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NADPH Oxidase 1 Overexpression Enhances Invasion via Matrix Metalloproteinase-2 and Epithelial–Mesenchymal Transition in Melanoma Cells

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NADPH oxidase 1 (Nox1) is a member of the NADPH oxidase family that has not been well characterized in the melanocytic cell lineage. Here we demonstrated that *Nox1* and *Nox4* were detected in melanocytic lineage, with only *Nox1* detected in normal human melanocytes and Nox4 in a subset of metastatic melanoma cell lines. The protein level and enzymatic activity of Nox1 was elevated in all melanoma cells as compared with normal melanocytes. Overexpression of GFP-Nox1 protein in Wm3211 primary melanoma cells increased invasion rate by 4- to 6-fold as measured by Matrigel invasion assay, whereas knocking down or inhibiting Nox1 decreased invasion by approximately 40–60% in Wm3211 and SK-Mel-28 cells. *Matrix metalloproteinase-2* (*MMP-2*) was increased by *Nox1* overexpression at the mRNA, protein, and activity levels, and decreased by *Nox1* knockdown. *MMP-2* promoter activity was also regulated by Nox1 knockdown. In addition, stable clones overexpressing *Nox1* exhibited an epithelial–mesenchymal transition (EMT) as examined by cell morphology and EMT markers; knockdown or inhibiting Nox1 led to a reversal of EMT. Supplementing MMP-2 to culture media did not induce EMT, suggesting that EMT induction by Nox1 was not through *MMP-2* upregulation. In summary, Nox1 was overexpressed in all melanoma cell lines examined, and enhanced cell invasion by MMP-2 upregulation and EMT induction.

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INTRODUCTION

NADPH oxidase (EC 1.6.3.–) is a family of enzymes that catalyze transfer of an electron from NAD(P)H to molecule oxygen to generate superoxide or hydrogen peroxide. It consists of seven members, represented by their catalytic subunits: NADPH oxidase 1 (Nox1), Nox2 (gp91phox), Nox3, Nox4, Nox5, Duox1, and Duox2. These enzymes are expressed differentially in various tissues and organs (Lambeth, 2004; Sumimoto, 2008). Nox2 is the founding member of this enzyme family that has been extensively studied in phagocytes and cardiovascular cells (Giannopoulou *et al.*, 2008; Krause and Bedard, 2008; Rada *et al.*, 2008). Nox1 is the enzyme closest to Nox2 in a phylogenetic tree of this family (Kawahara and Lambeth, 2007).

Nox1 requires a membrane subunit p22phox, Noxa1, and Noxo1 for its full activity (Banfi *et al.*, 1999; Ambasta *et al.*, 2004; Hanna *et al.*, 2004). *Nox1* is expressed in colon epithelial cells, vascular smooth muscle cells, endothelium, uterus, placenta, prostate, osteoclasts, and retinal pericytes, and serves as an important signal molecule downstream of platelet-derived growth factor, angiotensin II, and tumor necrosis factor-α (Brown and Griendling, 2009). Recently, overexpression of Nox1 was reported in colon cancer, gastric cancer, and prostate cancer (Suh *et al.*, 1999; Kamata, 2009). To our best knowledge, expression of *Nox1* was not reported in melanoma, although expression of *Nox4* has been reported previously (Brar *et al.*, 2002; Govindarajan *et al.*, 2007; Yamaura *et al.*, 2009).

An early study revealed that *Nox1* overexpression induced transformation in NIH3T3 cells (Suh *et al.*, 1999); subsequent studies showed that *Nox1* was required for K-ras-mediated transformation of normal rat kidney cells (Komatsu *et al.*, 2008). Hence, Nox1 plays an important role in cancer development. In colon cancer cells, reactive oxygen species (ROS) generated by Nox1 contributed to cell invasion

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DPI, diphenyl-iodonium chloride; EMT, epithelial–mesenchymal transition; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; NAC, N-acetyl-cysteine; NHM, normal human melanocytes; Nox1, NADPH oxidase 1; ROS, reactive oxygen species; RT–PCR, reverse transcriptase–PCR; WGA, wheat-germ agglutinin

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through upregulating matrix metalloproteinase-9 (MMP-9) (Shinohara *et al.*, 2010). MMPs are a family of proteinases with various substrates and functions, including angiogenesis, tissue invasion, inflammation, and tumor metastasis (Hadler-Olsen *et al.*, 2010; Kessenbrock *et al.*, 2010). In melanoma, both MMP-2 and MMP-9 were involved in cell invasion (van den Oord *et al.*, 1997; Simonetti *et al.*, 2002); however, only overexpression of *MMP-2* is correlated with progression and survival (Vaisanen *et al.*, 1998, 2008; Orimoto *et al.*, 2008; Malaponte *et al.*, 2010). To our best knowledge, regulation of *MMP-2* by Nox1 has not been reported in cancer cells including melanoma.

Epithelial–mesenchymal transition (EMT) is a pathway facilitating cell invasion, which is characterized by loss of epithelial and gain of mesenchymal morphology, accompanied by loss of E-cadherin, gain of N-cadherin, vimentin, and other markers (Pla *et al.*, 2001; Singh and Settleman, 2010). MMP-3 and MMP-9 may contribute to EMT via modifying the extracellular matrix components (Radisky *et al.*, 2005; Blavier *et al.*, 2010); whether *MMP-2* played such a role is not clear. Although *Nox1* played a role indirectly in modifying cell adhesion molecules (Sadok *et al.*, 2009), it is not clear whether *Nox1* mediates EMT process.

RESULTS

Nox1 is overexpressed in melanoma cell lines

The Nox1 expression levels and total Nox activities in normal human melanocytes (NHMs) and melanoma cell lines were examined. As shown in Figure 1a, compared with NHMs, Nox1 protein levels were increased in all melanoma cell lines. Figure 1b shows the densitometry-quantified Nox1 protein levels (first normalized to α-tubulin loading control and then to NHMs). Figure 1c shows total Nox enzymatic levels as analyzed using NADH as a substrate and lucigenin as the electron acceptor for superoxide anions (Cui and Douglas, 1997; Cai *et al.*, 2007). All melanoma cell lines exhibited higher Nox activities than NHMs. This enzyme assay does not distinguish among Nox family members; hence, it reflects total Nox activity based on superoxide generation. Therefore, reverse transcriptase–PCR (RT–PCR) was performed to identify individual Nox members. As shown in Figure 1d, *Nox1* mRNA was detected in normal melanocytes and all melanoma cell lines examined. Consistently, mRNAs for Nox1 subunits *Noxo1* and *p22phox* were also detected in NHMs and melanoma cell lines. In contrast, *Nox4* mRNA was not detected in NHMs or primary melanoma cell lines Wm3211 and Wm115 (Figure 1d); instead, it was only detected in three metastatic cell lines A375, c8161, and c8146A (Figure 1d). The HaCaT cells and human umbilical vein endothelial cells were used as positive controls for *Nox1* and *Nox4*, whereas *β-actin* was used as a loading control. We further examined the mRNA accumulation of all seven members of Nox family in NHMs and c8146A melanoma cell lines and confirmed again that the only two members expressed in melanocytic lineage were *Nox1* and *Nox4*, whereas only *Nox1* mRNA was detected in NHMs (Figure 1e).

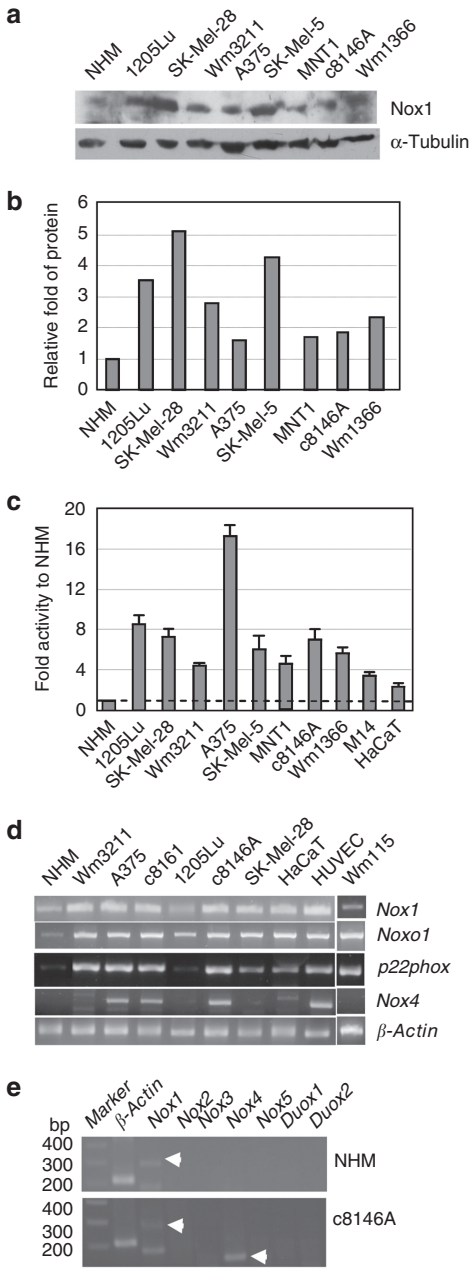

Figure 1. Expression of NADPH oxidase 1 (Nox1) in melanocytes and melanoma cells. (**a**) Western blot analysis of Nox1 protein in normal human melanocytes (NHMs) and melanoma cell lines. (**b**) Quantitative Nox1 protein accumulation in NHMs and melanoma cell lines. Protein accumulation was quantified by a densitometer and first normalized to α-tubulin, and then to NHM. (**c**) Relative NADPH oxidase activity in NHMs and melanoma cell lines and HaCaT cells. The Nox activities in other cell lines were compared with that in NHM cells. (**d**) Reverse transcriptase–PCR (RT–PCR) analysis of *Nox1*, *Noxo1*, *p22phox*, and *Nox4* genes in NHMs and melanoma cell lines, HaCaT cells, and human umbilical vein endothelial cells (HUVECs) were used as positive controls. β-Actin was used as internal control. (**e**) RT–PCR analysis of Nox family genes in NHMs and c8146A melanoma cell lines.

Subcellular localization of Nox1

To determine the subcellular localization of Nox1, a GFP-tagged Nox1 construct pEGFP-Nox1 was generated and transfected into Wm3211 cells. Two stable clones (Clone nos.

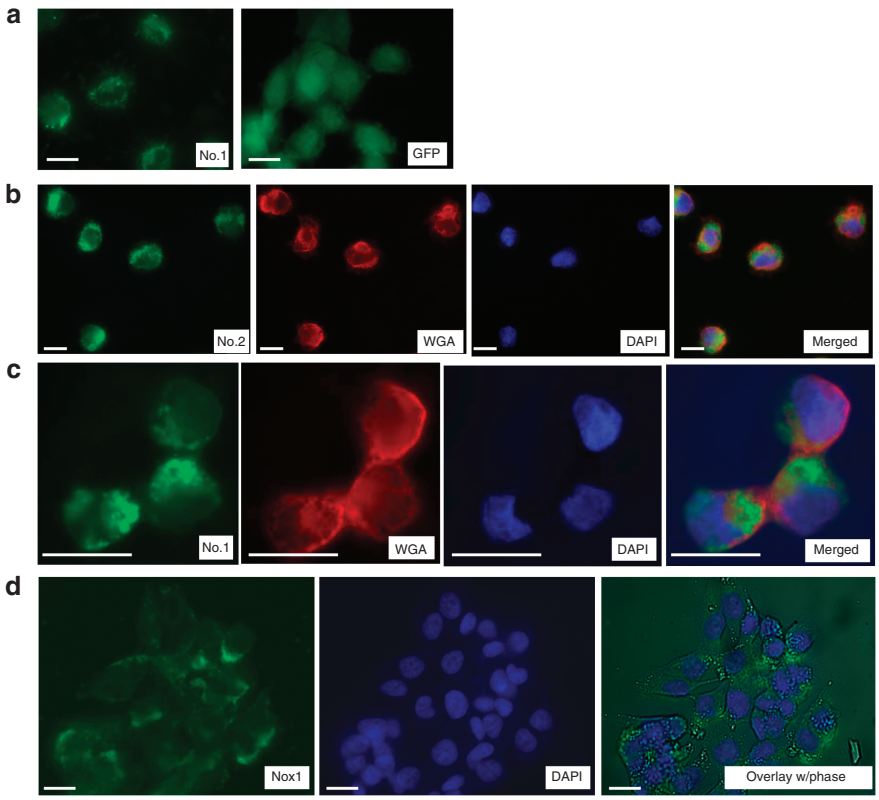

Figure 2. Subcellular localization of NADPH oxidase 1 (Nox1). (**a**) GFP-Nox1 overexpression Clone no. 1 showing the GFP fluorescence around cells, in contrast to clones expressing GFP protein alone. (**b**) GFP-Nox1 signal from Clone no. 2 cells (green), plasma-membrane marker wheat-germ agglutinin (WGA)-Texas Red (red), 4′,6-diamidino-2-phenylindole (DAPI) staining (blue), and their overlaying photos (processed by Image J, ×40 objectives). (**c**) GFP-Nox1 signals from cell Clone no.1 (green), WGA-Texas Red, DAPI staining, and their overlaying photos (×63 objectives). (**d**) Immunofluorescence staining of Nox1 in Wm3211 cells. Cells were fixed and stained with Nox1 primary antibody, and then FITC-conjugated secondary antibody. Phase-contrast photos were taken and overlayed with Nox1 and DAPI signal. Bar = 10 μm.

1 and 2) were established. The green fluorescence signal from GFP-Nox1 fusion protein in Clone no. 1 cells was located around the cell nucleus, distinct from what was observed in cells expressing GFP protein alone, where fluorescence signal was diffusely present throughout the cells (Figure 2a). Co-staining with 4′,6-diamidino-2-phenylindole (DAPI) and plasma-membrane marker wheat-germ agglutinin (WGA) (Flesch *et al.*, 1998) conjugated with Texas Red revealed that the Nox1 signal was mainly localized in the cytosolic compartment, with minor signal overlapping with WGA-Texas Red fluorescence (Figure 2b). When examined under a microscopy with ×63 objective oil lens, the cytosolic and partial plasma-membrane localization was more obvious (Figure 2c). No GFP-Nox1 signal was observed in the nucleus (Figure 2c). The cytosolic and partial plasma-membrane localization of Nox1 was further confirmed by immunofluorescence using anti-Nox1 antibody and FITC-conjugated secondary antibody. The majority of the Nox1 green fluorescence was within the cytosol, and again a portion of the signal was on the plasma membrane (Figure 2d).

Overexpression of Nox1 in Wm3211 cells led to increased invasion

As shown in Figure 2, overexpression of GFP-Nox1 showed green fluorescence signal in a pattern distinct from that of GFP alone; however, we were unable to detect the GFP-Nox1 protein on a western blot, presumably due to low levels of expression of this fusion protein, or disruption of the epitope recognized by the antibody. Yet, when total Nox activity was measured, both no. 1 and no. 2 cells showed higher level (3∼8-fold) compared with the parental Wm3211 cells (Figure 3a). Consistently, these clones also exhibited higher levels of superoxide (2.5- to 3.1-fold) as measured by dihydroethidium fluorescence (Figure 3b). To examine whether Nox1-mediated ROS has a particular role in melanoma invasion, Matrigel invasion assay was performed. As shown in Figure 3c and d, Clone nos. 1 and 2 showed 3.6- and 4.5-fold higher invasion rate than the control Wm3211 cells, whereas the negative control GFP clone did not show a significant increase of invasion. To rule out clone variation, GFP-Nox1 was transiently expressed in Wm3211 cells and the invasion rate was compared with that in cells overexpressing GFP alone. GFP signal was verified using a fluorescence microscope (Figure 3e). There was an average of 60 cells per view under a microscope for GFP-Nox1 overexpressed cells, whereas there was only an average of 14 cells per view for cells transfected with GFP vector alone (Figure 3e and f), representing an average of 4.3-fold increase of invasion driven by GFP-Nox1 expression.

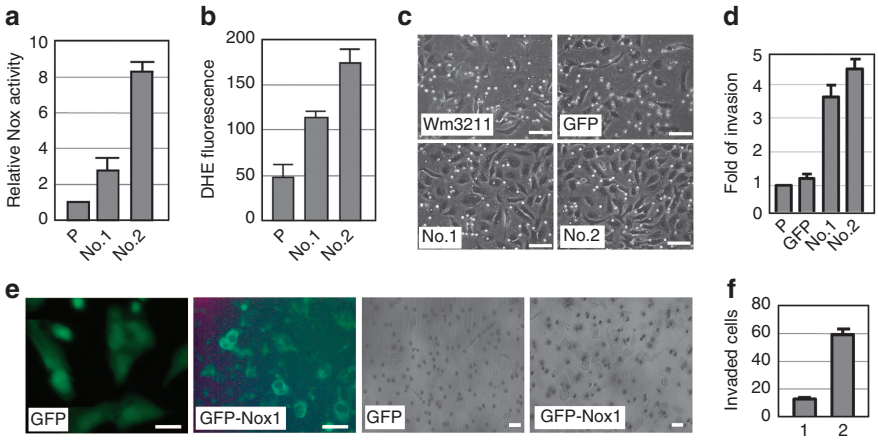

Figure 3. Overexpression of NADPH oxidase 1 (Nox1) enhanced cell invasion capacity. (**a**) Total Nox activities in Wm3211 (P), and no. 1 and no. 2 cells. (**b**) Superoxide levels in Wm3211 (P), and no. 1 and no. 2 cells (average of at least three measurements). (**c**) Invasion assay for Wm3211, GFP clone, and no. 1 and no. 2 clones. (**d**) Relative invasion capacity of GFP cells, and no. 1 and no. 2 cells compared with that of control Wm3211 cells. (**e**) Overexpression of GFP-Nox1 in a transient transfection experiment; GFP vector was used as a control. These cells were used for invasion assay 48 hours post-transfection, the invaded cells were photographed (right panels), and cell number was counted in five randomly picked views under a microscope. (**f**) The average invaded cell numbers were graphed. 1, GFP; 2, GFP-Nox1. Bar = 10 μm. DHE, dihydroethidium.

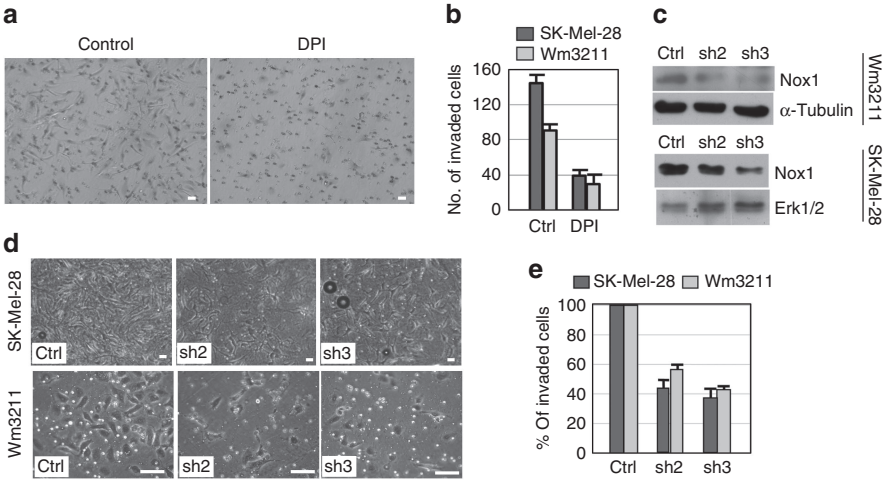

Figure 4. Inhibiting or knockdown of NADPH oxidase 1 (Nox1) decreased invasion capacity. (**a**) Invasion assay of diphenyl-iodonium chloride (DPI) (5 μM)-treated SK-Mel-28 cells. DPI was then included in the media during the incubation period. (**b**) Graphed average invaded cell numbers from **a** for SK-Mel-28 and for Wm3211 cells. Ctrl, control; DPI, treated. (**c**) Western blot analysis of Nox1 protein in Wm3211 with selected pRS-GFPi (Ctrl), pRS-sh2, and pRS-sh3 cells (sh2 and sh3) in Wm3211 and SK-Mel-28 cells. (**d**) Invasion assay for cells in **c**. Photos show representative pictures of invaded cells from Ctrl, sh2-, and sh3-transfected cells. (**e**) Graphed normalized invasion rate for cells from **d**. Bar = 10 μm.

Knocking down or inhibiting Nox1 led to decreased invasion

To further confirm the role of *Nox1* in melanoma invasion, melanoma cells were pretreated with a Nox inhibitor diphenyl-iodonium chloride (DPI) at 5 μM for 4 hours before invasion assay. DPI treatment decreased the number of invaded cells for both Wm3211 and SK-Mel-28 (Figure 4a and b). Furthermore, plasmids expressing short-hairpin RNAs targeting two different sequences in Nox1 coding region were constructed (pRS-sh2 and pRS-sh3) and transfected into Wm3211 and SK-Mel-28 cells, and then selected with puromycin for 2 weeks. The shRNA targeting GFP sequence (pRS-GFPi) was used as a control. Nox1 protein knockdown (70–80%) was confirmed by western blot in both cell lines (Figure 4c). Decreased invasion rates (40–60%) were observed in both cell lines transfected with either pRS-sh2 or pRS-sh3 plasmids, as compared with the control cells (Figure 4d and e).

Nox1* regulates *MMP-2

MMP-2 and *MMP-9* are among the MMPs characterized in melanoma cells for their key roles in invasion (Leslie and Bar-Eli, 2005). We examined the expression levels of both MMPs in response to Nox1 regulation. As shown in Figure 5a, the gelatinase activity assay indicated that the 72-kDa MMP-2 activity increased in culture media of Clone no. 1 and no. 2 cells, as compared with Wm3211 cells, and decreased in

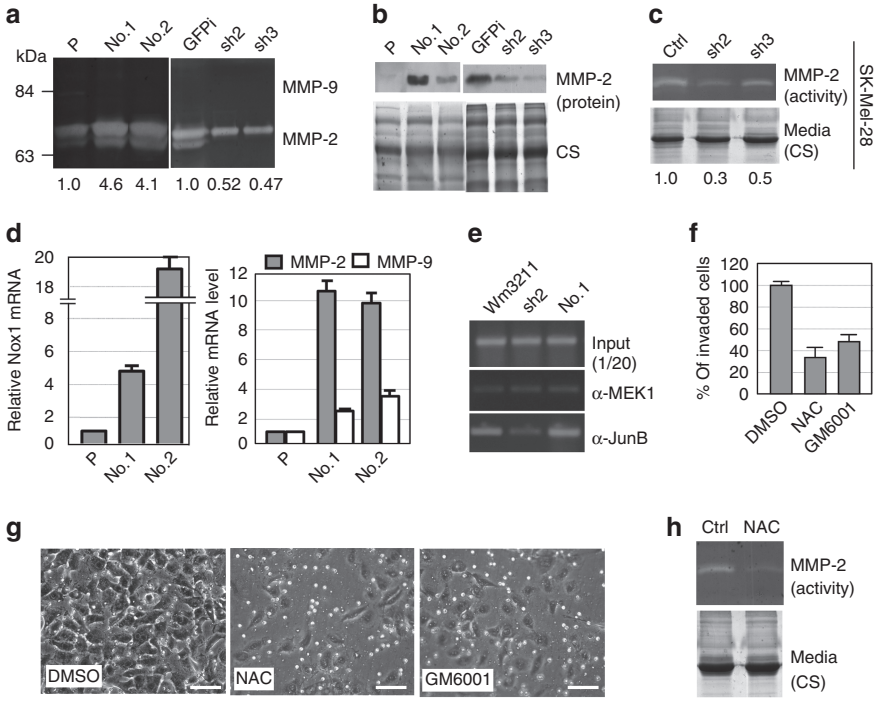

Figure 5. NADPH oxidase 1 (Nox1) regulates matrix metalloproteinase-2 (MMP-2). (**a**) Zymography assay for gelatinase activities in Wm3211 cells. The MMP-2 activity was quantified by a densitometer and the relative activity is shown below the photos. P, Wm3211. (**b**) Cell culture media were concentrated and analyzed by (top) western blot or for (bottom, loading control) Coomassie blue staining (CS). (**c**) MMP-2 activity decreased in SK-Mel-28 cells with Nox1 knockdown. (**d**) Quantitative real-time reverse transcriptase–PCR (qRT–PCR) analysis of Nox1, MMP-2, and MMP-9 transcripts in Wm3211 (P), and no. 1 and no. 2 cells. (**e**) Chromatin immunoprecipitation (ChIP) analysis of JunB binding to MMP-2 promoter. (**f**) Invasion capacity of control (dimethylsulfoxide (DMSO)) cells and *N*-acetyl-cysteine (NAC)- or GM6001-treated cells. (**g**) The representative pictures of the invaded cells in the above treatments. (**h**) Decreased MMP-2 activity in NAC-treated Wm3211 cells. Bar = 10 μm.

cells with Nox1 knockdown (Figure 5a). The MMP-9 activity was not detected in these cells (Figure 5a). Quantification by densitometry indicated that the MMP-2 activity increased about 4- to 5-fold in Clone nos. 1 and 2, and then decreased about 50% in sh2 and sh3 cells (Figure 5a). Western blot analysis confirmed that in Clone nos. 1 and 2, the secreted MMP-2 protein accumulated to higher levels than that in the control Wm3211 cells, and lower levels in sh2 and sh3 cells (Figure 5b). Duplicated gels stained with Coomassie blue (partial) is shown as loading controls (Figure 5b). In SK-Mel-28 cells with Nox1 knockdown (sh2 and sh3), MMP-2 activity was also decreased about 50–70% as compared with the control parental cells (Figure 5c). Quantitative RT–PCR analysis indicated that the MMP-2 mRNA increased about 10-fold in Clone no. 1 and no. 2 cells as compared with that in the parental cells. A small increase (2.5- to 3.5-fold) of *MMP-9* mRNA was also observed in these cells (Figure 5d). Overexpression of Nox1 was further confirmed in these clones as the Nox1 mRNA accumulated to about 5- to 19-fold in Clone nos. 1 and 2 as compared with Wm3211 cells (Figure 5d). Analysis of the *MMP-2* promoter reporter activity in sh2 and sh3 cells indicated that the knockdown of Nox1 decreased *MMP-2* promoter activity by 30–40% (Supplementary Figure S1a online). These data suggest that regulation of *MMP-2* by *Nox1* is likely through a transcriptional mechanism. As *MMP-2* is a well-documented transcriptional target for AP-1 transcriptional factors, we also examined the AP-1 transcription activity in Clone no. 1 and no. 2 cells. A reporter plasmid for AP-1 activity, pAP-1-SEAP, was transiently transfected into Wm3211 cells together with pRL-SV40 luciferase reporter plasmids (internal control). Our results showed that normalized AP-1 activities in Clone nos. 1 and 2 were about 2.5- to 4.0-fold of that in the parental Wm3211 cells (Supplementary Figure S1b online). Further examination of AP-1 transcription factors in *Nox1* overexpression clones indicated that among all AP-1 members, only JunB showed increased protein accumulation in Clone no. 1 and no. 2 cells (data not shown). Chromatin immunoprecipitation experiments using antibody against JunB showed that binding of JunB to MMP-2 promoter decreased in sh2 cells to nearly background level and increased about 2-fold in Nox1-overexpressing Clone no. 1 cells (Figure 5e).

To further confirm that Nox1-generated ROS and upregulated MMP-2 played key roles in its ability to stimulate invasion, Clone no. 1 cells were treated with the ROS scavenger *N*-acetyl-cysteine (NAC, 2 mM, 24 hours) or MMP inhibitor GM6001 (10 μM, 4 hours) before invasion assay. Our results showed that both NAC and GM6001 reduced invasion rate in these cells, by approximately 67 and 50%, respectively (Figure 5f and g). Zymography assay indicated that NAC treatment led to a decreased (about 65%) MMP-2 activity in Wm3211 cells (Figure 5h). Quantitative RT–PCR

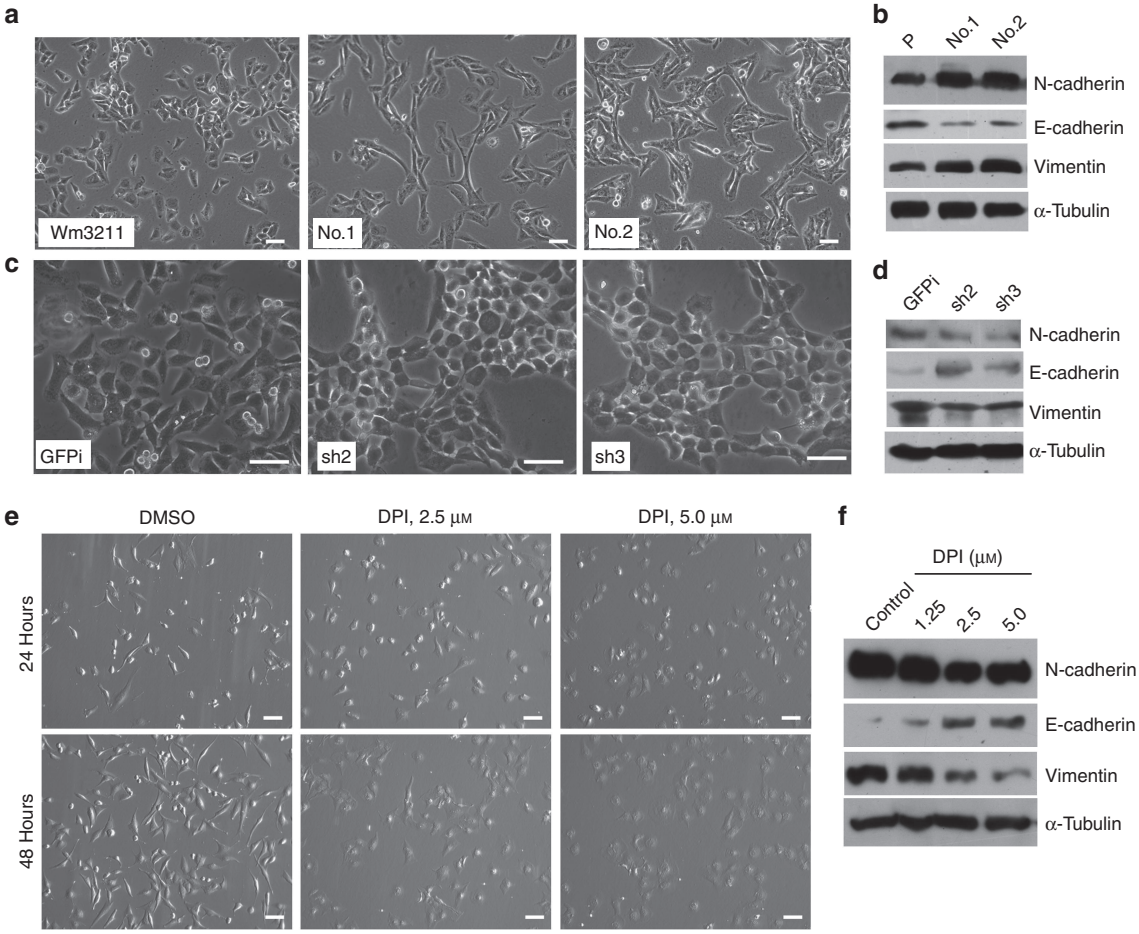

Figure 6. NADPH oxidase 1 (Nox1) induced epithelial–mesenchymal transition (EMT). (**a**) EMT morphological changes induced by Nox1 overexpression. (**b**) Western blot analysis showing changes in N-cadherin, vimentin, and E-cadherin in Clone no. 1 and no. 2 cells. (**c**) Knockdown of Nox1 led to a reversed EMT changes in Wm3211 cells. (**d**) Western blot analysis showing changes in N-cadherin, vimentin, and E-cadherin in sh2 and sh3 cells. (**e**) Inhibiting Nox1 by diphenyl-iodonium chloride (DPI) also led to a reversed EMT changes in SK-Mel-28 cells. (**f**) Western blot analysis showing changes in N-cadherin, vimentin, and E-cadherin in DPI-treated cells.

revealed that this regulation is likely also through transcription because the accumulation of MMP-2 transcripts decreased nearly 70% in the NAC-treated cells (Supplementary Figure S2 online).

Nox1 regulates EMT

In addition to MMPs, EMT also plays an important role in cell migration and invasion. We observed that Clone nos. 1 and 2 showed an elongated, mesenchymal morphology as compared with the parental Wm3211 cells (Figure 6a), which mimicked a transforming growth factor-β-induced EMT morphological change ((Supplementary Figure S3a online). Western blot analysis indicated that EMT markers N-cadherin and vimentin were increased in Clone no. 1 and no. 2 cells as compared with Wm3211 cells, and E-cadherin decreased (Figure 6b). In contrast, Wm3211 cells with Nox1 knockdown (sh2 and sh3 cells) showed a reversed EMT morphology: the cells were more epithelial-like as compared with the control GFPi cells (Figure 6c). Consistently, western blot analysis showed that the sh2 and sh3 cells exhibited reversed EMT marker changes: increased E-cadherin and decreased N-cadherin and vimentin (Figure 6d). Furthermore, when Nox1 was inhibited by DPI in SK-Mel-28 cells, these cells also showed a dose- and time-dependent reversal of EMT phenotype (Figure 6e). Western blot analysis indicated that N-cadherin and vimentin were decreased, and E-cadherin increased in the DPI-treated cells as compared with control cells (Figure 6f). Knocking down Nox1 in SK-Mel-28 cells led to similar reversed EMT morphological changes where cells became flatter, less elongated, and more epithelial-like (Supplementary Figure S4 online). It was reported that some MMPs were able to induce EMT in certain cell lineages (Tan *et al.*, 2010; Lin *et al.*, 2011). To test whether Nox1-upregulated MMP-2 played a role in EMT induction in Wm3211 cells, we added purified active MMP-2 into cell culture media and incubated for up to 48 hours; no induction of EMT was observed (Supplementary Figure S3b online).

DISCUSSION

The Nox gene family plays key roles in regulating many physiological processes including cardiovascular function and immune responses. An early study identified that Nox4

played a role in melanoma proliferation (Brar *et al.*, 2002). A few studies subsequently confirmed the role of *Nox4* in melanoma cell cycle progression and invasion (Govindarajan *et al.*, 2007; Yamaura *et al.*, 2009). Expression of other Nox family members was not reported, although Nox enzyme activity was shown to be important for melanoma cell differentiation (Zhao *et al.*, 2008). In this study, we report expression of *Nox1* and its subunits *p22phox* and *Noxo1* in normal human melanocytes and melanoma cell lines, and characterized its function in promoting melanoma invasion via upregulating MMP-2 and induction of EMT.

Nox1 expression was elevated in all melanoma cell lines including early radial growth phase cell line Wm3211; however, the protein level did not correlate with melanoma progression. This observation suggests that upregulation of Nox1 is an early event in melanoma transformation. In contrast, Nox4 was only detected in three metastatic cell lines, which is consistent with previous study showing that Nox4 was downstream of AKT pathway driving for melanoma invasion (Govindarajan *et al.*, 2007).

Studies from our laboratory and others have demonstrated that ROS levels are higher in melanoma cells than in NHMs (Meyskens *et al.*, 1997; Sander *et al.*, 2003; Fried and Arbiser, 2008). The source of ROS is multiple and includes various physiological procedures or organelles, such as biosynthesis of melanin (Slominski *et al.*, 2004), altered tumor metabolism, and immune reactions (Komarov *et al.*, 2005; Wittgen and van Kempen, 2007). Our observation that *Nox1* is ubiquitously overexpressed in all melanoma cell lines suggests that *Nox1* overexpression may contribute to the observed high ROS levels in melanoma cells.

The subcellular localization of Nox1 is still under debate. The protein contains six transmembrane domains; therefore, it is likely a membrane protein. Here we show that a GFP-tagged Nox1 protein is indeed partially localized to plasma membrane. However, the majority of the signal was localized to the cytoplasmic compartment. Further experiments are needed to determine whether the protein is localized to certain membrane organelles such as Golgi body or endoplasmic reticulum.

To understand the mechanism of Nox1-induced melanoma invasion, two key MMPs (MMP-2 and MMP-9) in melanoma invasion were examined. *MMP-2* was identified as the major effector of Nox1 in melanoma invasion, which is different from that in colon cancer cells where MMP-9 was a main effector (Kim *et al.*, 2009). The *Nox1*-induced *MMP-2* is likely through an ROS-dependent transcriptional mechanism, mediated by the AP-1 family transcription factors (Bergman *et al.*, 2003; Kajanne *et al.*, 2007; Singh *et al.*, 2010). The *Nox1/AP-1/MMP-2* signal pathway is consistent with previous studies showing that AP-1 and MMP-2 played important roles in melanoma progression (Yamanishi *et al.*, 1991; Yang and Meyskens, 2005; Malaponte *et al.*, 2010); we are actively investigating the interplay between Nox gene family and AP-1 family to understand their interaction in melanomagenesis.

Nox1 regulates colon cancer cell migration through α2/α3 integrins (Sadok *et al.*, 2009); however, it was not clear whether *Nox1* induced morphological changes such as EMT, which enabled cancer cells to adapt to a different morphology and to facilitate migration and invasion. We observed that overexpression of *Nox1* induced an EMT change and knockdown or inhibiting Nox1 led to a reversal of EMT, suggesting that Nox1 induced cell invasion through multiple mechanisms. Although MMPs, in particular MMP-9 and MMP-3, were able to induce EMT in some cells, MMP-2 did not show such ability in Wm3211 melanoma cells (Supplementary Figure S3b online). Hence, the Nox1-induced cell invasion is likely through induction of MMP-2 and EMT independently.

In summary, we compared the expression of *Nox1* in melanocytes and melanoma cell lines. Nox1 protein level and enzymatic activity are both higher in melanoma cells than in NHMs, suggesting that Nox1 may contribute to high ROS levels in melanoma cells. We further demonstrated that *Nox1* overexpression in melanoma cells enhanced cell invasion via two seemingly independent mechanisms: (1) upregulation of *MMP-2* at the transcriptional level, likely through the AP-1 transcription factor; and (2) induction of EMT. Other functions of Nox1 in NHMs and melanoma cells warrant further investigation because of its direct association with superoxide generation and hence modulating the cellular redox status and cell signaling events.

MATERIALS AND METHODS

Cell culture and invasion assay

All melanoma cell culture conditions were the same as described previously (Liu *et al.*, 2009). Normal human melanocytes were isolated from newborn foreskin, followed by a protocol approved by University of California Internal Review Board. Purified MMP-2 protein and GM6001 were purchased from Enzo Life Sciences (Farmingdale, NY). NAC was purchased from Sigma, St Louis, MO. The Matrigel invasion assay was carried out according to the manufacturer's instruction (Becton, Dickinson and Company, Franklin Lakes, NJ).

RT–PCR, quantitative real-time RT–PCR, and chromatin immunoprecipitation

The primers for all genes used in this study are listed in Supplementary Table S1 online. For both PCR and quantitative real-time RT–PCR, β-actin was used as internal control. The relative mRNA level was calculated by ΔΔCt method as described before (Liu *et al.*, 2010). The chromatin immunoprecipitation protocol followed one described previously (Liu and Lee, 2006).

Western blot and zymography assay

A modified protein extraction method and polyacrylamide gel system was used to analyze Nox1 protein. Cells were lysed in RIPA 250 buffer by sonication (without centrifugation) (Liu *et al.*, 2010). A 2 × urea buffer (25 mM Tris, pH 6.8, 12 M urea) was added to cell lysate at 1:1 (V:V) ratio, and placed on ice for 10 minutes. The cell lysate was further mixed with a 2 × urea loading buffer (25 mM Tris, pH 6.8, 8 M urea, 30% glycerol) and loaded to a urea–SDS-PAGE gel (containing 6 M urea) without boiling. Western blot analysis followed a standard procedure. The antibody for Nox1 was H-75, which was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). E-cadherin, N-cadherin, and vimentin antibodies were obtained from

BD Biosciences, San Diego, CA. α-Tubulin antibody was from Sigma. Gelatin-containing gels and developing kits were purchased from Bio-Rad (Hercules, CA). Zymography assay was performed as per the manufacturer's instruction.

DNA constructs, SEAP assay, and luciferase assay

The Nox1 cDNA was purchased from Open Biosystems, Lafayette, CO. The coding region was then PCR amplified (primers: Nox1U and Nox1L) (Supplementary Table S1 online) and cloned into pEGFP-N2 vector via *Eco*RI and *Hind*III sites, resulting in pEGFP-Nox1. The pRS-sh2 and pRS-sh3 were constructed from synthesized DNA oligos sh2A-sh2B and sh3A-sh3B (Supplementary Table S1 online). The MMP-2 promoter was cloned using primers prom2a and prom2b (Supplementary Table S1 online) via *Nhe*I and *Bgl*II into pGL4.10 vector, resulting in pGL4-MMP2. Dual luciferase assay was performed according to the manufacturer's instruction (Promega, Madison, WI). The pAP-1-SEAP was purchased from Clontech (Mountain View, CA) and the SEAP assay was performed using a Great EscAPe SEAP chemiluminescence detection kit from Clontech.

Cell staining and fluorescence microscopy

Cells were fixed in 4% formaldehyde, stained with WGA-conjugated to Texas Red (Molecular Probes, Grand Island, NY) at room temperature for 10 minutes, washed, then permeabilized with saponin, and finally stained with DAPI for 10 minutes. For immunofluorescence, Nox1 antibody was diluted 1:50 and incubated at 4 °C overnight. Fluorescence was viewed under a Zeiss fluorescence microscope.

Nox activity assay and superoxide detection

Nox activity assay was modified based on previous reports (Cui and Douglas, 1997). Briefly, 50 μg of lysate (10 μl) was added into 90 μl of assay buffer and luminescence was read every 4 seconds for 1.5 minutes in an ML 3000 Microtiter Plate Luminometer from Dynatech Laboratories (Chantilly, VA). Same reaction was also carried out in the presence of 10 μM DPI. The difference of reaction velocities (slopes) with or without DPI was calculated and expressed as relative Nox activities. Each reaction was carried out for at least three times. Assay for superoxide followed a previous study (Cai *et al.*, 2007).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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