza, Walkersville, MD) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were grown to confluence and then the medium was changed without a growth factor (EBM-2 medium with 0.5% FBS) and cultured overnight. Cells were treated with or without 100 nM 5alpha-dihydrotestosterone (DHT) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and 10 ng/ml human recombinant VEGF (Sigma-Aldrich, Inc., Saint Louis, MO) for 15 min [11]. Cultured cells with various treatments were washed twice with cold PBS and harvested after being lysed for 30 min at 4°C in a lysis buffer (Cell Signaling

Technology, Beverly, MA). Supernatants obtained by centrifugation were used for Western blot analysis and immunoprecipitation. The procedure for Western blot analysis was described previously [9, 12]. Antibodies against phosphorylated Akt (Ser473), total Akt, phosphorylated eNOS (Ser1177) and GAPDH were purchased from Cell Signaling Technology (Beverly, MA). Total eNOS was purchased from BD Bioscience (San Jose, CA).

Small interfering RNA (siRNA) experiments

HUVECs were plated in 12-well plates (1×10^5 per well) in EGM-2 medium without antibiotics. Twenty-four hours later, cells were transfected with siRNA targeting *AR* and nontargeting siRNA (negative control) using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA). The siRNA of *AR* (Stealth RNAi siRNA; Invitrogen, Carlsbad, CA) and negative control duplexes (Stealth Select RNAi; Invitrogen, Carlsbad, CA) were purchased. According to the manufacturer's protocol, 2 μl of lipofectamine RNAiMAX and 2 pmol RNAi duplex were added directly to 200 μl of Opti-MEM I Reduced Serum Media (Invitrogen, Carlsbad, CA) per well and incubated for 15 min. The RNAi duplex-Lipofectamine RNAiMAX complexes were added to each well containing cells, and the cells were incubated. The medium was changed without a growth factor (EBM-2 medium with 0.5% FBS) at 24 hr after transfection, and the transfected cells were stimulated with 10 ng/ml VEGF and 100 nM DHT for 15 min at 36 hr after transfection. Then the cells were washed with PBS and proteins were isolated from HUVECs. At 36 hr after transfection with this siRNA, mRNA and protein expression levels of AR were reduced to 13% and 15% of the control levels, respectively (data not shown).

Immunoprecipitation

The lysates of treated HUVECs, including 500 μg protein, were precleaned with protein A/G agarose beads (Santa Cruz Biotechnology, Inc., CA) and rabbit IgG antibody (Cell Signaling Technology, Beverly, MA) at 4°C for 1 hr, and then supernatants were collected. The lysates were incubated with the first protein-specific antiserum (anti-AR antibody (Santa Cruz Biotechnology, Inc., CA) and anti-KDR (VEGFR2) antibody (Cell Signaling Technology, Beverly, MA)) at 4°C overnight and then gently agitated with agarose beads for 1 hr. Immune complexes were collected by centrifugation followed by 3 washes with lysis buffer and boiling in sample buffer for 5 min. The immunoprecipitated proteins were subjected to Western blot analysis. Antibodies against anti-Src and PI3kinase p85 were purchased from Cell Signaling Technology (Beverly, MA).

HaloTag pull-down assay

HUVECs were grown to 80% confluency and transfected with plasmid DNA encoding HaloTag plus human *AR* cDNA (Kazusa DNA Res. Inst., Chiba, Japan) and control HaloTag protein (Promega Corporation, Madison, WI) using Lipofectamine LTX Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. At 24 hr after transfection, the transfected cells in EGM-2 medium including VEGF were stimulated with 100 nM DHT for 15 min and harvested. Cell lysates including HaloTag-AR fusion protein and control lysates were prepared using dedicated lysis buffer and protease inhibitor cocktail (Promega Corporation), HaloTag Pull-down assay was performed with HaloLink magnetic beads (Promega Corporation) according to the manufacturer's protocol. Cell lysates were incubated with prepared HaloLink

magnetic beads for overnight at 4°C, washed 4 times and resuspended in SDS gel loading buffer. Samples were heated to 95°C for 5 min, remove supernatant and load on an SDS-PAGE gel and analyzed by Western blot using anti-KDR (VEGFR2), Src and PI3kinase p85 antibody (Cell Signaling Technology) and anti-HaloTag antibody (Promega Corporation).

***In situ* proximity ligation (*in situ* PLA) assay**

HUVECs grown on a culture slide (BD Biosciences, Bedford, MA) were starved without a growth factor overnight and incubated in the presence or absence of 10 ng/ml VEGF and 100 nM DHT for 10 min. The cells were washed with chilled PBS and fixed with 4% paraformaldehyde for 30 min, permeabilized by 0.5% Triton X-100, and thereafter subjected to *in situ* PLA using Duolink II PLA probe anti-Rabbit PLUS, anti-Mouse MINUS and Detection Reagents (Olink Bioscience, Uppsala, Sweden) according to the manufacturer's instructions. Briefly, slides were blocked and incubated overnight with primary antibodies from two different species directed against mouse anti-KDR (VEGFR2) (Cell Signaling Technology) and rabbit anti-AR (Abnova Corporation, Taipei, Taiwan). The slides were then incubated with secondary antibodies (anti-mouse and anti-rabbit) conjugated with PLA probes PLUS and MINUS, and the two oligonucleotides and ligase were added. The oligonucleotides would hybridize to the two PLA probes and join to a closed circle if they were in close proximity. Nucleotides and fluorescently labeled oligonucleotides were added together with polymerase, and the PLA signals were visible as a distinct fluorescent spot. Next, filamentous actin staining was performed. Fluorescent phallotoxin staining solution (Alexa Fluor 488 phalloidin, Molecular Probes, Inc., Eugene, OR) was placed on the slide for 20 min at 37°C and washed

two times with PBS. The cells were air-dried and then mounted in mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA) and analyzed in a confocal microscope (Nikon Corporation, Tokyo, Japan). We chose randomly five microscopic fields in four independent preparations individually and evaluated the density of PLA signals per cell (magnification, 400×) [13].

Statistical analysis

Values for each parameter within a group are expressed as means ± SEM. For comparisons among groups, statistical significance was assessed with one-way ANOVA, and the significance of each difference was determined by *post-hoc* testing using Tukey's honestly significant difference test or Games-Howell method. These analyses were performed by using Excel (Microsoft Office Excel 2007; Microsoft, Richmond, CA) and PASW Statistics 18.0 (IBM SPSS Japan Inc., Tokyo, Japan). Statistical significance was considered at $P < 0.05$.

Supplemental References

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Figure legend

Supplemental Figure 1.

Reduced arteriolar numbers in both male and female *AR*KO mice after ischemia

(A and C) α-SMA immunohistochemical staining to determine arterioles in ischemic and nonischemic thigh adductor muscles in WT and *AR*KO mice at day 21 after surgery (A: male mice, C: female mice). Scale bar indicates 100 μm. (B and D) Quantification of arteriolar numbers is expressed as αSMA-positive cell number per square millimeter in WT and *AR*KO mice (B: male mice, D: female mice). n=16 in each group. ** using Dunn's test following the Kruskal-Wallis test. Bars represent mean values in each group.

Supplemental Figure 1