SDF-1α stimulates JNK3 activity via eNOS-dependent nitrosylation of MKP7 to enhance endothelial migration

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Edited by Thomas Michel, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, and accepted by the Editorial Board January 30, 2009 (received for review September 24, 2008)

The chemokine stromal cell-derived factor-1α (SDF-1α) is a pivotal player in angiogenesis. It is capable of influencing such cellular processes as tubulogenesis and endothelial cell migration, yet very little is known about the actual signaling events that mediate SDF-1α-induced endothelial cell function. In this report, we describe the identification of an intricate SDF-1α-induced signaling cascade that involves endothelial nitric oxide synthase (eNOS), JNK3, and MAPK phosphatase 7 (MKP7). We demonstrate that the SDF-1α-induced activation of JNK3, critical for endothelial cell migration, depends on the prior activation of eNOS. Specifically, activation of eNOS leads to production of NO and subsequent nitrosylation of MKP7, rendering the phosphatase inactive and unable to inhibit the activation of JNK3. These observations reinforce the importance of nitric oxide and *S*-nitrosylation in angiogenesis and provide a mechanistic pathway for SDF-1α-induced endothelial cell migration. In addition, the discovery of this interactive network of pathways provides novel and unexpected therapeutic targets for angiogenesis-dependent diseases.

The CXC chemokines have recently emerged as key regulators of angiogenesis because of their ability to enhance the migratory capacity of endothelial cells and facilitate the homing of endothelial progenitor cells (EPCs) to ischemic tissues (1, 2). Stromal cell-derived factor-1-α (SDF-1α) (also called CXCL12) is one of the most potent proangiogenic CXC chemokines. Both in vivo and in vitro data support a critical role for SDF-1α in angiogenesis. SDF-1α treatment of endothelial cells induces tube-like structure formation and migration (3, 4), and SDF-1α increases tubulogenesis of microvascular endothelial cells via enhanced expression of VEGF and FGF and subsequent endothelial cell migration (5). SDF-1α is required for angiogenesis associated with wound healing under conditions in which SDF-1α expression inversely correlates with oxygen tension (6, 7). Likewise, hypoxia-inducible factor (HIF-1)-induced SDF-1α expression in ischemic tissue increases the adhesion, migration, and homing of circulating progenitor cells that express the SDF-1α receptor CXCR4, thereby promoting tissue regeneration (8).

Further evidence that SDF-1α acts as a key regulator of angiogenesis comes from observations that CXCR4, also called Fusin or leukocyte-expressed seven-transmembrane-domain receptor (LESTR), and its coreceptor CXCR7 are required for normal cardiac ventricular septum formation and vascular development (9–14). CXCR4 is selectively expressed in vascular endothelial cells and EPCs (8, 15), and its expression is induced by proangiogenic factors such as FGF2 and VEGF (16). Although the critical roles of SDF-1α/CXCR4 in endothelial cell migration are well recognized, relatively little is known about the signal transduction pathways that mediate these effects.

SDF-1α-induced EPC migration is mediated in part through activation of the PI3K/Akt/endothelial nitric oxide synthase (eNOS) signal transduction pathway (17). However, the specific role of NO generated by eNOS in SDF-1α-induced neovascularization is still poorly understood and the mechanism by which eNOS regulates SDF-1α-induced endothelial migration is not known. Here, we characterize the participation of eNOS in the regulation of SDF-1α-mediated endothelial cell activation. We demonstrate that JNK3, which has previously been predominantly linked to neuronal signaling (18), is expressed in endothelial cells and is a key mediator of eNOS-dependent SDF-1α-induced endothelial cell migratory responses. MAPK phosphatase 7 (MKP7) (or DUSP16), a JNK3 phosphatase that binds to the JNK3 adaptor protein β-arrestin2, is a critical link in this eNOS-dependent JNK3 activation, undergoing nitrosylation and enzymatic inhibition after SDF-1α administration. The regulation of JNK3 by eNOS through the regulation of MKP7 activity via catalytic nitrosylation provides a missing link between eNOS and JNK3 for the coordinated regulation of endothelial cell migration induced by SDF-1α.

Results

eNOS Is Activated by SDF-1α and Is Required for Endothelial Cell Migration Induced by SDF-1α. SDF-1α-induced EPC migration is blocked by eNOS and PI3K inhibitors, suggesting that the PI3K/Akt/eNOS signaling axis is required for SDF-1α-dependent progenitor cell activation (17). However, the exact roles of eNOS and nitric oxide in the regulation of SDF-1α-mediated migration in mature endothelial cells are not known. To examine this, we first asked the following questions: Does the addition of SDF-1α to mature endothelial cell cultures result in eNOS activation? And, if so, does eNOS play a role in the SDF-1α-induced migration of endothelial cells?

The degree of SDF-1α-induced activation of eNOS was examined in bovine aortic endothelial cells (BAECs) by monitoring phosphorylation of the Ser-1777 activation site in eNOS. Our results demonstrated that eNOS was phosphorylated by SDF-1α in a time- and dose-dependent manner. Phosphorylation of Ser-1777 peaked at 5 min after SDF-1α stimulation (Fig. 1*A*) and increased in a dose-dependent fashion over a SDF-1α concentration range of 50–500 ng/mL (Fig. 1*B*), which approximates physiologically relevant SDF-1α concentrations.

To determine whether eNOS activation is required for SDF-1α-mediated endothelial cell migration, we performed scratch wound-healing and Boyden chamber migration assays in the presence of the NOS-specific inhibitor N^G-monomethyl-L-arginine (L-NMMA) (19). Confluent BAECs were pre-

Author contributions: X.P. and C.P. designed research; X.P., Y.W., and J.E.F. performed research; X.P., Y.W., J.E.F., and C.P. analyzed data; and X.P., A.L.P., and C.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. T.M. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0809568106/DCSupplemental.

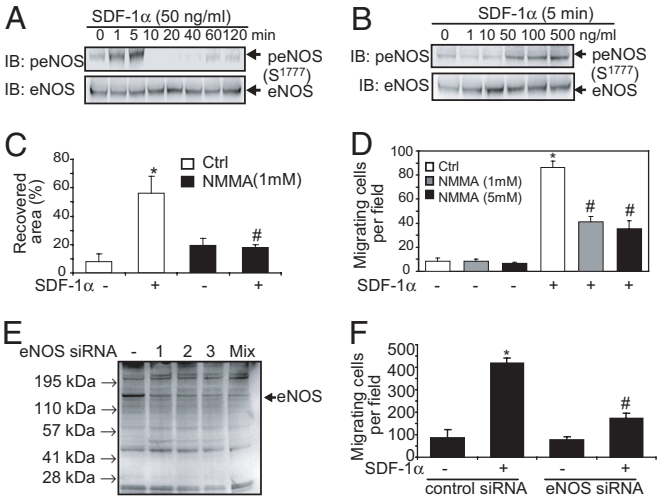

Fig. 1. eNOS is activated by SDF-1α. (*A*) Western blot analysis of eNOS activity in BAECs treated with 50 ng/mL SDF-1α for indicated time periods. (*B*) Western blot analysis of BAECs treated with SDF-1α with the indicated dosages for 5 min. (*C*) Results of a scratch wound-healing assay performed on BAECs pretreated with N^G-monomethyl-L-arginine (L-NMMA) with or without the addition of 50 ng/mL SDF-1α. *, $P < 0.05$, compared with control cells without SDF-1α. #, $P < 0.05$, compared with control cells with SDF-1α. (*D*) Results of the Boyden chamber assay with BAECs pretreated with L-NMMA and stimulated with 50 ng/mL SDF-1α. *, $P < 0.05$, compared with control cells without SDF-1α. #, $P < 0.05$ compared with control cells with SDF-1α. (*E*) Western blot analysis of eNOS protein level in BAECs transfected with eNOS siRNA1, -2, -3, or a mixture of the three. (*F*) Results of a Boyden chamber assay using BAECs transfected with eNOS siRNA or control siRNA and stimulated with 50 ng/mL SDF-1α. *, $P < 0.05$, compared with control cells without SDF-1α. #, $P < 0.05$ compared with control cells with SDF-1α.

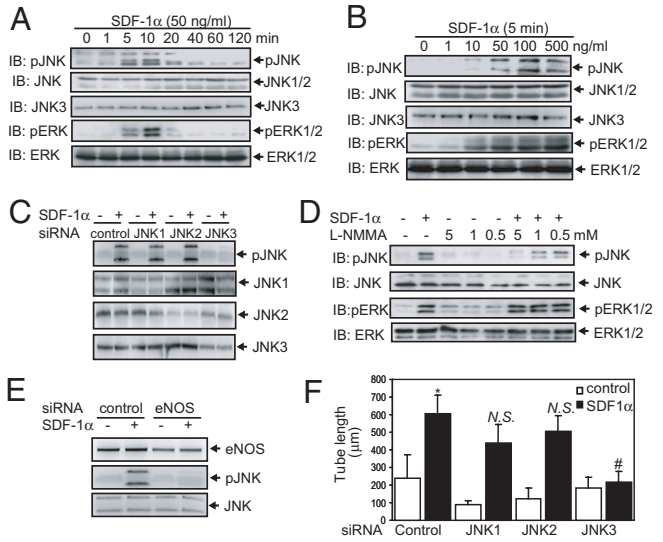

Fig. 2. JNK3 acts as a downstream mediator for eNOS activation induced by SDF-1α. (*A*) Western blot analysis of BAECs treated with 50 ng/mL SDF-1α for indicated time period. (*B*) Western blot analysis of BAECs treated with SDF-1α at the indicated dosages for 5 min. (*C*) Western blot analysis of BAECs transfected with siRNAs for JNK1, JNK2, or JNK3 and treated with 50 ng/mL SDF-1α for 10 min. (*D*) Western blot analysis of BAECs preincubated with L-NMMA and then activated with 50 ng/mL SDF-1α for 5 min. (*E*) Western blot analysis of BAECs transfected with a mixture of eNOS siRNA1–3 and treated with 50 ng/mL SDF-1α for 10 min. (*F*) Results from a Matrigel angiogenesis assay with BAECs transfected with JNK1, JNK2, or JNK3 siRNAs. *, $P < 0.05$, compared with control cells without SDF-1α. #, $P < 0.05$, compared with control cells with SDF-1α. *N.S.*, not significant.

treated for 1 h with 1 or 5 mM L-NMMA or vehicle before each assay. In the wound-healing assay, SDF-1α increased BAEC migration toward the wounded area from 8.09 ± 5.42% to 56.0 ± 12.0%, whereas L-NMMA pretreatment inhibited cell migration (from 56.0 ± 12.0% to 17.7 ± 2.4%) (Fig. 1*C*) and significantly decreased wound recovery. Similarly, cell migration in Boyden chambers was induced by SDF-1α (from 8 ± 3 to 86 ± 5 cells per field), but this increase in migration was inhibited by the preincubation of BAECs with L-NMMA (Fig. 1*D*). The involvement of eNOS in SDF-1α-induced endothelial cell migration was confirmed by treatment of cells with siRNAs specific for eNOS. Endothelial cells were transfected with 1 of 3 different bovine eNOS-specific siRNAs, or mixture of all three, and 72 h later cell lysates were examined by Western blot analysis for levels of eNOS protein. Each separate siRNA entity and the mixture of all three were found to be similarly effective at suppressing expression of endogenous eNOS in BAECs (Fig. 1*E*). Likewise, transfection of cells with eNOS-specific siRNA resulted in a significant decrease in migration of cells in response to SDF-1α treatment (Fig. 1*F*). Therefore, inhibiting eNOS activity by either siRNA or a protein-specific inhibitor is significantly detrimental to SDF-1α-induced endothelial cell migration, indicating that eNOS is activated downstream of SDF-1α in mature endothelial cells and that this activation is required for SDF-1α-stimulated endothelial cell migration.

JNK3, but Not JNK1 or JNK2, Is Activated by SDF-1α. MAPKs, including ERK1/2 and p38, have been reported to enhance angiogenesis (20, 21). However, the role of JNK in angiogenesis remains controversial, with studies showing both positive and negative outcomes of JNK activation. To determine which MAPKs are involved in SDF-1α-induced endothelial cell migration, we screened the activities and expression levels of ERK1/2, p38, and JNK after treatment of BAECs with SDF-1α. SDF-1α (50 ng/mL) potently induced JNK and ERK1/2 activities in BAECs (Fig. 2*A*; note that multiple bands in JNK blots represent splice variants of JNK). SDF-1α activated both JNK and ERK1/2 in a dose-dependent manner (Fig. 2*B*). When comparing the time course of SDF-1α-induced activation of JNK and ERK1/2 to that of eNOS, the peak of their activation lagged behind eNOS (compare Fig. 1*A* with Fig. 2*A*), suggesting that JNK and ERK1/2 may act downstream of eNOS to transduce the migratory events induced by SDF-1α in endothelial cells.

Protein and mRNA for all three JNK isoforms, JNK1, JNK2, and JNK3, can be found in BAECs (Fig. S1 *A* and *B*). To determine which JNK protein(s) was activated by SDF-1α in these cells, we used a previously described assay (22). Exogenous JNK1, JNK2, and JNK3 proteins containing Flag or GFP epitopes were transiently expressed in BAECs and their activities were determined by Western blot analysis with a phospho-JNK antibody after SDF-1α treatment. Interestingly, only phosphorylation of JNK3 (but not JNK1 or JNK2) was increased after treatment of endothelial cells with SDF-1α (Fig. S1*C*). To confirm the finding that JNK3 is the only JNK entity activated by SDF-1α, we transfected cells with siRNAs for each of the JNK proteins and then tested the effect of each siRNA for the ability to suppress SDF-1α-induced JNK activation. SDF-1α significantly enhanced JNK phosphorylation in the cells transfected with control, JNK1, and JNK2 siRNAs (Fig. 2*C*). However, the increase of JNK phosphorylation was abrogated in the cells transfected with JNK3 siRNA. These results suggest that different JNK isoforms might have different cellular functions in endothelial cells because they are being differentially regulated under these conditions, with JNK3 specifically mediating effects downstream of SDF-1α signaling. Because JNK3 was the only SDF-1α-activated JNK in endothelial cells in our studies (Fig. 2*C*

and Fig. S1*C*), we consider the phospho-JNK signals in Western blots with anti-phospho-JNK antibody as the signals for activated JNK3.

JNK3 Acts as a Downstream Mediator of SDF-1α-Induced eNOS Activation. Nitric oxide produced by eNOS activates cGMP-dependent protein kinase to mediate VEGF-induced raf-1 and ERK1/2 activation, resulting in VEGF-induced angiogenesis (for review, see ref. 23). Our data indicate that eNOS is also a requisite mediator of SDF-1α-induced endothelial cell migration (Fig. 1), yet the mechanisms whereby eNOS-dependent regulation of SDF-1α signaling in endothelial cell migration occurs is unclear. To determine whether JNK3 and/or ERK1/2 are targets of the NO produced by SDF-1α-induced eNOS activation, L-NMMA was used to inhibit eNOS activation. Interestingly, JNK3 (but not ERK1/2) activation induced by SDF-1α was specifically inhibited by 0.5≈5 mM L-NMMA (Fig. 2*D*), indicating that SDF-1α-induced JNK3 activation is reliant on eNOS-dependent NO generation. To further confirm JNK3 is the target for NO produced by SDF-1α-induced eNOS activation, eNOS siRNAs were transfected into BAECs to knockdown endogenous eNOS protein. As predicted by the previous results (Fig. 2*D*), JNK3 was activated by SDF-1α in the cells transfected with control siRNA, but it was inhibited in the cells transfected with eNOS siRNA (Fig. 2*E*). These results support the contention that eNOS is a required mediator for JNK3 activation induced by SDF-1α.

JNKs mediate cell migration through the regulation of focal adhesion assembly and microtubule and actin dynamics [for review, see Huang et al. (24)]. JNK3 has previously been described as a relatively brain-selective JNK isoform and is suggested to be an important therapeutic target for Alzheimer's disease, Parkinson's disease, and stroke [for review, see Resnick and Fennell (18)]. However, the role of JNK3 in endothelial cell migration has not yet been evaluated. Because JNK3 was the only SDF-1α-activated JNK in endothelial cells in our studies (Fig. 2*C* and Fig. S1*C*), we were able to use the general JNK inhibitor SP600125 to test the role of JNK3 in SDF-1α-induced endothelial cell migration. As shown in Fig. S1*D*, SP600125 (10 or 50 μM) significantly inhibited SDF-1α-mediated endothelial cell migration (from 86 ± 5 to 27 ± 3 cells with 10 μM SP600125 or 20 ± 2 cells with 50 μM SP600125, respectively). Interestingly, in the absence of SDF-1α treatment, cell migration was also inhibited by SP600125, suggesting that there is a low level of tonic JNK signaling, probably elicited by endogenous SDF.

Endothelial cell migration is a critical step for tube formation during angiogenesis. To test the importance of JNK3 in highly ordered cell assembly processes, we examined the effect of JNK knockdown with siRNAs on the formation of capillary-like tubes in Matrigel. BAECs were transfected with JNK1 siRNA, JNK2 siRNA, JNK3 siRNA, or control siRNA. After 72 h, cells were plated on Matrigel in medium containing 50 ng/mL SDF-1α. Treatment with SDF-1α significantly enhanced tube formation (Fig. S1*E*), with the tube length increasing from 238 ± 136 to 605 ± 106 μm. In cells expressing either JNK1 siRNA or JNK2 siRNA, tube formation was also increased by SDF-1α from 88 ± 23 to 438 ± 108 μm or 121 ± 61 to 504 ± 93 μm, respectively (Fig. 2*F*). In contrast, tube formation was significantly inhibited by ≈90.3% in JNK3 siRNA-expressing cells. These data once again implicate JNK3 as the critical JNK moiety involved in SDF-1α-dependent angiogenesis.

MKP7 Activity Is Inhibited by *S*-Nitrosylation. The preceding experiments indicate that eNOS and its downstream effector JNK3 are required for SDF-1α-induced endothelial cell migration. However, the molecular mechanisms of eNOS-dependent regulation of JNK3 activity are not intuitively obvious. In the course of exploring various potential regulatory mechanisms, we considered the possibility that regulation of JNK3 by eNOS was determined by suppression of dephosphorylation activities. When cells were incubated with sodium orthovanadate, an inhibitor of protein tyrosine and dual-specificity protein phosphatases, JNK phosphorylation increased both under basal conditions and, most prominently, after SDF-1α treatment (Fig. 3*A*). These observations suggested to us that JNK phosphatases may be targets for determining JNK3 activation by eNOS. MKP7, a member of the dual-specificity family of protein phosphatases, interacts with the JNK3 scaffold protein β-arrestin2 (25). Located within the N-terminal catalytic domain of MKP7, the Cys^{244} residue is highly sensitive to oxidation because of its low pK_a, and this oxidation is required for decreased MKP7 activity and sustained activation of JNK3 under certain conditions (26). Given that eNOS is the major source of nitric oxide production in endothelial cells and in some circumstances can regulate protein function via *S*-nitrosylation of cysteine residues, we tested (*i*) whether MKP7 could be *S*-nitrosylated by nitric oxide and (*ii*) whether *S*-nitrosylation of MKP7 would affect its ability to regulate JNK3 activity. An in vitro biotin switch nitrosylation assay demonstrated that MKP7 could indeed be *S*-nitrosylated by the exogenous nitric oxide donor nitrosoglutathione (GSNO) (Fig. 3*B*). To determine whether this *S*-nitrosylation event affected MKP7 activity, we performed an in vitro phosphatase

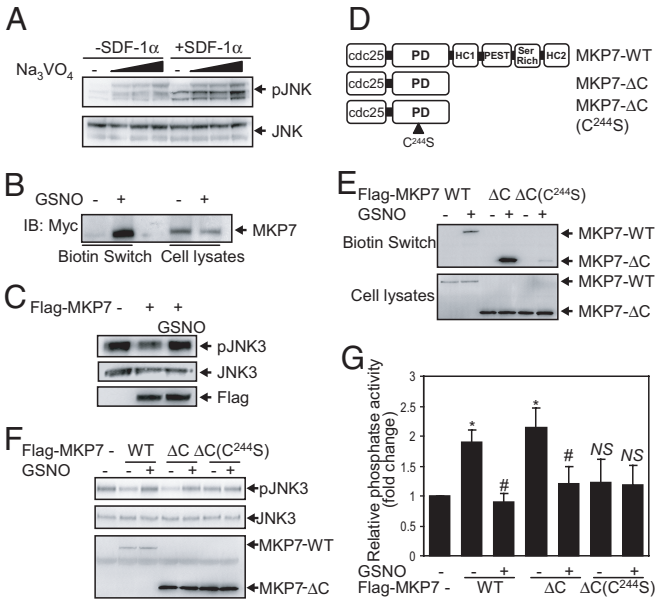

Fig. 3. MKP7 is nitrosylated by nitric oxide. (*A*) Western blot analysis to detect phosphorylated JNK and total JNK protein in BAECs pretreated with Na_3VO_4 (10, 100, and 500 μM) and then treated with 50 ng/mL SDF-1α for 10 min. (*B*) Results of a biotin switch assay on HEK293T cells transfected with Myc-MKP7 and treated with the nitric oxide donor nitrosoglutathione (GSNO) (500 μM) for 10 min at room temperature. (*C*) Results of an in vitro phosphatase assay using active JNK3 protein as the substrate on lysates of HEK293T cells transfected with Myc-MKP7. (*D*) Schematic structure of MKP7 mutant constructs. (*E*) Biotin switch analysis of HEK293T cells transfected with the indicated Flag-tagged MKP7 constructs and then treated with 50 μM GSNO for 10 min at room temperature. (*F*) Results of an in vitro phosphatase assay using active JNK3 protein as the substrate on HEK293T cells transfected with Flag-tagged MKP7 and then treated with 200 μM GSNO for 10 min. (*G*) Quantitative analysis of results from in vitro phosphatase assay of MKP7 based on three independent experiments. *, $P < 0.05$, compared with control cells. #, $P < 0.05$, compared with the same cells without GSNO treatment. *NS*, not significant, compared with control cells or the same cells without GSNO treatment, respectively.

activity analysis by using activated JNK3 protein in a reaction with MKP7-concentrated cell lysates. The ectopically expressed MKP7 protein exhibited strong phosphatase activity as demonstrated by the decrease in JNK3 phosphorylation (Fig. 3*C*). However, nitrosylation of MKP7 by GSNO markedly inhibited MKP7 phosphatase activity (Fig. 3*C*). To determine whether Cys^{244} was the targeted residue of MKP7 for this nitrosylation, mutant constructs of MKP7 were generated (Fig. 3*D*). As shown in Fig. 3*E*, both MKP7-WT and MKP7-ΔC (amino acids 1–317, retaining the catalytic domain) were strongly nitrosylated by GSNO. However, the nitrosylation of MKP7-ΔC($C^{244}S$) by GSNO was markedly attenuated compared to that of MKP7-ΔC (Fig. 3*E*). The activities of these mutants were also evaluated by using an in vitro phosphatase assay. Both MKP7-WT and MKP7-ΔC possessed strong phosphatase activities as detected by the decreased phosphorylation of JNK3, and their activities decreased dramatically after the treatment with GSNO (Fig. 3 *F* and *G*). In contrast, the phosphatase activity of MKP7-ΔC($C^{244}S$) was much lower than that of MKP7-WT or MKP7-ΔC protein, and the activity after GSNO treatment was essentially unchanged. Collectively, these observations indicate that Cys^{244} located in the catalytic domain of MKP7 is nitrosylated and that *S*-nitrosylation of the MKP7 protein decreases its activity, providing mechanistic support that MKP7 is the mediator for regulation of JNK3 activity by nitric oxide.

MKP7 *S*-Nitrosylation Induced by SDF-1α Is Required for JNK3 Activation and Cell Migration. Because our in vitro data suggested that MKP7 is nitrosylated by nitric oxide and that this nitrosylation inhibits its phosphatase activity, we next tested the relationship between SDF-1α stimulation and MKP7 *S*-nitrosylation in BAECs. As a first step, we investigated whether SDF-1α treatment of BAECs would result in *S*-nitrosylation of MKP7. SDF-1α treatment of cells increased MKP7 nitrosylation (Fig. 4*A*), similar to the increase of MKP7 nitrosylation observed previously with GSNO. eNOS siRNA was also used to investigate the effect on MKP7 nitrosylation after SDF-1α treatment in BAECs. BAECs were transfected with eNOS siRNA or control siRNA and Flag-tagged MKP7. After 72 h, cells were treated with SDF-1α and a biotin switch assay was performed. Consistent with the result obtained with the eNOS inhibitor L-NMMA (Fig. 4*A*), eNOS protein knockdown with eNOS siRNA blocked MKP7 nitrosylation after SDF-1α treatment, compared to the cells transfected with control siRNA (Fig. 4*B*). Next, we used JNK3 phosphorylation as a readout for determining whether SDF-1α activation of JNK3 could be inhibited by MKP7. Using the MKP7 mutants, we determined that the SDF-1α-induced JNK3 activation was substantially inhibited by MKP7-WT or MKP7-ΔC, but not by MKP7-ΔC($C^{244}S$) (Fig. 4*C*). Interestingly, when cells were made to express MKP7-ΔC($C^{244}S$) to block the endogenous MKP7 phosphatase activity, JNK3 activity increased even under unstimulated conditions (Fig. 4*C*). Similarly, the mutants of full-length MKP7, MKP7-$C^{244}S$ and MKP7-$C^{244}A$, failed to decrease JNK3 activity; instead, JNK3 activity increased under both basal and SDF-1α-treated conditions, compared with control cells without SDF-1α treatment (Fig. 4*D*). These results clearly demonstrate that, after SDF-1α treatment of BAECs, MKP7 is nitrosylated at Cys^{244} and that this event in turn is required for SDF-1α-induced JNK activation. To confirm that Cys^{244} in MKP7 is the target of eNOS-induced nitrosylation, we repeated the experiment detailed in Fig. 2*D*, in which the eNOS inhibitor L-NMMA was used to inhibit SDF-1α-induced JNK3 activation, by using MKP7-$C^{244}S$ as a dominant-negative construct. When MKP7-$C^{244}S$ was expressed in the cells, the inhibitory effect of L-NMMA was completely relieved (Fig. 4*E*). This result strongly implies that endogenous MKP7 is the mediator for eNOS-dependent JNK3 activation. Together with the data presented previously that overexpressed MKP7 could be nitrosylated by nitric oxide and eNOS

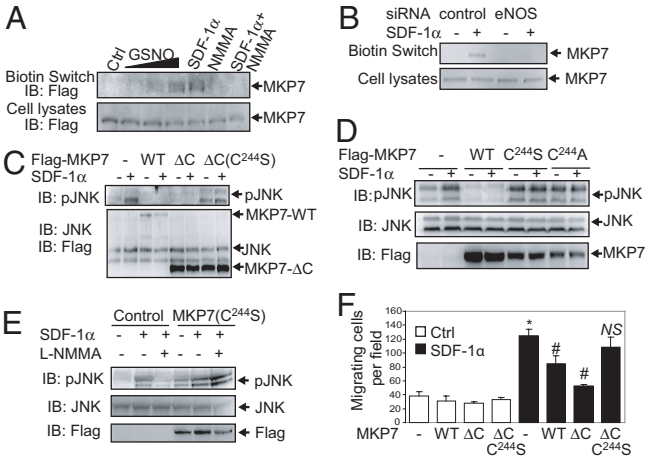

Fig. 4. MKP7 nitrosylated at Cys^{244} is required for SDF1α-induced cell migration. (*A*) Biotin switch analysis of BAECs transfected with Flag-tagged MKP7 and incubated with 1 mM L-NMMA followed by treatment with 50 ng/mL SDF-1α for 5 min. (*B*) Biotin switch analysis of BAECs transfected with a 100 pmol of eNOS siRNA mixture and 2 μg of Flag-MKP7 and subsequently treated with SDF-1α for 5 min. (*C*) Western blot analysis of BAECs transfected with Flag-tagged MKP7 constructs and activated with 50 ng/mL SDF-1α for 5 min. JNK activation was determined by the detection of phospho-JNK level in the cell lysates. (*D*) Western blot analysis of BAECs transfected with Flag-tagged MKP7 constructs and activated by 50 ng/mL SDF-1α for 5 min. (*E*) Western blot analysis of BAECs transfected with Flag-tagged MKP7-$C^{244}S$ or pCMV empty vector as control, and 2 days later pretreated with 1 mM L-NMMA for 1 h and treated with 50 ng/mL SDF-1α for 5 min. (*F*) Boyden chamber analysis of BAECs transfected with Flag-tagged MKP7 constructs using 50 ng/mL SDF-1α as the chemoattractant. *, $P < 0.05$, compared with control cells without SDF-1α. #, $P < 0.05$, compared with control cells with SDF-1α. *NS*, not significant, compared with control cells with SDF-1α.

siRNA inhibited its nitrosylation, it supported that endogenous MKP7 could be nitrosylated by nitric oxide generated by active eNOS, which in turn regulates JNK3 activity.

To ascertain the functional consequences of MKP7 *S*-nitrosylation, we performed endothelial cell migration assays using the various MKP7 mutants. As shown in Fig. 4*F*, SDF-1α treatment increased endothelial cell migration from 38 ± 7 to 125 ± 9 cells per field. Expression of MKP7-WT or MKP7-ΔC in BAECs significantly decreased cell migration from 125 ± 9 to 84 ± 12 or 53 ± 2 cells per field, respectively; however, SDF-1α-induced cell migration was not inhibited by MKP7-ΔC($C^{244}S$) (from 125 ± 9 to 109 ± 14 cells per field). Although cells transfected with MKP7-ΔC($C^{244}S$) exhibited higher JNK3 activity under basal conditions (Fig. 4*C*), cell migration was not increased (Fig. 4*F*), suggesting that JNK3 activity is required but probably not sufficient for SDF-1α-induced cell migration and that other mediators responsive to SDF-1α, such as ERK1/2, may also be required for the highly coordinated process of cell migration. Collectively, these data indicate that MKP7 nitrosylation by nitric oxide after SDF-1α-induced eNOS activation provides a critical checkpoint for JNK3 activation and subsequent endothelial cell migration after SDF-1α treatment. Furthermore, these studies articulate a mechanism through which MKP7 is a key participant in the coordination of events mediated by eNOS and JNK3 after activation by SDF-1α.

Discussion

The major finding of this study is the discovery that SDF-1α activates JNK3 in endothelial cells and that this activation is required for SDF-1α-induced endothelial cell migration. In addition, we demonstrate that JNK3 activation is eNOS dependent. Specifically, MKP7—a JNK3 phosphatase—can be nitrosylated and inhibited by nitric oxide after SDF-1α-induced eNOS activa-

tion. The inhibition of MKP7 activity by SDF-1α treatment is critical for JNK3 activation and the resultant endothelial migration. These observations reinforce the importance of nitric oxide and *S*-nitrosylation in cell migration and angiogenesis. In addition, the findings provide mechanistic insight into the signaling pathways responsible for SDF-1α-induced JNK3 activation of endothelial cells, strongly suggesting the need for cross-talk between eNOS and MAP kinases through the "bridge" molecule—MKP7. The critical roles of MKP7 and JNK3 in SDF-1α-induced cell migration provide unique and unexpected therapeutic targets for angiogenesis-dependent diseases.

SDF-1α and its receptor CXCR4 are considered to be critical for endothelial migration and in vivo neovascularization (5, 15, 16). SDF-1α activates Akt/eNOS and MAP kinases including ERK1/2 and p38 in different cell types (including endothelial cells and EPCs), resulting in a vast array of consequences including cell migration, apoptosis, and cell survival (27–30). However, until now, no detailed description of the signaling pathways involved in SDF-1α-dependent angiogenesis have been published. Likewise, although eNOS and its generated nitric oxide have long been considered critical for SDF-1α-mediated endothelial cell migration and angiogenesis, the exact mechanisms by which eNOS regulates SDF-1α-dependent endothelial cell migration have until now been a mystery. Our results demonstrate that JNK3, but not the other MAP kinases ERK1/2 or p38, acts downstream of eNOS to promote endothelial cell migration. This discovery increases our understanding of the role that eNOS and nitric oxide play in SDF-1α-induced endothelial activation. However, this JNK3/eNOS-dependent pathway is not the only SDF-1α-induced migration mechanism used by endothelial cells. Our results also point to an eNOS-independent pathway for MAP kinase-associated cell migration mediated by ERK1/2, which was significantly activated by SDF in BAECs (Fig. 2 *A* and *B*). It has been shown that SDF-1α can activate p38 in other cultured cell types (28), but we did not detect p38 activation by SDF-1α, suggesting cellular specificity in the regulation of p38 by SDF-1α. The requirement of both eNOS-dependent and -independent MAP kinase activation pathways for endothelial cell migration is consistent with the complex nature of cellular migration and angiogenesis in general. The eNOS-dependent and -independent pathways coordinating the migratory responses of endothelial cells in response to SDF-1α are an avenue of further investigation.

S-Nitrosylation of proteins by nitric oxide is one of the major avenues of nitric oxide regulation of multiple cellular responses, including DNA repair, host defense, blood pressure control, and neurotransmission (31). More than 100 proteins have been reported to be *S*-nitrosylated in cells, including JNK1 and JNK3, resulting in either inhibition or activation of protein function (32). JNK1 activity is suppressed after nitrosylation on Cys^{116} of JNK1 by nitric oxide generated after IFN-γ administration in macrophages (33). Conversely, nitric oxide mediates ERK and JNK activation during hypoxia in neuronal cells (34) and increases nitrosylation and phosphorylation of JNK3 in hippocampal CA1 cells (35). In our study, JNK3 activity was positively regulated by *S*-nitrosylation, although the target of this *S*-nitrosylation appears to be the JNK3 phosphatase, MKP7, and not JNK3 directly (Fig. 3). The inhibitory effect of NMMA was relieved by expression of the dominant-negative form of MKP7 (Fig. 4*E*), which suggests that JNK3 or its upstream kinases are not the dominant targets of eNOS and nitrosylation.

EPCs, improving neovascularization, are increasingly considered as therapeutic tools for the prevention of vascular diseases, and gene-targeted endothelial progenitor cells are considered potential carriers of targeted therapies for cancers (36, 37). SDF-1α is known to be a key player in EPC mobilization and homing processes in animal models, similar to other cytokines such as granulocyte colony-stimulating factor, granulocyte–monocyte colony-stimulating factor, and $VEGF_{165}$ (38). Although future experiments are still needed to determine whether eNOS and JNK3 contribute coordinately to EPC-mediated neovascularization, this study provides insights into the signaling pathways responsible for SDF-1α-dependent angiogenesis and shed more light on the therapeutic options for cell-based neovascularization therapies against vascular diseases, stroke, and cancer.

Materials and Methods

Reagents. Recombinant human SDF-1α protein was obtained from R&D Systems. Antibodies to pJNK, JNK, peNOS, eNOS, pAkt, Akt, pERK1/2, and ERK1/2 were purchased from Cell Signaling. JNK3 antibody and the active JNK3 purified protein were purchased from Upstate. MKP7 antibody was purchased from Novus Biologicals. The cDNA constructs, including Flag-JNK1, Flag-JNK2, and Flag-JNK3, were kindly provided by Roger J. Davis (University of Massachusetts, Worcester, MA).

cDNA Constructs. cDNA clone MGC:50665 (IMAGE: 4400399) containing human MKP7 cDNA was purchased from Invitrogen. The Myc- or Flag-tagged MKP7 cDNA constructs were generated by PCR-based cloning with IMAGE cDNA clone of human MKP7 cDNA into pCMV-Tag-3B or pCMV-Tag-2B vector (Stratagene), respectively. The mutants of MKP7 constructs were generated by PCR-based mutagenesis for deletion or point mutation.

Cell Culture and Transient Transfection. For transient expression experiments with BAECs, 50–70% confluent cells were transfected for 3 h with 2 μg of plasmids by using 8 μL of Lipofectamine and 8 μL of Plus reagent (Invitrogen). One day later, cells were serum starved overnight, and then treated with SDF-1α. For transient expression experiments with 293 cells, 90–100% confluent 293 cells were transfected 24 h after plating with 2 μg of plasmids by using 15 μL of Lipofectamine 2000 (Invitrogen).

siRNA Design and Transient Transfection. siRNAs for bovine eNOS, JNK1, JNK2, and JNK3 were designed with BLOCK-iT RNAi designer (www.invitrogen.com). Sequences are available in *SI Materials and Methods*. The transfection of siRNA into BAECs was performed with Nucleofector electroporation system following the manufacturer's protocol for HUVEC (Amaxa).

Immunoprecipitation and Western Blotting Analysis. Cells were harvested in lysis buffer and equal amounts of proteins were incubated with a specific antibody overnight at 4 °C with gentle rotation. Protein A/G Plus-agarose beads (Santa Cruz Biotechnology) were used to pull down the antibody complexes. Immune complexes were then separated by SDS/PAGE and analyzed by Western blotting.

Biotin Switch Assay. Experiments were performed following the methods previously described in ref. 39.

In Vitro Phosphatase Assay. In vitro phosphatase reactions were carried out on Flag-MKP7 protein immunoprecipitated from transfected cells, and results were visualized by using Western blot analysis with phosphospecific JNK and JNK antibodies.

Boyden Chamber Assay. Boyden chamber assays were performed as described in refs. 40 and 41.

Wound-Healing Assay. For detection of cell migration, a wound-healing assay was performed as described in ref. 40.

In Vitro Matrigel Angiogenesis Assay. Endothelial cell tube formation was analyzed with the Matrigel-based tube formation assay as previously described in refs. 40 and 41.

The methods in detail are available in *SI Materials and Methods*.

Statistical Analysis. Data are shown as mean ± SD for 3 or 4 separate experiments. Differences were analyzed by Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

ACKNOWLEDGMENTS. C.P. is an Established Investigator of the American Heart Association and a Burroughs Wellcome Fund Clinician Scientist in Translational Research. This work was supported by National Institutes of Health Grants HL 61656, HL 03658, and HL 072347 (to C.P.).

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