**SUPPLEMENTAL METHODS**

**MKP-1 PROMOTES NEOVASCULARIZATION AND ANGIOGENIC GENE EXPRESSION**
**Boerckel et al. ATVB**

Animals

MKP-1-deficient mice[1] (MKP-1$^{-/-}$, KO) were obtained from Dr. Yusen Liu of the Ohio State University (Columbus, OH) and backcrossed over 10 generations on a C57Bl6 background (The Jackson Laboratory, stock 000664). Heterozygous mice were bred to produce littermate pairs of wild type (MKP-1$^{+/+}$, WT) and knockout (MKP-1$^{-/-}$, KO) mice. A total of 56 mice were used in this study. Of this number, two mice exsanguinated during surgery and four mice were removed for insufficient Microfil perfusion or inefficient snap-freezing and these samples were excluded from analysis. All protocols were approved by the Cleveland Clinic Institutional Animal Care and Use Committee (protocol #2010-0333).

Genotyping

Mice were genotyped by polymerase chain reaction (PCR) as described previously[2]. Briefly, tissue samples were digested overnight by proteinase K incubation at 55C. DNA was purified by filtration using the Dneasy Blood & Tissue Kit (Qiagen 69506), and 4 ul extract was mixed with primers at 0.4μM and REDExtract-N-Amp Tissue PCR kit (Sigma XNAT-1KT) according to manufacturers instructions. PCR was conducted at denaturation temperature of 94C, annealing temperature of 62C, and extension temperature of 72C for 34 cycles. Primer sets were specific for MKP-1 and neomycin:
MKP-1 forward-1:
5'-CCAGGTACTGTGTCGGTGGT-GC-3',
MKP-1 forward-2 (Neo):
5'-TGCCTGCTCTTTACTGAAGGCTC-3',
MKP-1 reverse:
5'-CCTGGCACAATCCTCCTAGAC-3'.

Hindlimb Ischemia Model

Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (15 mg/kg). WT and KO mice underwent surgical induction of hindlimb ischemia as described previously[3]. Briefly, the left femoral artery and vein were blunt dissected away from the sciatic nerve and ligated using non-absorbable 6-0 silk sutures proximal to the profunda femoris artery; a second ligation was performed proximal to the popliteal branch point. The length of artery and vein was then excised between the ligation points, resulting in ischemia of the distal hindlimb. The right limb was used as an unoperated internal control. Following surgery, animals received analgesic injections of buprenorphine (0.1 mg/kg), subcutaneously, twice daily for 3 days. Recovery was evaluated by laser Doppler perfusion imaging (LDPI) and immunostaining (see below).

Fractalkine Delivery to Ischemic Hindlimbs

WT and MKP-1 KO mice (N=6 per group) received hindlimb ischemia as described above. Immediately after surgery, 5μg recombinant mouse fractalkine (R&D Systems), reconstituted at 50ug/ml, was intramuscularly injected into
WT and MKP-1 KO mice (N=6 per genotype) received hindlimb ischemia as described above, and were then injected intramuscularly immediately after surgery with 5μg recombinant mouse fractalkine, reconstituted at 50ug/ml, divided evenly between GC and SM muscles, as described previously in rats[13]. Perfusion recovery was evaluated over 14 days by LDPI and immunostaining at day 7 (see below).

Laser Doppler Perfusion Imaging

Blood flow to the paw was evaluated by LDPI immediately prior to and after surgery (d0), and at days 3, 7, 14, 21, and 28 post-surgery (N = 11 to 20 for each genotype). Mice were anesthetized as described above and placed in a supine position in a Moor LDI2 laser Doppler imaging system (Moor Instruments). The limbs were scanned, and blood flow to the skin was evaluated as average perfusion in a region of interest encompassing the paw, ending at the

ankle joint. Data were normalized to the contralateral control.

MicroCT angiography

Microcomputed tomography (microCT) angiography was performed at days 7 and 28 to quantitatively analyze the three-dimensional vascular network morphology within the hindlimb, as described previously [3-5]. The vasculature was perfused through the left ventricle with sequential solutions of 25U/ml heparin in 0.9% saline, neutral buffered formalin, saline, and radiopaque contrast agent (Microfil MV-122, Flow Tech). Limbs were then decalcified for two weeks in CalExII (Fisher Scientific) and imaged on an eXplore Locus Micro-CT (GE Healthcare) at 20 um resolution (360 projections, 80kV, 500uA).

Contralateral reconstructed volumes were subsequently co-registered to enable delineation of a single, consistent volume of interest (VOI) for analysis and parameter comparison between ischemic and control limbs. In the hindlimb ischemia model, vascular growth in the thigh occurs primarily through arteriogenesis (collateralization) stemming from increased wall shear stress in collateral vessels, whereas it proceeds predominantly through angiogenesis in the lower hindlimb (calf) as a result of tissue ischemia [6,7]. Therefore, these two regions of interest (ROI) were selected for independent analysis. Vasculature in these registered volumes were segmented using a local neighborhood equalization (Fourier spectral filters) and subsequently thresholded to generate binary volumes.

Histology & immunofluorescence

Histological and immunofluorescence analysis of tissue morphology, vascularity, gene expression, and inflammation was performed at days 7, 14, and 28. Animals were euthanized by $CO_2$ inhalation and the gastrocnemius (GC) and soleus (SM) muscles of each hindlimb were excised. Samples (N = 3-8 per genotype) were snap frozen in isopentane and embedded in OCT prior to transverse sectioning at 8 µm for histology (Haematoxylin & Eosin, H&E) and immunohistochemistry (IHC). IHC samples were fixed in either 4% paraformaldehyde or -20°C acetone and blocked in 2% horse serum with 0.3% Triton X-100 prior to incubation overnight with primary antibodies for CD68 (Abcam ab125212, 1:500 dilution, PFA fixation), CD3 (Abcam ab5690, 1:100 dilution, acetone fixation), and fractalkine (R&D Systems AF365, 5µg/ml, PFA fixation), VEGF (Abcam ab46154, 1:200 dilution, PFA fixation), αSMA (Abcam ab5694, 1:200 dilution, acetone fixation), MKP-1 (V15, Santa Cruz sc-1199, dilution 1:50). Secondary antibodies were Alexa Fluor 488- or 568-conjugated IgG. Endothelial cells were labeled with FITC-conjugated Lectin from Bandeiraea simplicifolia (BS-1 lectin, aka isolectin B4, Sigma L2895, 10µg/ml, acetone or PFA fixation). Nuclei were counterstained with DAPI. Staining was verified in positive control brain or spleen tissue, and negative controls lacking primary antibody were included to rule out non-specificity.

Cell isolation and culture

HUVEC were isolated by trypsin digestion from fresh umbilical veins as described previously[8,9]. Briefly, segments of 1-3-day-old umbilical cords were drained, and rinsed with phosphate-buffered saline (PBS). The open end of the cord was sealed, and the cord was distended with 0.06% trypsin/0.2% EDTA and incubated at 37°C for 15 min. The cord was massaged, cut, and the trypsin digest was collected. The cells were then washed and seeded directly into assay plates at approximately 105 cells/$cm^2$. Cells were maintained in MCDB/F12 supplemented with 15% fetal bovine serum (FBS), 0.009% heparin, and 0.015% endothelial growth supplement (ECGS) and used between passages 3 and 5. After passage, cells were transfected for 3-4 hours using Targefect reagents with control, MKP-1, or fractalkine siRNA (Table 1), and used for experiments at 30-48 hours post-transfection.

Murine aortic endothelial cells (MAEC) were isolated from MKP-1 knockout or littermate wild type mice as previously described[10]. Briefly, mice were anesthetized as described above. The thoracic cavity was exposed and the thoracic aorta was cleared of fat and connective tissue and was cut into 3mm long rings. Aortic segments were then placed on Matrigel in 6-well

tissue culture plates and incubated in DMEM supplemented with 15% FBS, 1% penicillin-streptomycin, 0.009% heparin, and 0.015% ECGS. EC outgrowth was observed over 4days and vessel rings were removed. Cells were then passaged as described above.

Prior to treatment, cells were serum starved for 2 hours in MCDB or DMEM and treated with MCDB/DMEM containing 50ng/ml recombinant human vascular endothelial growth factor-165 (VEGF, R&D Systems 293-VE/CF). Cells were depleted of MKP-1 or fractalkine by transfection with small interfering RNA (siRNA) using Targefect reagents (TargetingSystems). SiRNA oligonucleotides were purchased from Ambion/Applied biosystems: Silencer Select Negative Control siRNA (4390844), MKP-1 (DUSP1 4390824), and fractalkine (CX3CL1 4392420).

Endothelial cell wound migration assay

HUVEC, transfected with control, MKP-1, or fractalkine siRNA as indicated, or MAEC isolated from KO and WT mice, were cultured to 90% confluence in 6-well plates. The cell monolayer was then scratched with a 200ul pipette tip along a straight edge leaving a linear "wound" region. This was performed twice per well at orthogonal angles, allowing for four fields of view per well for quantification. The cells were then serum starved for two hours and treated overnight as described. Cellular migration into the wound gap was evaluated in ImageJ (NIH) by quantification of remaining acellular area, normalized to the original area of the wound.

Tube formation assay

HUVEC transfected with control or MKP-1 siRNA were cultured on Matrigel & VEGF-stimulated tubular network formation was evaluated over 6-8 hours. Briefly, 200 µl growth factor-reduced Matrigel (R&D Systems 356231) was pipetted into 48-well plates at 4°C and then brought to 37°C to induce polymerization of the matrix. Serum starved HUVEC were then deposited on the solidified matrix at a density of 50,000 cells/cm$^2$. Cells were treated with VEGF at a concentration of 50 ng/ml. Tubular network formation was imaged by labeling with 1 uM calcein (Invitrogen C1430) and quantified using a custom analysis package (ImageIQ).

Proliferation assay

VEGF-induced proliferation of control or MKP-1-depleted HUVEC was evaluated using the bromodeoxyuridine (BrdU)-incorporation assay according to manufacturer's instructions (Cell Signaling Technology 6318). Cells were serum-starved overnight and treated for 12 hours with 50ng/ml VEGF or vehicle, with BrdU incorporation between hours 8 and 12[11].

Chromatin Immunoprecipitation-sequencing

To identify potential gene targets of MKP-1-mediated chromatin modification, chromatin immunoprecipitation (ChIP)-sequencing was performed using HUVEC transfected to express myc-tagged CS-MKP-1. Cells were then serum starved 2 hours and treated with either vehicle or 50ng/ml VEGF for 10 minutes, crosslinked with 1% formaldehyde, washed, and lysed with SDS lysis buffer. Lysates were sonicated to fragment the DNA into ~500bp segments, and CS-MKP-1 and its binding partners were immunoprecipitated under chromatin binding buffer conditions using an anti-myc antibody. MKP-1 complex-associated DNA were then cloned in p-Bluescript and sequenced.

RT-PCR analysis of gene expression

HUVEC transfected with control or MKP-1 siRNA were serum starved 2 hrs and treated with 50ng/ml VEGF**.** Fractalkine expression was evaluated at 6 hours by quantitative RT-PCR. Total RNA was extracted using the RNeasy kit (Qiagen). 1ug of total RNA was reverse transcribed using a cDNA synthesis kit (TaqMan, Applied Biosystems). Real time RT-PCR was then performed using Power SYBR green reagents (Applied Biosystems) and the following primers: fractalkine forward: 5'-CAT CAT GCG GCA AAC GCG CA-3'; fractalkine reverse: 5'-AGC AGC CTG GCG GTC CAG AT-3' (yielding a product of 116 bp). Results were normalized to the housekeeping gene GAPDH, evaluated with the following primers: GAPDH forward: 5'-TCA ACA GCG ACA CCC ACT CC-3'; GAPDH reverse: 5'-TGA GGT CCA CCC TGT TG-3'. Detection was accomplished with an optical real time PCR

system (Applied Biosystems) and quantification was assessed using the ΔΔCT method[12].

Chromatin Immunoprecipitation

HUVEC transfected with either control or MKP-1 siRNA were treated with 50ng/ml VEGF for 0, 30, 60, and 90 minutes and chromatin was immunoprecipitated using antibodies against either phospho-H3S10 (Abcam ab32107) or H3 (Abcam ab1791), and associated DNA was amplified by real time polymerase chain reaction (RT-PCR) using primers targeting the region of DNA identified to immunoprecipitate with MKP-1-CS.

FKN exon 3 #4 F:
5'-TCCTCCCTTGTAGCTTGGAGA-3'
FKN exon 3 #4 R:
5'-CATTTCGAGTTAGGGCAGCAG-3'

Statistical analysis.

All data are presented as mean ± standard error of the mean (SEM) from at least three independent experiments. Nonlinear regression analysis was performed in GraphPad Prism to fit LDPI perfusion ratio data to a one-phase exponential recovery model ($y = S\ e^{-t/\tau}+P$); regression parameters $S$ (span), $\tau$, (time constant), and $P$ (plateau) were calculated from raw data and comparison between groups was evaluated by F test with significance threshold at $p \leq 0.05$, N = 8-21 samples per time point per group for data from Figure 1, N = 6 per group for LDPI data from Figure 6. Analyses of other comparisons were accomplished using Student's t-test for single comparisons or analysis of variance (ANOVA) with Bonferroni's post-hoc analysis for multiple comparisons, as appropriate. A p-value $\leq 0.05$ was considered significant.

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