Reactive Oxygen Generated by NADPH Oxidase 1 (Nox1) Contributes to Cell Invasion by Regulating Matrix Metalloprotease-9 Production and Cell Migration*§

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A mediating role of the reactive oxygen species-generating enzyme Nox1 has been suggested for Ras oncogene transformation phenotypes including anchorage-independent cell growth, augmented angiogenesis, and tumorigenesis. However, little is known about whether Nox1 signaling regulates cell invasiveness. Here, we report that the cell invasion activity was augmented in K-Ras-transformed normal rat kidney cells and attenuated by transfection of Nox1 small interference RNAs (siRNAs) into the cells. Diphenyleneiodonium (DPI) or Nox1 siRNAs blocked up-regulation of matrix metalloprotease-9 at both protein and mRNA levels in K-Ras-transformed normal rat kidney cells. Furthermore, DPI and Nox1 siRNAs inhibited the activation of IKKα kinase and the degradation of IκBα, suppressing the NFκB-dependent matrix metalloprotease-9 promoter activity. Additionally, epidermal growth factor-stimulated migration of CaCO-2 cells was abolished by DPI and Nox1 siRNAs, indicating the requirement of Nox1 activity for the motogenic effect of epidermal growth factor. This Nox1 action was mediated by down-regulation of the Rho activity through the low molecular weight protein-tyrosine phosphatase-p190RhoGAP-dependent mechanism. Taken together, our findings define a mediating role of Nox1-generated reactive oxygen species in cell invasion processes, most notably metalloprotease production and cell motile activity.

The gene family of a flavoprotein (gp91phox) homologs, so-called Nox (Nox1–5 and Duox1 and -2), generates reactive oxygen species (ROS) as a mediator of physiological processes including growth, apoptosis, and inflammation (1). Overproduction of intracellular ROS has been considered as a risk factor in cancer development. In this context it should be noted that aberrant activation of the Nox activity benefits transformation phenotypes of a subset of cancer cells (2); that is, Nox1 in Ras-transformed cells (3), Nox4 in pancreatic cancer (4) and melanoma cells (5), and Nox5 in esophageal adenocarcinoma cells (6). With regard to Nox1, Ras oncogene up-regulated the Nox1 expression by activating GATA-6 through mitogen-activated extracellular signal-regulated kinase (MEK)-extracellular signal-regulated kinase (ERK)-dependent phosphorylation (7). Increased generation of Nox1-derived ROS was functionally required for Ras transformation phenotypes (3), including morphological alteration and decreased cell adhesion (8), vascular endothelial growth factor (VEGF) production and tumor angiogenesis (9), and tumorigenesis (3). In addition, a good correlation between the elevated Nox1 expression and K-Ras activation mutations was observed with both human colorectal cancer specimen and the intestinal epithelium of K-RasVal12 transgenic mice (10), which is consistent with the above-mentioned notion that Nox1 is a key player in Ras oncogene-mediated transformation process.

Tissue invasiveness of transformed cells is thought to be a crucial step to metastatic state. This process is accompanied by enhanced production of matrix metalloproteases (MMPs) and stimulation of cell migration. MMPs degrade extracellular matrix proteins that constitute connective tissues and consist of several isoforms on the basis of the structure and substrate specificity (11). Cell migration is essential for the organization and maintenance of tissue integrity and plays a role in wound healing, inflammation, and invasiveness through extracellular matrix (12). Currently, little is known of how Nox1 signaling directs protease production and cell motogenesis during malignant cell transformation.

Ras-transformed cells are highly metastatic, and Ras oncogene is able to stimulate both matrix metalloprotease production and cell migration (13). Furthermore, the epidermal growth factor (EGF) receptor plays a regulatory role in basal migration of colon cancer cells (14, 15) as well as wound repair of the colonic epithelium (16). Therefore, we addressed the involvement of Nox1 in matrix metalloprotease production and cell invasion by using Ras-transformed cells and EGF-stimulated colon cancer cells as a model system. Our results show that Nox1 mediates RasVal12-induced MMP-9 production via the NFκB signaling pathway and that Nox1 exerts a mediating role in RasVal12- or EGF-dependent cell migration through the
Zymography—The conditioned medium was collected after treatment of cells with drugs and concentrated by an Ultracent-30 (Tokoh). Equal amounts of samples were loaded onto a SDS-PAGE gel containing gelatin (0.5 mg/ml), and the gels were incubated in a solution (50 mM Tris-HCl, pH 8.0, 0.5 mM CaCl₂, 1 mM ZnCl₂, and 1% Triton X-100) for 16 h at 30°C according to a published method (21) with slight modifications. The areas representing for the gelatinolytic activity were visualized by negative staining with Coomassie Brilliant Blue.

Measurement of the Rho Activity—GST-Rho binding domain proteins derived from rhoetokin were prepared as described previously (8). Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, 1 mM dithiothreitol, 1% glycerol, 1 mM phenylmethylsulfonyl fluoride), and lysates were incubated with GST-Rho binding domain-coupled glutathione S-transferase resins for 90 min at 4°C. The active Rho-GTP retained to the resins were analyzed by immunoblotting with anti-Rho antibodies.

5′-Fluorescein Iodoacetamide Labeling—Labeling was performed as described previously (8). Cell lysates were labeled with 10 μM 5′-fluorescein iodoacetamide (Molecular Probes, Eugene, OR) for 90 min at 4°C, and the reaction products were dialyzed against phosphate-buffered saline to remove free 5′-fluorescein iodoacetamide.

Invasion and Migration Assays—The invasion assay was performed by using Matrigel-coated Boyden chambers (BD Biosciences) according to the company’s protocol. Cells (5 × 10⁴) were inoculated into the top of the chamber, and the NRK serum-free-conditioned medium was added to the bottom of the chamber. After 20 h of incubation, the invaded cells were stained with trypan blue and counted. Migration was assayed by using Matrigel-uncoated Boyden chambers (Nalge Nunc, Rochester, NY). Cells (5 × 10⁴) were transfected with indicated vectors, and 24 h later, transfected cells were replated to the chambers in the Dulbecco’s modified Eagle’s medium plus 0.1% bovine serum albumin medium. In some experiments, cells were infected with Ad-HA-LMW-PTPCII25 after transfection as described previously (8). EGF (100 ng/ml) in the same medium was used as a cue. After 48 h of incubation, migrating cells were counted.

RESULTS

Nox1 Mediates Oncogenic Ras-induced Cell Invasion—To evaluate whether Nox1 is involved in cell invasion, we first examined a mediating role of Nox1 in Ras-induced cell invasion. To this end, NRK cells, KNRK cells, and KNRK cells carrying Nox1 siRNAs (N-7), and KNRK cells carrying scrambled siRNAs (Neg-1) were compared for the invasion activity by using the Matrigel invasion assay. As shown in Fig. 1, the number of invading cells was markedly increased in KNRK cells compared with that in NRK cells, and the invasive activity of N-7 cells was reduced compared with that in Neg-1 cells. In addition, Nox1 siRNAs did not influence the low invasion activity of NRK cells. In our previous study, the Nox1 activity was enhanced due to K-RasV12-induced up-regulation of Nox1 in KNRK cells, and silencing of Nox1 siRNAs in KNRK cells suppressed the Nox1 activity (3). Thus, the alteration of cell invasiveness correlates well with that of the Nox1 activity, and these data support the
notion that the Nox1 signaling mediates the activated Ras-induced cell invasion activity. Given that activation of both extracellular matrix protease production and cell motility contribute to increased cell invasiveness, we investigated the role of Nox1 in these two biological processes in the subsequent studies.

Nox1 Mediates Oncogenic Ras-induced MMP-9 Production—It is well documented that MMP-9 and MMP-2 are generated in response to oncogenic activation of Ras (21). To gain insight into the regulatory role of Nox1 in oncogenic Ras-induced metalloprotease production and cell motility contribute to increased cell invasiveness, we investigated the role of Nox1 in these two biological processes in the subsequent studies.

Nox1 Mediates Oncogenic Ras-induced MMP-9 Production—It is well documented that MMP-9 and MMP-2 are generated in response to oncogenic activation of Ras (21). To gain insight into the regulatory role of Nox1 in oncogenic Ras-induced metalloprotease production, we compared NRK cells with KNRK cells for the proteolytic enzyme secretion. Zymographic analysis showed that latent MMP-9 was predominantly secreted and, to a lesser extent, latent MMP-2 in the conditioned medium was isolated from KNRK cells, whereas little or no MMP-9 and MMP-2 were produced in NRK cells (Fig. 2A). Treatment of KNRK cells with a flavoprotein inhibitor, DPI, or anti-oxidants, NAC and vitamin E, resulted in an inhibition of the MMP-9 and MMP-2 activities. In addition, N-7 cells markedly reduced the level of MMP-9 and MMP-2, whereas Neg-1 cells maintained the activities, indicating the involvement of Nox1 in their expression (Fig. 2A). In contrast, the expression of Type I matrix metalloprotease (MT1-MMP) was not affected by Ras activation (data not shown). As MMP-9 was predominantly secreted in KNRK cells, we focused on the regulatory mechanism of MMP-9 expression in the subsequent study. Reverse transcription-PCR analysis was performed to confirm the above results at the mRNA level. The MMP-9 mRNA level was significantly increased in KNRK cells as compared with that in NRK cells (Fig. 2B). Furthermore, DPI and NAC treatment lowered the expression of MMP-9 mRNAs in KNRK cells. The synthesis of MMP-9 mRNAs was consistently suppressed in N-7 cells compared with that in Neg-1 cells (Fig. 2B). Thus, the data indicate that the levels of MMP-9 protein were changed upon Nox1 inhibition, correlating with those of MMP-9 mRNAs and that the regulation of MMP-9 production by Nox1 was through the transcriptional control.

To understand whether the sole activation of the Nox1 activity can turn on the synthesis of MMP-9, NRK cells were cotransfected with Nox1 and its adaptors, NOXO1 and NOXA1, and the conditioned media were subjected to zymography. As shown in Fig. 2A, overexpression of Nox1 and its adaptor proteins stimulated ROS production (see Fig. 4A) and thereby enhanced MMP-9 production (Fig. 2A), indicating that at least the Nox1 activity alone is sufficient for induction of MMP-9. This is in sharp contrast to induction of VEGF that seemingly requires not only Nox1 but also an additional signaling pathway triggered by activated Ras (9).

NFκB Mediates Oncogenic Ras-induced MMP-9 Expression via Nox1—We next wished to dissect the biochemical signaling pathway linking Nox1 to the transcriptional regulatory system for MMP-9. Although the cis-acting element of the MMP-9 promoter encompasses several transcription factor binding motifs (17), we focused on the molecular pathway involved in NFκB-controlled MMP-9 transcription for the following rea-
3). Expression of transfected Mn-SOD was verified by immunoblotting (supplemental Fig. 2). These data collectively indicate that ROS generated by Nox1 is increased due to up-regulation of Nox1 in KNRK cells because our previous study shows that the superoxide generation and NFκB targets of the Ras signaling pathway in Ras-mediated oncogenesis, and NFκB is activated in colon adenocarcinoma cells over-expressing Nox1 (23). A reporter pGL-MMP-9-670 bearing a proximal 670-bp MMP-9 promoter fragment that harbors a NFκB binding site and a NFκB-site-mutated reporter pGL-MMP-9-670kBmt were constructed (supplemental Fig. 1) and transfected into NRK and KNRK cells. The luciferase activity assay showed that the MMP-9 promoter activity was markedly increased in KNRK cells, whereas it remained at the basal level in NRK cells (Fig. 3A). In contrast, mutagenic disruption of the NFκB binding site reduced the promoter activity by 60%, suggesting that NFκB is responsible, at least in part, for oncogenic Ras-induced transcriptional activation of the MMP-9 gene.

To determine whether Nox1 regulates the NFκB-dependent transcriptional activity of the MMP-9 promoter in response to Ras activation, KNRK cells were treated with DPI after transfection with a pGL-MMP-9-670 reporter and subjected to luciferase activity assay. The promoter activity was abrogated by DPI in an incubation time-dependent manner (Fig. 3B). Furthermore, a significant decrease in the MMP-9 promoter activity was detected in N-7 cells as compared with Neg-1 cells (Fig. 3C). As an additional means to demonstrate the regulatory effect of ROS on the MMP-9 promoter activity, we transfected Mn-SOD, a scavenger of superoxide into KNRK cells, together with the pGL-MMP-9-670 reporter. The promoter activity was significantly reduced upon transfection of Mn-SOD (Fig. 3D). Because our previous study shows that the superoxide generation is increased due to up-regulation of Nox1 in KNRK cells (3), these data collectively indicate that ROS generated by Nox1 are required for NFκB-mediated response of the MMP-9 expression to oncogenic activation of Ras. To understand whether up-regulation of the Nox1 activity alone can initiate transcription of MMP-9, NRK cells were co-transfected with Nox1, NOXO1, and NOXA1 and analyzed for the MMP-9 promoter activity assay. Overexpression of Nox1 and its adaptors enhanced superoxide production (Fig. 4A) and thereby induced the activity of a MMP-9-luciferase reporter (Fig. 4B). This correlated with the zymographic assay data (Fig. 2A), suggesting that the Nox1 activity per se may recapitulate the inducing effect of Ras on MMP-9 expression.

To further establish that Nox1 is a mediator of Ras-induced NFκB signaling, we determined whether Nox1 controls the activity of IKKα kinase that phosphorylates IκBα, a key negative regulator of NFκB signaling. IKKα was immunoprecipitated from both NRK and KNRK cells, and the immunoprecipitates were subjected to in vitro kinase activity assay utilizing GST-IκBα as a substrate. The data indicate that the IKKα kinase activity was enhanced upon Ras transformation (Fig. 5A). Furthermore, treatment of KNRK cells with both DPI and NAC inhibited the IKKα activity (Fig. 5B). Similarly, ablation of Nox1 with Nox1 siRNAs blocked the IKKα activity (Fig. 5C). Because phosphorylation of IκBα by IKKα is thought to cause degra-
Nox1 Regulates Cell Migration of Ras-transformed Cells—Oncogenic Ras is also known to potently stimulate cell migration and contribute to tissue invasiveness (25), and as shown in Fig. 1, it is evident that this Ras bioactivity is mediated by Nox1. Our previous study demonstrated that Nox1 mediated oncogenic-Ras-induced disruption of actin stress fiber formation and loss of cell adhesion by inactivating the LMW-PTP-p190RhoGAP-Rho axis (8). To test whether Nox1 regulates cell migration via a similar signaling pathway, the involvement of LMW-PTP in the cell migratory activity was examined. When transfected with the dominant negative LMW-PTP C12S mutant, the migration activity of N-7 cells was readily restored, whereas that of Neg-1 cells was not changed (Fig. 6). This mutant was no longer accessed by Nox1 due to substitution of redox-sensitive cysteine-12 to serine, and hence, it maintained the p190RhoGAP activity, suppressing Rho (8). Thus, the available data support the idea that Nox1 can exert a critical regulatory role on cell migration through LMW-PTP.

Nox1 Down-regulates Rho Activity in CaCO-2 Cells—To further substantiate the involvement of Nox1 in cell motogenesis, we next addressed this Nox1 bioactivity in another biological system, EGF-induced migration of colon cancer cells. We first examined whether Nox1-generated ROS affect the activity of Rho, a key regulator of cytoskeletal contractility. Because overexpression of NOXO1 and NOXA1 (Fig. 7B) is able to increase superoxide production in Nox1-abundant CaCO-2 cells (26), HA-NOXO1 and HA-NOXA1 were co-transfected into CaCO-2 cells, and cell lysates were subjected to the Rho activity assay. Overexpression of NOXO1 and NOXA1 increased ROS production that can be inhibited by DPI and NAC (supplemental Fig. 5). GST-Rho binding domain pulldown assays showed that the Rho activity was significantly down-regulated upon overexpression of NOXO1 and NOXA1 (Fig. 7A). When cells were treated with either DPI or NAC, the suppressive effect of overexpressed NOXO1 and NOXA1 on Rho was removed (Fig. 7A). Furthermore, we found that ablation of Nox1 by Nox1 siRNA prevented inhibition of the Rho activity by ectopic expression of NOXO1 and NOXA1 (Fig. 7B). Transfection of Nox1 siRNAs was demonstrated to effectively decrease the expression level of Nox1 proteins (supplemental Fig. 6). Taken together, these results indicate the suppression of the Rho activity by Nox1-generated ROS in CaCO-2 cells, similar to that in KNRK cells (8).
Reactive Oxygen Generated by Nox1 Contributes to Cell Invasion

Because Rac1 is believed to act as a critical molecular switch for the oxidase activity of Nox1 (27), we next examined whether Rac1 controls the Rho activity through Nox1. Transfection of the dominant active Rac1QL mutant decreased the amount of active Rho-GTP complexes, whereas treatment of Rac1QL-transfected cells with DPI or NAC restored the Rho activity (Fig. 8A). Similarly, silencing of Nox1 by Nox1 siRNAs blocked Rac1QL-induced down-regulation of the Rho activity (Fig. 8B). Moreover, transfection of Rac1QL increased the intracellular ROS level, whereas additional transfection of Nox1 siRNAs prevented Rac1QL-induced stimulation of ROS synthesis (Fig. 8C). This indicates that Rac1 stimulates Nox1-catalyzed ROS production and thereby suppresses the Rho activity. When cells transfected with both Rac1QL and Nox1 siRNAs were treated with 0.5 mM H$_2$O$_2$ for 10 min, lysates were subjected to the Rho activity assay. NAC restored the Rho activity (Fig. 8D) whereas treatment of Rac1QL-transfected cells with DPI or non-receptor tyrosine kinases, which in turn leads to down-regulation of Rac1QL (29). We, therefore, reasoned that the observed negative regulation of Rho by Nox1 upon EGF stimulation could be mediated by EGF-induced activation of p190RhoGAP. Immunoblotting analysis indicated that EGF treatment increased tyrosine phosphorylation of p190RhoGAP, whereas Nox1 siRNAs markedly blocked the stimulatory effect of EGF on p190RhoGAP phosphorylation (Fig. 9B). From these results, it is conceivable that Nox1 mediates the EGF receptor-stimulated activation of p190RhoGAP and subsequently inhibits the Rho activity. To determine whether Nox1 is necessary for EGF-induced migration of cells, CaCO-2 cells were transfected with either Nox1 siRNAs or scrambled siRNAs and subjected to migration assay. Nox1 siRNAs impaired the ability of EGF receptor to stimulate cell migration (Fig. 10A), indicating the mediating role of Nox1 in EGF-induced motogenesis.

Nox1 Mediates EGF-induced Down-regulation of Rho in CaCO-2 Cells—The EGF receptor is expressed on the basolateral membrane of intestinal epithelial cells and plays a mediating role in the augmented migration of colon cancer cells (16). We, therefore, investigated whether the Nox1-Rho axis is engaged in EGF receptor-mediated cell movement. Although EGF treatment of CaCO-2 cells rapidly suppressed the Rho activity, transfection of Nox1 siRNAs antagonized EGF-induced inhibition of Rho (Fig. 9A).

p190RhoGAP is activated after tyrosine phosphorylation by receptor or non-receptor tyrosine kinases, which in turn leads to down-regulation of Rho (29). We, therefore, reasoned that the observed negative regulation of Rho by Nox1 upon EGF stimulation could be mediated by EGF-induced activation of p190RhoGAP. Immunoblotting analysis indicated that EGF treatment increased tyrosine phosphorylation of p190RhoGAP, whereas Nox1 siRNAs markedly blocked the stimulatory effect of EGF on p190RhoGAP phosphorylation (Fig. 9B). From these results, it is conceivable that Nox1 mediates the EGF receptor-stimulated activation of p190RhoGAP and subsequently inhibits the Rho activity. To determine whether Nox1 is necessary for EGF-induced migration of cells, CaCO-2 cells were transfected with either Nox1 siRNAs or scrambled siRNAs and subjected to migration assay. Nox1 siRNAs impaired the ability of EGF receptor to stimulate cell migration (Fig. 10A), indicating the mediating role of Nox1 in EGF-induced motogenesis.

We next investigated whether LMW-PTP acts as a sensor for Nox1-generated ROS that transmits an activation signal to p190RhoGAP. By utilizing the 5'-iodoacetamide fluorescein-labeling approach, in which iodoacetamide derivative competes with intracellular H$_2$O$_2$ in attacking a redox-sensitive cysteine-SH residue (8), we analyzed a Nox1-induced oxidation state of LMW-PTP. When NOXO1 and NOXA1 were co-transfected into CaCO-2 cells, the labeling of exogenously expressed HA-LMW-PTP was suppressed as com-
pared with that in control vector-transfected cells, and apocynin, a Nox inhibitor treatment, removed the suppressive effect (Fig. 10B). The data suggest that the cysteine-SH of LMW-PTP was oxidized by Nox1-generated ROS. Additionally, stimulation of migration with EGF was blocked in Nox1 siRNA-transfected cells, whereas forced expression of the LMW-PTP<sup>C12S</sup> mutant restored EGF-mediated motogenesis in Nox1 siRNA-transfected cells (Fig. 10C). In contrast, overexpression of LMW-PTP<sup>C12S</sup> did not alter the motogenic effect of EGF on scrambled siRNA-transfected cells because endogenous LMW-PTP was inactivated by EGF via Nox1. The data suggest that LMW-PTP mediates EGF receptor-Nox1-dependent motogenesis.

**DISCUSSION**

The ability of tumors to invade the neighboring extracellular matrix is critical for the metastases, which is primarily accompanied by augmented matrix metalloprotease production and cell motility. We show here that Nox1-generated ROS mediates oncogenic Ras-induced MMP-9 production. The mechanism by which Nox1 modulates the expression of this proteolytic enzyme involves, at least in part, the NFκB signaling pathway. Nox1-derived ROS stimulated IKK activity, driving concomitant degradation of IκBα and activation of NFκB, which in turn up-regulated the MMP-9 promoter activity. Given that the interconnection between Ras and Nox1 in regulation of MMP-9 expression was not previously addressed, our finding is significant in that it establishes the role of Nox1 as a mediator of Ras-induced MMP-9 activity. Identification of a putative sensor for Nox1-generated ROS in this process has to await further study. Of note, unlike the induction of VEGF, where the sole activation of the Nox1 system is insufficient (9), the Nox1 signal alone seems to recapitulate the ability of K-RasVal12 to induce the MMP-9 expression. This was not clarified in an earlier report because of the presence of RasVal12 in Nox1-transfected cells (31, 32). One possible explanation for the distinct Nox1 actions is that VEGF induction requires more complex interplay between Nox1 and other components in a Ras signaling network than MMP-9 production. Alternatively, VEGF synthesis may have a higher threshold for induction by ROS than MMP-9 expression. Secretion of latent MMP-2, although to a lesser extent, was also increased upon Ras transformation and suppressed by Nox1 siRNAs. Nox1 may also affect MMP-2 synthesis, but the detailed mechanism remains to be determined in the future study.

**FIGURE 9.** Nox1 mediates EGF-induced down-regulation of Rho. A, CaCO-2 cells were co-transfected with HA-RhoA, scrambled siRNAs, or Nox1 siRNAs and 48 h later stimulated with EGF (100 ng/ml) for 10 min. Lysates were subjected to the Rho activity assay. Expression of transfected Rho was monitored by immunoblotting with anti-HA antibodies. B, CaCO-2 cells were transfected with scrambled siRNAs or Nox1 siRNAs and 48 h later stimulated with EGF (100 ng/ml) for 10 min. Lysates were immunoprecipitated with anti-p190RhoGAP antibodies, and the immunoprecipitates were probed with immunoblotting using anti-phosphotyrosine antibodies or anti-p190RhoGAP antibodies. In A and B, silencing of Nox1 by Nox1 siRNAs was confirmed as described in Supplemental Fig. 5 (data not shown).

**FIGURE 10.** Nox1 mediates EGF-induced cell migration involving LMW-PTP. A, CaCO-2 cells were transfected with scrambled siRNAs or Nox1 siRNAs and 48 h later replated into Matrigel chambers. Cells were then stimulated with EGF (100 ng/ml) for 24 h, and the number of migrated cells was determined. *, p < 0.05 versus scrambled, EGF(-); **, p < 0.05 versus scrambled, EGF(+). B, CaCO-2 cells were transfected with HA-NOXO1, HA-NOXA1, or control (Cont) vectors and 4 h later infected with Ad-HA-wt-LMW-PTP. Lysates were prepared 48 h after infection, and proteins were labeled with 5′-fluorescein iodoacetamide. The labeled HA-wt-LMW-PTP was immunoprecipitated (IP) with anti-fluorescein antibodies (anti-Fluo: Molecular Probe) followed by immunoblotting (IB) with anti-HA antibodies. Expression of transfected proteins was monitored by immunoblotting. Ectopic expression of HA-LMW-PTP<sup>C12S</sup> was monitored by immunoblotting. C, CaCO-2 cells were transfected with scrambled siRNAs or Nox1 siRNAs, 4 h later infected with either Ad-HA-LMW-PTP<sup>C12S</sup> or control virus, and 48 h later subjected to cell migration assay. *, p < 0.02 and #, p > 0.05 versus scrambled, control; **, p < 0.05 versus Nox1 siRNA, control.
The potential involvement of Nox enzymes in MMP-9 expression has been reported in other systems as well; Nox1 in doxorubicin-treated cardiac myocytes (30) and Nox2 (via induction of p47phox and p67phox) in tumor promoter agent-stimulated keratinocytes (33). Thus, these observations together with ours suggest that Nox oxidase-based ROS plays a pivotal role in the regulation of MMP-9 production involved in a variety of biological processes ranging from tumor invasion to tissue remodeling.

We also found that Nox1 signals the cell migratory activity in both Ras-transformed cells and EGF-stimulated colon adenocarcinoma cells via a similar signaling pathway despite distinct biological systems. Our previous data suggested that Nox1-generated H₂O₂ oxidized and inactivated LMW-PTP, which caused inhibition of the Rho activity through activation of p190RhoGAP, possibly leading to disassembly of actin stress fibers and loss of focal adhesion (8). The present observation indicates that this Nox1 signaling linked to Rho is also involved in cell motility of KNRK cells as well as EGF-stimulated CaCO-2 cells, implicating its wide role in cytoskeletal rearrangements. In particular, in light of frequent overexpression of RhoGTPase signaling, and its relevance to our study is unclear (36). However, this study did not explore the involvement of NOX1 in cell migration.

In summary, our study revealed a sequence of events involved in Nox1-mediated cancer cell invasiveness: matrix metalloprotease production and cell migration. Given that tumor progression to the metastatic phenotype largely relies on invasiveness of tumor cells, inhibition of Nox1 may provide a pharmacological means to intervene in cancer progression.

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