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Nox4 involvement in TGF-beta and SMAD3-driven induction of the epithelial-to-mesenchymal transition and migration of breast epithelial cells

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Abstract

The epithelial-to-mesenchymal transition (EMT) is the development of increased cell plasticity that occurs normally during wound healing and embryonic development and can be coopted for cancer invasion and metastasis. TGF-beta induces EMT but the mechanism is unclear. Our studies suggest Nox4, a member of the NADPH oxidase (Nox) family, is a source of reactive oxygen species (ROS) affecting cell migration and fibronectin expression, an EMT marker, in normal and metastatic breast epithelial cells. We found TGF-beta induces Nox4 expression (mRNA and protein) and ROS generation in normal (MCF10A) and metastatic (MDA-MB-231) human breast epithelial cells. Conversely, cells expressing a dominant-negative form of Nox4 or Nox4-targeted shRNA showed significantly lower ROS production upon TGF-beta treatment. Expression of a constitutively active TGF-beta receptor type I significantly increased Nox4 promoter activity, mRNA and protein expression, and ROS generation. Nox4 transcriptional regulation by TGF-beta was SMAD3-dependent based on the effect of constitutively active SMAD3 increasing Nox4 promoter activity, whereas dominant-negative SMAD3 or SIS3, a SMAD3-specific inhibitor, had the opposite effect. Furthermore, Nox4 knockdown, dominant-negative Nox4 or SMAD3, or SIS3 blunted TGF-beta induced wound healing and cell migration, whereas cell proliferation was not effected. Our experiments further indicate Nox4 plays a role in TGF-beta regulation of fibronectin mRNA expression, based on the effects of dominant-negative Nox4 in reducing fibronectin mRNA in TGF-beta treated MDA-MB-231 and MCF10A cells. Collectively, these data indicate Nox4 contributes to NADPH oxidase-dependent ROS production that may be critical for progression of the EMT in breast epithelial cells, and thereby has therapeutic implications.

Keywords

NADPH oxidase 4 (Nox4); Cell Migration; TGF-beta signaling; Epithelial-to-Mesenchymal Transition (EMT)

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Disclosure

The authors declared no conflict of interest.

Introduction

Recent observations suggest reactive oxygen species (ROS) play important roles in TGF-beta-induced epithelial-to-mesenchymal transition (EMT) and cell mobility of many cell types; however, little is known about redox-dependent TGF-beta-induced mechanisms in breast epithelial cells [1, 2]. EMT is a common process during embryonic development, wound healing, and organ fibrosis in which epithelial cells undergo morphological changes resulting in increased cell plasticity and mobility as they transition into a mesenchymal-like cell phenotype. Recently, the EMT has been a focus of tumor cell migration and invasion. Hallmarks of cells undergoing EMT include cytoskeletal reorganization, loss of polarization, disruption of cell-cell junctions, and alterations in ECM production. Tumor microenvironments secrete soluble growth factors, inflammatory cytokines (TGF-beta, HGF, VEGF, PDGF), and matrix proteins that activate epithelial cells to induce specific EMT transcriptional programs. TGF-beta is established as a contributor to EMT progression. Smooth muscle actin (SMA) and fibronectin expression are established indicators of EMT progression, both of which are modulated in a redox-dependent manner [2, 3].

Redox mediated signaling has become a focus toward understanding EMT progression. Oxidative stress is primarily associated with cytotoxicity, however, ROS are now recognized as important regulators of signaling pathways and gene transcription [4, 5]. ROS can act through oxidative modification of protein tyrosine phosphatases, redox-dependent transcription factors, and DNA [6–9]. Major sources of cellular ROS include NADPH oxidase enzymes (Nox), mitochondrial electron transport, xanthine oxidase, and nitric oxide synthase [3]. Evidence suggests the Nox family of ROS generating enzymes (Nox1–5 and Duox1–2) are significant players in redox-mediated signaling [10]. In particular, Nox4 was identified as a TGF-beta-responsive oxidase implicated in cytoskeletal alterations in endothelial cells, osteoblast differentiation, pulmonary fibroblast proliferation, idiopathic pulmonary fibrosis, and enhanced oxidative stress in hepatitis C virus infection [11–16].

Nox4 is a 578 amino acid, six transmembrane domain flavocytochrome that functions in generating superoxide by transporting electrons from cytosolic NADPH across biological membranes to molecular oxygen. Nox4 has 39% homology to Nox-2 (gp91^{phox}), the Nox family prototype. Originally described in the kidney, Nox4 mRNA is also detected in other human and murine tissues including bone, vascular tissue, heart, liver, and lung [17–22]. Nox4 is primarily localized in perinuclear regions and the ER but is also detected at the plasma membrane, focal adhesions, and associated with mitochondria [9, 22–27]. Although Nox4 expression is regulated by TGF-beta in many cell types, little is known about its role in the EMT of breast epithelial cells.

In a previous study Nox family genes were surveyed in several human cancer cell lines and in patient tumors and adjacent normal tissues [28]. Their results showed increased Nox4 in breast cancer cell lines as well as in patient tumor samples. In addition, Graham, *et al.*, reported high expression of Nox4 in invasive and non-invasive cell lines as well as in patient breast tumor samples [29]. Furthermore, their report indicates overexpression of Nox4 in MCF12A or MDA-MB-435 cells resulted in cellular senescence, resistance to apoptosis, and tumorigenic transformation. Interestingly, several studies reported the presence of senescent cells in tumor microenvironments. Although resistance to mitogenic stimuli is characteristic of senescent cells, these cells remain metabolically active and capable of secreting growth factors that activate tumor cells [30].

Here we identify Nox4 as the principle source of TGF-beta-induced superoxide production in MCF10A and MDA-MB-231 epithelial cells, and we show Nox4 functions as an

important player in EMT-related events including cell migration and fibronectin gene regulation.

Materials and Methods

Cell culture

Immortalized human MCF10A and human metastatic MDA-MB-231 breast epithelial cell lines were obtained from the ATCC (Rockville, MD). MCF10A cells were maintained in Dulbecco's minimal essential medium/F-12 (DMEM/F12) (Invitrogen, Carlsbad, CA) supplemented with 5% horse serum (Sigma, St. Louis, MO), 500ng/ml hydrocortisone (Sigma), 20ng/ml EGF (R and D Systems, Minneapolis, MN), 100ng/ml cholera toxin (Sigma), 10µg/ml insulin (Sigma), and 100 mg/ml of penicillin-streptomycin (Invitrogen). MDA-MB-231 cells were maintained in Dulbecco's minimal essential medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (HyClone/Thermo Scientific, Logan, UT) and 100 mg/ml of penicillin-streptomycin. All cells were grown in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Plasmids and primers

Vectors encoding truncated, dominant-negative Nox4 (Nox4-DN; residues 1–305), pGL3-Nox4 (–1848), and Nox4 shRNA plasmids were previously described [16]. Human Nox4 was generated using full-length Nox4 (GenBank accession number NM_016931) cDNA subcloned into pcDNA3.3. The HA-tagged constitutively active TGF-beta receptor type-1 (T204D) cDNA was subcloned into pcDNA3.1 TOPO (Invitrogen). The full-length receptor was amplified from MCF10A cDNA using the following primers: forward primer 5'-CACCATGGAGGCGGCGGTCTGCTCC-3', and reverse primer 5'-TTACTAGGCGTAATCGGGGACATCATAGGGGTACATTTTGATGCCTTCCTGTTGACT-3' (HA epitope tag underlined in reverse primer). Site directed mutagenesis was performed for mutation of TGF-beta receptor-1 residue 204 from threonine to aspartate with the following primers: forward primer 5'-AGAACAATTGCGAGAGATATTGTGTACAAGAA-3', and reverse primer 5'-TTCTTGTAACACAATATCTCTCGCAATTGTCT-3' (mutated sequence underlined). All constructs were verified by automated DNA sequencing (Macrogen, Rockville, MD).

Transient transfections

MCF10A cells were transfected with Fugene 6 (Roche, Indianapolis, IN, USA) using manufacturer's protocols. Briefly, 2.5×10^5 cells were seeded in 6-well tissue culture dishes (BD Biosciences) 24 hours before transfection. Transfection mixtures were incubated at RT for 20 minutes in serum-free DMEM and then added drop-wise to cells. MDA-MB-231 cells were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's protocols. Briefly, 2×10^5 cells were seeded on 6-well plates 24 hours before transfection. The transfection mixtures were incubated at RT for 20 minutes in serum-free DMEM and added drop-wise to cells containing serum-free medium. After 4 hours, the medium was changed to DMEM containing serum.

ROS detection

Cells were collected by trypsinization and washed twice with Hank's Balanced Salt Solution (HBSS; Invitrogen). Cells were treated 10 min at 37°C with or without 10 µM diphenylene iodonium chloride (DPI) (Sigma). Kinetic ROS measurements were performed by chemiluminescence in 96-well plates at 37°C over a 1 hr time course using a Luminoskan luminometer (Thermo, Waltham, MA, USA). Extracellular superoxide production was measured as superoxide dismutase-inhibitable chemiluminescence detected using Diogenes

reagent (National Diagnostics, Atlanta, GA, USA). Extracellular H₂O₂ was measured by a luminol/HRP-based chemiluminescence assay. Briefly, the cells were collected as above and treated with or without 10 μM DPI (37°C for 10 min). An equal volume of HBSS containing 1 mM luminol and 20 U/ml HRP was added following DPI treatment. Luminescence was measured in 96-well plates at 37°C over a 1 hr time course using a Luminoskan luminometer.

RNA isolation and RT-PCR

Total RNA from MCF10A or MDA-MB-231 cells was extracted from cells with Trizol (Invitrogen). One microgram of total RNA was used for ThermoScript RT-PCR. Both were conducted according to manufacturer's protocols (Invitrogen).

Quantitative real-time RT-PCR

Gene expression was quantified by real-time PCR using an ABI Prism 7500 RT-PCR System (Applied Biosystems). One microgram of total cellular RNA was reverse transcribed with ThermoScript RT-PCR kit (Invitrogen). SYBR Green PCR mix (Invitrogen) was used to detect mRNA expression with the following human specific primers: (Fibronectin) forward 5'-CGAGCTTCCCCAACTGGTAACCC-3', reverse 5'-GGTGGCACCTCTGGTGAGGC-3'; (GAPDH) forward 5'-AGCCACATCGCTCAGACAC-3', reverse 5'-GCCCAATACGACCAAATCC-3'. Primers designed to detect human Nox1-5, Duox1 or 2, and p22^{phox} were used as previously described [16].

Antibodies and immunoblotting analysis

MCF10A cell lysates were processed for Western blotting as previously described [16] and probed with the following antibodies: anti-rabbit polyclonal Nox4 (Novus Biologicals); mouse monoclonal anti-V5 (Invitrogen); mouse monoclonal anti-HA (Sigma); rabbit monoclonal anti-phospho-SMAD3, rabbit polyclonal anti-phospho-SMAD2, rabbit monoclonal anti-SMAD3, and mouse monoclonal anti-SMAD2 (Cell Signaling); rabbit polyclonal anti-beta Tubulin H-235 (Santa Cruz).

Luciferase assay

The human Nox4 promoter (-1848) was subcloned into pGL3 reporter plasmid as previously described [16]. Luciferase assays were performed according to manufacturer's protocols (Promega). Briefly, 1 × 10⁵ MCF10A cells were seeded per well in six-well dishes. 24 hours later, each well was transfected with 2 μg total DNA using 4 μL FuGENE. Luciferase expression was assayed 48 hours later by lysing cells in 200 μL cell lysis buffer, aliquoting 20 μL lysate in triplicate in 96-well plates, and injecting 100 μL luciferase assay buffer per well; luciferase activities from multiple transfections were normalized to cells transfected with pGL-basic vector, which lacked the Nox4 reporter sequence. Assays were performed using a Luminoskan luminometer.

Wound Closure Assay

2 × 10⁵ MDA-MB-231 cells were seeded on 6-well tissue culture plates 24 hours before transfection. The cells were transfected with 2 μg of either control vector (empty or GFP-expressing) or vectors expressing Nox4-DN, Nox4-targeted or control shRNA, TGFBR1(T204D), SMAD3-CA, or SMAD3-DN proteins plasmids. Wounding was performed by scratching cell monolayers with a 200 μl plastic pipette tip 48 hours post-transfection. For treated cells, either 5 ng/mL TGFbeta or 10 μM SIS3 (SMAD3 inhibitor) were added immediately following the scratch. Images of scratched monolayers were monitored under phase contrast microscopy just before addition of reagents (0 hr) and 6

hours (MDA-MB-231) or 24 hours (MCF10A) later. Experiments were repeated three to five times.

Matrigel Cell Migration Assay

MDA-MB-231 cells were seeded and transfected as described above, then trypsinized and washed 24 hours later. 1.5×10^5 transfected cells were seeded in upper chambers of 6-well BD Biocoat matrigel transwell culture plates (BD Biosciences) and incubated with lower chambers containing 1.5ml of DMEM medium with TGF-beta (5ng/ml). After 24 hours, non-migrating cells were scraped away and migrating cells were stained with Diff Stain (IMEB, San Marcos, CA). Invading cells were counted from 10 random fields. Matrigel experiments were repeated three times.

Cell proliferation assay

Cell proliferation was assessed by the Click-iT EdU Imaging Kit according to manufacturer's instructions (Invitrogen). Briefly, cells after EdU-labeling (1hr exposure), fixation and permeabilization were stained with anti-EdU Alexa Fluor 555. Cell densities were determined by counting DAPI-stained nuclei in fields, using a Leica fluorescence microscope (average n=6–10 fields/sample), after 24-hour treatments with TGF-beta and normalized relative to densities of samples before TGF-beta treatments. The proportion of proliferating cells was calculated based on the number of EdU-positive cells divided by the number of DAPI-stained nuclei (20× magnification; n=6–10).

Statistical Analysis

Data are represented as the means $\pm$ standard deviations of the results of at least three independent experiments. Student's t test was used to calculate significance values. Significant values are indicated as (*) P value of <0.05 or (**) P value of <0.01.

Results

TGF-beta induces NADPH oxidase-dependent superoxide production in normal and in metastatic human breast epithelial cells

To investigate the effect of TGF-beta on NADPH oxidase-dependent ROS production in human breast epithelial cells, we treated immortalized (MCF10A) or metastatic (MDA-MB-231) cell lines with TGF-beta for 24 hours. We found MCF10A cells produced a significant amount of extracellular NADPH oxidase-dependent superoxide when treated with increasing concentrations of TGF-beta. Superoxide production was inhibited with DPI, a selective inhibitor of Noxes and other flavoenzymes (Fig. 1A, left). Interestingly, detection of extracellular H_2O_2 by luminol/HRP was not inhibited by DPI in cells treated with TGF-beta (Fig. 1A, right), suggesting the prevalent H_2O_2 source is not a Nox enzyme. Similarly, the effect of TGF-beta on MDA-MB-231 cells resulted in increased DPI-sensitive superoxide production, whereas, H_2O_2 generation was unaffected by TGF-beta or DPI treatment (Fig. 2B). These results indicate TGF-beta treatment of both normal and metastatic breast epithelial cells results in NADPH-oxidase dependent superoxide production at the plasma membrane.

To determine if induction of a member of Nox family enzymes is involved in TGF-beta-mediated ROS production, we measured Nox gene mRNA levels by quantitative PCR. Nox4 mRNA was increased upon TGF-beta treatment of MCF10A and MDA-MB-231 cells (Fig. 1C), while expression of other Nox family members showed no significant change. Increased Nox4 mRNA was further confirmed by semi-quantitative PCR in both cell lines (Fig. 1D). Nox4 protein expression was also upregulated in TGF-beta treated cells compared to untreated control cells (Fig. 1E). Activation of TGF-beta signaling was confirmed by

immuno-detection of phosphorylated SMAD2 and SMAD3. Basal levels of SMAD proteins were not affected by TGF-beta. Our results indicate TGF-beta increases NADPH oxidase-dependent superoxide release, which correlates with a significant up-regulation of Nox4 mRNA and protein expression in both normal and metastatic breast epithelial cells.

Dominant-negative Nox4 and Nox4-specific gene knockdown reduce TGF-beta-induced superoxide generation

To determine the role of Nox4 as a TGF-beta responsive superoxide generator, we constructed a Nox4 dominant-negative cDNA with deleted C-terminal FAD and NADPH domains. We observed that overexpression of Nox4-DN diminished superoxide production compared to cells overexpressing empty vector control in MCF10A or MDA-MB-231 cells (Fig 2A left and right panels). These results were further confirmed by knockdown of endogenous Nox4 with Nox4-specific shRNA. Cells transfected with Nox4-shRNA plasmids showed a significant reduction of Nox4 expression at both the mRNA and protein level (Fig. 2B). Furthermore, decreased expression of endogenous Nox4 resulted in reduced TGF-beta-mediated superoxide generation in MCF10A and MDA-MB-231 cells (Fig. 2C, left and right panels). Collectively, these data indicate Nox4 is the primary source of TGF-beta-induced ROS in both normal and metastatic breast epithelial cells.

Constitutive activation of TGF-beta receptor-1 modulates Nox4 expression and superoxide generation

To further validate the role of TGF-beta mediated Nox4 induction, MCF10A cells were transfected with an HA epitope-tagged constitutively active TGF-beta type 1 receptor (T204D) cDNA construct. Interestingly, MCF10A cells expressing T204D produced a significant level of DPI-sensitive superoxide in the absence of TGF-beta treatment compared to control (Fig. 3A). Superoxide generation was further increased upon TGF-beta stimulation in both T204D and vector control cells. Constitutive activation of TGF-beta receptor-1 also affected Nox4 protein levels. TGF-beta stimulated MCF10A cells increased Nox4 protein in control and T204D transfected cells (Fig. 3B). Untreated T204D cells also resulted in an up-regulation of Nox4 expression. These results correlate with the T204D-dependent superoxide production in panel A. Subsequent immunoblotting with phospho-SMAD3, total SMAD3, and HA antibodies confirmed TGF-beta signaling activation and T204D expression. Real-time quantitative PCR of MDA-MB-231 cells expressing T204D also revealed Nox4 mRNA was substantially upregulated in the presence or absence of TGF-beta in this metastatic cell line (Fig. 3C).

TGF-beta/SMAD3 signaling modulates the human Nox4 promoter in breast epithelial cells

We previously reported TGF-beta treated HepG2 hepatocytes significantly increased Nox4 promoter activity indicating Nox4 expression is regulated by TGF-beta at the transcriptional level [16]. Here, we further confirmed our results in MCF10A cells expressing the human Nox4 promoter (-1848 bp) linked to a luciferase reporter plasmid co-transfected with empty vector or T204D. Nox4 promoter-driven luciferase activity was significantly increased when stimulated by TGF-beta in both control and T204D cells (Fig. 4A). To demonstrate the activating contribution of SMAD3 on the Nox4 promoter, we selectively inhibited SMAD3 by pretreating MCF10A cultures transfected with T204D or vector control with SIS3, a SMAD3 specific inhibitor, 24 hours before assaying luciferase activity. As expected, SIS3 treatment significantly reduced luciferase activity compared to untreated cells (Fig. 4B). Similar inhibition was observed by transfecting MCF10A cultures with a SMAD3 dominant-negative (SM3-DN) construct compared to SMAD3 wild-type (SM3-WT), whereas transfection of a constitutively active variant (SM3-CA) induced significantly higher expression compared to both wild-type and vector control (Fig. 4C). Collectively, these data indicate the TGF-beta/SMAD3 signaling pathway positively regulates the Nox4 promoter.

However, we cannot rule out involvement of other transcription factors involved in TGF-beta regulation of the Nox4 promoter.

Nox4 contributes to TGF-beta-mediated cell migration

Excess ROS production is known to play a role in EMT gene regulation and cell migration. In order to determine if Nox4 is involved in TGF-beta mediated migration of breast epithelial cells, we performed a wound-healing assay on confluent MDA-MB-231 and MCF10A cells and examined the effects of Nox4-DN or Nox4-shRNA. TGF-beta treated vector control cells displayed an advanced stage of wound closure at 6 hrs (MDA-MB-231 cells; upper panel) or 24 hrs (MCF10 cells; lower panel), whereas cells expressing Nox4-DN (MDA-MB231) or Nox4-shRNA (MCF-10A) displayed minimal wound closure in the presence of TGF-beta (Fig. 5A).

To further confirm involvement of Nox4 in cell migration, we utilized the Matrigel migration assay on MDA-MB-231 cells. Vector control or Nox4-DN-transfected cells were seeded in the upper chambers of transwells atop a porous membrane coated with a thin layer of Matrigel. The chamber was then placed in serum-free medium with or without TGF-beta. Vector-transfected cells showed enhanced migration through the Matrigel and porous membrane after 24 hours when incubated with TGF-beta containing medium in comparison to untreated, whereas cells over-expressing Nox4-DN did not show a significant increase in migration in response to TGF-beta (Fig. 5B).

TGF-beta has also been shown to enhance cell proliferation [31]; therefore, we examined whether Nox4 is involved in TGF-beta-mediated cell proliferation of MDA-MB-231 cells. Cell proliferation after 24 hours of TGF-beta treatment was determined either by measuring relative changes in cell density (DAPI-stained nuclei/ surface area) or by monitoring EdU incorporation for 1 hour after 24 hour TGF-beta treatment. Expression of dominant-negative Nox4 did not affect the increases in cell density or the number of EdU-positive cells (Fig. 5C).

Lastly, we examined whether SMAD3 inhibition would abrogate cell migration in MDA-MB-231 cells in a manner similar to transfection of the Nox4-DN construct. Cultures transfected with the T204D and empty vector or with T204D and constitutively active Smad3 (SM3-CA) showed enhanced wound closure after 6 hours compared to cultures transfected with T204D and dominant negative Smad3 (SM3-DN) (Fig. 5D). Treatment with SIS3 similarly inhibited wound closure in cells transfected with T204D, whereas no treatment resulted in wound closure after 6 hours, and cells transfected with vector alone showed minimal cell migration (Fig. 5E). Vector control cells treated without vs. with SIS3 provide an indication of the basal SMAD3 activity that contributes to cell migration in MDA-MB-231 cells. Together, these results indicate Nox4 via Smad3 is involved in TGF-beta-mediated cell migration but not cell proliferation.

Nox4 modulates TGF-beta-mediated fibronectin expression

It has been well established that TGF-beta induces fibronectin in migrating cells and in cells undergoing EMT [32]. Nox4 has been implicated in the modulation of matrix protein gene regulation including fibronectin and smooth muscle actin in cardiac fibroblasts, mesangial cells, and pulmonary fibroblasts [12, 33–35]. Recently, Nox4 was shown to be involved in TGF-beta/SMAD3-dependent gene regulation of fibronectin and smooth muscle actin in human lung mesenchymal cells [35]. We investigated the involvement of Nox4 in breast epithelial cell migration by analyzing fibronectin expression in TGF-beta treated cells transfected with Nox4-DN or Nox4-shRNA. Compared to the vector control, Nox4-DN expression decreased TGF-beta-induced fibronectin mRNA in both MDA-MB-231 cells

(left panel) and MCF10A cells (right panel) as indicated by quantitative real-time PCR (Fig. 6A). Similarly, shRNA-mediated knockdown of endogenous Nox4 significantly reduced TGF-beta-mediated fibronectin expression compared to control shRNA transfected cells (Fig. 6B) in both cell models. Finally, we examined whether Nox4 cDNA overexpression can induce fibronectin expression in the absence of TGF-beta treatments (Fig. 6C). The overexpressed Nox4 mRNA levels exceeded those of endogenous Nox4 induced by TGF-beta in both cell lines, however fibronectin mRNA levels were only marginally affected in the MCF10A cells. Collectively, our data indicate an important role for Nox4 in TGF-beta-induced breast epithelial cell migration and fibronectin expression, but that Nox4 overexpression itself does not appear to be sufficient to mediate these effects.

Discussion

TGF-beta-induced oxidative stress has been implicated in regulating EMT progression, including gene expression, cytoskeletal rearrangement, and cell mobility. Here we show Nox4 contributes to TGF-beta-mediated cell migration and fibronectin expression of normal (MCF10A) and metastatic (MDA-MB-231) breast epithelial cells. We found TGF-beta induced NADPH oxidase-dependent superoxide release in both MCF10A and MDA-MB-231 breast epithelial cells (Fig. 1A and B). We show Nox4 mRNA and protein expression was substantially increased upon TGF-beta stimulation (Fig. 1C-E). Furthermore, expression of constitutively active TGF-beta receptor type-1 increased NADPH oxidase-dependent superoxide release, and Nox4 mRNA, protein, and promoter activity (Figs. 3 and 4A). We further show that wild type and constitutively active SMAD3 significantly increase Nox4 promoter activity, whereas dominant-negative SMAD3 and SIS3, a SMAD3-specific inhibitor, decreased promoter activity (Fig. 4B and C). In contrast, TGF-beta-mediated superoxide was significantly reduced in MCF10A and MDA-MB-231 cells expressing a dominant-negative form of Nox4 or Nox4-specific shRNA (Fig. 2), indicating Nox4 is the primary source of superoxide generation. Moreover, wound closure and cell migration assays of epithelial cells expressing dominant-negative Nox4 or Nox4-shRNA were significantly reduced in response to TGF-beta treatment (Fig. 5A and B), indicating Nox4 is involved in TGF-beta regulation of breast epithelial cell migration. Inhibition of endogenous SMAD3 by transfection of a dominant-negative construct (Fig. 5D) or SIS3 treatment (Fig. 5E) also substantially reduced cell migration further suggesting SMAD3 is a key regulator of Nox4-mediated cell migration. Interestingly, dominant-negative Nox4 had no effect on TGF-beta-mediated cell proliferation (Fig. 5D), indicating TGF-beta modulation of Nox4 plays important roles in cell migration rather than cell proliferation. We also found TGF-beta up-regulation of fibronectin, an indicator of EMT progression, was reduced in dominant-negative Nox4 or Nox4 shRNA transfected breast epithelial cells (Fig. 6), suggesting Nox4 is involved in redox-dependent fibronectin gene regulation during EMT. Taken together, our results indicate Nox4 plays an important role in breast epithelial cell migration and fibronectin expression driven by a TGF-beta/SMAD3 signaling mechanism, as summarized in Figure 7.

Previous reports indicate TGF-beta modulates Nox4 in many cell types including vascular endothelial cells, kidney fibroblasts, hepatocytes, and lung epithelial cells [36–38], however, little is known about TGF-beta mediated ROS generation in breast epithelial cells. Breast cancer epithelial cells are known to secrete high levels of soluble TGF-beta into the tumor microenvironment allowing for paracrine and autocrine TGF-beta signaling [39, 40]. While TGF-beta can have pro-apoptotic effects on specific cell types, MDA-MB-231 and MCF10A breast epithelial cells are resistant to TGF-beta-mediated growth inhibition [31, 39, 41]. Rather, breast epithelial cells respond to TGF-beta by increasing EMT gene expression and enhancing their migratory and metastatic potential. Tobar *et al.* recently reported Nox4-dependent ROS in migration of MCF-7 cells, a mildly invasive breast

epithelial line [36]. They demonstrated that TGF-beta is a soluble factor secreted by RMF-EG mammary fibroblasts that increased Nox4 mRNA, ROS production and cell migration of co-cultured MCF-7 cells. Here we show TGF-beta-mediated cell migration is activated through a TGFBR1 → SMAD3 → Nox4 signaling pathway in both normal and highly metastatic breast epithelial cells.

Nox4 has been implicated in EMT gene regulation and migration of other cell types. Bondi et al. reported TGF-beta induced Nox4-dependent modulation of EMT markers alpha-smooth muscle actin (SMA) and fibronectin during conversion of rat kidney fibroblast to a myofibroblast phenotype [42]. Nox4 was also reported to have a promigratory effect on angiotensin-II-treated vascular adventitial myofibroblasts [43]. Recently, Nam et al. described ROS generated from Nox1 and Nox4 involved in migration of human keratinocytes co-treated with HGF and TGF-beta [37], and Pendyala et al. indicated Nox4 is involved in hyperoxia-induced pulmonary artery endothelial cell migration [44]. Also, Meng et al. reported Nox4 and Rac1 mediate IGF-1-induced ROS production and cell migration in vascular smooth muscle cells [45]. They also demonstrated IGF-1-induced matrix metalloproteinase (MMP)-2 and 9 activities, indicative of EMT progression, were inhibited by knockdown of Nox4 or Rac1 inactivation through independent mechanisms.

Several ECM proteins are modulated under the control of TGF-beta including increased fibronectin expression. Fibronectin is important for directing cell attachment and migration during the cell reorganization and migratory process. Previous reports implicated Nox4 in cytoskeletal alterations and regulation of fibronectin expression. Under high glucose conditions, the diabetic rat kidney showed Nox4-derived ROS can mediate renal hypertrophy and fibronectin expression [33, 34]. Also, Hu et al. reported expression of dominant-negative Nox4 in HUVEC cells blocked the effect of TGF-beta on filopodia formation and F-actin assembly [13]. In vascular smooth muscle cells, p22phox association with poldip2 appears to increase Nox4 activity, which was correlated with cytoskeletal remodeling and focal adhesion turnover [46]. Our study not only demonstrates Nox4 is involved in TGF-beta-induced breast epithelial cell migration by wound healing and matrigel assays, but also provides evidence that Nox4 plays a role in EMT and cell mobility by modulating fibronectin expression.

The role of SMAD proteins, particularly SMAD3, during TGF-beta-induced EMT has been well established. Previous observations showed in SMAD3 knockout mice that EMT of lens epithelium after injury is blocked in the absence of SMAD3, along with decreased expression of EMT markers including, snail, SMA, and collagen [47]. Another study using selective knockdown of SMAD2 and SMAD3 demonstrated that TGF-β-induced EMT is SMAD3 dependent in lung cancer cells [48]. Moreover, Sturrock et al. showed TGF-beta-induced Nox4 mRNA and ROS were decreased in pulmonary smooth muscle cells overexpressing either dominant-negative SMAD2 or SMAD3 [49]. By comparing constitutively active SMAD3 (C-terminal pseudo-phosphorylated) to dominant-negative SMAD3 (deletion of the MH2 domain), we are the first to demonstrate that phosphorylation of the C-terminus of SMAD3 positively regulates the human Nox4 promoter in breast epithelial cells. While we cannot rule out involvement of other transcription factors in response to TGF-beta, our findings provide an important foundation for identifying SMAD binding elements (SBE) and other key components involved in TGF-beta regulation of the Nox4 promoter.

The precise mechanisms of Nox4-derived ROS in TGF-beta-mediated cell migration and EMT progression of breast epithelial cells warrants further investigation. It is possible that Nox4-dependent ROS alter the activation states of signaling components or affect ECM proteins involved in EMT processes by direct protein oxidation. ROS have been shown to

modulate cell signaling by altering oxidation states of proteins such as tyrosine phosphatases (PTP1B, SHP2, PTEN, and LMW-PTP), redox sensitive transcription factors (NF κ -B, p53, and AP-1), receptor and non-receptor tyrosine kinases (EGFR, PDGFR, and Src), and cytoskeletal proteins (beta-actin or associated proteins) [50–53]. Moreover, a previous study reporting a proteomic screen for cysteine glutathionylated proteins revealed several cytoskeletal proteins including actin, cofilin, vimentin, and profilin are redox sensitive[54]. Nox4 subcellular localization has been described in many places including the plasma membrane, ER, perinuclear, and nuclear compartments. Nox4 was recently reported to co-localization with F-actin at sites of invadopodia; and Nox4 knockdown resulted in reduced invadopodia formation in Src transformed 3T3 cells [55]. Determination of TGF-beta-induced Nox4 localization will provide further insight into Nox4-dependent redox mechanisms involved in EMT progression and cell motility.

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Abbreviations

ROS	Reactive oxygen species
TGF-beta	Transforming growth factor-beta
EMT	Epithelial-to-mesenchymal transition
ECM	Extracellular matrix

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Highlights

- We show that Nox4 is involved in the epithelial-to-mesenchymal transition.
- TGF-beta induces Nox4-dependent ROS generation and migration of epithelial cells.
- Inhibition of Nox4 or SMAD3 attenuates TGF-beta-mediated cell migration.
- Inhibition of SMAD3 significantly reduces TGF-beta-induced Nox4 promoter activity.
- Nox4 is involved in TGF-beta-induced fibronectin expression.

Figure 1. TGF-beta induces Nox4-dependent superoxide generation in normal and metastatic human breast epithelial cells

(A) Left, MCF10A cells were treated with increasing concentrations of TGF-beta for 24 hours. Cells were assayed for superoxide generation with Diogenes luminescence for 1 hour in the absence or presence of the NADPH oxidase/ flavoenzyme inhibitor DPI (10 μ M) (n = 3, each experiment in triplicate). Units are represented in relative light units (RLU). Right, MCF10A cells were treated for 24 hours as in left panel and assayed for H₂O₂ release with luminol and horseradish peroxidase (HRP) for 1 hour with or without DPI (10 μ M) (n = 3, in triplicate). (B) MDA-MB-231 cells were treated as described in panel A for 24 hours and then assayed for superoxide (left) or H₂O₂ [56] release as described above. (C) Real-time

quantitative PCR (qPCR) analysis of NADPH oxidase gene expression patterns in TGF-beta treated MCF10A and MDA-MB-231 cells. Twenty-four hours after TGF-beta (MCF10A, 10 ng/ml)(MDA-MB-231, 5 ng/ml) stimulation, total RNA was collected and reverse transcribed. Total cDNA was used for qPCR with human Nox-specific primers (see materials and methods). Units are described as Nox mRNA relative to GAPDH mRNA expression as a reference (MCF10A, n=4 in triplicate) (MDA-MB-231, n=3 in triplicate). (D) Human Nox4 and actin primers were used for PCR amplification of total cDNA reverse-transcribed from MCF10A or MDA-MB-231 total RNA collected from cells treated with or without TGF-beta for 24 hours. (E) Immunoblot analysis of MCF10A and MDA-MB-231 cells treated 24 hours with TGF-beta shows induction of Nox4 protein levels along with enhanced SMAD2 and SMAD3 phosphorylation. Total cell lysates (40 µg) were analyzed by Western blotting by sequentially probing with anti-Nox4, anti-phospho-SMAD3, anti-phospho-SMAD2, anti-SMAD3, and anti-SMAD2 antibodies. * p <0.05; **p <0.01

Figure 2. Dominant negative Nox4 and shRNA-mediated Nox4 gene knockdown suppress TGF-beta-induced superoxide production in normal and metastatic breast epithelial cells
 (A) MCF10A (left panel) and MDA-MB-231 (right panel) cells were transfected with vector alone or Nox4-DN cDNA. Twenty-four hours after transfection, cells were treated with TGF-beta (MCF10A, 10 ng/ml; MDA-MB-231, 5 ng/ml) for 24 hours. Cells were then collected and assayed for superoxide generation with or without DPI (10 μ M) ($n = 3$, in triplicate). (B) MCF10A and MDA-MB-231 cells were transfected with non-targeting control shRNA or Nox4-specific shRNA plasmids (2 μ g) for 48 hours. Nox4 mRNA expression was quantified by qPCR using GAPDH as an internal control. Nox4 protein expression was analyzed by Western blotting by sequentially probing with anti-Nox4 and

anti-tubulin antibodies. (C) MCF10A and MDA-MB-231 cells were transfected as in panel B and assayed for superoxide production with Diogenes reagent for 1 hour in the absence or presence of DPI (10 μ M) (n = 3, in triplicate).

Figure 3. Constitutively active type 1 TGF-beta receptor enhances superoxide production and Nox4 expression in MCF10A and MDA-MB-231 cells

(A) MCF10A cells were transfected with vector alone or constitutively active HA-tagged TGF-beta receptor type1 (TBR1 (T204D)). Twenty-four hours post-transfection, cells were left untreated or treated with TGF-beta (10 ng/ml) for 24 hours. Cells were collected and assayed for superoxide production with Diogenes reagent for 1 hour in the absence or presence of DPI (10 μ M) (n = 3, in triplicate). (B) Nox4 induction by TGF-beta and TBR1 (T204D) correlates with enhanced SMAD3 phosphorylation. MCF10A cells were transfected and treated as described in panel A. Forty micrograms of total cell lysate were analyzed by Western blotting. The immunoblot was probed sequentially with antibodies

against Nox4, HA-tag, phospho-SMAD3, and total SMAD3. (C) MDA-MB-231 cells were transfected with vector alone or T204D for 24 hours. Twenty-four hours post-transfection, transfected cells were left untreated or treated with TGF-beta (5 ng/ml) for an additional 24 hours. Nox4 specific primers were used for quantitative real-time PCR. Nox4 mRNA expression was normalized to GAPDH as an internal control (n = 3). Units are described as fold change of Nox4 mRNA expression compared to vector untreated samples. * p <0.05; **p <0.01, relative to untreated vector control.

Figure 4. TGF-beta/SMAD3 signaling modulates the human Nox4 promoter in breast epithelial cells

(A) MCF10A cells were co-transfected with Nox4 promoter luciferase reporter plasmid (pGL3-Nox4 (-1848)) and vector plasmid or T204D plasmid for 24 hours followed by TGF-beta (5 ng/ml) treatment or left untreated for 24 hours. Total cell lysates were collected and assayed for luciferase activity by luminescence as described in the materials and methods section (n = 4, in triplicate). Luciferase activity is described as fold change in relative light units (RLU) compared to the vector untreated cells. (B) MCF10A cells were transfected as in panel A and treated with SIS3 (10μM) for 24 hours (n=2). (C) MCF10A cells were co-transfected with Nox4 promoter luciferase reporter plasmid and vector control plasmid,

SMAD3 wildtype (SM3-WT), SMAD3 constitutively active (SM3-CA), or SMAD3 dominant negative (SM3-DN) constructs for 48 hrs (n=3). * $p < 0.05$; ** $p < 0.01$, relative to untreated vector control. Total cell lysates were collected and assayed for luciferase activity by luminescence as described in the materials and methods section.

Figure 5. Nox4 is involved in TGF-beta/SMAD3-induced cell migration

(A) MDA-MB-231 (upper panel) and MCF10A (lower panel) cells were transfected with control vector, Nox4-DN (upper), or Nox4 shRNA (lower) plasmids and grown to confluence for 48 hours. A 200 μ l pipet tip was then used to create a wound and cells were left untreated or treated with TGF-beta (5ng/ml) for 6 hours (MDA-MB-231) or 24 hours (MCF10A). Shown are representative phase contrast images recorded at 10 $\times$ magnification at 0 and 6 or 24 hours from one experiment out of three, which gave similar results. (B) Empty vector or Nox4-DN transfected MDA-MB-231 cells were seeded in the upper chamber of a matrigel transwell and incubated in the lower chamber with DMEM medium containing TGF-beta 5ng/ml. After 24 hours, the migrating cells were stained with Diff

Stain (IMEB, San Marcos, CA). Data obtained from invading cells counted in 10 random fields analyzed from four experiments. $**p < 0.01$. (C) Cell proliferation of MDA-MD-231 cells transfected with empty vector or Nox4-DN and treated with or without TGF- β 5ng/ml for 24 hours was determined by measuring cell densities and Click-iT EdU incorporation assays. Cell densities after 24 hours was determined by DAPI stained cell counts (upper panel). Proliferating cells were quantified as the ratio of EdU-positive cell number and DAPI-stained total cell number (lower panel). (D) MDA-MB-231 cells were co-transfected with T204D and vector control, or dominant negative Smad3 (SM3-DN), or constitutively active Smad3 (SM3-CA). After 48 hours, a 200 μ l pipet tip was used to create a wound, which was imaged immediately and again 6 hours later. The quantification of wound closure is expressed as the average distance of wound closure compared to the SM3-CA (n=3). $*p < 0.05$, relative to SM3-CA. (E) MDA-MB-231 cells were transfected with vector control or T204D vector for 48 hours. Wound assays were then performed as in panels A and D following treatments with either DMSO or 10 μ M SIS3.

Figure 6. Nox4 modulates TGF-beta-induced fibronectin expression in breast epithelial cells
 (A) MDA-MB-231 (left panel) cells were transfected with empty vector alone or Nox4-DN for 24 hours followed by treatment with or without TGF-beta (5ng/ml) up to 48 hours. Fibronectin transcripts were quantified relative to GAPDH by qPCR amplification of reverse-transcribed total cellular cDNA (n = 3). MCF10A cells (right panel) were treated as in the left panel and assayed for fibronectin expression 24 hours after TGF-beta treatment (10ng/ml) (n=3). (B) Quantitative PCR analysis of fibronectin expression was determined from MDA-MB-231 (left panel) or MCF10A (right panel) cells transfected with control shRNA or Nox4- shRNA plasmids for 24 hours. Results are described as fold change in fibronectin mRNA relative to vector untreated expression using GAPDH as an internal

control. Data were obtained from three separate experiments performed in triplicate ($p < 0.05$ for matched time points as indicated with *). (C) MDA-MB-231 (left panel) or MCF10A (right panel) cells were transfected with human Nox4 cDNA or empty vector for 24 hours followed by TGF-beta treatment (MDA-MB-231, 5ng/ml) (MCF10A, 10ng/ml) for 24 hours (n=3). * $p < 0.05$; n.s.= not significant

Figure 7. Nox4 modulates TGF-beta/SMAD3-mediated breast epithelial cell migration

TGF-beta induces breast epithelial Nox4 expression and Nox4-dependent ROS generation in a TGF-beta/SMAD-3 dependent manner. TGF-beta-mediated wound healing, cell migration, and fibronectin expression is Nox-4 dependent. Epithelial cell migration and wound closure is diminished by the expression of inactive Nox4 (Nox4-DN), Nox4-specific shRNA, or inhibition of TGF-beta-induced SMAD3 (SMAD3-DN or SIS3). Furthermore, inactivation of Nox4 by Nox4-DN or Nox4 shRNA reduces TGF-beta-mediated fibronectin expression.