The American Journal of Pathology, Vol. 177, No. 5, November 2010

DOI: 10.2353/ajpath.2010.100446

Cardiovascular, Pulmonary and Renal Pathology

Hypermethylation of *PTGER2* Confers Prostaglandin E_2 Resistance in Fibrotic Fibroblasts from Humans and Mice

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Idiopathic pulmonary fibrosis (IPF) is a devastating lung disease that is characterized by excessive proliferation of fibroblasts. The lipid mediator prostaglandin E_2 (PGE_2) has the capacity to limit fibrosis through its inhibition of numerous functions of these fibroblasts; however, in the setting of fibrosis, fibroblasts have been shown to be resistant to PGE_2. We have linked such resistance to decreased expression levels of the E prostanoid 2 (EP2) receptor. In this study, in fibroblasts from both mice and humans with pulmonary fibrosis, we show that DNA hypermethylation is responsible for diminished EP2 expression levels and PGE_2 resistance. Bisulfite sequencing of the prostaglandin E receptor 2 gene (*PTGER2*) promoter revealed that fibrotic fibroblasts exhibit greater *PTGER2* methylation than nonfibrotic control cells. Treatment with the DNA methylation inhibitors 5-aza-2′-deoxycytidine and zebularine as well as DNA methyltransferase-specific siRNA decreased *PTGER2* methylation, increased EP2 mRNA and protein expression levels, and restored PGE_2 responsiveness in fibrotic fibroblasts but not in nonfibrotic controls. *PTGER2* promoter hypermethylation was driven by an increase in Akt signal transduction. In addition to results described for the *PTGER2* promoter, fibrotic fibroblasts also exhibited increased global DNA methylation. These findings demonstrate that the down-regulation of *PTGER2* and consequent PGE_2 resistance are both mediated by DNA hypermethylation; we identified increased Akt signal transduction as a novel mechanism that promotes DNA hypermethylation during fibrogenesis. *(Am J Pathol 2010, 177:2245–2255; DOI: 10.2353/ajpath.2010.100446)*

Idiopathic pulmonary fibrosis (IPF) is a devastating lung disease characterized by excess accumulation of extracellular matrix in the lung, resulting in architectural distortion and impaired gas exchange.[1,2] Fibroblasts are responsible for the generation of excess extracellular matrix and are therefore essential to fibrotic scarring.[3,4] Because of the general paucity of inflammation on lung histology and disappointing therapeutic responses to antiinflammatory agents, mechanistic research in IPF has increasingly focused on a better understanding of the excessive fibroproliferative response.[5,6]

Prostaglandin E_2 (PGE_2) is a lipid mediator derived from the cyclooxygenase metabolism of arachidonic acid that potently inhibits virtually all pertinent functions of fibroblasts.[7–12] Its significance as an antifibrotic mediator is supported by the facts that patients with pulmonary fibrosis have lower levels of PGE_2 in lung lavage fluid,[13] fibroblasts of patients with IPF exhibit diminished PGE_2 synthesis,[14] and mice genetically deficient in generation of PGE_2 exhibit worse pulmonary fibrosis in experimental models.[15] These findings support exogenous administration of PGE_2 as a potential therapeutic modality in this disease, just as prostacyclin analogs are used for the treatment of pulmonary arterial hypertension.[16] However, we have also shown that fibroblasts from patients with IPF[17] and mice with experimental fibrosis[18] are resistant to the antifibrotic actions of exogenous PGE_2. In patients, the degree of fibroblast PGE_2 resistance has been correlated with impairment of lung function.[17] In mice, this resistance may help to explain the development of experimental lung fibrosis despite the fact that, unlike pa-

Supported by a Parker B. Francis Fellowship, an American Thoracic Society Fellows Career Development Award, and grant K08 HL094657 01 from the National Heart, Lung, and Blood Institute (S.K.H.) and R01 HL094311 (M.P.-G.).

Accepted for publication July 22, 2010.

Supplemental material for this article can be found on *http://ajp.amjpathol.org*.

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tients with IPF, they manifest increased lung levels of PGE_2 after injury.

We have previously shown that the PGE_2 resistance in fibroblasts from mice with experimental fibrosis and from some patients with IPF is due to decreased expression of the E prostanoid 2 receptor (EP2), the major G protein–coupled receptor responsible for the antifibrotic actions of PGE_2 [17,18]. The mechanism for diminished EP2 expression in these cells is unknown. Here we tested the hypothesis that DNA methylation is responsible for decreased EP2 expression. Although DNA hypermethylation has been extensively linked to the pathogenesis of cancer,[19,20] there is little known about its role in pulmonary fibrosis. Both the human *PTGER2* and mouse *Ptger2* promoters contain numerous CpG dinucleotides susceptible to methylation.[21,22] Our studies in two different mouse models and in IPF patients identify hypermethylation of the *PTGER2* promoter as a novel mechanism that accounts for PGE_2 resistance in fibrotic fibroblasts and contributes to excessive fibroblast activation in pulmonary fibrosis. We also link hypermethylation of *PTGER2* with PTEN suppression/Akt activation, signaling abnormalities known to be characteristic of IPF fibroblasts.[23] Inhibition of DNA methylation restores EP2 expression and PGE_2 responsiveness, opening the door for novel therapies in this deadly disease.

Materials and Methods

Cell Isolation and Culture

Lungs were collected from mice at day 21 after intratracheal instillation of 50 μl of phosphate-buffered saline or bleomycin (0.00135 U/g body weight, Sigma-Aldrich, St. Louis, MO). Fibroblasts were grown from lungs minced and cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 100 U/ml penicillin/streptomycin as previously described.[18] Mouse fibroblasts were studied between passages 2–4. Embryonic wild-type and *Pten*$^{-/-}$ fibroblasts were isolated as previously described.[10] All animal studies were approved by the University of Michigan Institutional Animal Care and Use Committee.

IPF fibroblasts were cultured from lung biopsy specimens of patients diagnosed with IPF whose tissue histopathology showed usual interstitial pneumonia, as previously described.[17] Control adult human lung fibroblasts were cultured from the margins of lung tissue resected for lung cancer that displayed normal lung histology. All human fibroblasts were cultured in DMEM supplemented with 10% FBS and studied between passages 4–9. All patients received informed consent; the studies were approved by the University of Michigan Institutional Review Board. Primary normal fetal lung fibroblasts (IMR-90) were obtained from the Coriell Institute for Medical Research (Camden, NJ).

For DNA methylation inhibitor studies, cells were plated at 30 to 50% confluence and treated with 5-aza-dC (Sigma-Aldrich) or zebularine (EMD Chemicals, Gibbstown, NJ) at the indicated concentrations for 72 hours in DMEM with 10% FBS. The doses of inhibitors used were based on previously published reports[24,25] and were not found to induce cell toxicity in our studies. For studies with the PI3 kinase inhibitor and Akt inhibitor, cells were treated for 48 hours with LY294002 and Akt-I (EMD Chemicals), respectively.[26]

Bisulfite Modification and Pyrosequencing

DNA was isolated from $\sim 1 \times 10^6$ cells using the Dneasy kit (Qiagen, Valencia, CA). One microgram of genomic DNA was used for bisulfite conversion using the DNA Methylation Gold Kit (Zymo Research, Orange, CA). After bisulfite conversion, the *PTGER2* promoter was amplified with specific biotin-labeled primers (EpigenDx Inc., Worcester, MA). Amplicons were then isolated with beads, denatured, and annealed with specific sequencing primers. Pyrosequencing was then performed on those amplicons using the Pyromark Q24 (Qiagen), with methylation quantitated during the sequencing-by-synthesis reaction as previously described.[27] The murine *Ptger2* promoter was amplified using primers 5′-AGGAAGGAAGATTTTATGGGTTAG-3′ (forward) and 5′-ACTTACCAAAACAACTACTCCCTC-3′ (reverse) for CpG sites 1–5, and 5′-TTGTTAGGGTAGGTGAGGTATAGA-3′ (forward) and 5′-TTCCAAACAAATACCAAACAATC-3′ (reverse) for CpG sites 6–13. The sequencing primers include: 5′-TGGGTTAGTTTAGGGTGA-3′ (for CpG sites 1–2), 5′-TTATTGGATTTGTCTTTTGA-3′ (for CpG sites 3–4), 5′-TAGAGTTTTGTTAGTGTGTG-3′ (for CpG site 5), 5′-GGGTAGGTGAGGTATAGAA-3′ (for CpG sites 6–9), 5′-AATAAGTCTGTTTTTGGTG-3′ (for CpG sites 10–12), and 5′-TGGAGTTTTGGGGTTACTAGAT-3′ (for CpG site 13). The human *PTGER2* promoter was amplified using primers 5′-GATATTAGTATTTGTAAGGTTTGGTTAGTA-3′ (forward) and 5′-AAATCCAAAACCCCCTTC-3′ (reverse). The sequencing primers include: 5′-TTGTAAGGTTTGGTTAGTAT-3′ (for CpG sites 1–13) and 5′-GGGTAGGAGYGGGAGT-3′ (for CpG sites 14–23).

Global DNA Methylation Assay

Global DNA methylation levels were assayed using the Methylamp Global DNA Methylation Quantification Ultra Kit (Epigentek, Brooklyn, NY). Genomic DNA (200 ng) was added to 96-well strips, and levels of methylated DNA were quantified through an ELISA-based reaction using 5-methylcytosine antibody per manufacturer's protocol.

Semiquantitative Real-Time RT-PCR

RNA was isolated from cells using Trizol (Invitrogen). Semiquantitative levels of EP2 mRNA were measured by real-time RT-PCR on the Applied Biosystems ABI Prism Thermocycler (Carlsbad, CA) using human and mouse EP2 and β-actin primers and probes with the sequences as follows: mouse EP2 forward: 5′-TGCGCTCAGTCCTCTGTTGT-3′; mouse EP2 reverse: 5′-TGGCACTGGACTGGGTAGAAC-3′; mouse EP2 probe: 6FAM-5′-CACTGAGAACACAAGAAGCTCAGCAAACAT-TAMRA-3′; mouse β-actin forward: 5′-CTGCCTGACGGCCAAGTC-3′; mouse β-actin reverse: 5′-CAAGAAGGAAGGCTGGAAAAGAG-3′; mouse β-actin probe: 6FAM-5′-AACGAGAGGTTCCGATGCCCTG-TAMRA-3′; human EP2 forward: 5′-GGTGCTCGC-

CTGCAACTTC-3′; human EP2 reverse: 5′-TCCGCAGCG-GCTTCTC-3′; human EP2 probe: 5′-6FAM-TCCGC-ATGCACCGCCGAA-TAMRA-3′; human β-actin forward: 5′-GCCACGGCTGCTTCCA-3′; human β-actin reverse: 5′-GAACCGCTCATTGCCATTG-3′; human β-actin probe: 5′-6FAM-TCCCTGGAGAAGAGCTACGAGCTG-TAMRA-3′. Semiquantitative levels of DNMT enzymes were measured using SYBR Green (Applied Biosystems) with primers: mouse DNMT1 forward: 5′-TACCTGGACGACCCTGAC-CTC-3′; mouse DNMT1 reverse: 5′-CGTTGCATCAAAGAT-GGACA-3′; mouse DNMT3a forward: 5′-TATTGATG-AGCGCACAAGAGAGC-3′; mouse DNMT3a reverse: 5′-GGGTGTTCCAGGGTAACATTGAG-3′; mouse DNMT3b forward: 5′-GGCAAGTTCTCCGAGGTCTCTG-3′; mouse DNMT3b reverse: 5′-TGGTACATGGCTTTTCGATAGGA-3′.

Immunoblotting

Cell lysates were collected in lysis buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor (Roche) and phosphatase inhibitor (EMD Chemicals) cocktails. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies against EP2 (1:500, Cayman Chemicals, Ann Arbor, MI), collagen I (1:500, CedarLane, Ontario, Canada), DNMT1, -3a, and -3b (all at 1:250, Imgenex, San Diego, CA), or α-tubulin (1:1000, Sigma-Aldrich). Membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies and developed with enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ). Densitometry was performed on visualized bands using NIH Scion Image (National Institutes of Health, Bethesda, MD).

Collagen and Proliferation Assays

For collagen assays, cells were serum starved overnight and treated with 500 nmol/L PGE_2 (Cayman Chemicals) in SFM4MegaVir medium (Hyclone, Logan, UT) for 18 hours. Lysates were isolated in lysis buffer and analyzed for collagen I by immunoblotting. For proliferation assays, cells were plated in 96-well plates (1×10^4 cells per well) and treated with 500 nmol/L PGE_2 in SFM4MegaVir medium supplemented with [^{3}H]-thymidine for 18 hours. Cells were harvested and [^{3}H]-thymidine uptake was measured by scintillation counting.

siRNA

Cells were grown to 30 to 50% confluence and transfected with siRNA against DNMT1, -3a, and -3b (Qiagen) using Lipofectamine LTX (Invitrogen). Cells were cultured for 72 hours and collected for RNA and protein.

Data analysis

All data are presented as mean ± SE and were analyzed by analysis of variance or Student's *t*-test, as appropriate, using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA) with $P < 0.05$ defined as statistically significant.

Results

The PTGER2 Promoter Is Hypermethylated in Fibrotic Lung Fibroblasts

We first sought to determine whether the *PTGER2* promoter is hypermethylated in a mouse model of pulmonary fibrosis. Intratracheal injection of bleomycin (0.00135 U/g body weight) in C57BL/6 mice is well known to induce lung injury which results in pulmonary fibrosis by day 21.[28] Fibroblasts isolated from the lungs of bleomycin-treated mice have been shown to be resistant to the suppressive actions of PGE_2 on collagen expression and cell proliferation, and this was attributed to a decrease in EP2 mRNA and protein expression.[18] To determine whether fibrotic fibroblasts exhibit increased *Ptger2* promoter methylation, we performed bisulfite sequencing of the *Ptger2* promoter in fibroblasts isolated on day 21 from both bleomycin- and saline-treated mice. Using pyrosequencing, we assayed the methylation levels of 13 CpG dinucleotide sites located in the first 420 bp upstream of the *Ptger2* transcription start site, focusing on this region because of its proximity to the transcriptional start site and its high density of CpGs (Figure 1A). Compared to fibroblasts from saline control mice, fibroblasts from bleomycin-treated mice exhibited increased DNA methylation at many of the 13 CpG dinucleotide sites (Figure 1B), with

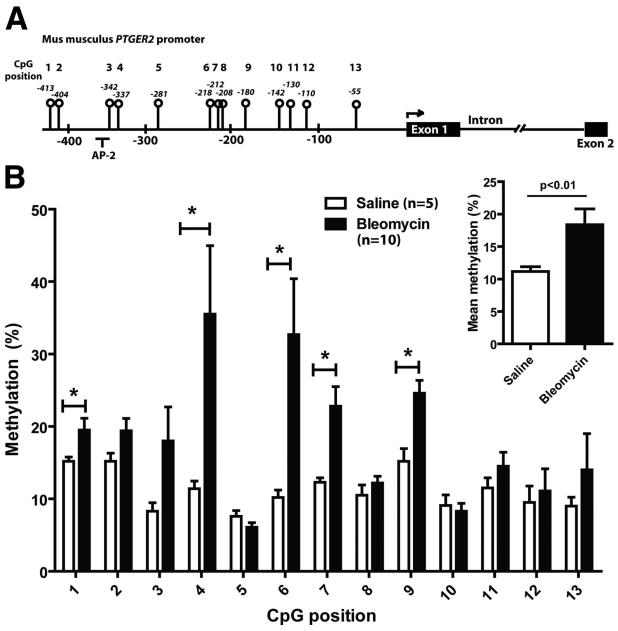

Figure 1. DNA methylation of *Ptger2* promoter in mouse lung fibroblasts. Fibroblasts from mice treated intratracheally with saline ($n = 5$) or bleomycin ($n = 10$) were cultured and assayed for *Ptger2* promoter methylation by bisulfite conversion and pyrosequencing. **A:** Schematic of the mouse *Ptger2* gene and promoter, which has 13 CpG sites in the 420 bp upstream of the transcription start site. **B:** Percent methylation at each CpG site is shown graphically in cells from saline- and bleomycin-treated animals. The mean methylation values at all 13 sites for each group are shown in the **inset**. $^*P < 0.05$.

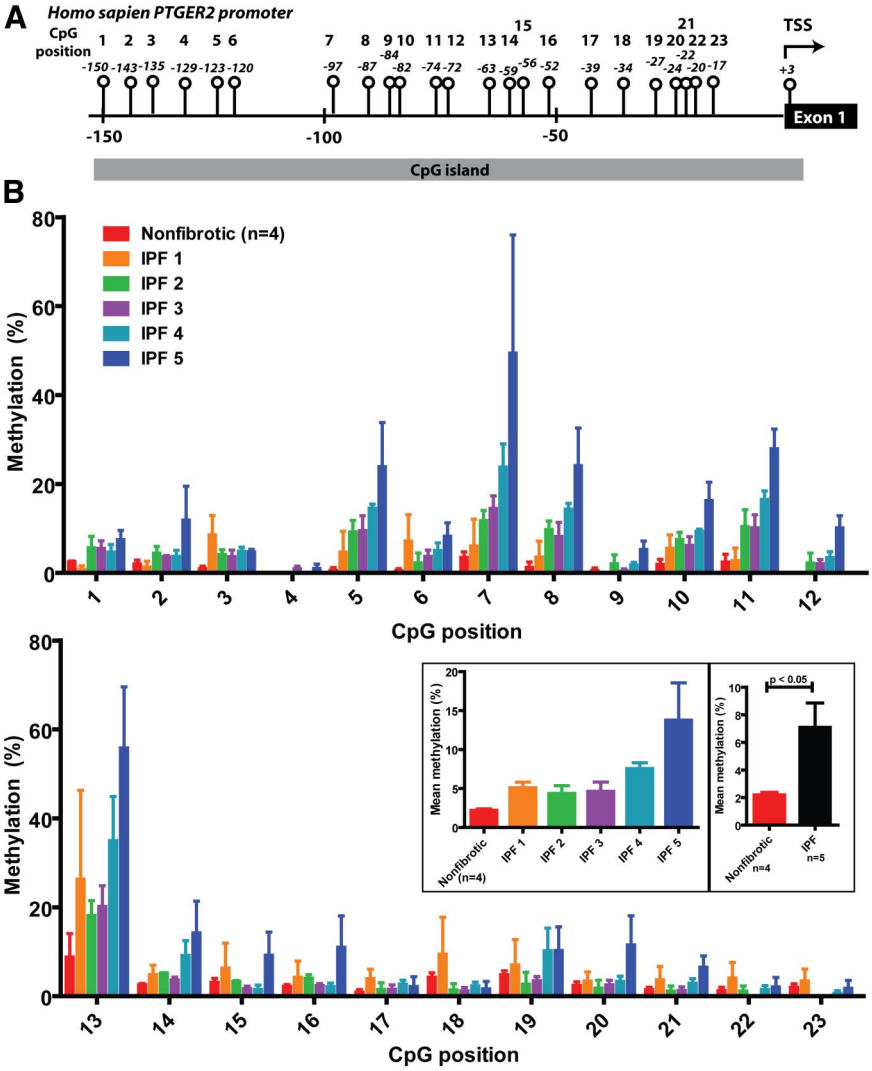

Figure 2. Methylation of *PTGER2* promoter in IPF fibroblasts. **A:** Schematic of the human *PTGER2* gene and promoter, showing 23 CpG dinucleotides in the first 150 bp upstream of the transcriptional start site. **B:** DNA methylation analysis by bisulfite conversion and pyrosequencing of the 23 CpG dinucleotides was performed in four different nonfibrotic and five different IPF fibroblast cell lines; the mean ± SE values for the group of four nonfibrotic lines and for each of the IPF lines are depicted. Methylation levels represent two to four independent experiments for each line, with mean ± SE shown for each line. The mean methylation values at all 23 sites for each line are shown in the **inset**. The mean methylation at all 23 sites for the groups of nonfibrotic and fibrotic cell lines are also shown in the adjacent **inset**.

certain sites (−413, −337, −218, −212, −180) being particularly highly methylated. Fibroblasts from bleomycin-treated mice showed increased mean DNA methylation over all 13 sites (inset).

To determine whether the *PTGER2* promoter is also hypermethylated in IPF cells, we performed bisulfite sequencing of the first 150 bp upstream of the *PTGER2* transcriptional start site, which includes 23 CpGs (Figure 2A). We chose to focus on this region because it met criteria for a CpG island,[29] and it has been shown to be hypermethylated in several cancers.[30,31] Comparing cell lines from five different patients with IPF to cell lines from four different nonfibrotic control patients, IPF fibroblasts exhibited hypermethylation of the *PTGER2* promoter (Figure 2B). Although the mean degree of methylation of the entire group of IPF fibroblast lines exceeded that of the nonfibrotic control group (Figure 2B, inset), some lines (eg, IPF 4 and IPF 5) exhibited a greater mean degree of methylation, and had a higher percentage of methylation at particular sites (ie, −123, −97, −87, −74, −63), than did others.

Inhibition of DNA Methylation Restores EP2 Gene Expression in Fibrotic Lung Fibroblasts

To determine whether the increased DNA methylation observed in fibroblasts from both bleomycin-injured mice and patients with IPF is responsible for decreased EP2 expression, we treated cells for 72 hours with the DNA methylation inhibitors 5-aza-2′-deoxycytidine (5-aza-dC) or zebularine and assayed EP2 mRNA and protein expression. The doses of inhibitors used were based on previously published reports[24,25] and from assays showing that these effected a decrease in global DNA methylation (see Supplemental Figure S1, A and B at *http://ajp.amjpathol.org*). To confirm that these inhibitors decreased DNA methylation of the *Ptger2* promoter, we performed bisulfite sequencing in fibroblasts from bleomycin-treated mice and observed a decrease in mean methylation of the 13 CpG sites in cells treated with 5-aza-dC and zebularine (see Supplemental Figure S1C at *http://ajp.amjpathol.org*).

Data in Figure 3A confirm our previous report[18] that fibroblasts from bleomycin-injured mice exhibit reduced

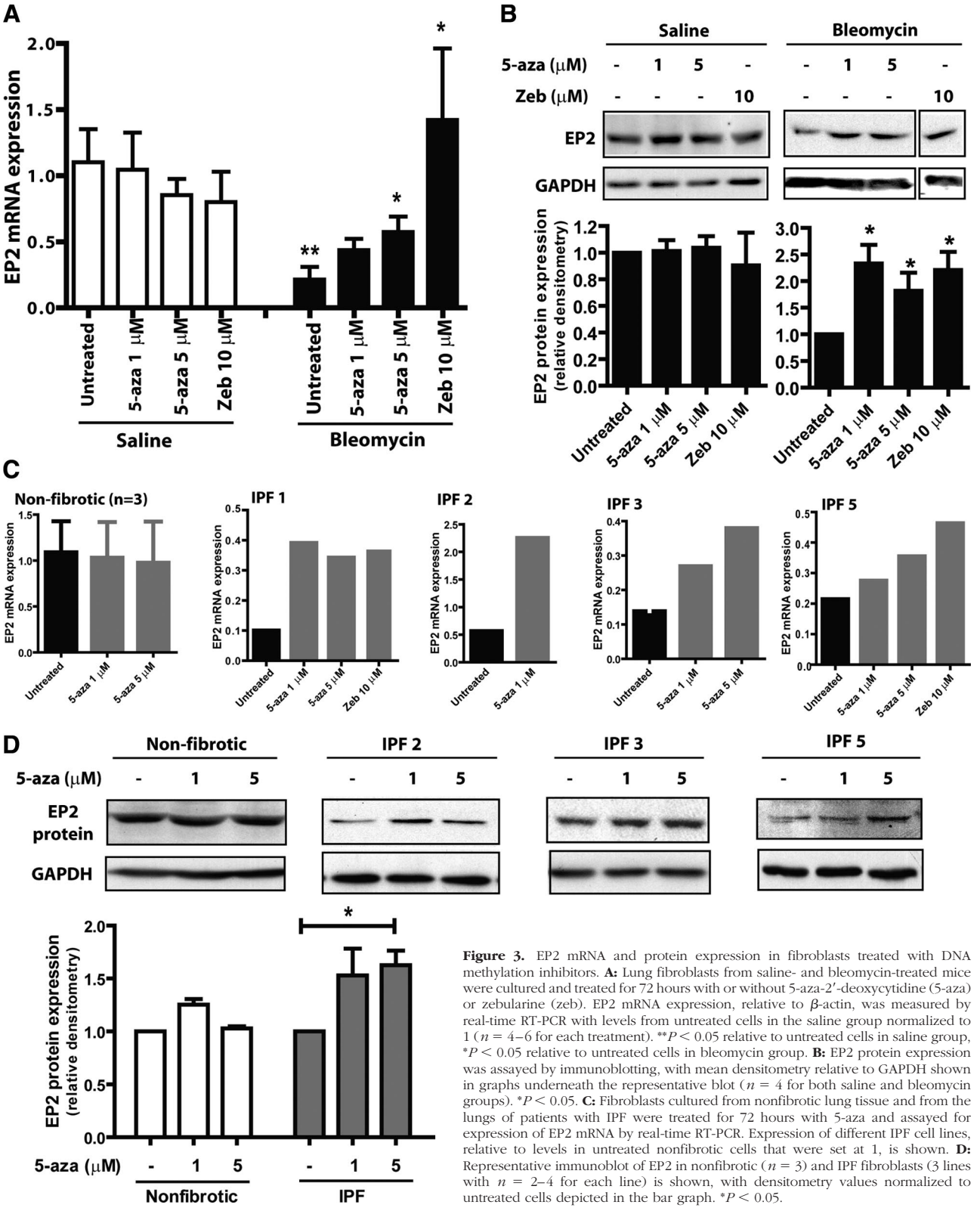

Figure 3. EP2 mRNA and protein expression in fibroblasts treated with DNA methylation inhibitors. **A:** Lung fibroblasts from saline- and bleomycin-treated mice were cultured and treated for 72 hours with or without 5-aza-2′-deoxycytidine (5-aza) or zebularine (zeb). EP2 mRNA expression, relative to β-actin, was measured by real-time RT-PCR with levels from untreated cells in the saline group normalized to 1 ($n = 4–6$ for each treatment). $^{**}P < 0.05$ relative to untreated cells in saline group, $^{*}P < 0.05$ relative to untreated cells in bleomycin group. **B:** EP2 protein expression was assayed by immunoblotting, with mean densitometry relative to GAPDH shown in graphs underneath the representative blot ($n = 4$ for both saline and bleomycin groups). $^{*}P < 0.05$. **C:** Fibroblasts cultured from nonfibrotic lung tissue and from the lungs of patients with IPF were treated for 72 hours with 5-aza and assayed for expression of EP2 mRNA by real-time RT-PCR. Expression of different IPF cell lines, relative to levels in untreated nonfibrotic cells that were set at 1, is shown. **D:** Representative immunoblot of EP2 in nonfibrotic ($n = 3$) and IPF fibroblasts (3 lines with $n = 2–4$ for each line) is shown, with densitometry values normalized to untreated cells depicted in the bar graph. $^{*}P < 0.05$.

EP2 expression but also show that zebularine and 5-aza-dC increased EP2 mRNA expression in fibroblasts from bleomycin-treated mice but not from saline-treated controls. The increase in EP2 mRNA by zebularine in fibroblasts from bleomycin-treated mice was sufficient to fully restore the expression level to that of nontreated cells from saline-treated mice. Immunoblot analysis confirmed the increase in EP2 mRNA expression at the protein level. Treatment with zebularine and 5-aza-dC resulted in a twofold increase in EP2 protein expression in

fibroblasts from bleomycin-treated mice, with EP2 expression in cells from saline-treated mice being unaffected (Figure 3B).

Fibroblasts from IPF patients exhibited a similar increase in EP2 mRNA and protein expression with 5-aza-dC and zebularine treatment. 5-aza-dC increased EP2 mRNA (Figure 3C) and EP2 protein expression (Figure 3D) in all of the IPF cell lines we examined, whereas it did not significantly change EP2 expression in the nonfibrotic controls. Increased EP2 mRNA was also observed with zebularine treatment of a more limited subset of 2 IPF lines.

Several isoforms of DNA methyltransferase (DNMT)—1, -3a, and -3b—can mediate *in vivo* DNA methylation.[32] To determine which DNMT is responsible for maintaining *PTGER2* hypermethylation and silencing of EP2 expression, we transfected fibrotic lung fibroblasts from bleomycin-injured mice and IPF patients with DNMT-specific siRNAs. We confirmed silencing of the intended siRNA target by real-time RT-PCR and immunoblotting (see Supplemental Figure S2A-C at *http://ajp.amjpathol.org*). As shown in Figure 4, A and B, silencing of DNMT3b, and to a lesser extent DNMT1, resulted in increased EP2 mRNA and protein expression in fibroblasts from bleomycin-injured mice. By contrast, silencing of DNMT3a, but not DNMT1 or DNMT3b, resulted in increased EP2 expression in IPF fibroblasts (Figure 4, C and D).

Inhibition of DNA Methylation Restores PGE_2 Responsiveness in Fibrotic Lung Fibroblasts

The EP2 receptor is responsible for most of the inhibitory actions of PGE_2 in fibroblasts,[12,33,34] and its decreased expression in fibrotic lung fibroblasts was correlated with the cells' resistance to PGE_2 inhibition of collagen synthesis and proliferation.[17,18] To determine the functional consequence of restoration of EP2 expression by DNA methylation inhibitors, human and murine fibrotic lung fibroblasts were pretreated with 5-aza-dC and zebularine and then assayed for their responsiveness to PGE_2 by measuring collagen I expression and cell proliferation. In nonfibrotic human cells and fibroblasts from saline control mice, PGE_2 readily inhibited collagen synthesis and its actions were not influenced by pretreatment with 5-aza-dC and zebularine (Figure 5, A and B). By contrast, fibroblasts from bleomycin-treated mice were resistant to the suppressive effects of PGE_2 at baseline and in fact demonstrated a modest degree of stimulation in response to the prostanoid, presumably due to unopposed activation of EP3, as previously reported.[18] In parallel with the increase in EP2 receptor expression demonstrated in Figure 3, treatment with 5-aza-dC or zebularine restored the ability of PGE_2 to inhibit collagen synthesis (Figure 5, A and C) and cell proliferation (Figure 5E) in these cells. IPF fibroblasts at baseline were also resistant to the ability of PGE_2 to reduce collagen expression and cell proliferation below the no-PGE_2 control levels (Figure 5, B and D), in agreement with our previous findings.[17] However, pretreatment with 5-aza-dC or zebularine restored the capacity of PGE_2 to inhibit collagen expression (Figure 5, B and D) and cell proliferation (Figure 5F) in these cells. These findings show that inhibition of DNA methylation of *PTGER2* increases EP2 expression and, importantly, restores PGE_2 responsiveness in fibrotic lung fibroblasts.

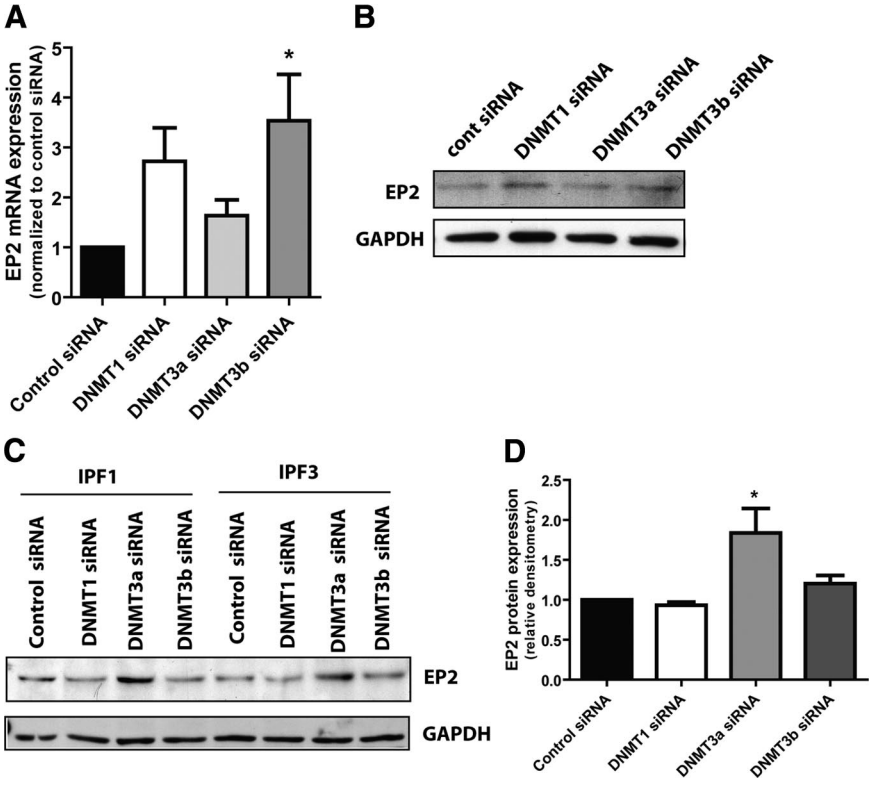

Figure 4. Expression of EP2 in fibrotic lung fibroblasts treated with DNMT-specific siRNA. Fibroblasts from bleomycin-injured mice were treated with DNMT-specific siRNA and assayed at 72 hours for EP2 mRNA (**A**, $n = 3$) and protein (**B**) expression. Representative blot of three independent experiments is shown. **C:** Fibroblasts from patients with IPF were treated with DNMT-specific siRNA and assayed at 72 hours for EP2 protein expression. Representative blot from two cell lines is shown with mean densitometric values of three different cell lines shown graphically in **D**. $^*P < 0.05$.

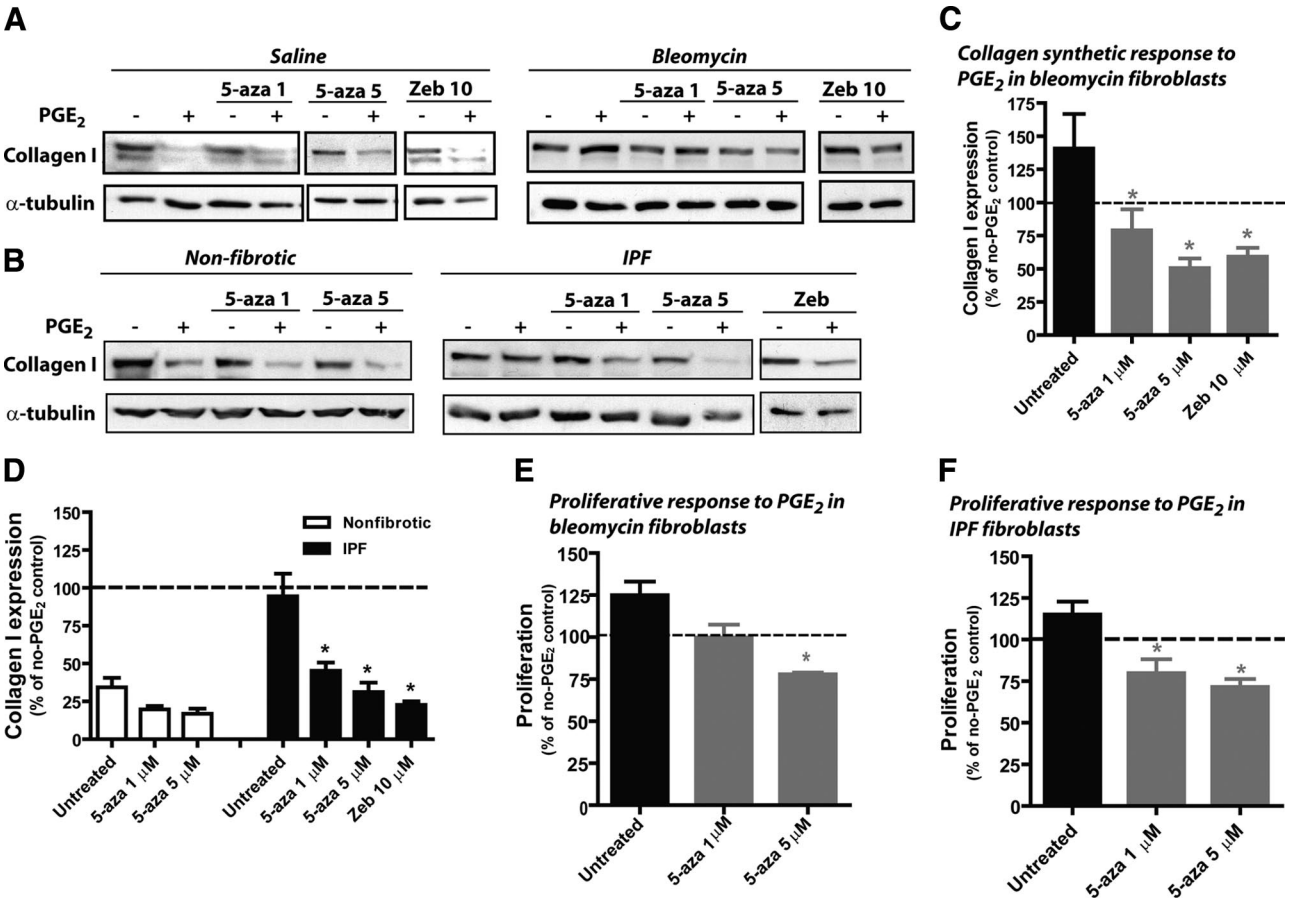

Figure 5. Effect of DNA methylation inhibitors on PGE_2 suppression of collagen expression and cell proliferation in lung fibroblasts. Lung fibroblasts were pretreated for 72 hours with 5-aza-2′-deoxycytidine (5-aza, μM) or zebularine (zeb, μM). Cells were then treated with PGE_2 (500 nmol/L) for 20 hours and assayed for collagen I expression by immunoblot analysis or proliferation. **A:** Representative immunoblot of cells from saline- or bleomycin-treated mice ($n = 4$ each) is shown. **C:** Densitometric analysis of collagen immunoblots normalized to α-tubulin ($n = 4$) in bleomycin fibroblasts treated $\pm$ PGE_2 were performed, with results expressed as a percentage of no-PGE_2 control. **B:** Representative immunoblot from nonfibrotic ($n = 3$) and IPF fibroblasts (three lines with $n = 2$–4 for each line) is shown, with mean effects of PGE_2 as determined from densitometic analysis shown in **D**. **E:** Mean proliferative response to PGE_2 in bleomycin fibroblasts is expressed as a percentage of the no-PGE_2 control ($n = 3$). **F:** Mean proliferative response to PGE_2 in IPF fibroblasts is expressed as a percentage of the no-PGE_2 control ($n = 4$). $^*P < 0.05$ for all graphs.

PTGER2 Promoter Hypermethylation Is Mediated by Decreased PTEN Activity and Increased Akt Activity

PTEN has emerged as a critical brake on fibrogenesis.[23,35] PTEN dephosphorylates phosphatidylinositol-3-phosphate and thus opposes the activation of Akt, which is itself involved in fibroblast activation and prosurvival pathways. We recently reported that EP2 expression was decreased in embryonic fibroblasts from *Pten*$^{-/-}$ mice and that EP2 expression could be upregulated both by increasing PTEN and decreasing Akt activity,[26] suggesting a novel mechanism that might contribute to the antifibrotic actions of PTEN. We therefore sought to determine whether the Akt signaling pathway is responsible for the hypermethylation of *PTGER2* in fibrotic cells.

Using bisulfite sequencing, we found that *Pten*$^{-/-}$ embryonic fibroblasts, whose expression of EP2 is greatly diminished, exhibited a much greater degree of *Ptger2* promoter methylation than did wild-type embryonic fibroblasts (Figure 6A). This degree of methylation greatly exceeded that observed in fibroblasts from bleomycin-treated mice. To determine whether excessive Akt signaling is responsible for *Ptger2* hypermethylation in fibroblasts from bleomycin-injured mice, we tested the effect of an Akt inhibitor, Akt-I, and an inhibitor of the upstream PI3 kinase, LY294002, on *Ptger2* methylation. As shown in Figure 6B, both inhibitors decreased the mean methylation of the *Ptger2* promoter. DNA methylation of *PTGER2* also decreased substantially in two separate IPF fibroblast lines treated with these inhibitors (Figure 6C). Inhibition of PI3 kinase and Akt also decreased global DNA methylation in these cells (see Supplemental Figure S3 at *http://ajp.amjpathol.org*). These data identify increased Akt signaling as a potential determinant of hypermethylation of *PTGER2* in fibrotic lung fibroblasts.

Fibrotic Lung Fibroblasts Exhibit Increased Global Levels of DNA Methylation

Our studies identify *PTGER2* to be hypermethylated in fibrotic lung fibroblasts, and others have recently shown that another gene, Thy-1, is also hypermethylated in fi-

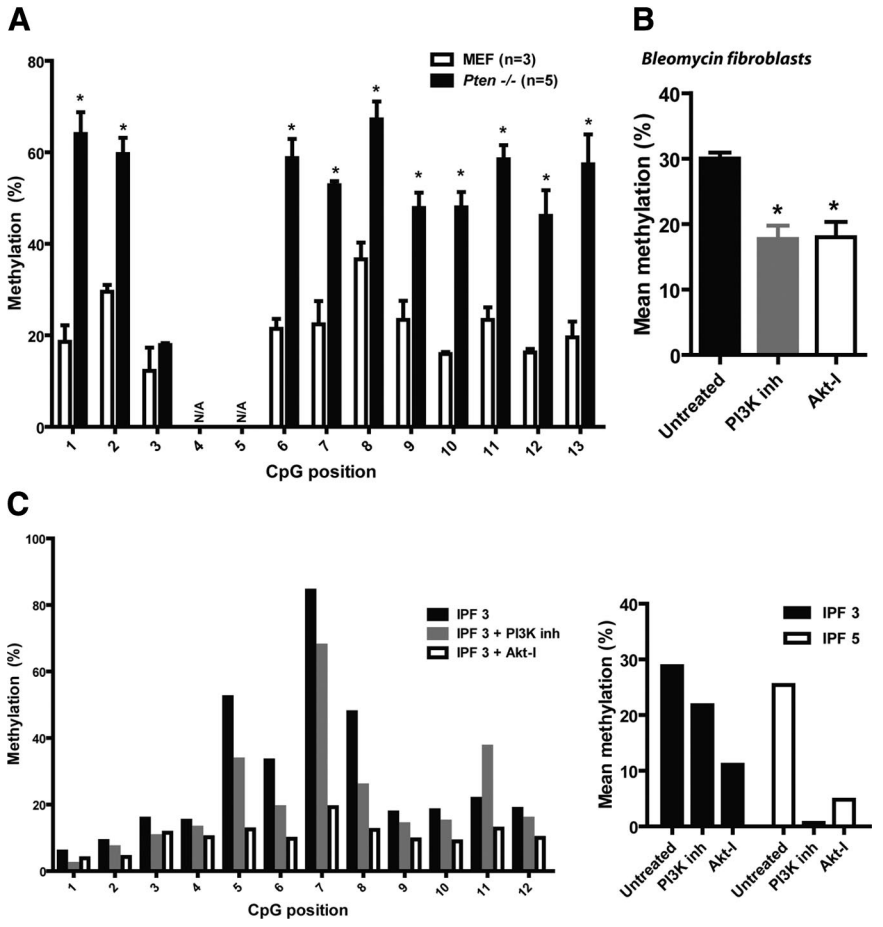

Figure 6. Ptger2 DNA methylation in *Pten*$^{-/-}$ fibroblasts and in fibrotic cells treated with a PI3K inhibitor or Akt inhibitor. **A:** Bisulfite sequencing of the *Ptger2* promoter was performed in *Pten*$^{-/-}$ and wild-type murine embryonic fibroblasts (MEF). The percent methylation of individual CpG sites is depicted. *$P < 0.05$. **B:** Fibroblasts from mice treated with bleomycin were cultured and treated with the PI3K inhibitor, LY294002 (10 μmol/L), or the Akt inhibitor, Akt-I (5 μmol/L). Methylation of the *Ptger2* promoter was determined by bisulfite pyrosequencing, with mean methylation of the 13 CpG sites shown ($n = 3$). *$P < 0.05$. **C:** Bisulfite sequencing of the human *PTGER2* promoter was performed in IPF fibroblasts treated for 48 hours with and without the PI3K inhibitor, LY294002 (10 μmol/L), or the Akt inhibitor, Akt-I (5 μmol/L). Shown is the percent methylation of individual CpG sites from a representative line, with the mean methylation from two IPF lines shown on the **right**.

broblasts from patients with IPF.[36] To determine whether fibrotic fibroblasts exhibit a higher level of global DNA methylation, we used an ELISA-based assay for methylated genomic DNA and observed that fibroblasts from lungs of bleomycin-injured mice and IPF patients exhibited increased global DNA methylation compared to nonfibrotic cells (Figure 7, A and B). Additionally, in cells from bleomycin-treated mice, we observed a trend for increased DNMT3a and DNMT3b mRNA and protein expression (see Supplemental Figure S4, A and B at *http://ajp.amjpathol.org*). IPF fibroblasts also exhibited an increase in DNMT3a expression (see Supplemental Figure S4C at *http://ajp.amjpathol.org*) compared to nonfibrotic controls. These findings suggest that hypermethylation of genes other than *PTGER2* is a characteristic of fibrotic fibroblasts, likely as a consequence of excessive DNMT actions.

Discussion

Although epigenetic alterations have been implicated in the development of many cancers,[19,20] the role of epigenetic changes in IPF and other lung diseases remains relatively underappreciated. Here, we show an important role for DNA hypermethylation in mouse models of pulmonary fibrosis and in its most common variety in humans, IPF. PGE_2 resistance is a characteristic of the fibrotic cell phenotype that disrupts normal feedback control on fibroblasts and thereby contributes to fibrogenesis.[17] In both animal models and patients with pulmonary fibrosis, we found that PGE_2 resistance can be explained by DNA hypermethylation of *PTGER2* with sub-

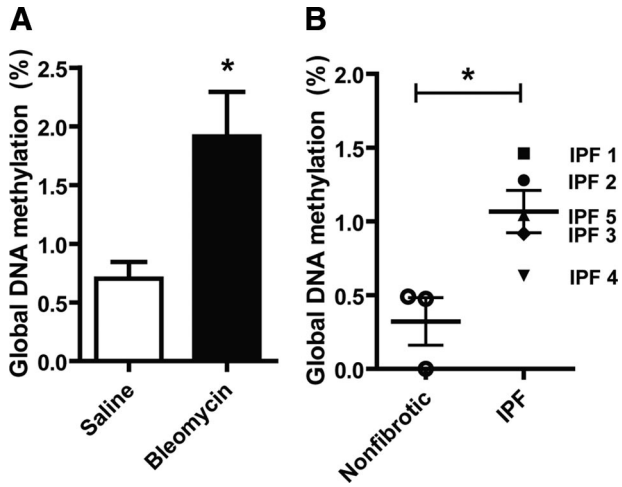

Figure 7. Global DNA methylation levels in fibrotic and nonfibrotic lung fibroblasts. **A:** Fibroblasts from saline- ($n = 8$) and bleomycin-treated ($n = 11$) mice were cultured and assayed for global DNA methylation as described in *Materials and Methods*. **B:** Global DNA methylation levels were assayed in patient-derived nonfibrotic ($n = 3$) and IPF ($n = 5$) fibroblasts. *$P < 0.05$.

sequent silencing of its gene product, EP2, which is responsible for the antifibrotic actions of this prostanoid. Reversal of this hypermethylation by pharmacological or molecular approaches restored EP2 expression and PGE_2 responsiveness. Lung fibroblasts from IPF patients have previously been shown to exhibit decreased PTEN expression and activity and therefore unchecked PI3 kinase and Akt activity,[35] and we observed that inhibition of Akt and PI3 kinase in these cells as well as in fibroblasts from mouse models of pulmonary fibrosis reverses the increased *PTGER2* methylation in these cells. These findings demonstrate a new and critical role for DNA methylation in the pathogenesis of pulmonary fibrosis and illustrate a critical role for PTEN/Akt in regulating gene-specific DNA methylation.

That fibroblasts from both humans and mice with pulmonary fibrosis exhibit *PTGER2* promoter hypermethylation demonstrates the importance of this shared pathogenic mechanism. Mouse models of pulmonary fibrosis, especially that elicited by intrapulmonary administration of bleomycin, have been criticized for failing to approximate certain aspects of human disease. Here, we observed that *PTGER2* promoter methylation is increased in fibroblasts from both species. Our findings underscore the importance of PGE_2 as an antifibrotic mediator, as the target of DNA hypermethylation in both species is a gene target critical to the antifibrotic signaling of PGE_2. In mice, this finding does not represent a specific response to bleomycin, as diminished lung fibroblast EP2 expression was also observed in *Pten*$^{-/-}$ mice and in another experimental model of pulmonary fibrosis using intratracheal administration of FITC.[18] In humans, *PTGER2* hypermethylation appears specific for IPF, as we have previously shown that EP2 expression is normal in fibroblasts from patients with other fibrotic lung disorders.[17]

It has been shown that Akt can stabilize[37] and phosphorylate[38] DNMT1, maintaining global DNA methylation.[39] In accordance with data that show Akt signaling to be increased in IPF fibroblasts,[23] we observed greater levels of global DNA methylation in fibrotic lung fibroblasts. This includes methylation at certain gene promoters, in this case, the *PTGER2* promoter. However, methylation at the *PTGER2* promoter appears mediated by DNMT3a and DNMT3b isoforms in fibrotic human and mouse cells, respectively. These findings suggest that Akt may modulate the expression or activity of these other isoforms. To our knowledge, this is the first observation of two different DNMT isoforms being responsible for maintaining methylation of the same gene in two different species. The participation of DNMT3a and DNMT3b in *PTGER2* methylation in the two species parallels their increased expression in cells from the human disease and from mouse models, respectively. More studies are needed to understand how these enzymes are upregulated in a species-specific manner in fibrotic lung disease.

Our data in individual IPF cell lines suggest that transcription of *PTGER2* is sensitive to even modest changes in methylation of its promoter. Of note, *Pten*$^{-/-}$ fibroblasts, which possess even lower levels of EP2 expression[26] than those we observed in fibroblasts from bleomycin-treated mice, exhibited a higher degree of *Ptger2* methylation, suggesting a possible dose-response relationship between levels of methylation and gene expression. The *Pten*$^{-/-}$ fibroblasts thus represent a second mouse model of human IPF in which *Ptger2* methylation results in decreased EP2 expression and a profibrotic phenotype.

We have previously shown that fibroblast lines from individual patients with IPF exhibit variable degrees of PGE_2 resistance[17] which correlate with the severity of lung function impairment. It is thus not surprising that we also observed heterogeneity in the degree of *PTGER2* DNA hypermethylation among IPF patient cell lines. Unfortunately, limitations in the availability of biopsy-derived primary cells from patients with IPF preclude us from being able to correlate the degree of methylation with either progression of the disease or the aggressiveness of the fibrotic phenotype. The CpG sites we interrogated were chosen based on their high density, proximity to the transcriptional start site, and demonstrated hypermethylation in neuroblastomas[30] and non-small cell lung cancer cells,[31] but it is unknown whether methylation of certain CpG positions is more critical to EP2 expression than methylation at other sites. This, along with coexisting histone modifications that may be associated with DNA methylation but which we did not investigate here, may explain the variability in EP2 expression and mean methylation observed in IPF cell lines.

Increased global DNA methylation and expression of certain DNMT isoforms imply that other genes besides *PTGER2* may be hypermethylated and that the methylation machinery may be dysregulated in pulmonary fibrosis. It has already been shown that Thy-1, expressed at lower levels in fibroblastic foci,[40] is hypermethylated in IPF.[36] Both viruses[41] and cigarette smoking[42] have been implicated as risk factors in the development of IPF, and both have been shown to promote DNA methylation.[43,44] The predilection of IPF for the elderly[45] could reflect the fact that acquired epigenetic defects are known to accumulate with age.[46] High throughput and next-generation sequencing technology will be necessary to determine other genes that may be hypermethylated in IPF.

In conclusion, multiple lines of evidence point to the importance of PGE_2 as a critical antifibrotic mediator whose synthesis and actions are dysregulated in pulmonary fibrosis. Diminished COX-2 expression, and thus PGE_2 synthesis, has been shown to be critical in pulmonary fibrosis[14,15] and recently attributed to histone deacetylation at the COX-2 promoter.[47] Here we now show that resistance to PGE_2 in fibrotic lung fibroblasts is attributable to another epigenetic mechanism, DNA methylation. This hypermethylation of the *PTGER2* promoter can be observed in cells from both mice and humans with pulmonary fibrosis and is mechanistically driven by decreased PTEN and increased Akt signaling. Inhibition of DNA methylation by DNMT inhibitors and siRNA restored EP2 expression and sensitivity to the antifibrotic actions of PGE_2. This suggests the possibility that PGE_2 may be combined with methylation inhibitors, which are currently approved for treatment of myelodysplastic syndrome[48] and are under investigation in other

neoplasms,[49] as a potentially novel approach to the treatment of IPF.

Acknowledgments

We thank Raymond Yung, Colin Delaney, and Robert Hinderer for their assistance with pyrosequencing. We thank Bethany Moore for her assistance with mice.

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