The epithelial–mesenchymal transition (EMT) has been implicated in various pathophysiological processes, including cancer cell migration and distant metastasis. Reactive oxygen species (ROS) and insulin receptor substrate-1 (IRS-1) are important in cancer progression and regulation of EMT. To explore the biological significance and regulatory mechanism of EMT, we determined the expression, the biological function and the signaling pathway of prostate transmembrane protein, androgen induced-1 (TMEPAI), during the induction of EMT and cell migration. Transforming growth factor (TGF)-β1 significantly upregulated the expression of TMEPAI during EMT in human lung adenocarcinoma. Depletion of TMEPAI abolished TGF-β1-induced down-regulation of ferritin heavy chain and the subsequent generation of ROS, thus suppressing TGF-β1-induced EMT and cell migration. In addition, increased ROS production and overexpression of TMEPAI downregulated the level of IRS-1. Both the addition of H2O2 and IRS-1 small interfering RNA rescued the ability of TGF-β1 to induce EMT in TMEPAI-depleted cells. Remarkably, the levels of TMEPAI in lung tumor tissues are very high, whereas its expression in normal lung epithelium is very low. Moreover, TMEPAI expression was positively correlated with the cell mesenchymal phenotype and migration potential. Our work reveals that TMEPAI contributes to TGF-β1-induced EMT through ROS production and IRS-1 downregulation in lung cancer cells.

Introduction

The epithelial–mesenchymal transition (EMT) is a fundamental biological event that plays important roles in embryonic development, chronic fibrosis and cancer progression (1–3). EMT is characterized by distinct morphological changes, which is associated with the reorganization of cytoskeleton, including the loss of cell–cell adhesion proteins and the gain of mesenchymal proteins. The epithelial cells undergoing EMT acquire cell motility and invasive capacity, which is a crucial step of cancer progression. During the process of EMT, the cells respond and adapt to particular environmental cues. It is an exemplary form of epithelial cell plasticity, manifested by alterations of the cell identity, behavior and fate including escape from apoptosis, senescence and immune defense (4–6). Recent evidence shows that EMT is related to senescence. Both EMT and senescence events in cancer progression share several regulating factors, and the inhibition of senescence is accompanied by the activation of EMT. Cancer cells utilize these two crossing paths for the metastatic cascade to overcome the growth-limiting aspects (7–10). Despite the involvement of complex molecular mechanisms, most signals triggering EMT downregulate the expression of E-cadherin by transcriptional repression or protein degradation (11,12).

Abbreviations: DMEM, Dulbecco’s modified Eagle’s medium; EMT, epithelial–mesenchymal transition; FHC, ferritin heavy chain; IRS-1, insulin receptor substrate-1; NAC, N-acetyl cysteine; ROS, reactive oxygen species; siRNA, small interfering RNA; TGF, transforming growth factor; TMEPAI, transmembrane protein, androgen induced-1.

Recent studies have revealed that EMT can be induced by factors such as hypoxia, reactive oxygen species (ROS) and nitric oxide (13–15). ROS are important for maintaining redox homeostasis and involved in various signaling pathways governing cell proliferation, apoptosis and differentiation. Excessive ROS production can lead to oxidative stress, resulting in diverse human diseases including cancers (3,16–18). Because EMT can be tightly controlled by the intracellular redox state, further investigation of the molecular mechanism involved in oxidative stress during tumor growth and metastasis will be instrumental for understanding cancer biology and aiding in the development of cancer therapies.

Transforming growth factor-β (TGF-β) is a potent pleiotropic factor implicated in a variety of cellular functions. TGF-β-induced EMT is implicated in embryonic development, fibrotic diseases, wound healing and tumor metastasis. In recent years, reports from our laboratory have identified several molecules, including those regulating the intracellular redox state or energy metabolism, to be important in TGF-β1-induced EMT (15,19,20). Insulin receptor substrate-1 (IRS-1) and FOXA2 are EMT suppressors, and they play an important role in maintaining the epithelial phenotype in lung cancer cells (21,22). TGF-β increases the labile iron pool by downregulating the expression of ferritin heavy chain (FHC), thus increasing the production of ROS and subsequently EMT. The adenosine monophosphate-activated protein kinase, which is involved in cellular energy homeostasis, is required for TGF-β1-induced EMT.

The prostate transmembrane protein androgen induced-1 (TMEPAI), also termed solid tumor-associated gene 1 (STAG1), is usually overexpressed in several types of epithelial tumors. Amplification of the TMEPAI gene and/or upregulation of the TMEPAI protein are observed in breast, prostate, colon, ovarian and other cancers (23–25). TMEPAI was originally identified as an androgen-inducible gene that is predominantly expressed in the prostate tissue (24). Subsequent studies revealed that TMEPAI is also a direct target of TGF-β signaling; its expression is highly upregulated after TGF-β treatment (25). TMEPAI is involved in growth arrest of prostate cancer cells (24) and is reportedly required for TGF-β-induced cell growth and motility in breast cancer cells (26). Nevertheless, it has also been reported that TMEPAI can negatively regulate TGF-β signaling by perturbing the recruitment of Smad2 or Smad3 to TGF-β receptor I (27). It is not clear whether the TMEPAI-mediated signal is cell type and/or context dependent. Several reports have shown that the expression of TMEPAI is dramatically induced by TGF-β. However, the role of TMEPAI in TGF-β1-induced EMT and cancer progression is unknown.

In this study, we investigated the TGF-β1-mediated regulation of TMEPAI expression, the role and the putative underlying mechanism of TMEPAI in TGF-β1-induced EMT, as well as cell migration and the progression of lung cancer.

Materials and methods

Cell culture

A549, H358, HCC827, H446 and H1299 human lung cancer cells, SK-MES-1 human lung squamous cancer cell line, HPAEpiC human pulmonary alveolar epithelial cells and 293T cells were purchased from the American Type Culture Collection (Manassas, VA). A549 and 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Scientific HyClone, Rockford, IL) containing 10% fetal bovine serum and supplied with penicillin (100 units/ml) and streptomycin (100 μg/ml; Life Technologies Gibco, Grand Island, NY); NCI-H520, H358, HCC827, H446, H1299 and HPAEpiC cells were cultured in RPMI medium 1640 (Thermo Scientific Hyclone) containing the same supplements; SK-MES-1 was cultured in modified Eagle’s medium/Earle’s balanced salt solution (Thermo Scientific HyClone) supplied with 0.1 mM non-essential amino acids, penicillin (100 U/ml) and streptomycin (100 μg/ml; Life Technologies Gibco, Grand Island, NY).
Technologies Gibco). All of the cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Reagents

Human recombinant TGF-β1 was purchased from Chemi-Con (Rosemont, IL). The antibody against TMEPAI was purchased from Abnova (Taipei, Taiwan). Antibodies against E-cadherin and N-cadherin were obtained from BD Biosciences (Franklin Lakes, NJ). Antibodies against IRS-1, Vimentin, myc and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against tubulin, fibronectin and the antioxidant N-acetyl cysteine (NAC), SB431542 and a 3% H₂O₂ solution were purchased from Sigma (St Louis, MO). Chemical small interfering RNAs (siRNAs) were synthesized by Shanghai GenePharma, Ltd (Shanghai, China).

Reverse transcription and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Tiangen, Beijing, China) according to the manufacturer’s instructions. Then, mRNA was reverse transcribed at 42°C for 30 min using ReverTra Ace-α (Toyobo, Osaka, Japan).

Real-time PCR was performed using Power SYBR Green PCR Master Mix with Applied Biosystems 7500 Fast Real-Time PCR system. The primers were used as follows: for TMEPAI, forward 5’-TGTCAAGGCAA CGGAATCCC-3’ and reverse 5’-CAGGAGCAATGCCGATGGGC-3’; for GAPDH, forward 5’-AGAAGGGCTGCGCTATTGG-3’ and reverse 5’-AGGCGGATCCACAGTCTTC-3’.

Plasmid construction

The primers used for amplification of TMEPAI were as follows: forward 5’-CCGAAATCTCCATGCACCGCTTGATGGG-3’ and reverse 5’-CCAGCT TGAGCAGGGTGTCCTTTCTGTTTATCCTTCT-3’. The TMEPAI-complete cDNA was cloned into pcDNA3.1(−)/myc-his A at the Bgl II and HindIII sites.

Generation of stably transfected cell lines

pPGKsuper was used for the expression of short interfering RNA. The target sequences of TMEPAI were 5’-AACAGAACACCTTCTGCAGACT-3’ and 5’-AAGGGGAGAAATGTCGGAATTT-3’ respectively. Oligonucleotides were inserted into the pPGKsuper vector at the Bgl II and HindIII sites. A549 cells were transfected with empty vector or TMEPAI-RNAi plasmids using Lipofectamin-2000 (Invitrogen, NY). After transfection (48h), cells were selected with 1100 mg/ml G418. The TMEPAI knockdown efficiency of each monoclone was determined by western blotting using the TMEPAI antibody.

Synthetic siRNAs for TMEPAI and IRS-1

Chemical synthetic siRNA sequences are as follows: for TMEPAI, 5’-GCCUGGAGUUUGUUCAGAUCC-3’ and 5’-GAUCUGAACAAACACUCACUG GCCT-3’; 5’-GCAAGAAGAAGGAUAAACATT-3’ and 5’-UGUUUAAUCUCUCUUUG-3’. For IRS-1, 5’-CGCGAUCAGGUGGAUAAU-3’ and 5’-UAAUCUCCACUUGGAGGT-3’.

Preparation of cell lysates and western blotting

Cells were lysed in lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium dodecyl sulfate, 0.1% sodium orthovanadate, 1 mM PMSF, 1 μg/ml aprotinin). Supernatants were collected and protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Equivalent amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). After blocking with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with appropriate primary antibodies. Then, the membranes were washed with Tris-buffered saline containing 0.1% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies. The immunoreactive protein bands were detected by enhanced chemiluminescence. Primary antibodies were diluted 1:2500, and secondary antibodies were diluted 1:10 000 for use.

Immunofluorescent staining

Cells were cultured on glass slides and treated as indicated. The slides were quickly washed with phosphate-buffered saline to terminate the reaction, followed by fixing with 4% paraformaldehyde and permeabilizing in 0.1% Triton X-100. After blocking with 5% bovine serum albumin, the samples were probed with appropriate primary and secondary antibodies. The nuclei were stained with 4’,6-diamidino-2-phenylindole. F-actin was stained with phalloidin. The fluorescence was visualized using a Leica confocal microscope (Mannheim, Germany).

Cell migration assays

For wound healing assays, cells were seeded in 35 mm tissue culture dishes. A wound was incised in the central area of the confluent culture, followed by careful washing of the detached cells and addition of fresh medium. Pictures were taken of the wounded area 24 and 48h later using a Canon EOS 50D digital camera.

Transwell assays were performed in 6.5 mm diameter and 8.0 μm pore polycarbonate filter transwell plates (Corning Costar, NY). Cells (5 x 10⁵) were suspended with DMEM containing 0.5% fetal calf serum and seeded into the upper well of the chamber; the lower well was filled with DMEM containing 10% fetal calf serum as a chemoattractant. After incubation for 12h at 37°C in the presence of 5% CO₂, the non-migrated cells were removed from the upper surface of the transwell. Cells that migrated to the bottom surface of the filter were fixed, stained and counted.

ROS production measurement

Adherent cells or cells trypsinized and suspended in 0.2 ml serum-free DMEM were incubated with 10 μm/1 ml 2’, 7’-dichlorofluorescein diacetate at 37°C for 20 min and then washed twice with serum-free DMEM. The fluorescence of the cells was examined with a fluorescent microscopy (Olympus, Japan) or measured with a flow cytometer (Becton Dickinson FACS Calibur). 2’,7’-Dichlorofluorescein was excited at 488 nm, and fluorescence was measured at 525 nm. The mean fluorescence intensity per cell was used for comparison.

Immunohistochemical analysis

Thirty pairs of human lung carcinoma tissues and the relevant adjacent tissues were purchased from Outo Biotechnology (Shanghai, China). The sections were dewaxed, rehydrated, pretreated and treated with H₂O₂. The sections were incubated with TMEPAI antibody (1:150), E-cadherin antibody (1:500) or N-cadherin antibody (1:150) at 4°C overnight and secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG) for 30 min at room temperature. After washing, the bound antibody was visualized using diaminobenzidine/H₂O₂ substrate, and the nuclei were stained with hematoxylin. The photographs were taken at ×100 magnification using an Olympus microscopy.

Statistical analyses

For statistical analysis, quantitative data from at least three experiments were compared and are expressed as the mean ± SD. Statistical significance was determined with the Student’s t-test or an analysis of variance. The level of statistical significance was set at P < 0.05.

Results

TGF-β induces the expression of TMEPAI during EMT

Because TGF-β1 plays a critical role during cancer progression, we hypothesized that the genes regulated by TGF-β1 may contribute to the metastasis of certain tumors. To better understand the molecular mechanism of TGF-β1-induced EMT and cell motility, we first identified the genes that are regulated by TGF-β in the A549 human lung adenocarcinoma cell line. We found that the mRNA level of TMEPAI was dramatically increased after TGF-β1 treatment in both a time- and dose-dependent manner (Figure 1A). The induction of TMEPAI by TGF-β1 was also confirmed by immunoblotting (Figure 1B). As seen in Figure 1C, the upregulation of TMEPAI by TGF-β1 occurred rapidly and was a sustained event that can be completely blocked by SB431542, a selective inhibitor of TGF-β type I ALK5, suggesting a role of TMEPAI in TGF-β1-induced EMT.

To further understand the relationship between TMEPAI and EMT, we examined four other human lung cancer cell lines: HCC827, H358, H446 and H1299. These lung cancer cell lines were derived from either primary or metastatic tumors and therefore exhibit variable degrees of malignancy. As shown in Figure 1D, HCC827 and H358 cells displayed strong epithelial characteristics, whereas H446 and H1299 cells exhibited a strong mesenchymal phenotype. Compared with other four cell lines, A549 cells appeared to resemble both epithelial and mesenchymal phenotypes. The expression of TMEPAI in these lung cancer cell lines was closely related to the epithelial/mesenchymal properties of these cells. TMEPAI was highly expressed in the cells with relatively strong mesenchymal characteristics. As shown in Figure 1E, TGF-β1 only induced EMT in H358 cells. Strikingly, TGF-β1 also significantly increased the expression of TMEPAI in H358 but not in HCC827 (Figure 1F). These
results indicate that the induction of TMEPAI confers epithelial cell plasticity, and its level of expression determines the cellular response to the EMT-inducing factor.

**TMEPAI is required for TGF-β1-induced EMT**

To determine if TMEPAI is involved in TGF-β1-induced EMT, we designed short interfering RNAs targeting two different sites in the human TMEPAI gene and knocked down endogenous TMEPAI in A549 cells. As shown in Figure 2A, PGK vector control cells displayed a spindle-like and scattered phenotype 48h after TGF-β1 treatment, whereas the morphology of TMEPAI-depleted cells remained unchanged. The inhibitory effect of TMEPAI depletion on TGF-β1-induced EMT was further determined by the detection of the epithelial marker E-cadherin and the mesenchymal marker N-cadherin.

EMT is a coordinated process accompanied by a series of biological changes, including collapse of the cell junction complex and...
TMEPAI regulates ROS and IRS-1 during EMT

reorganization of the cytoskeleton. We observed that E-cadherin was eliminated from the cell membrane after TGF-β1 treatment in cells expressing the PGK empty vector, whereas depletion of TMEPAI abolished this effect (Figure 2B). Immunofluorescence staining of F-actin indicated that silencing TMEPAI also inhibited F-actin reorganization during EMT (Figure 2C).

Epithelial cells are highly motile after undergoing EMT. Because TMEPAI depletion inhibited TGF-β1-induced EMT, we explored whether knockdown of TMEPAI would also affect the capacity of cells to migrate. As expected, TGF-β1-induced cell migration was abolished in the absence of TMEPAI, as demonstrated by transwell assays, Columns, average number of cells migrated per field from three independent experiments; bars, SD; **P ≤ 0.01. (E) Cell migration of control and TMEPAI-RNAi cells with or without TGF-β1 treatment was examined by wound healing assays. Cell morphology was examined at the indicated time points after a scratch was made. (F) Cell migration of H358 and HCC827 cells in response to 5 ng/ml TGF-β1 was examined by wound healing assays.

Fig. 2. TMEPAI is required for TGF-β1-induced EMT in A549 cells. (A) Cells transfected with empty vector- or TMEPAI-RNAi-containing plasmid were treated with 2.5 ng/ml TGF-β1 for 48 h, cell morphology (left) was examined, and EMT was determined by immunoblotting for E-cadherin and N-cadherin (right). (B and C) Immunostaining of E-cadherin and F-actin in control and TMEPAI-RNAi cells treated with 2.5 ng/ml TGF-β1 for 48 h. Nuclei were stained with 4',6-diamidino-2-phenylindole. Representative results from three independent experiments are shown. (D) Cell migration of control and TMEPAI-RNAi cells in response to TGF-β1 was examined by transwell assays. (E) Cell migration of control and TMEPAI-RNAi cells with or without TGF-β1 treatment was examined by wound healing assays. (F) Cell migration of H358 and HCC827 cells in response to 5 ng/ml TGF-β1 was examined by wound healing assays.

TMEPAI is involved in EMT and cell motility, we determined whether IRS-1 and FHC are downstream targets of TMEPAI. Strikingly, the downregulation of both IRS-1 and FHC by TGF-β1 treatment was reversed in TMEPAI-depleted cells (Figure 3B). These results confirm our hypothesis that TMEPAI is an upstream factor inhibiting IRS-1 and FHC. TMEPAI modulates TGF-β1-induced EMT and cell migration by downregulating the expression of IRS-1 and FHC.

Downregulation of FHC increases the cellular labile iron pool, consequently increasing the production of ROS, an essential factor for TGF-β1-induced EMT. Our finding that TMEPAI modulates TGF-β1-induced EMT via the inhibition of FHC expression also prompted us to explore whether TMEPAI exerts its effect through ROS production during EMT. As shown in Figure 3C and Supplementary Figure 2, available at Carcinogenesis Online, TGF-β1 induced significant elevation of intracellular ROS levels in A549 cells, and TGF-β1-induced cell migration was completely abolished by the ROS scavenger, NAC (Figure 3D), supporting the role of ROS. In addition, the ROS level in both TGF-β1-treated and control cells was greatly reduced by TMEPAI depletion (Figure 3E and Supplementary Figure 3, available at Carcinogenesis Online), and the EMT phenotype was rescued by treatment with H₂O₂ (an oxidant that can increase ROS) in TMEPAI-knockdown cells (Figure 3F). These results indicate that TMEPAI participates in TGF-β1-induced EMT by promoting ROS generation.

Induction of EMT through TMEPAI-mediated ROS production and IRS-1 downregulation

IRS-1 and FHC molecules are critical for EMT induction (19,21). TGF-β1-mediated downregulation of IRS-1 and FHC is apparent at relatively later stages of EMT (Figure 3A), suggesting that these molecules are regulated by upstream signaling events. To understand how TMEPAI regulates IRS-1 during EMT, we observed that E-cadherin was eliminated from the cell membrane after TGF-β1 treatment in cells expressing the PGK empty vector, whereas depletion of TMEPAI abolished this effect (Figure 2B). Immunofluorescence staining of F-actin indicated that silencing TMEPAI also inhibited F-actin reorganization during EMT (Figure 2C).

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TMEPAI also downregulates IRS-1 expression, implying that in addition to FHC, IRS-1 is also involved in the TMEPAI-mediated effect. Depletion of IRS-1 by transfecting synthetic IRS-1 siRNA into TMEPAI-depleted cells restored EMT that was abolished by TMEPAI depletion (Figure 4A), confirming that IRS-1 is another downstream target of TMEPAI in controlling EMT. Next, we investigated whether there...
TMEPAI regulates ROS and IRS-1 during EMT

is any relationship between the two downstream targets of TMEPAI: ROS and IRS-1. To examine the effect of ROS on IRS-1 expression, cells were treated with NAC to inhibit ROS generation. Our results show that NAC inhibited TGF-β1-induced IRS-1 downregulation but not TMEPAI upregulation (Figure 4B). Consistently, H2O2 treatment significantly restored TGF-β1-induced downregulation of IRS-1 protein in TMEPAI-depleted cells (Figure 4C). Furthermore, in 293T cells, which express no detectable IRS-1, ectopically expressed IRS-1 was markedly inhibited by overexpression of TMEPAI (Figure 4D). These data suggest that ROS functions downstream of TMEPAI and upstream of IRS-1.

TMEPAI is overexpressed in human lung squamous carcinoma

Because TMEPAI contributed to EMT in lung adenocarcinomas, we explored whether TMEPAI is also involved in TGF-β1-induced EMT in lung squamous cell carcinoma. We employed two human lung squamous carcinoma cell lines SK-MES-1 and NCI-H520. As seen in Figure 5A, TGF-β1 significantly induced the expression of TMEPAI in SK-MES-1 cells, which correlated with its ability to induce EMT. In contrast, TGF-β1 could not induce the expression of TMEPAI in NCI-H520 cells, which correlated with its inability to induce EMT. The EMT features in SK-MES-1 cells could also be inhibited by TMEPAI siRNAs (Figure 5B). These data support the role of TMEPAI in conferring the epithelial plasticity that is required for TGF-β1-induced EMT.

Abnormal expression of TMEPAI has been observed in several types of carcinomas, but the expression of TMEPAI in lung cancers has not been determined. To determine the clinical relevance of our findings, we analyzed the expression of TMEPAI in human lung squamous carcinomas ranging from Stage I to Stage III and their matched adjacent normal tissues. TMEPAI expression was determined along with the EMT markers E-cadherin and N-cadherin. TMEPAI expression in the normal lung epithelium was very low, which is consistent with previous reports that TMEPAI is predominantly expressed in the prostate tissue. The well-organized epithelial structures were gradually disintegrated as the progression of tumors. However, we detected a significant increase in the expression of TMEPAI in squamous cell lung cancers regardless of the stage of disease progression (Figure 5C). The increased expression of TMEPAI in human lung cancer cells was confirmed by comparing TMEPAI protein levels in A549 cells and the HPAEpiC human pulmonary alveolus epithelial cell line (Figure 5D). The concentration of TGF-β in plasma, sufficient to induce EMT in vivo, also increases during cancer progression (28,29). Unlike tumor cells, normal epithelial cells do not undergo EMT to metastasize to distal sites, implying that the cellular context contributes to the cellular response to TGF-β. When HPAEpiC cells were treated with TGF-β1, no EMT was induced, as shown by cell morphology and molecular markers (Figure 5E). Interestingly, TGF-β1 was also unable to induce the expression of TMEPAI in the HPAEpiC cell line (Figure 5F). Because ROS production plays an important role during EMT and cancer progression, we also examined ROS levels before and after TGF-β1 treatment in A549 and HPAEpiC cells. As shown in Figure 5G, TGF-β1 significantly elevated ROS generation in A549 cells, but ROS levels in HPAEpiC cells remained unchanged after TGF-β1 treatment. The above results demonstrate that the fundamentally different response of A549 and HPAEpiC cells to TGF-β1 in EMT may depend on the...
pronounced differences of TMEPAI expression and subsequent ROS production levels.

**Discussion**

TMEPAI was originally discovered as an androgen-regulated gene in 2000 (24). The biological function of TMEPAI has mostly been studied in androgen signaling or prostate cancer, but the novel function of TMEPAI remains largely unknown. In this study, we found for the first time that increased expression of TMEPAI plays an important role in TGF-β1-induced EMT in lung cancer cells. Knocking down TMEPAI inhibited cell junction collapse and cytoskeleton reorganization, leading to the inhibition of TGF-β1-induced cell migration. It has recently been shown that knockdown of TMEPAI in breast cancer cells abolishes TGF-β1-induced cell migration (26). This study revealed that TMEPAI regulates TGF-β1-induced cell migration by modulating EMT (Figure 6).
TMEPAI regulates ROS and IRS-1 during EMT

The integrity of epithelial cells can be modified by altering the process of EMT, a manifestation of epithelial cell plasticity. The plasticity of tumor cells may explain the prometastatic effect of EMT during cancer progression. EMT both drives the epithelial cells toward a mesenchymal state and causes fundamental changes in cell behavior and identity, including migration, invasion, proliferation and sterness, all related to tumor onset and cancer progression. The observation that there is increased expression of TMEPAI in very early- and late-stage lung tumors implies that increased expression of TMEPAI may confer antiapoptotic and stemness properties to further promote tumorigenesis and progression. The observation that TMEPAI is abundantly expressed in cells with strong mesenchymal characteristics and EMT can only occur when the expression of TMEPAI is induced suggests that high expression of TMEPAI is also required for epithelial cells to achieve a mesenchymal state and migration capacity. The finding that increased TMEPAI expression is essential for the epithelial/mesenchymal plasticity of lung cancer cells provides a novel molecular link between epithelial plasticity and lung cancer cell migration and metastasis.

The zinc finger protein family members SNAI1/Snail, SNAI2/Slug, ZEB1 and ZEB2, as well as the basic-helix-loop-helix transcription factor Twist, are important regulators of EMT via the repression of E-cadherin expression through binding to the E-box region of the E-cadherin promoter (30). Previous studies have demonstrated that Snail and Slug are significant EMT inducers that are correlated with E-cadherin promoter (31,32). We also found the induction of Slug, the E-cadherin transcription suppressor, was inhibited by TMEPAI knockdown (data not shown).

ROS can be generated by mitochondrial oxidative phosphorylation and intracellular responses to cytokines and other stimuli. Although oxidative stress due to excessive ROS generation is implicated in various diseases, recent evidence has shown that ROS also act as critical signaling molecules during signal transduction. ROS can crosstalk with multiple signaling pathways, such as MAPK, PI3K and NF-κB (33–37). Studies have indicated that ROS are involved in tumorigenesis and progression; exploring how ROS are generated and participated in cancer progression is an emerging field of research. We previously demonstrated that increased ROS production can be induced by increases in the labile iron pool during the process of TGF-β1-induced FHC downregulation (19). In this study, we report that TMEPAI is required for TGF-β1-induced FHC downregulation and ROS generation during EMT induction. Knockdown of TMEPAI significantly blocked TGF-β1-induced ROS generation and cell migration. However, increasing the intracellular level of ROS rescued this phenotype, indicating that ROS production can be tightly modulated by TMEPAI.

IRS-1 has been regarded as an adaptor protein, but it was recently shown to be a critical molecule in maintaining the epithelial phenotype of cells, which can be downregulated by TGF-β1-induced EMT in lung cancer cells (21). However, the mechanism by which TGF-β1 decreases IRS-1 levels has not yet been characterized. In this study, TMEPAI was shown to be essential in the downregulation of IRS-1 during TGF-β1-induced EMT. IRS-1 can be downregulated by TMEPAI-mediated FHC downregulation and resultant ROS generation. TMEPAI is a neural precursor cell expressed, developmentally downregulated 4, E3 ubiquitin protein ligase (NEDD4)-binding protein involved in ubiquitin degradation (38,39); thus, TMEPAI putatively downregulates IRS-1 through TMEPAI-induced IRS-1 degradation. TMEPAI has not yet been reported to function as a transcription factor, so it is not clear whether TMEPAI can suppress transcription of the IRS-1 gene.

Dissecting the TGF-β signaling pathways is important for exploring the mechanisms by which TGF-β promotes cancer metastasis. We found that TMEPAI expression is induced at very early stages of tumor progression by TGF-β and is essential for the biological functions of TGF-β in lung cancer cells. It is not clear whether TMEPAI overexpression alone is enough to induce EMT in lung cancer cells. Due to unknown reasons, we failed to obtain stably TMEPAI-transfected cell clones to express ectopic TMEPAI proteins. However, transient overexpression of TMEPAI in 293T cells decreased the expression of IRS-1, which is in line with the presented findings.

Carcinomas account for >90% of all human cancers, and lung cancer is one of the most common and lethal cancers worldwide. Our data provide insight into the molecular mechanism of EMT induction during lung cancer progression, which may be of significance in developing new strategies for clinical diagnosis and therapeutic treatment for lung cancers.

Supplementary material
Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

Funding
Chinese Ministry of Science and Technology (2011CB966200); Natural Science Foundation of China (30730023).

Acknowledgements
We thank Dr C.Ronald Kahn for the kind gift of the wild-type IRS-1 plasmid and members of our laboratory for helpful discussions and suggestions.

Conflict of Interest Statement: None declared.

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Received November 7, 2012; revised February 27, 2013; accepted April 20, 2013