

REACTIVE OXYGEN GENERATED BY NADPH OXIDASE (NOX) 1 REGULATES MATRIX METALLOPROTEASE-9 PRODUCTION AND CELL MIGRATION

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A mediating role of the reactive oxygen species (ROS)-generating enzyme Nox1 has been suggested for Ras oncogene transformation phenotypes including anchorage-independent cell growth, augmented angiogenesis, and tumorigenesis. But, little is known about whether Nox1 signaling regulates cell invasiveness. Here, we report that abrogation of Nox1-derived ROS by diphenylene iodonium (DPI) or Nox1 small-interference RNAs (siRNAs) blocked upregulation of matrix metalloprotease (MMP)-9 at both protein and mRNA levels in K-Ras-transformed normal rat kidney (KNRK) cells. Furthermore, DPI and Nox1 siRNAs inhibited the activation of IKK α kinase and the degradation of I κ B α , attenuating the NF κ B-dependent MMP-9 promoter activity. These Nox1 inhibitory agents also decreased the invasiveness of KNRK cells in matrigel assay. Additionally, epidermal growth factor (EGF) stimulated-migration of CaCO-2 cells was abolished by DPI and Nox1 siRNAs, indicating the requirement of Nox1 activity for the motogenic effect of EGF. This Nox1 action was mediated by down-regulation of the Rho activity through the low molecular weight protein tyrosine phosphatase (LMW-PTP)-p190RhoGAP-dependent mechanism. Taken together, our findings define a mediating role of Nox1-generated ROS 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) generate 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): 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 upregulated 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 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 MMP-9 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 repairing of the colonic epithelium (16). Therefore, we addressed the involvement of Nox1 in MMP-9 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 NF κ B signaling pathway, and that Nox1 exerts a mediating role in RasVal12- or EGF-dependent cell migration through the LMW-PTP-p190 RhoGAP-Rho signaling pathway. Thus, Nox1 could be a critical component of the regulatory machinery for cell invasiveness associated with tumor progression.

Experimental procedures

Cell Culture and Materials- Normal rat kidney fibroblast (NRK) cells, Kirstein-Ras-transformed NRK (KNRK) cells, and CaCO-2 cells were purchased from American Type Culture Collection (Manassas, VA). KNRK cell lines which stably expressed scrambled siRNA (Neg-1) and Nox1 siRNA (N-7) were described in the previous study (3). Rabbit anti-IKK α antibodies were purchased from Cell Signaling Technology (Beverly, MA) and Rabbit anti-RhoA antibodies from UBI (Lake Placid, NY). Rabbit anti-I κ B α antibodies and GST-I κ B α (1-100) were gifts from Dr. N. Rice and Dr. H. Nakano, respectively. Diphenylene iodonium (DPI)

was purchased from CalBiochem (La Jolla, CA) and EGF, mouse anti-HA antibodies, N-acetyl-L-Cysteine (NAC), and vitamin E from Sigma-Aldrich (Milwaukee, WI). Rabbit anti-Nox1 antibodies were used as described previously (4). pSilencer-human Nox1 siRNAs were constructed as described previously (4).

Plasmids- The NheI/NcoI fragment (nt-670 to +22) of the human MMP-9 promoter (17) was inserted into the reporter plasmid pGL-3 Basic (Promega, Madison, WI) to generate pGL-MMP-9-670. pGL-MMP-9-670kBmt (the NF- κ B site AGTGGAATCCCCA is mutated to AGTTCTCGAGCCCCA) was constructed by PCR-based mutagenesis (7). HA-Rac1Q61L cDNA was subcloned into pEFBOS. pHBMnSOD was provided by Dr. K Hirose (18) and pcDNA3.0-Nox1, pEFBOS-HA-NOXO1, and pEFBOS-HA-NOXA1 by Dr. H. Sumimoto. Adenoviral construct (Ad-HA-LMW-PTP^{C12S}) was described previously (19).

Immunoprecipitation and immunoblotting- Cells were lysed in RIPA buffer (8) unless specified, and lysates were subjected to immunoblotting or immunoprecipitation as described (8).

Transfection- Transfection was performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells ($5 \times 10^5 \sim 10^6$) were transfected with 2~4 μ g of the indicated plasmid DNAs.

Luciferase Assay- Cells were transfected with 2 μ g of luciferase reporters and 2 μ g of various expression vectors with and without drug treatments. Cells were lysed after 48h and the promoter activity was assayed as described previously (7).

IKK Assay- The activity of IKK was measured as described previously (20). The cells were treated with chemical inhibitors for 6h and lysed for 30min. The lysates were immunoprecipitated with anti-IKK α antibodies and the immunoprecipitates were incubated with 1 μ g of GST-I κ B α (1-100) and

370 kBq of [γ - 32 P] ATP(110 TBq/mmol: GE Healthcare Bio-Sciences) in kinase buffer for 1h at 30°C. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), followed by autoradiography.

RT-PCR- Total RNAs were extracted from cells by using Trizol (Invitrogen). Following reverse transcription, PCR for MMP-9 was performed by using the primers (forward 5'-CGGTCGGTATTGGAAGTTCTCG-3' and reverse 5'-GCTGAAGCAAAAGAGGAGCCTTA-3') as described previously (7).

Zymography- The conditioned medium was collected after treatment of cells with drugs and concentrated by an ULTRACENT-30 (TOSOH, Tokyo). The samples were loaded onto a SDS-PAGE gel containing gelatin (0.5mg/ml) and the gels were incubated in a solution (50mM Tris-HCl pH8.0, 0.5mM CaCl₂, 1 μ M ZnCl₂, and 1% TritonX-100) for 16h at 30°C, according to the 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 (RBD) proteins derived from rhotekin were prepared as described previously (8). Cells were lysed in lysis buffer (50mM Tris-HCl, PH7.2, 100mM NaCl, 5mM MgCl₂, 1% NP-40, 1mM dithiothreitol, 1% glycerol, 1mM phenylmethylsulfonyl fluoride) and lysates were incubated with GST-RBD-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 μ M 5'-fluorescein iodoacetamide (5'-IAF) (Molecular Probes, Eugene, OR) for 90min at 4°C and the reaction products were dialyzed against PBS to remove free 5'-IAF.

Invasion and Migration Assays -The invasion assay was performed by using matrigel-coated Boyden chambers (BD Biosciences, San Jose, CA) according to the company's protocol. Cells (5×10^5) were inoculated into the top of the chamber and the NRK serum-free conditioned medium was added to the bottom of the chamber. After 20h 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 (5×10^5) were transfected with indicated vectors and 24h later, transfected cells were replated to the chambers in the DMEM plus 0.1% BSA medium. In some experiments, cells were infected with Ad-HA-LMWPTP^{C125} following transfection as described previously (8). EGF (100ng/ml) in the same medium was used as a cue. After 48h 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 the invasion activity of K-RasVal12-transformed normal rat kidney (KNRK) cells. To this end, KNRK cells carrying Nox1 siRNAs (N-7) and KNRK cells carrying scrambled siRNAs (Neg-1) were analyzed in the matrigel invasion assay. We previously established these cell lines and demonstrated that the Nox1 activity was elevated due to K-RasVal12 induced-upregulation of Nox1 in Neg-1 cells, whereas the Nox1 activity was suppressed by Nox1 siRNAs in N-7 cells (3). Neg-1 cells exhibited a 5-fold increase in the number of invading cells as compared with normal rat kidney (NRK) cells (Fig. 1). In contrast, the invasive activity of N-7 cells was significantly abrogated. Thus, 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 is a major matrix metalloprotease 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 MMP-9 production. Zymographic analysis showed increased amount of MMP-9 in conditioned media isolated from KNRK cells as compared with that in NRK cells (Fig. 2A). Treatment of KNRK cells with a flavoprotein inhibitor, DPI, or anti-oxidants, NAC or vitamin E resulted in an inhibition of the MMP-9 activity. In addition, N-7 cells exhibited markedly reduced level of the MMP-9 activity, while Neg-1 cells maintained the activity (Fig. 2A). Thus, we conclude that activated Ras-dependent induction of MMP-9 production requires the Nox1 activity. RT-PCR analysis was performed in order to confirm the above results at the mRNA level. 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 co-transfected 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 (Fig. 4A) and thereby enhanced MMP-9 production (Fig. 2B), indicating that 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. Because our data demonstrated that Nox1 controlled K-RasVal12-induced MMP-9 expression at the transcriptional level, we 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 because of the following reasons. Both NFκB (22) and Nox1 (3,7) are critical downstream targets of the Ras signaling pathway in Ras-mediated oncogenesis, and NFκB is activated in colon adenocarcinoma cells overexpressing Nox1 (23). A reporter pGL-MMP-9-670 bearing a proximal 670bp MMP-9 promoter fragment that harbors a NFκB binding site and a NFκB-site-mutated reporter pGL-MMP-9-670κBmt were constructed (Supplementary 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 following 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 MnSOD, a scavenger of superoxide into KNRK cells together with the pGL-MMP-9-670 reporter. The promoter activity was significantly reduced upon transfection of MnSOD (Fig. 3D). Since our previous study shows that the superoxide

generation is increased due to upregulation of Nox1 in KNRK cells (3), these data collectively indicate that ROS generated by Nox1 are required for NF κ B-mediated response of the MMP-9 expression to oncogenic activation of Ras. To understand whether upregulation 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 NF κ B signaling, we determined whether Nox1 controls the activity of IKK α kinase that phosphorylates I κ B α , a key negative regulator of NF κ B signaling. IKK α was immunoprecipitated from both NRK and KNRK cells and the immunoprecipitates were subjected to *in vitro* kinase activity assay utilizing GST-I κ B α as a substrate. The data indicate that the IKK α kinase activity was enhanced upon Ras transformation (Fig. 5A). Furthermore, treatment of KNRK cells with both DPI and NAC inhibited the IKK α activity (Fig. 5B). Similarly, ablation of Nox1 with Nox1 siRNAs blocked the IKK α activity (Fig. 5C). Because phosphorylation of I κ B α by IKK α is thought to cause degradation of I κ B α , resulting in NF κ B activation (24), the levels of I κ B α expression were examined. Immunoblotting analysis showed that the amount of I κ B α protein was decreased in KNRK cells (Fig. 5D). In contrast, the normal I κ B α level was regained after KNRK cells were exposed to DPI for 6h (Fig. 5E). Simultaneously, the expression of I κ B α was increased in N-7 cells compared with that in Neg-1 cells (Fig. 5F). Taken together, these results suggest that the signal initiated by Nox1-derived ROS induces the IKK α activation, leading to preferential degradation of I κ B α .

Nox1 regulates cell migration of Ras transformed cells. Oncogenic Ras is also known to potently stimulate cell migration, contributing 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 in KNRK cells, upregulated Nox1 inactivated LMW-PTP, a negative regulator of p190RhoGAP through oxidation of redox-sensitive cysteine-12 (8). This subsequently sustained the p190RhoGAP activity and thereby suppressed the Rho activity, leading to loss of both focal contacts and actin stress fiber formation (8). Since control of actin filament dynamics is essential for cell movement, Nox1-regulated cell motility could also be mediated by the above Nox1-LMW-PTP redox signaling linking to the Rho pathway. Indeed, as shown in Fig. 6A, the migration activity of N-7 cells was readily restored when transfected with the dominant negative LMW-PTP^{C12S} mutant. This mutant was no longer accessed by Nox1 due to substitution of cysteine-12 to serine and hence maintained the p190RhoGAP activity, resulting in suppression of Rho (8). Thus, the available data support the idea that Nox1 can exert a critical regulatory role on cell migration.

Nox1 down-regulates the 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. Since overexpression of NOXO1 and NOXA1 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 (Supplementary Fig. 5). GST-RBD pull down 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 (Supplementary 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).

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 exposed to H₂O₂, the Rho activity was reduced, implicating that exogenously added H₂O₂ mimics the repressive effect of Nox1-generated ROS on Rho (Fig.8B). Collectively, these results support the notion that Nox1-derived ROS signals down-regulation of the Rho activity in CaCO-2 cells.

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 basal migration of colon cancer cells (16). Because the Rho GTPase regulates the motogenic factor-induced cell migration by balancing the opposing effects of cell body contraction and adhesion (28), we investigated whether the

Nox1-Rho axis is engaged in the EGF receptor-mediated cell movement. While EGF treatment of CaCO-2 cells rapidly suppressed the Rho activity, transfection of Nox1 siRNA antagonized EGF-induced inhibition of Rho (Fig. 9A).

p190RhoGAP is activated following 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 using Boyden chambers. Nox1 siRNAs impaired the ability of EGF receptor to stimulate cell migration (Fig.6B), 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 H₂O₂ for attacking a redox-sensitive cysteine-SH residue (8), we analyzed 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 compared with that in control vector-transfected cells and apocynin, a Nox inhibitor treatment removed the suppressive effect (Fig. 6C). 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^{C12S} mutant restored EGF-mediated motogenesis in Nox1 siRNA-transfected cells (Fig. 6D). In contrast, overexpression of LMW-PTP^{C12S} 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 NF κ B signaling pathway. Nox1-derived ROS stimulated the IKK α activity, driving concomitant degradation of I κ B α and activation of NF κ B, which in turn upregulated 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 due to the presence of RasVal12 in Nox1-transfected cells (31, 32). One possible explanation for 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.

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 p47^{phox} and p67^{phox}) 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 the 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 the EGF receptor in colon cancer (34), identification of Nox1 as a signaling component of EGF-regulated motility has significance in understanding both the biology and pathology of colon cancer. Previously, the direct migration-inducing activity of H₂O₂ was suggested for neutrophils (35). H₂O₂ may trigger cell migration by diffusing into cytoplasm and modulating intracellular redox-sensitive proteins. This view could be supported by our current finding. Recently, Nox1 activation by arachidonic acid through 12-lipoxygenase and protein kinase C δ was also reported to augment migration of CaCO-2 cells (36). However, this study did not explore the involvement of the RhoGTPase signaling and its relevance to our study is unclear at present.

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 cancer progression.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Inhibition of Nox1 suppresses oncogenic Ras-induced cell invasion. NRK, Neg-1, and N-7 cells were subjected to cell invasion assays as described in Materials and Methods. Histograms show the number of invading cells (means \pm S.D. n=3).

Fig. 2. Nox1 mediates oncogenic Ras-induced MMP-9 production. **A.** KNRK cells that had been treated with 10mM NAC, 10 μ M DPI, and Vit E for 24h (*left*), Neg-1 and N-7 cells (*middle*) or NRK cells that had been transfected with a mixture of pcDNA3.0-Nox1, pEF-BOS-HA-NOXO1, and pEF-BOS-HA-NOXA1 or control vectors (*right*) were subjected to be the MMP-9 activity assay using zymography. Histograms show the intensity of visualized bands. **B.** Total RNAs were extracted from NRK, Neg-1, or N-7 cells and additionally from KNRK cells treated with 10 μ M DPI or 10mM NAC for 2h. The levels of MMP-9 mRNAs were quantified by RT-PCR. GAPDH was used as a loading control.

Fig. 3. Nox1 is required for the Ras-induced NF κ B-dependent MMP-9 promoter activity. **A.** NRK and KNRK cells were transfected with reporter constructs and luciferase assay was performed 48h later. The data indicate means \pm S.E. (n=3). **B.** KNRK cells were transfected with pGL-MMP-9-670, 48h later treated with DPI for indicated time intervals, and subjected to luciferase assay. The data indicate means \pm S.E. (n=3). **C.** Neg-1 and N-7 cells were transfected with pGL-MMP-9-670 and luciferase assay was performed 48h later. The data indicate means \pm S.E. (n=3). **D.** KNRK cells were transfected with pGL-MMP-9-670 together with either pHB or pHBMnSOD and luciferase assay was performed. The data indicate means \pm S.E. (n=3). Expression of transfected MnSOD was verified by immunoblotting (Supplementary Fig. 2).

Fig. 4. Augmented Nox1 signaling stimulates the NF κ B-dependent MMP-9 promoter activity. NRK cells were transfected with control vectors or a mixture of pcDNA3.0-Nox1, pEF-BOS-HA-NOXO1, and pEF-BOS-HA-NOXA1. Luciferase assay (**B**) and luminol assay (**A**) were performed 48h after transfection. The data indicate means \pm S.E. (n=3). Expression of transfected proteins was verified by immunoblotting (Supplementary Fig 3).

Fig. 5. Nox1 mediates Ras-induced activation of IKK α and degradation of I κ B α . **A.** GST-I κ B α (1-100) proteins were expressed and purified (Supplementary Fig. 4). Cell lysates prepared from NRK and KNRK cells were immunoprecipitated with anti-IKK α antibodies, the immunoprecipitates were incubated with GST-I κ B α (1-100) and [γ -³²P] ATP, and the reaction products were analyzed by SDS-PAGE, followed by autoradiography. **B.** KNRK cells were lysed

following treatment with either 10 μ M DPI or 10mM NAC for 6h. Cell lysates were subjected to immunoprecipitations and the kinase activity assay as in **A**. **C**. Cell lysates prepared from Neg-1 and N-7 cells were subjected to immunoprecipitations and the kinase activity assay as in **A**. Through **A–C**, the amounts of IKK α in the immunoprecipitates were examined by immunoblotting. **D**. The levels of I κ B α in NRK and KNRK cells were determined by immunoblotting with anti-I κ B α antibodies. **E**. KNRK cells were untreated or treated with 10 μ M DPI for 6h and the levels of I κ B α were analyzed as in **D**. **F**. The levels of I κ B α in Neg-1 and N-7 cells were determined by immunoblotting as in **D**. Through **D–F**, β - actin was used as a loading control.

Fig. 6. Nox1 regulates the cell migration activity involving LMW-PTP. **A**. LMW-PTP, downstream of Nox1, mediates cell migration of KNRK cells. Neg-1 and N-7 cells were infected with Ad-HA-LMW-PTP^{C12S} for 24h and subjected to cell migration assay. The data represent means \pm S.D. (n=3). P* <0.05 and P** and P⁺>0.5 (statistically not significant) versus control, LMW-PTP^{C12S}, respectively. **B**. Nox1 mediates EGF-induced cell migration. CaCO-2 cells were transfected with scrambled siRNAs or Nox1 siRNAs and 48h later, replated into matrigel chambers. Cells were then stimulated with EGF (100ng/ml) for 24h and the number of migrated cells was determined. The data represent means \pm S.D. (n=3). P* <0.05 versus scrambled, EGF(-) and p** <0.05 versus scrambled, EGF(+). **C**. CaCO-2 cells were transfected with HA-NOXO1, HA-NOXA1, or control vectors and 4h later infected with Ad-HA-wt-LMW-PTP. Lysates were prepared 48h after infection and proteins were labeled with 5' -IAF. The labeled HA-wt-LMW-PTP was immunoprecipitated with anti-Fluorescein antibodies (anti-Fluo: Molecular Probe), followed by immunoblotting with anti-HA antibodies. Expression of transfected proteins was monitored by immunoblotting. **D**. LMW-PTP mediates EGF-induced cell migration of CaCO-2 cells. CaCO-2 cells were transfected with scrambled siRNAs or Nox1 siRNAs, 4h later infected with either Ad-HA-LMW-PTP^{C12S} or control virus, and 48h later subjected to cell migration assay as in **B**. The data represent means \pm S.D. (n=3). P* <0.05 and P⁺>0.5 (statistically not significant) versus scrambled, control. P** <0.05 versus Nox1 siRNA, control. In **A** and **C**, ectopic expression of HA-LMW-PTP^{C12S} was monitored by immunoblotting.

Fig. 7. Nox1-derived ROS downregulate the Rho activity. **A**. CaCO-2 cells were co-transfected with HA-NOXO1, HA-NOXA1, or control vectors and 48h later, treated with 10 μ M DPI for 2h or 10mM NAC for 30 min. Cell lysates were subjected to the Rho activity assay. **B**. CaCO-2 cells were transfected with HA-RhoA, HA-NOXO1, HA-NOXA1, scrambled siRNAs and Nox1 siRNAs and cell lysates were prepared. The Rho activity assay was performed. Suppression of Nox1 by Nox1 siRNAs was confirmed (Supplementary Fig. 6). In **A** and **B**, ectopic expression of transfected proteins was monitored by immunoblotting with anti-RhoA or anti-HA antibodies.

Fig. 8. Rac1 suppresses the Rho activity through a Nox1-dependent manner. **A**. CaCO-2 cells were co-transfected with HA-RhoA, Rac1QL, or control vectors and 48h later, treated with 10 μ M DPI for 2h or 10mM NAC for 30min. Cell lysates were subjected to the Rho activity assay. **B**. CaCO-2 cells were co-transfected with HA-RhoA, Rac1QL, scrambled siRNAs or Nox1 siRNAs and lysed. In some experiments, cells were co-transfected with Rac1QL and Nox1 siRNAs and 48h later, treated with 0.5mM H₂O₂ for 10min. Lysates were subjected to the Rho activity assay. **C**. CaCO-2 cells were co-transfected with Rac1QL, control vectors, scrambled siRNAs, or Nox1 siRNAs. ROS production was measured as described. The data represent means \pm S.D. (n=3). Through **A–C**, ectopic expressions of transfected proteins were monitored by immunoblotting. Silencing of Nox1 by Nox1 siRNAs was confirmed as described in Supplementary Fig. 5 (data not shown).

Fig. 9. Nox1 mediates EGF-induced downregulation of Rho. **A**. CaCO-2 cells were co-transfected with HA-RhoA, scrambled siRNAs or Nox1 siRNAs and 48h later, stimulated with

EGF (100ng/ml) for 10min. 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 48hr later, stimulated with EGF (100ng/ml) for 10min. 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 Supplementary Fig. 5 (data not shown).

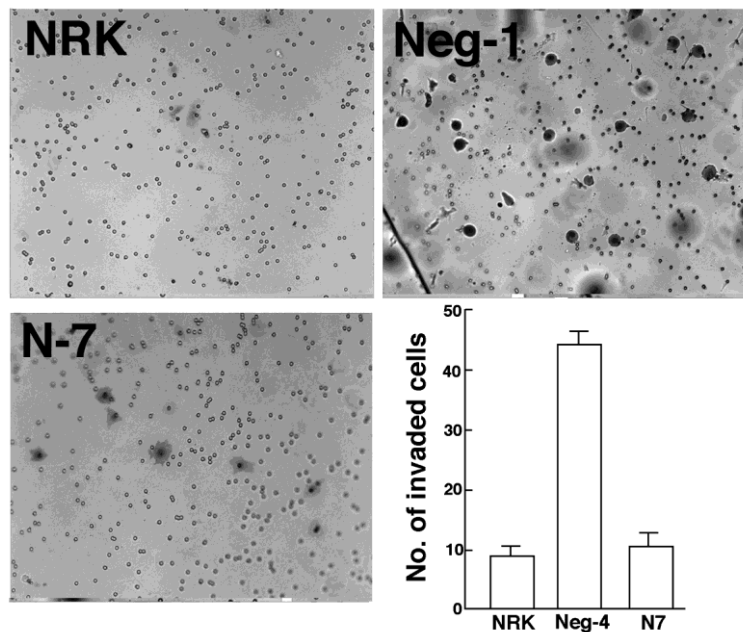


Fig.1

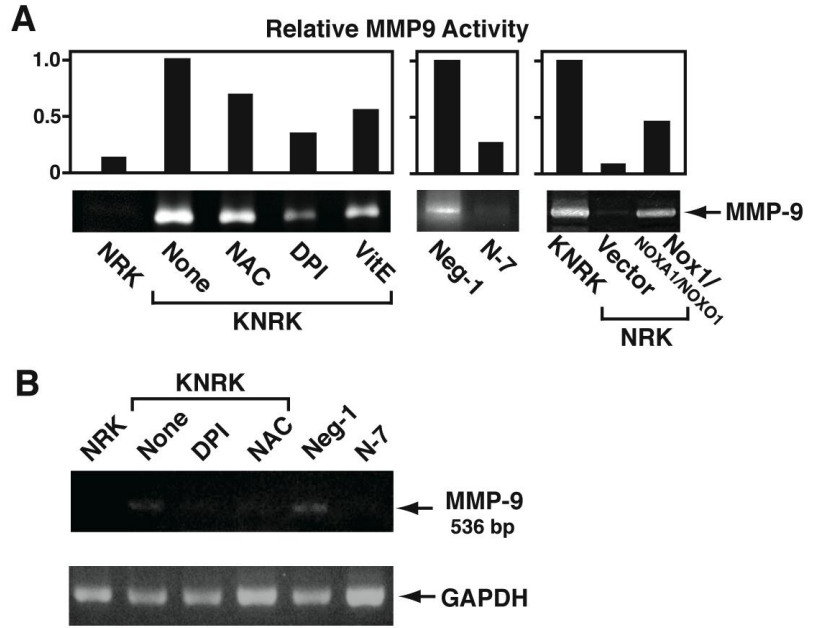


Fig. 2

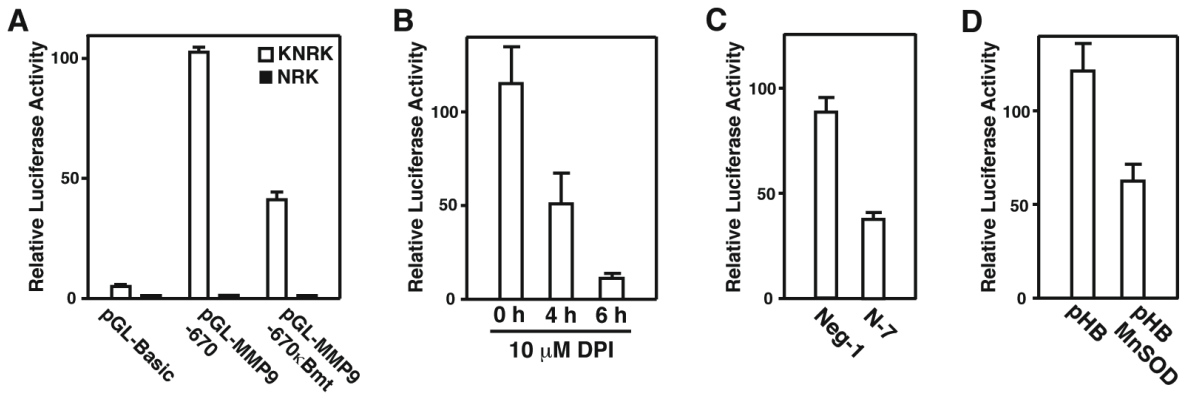


Fig. 3

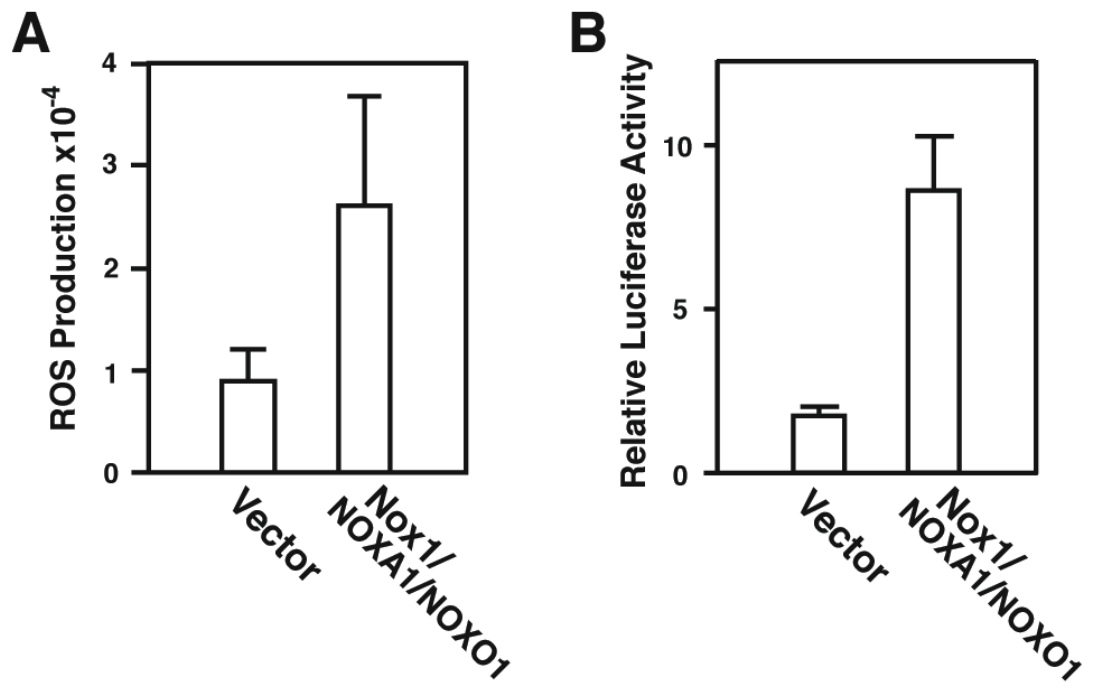


Fig. 4

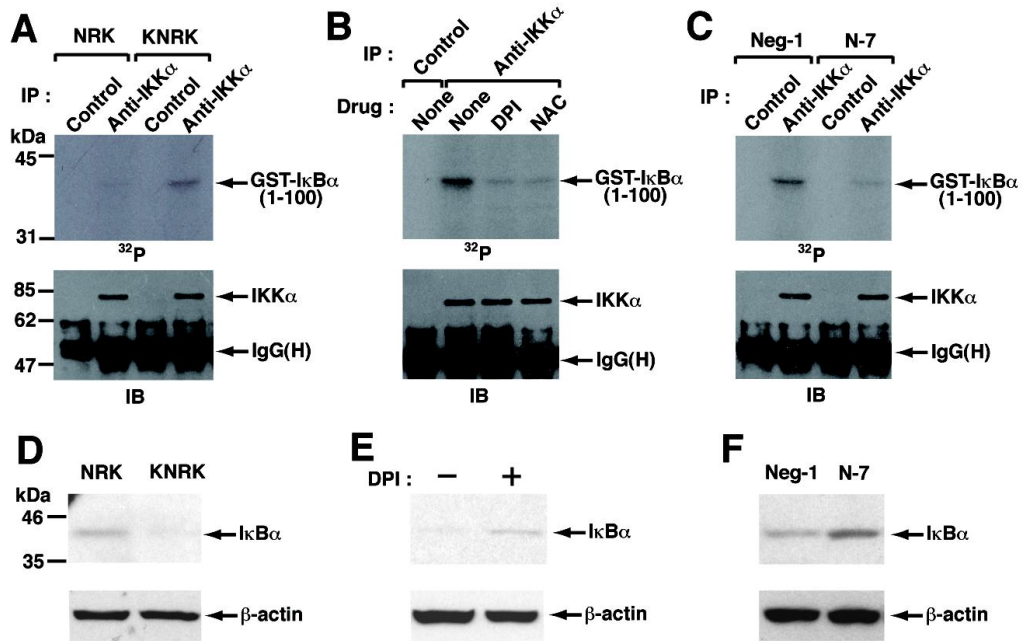


Fig. 5

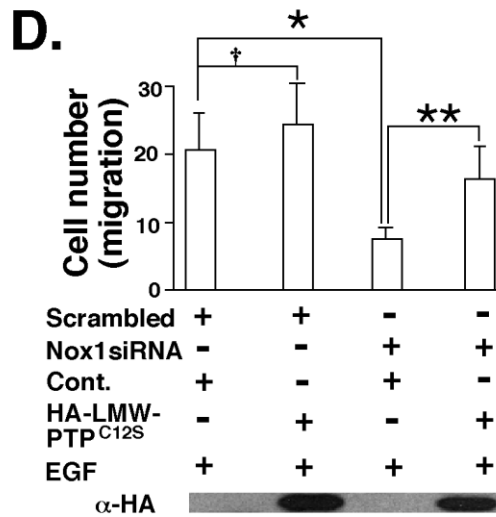
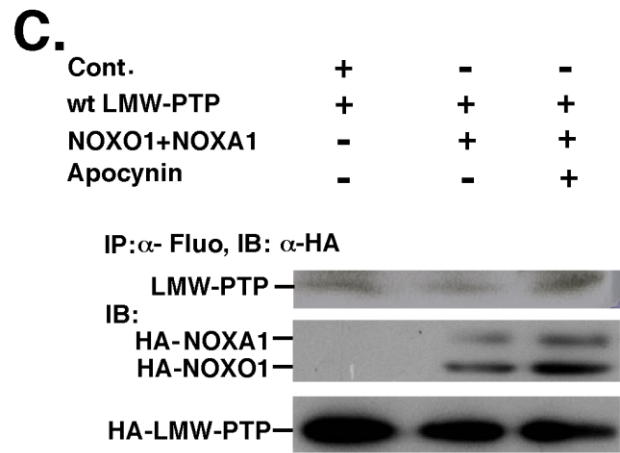
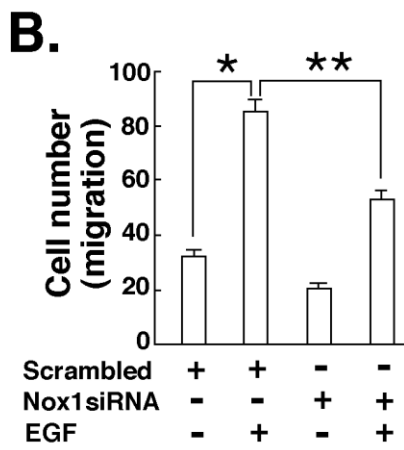
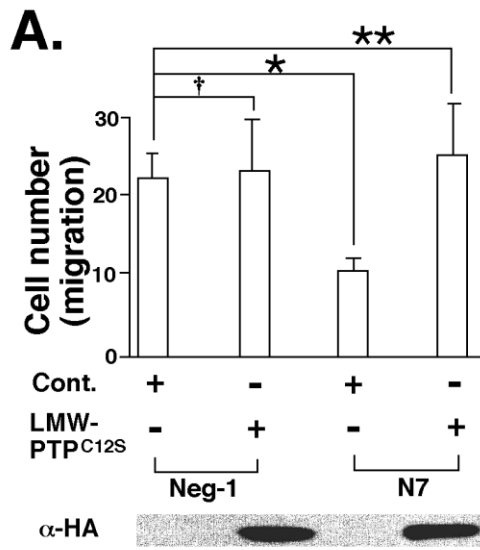


Fig.6

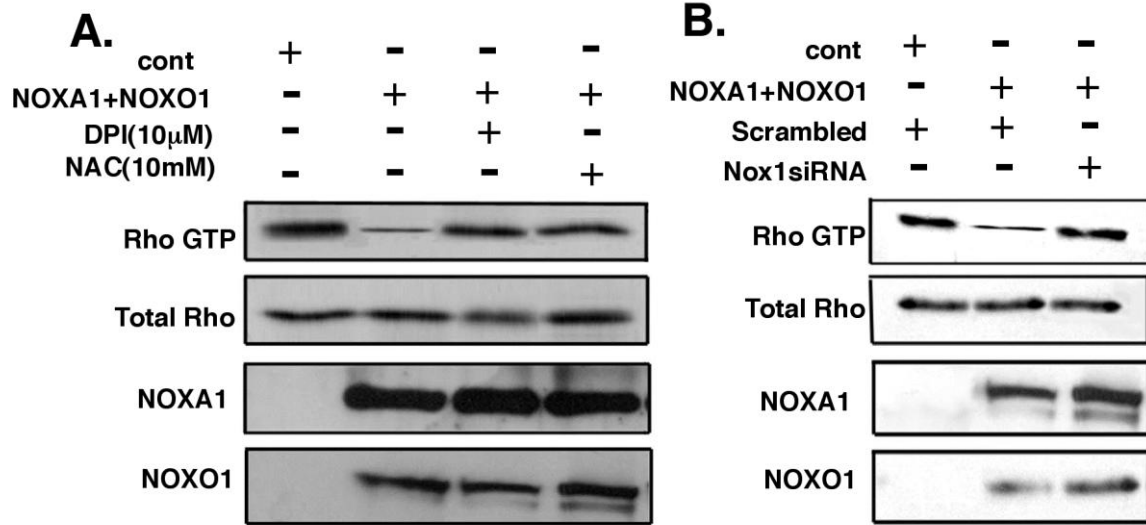
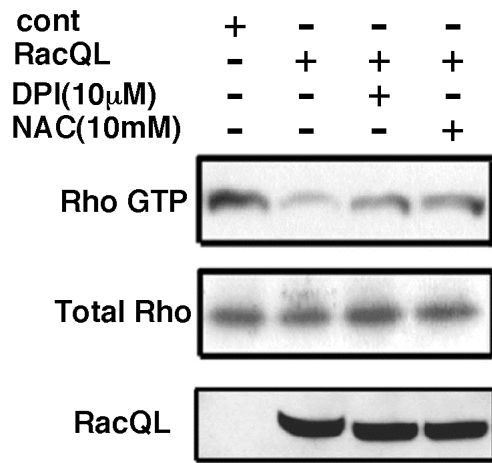
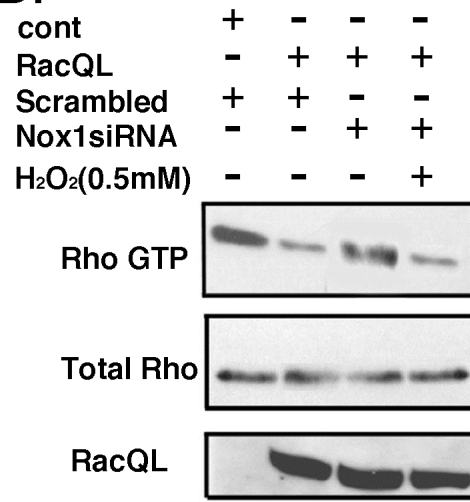


Fig.7

A.



B.



C.

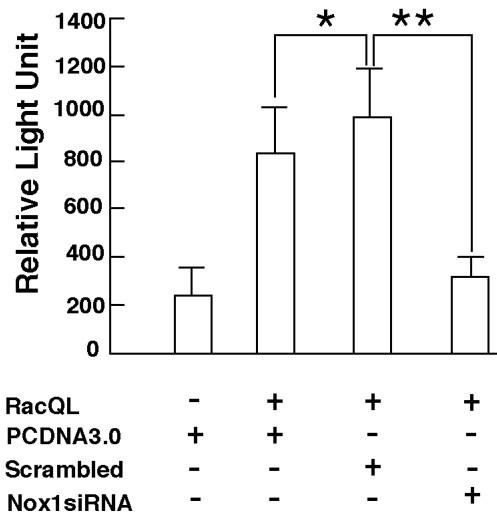


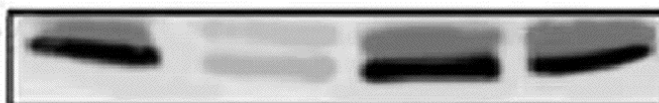
Fig.8

Fig.9

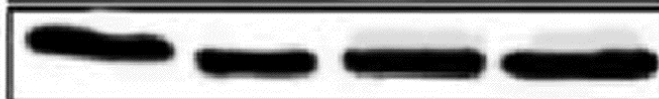
A.

EGF	-	+	-	+
Scrambled	+	+	-	-
Nox1siRNA	-	-	+	+

Rho GTP



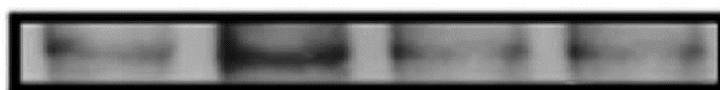
Total Rho



B.

EGF	-	+	-	+
Scrambled	+	+	-	-
Nox1siRNA	-	-	+	+

phospho-
p190RhoGAP



p190RhoGAP

