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## NADPH oxidase- generated ROS are required for SDF-1 $\alpha$ -stimulated angiogenesis Short title: NOX is an angiogenic regulator

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### Abstract

**Objective**—Reactive oxygen species (ROS) act as signaling molecules during angiogenesis, however, the mechanisms used for such signaling events remain unclear. Stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) is one of the most potent angiogenic chemokines. Here we examined the role of ROS in the regulation of SDF-1 $\alpha$ -dependent angiogenesis.

**Approach and results**—Bovine aortic endothelial cells (BAECs) were treated with SDF-1 $\alpha$  and intracellular ROS generation was monitored. SDF-1 $\alpha$  treatment induced BAEC migration and ROS generation, with the majority of ROS generated by BAECs at the leading edge of the migratory cells. Antioxidants and NADPH oxidase (NOX) inhibitors blocked SDF-1 $\alpha$ -induced endothelial migration. Furthermore, knockdown of either NOX5 or p22phox (a requisite subunit for NOX1/2/4 activation) significantly impaired endothelial motility and tube formation, suggesting that multiple NOXs regulate SDF-1 $\alpha$ -dependent angiogenesis. Our previous study demonstrated that JNK3 activity is essential for SDF-1 $\alpha$ -dependent angiogenesis. Here, we identified that NOX5 is the dominant NOX required for SDF-1 $\alpha$ -induced JNK3 activation and that NOX5 and MKP7 (the JNK3 phosphatase) associate with one another but decrease this interaction upon SDF-1 $\alpha$  treatment. Furthermore, MKP7 activity was inhibited by SDF-1 $\alpha$  and this inhibition was relieved by NOX5 knockdown, indicating that NOX5 promotes JNK3 activation by blocking MKP7 activity.

**Conclusions**—We conclude that NOX is required for SDF-1 $\alpha$  signaling and that intracellular redox balance is critical for SDF-1 $\alpha$ -induced endothelial migration and angiogenesis.

### Keywords

Reactive oxygen species; NADPH oxidase; SDF-1 $\alpha$ ; migration; angiogenesis

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### DISCLOSURES

None.

## INTRODUCTION

Reactive oxygen species (ROS) are generated not only as by-products of mitochondrial metabolism, but also by a variety of cellular enzyme systems including NADPH oxidase (NOX), uncoupled endothelial nitric oxide synthase (eNOS), xanthine oxidase and arachidonic acid metabolizing enzymes. When cellular production of ROS exceeds the antioxidant capacity of cardiovascular cells, proteins, lipids and nucleic acids become damaged and may eventually contribute to the development of cardiovascular diseases such as atherosclerosis, hypertension, diabetic cardiovascular complications and ischemic-reperfusion injury. Conversely, low concentrations of ROS play a critical role in regulating cardiovascular functions such as angiogenesis and tissue repair.<sup>1-3</sup> ROS are required for VEGF-induced endothelial migration, proliferation and tube formation.<sup>4, 5</sup> During ischemia and reperfusion, ROS generation promotes capillary tube formation in human microvascular endothelial cells<sup>6</sup> and the heart<sup>7</sup>, whereas inhibiting ROS through treatment with antioxidants or superoxide dismutases blocks vascularization and growth of tumors.<sup>8, 9</sup> The precise molecular mechanisms, however, by which ROS mediate angiogenic responses are incompletely understood.

NOX is an important enzymatic source of ROS. There are seven Nox genes identified in mammalian organisms - Nox1-5 and Duox (Dual Oxidase) 1-2. NOXs are expressed in endothelial cells and other cardiovascular cells and regulate various functions such as cell survival, growth, apoptosis, differentiation, angiogenesis and contractility.<sup>10</sup> The NOX enzymes are heteroprotein complexes (except NOX5) with different regulatory mechanisms, tissue distribution and subcellular localization and downstream targets. A membrane regulatory subunit, p22phox, is associated with NOXs 1, 2 and 4, and is required for their activity.<sup>10</sup> NOXs 1 and 2 share a common overall structure with a very short cytoplasmic N-terminus, that is required for activation<sup>11-13</sup>, and six transmembrane domains.<sup>14, 15</sup> In contrast, NOX4 is constitutively active and is regulated by gene expression.<sup>16</sup> Interestingly, unlike other NOXs, NOX5 possesses a longer cytoplasmic N-terminus containing Ca<sup>2+</sup>-binding motifs, resulting in its activation by Ca<sup>2+</sup> elevation.<sup>17</sup> ROS generated by NOXs 1, 2 and 4 mediate angiogenic effects in response to angiogenic factors such as VEGF and angiotensin II.<sup>10</sup> However, the exact function of these NOXs and the underlying mechanisms by which they mediate their actions remain unknown, partly due to inconsistent published observations and context-dependent efficacy.

Stromal cell-derived factor 1 $\alpha$  (SDF-1 $\alpha$ , also called CXCL12) is one of the most potent angiogenic CXC chemokines. Our previous studies have shown that SDF-1 $\alpha$  requires MKP7 S-nitrosylation to activate JNK3 and promote endothelial migration and angiogenesis.<sup>18</sup> MKP7 belongs to a subgroup of protein tyrosine phosphatases (PTPs), which are widely recognized as targets of ROS that can be oxidized at redox-sensitive cysteine residues resulting in the inhibition of phosphatase activity following growth factor treatment.<sup>19-21</sup> MKP7 possesses a critical cysteine in its catalytic pocket that is highly sensitive to oxidation due to its low *pKa*.<sup>22</sup> Therefore, we hypothesized that NOX-generated ROS may oxidize and inhibit MKP7, thereby regulating SDF-1 $\alpha$ -dependent JNK3 activity and angiogenesis. By performing a series of biochemical and cell biological assays, we have developed strong

evidence that suggests that NOXs, including NOX5, are novel positive regulators of SDF-1 $\alpha$ -induced endothelial migration and angiogenesis.

## MATERIAL AND METHODS

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

## RESULTS

### SDF-1 $\alpha$ induces transient generation of ROS in BAECs

Our previous studies demonstrated that MKP7 S-nitrosylation and subsequent inhibition is required for SDF-1 $\alpha$ -induced endothelial migration and angiogenesis.<sup>18</sup> MKP7 activity can also be modified and inhibited through oxidation of a critical redox sensitive cysteine in its catalytic pocket.<sup>22</sup> NOXs 1, 2 and 4 are the major source of ROS in endothelial cells.<sup>23</sup> In addition, they regulate angiogenesis in response to growth factors such as VEGF and angiotensin II.<sup>10</sup> However, the exact function of these NOXs remain elusive and whether NOX-generated ROS regulate SDF-1 $\alpha$ -dependent signaling is unknown. We hypothesized that NOX-generated ROS may be another mechanism by which MKP7 activity is inhibited during SDF-1 $\alpha$ -dependent angiogenesis. To investigate the role of NOX-generated ROS in SDF-1 $\alpha$ -dependent angiogenesis, we first examined ROS generation in BAECs following SDF-1 $\alpha$  treatment. The fluorescent dyes CM-H<sub>2</sub>DCFDA and dihydroethidium (DHE) were used to monitor and determine the level of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide (O<sub>2</sub><sup>·-</sup>) production, respectively, in BAECs following treatment with SDF-1 $\alpha$ . When cells were treated with SDF-1 $\alpha$  for 5 minutes, an increase in fluorescent DCF signal was detected in BAECs by confocal microscopy (Figure 1A) and by flow cytometry (from 0.01% cells positive under control conditions to 15.45% cells in response to SDF-1 $\alpha$ ; Figure 1B & SIA). Moreover, this SDF-1 $\alpha$ -induced DCF signal was inhibited by PEG-catalase, but not the inhibitors of nitric oxide synthase (NOS)- L-NAME and that of cyclooxygenase-diclofenac (Figure SIB, C, D and E), suggesting that this detected DCF signal is due to H<sub>2</sub>O<sub>2</sub> generation, but not the formation of peroxynitrites or arachidonate metabolites. SDF-1 $\alpha$  treatment of BAECs also resulted in O<sub>2</sub><sup>·-</sup> generation, detected by the appearance of fluorescent ethidium generated from oxidation of DHE. SDF-1 $\alpha$ -induced superoxide production rapidly increased ~2 fold, peaking at 5-minutes after the SDF-1 $\alpha$  treatment began (Figure 1C). Moreover, the superoxide level was induced in a dose- dependent manner (Figure 1D). To determine whether ROS were generated during SDF-1 $\alpha$  -induced endothelial cell migration, a wound scratch assay was performed to visualize the active migratory process of BAECs following SDF-1 $\alpha$  treatment. Consistent with the observations above, SDF-1 $\alpha$  treatment of BAECs resulted in generation of a fluorescent DCF signal that was most prominent at the leading edge of the BAECs where active migration takes place (Figure 1E). These data suggest that ROS are generated in response to SDF-1 $\alpha$  treatment in BAECs and that this ROS generation correlates with the SDF-1 $\alpha$ -dependent migratory response.

### Antioxidants inhibit SDF-1 $\alpha$ -induced JNK3 activation and endothelial migration

To determine whether the ROS generation seen at the leading edge of the migrating BAECs is necessary for SDF-1 $\alpha$ -induced migration, the wound scratch assay was repeated in the presence of the two antioxidants NAC and ebselen. As shown in Figure 2A, SDF-1 $\alpha$ -induced BAEC migration was significantly inhibited by pretreatment of cells with either NAC or ebselen, indicating that ROS generation is required for SDF-1 $\alpha$ -induced cell migration.

### NOXs is required for SDF-1 $\alpha$ -induced angiogenesis

Recent evidence indicates that NOXs are the major sources of ROS in endothelial cells.<sup>23</sup> Given our observation that SDF-1 $\alpha$  treatment of BAECs resulted in ROS generation that was associated with SDF-1 $\alpha$ -induced cell migration, we next examined whether NOXs were also involved in these events. Using the Boyden chamber migration assay, we observed that SDF-1 $\alpha$  treatment increased BAEC migration from 13 $\pm$ 1 to 48 $\pm$ 1 cells per field (Figure 2B), but that SDF-1 $\alpha$ -induced migration could be completely inhibited by the two general NOX inhibitors DPI and apocynin (Figure 2B), indicating that NOX enzyme-dependent ROS generation is essential for SDF-1 $\alpha$ -mediated BAEC migration.

Four different NOX enzymes (NOX1, 2, 4 and 5) are expressed in vascular endothelial cells such as BAECs (Figure SIIA).<sup>10</sup> NOX 1, 2 and 4 have each been linked to angiogenic responses, however, the underlying molecular mechanisms behind these events are not completely understood. Furthermore, since previous experiments have been largely performed in rodent cells, which do not express NOX5, the role for NOX5 in angiogenesis has yet to be determined. Therefore, to determine which NOX was responsible for SDF-1 $\alpha$ -induced angiogenesis, we first examined the effect of knockdown of NOX5 or p22phox, a crucial component of NOX1/2/4 active enzyme complexes<sup>10</sup>, on SDF-1 $\alpha$ -induced endothelial migration in BAECs. p22phox or NOX5 siRNA specifically decreased the RNA and protein levels of p22phox or NOX5 respectively, but not other NOXs in BAECs (Figure SIIB, C). Similar to antioxidants or NOX inhibitors, knockdown of either NOX5 or p22phox decreased superoxide generation (Figure SIID). More excitingly, their knockdown blocked endothelial migration induced by SDF-1 $\alpha$  (Figure 2C), suggesting that multiple NOXs, including NOX5, are involved in SDF-1 $\alpha$ -induced endothelial migration. To assess the effect of NOX depletion on tube formation, BAECs were transfected with siRNA specific for either NOX5 or p22phox and the effect on SDF-1 $\alpha$ -induced tube formation in Matrigel was analyzed. 72 hours following transfection with p22phox siRNA, NOX5 siRNA or control siRNA, BAECs cells were plated on Matrigel in medium containing 100 ng/ml SDF-1 $\alpha$ . SDF-1 $\alpha$  treatment significantly enhanced tube formation in BAECs transfected with control siRNA, with the tube number increasing from 12 $\pm$ 3 to 68 $\pm$ 10 tubes per field (Figure 2D). In contrast, SDF-1 $\alpha$ -induced tube formation was significantly blocked in BAECs transfected with either p22phox siRNA or NOX5 siRNA, by 4.5- or 3.4-fold respectively (Figure 2D). To confirm this anti-angiogenic effect of p22phox and NOX5 siRNAs, we also performed a spheroid-sprouting angiogenesis assay using BAECs treated with SDF-1 $\alpha$ . This assay measures the sprouting and network formation of gel-embedded aggregated endothelial cells. Endothelial spheroids were prepared with BAECs transfected with different siRNAs and then stimulated with SDF-1 $\alpha$ . The total number of sprouts from

each spheroid was counted and compared with control cells. As shown in Figure SIII E, SDF-1 $\alpha$ -treated BAECs showed a 3.4-fold increase in sprouts/spheroids when compared with control BAECs. However, this increase in SDF-1 $\alpha$ -induced sprout formation was inhibited in BAECs transfected with either p22phox or NOX5 siRNA. The data collected from the Matrigel and spheroid-sprouting angiogenesis assays strongly suggest that multiple NOXs, including NOX5 and probably NOX1/2/4, are critical mediators of SDF-1 $\alpha$ -dependent angiogenesis in BAECs.

### **NOX5, but not NOX1/2/4, is required for JNK3 activation induced by SDF-1 $\alpha$ .**

We have previously demonstrated that JNK3 is an important component of SDF-1 $\alpha$ -mediated endothelial migration and angiogenesis<sup>18</sup>. Since the data above demonstrates that NOX-generated ROS also play an essential role in SDF-1 $\alpha$ -dependent angiogenesis (Figure 2), we tested whether SDF-1 $\alpha$ -induced JNK3 activation was influenced by NOX-dependent ROS generation. All three antioxidants- BHA, NAC and ebselen- inhibited SDF-1 $\alpha$ -induced JNK phosphorylation in BAECs (Figure 3A and SIII A). SDF-1 $\alpha$ -induced JNK3 activation was also inhibited by the NOX inhibitors DPI and apocynin (Figure 3B). To test which NOX isoform was involved in SDF-1 $\alpha$ -induced JNK3 activation, BAECs were transfected with p22phox siRNA, NOX5 siRNA or control siRNA. Activation of SDF-1 $\alpha$ -induced JNK3 activation was dramatically decreased by knockdown of NOX5, but not by knockdown of p22phox (Figure 3C). Furthermore, ectopic expression of NOX5 in BAECs was sufficient to activate JNK3 at baseline and augmented JNK3 activation following SDF-1 $\alpha$  stimulation (Figure 3D). The inhibitory effect of NOX inhibitors and NOX5 siRNAs on SDF-1 $\alpha$ -induced JNK3 activation was further confirmed in human vascular endothelial cells (HUVECs; Figure SIII B, C and D). Moreover, we also observed that ERK activation in response to SDF-1 $\alpha$  was also blocked by NOX inhibitors and NOX5 siRNAs, but not p22phox siRNAs in both BAECs and HUVECs (Figure SIII C, E and F). In summary, these data suggest that NOX5, but not NOX1/2/4, is required and sufficient for SDF-1 $\alpha$ -induced JNK3 and ERK activation. In addition to the effects seen by SDF-1 $\alpha$ , we also observed other cytokines, including TNF1 $\alpha$  and interleukin 1 $\beta$  (IL1 $\beta$ ), but not bFGF, evoke an increase in superoxide generation in BAECs (Figure SIV A, B and C). Furthermore, the activation of JNK by TNF1 $\alpha$  was inhibited by NOX5 siRNA, but not p22phox siRNA (Figure SIV D). The activation of NOX1, 2 and 4 and ROS generation regulate VEGF-induced angiogenesis<sup>24</sup>. However, the involvement of NOX5 has not been tested. To assess the effect of NOX5 depletion on VEGF-induced tube formation, HUVECs were transfected with NOX5 siRNA. VEGF increased the formed tube number in control cells. However, the knockdown of NOX5 inhibited tube formation in response to VEGF significantly (Figure SIV E, F). Taken together, we speculate that the NOX5-dependent signaling pathway may represent a relatively general mechanism for growth factors and cytokines.

ROS induce the expression of numerous genes including those that code for antioxidant proteins. One such antioxidants, heme oxygenase-1 (HO-1) has previously been linked to SDF-1 induced angiogenesis<sup>25</sup>. Therefore, we were interested to test whether HO-1 expression in BAECs is also regulated by NOXs. Excitingly, when BAECs were treated with SDF-1 $\alpha$  for 8 hours, the protein level of HO-1 increased dramatically, which is

consistent with a previous report<sup>25</sup>. However, the induction in HO-1 protein level was inhibited in cells that were transfected with NOX5 siRNAs, but not p22phox siRNAs (Figure SV), indicating that HO-1 is likely one of the downstream mediators for NOX5's angiogenic effect in BAECs.

### Hyperglycemia disrupts SDF-1 $\alpha$ -dependent ROS generation and JNK3 activation

Increased oxidative stress is associated with many chronic diseases, including diabetes and related cardiovascular diseases<sup>26</sup>. SDF-1 $\alpha$  is a dominant angiogenic factor in multiple pathological conditions such as diabetic retinopathy<sup>27</sup>. Therefore, we investigated the effect of elevated ROS level on SDF-1 $\alpha$ -dependent signaling when BAECs were cultured under elevated glucose conditions. Comparing the cells cultured under normal glucose concentration (7 mM glucose), the incubation of cells with ~3.3-fold higher glucose level for three days significantly increased the intracellular ROS level (Figure 4A). Reports have demonstrated that NOX1/2/4, but not NOX5, are responsible for the elevation of ROS level in response to high glucose in vascular cells<sup>26, 28, 29</sup>. Interestingly, when cells were cultured under normal glucose concentrations, SDF-1 $\alpha$  increased JNK3 activation and antioxidant-ebesen blocked it. However, when cells were cultured with a higher concentration of glucose, the response of JNK3 activation to SDF-1 $\alpha$  was blunted (Figure 4B). Together, these data suggest that SDF-1 $\alpha$ -dependent NOX5 activation and its downstream signaling are inhibited under hyperglycemic conditions and that the regulatory roles of NOX1/2/4 and NOX5 are likely altered when cellular glucose homeostasis is disrupted.

### NOX5 is associated with MKP7 in BAECs and inhibits MKP7 activity

The preceding experiments indicate that NOX5 is the dominant NOX responsible for SDF-1 $\alpha$ -induced JNK3 activation. However, the molecular mechanisms underlying this NOX5-dependent signaling event are not clear. *S*-nitrosylation of the catalytic cysteine of MKP7, which blocks its phosphatase activity, is required for the SDF-1 $\alpha$ -induced activation of JNK3.<sup>18</sup> Interestingly, the labile cysteine at the catalytic center of MKPs is also susceptible to oxidation.<sup>22</sup> Indeed, oxidation of this cysteine by TNF $\alpha$ -generated ROS is critical for activation of JNK1 and the apoptotic effects of TNF $\alpha$ .<sup>22</sup> This prompted us to examine whether ROS, generated by NOX5 could result in oxidation of MKP7. In order for NOX5-generated ROS to efficiently oxidize MKP7, NOX5 and MKP7 would need to be in close proximity<sup>30</sup>. To determine the relative localization of MKP7 and NOX5, Flag-tagged MKP7 and GFP-tagged NOX5 were transfected into BAECs and their localization determined using confocal microscopy. Both flag-tagged MKP7 (Figure 5B) and GFP-tagged NOX5 co-localized to the plasma membrane and cytoplasm of BAECs (Figure 5A), indicating a spatial proximity that would be favorable for NOX5 oxidation of MKP7. To determine whether these two proteins associate directly, immunoprecipitation assays were performed on transfected cells. As shown in Figure 5C, Flag-tagged MKP7 was detected in a protein complex immunoprecipitated with Myc-tagged NOX5 but not Myc-p22phox, suggesting that MKP7 was associated with NOX5 specifically in HEK 293 cells. The reverse co-immunoprecipitation experiment confirmed the association of MKP7 with NOX5 (Figure SVIA). Co-immunoprecipitation of endogenous MKP7 and NOX5 from BAECs confirmed that, under basal conditions, MKP7 and NOX5 were associated together (Figure 5D). However, following SDF-1 $\alpha$  treatment, the proteins dissociated, suggesting a possible

mechanism for reversible SDF-1 $\alpha$  regulation of MKP7 (Figure 5D, E). To identify which region of MKP7 was responsible for NOX5 binding, a series of MKP7 deletion mutants (Figure 5B) were engineered. Using co-immunoprecipitation experiments once again, we determined that MKP7 (318–665) but not MKP7 (1–317) was associated with NOX5 (Figure SVIB). Further mutations of the 318–665 fragment revealed that MKP7 (415–440; indicated by black arrow in Figure 5B) was required for association with NOX5 (Figure 5F). Taken together, these data demonstrate that MKP7 and NOX5 associate in BAECs and that this association is dynamically regulated by SDF-1 $\alpha$ .

Having established that NOX5 and MKP7 do associate with one another in BAECs, we next sought to determine whether ROS generated from SDF-1 $\alpha$ -induced activation of NOX5 oxidizes MKP7, resulting in the inhibition of MKP7 phosphatase activity and subsequent activation of JNK3. An in vitro MKP7 activity analysis was performed using active JNK3 protein and MKP7 concentrated from cell lysates following our established protocol<sup>18</sup>. At baseline, Flag-tagged MKP7 protein demonstrated strong phosphatase activity as shown by the decrease in JNK3 phosphorylation (Figure 6A). Similar to our previous report<sup>18</sup>, the treatment of SDF-1 $\alpha$  blocked 42.8% of MKP7 phosphatase activity (Figure 6A and B). However, when cells were treated with the NOX inhibitor DPI, MKP7 retained high phosphatase activity at both baseline and following SDF-1 $\alpha$ -treatment of cells (Figure 6A and B). Similarly, NOX5 knockdown via transfection of cells with NOX5 specific siRNAs relieved the inhibitory effect of SDF-1 $\alpha$  on MKP7 phosphatase activity (Figure 6C and D). These data suggest that ROS generated by NOX5 inhibits MKP7 phosphatase activity, offering one plausible mechanism by which NOX5 promotes SDF-1 $\alpha$ -induced JNK3 activation.

Our previous study has demonstrated that MKP7 can be nitrosylated by nitric oxide generated by eNOS after eNOS is activated in response to SDF-1 $\alpha$  treatment.<sup>18</sup> Similar to eNOS activation leads to the inhibition of MKP7 phosphatase activity<sup>18</sup>, NOX5 activation blocked MKP7 activity and enhanced JNK3 activation. Therefore, we tested whether the blockage of both eNOS and NOX5 activities would result in synergistic inhibitory effects on JNK3 activation. BAECs were transfected with both siRNAs of eNOS and NOX5 and the changes of JNK3 activity were determined. Interestingly, the knockdown of NOX5 or eNOS blocked the activation of JNK3. However, the knockdown of both NOX5 and eNOS did not further significantly inhibit the activation of JNK3 (Figure SVII). It suggests that NOX5 and eNOS might work on the same pathway upstream of JNK3 activation. Reports have shown that NOX5 induces eNOS activity possibly by enhancing eNOS:hsp90 binding or increasing intracellular calcium level<sup>31</sup>. Another regulatory mechanism for their interaction could be that both eNOS and NOX5 regulate MKP7 activation.

Taken all together, our data identify NOX5 as a novel component of SDF-1 $\alpha$ -dependent JNK3 activation and support the hypothesis that NOX-generated ROS are important mediators of SDF-1 $\alpha$ -dependent angiogenesis. More importantly, both nitrous and oxidative stresses, and therefore the intracellular redox status, are important for SDF-1 $\alpha$ -dependent signaling and endothelial activation in response to pathophysiologic conditions such as hyperglycemia.

## DISCUSSION

NOXs are well accepted as being as novel players in the regulation of angiogenesis. However, the underlying mechanisms by which they exert their actions have not been completely elucidated. Using NOX inhibitors and NOX5-specific siRNA, we have demonstrated a requisite role for NOX5 in the regulation of SDF-1 $\alpha$ -dependent JNK3 activation, and subsequent endothelial activation and angiogenesis. This is the first report demonstrating that NOX-generated ROS are required for the angiogenic effects of SDF-1 $\alpha$  in endothelial cells. Moreover, we observed that multiple cytokines including SDF-1 $\alpha$ , TNF1 $\alpha$  and IL1 $\beta$  induced superoxide generation and the activation of JNK by TNF1 $\alpha$  was inhibited by NOX5 siRNA (Figure SIV). This recapitulates previous evidence demonstrating that NOX-generated ROS play an important role in angiogenic signaling in response to growth factors and cytokines.<sup>10</sup> Given the lack of NOX5 expression in rodents, the functional roles of NOX5 have not been well studied. However, our data hints at an unexpected role for NOX5 in the regulation of human pathophysiology that is associated with angiogenesis.

Increased oxidative stress is associated with many chronic diseases, including diabetes and related cardiovascular diseases<sup>26</sup>. Our observation that SDF-1 $\alpha$ -dependent JNK3 activation was blunted under hyperglycemic conditions (Figure 4) indicates that NOX-dependent ROS generation normally required for angiogenesis is dysregulated in a microenvironment where significantly higher levels of oxidative stress are present. Therefore, both beneficial and detrimental roles of ROS, whose generation might be due to the activation of different enzyme and non-enzyme systems, on endothelial pathophysiology need further clarification to improve the efficacy and safety of therapeutic strategies involving use of antioxidants and NOX inhibitors. In addition, NOX5 is up-regulated in some cancers, such as prostate cancer, esophageal adenocarcinoma and hairy cell leukemia.<sup>32-34</sup> DPI, a general NOX inhibitor, potently inhibits the migration, proliferation and invasion of prostate cancer cells by modulating the activity of growth signaling pathways including ERK1/2, p38 and causing cell cycle arrest.<sup>35</sup> Based on our finding that NOX-generated ROS participate in angiogenic events, it is also possible that DPI blocks angiogenesis within the tumor, thereby increasing its usefulness as an anti-tumor therapy. Likewise, blockade of the SDF-1 $\alpha$ /CXCR4 signaling axis, including NOX5, may represent a promising additional or alternative target for the treatment of some forms of cancer such as rectal carcinoma and colorectal cancer in which SDF-1 $\alpha$  levels are seen to increase following reoccurrence of tumors after chemo- and anti-VEGF therapy<sup>36, 37</sup>. Targeting NOX5 in the SDF-1 $\alpha$  pathway may also be useful in treatment against atherosclerosis. In the vascular system, NOX5 is predominantly expressed in endothelial cells, but is also detected in smooth muscle cells. In atherosclerotic aortas, the expression level of endothelial NOX5 is increased<sup>38</sup>. Given our data demonstrating that NOX5 is involved in angiogenic responses in endothelial cells, it is quite possible that the elevation in NOX5 expression in atherosclerotic lesions exacerbates plaque formation by promoting new vessel formation. Blockade of NOX5 may therefore be one mechanism by which the development of atherosclerotic plaques can be cured.

The data presented in this report is the first demonstration that NOXs are activated by the SDF-1 $\alpha$ /CXCR4 signaling axis. All forms of NOX (NOX 1, 2, 4 and 5) are expressed in



endothelial cells.<sup>10</sup> Previous studies have demonstrated that p22phox is required for activity of NOX1, NOX2 and NOX4.<sup>10</sup> Since knockdown of p22phox also blocked SDF-1 $\alpha$ -induced endothelial migration and angiogenesis, we conclude that NOX1, NOX2 and/or NOX4, in addition to NOX5, can be activated by SDF-1 $\alpha$  in endothelial cells. That said, their activation and downstream effectors are very likely to be different based on their individual molecular structures. NOX5 possesses additional cytosolic N-terminal EF-hands for binding calcium, rendering it able to be activated by calcium, probably due to the fact that the binding of calcium to these EF-hands relieves the auto-inhibitory loop on the activity domain of NOX5.<sup>39</sup> CXCR4, a G-protein coupled receptor, is ligated by SDF-1 $\alpha$  and its activation initiates a series of signaling processes including an increase in calcium influx.<sup>40</sup> Alternative calcium-independent mechanisms have also been reported to promote calcium sensitization and activation of NOX5.<sup>39</sup> Whether calcium influx or other mechanisms of calcium sensitization is required for NOX5 activation by SDF-1 $\alpha$  remains to be determined. In contrast to NOX5, the activation of NOX1, NOX2 and NOX4 are regulated differentially by small G proteins and multiple kinases. Rac GTPases are required for both NOX1 and NOX2 activity.<sup>41–44</sup> PKC, PI3K and Src are kinases that regulate the assembly of the NOX2 oxidase complex by recruiting p47phox bound to p67phox and p40phox; Akt, PKC and PKA negatively modulate NOX1 activity<sup>10</sup>. Unlike NOX1 and NOX2, NOX4 exhibits constitutive activity. NOX4 agonists acutely increase its activity, probably through the fast induction of protein expression, increasing the available NADPH pool, or by promoting NOX4's association with its receptors.<sup>10</sup> The diverse range of activators and repressors of NOX 1, 2 and 4 activity suggest that numerous upstream mediators could be activated in response to SDF-1 $\alpha$ . Therefore, further experiments are needed to characterize the detailed molecular mechanisms by which NOX1, NOX2 and NOX4 are activated by SDF-1 $\alpha$ .

The downstream mediators of NOX1, NOX2, NOX4 and NOX5 responsible for promoting the effects of SDF-1 $\alpha$ -induced endothelial migration and angiogenesis are unknown. By using NOX inhibitors and specific siRNAs of NOX5 and p22phox, we have demonstrated that JNK3 and ERK are specific mediators for NOX5 in the regulation of SDF-1 $\alpha$ -induced angiogenesis (Figure 2, 3, SII, SIII). Moreover, we observed that the induction of HO-1, a known mediator of SDF-1 $\alpha$ -induced angiogenesis, is mediated by NOX5 (Figure SV). However, whether JNK3 and ERK act as upstream mediators in response to SDF-1 $\alpha$  to induce HO-1 expression still needs further investigation. Other than the downstream mediators that are mentioned above, multiple other pathways have been characterized as downstream mediators of NOX1/2/4 in the regulation of cell proliferation and migration. For example, Akt is reported to mediate NOX-promoted cell proliferation responses<sup>6, 45–47</sup>, whereas the PPAR $\alpha$  pathways have been implicated as downstream effectors for NOX1's effect on cell migration.<sup>48</sup> The interaction of NOX2 and the scaffold protein IQGAP1 leads to the accumulation of NOX2 at the leading edge of migrating endothelial cells<sup>49</sup> and NOX4 is reported to regulate eNOS expression in endothelial cells.<sup>50</sup> Whether these pathways are involved in cell migration and proliferation associated with SDF-1 $\alpha$  activation of these NOXs still needs further investigation.

The data presented in this report is a continuation of our recent observations that JNK3 is a major mediator of SDF-1 $\alpha$ -dependent endothelial migration, and that MKP7, the JNK3

phosphatase, is nitrosylated by nitric oxide and this nitrosylation leads to inhibition of its phosphatase activity.<sup>18</sup> Here, we present data demonstrating that MKP7 activity is also inhibited by NOX inhibitors or NOX5 siRNAs, suggesting that the intracellular redox status is critical for maintaining cellular functions for endothelial cells. Cysteine oxidation is one important mechanism by which ROS modulate protein function and the oxidation of the catalytic cysteine on MKP7 has been reported to be required for TNF $\alpha$ -induced JNK activation.<sup>22, 51</sup> Therefore, we propose that MKP7 is oxidized by NOX5-generated ROS following SDF-1 $\alpha$  administration, which in turn inhibits MKP7 phosphatase activity and sustains JNK3 activation. All MKPs share this cysteine-containing catalytic motif, suggesting they are similarly regulated by the intracellular redox status. In addition to cysteine oxidation, other amino acids such as lysine, proline, arginine and threonine can also be oxidized, and these modifications are recognized as being important for the pathogenesis of many degenerative diseases.<sup>52</sup> Future studies aimed at further characterizing the role of protein oxidation may provide essential information that helps to explain the pathophysiologic mechanism of oxidative stress.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## ABBREVIATIONS

<b>ROS</b>	reactive oxygen species
<b>SDF-1<math>\alpha</math></b>	stromal cell-derived factor-1 $\alpha$
<b>BAEC</b>	bovine aortic endothelial cell
<b>NOX</b>	NADPH oxidase
<b>eNOS</b>	endothelial nitric oxide synthase
<b>Duox</b>	dual Oxidase
<b>PTP</b>	protein tyrosine phosphatase
<b>BHA</b>	butylated hydroxyanisole
<b>NAC</b>	N-acetyl-L-cysteine
<b>Ebs</b>	ebselen
<b>DPI</b>	diphenylene iodoniu
<b>Apo</b>	apocynin

<b>DCF</b>	dichlorofluorescein
<b>DHE</b>	dihydroethidium
<b>HUVEC</b>	human vascular endothelial cells
<b>HO-1</b>	heme oxygenase-1

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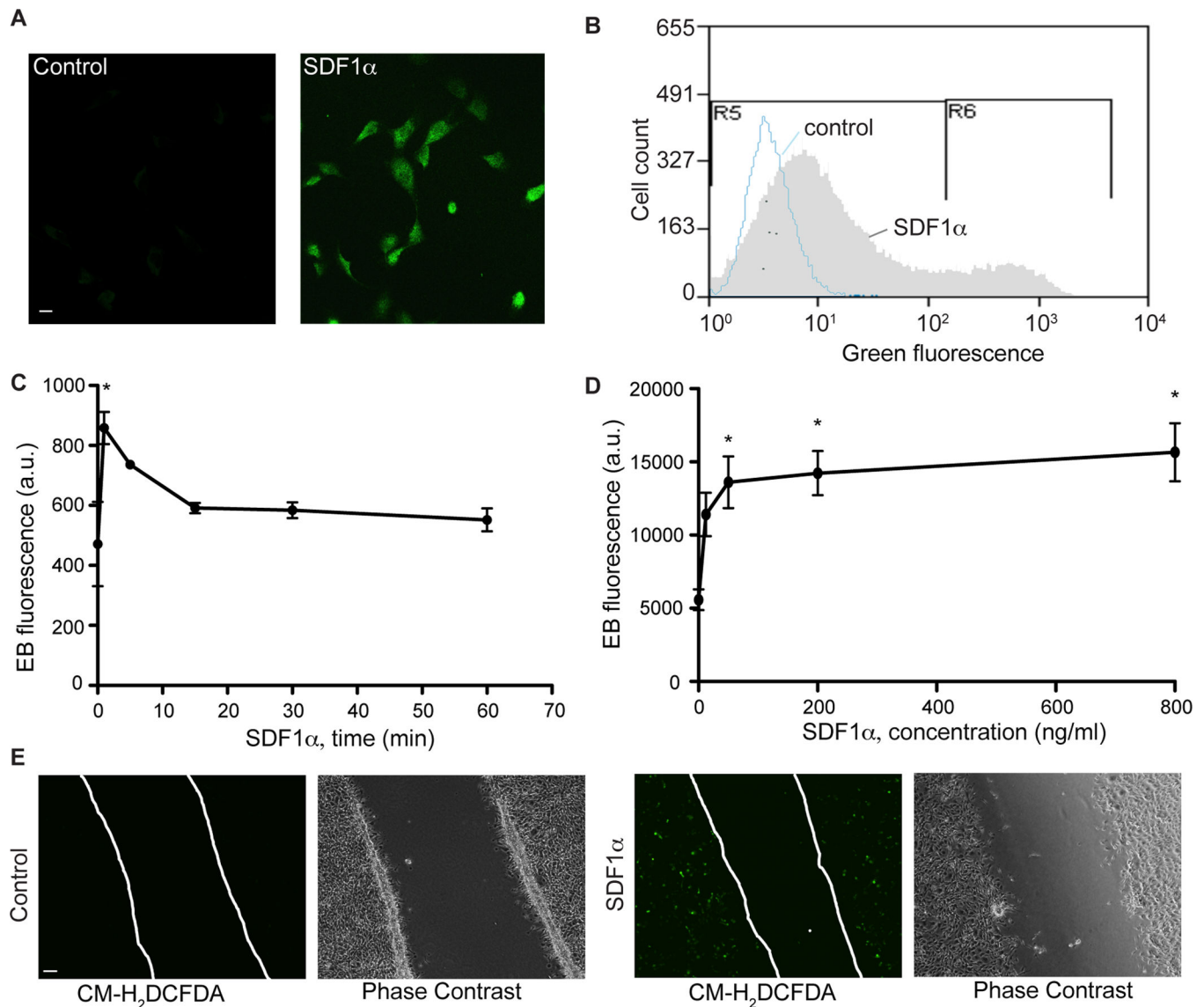
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### SIGNIFICANCE

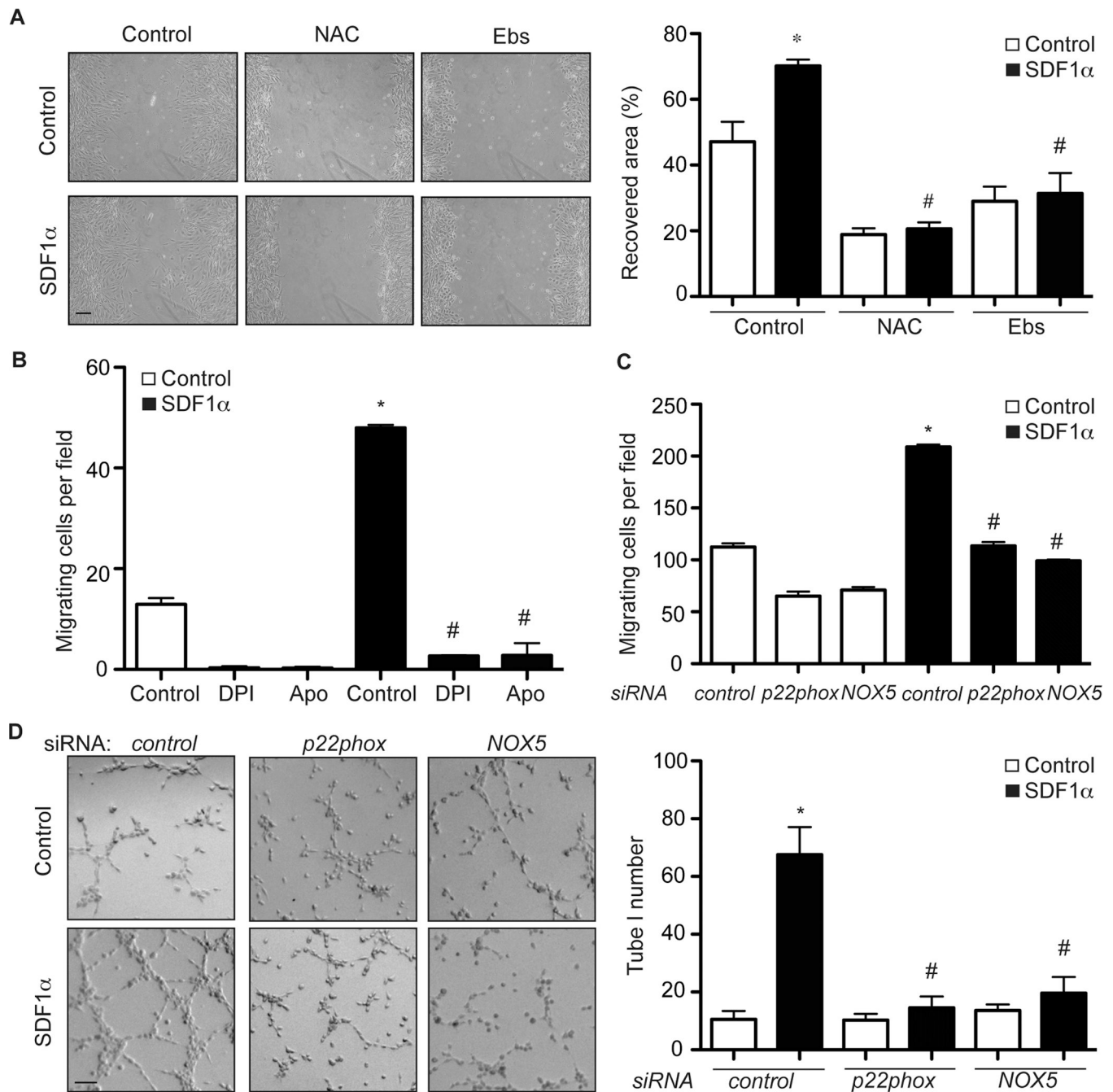
NOXs are well accepted as being novel players in the regulation of angiogenesis. However, the underlying mechanisms by which they exert their actions have not been completely elucidated. Using NOX inhibitors and NOX5-specific siRNA, our data identify NOX5 as a novel component of SDF-1 $\alpha$ -dependent JNK3 activation and support the hypothesis that NOX-generated ROS are important mediators of SDF-1 $\alpha$ -dependent angiogenesis. More importantly, both nitrous and oxidative stresses, and therefore the intracellular redox status, are important for SDF-1 $\alpha$ -dependent signaling and endothelial activation in response to pathophysiologic conditions such as hyperglycemia.



**Figure 1.**

ROS are generated following SDF-1 $\alpha$  treatment. BAECs were treated with SDF-1 $\alpha$  (100 ng/ml) for 5 minutes and ROS production was detected by CM-H<sub>2</sub>DCFDA (A-B) or DHE staining (C-D). **A**, Representative images of DCFDA-stained cells. Scale bar, 10  $\mu$ m. **B**, Representative flow cytometry data obtained for H<sub>2</sub>O<sub>2</sub> measurement that was detected as green fluorescence after CM-H<sub>2</sub>DCFDA staining. This is an overlay picture of two samples—the control (in blue) and 100 ng/ml SDF-1 $\alpha$ -treated for 5 minutes (in gray). **C–D**, BAECs were treated with SDF-1 $\alpha$  in 100 ng/ml for different time periods (C) or different concentrations for 5 minutes (D). O<sub>2</sub><sup>-</sup> production was quantified as ethidium bromide fluorescence. \*,  $P < 0.05$ , compared to control BAECs.  $n = 3$ . **E**, Representative images of an endothelial cell monolayer with a scratch-wound. Cells were treated with SDF-1 $\alpha$  (100 ng/ml) for 5 minutes and fixed for CM-H<sub>2</sub>DCFDA staining. Scale bar, 50  $\mu$ m.

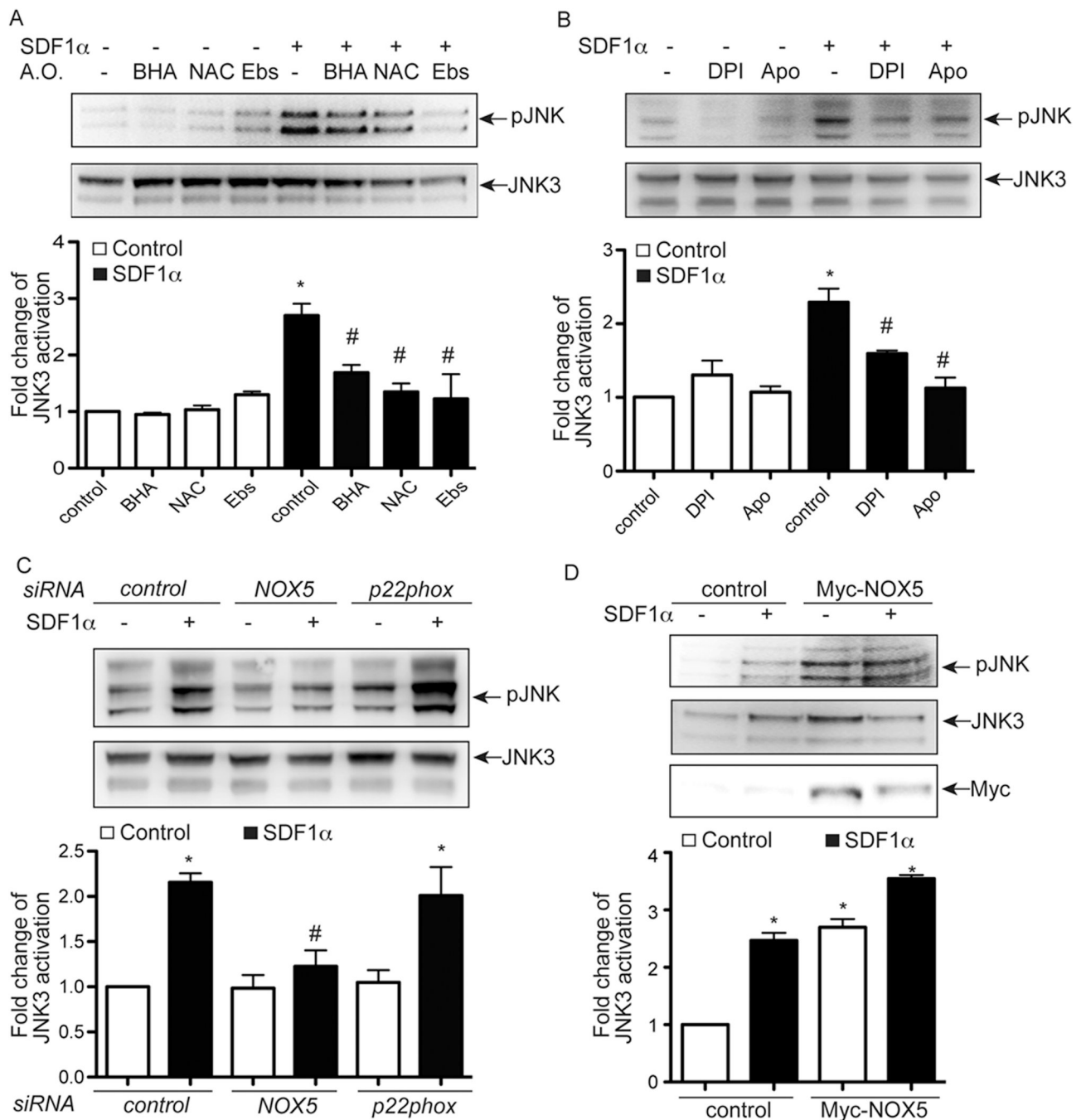




**Figure 2.**

NOXs inhibit SDF-1 $\alpha$ -induced migratory and angiogenic responses in BAECs. **A**, A wound healing assay was performed with BAECs. Cell monolayers were scratched, incubated with NAC (10 mM) or ebselen (Ebs, 40  $\mu$ M) for 30 minutes and then treated with SDF-1 $\alpha$  (100 ng/ml). 24 hours later, cells were fixed and images were taken. The level of cell migration into the scratch-wound was quantified as the recovered area. \*,  $P < 0.05$ , compared to control cells; #,  $P < 0.01$ , compared to control cells that were treated with SDF-1 $\alpha$ .  $n = 3$ . **B**, Boyden chamber analysis of BAEC migration was performed using 100 ng/ml SDF-1 $\alpha$  as the

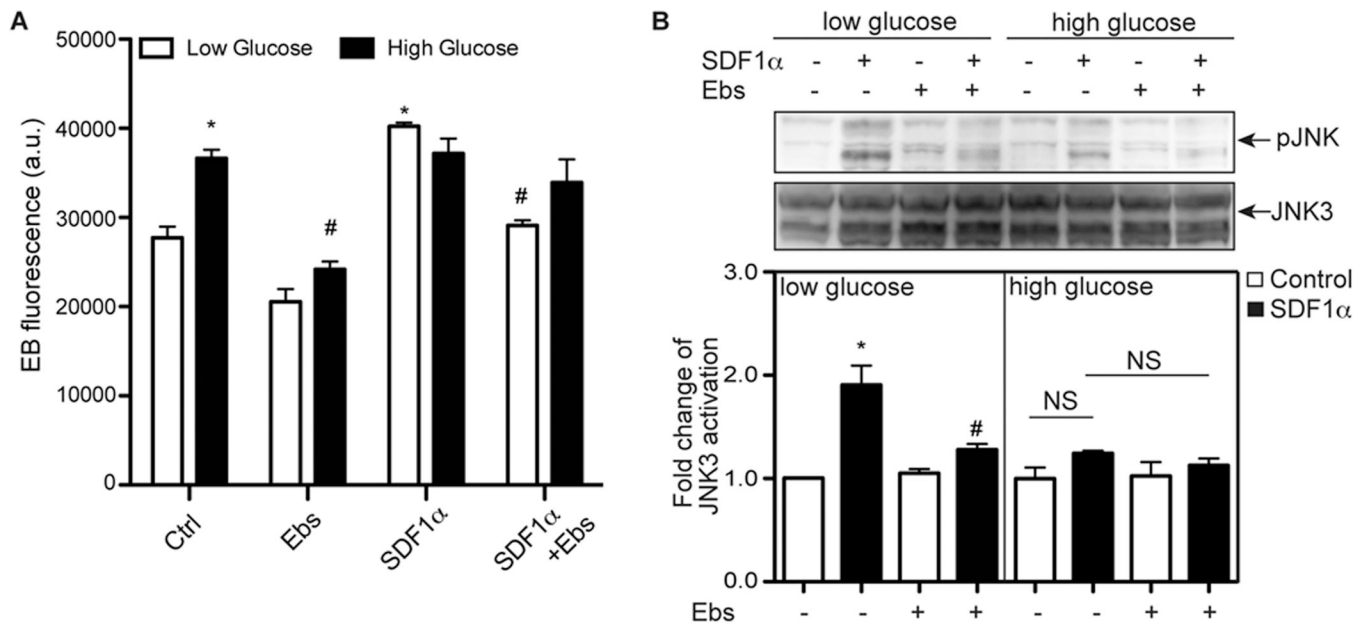
chemoattractant. \*,  $P < 0.001$ ; compared to control cells without SDF-1 $\alpha$ . #,  $P < 0.001$ ; compared to control cells with SDF-1 $\alpha$ . n=3. **C**, Boyden chamber analysis of BAEC migration was performed using 100 ng/ml SDF-1 $\alpha$  as the chemoattractant. BAECs were transfected with p22phox, NOX5 or control siRNA. \*,  $P < 0.01$ ; compared to control cells without SDF-1 $\alpha$ . #,  $P < 0.01$ ; compared to control cells with SDF-1 $\alpha$ . n=3. **D**, In vitro Matrigel angiogenesis assays were performed with BAECs that were transfected with p22phox, NOX5 or control siRNAs. 100 ng/ml SDF-1 $\alpha$  was used to induce tube formation. Images of formed tubes were taken and tube numbers were counted. \*,  $P < 0.001$ ; compared to control cells without SDF-1 $\alpha$ . #,  $P < 0.05$ ; compared to control cells with SDF-1 $\alpha$ . Scale bar, 50  $\mu$ m.



**Figure 3.**

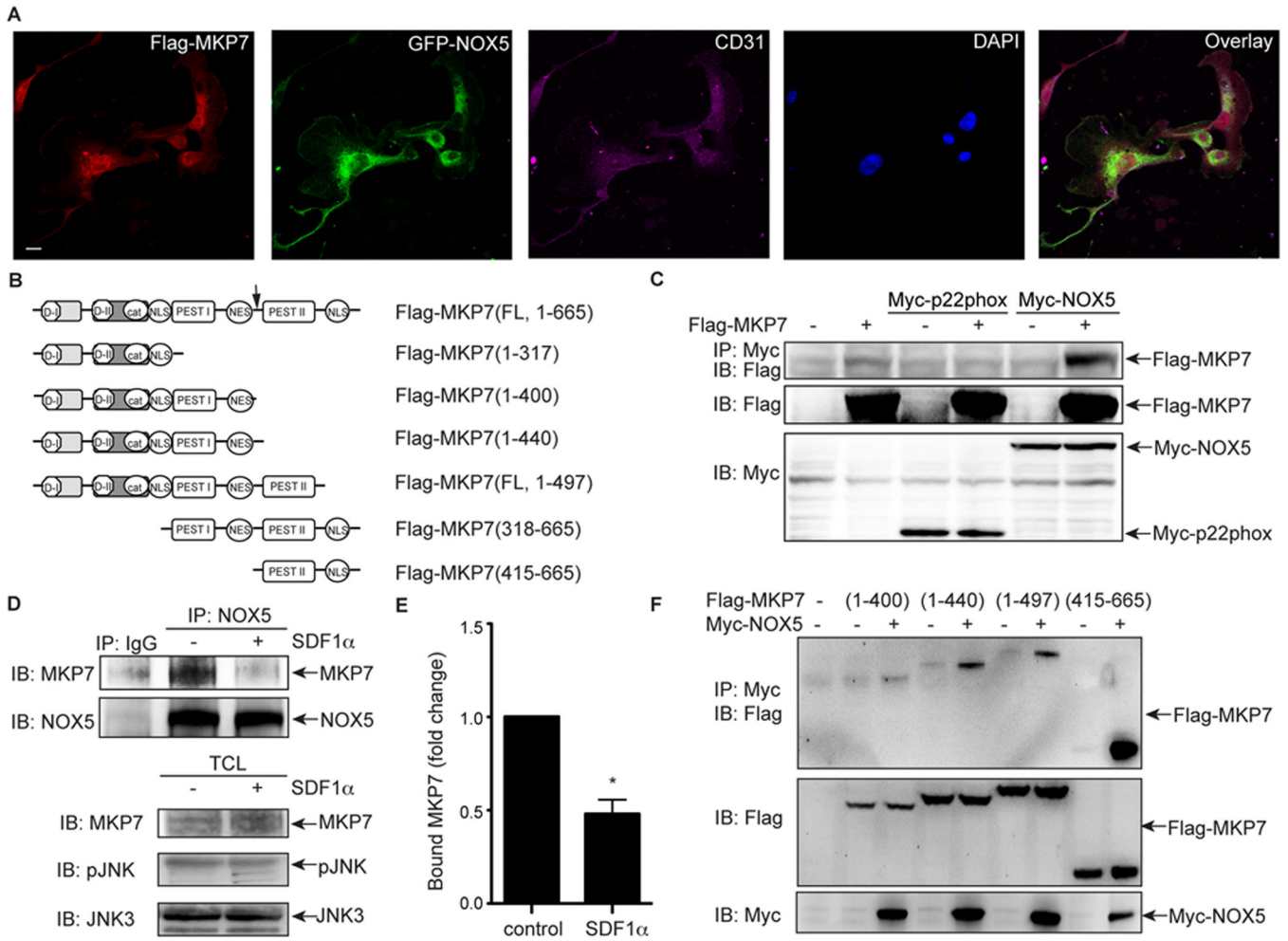
NOX5 is required for SDF-1 $\alpha$ -induced JNK3 activation in BAECs. **A**, BAECs were incubated with BHA (200  $\mu$ M), NAC (10 mM) or ebselen (Ebs, 40  $\mu$ M) for 30 minutes and then treated with SDF-1 $\alpha$  (100 ng/ml) for 10 minutes to activate JNK3. Cell lysates were used for Western blotting to detect the activation of JNK3 using the phospho-JNK antibody. The phosphorylation of JNK was interpreted as JNK3 phosphorylation since JNK3 is the major JNK moiety activated by SDF-1 $\alpha$ - in endothelial cells in our system.<sup>18</sup> **B**, BAECs were incubated with NOX inhibitors DPI (10  $\mu$ M) or apocynin (Apo, 5 mM) for 30 minutes

and then treated with SDF-1 $\alpha$  (100 ng/ml) for 10 minutes to activate JNK3. Cell lysates were used for Western blotting to detect the activation of JNK3. **C**, BAECs were transfected with p22phox siRNA, NOX5 siRNA or control siRNA. Three days later, cells were treated with SDF-1 $\alpha$  (100 ng/ml) for 10 minutes to activate JNK3. Cell lysates were used for Western blotting to detect the activation of JNK3. **D**, BAECs were transfected with Myc-tagged NOX5 or control vector. Two days later, cells were treated with SDF-1 $\alpha$  (100 ng/ml) for 10 minutes to activate JNK3. Cell lysates were used for Western blotting to detect the activation of JNK3. \*,  $P < 0.05$ ; compared to control cells without SDF-1 $\alpha$ ; #,  $P < 0.05$ ; compared to control cells with SDF-1 $\alpha$ ; n=3.

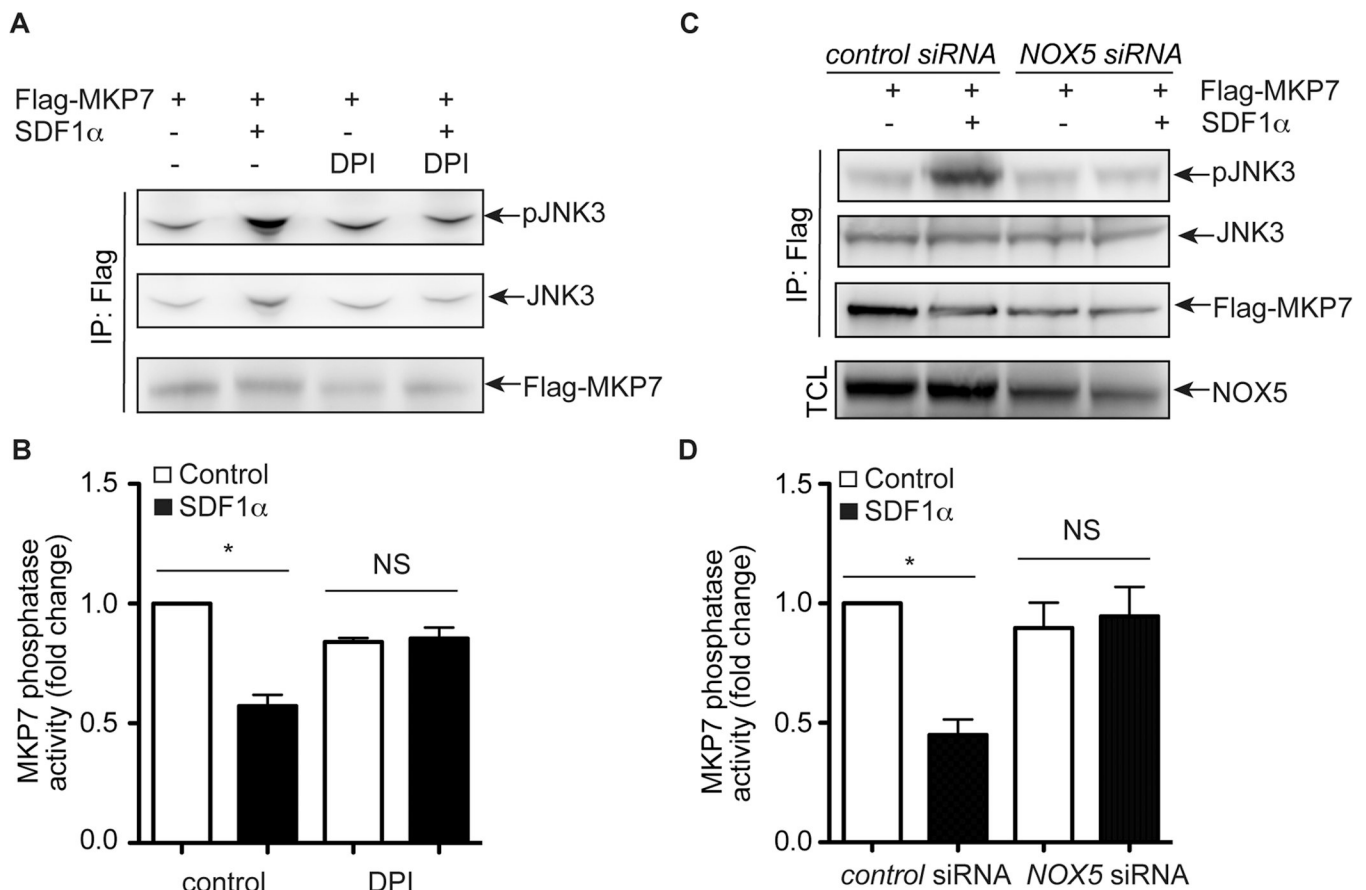


**Figure 4.**

The response to SDF-1 $\alpha$  is blunted when BAECs were cultured in the presence of high glucose concentrations. BAECs were cultured in media containing either normal glucose concentration (7 mM glucose + 23 mM mannitol) or high glucose concentration (30 mM glucose). Three days later, cells were incubated with ebselen (Ebs, 40  $\mu$ M) for 30 minutes and then treated with SDF-1 $\alpha$  (100 ng/ml) for 5 minutes to determine ROS generation and for 10 minutes to determine JNK3 activation. **A**, O<sub>2</sub><sup>-</sup> production was quantified as ethidium bromide fluorescence. **B**, JNK3 activation was determined by Western blotting. \*,  $P < 0.05$ , compared to control BAECs with low glucose and without SDF-1 $\alpha$ . #,  $P < 0.05$ ; compared to control cells without ebselen; NS, not significant; n=3.



**Figure 5.** NOX5 is associated with MKP7. **A**, BAECs were transfected with Flag-MKP7 and GFP-NOX5. Two days later, cells were fixed and analyzed by confocal microscopy. Scale bar, 5µm. **B**, A schematic representation of full length Flag-MKP7(1–665) and its deletion mutant constructs. The black arrow indicates the fragment of MKP7 responsible for its minimal association with NOX5. **C**, HEK293 cells were transfected with Flag-MKP7, Myc-NOX5 or Myc-p22phox constructs. One day later, total cell lysates were used for immunoprecipitation for Myc-NOX5 and Myc-p22phox, and immunoblotting for Flag-MKP7. **D**, BAECs were treated with SDF-1α (100 ng/ml) for 5 minutes and harvested for immunoprecipitation for NOX5 and immunoblotting for MKP7. TCL, total cell lysates. **E**, The MKP7 proteins associated to NOX5 in BAECs were quantified as the fold change, compared to the control cells. \*,  $P < 0.05$ ; compared to control cells without SDF-1α;  $n = 3$ . **F**, HEK293 cells were transfected with different Flag-MKP7 mutants and Myc-NOX5 constructs. One day later, total cell lysates were used for immunoprecipitation for Myc-NOX5 and immunoblotting for Flag-MKP7.



**Figure 6.**

MKP7 activity is regulated by NOX5. **A**, Results of an in vitro phosphatase assay using active JNK3 protein as the substrate. BAECs were transfected with Flag- MKP7 and then treated with DPI (10  $\mu$ M) for 30 minutes and followed by SDF-1 $\alpha$  (100 ng/ml) treatment for 5 minutes. The cell lysates concentrated for Flag-tagged MKP7 protein were used for in vitro phosphatase assays. **B**, Quantitative analysis of results from in vitro phosphatase assays of MKP7 in Figure 5A based on three independent experiments. \*,  $P < 0.05$ ; compared to control cells. NS, not significant. **C**, Results of an in vitro phosphatase assay using active JNK3 protein as the substrate. BAECs were transfected with Flag-MKP7, and NOX5 or control siRNA and then treated with SDF-1 $\alpha$  for 5 minutes. The cell lysates concentrated for Flag-tagged MKP7 protein were used for in vitro phosphatase assays. **D**, Quantitative analysis of results from in vitro phosphatase assays of MKP7 in Figure 5C based on three independent experiments. \*,  $P < 0.05$ ; compared to control cells. NS, not significant.