Nox4-generated ROS Regulate TGF-β1-induced Motility of Colon Cancer Cells through the Low Molecular Weight Protein Tyrosine Phosphatase-Rho Signaling Pathway

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We investigated the role of NADPH oxidase (Nox) 4 in colon cancer development. The expression of Nox4, unlike Nox1, was specifically induced in colon carcinoma and was quite similar to that of transforming growth factor-β (TGF-β)1. TGF-β1 treatment of colon cancer cells upregulated the Nox4 expression and ROS production through SMAD3. Knockdown of Nox4 suppressed TGF-β1-induced cell motility. Nox4 modulated the Rho activity through redox regulation of the low molecular weight protein tyrosine phosphatase (LMW-PTP)-p190RhoGAP pathway upon TGF-β1 stimulation. These finding suggests that Nox4 contributes to TGF-β1-dependent motility of colon carcinoma through the LMW-PTP-p190RhoGAP-Rho signaling pathway. *Shinshu Med J 63 : 281—293, 2015*

I Introduction

The NADPH oxidase (Nox) family represent major cellular sources of ROS for signaling purposes and consist of Nox1~5 and Duox1 and 2[1]. Among them, H_2O_2-generating Nox4 is localized to the membrane of intracellular compartments such as the endoplasmic reticulum, mitochondria, and perinuclear regions and is expressed in a variety of tissues[2]. While it exerts mediating roles in many physiological processes including vascularization and glucose metabolism, aberrant control of Nox4-derived ROS has been implicated in human diseases including carcinogenesis[3]. For example, Nox4-mediated ROS formation contributes to proliferation of melanoma cells[4], motility of breast cancer cells[5], and migration of glioblastoma cells[6]. With regard to the relationships of Nox isoforms with colon cancer, Nox1 has been extensively studied. Although Nox1 was shown to be upregulated in colon cancer cells[7)8)] and to mediate their growth[9], the functional relevance to colon carcinogenesis still remains under debate[8)10)]. In contrast to Nox1 which is abundant in the intestine, Nox4 is not expressed in the normal colon but is expressed to some extent in a subset of colon cancer cell lines including CaCO2 and SW620[11)12)]. Given that Nox4 is a transcriptionally controlled constitutive enzyme, altered expression of Nox4 may have significant implications in carcinogenesis. In this regard, a recent finding is noteworthy for demonstrating that Nox4 is upregulated in rat colon tumors induced by a heterocyclic amine carcinogen[13]. More recently, the

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elevated expression of Nox4 was detected in association with relapse in stage II-colon cancer[12]. These observations raise the possibility that Nox4 might participate in some steps of colon cancer progression, but the detailed mechanism of Nox4 action has not been defined.

In this study, we evaluated the protein level of Nox4 in human colon neoplasms with various grade of differentiation and investigated the Nox4 signaling pathway involved in the colon cancer development. Our results for the first time demonstrated that Nox4 overexpression, distinct from Nox1, was induced in adenocarcinomas, and that Nox4-generated ROS mediated TGF-β1-induced motility of colon cancer cells by controlling the LMW-PTP-Rho axis.

II Materials and Methods

A Cell lines and materials

HCT-116 and RKO cells were obtained from American Type Culture Collection (Manassas, VA, USA). TGF-β1 was purchased from R & D Systems (Minneapolis, MN, USA), N-acetylcysteine (NAC) from Calbiochem (LaJolla, CA, USA), BIAM from Molecular Probes (Eugene, OR, USA), rabbit anti-Nox1 antibodies from Abcam (Cambridge, MA, USA), rabbit anti-Nox4 antibodies (#58851) from Novus (Littleton, CO, USA), rabbit anti-TGF-β1 antibodies from BioVision (Mountain View, CA, USA), mouse anti-phospho tyrosine and rabbit anti-Rho antibodies from Millipore (Tempecula, CA, USA), rabbit anti-p190RhoGAP antibodies from Cell Signaling (Lake Placid, NY, USA), and rabbit anti-HA antibodies from Sigma Aldrich (St. Louis, MO, USA). Mouse monoclonal anti-Nox1 antibodies were provided by dia DeXus (South San Francisco, CA, USA) and rabbit anti-Nox1 antibodies by Dr. D. Lambeth. Rabbit anti-Nox4 antibodies were provided by Drs. A. Shah and J. Goldstein. pcDNA3.0-Nox1, adenovirus (Adv) HA-LMW-PTP and HA-LMW-PTP^{C12S} and GST-PAK-1 expression vectors were described previously[14].

B Tissue specimens of colorectal cancer

Surgical specimens were obtained from 70 cases of primary colorectal adenoma and adenocarcinoma patients who underwent surgical resection at Shinshu University Hospital, Matsumoto, Japan from 1999-2004 after informed consent was obtained. These patients included 31 cases of tubular adenomas and 39 cases of adenoma/carcinomas which had not received any therapeutic agents or irradiation before surgery. Tissue samples from colorectal adenomas or carcinomas and the cut end of the normal-looking mucosa were examined in this study. Histological diagnosis of colorectal cancer was made according to the World Health Organization histologic classification.

C Immunohistochemistry

Formalin-fixed tissue samples were embedded in paraffin, deparaffinized, and retrieved in Tris-EDTA pH8.0 by microwave for 30 min as described previously[15]. The tissue sections were stained with the indicated antibodies by using immunoglobulins conjugated with horseradish peroxidase (HRP) (Dako Japan, Tokyo, Japan) and 3, 3'-diaminobenzidine tetrahydroxychloride (BD Bioscience, San-Jose, CA, USA). Counterstaining was performed with hematoxylin.

D Scoring

Tissue specimens in which $>5 \%$ of the cells were positive for each primary antibody were defined as positive, and others were defined as negative, according to criteria previously described[15]. In the positive group, immunohistochemical results were further categorized into 3 groups : weak ($+1$), medium ($+2$), and strong ($+3$) based on staining intensity and summarized in the histograms.

E siRNAs and RT-PCR

pSilencer-hygro Nox4 siRNA vector was constructed as described previously[6]. SMAD3 siRNAs (sense : 5'- CUGUGUGAGUUCGCCUUCATT - 3', anti-sense : 5'-UGAAGGCGAACUCACACAGTT-3') were synthesized. RT-PCR was carried out as described[8], and PCR was performed under the condition of 35 cycles of 94 ℃ for 30 sec, 55 ℃ for 30 sec, and 72 ℃ for 1 min according to the protocol by Promega (Madison, WI, USA). Noxs and SMAD3 primers (sense

5'-GGAGCAGGAATTGGGGTCAC-3' and antisense 5'-TTGCTGTCCCATCCGGTGAG-3' for Nox1 ; sense 5'-CTCAGCGGAATCAATCAGCTGTG-3' and antisense 5'-AGAGGAACACGACAATCAGCCTTAG-3' for Nox4 ; sense 5'-GGTCAAGAGCCTGGTCAAGA -3' and anti-sense 5'-TTGAAGGCGAACTCACACAG -3' for SMAD3) were used.

F Transfection and Virus infection

Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), Lipofectamine RNAi Max (Invitrogen), or DharmaFECT (Waltham, MA, USA) were used for transfection.. Infection with adenovirus vectors was performed as described previously[14].

G Measurement of ROS production

Cell suspensions were incubated with 200μM luminol and 1 unit horseradish peroxidase for 10 min at $37\,°$C as described[6]. Luminescence was quantified by Lmax (Molecular Devices, San Diego, CA, USA).

H Rho activity assay

Cell lysates were pulled down by using GST-PAK1-coupled glutathione-sepharose beads, and the amount of active GTP・Rho retained on the beads was determined by immunoblotting using anti-Rho antibodies as described[14].

I Labeling with BIAM

Cells were lysed in phosphate buffer (pH 7.0) containing 20 mM NEM, an alkylator of cysteine-SH residues, reduced by 20 mM DTT and labeled with 20μM BIAM as described[16]. Labeled proteins were analyzed by immunoprecipitation with anti-HA antibodies, followed by SDS-PAGE and HRP-streptavidin staining.

J Migration Assay

Migration assay was performed by using Boyden chambers (BD Bioscience) as described previously[14]. Cells were plated to the chambers of 24-well plates in serum-free DMEM, and TGF-β1 (10 ng/ml) was used as a cue. Migrating cells were counted after 24h.

K Statistical analysis

Data were shown as mean ± S.D. of the results of at least three independent experiments. Statistical analysis was performed with Student's t test. Differences with P-values of <0.05 were considered to be statistically significant.

Ⅲ Results and Discussion

A Differential expression of Nox4 and Nox1 in normal mucosa and adenoma-carcinoma sequence of human colon

To address the role of Nox4 in colorectal cancer, we first performed comparative immunohisto-chemical analyses of Nox4 and Nox1 expression in normal colorectal mucosa as well as adenoma-carcinoma sequence during tumorigenesis of colorectum. To date, no histological study is available that provides detailed relationships between Nox4 expression and the grade of human colon epithelial neoplasms. In contrast to Nox1 which is abundant in the normal colon, Nox4 expression was not observed in either normal mucosa or tubular adenomas (**Fig. 1A, 1C**). However, unexpectedly, a high level of Nox4 was expressed in the cytoplasmic region of well and moderately differentiated adenocarcinomas, indicating that induction of the Nox4 expression takes place at the advanced stages of colorectal cancer (**Fig. 1A, 1C**). Immunoblotting analyses also showed that the expression of Nox4 proteins was elevated in colon tumors compared to adjacent normal tissues (**Fig. S1**), which is in agreement with the previous observation[13]. We also confirmed that the two independent antibodies- one from Novus (**Fig. 1A**) and the other from Dr. J. Goldstein (**Fig. S2B**)-yielded a similar staining pattern in normal mucosa and well-differentiated adenocarcinomas, which reinforces our finding of specific Nox4 expression associated with colorectal carcinoma.

Meanwhile, the precise relationships between the expression pattern of Nox1 proteins and the grade of differentiation have not been examined, although analysis of Nox1 mRNA expression in colon carcinomas by *in situ* hybridization was previously reported[7]. Nox1 proteins were weakly detected along lower crypts of normal mucosa (**Fig. S3A**), and its expression level increased in association
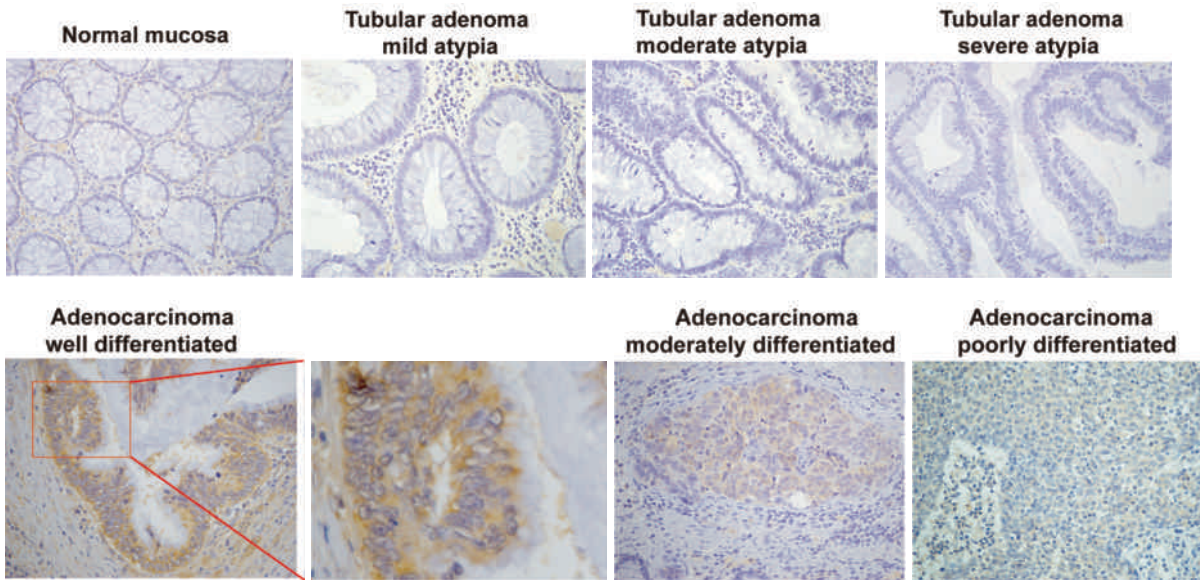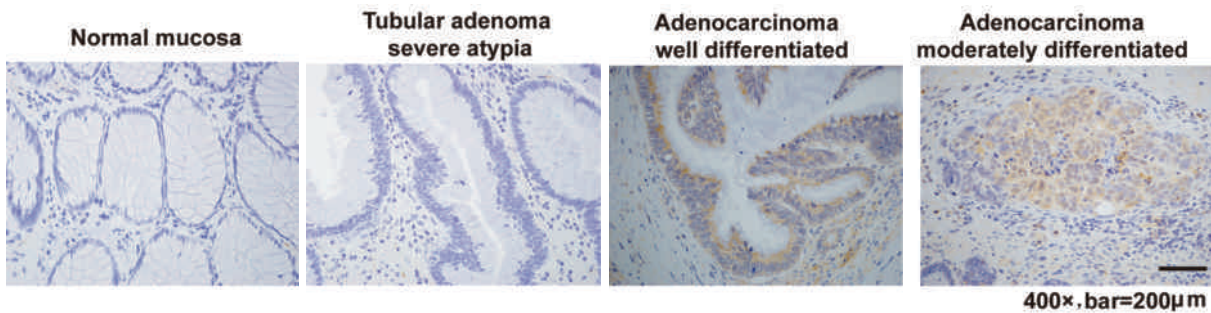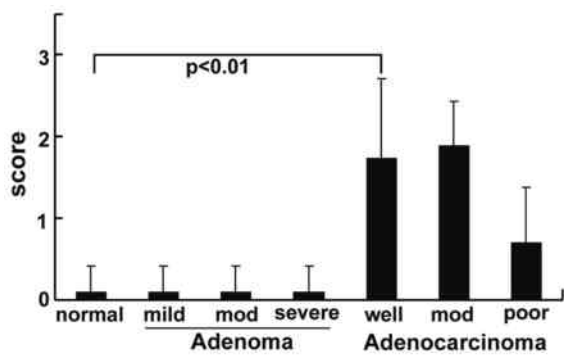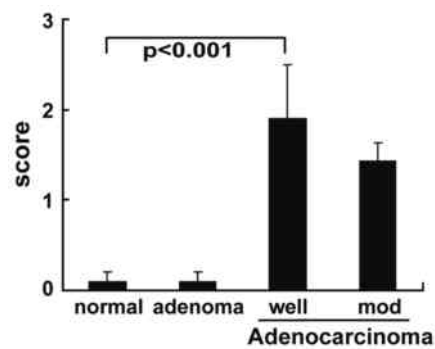
Fig. 1 Expression of Nox4 is upregulated in human colon tumors and correlates with expression of TGF-β1.
(A) The specimens from human normal colon tissues, tubular adenomas (mild, moderate, and severe atypia), and adenocarcinomas (well differentiated, moderately differentiated, and poorly differentiated type) were subjected to immunostaining with rabbit anti-Nox4 antibodies from Novus. (B) The specimens from normal colon tissues, tubular adenomas, and adenocarcinomas (well differentiated and moderately differentiated type) were immunostained with anti-TGF-β1 antibodies. (C) Frequency of Nox4 expression in normal colon tissues and colon tumor samples. Samples from 10 patients were examined for each grade of tumor and normal tissues. Frequency of Nox4 expression was scored as described in Materials and Methods. The histograms represent mean ± S. D. (n=10). (D) Frequency of the TGF-β1 expression in colon tumors was estimated as in the case of Nox4 (see Materials and Methods). The histograms represent mean ± S.D. (n=5).

with the degree of atypia of tubular adenomas (**Fig. S3A, S3B**). The expression of Nox1 proteins reached a maximum level in the apical cell surface of well differentiated adenocarcinomas but decreased as cancer progressed through moderately and poorly differentiated adenocarcinomas (**Fig. S3A, S3B**).

Taken together, the data revealed the striking difference in expression pattern between Nox4 and Nox1 during colon cancer development : Nox4 was specifically expressed at the stages of colon carcinomas, while increased expression of Nox1 occurred even in adenomas during neoplastic progression.

B TGF-β1 is expressed in colon cancer and induces both Nox4 expression and ROS production

We next examined the regulatory mechanism of Nox4 induction, which is closely associated with progression of colon carcinomas. Gene expressions are frequently influenced by stimuli such as cytokines and growth factors, which are secreted from the colon cancer cells and surrounding stromal cells, via either autocrine or paracrine signalings. Among them, TGF-β1 acts as a stimulator of both cell growth and invasion during colorectal cancer promotion, and its intense expression correlates with pathological progression to metastasis and poor prognosis[17]. We therefore reasoned that TGF-β1 might be involved in the regulation of Nox4 expression in colon carcinoma. To test this possibility, we evaluated the expression pattern of TGF-β1 in normal colon and tumor samples. Interestingly, well and moderately differentiated adenocarcinomas exhibited strong staining of TGF-β1, whereas normal mucosa and tubular adenomas displayed no TGF-β1 staining (**Fig. 1B**). Of note, the staining pattern of TGF-β1 remarkably resembled that of Nox4 in terms of association with grade of colon carcinoma and the localization (**Fig. 1A-1D**). This prompted us to determine whether TGF-β1 induces Nox4 expression. To this end, RKO and HCT-116 colon carcinoma cells were treated with TGF-β1 and subjected to analysis of Nox4 expression by RT-PCR. TGF-β1 specifically upregulated the expression of Nox4 mRNAs, whereas the expression of Nox1 was not affected (**Fig. 2A**). This was also

verified by immunoblotting analysis of the protein levels of Nox4 and Nox1 (**Fig. S4**). Furthermore, pretreatment of RKO cells with SIS3, a specific inhibitor of the SMAD3 transcription factor significantly suppressed the TGF-β1-induced expression of Nox4 mRNAs (**Fig. 2B**). Knockdown of SMAD3 by siRNAs also suppressed the expression of both Nox4 mRNAs and Nox4 proteins (**Fig. S5**). Thus, we conclude that TGF-β1 regulates the Nox4 expression through the SMAD3 pathway in colon cancer cells.

In addition, we found that TGF-β1 treatment enhanced ROS generation in the cells, and that the stimulatory effect of TGF-β1 on ROS synthesis was suppressed by transduction of SMAD3 siRNAs or addition of SIS3 (**Fig. 2C**). RT-PCT and immunoblotting analyses indicated that SMAD3 siRNAs suppressed the expression of SMAD3 (**Fig. 2D**). The results support that the view that TGF-β1 stimulates ROS production by inducing Nox4 expression via SMAD3.

C Nox4 mediates TGF-β1-induced migration of colon cancer cells

Because Nox4 but not Nox1 was found to participate in TGF-β1 signaling, we focused on the regulatory role of Nox4 signaling in TGF-β1-dependent cell motility in the subsequent study. We first examined the involvement of Nox4 in TGF-β1-induced migration of colorectal cancer cells. Both HCT-116 and RKO cells were treated with TGF-β1 following transfection of Nox4-specific siRNAs or scrambled siRNAs and tested for their ability to migrate. The number of migrating cells was markedly enhanced by TGF-β1 treatment, whereas TGF-β1-promoted cell migration was attenuated by Nox4 knockdown (**Fig. 3A**). Immunoblotting analysis demonstrated efficient ablation of endogenous Nox4 by Nox4 siRNAs (**Fig. 3B**). The data point to an important role of Nox4-dependent mechanism in TGF-β1-induced motility of colon cancer cells.

To further analyze the Nox4 signaling pathway leading to cell motogenesis, we first determined whether Nox4-generated ROS modulate the activity of Rho, a key regulator of cytoskeletal contracti-
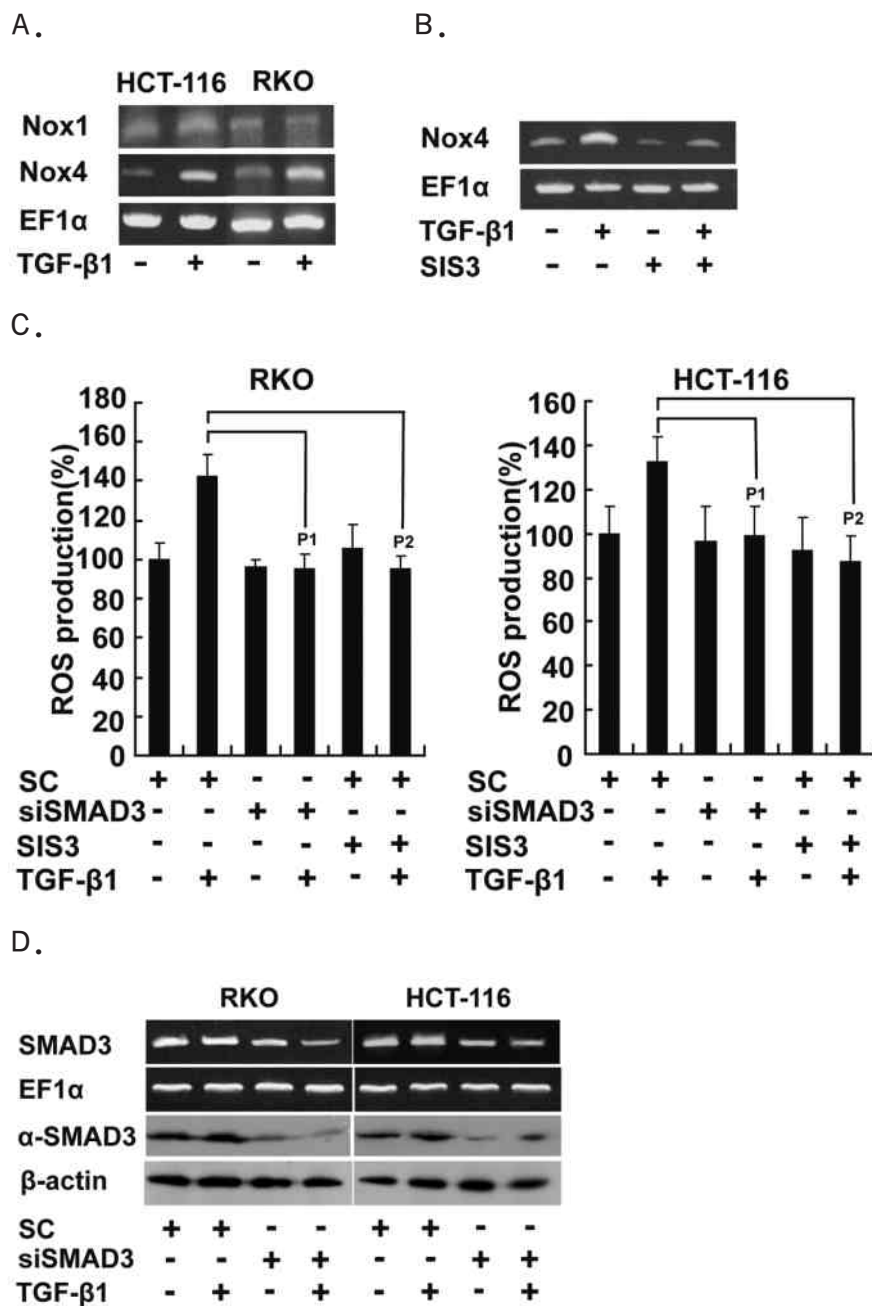
Fig. 2 TGF-β1 induces both Nox4 expression and Nox4-derived ROS production in colon cancer cells. (A) RKO and HCT 116 cells were treated with TGF-β1 (10 ng/ml) for 24h. Expressions of Nox1 and Nox4 mRNAs were analyzed by RT-PCR. (B) RKO cells were treated with TGF-β1 (10 ng/ml) in the absence or presence of a SMAD3 inhibitor, SIS3 (10μM) for 24 h and subjected to RT-PCR analysis. (C) RKO and HCT116 cells (5x10⁴) were treated with TGF-β1 and SIS3 as in (B) or transfected with SMAD3 siRNAs and scrambled siRNAs(sc) prior to TGF-β1 treatment for 24h. The cells were then subjected to luminol-based ROS assay. The data represent mean ± S.D (n=3) in 3 separate experiments. P1 or P2 <0.05 versus sc, TGF-β1. (D) RKO and HCT116 cells were transfected with SMAD3 siRNAs or scrambled controls, treated with TGF-β1 as described in (C), and subjected to RT-PCR and immunoblotting with rabbit anti-SMAD3 antibodies. EF1α and β-actin are loading controls.
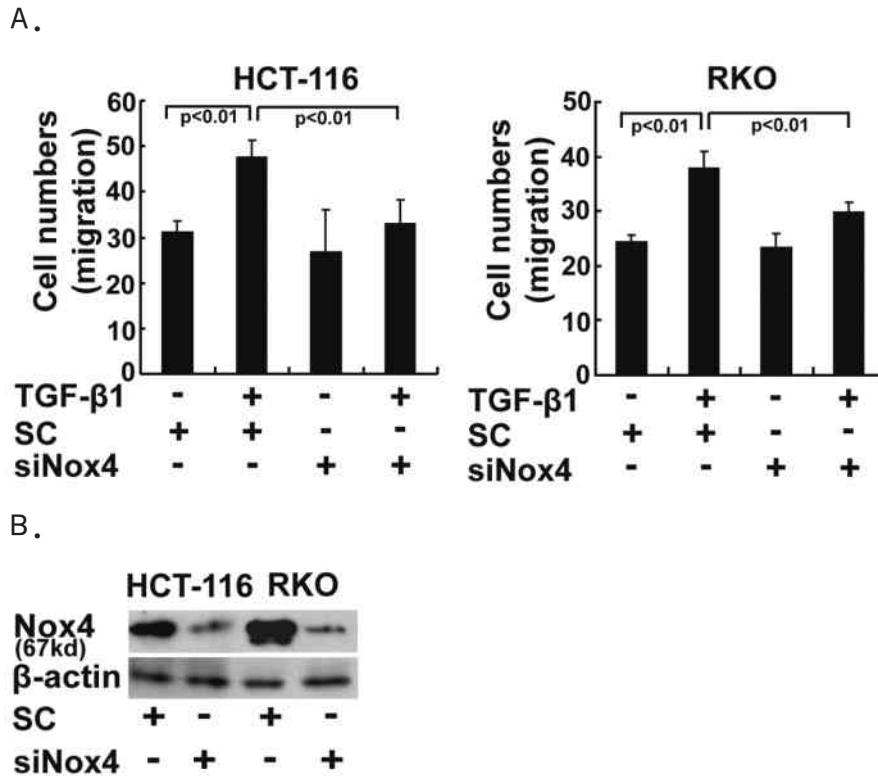
Fig. 3   Nox4 mediates TGF-β1-induced migration of colon cancer cells.
(A) RKO and HCT-116 cells were transfected with Nox4 siRNAs or scrambled siRNAs. Transfected cells were replated 48h later, treated with TGF-β1 (10 ng/ml), and subjected to migration assay as described in Materials and Methods. The numbers of migrating cells were determined. The data represent mean ± S.D. (n=3) in three separate experiments. (B) Alternatively, the expression of Nox4 in transfected cells was analyzed by immunoblotting with anti-Nox4 antibodies. β-actin is a loading control.

lity. GST-Rho binding domain pull-down assays indicated that the amount of active GTP-bound Rho was decreased upon TGF-β1 treatment of RKO cells (**Fig. 4A**), whereas the suppressive effect of TGF-β1 on Rho was abolished when cells were transfected with Nox4 siRNAs (**Fig. 4A**). Consistently, SIS3 and NAC, an antioxidant, interfered with the suppressive action of TGF-β1 on Rho (**Fig. S6**). These results indicate that Nox4 signaling mediates TGF-β-induced cell motility by negatively regulating the Rho activity.

p190RhoGAP is activated upon tyrosine phosphorylation by receptor or non-receptor tyrosine kinases, thereby down-regulating Rho[18]. We therefore speculated that TGF-β1-mediated negative regulation of Rho could be caused by activation of p190RhoGAP in response to TGF-β1 stimulation. Immunoblotting analyses demonstrated that TGF-β1 treatment elevated tyrosine phosphorylation of p190RhoGAP, whereas Nox4 siRNAs abolished the stimulatory effect of TGF-β1 on p190RhoGAP phosphorylation. This suggests that Nox4 mediates TGF-β1-induced activation of p190RhoGAP (**Fig. 4B**), although the nature of a putative tyrosine kinase involved is unknown at present.

Given that low molecular weight-protein tyrosine-phosphatase (LMW-PTP) deactivates tyrosine-phosphorylated p190RhoGAP[19] and H_2O_2 inactivates LMW-PTP through oxidation of its redox-sensitive Cys-12 and -17 residues in its catalytic pocket[20], we next investigated whether Nox4-generated ROS transmit an activation signal to p190RhoGAP via LMW-PTP. To this end, we tested the ability of Nox4 to oxidize LMW-PTP. Cells were transfected with LMW-PTP, and cell lysates were processed for BIAM labeling, in which redox-
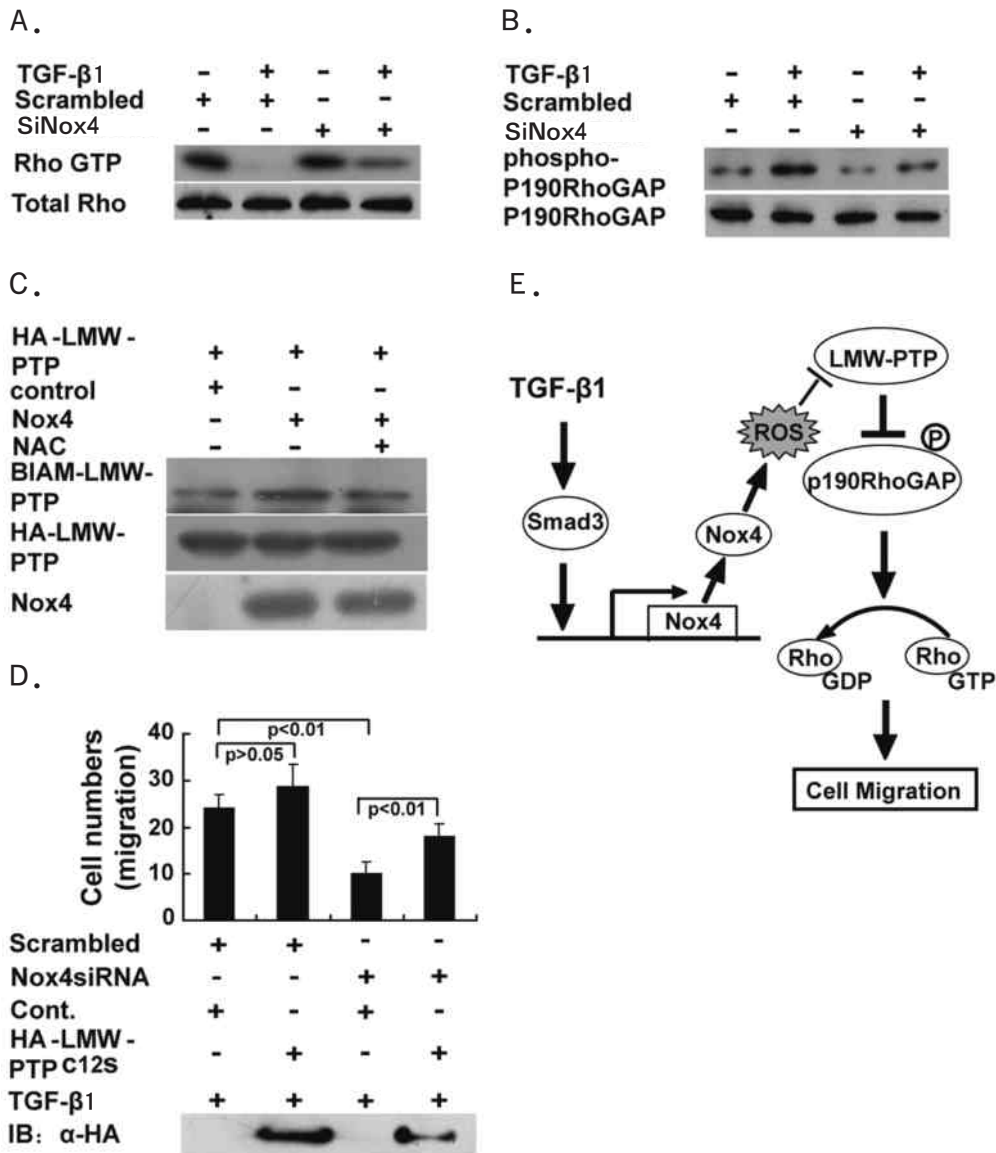
A.

TGF-β1	−	+	−	+
Scrambled	+	+	−	−
SiNox4	−	−	+	+

Rho GTP

Total Rho

B.

TGF-β1	−	+	−	+
Scrambled	+	+	−	−
SiNox4	−	−	+	+

phospho-
P190RhoGAP

P190RhoGAP

C.

HA-LMW-PTP	+	+	+
control	+	−	−
Nox4	−	+	+
NAC	−	−	+

BIAM-LMW-PTP

HA-LMW-PTP

Nox4

E.

D.

Scrambled	+	+	−	−
Nox4siRNA	−	−	+	+
Cont.	+	−	+	−
HA-LMW-PTP^{C12S}	−	+	−	+
TGF-β1	+	+	+	+

IB: α-HA

Fig. 4　Nox4 mediates TGF-β1-induced cell migration via the LMW-PTP-p190RhoGAP-Rho pathway. (A) TGF-β1 downregulates the Rho activity through Nox4. RKO cells were transfected with scrambled siRNAs or Nox4 siRNAs, treated with TGF-β1 (10 ng/ml) for 10 min, and processed for cell lysate preparation. Lysates were subjected to the Rho activity assay as described in Materials and Methods. The total Rho was monitored by immunoblotting with anti-Rho antibodies. (B) RKO cells were transfected with scrambled siRNAs or Nox4 siRNAs and treated with TGF-β1 (10 ng/ml) for 10 min. Lysates were subjected to immunoblotting analysis with anti-phospho-p190RhoGAP and p190RhoGAP antibodies. (C) RKO cells were transfected with pcDNA3.0-Nox4 or control vectors, infected with adv-HA-LMW-PTP, and 48h later treated with or without NAC (10mM) for 30 min. BIAM labeling of LMW-PTP, following alkylation and reduction, was performed as described in Materials and Methods. Expressions of transfected LMW-PTP and Nox4 were monitored by immunoblotting with anti-HA antibodies and anti-Nox4 antibodies. (D) RKO cells were transfected with scrambled siRNAs or Nox4 siRNAs, infected with adv-HA-LMW-PTP^{C12S} or control viruses, and subjected to migration assay using TGF-β1 (10 ng/ml). Expression of introduced LMW-PTP^{C12S} was monitored by immunoblotting with anti-HA antibodies. The data represent mean ± S. D. (n=3) in four separate experiments. (E) Schematic model for Nox4-mediated TGF-β1-induced cell migration. TGF-β1 induces Nox4 expression possibly in a SMAD3-dependent manner. Nox4-derived ROS oxidize and inactivate LMW-PTP, which results in accumulation of activated, tyrosine phosphorylated p190RhoGAP. This causes downregulation of Rho, leading to cell migration of colon cancer cells.

sensitive cysteine residues oxidized by intracellular H_2O_2 are protected from postlysis alkylation with NEM and reacted with BIAM following reduction with DTT[16)21)]. Co-transfection of Nox4 promoted BIAM labeling of LMW-PTP, and treatment with NAC decreased the labeling (**Fig. 4C**). Introduction of Nox4 siRNAs also attenuated BIAM-labeling of LMW-PTP in RKO cells (**Fig. S7**). This is consistent with the notion that LMW-PTP is oxidized by Nox4-generated ROS, serving as their sensor. To test the involvement of LMW-PTP in TGF-β1-induced cell migration, cells were transfected with Nox4 siRNAs and/or LMW-PTP[C12S], a catalytically inactive, Cys-12 to Ser mutant of LMW-PTP[22)] and subjected to cell migration assay. Stimulation of migration with TGF-β1 was blocked in Nox4 siRNA-transfected cells, whereas forced expression of LMW-PTP[C12S] restored TGF-β1-induced motogenesis in Nox4 siRNA-transfected cells (**Fig. 4D**). The motogenic effect of TGF-β1 on scrambled siRNA-transfected cells was not significantly altered by introduction of LMW-PTP[C12S], possibly because endogenous LMW-PTP had been inactivated by TGF-β1 via Nox4. The data demonstrate that LMW-PTP mediates TGF-β1-induced, Nox4-dependent cell migration.

In summary, our study revealed that Nox4 is differentially expressed from Nox1 during the course of colon tumor development : Nox4 expression is specifically induced in colon carcinomas, while Nox1 expression begins to increase in adenomas even before malignant colon tumor. Another important finding is that Nox4 expression in colon primary tumors is upregulated similar to that of TGF-β1, and that Nox4 but not Nox1 is induced by TGF-β1 in colon cancer cells. Further-

more, Nox4 mediated TGF-β1-triggered motility of colon cancer cells via the LMW-PTP-Rho pathway. Supporting this observation, Nox4 is known to localize to focal adhesions, thereby coordinating cell adhesion and migration together with Src tyrosine kinases[23)]. We postulate that TGF-β1 induces the Nox4 expression in the colon cancer cells, and that Nox4-derived ROS subsequently transmit TGF-β1 signaling through the LMW-PTP-RhoGAP-Rho axis to accelerate cell migration, thereby contributing to the invasiveness of colon cancer cells (**Fig. 4E**). Similar to our finding, TGF-β1-secreting stromal cells activated Nox4-dependent migration of breast cancer cells when cocultured[5)]. Although Bauer KM et al[12)] have suggested the involvement of Nox4 in the motility of colon cancer, no detailed mechanistic study has been provided. Thus, our study is significant in the sense that it revealed the Nox4 signaling mechanism responsible for the motility of colorectal cancer.

Ⅳ Conflict of Interest

The authors declare no conflict of interest in this work.
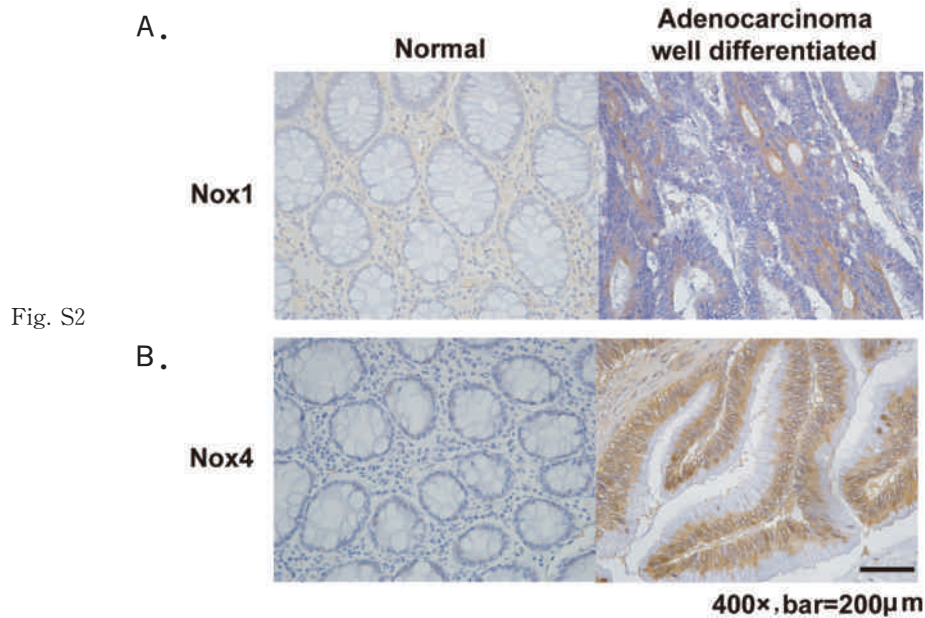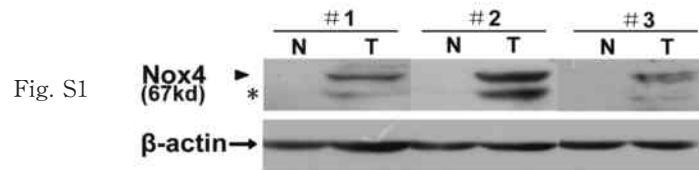
Ⅴ Acknowledgments

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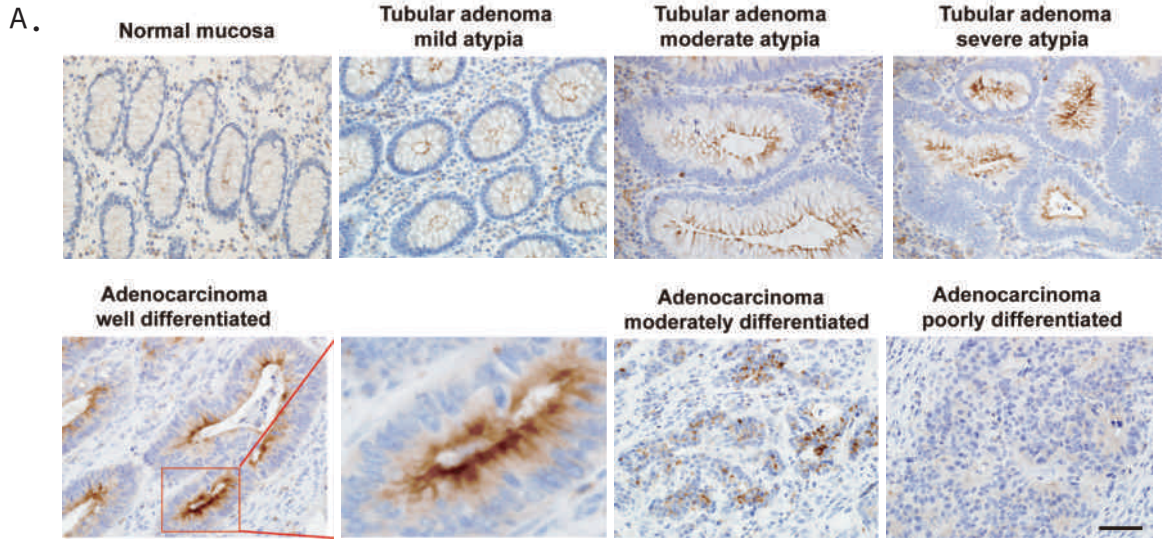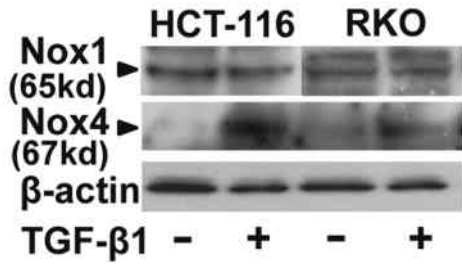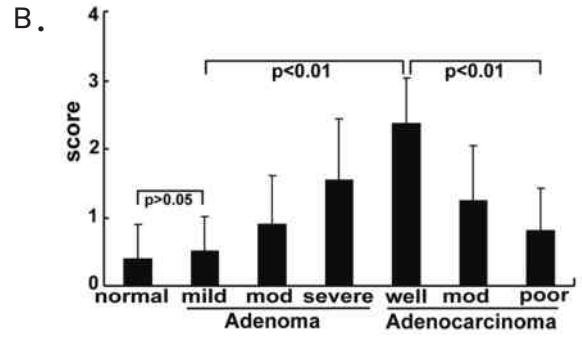
Supplementary Materials

Fig. S1

Fig. S2

400×, bar=200μm

A.

Normal mucosa	Tubular adenoma mild atypia	Tubular adenoma moderate atypia	Tubular adenoma severe atypia

Fig. S3

Adenocarcinoma well differentiated		Adenocarcinoma moderately differentiated	Adenocarcinoma poorly differentiated

B.

Fig. S4

HCT-116   RKO

Nox1 (65kd)
Nox4 (67kd)
β-actin

TGF-β1   −   +   −   +

Fig. S5

RKO

Nox4
EF1α
Nox4 (67kd)
β-actin

SC   +   +   −   −
siSMAD3   −   −   +   +
TGF-β1   −   +   −   +

Fig. S6

Rho GTP
Total Rho

TGF-β1   −   +   −   +   −   +
SIS3   −   −   +   +   −   −
NAC   −   −   −   −   +   +

Fig. S7

BIAM-LMW-PTP
HA-LMW-PTP
Nox4 (67kd)

SC   +   +   −
TGF-β1   −   +   +
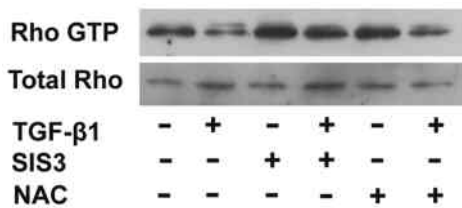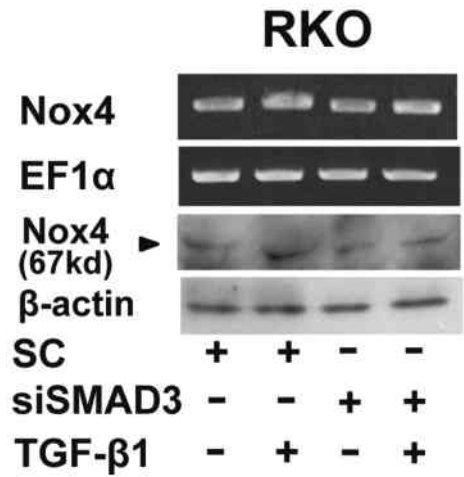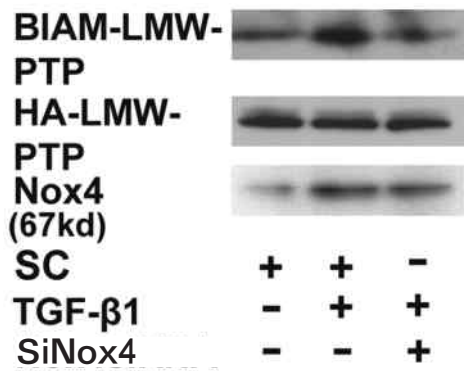SiNox4   −   −   +

Fig. S1   Immunoblotting analysis of Nox4 expression in colon tumors.
 Fractionation of colon tissues and immunoblotting analysis using anti-Nox4 antibodies were performed as described
   previously [1]. Cell lysates were prepared from normal colon tissues (N) or colon tumors (well differentiated
   adenocarcinomas) (T) from three patients. β-actin is a loading control. ＊indicates the degraded Nox4 proteins.
[1]   Komatsu D, Kato M, Nakayama J, Miyagawa S, Kamata T : NADPH oxidase 1 plays a critical mediating role
   in oncogenic Ras-induced vascular endothelial growth factor expression. Oncogene 27 : 4724-4732, 2008

Fig. S2   Validation of immunohistochemical data on Nox4 and Nox1 expressions in colon tumors by using alternative
       sources of the antibodies.
 To verify the immunohistochemical data on Nox4 (Fig. 1A) and Nox1 (Fig. S2A) expressions, tissue sections from
normal colon and well-differentiated adenocarcinomas were immunostained with the rabbit anti-Nox4 antibody
provided by Dr. J. Godstein, Thomas Jefferson University (A) and the rabbit anti-Nox1 antibody provided by Dr. J.
Lambeth, Emory University (B). The data indicate that Dr. Goldstein's antibodies and Dr. Lambeth's antibodies
exhibited staining patterns similar to those obtained with the Novus Nox4 antibody (Fig. 1A) and the dia DeXus Nox1
antibody (Fig. S2), respectively.

Fig. S3   Expression of Nox1 in human colon tumors.
 (A) The specimens from human normal colon tissues, tubular adenomas (mild, moderate, and severe atypia), and
adenocarcinomas (well differentiated, moderately differentiated, and poorly differentiated type) were subjected to
immunostaining with mouse monoclonal anti-Nox1 antibodies. (B) Frequency of the Nox1 expression in normal colon
tissues and colon tumor samples. Samples of 10 patients were examined for each grade of tumors and normal tissues.
Frequency of the Nox1 expression was scored as described in Materials and Methods. The histograms represent
mean ± S. D. (n=10).

Fig. S4   TGF-β induces the expression of Nox4 proteins.
 RKO and HCT116 cells were treated with TGF-β1 (10 ng/ml) for 24h, and expressions of Nox4 and Nox1 proteins
were analyzed by immunoblotting with rabbit anti-Nox4 antibody provided by Dr. A. Shah and rabbit anti-Nox1
antibody from Abcam, respectively. The data indicated that Nox4 proteins but not Nox1 proteins were induced by
TGF-β1, which is in agreement with the results of RT-PCR analysis (Fig. 2). β-actin is a loading control.

Fig. S5   Knockdown of SMAD3 suppresses TGF-β1-induced expression of Nox4 mRNAs and Nox4 proteins.
 RKO cells were transfected with SMAD3 siRNAs or scrambled siRNAs (se), 48h later treated with TGF-β1 (10 ng/
ml) for 24h, and subjected to both PCR analysis and immunoblotting using rabbit-anti Nox4 antibodies from Dr.
Goldstein. The data indicated that knockdown of SMAD3 by siRNAs suppressed TGF-β1-induced expression of Nox4
mRNAs andNox4 proteins, which is consistent with the results of RT-PCR analysis (Fig. 2B).

Fig. S6   SIS3 and NAC block TGF-β1-dependent regulation of Rho.
 RKO cells were treated with TGF-β1 (10 ng/ml) in the presence or absence of SIS3 (10μM) for 24h or NAC (10mM)
for 30 min and subjected to the Rho activity assay as described in Materials and Methods. The total Rho was monitored
by immunoblotting. The data indicated that treatment with SIS3 and NAC abolished TGF-β1 –induced suppression of
the Rho activity, which is consistent with the idea that TGF-β/SMAD3-induced Nox4 mediates the regulation of Rho.

Fig. S7   Nox4 knockdown suppresses oxidation of LMW-PTP.
 RKO cells were transfected with pSilencer-Nox4 siRNA or pSilencer-scrambled siRNA vectors and infected with
Adv-HA-LMW-PTP. 48h later, cell lysates were prepared and subjected to BIAM labeling of LMW-PTP as described
in Materials and Methods. Expressions of transfected LMW-PTP and Nox4 were monitored as in Fig. 4C and Fig. S4,
respectively.