Fibrotic Lung Fibroblasts Show Blunted Inhibition by cAMP Due to Deficient cAMP Response Element-Binding Protein Phosphorylation

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ABSTRACT

Pulmonary fibroblasts regulate extracellular matrix production and degradation; thus, they are critical for maintenance of lung structure, function, and repair. In pulmonary fibrosis, fibroblasts produce excess collagen and form fibrotic foci that eventually impair lung function, but the mechanisms responsible for these alterations are not known. Receptors coupled to the stimulation of cAMP production can inhibit activation of fibroblasts and thereby are antifibrotic. To test whether this signaling pathway is altered in pulmonary fibrosis, we compared the ability of normal adult human pulmonary fibroblasts to generate and respond to cAMP with that of cells isolated from lungs with idiopathic pulmonary fibrosis. Serum- and transforming growth factor (TGF-)β/H9252-stimulated cell proliferation was inhibited 50% by forskolin and 100% by prostaglandin (PG) E2 in the normal cells but substantially less in the diseased cells. Collagen synthesis was also inhibited 50% by the same drugs in the normal cells but significantly less so in the diseased cells, despite responding with similar increases in cAMP production. Although expression of protein kinase A (PKA) and cAMP-stimulated PKA activity were similar in both the normal and diseased cell types, forskolin- and PGE2-stimulated cAMP response element-binding protein (CREB) phosphorylation was decreased in the diseased cell lines compared with the normal cells. cAMP-mediated activation and TGF-β-mediated inhibition of CREB DNA binding was also diminished in the diseased cells. Thus, pulmonary fibroblasts derived from patients with pulmonary fibrosis are refractory to the inhibition by cAMP due to altered activity of components distal to the activity of PKA, in particular the phosphorylation of CREB.

Fibroblasts play a critical role in the homeostasis of extracellular matrix (ECM) in the lung. Recruitment, accumulation, and activation of pulmonary fibroblasts contribute not only to normal wound healing but also to the development of interstitial lung diseases, such as idiopathic pulmonary fibrosis (IPF). No effective therapy currently exists for pulmonary fibrosis (Giri, 2003). The pathogenesis and biochemical mechanisms that underlie pulmonary fibrosis are incompletely understood, but key pathological features are an increased number of activated fibroblasts, abnormal tissue remodeling, re-epithelialization, and increased collagen deposition (Chapman, 2004). Fibrosis seems to strongly correlate with the presence and number of foci in which fibroblasts assume a migratory, then a proliferative, and ultimately a profibrotic, myofibroblastic phenotype (Selman et al., 2004).

The second messenger cAMP can regulate fibroblasts in many tissues. Cellular levels of cAMP represent the balance between formation, which is regulated by G protein-coupled receptors that stimulate (via Gs) or inhibit (via Gi) adenylyl cyclase (AC), and degradation, which occurs via cyclic nucleotide phosphodiesterase (PDE). Increases in cAMP influence growth, death, and differentiated cell functions, primarily (although not exclusively; de Rooij et al., 1998) by promoting phosphorylation of proteins via the activation of cAMP-dependent protein kinase (protein kinase A; PKA). PKA-mediated phosphorylation of cAMP response element-binding protein (CREB) and CREB-mediated regulation of transcription via interaction with cAMP-response elements is a major pathway that alters gene expression (Montminy, 1997).

Certain prostaglandins, such as prostacyclin and related...
analogs prostaglandin (PG) E₂ and PG D₂, inhibit pulmonary fibroblast migration, proliferation, and collagen synthesis (Keerthisingam et al., 2001; Kohyama et al., 2002; Vancheri et al., 2004). Those agents all activate receptors coupled to Gαi, implying that cAMP is a negative regulator of fibroblast function. Some evidence suggests the importance of the Gαi-AC-CAMP pathway in IPF. This includes results showing compromise of this pathway after experimental pulmonary fibrosis induced by the cancer chemotherapeutic drug bleomycin (Giri et al., 1987) and the diminished capacity to generate PGE₂ of fibroblasts from IPF patients due to insufficient induction of cyclooxygenase expression (Wilborn et al., 1995; Keerthisingam et al., 2001). Moreover, elevation of cAMP production inhibits cell proliferation, collagen synthesis, and the differentiation of pulmonary fibroblasts into profibrogenic myofibroblasts (Kolodski et al., 2003; Liu et al., 2004).

One mechanism by which cAMP may regulate fibrogenicity is via interaction with the TGF-β signaling pathway. Activation by TGF-β of SMAD proteins, such as SMAD3/4, regulates gene expression by promoting SMAD translocation to the nucleus, where SMAD proteins function as transcription factors (Chen et al., 2003). TGF-β/SMAD signaling enhances transcription of numerous genes, including connective tissue growth factor, plasminogen activator inhibitor-1, and various collagen genes (Leask et al., 2003). Recent work suggests that activation of the cAMP/PKA signaling pathway inhibits SMAD-mediated transcription by abolishing SMAD interaction with key transcriptional activators (Schiller et al., 2003).

In the present study, we tested the hypothesis that generation of and/or response to cAMP contributes to altered pulmonary fibroblast function in pulmonary fibrosis. Accordingly, we compared the responses of a human adult pulmonary fibroblast cell line, LL47, isolated from nonfibrotic lung (normal) and cells isolated from the lungs of patients with IPF (diseased). We found that normal and diseased cells generate similar levels of cAMP but that cAMP-elevating agents only inhibit collagen synthesis in the normal fibroblasts and display reduced inhibition of cell proliferation in the diseased fibroblasts. Although normal and diseased cells have similar levels of expression and activity of PKA, the diseased cells displayed less phosphorylation and DNA binding of CREB in response to cAMP-elevating agents. Reduced activation of CREB is predicted to result in greater TGF-β/SMAD-mediated transcription of profibrotic genes via decreased competition for transcriptional coactivators required for full SMAD3/4 and CREB transcription. These findings imply that a key defect in pulmonary fibroblasts from fibrotic lung is the regulation of the phosphorylation state of CREB.

Materials and Methods

Materials and Cell Culture. Primary antibodies for collagen I, collagen III, PKA catalytic subunit, and β-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primary antibody for serine 133-phosphorylated CREB (pCREB) was obtained from Chemicon International (Temecula, CA). Primary antibody for α-smooth muscle (α-SM) actin was obtained from Cymbus Biotechnology (West Lehigh, UK). Primary antibody for PKA RII subunit was obtained from Upstate Biotechnology (Charlottesville, VA). PKA inhibitor peptide was obtained from U.S. Biochemical Corp. (Cleveland, OH). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO). Human adult pulmonary fibroblasts (LL47, LL29, and LL97A cells) were obtained from American Type Culture Collection (Manassas, VA) and were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin in a 37°C incubator with 5% CO₂.

Cells were used between passages 6 and 14 for all experiments.

Measurement of cAMP Accumulation. Cells were washed three times with serum and NaHCO₃-free Dulbecco’s modified Eagle’s medium supplemented with 20 mM HEPES, pH 7.4, and equilibrated for 30 min. Assay for cAMP accumulation was performed by incubation with drugs of interest and 0.2 mM β-isobutyl-1-methylxanthine, a PDE inhibitor, for 10 min. To terminate reactions, assay medium was aspirated, and 200 µl of lysis buffer (GE Healthcare, Piscataway, NJ) was added. cAMP content of the extract was quantified using the Biotrak EIA kit (GE Healthcare). Data were normalized to the amount of protein per sample, as determined using a dye-binding protein assay (Bio-Rad, Hercules, CA).

Assays of Cell Proliferation. DNA synthesis was assessed by determination of [³H]thymidine incorporation into trichloroacetic acid (TCA)-precipitable material. Cells were suspended in Dulbecco’s modified Eagle’s medium containing 10% FBS, seeded (10⁵ cells/well) in 24-well plates, grown to 60 to 70% confluence, and synchronized by serum deprivation in MEM containing 0.25% FBS for 24 h. MEM was then supplemented with 2.5% FBS (except for unstimulated conditions, when 0.25% FBS was used) for 24 h along with 0.5 µCi of [³H]thymidine/well and drugs of interest. The medium was removed, the cells were washed with ice-cold phosphate-buffered saline, and then incubated with 7.5% TCA for 1 h at 4°C to precipitate the DNA. After 1 h, cells were washed with 7.5% TCA and then 75% ethanol, and samples were left to dry at room temperature. The TCA-precipitated counts were determined by liquid scintillation counting.

Assays of Collagen Synthesis. [³H]Proline incorporation was measured according to a method modified from that of Guarda et al. (1993) as described previously (Ostrom et al., 2003). Briefly, cells were seeded in 12-well plates and then serum-starved in 0.25% FBS for 24 h. [³H]Proline incorporation was assayed by adding 1 µCi/well of [³H]proline (PerkinElmer Life and Analytical Sciences, Boston, MA) along with, where indicated, drugs of interest and 2.5% FBS for 24 h. Cells were then trypsinized, and the protein was precipitated with 10% TCA. The collagenase-sensitive [³H]proline in the supernatant was determined by liquid scintillation counting.

Reverse-Transcriptase-Polymerase Chain Reaction. Total RNA was extracted from cells grown to 80 to 90% confluence on 10-cm plates using TRIzol reagent (Invitrogen, Carlsbad, CA) and RNeasy RNA isolation kit (QIAGEN, Valencia, CA). A DNase reaction was performed initially to confirm that single PCR products resulted from DNA templates. PCR products were analyzed by agarose gel electrophoresis and visualized under UV light with ethidium bromide. RT-PCR was performed initially to confirm that single PCR products resulted from reactions with each primer pair. Suitable primers were then used in real-time PCR reactions with SYBR Green (Bio-Rad QI Taq SYBR Green). The primers were designed based on GenBank sequences, as described previously (Liu et al., 2004). The thermal profile for all real-time PCR reactions was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60 to 62°C for 1 min. Fluorescence data from each sample was analyzed by the 2⁻¹ΔCt method using the vehicle-treated control as the calibrator: ΔCt = Ct sample - Ct calibrator, where ΔCt = Ct sample - Ct calibrator. GI (calibrator sample) and ΔCt (β-actin (calibrator sample)). GI is the gene of interest, and Ct is the
cycle threshold (the cycle number where the fluorescent signal crosses an arbitrary intensity threshold).

**Immunoblot Analysis.** Whole-cell lysates were obtained from treated or untreated cells by scraping cells in modified radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, plus mammalian protease inhibitor cocktail; catalog no. P-8340, Sigma-Aldrich) and homogenizing by sonication. For immunoblot of pCREB, cells were lysed in 2× SDS sample buffer and boiled for 5 min. Equal protein amounts of the lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA) by electroblotting. Membranes were blocked in 20 mM phosphate-buffered saline with 3% nonfat dry milk and incubated with primary antibody (see Materials and Methods) overnight at 4°C. Bound primary antibodies were visualized using appropriate secondary antibody with conjugated horseradish peroxidase (Santa Cruz Biotechnology, Inc.) and enhanced chemiluminescence reagent (Pierce Chemical, Rockford, IL). The amount of protein per sample was determined using a dye-binding protein assay (Bio-Rad).

**PKA Activity Assay.** PKA activity was determined by an enzyme-linked immunosorbent assay-based assay using crude whole-cell lysates. Cells were scraped from the plate in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630 plus mammalian protease inhibitor cocktail as described above), incubated for 10 min, and then homogenized in a Dounce (glass-glass) homogenizer for 20 s on ice. Homogenates were then centrifuged at 3000 g for 10 min. Protein concentration in the nuclear extract was determined using the Bio-Rad dye-binding protein assay. Samples were then centrifuged at 12,900 g to resulting pellet and incubated at 4°C with slow rocking for 2 h. Dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride was added to the resulting supernatant. PKA activity was measured in the resulting supernatant using a PKA assay kit (StressGen Biotechnologies, San Diego, CA), according to the manufacturer's instructions. PKA activity was stimulated by the addition of 0.01 to 5 μM cAMP for 5 min at 37°C. Greater than 95% of the measured kinase activity was PKA activity, as determined by pretreating lysates for 20 min with 10 nM PKA inhibitor peptide.

**Electromobility Shift Assays.** Cells were starved with 0.25% FBS for 24 h, and then cells were treated with either 10 μM forskolin or vehicle for 20 min. TGF-β1 (10 ng/ml) or vehicle was then added for another 20 min. Cells were lysed in lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonfonyl fluoride), incubated at 4°C for 5 min, and then centrifuged at 5000g for 10 min. Nuclear extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 400 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonfonyl fluoride) was added to the resulting pellet and incubated at 4°C with slow rocking for 2 h. Samples were then centrifuged at 12,900g for 10 min. Protein concentration in the nuclear extract was determined using the Bio-Rad dye-binding protein assay. Binding reactions with biotin-labeled probes were run and separated by electrophoresis on 6% polyacrylamide gels as per the manufacturer's protocol (Panomics, Redwood City, CA), and then visualized by chemiluminescence. The specificity of each probe-protein complex was confirmed by supershift assay using a CREB binding protein 1 (CBP-1) antibody.

**Data Analysis and Statistics.** Data are presented as the mean ± S.E.M. or as representative images of at least three separate experiments. Statistical comparisons (t tests and one-way analysis of variance) and graphics were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA).

**Results**

cAMP-Elevating Agents Inhibit Pulmonary Fibroblast Proliferation and Collagen Synthesis. Previous work indicates that cAMP-elevating agents inhibit cell proliferation and collagen synthesis of pulmonary fibroblasts (Lindenschmidt and Witschi, 1985; Liu et al., 2004). To determine whether fibroblasts from fibrotic lung are less responsive to inhibition by cAMP-elevating agents, we compared [3H]thymidine incorporation (an index of DNA synthesis) and collagenase-sensitive [3H]proline incorporation (an assay of collagen synthesis) in LL47 (normal) and LL29 (diseased) cells. Both cell types were serum-starved for 24 h in 0.25% FBS and then cultured for 24 h with 2.5% FBS (a maximally effective concentration of serum; Liu et al., 2004) and either vehicle or various concentrations of cAMP-elevating agonists. In LL29 cells 2.5% FBS was less effective in stimulating [3H]thymidine incorporation than in LL47 cells (Fig. 1), consistent with observations that cells from fibrotic lung are less proliferative (Ramos et al., 2001). More impressive differences were noted when we tested agents that directly increase AC activity (e.g., forskolin) or that activate G protein-coupled receptors coupled to Gs. Forskolin (1 μM) and the prostaglandin E receptor agonist PGE₂ (1 μM) inhibited FBS-stimulated [3H]thymidine incorporation in LL47 cells by 79 ± 15 and 114 ± 16%, respectively. Isoproterenol (1 μM), an agonist for β-adrenergic receptors linked to increases in cAMP, also inhibited [3H]thymidine incorporation (Table 1). These responses were similar in magnitude to those measured in another normal pulmonary fibroblast cell line, WI-38 (Liu et al., 2004). None of the agents changed cell number. Thus, numerous approaches that increase...
cAMP levels inhibit DNA synthesis in normal pulmonary fibroblasts.

By contrast, [3H]thymidine incorporation in LL29 (diseased) cells was not significantly inhibited by forskolin (Fig. 1; Table 1). [3H]Thymidine incorporation was inhibited by PGE2 and isoproterenol, but this inhibition was less in LL29 cells than in LL47 cells (Fig. 1; Table 1). Therefore, forskolin, PGE2, and isoproterenol are less effective at inhibiting proliferation, as assessed by [3H]thymidine incorporation, of LL29 (diseased) cells than the normal LL47 fibroblasts.

We measured collagenase-sensitive [3H]proline incorporation, an assay of collagen synthesis, to examine a differenti-ated function of the fibroblasts. As with [3H]thymidine incorporation, LL29 cells responded to a smaller extent to stimulation with 2.5% serum than did LL47 cells (Fig. 2). The reasons for this reduced responsiveness of the diseased cells are not clear, but they may be due to a chronic desensitization to trophic stimuli. Forskolin and PGE2 inhibited collagenase-sensitive [3H]proline incorporation in LL47 cells, with PGE2 yielding inhibition at or below the basal activity of the cells (Fig. 2). Isoproterenol was nearly as efficacious as forskolin in inhibiting FBS-stimulated [3H]proline incorporation, maximally inhibiting this response 93 ± 8% in LL47 cells (Table 1). Forskolin also inhibited TGF-β1-stimulated collagen synthesis 47 ± 12%. By contrast with the results for LL47 cells, collagenase-sensitive [3H]proline incorporation in LL29 (diseased) cells was not inhibited by forskolin, PGE2 (Fig. 2), or isoproterenol (Table 1). Forskolin-mediated inhibition of collagenase-sensitive [3H]proline incorporation exhibited a biphasic response in LL47 cells (Fig. 2, bottom). These data were best fit using a two-site model, with −log IC50 values of 8.6 ± 1.3 nM and 5.1 ± 0.5 μM for the high- and low-potency components, respectively. In LL29 cells, forskolin only exhibited a single site concentration response in inhibiting collagen synthesis, with a −log IC50 of 5.6 ± 0.4 μM. Thus, LL29 cells are less responsive to the inhibition of collagen synthesis by multiple cAMP-elevating agents primarily due to a lack of high-potency responses.

**Regulation of cAMP Production in Human Pulmonary Fibroblasts.** To determine whether diseased cells produce less cAMP, we measured cAMP accumulation in LL47 and LL29 human pulmonary fibroblasts. Forskolin and PGE2 each significantly (p < 0.05) stimulated cAMP production in the presence of a nonselective PDE inhibitor, 3-isobutyl-1-methylxanthine, in both LL47 and LL29 cells (Fig. 3). Forskolin and PGE2 displayed similar EC50 values in both LL47 and LL29 (−log EC50 for forskolin: LL47 = 6.47 ± 0.14, LL29 = 6.46 ± 0.16; for PGE2: LL47 = 5.87 ± 0.18, LL29 = 5.88 ± 0.13; p > 0.1 for each comparison). LL29 cells displayed slightly increased basal cAMP production (0.8 ± 0.06 at ASPET Journals on July 8, 2017 jpet.aspetjournals.org Downloaded from
pmol/mg protein for LL47 cells, 1.2 ± 0.08 pmol/mg protein for LL29 cells; p < 0.05) and slightly increased response to multiple concentrations of both agonists (Fig. 3). Both normal and diseased cells responded with similar increases in cAMP production when stimulated with isoproterenol (1 μM) or PGE₂ (1 μM) as described in Materials and Methods. Each point represents the mean ± S.E. of six experiments, and the line represents the fit of each data set using nonlinear regression analysis. *p < 0.05 comparing cell types by t test.

**Effect of Forskolin and PGE₂ on Collagen Isoform Expression.** We used quantitative real-time RT-PCR to examine whether increased cellular cAMP levels alter expression of specific collagen isoforms in LL47 and LL29 cells and in addition, tested LL97A cells, another normal pulmonary fibroblast cell line isolated from fibrotic lung (Liu et al., 2004). Thus, compared with normal pulmonary fibroblasts, fibroblasts cultured from human fibrotic lung are not deficient in their ability to generate cAMP.

![Fig. 3.](image)

**Collagen Expression.** We isolated total RNA from LL47 and LL29 cells treated with either vehicle or PGE₂ (1 μM) for 24 h, and quantified the level of mRNA expression of collagen types Iα1, Iα2, and IIIα1. We found that the level of mRNA for all three collagens was 9- to 20-fold higher in the diseased cells than in the normal LL47 cells. Treatment of LL47 cells with PGE₂ decreased collagen type Iα1 and IIIα1 by 74 to 87%; by contrast, PGE₂ either had no effect or increased mRNA levels of these same genes in LL29 and LL97A cells with LL97A cells showing greater increases than did the LL29 cells (Table 2). Thus, normal pulmonary fibroblasts respond to PGE₂ with decreased collagen mRNA expression, whereas two different lines of fibroblasts isolated from patients with IPF show unaltered or increased expression of collagen mRNA in response to PGE₂.

**Immunoblot analysis.** We performed SDS-PAGE and immunoblot analyses of whole-cell lysates from LL47 and LL29 cells and revealed that both collagens are increased by treatment of LL47 and LL29 cells (Fig. 4). These data are consistent with those obtained from expression analysis in responses to PGE₂.

**PKA Expression and CREB Phosphorylation.** We thus examined expression and activity of PKA, the major mediator of cAMP action. PKA activity was equivalent in a second diseased cell line, LL97A (data not shown). These data suggest that differences in PKA expression and activity do not account for the difference in responses to cAMP-elevating agents.

**TABLE 2**

Quantitative RT-PCR analysis of collagen gene expression in pulmonary fibroblast cells treated with either vehicle or PGE₂ (1 μM). Total RNA was isolated from cells and RT-PCR was performed as described under Materials and Methods. Fluorescence data from each PCR reaction were analyzed using the 2⁻^Δ^ΔCt method (see Materials and Methods). Data are presented as the mean ± S.E. of the fold change in response to PGE₂ vs. vehicle-treated, such that 1 is unchanged, >1 is increased, and <1 is decreased mRNA abundance.

<table>
<thead>
<tr>
<th>Collagen</th>
<th>LL47 Cells</th>
<th>LL29 Cells</th>
<th>LL97A Cells</th>
</tr>
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<tbody>
<tr>
<td>Iα1</td>
<td>2.56 ± 0.88</td>
<td>5.69 ± 1.21</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Iα2</td>
<td>2.56 ± 0.88</td>
<td>5.69 ± 1.21</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>IIIα1</td>
<td>2.56 ± 0.88</td>
<td>5.69 ± 1.21</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>

*Data are presented as the mean ± S.E. of the fold change in response to PGE₂ vs. vehicle-treated, such that 1 is unchanged, >1 is increased, and <1 is decreased mRNA abundance.*
noreactivity at 5 min, the earliest time point we measured; forskolin-stimulated pCREB immunoreactivity remained elevated for 3 h, whereas response to PGE$_2$ was increased for 1 h. By contrast, neither forskolin nor PGE$_2$ significantly elevated pCREB immunoreactivity over basal levels in LL29 cells at any time point measured. The levels of pCREB immunoreactivity decreased during the 24-h incubation under all conditions, an effect not attributable to decreased cell
The present results demonstrate that fibroblasts isolated from fibrotic lung lack responsiveness to a key negative regulator of cell proliferation and collagen synthesis, cAMP, despite generating equivalent or higher levels of this second messenger compared with normal pulmonary fibroblasts. We assessed several types of agents (an AC activator and receptor agonists) and two stimulatory signals (serum and TGF-β) using multiple experimental approaches that all yielded results consistent with this conclusion. We did not observe differences in levels of PKA expression or activity between normal and diseased pulmonary fibroblasts. Instead, our data implicate differences in events distal to cAMP/PKA, in particular the phosphorylation and activation of CREB, as accounting for the decreased response to cAMP-elevating agents in cells from fibrotic lung. Diseased cells displayed elevated basal pCREB levels and CREB binding with no detectible increases in response to increases in cAMP and activation of PKA. In normal pulmonary fibroblasts, the increase in pCREB recruits coactivators such as CBP-1 and p300, sequestering and preventing them from acting as co-factors with transcriptional elements such as SMAD3/4, which stimulate profibrotic gene programs (Shen et al., 1998). The lack of cAMP-stimulated increases in pCREB levels in fibroblasts from fibrotic lung likely blunts the recruitment of CBP-1 and presumably allows SMAD3/4-mediated transcription to go unchecked. Such a model is consistent with transcriptional regulation that has been demonstrated in other cell types (Ghosh et al., 2001; Schiller et al., 2003) and with defects that have been described in scleroderma, a disorder associated with enhanced fibrosis in the skin, lung, and other sites (Holmes et al., 2003; Asano et al., 2004). Fibroblasts cultured from such patients produce greater amounts of prostaglandins (which increase cAMP production on TGF-stimulated collagen production via CREB-mediated inhibition of cell proliferation and collagen synthesis.

**Discussion**

Recent reports indicate that cAMP-elevating agents inhibit TGF-β₁-induced collagen gene transcription via CREB-mediated transcriptional regulation by TGF-β acting via SMAD3/4 (Shen et al., 1998). We used electromobility shift assays to assess impact of increased cAMP production on TGF-stimulated collagen production via effects on transcriptional coactivators and CREB binding to TGFβ1 receptor. Forskolin treatment stimulated CREB DNA binding in normal cells but had no effect in diseased cells, although the diseased cells had an elevated basal level of CREB binding (Fig. 7). TGF-β treatment did not alter basal CREB binding in either the LL29 or LL47 cells but inhibited forskolin-stimulated CREB binding in the LL47 cells. These data are consistent with the idea that SMAD3/4 and CREB compete for transcriptional cofactors and imply that cAMP-PKA signaling reduces expression of profibrotic genes by competing with SMAD3/4 for transcriptional coactivators such as CBP-1 and p300 (Ghosh et al., 2001). Because the diseased cells (in spite of having higher basal CREB binding) are unresponsive to either forskolin or TGF-β, we conclude that diminished activation of CREB, deficient stimulation of transcriptional cofactor binding, and decreased inhibition of growth factor-stimulated gene transcription contribute to the refractoriness of fibroblasts from human fibrotic lung to cAMP-mediated inhibition of cell proliferation and collagen synthesis.

![Fig. 5. PKA expression and PKA activity are similar in normal and diseased cells. A, whole-cell lysates from LL47 and LL29 cells were separated by SDS-PAGE and analyzed by immunoblot analysis using antibodies specific for PKA regulatory II subunit (RII), PKA catalytic subunit (cat), or β-actin (used to normalize for loading). Representative images of three experiments are shown (top). Immunoreactive bands were quantified then adjusted for the density of individual β-actin loading controls and are shown as the mean ± S.E. of all experiments (bottom). B, PKA activity was measured (as under Materials and Methods) in whole-cell lysates from LL47, LL29, and LL97A cells incubated with vehicle (basal) or the indicated concentrations of cAMP. Each column represents the mean ± S.E. of three experiments. * p < 0.05 compared with basal (no cAMP) by paired t test. Basal PKA activity and that stimulated by 10 nM or 1 μM cAMP was similar in LL47 and LL29 cells (p = 0.10, 0.15, and 0.14, respectively).](image-url)
levels) but are refractory to their action in terms of inhibition of formation of connective tissue growth factor, which is produced in response to TGF receptor activation (Stratton et al., 2001).

Several transcription factors, including CREB, CCAAT binding factor, nuclear factor 1, activator protein 1, and Sp1 are required for expression of collagen genes in fibroblasts (Li et al., 1995; Hasegawa et al., 1996; Fabbro et al., 1999). Thus, although our studies implicate cAMP as a regulator of transcription of collagen genes, transcriptional regulation of pulmonary fibroblasts likely occurs by factors in addition to CREB and SMAD3/4 and contributes...
to modulation of collagen synthesis and cell growth in these cells.

LL47, LL29, and LL97A cells as well as WI-38 cells (Liu et al., 2004) display many of the same qualities as primary human pulmonary fibroblasts: they differentiate into myofibroblasts and produce collagen upon stimulation with serum, responses that are inhibited by exposure to agents that increase cAMP levels (Fine et al., 1989; Kolodsiick et al., 2003). Furthermore, several responses that we measured mirror those reported for primary fibroblasts isolated from human lung, including reduced growth rate of cells isolated from fibrotic lung (Ramos et al., 2001). Therefore, the cell lines that we studied here seem to be useful cell culture models of normal and fibrortic pulmonary fibroblasts.

A link between PGE₂ and the cellular anatomy of pulmonary fibrosis has been implied from studies of human samples and animal models of the disease (Wilborn et al., 1995; Ogushi et al., 1999; Vancheri et al., 2000). Such studies derived from early findings that prostanoids, particularly PGE₂, regulate pulmonary fibroblast proliferation (Fine and Goldstein, 1987). Fibrotic lung fibroblasts have decreased expression of cyclooxygenase and reduced PGE₂ production compared with normal pulmonary fibroblasts (Wilborn et al., 1995; Vancheri et al., 2000; Keerthisingam et al., 2001). PGE₂ seems to act in large part via the stimulation of cAMP production; cAMP, in turn, seems to act at the level of gene transcription and by inhibiting synthesis of certain growth factors (Fine and Goldstein, 1987; Duncan et al., 1999). Our results identify another possible alteration in fibroblasts that participates in pulmonary fibrosis: fibrortic fibroblasts have reduced capacity to respond to PGE₂ with changes in gene expression.

PGE₂ is more efficacious than forskolin at inhibiting cell proliferation and collagen synthesis in pulmonary fibroblasts, despite the fact that forskolin stimulated higher levels of cAMP production. Even when tested at higher concentrations than in some of the studies here, forskolin generally did not lead to a level of inhibition equal to that induced by PGE₂. In addition, PGE₂ inhibited cell proliferation in diseased LL29 cells, albeit to a lesser extent than in normal fibroblasts, whereas other cAMP-elevating agents, including forskolin, were much less effective in this regard. This heterogeneity in responses mediated by PGE₂, forskolin and other agents may reflect involvement of a signaling pathway other than AC stimulation by PGE₂, an idea supported by previous studies (Fine et al., 1992; Seternes et al., 1999). PGE₂ can activate EP₂ and EP₃ receptors (coupled to Gₛ and adenylyl cyclase activation) and in addition, EP₁ and EP₃ receptors (prototypically coupled to Gₛ and activation of phospholipase C). In other types of fibroblasts, EP₂ receptors are critical for PGE₂-mediated inhibition of collagen synthesis and IP receptors can mediate inhibition of fibroblasts by prostanoids (Choung et al., 1998). Other, nontraditional signaling pathways activated by EP receptors perhaps contribute to the disparate response to PGE₂ compared with other agents that we tested.

Giri et al. (1987) described that signaling via the β-adrenergic receptor/adenylyl cyclase system is defective in a rat model of bleomycin-induced pulmonary fibrosis (Giri et al., 1987). Although these observations differ from our current results (in that we do not observe differences in cAMP generation, Fig. 3), Giri et al. (1987) reached a similar conclusion regarding impairment in the cAMP pathway as partly responsible for the fibrogenic response. Therefore, fibrogenesis in human lung may share some commonality with experimentally induced pulmonary fibrosis in rodents.

Matrix metalloproteinases and the tissue inhibitors of metalloproteinases play central roles in regulating ECM, and, by association, in the fibrogenic response. cAMP-elevating agents may exert some of their in vivo antifibrotic actions via changes in matrix metalloproteinases and tissue inhibitors of metalloproteinase expression that lead to altered ECM degradation (Liu et al., 2004) and by accelerating the degradation of newly synthesized collagen (Baum et al., 1980). Our results showing the ability of cAMP to inhibit collagenase-sensitive [³H]proline incorporation does not seem attributable to stimulatory effects on collagen degradation, since agents that increase cAMP directly inhibit collagen gene expression (Table 2) and reduce levels of the procollagen peptide cleaved during collagen processing into ECM (a measure of collagen synthesis not dependent upon incorporation of proline) (Liu et al., 2004). Thus, although cAMP-elevating
agents can be able to enhance ECM degradation, such activity does not seem to account for the inhibition of collagen synthesis in our experimental paradigm.

Pathogenesis of pulmonary fibrosis is related to the presence and activity of myofibroblasts, a differentiated and active interstitial fibroblast (Kuhn and McDonald, 1991; Zhang et al., 1994). We and others have reported that increases in cellular AMP levels inhibit myofibroblast differentiation (as defined by expression of α-SM actin) and have speculated that this effect may be important in attenuating fibrosis (Kolodsiick et al., 2003; Liu et al., 2004). The present data indicating that cAMP-mediated inhibition of myofibroblast differentiation is equally robust in diseased pulmonary fibroblasts as in control fibroblasts (Fig. 4) implies that cAMP-mediated inhibition of collagen synthesis and cell proliferation occur through different signaling pathways. Our data also imply that inhibition of collagen synthesis by cAMP is not a result of its inhibition of myofibroblast differentiation but more likely a consequence of inhibition of collagen gene transcription.

We conclude that a defect exists in the cAMP/PKA/CREB signaling pathway regulating collagen synthesis and cell proliferation in LL29 and LL97A pulmonary fibroblasts cultured from fibrotic human lung. This defect is distal to the generation of cAMP and activation of PKA but seems to occur at the level of CREB transcriptional activity, explaining at least some of the observed reductions in collagen synthesis. We conclude that a defect exists in the cAMP/PKA/CREB signaling pathway regulating collagen synthesis and cell proliferation in LL29 and LL97A pulmonary fibroblasts cultured from fibrotic human lung. This defect is distal to the generation of cAMP and activation of PKA but seems to occur at the level of CREB transcriptional activity, explaining at least some of the observed reductions in collagen synthesis.