Alveolar Epithelial Cells Undergo Epithelial-to-Mesenchymal Transition in Response to Endoplasmic Reticulum Stress

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Expression of mutant surfactant protein C (SFTPC) results in endoplasmic reticulum (ER) stress in type II alveolar epithelial cells (AECs). AECs have been implicated as a source of lung fibroblasts via epithelial-to-mesenchymal transition (EMT); therefore, we investigated whether ER stress contributes to EMT as a possible mechanism for fibrotic remodeling. ER stress was induced by tunicamycin administration or stable expression of mutant (L188Q) SFTPC in type II AEC lines. Both tunicamycin treatment and mutant SFTPC expression induced ER stress and the unfolded protein response. With tunicamycin or mutant SFTPC expression, phase contrast imaging revealed a change to a fibroblast-like appearance. During ER stress, expression of epithelial markers E-cadherin and Zonula occludens-1 decreased while expression of mesenchymal markers S100A4 and α-smooth muscle actin increased. Following induction of ER stress, we found activation of a number of pathways, including MAPK, Smad, β-catenin, and Src kinase. Using specific inhibitors, the combination of a Smad2/3 inhibitor (SB431542) and a Src kinase inhibitor (PP2) blocked EMT with maintenance of epithelial appearance and epithelial marker expression. Similar results were noted with siRNA targeting Smad2 and Src kinase. Together, these studies reveal that induction of ER stress leads to EMT in lung epithelial cells, suggesting possible cross-talk between Smad and Src kinase pathways. Dissecting pathways involved in ER stress-induced EMT may lead to new treatment strategies to limit fibrosis.

Idiopathic pulmonary fibrosis (IPF) is a fatal lung disease characterized by extensive architectural distortion and progressive parenchymal fibrosis. A substantial body of work supports the concept that alveolar epithelial cells (AECs) play an essential role in the development of IPF, but the specific mechanisms linking AECs to lung fibrosis are not well understood. Genetic studies have indicated that mutations in the gene encoding surfactant protein C (SFTPC) can lead to familial interstitial pneumonia, which is the familial form of IPF. Mutations in the carboxyl terminal region of the gene result in a product that cannot be processed normally in type II AECs, leading to accumulation of misfolded prosurfactant protein C in the endoplasmic reticulum (ER), ER stress, and activation of the unfolded protein response (UPR). In addition, our group and others have reported that ER stress and UPR activation are found in the alveolar epithelium in lung biopsies from patients with familial interstitial pneumonia in the absence of SFTPC mutation as well as sporadic IPF. Taken together, available data indicate that ER stress may be important in the pathogenesis of IPF; however, the means by which ER stress contributes to lung fibrosis is not currently identified.

With ER stress, UPR pathways are activated as a means to help the cell abrogate the untoward effects of protein accumulation in the ER. The three arms of the UPR include processes designed to attenuate protein translation, increase the production of metabolism and redox proteins, enhance production of protein folding chaperones, and up-regulate the production of protein degradation enzymes. Excessive ER stress, however, can lead to apoptosis. Several studies related to disease pathogenesis in settings where ER stress appears important have focused on apoptosis as the link between ER stress and disease. However, some recent studies have suggested that, in addition to effects on apoptosis, ER stress may induce pathways involved in cell differentiation, raising the intriguing possibility that cells might respond to ER stress by changing their phenotype to one that reduces expression of the offending protein or is more equipped to handle the consequences of ER stress.

Epithelial-to-mesenchymal transition (EMT) is thought to contribute to fibrotic remodeling in a number of organs, including the lungs, by increasing the population of cells responsible for collagen production and matrix deposition. In alveolar epithelial cells; UPR, unfolded protein response; TM, tunicamycin; TGFβ1, TGFβ type I receptor; α-SMA, α-smooth muscle actin; EDEM, ER degradation enhancing α-mannosidase-like protein; IR-E-1, inositol-requiring enzyme 1; DMSO, dimethyl sulfoxide.
these studies, we investigated whether ER stress and UPR pathway activation could induce EMT in type II AECs in culture. ER stress was induced in AECs in two different ways: 1) stable expression of the L188Q SFTP C genetic mutation (mutant SFTP C) and 2) exposure to tunicamycin (TM), an antibiotic agent that disrupts protein glycosylation in the ER. We found that induction of ER stress by either means results in EMT. This effect is mediated through Smad2/3 and Src kinase activation and is reversible after discontinuation of the ER stress-inducing condition. Together, these data indicate that ER stress in epithelial cells may contribute to fibrosis by inducing EMT and thus adding to the effector fibroblast population in the lungs.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant Dickkopf1 (DKK1) was purchased from R&D Systems (Minneapolis, MN). Tunicamycin was purchased from Sigma-Aldrich. TGFβ1 type I receptor inhibitor (SB431542), extracellular signal-regulated kinases 1/2 (ERK1/2) inhibitor (U-0126), Src family tyrosine kinase inhibitor (PP2), and JNK inhibitor (SP600125) were purchased from Tocris Bioscience (Ellsville, MO).

Antibodies used were as follows: S100A4 rabbit polyclonal antibody (obtained from Dr. Eric Neilson, Vanderbilt University, Nashville, TN); E-cadherin mouse monoclonal antibody (BD Biosciences); Zonula occludens 1 (ZO-1) rabbit polyclonal, phosphorylated eukaryotic initiation factor 2α (p-eIF2α) rabbit polyclonal, eIF2α mouse monoclonal (Invitrogen); phosphorylated Smad2 rabbit polyclonal, phosphorylated ERK1/ERK2 MAPK rabbit polyclonal, ERK1/ERK2 MAPK rabbit polyclonal, phosphorylated Src (Tyr-416) rabbit polyclonal, Src rabbit monoclonal, phosphorylated p-JNK rabbit polyclonal, JNK rabbit polyclonal, BiP (ER chaperon immunoglobulin heavy chain-binding protein) rabbit polyclonal, Snail rabbit polyclonal (Cell Signaling, Danvers, MA); α-smooth muscle actin (α-SMA) rabbit polyclonal (Abcam, Cambridge, MA); ER degradation enhancing α-mannosidase-like protein (EDEM) goat polyclonal, phosphorylated Smad2/3 rabbit polyclonal, ZEB1 rabbit polyclonal, ZEB2/SIP1 rabbit polyclonal, N-cadherin rabbit polyclonal, E12/E47 rabbit polyclonal, PKR-like endoplasmic reticulum kinase (PERK) rabbit polyclonal, activating transcription factor 6 (ATF6) rabbit polyclonal, inositol-requiring enzyme 1α (IRE-1α) rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA); vimentin mouse monoclonal (Millipore, Temecula, CA); and actin rabbit polyclonal (Sigma-Aldrich).

Cell Culture Studies—The A549 human lung adenocarcinoma cell line, MLE-12 mouse lung epithelial cell line, and RLE6TN cells, a rat type II alveolar epithelial cell line, were purchased from American Type Culture Collection (Manassas, VA). RLE6TN cells were maintained at 37 °C in Ham-F12 (Invitrogen) with 2 ml l-glutamine, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin. A549 cells and MLE-12 cells were maintained in DMEM (Invitrogen) with 2 ml l-glutamine, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin. Expression constructs containing wild-type human SFTP C and mutant SFTP C with the exon 5 and 128 Thr→Ala mutation (L188Q SFTP C) were developed as described previously (2, 6). RLE6TN (rat type II AEC line) cells were stably transfected with WT SFTP C and mutant SFTP C constructs using FuGENE 6 transfection reagent (Roche Diagnostics). G418 selected clones were pooled and used for the present study. Untransfected, empty vector-transfected, WT SFTP C-transfected, and mutant SFTP C-transfected RLE6TN cells were grown in six-well culture plates at 50–70% confluence in complete medium containing 10% FBS for 24 h and then changed to 0.5% FBS for up to 96 h. In experiments with TM, untransfected A549 cells, MLE-12 cells, and RLE6TN cells were grown in complete media with 10% FBS and treated with 0.5 µg/ml TM diluted in DMSO for 48 h. DMSO-treated cells were used as vehicle controls. Cell viability in AECs treated with various concentrations of TM (0.01–10 µg/ml) for 48 h was estimated using CellTiter-Glo Luminescent Cell Viability assay (Promega, Madison, WI) as per the manufacturer’s instructions. For pathway inhibitor studies, RLE6TN cells were maintained in the same culture conditions above, with each inhibitor added at a concentration of 5 µM, 1 h prior to the addition of TM.

Small Interfering RNA—RLE6TN cells were seeded at 40–50% confluence in six-well plates. siRNA constructs were obtained as siGENOME SMARTpool reagents from Dharmacon (Lafayette, CO) as follows: siGENOME SMARTpool Smad2 (M-091698-00-0010), siGENOME SMARTpool Src (M-M-080144-01-0001), siGENOME SMARTpool IRE-1α (L-103829-01-0010), siGENOME SMARTpool PERK (M-092012-00-0010), siGENOME SMARTpool ATF6 (D-001206-13-20), and nontargeting siRNA control siControl Non-targeting siRNA pool (D-001206-13-20). Cells were transfected with 50 to 100 nM siRNA in Lipofectamine RNAiMax reagent in serum-free conditions per the manufacturer’s instructions. Twenty-four hours after transfection, the cells were switched into a medium containing 10% fetal calf serum and were treated with 0.1- 0.5 µg/ml tunicamycin for 24–48 h.

Western Blotting—Cells were washed two times with ice-cold PBS and then lysed with Cellytic M (Sigma-Aldrich) in the presence of protease inhibitor mixture (Sigma-Aldrich) and incubated for 15 min at room temperature. The cell lysate was centrifuged for 10 min at 14,000 × g at 4 °C. Supernatant was stored at −70 °C until further use. The total protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce) following the manufacturer’s instructions using bovine serum albumin standards. Western blotting was done with the NuPAGE system (Invitrogen). 30 µg of protein were resolved on 10% NuPAGE Bis-Tris gels using MOPS buffer. The proteins were transferred to Hybond nitrocellulose membranes (Amersham Biosciences) in NuPAGE transfer buffer. The membrane was blocked for 1 h at room temperature in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, Nebraska) and immunoblotted at 4 °C overnight with the primary antibody. Detection was done using fluorescent-tagged secondary antibodies coupled to either IRDye 700 infrared dye or IRDye 800 infrared dye. The membrane was washed with PBS and captured with an Odyssey Infrared imaging system (LI-COR Biosciences).

Immunofluorescence Staining—Cells were cultured on eight-well chamber slides for immunofluorescent staining. The cells were fixed in 2% paraformaldehyde for 15 min at
room temperature. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. After two washes with PBS, the cells were blocked with 5% normal donkey serum in PBS for 1 h in a humidified chamber. Cells were incubated with indicated primary antibodies overnight at 4 °C, washed three times with PBS, and incubated with fluorescent secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). Nuclear staining was done with DAPI using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Phase contrast and fluorescent microscopy was performed using an Olympus IX81 Inverted research microscope configured with an Olympus IX2 biological disk-scanning unit (Olympus, Tokyo, Japan).

**Results**

**Tunicamycin and Mutant L188Q SFTPC Induce ER Stress and EMT**—In initial studies, we sought to determine the optimal concentration of TM for induction of ER stress without excessive cell death. Thus, we generated a dose response curve in A549, MLE-12 and RLE6TN cells with 0.01–10 μg/ml TM for 48 h (Fig. 1A and supplemental Fig. 1) and assessed cell viability. Because 0.1–0.5 μg/ml of TM induced <25% cell death, we used these concentrations for our experiments. We then performed further studies in RLE6TN cells to determine whether TM treatment results in measurable ER stress/UPR pathway markers in RLE6TN cells transfected with empty vector or cells stably expressing WT or mutant SFTPC. D and E, densitometry results from three independent experiments. *, p < 0.05 compared with vehicle (DMSO) or WT SFTPC.
strate that in vitro treatment of AECs using sublethal concentrations of TM results in ER stress and UPR activation.

As another means of inducing ER stress, we established stable expression of mutant L188Q SFTPC in AECs. We generated expression constructs that contain WT and mutant human SFTPC (exon 5 and 128 Thr→Ala mutation that results in the L188Q substitution), and transfected these constructs into RL6E6TN cells. Stable expressing clones were picked and pooled by G418 selection. Next, we examined expression levels of WT SFTPC and mutant SFTPC in stable cell lines by real-time PCR and found similar levels of transgene expression (supplemental Fig. 2). As with TM studies, we assessed whether expression of mutant SFTPC resulted in ER stress with increased production of UPR proteins. Levels of BiP, EDEM, and pEF2α were low in empty vector-transfected AECs (Fig. 1C). Although WT SFTPC expression resulted in similar expression of BiP, EDEM, and pEF2α compared with empty vector-transfected cells, the expression of these proteins was increased in AECs expressing mutant SFTPC. After establishing models for ER stress induction in AECs, we treated AECs with TM (0.5 μg/ml) or vehicle (DMSO) for 48 h and observed for changes in cell morphology under phase contrast microscopy. Interestingly, phase contrast images revealed that cells changed their morphology from a cobblestone-like appearance to an elongated fibroblast-like appearance with TM treatment, whereas vehicle-treated control cells remained cobblestone in morphology (Fig. 2E). Similar changes in morphology were observed in A549 cells and MLE-12 cells (data not shown). After this observation, we evaluated further for EMT by analyzing expression of epithelial and mesenchymal markers. Western blots from TM-treated AECs revealed a significant loss of the epithelial marker E-cadherin compared with vehicle-treated AECs, whereas the fibroblast markers α-SMA, vimentin, and N-cadherin were significantly increased in TM-treated AECs compared with vehicle-treated cells (Fig. 2, A and B, and supplemental Fig. 3). Immunofluorescent staining with ZO-1 and E-cadherin revealed a cortical pattern at cell-cell junctions in vehicle-treated AECs, whereas ZO-1 and E-cadherin disappeared from cell-cell junctions in TM-treated cells (Fig. 2E). Previously, we have used S100A4, also referred to as FSP1 (fibroblast-specific protein 1), as a marker for fibroblasts in bleomycin-induced lung fibrosis (21, 22). Here, TM-treated cells gained S100A4 expression as detected by immunofluorescent staining, whereas vehicle-treated cells did not have S100A4 expression (Fig. 2E).

We then evaluated whether EMT was observed in AECs expressing mutant SFTPC. As with TM treatment, AECs expressing mutant SFTPC had an elongated fibroblast-like appearance on phase contrast microscopy (Fig. 2F) as well as a significant loss of E-cadherin expression compared with WT SFTPC expressing cells (Fig. 2, C and D). α-SMA Western blots showed a similar expression pattern in mutant SFTPC- and WT SFTPC-expressing cells (Fig. 2, C and D). On the other hand, Western blots for vimentin and N-cadherin showed increased expression in mutant SFTPC compared with WT SFTPC-expressing cells. Immunofluorescent staining with ZO-1 and E-cadherin revealed a cortical pattern at cell-cell junctions in WT SFTPC and empty vector-transfected AECs, whereas this cortical pattern was not observed in mutant SFTPC-expressing cells. Immunofluorescent staining for S100A4 expression revealed that mutant SFTPC-transfected cells gained S100A4 expression consistent with a fibroblast phenotype, whereas S100A4 expression was minimal in WT SFTPC-expressing and empty vector-transfected cells (Fig. 2F). Together, these studies show that acute and chronic ER stress can induce EMT in AECs as assessed by cell appearance, reduction of epithelial markers, and acquisition of mesenchymal cell markers.

ER Stress-induced EMT Is Associated with Up-regulation of Multiple Signaling Pathways—We sought to determine which EMT-related pathways are activated following ER stress by testing candidate pathways that have been shown to regulate EMT in other settings. Among key EMT pathways, the TGFβ pathway has received considerable attention (23–26). Binding of TGFβ to its cell surface receptors results in Smad pathway activation with phosphorylation of Smad2/3, which then forms a complex with Smad4 and migrates to the nucleus to regulate transcription of multiple genes, including some involved with EMT. In our cell models, we tested whether ER stress resulted in increased Smad2/3 phosphorylation. Western blots revealed that phosphorylated Smad2/3 (pSmad2/3) increased markedly in AECs exposed to TM compared with vehicle-treated cells and in mutant SFTPC-expressing AECs compared with WT SFTPC-expressing cells (Fig. 3A). ELISA for TGFβ1 from the media collected from AECs exposed to TM failed to show an increase in TGFβ1 concentration (data not shown), suggesting that the observed increase in intracellular pSmad2/3 was not dependent on increased TGFβ1 production.

Because non-Smad pathways downstream of TGFβ have also been implicated in EMT, we evaluated MAPK activation in AECs after induction of ER stress. As shown in Fig. 3A, phosphorylated forms of JNK and ERK1/ERK2 were increased in AECs exposed to TM compared with vehicle-treated AECs and in mutant SFTPC-expressing AECs compared with WT SFTPC-expressing AECs. In contrast, no differences in p38 phosphorylation were identified (data not shown).

The Wnt/β-catenin pathway is another pathway that has been shown to be involved in EMT (23–25). Thus, we next tested for nuclear and cytoplasmic localization of β-catenin in ER stress-induced EMT by immunofluorescent staining. As seen in Fig. 3B, AECs treated with TM had prominent nuclear localization of β-catenin, as opposed to cell membrane staining in vehicle-treated cells. Mutant SFTPC-expressing AECs had a similar staining pattern to that seen in TM-treated cells (Fig. 3B). Because a recent study by Kim et al. (28, 29) implicated β-catenin-pSmad2 complex formation in AECs undergoing EMT, we performed dual immunofluorescence staining that revealed co-localization of phosphorylated Smad2/3 and β-catenin in the nucleus of AECs undergoing ER stress (Fig. 3B). This finding suggests that these two pathways might be involved in ER stress-induced EMT.

We also evaluated activation of the Src pathway by testing for phosphorylation of Src (tyrosine 416). Src family protein kinases are known to regulate cell differentiation and have been implicated in EMT in thyroid epithelial cells (30). Western blots for phosphorylated Src (Tyr-416) revealed increased pSrc in AECs exposed to TM compared with vehicle-treated AECs, as
well as in mutant SFTPC-expressing AECs compared with controls (Fig. 3A). As shown for pSrc, differences in activation of most of the pathways tested was greater between TM-treated cells and controls than between WT and mutant SFTPC-expressing cells. This appears due to the effects of WT SFTPC expression because untreated RLE6TN cells and empty vector-transfected RLE6TN cells used as controls for these experiments (data not shown) appeared similar to the vehicle (DMSO) treated controls shown in Fig. 3A (far left column).

Loss of E-cadherin is one of the hallmarks of EMT, and its decreased expression is mediated by transcription factors that bind E-box elements in the E-cadherin promoter, greatly suppressing expression of E-cadherin (31). Hence, we evaluated for the expression of ZEB1, ZEB2/SIP1, E12/E47, and Snail in

![Image of Western blots and bar charts showing the expression of E-cadherin, α-SMA, vimentin, and N-cadherin in RLE6TN cells treated with TM or vehicle control for 48 h, and in cells stably expressing WT or mutant SFTPC.](image-url)
AECs. Of these transcription factors, we did not see expression of Snail and E12/E47 in either RLE6TNs treated with TM or in mutant SFTPC-expressing cells (data not shown). On the other hand, ZEB1 and ZEB2/SIP1 expression increased significantly in TM-treated AECs compared with vehicle (DMSO)-treated controls (Fig. 3A). Similarly, mutant SFTPC-expressing cells had increased expression of ZEB1 and ZEB2/SIP1 compared with WT SFTPC-expressing cells (Fig. 3A).

**ER Stress-induced EMT Is Mediated through Intracellular Smad and Src Kinase Pathways**—Because ER stress leads to activation of a number of pathways that could regulate EMT in AECs, we asked whether modulating these pathways would prevent or reverse ER stress-induced EMT. We first investigated whether autocrine or paracrine mediated extracellular signaling through the TGFβ or Wnt/β-catenin pathways is involved in ER stress-associated EMT. However, neither administration of a neutralizing TGFβ1 antibody nor treatment with DKK1 (to block Wnt signaling) affected EMT following TM treatment (data not shown).

Next, we performed studies using specific intracellular inhibitors to block the pathways analyzed above, including the TGFβR1-Smad inhibitor SB431542 (which blocks phosphorylation of TGFβR1, preventing downstream Smad activation); the ERK1/ERK2 inhibitor U0126 (which is a selective noncompetitive inhibitor); the Src kinase inhibitor PP2 (a selective competitive inhibitor); and the pJNK inhibitor SP600125 (a selective competitive inhibitor). For these experiments, AECs were pretreated with an individual inhibitor for 1 h followed by addition of TM for up to 96 h. Vehicle or inhibitor treatment of cells in the absence of TM did not affect cell morphology or viability. Despite blocking activation of the target pathway, neither the ERK1/ERK2 inhibitor U0126 nor the pJNK inhibitor SP600125 had an observable effect on the TM-induced EMT phenotype (data not shown). In TM-treated cells exposed to either the Smad inhibitor SB431542 or the Src kinase inhibitor PP2, more RLE6TN cells maintained an epithelial cell appearance on phase microscopy (data not shown), and Western blots revealed a partial effect of PP2 to restore E-cadherin expression and a partial effect of SB431542 to attenuate TM induced /-SMA expression and up-regulate E-cadherin expression (Fig. 4A). Based on these data, we performed subsequent studies with these two inhibitors in combination. TM-treated cells exposed to the combination of SB431542 and PP2 had preserved epithelial cell appearance (Fig. 4B), restoration of basal E-cadherin expression, and elimination of inducible /-SMA expression (Fig. 4A). Furthermore, SB431542 and PP2 in combination attenuated TM-induced expression of S100A4, loss of ZO-1, and nuclear localization of /-catenin (Fig. 4B), although neither inhibitor alone had these effects.

To further evaluate the contributions of these two pathways to ER stress-induced EMT, we performed siRNA studies against both Smad2 and Src kinase in RLE6TN cells. For both targets, siRNA transfection resulted in appropriate knockdown of the protein (Fig. 4C). As was seen with the small molecule pathway inhibitors, siRNA targeting of Smad2 and Src kinase led to a relative preservation of E-cadherin expression and attenuation of /-SMA expression after TM treatment (Fig. 4D). Considering the results of both the small molecule inhibitor studies and the siRNA knockdown studies, the effects of Smad2 inhibition appeared to be the more prominent in attenuating...
ER stress-induced EMT. However, inhibiting both pathways together had an additive effect.

Next, we wanted to determine which of the three UPR arms was involved in ER stress-induced EMT. For these studies, we performed siRNA studies targeting each of the three ER transmembrane sensors: PERK, ATF6, and IRE-1. With siRNA targeting of PERK and ATF6, cells were viable in the unstressed state; however, with the addition of TM, marked cell toxicity was noted within 24 h. With IRE-1α siRNA targeting, cells were viable at 24 h following TM exposure, demonstrating knockdown of the IRE-1 target (Fig. 5A and supplemental Fig. 4). Furthermore, siRNA targeting of IRE-1α led to an attenuation of tunicamycin-induced Smad2 and Src phosphorylation (Fig. 5B and supplemental Fig. 4), the two pathways implicated in ER stress-induced EMT above. Taken together, the small molecule inhibitor and siRNA studies indicate that both the Smad and Src kinase pathways are involved in ER stress-induced EMT in AECs. The IRE-1 arm of the UPR appears to be involved in activating these signaling pathways.

We also asked whether Smad2 and Src pathways are activated simultaneously or whether one might be activated upstream of the other. Western blots from TM-treated cells at different time points showed that Smad2/3 phosphorylation was increased at 2 h after TM treatment, rising further from 4 to 12 h and remaining elevated even at 72 h compared with vehicle (DMSO) treated controls. In contrast, pSrc was present at low levels at baseline, increased at 12 h after TM treatment, and remained strongly elevated through 72 h compared with controls (supplemental Fig. 5). Thus, both pathways were up-regulated by 12 h, which is well ahead of the time that morphologic changes of EMT were observed.

Tunicamycin-induced EMT Is Reversible—We wondered whether EMT observed in AECs treated with TM was a stable or a transient phenotype. For these studies, we treated AECs with TM for 48 or 96 h.

FIGURE 4. ER stress-induced EMT is attenuated by blocking Smad2/3 and Src kinase pathway. A, Western blot for E-cadherin and α-SMA in whole cell lysates of RLE6TN cells exposed to TM (0.5 μg/ml) in the presence of the Smad2/3 pathway inhibitor SB431542 (5 μM) and/or Src kinase inhibitor PP2 (5 μM) for 96 h. B, phase contrast images and immunofluorescent staining for ZO-1, S100A4, and β-catenin in RLE6TN cells treated with vehicle (DMSO), TM, or TM and a combination of SB431542 and PP2 inhibitors. C, Western blot for Smad2 and Src in whole cell lysates from RLE6TN cells transfected with siRNA targeting Smad2, Src, or nontarget control siRNA. Cells were subsequently exposed to TM or vehicle for 48 h. D, Western blot for E-cadherin and α-SMA in RLE6TN cells transfected with targeting siRNAs against Smad2 and/or Src followed by exposure to TM or vehicle for 48 h.

FIGURE 5. IRE-1 is involved in ER stress-induced Smad 2/3 and Src pathway activation. Western blots for IRE-1 (A) and phosphorylated (B) and total Smad2/3 and Src from whole cell lysates of RLE6TN cells transfected with nontarget control siRNA or siRNA targeting IRE-1α followed by exposure to TM (0.5 μg/ml) or vehicle (DMSO) for 24 h.
FIGURE 6. ER stress-induced EMT is reversible. A, RLE6TN cells were exposed to TM (0.5 μg/ml) for 48 h followed by removal of TM by washing the cells and adding fresh culture medium. Cell phenotype was assessed 72 h after removing TM by phase contrast microscopy (A) and Western blot (B) for E-cadherin and α-SMA. Data are representative of three separate experiments.

DISCUSSION

In these studies, we have provided one potential explanation for the profibrotic effects of ER stress by demonstrating that induction of ER stress in AECs results in EMT. RLE6TN cells undergo EMT following short term administration of the anti-biotic agent TM or with chronic expression of mutant L188Q SFTPC. In AECs, ER stress-induced EMT is regulated through the Smad2/3 and Src kinase pathways. In addition, TM discontinuation studies suggest that ER stress-induced EMT may be reversible if the offending stimulus can be removed.

Our findings demonstrating ER stress-induced EMT in AECs is consistent with a study by Ulianich et al. (30) in which thyroid epithelial cells exposed to TM or thapsigargin exhibited changes consistent with EMT. In another study, cyclosporine-induced ER stress was noted to result in EMT in normal human renal epithelial cells (32). Taken together, available information suggests that EMT might be a common response by epithelial cells to substantial or prolonged ER stress. In cases in which ER stress is induced by a misfolded protein product like mutant SFTPC, EMT may represent an escape mechanism for these stressed cells to reduce production of the offending protein.

The ER is an important organelle that provides a unique environment for normal protein synthesis and folding. Under normal unstressed conditions, chaperone proteins such as BiP facilitate proper folding to maintain homeostasis. However, accumulation of misfolded or mutant proteins results in ER stress and activation of the UPR. The UPR is a genomic response designed to improve cellular function and prevent cell death (33). This is achieved by the activation of the ER trans-membrane sensors PERK, ATF6, and IRE-1. These pathways are activated to reduce overall protein translation and increase expression of metabolism and redox proteins designed to protect the cell (downstream of PERK), enhance the expression of ER chaperones to assist with protein folding (downstream of ATF6), and degrade the accumulated proteins and mRNA (downstream of IRE-1) (7–14, 34). The UPR can also activate a number of other signaling pathways. IRE-1 activates JNK via TNF receptor-associated factor 2 recruitment and activation (35). ATF6 can induce phosphorylation of p38 MAPK in response to the ER stress-inducing agent azetidine (36). PERK activates p38 and JNK kinases when calcium homeostasis is altered by thapsigargin and dithiothreitol but not by TM (37, 38). In our studies, however, we did not find that these known signaling pathway interactions were involved in ER stress-induced EMT. Instead, our data indicate that endogenous Smad2/3 activation and Src activation are critical for EMT in AECs. Our results also suggest that ER stress-induced EMT is mediated at least in part through the IRE-1 arm of the UPR. However, the precise mechanisms linking UPR pathways to Smad and Src activation require future investigation.

Although numerous pathways have been proposed to be involved in EMT, in the lungs most studies have focused on TGFβ signaling in this process. AECs, including RLE6TN cells, have been shown to undergo EMT in vitro and in vivo with exposure to TGFβ (19, 20), and inhibition of the Smad pathway attenuates this response in vitro (39). The Wnt/β-catenin pathway is another pathway linked to EMT in lungs (23–25), and previous reports have suggested that β-catenin and Smad pathways interact (29, 40). In our studies, we found co-localization of pSmad2/3 and β-catenin in the nucleus of AECs after induction of ER stress. Interestingly, however, efforts to modulate each of these two pathways by exposure to a neutralizing TGFβ antibody or DKK1 did not impact EMT, suggesting that extracellular signaling through these pathways is not a driving force for ER stress-induced EMT. Our findings are similar to a previous report by Kim et al. (19) showing that fibronectin driven Smad2 activation and EMT were not blocked by soluble TGFβ receptor or addition of TGFβ-neutralizing antibodies; however, β6 integrin null AECs failed to undergo fibronectin-in-duced EMT. These findings suggested that αvβ6 integrin related activation of TGFβ was required for EMT in this setting. More recently, Garmaszegi et al. (41) demonstrated that extra-cellular matrix molecules could activate Smad2 and Src independent of TGFβ through interactions between TGFβ type II receptor and β1 integrins. This study suggested that ligand-independent mechanisms involving interactions at the cyto-
plasmic membrane can intrinsically activate Smad signaling pathways. Considering the complexity of EMT processes, it is not surprising a variety of signaling mechanisms may be involved in shifting cell phenotype and, therefore, modulation of more than one pathway might be necessary to block or reverse this phenotype shift.

These studies help to clarify the processes by which ER stress could contribute to fibrotic remodeling in the lungs. When UPR mechanisms fail or if ER stress is too severe, it can lead to altered cell function, growth arrest, and cell death through apoptosis (15–18). Studies from our lab and others (2, 6, 27) have shown that mutant SFTPC expression leads to enhanced AEC apoptosis, which has been implicated as a causative factor in human and experimental pulmonary fibrosis. Our current studies add to this picture by adding EMT to the repertoire of ER stress responses of AECs. Expansion of the effector fibroblast population via EMT may be another way in which AECs involved in shifting cell phenotype and, therefore, modulation of more than one pathway might be necessary to block or reverse this phenotype shift.

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