Mitogen-Activated Protein Kinase Phosphatase-1 Promotes Neovascularization and Angiogenic Gene Expression

Joel D. Boerckel, Unnikrishnan M. Chandrasekharan, Matthew S. Waitkus, Emily G. Tillmaand, Rebecca Bartlett, Paul E. DiCorleto

Objective—Angiogenesis is the formation of new blood vessels through endothelial cell sprouting. This process requires the mitogen-activated protein kinases, signaling molecules that are negatively regulated by the mitogen-activated protein kinase phosphatase-1 (MKP-1). The purpose of this study was to evaluate the role of MKP-1 in neovascularization in vivo and identify associated mechanisms in endothelial cells.

Approach and Results—We used murine hindlimb ischemia as a model system to evaluate the role of MKP-1 in angiogenic growth, remodeling, and arteriogenesis in vivo. Genomic deletion of MKP-1 blunted angiogenesis in the distal hindlimb and microvascular arteriogenesis in the proximal hindlimb. In vitro, endothelial MKP-1 depletion/deletion abrogated vascular endothelial growth factor–induced migration and tube formation, and reduced proliferation. These observations establish MKP-1 as a positive mediator of angiogenesis and contrast with the canonical function of MKP-1 as a mitogen-activated protein kinase phosphatase, implying an alternative mechanism for MKP-1–mediated angiogenesis. Cloning and sequencing of MKP-1–bound chromatin identified localization of MKP-1 to exonic DNA of the angiogenic chemokine fractalkine, and MKP-1 depletion reduced histone H3 serine 10 dephosphorylation on this DNA locus and blocked fractalkine expression. In vivo, MKP-1 deletion abrogated ischemia-induced fractalkine expression and macrophage and T-lymphocyte infiltration in distal hindlimbs, whereas fractalkine delivery to ischemic hindlimbs rescued the effect of MKP-1 deletion on neovascular hindlimb recovery.

Conclusions—MKP-1 promoted angiogenic and arteriogenic neovascular growth, potentially through dephosphorylation of histone H3 serine 10 on coding-region DNA to control transcription of angiogenic genes, such as fractalkine. These observations reveal a novel function for MKP-1 and identify MKP-1 as a potential therapeutic target.

Key Words: angiogenesis effect  MKP-1 phosphatase

Angiogenesis, the process of endothelial cell (EC) sprouting from existing vessels to form new vessels, is essential for myriad physiological processes and pathological conditions, including development and growth, regeneration and repair, and tumor growth and metastasis. Despite intense study, the mechanisms underlying the activation, progression, and regulation of angiogenesis remain incompletely understood. The vascular endothelial growth factor (VEGF) has been identified as one of the most potent cytokines capable of initiating and maintaining this process and has long been a target for both pro- and antiangiogenic therapies. Despite its potency in model systems, both delivery of VEGF, through recombinant proteins or gene therapy, and inhibition of VEGF for cancer therapy have had mixed clinical success. Thus, elucidating the molecular and regulatory mechanisms underlying this process is essential for both therapeutic formation of new vessels that feed growing and regenerating tissues, as well as prevention of aberrant vascular growth that drives tumor size and aggression. One of the key mechanisms that regulates the cellular response to growth factors, cytokines, and environmental stresses is the mitogen-activated protein kinase (MAPK) signaling pathway. This pathway consists of several families of primary kinase effectors, extracellular-related kinase, p38, and c-Jun N-terminal kinase. These MAPK are required for vascular growth and development and positively mediate angiogenic processes in ECs, including migration, proliferation, and tube formation. They are deactivated through Thr/Tyr dephosphorylation by the nuclear phosphatase, MAPK phosphatase-1 (MKP-1; also known as DUSP1 or CL100).

In this study, we investigated the role of MKP-1 in angiogenic and arteriogenic neovascularization. Given the requirement of MAPK activation for vascular development and angiogenesis, we previously hypothesized that MKP-1 negatively regulates angiogenesis; surprisingly, however, we found that MKP-1 positively mediated EC migration and aortic ring sprouting in response to VEGF stimulation in vitro.
suggesting that MKP-1 may also have a noncanonical function that plays a positive role in neovascularization independently or concurrently with its action on MAPK.

Further recent observations from our laboratory suggested a potential effector of this putative noncanonical signaling: MKP-1–mediated chromatin modification. Using a substrate trap cysteine-to-serine (C259S) mutant of MKP-1 (CS-MKP-1), which results in stable binding of MKP-1 to its substrates, we identified MKP-1 as the only known mammalian histone H3 serine 10 (H3S10) phosphatase, which is required for VEGF-induced H3S10 dephosphorylation. Here, we describe the effect of genomic deletion of MKP-1 on angiogenic and arteriogenic recovery from hindlimb ischemia in vivo and demonstrate a positive role for endothelial MKP-1 in angiogenic gene expression associated with MKP-1–mediated exonic histone H3 dephosphorylation on the angiogenic and inflammatory gene, fractalkine.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
MKP-1 knockout and littermate wild-type mice (n=11–20 per group) underwent surgical induction of hindlimb ischemia, and angiogenic and arteriogenic recovery were evaluated >28 days by longitudinal laser Doppler perfusion imaging (Figure 1A and 1B). Exponential curve–fit analysis demonstrated that MKP-1 knockout mice featured significantly slower recovery when compared with wild type (τ½=4.44±0.99 days; τ∞=13.7±4.08 days; P=0.04) but recovered to the same plateau by day 28 (P½=0.48±0.03, P∞=0.54±0.09; P=0.58). The perfusion ratio was significantly lower in knockout limbs at days 3 and 7, and knockout mice recovered to wild-type levels by day 28.

On the basis of these observations, we selected days 7 and 28 for quantitative micro–computed tomography (CT) angiography analysis. Angiogenesis and arteriogenesis are linked in the hindlimb ischemia model because sufficient collateralization of the upper hindlimb is necessary for perfusion and angiogenesis of the lower hindlimb and sufficient capillary networks are required for arteriogenesis and collateralization. Thus, angiogenesis and collateral arteriogenesis act in concert to establish a functional vascular network in both proximal and distal parts of the ischemic limb. However, although these mechanisms of new vessel formation cannot be spatially divorced, they are dominant in different regions of the limb, as has been described previously. Vascular growth in the thigh occurs primarily through arteriogenesis 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 hypoxia. Therefore, these 2 regions of interest were selected for independent analysis. In the distal hindlimb, wild-type mice featured a biphasic recovery characterized by increased vascularity above contralateral controls at day 7 (P<0.05 versus control) and remodeling to control values by day 28 (P>0.05 versus control). In contrast, vascular parameters of knockout mice failed to exceed unoperated controls at either time point, with significantly lower vascularity when compared with ischemic wild-type limbs at day 7 and equivalent values at day 28 (Figure 1C–1G; unoperated control values indicated by dotted lines). In the proximal hindlimb, there were no differences in vascular parameters between genotypes or between control and ischemic limbs, with the exception of connectivity at day 7, in which ischemic limbs of wild-type mice had significantly greater connectivity than the contralateral control and ischemic limbs of knockout mice (Figure 1 in the online-only Data Supplement).

Because micro-CT angiography is limited by resolution to assess patent vessels of ≥20 μm in diameter, microvascular angiogenesis and arteriogenesis were evaluated in both regions of interest by immunofluorescent staining for capillaries (EC marker BS-1 lectin) and arterioles (αSMAs+, BS-1 lectin-stained vessels) at days 7, 14, and 28. In the gastrocnemius muscle of the lower hindlimb, ischemia significantly increased capillary density in wild-type mice when compared with controls (Figure 2A and 2B). This increase was delayed in MKP-1 knockout mice, with significantly lower values at days 7 and 14 but recovery to wild-type values by day 28. In contrast, capillary density was not significantly altered by MKP-1 deletion in the soleus muscle (SM) of the upper hindlimb (Figure 2C and 2D). Arteriole formation was not affected by MKP-1 deletion in the gastrocnemius muscle (Figure 2E and 2F), but in the SM muscle of the upper hindlimb, arteriolar density was significantly higher in wild-type mice when compared with knockout and controls at day 7 (Figure 2G and 2H).

To evaluate the time course of MKP-1 expression in ischemic hindlimbs, SM and gastrocnemius sections of wild-type mice were immunostained for MKP-1 at days 7, 14, and 28. MKP-1 was induced by hindlimb ischemia in both regions at day 7 and equivalent values at day 28 (Figure 3A–3G; P=0.54). MKP-1 depletion by siRNA (Figure 3A) completely abolished VEGF-induced human umbilical vein EC (HUVEC) migration (Figure 3B and 3C). Similarly, mouse aortic ECs (MAEC) from knockout mice exhibited the same migratory deficit (Figure IIIA and IIB in the online-only Data Supplement). MKP-1 depletion significantly reduced HUVEC proliferation overall although cells remained responsive to VEGF stimulation (Figure 3D). MKP-1 depletion also abrogated VEGF-induced HUVEC tube formation on Matrigel with reduced length, number of nodes, and number of branches without altering cell number (Figure 3E and 3F).

Next, to identify putative gene targets for MKP-1 regulation through chromatin interactions in HUVEC, we
immunoprecipitated CS-MKP-1–bound chromatin and cloned and sequenced associated DNA. Although sequencing of vehicle-treated samples returned only pBluescript plasmid, in VEGF-treated samples, 3 independent clones were identified; these fragments overlapped one another on the proximal end of exon 3 of the angiogenic and inflammatory gene, fractalkine, also known as cx3cl1 (Figure 4A).

To test whether MKP-1 specifically dephosphorylates H3S10 on this gene locus, we immunoprecipitated phospho-H3S10–bound chromatin in control or MKP-1–depleted HUVEC and performed real-time polymerase chain reaction for the proximal region of fractalkine exon 3. MKP-1 depletion prevented dephosphorylation of H3S10 on this gene locus at 0 and 30 minutes after VEGF treatment, returning to control levels by 90 minutes, but remained dephosphorylated at all time points in control cells (Figure 4B). In contrast, binding of total histone H3 to this DNA locus was not affected by MKP-1 depletion (Figure 4C).

Next, we evaluated fractalkine induction by VEGF in HUVEC. In cells depleted of MKP-1, VEGF-induced fractalkine expression was completely ablated at both the message (Figure 4D) and the protein (Figure 4E) levels. Similarly,
MKP-1–null MAEC exhibited reduced VEGF-induced fractalkine expression (Figure IIIC in the online-only Data Supplement). Functionally, fractalkine-depleted HUVEC were no longer significantly responsive to VEGF-induced migration (Figure 4F and 4G), and treatment of HUVEC (Figure 4H) or MAEC (Figure IIID and IIIE) with recombinant fractalkine (100 ng/mL) rescued the effect of MKP-1 deletion on EC migration.

Figure 2. Immunofluorescent evaluation of microvascular capillaries and arterioles. Shown are representative micrographs and quantification of endothelial cell (BS-1 lectin, A–D), and arteriole (αSMA+, BS1-lectin++; E–H) staining in transverse sections of gastrocnemius (GC; calf) and soleus (SM; thigh) muscles of wild-type (WT) and mitogen-activated protein kinase phosphatase-1 (MKP-1) knockout (KO) mice (n=3–8 per time point per genotype). Capillary density was significantly lower in GC muscles of KO mice at days 7 and 14 but equivalent to WT at day 28, whereas arteriole density was lower in SM muscles of KO mice at day 7. *P≤0.05 vs KO at same time point. Scale bar, 25 μm.
To verify that MKP-1 mediated fractalkine gene regulation in vivo, we evaluated fractalkine protein expression in both gastrocnemius and SM muscles of control and ischemic hindlimbs of wild-type and MKP-1 knockout mice at days 7, 14, and 28 by immunofluorescence. Fractalkine staining colocalized with ECs in the ischemic hindlimbs of both wild-type and knockout mice (Figure IV in the online-only Data Supplement), demonstrating that ECs in the ischemic hindlimb express fractalkine although fractalkine staining was not exclusive to ECs, consistent with its role as a soluble signaling molecule in its cleaved form. In gastrocnemius muscle of the lower hindlimb, fractalkine expression was significantly induced over control levels in ischemic limbs of wild-type, but not knockout, mice at day 7, confirming the MKP-1 dependence of fractalkine expression. Over time, expression decreased to negligible levels in both genotypes by day 28 (Figure 5A and 5B). Background

**Figure 3.** Effect of mitogen-activated protein kinase phosphatase-1 (MKP-1) depletion on in vitro angiogenesis. Human umbilical vein endothelial cells were depleted of MKP-1 by siRNA (A) and serum starved 2 hours before treatment with 50 ng/mL vascular endothelial growth factor (VEGF). VEGF-induced migration (B and C), proliferation (D), and tube formation (E and F) were evaluated using the scratch wound, BrdU, and Matrigel assays, respectively. Representative migration images in B show initial wound (top) and migration >11 hours (bottom) in VEGF-treated samples. Representative tube formation images in E show tube formation >6 hours after VEGF treatment. MKP-1 depletion abrogated VEGF-induced migration and tube formation and reduced overall proliferation but did not block the proproliferative effect of VEGF. *P≤0.05, **P≤0.01. Scale bars, 100 μm. NS indicates not significant.
Figure 4. Mitogen-activated protein kinase phosphatase-1 (MKP-1)–chromatin interactions and fractalkine regulation. Human umbilical vein endothelial cells (HUVECs) were transfected with substrate trap mutant CS-MKP-1 (myc-tagged) and treated 10 minutes with 50 ng/mL VEGF or vehicle; chromatin was immunoprecipitated using an antimyc antibody, and associated DNA were cloned and sequenced. In VEGF-treated samples, 3 independent clones were identified overlapping exon 3 of the angiogenic chemokine, fractalkine, as illustrated (A). Vehicle-treated samples had no DNA binding. HUVEC depleted of MKP-1 by siRNA were treated with 50 ng/mL VEGF during a 90-minute time course, and chromatin was immunoprecipitated using antibodies against phospho-histone H3S10 (B) or H3 (C) on this DNA locus. MKP-1 depletion enriched the DNA for p-H3S10 at 0 and 30 minutes after VEGF treatment but had no effect on total H3 binding. Fractalkine mRNA (D) and protein (E) expression were induced under control conditions but completely ablated when MKP-1 was depleted. HUVEC depleted of fractalkine (F) exhibited a blunted migratory response to VEGF (G), but treatment with recombinant fractalkine rescued the migratory deficit of MKP-1–depleted cells (H). *P≤0.05, **P≤0.01. NS indicates not significant.
Fractalkine levels were moderately elevated in control limbs at day 7 when compared with days 14 and 28, suggesting a systemic circulation of cytokines that resulted in contralateral induction although this was insufficient to cause inflammatory cell infiltration in distal knockout or control limbs (Figure 5G, 5H, 5J, and 5K). This may suggest a threshold expression level necessary for inflammatory cell infiltration, which was not met in either control or knockout limbs at any time point. In SM muscle of the upper hindlimb, fractalkine expression was not significantly induced in either ischemic or control limbs of either genotype (Figure 5C). Although monocyte/macrophage infiltration was significantly induced by ischemia at day 7 in SM sections, it was less robust than in gastrocnemius muscle, and there were no differences between wild-type and MKP-1 knockout mice (Figure 5I). T-lymphocyte staining in SM sections was minimal and exhibited no differences between genotypes (Figure 5L).

Next, we quantified the time course of VEGF protein expression in gastrocnemius and in SM muscles of control and ischemic hindlimbs of wild-type and MKP-1 knockout mice. In the gastrocnemius muscle, VEGF expression was induced by ischemia in wild-type mice although expression did not differ significantly from knockout levels (Figure 5D and 5E). There were no differences at days 14 or 28. In wild-type SM muscle,
VEGF was significantly induced by hindlimb ischemia at day 7 and decreased through day 28, whereas in knockout mice VEGF expression was not elevated at day 7 but increased over time (Figure 5F). VEGF expression levels were lower in SM than that in gastrocnemius muscles for both genotypes.

Fractalkine is a potent chemotactic agent for inflammatory leukocytes, so to evaluate the role of MKP-1 in the time course of inflammation, gastrocnemius and SM sections from wild-type and MKP-1 knockout mice were probed for monocytes/macrophages (CD68; Figure 5G–5I) and T-lymphocytes (CD3; Figure 5J and 5K) at 7, 14, and 28 days. In gastrocnemius muscle, monocyte/macrophage and T-lymphocyte infiltration were minimal in control limbs and in ischemic limbs of knockout mice but was significantly elevated in ischemic limbs of wild-type mice at all time points evaluated (Figure 5G, 5H, 5J, and 5K). In contrast, in SM muscle, monocyte/macrophage infiltration was elevated at day 7 in both wild-type and knockout mice and diminished to negligible levels by day 28. T-lymphocyte staining was minimal in all groups at all time points in SM sections. Immunofluorescence specificity was demonstrated in positive and negative controls (Figure V in the online-only Data Supplement).

Finally, to test whether delivery of recombinant fractalkine would rescue the effect of MKP-1 deletion, wild-type and MKP-1 knockout mice (n=6 per genotype) received hindlimb ischemia and were then injected intramuscularly immediately after surgery with 5-µg recombinant mouse fractalkine, divided evenly between gastrocnemius and SM muscles, as described previously in rats. Although fractalkine delivery unexpectedly had no effect in wild-type mice, perfusion recovery of MKP-1 knockout mice was restored to wild-type untreated levels (data reproduced from Figure 1 in gray), with no significant differences between wild-type–untreated, wild-type–treated, or knockout-treated groups at any time point (Figure 6A and 6B). Exponential curve–fit analysis revealed no differences in characteristic time scales between fractalkine-treated wild-type and fractalkine-treated knockout mice (t50=9.29 days; τ90=8.03 days; P=0.83). Gastrocnemius and SM muscles at day 7 after ischemia (n=3 per group) were sectioned and immunostained for capillaries (Figure 6C), arterioles (Figure 6D), fractalkine (Figure 6E), monocyte/macrophages (Figure 6F), and T-lymphocytes (Figure 6G). For each measure, there were no differences between wild-type or MKP-1 knockout mice.

**Discussion**

In this study, we evaluated the role of the nuclear phosphatase, MKP-1, in neovascularization using hindlimb ischemia as a model system to evaluate angiogenesis, arteriogenesis, and vascular remodeling. We found that MKP-1 was transiently induced by hindlimb ischemia and positively mediated neovascular growth in vivo and in vitro, in part through regulation of the angiogenic and inflammatory chemokine, fractalkine.

Hindlimb ischemia recovery can be characterized as an underdamped feedback control system with a biphasic response profile featuring an initial phase of vessel growth peaking above contralateral controls and a later phase of vessel rarefaction and remodeling in which unnecessary and inefficient vessels are pruned away without changes in functional perfusion. In this study, wild-type mice followed this underdamped biphasic profile, with vascular parameters elevated above contralateral controls at day 7 and normalized by day 28. In contrast, MKP-1 knockout mice responded as an overdamped system, failing to supersede control values at any time point.

These data demonstrate a critical role for MKP-1 in the early growth phase of neovascular growth, attributable to a combination of angiogenesis and arteriogenesis. These processes can be evaluated by 3-dimensional vascular network analysis and immunostaining for both capillary and arteriolar formation. MKP-1 deletion inhibited hypoxia-induced angiogenesis in the distal hindlimb but did not affect arteriolar formation in this region. In the proximal hindlimb, MKP-1 knockout limbs did not show differences in capillary density but exhibited transiently reduced arteriogenesis, as measured by arteriolar staining. The MKP-1–associated defect in arteriogenesis was largely limited to the microvasculature because micro-CT angiography did not detect differences in vascular network parameters besides connectivity. However, of the micro-CT–based parameters, connectivity has been shown to correlate best with functional perfusion, suggesting a potential functional importance of MKP-1 in arteriogenesis. Together, these data implicate MKP-1 as an important regulator of the early response to ischemia, with downstream effects mirroring the kinetics of MKP-1 expression in the ischemic limb. These data are consistent with reports that MKP-1 is induced in ischemic tumor microenvironments and is required for VEGF expression and maintenance of vascular density in hypoxia-exposed lung. Importantly, the early angiogenic response is critical for prevention of irreversible myocardial necrosis in ischemic heart disease and is an indicator of functional outcome and mediates revival of tumor cells from dormancy, highlighting the relevance of these findings beyond accelerated recovery from peripheral artery disease.

To evaluate the molecular mechanisms by which MKP-1, a negative regulator of MAPK, may serve a positive role in angiogenic function of ECs, we evaluated the effect of MKP-1 depletion/deletion on HUVEC/MAEC angiogenesis in vitro. We found that endothelial MKP-1 is required for VEGF-induced migration and tube formation and positively contributed to EC proliferation independently of VEGF. Initially, these data seem to conflict with a recent report that MKP-1 overexpression reduced EC motility and proliferation. In those experiments, however, MKP-1 was expressed to supraphysiological levels, which may lead to nonphysiological outcomes. Indeed, in that report, depletion of MKP-1 reduced VEGF-stimulated proliferation, consistent with the proangiogenic role described here. These data are consistent with our previous observations that MKP-1 is required for VEGF-induced EC migration and aortic ring sprouting. Together, this study implicates MKP-1 as an important regulatory molecule in angiogenesis and suggests that tight control of MKP-1 levels is required for proper EC function.

Our previous discovery that MKP-1 is an H3S10 phosphatase led us to hypothesize that MKP-1 may regulate EC function through chromatin modification. Using chromatin immunoprecipitation, we identified MKP-1 association...
with exon 3 of the angiogenic chemokine fractalkine and found that MKP-1 dephosphorylated H3S10 on this DNA locus and was required for fractalkine induction by VEGF. This histone modification occurred in exonic DNA, immediately proximal to the stop codon, rather than in the promoter region, suggesting that MKP-1 may regulate transcriptional arrest and progression rather than initiation. These data support the hypothesis that histone tail modifications downstream of the promoter also contribute to the histone code, regulating not only transcription initiation and transcription factor recruitment but also elongation and propagation of chromatin disruption. Such coding-region histone modification for gene regulation has been reported previously. For example, H3 and H4 acetylation within the coding region of the angiogenic VEGF-cofactor, CCN1 (CCN family member 1), mediate chromatin folding, and transcription. In addition, H3S10 phosphorylation is required for the initiation of chromatin condensation during mitosis, as well as priming H3 for subsequent Lys14 acetylation, a switch between permissive and repressive chromatin, consistent with the observations seen here. Furthermore, H3S10 phosphorylation within the FOSL1 enhancer has been shown to trigger a

Figure 6. Rescue of mitogen-activated protein kinase phosphatase-1 (MKP-1) phenotype by recombinant fractalkine (FKN) delivery. Ischemic wild-type (WT) and MKP-1 knockout (KO) mice (n=6 per group) were intramuscularly injected immediately after surgery with 5-μg recombinant mouse fractalkine, divided evenly between gastrocnemius (GC) muscle and soleus muscle (SM). Recovery >14 days was evaluated by laser Doppler perfusion imaging (A and B), and GC and SM muscles at day 7 (n=3 per group) were immunostained for capillaries (C), arterioles (D), fractalkine (E), monocytes/macrophages (F), and T-lymphocytes (G). Fractalkine delivery rescued the effect of MKP-1 deletion on perfusion recovery, and there were no differences in fractalkine-treated samples between genotypes for immunostained parameters.
cascade required for transcription elongation.\textsuperscript{41} Thus, MKP-1 dephosphorylation of H3S10 may alter chromatin structure and accessibility to transcriptional machinery, allowing transcriptional progression and gene expression; however, in the absence of MKP-1, chromatin may be closed and transcription arrested. Consistent with these molecular signaling links, treatment of both HUVEC and MAEC with recombinant fractalkine rescued the migratory deficit caused by MKP-1 deletion, demonstrating the importance of this factor in MKP-1–mediated EC activation.

Given the ubiquity of histone H3, fractalkine may be representative of more general gene regulation by MKP-1. Therefore, we explored gene regulation of fractalkine as a prototype for MKP-1–mediated angiogenic gene expression. Fractalkine\textsuperscript{43} is a multifunctional chemokine, expressed primarily by ECs, that exists in both membrane-bound and soluble forms, the latter derived by proteolytic cleavage.\textsuperscript{25,26} It exerts its angiogenic effect through cleavage from the endothelial surface and signaling through its receptor, CX3CR1 on nearby ECs.\textsuperscript{10,25–28} In the context of hindlimb ischemia, activated CX3CR1 induced HIF-1α (hypoxia inducible factor-1α) expression, VEGF production, and signaling through VEGF receptor 2,\textsuperscript{28} consistent with the timecourse and MKPO-1 dependence of VEGF expression in the present study.

Fractalkine mRNA has been shown to be upregulated in ischemic hindlimbs, peaking at 1 to 3 days after surgery, and, in that study, delivery of recombinant fractalkine stimulated hindlimb recovery in a rat hindlimb ischemia model.\textsuperscript{29} In a separate study, fractalkine induced angiogenesis in the chick chorioallantoic membrane and mouse subcutaneous pocket models with similar strength as recombinant VEGF.\textsuperscript{41,42} In the present study, we found that MKP-1 knockout mice exhibited reduced fractalkine expression associated with deficient neovascularization, and fractalkine treatment rescued the effect of MKP-1 deletion, with no differences in functional perfusion recovery or day 7 microvascularity. However, in contrast to the rat study, exogenous fractalkine delivery failed to enhance perfusion recovery in wild-type mice, suggesting that in ischemic mice, endogenous fractalkine levels are sufficient to mediate neovascular recovery. Together, these data confirm the role of fractalkine in MKP-1–mediated neovascularization.

In addition to its angiogenic functions, fractalkine also mediates chemotaxis and firm adhesion of monocytes and T-lymphocytes to activated endothelium,\textsuperscript{25–27} cells that play important roles in recovery from hindlimb ischemia.\textsuperscript{45–46} Consistent with these observations, we found that MKP-1 deletion dramatically reduced both monocyte/macrophage and T-lymphocyte invasion in the distal hindlimb. In the proximal hindlimb, however, inflammatory cell invasion was less than in the distal limb, and there were no differences between genotypes, which may partially explain the merely modest effect of MKP-1 deletion on arteriogenesis in that region of interest. On fractalkine delivery, there were no differences between genotypes in monocyte/macrophage or T-lymphocyte invasion in either region of interest, suggesting that this effect is mediated, at least in part, by MKP-1–regulated fractalkine expression.

Together, these data implicate a dual role for MKP-1 in neovascularization: through EC migration, proliferation, and tube formation and through inflammatory cell infiltration. This is consistent with our previous findings in the ApoE\textsuperscript{−/−} background that MKP-1 mediates macrophage activation.\textsuperscript{21} In that study, MKP-1 null mice featured decreased atherosclerotic lesion size, decreased inflammatory cytokines (interleukin-1α, tumor necrosis factor α), and increased levels of anti-inflammatory interleukin-10 in the circulation, as well as impaired macrophage spreading, migration, and tissue infiltration.\textsuperscript{21} Mechanistically, defective extracellular-related kinase-1/2 signaling was implicated in the reduced migration of MKP-1– and ApoE\textsuperscript{−/−} macrophages through pharmacological MeK1/2 kinase inhibition.\textsuperscript{21} Thus, MKP-1 may play dual roles in recovery from hindlimb ischemia, involving both its canonical function as a MAPK phosphatase in inflammatory cells and its chromatin-modifying function in ECs.

In addition, increasing evidence suggests that chemokine pathways, such as CCL2/CCR2 and fractalkine/CX3CR1, control inflammatory cell mobilization from the bone marrow to the bloodstream.\textsuperscript{47,48} For example, Cochain et al\textsuperscript{48} showed that although the CCL2/CCR2 axis regulated recruitment of Ly6C\textsuperscript{lo} monocytes to the circulation and contributed to hindlimb ischemia recovery, CX3CR1−/− mice exhibited a reduction in only Ly6C\textsuperscript{lo} monocytes and did not present a deficit in hindlimb neovascularization. Future studies will evaluate the role of MKP-1 in regulation of other chemokine pathways and MKP-1/chemokine-mediated monocyte mobilization and their role in neovascularization.

These data do not rule out the possibility that canonical MAPK deactivation may contribute to the functional effects observed here because numerous studies have verified the MAPK phosphatase activity of MKP-1 in ECs.\textsuperscript{18,49,50} Indeed, we have previously shown that VEGF induction of MKP-1 is c-Jun N-terminal kinase MAPK dependent, and VEGF-induced MKP-1 regulates phosphorylation of both c-Jun N-terminal kinase and p38, but not extracellular-related kinase-1/2, in HUVEC.\textsuperscript{18} Thus, the observations described here may result from a combination of these various MKP-1 substrates, which may explain the blunted angiogenic growth phase concurrent with decreased fractalkine expression but eventual recovery, perhaps because of prolonged MAPK activation.

Together, these data reveal a novel role for MKP-1 in angiogenic gene expression and neovascularization in vivo, identify a putative epigenetic mechanism for MKP-1–mediated gene regulation in ECs, and validate MKP-1 as a potential therapeutic target that can be activated specifically through combinatorial growth factor stimulation.\textsuperscript{49,51}

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Disclosures
None.

References
Angiogenesis is critical to many physiological and pathophysiological processes, and despite intense study, the regulatory mechanisms controlling this process remain incompletely understood. Mitogen-activated protein kinase phosphatase-1 (MKP-1) is typically considered an off-switch for the mitogen-activated protein kinases, signaling molecules that promote angiogenesis. Here, however, we show in the hindlimb ischemia model that MKP-1 positively mediates the early growth phase of neovascularization, a process critical in diseases, such as myocardial ischemia and tumor revival. We show that MKP-1 binds to DNA of the angiogenic chemokine, fractalkine, dephosphorylates histone H3 on this DNA locus, and is required for expression of this gene in vitro and in vivo. Finally, the deficit in neovascular recovery from hindlimb ischemia in MKP-1 knockout mice is rescued by in vivo fractalkine delivery. Together, these observations identify MKP-1 as a potential therapeutic target in vascular regeneration and disease.
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SUPPLEMENTAL METHODS

MKP-1 PROMOTES NEOVASCULARIZATION AND ANGIOGENIC GENE EXPRESSION
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Animals
MKP-1-deficient mice (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. Briefly, tissue samples were digested overnight by proteinase K incubation at 55°C. 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'-TGCCCTGCTCTTTACTGAAGGCTC-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. 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 rats13. 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 LD12 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. 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. 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 CO₂ 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, acetic acid 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, acetic acid 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, acetic acid 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. 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/cm². Cells were maintained in MCDB/F12 supplemented with 15% fetal bovine serum (FBS), 0.009% heparin, and 0.015% endothelial growth supplement (EGS) 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. 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 4 days 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 50 ng/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 (Targeting Systems). 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 200 ul 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/cm². 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 50 ng/ml VEGF or vehicle, with BrdU incorporation between hours 8 and 12.

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 50 ng/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\textsuperscript{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'-TCCTCCCTGTAGCTTGGAGA-3'
FKN exon 3 #4 R:
5'-CATTTGAGTTAGGGCAGCAG-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.

References:

SUPPLEMENTAL DATA

MKP-1 PROMOTES NEOVASCULARIZATION AND ANGIOGENIC GENE EXPRESSION
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Supplemental Figure I. MicroCT angiography of proximal hindlimb region of interest. 3D reconstructions of WT and KO vascular structures in the thigh region at day 7 (A, C) and day 28 (B, D) post-surgery (N = 4-9 per time point per genotype). Quantification of vascular network parameters (E) revealed no significant differences in arteriogenic network formation between WT and KO mice, except for connectivity at day 7 (*p ≤ 0.05 vs. KO at same time point, #p ≤ 0.05 vs. control at same time point). At day 28, there were no significant differences between genotypes or compared with controls.

Supplemental Figure II. Quantification of MKP-1 immunostaining in WT hindlimbs. Gastrocnemius (GC; A) and soleus (SM; B) muscle sections from WT mice were stained for MKP-1 at 7, 14 and 28 days. MKP-1 was elevated at day 7 post surgery, and expression decreased over time. *p ≤ 0.05 vs control limb at same time point.
**Supplemental Figure III.** Mouse aortic endothelial cell (MAEC) migration and fractalkine gene expression. Serum starved MAEC were scratched, treated with 50 ng/ml VEGF, and cells were allowed to migrate for 11 hrs (A). MKP-1 KO cells featured significantly reduced migration into the gap compared with cells from WT mice (B). Scale bar: 50 µm. Serum-starved MAEC, treated for 6 hrs with 50ng/ml, were then immunoblotted for fractalkine and GAPDH expression (C). MAEC were taken from 6 independent mice (3 WT, 3 KO). Fractalkine expression was elevated in WT mouse cells compared to KO. MAEC were then treated with vehicle, VEGF (50ng/ml), or VEGF (50ng/ml) + recombinant mouse fractalkine (100ng/ml), and migration was evaluated over 11 hrs (D, E). Fractalkine treatment rescued the effect of MKP-1 deletion. *p ≤ 0.05, NS = not significant.
Supplemental Figure IV. Fractalkine & endothelial cell co-localization. To demonstrate that ischemic hindlimb endothelial cells express fractalkine, sections were co-stained with BS-1 lectin (green) and fractalkine (red). Nuclei were co-stained with DAPI (blue). Red and green signal co-localized in both patent vessels (left column) and microvessels (right column), though fractalkine staining was also evident in hematocytes, consistent with the known function of fractalkine as a cleaved signaling molecule that interacts with CX3CR1-expressing cells. Despite positive staining in ECs, fractalkine expression was also markedly lower in KO mice compared to WT littermates, indicative of MKP-1 regulation of fractalkine gene expression. Scale bar: 50 µm.
Supplemental Figure V. Immunofluorescent tissue staining: positive and negative controls. Verification of antibody staining was evaluated by positive control tissue staining for CD31 and fractalkine in WT mouse brain tissue (A, B) and CD3 and CD68 in WT mouse spleen tissue (C, D). Lack of non-specific antibody-tissue binding was verified using negative controls of adjacent sections of WT gastrocnemius muscle lacking primary antibody but incubated with secondary Alexa Fluor 488 and 568 (E, F). H&E staining of GC sections illustrated the extent of inflammatory cell infiltration in WT tissue, but not KO (G). Scale bar: 50 µm.