Nox4-derived ROS Signaling Contributes to TGF-β-induced Epithelial-mesenchymal Transition in Pancreatic Cancer Cells

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Abstract. Transforming growth factor (TGF)-β induces epithelial-mesenchymal transition (EMT) in pancreatic adenocarcinoma. In this study, we investigated how NADPH oxidase (Nox) 4-generated reactive oxygen species (ROS) regulate TGF-β-induced EMT in pancreatic cancer cells. Materials and Methods: Pancreatic cancer cells were transfected with Nox4 siRNAs or PTP1B mutants and subjected to TGF-β-induced EMT assay. Expression of Nox4, TGF-β, and N-cadherin was immunohistochemically-examined with patient tumor samples. Results: Treatment of pancreatic cancer cells with TGF-β induced Nox4 expression, indicating that Nox4 represents a major source for ROS production. The Nox4 inhibitor diphenylene iodonium and Nox4 siRNAs blocked TGF-β-induced EMT phenotype including morphological changes, augmented migration, and altered expression of E-cadherin and Snail. Furthermore, PTP1B as a redox-sensor for Nox4-derived ROS participated in TGF-β-promoted EMT. Nox4, TGF-β, and N-cadherin were up-regulated in tumors from pancreatic cancer patients. Conclusions: These findings suggest that Nox4-derived ROS, at least in part, transmit TGF-β-triggered EMT signals through PTP1B in pancreatic cancer.

Epithelial-mesenchymal transition (EMT) is the process that converts polarized epithelial cells, tightly bound to each other, into fibroblast cells with migratory potential (1). During EMT, epithelial cells undergo numerous biological and biochemical changes in order to acquire a fibroblast-like phenotype. These include acquisition of both spindle-like morphology and cell-to-cell dissociation, up-regulation of mesenchymal markers (N-cadherin and vimentin), down-regulation of epithelial markers (E-cadherin and ZO-1), and induction of specific transcription factors (Snail and Twist) (2). EMT is vital for morphogenesis during embryonic development and is also implicated in tumor cell invasion and migration. EMT associated with cancer is promoted by growth factors, cytokines, and matrix proteins secreted in tumor microenvironments.

Pancreatic cancer, which is characterized by strong chemoresistance and high metastatic potential, is one of the most deadly cancer types. The critical role of EMT has been implicated in aggressive malignancy and lethality of pancreatic carcinoma (3). In pancreatic adenocarcinoma, TGF-β is the major inducer of EMT, which is a prerequisite for tumor invasiveness and metastasis (4), and overexpression of TGF-β is associated with poor prognosis (5). On this context, understanding of the signaling pathways responsible for TGF-β-induced EMT would be important to prevent malignant progression of pancreatic cancer.

Accumulating evidence indicates that reactive oxygen species (ROS) generated by NADPH oxidase (Nox) family enzymes act as signaling molecules in various physiological and pathophysiological events (6). The Nox family consists of Nox1-5 and Duox-1 and-2, catalyzes oxidation of NADPH to produce superoxide, and exerts specific biological roles in different tissues. Among them, Nox4 participates in regulation of glucose metabolism (7) and vascularity (8). Dysfunction of Nox4 signaling is implicated in cancer (9, 10), diabetes (11), and cardiovascular diseases (12). With respect to pancreatic cancer, we and others have previously described that Nox4-derived ROS sustain growth of pancreatic cancer cells, at least in part, through the cell survival pathway (13, 14). Given the pleiotropic functions of Nox4 isoforms, there is a strong possibility that Nox4 might have an additional role in development of pancreatic cancer. In this regard, it is noteworthy that TGF-β-up-regulated Nox4 contributes to induction of EMT in metastatic breast cancer cells (15). However, it is not known whether this Nox4-mediated scheme similarly functions in the EMT process of other malignant cancers including pancreatic carcinoma, nor are the signaling events that follow Nox4 upregulation and ROS generation defined.
In the present study, we investigated whether Nox4 plays a regulatory role in TGF-β-promoted EMT in pancreatic cancer. Our data indicated that Nox4-generated ROS are critically involved in TGF-β-induced EMT in pancreatic cancer cells by controlling p38MAPK and PTP1B activities. The study provides further insight into redox-mediated signaling of EMT during cancer development.

**Materials and Methods**

**Cell culture and materials.** A human pancreatic adenocarcinoma cell line, moderately differentiated Panc-1, was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained at 37˚C under 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum. Diphenylene iodonium (DPI) was purchased from Calbiochem (La Jolla, CA, USA), recombinant human TGF-β1 from R&D Systems (Minneapolis, MN, USA), N-acetylcystein (NAC) and mouse anti-HA antibodies from Sigma (Milwaukee, WI, USA), anti-E-cadherin antibodies from BD Biosciences (Franklin Lakes, NJ, USA), mouse anti-Snail antibodies from Cell Signaling (Danvers, MA, USA), and rabbit anti-Nox4 antibodies from NOVUS (Littleton, CO, USA). SB203580, an inhibitor of p38 MAP-kinase was purchased from Merck Millipore (Darmstadt, Germany). 5'-iodoacetamide fluorescein (IAF) and anti-fluorescein antibodies were purchased from Molecular Probes (Eugene, OR, USA).

**Construction of Nox4 siRNAs and plasmids.** DNA oligonucleotides encoding for Nox4siRNAs were subcloned into the HI promoter vector (provided by Dr. Z. Zhang, Indiana University School of Medicine). All construction was carried out using the Clontech In-Fusion HD cloning system. DNA oligonucleotides were designed as follows: Nox4 siRNA(1): 5’-CAGAACATTCCATATTAC-3’; Nox4 siRNA(2): 5’-ACITTTGTTGAAGCTGAT-3’. All of the constructs were verified by sequencing. Universal scrambled siRNAs, which have no homology to mouse, rat, or human cDNA databases, were used as controls (supplied by Ambion). pCN-HA-PTP1B and pCN-PA-PTP1B-D181A expression vectors (Eugene, OR, USA) were used to investigate whether PTP1B is a redox sensor for Nox4.

**Immunostaining.** Cells were fixed with 4% parafolmaldehide, permeabilized with 0.2% Triton-X, and stained with rabbit anti-E-cadherin antibodies and FITC-conjugated anti-rabbit IgG. Stained cells were observed under a confocal laser-scanning microscope (Carl Zeiss, Thornwood, NY, USA).

**Real time-polymerase chain reaction.** Total RNA preparation and reverse transcription were performed as described previously (14). PCR for human Nox-4 genes was carried out using the following primers: forward 5’-CTCACGGGATAACTACGCTGTG-3’ and reverse 5’-AGAGGAAACGACAATCGCCCTTAG-3’.

**Transfection and immunoblotting.** Cells were transfected with the indicated expression vectors by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were lysed in lysis buffer and subjected to immunoblotting with appropriate antibodies as described previously (14). Protein bands were visualized by ECLPLUS or ECLPrime (Amersham, Piscataway, NJ, USA).

Measurement of the intracellular ROS levels. ROS production was measured by using luminol luminescence as described previously (14). Cells were transfected with scrambled siRNA or siNox4RNAs. Transfected cells (1x10^5) were re-plated into 96-well plates, treated with TGF-β for 10 min, and incubated with Hank’s buffer containing 200 μM luminol and 0.8 units horseradish peroxidase for 5 min. Luminescence intensity was quantified by Lumax (Molecular Devices, Japan).

5’-IAF labeling. Cells were lysed in the lysis buffer (50 mM MES-NaOH, pH 6.5, 0.5% Triton X-100, 1mM PMSF), and 12 μM IAF were added to the lysate for 60 min at 4˚C under anaerobic condition, as described previously (17, 18). The labeled proteins were subjected to immunoprecipitation with anti-fluorescein antibodies, followed by immunoblotting with anti-PTP1B antibodies.

**Migration assay.** Migration was assayed by using Matrigel-coated Boyden chambers (Nalgé Nunc, Rochester, NY) as described previously (19). Cells were transfected with indicated vectors and, 48 h later, they were inoculated into the top of the chamber in DMEM plus 0.1% BSA and TGF-β (10 ng/ml) DMEM containing 10% FBS was added to the bottom of the chamber. After 16 h incubation, the migrating cells were stained with trypan blue and counted.

**Patients and tissue samples.** The Human Ethics Review Committee of Shinshu University School of Medicine approved the study protocol. Pancreatic carcinoma and benign tissue samples were obtained from patients with primary pancreatic cancer who were operated on at Shinshu University Hospital between March, 2010 and July, 2011. Paraffin sections from tissue samples were prepared for analysis.

**Immunohistochemistry.** Paraffin sections were de-paraffinized and dehydrated as described previously (20). Antigen retrieval was performed with microwave treatment in 10 mM citric acid buffer (pH 6.0) for Nox4 staining and 1 mM EDTA (pH 8.0) for N-cadherin staining. The sections were blocked with 3% BSA, stained with Nox4 or N-cadherin antibodies overnight at 4˚C and developed with labelled polymer (DAKO) and DAB (DAKO). Samples were counterstained with hematoxylin. The staining index was evaluated as the sum of the intensity score (0, no staining; 1+, weak; 2+, moderate; 3+, strong) and the distribution score (0, <5% of cells; 1+, <10% of cells; 2+, between 10% and 50% of cells; 3+, staining of >50% of cells) as described previously (20).

**Statistics.** Differences or correlations between two groups were assessed by Student’s t-test. Differences with values p<0.05 were considered to be statistically significant.

**Results**

**TGF-β promotes EMT, and DPI inhibits its effect in Panc-1 cells.** Type-1 TGF-β is well-documented to play a critical role in induction of EMT during pancreatic cancer progression. To determine whether ROS derived from Nox family enzymes affect TGF-β-induced EMT, pancreatic cancer Panc-1 cells were treated with type-1 TGF-β and the inhibitor of flavoprotein-dependent oxidase DPI, and subsequent cell morphological changes were examined. Consistent with other reports, the number of cells displaying spindle-like shape was increased and cell-to-cell contact was...
attenuated upon TGF-β treatment (Figure 1A) (21). In parallel with this, immunofluorescence staining showed that expression of an epithelial cell marker E-cadherin was repressed (Figure 1A). In contrast, TGF-β-induced morphological alteration was inhibited when cells were exposed to DPI or the chemical antioxidant NAC, and the TGF-β-induced down-regulation of E-cadherin was prevented by DPI treatment (Figure 1A). Immunoblotting analysis demonstrated that TGF-β treatment reduced the expression level of E-cadherin, whereas it up-regulated Snail, a transcription factor negatively-regulating E-cadherin expression (22) (Figure 1B). Furthermore, DPI treatment abolished these TGF-β activities, confirming the above cell biological observations. Taken together, these results indicate that DPI blocks TGF-β-induced EMT.

TGF-β stimulates Nox4-mediated ROS production. Because TGF-β-induced EMT was down-regulated by DPI and NAC, this implies the involvement of ROS derived from Nox isoforms in TGF-β-dependent EMT. Therefore, RT-PCR analysis of mRNAs of Nox family enzymes was performed to explore which Nox isotype responds to TGF-β. The obtained data showed that Nox4 expression was specifically induced by TGF-β treatment, indicating that Nox4 represents a major source for ROS production. We next assessed whether TGF-β regulates Nox4-dependent ROS production in Panc-1 cells. To this end, pSilencer vectors carrying Nox4-specific siRNAs [siNox4(1)] and [siNox4(2)] were created and tested for the ability to suppress Nox4 expression. Immunoblotting analysis showed
that the protein levels of Nox4 were decreased by transfection of Nox4siRNA(2) but not Nox4siRNA(1) (Figure 2B). We, therefore, used Nox4 siRNA(2) for the subsequent Nox4-knockdown study. Luminol assay showed that ROS generation was increased in TGF-β-stimulated cells, and that transfection of Nox4 siRNAs removed a net increase in TGF-β-induced ROS production compared with that of scrambled siRNAs (Figure 2C), indicating that TGF-β up-regulates Nox4 expression, which in turn increases the intracellular ROS level.

**Figure 3.** Inhibition of Nox4 suppresses TGF-β-induced EMT (A) and (B). Panc-1 cells were transfected with siNox4(2) or scrambled siRNAs for 48 h, treated with TGF-β (10 ng/ml) for 48 h and immunostained with anti-E-cadherin antibodies. Phase-contrast (A) and immunofluorescence (B) images of cells are shown. The histogram shows the number of cells with spindle-like shape [mean±S.D. (n=3)]. (C) Panc-1 cells were transfected with siNox4(2) or scrambled siRNAs for 48 h, and cell lysates were subjected to immunoblotting using anti-E-cadherin and anti-Snail antibodies. β-actin is a loading control. (D) Panc-1 cells were transfected with siNox4(2) or scrambled siRNAs for 48 h and re-plated to 24-well plates. Cell migration assays were performed in the presence or absence of TGF-β (10 ng/ml).

Nox4 mediates TGF-β-induced EMT. We next investigated whether Nox4 is involved in regulation of TGF-β-induced EMT. Transfection of Nox4 siRNAs into Panc-1 cells prevented induction of both spindle-like morphological changes and cell-cell dissociation by TGF-β (Figure 3A). Then, the effect of Nox4 siRNAs on E-cadherin expression was determined by immunostaining. Ablation of endogenous Nox4 by Nox4 siRNAs attenuated the repressive effect of TGF-β on E-cadherin (Figure 3B), restoring E-cadherin expression. Consistently, immunoblot analysis revealed that Nox4 siRNAs...
inhibited down-regulation of E-cadherin and concomitant up-regulation of Snail by TGF-β (Figure 3C). Since TGF-β-induced EMT is known to precede augmented cell motility and invasiveness, we determined whether Nox4 is involved in TGF-β-induced cell migration. Cell migration assays in vitro indicated that TGF-β enhanced the motility of Panc-1 cells, while introduction of Nox4 siRNA(2) into the cells blocked cell motility (Figure 3D). Thus, the obtained data suggested that Nox4 is a vital regulator of cell migration associated with EMT in pancreatic cancer cells.

p38MAPK may link Nox4 to Snail. We next attempted to dissect the signaling pathway downstream of the TGF-β-Nox4 axis leading to EMT. Previously, Nox4 has been suggested to transmit signals through stress-responsive p38MAPK in TGF-β-induced smooth muscle α-actin organization and TGF-β-promoted hepatic inflammation (23, 24). We addressed whether Nox4 similarly regulates EMT via activation of the p38MAPK pathway. Treatment with the p38MAPK inhibitor SB203580 prevented both the increased expression of Snail and the decreased expression of E-cadherin in TGF-β-stimulated Panc-1 cells (Figure 4A). Moreover, TGF-β enhanced phosphorylation of p38MAPK, and Nox4siRNAs repressed this TGF-β action (Figure 4B). These data are consistent with the idea that Nox4 regulates the expression of Snail and E-cadherin through activation of p38MAPK in response to TGF-β.

TGF-β-regulated expression of E-cadherin involves PTP1B. Signaling through Nox enzyme-derived ROS is considered to be mediated by redox-sensitive signaling molecules such as protein tyrosine phosphatases (PTPs) (25). With respect to PTPs biochemically linked to Nox4, PTP1B has been well-characterized. PTP1B acts as a critical signal terminator for epidermal growth factor (EGF) receptor signaling, and Nox4 antagonizes the PTP1B action through its oxidation (12). To investigate the role of PTP1B in TGF-β-induced EMT, we utilized PTP1B-D181A, the substrate-trapping mutant of PTP1B (26). Panc-1 cells were transduced with wild-type PTP1B and PTP1B-D181A and treated with TGF-β. Immunoblotting analysis showed that overexpression of wild-type PTP1B significantly blocked TGF-β-induced down-regulation of E-cadherin expression, whereas PTP1B-D181A did not affect the antagonistic effect of TGF-β on E-cadherin expression (Figure 5A). To test whether PTP1B acts as a sensor for Nox4-derived ROS, we examined the oxidation state of PTP1B by utilizing the 5′-IAF-labeling method, where the iodoacetamide derivative competes with intracellular H₂O₂ in reacting with a nucleophilic thiol of redox-sensitive cysteine (Cys-SH) residue (17). TGF-β treatment markedly suppressed the labeling of PTP1B compared with controls, whereas it was readily restored by the addition of DPI (Figure 5B). Because among the Nox family, Nox4 expression is specifically induced

![Figure 5. PTP1B is involved in TGF-β-induced, Nox4-dependent regulation of E-cadherin expression. (A). Panc-1 cells were transfected with HA-wild-type (wt) PTP1B, HA-PTP1B-D181A mutant, or control vector and treated with TGF-β (10 ng/ml) for 48 h, and subjected to immunoblotting with anti-E-cadherin and anti-HA antibodies. β-actin is a loading control. The histogram shows relative band intensity of E-cadherin (mean±S.D. (n=3)). (B). DPI inhibits TGF-β-induced oxidization of PTP1B. Panc-1 cells were transfected with HA-wPTP1B, treated with TGF-β (10ng/ml) in the presence or absence of DPI (10 μM) for 24 h and subjected to 5′-IAF labeling. Labeled PTP1B was detected as described in Materials and Methods. The amount of PTP1B in the lysate was monitored by immunoblotting with anti-HA antibodies. N.S.; Not significant.](image-url)
by TGF-β (Figure 2A), the data suggest that Cys-SH of PTP1B was oxidized by Nox4-generated ROS. Based on these observations, we propose that PTP1B negatively regulates TGF-β signaling, thereby acting as a sensor for Nox4-derived ROS.

Nox4 and N-cadherin are up-regulated in pancreatic cancer patient samples. There was a significant correlation between up-regulation of a neural cell adhesion molecule N-cadherin and TGF-β-induced EMT in metastatic liver tumors (27), and TGF-β treatment of Panc-1 cells caused an increase in the levels of N-cadherin as well as in those of vimentin (21, 27). To further substantiate the involvement of Nox4 in EMT, expression patterns of Nox4, N-cadherin, and TGF-β in primary pancreatic tumors were immunohistochemically examined. High levels of Nox4, N-cadherin, and TGF-β were present in pancreatic adenocarcinoma with high frequency (~80%), whereas in non-cancerous tissues, only marginal immunoreactivity to Nox4 was detected, and neither N-cadherin nor TGF-β was stained (Figure 6A and B). The results indicate that pancreatic tumors elevate the expression level of Nox4, N-cadherin, and TGF-β, implying the association of Nox4 with EMT process.

Discussion

The ROS signaling pathway has been implicated in EMT in both physiological and pathophysiological conditions (28), but the detailed ROS function in the process of EMT remains undefined. In the present study, we provided evidence that Nox4-derived ROS are essential for TGF-β-induced EMT in pancreatic cancer cells: a) TGF-β up-regulates Nox4 expression and Nox4-catalyzed ROS production, b) Nox4-derived ROS
mediate TGF-β-induced morphological transformation from epithelial to fibroblast-like cells, repression of E-cadherin expression, and gain of Snail expression. c) Nox4 is also required for TGF-β-induced cell motility. d) Nox4 transmits EMT signals through p38MAPK and PTP1B.

TGF-β transduces both SMAD-dependent signals through phosphorylation of SMAD and SMAD-independent signals through protein kinases including ERK, Tak-1, and PI3K, which results in the transcription of EMT-related target genes (29). In addition, we found that p38MAPK transmits activation signals from the TGF-β-Nox4 axis, which in turn alters the expression of Snail and E-cadherin as EMT markers. Under normal circumstances, similar biochemical links between Nox4 and p38MAPK have been observed in TGF-β-induced smooth muscle α-actin in aortic smooth cells (23). Although the mechanism underlying Nox4-mediated activation of p38MAPK is currently unknown, several possibilities are conceivable. These include inhibition of the associated phosphatase such as mitogen kinase phosphatase-1 (30) and activation of the upstream p38MAPK kinase, Tak1 (31) by Nox4-generated ROS.

Biological effects of TGF-β are also known to depend on tyrosine kinases such as EGF receptor kinase and Src kinase (32, 33). Because tyrosine phosphorylation status is controlled through the balance between phosphorylation by protein tyrosine kinases and de-phosphorylation by protein tyrosine phosphatases (PTPs), it is possible that PTPs serve as modulators of TGF-β signaling. Among PTPs, the biochemical property of PTP1B is well-characterized. The PTP1B activity is regulated by reversible oxidation of the redox sensitive cysteine-215 residue in the vicinity of its active center (34). ROS generated by Nox4 in response to EGF have been shown to oxidize and inactivate PTP1B that provides an inhibitory constraint, thereby sustaining tyrosine phosphorylation and stimulating EGF receptor-mediated signaling (12). Consistently, our data suggest that Nox4-generated ROS mediate TGF-β-promoted EMT by inhibiting PTP1B as a negative regulator of EMT signaling. It would be of great interest to identify a tyrosine-phosphorylated substrate protein for PTP1B in a future study. Although a recent study has also reported that TGF-β-driven EMT in breast cancer cells involves Nox4 (15), it has not been clarified which pathway downstream of Nox4 mediates the Nox4 action. Thus, our finding that p38MAPK and PTP1B are potential mediators for the Nox4 action provides new insight into the signaling pathway involved in TGF-β-induced, Nox4-dependent EMT.

The notion that Nox4 redox signaling controls TGF-β-promoted EMT appears to be supported by an immunohistochemical study on the expression of Nox4 and N-cadherin, a mesenchymal marker in pancreatic cancer patient samples. Our data indicated that Nox4 expression was significantly upregulated in pancreatic tumors compared to non-cancerous tissues. In parallel with this, the increased expression of N-cadherin and TGF-β was observed in tumor tissues, as reported earlier (27), implying the close association of Nox4 with EMT.

In conclusion, our findings suggest that Nox4 serves as a critical redox signal transducer that regulates TGF-β-induced EMT in pancreatic cancer cells and further emphasizes the importance of intracellular redox control in cancer biology (Figure 7). Because acquisition of EMT phenotype is closely associated with invasion and metastasis of cancer cells, Nox4 could be a critical limiting factor in malignant progression of pancreatic adenocarcinoma.

Acknowledgements

We thank Dr. Z. Zhang for providing PTP1B plasmid DNAs, Dr. J. Nakayama for valuable discussion, and Dr. M. Kawakubo for technical assistance in confocal microscopy. We are also indebted to F. Ushiyama for assistance in manuscript preparation. This work was supported by Grant-in-Aid for Scientific Research for Japan Society for promotion of Science (T. K.: 22300328).

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Received August 12, 2013
Revised September 17, 2013
Accepted September 18, 2013