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Human peroxiredoxin 1 modulates TGF-β1-induced epithelial–mesenchymal transition through its peroxidase activity

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ABSTRACT

The epithelial-to-mesenchymal transition (EMT), which is induced by transforming growth factor-β1 (TGF-β1), is an important event that allows cancer cells to obtain invasive and metastatic characteristics. Although human peroxiredoxin 1 (hPrx1) has been implicated in tumor progression (e.g., invasion and metastasis), little is known about the role of hPrx1 in the EMT process during tumorigenesis. Here, we investigated the regulatory effect of hPrx1 during TGF-β1-induced EMT in A549 lung adenocarcinoma cells. We observed that high hPrx1 levels downregulated E-cadherin expression, and low hPrx1 levels upregulated E-cadherin expression, suggesting that the hPrx1 level may be correlated with EMT. Knockdown of hPrx1 significantly inhibited TGF-β1-induced EMT and cell migration, whereas hPrx1 overexpression enhanced TGF-β1-induced EMT and cell migration. In contrast to wild-type hPrx1, a peroxidase-inactive hPrx1 mutant (hPrx1-C51S) resulted in markedly increased E-cadherin expression. Moreover, hPrx1 regulated the expression of two E-cadherin transcriptional repressors, Snail and Slug. These findings provide new insight into the role of hPrx1 in regulating TGF-β1-induced EMT.

1. Introduction

The epithelial–mesenchymal transition (EMT) is a morphological event characterized by the loss of epithelial features and the acquisition of mesenchymal features [1,2]. EMT induction is characterized by cell–cell junction dissolution, cytoskeletal rearrangement, increased cell motility, and synthesis of extracellular matrix [3]. The EMT process is tightly regulated by signaling molecules and involves the downregulation of epithelial markers, such as E-cadherin and zonula occludens-1, and the upregulation of mesenchymal markers, such as fibronectin, N-cadherin, and vimentin [4,5].

Transforming growth factor (TGF)-β1, a multipotent cytokine, participates in cell apoptosis, differentiation, and proliferation. It is also a tumorigenesis indicator and a primary inducer of EMT. Transcription factors such as Snail, Slug, Twist1, and ZEB1 play critical roles in TGF-β1-induced EMT in epithelial cells; their overexpression is involved in the repression of E-cadherin expression and the induction of EMT [6–8].

Peroxiredoxins (Prxs) are a ubiquitous family of antioxidant enzymes that eliminate various peroxide substrates [9,10]. Mammalian cells express six Prx isoforms (Prx1–6), which are divided into three groups based on their number of conserved Cys residues and catalytic mechanism: 2-Cys Prxs (Prx1–4), atypical 2-Cys Prx (Prx5), and 1-Cys Prx (Prx6). All Prxs have a conserved N-terminal cysteine residue that undergoes a cycle of peroxide-dependent oxidation and reduction during the catalytic reaction. Upon peroxidatic Cys oxidation, 2-Cys Prxs are structurally converted from a low-molecular-weight species that acts as a peroxidase to a high-molecular-weight complex that functions as a chaperone [11,12].

Human Prx1 (hPrx1) is a member of the 2-Cys Prx subfamily and is present mainly in the cytosol [13]. In addition to its peroxidase and chaperone functions, hPrx1 also enhances natural killer cell cytotoxicity and inhibits oncogenic proteins such as c-myc and c-Abl [14–16]. Previous studies have suggested that hPrx1 is associated with carcinogenesis based on its overexpression in human cancers, including pancreatic, follicular thyroid, oral, breast, and lung cancers [17–19]. Specifically, augmented hPrx1 expression was found in lung cancer tumors, and Prx1 downregulation led to reduced cell growth and metastasis in lung adenocarcinoma [20–22].

Although the significance of hPrx1 has been observed in tumor progression, including invasion and metastasis, little is known about the role of hPrx1 in TGF-β1-induced EMT during tumorigenesis. In this study, we demonstrate that hPrx1 accelerates TGF-β1-induced EMT through its peroxidase function in A549 lung carcinoma cells.

2. Materials and methods

2.1. Materials

Cell culture reagents were purchased from WelGENE (Daegu, Korea); Lipofectamine 2000 and TRIzol reagents, from Invitrogen (Carlsbad, CA) and recombinant human TGF-β1, from PeproTech

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(Rocky Hill, NJ). Antibodies against E-cadherin and N-cadherin were from BD Biosciences (Franklin Lakes, NJ). Anti-hPrx1 antibody and an enhanced chemiluminescence system (ECL) were purchased from AB Frontier (Seoul, Korea). Actin and secondary antibodies were from Thermo Scientific (Rockford, IL). Control and hPrx1 small interfering RNAs (siRNAs) were from Genolution Pharmaceuticals (Seoul, Korea). A high-capacity cDNA reverse transcription kit was purchased from Applied Biosystems (Carlsbad, CA); and a SYBR Green Quantitative PCR kit, from Bio-Rad (Berkeley, CA).

2.2. Cloning, cell culture, and transfection

The coding region of the human Prx1 gene was cloned from a human placental cDNA library, and point-mutated hPrx1 (C51S) was generated by polymerase chain reaction (PCR)-mediated mutagenesis. The resulting fragment was ligated into pCS4-3myc vector and verified by sequencing. The human lung adenocarcinoma epithelial cell line (A549) was maintained in RPMI-1640 culture medium containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL). The cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. Transfections were performed with 20 nM siRNA or pCS4-myc-hPrx1 plasmid using Lipofectamine 2000 according to the manufacturer's instructions. The sequences for the two hPrx1 siRNA (si-hPrx1) oligos were as follows: si-hPrx1#1, sense 5′-GCCGAAUUGUGGUGUCUUAUU-3′ and antisense 5′-UAAGACACCACAAUUCGGCUU-3′ and si-hPrx1#2, sense 5′-AUCAUACAAACCAGUAGCCUU-3′ and antisense 5′-GGCUACUGGUUUGUAUGAUUU-3′. Cell morphology was observed under an inverted phase-contrast microscope.

2.3. Luciferase reporter assay

Cells in six-well plates were transfected with 500 ng of E-cadherin luciferase reporter construct. Where indicated, cotransfection was performed with pCS4-myc vector, pCS4-myc-hPrx1, or pCS4-myc-hPrx1-C51S, or with a siRNA. All cells were cotransfected with 200 ng of β-galactosidase plasmid to adjust for variation in transfection efficiency. At 48 h after transfection, luciferase activity was determined using a luminometer and a luciferase assay system (Promega, Madison, WI) according to the manufacturer's protocol. The luciferase assay was performed in triplicate for three independent experiments.

2.4. Western blot analysis

The cells were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 50 mM EDTA, 1% Triton X-100) at 4 °C for 10 min. The cell lysates were clarified by centrifugation at 12,000 rpm for 15 min, and the supernatants were collected. Protein concentrations in the supernatants were determined using the Bradford method. Equal amounts of lysate proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a nitrocellulose membrane. The membranes were incubated with primary antibodies against E-cadherin, N-cadherin, hPrx1, and β-actin. After the membranes were washed, they were incubated with a horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized using an ECL system.

2.5. Cell migration assay

A549 cells were grown to 40% confluency in six-well plates and were transiently transfected with control siRNA and hPrx1#2 siRNA, or with pCS4-myc and pCS4-myc-hPrx1 using Lipofectamine 2000. The cells were scratched and incubated with or without TGF-β1 for 72 h. Cell morphology was observed under an inverted phase-contrast microscope. Five random fields were photographed, and migrated cells were counted.

2.6. Quantitative real-time PCR

Total RNA was isolated from transfected cells using TRIzol reagent and was treated with DNase I. First-strand cDNA was synthesized from 1 μg of total RNA using a high-capacity cDNA reverse transcription kit. Snail and Slug mRNA levels were quantified by real-time PCR using a SYBR Green quantitative PCR kit (Bio-Rad) and a C1000™ thermal cycler, following the manufacturer's protocol. PCR primer sequences were as follows: human Snail, forward 5′-CCCCAATCGGAAGCCTAACT-3′ and reverse 5′-CGTAGGGCTGCTGGAAGGTA-3′ and human Slug, forward 5′-CCATTCCACGCCCAGCTA-3′ and reverse 5′-CTCACTCGCCCCAAAGATGA-3′. Each sample was tested in triplicate, and expression of each target was normalized to that of the human cyclophilin gene.

3. Results

3.1. hPrx1 regulates E-cadherin expression in A549 lung cancer cells

To determine the regulatory effect of hPrx1 during TGF-β1-induced EMT, we examined the effect of hPrx1 on the expression level of an epithelial marker protein, E-cadherin. After transfection of A549 cells with control siRNA or hPrx1 siRNA, we detected E-cadherin protein expression by Western blot analysis. As shown in Fig. 1A, E-cadherin expression was significantly increased in

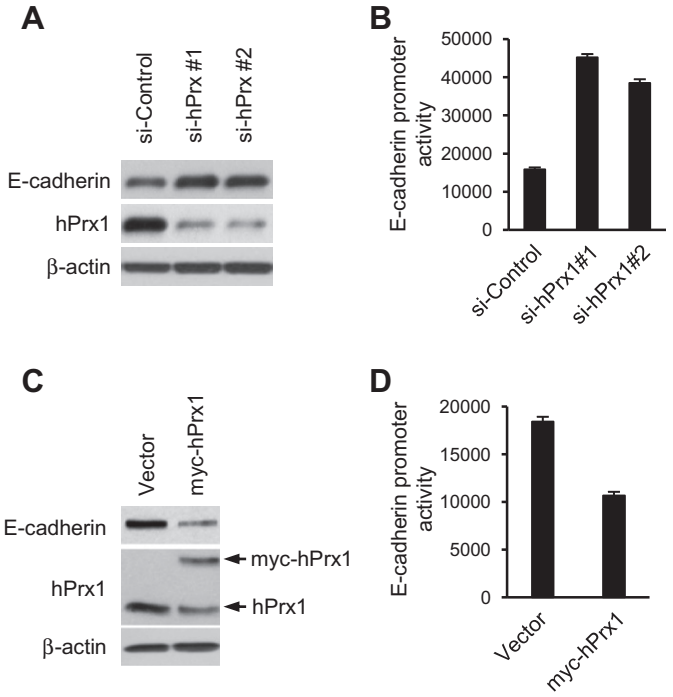

Fig. 1. hPrx1 protein level regulates E-cadherin expression in A549 cells. (A) Western blotting was performed to detect hPrx1 protein expression in control siRNA-transfected (si-Control) and hPrx1 siRNA-transfected (si-hPrx1#1 and si-hPrx1#2) A549 cells. (B) Cells were cotransfected with pE-cadherin-Luc and si-Control, si-hPrx1#1 or si-hPrx1#2. Luciferase activity assays were performed. (C) After transfection with pCS4-myc vector (vector) or pCS4-myc-hPrx1 (myc-hPrx1), myc-hPrx1 protein expression was examined by Western blotting. (D) Luciferase activity assays were performed in cells that co-expressed pE-cadherin-Luc with pCS4-myc vector or pCS4-myc-hPrx1. Data in (B) and (D) are means ± SD of three independent experiments.

hPrx1 knockdown cells as compared with control siRNA cells. In addition, hPrx1 knockdown also increased E-cadherin promoter activity (Fig. 1B). Conversely, hPrx1 overexpression resulted in decreased E-cadherin protein levels and low E-cadherin promoter activity (Fig. 1C and D). These results reveal a correlation between the hPrx1 level and the E-cadherin expression level, suggesting that hPrx1 may be involved in TGF-β1-induced EMT to E-cadherin regulation.

3.2. hPrx1 knockdown blocks TGF-β1-induced EMT and cell migration

To investigate whether hPrx1 is involved in TGF-β1-induced EMT, we transiently silenced its expression in A549 cells with siRNA transfection. After TGF-β1 treatment, we observed morphological changes from an epithelial phenotype to a spindle-like mesenchymal phenotype. TGF-β1 induced EMT-related morphological changes in siRNA control cells, whereas hPrx1 knockdown strikingly abolished TGF-β1-induced EMT (Fig. 2A). Moreover, to confirm the mesenchymal phenotype, we examined the expression levels of two EMT markers, E-cadherin and N-cadherin. Upon TGF-β1 treatment, hPrx1 downregulation did not lead to E-cadherin reduction and N-cadherin induction similar to that in control cells (Fig. 2B). Previous studies have demonstrated that tumor cells with an EMT phenotype are more motile [23,24]. To further characterize this phenomenon in A549 cells, we performed a wound healing assay. Consistent with the above EMT results, hPrx1 knockdown inhibited TGF-β1-induced cell migration when compared with control cells (Fig. 2C). We obtained similar results in the TGF-β1-induced EMT and cell migration experiments using si-hPrx1#1 (data not shown). These results indicate that hPrx1 downregulation is critical in maintaining an epithelial phenotype and in suppressing EMT in A549 cells.

3.3. hPrx1 overexpression accelerates TGF-β1-induced EMT and cell migration

To further confirm the role of hPrx1 in EMT, we examined the effect of exogenous hPrx1 overexpression. Compared with control vector transfection, myc-hPrx1 overexpression accelerated the EMT morphological changes mediated by TGF-β1 treatment (Fig. 3A). E-cadherin downregulation and N-cadherin upregulation induced by TGF-β1 were enhanced in hPrx1-overexpressing A549 cells (Fig. 3B). Moreover, hPrx1 overexpression led to high migratory activity of cells and earlier wound closure after 72 h of TGF-β1 treatment (Fig. 3C). These results suggest that hPrx1 is required for TGF-β1-induced EMT and cell migration in A549 cells.

3.4. hPrx1 regulates E-cadherin expression through its peroxidase activity

The N-terminal conserved cysteine (Cys51) of hPrx1 is susceptible to oxidation and is an active site critical for peroxidase enzyme activity [11]. A recent study showed that hPrx1 peroxidase activity is important in preventing Ras- and ErbB-2-induced transformation [25]. To test the role of the catalytic hPrx1 cysteine (Cys51) in EMT, we generated a mutant construct, hPrx1-C51S, in which Cys51 was substituted with a Ser residue. We compared E-cadherin promoter activity in cells transfected with hPrx1-C51S and in cells overexpressing wild-type hPrx1 (hPrx1-WT). As shown in Fig. 3D, hPrx1-WT overexpression resulted in decreased E-cadherin promoter activity, whereas E-cadherin promoter activity in hPrx1-C51S-transfected cells was significantly increased, to about sixfold that with hPrx1-WT. These results suggest that hPrx1 peroxidase function is required for the regulation of E-cadherin expression in TGF-β1 signaling.

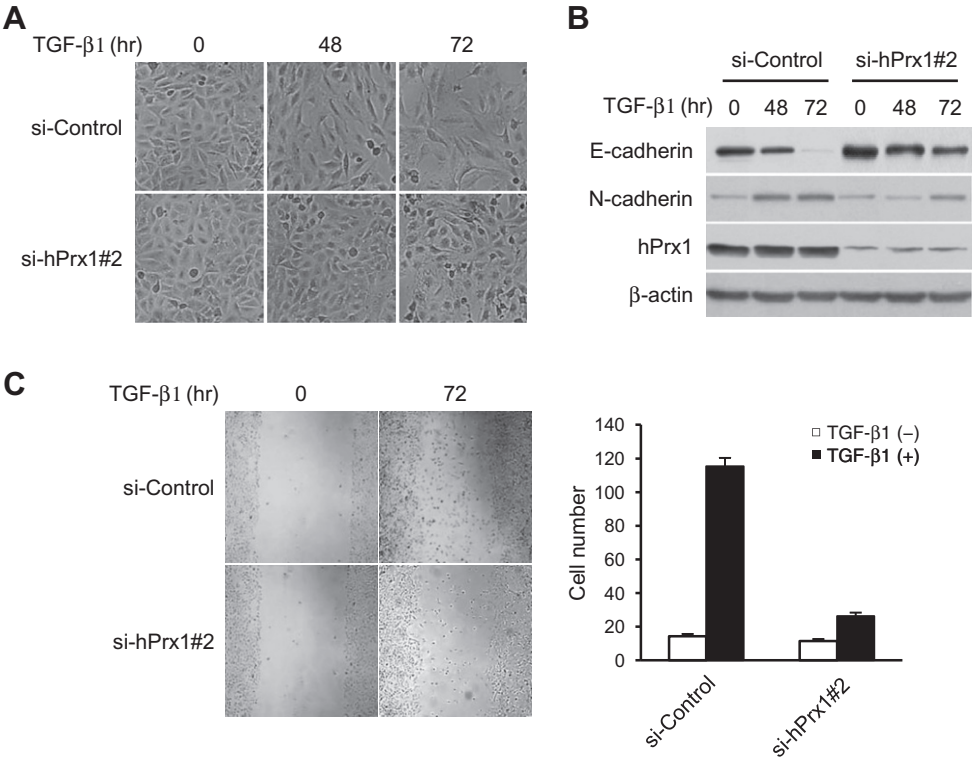

Fig. 2. hPrx1 knockdown suppresses TGF-β1-induced EMT and cell migration in A549 cells. (A) The effect of hPrx1 knockdown on TGF-β1-induced EMT was examined as cell morphological changes at the indicated times after treatment with 5 ng/mL TGF-β1. (B) The expression levels of E-cadherin and N-cadherin were analyzed by Western blotting. β-actin was used as a loading control. (C) Cells transfected with si-Control or si-hPrx1#2 were scratched and incubated with or without TGF-β1 treatment for 72 h (Left). Quantitation of wound healing is shown. The sum of five random fields was counted (right).

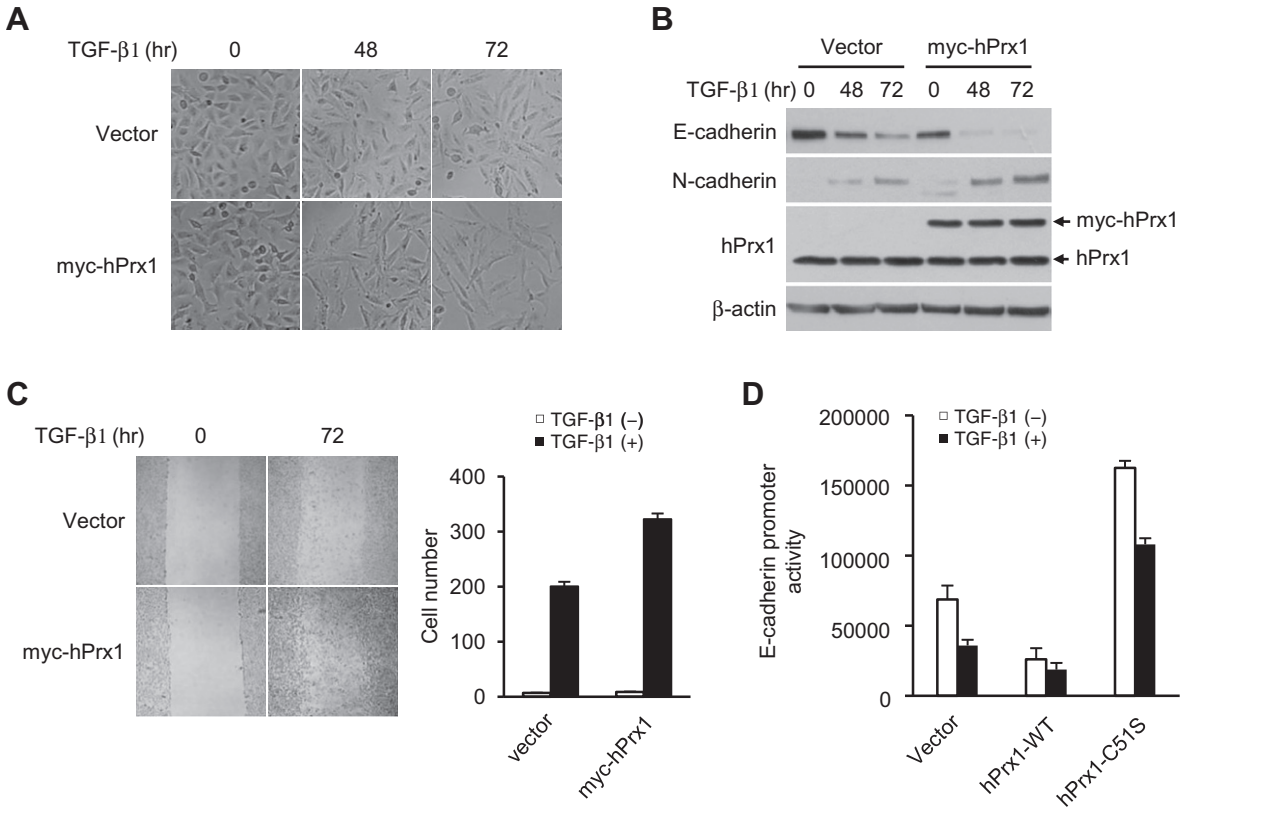

Fig. 3. hPrx1 overexpression enhances TGF-β1-induced EMT and cell migration, and regulates E-cadherin expression via its peroxidase activity. (A) The effect of hPrx1 overexpression on TGF-β1-induced EMT was examined as cell morphological changes at the indicated times after treatment with 5 ng/mL TGF-β1. (B) The expression levels of E-cadherin and N-cadherin were analyzed by Western blotting. β-actin was used as a loading control. (C) Cells transfected with pCS4-myc vector or pCS4-myc-hPrx1 were scratched and incubated with or without TGF-β1 treatment for 72 h (left). Quantitation of wound healing is shown. The sum of five random fields was counted (right). (D) Cells cotransfected with E-cadherin-Luc and vector, myc-hPrx1 plasmid, or myc-hPrx1-C51S plasmid were treated with or without TGF-β1 for 24 h. Luciferase activity assays were performed. Data are the means ± SD of three independent experiments.

3.5. hPrx1 regulates Snail and Slug expression

Snail and Slug transcription factors act as repressors of E-cadherin expression, and their expression induces EMT [26]. To address the mechanism of E-cadherin regulation by hPrx1 in EMT, we determined whether hPrx1 is required for Snail or Slug expression. Quantitative real-time PCR results revealed that hPrx1 knockdown repressed the expression of both Snail and Slug (Fig. 4), indicating that hPrx1 regulates E-cadherin expression through Snail and Slug.

4. Discussion

The EMT process is a morphological event crucial to tumor progression in physiological and pathological states [27,28]. TGF-β1 plays a key role in EMT induction [1,29]. Although hPrx1 is known to be involved in cancer progression, its role in EMT has not been examined, and no evidence has been reported.

In this study, the hPrx1 protein level modulated the expression level of the epithelial marker E-cadherin, implying a potential role of hPrx1 in TGF-β1-mediated biological functions. The findings on hPrx1 regulation in TGF-β1-induced EMT provide evidence of a novel biological function of hPrx1. Our data show that hPrx1 downregulation is critical in maintaining the epithelial phenotype of A549 cells, and thus, hPrx1 acts as an EMT inducer. The most typical TGF-β1-induced mesenchymal phenotype is generally observed 48 h after treatment; EMT cell morphological changes in hPrx1 knockdown cells did not occur until 72 h of TGF-β1 treatment. In addition, a correlation between hPrx1 and E-cadherin expression was observed. We did not observe transcriptional or translational regulation of hPrx1 expression by TGF-β1 (data not shown), but hPrx1 is required for EMT as shown by hPrx1 knockdown and overexpression experiments.

Fig. 4. hPrx1 downregulation suppresses Snail and Slug expression. Quantitative RT-PCR was performed to detect Snail and Slug mRNA in si-Control-transfected and si-hPrx1#2-transfected A549 cells. The human cyclophilin gene was used for normalization. The results are shown as fold changes compared with si-Control-transfected cells. Representative results from three independent experiments are shown.

The peroxidase function of hPrx1 is involved in regulating Ras-induced transformation [25]. Interestingly, when we examined the effect of a peroxidase-inactive mutant, hPrx1-C51S, on E-cadherin expression, we found that hPrx1-C51S overexpression markedly enhanced E-cadherin expression by a dominant-negative mechanism. These results suggest that hPrx1 Cys51 may have an important function in regulating the EMT process. Furthermore, Snail and Slug are key EMT inducers with the ability to repress E-cadherin transcription. Our data showed for the first time that hPrx1 can regulate Snail and Slug gene transcription to induce EMT by an unknown mechanism.

In conclusion, we provided evidence for a functional role of hPrx1 in TGF-β1-induced EMT in lung cancer cells. The regulation of EMT induction by hPrx1 may be a potential target for therapeutic strategies directed against cancer metastasis.

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