SUPPLEMENTAL MATERIAL.

Detailed Methods

Cell culture, shear stress and cyclic stretch assays PECAM-1 knockout (PE-KO) cells and cells reconstituted (PE-RC) with full-length PECAM-1 were prepared as described.[1, 2] Levels of PECAM-1 in reconstituted cells are similar to wild-type levels.[2] Laminar shear stress assay was described previously[3]. Briefly, confluent cells were incubated in starvation media (full medium with 0.5% fetal bovine serum) overnight before flow experiments. Cells on 10μg/ml fibronectin-coated slides were exposed to shear stress using the cone and plate flow chamber system for 24h at 12 dynes/cm^2. Mechanical stretch assay was described previously.[4] Briefly, cells were plated on 6-well Bioflex 10μg/ml fibronectin-coated plates (Flexcell International, Hillsborough, NC). Uniform biaxial strain was applied (15% magnitude, 1Hz for 24 hours) using the Flexcell FX-4000 system. Static controls were cells not subjected to shear stress or cyclic stretch. To visualize F-actin filaments and cell boarders, cells were stained with TRITC-labeled phalloidin (1:100) and β-catenin (1:100, Sigma-Aldrich, St. Louis, MO). The angle of actin filaments relative to the direction of stretch was measured using *NIH Image J* package. The angle average and S.E were calculated to compare the F-actin alignment in both genotypes.

Western blotting Cells were washed with PBS and lysed in 10x sample buffer (1ml/100mm dish, or 125ul/well). Immunoblotting was performed using anti-ephrin B2 (1:300, R & D systems, Minneapolis, MN) and mouse anti-α-tubulin (1:1000, Sigma-Aldrich) and Alexa 680-conjugated donkey anti–goat (Invitrogen, Carlsbad, CA) followed by IRDye800-conjugated goat anti–mouse antibodies (Rockland, Gilbertsville, PA). Membranes were scanned with an Odyssey laser scanner and quantified with Image J package.

Animals Wild-type C57/BL6 (PECAM-1$^{+/+}$) mice were purchased from Charles River Laboratories International, Inc (Wilmington, MA). PECAM-1$^{-/-}$ C57BL/6 mice were kindly provided by Dr. P. Newman (Blood Research Institute, Blood Center of Wisconsin, Milwaukee, WI). PECAM-1$^{+/+}$ C57BL6 mice and PECAM-1$^{-/-}$ mice had been backcrossed for >12 generations onto a C57BL6 background.[5, 6] The animals were bred in house and used in accordance with the guideline of the National Institute of Health and for the care and use of laboratory animals (approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill). Male PECAM-1$^{-/-}$ and age-matched littermates (PECAM-1$^{+/+}$, 10-14 weeks) were used for all experiments. To genotype animals, DNA was isolated from ears at weaning and PCR performed. All analyses were conducted by observers blinded to animal phenotype.

Unilateral hindlimb ischemia The surgery procedure was performed on the right side as described previously.[7] Briefly, animals were anesthetized with 1.125% isoflurane supplemented with oxygen, and body temperature was maintained at 37°C. Hair was removed from the hindquarters with a depilating cream. The femoral artery was exposed aseptically through a 2mm incision and isolated from vein and nerve. The femoral artery was ligated with 7-0 ligatures proximal to the bifurcation of the popliteal artery and distal to the lateral caudal femoral artery (LCFA) for the less severe ischemia mode. The incision was closed after the wound was irrigated with sterile saline.

Laser-Doppler Imaging The animals were placed for 5min at a 37°C chamber before the measurements to avoid vasoconstriction by anesthetic heat loss. A Laser Doppler imager was used to estimate relative blood flow. Ratios of occluded over non-occluded values were compared. Noninvasive measurements of superficial hindlimb perfusion were obtained before ligation, immediately after ligation (acute), 7 and 21 days after ligation.[8] Plantar perfusion was quantified within anatomically defined regions of interest (ROIs). All ROIs were drawn by an

investigator blind to animal genotype. Data are reported as perfusion ratios of ligated vs. sham control side.

Postmortem angiography Arterioles and collaterals were counted as described previously.[7, 9] Briefly, mice were perfusion-fixed with 4% paraformaldehyde (PFA) at 100 mmHg. The vasculature was injected with barium sulfate to provide X-ray arteriograms. Films were digitized, and index of pre-existing collateral number in the acutely ligated limb was measured by counting arteries crossing a Rentrop-like line beginning at the midpoint between the proximal and distal ligations of the femoral artery and extending to the posterior edge of the thigh.

Morphometry Collateral arteries were harvested from mice as described previously.[7] Briefly, animals were transcardially perfused at 100 mmHg with PBS containing 10 nmol/l sodium nitroprusside and 10U/ml heparin 3 weeks after hindlimb ischemia. PBS was followed by 2% PFA for 20 min. We harvested the anterior and posterior *gracilis* muscles which contain two preexisting collaterals. The midzone of the muscles (i.e., the 5-mm-wide centermost section) was trimmed. A section of the calf (*gastrocnemius/soleus*) muscle was also harvested for the examination of capillary density (described below). Samples were embedded in paraffin and 5μm thick cross sections were H.E. stained. Lumen diameter of collateral arteries was measured as previously described.[8] Briefly, HE stained cross-sections within 0.5mm from the midzone of the collateral arteries in anterior and posterior *gracilis* muscles were digitized at 60x magnification. Lumen circumference (C) was measured interactively using NIH Image J package. Lumen diameter (D) was calculated from the circumference as $D=c/\pi$. For each mouse, four arteries were studied, two from the surgery-operated side and two from the sham control side. At least 4 cross-sections from each mouse were measured and the average lumen diameter of collateral vessel was used. Samples were selected by an observer blinded to wild-type vs. PECAM-$1^{-/-}$ genotype.

Silver staining The staining procedure was previously described and slightly modified [10]. Mice were euthanized and perfused sequentially with 5ml PBS, 5ml 0.2% AgNO3 and 5ml PBS through abdominal aorta using 5ml syringes, followed by 60min fixation with 4% paraformaldehyde at 100mm Hg pressure. Femoral arteries and *gracilis* muscles were isolated from fixed tissue and cleared sequentially with 70%, 95%, 100% ethanol, and methyl salicylate (Sigma-Aldrich) for 60min each. Cleared arteries and muscles were mounted with methyl salicylate on glass slides and examined under microscope.

Immunohistochemistry We used antibodies to NFκB (1:200, BD Pharmingen, San Diego, CA), ICAM-1 (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA), VCAM-1 (1:200, Santa Cruz), CD45 (1:100, BD Pharmingen), PCNA (1:1000, Abcam, Cambridge, MA), ephrin B2 (1:200, R & D systems, Minneapolis, MN), MCP-1 (1:200, Abcam), smooth muscle cell α-actin (SMC α-actin, 1: 1000, Sigma-Aldrich). Antigen retrieval was performed for cross sections with Retrogen (BD Pharmingen), except for NFκB, PCNA and ICAM-1 antibodies. Thyramide signal amplification (TSA, Perkins Elmar Inc, Waltham, MA) was performed for NFκB, ICAM-1, VCAM-1 and ICAM-1, CD45, ephrin B2 and MCP-1, per manufacturer's instruction. Briefly, a primary antibody was incubated at 4°C overnight, followed by 60 minutes for biotinylated secondary antibody (1:500), and 30 minutes for ABC complex (Elite ABC kit, Vector Laboratories, Burlingame, CA). Cy3-thyamide was used to visualize the peroxidase-binding sites. To visualize collateral media, the sildes were further incubated with mouse anti-SMC α-actin for 2 hours, followed by the incubation of Cy5-goat anti-mouse Ig G (1:100) for one hour. The slides were counter stained with DAPI to visualize cellular nuclei.

Leukocyte density Leukocytes in the adventitia and periadventitia of preexisting collaterals were detected with CD45 antibody as described above. CD45-positive cells having a blue nucleus surrounded by Cy3 fluorescence (from Cy3-thyramide) on their surface were counted by an observer blinded to the identity of the randomly arranged slides. Average leukocyte density was determined from 4 sections from each animal.

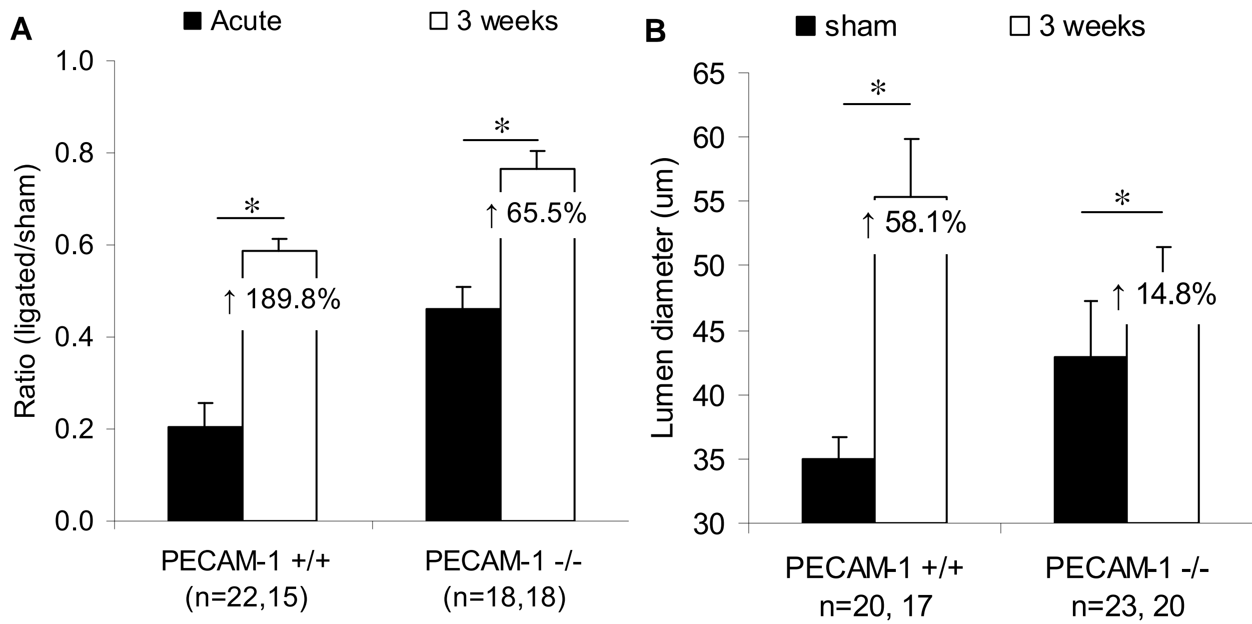
Capillary density Capillary density was counted as described previously.[7, 8] Briefly, muscle fibers were harvested from the right and left side, from the *m. adductor* and *m. gastrocnemius*. The plasma membrane of capillary ECs in tissue sections was labeled with Alexa 633-conjugated wheat germ albumin (WGA, Invitrogen, CA). Micrographs were obtained with the Nikon fluorescence microscope using a 20x objective lens. Digitized images were analyzed with an image analysis package, Image J. The total number of capillaries was counted on 5 random optical fields for each mouse. Results were expressed in capillaries per square millimeters.

Real-time reverse transcription PCR Total RNA was extracted from static or stretched ECs with Trizol reagents (Invitrogen, Inc, Carlsbad, CA). Total RNA (5μg) was reverse-transcribed into cDNA with (0.5μg) dT17 and SuperScript II (Invitrogen) per manufacturer's instruction. The primer pairs were as following ("-F": forward, "-R": reverse): α-tubulin-F,TGC AAC CAT CAA GAC AAA GC; α-tubulin-R,CAC AGT GGG AGG CTG GTA GT; ephrin B2-F, CAG CTT GTT TAA CGG CAG TGT; ephrin B2-R,CAG CAA TTT GGC AAC CTT TT; MCP-1-F,TGC ATC CAC TAC CTT TTC CA; MCP-1-R,AAG GCA TCA CAG TCC GAG TC. SYBR Green I based real-time PCR (Absolute SYBR Green ROX Mix, Thermo Fisher Scientific, Surrey, UK) was performed in a Rotor Gene thermal cycler (Qiagen, Foster City, CA) with the following thermal parameters: 95°C 15 min, followed by 40 cycles of 95°C for 30sec, 60°C for 30sec, 72°C for 30sec. Data were analyzed using relative real-time PCR quantification based on the ΔΔCt method.[11] α-tubulin was the endogenous reference gene for ephrin B2 and MCP-1, and the control was static ECs.

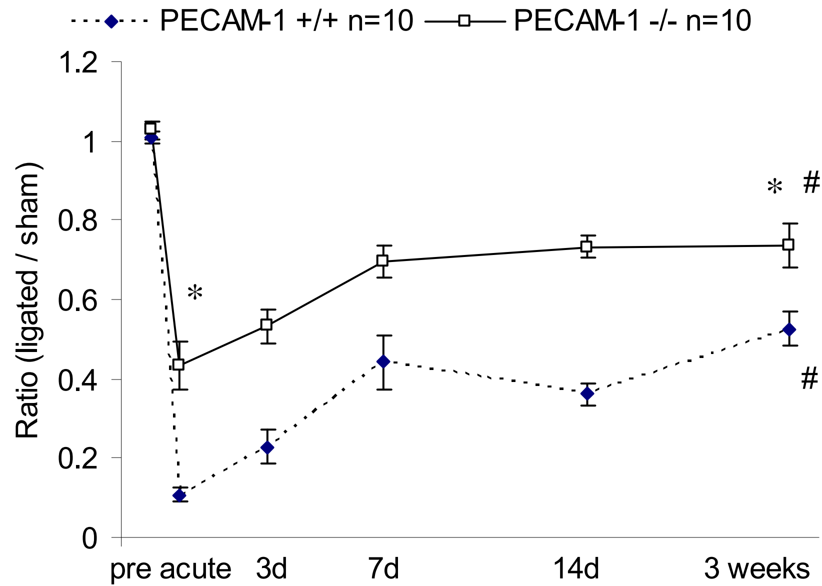
Statistical analysis Values are presented as means ± SE. Differences was determined by Student t-test (between two groups) and one-way ANOVA (among multiple groups). A value of $P<0.05$ was considered to indicate statistical significance.

Supplemental Figures and Figure legends

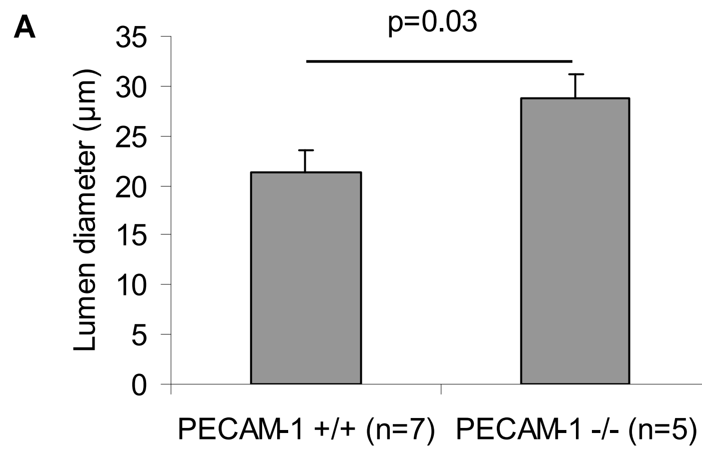
Online Figure I. Perfusion recovery and collateral diameter changes. (**A**) Ratio of plantar perfusion (ligated vs. sham control side) acutely and 3 weeks after ligation. (**B**) Collateral lumen diameter of sham controls and 3 weeks after ligation. Values are means ± SE. * p<0.05.

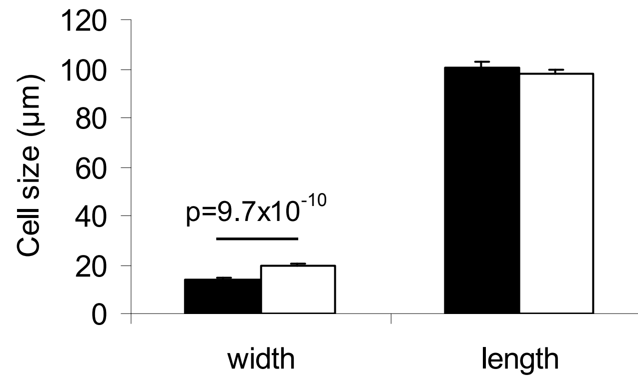


Online Figure II. Plantar perfusion after severe hindlimb ischemia. Proximal lateral caudal femoral artery ligation was performed. Ratio of plantar perfusion (ligated vs. sham control side) quantified from the Doppler images. Values are means ± SE. * p<0.05, compared with the respective time point of PECAM-1$^{+/+}$; # p<0.05, compared with the respective acute time point.

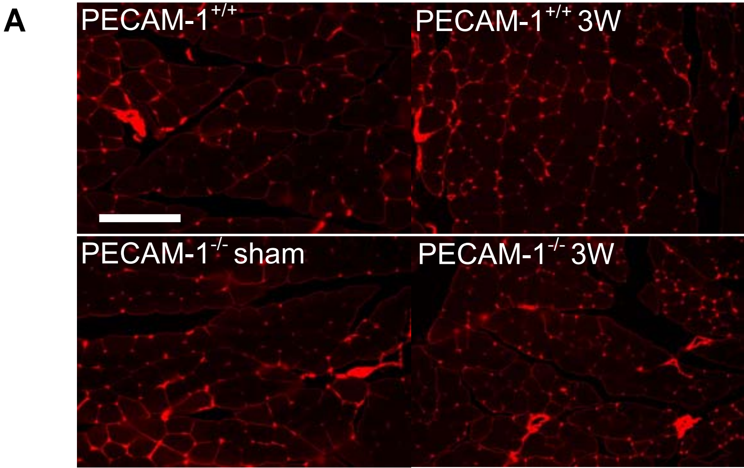


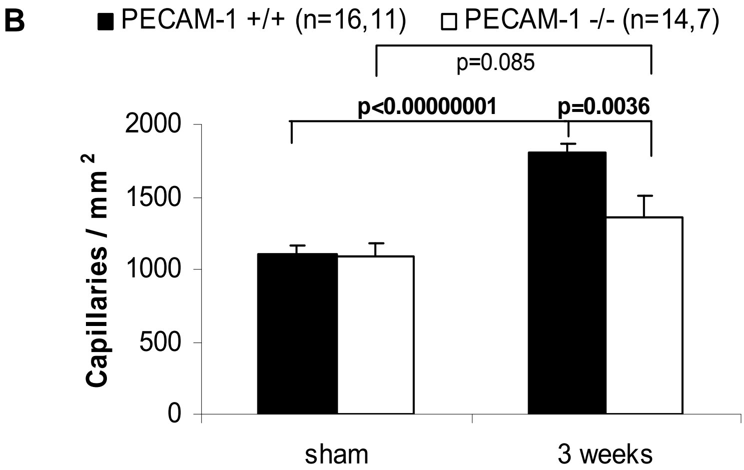
Online Figure III. Intestinal collateral diameter and EC size. Intestinal collateral caliber (**A**) and EC size (**B**) are quantified of in both genotypes. Values are means ± SE.





Online Figure IV. **Capillary density in *gastrocnemius* muscle during arteriogenesis.** (**A**) Capillary density in *gastrocnemius* muscle 3 weeks after hindlimb ischemic surgery. Sham control is the non-surgery side. Cross-sections were stained with Alexa 633-labeled WGA (red) and DAPI (blue). Images were taken with a 20x objective. Scale bar: 100μm. (**B**) Capillary density was counted from the images and values are means ± SE; n, number of animals.





Supplemental References

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