Biomarkers, Genomics, Proteomics, and Gene Regulation

Regulation of Myofibroblast Differentiation by Poly(ADP-Ribose) Polymerase 1

Biao Hu,* Zhe Wu,* Polla Hergert,† Craig A. Henke,‡ Peter B. Bitterman,§ and Sem H. Phan*}

From the Department of Pathology,* University of Michigan Medical School, Ann Arbor, Michigan; and the Department of Medicine,† University of Minnesota, Minneapolis, Minnesota

Poly(ADP-ribosylation) (PARylation) is a post-translational protein modification effected by enzymes belonging to the poly(ADP-ribose) polymerase (PARP) superfamily, mainly by PARP-1. The key acceptors of poly(ADP-ribose) include PARP-1 itself, histones, DNA repair proteins, and transcription factors. Because many of these factors are involved in the regulation of myofibroblast differentiation, we examined the role of PARylation on myofibroblast differentiation. Overexpression of PARP-1 with an expression plasmid activated expression of the α-SMA gene (Actα2), a marker of myofibroblast differentiation in lung fibroblasts. Suppression of PARP-1 activity or gene expression with PARP-1 inhibitors or siRNA, respectively, had the opposite effect on these cells. PARP-1-deficient cells also had reduced α-SMA gene expression. DNA pyrosequencing identified hypermethylated regions of the α-SMA gene in PARP-1-deficient cells, relative to wild-type cells. Interestingly, and of potential relevance to human idiopathic pulmonary fibrosis, PARP activity in lung fibroblasts isolated from idiopathic pulmonary fibrosis patients was significantly higher than that in cells isolated from control subjects. Furthermore, PARP-1-deficient mice exhibited reduced pulmonary fibrosis in response to bleomycin-induced lung injury, relative to wild-type controls. These results suggest that PARylation is important for myofibroblast differentiation and the pathogenesis of pulmonary fibrosis. (Am J Pathol 2013, 182: 71–83; http://dx.doi.org/10.1016/j.ajpath.2012.09.004)

Suitably activated fibroblasts can give rise to a differentiated phenotype characterized by induction of α-smooth muscle actin (α-SMA), which is incorporated into stress fibers and are therefore aptly identified as myofibroblasts.1–3 These differentiated cells are known to play important roles in lung development, carcinogenesis, wound healing, tissue repair, and fibrosis.1–6 Although the tissue fibroblast is a well-established precursor cell for the myofibroblast, there is evidence that other cell types could give rise to this differentiated phenotype.7 The mechanism underlying genesis of the myofibroblast is incompletely understood, however, and it appears to be quite complex, involving diverse signaling pathways, transcription factors, and epigenetic processes.3,8–11 Given the important function of this cell in these processes and in related diseases, a more complete understanding of myofibroblast differentiation would be of value in providing new insights into novel approaches of how to regulate myofibroblast genesis as a means of controlling these processes and/or treatment of related diseases.

Poly(ADP-ribosylation) (PARylation) is a post-translational protein modification catalyzed by enzymes belonging to the poly(ADP-ribose) polymerase (PARP) superfamily, mainly by PARP-1.12,13 PARP-1 uses NAD+ as the ADP donor to generate the long branching poly(ADP-ribose) polymer covalently attached to glutamate or aspartate residues of suitable acceptor proteins.14 The known key acceptors of poly(ADP-ribose) include PARP-1 itself (automodification), histones, DNA repair proteins, and transcription factors.15,16 Inhibition of PARylation in oxidative stress-related pathologies such as arthritis, colitis, diabetes, and shock has emerged as a very effective anti-inflammatory intervention in animal models.17–22

Supported in part by NIH grants HL28737, HL31963, HL52285, HL77297 (S.H.P.), and HL91775 (C.A.H. and S.H.P.). This work made use of the University of Michigan’s Pathology Flow Cytometry Core Laboratory and Cell and Molecular Biology Core of the Michigan Diabetes Research and Training Center, funded by the National Institute of Diabetes and Digestive and Kidney Diseases (DK020572).
Protective effects of PARP inhibition or PARP-1 deficiency in asthma models have also been observed. Treatment of hamsters with the PARP inhibitor nicotinamide significantly decreased bleomycin-induced lung fibrosis and PARP activities. Additionally, various studies have demonstrated PARylation of Smad3 and DNA methyltransferase 1 (Dnmt1), known regulators of myofibroblast differentiation. However, the precise role of PARP-1 in the regulation of myofibroblast differentiation has not been determined.

In the present study, we investigated the role of PARP-1 in the regulation of myofibroblast differentiation and evaluated its potential in vivo importance in a model of pulmonary fibrosis. PARP-1 activated myofibroblast differentiation, whereas its inhibition or deficiency had the opposite effect, as determined by α-SMA expression. Further studies indicated that PARP-1 suppressed DNA methylation in the α-SMA gene (Acta2) and affected the binding of Smad3 to the Smad3 binding element in the α-SMA promoter. In addition, lung fibroblasts isolated from idiopathic pulmonary fibrosis (IPF) patients exhibited significantly increased PARP-1 expression and activity relative to lung fibroblasts isolated from control subjects. Immunostaining of the fibroblastic foci of lung tissue sections from IPF patients showed induced PARP-1 expression in α-SMA-positive cells. Finally, in vivo mice deficient in PARP-1 expression exhibited attenuated pulmonary fibrosis in response to bleomycin-induced injury. These results suggest that PARP-1 plays an essential role in the regulation of myofibroblast differentiation, with consequent effect on pulmonary fibrosis.

Materials and Methods

Ethics

Use of human tissue and animal care were conducted in accordance with the NIH guidelines for survival Rodent Surgery, the Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals, 7th edition (1996). The study was reviewed and approved by the University of Michigan Institutional Biosafety Committee and the University Committee on Use and Care of Animals.

Animals and Cell Culture

Pathogen-free female Fischer 344 rats, C57/BL6 mice, and PARP-1−/− deficient mice (7 to 8 weeks old) were used; rats were purchased from Charles River Breeding Laboratories (Wilmington, MA) and mice were from the Jackson Laboratory (Bar Harbor, ME). Fibroblasts were isolated by enzymatic digestion, as described previously, and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% plasma-derived serum (Cocalico Biologicals, Reamstown, PA), antibiotics, 1% ITS (insulin, transferrin, selenium) (Sigma-Aldrich, St. Louis, MO), 5 ng/mL platelet-derived growth factor (PDGF; R&D Systems, Minneapolis, MN), and 10 ng/mL epidermal growth factor (EGF; R&D Systems). The adherent cells were then trypsinized and passaged at least three times before use, to ensure >99% purity.

Pulmonary fibrosis was induced by the endotracheal injection of bleomycin (Blenoxane; 1.5 U/kg; Mead Johnson, Princeton, NJ) in sterile PBS for each mouse, as described previously. The control group received the same volume of sterile PBS only. For evaluation of the fibrotic response, animals were sacrificed and the lungs were removed for extracting RNA, for fibroblast isolation 7 days after bleomycin treatment, and for hydroxyproline assay and Western blot analysis 21 days after bleomycin treatment. For all other experiments, fibroblasts isolated from normal healthy animals were used.

To evaluate the role of PARP-1 in human cells, five primary cultured fibroblast lines from IPF patients and five primary cultured human lung fibroblast lines from control subjects were used. The control or normal cells were defined as those obtained from histologically normal lung tissue distal from tumor margins of lung resections. All cells were established from lungs removed at the time of transplantation or death and maintained in high-glucose DMEM containing 10% fetal calf serum between passages 6 and 10, as described previously. Cells from each individual donor were cultured separately and were analyzed individually, without mixing. The diagnosis of IPF was supported by history, physical examination, pulmonary function tests, and high-resolution chest computed-tomography findings typical of IPF. All patients fulfilled the criteria for the diagnosis of IPF as established by the American Thoracic Society and the European Respiratory Society. Diagnoses were confirmed by microscopic analysis of lung tissue, which demonstrated the characteristic morphological findings of interstitial pneumonia. Use of human tissues was approved by the Institutional Review Boards of the University of Minnesota and of the University of Michigan.

TGF-β Treatment

Recombinant TGF-β1 (240-B-002; R&D Systems, Minneapolis, MN) was dissolved in sterile 4 mmol/L HCl containing 1 mg/mL bovine serum albumin, aliquoted, and stored at −80°C until use. Cells were washed with 1× PBS and incubated with 4 ng/mL TGF-β or the same amount of the dissolving buffer in conditioned medium (Dulbecco’s modified Eagle’s medium containing 0.5% plasma-derived serum) for the indicated time.

Plasmids and Constructs

The rat −2880 to +32 promoter region, previously amplified by PCR, was cloned into promoterless pGL3-basic vector to form the α-SMApro-Luc construct, in which luciferase reporter gene expression was controlled by the α-SMA gene promoter.
The rat PARP-1 cDNA constructs, the lentivirus-based shRNA constructs specific for PARP1 gene, and the negative control shRNA construct were purchased from Thermo Fisher Scientific (Huntsville, AL).

DNA Pyrosequencing Analysis
DNA pyrosequencing was performed essentially as described previously. Genomic DNA was extracted from cells using a Wizard genomic DNA extraction kit (Promega, Madison, WI), and 1 μg of the genomic DNA was bisulfite-modified using a Zymo Research EZ Methylation Gold kit (Zymo Research, Irvine, CA), according to the manufacturer’s protocol. The bisulfite-modified sample DNA was then 10-fold diluted, and 1 μL of diluted DNA was used in PCR reactions with 3 μL 10× PCR buffer, 200 μmol/L of dNTPs, 6 pmol forward primer, 6 pmol reverse primer, 3 mmol/L MgCl₂, and 0.75 U HotStarTaq DNA polymerase (205203; Qiagen, Valencia, CA) in a final volume of 30 μL (adjusted as necessary with double-distilled H₂O). The PCR cycling conditions were as follows: 95°C for 15 minutes; 45 cycles of 95°C for 30 seconds, 51°C for 30 seconds, and 72°C for 30 seconds; 72°C for 10 minutes; and 4°C until completion. The PSQ 96HS system (currently named PyroMark Q96 MD) (Qiagen) was used for the pyrosequencing analysis, according to the manufacturer’s protocol. Primers corresponding to the mouse α-SMA gene promoter region and first intronic region, respectively, were synthesized at EpigenDx (Hopkinton, MA) for use in the DNA pyrosequencing.

Transfection and Reporter Gene Assay
All transient transfections of cells were performed using FuGENE 6 reagent (Roche Applied Science, Indianapolis, IN), according to the manufacturer’s instructions and as described previously. Supercoiled DNA was isolated with an endotoxin-free column kit (Qiagen). Unless otherwise indicated, 2 μg DNA of the α-SMA promoter-luciferase construct of interest and 100 ng plasmid pRL-SV40 control vector (used for normalization) were cotransfected per culture into rat lung fibroblasts in serum-free DMEM. At 4 hours after transfection, the medium was replaced with DMEM containing 10% plasma-derived serum and treated with 4 ng/mL TGF-β or buffer only. The cells were harvested 48 hours after transfection, and the activity of firefly or Renilla luciferase was measured using a dual luciferase assay system (Promega). The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of Renilla luciferase. Experiments with each construct were repeated two to four times; data are expressed in relative light units as means ± SEM.

Electrophoretic Mobility Shift Assay
A double-stranded oligonucleotide probe corresponding to the region between −552 and −513 in the rat α-SMA gene promoter containing the Smad3 binding elements was synthesized (Integrated DNA Technologies, Coralville, IA). The sequence of the sense strand was 5’-TACAGACTTCA-TTGATATACACACAGACTCCAGACTAC-3’. It was radiolabeled with T4 polynucleotide kinase and γ-[32P]ATP and was incubated with 50 ng recombinant Smad3, as described previously, or with 2 μg nuclear extract from mouse lung fibroblasts isolated from either wild-type (WT) or PARP-1-deficient knockout (KO) mice. Selected samples were pretreated with high-specific-activity PARP-1 enzyme (4668-100-01; Trevigen, Gaithersburg, MD) in 1× PARP buffer (4671-096-02; Trevigen) containing the NAD⁺ substrate of PARP-1 before addition of the 32P-labeled probe (20,000 to 30,000 counts/minute). The labeled probe was incubated with the proteins in Dignam’s buffer C (20 mmol/L HEPES, pH 7.9, 0.42 mol/L NaCl, 1 mmol/L EDTA, 0.1 mmol/L EGTA, 1.0 mmol/L dithiothreitol, 100 μmol/L sodium orthovanadate, and protease inhibitors) in a final volume of 15 μL for 20 minutes at room temperature. The samples were then analyzed by electrophoresis on 4% non-denaturing polyacrylamide gels at 150 V in 1× Tris-acetate-EDTA buffer (40 mmol/L Tris-acetate and 1 mmol/L EDTA, pH 8.3, at room temperature). After electrophoresis, the gels were dried and exposed on X-ray film for 24 hours.

RNA Analysis by qPCR
Quantitative real-time PCR (qPCR) for RNA analysis to assess gene expression was performed using an Applied Biosystems GeneAmp 7500 sequence detection system (Life Technologies, Foster City, CA), as described previously. The amount of target, normalized to endogenous reference and relative to a calibrator, is given by 2−ΔΔCT, where CT is the cycle number at which DNA amplification is detected. All required primers and probes were purchased from Applied Biosystems (Life Technologies). Results are expressed as 2−ΔΔCT, with 18S rRNA as the reference. The specific calibrator for each assay was the appropriate control group mean value.

Western Blot Analysis
The anti-α-SMA and anti-procollagen I antibodies were purchased from Sigma-Aldrich. All other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Equal amounts of cell protein extracts were loaded onto 12% SDS polyacrylamide gels and transferred onto Hybond-P membranes (GE Healthcare, Little Chalfont, UK) for Western blotting, as described previously. Blots were scanned and digitized, and band intensities were quantified using Carestream Molecular Imaging software version 5.0.2.30 (Carestream Health, Rochester, NY).

Morphological Analysis
Morphological analysis was performed on formalin-fixed, paraffin-embedded lung tissue sections stained with
Masson’s trichrome using an Accustain trichrome stain kit (Sigma-Aldrich), according to the manufacturer’s protocol.39

Hydroxyproline Assay

As a measure of fibrosis, total lung hydroxyproline content was determined by colorimetric assay after acid hydrolysis, as described previously.36,40 Briefly, the whole lung was removed from the animal and washed with 1× PBS. Lungs were homogenized in 2 mL 0.5 mol/L glacial acetic acid, dried in a centrifugal evaporator, and then baked in 6 mol/L HCl at 110°C overnight. The hydrolysates were filtered and assayed colorimetrically at 550 nm with 4-(dimethylamino)benzaldehyde to quantify hydroxyproline. Hydroxyproline content was expressed on a mass basis (μg/lung).

PARP Assay

The PARP activity of human lung fibroblasts was tested with an HT colorimetric PARP/apoptosis assay kit (4684-096-K; Trevigen), according to the manufacturer’s protocol. Briefly, cultured human lung fibroblasts isolated from either control subjects or patients with IPF34 were lysed in cell extraction buffer prepared from the kit. The protein extracts were then incubated with PARP substrate cocktail together with histone-coated strip wells. To confirm specificity, 3-aminobenzamide (3-AB; 10 mmol/L) was added to sample lysates before assay. The poly(ADP-ribose) on the strip wells was then detected by monoclonal anti-poly(ADP-ribose) antibody and HRP-conjugated goat anti-mouse IgG antibody, with stringent washing for each step. TACS-Sapphire colorimetric substrate from the kit was then added, and PARP activity was detected by chemiluminescence.

Immunohistology

Lung tissue was removed and fixed in 10% formalin, paraffin-embedded, cut into 5-μm sections, and mounted onto polylysine slides. The sections were deparaffinized in xylene, rehydrated through a graded alcohol series of PBS. Lungs were scanned with a scanner in the animal and washed with 1× PBS. Lungs were homogenized in 2 mL 0.5 mol/L glacial acetic acid, dried in a centrifugal evaporator, and then baked in 6 mol/L HCl at 110°C overnight. The hydrolysates were filtered and assayed colorimetrically at 550 nm with 4-(dimethylamino)benzaldehyde to quantify hydroxyproline. Hydroxyproline content was expressed on a mass basis (μg/lung).

Statistical Analysis

Statistical analysis was performed using analysis of variance followed by post hoc testing using Scheffé’s test. Data are expressed as means ± SEM, unless otherwise indicated. Differences between means of various treatment and control groups were assessed for statistical significance by analysis of variance followed by post hoc analysis using Scheffé’s test for comparison between any two groups. A P value of <0.05 was considered statistically significant.8,10,11,36,41

Results

PARP-1 Activates Myofibroblast Differentiation

The importance of PARP-1 as a regulator of myofibroblast differentiation was first evaluated by examining the effect of the PARP-1 inhibitor 3-AB on α-SMA expression in primary culture-derived rat lung fibroblasts by qPCR. Selected samples were also treated with TGF-β, a known inducer of myofibroblast differentiation. Treatment with 3-AB led to dose-dependent inhibition of α-SMA gene expression, even in cells treated with TGF-β (Figure 1A). No appreciable cell toxicity was observed after the 3-AB treatment at experimental doses, as determined by Trypan Blue exclusion assay (data not shown) or by visual inspection of the cells by phase-contrast microscopy (Figure 1B).

To confirm that this effect was specifically due to PARP-1 inhibition, PARP-1 shRNA plasmid (pGIPZ-PARP-1), PARP-1 cDNA expression construct (pExpress1-PARP-1), or the corresponding vector control plasmids were transfected with FuGENE 6 reagent into rat lung fibroblasts. Induced overexpression by the PARP-1 cDNA caused a significant increase in α-SMA mRNA levels, even in cells treated with TGF-β (Figure 2A). Suppression of PARP-1 expression by the shRNA had the opposite effect on α-SMA expression. Treatment with TGF-β induced PARP1 gene expression, and the stimulatory effect of TGF-β on α-SMA expression was enhanced or blunted by PARP-1 overexpression or underexpression, respectively. Similar effects were noted when α-SMA expression was analyzed by Western blotting, although the effects were not as great because of the
considerable level of α-SMA protein already present basally (Figure 2, B–E). In addition, there was nearly a doubling in PARP-1 protein by the expression plasmid, whereas the shRNA completely suppressed the TGF-β-induced doubling of PARP-1 protein, which resulted in an approximately 50% reduction in TGF-β-induced α-SMA expression. The PARP-1 shRNA caused a smaller but statistically significant reduction of basal PARP-1 protein levels.

These effects on α-SMA mRNA and protein suggest that PARP-1 regulates α-SMA expression at the transcriptional level. To examine this possibility, an α-SMApro-Luc promoter construct driving a luciferase reporter gene was transfected into fibroblasts. The cells were then cotransfected with the PARP-1 shRNA plasmid, PARP-1 cDNA expression construct, or corresponding vector control plasmids. Consistent with the mRNA data (Figure 2), α-SMA promoter activity was significantly stimulated by PARP-1 overexpression (Figure 3A) but was suppressed by PARP-1 deficiency induced by the shRNA (Figure 3B); neither the absence nor the presence of TGF-β had any effect. These findings confirmed that regulation of α-SMA expression by PARP-1 was at the transcriptional level.

PARP-1 Enhances Complex Formation with Smad3 Binding Element in the α-SMA Promoter

TGF-β induces binding of Smad3 to the Smad3 binding consensus sequence in the α-SMA gene promoter and enhances α-SMA gene expression. To test whether PARP-1 affects this binding interaction, nuclear extracts from either WT or PARP-1–deficient mouse lung fibroblasts were analyzed by electrophoretic mobility shift assay using a probe containing the Smad3 binding consensus sequence from the α-SMA gene promoter. Four major complexes were detectable with nuclear extracts from WT and PARP-1–deficient mouse lung fibroblasts (Figure 4A). Band intensities were weaker in the samples from deficient mice; however, when these samples were pretreated with PARP-1, band intensities were enhanced almost to the WT level. Thus, PARP-1 appears to be essential for optimal complex formation with this probe. Incubation of the probe with recombinant Smad3 resulted in a single complex (Figure 4B), and pretreatment with PARP-1 did not cause a noticeable change in band intensity, suggesting that additional factors in the nuclear extract are necessary for PARP-1–mediated enhancement of complex formation.

PARP-1 Deficiency Induces DNA Hypermethylation of α-SMA Gene CpG Islands

Previous studies have demonstrated the presence of CpG islands in the rat α-SMA gene (GenBank accession no. NC_005100.3 and no. JX430794.1), as well as showing that methylation suppressed gene expression. Similar CpG islands were identified in mouse Acta2 by sequence analysis (Supplemental Figure S1). Given that PARP-1 and Dnmt1 are associated in vivo and that ADP-ribose polymers modulate Dnmt1 activity, the effect of PARP-1 on the methylation of the α-SMA gene was examined. Genomic DNA from either PARP-1–deficient (KO) or WT murine lung fibroblasts was isolated and analyzed for methylation status of the identified α-SMA gene CpG sites in the promoter and intronic regions by DNA pyrosequencing, as described previously. The analysis indicated a greater than
twofold increase in DNA methylation status of 8/9 CpG sites identified in the α-SMA gene (Figure 5). In the majority of these sites, especially in the intronic regions, the increase was more than fourfold. These findings confirm the importance of PARP-1 in regulating α-SMA gene methylation, which is known to profoundly affect gene expression. Thus, in addition to regulation at the transcriptional level, PARP-1 may regulate myofibroblast differentiation at least in part through this epigenetic mechanism.

In Vivo Effects of PARP-1 Deficiency

The myofibroblast arises de novo at sites of tissue injury undergoing repair to play a key role in the subsequent fibrotic phase. This process is characterized by induction of α-SMA expression, which was demonstrated in vitro to be regulated in part by PARP-1 (Figures 1–5). To determine whether these in vitro effects of PARP-1 on myofibroblast differentiation have any in vivo or pathophysiological significance, we examined the effects of PARP-1 deficiency on myofibroblast differentiation in a model of lung injury and fibrosis using PARP-1 KO mice. In this model, endotracheal injection of bleomycin into WT mice causes acute lung injury, followed by significant fibrosis associated with induction of myofibroblast differentiation (as monitored by α-SMA expression).3,42 In the present study, the expected fibrosis (as measured by lung hydroxyproline content) was observed in WT mice; in KO mice, fibrosis was significantly reduced, by >50% (Figure 6A). This diminished fibrosis in response to PARP-1 deficiency was accompanied by similar reductions in lung α-SMA and type I collagen mRNA levels in KO mice (Figure 6B). The significantly diminished mRNAs for both α-SMA and type I collagen in the KO mice were also reflected in their protein levels (as analyzed by Western blotting) (Figure 6C). Finally, histopathological evaluation confirmed the reduction in fibrosis in the KO mice (Figure 7). Although lungs from WT mice showed the typical marked distortion of alveolar architecture, matrix deposition, and interstitial fibrosis, those from KO mice were largely spared, and fibrotic lesions were much smaller and less extensive.

To determine whether the reduced fibrotic response in PARP-1−deficient mice was related to suppression of myofibroblast differentiation, α-SMA expression in mouse lung fibroblasts was analyzed by Western blotting. The reduced collagen expression in PARP-1−deficient mouse lung
fibroblasts correlated with a reduction in α-SMA expression in lung, which was only slightly stimulated by bleomycin treatment in PARP-1—deficient fibroblasts (in contrast to the robust response in WT cells) (Figure 8). Thus, PARP-1 deficiency had significant in vivo consequences, manifested by reduced myofibroblast differentiation and pulmonary fibrosis as defined biochemically, by matrix gene expression, and histopathologically.

To further examine whether these findings in a rodent model could also be relevant to human disease, PARP-1 expression and PARP activity were analyzed in lung fibroblasts isolated from control subjects or from patients with IPF. The levels of PARP-1 and α-SMA mRNAs in cells from IPF patients were significantly higher than those in the control cells (Figure 9A), confirming the association between PARP-1 and myofibroblast differentiation. The elevated expression of PARP-1 in cells from IPF patients

![Figure 3](image_url)

**Figure 3** PARP-1 regulation of α-SMA gene transcription. PARP-1 cDNA plasmid, PARP-1 shRNA expression construct, or empty vector (Vector only) were transfected together with the WT rat α-SMA promoter-luciferase fusion plasmid, pGL3-α-SMAPro-Luc, into rat lung fibroblasts. Cells were treated with buffer only (None) or TGF-β and analyzed for α-SMA promoter activity. The effects of PARP-1 cDNA (A) or PARP-1 shRNA (B) on α-SMA promoter activity were determined in terms of luciferase activities. Data are expressed as means ± SEM, normalized to the respective Renilla luciferase control activity (to correct for transfection efficiency). n = 3. *P < 0.05 versus control.

![Figure 4](image_url)

**Figure 4** Binding of Smad3 to Smad3 binding element in the rat α-SMA gene. Double-stranded oligo DNA primers containing the Smad3 binding element in the rat α-SMA gene were synthesized and labeled with 32P for use in an electrophoretic mobility shift assay. Nuclear extracts (2 μg) from PARP-1—deficient (KO) or WT mouse lung fibroblasts (A) or 50 ng of recombinant Smad3 (B) were incubated with the 32P-labeled probe and then analyzed by gel electrophoresis. Nuclear extracts from PARP-1 KO cells (A) or purified Smad3 (B) were pretreated with PARP-1 enzyme without or with the PARP-1 cofactor (NAD+), or with buffer only, before addition of the probe. Samples with the PARP-1 treatment only (without nuclear extract or Smad3) were also incubated with the 32P-labeled probe, as controls. To document binding specificity, excess cold probe (100×) was added to the reaction mixture. The protein-DNA complexes formed by nuclear extract (A) or Smad3 (B) with the labeled oligo DNA were indicated by arrows.
was reflected in the level of PARP activity in fibroblasts isolated from IPF patients, which was more than threefold higher than that in fibroblasts isolated from control subjects (Figure 9B). The specificity of the assay was confirmed by susceptibility to inhibition by 3-AB.

To confirm induction and localization of PARP-1 expression in vivo in IPF, lung tissue sections from control and IPF patients were immunostained with antibodies to PARP-1 and α-SMA. This approach revealed induction of PARP-1 nuclear staining in cells within fibroblastic foci in IPF lung sections (Figure 9C). The PARP-1 nuclear staining colocalized with cells with cytoplasmic α-SMA staining, consistent with induction of PARP-1 in myofibroblasts in these fibroblastic foci. Control lung sections showed normal alveolar architecture without detectable staining for PARP-1, whereas α-SMA staining was detectable in smooth muscle cells of the vasculature and airways (Figure 9C). These findings confirmed the in vitro studies using isolated lung fibroblasts, suggesting the potential importance of PARP-1 in genesis of the myofibroblast in IPF.

**Discussion**

PARP-1 is widely involved in the regulation of DNA repair, DNA replication, modulation of chromatin structure, and apoptosis. All of these processes are of potential importance in development and cell differentiation. Myofibroblasts are also important in development, cancer, tissue repair, remodeling, and fibrosis. Myofibroblasts commonly arise from tissue fibroblasts, although other precursor cells have also been identified. Differentiation from these precursor cells is manifested by induction of α-SMA expression and its incorporation into stress fibers. Extensive studies have revealed a complex differentiation mechanism involving diverse signaling pathways, transcription, and epigenetic factors. Although understanding of this mechanism remains incomplete, it is of considerable interest, given the importance of the myofibroblast in...
development, tissue fibrosis, and cancer.1–4,6,7 In the present study, we hypothesized that PARP-1 may regulate α-SMA gene expression. This hypothesis was first tested in vitro, to see whether inhibition of PARP activity could affect myofibroblast differentiation in isolated rodent lung fibroblasts. The inhibitor 3-AB had a significant inhibitory effect on α-SMA expression, and thus also on myofibroblast differentiation. This effect was confirmed to be due to PARP-1, because induced overexpression using a PARP-1 expression plasmid stimulated α-SMA expression but suppression by transfection of PARP-1 shRNA caused a significant reduction in α-SMA expression. Use of an α-SMA promoter-reporter gene construct revealed that these effects on both fibroblast PARP-1 mRNA and protein levels were due to regulation at the transcriptional level. This is consistent with previous reports that poly(ADP-ribose) can modulate gene transcription by interfering directly with transcription factors and cofactors. For example, PARP-1 interacts with and stimulates NF-κB in the absence of poly(ADP-ribose) synthesis.44–46 NF-κB is a transcription factor with regulatory activity on myofibroblast differentiation.47–49 PARP-1 also binds specifically to both TEF-1 and the flanking sequences of the MCAT1 element.50 PARP-1 communoprecipitates with TEF-1 from muscle nuclear extracts and can ribosylate TEF-1 in vitro (ADP ribosylation).50 Inhibition of PARP enzymatic activity represses expression of an MCAT1-dependent promoter.50 Two MCAT elements are also found in the α-SMA gene promoter.51,52 Interestingly, their interaction with TEF-1 regulates the transcription of the α-SMA gene in a cell-specific manner.51,52 Mutations of MCAT elements selectively abolish the α-SMA transcriptional activity in myofibroblasts within granulation tissue of skin wounds.52

Another key regulator of α-SMA gene transcription is Smad3, a key factor of the TGF-β signaling pathway. Electrophoretic mobility shift assays revealed that complex formation with a probe containing the Smad3 binding consensus sequence or element (SBE) was reduced when PARP-1 was deficient in the nuclear extract sample. This reduction was reversed by preincubation of the nuclear extract with PARP-1, thus indicating that PARP-1 was essential for optimal complex formation with the SBE. However, PARP-1 pretreatment of recombinant Smad3 did not appear to affect its binding to the SBE significantly. A possible explanation is that modification of Smad3 by PARP-1 requires phosphorylation of Smad3, which would

---

**Figure 7** Effect of PARP-1 deficiency on histopathology. PARP-1−deficient and WT mice were treated with bleomycin to induce lung injury or with saline as a control. On day 21 after treatment, lung tissue sections were obtained and stained with Masson's trichrome. Representative sections from WT mice treated with saline (A and E) or bleomycin (B and F) and from PARP-1 KO mice treated with saline (C and G) or bleomycin (D and H) show reduced fibrosis in the KO mice. Original magnification, ×100 (A–D); ×400 (E–H).

**Figure 8** Effect of PARP-1 deficiency on lung fibroblasts. A: Lung fibroblasts isolated from either saline- or bleomycin-treated WT or PARP-1−deficient mice (7 days after bleomycin treatment) were analyzed for α-SMA and α1(I) procollagen proteins by Western blotting. Each lane represents a sample from a single animal. GAPDH was used as a loading control, after stripping of the membrane. B: Relative density of the bands was quantified from three independent experiments, normalized to the density of corresponding GAPDH bands. Data are expressed as means ± SEM. n = 3. *P < 0.05.

---

The American Journal of Pathology ■ ajp.amjpathol.org
not occur in the absence of the requisite kinase in the setting of the experiment using the recombinant Smad3. This possibility was suggested in a study in smooth muscle cells, in which a similar effect of PARP-1 on Smad3 modification and binding to the SBE occurred only with phosphorylated Smad3. In contrast to the recombinant Smad3 experiment, the nuclear extract samples likely contained phosphorylated Smad3, because of endogenous TGF-β signaling; thus, PARP-1 should be able to interact with this phosphorylated form of Smad3. Alternatively, other factors present in the nuclear extract may be necessary for PARP-1 interaction or regulation of Smad3 binding to the SBE. The modification of Smad3 by PARP-1 and its functional effects appear to be cell specific. Thus, in smooth muscle cells and fibroblasts in the present study, PARP-1 was found to enhance Smad3 binding and activate its target gene, which is in contrast to the attenuation by PARP-1 of the TGF-β signaling in HaCaT keratinocytes by blunting Smad3 binding to the SBE. In the latter study, inhibition of epithelial-mesenchymal transition by PARP-1 was also noted; however, this finding is contradicted by another study showing that PARP-1 is essential for epithelial-mesenchymal transition. Nevertheless, it appears that, at least in mesenchymal cells capable of z-SMA expression, PARP-1 functions as an activator of z-SMA gene transcription.

In addition to regulation of transcription factors, PARP-1 is involved in modulation of chromatin structure and epigenetic control. PARylation of histone by PARP-1 causes relaxation of chromatin superstructure and dissociation of histone-DNA complexes, which increases the accessibility of the trans-acting factors and RNA polymerase to DNA to enable gene transcription. Moreover PARP-1 associates with DNA methyltransferases 1 and modulates its activity. Recent reports suggest that DNA methylation may play significant roles in pulmonary fibrosis. The present results indicate that PARP-1 deficiency in cells from PARP-1 KO mice resulted in hypermethylation of the z-SMA gene, as shown by quantitative DNA pyrosequencing, and thus provide direct evidence for the first time of the importance of PARP-1 in the regulation of DNA methylation.

Increased DNA methylation due to overexpression of DNA methyltransferase leads to suppression of z-SMA gene expression, whereas inhibition of DNA methyltransferase activity or knockdown of DNA methyltransferase causes significant induction of z-SMA in fibroblasts. In the present study hypermethylation of the z-SMA gene was observed in PARP-1−/− deficient cells, which correlated with significantly reduced z-SMA gene expression. Thus, our data suggest that PARP-1 has a significant role in regulation of myofibroblast differentiation at the transcriptional and epigenetic levels. It remains unclear, however, whether the transcriptional regulation is via direct mechanisms, in which PARP-1 itself is interfering with the binding or activity of transcription factors that regulate the z-SMA promoter, and/or via more distant mechanisms involving upstream signaling pathways targeting both the z-SMA gene and other
PARP-1 Activates z-SMA Gene Expression

differe...
poly(ADP-ribose) polymerase-1 prevents lung inflammation in a murine model of asthma. Am J Respir Cell Mol Biol 2003, 28:322–329


