The aberrant expression of transforming growth factor (TGF)-β1 in the tumor microenvironment and fibrotic lesions plays a critical role in tumor progression and tissue fibrosis by inducing epithelial-mesenchymal transition (EMT). EMT promotes tumor cell motility and invasiveness. How EMT affects motility and invasion is not well understood. Here we report that HDAC6 is a novel modulator of TGF-β1-induced EMT. HDAC6 is a microtubule-associated deacetylase that predominantly deacetylates nonhistone proteins, including α-tubulin, and regulates cell motility. We showed that TGF-β1-induced EMT is accompanied by HDAC6-dependent deacetylation of α-tubulin. Importantly, inhibition of HDAC6 by small interfering RNA or the small molecule inhibitor tubacin attenuated the TGF-β1-induced EMT markers, such as the aberrant expression of epithelial and mesenchymal peptides, as well as the formation of stress fibers. Reduced expression of HDAC6 also impaired the activation of SMAD3 in response to TGF-β1. Conversely, inhibition of SMAD3 activation substantially impaired HDAC6-dependent deacetylation of α-tubulin as well as the expression of EMT markers. These findings reveal a novel function of HDAC6 in EMT by intercepting the TGF-β-SMAD3 signaling cascade. Our results identify HDAC6 as a critical regulator of EMT and a potential therapeutic target against pathological EMT, a key event for tumor progression and fibrogenesis.

Epithelial-mesenchymal transition (EMT) is defined as a series of events through which epithelial cells lose many of their epithelial characteristics and acquire properties that are typical of mesenchymal cells (1). Aberrant EMT has been well documented in chronic fibrosis in multiple organs and carcinoma progression. During progression to metastatic competence, carcinoma cells acquire mesenchymal gene expression patterns and properties through EMT, which results in coordinated alterations in adhesive properties, activation of proteolysis and motility, and competence to metastasize and establish secondary tumors at distant sites (1). Fibrosis is characterized by an increased number of myofibroblasts that deposit interstitial extracellular matrix. Mounting evidence indicates that a significant fraction of these myofibroblasts arise from the resident epithelial cells via EMT during renal and lung fibrogenesis (2–6).

Family members of transforming growth factor (TGF)–β are among the most potent inducers of EMT in a variety of physiological and pathological contexts (7). The aberrant expression of TGF-β1 is well documented in the tumor microenvironment and fibrotic lesions where TGF-β1 is widely believed to promote tumor progression and tissue fibrosis (7). Several recent studies have elegantly demonstrated EMT of lung alveolar epithelial cells in biopsies from patients with idiopathic pulmonary fibrosis and in experimental pulmonary fibrosis (4, 5, 8). A host of evidence indicates an essential role for SMAD3 in the expression of a panel of EMT-related genes upon translocation into the nucleus (9). The molecular mechanisms underlying TGF-β1–induced EMT are not completely understood and are under intensive investigation.

HDAC and histone acetyltransferase (HAT) regulate acetylation of lysine residues, which has emerged as a key component of the cellular signaling network that coordinates fundamental cellular processes. Eighteen human HDACs have been identified to date and are divided into four classes. HDAC6 belongs to class II and distinguishes itself from other family members in that it contains two HDAC domains and a ubiquitin-binding domain and results in an increase in cell motility (13, 14). HDAC6 deacetylates α-tubulin via a process that requires its second HDAC domain and regulates their processing through its BUZ domain and interaction with chaperones (15–17). Moreover, HDAC6 deacetylates heat shock protein 90, which is essential for heat shock protein 90-mediated maturation of the glucocorticoid receptor (18, 19). A recent study has demonstrated up-regulation of HDAC6 expression by estrogen in estrogen receptor-positive breast cancer cells and further suggests that HDAC6 mediates an...
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estrogen-induced increase in cell motility (20). The exact role for HDAC6 in tumor progression, however, remains unclear.

The current study investigated a role of HDAC6 in TGF-β1-induced EMT. We showed that TGF-β1 induced HDAC6-dependent deacetylation of α-tubulin in human lung epithelial cells, which was concurrent with the expression of EMT markers. Inhibition of HDAC6 attenuated the TGF-β1-induced expression of EMT markers as well as activation of SMAD3. In addition, inhibition of SMAD3 activation abrogated HDAC6-dependent deacetylation of α-tubulin and the expression of EMT markers induced by TGF-β1.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids—Tubacin, an HDAC6-selective inhibitor, and the inactive structural homologue niltubacin were kindly provided by Dr. S. L. Schreiber at Broad Institute of Harvard and MIT (14). SIS3, a selective inhibitor of TGF-β1-dependent phosphorylation of SMAD3 (21), was purchased from EMD Biosciences (San Diego, CA). pRS-SMAD3, a retroviral vector expressing SMAD3-specific siRNA and the negative control vector were provided by Dr. X-F. Wang (Duke University Medical School) and Dr. X-F. Feng (Baylor College of Medicine) (22). Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Promega (Madison, WI) provided the pR-LTK vector expressing Renilla luciferase regulated by the herpes simplex virus thymidine kinase promoter. 3TP-LUX, a luciferase reporter, which contains multiple copies of SMAD3 binding sites was a gift from Dr. J. Massague at Sloan-Kettering Institute (23).

Cell Culture—A549 cells, a human lung adenocarcinoma cell line with features of type II alveolar epithelium, was obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (v/v), 100 μg/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 5% CO₂. A549 cells were serum-starved in Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum for 24 h prior to exposure to TGF-β1 (2.5 ng/ml or the indicated doses) for the indicated durations. To inhibit SMAD3 or HDAC6, A549 cells were pretreated with SIS3 (5 μM for 1 h) or tubacin (5 μM for 6 h), followed by exposure to TGF-β1. A549 variants with intact HDAC6 expression (pS) or reduced HDAC6 expression by RNA interference (KD) were generated using retroviral siRNA expression vectors as previously described (24). The following primary antibodies were used for immunoblots: rabbit polyclonal antibodies specific for E-cadherin (CS-4065), phosphorylated SMAD2 (CS-3101), phosphorylated SMAD3 (CS-9514), and α-tubulin (CS-2144) from Cell Signaling Technology (Danvers, MA) at 1:1,000; mouse monoclonal antibodies specific for vimentin (V9) used at 1:500; acetylated α-tubulin (6-11B-1) at 1:4,000 from Sigma. The secondary antibodies (IRDye800- or IRDye700-conjugated anti-rabbit or anti-mouse IgG, Licor Biosciences, Lincoln, NE) were used at a dilution of 1:15,000 and detected using an Odyssey Infrared Imaging System (Licor Biosciences). The ratios of the proteins of interest over the loading control α-tubulin were determined by densitometry using ImageJ (National Institutes of Health), and a -fold change of each protein upon treatment was obtained by setting the values from the control groups to 1.

Isolation of Nuclear Extracts—Nuclear extracts were isolated from A549 cells exposed to TGF-β1 (2.5 ng/ml) for 1 h as previously described (25). An equal amount of total protein was loaded for immunoblots.

Immunofluorescence—The formation of stress fibers was visualized using Alexa 488-conjugated phalloidin (Invitrogen). Briefly, A549 cells were cultured in 8-well chamber slides and exposed to TGF-β1 (2.5 ng/ml) for 48 h. The cells were then fixed in 4% paraformaldehyde/phosphate-buffered saline for 5 min, followed by incubation with phalloidin. The digital images were captured using a Nikon Eclipse 80i microscope along with the accompanying program IPLab, version 3.6.5 (Nikon). Immunofluorescence was carried out to examine the distribution of SMAD3 (antibody at 1:50 dilution) and acetylated α-tubulin (antibody at 1:500 dilution) using the appropriate primary antibodies on A549 cells exposed to TGF-β1 (2.5 ng/ml). Alexa 488- and 594-conjugated secondary antibodies (at 1:1500 dilution) were used to visualize SMAD3 and acetylated α-tubulin, respectively. The nucleus was stained using 4’,6-diamidino-2-phenylindole-containing mounting medium VectorShield (Vector Laboratories, Burlingame, CA). The subcellular distribution of SMAD3 and acetylated α-tubulin was analyzed using a Leica DMRXA deconvolution microscope (Morphology and Imaging Core of the Gene Therapy Program at the Louisiana State University Health Sciences Center). The morphological changes of A549 cells upon exposure to TGF-β1 were assessed by staining with hematoxylin and eosin.

Transient Transfection and Luciferase Reporter Assays—A549 cells and its variants were transfected with either the reporter constructs or siRNAs using Lipofectamine 2000 (Invitrogen), as previously described (26). The siRNAs targeting human HDAC6 (HDAC6si, ID number 120450; Ambion, Austin, TX) or SMAD3 (pRS-SMAD3) or the negative control siRNA (CTLSi or pRS-Vec) were transfected to knock down HDAC6 or SMAD3 expression. For luciferase reporter assays, the cells were serum-starved for 24 h, followed by exposure to TGF-β1 (2.5 ng/ml) for 24 h. The treated cells were harvested and assayed for luciferase activity using the Dual-Luciferase Reporter Assay system (Promega) as per the provider’s instructions. Variation in transfection efficiency was monitored and normalized to Renilla luciferase activity from the co-transfected pRL-TK. A -fold change of normalized luciferase activity was obtained by setting the values from the untreated cells to 1.

Quantitative Reverse Transcription PCR—Quantitative RT-PCR was performed to determine miRNA levels of genes of interest in A549, and its variants that were subjected to the indicated
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RESULTS

HDAC6-dependent Deacetylation of α-Tubulin during TGF-β1-mediated EMT—To investigate the role of HDAC6 in EMT, we employed A549 cells, a human lung epithelial cell line that has been demonstrated to undergo EMT upon exposure to TGF-β1 (27). First, we examined the expression of EMT markers in the cells stimulated with TGF-β1 (2.5 ng/ml) through 48 h. TGF-β1 substantially reduced the protein levels of E-cadherin, an epithelial cell marker (Fig. 1A), and ablated the membrane expression of E-cadherin as demonstrated by immunofluorescence (data not shown). In contrast, TGF-β1 induced a significant increase in vimentin, a mesenchymal cell marker (Fig. 1A). Equal loading of each sample was confirmed by immunoblotting for α-tubulin, a housekeeping gene (Fig. 1B). The primers used were as follows: human 36B4 (NM_001002), forward primer (positions 97−116; 5'-CGACCTGGAAGTGCCAATCAG-3') and reverse primer (positions 205 to 188; 5'-CACGAGGAGTCAGGAAGG-3'), and reverse primer (positions 97−116; 5'-CACGAGGAGTCAGGAAGG-3') and reverse primer (positions 347 to 327; 5'-AGGCGCAGTCACTGAGGAG-3') and reverse primer (positions 2288−2308; 5'-GAATGC-3') and reverse primer (positions 347 to 327; 5'-AGGCGCAGTCACTGAGGAG-3') and reverse primer (positions 2288−2308; 5'-GAATGC-3'), and reverse primer (positions 2288−2308; 5'-GAATGC-3'), and reverse primer (positions 779 to 758; 5'-AATGGTCTCTTCCAGTGGAGG-3') and reverse primer (positions 1036 to 1018; 5'-AGGCTTCGACTGACTCC-3').

The results that were obtained from three independent experiments were presented as mean ± S.D. Statistical evaluation was performed between individual treatments, except that the protein levels of α-SMA, α-tubulin, and acetylated α-tubulin were examined in the primary cultures of human lung fibroblasts and arterial endothelial cells exposed to TGF-β1 (2.5 ng/ml) for 48 h, and protein levels of α-tubulin and acetylated α-tubulin were examined in A, C, culture conditions were similar to those in A, except that the protein levels of α-SMA, α-tubulin, and acetylated α-tubulin were examined in the primary cultures of human lung fibroblasts and arterial endothelial cells exposed to TGF-β1 (2.5 ng/ml) for 48 h, and protein levels of α-tubulin and acetylated α-tubulin were examined in A, C, culture conditions were similar to those in A, except that the protein levels of α-SMA, α-tubulin, and acetylated α-tubulin were examined in the primary cultures of human lung fibroblasts and arterial endothelial cells exposed to TGF-β1 (2.5 ng/ml) for 48 h, and protein levels of α-tubulin and acetylated α-tubulin were examined in A, C, culture conditions were similar to those in A, except that the protein levels of α-SMA, α-tubulin, and acetylated α-tubulin were examined in the primary cultures of human lung fibroblasts and arterial endothelial cells exposed to TGF-β1 (2.5 ng/ml) for 48 h, and protein levels of α-tubulin and acetylated α-tubulin were examined in A, C, culture conditions were similar to those in A, except that the protein levels of α-SMA, α-tubulin, and acetylated α-tubulin were examined in the primary cultures of human lung fibroblasts and arterial endothelial cells exposed to TGF-β1 (2.5 ng/ml) for 48 h, and protein levels of α-tubulin and acetylated α-tubulin were examined in A, C, culture conditions were similar to those in A, except that the protein levels of α-SMA, α-tubulin, and acetylated α-tubulin were examined in the primary cultures of human lung fibroblasts and arterial endothelial cells exposed to TGF-β1 (2.5 ng/ml) for 48 h, and protein levels of α-tubulin and acetylated α-tubulin were examined in A, C, culture conditions were similar to those in A, except that the protein levels of α-SMA, α-tubulin, and acetylated α-tubulin were examined in the primary cultures of human lung fibroblasts and arterial endothelial cells exposed to TGF-β1 (2.5 ng/ml) for 48 h, and protein levels of α-tubulin and acetylated α-tubulin were examined in A, C, culture conditions were similar to those in A, except that the protein levels of α-SMA, α-tubulin, and acetylated α-tubulin were examined in the primary cultures of human lung fibroblasts and arterial endothelial cells exposed to TGF-β1 (2.5 ng/ml) for 48 h, and protein levels of α-tubulin and acetylated α-tubulin were examined in A, C, culture conditions were similar to those in A, except that the protein levels of α-SMA, α-tubulin, and acetylated α-tubulin were examined in the primary cultures of human lung fibroblasts and arterial endothelial cells exposed to TGF-β1 (2.5 ng/ml) for 48 h, and protein levels of α-tubulin and acetylated α-tubulin were examined in A, C, culture conditions were similar to those in A, except that the protein levels of α-SMA, α-tubulin, and acetylated α-tubulin were examined in the primary cultures of human lung fibroblasts and arterial endothelial cells exposed to TGF-β1 (2.5 ng/ml) for 48 h, and protein levels of α-tubulin and acetylated α-tubulin were examined in A, C, culture conditions were similar to those in A, except that the protein levels of α-SMA, α-tubulin, and acetylated α-tubulin were examined in the primary cultures of human lung fibroblasts and arterial endothelial cells exposed to TGF-β1 (2.5 ng/ml) for 48 h, and protein levels of α-tubulin and acetylated α-tubulin were examined in A, C, culture conditions were similar to those in A, except that the protein levels of α-SMA, α-tubulin, and acetyl...
stress fibers, which are typically rich in the cells of mesenchymal lineage (Fig. 1D). These results validated A549 as an experimental system to examine the role of HDAC6 in EMT.

We also measured the levels of acetylated α-tubulin (Fig. 2A), a well established substrate of HDAC6 (13). The expression of acetylated α-tubulin declined as early as 24 h after exposure to TGF-β1 (2.5 ng/ml) and was reduced to 46 ± 4% of the base line by 48 h (Fig. 2A, average and S.D. values from three independent experiments; p < 0.01). The reduction persisted through 72 h (data not shown). Moreover, acetylated α-tubulin declined in a dose-dependent manner following treatments with increasing doses of TGF-β1 (0–5 ng/ml) for 48 h (Fig. 2B). We further examined the levels of acetylated α-tubulin in primary cultures of human pulmonary arterial endothelial cells and fibroblasts stimulated with TGF-β1 (3 ng/ml) for 48 h. Both cell types exhibited a robust induction of α-smooth muscle actin (α-SMA) in response to TGF-β1 (Fig. 2C), and disparate responses of acetylated α-tubulin were observed in these two cell types exposed to TGF-β1. Similar to A549 cells, the endothelial cells exhibited a 50% reduction in acetylated α-tubulin in response to TGF-β1, whereas the fibroblasts displayed little change in the levels of acetylated α-tubulin (Fig. 2C; average from two independent experiments). To test whether deacetylation of α-tubulin was dependent upon HDAC6, tubacin, a small molecule inhibitor of HDAC6 (14), was utilized to inhibit HDAC6 activity. Tubacin (5 μM) elevated the expression of acetylated α-tubulin to 1.7-fold over the base line in the absence of TGF-β1 (Fig. 2D; average from two independent experiments). More importantly, tubacin restored the levels of acetylated α-tubulin to 94% of the base line in the presence of TGF-β1 (2.5 ng/ml) (Fig. 2D). In contrast, niltubacin, an inactive structural homologue to tubacin, exhibited little effect on the levels of acetylated α-tubulin (Fig. 2D). To further confirm the HDAC6-dependent deacetylation of α-tubulin, an A549 variant that stably expresses HDAC6-targeting siRNA (KD) and the matching control (pS) were exposed to TGF-β1 (1 and 2.5 ng/ml) for 48 h. Quantitative RT-PCR for HDAC6 revealed a more than 70% reduction in HDAC6 transcript regardless of the presence of TGF-β1 in KD but not pS cells (data not shown). Immunoblots for HDAC6 confirmed siRNA-mediated knockdown of HDAC6 (Fig. 2E). When compared with pS cells, KD cells exhibited higher basal levels of acetylated α-tubulin, 1.2-fold over the base line, and restored acetylated α-tubulin to 97% of the base line in the presence of TGF-β1 (Fig. 2E; average from two independent experiments). Similar profiles of acetylated α-tubulin were observed when the cells were transiently transfected with HDAC6-targeting siRNA (data not shown). In contrast to HDAC6-dependent deacetylation of α-tubulin in response to TGF-β1, TGF-β1 did not alter the expression of HDAC6 at either the mRNA or protein levels (Fig. 2E) (data not shown). Taken together, these results demonstrate HDAC6-dependent deacetylation of α-tubulin during TGF-β1-induced EMT.

Requirement of HDAC6 for the Expression of EMT Markers—Our findings described above indicated up-regulated activity of HDAC6 in conjunction with TGF-β1-induced expression of EMT markers, which implicated a role of HDAC6 in EMT. Therefore, we examined the expression of EMT markers in the same cell lysates that exhibited HDAC6-dependent deacetylation of α-tubulin (Fig. 2E). Expression of HDAC6 targeting siRNA in KD cells attenuated the expression of EMT markers induced by TGF-β1, as evidenced by 1) restored expression of E-cadherin, up to 81% of the base line versus a decrease to 18% of the base line by TGF-β1 in pS cells, 2) diminished induction of vimentin to a 3 ± 1.8-fold increase over the base line versus a 6.8-fold increase over the base line by TGF-β1 (Fig. 3A; average from two independent experiments).
tein was verified by immunoblots for α-tubulin, as illustrated in Fig. 2E. Consistently, KD cells exhibited reduced alteration of E-cadherin and vimentin transcripts in response to TGF-β1 (2.5 ng/ml) when compared with that of pS cells. Repressed expression of E-cadherin mRNA by TGF-β1 was partially rescued in KD cells as demonstrated by a 30 ± 5% reduction in KD cells versus an 85 ± 22% reduction in pS cells (Fig. 3B, average and S.D. from three independent experiments, p < 0.05). Activated expression of vimentin mRNA by TGF-β1 was substantially inhibited in KD cells as illustrated by a 1.8 ± 0.3-fold increase in KD cells versus a 3.2 ± 0.2-fold increase in pS cells (Fig. 3B, average and S.D. from three independent experiments, p < 0.01). Two other HDAC6-inhibitory approaches were undertaken to confirm the requirement of HDAC6 for the maximal expression of EMT markers. First, the HDAC6-selective inhibitor tubacin (5 μM) was employed. Tubacin, but not niltubacin, restored the expression of E-cadherin with a modest 24% reduction in the mRNA levels of E-cadherin by TGF-β1 (2.5 ng/ml), whereas TGF-β1 repressed the expression of E-cadherin to a 71% reduction in the absence of tubacin (Fig. 3C). On the other hand, TGF-β1-induced expression of vimentin was significantly inhibited by tubacin from a 3.4-fold increase in the absence of tubacin down to a 2.2-fold increase in the presence of tubacin (Fig. 3C). Consistently, tubacin restored the protein levels of E-cadherin in the presence of TGF-β1 to 83% of the base line in contrast to a reduction to 15–20% of the base line in the TGF-β1 with or without niltubacin groups (Fig. 3D; average from two independent experiments). On the other hand, induction of vimentin was substantially reduced to a 2.9-fold increase by tubacin versus a 6–7-fold increase over the base line by TGF-β1 with or without niltubacin. Second, transient transfection of a second pair of HDAC6-specific siRNA oligonucleotides (30 nm), but not the control siRNA, efficiently compromised TGF-β1-altered expression of E-cadherin and vimentin at the mRNA levels (Fig. 3E). In addition, we examined the role of HDAC6 in the expression of another hallmark feature of EMT, the formation of stress fibers. KD cells exhibited little formation of stress fibers after exposure to TGF-β1 (2.5 ng/ml) as compared with the robust induction of stress fibers in pS cells (Fig. 3F). In concert, these results demonstrate a requirement of HDAC6 for the maximal expression of a panel of EMT markers induced by TGF-β1.

We expanded our analyses to the expression of a broad range of genes that are regulated by TGF-β1 using a targeted RT2 Profiler PCR array for human TGF-β and BMP signaling pathways. Among 84 genes tested in the array, activation of 13 genes by TGF-β1 was substantially inhibited in KD cells when compared with that in pS cells (Table 1). The profiles of three genes with direct relevance to EMT were confirmed individually by quantitative RT-PCR. Consistent with the results from the array, the increase in the transcripts encoding PAI-1, collagen type I α1, and PDGF-B was reduced to 15–40% of that observed in pS cells induced by TGF-β1 (Fig. 4A). We further tested the role of HDAC6 in the expression of TGF-β1-activated genes in a mouse lung epithelial cell line, C10 cells. Exposure to TGF-β1 (2.5 ng/ml) for 24 h elevated the mRNA levels of PDGF-B, CTGF, and TIMP1 to a 3.4-, 3.8-, and 5.1-fold increase, respectively (Fig. 4B). Tubacin, but not niltubacin, completely abrogated the increase in all three genes in the presence of TGF-β1 (Fig. 4B). These findings further strengthen the concept that HDAC6 is a key mediator of TGF-β1 signaling.

**Interplay between HDAC6 and SMAD3 Signaling—**SMAD3 is essential for TGF-β1-induced EMT (9). Microtubule structure has been suggested in regulation of SMAD signaling (28), which prompted us to investigate a role of HDAC6 in SMAD3 signaling. Phosphorylation of SMAD2/3 was examined in pS and KD cells exposed to TGF-β1 (2.5 ng/ml) for 30 min. KD cells displayed diminished activation in SMAD3, a 3.4-fold increase in phosphorylated SMAD3 versus a 7.8-fold increase in pS cells by TGF-β1, whereas SMAD2 was phosphorylated to a similar extent in pS and KD cells (Fig. 5A; average from two independent experiments). pS and KD cells displayed similar levels of

**TABLE 1**

**Reduced expression of TGF-β1-activated genes in response to HDAC6-targeting siRNA**

| Genes   | pS Control | TGF-β1 | KD Control | TGF-β1 |
|---------|------------|--------|------------|--------|
| Nog     | 1          | 46.6   | 0.7        | 7.1    |
| COLIA1  | 5          | 99.2   | 1.6        | 6.6    |
| GDF6    | 1          | 110.7  | 1.2        | 2.1    |
| IGF1    | 5          | 30.5   | 0.5        | 0.7    |
| JUN     | 1          | 16.2   | 0.5        | 0.8    |
| HINB    | 2          | 26.2   | 0.2        | 3.4    |
| LTBP2   | 1          | 24.2   | 1.3        | 4.5    |
| PDGFB   | 1          | 50.0   | 0.4        | 2.1    |
| PLAU    | 1          | 7.7    | 1.5        | 3.1    |
| TGF-BII | 1          | 6.9    | 0.6        | 1.6    |
| TGF-B1  | 1          | 22.2   | 0.4        | 11.3   |
| TGF-BR1 | 1          | 11.1   | 0.8        | 1.5    |
| PAI-1   | 1          | 100.6  | 0.6        | 7.4    |

**FIGURE 4. HDAC6 is required for the expression of TGF-β1-induced genes.** A, the A549 cell variants pS and KD were exposed to TGF-β1 for 24 h. Quantitative RT-PCR was performed for the indicated genes on the total cellular RNA isolated from the treated cells. The fold change of each transcript was obtained by setting the values of the unexposed pS cells to 1. B, serum-starved C10 cells were exposed to TGF-β1 (2.5 ng/ml) in the presence or absence of tubacin (5 μM) for 24 h. The expression of the indicated genes was analyzed, similar to A. N, cells exposed to niltubacin. Error bars, S.D. from three independent experiments.
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FIGURE 5. HDAC6 is required for SMAD3 activation. A, total cellular protein was extracted from A549 variant pS and KD cells exposed to TGF-β1 (2.5 ng/ml) for 30 min. Immunoblots were carried out for phosphorylated SMAD2, phosphorylated SMAD3, and total SMAD3. B, the reporter constructs 3TP-LUX and RL-TK were cotransfected into A549 pS and KD cells. The transfected cells were then serum-starved, followed by exposure to TGF-β1 (2.5 ng/ml) for 24 h, and expression of the luciferase reporters was measured on cell lysates from the treated cells. The fold change of SMAD3-responsive 3TP-LUX activity was obtained by normalizing against RL-TK activity and setting the values of the unexposed pS cells to 1. C, nuclear extracts were prepared from pS and KD cells exposed to TGF-β1 for 1 h and immunoblotted for SMAD3 and histone H3. D, pS and KD cells were treated similarly to C. Immunofluorescence was carried out for SMAD3 and acetylated α-tubulin. The nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI). E, serum-starved A549 cells were exposed to TGF-β1 (2.5 ng/ml) for 1 h. Immunofluorescence was carried out for SMAD3 and acetylated α-tubulin. Deconvolution immunofluorescence microscopy was used to analyze subcellular distribution of SMAD3 and acetylated α-tubulin. Representative images were presented. Pseudocolors were assigned to SMAD3 (green), acetylated α-tubulin (red), and the nucleus (blue). CTL, control.

SMAD3 and TGF-β receptor type I. The results led us to investigate whether there is a requirement of HDAC6 for SMAD3-dependent transcriptional activation. A reporter construct, 3TP-LUX, that harbors multiple copies of SMAD3 binding sites (23) was transfected into pS and KD cells to assess SMAD3-dependent transcriptional activation. In pS cells, TGF-β1 (2.5 ng/ml) stimulated a 4.9 ± 0.4-fold increase in the reporter activity (Fig. 5B). In contrast, the reporter activity in KD cells was only approximately 20 ± 8% of that in pS cells in the absence of TGF-β1. Moreover, TGF-β1-mediated activation of the reporter was elevated to only 82 ± 15% of what was observed in the unstimulated pS cells (Fig. 5B; average and S.D. from three independent experiments, p < 0.01). Since TGF-β1-stimulated phosphorylation of SMAD3 leads to nuclear translocation of SMAD3, we compared nuclear accumulation of SMAD3 in pS and KD cells exposed to TGF-β1 (2.5 ng/ml) for 1 h. TGF-β1-stimulated nuclear accumulation of SMAD3 with a 2.4-fold increase over the base line in pS cells (Fig. 5C; average from two independent experiments). In contrast, KD cells exhibited reduced nuclear accumulation of SMAD3 in the absence of TGF-β1, 64% of the base line observed in pS cells. In the presence of TGF-β1, KD cells demonstrated 88% of the base line occurring in pS cells. Reduced nuclear accumulation of SMAD3 was further confirmed by immunofluorescence for SMAD3. TGF-β1 induced nuclear translocation of SMAD3 (green) in pS cells, as evidenced by a shift from diffuse cytoplasmic and nuclear stain of SMAD3 to a predominant nuclear distribution of SMAD3 (Fig. 5D, green). In contrast, SMAD3 remained diffuse in KD cells exposed to TGF-β1. Since previous reports suggest a role of microtubule structure in modulating localization of SMAD3 (28), we speculated that acetylated α-tubulin might bind to SMAD3 directly and modulate its cytoplasm and nuclear distribution. Acetylated α-tubulin was simultaneously stained and analyzed by deconvolution immunofluorescence microscopy. We did not detect direct binding of SMAD3 to acetylated α-tubulin, regardless of the presence of TGF-β1 (Fig. 5E).

As demonstrated above (Figs. 1 and 2), HDAC6-dependent deacetylation of α-tubulin was concurrent with the expression of EMT markers induced by TGF-β1. Thus, we investigated the role for SMAD3 in HDAC6-dependent deacetylation of α-tubulin. Activation of SMAD3 by TGF-β1 (2.5 ng/ml) was selectively inhibited by pretreating the cells with SIS3 (5 μM), a selective inhibitor of SMAD3 activation. SIS3 selectively reduced phosphorylation of SMAD3 to a 2.4-fold increase over the base line versus a 7.5-fold increase by TGF-β1 with or without DMSO (Fig. 6A; average from two independent experiments). Phosphorylation of SMAD2 by TGF-β1 remained intact in the presence of SIS3. Consistent with the established requirement of SMAD3 in EMT, SIS3 inhibited TGF-β1-induced alterations in the expression of E-cadherin (restored up to 74% of the base line) and vimentin (reduced to a 2.6-fold increase over the base line versus a 5.7-fold increase by TGF-β1 alone) (Fig. 6B; average from two independent experiments). More importantly, reduced EMT was accompanied by restored levels of acetylated α-tubulin to 72% of the base line in the presence of TGF-β1 (Fig. 6B). Accordingly, transfection of a SMAD3-targeting siRNA vector (pRS-SMAD3), but not the control siRNA (pRS-Vec), reduced the expression of EMT markers and restored the levels of acetylated α-tubulin to 73 ± 4% of the base line (Fig. 6C; average and S.D. from three independent experiments, p value < 0.01). Similar to HDAC6 knockdown (Fig. 3F), SIS3 (5 μM) substantially inhibited the formation of stress fibers induced by TGF-β1 (Fig. 6D). Taken together, these results suggest that there is interplay...
demonstrates HDAC6-dependent deacetylation of 
lates diverse and fundamental cellular processes (13). Our study 
between acetylation and microtubules, a structure that regu-
Apendent deacetylation of HDAC6 is required for the maximal expression of a panel of 
expression of EMT markers in human lung epithelial cells. HDAC6 as a deacetylase for 
effects related to EMT.

FIGURE 6. SMAD3 activation is required for TGF-β1-induced EMT and deacetylation of α-tubulin. 
A, serum-starved A549 cells were pretreated with SIS3 (μM) for 1 h followed by exposure to TGF-β1 (2.5 ng/ml) 
for 30 min. Immunobots were carried out for SMAD3 and phosphorylated SMAD2/3. B, culture conditions were 
similar to those in A, except that the cells were exposed to TGF-β1 (2.5 ng/ml) for 48 h. Immunobots were 
performed to assess the expression of the indicated proteins. C, A549 cells were transfected with either the 
control (SR) or the SMAD3-specific siRNA-expressing (SMAD3si) vector followed by treatments as described in B. The abundance of the indicated proteins was determined by immunobots. D, serum-starved A549 p53 and 
KD cells were exposed to TGF-β1 with or without SIS3 for 48 h. The formation of stress fibers was visualized 
using Alexa 488-conjugated phalloidin. TGF-β1 + S, cells exposed to TGF-β1 and DMSO. CTL, control.

between HDAC6 and SMAD3 in the cells undergoing TGF-β1-induced EMT.

DISCUSSION

EMT contributes to tumor progression and tissue fibrosis. The current study explores the molecular mechanisms that 
mediate TGF-β1-induced EMT. TGF-β1 induces HDAC6-dep
ependent deacetylation of α-tubulin that correlates with the expression of EMT markers in human lung epithelial cells. HDAC6 is required for the maximal expression of a panel of 
EMT markers and activation of SMAD3 by TGF-β1. Furthermore, our results suggest interplay between SMAD3 signaling 
and HDAC6 during EMT. These results warrant further invesigation of HDAC6 as a therapeutic target against pathological 
effects related to EMT.

Reversible acetylation of lysine residues has emerged as a major mechanism to regulate protein activity. Identification of 
HDAC6 as a deacetylase for α-tubulin highlights a connection between acetylation and microtubules, a structure that regulates diverse and fundamental cellular processes (13). Our study demonstrates HDAC6-dependent deacetylation of α-tubulin in a human lung epithelial cell line exposed to TGF-β1. More importantly, deacetylation of α-tubulin is concurrent with the expression of EMT markers (Figs. 2 and 4). In addition, blockade of SMAD3 activation by SIS3 or siRNA attenuates deacetylation of α-tubulin as well as the expression of EMT markers (Fig. 6). These findings strongly suggest that HDAC6-dependent deacetylation of α-tubulin is an integral component of TGF-β1-induced EMT. The findings are also confirmed using a murine nontransformed epithelial cell line (Fig. 4B), thus showing that our findings are not limited to malignant epithelial cells. Our results using primary human lung endothelial cells exposed to TGF-β1 further support this notion. Endothelial cells have been reported to transition to α-SMA-positive mesenchymal cells upon exposure to TGF-β1 (29). Not surpris

ingly, human lung endothelial cells respond to TGF-β1 with a robust increase in α-SMA. More interestingly, activation of the α-SMA expression correlates nicely with deacetylation of α-tubulin (Fig. 2C, left). In contrast, deacetylation of α-tubulin is absent in human lung fibroblasts that undergo TGF-β1-induced transdifferentiation into myofibroblasts (30), as evidenced by activation of α-SMA expression (Fig. 2C, right). Results from the three cell types employed in this study suggest that TGF-β1 regulates HDAC6-dependent deacetylation of α-tubulin in a cell type- and context-dependent manner. Given that overexpression of HDAC6 leads to deacetylation of α-tubulin and increased cell motility (13, 14), it is conceivable that TGF-β1-induced HDAC6-dependent deacetylation of α-tubulin contributes to increased motility, an important biological consequence of EMT. TGF-β1 appears to up-regulate HDAC6 activity via post-translational mechanisms, because neither the mRNA nor the protein levels of HDAC6 are altered by TGF-β1 (Fig. 2 and data not shown). A recent report indicates that Aurora-A kinase activates α-tubulin deacetylase activity of HDAC6 via phosphorylation (31). The study further suggests that Aurora A kinase directly phosphorylates HDAC6, although the phosphorylation site remains unidentified. Interestingly, two potential phosphorylation residues are predicted using a probability-based approach based on the mass spectrometry profile of HDAC6 (available on PhosphoSitePlus™). One of the two sites, serine 22, resides in a mitogen-activated protein kinase consensus site PQSP, which raises the possibility that mitogen-activated protein kinases modulate HDAC6 activity via phosphorylation of serine 22 during TGF-β1-induced EMT. It is also noteworthy that a second deacetylase SIRT2 has been identified as an α-tubulin deacetylase (32). Intriguingly, SIRT2 co-immunoprecipitates with HDAC6 in the study, which raises the possibility that the two enzymes cooperate to deacetylate α-tubulin. Thus, it would be appealing to test whether TGF-β1 up-regulates HDAC6 activity via post-translational modifications and/or coordination with SIRT2.

Given the importance of pathological EMT in tumor progression and tissue fibrosis, dissection of the signaling network that mediates EMT can lead to novel therapeutic targets to treat these maladies. The current study unravels a pivotal role of HDAC6 for the maximal and full spectrum expression of EMT markers induced by TGF-β1 in human lung epithelial cells (Figs. 3 and 4). A limited panel of EMT markers evaluated in our studies exhibited sensitivity to inhibition of HDAC6 by both pharmacologic and molecular approaches. EMT is defined by coordinated alteration in diverse facets of cellular appearance and behavior, which is orchestrated by an extremely complex
extracellular and intracellular signaling network. Given the complexity of this extreme example of cell plasticity, it is likely that HDAC6 mediates EMT in response to only certain stimuli and in only certain cellular contexts. Moreover, HDAC6 might be essential for expression of a selected group of EMT markers but dispensable for others. For instance, HDAC6-deficient mice are viable, which suggests that HDAC6 is not required for EMT essential for embryonic development.\(^3\) Our study warrants extensive analysis of the role of HDAC6 in the expression of a broad range of EMT markers and in diverse cellular contexts.

Our results further suggest that the role of HDAC6 in the cellular response to TGF-\(\beta\)-1 is probably beyond regulation of mere EMT markers. In addition to EMT markers, such as PAI-1 and collagen Type 1 \(\alpha_1\), inhibition of HDAC6 substantially impairs activation of a panel of other classical TGF-\(\beta\)-1-responsive genes that have not been directly connected to EMT (e.g. CTGF, TIMP1, and LTBP2) (Table 1 and Fig. 4). In line with this concept, TGF-\(\beta\)-1 induces deacetylation of \(\alpha\)-tubulin in endothelial cells, which is associated with increased permeability of the endothelium and an impaired blood vessel barrier (33). These observations implicate HDAC6 as a mediator for other TGF-\(\beta\)-1-mediated pathology.

TGF-\(\beta\)-1 induces EMT via activation of both canonical and noncanonical pathways. As a pivotal component of the canonical pathway, SMAD3 is essential for TGF-\(\beta\)-1-induced EMT (9). Interestingly, emerging evidence implicates that microtubule structure regulates SMAD activity (28, 34, 35). Our study suggests that there is interplay between SMAD3 and HDAC6. The expression of HDAC6-specific siRNA impairs phosphorylation of SMAD3 by TGF-\(\beta\)-1, whereas phosphorylation of SMAD2 is spared (Fig. 5A). Our results further demonstrate the requirement of HDAC6 for TGF-\(\beta\)-1-mediated activation of a luciferase reporter that is controlled by SMAD3-responsive elements (Fig. 5B). Previous studies suggest that binding of SMADs to microtubules keeps SMADs in their inactive stage, and TGF-\(\beta\)-1 can trigger the release of SMADs from microtubules and the subsequent phosphorylation of SMADs. Our results suggest that HDAC6 is required for nuclear accumulation of SMAD3 by TGF-\(\beta\)-1 (Fig. 5). However, HDAC6-dependent deacetylation of acetylated \(\alpha\)-tubulin may not have a direct role in nuclear translocation of SMAD3, since we did not detect direct binding of SMAD3 to acetylated \(\alpha\)-tubulin (Fig. 5). A recent study has reported the requirement of HDAC6 for EGF-induced nuclear localization of \(\beta\)-catenin (36). Deacetylation of lysine 49 in \(\beta\)-catenin is suggested to be essential for nuclear localization of \(\beta\)-catenin. It is conceivable that HDAC6 regulates SMAD3 nuclear localization via deacetylating SMAD3 or SMAD3-interacting proteins. Further studies are necessary to determine the molecular mechanisms underlying HDAC6-mediated modulation of SMAD signaling. On the other hand, inhibition of SMAD3 activation attenuates HDAC6-dependent deacetylation of \(\alpha\)-tubulin as well as the expression of the EMT markers (Fig. 5, C and D), which suggests that SMAD3 mediates TGF-\(\beta\)-1 induction of HDAC6 activity. Since inhibition of SMAD3 does not abolish TGF-\(\beta\)-1-induced EMT and deacetylation of \(\alpha\)-tubulin completely, it is likely that activation of noncanonical pathways also plays a role in TGF-\(\beta\)-1-mediated activation of HDAC6 as well as EMT. Nevertheless, our results implicate a co-dependence of HDAC6 and SMAD3 in that HDAC6 mediates activation of SMAD3, an early event occurring within 30 min after exposure to TGF-\(\beta\)-1 and SMAD3 activation, which in turn mediates HDAC6-dependent deacetylation of \(\alpha\)-tubulin, a rather late event.

In conclusion, the current study demonstrates that HDAC6 activity is required for the expression of EMT markers induced by TGF-\(\beta\)-1 in human lung epithelial cells. Our results also reveal interplay between HDAC6 and SMAD3 during TGF-\(\beta\)-1-induced EMT. These findings suggest a novel function for HDAC6 as a regulator of EMT through interplay with SMAD3 signaling. Further investigation of the HDAC6-dependent pathway in EMT may potentially lead to novel therapeutic targets against tumor progression and fibrogenesis.

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