TGF-β1 stimulates human AT₁ receptor expression in lung fibroblasts by cross talk between the Smad, p38 MAPK, JNK, and PI3K signaling pathways

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fibroblasts (33). Together, these studies suggest that there is a “positive feedback loop” between ANG II and TGF-β1 that results in the amplification of their proinflammatory effects.

Currently, very little information is known regarding the mechanisms by which TGF-β1 enhances human AT₁R (hATıR) gene expression (33). Therefore, the following study was initiated to investigate these mechanisms. We demonstrated that in primary cultured human fetal pulmonary fibroblasts (hPFBs), TGF-β1 treatment (4 ng/ml) maximally stimulated hATıR steady-state mRNA levels at 4 h. Maximal protein and ANG II–induced signaling occurred 8 h after TGF-β1 treatment. Together, our data support the hypothesis that TGF-β1 treatment enhances AT₁R expression by the synergistic interaction between the Smad and specific kinase signaling pathways that are simultaneously activated by activin receptor-like kinase (ALK5).

EXPERIMENTAL PROCEDURES

**Chemicals, reagents, and antibodies.** ANG II, actinomycin D (Act D), and cycloheximide were purchased from Sigma (St. Louis, MO). 125I-[Sar¹, Ile⁴]-ANG II was purchased from the Peptide Radiodination Service Center (University of Mississippi, University, MS). Human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). PD-98059, LY-294002, SB 203580, U0126, SP600125, and SB 431542 were purchased from Calbiochem (San Diego, CA). Tubulin, ERK1/2, phospho-ERK1/2, Smad2, phospho-Smad2, Smad3, phospho-Smad3, Smad4, MAPK1, MEK1, phospho-tidylinositol 3-kinase (PI3K), p38 MAPK, and JNK antibodies were purchased from Cell Signaling (Beverly, MA). ON-TARGETplus SMARTpool human Smad2 (L-003661), Smad3 (L-020127), Smad4 (L-003902), ALK5 (L-003929), MAPK1 (MEK1, L-003555), p38 MAPKα (MAPK14, L-003512), JNK1 (MAPK8, L-003514), PI3K (PIK3CA, L-003018), and siControl (D-001810) small interference RNAs (siRNAs) were purchased from Dharmacon (Lafayette, CO). The negative control siRNA has at least four mismatches to every known human gene and was microarray tested for “off” target activity. The negative control siRNA has at least four mismatches to every known human gene and was microarray tested for “off” target activity. The negative control siRNA has at least four mismatches to every known human gene and was microarray tested for “off” target activity.

Cell culture. hPFBs were established in primary culture from human neonatal lung tissue obtained at autopsy and were the enzyme-dispersed tissue fragments adhered to Primaria culture plates (IBA, Gottingen, Germany) as instructed by the manufacturer (magnets present represent specific [total minus nonspecific, i.e., total non–radiolabeled RNA, 500 – 800 cpm; and nonspecific ~500 – 800 cpm] binding and have been normalized with protein content. To ensure that TGF-β1 treatment did not modulate the content of the AT₁Rs, radioligand saturation isotherm experiments and Scatchard analysis were also performed as previously described (25, 26).

Western blot analyses. hPFB cells were plated and treated as described in Cell culture. These cells were subsequently lysed with RIPA buffer with freshly added protease and phosphatase inhibitors. Equal amounts (10 μg/ml) of cell lysate were separated by 10% SDS-PAGE. Following transfer to nitrocellulose membrane and blocking with 5% nonfat milk, the blots were incubated with the appropriate antibody. The immunoblots were then incubated with a secondary antibody conjugated with horseradish peroxidase and visualized with enhanced chemiluminescence, and the autoradiograph was quantitated by densitometric analysis.

Nuclear run-on assays. hPFB cells were plated on six-well dishes, grown to 70–80% confluence, washed once with PBS, and serum-starved for 24 h. The cells were then stimulated with, or without, 4 ng/ml TGF-β1 for 4 h. The cells were lysed, and nuclei were isolated for reconstitution. The nuclei (~2 × 10⁶/reaction) were utilized to perform the in vitro transcription in a reaction mixture containing 40% glycerol, 50 mM KCl, pH 8.0, 5 mM MgCl₂, 0.1 mM EDTA, 25 mM of CTP, GTP, ATP, and UTP at 30°C for 30 min. RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. The RNA was subsequently treated with RNase-free DNase I and cDNA was synthesized from the “run-on” transcripts using oligo(dT) (for hATıR amplification) or 18S gene-specific primer. The expression of hATıR mRNA relative to 18S rRNA was determined using SYBR green real-time quantitative PCR assay as described (26). Relative gene expression was calculated as 2^(-ΔΔCT) and was multiplied by 10⁶ to simplify data presentation. The hATıR-specific primers used were as follows: sense primer, 5’-CACCACTGTGGTGTGCTGACTGC-3’; antisense primer, 5’-CAAGTTAGAGGATTTGCTCTG-3’. hATıR mRNA half-life assays. hPFB cells were plated on six-well dishes, grown to 70–80% confluence, washed once with PBS, and serum-starved for 24 h. The cells were then stimulated with or without 4 ng/ml TGF-β1 for 4 h. The cells were then either collected (time 0, control) or treated with 3 μg/ml Act D to further block transcription of mRNA, and the cells were subsequently harvested at 1, 4, and 8 h. Total RNA was isolated and subjected to Northern blot analyses as described above. Alternatively, total RNA was utilized for real-time PCR experiments utilizing hATıR and 18S gene-specific primers as described in Nuclear run-on assays.

Transfection. siRNAs (i.e., Smart Pool siRNAs from Dharmacon) were transfected into hPFB cells by using magnet-assisted transfection (IBA, Gottingen, Germany) as instructed by the manufacturer (magnets were purchased separately from Engineered Concepts, Birmingham, AL). hPFB cell transfection conditions were optimized using a fluorescent-labeled double-stranded RNA oligomer designated BLOCK-IT fluo-
were imaged by confocal microscopy for GFP expression. hPFBs were transfected with a pGFP construct by using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. The cells were subsequently treated with or without cycloheximide (10 μg/ml), and the cells were imaged by confocal microscopy for GFP expression.

**Statistical analysis.** All data are means ± SE. When comparisons were made between two different groups, statistical significance was determined using Student's t-test. When multiple comparisons were made, statistical significance was determined using one-way ANOVA followed by Tukey's posttest. All statistical analyses were performed using the software package Prism 4.0b (GraphPad Software, San Diego, CA).

**RESULTS**

**TGF-β1 upregulates hAT1R steady-state mRNA and protein levels in human primary lung fibroblasts.** Gene expression profiling of adult human lung fibroblasts treated with TGF-β1 demonstrated that the hAT1R gene was an important target (33). To begin to investigate the mechanisms by which TGF-β1 upregulates hAT1R gene expression, hPFBs were incubated with this stimulus for the times indicated (Fig. 1A). Northern blot experiments demonstrated that hAT1R steady-state mRNA levels were maximally increased at 4 h (i.e., ∼6-8 fold vs. nonstimulated level) and returned to basal conditions by 24 h (Fig. 1, A and B). To determine the optimal dose for TGF-β1-induced hAT1R mRNA upregulation, hPFBs were treated with increasing concentrations of TGF-β1 for 4 h and total RNA was isolated. Northern blot analyses demonstrated that 4 ng/ml TGF-β1 maximally stimulated hAT1R mRNA levels (Fig. 2, A and B).

To investigate whether the increase in hAT1R steady-state mRNA levels equated to enhanced receptor expression, hAT1R density was quantitated by performing whole cell radioreceptor binding assays. AT1R binding assays demonstrated that TGF-β1 (4 ng/ml) stimulation of hPFBs maximally increased hAT1R density (∼5-fold) at 8 h after treatment (Fig. 3A). To ensure that TGF-β1 treatment did not modulate the affinity of ANG II for the hAT1R, saturation isotherm experiments were performed and Scatchard analysis was conducted. These experiments demonstrated that although the maximal binding capacity (Bmax) values differed in the TGF-β1-treated hPFB cells (i.e., 8 h) compared with the nontreated cells (Bmax: 584 ± 52 vs. 97 ± 23 fmol/mg protein), the Kd value (1.25 ± 0.31 μM) did not change (data not shown). To determine whether the enhanced hAT1R protein expression levels also resulted in augmented ANG II-induced signal transduction, TGF-β1-treated or nontreated hPFBs were activated with 0.1 μM ANG II for 5 min, and phospho-ERK1/2 levels were determined. These results demonstrated that hPFBs treated with TGF-β1 exhibited an approximately fourfold increase in ANG II-induced phospho-ERK1/2 levels (Fig. 3, B and C). Together, these experiments demonstrated that TGF-β1 treatment not only increased hAT1R expression but also enhanced ANG II-induced signaling via the hAT1R.

**TGF-β1 transcriptionally upregulates the hAT1R gene.** Accumulation of hAT1R mRNAs within 4 h of TGF-β1 treatment suggested that a transcriptional mechanism may be involved. To test this hypothesis, hPFBs were pretreated with the transcriptional inhibitor Act D (5 μg/ml) for 1 h before a 4-h combined TGF-β1/Act D treatment. The data shown in Fig. 4A demonstrate that Act D pretreatment prevented TGF-β1 activation of the hAT1R gene. This inhibition was not a result of Act D toxicity, since GAPDH steady-state mRNA levels were not influenced by this reagent (Fig. 4A).

To investigate whether protein synthesis was required for TGF-β1 induction of the hAT1R gene, hPFBs were pretreated with the protein translation inhibitor cycloheximide 1 h before a 4-h combined TGF-β1/cycloheximide (10 μg/ml) treatment. These experiments demonstrated that hAT1R steady-state mRNA levels increased in a TGF-β1-dependent manner irrespective of cycloheximide pretreatment, indicating that new protein synthesis is not required for TGF-β1 induction of the hAT1R gene expression (Fig. 4B). To ensure that the concentration of cycloheximide utilized did inhibit protein synthesis, control experiments were performed utilizing GFP-transfected hPFBs treated with or without cycloheximide for 4 h. Imaging of the transfected cells revealed no reporter gene expression in hPFBs treated with cycloheximide (data not shown). These data suggest that the hAT1R gene is a direct TGF-β1 transcription target and that new protein synthesis is not necessary to activate the hAT1R gene.

To validate this hypothesis, hPFBs were incubated for 4 h with 4 ng/ml TGF-β1 or vehicle before nuclear hnRNA de
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Fig. 2. TGF-β1 stimulation upregulates hAT1R steady-state mRNA levels in hPFBs in a dose-dependent manner. hPFBs were grown to 70–80% confluence, washed twice, and serum-starved for 24 h. A: hPFBs were treated with TGF-β1 at the concentrations indicated for 8 h, and total RNA was isolated. RNA samples (20 μg) were fractionated, blotted, and probed with a radiolabeled hAT1R cDNA. The Northern blot was stripped and hybridized with a labeled GAPDH cDNA probe. Data are representative of 3 separate experiments. B: TGF-β1 dose response of hAT1R and GAPDH Northern hybridization signal intensity was quantitated by densitometric analysis. Each point represents the relative hybridization signal (±SE) normalized to untreated hPFBs from 3 separate experiments. *P < 0.01, TGF-β1-treated vs. nontreated hPFBs.

Fig. 3. TGF-β1 stimulation increases AT1R protein levels and ANG II-induced ERK1/2 activation. hPFBs were grown to 70–80% confluence, washed twice, and serum-starved for 24 h. A: hPFBs were subsequently incubated with TGF-β1 (4 ng/ml) for the times indicated, and AT1R radioreceptor binding assays were performed as described in EXPERIMENTAL PROCEDURES. Data are expressed as relative increase over nontreated (i.e., 0 h) hPFBs. Error bars represent SE of 3 independent experiments. *P < 0.01, TGF-β1-treated vs. nontreated hPFBs. B: hPFBs were serum-starved for 24 h, incubated with TGF-β1 (4 ng/ml, 8 h), and further activated with 0.1 μM ANG II for 5 min, and phospho-ERK1/2 activation was determined by Western blot analysis. The blot was stripped and reprobed with an ERK1/2-specific antibody. Data are representative of 3 separate experiments. C: Each autoradiograph was quantitated by densitometric analysis, and ERK1/2 phosphorylation (p) was normalized with ERK1/2 protein levels and plotted as relative increase of ANG II-induced pERK1/2 over non-ANG II-induced pERK1/2 values. Error bars represent SE of 3 independent experiments. *P < 0.01, TGF-β1-treated vs. nontreated hPFBs.

Fig. 4. The translocation of TβRII is a critical event for TGF-β1-induced transcriptional activation. A: hPFBs were treated with SB 431542 (1 μM, 1 h) followed by addition of TGF-β1 (4 ng/ml) for 1.5 h. hPFBs were subsequently incubated with Act D (1 μg/ml, 4 h), and then total RNA was isolated. Northern blot analysis was performed using a labeled hAT1R cDNA. The Northern blot was stripped and hybridized with a labeled GAPDH cDNA probe. Data are representative of 3 separate experiments. *P < 0.01, TGF-β1-treated vs. nontreated hPFBs. B: hPFBs were serum-starved for 24 h, incubated with TGF-β1 (4 ng/ml) for 8 h, and further activated with 0.1 μM ANG II for 5 min, and phospho-ERK1/2 activation was determined by Western blot analysis. The blot was stripped and reprobed with an ERK1/2-specific antibody. Data are representative of 3 separate experiments. C: Each autoradiograph was quantitated by densitometric analysis, and ERK1/2 phosphorylation (p) was normalized with ERK1/2 protein levels and plotted as relative increase of ANG II-induced pERK1/2 over non-ANG II-induced pERK1/2 values. Error bars represent SE of 3 independent experiments. *P < 0.01, TGF-β1-treated vs. nontreated hPFBs.
TGF-β1 stimulation (4 ng/ml, 4 h) and Northern blot analyses and radioreceptor binding assays were subsequently performed. Pretreatment with SB 431542 completely abolished the TGF-β1-mediated increases in hAT1R mRNA expression (Fig. 6A) and hAT1R protein levels (data not shown). To validate the specificity of SB 431542 in hPFB cells, Western blot experiments were performed utilizing phospho-Smad2 and -Smad3 antibodies. These experiments demonstrated that TGF-β1 treatment led to the phosphorylation of Smad2 and Smad3 and that pretreatment of hPFBs with SB 431542 resulted in attenuated phosphorylation levels (Fig. 6B). To further confirm the involvement of ALK5 in specifically mediating the TGF-β1 response, hPFBs were transduced with an adenovirus expressing a constitutively active ALK5 (caALK5-GFP) (29, 40), and Northern blot analyses and radioreceptor binding assays were again performed. Importantly, forced expression of caALK5 resulted in augmented expression of hAT1R mRNAs (Fig. 6C) and hAT1R protein levels (data not shown), thus confirming the importance of ALK5 in this process. Control Western blot analyses were also performed to demonstrate that Smad2 and Smad3 were properly activated with the forced expression of caALK5 (Fig. 6D).

To further investigate whether TGF-β1 stimulation of hAT1R gene expression is mediated by a Smad-dependent pathway, hPFBs were transfected with control, ALK5-, Smad2-, Smad3-, or Smad4-specific siRNAs (25 nM final concentrations) and subsequently stimulated with TGF-β1 (4 ng/ml, 8 h). Importantly, ANG II radioreceptor binding assays demonstrated that knockdown of ALK5, Smad2, Smad3, or Smad4 attenuated TGF-β1 stimulation of hAT1R expression (Fig. 7A). In contrast, transfection of control siRNA had no effect on TGF-β1-mediated changes in hAT1R density. Northern blot analyses of siRNA transfected cells treated with TGF-β1 (4 ng/ml, 4 h) also demonstrated that each siRNA specifically reduced the hAT1R steady-state mRNA levels (data not shown). To confirm that the various siRNAs knocked down their appropriate target, Western blot analyses were performed. Immunoblotting results demonstrated that ALK5, Smad2, Smad3, and Smad4 protein levels were specifically reduced (Fig. 7B).

**TGF-β1 stimulation of hPFB also enhances hAT1R mRNA expression by activating specific kinase signaling pathways.** Although the Smad pathway is the main mediator of TGF-β1 signaling, recent studies have implicated other pathways such as ERK1/2, p38 MAPK, PI3K, JNK, and PKC as either mediators or modulators of TGF-β1-dependent biological effects (reviewed in Refs. 11, 28). To begin to investigate whether TGF-β1 activation of these kinase signaling pathways also plays a role in the augmentation of hAT1R gene expres-
In this study, we have analyzed the mechanisms by which TGF-β1 regulates hAT1R gene expression in hPFBs. For the first time, we have demonstrated that TGF-β1 stimulation of hPFBs activates hAT1R gene transcription, which, in turn, leads to a robust upregulation of hAT1R steady-state mRNA levels (~6- to 8-fold). Importantly, the increased hAT1R mRNA levels resulted in augmented hAT1R protein densities (~5-fold increase 8 h after TGF-β1 treatment), which correlated with enhanced ANG II-induced signaling via the hAT1R.
Computer analysis (MatInspector; http://www.genomatix.com) of several thousand base pairs of the hAT1R promoter sequence demonstrated that several putative Smad binding elements are harbored in this region, including one binding site at −1355 bp and another at +50 bp (with respect to the transcription initiation start site; Ref. 48). We were unable to activate hAT1R promoter luciferase constructs that encompassed both of the sites described above with TGF-β1 (data not shown). Since we have clearly shown that TGF-β1 transcriptionally activates the hAT1R gene, we hypothesize that the appropriate Smad binding element(s) needed for TGF-β1 activation is(are) missing in our reporter constructs. Therefore, we are currently generating new reporter constructs that harbor additional putative Smad binding sites further upstream within the hAT1R promoter region.

Many studies have demonstrated that in addition to Smads, other signaling pathways such as ERK1/2, p38 MAPK, JNK, PI3K, Rho/ROCK, PKC, and CaMKII have also been shown to mediate TGF-β1 function (reviewed in Refs. 11, 28). We present evidence that PI3K, p38 MAPK, and JNK signaling pathways are critical to TGF-β1-mediated induction of hAT1R expression in hPFBs. This conclusion was based on the obser-

Fig. 8. TGF-β1-induced hAT1R expression can be attenuated by PI3K, p38 MAPK (p38K), and JNK pharmacological inhibitors. A: hPFBs were grown to 70–80% confluence, washed twice, and serum-starved for 24 h. Cells were preincubated for 30 min with the following inhibitors at the indicated concentrations: PD-98059 (10 μM, MAPK inhibitor), LY-294002 (5 μM, phosphatidylinositol 3-kinase (PI3K) inhibitor), SB 203580 (1 μM, p38K inhibitor), SP600125 (10 μM, JNK inhibitor), R031-8425 (10 μM, PKC inhibitor), and U0126 (10 μM, ERK inhibitor). After pretreatment with the various inhibitors, cells were stimulated with 4 ng/ml TGF-β1 for 4 h. Total RNA was isolated, and Northern analysis was performed. Error bars represent SE of 3 independent experiments. *P < 0.01, TGF-β1 + inhibitor vs. TGF-β1.

We also present evidence that the ubiquitously expressed TGF-β1 type I receptor, ALK5, mediates TGF-β1 activation of the hAT1R gene. This conclusion was based on the observation that forced expression of caALK5 mimicked TGF-β1 stimulation of the hAT1R gene and that TGF-β1 augmentation of hAT1R gene expression was attenuated by an ALK5-specific pharmacological inhibitor or by transfection of an ALK5-specific siRNA.

It is well established that once ALK5 is activated by TGF-β1, the downstream receptor-regulated Smads (R-Smads) are phosphorylated at two distal serine residues located in the COOH terminus. Subsequently, the phosphorylated R-Smads (Smad2 and Smad3) associate with a core-Smad, Smad4, and enter the nucleus to modulate the transcription of TGF-β1-responsive genes (11, 36). Consensus DNA-binding sequences for R-Smad/Smad4 complexes have been identified that contain the palindromic GTCTAGAC, half-sites of this sequence, or CAGA motifs (10, 14, 45–47). R-Smad/Smad4 complexes are capable of binding to DNA alone, but they do so with low affinity, and their interaction with additional transcription factors is required for target gene regulation (12, 42, 46, 47).

We present siRNA knockdown evidence that Smad2/3 and Smad4 are involved in the TGF-β1 induction of the hAT1R gene.

Fig. 9. TGF-β1-induced hAT1R expression can be attenuated by PI3K, p38K, and JNK siRNAs. A: hPFBs were grown to 30–60% confluence and transiently transfected with control, MAPK1-, PI3K-, p38K-, or JNK-specific siRNAs (25 nM final concentration). Forty-eight hours after transfection, hPFBs were serum-starved for an additional 24 h and subsequently stimulated with TGF-β1 (4 ng/ml, 8 h), and AT1R radioreceptor binding assays were performed. Error bars represent SE of 3 independent experiments. *P < 0.01, TGF-β1/p38K, TGF-β1/PI3K, TGF-β1/JNK siRNA-treated vs. TGF-β1-treated hPFBs. B: hPFBs were transfected and treated as described in A; however, cells were lysed and subjected to immunoblotting with the antibodies indicated. Tubulin immunoblots were performed as a protein loading control. Data are representative of 3 separate experiments.
vation that blockade of PI3K, p38 MAPK, and JNK signaling with any of the pharmacological inhibitors LY-294002, SB 203580, or SP600125 or kinase-specific siRNAs significantly attenuated expression of the hAT1R gene. In contrast, PD-98059 or U0126, two well-known inhibitors of the ERK1/2 signaling pathways, and blockade of PKC signaling with the inhibitor R031-8425 demonstrated that these pathways were not essential for the TGF-β1-mediated upregulation of the hAT1R gene, since pretreatment with these inhibitors did not block the TGF-β1 response. The utilization of a MAPK1-specific siRNA confirmed that this signaling pathway was not involved. Although PKC-specific siRNAs were not utilized in this study, other investigators (11, 28) have utilized 10 μM R031-8425 to successfully inhibit the PKC signaling pathways. Together, our studies suggest that TGF-β1-mediated induction of hAT1R expression results from the ability of TGF-β1 to activate Smads, PI3K, p38 MAPK, and JNK since the elimination of any of these signaling pathways attenuates the potential for TGF-β1 to stimulate hAT1R expression. Therefore, we conclude that the Smad and kinase signaling pathways do not act independently but involve some level of intracellular cross talk or scaffolding.

In support of our hypothesis, several studies from other investigators have suggested that the PI3K signaling pathway can be modulated by TGF-β1 (1, 17, 34, 43). The activation of PI3K signaling by TGF-β1 may be direct, since coimmunoprecipitation between the p85 subunit of PI3K and both TβRI and TβRII has been demonstrated in airway smooth muscle cells (17). In addition, it has been shown that the activated TβRII serine-threonine kinase can potently induce PI3K activity (43). Interestingly, Bakan et al. (1) showed that the PI3K inhibitor LY-294002 blocked TGF-β1-induced Smad2 phosphorylation in breast cancer cells, suggesting that Smad proteins may be potential targets of the TGF-β3 pathway. Furthermore, Runyan et al. (34) demonstrated that TGF-β1 stimulated the PI3K/Akt pathway, which, in turn, augmented the ability of Smad3 to transcriptionally activate collagen I expression in human mesangial cells. These investigators also demonstrated that TGF-β1 activation of PI3K resulted in the phosphorylation of Smad3 at serine residues other than the direct TβRI/ALK5 target site located in the COOH terminus.

In further support of our observations, it has been shown that TGF-β1 can activate the p38 kinase and JNK signaling pathways, possibly through the activation of TGF-β-activated kinase (TAK1/MAP3K7) (15, 44). Recent studies by Kamaraju and Roberts (15) demonstrated that the p38 MAPK pathway is activated by TGF-β1, and this activation results in the phosphorylation of Smad2/3 in the linker region. Since COOH-terminal phosphorylation of Smad2 and 3 was not affected by p38 MAPK inhibitors, these investigators surmised that these pathways were activated in parallel and independently of one another. In addition, they concluded that hierarchically, the p38 MAPK pathway is likely upstream to Smad signaling and modulates Smad function through phosphorylation in the linker.
regions of Smad2 and Smad3. Finally, Yoshida et al. (44) demonstrated that in hepatic stellate cells treated with TGF-β1, JNK and p38 MAPK were activated. Importantly, they demonstrated that the activated kinases could directly phosphorylate Smad2 and Smad3 in their linker regions, which in turn resulted in augmented PAI-1 transcription rates. Together, these studies demonstrate that there is interdependence between Smad signaling and specific kinase pathways that may involve the phosphorylation of specific amino acids localized in the linker region of Smad2 and Smad3. On the basis of these studies, we hypothesize that in hPFBs, TGF-β1 stimulation of hAT1R expression arises from the synergy of the activation of Smads and the PI3K, p38 MAPK, and JNK signaling pathways, which, in turn, results in the phosphorylation of Smad2/3 at multiple sites (Fig. 10). Once the R-Smad/Smad4 complex is formed and translocated into the nucleus, we speculate that hyperphosphorylation of Smad2/3 in the linker and COOH-terminal regions allows for the recruitment of specific transcriptional coactivators that are required to stimulate the expression of a novel subset of TGF-β1-inducible genes (i.e., the hAT1R gene) (Fig. 10).

An alternative explanation for intracellular cross-talk among the Smad, PI3K, p38 MAPK, and JNK pathways for TGF-β1-stimulated hAT1R expression is that each kinase pathway phosphorylates specific transcriptional coactivators that are necessary for the activation of the hAT1R gene (Fig. 10). In support of this model, it has been demonstrated that the downstream targets of JNK include the transcription factors c-Jun, ATF-2, ELK-1, and p53 (27). Targets of the p38 MAPK include multiple transcription factors such as MEF2, ATF-2, c-Jun, ATF-2, ELK-1, and p53 (27). Together, these studies clearly demonstrate that the kinase pathways that are activated by TGF-β1 can stimulate the phosphorylation of many transcription factors; therefore, we speculate that the Smad, PI3K, p38 MAPK, and JNK pathways merge their signals within the nucleus to mediate TGF-β1-stimulated hAT1R gene expression (Fig. 10). Therefore, if one kinase pathway is inhibited, the transcriptional complex would be missing a key component and hAT1R gene expression would not be activated by TGF-β1.

Our current studies cannot distinguish between the two proposed models and must be the focus of future research.

Convincing evidence indicates that ANG II, via the AT1R, activates the TGF-β1 axis in the lung by both direct and indirect mechanisms (2, 18, 21–23, 32, 39). For example, ANG II is mitogenic for human fetal and adult lung fibroblasts in vitro, and this response is attenuated by anti-ANG II antibodies, suggesting that the mitogenic effect is mediated by autocrine production of TGF-β1 (23, 24). TGF-β1 is a potent profibrotic cytokine: it enhances fibroblast chemotaxis and proliferation and induces extracellular matrix synthesis; therefore, TGF-β1 has a decisive role in pulmonary fibrotic diseases (2, 41). In a variety of forms of pulmonary pathology, including chronic lung disease of prematurity as well as several forms of acute and chronic adult lung disease, the expression of TGF-β1 is increased (2). Interestingly, TGF-β1 has now been shown to activate specific components of the RAS axis (18, 41). Specifically, TGF-β1 stimulates angiotensinogen gene expression in proximal tubular cells (3). Furthermore, TGF-β1 was shown to enhance hAT1R gene expression in lung fibroblasts (33), adrenal cells (19), and trophoblasts (38). Our current findings have extended these observations by demonstrating that TGF-β1 augmentation of hAT1R protein levels results from the interdependence of ALK5 activation of Smad and specific kinase signaling pathways. These studies support the existence of a self-potentiating loop between the RAS and TGF-β1 system. The clinical implications of these studies are highly significant, since the resulting amplification of the profibrotic effects of both systems may play a role in mediating the pathogenesis of pulmonary fibrosis. Although ACE inhibitors and ARBs can block experimental models of lung fibrosis (18), there are currently no published prospective or retrospective studies regarding the use of these drugs in humans with pulmonary fibrosis. Therefore, it is still unclear whether inhibition of the RAS would indeed have beneficial effects on lung fibrosis. Together, these studies may suggest the need to pharmacologically inhibit both systems to treat fibrotic diseases.

REFERENCES
