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Role of Nox2-Based NADPH Oxidase in Bone Marrow and Progenitor Cell Function Involved in Neovascularization Induced by Hindlimb Ischemia

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Abstract

Bone marrow (BM) is the major reservoir for endothelial progenitor cells (EPCs). Postnatal neovascularization depends on not only angiogenesis but also vasculogenesis, which is mediated through mobilization of EPCs from BM and their recruitment to the ischemic sites. Reactive oxygen species (ROS) derived from Nox2-based NADPH oxidase play an important role in postnatal neovascularization; however, their role in BM and EPC function is unknown. Here we show that hindlimb ischemia of mice significantly increases Nox2 expression and ROS production in BM-mononuclear cells (BMCs), which is associated with an increase in circulating EPC-like cells. Mice lacking Nox2 show reduction of ischemia-induced flow recovery, ROS levels in BMCs, as well as EPC mobilization from BM. Transplantation of wild-type (WT)-BM into Nox2-deficient mice rescues the defective neovascularization, whereas WT mice transplanted with Nox2-deficient BM show reduced flow recovery and capillary density compared to WT-BM transplanted control. Intravenous infusion of WT- and Nox2-deficient BMCs into WT mice reveals that neovascularization and homing capacity are impaired in Nox2-deficient BMCs in vivo. In vitro, Nox2-deficient c-kit^{+}Lin^{-} BM stem/progenitor cells show impaired chemotaxis and invasion as well as polarization of actins in response to stromal derived factor (SDF), which is associated with blunted SDF-1–mediated phosphorylation of Akt. In conclusion, Nox2-derived ROS in BM play a critical role in mobilization, homing, and angiogenic capacity of EPCs and BM stem/progenitor cells, thereby promoting revascularization of ischemic tissue. Thus, NADPH oxidase in BM and EPCs is potential therapeutic targets for promoting neovascularization in ischemic cardiovascular diseases.

Keywords

NADPH oxidase; reactive oxygen species; angiogenesis; vasculogenesis; neovascularization; stromal derived factor; endothelial progenitor cells

Neovascularization is an important repair mechanism to rescue tissue from critical ischemia.[1] It is a key process involved in normal development and wound repair as well as various

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Disclosures
None.

pathophysiologies such as ischemic heart disease and peripheral artery disease. Postnatal new blood vessel formation involves not only angiogenesis but also vasculogenesis which is mediated through mobilization of endothelial progenitor cells (EPCs) and stem/progenitor cells from bone marrow (BM) as well as their homing to the ischemic tissues.[1] Intravenous infusion of BM mononuclear cells (BMCs) and progenitor cells augments ischemia-induced neovascularization.[2] BM microenvironment, which consists of macrophages, fibroblasts, and endothelial cells (ECs), is important for maintenance and mobilization of stem/progenitor cells.[3–5] Understanding mechanisms by which EPCs and BM progenitor cells are mobilized and homed to the site of neovascularization in response to ischemia is essential for development of new therapeutic strategies for ischemic cardiovascular diseases.

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) play an important role in normal cell growth, migration, differentiation, apoptosis, and senescence.[6,7] Although excess amounts of ROS are toxic, low levels of ROS produced in response to tissue ischemia serve as intracellular signaling molecules to prevent tissue injury and promote angiogenesis.[8,9] In ECs, NADPH oxidase is one of the major sources of ROS,[10] and it consists of catalytic subunits (Nox1, Nox2, and Nox4), p22phox, p47phox, p67phox, and the small GTPase Rac1. The endothelial NADPH oxidase is activated by numerous stimuli including angiogenesis growth factors, cytokines, shear stress, hypoxia, and G protein– coupled receptor agonists.[9] ROS derived from NADPH oxidase stimulate diverse redox signaling pathways leading to angiogenic-related responses in ECs.[9] We previously reported that Nox2-derived ROS play an important role in VEGF signaling linked to EC migration and proliferation, as well as reparative angiogenesis in response to hindlimb ischemia in vivo.[11,12] Increasing evidence suggests that ROS are generated by NADPH oxidase in EPCs and stem/progenitor cells,[13–15] which are involved in differentiation, proliferation, senescence, or apoptosis depending on cell types and amount of ROS. However, the role of Nox2-based NADPH oxidase in BM and EPCs/progenitor cell function linked to postnatal vasculogenesis remains unknown.

Here we demonstrate that hindlimb ischemia of mice increases expression of Nox2-based NADPH oxidase and ROS production in BM. Mice lacking Nox2 show impaired ischemia-induced blood flow recovery and neovascularization, which is associated with reduction of ROS production in BM as well as number of EPCs in peripheral blood. Defective neovascularization in $Nox2^{-/-}$ mice is rescued by transplantation of BM from WT mice. Intravenous infusion of $Nox2^{-/-}$ BMCs show impaired homing ability to sites of neovascularization. Mechanically, chemotaxis and invasion as well as actin polymerization induced by chemokine, stromal cell derived factor-1α (SDF-1 α) are inhibited in $Nox2^{-/-}$ BM stem/progenitor cells, which is associated with blunted phosphorylation of Akt. These findings suggest that Nox2-derived ROS play an essential role in regulating redox state in BM which is required for reparative EPC and progenitor cells mobilization from BM as well as their homing and neovascularization capacity, thereby promoting revascularization and tissue repair in response to ischemic injury.

Materials and Methods

Isolation of BM mononuclear cells and BM stem/progenitor cells, mouse hindlimb ischemia model, immunocytochemical analysis of hindlimb tissues, measurement of ROS production in BM, BM transplantation, measurement of circulating EPCs and vascular progenitor cells levels using EPCs culture assay and FACS analysis, chemotaxis and invasion assay, phalloidin staining in BM progenitor cells, cell-matrix adhesion assay, Western blotting, and statistical analyses are described in the Material and Methods section in the online Data Supplement (available online at http://circres.ahajournals.org).

Results

ROS Levels and Nox2 Expression Are Increased in BMCs in Response to Hindlimb Ischemia

To assess the role of ROS in BM, we examined effects of hindlimb ischemia on ROS levels in BMCs. Figure 1 shows that ROS level in BMCs was markedly increased at 3 and 7 days after hindlimb ischemia peaking at day 3, as measured by lucigenin (Figure 1A) and dihidrorhodamine (DHR) fluorescence assays with FACS analysis (Figure 1B). Of note, lucigenin- and DHR-sensitive ROS production were abolished by the presence of superoxide ($O_2^{\cdot-}$) dismutase and catalase, respectively (data not shown), suggesting that both $O_2^{\cdot-}$ and H_2O_2 are increased in BM after hindlimb ischemia. To assess the role of Nox2-based NADPH oxidase in BM, we next examined the expression of Nox2 and other NADPH oxidase components in BMCs with and without hindlimb ischemia using real-time PCR. Figure 2A shows that mRNAs for Nox2 and other components including p22phox, p47phox, and p67phox were significantly increased in BM with peak at 3 days after ischemia. Nox2 protein expression was also markedly increased in BMCs after hindlimb ischemia (data not shown). We found that Nox2 was the most highly expressed Nox isoforms compared to Nox1 and Nox4 in BMCs, as measured by real-time PCR. There was no significant increase of Nox1 and Nox4 mRNAs in BMCs in response to ischemia (data not shown). Moreover, basal and ischemia-induced $O_2^{\cdot-}$ productions were almost completely abolished in Nox2 KO BMCs (Figure 2B). These results suggest that Nox2-based NADPH oxidase is the major source of ROS produced in BM in basal state and after hindlimb ischemia.

Ischemia-Induced Increase in EPC-Like Cells in Peripheral Blood Is Inhibited in Nox2$^{-/-}$ Mice

To examine whether Nox2-derived ROS are involved in EPC mobilization from BM, we analyzed the number of c-kit$^+$Flk-1$^+$ cells in peripheral blood (PB) in WT and Nox2$^{-/-}$ mice. FACS analysis shows a significant reduction in the number of c-kit$^+$Flk-1$^+$ cells in PB at 3 days after ischemia in Nox2$^{-/-}$ mice compared with WT mice (Figure 3A and 3B). This finding was further confirmed by the decrease of number of circulating Sca-1$^+$Flk$^+$ cells and Sca-1$^+$Lin$^-$ cells after ischemia in Nox2$^{-/-}$ mice (supplemental Figure I). Moreover, number of DiI-acLDL and BS lectin double positive EPCs obtained by culture of PB-derived mononuclear cells after hindlimb ischemia was significantly decreased in Nox2$^{-/-}$ mice (Figure 3C and 3D). Of note, the number of EPC-like cells is increased in Nox2$^{-/-}$ mice under normoxic conditions presumably because of compensatory mechanism induced by the absence of Nox2. These results suggest that Nox2-based NADPH oxidase plays an important role in reparative mobilization of BM-derived EPCs and stem/progenitor cells in response to ischemia.

Nox2$^{+/+}$ Bone Marrow Rescues Impairment of Neovascularization in Nox2$^{-/-}$ Mice

To assess the role of Nox2 in BM function involved in neovascularization in vivo, we performed BM transplantation between WT and Nox2$^{-/-}$ mice to make BM chimera mice. Figure 4 show that Nox2$^{-/-}$ mice without BM transplantation showed significant reduction of limb blood flow recovery and capillary density after ischemia compared with WT mice, which is consistent with our previous report.[12] Transplantation of WT BMCs into the irradiated Nox2$^{-/-}$ mice rescued the impaired ischemia-induced blood flow recovery (Figure 4A and 4B) and reduced capillary formation in ischemic muscle (Figure 4C and 4D). In contrast, WT mice transplanted with Nox2$^{-/-}$ showed opposite effects (Figure 4). These data suggest that Nox2-based NADPH oxidase induced in BM after tissue ischemia plays a critical role in BM function, thereby regulating reparative neovascularization.

Nox2 Is Involved in Homing and Neovascularization Capacity of BM-Derived Cells In Vivo

To examine the role Nox2 in neovascularization capacity of BM-derived cells, we performed intravenous infusion of BMCs derived from WT and Nox2$^{-/-}$ mice into WT mice at 1 day after

induction of hindlimb ischemia. Figure 5A shows that WT-BMC injection into WT mice significantly augmented blood flow recovery at 14 days after operation, whereas $Nox2^{-/-}$ BMC infusion showed no significant effect. Of note, pretreatment of $Nox2^{-/-}$ BMCs with 5 μmol/L H_2O_2 rescued defective neovascularization capacity of $Nox2^{-/-}$ BMCs (supplemental Figure II). In addition, Nox2 deficiency did not affect the number of Sca-1^+lk-1^+cells, Sca-1^+in$^-$ cells or expression of $CXCR4^+$ cells in BMCs (data not shown). To examine the role of Nox2 in homing capacity of BMCs, we intravenously injected DiI-labeled WT and Nox2 KO BMCs at 1 day after ischemia. Figure 5B shows that $Nox2^{-/-}$ BMCs reduced ability to home to ischemic tissues in comparison to WT BMCs. These results suggest that Nox2 is required for proangiogenic and homing capacity of BMCs, thereby promoting neovascularization of ischemic tissue in vivo.

Nox2 Is Involved in Chemotaxis, Invasion, and Polarization of Actin in BM Progenitor Cells In Vitro

Because homing capacity of EPCs and stem/progenitor cells is regulated by various processes such as cell adhesion, chemotaxis, and invasion, we next examined whether Nox2 is involved in these responses using BM-MNCs and c-kit$^+$Lin$^-$ BM progenitor cells which play an important role in revascularization of ischemic tissue.[16] Adhesion capacity of c-kit$^+$Lin$^-$ BM cells to various extracellular matrix proteins was not affected in $Nox2^{-/-}$ cells (supplemental Figure III). To determine the role of Nox2 in chemotaxis and invasion of BM progenitor cells, we performed transwell migration assay toward chemokine SDF-1α, an exclusive ligand of CXCR4 receptor, which is potent stimulator for migration and homing of stem/progenitor cells. Both migration (Figure 6A) and invasion capacity (Figure 6B) of ckit$^+$Lin$^-$ BM cells toward SDF-1α were significantly inhibited in $Nox2^{-/-}$ cells. We confirmed that pretreatment of $Nox2^{-/-}$ BM c-kit$^+$Lin$^-$ cells with 5 μmol/L H_2O_2 improved the impaired migration capacity of these cells, whereas it had no significant effect on WT-BM cells (supplemental Figure IV). SDF-1α–induced actin polymerization plays an important role in hematopoietic stem/progenitor cell migration.[17,18] Furthermore, immuno-fluorescence analysis of ckit$^+$Lin$^-$ BM cells revealed that SDF-1α–induced actin polarization, as visualized by FITC-phalloidin staining, was inhibited in $Nox2^{-/-}$ cells (Figure 7). These data suggest that Nox2 is necessary for chematoxis, invasion capacity at least in part by regulating actin polymerization, but not cell-matrix adhesion capacity of BM progenitor cells, in vitro.

Nox2 Is Involved in Phosphorylation of Akt in BMCs

Akt plays an important role in migration of BM progenitor cells.[19] To gain insight into the mechanism by which Nox2-derived ROS are involved in BMC migration, we examined whether Nox2 is involved in SDF-1α–stimulated Akt phosphorylation in BMCs. Figure 8 shows that phosphorylation of Akt in response to SDF-1α was significantly inhibited in $Nox2^{-/-}$ BMCs, whereas proliferation signal, ERK1/2 phosphorylation was not affected in $Nox2^{-/-}$ cells. This result suggests that function of CXCR4 is intact in $Nox2^{-/-}$ BMCs and that Nox2-derived ROS are involved in Akt phosphorylation in BM stem/progenitor cells, thereby promoting their homing capacity.

Discussion

We previously reported that Nox2-derived ROS are involved in reparative angiogenesis induced by hindlimb ischemia; however, their roles in postnatal vasculogenesis remains unclarified The present study extends our previous study by providing novel evidence that: (1) Hindlimb ischemia of mice significantly increases expression of Nox2-based NADPH oxidase and ROS production in BM, which is associated with an increase in circulating EPC-like c-Kit$^+$Flk-1^+ cells; (2) Mice lacking Nox2 show reduction of ischemia-induced increase in ROS production in BM as well as EPC mobilization; (3) Transplantation of WT-BM into $Nox2^{-/-}$

mice rescues the defective neovascularization; (4) Intravenous infusion of WT- and $Nox2^{-/-}$ BMCs into WT mice reveals impaired neovascularization and homing capacity of $Nox2^{-/-}$ BMCs in vivo; (5) In vitro SDF-stimulated chemotaxis and invasion as well as polarization of actins are inhibited in $Nox2^{-/-}$ BM stem/progenitor cells, which is associated with blunted phosphorylation of Akt.

EPCs and stem/progenitor cells are mobilized from BM in response to ischemic injury, which contributes to postnatal neovascularization.[20–22] Using lucigenin assay, DHR fluorescence analysis, real-time PCR to detect expression of Nox2-based NADPH oxidase and $Nox2^{-/-}$ mice, here we demonstrate that hindlimb ischemia increases Nox2-dependent NADPH oxidase expression and ROS production in BM. Consistent with our data, previous reports show that NADPH oxidase components are expressed in various stem/progenitor cells including BM-derived human $CD34^+$ cells,[13,14] mouse embryonic stem cells,[15] skeletal muscle precursor cells,[23] and rat mesenchymal stem cells.[24] In these studies, ROS derived from NADPH oxidase are shown to be involved in differentiation, proliferation, senescence, or apoptosis. We found that Nox2 is the most highly expressed Nox isoforms in normoxic BMCs, and that there is no significant increase of Nox1 and Nox4 mRNA expression in BMCs after ischemia. Mechanism by which Nox2 is increased in BMCs in response to hindlimb ischemia remains unclear. BMCs consist of heterogeneous populations including myeloid cells, immature lymphoid cells, and stem/progenitor cells. Thus, it is possible that Nox2 is increased in particular cell types or number of Nox2 expressing cells is increased attributable to differentiation, which may contribute to the increase in Nox2 expression in BMC after ischemia. Detailed analysis of mechanism of Nox2 induction in BMCs in response to ischemia is an objective of future study. In the present study, functional role of Nox2-based NADPH oxidase in BM is demonstrated by the observation that ischemia-induced increase in the numbers of EPC-like c-Kit^+/$Flk\text{-}1^+$ cells as well as DiI-acLDL and lectin double positive EPCs in PB, are significantly decreased in $Nox2^{-/-}$ mice which show defective neovascularization. Consistent with our result, number of circulating stem/progenitor cells induced by hematopoietic cytokine is reduced in $Nox2^{-/-}$ mice.[25] Thus, Nox2 seems to play an important role in reparative mobilization of EPCs and stem/progenitor cells from BM in response to hindlimb ischemia.

The present study also demonstrates that transplantation of WT-BM into $Nox2^{-/-}$ mice rescue the defective neovascularization, whereas transplantation of $Nox2^{-/-}$ BM into WT mice shows opposite effects. This result suggests that Nox2-based NADPH oxidase expressed in BM is essential for BM function, which is required for maintenance and mobilization of EPCs as well as revascularization of ischemic tissues. Although excess amount of ROS is toxic to EPCs and stem/progenitor cells,[26] our results are consistent with the notion that optimal levels of ROS, which are balanced by ROS-generating and antioxidant enzymes, are required for normal BM or EPC function involved in postischemic neovascularization. [27–29] Mechanisms by which hindlimb ischemia increases ROS levels in BM are unclear. It has been shown that ischemic injury of skeletal muscle increases production of hematopoietic cytokines, thereby stimulating stem/progenitor cells mobilization from BM.[30] Of note, hematopoietic cytokines increase generation of ROS in progenitor cells, which in turn exits quiescent BM progenitor cells to promote cell-cycle progression.[31] Thus, it is conceivable that induction of ischemia in skeletal muscles enhances circulating cytokine levels, which may stimulate Nox2-dependent ROS production in BM, thereby promoting reparative mobilization of EPCs from BM and revascularization of ischemic tissues.

EPCs and stem/progenitor cells are embedded in a local BM microenvironment, the so-called stem cell niche, and they are mobilized to the circulation in response to cytokines. [22,32] This process is dependent on EPCs and stem/progenitor cell proliferation, migration, and differentiation in BM. It remains unknown how Nox2-derived ROS regulate EPCs mobilization

from BM. Most recent reports indicate that increased ROS in BM causes hematopoietic stem cells to exit from quiescent state, and drive to stimulate proliferation and differentiation[33] during the early steps of commitment. [34] Stem cell mobilization is mediated by proteinases such as elastase, cathepsin G, and matrix metalloproteinase-9 (MMP-9).[5] We found that there was no difference of MMP-9 expression and activity between WT and $Nox2^{-/-}$ BM before and after hindlimb ischemia (data not shown), suggesting that other targets of Nox2-derived ROS in the BM microenvironment are involved in mobilization of EPCs. Further studies are required to clarify the mechanism of how Nox2-derived ROS in BM can sense the ischemia-induced signals to promote EPCs and stem/progenitor cells mobilization.

BM-derived circulating EPCs and progenitor cells have the potential to home at the sites of ischemic tissues to contribute to revascularization. This homing is mediated through highly concerted mechanisms, which involve adhesion, chemotaxis, and invasion, followed by integration of cells into vascular structures.[35] The present study with intravenous infusion of WT- and $Nox2^{-/-}$ BMCs demonstrates that in vivo homing capacity of $Nox2^{-/-}$ BMCs to sites of ischemia and their neovascularization capacity are significantly reduced compared to WT cells. Of importance, $Nox2^{-/-}$ BMCs pretreated with low-dose H_2O_2 rescued impaired proangiogenic capacity of these cells, confirming that phenotype of $Nox2^{-/-}$ BMCs is specifically attributable to the lack of Nox2-derived ROS. Consistent with our data, Kubo et al[36] reported that intramuscular injection of BMCs pretreated with low-dose H_2O_2 enhances their angiogenic potency. By contrast, improvement by H_2O_2 in WT-BMCs was not observed in our study, presumably attributable to the possibility that WT-BMCs used in the present study have higher ROS levels and more active than those prepared by Kubo et al. These results suggest that Nox2-derived ROS are required for homing and angiogenic capacity of BMCs, thereby promoting neovascularization of ischemic tissues in vivo. Previous reports show that β2 integrin and phosphatidylinositol-3-kinase-γ (PI3Kγ) are involved in homing and neovascularization capacity of EPCs and BM progenitor cells by regulating adhesion of EPCs on fibronectin and ICAM-1.[37,38] In the present study, adhesion of BMCs to fibronectin and ICAM-1 is not affected in $Nox2^{-/-}$ cells, suggesting that β2 integrin- and PI3Kγ-mediated EPC adhesion capacity is independent of Nox2-derived ROS.

SDF-1α, acting through CXCR4 receptor, is the most effective chemoattractant for hematopoietic progenitor cells and EPCs, and plays an important role in homing and engraftment of these cells.[39,40] Our in vitro studies demonstrate that Nox2-deficiency impairs SDF-1–induced migration and invasion capacity of BM progenitor cells, which may contribute to defective homing and mobilization capacities of $Nox2^{-/-}$ BMCs in vivo. $Nox2^{-/-}$ BM progenitor cells pretreated with low-dose H_2O_2 improves the impaired migration capacity of these cells, further confirming that phenotype of $Nox2^{-/-}$ BMCs is attributable to lacking ROS levels. Mechanistically, SDF-1–induced actin polymerization and Akt phosphorylation, both of which are important for hematopoietic stem/progenitor cell migration,[17,18] are significantly inhibited in $Nox2^{-/-}$ BMCs. Of note, SDF-induced ERK1/2 phosphorylation, which plays a minor role for SDF-1–induced migration,[18] is not affected in $Nox2^{-/-}$ cells, suggesting that impairment of BMCs function is not attributable to the CXCR4 dysfunction. Thus, Nox2-derived ROS are selectively involved in the pathways linking CXCR4 to Akt, but not to ERK1/2, thereby promoting migration of BMCs and BM progenitor cells. To our knowledge, this is the first demonstration that Nox2-derived ROS are downstream mediators of CXCR4 signaling linked to migration of stem/progenitor cells. Consistent with our result, receptor tyrosine kinase activation by hematopoietic cytokines increases generation of ROS that are involved in cell proliferation of BM stem cells.[31] PI3Kγ is involved in NADPH oxidase–mediated oxidant generation[41] in ECs and Akt phosphorylation in EPCs.[42] ROS derived from NADPH oxidase regulate actin polymerization[43] and Akt phosphorylation[44–46] in ECs. Thus, one may speculate that PI3Kγ may be upstream mediator for Nox2-based NADPH oxidase in BM progenitor cells, and it is crucial in ROS-dependent actin polymerization and Akt

activation, thereby stimulating cell migration and homing of BMCs, which may contribute to postnatal neovascularization. This point requires further investigation in future study.

In summary, the present study demonstrates that Nox2-based NADPH oxidase expressed in BMCs and EPCs plays an essential role for mobilization of EPCs and stem/progenitor cells from BM and their homing capacity, which may contribute to revascularization of ischemic tissues. We also found that mobilization and homing defects of $Nox2^{-/-}$ BMCs are at least in part attributable to inhibition of chemotaxis and invasion function of BM progenitor cells by reduction of actin polarization and Akt phosphorylation in response to SDF-1α. Thus, NADPH oxidase in BM and EPCs is a potential therapeutic target for promoting neovascularization in ischemic cardiovascular diseases. Furthermore, our findings support the concept that ROS generated by NADPH oxidase serve as important signaling molecules to mediate EPCs and stem/progenitor cell function, which may contribute to promoting neovasculalization and tissue repair in response to injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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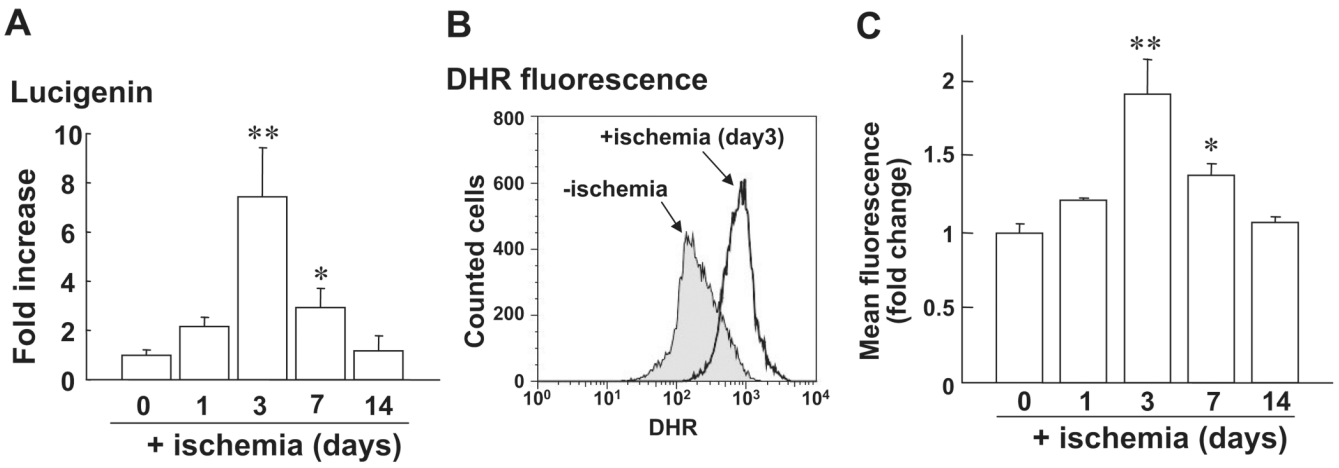

Figure 1.

Increase of ROS production in BMCs after hindlimb ischemia. Low-density BM-MNCs (BMCs) were separated by Histopaque 1077. A, $O_2^{\cdot -}$ production in BMCs after hindlimb ischemia on postoperative days 0, 1, 3, 7, and 14, as measured by lucigenin-enhanced chemiluminescence technique (n=4 in each time point). B, Dihydrorhodamine 123 (DHR) fluorescence in BMCs after hindlimb ischemia is measured by FACS analysis. Upper panel shows the representative histogram of fluorescence intensity, expressed by the logarithm (*x* axis) on 0 and 3 days after ischemia. Lower panel shows the averaged data, expressed as fold change of fluorescence over basal (the ratio on day 0 was set to 1) on various postoperative days (n=4, $^{**}P<0.05$, $^{**}P<0.01$ vs day 0).

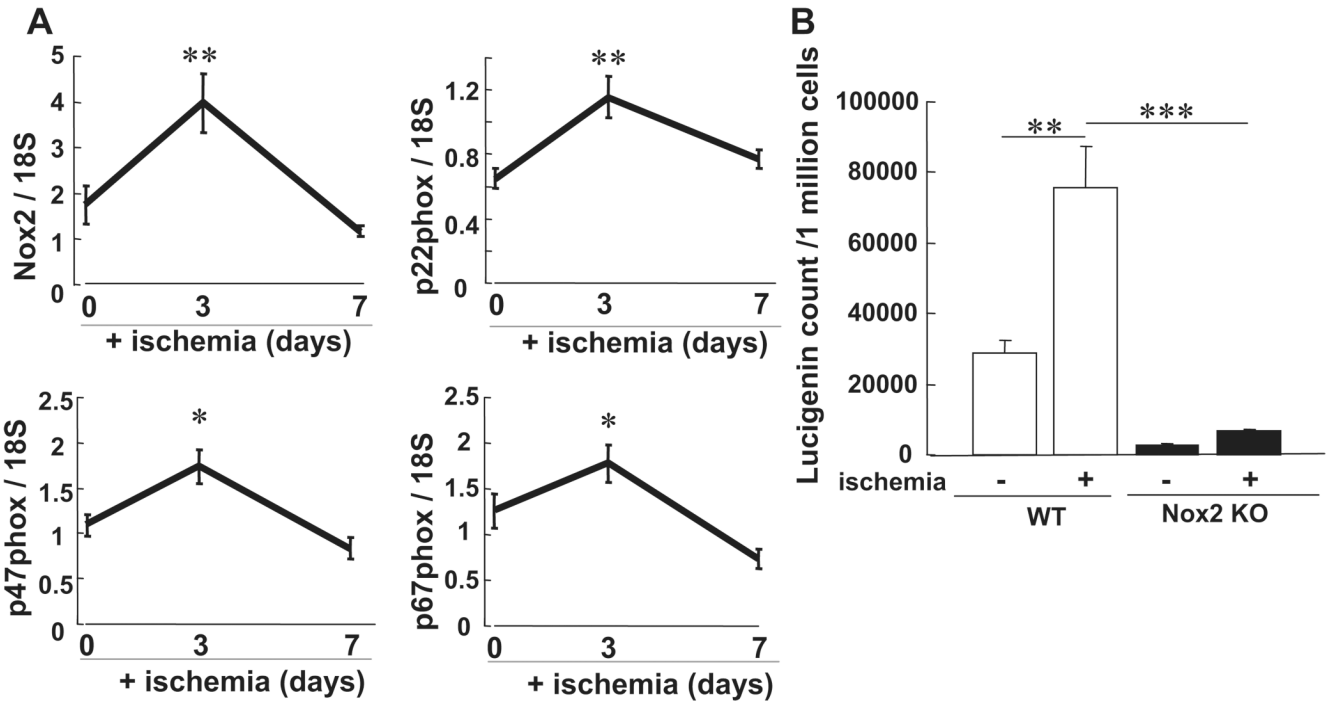

Figure 2.

Nox2-based NADPH oxidase is involved in ROS production in BMCs after hindlimb ischemia. A, The mRNA expression for Nox2 and its coupled NADPH oxidase components including p22phox, p47phox, and p67phox in BMCs at 0, 3, and 7 days after ischemia, as measured by real-time PCR. B, $O_2^{\cdot -}$ production in BMCs at 0 and 3 days after hindlimb ischemia in WT and Nox2$^{-/-}$ mice, as measured by lucigenin-enhanced chemiluminescence technique (n=4 to 6, ***P*<0.01 vs baseline, ****P*<0.001 vs WT).

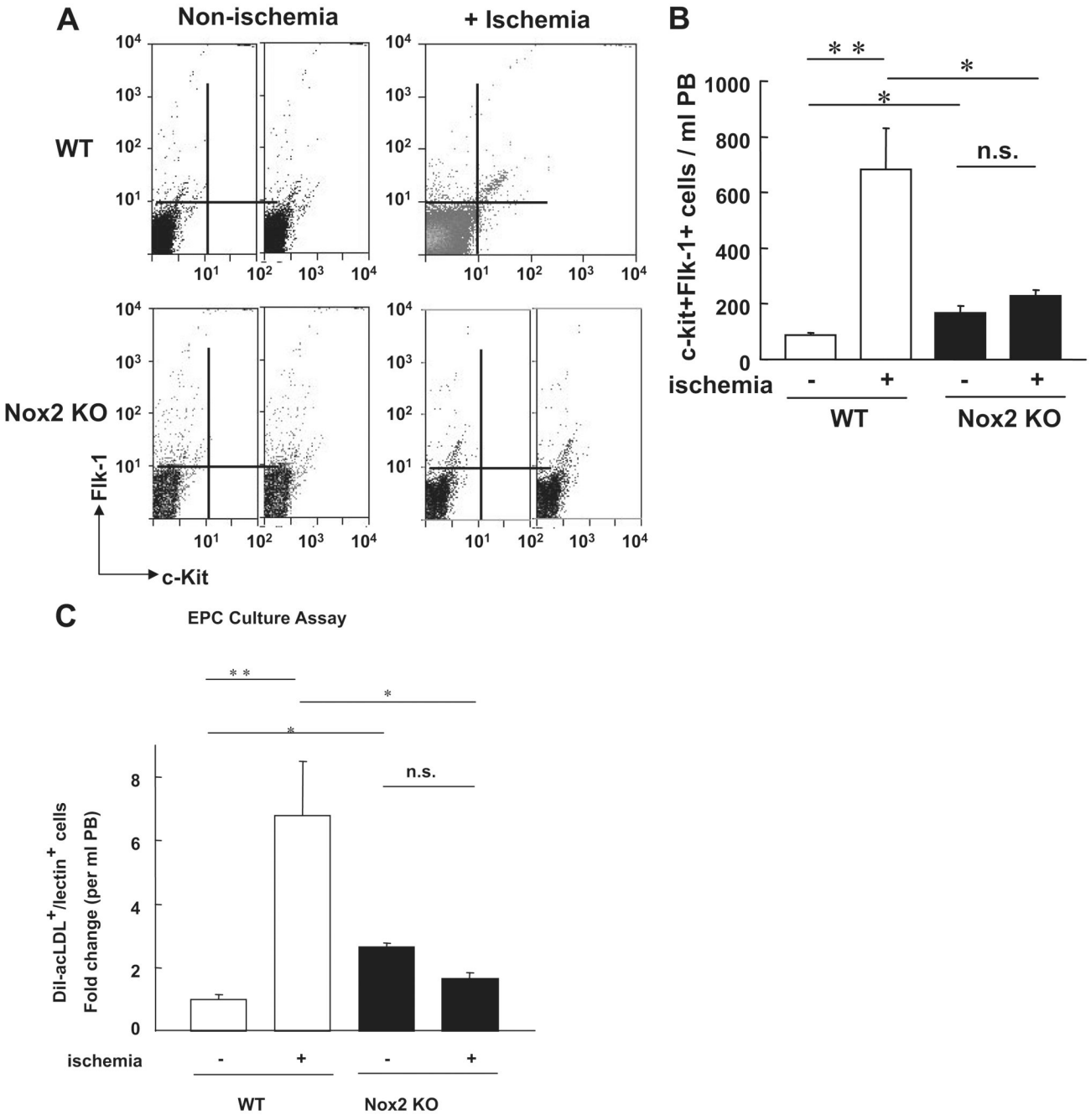

Figure 3.

The number of circulating EPC-like cells after hindlimb ischemia is inhibited in Nox2$^{-/-}$ mice. A, The number of EPC-like ckit^{+}Flk1^{+} cells in peripheral blood (PB) mononuclear cell fraction at 0 (+ nonischemia) and 3 days (+ ischemia) after hindlimb ischemia in WT and Nox2$^{-/-}$ mice, as measured by FACS analysis. B, Statistical analysis of ckit^{+}Flk1^{+} positive cells in PB (n=6, **P*<0.05, ***P*<0.01). C, EPC culture assay. Quantitative analysis of the numbers of DiI-acLDL and BS lectin double positive EPCs measured at 4 days after culture of PB-derived MNCs obtained from WT and Nox2$^{-/-}$ mice at 0 and 3 days after ischemia. Data are expressed as fold change over basal (the ratio on day 0 in WT mice was set to 1). (n=4 to 6, **P*<0.05, ***P*<0.01).

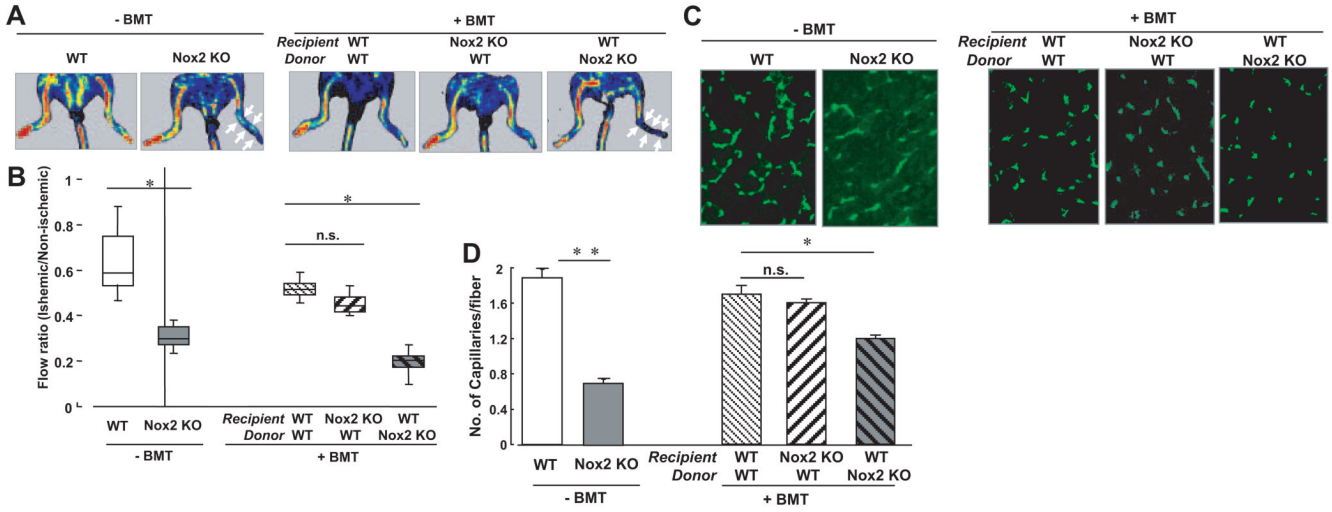

Figure 4.

Transplantation of BM from wild-type mice rescues the impaired neovascularization after hindlimb ischemia in Nox2$^{-/-}$ mice. The lethally irradiated recipient WT or Nox2$^{-/-}$ mice were transplanted with BMCs from WT or Nox2$^{-/-}$ mice and subjected to hindlimb ischemia at 6 to 8 weeks after BM transplantation. A, Representative picture of laser Doppler blood flow (LDBF) imaging at day 14 of ischemic (right) and nonischemic (left) limbs after ligation of femoral artery in WT and Nox2$^{-/-}$ mice without (−) or with (+) BM transplantation (BMT). Arrow indicates delayed blood flow recovery. B, Quantification of blood flow recovery, as determined by the ischemic/nonischemic LDBF ratio in each group (n=4 to 10, **P*<0.05). C, Representative pictures of immunostaining of isolectin B4 positive cells which represent capillaries in ischemic tissues obtained from WT and Nox2$^{-/-}$ mice without or with BMT, as described above. D, Quantitative analysis of capillary density, expressed as the number of capillaries per fiber in each group (n=4, **P*<0.05, ***P*<0.01).

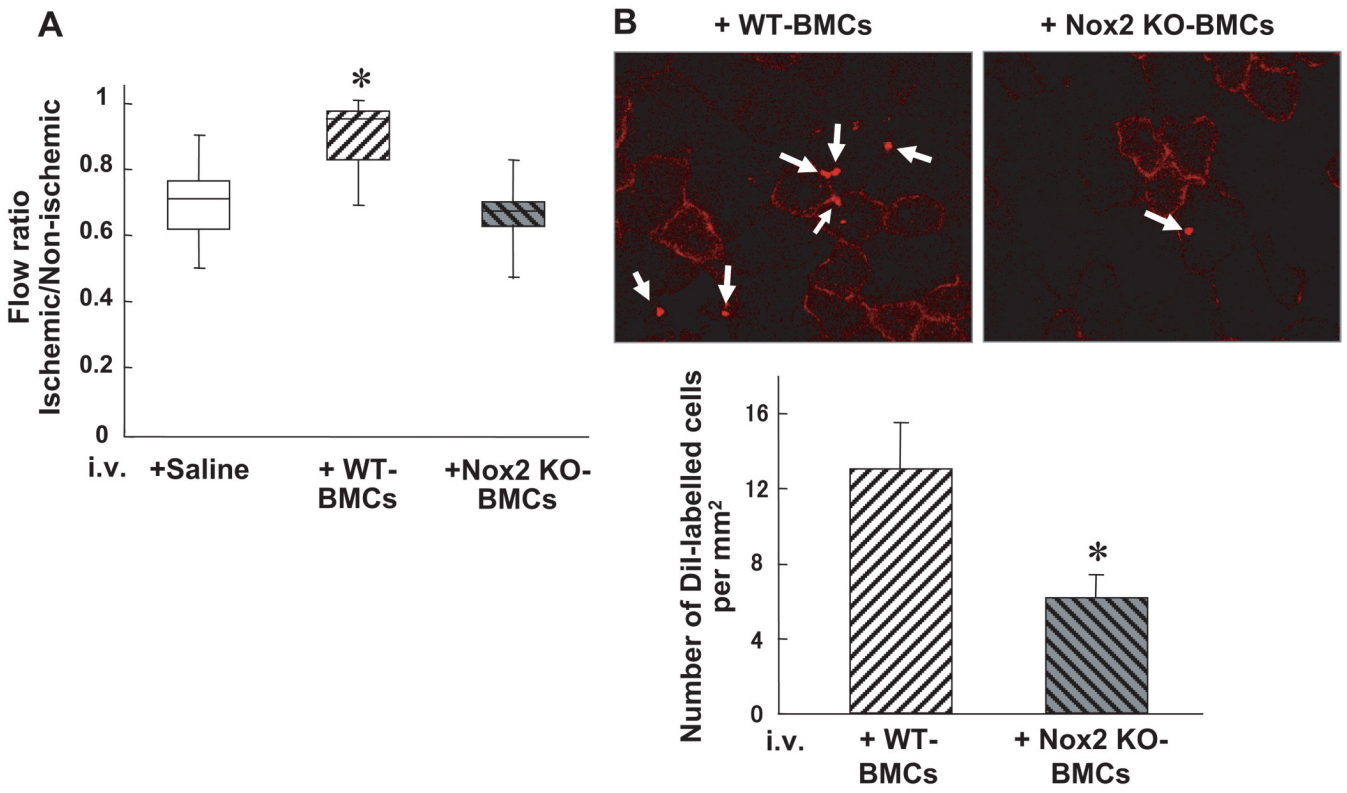

Figure 5.

Homing and neovascularization capacity are impaired in Nox2$^{-/-}$ BMCs in vivo. A, BMCs (3×10^6 cells) from WT or Nox2$^{-/-}$ mice, or saline control, were intravenously injected into WT mice at 24 hours after femoral artery ligation, and blood flow recovery, as assessed by the ischemic/nonischemic LDBF ratio, was measured at 14 days after hindlimb ischemia. (n=4, **P*<0.05 vs control). B, Upper panel, representative photographs of accumulated intravenously injected Dil-labeled WT- and Nox2$^{-/-}$ BMCs in ischemic adductor muscles. Lower panel, quantitative analysis of the number of accumulated Dil-labeled WT- and Nox2$^{-/-}$ BMCs in ischemic border zones of adductor muscle (n=3 in each group, **P*<0.05).

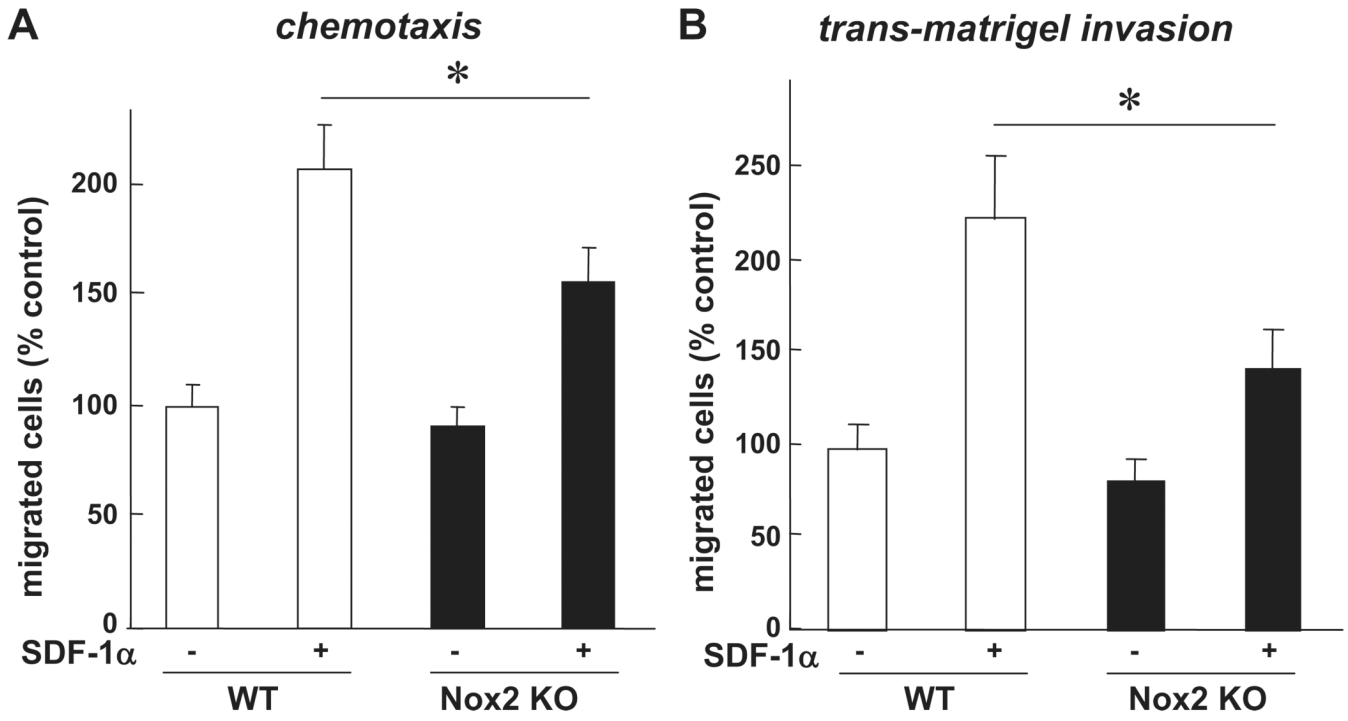

Figure 6.

Chemotaxis and transmatrigel invasion induced by SDF-1α are impaired in Nox2$^{-/-}$ BM stem/progenitor cells in vitro. Quantification of SDF-1α–induced migration (chemotaxis; A) and invasion through matrigel (B) of WT-and Nox2$^{-/-}$ BM c-kit^{+}Lin^{-} progenitor cells, as measured by transwell migration assay. Cells were stimulated with or without SDF-1α (300 ng/mL) for 3 hours. Bar graph represents averaged data, expressed as fold change in number of migrated cells to lower chamber, over that in unstimulated WT cells (control). (n=4, *P<0.05).

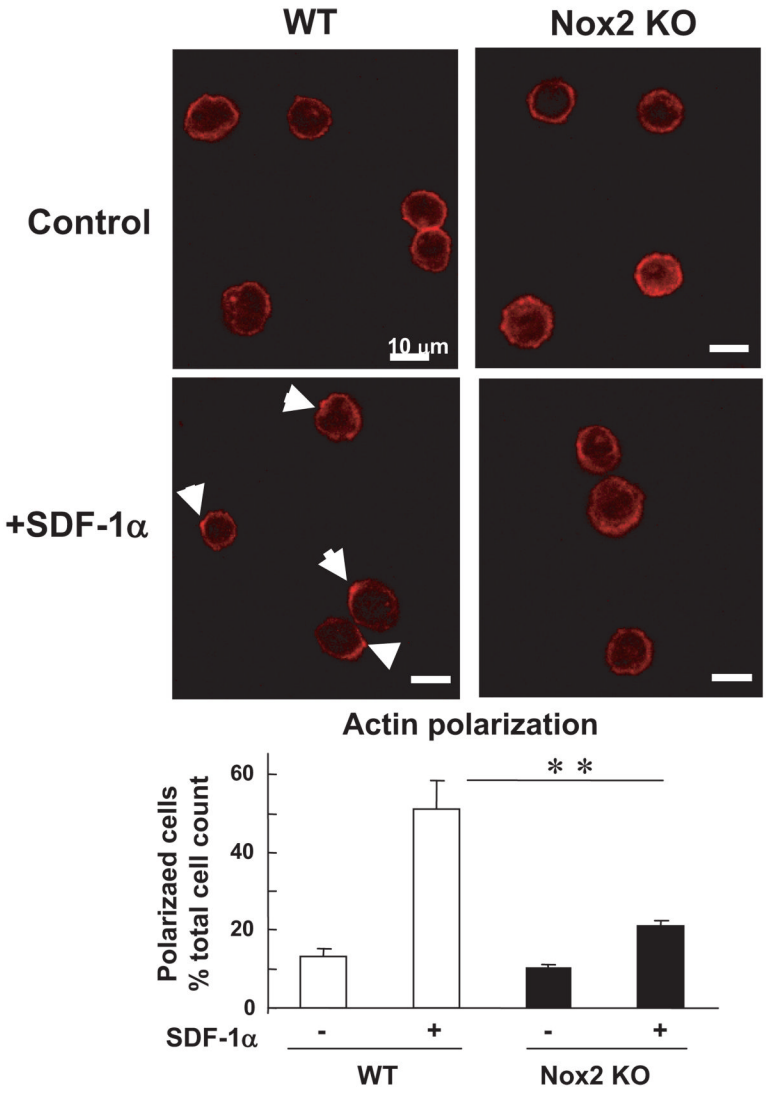

Figure 7.

SDF-1α–induced F-actin polarization is impaired in Nox2$^{-/-}$ BM stem/progenitor cells in vitro. Upper panel, freshly isolated WT- and Nox2$^{-/-}$ BM c-kit^{+}Lin^{-} cells in suspension were stimulated with or without SDF-1α for 5 minutes, fixed, and then stained for Alexa Fluor 568 conjugated phalloidin, which is visualized by confocal microscopy. Arrows indicate the polarized F-actin. Original magnification is ×630 and bars show 10 μm. Lower panel, quantitative analysis of SDF-induced F-actin polarization in WT- and Nox2$^{-/-}$ BM c-kit^{+}Lin^{-} cells. Data are expressed as percentage of the total 100 cells from 4 randomly selected fields (×100; n=4, ***P*<0.01).

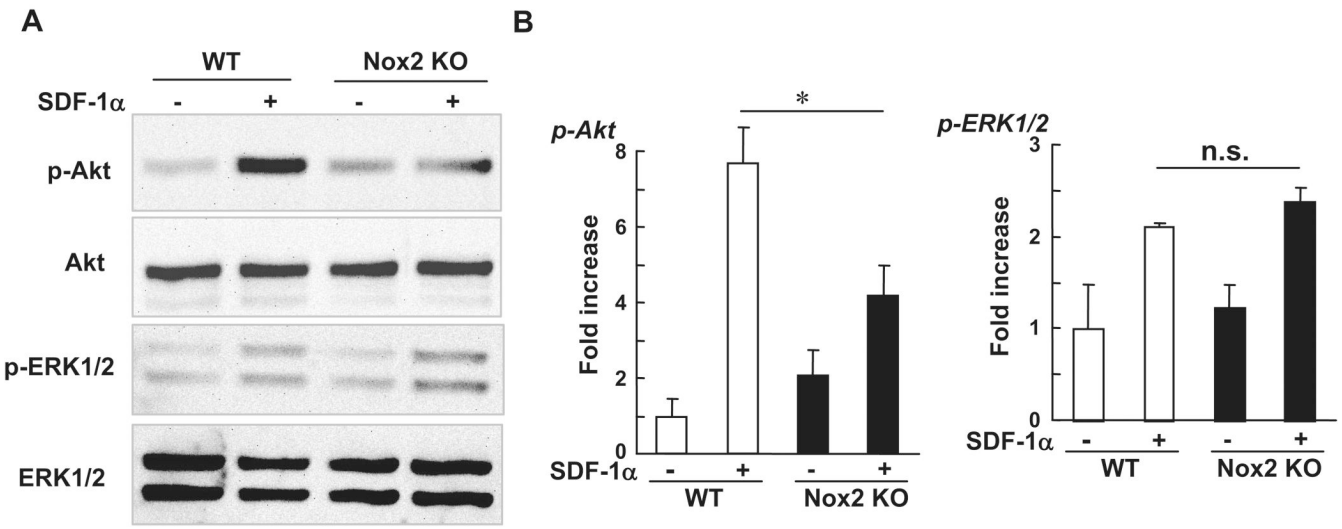

Figure 8.

Phosphorylation of Akt induced by SDF-1 is inhibited in Nox2$^{-/-}$ BMCs. Freshly isolated WT- and Nox2$^{-/-}$ BMCs were serum depleted for 4 hours and then stimulated with SDF-1α (300 ng/mL) for 5 minutes. Lysates were used for Western analysis with antiphospho-Akt (Ser473), phospho-ERK1/2, total Akt, or total ERK1/2 antibodies. Data are expressed as fold increase over the unstimulated WT cells (n=3, **P*<0.05).