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Regulation of Lung Injury and Fibrosis by p53-Mediated Changes in Urokinase and Plasminogen Activator Inhibitor-1

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Alveolar type II epithelial cell (ATII) apoptosis and proliferation of mesenchymal cells are the hallmarks of idiopathic pulmonary fibrosis, a devastating disease of unknown cause characterized by alveolar epithelial injury and progressive fibrosis. We used a mouse model of bleomycin (BLM)—induced lung injury to understand the involvement of p53-mediated changes in urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) levels in the regulation of alveolar epithelial injury. We found marked induction of p53 in ATII cells from mice exposed to BLM. Transgenic mice expressing transcriptionally inactive dominant negative p53 in ATII cells showed augmented apoptosis, whereas those deficient in p53 resisted BLM-induced ATII cell apoptosis. Inhibition of p53 transcription failed to suppress PAI-1 or induce uPA mRNA in BLM-treated ATII cells. ATII cells from mice with BLM injury showed augmented binding of p53 to uPA, uPA receptor (uPAR), and PAI-1 mRNA. p53-binding sequences from uPA, uPAR, and PAI-1 mRNA 3′ untranslated regions neither interfered with p53 DNA binding activity nor p53-mediated promoter transactivation. However, increased expression of p53-binding sequences from uPA, uPAR, and PAI-1 mRNA 3′ untranslated regions in ATII cells suppressed PAI-1 and induced uPA after BLM treatment, leading to inhibition of ATII cell apoptosis and pulmonary fibrosis. Our findings indicate that disruption of p53—fibrinolytic system cross talk may serve as a novel intervention strategy to prevent lung injury and pulmonary fibrosis. *(Am J Pathol 2013, 183: 131—143; http://dx.doi.org/10.1016/j.ajpath.2013.03.022)*

Idiopathic pulmonary fibrosis is a progressive and fatal lung disease that is refractory to current therapy. A better understanding of the underlying mechanisms is necessary for development of novel treatments. Dysregulated fibrinolysis and induction of p53 are often associated with lung injury and precede development of pulmonary fibrosis.[1] These changes occur in a mouse model of bleomycin (BLM)—induced lung injury and accelerated pulmonary fibrosis.[1] p53 Expression increases substantially in type II alveolar epithelial (ATII) cells after BLM- or cigarette smoke—induced lung injury,[1–3] in association with induction of plasminogen activator inhibitor-1 (PAI-1) and suppression of urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR) expression.

We have previously reported that p53 binds to 35-, 37-, and 70-nucleotide sequences within the 3′ untranslated region (UTR) of uPA, uPAR, and PAI-1 mRNAs.[4–6] Insertion of p53-binding sequences into the 3′ UTR of β-globin mRNA destabilizes this otherwise stable transcript,[4–6] which indicates that these sequences affect mRNA stability. Subsequent studies by other groups have demonstrated that p53 induction of PAI-1 expression requires p53 serine phosphorylation,[7] which facilitates interaction of the C-terminal domain with the PAI-1 3′ UTR. We found that the 3′ UTR binding sequences can independently compete with endogenous uPA, uPAR, or PAI-1 mRNA for binding to p53

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protein, leading to induction of uPA and uPAR[4,5] and attenuation of PAI-1 levels.[6] We also found that mice deficient in p53 or PAI-1 resist lung injury induced by BLM or cigarette smoke, whereas mice deficient in uPA remain highly susceptible to BLM-induced lung injury and pulmonary fibrosis.[1] Therefore, we hypothesized that simultaneous inhibition of p53 binding to uPA, uPAR, and PAI-1 mRNAs would reduce PAI-1 induction and restore uPA expression in ATII cells despite elevated p53 levels. These changes in PAI-1 and uPA levels are expected to reduce apoptosis of ATII cells and development of pulmonary fibrosis after BLM-induced lung injury.

In the present study, we tested our hypothesis using lentiviral constructs expressing p53-binding sequences from 3′ UTRs of uPA, uPAR, and PAI-1 mRNAs under the control of lung surfactant protein (SP)—B promoter. SP-B is expressed only in ATII and bronchiolar (Clara) epithelial cells in the lungs[8,9] and *SP-B* promoter directs transgene expression in ATII and Clara epithelial cells in mice.[10] Recombinant lentivirus was administered in mice before or after initiation of BLM-induced lung injury, and effects on uPA and PAI-1 levels, ATII cell apoptosis, and indices of lung fibrosis were determined. Results demonstrated that overexpression of p53-binding sequence reduced PAI-1 but augmented uPA expression. More important, BLM-induced ATII cell apoptosis and development of pulmonary fibrosis were reduced without inhibition of p53 expression in the lungs. Overall, our findings demonstrated for the first time that the increased interactions of p53 with the 3′ UTR of uPA, uPAR, and PAI-1 mRNAs contribute to lung injury and remodeling and that disrupting of these interactions reverses lung injury and prevents development of BLM-induced pulmonary fibrosis.

Materials and Methods

Cell Culture

ATII cells isolated from the mouse lungs were treated with 40 μg/mL BLM for 0 to 28 hours in ATII culture medium (ScienCell Research Laboratories, Carlsbad, CA). 293T cells were obtained from ATCC (Manassas, VA).

Construction of Recombinant Lentiviral Vectors

A chimeric cDNA containing the 35-, 37-, and 70-nucleotide p53-binding regions from the 3′ UTR of uPA, uPAR, and PAI-1 mRNAs[4–6] was synthesized. The p53-binding cDNA chimera was 5′-TAAACCTGAAATCCCCCTCTCTGCCCTGGCTGGATCCCTCTACGTACGACCTGTGACCAGCACTGTCTCAGTTTCACTTTCACTCTACGTACGCGTGCCCAGCTCTTCACCCCCCAATCTCTTGGTGGGGAGGGGTGTACCTAAATATTTATCATATCCTTG-3′. A cDNA fragment devoid of p53-binding sites was also synthesized and used as control. The non-binding cDNA control was 5′-ACCCCTTTGCCCTTCCCTCGGCTCCCAGCCCTACAGACTCTACGTACAGCATGTTCATTGCTGCCCCTTATGAAAAAGAGCTCTACGTACTCATCATCAATGACTGGGTGAAGACACACACAAAAGGTATGATCAGCAACTTGCTTGGGAAAGGAGCCGT-3′. In both cases, the binding or non-binding regions were separated by a 10-nucleotide unrelated sequence (underlined). Each of these cDNA sequences were ligated to a lentiviral vector DNA downstream of the human SP-B 5′ flanking DNA (−911/+41).[11] The lentiviral vector constructs were transfected into 293T cells using Invitrogen Lipofectamine 2000 (Life Technologies Corp., Grand Island, NY) to obtain phage particles and viral titers, measured per the manufacturer's protocol. In as much as expression of the SP-B promoter is cell-specific in ATII and Clara cells in the lungs, these constructs were used to express the chimeric mRNAs in ATII cells *in vivo*. The vector containing the SP-B 5′ flanking DNA linked to the luciferase gene was used in selected experiments to determine the cell-specific expression of luciferase.

Electrophoretic Mobility Shift Assay

Total cell extracts from H1299 cells stably expressing p53 were prepared according to published methods.[4–6] The electrophoretic mobility shift assay was performed as described previously.[12] In brief, protein-DNA complexes were formed by incubating 20-μg cell extracts with a phosphorus 32—labeled consensus p53-binding site oligonucleotide (5′-TACAGAACATGTCTAAGCATGCTGGGGACT-3′ 30 mer) in the presence or absence of a molar excess of an unlabeled oligonucleotide as competitor and resolved using polyacrylamide gel electrophorosis under nondenaturing conditions. Gels were exposed to X-ray film to obtain autoradiographs.

Luciferase Assay

H1299 cells stably transfected with pcDNA3.1 containing p53 cDNA or pcDNA3.1 alone were transiently transfected with a p53-luciferase reporter plasmid (PG13-luc; Addgene, Inc., Cambridge, MA) or plasmids expressing p53-binding or non-binding control 3′ UTR sequences (pReceiver; GeneCopoeia, Inc., Rockville, MD) using Lipofectamine 2000 (Life Technologies Corp., Grand Island, NY). After overnight incubation, luciferase activities of cell lysates were determined via chemiluminescent assay (Promega Corp., Madison, WI) and normalized to protein content of cell lysates.

Mice

Wild-type (WT) and p53-, uPA-, and PAI-1—deficient mice of C57BL-6 background were either bred at the University of Texas Health Science Center at Tyler or purchased from The Jackson Laboratory (Bar Harbor, ME). Antibodies to uPA, PAI-1, p53, cleaved and total caspase-3, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Model of BLM-Induced Lung Injury in Mice and Analysis of Lung Tissues

All animal experiments were approved by our Institutional Animal Care and Use Committee. Six-week-old WT and p53-, uPA-, and PAI-1−deficient female mice were used in the experiments. Lentiviruses were administered to anesthetized mice ($n = 7$) via the retro-orbital (i.v.) or intratracheal (i.t.) route, and 24 hours later BLM was administered via intranasal insufflation.[1,13,14] Mice were sacrificed on day 3 after BLM treatment, and lungs were inflated via i.t. instillation of Excell Plus solution (American MasterTech Scientific, Inc., Lodi, CA) at a constant pressure of 20 cm H_2O. Lung sections, 5 μm, from three animals in each group were subjected to TUNEL and H&E staining. Lung sections of mice sacrificed on day 21 were stained with Masson's trichrome for detection of matrix proteins. In some experiments, mice were administered lentivirus at 3 days after BLM injury, and their lungs were evaluated for changes in pulmonary fibrosis 21 days later. Lung sections from BLM-treated SP-C−dominant negative p53 (DNp53) mice and nontransgenic littermates were prepared on day 7 after BLM treatment.[15]

Isolation of ATII Cells from Mouse Lungs

Mouse ATII cells were isolated according to the method described previously.[1,2,16] ATII cell purity was confirmed via lithium carbonate staining.[2]

Preparation of ATII Cell Lysates and Western Immunoblotting

ATII cell lysates were prepared as described previously.[17] The proteins were separated using SDS-polyacrylamide gel electrophorosis on 10% gels and electroblotted to a nitrocellulose membrane. After blocking with 1% Tris-buffered saline−Tween 20 buffer, membrane was incubated with primary antibodies at 1:1000 dilutions at 4°C overnight, followed by reaction with goat anti-rabbit horseradish peroxidase−conjugated secondary antibody at 1:1000 dilution for 1 hour at room temperature. Protein bands were visualized using the enhanced chemiluminescence detection method.

Hydroxyproline Assay

Hydroxyproline content of whole mouse lungs was determined according to a previously described method[18] after minor modifications. In brief, lung homogenates were hydrolyzed in 6N HCl at 100°C for 24 hours. Hydrolysates were mixed with an equal volume of citrate/acetate buffer (pH 6.0) and 100 μL chloramine-T solution in a 96-well plate. The mixture was incubated for 20 minutes at room temperature; then 100 μL Ehrlich's solution was added, and incubation was continued at 65°C for 18 minutes. Absorbance at 550 nm was measured, and hydroxyproline content was determined from a standard curve.

Immunohistochemistry

Lungs were fixed under inflation as described in model of BLM-Induced lung injury in mice and analaysis of lung tissues, and 5-μm sections were deparaffinized using xylene, followed by incubation with 100% and 95% alcohol. Sections were then subjected to antigen retrieval via incubation in 10 mmol/L sodium citrate buffer (pH 6.0) at 95°C for 30 minutes and were incubated with hydrogen peroxide for 30 minutes to quench endogenous peroxidase. Sections were incubated overnight with antibodies or rabbit IgG,

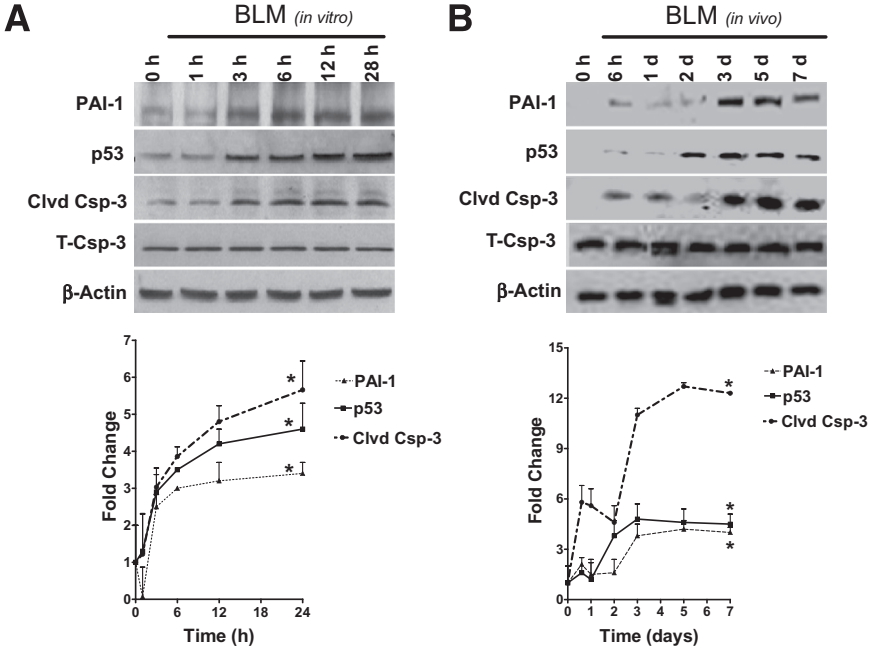

Figure 1 Effects of BLM treatment on temporal changes in the levels of PAI-1, p53, and apoptosis in ATII cells. Levels of PAI-1, p53, active caspase-3 (Clvd Csp-3), and total caspase-3 (T-Csp-3) to assess apoptosis in ATII cells treated *in vitro* with BLM for up to 28 hours (**A**) or in ATII cells isolated from mice that received BLM via intranasal instillation for up to 7 days (**B**) were analyzed via Western blotting. β-Actin levels were determined to correct for loading differences. Data shown in line graphs are means ± SD of three independent experiments. Differences between treatments are statistically significant ($*P < 0.05$).

which served as negative control, in PBS containing 0.1% Tween 20, and were processed for antigen detection (Lab Vision Corp., Fremont, CA).

Immunofluorescence Assay

Mouse lung sections, 5 μm, were deparaffinized using xylene, followed by incubation with 100% and 95% alcohol, and then were subjected to antigen retrieval via incubation in 10 mmol/L sodium citrate buffer (pH 6.0) at 95°C for 10 minutes. The sections were incubated overnight with respective antibodies or rabbit IgG, as negative control, in antibody dilution buffer. The sections were then incubated with fluorochrome-conjugated secondary antibody diluted in antibody dilution buffer. The lung sections were then examined via fluorescent microscopy to visualize ATII cell apoptosis.

Statistical Analysis

Statistical significance between two groups was analyzed using Student's *t*-test, and for multiple groups using one-way analysis of variance.

Results

Induction of p53 Expression by ATII Cells During Lung Injury

Increased alveolar epithelial damage and disordered fibrin turnover are commonly associated with fibrosing lung injury. We exposed ATII cells to BLM and analyzed changes in p53 levels, caspase-3 activation, and PAI-1 expression as indicators of ATII cell death and disruption of alveolar fibrinolysis. Exposure of ATII cells to BLM increased p53 and PAI-1 expression in a time-dependent manner, with maximum effect at 3 to 28 hours after exposure (Figure 1A). These changes were associated with increased apoptosis, as evident from the temporal accumulation of active or cleaved caspase-3. Similar results were also found *in vivo* (Figure 1B), with maximum induction of p53 and PAI-1 expression by ATII cells between 2 and 5 days after BLM-induced lung injury in mice. These results demonstrated that induction of p53 is associated with caspase-3 activation, which suggests links between p53, PAI-1 expression, and ATII cell apoptosis.

Because p53 controls the expression of both proapoptotic and antiapoptotic proteins to induce programmed cell death during injury, we examined the contribution of p53 to apoptosis resulting from BLM-induced lung injury. Transgenic mice expressing a transcriptionally inert DNp53 protein in ATII cells[15] were exposed to BLM, and lung sections were analyzed for apoptosis and p53 via TUNEL and immunohistochemical staining. Mice expressing DNp53 showed elevated levels of ATII cell apoptosis (Figure 2, A and B). SP-C—DNp53 mice also showed increased BLM-induced pulmonary fibrosis 2 weeks later, as reported previously.[15] In sharp contrast, compared with WT mice, p53-deficient mice exhibited minimal ATII cell apoptosis after BLM treatment.

We have previously found that although p53 induced PAI-1, it inhibited uPA and uPAR expression.[1,4–6] Therefore, it was of interest to determine whether transcriptional

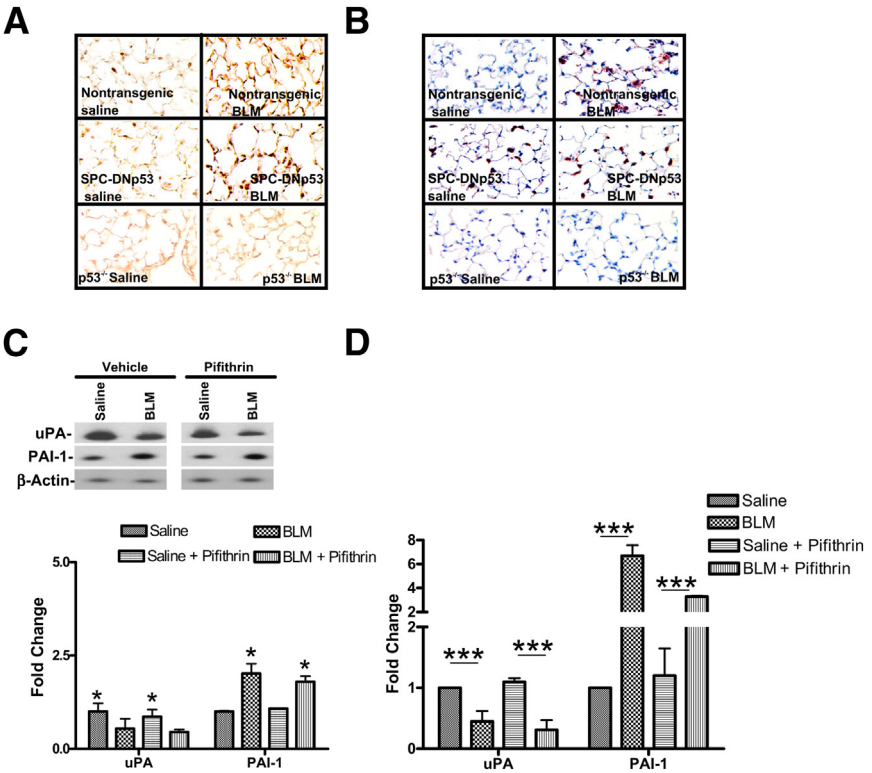

Figure 2 Role of p53 in ATII cell apoptosis in mice with BLM-induced injury. Lung sections from WT transgenic mice expressing DNp53 in ATII cells and p53-deficient mice treated with or without BLM were subjected to TUNEL staining to assess apoptosis (**A**) or to immunohistochemical staining for p53 (**B**). **C**: ATII cells isolated from WT mice were exposed to saline solution or BLM with or without pifithrin-α for 24 hours *in vitro*. Total RNA isolated from these cells was analyzed for uPA and PAI-1 mRNA via reverse transcription-PCR in the presence of ^{32}P-labeled deoxycytidine triphosphate. ^{32}P-labeled PCR amplicons were separated on urea or polyacrylamide gel and exposed to X-ray film. **D**: Total RNA was subjected to quantitative real-time PCR for uPA and PAI-1 mRNA after normalization with β-actin mRNA from the same sample. **C** and **D**: Shaded columns represent mean densities after normalization with corresponding densities of β-actin mRNA. Experiments were repeated at least three times. Differences between treatments are statistically significant ($^*P < 0.05$, $^{***}P < 0.005$).

mechanisms are involved in p53 regulation of PAI-1 and uPA. The effects of pifithrin-α, an inhibitor of p53-dependent transcription, on BLM-induced changes in uPA and PAI-1 mRNA in ATII cells were investigated. BLM treatment reduced mRNA levels of uPA, whereas it induced PAI-1 mRNA (Figure 2, C and D). However, pifithrin-α had no effect on uPA and PAI-1 levels, indicating that p53 alters their expression at the posttranscriptional level. Collectively, these data implicate elevated p53 levels on the changes in uPA and PAI-1 mRNA expression in BLM-induced lung injury and strongly suggest that the effect is independent of p53 transcriptional activity.

Specificity of p53-uPA and p53-PAI-1 3′ UTR mRNA Interaction

In as much as p53, through its C-terminal region, can concurrently bind to the regulatory elements in the 3′ UTRs of uPA,[4] uPAR,[5] and PAI-1[6] mRNAs, disrupting these interactions would be expected to simultaneously prevent p53-dependent inhibition of uPA and induction of PAI-1 expression. We further speculated that targeting these interactions simultaneously could provide better protection against ATII cell apoptosis. To investigate p53 interaction with uPA, uPAR, and PAI-1 mRNAs, we analyzed binding of recombinant p53 protein to synthetic mRNA fragments containing p53-binding sequences from the 3′ UTRs of uPA and PAI-1[4–6] via gel mobility shift assay. As shown in Figure 3, recombinant p53 protein formed a specific complex that was abolished by a 200-fold molar excess of the appropriate unlabeled 3′ UTR sequence or unlabeled chimeric mRNA containing uPA, uPAR, and PAI-1 3′ UTR sequences but not the control sequence (Figure 3, B and C). Interaction of p53 with the 3′ UTRs of uPA, uPAR, and PAI-1 is further supported by modeling studies that indicated that p53-binding regions in the 3′ UTR of uPA, uPAR, and PAI-1 mRNAs form a stable hairpin structure capable of binding the p53 C-terminal domain with nanomolar affinity (Figure 4).

p53 acts at transcriptional[19,20] and posttranscriptional[4-6] levels to control gene expression. Our results demonstrated that increased expression of p53-binding sequences from uPA, uPAR, and PAI-1 3′ UTRs reduced BLM-induced lung fibrosis. To determine whether the observed effects are due to interference with p53-binding to its target promoters, we analyzed the effects of uPA, uPAR, and PAI-1 3′ UTRs on p53 binding to its promoter binding sequence and p53 promoter activity. Using cell extracts from H1299 cells ($p53^{-/-}$) stably transfected with empty plasmid or p53 cDNA, we found that p53 DNA binding activity was unaffected by excess amounts of 3′ UTR p53-binding sequence (Figure 5, C and D), indicating that the 3′ UTR p53-binding sequence does not interfere with p53 DNA binding activity. In agreement with these results, we found that expression of a 3′ UTR p53-binding sequence had no effect on p53 promoter activity (Figure 5E). As expected, the control sequence had no effect on p53 promoter activity. Cells transfected with vector alone expressed negligible levels of luciferase activity, whereas those expressing p53 cDNA expressed luciferase at greater than 10-fold.

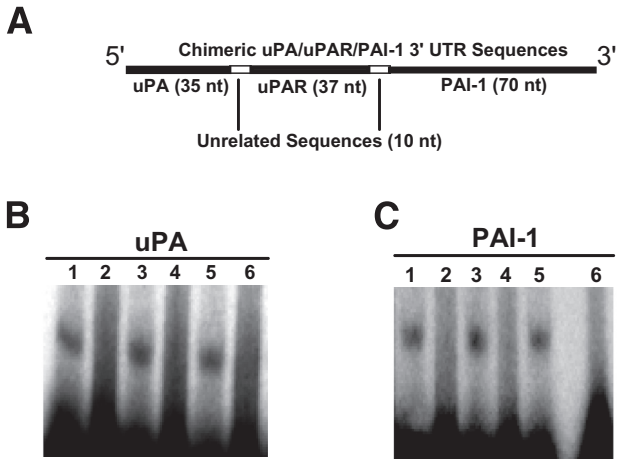

Figure 3 Inhibition of p53 binding to uPA and PAI-1 mRNA 3′ UTR by chimeric uPA/uPAR)/PAI-1 3′ UTR sequences. **A:** Schematic diagram shows p53-binding chimeric uPA/uPAR/PAI-1 mRNA 3′ UTR (**solid lines**) separated by unrelated 10-nucleotide (nt) regions (**open lines**). **B:** Purified recombinant p53 protein (purity >95%)[4–6] was incubated with ^{32}P-labeled uPA 3′ UTR sequences in the absence (**lane 1**) or presence of a 200-fold molar excess of unlabeled p53-binding 35-nt uPA 3′ UTR sequence (**lane 2**), 35-nt non—p53-binding control sequence (**lane 3**), chimeric p53-binding uPA/uPAR/PAI-1 3′ UTR sequence (**lane 4**), or chimeric non—p53-binding control uPA/uPAR/PAI-1 3′ UTR sequence (**lane 5**). The reaction mixtures were digested with RNAse T1 and heparin and subjected to gel mobility shift assay. The reaction mixture contains all of the reagents except recombinant p53 protein (**lane 6**). **C:** Recombinant p53 protein was incubated with ^{32}P-labeled PAI-1 mRNA 3′ UTR sequences in the absence (**lane 1**) or presence of a 200-fold molar excess of unlabeled p53-binding 70-nt PAI-1 3′ UTR sequence (**lane 2**), 70-nt non—p53-binding control sequence (**lane 3**), chimeric p53-binding uPA/uPAR/PAI-1 3′ UTR sequence (**lane 4**), or chimeric non—p53-binding control uPA/uPAR/PAI-1 3′ UTR sequence (**lane 5**). The reaction mixtures were digested with RNAse T1 and heparin and subjected to gel mobility shift assay. The reaction mixture contains all of the reagents except recombinant p53 protein (**lane 6**).

Chimeric p53 mRNA Binding Sequence Reverses BLM-Induced Expression of uPA and PAI-1 *in Vivo*

Results of both gel mobility shift assay (Figures 3 and 5) and modeling studies (Figure 4) indicated that p53 specifically interacts with the 3′ UTRs of the uPA and PAI-1 mRNAs. Therefore, we investigated whether blocking p53 binding to uPA and PAI-1 transcripts in vivo would alter the expression of uPA and PAI-1 during BLM injury. Lentiviral vectors containing cDNA encoding p53-binding or non-binding sequences driven by the *SP-B* promoter were generated (see *Materials and Methods*). Mice were administered lentivirus via intraorbital plexus and subjected to BLM treatment. In separate experiments, we assessed ATII cell—specific expression by analyzing luciferase expression in mice administered lentivirus containing luciferase cDNA driven by the *SP-B* promoter. Both lung sections as well as

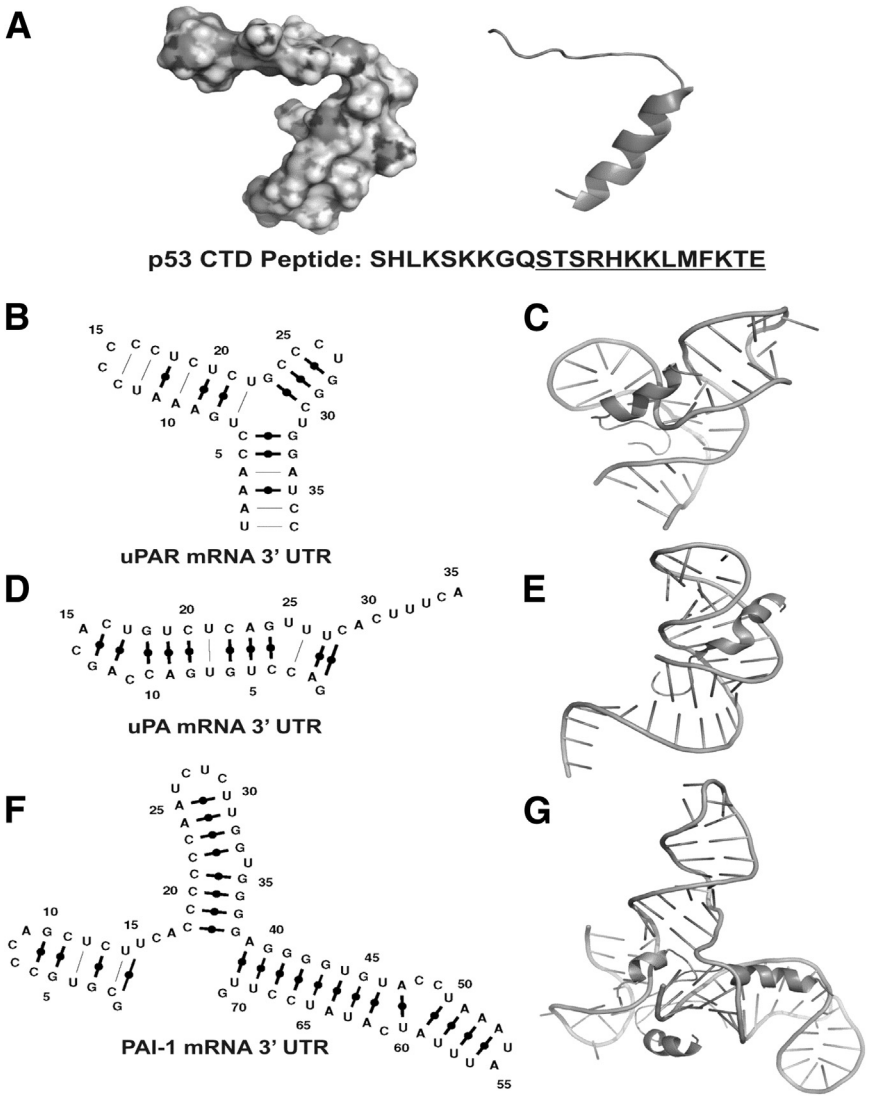

Figure 4 Molecular modeling of the p53 C-terminal domain binding to 3′ UTR of uPA/uPAR/PAI-1 mRNAs. **A:** The published 3D structure of the C-terminal domain of p53 (Protein Data Bank code 1DT7) is shown as a rendered surface (**left**) or ribbon (**right**). The amino acid sequence is indicated with the α-helical portion underlined. The C-terminal domain peptide was docked to the rendered 3D models of the identified UTRs for uPAR (**B** and **C**), uPA (**D** and **E**), and PAI-1 (**F** and **G**). Three different C-terminal domain docking configurations of similar binding energy were identified for the PAI-1 RNA (red, green, and magenta). In all cases, the 2D RNA structures with the lowest energy were first predicted using MC-Fold (**B**, **D**, and **F**). These folded structures were then submitted to MC-Sym (*http://www.major.iric.ca/MC-Pipeline*) to generate the 3D structures with the lowest energy (**C**, **E**, and **G**), which were used in docking procedures with the C-terminal domain peptide using Autodock Vina (Scripps Research Institute, La Jolla, CA). The web-based MC-Fold and MC-Sym pipeline was provided by the Institute for Research in Immunology and Cancer (University of Montreal, Montreal, QC, Canada).

isolated ATII cells from mice administered recombinant lentivirus showed increases in luciferase protein level and activity, indicating successful expression of luciferase in ATII cells (Figure 6, B–D).

Compared with a saline-treated control, mice exposed to BLM showed a reduction in ATII cell uPA transcripts and an increase in PAI-1 transcript (Figure 6E). Expression of chimeric p53-binding sequence, but not control sequence, reversed the effects of BLM to induce PAI-1 and suppress uPA mRNA levels. We next examined whether expression of chimeric p53 mRNA binding sequence inhibits BLM-induced p53 binding to endogenous uPA, uPAR, and PAI-1 transcripts. The binding was analyzed by determining the levels of uPA, uPAR, and PAI-1 transcripts using reverse transcription-PCR in co-immunoprecipitation experiments using anti-p53 antibody. As expected, ATII cells from mice exposed to BLM yielded higher levels of uPA, uPAR, and PAI-1 transcripts in p53-immune complexes when compared with ATII cells from saline-treated controls. However, expression of the chimeric p53-binding sequences significantly reduced levels of these transcripts in ATII cells, whereas expression of a non–p53-binding sequence had no effect (Figure 6F).

Increased ATII cell apoptosis is associated with higher levels of p53 and PAI-1 and lower levels of uPA in lung tissues of patients with diffuse alveolar damage,[1] which suggests links between p53, PAI-1, and uPA levels and control of ATII cell apoptosis. To determine the importance of altered p53 expression in human lung epithelial cell injury, we determined the effects of overexpression of p53-binding sequence on BLM regulation of PAI-1 and uPA levels and apoptosis in H441 lung epithelial cells. We found that BLM induced p53 and PAI-1 expression but inhibited uPA expression, with concomitant activation of caspase-3 (Figure 7A). However, overexpression of p53-binding sequence reversed BLM-induced PAI-1 expression and inhibition of uPA and prevented H441 cell apoptosis without affecting BLM-induced p53 levels (Figure 7A). We next determined the effects of BLM treatment on apoptosis of ATII cells and changes in p53, uPA, PAI-1, and cleaved caspase-3 levels in mice expressing control or chimeric p53

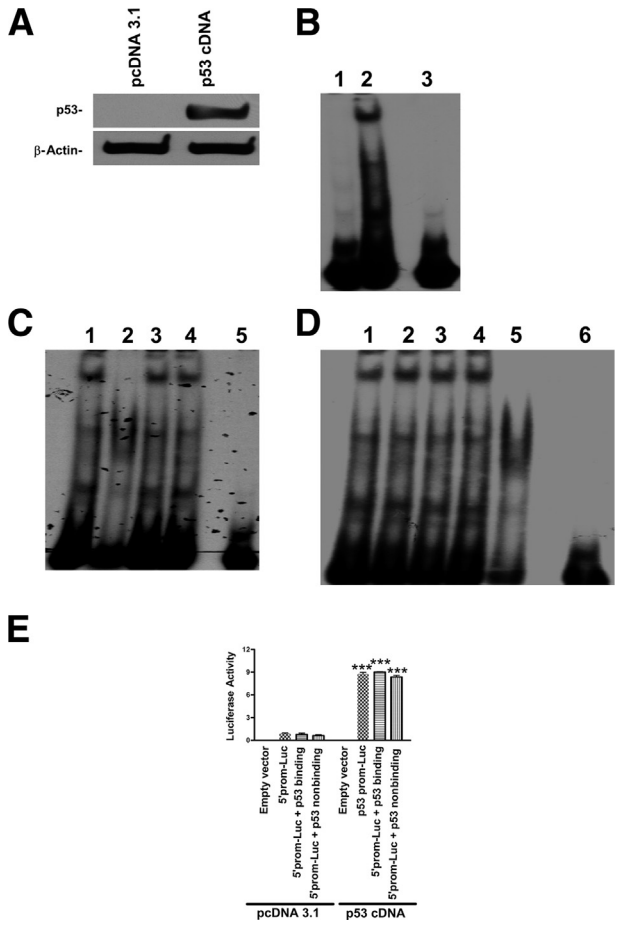

Figure 5 Effect of chimeric uPA/uPAR/PAI-1 3′ UTR sequences on promoter DNA consensus sequence binding and promoter activation by p53 protein. **A:** Stable H1299 cells expressing vector pcDNA3.1 alone or p53 cDNA in pcDNA3.1 were immunoblotted for p53 and β-actin proteins. **B:** Protein extracts (20 μg per lane) from stable H1299 cells expressing pcDNA3.1 (**lane 1**) or p53 cDNA in pcDNA3.1 (**lane 2**) were subjected to gel mobility shift assay using ^{32}P-labeled p53 promoter DNA consensus sequence. Free probe reaction mixture lacking protein extracts was incubated with ^{32}P-labeled p53 promoter DNA consensus sequence (**lane 3**). The reaction mixtures were separated via electrophoresis on a 5% native polyacrylamide gel with 0.25× Tris-borate-EDTA running buffer and autoradiographed. **C:** Protein extracts containing recombinant p53 protein (isolated from H1299 cells stably expressing p53 cDNA) were incubated with 1×10^5 cpm ^{32}P-labeled p53 promoter DNA consensus sequence in the absence (**lane 1**) or presence of 50-fold excess of unlabeled p53 consensus sequence (**lane 2**), 50-fold excess chimeric p53-binding sequence (**lane 3**), or chimeric non−p53-binding (**lane 4**) uPA/uPAR/PAI-1 3′ UTR sequences or Free probe (**lane 5**). The p53 protein and ^{32}P-labeled promoter DNA complexes were separated on a 5% native polyacrylamide gel and autoradiographed. **D:** H1299 cell extracts containing 20 μg recombinant protein were incubated with 1×10^5 cpm ^{32}P-labeled p53 promoter DNA consensus sequence in the absence (**lane 1**) or presence (**lanes 2, 3,** and **4**, respectively) of 100-, 200-, or 300-fold excess of unlabeled chimeric p53-binding 3′ UTR sequences, 50-fold excess of unlabeled p53 consensus sequence (**lane 5**), or Free probe (**lane 6**). Samples were subjected to gel mobility shift assay and autoradiographed. **E:** Stable H1299 cells expressing pcDNA3.1 vector control or p53 cDNA in pcDNA3.1 were transiently transfected with p53-binding promoter sequence construct containing a luciferase reporter gene (5′prom-Luc) alone or 5′prom-Luc co-transfected with chimeric p53-binding or non−p53-binding control sequences overnight in serum medium. These cells were lyzed in lysis buffer, and luciferase activity was measured using a chemiluminescence assay. Differences between treatments are statistically significant (***$P < 0.0005$) versus vector control.

mRNA binding sequences. BLM augmented p53 and PAI-1 protein levels, with a concurrent reduction in uPA protein in ATII cells (Figure 7B). Activation of caspase-3 was also observed, demonstrating ATII cell apoptosis. In sharp contrast, ATII cells isolated from mice expressing the chimeric p53-binding sequence exhibited abrogated responses to BLM; namely, uPA remained at normal levels, compared with saline control; caspase-3 activation was completely suppressed; and PAI-1 was significantly reduced. We also sought to determine whether the p53-binding chimeric sequence affects p53 transcriptional activity by determining changes in murine double minute 2 and Bax expression. MDM2 and Bax are regulated at the transcriptional level by p53.[21,22] We found increased expression of murine double minute 2 and Bax in ATII cells from BLM-treated mice that were unaffected by expression of p53-binding or non−p53-binding sequences (Figure 7B), which suggests that p53-binding sequence does not interfere with p53 transcriptional activation. Immunohistochemical analyses confirmed reduced levels of PAI-1 (Figure 7C). Despite a small increase in PAI-1 levels over those in saline controls, TUNEL staining (Figure 7D) showed minimal apoptosis. Immunofluorescence staining (Figure 7E) further confirmed that changes were restricted to ATII cells. These results indicated that expression of the chimeric p53-binding sequence suppresses ATII cell apoptosis and thus validates the contribution of cross talk between p53 and the fibrinolytic system in defining the extent of ATII cell damage. We next sought to interfere with BLM-induced p53 from binding to endogenous uPA, uPAR, and PAI-1 mRNA by i.t. instillation of p53-binding sequences. As shown in Figure 7F, i.t. administration of the p53-binding sequence likewise blocked BLM-induced ATII cell PAI-1 expression and apoptosis, whereas it increased uPA expression. TUNEL staining of ATII cells for apoptosis also showed significant suppression of BLM-induced ATII cell apoptosis after i.t. transduction of the p53-binding sequence (Figure 7G).

The ability of the p53-binding sequence to prevent pulmonary fibrosis was next examined by analyzing lung tissues for signs of fibrosis at 21 days after BLM injury. BLM-injured mouse lung tissues showed massive matrix protein deposits, as illustrated by blue Masson's trichrome staining (Figure 8A). However, mice expressing the p53-binding sequence displayed reduced matrix protein deposits in the lungs after exposure to BLM. Total hydroxyproline content of lung tissues of mice expressing the p53-binding sequence was also significantly decreased after BLM injury when compared with mice expressing the non−p53-binding sequence (Figure 8B). H&E-stained lung sections from mice expressing the p53-binding sequence demonstrated significant protection against alveolar condensation and fibrosis even at 21 days after BLM exposure (data not shown). Next, we wanted to determine whether expression of p53-binding sequence after inception of BLM injury would reduce pulmonary fibrosis. Therefore, mice were treated with lentivirus expressing p53-binding or non−p53-binding sequence at 3 days after initiation of BLM injury, and the

lungs were analyzed for fibrosis at 21 days after BLM treatment. Results demonstrated that mice expressing p53-binding sequences showed reduced matrix deposition (Figure 8C) and hydroxyproline content in lungs (Figure 8D) when compared with mice expressing non-binding sequences. Further, H&E staining of lung sections demonstrated significant protection against BLM injury and lung remodeling in mice expressing the p53-binding sequence, as indicated by preservation of lung architecture (data not shown).

Role of p53-Mediated Changes in uPA and PAI-1 Expression in ATII Cell Apoptosis and Pulmonary Fibrosis

Our results demonstrated that expression of the uPA/uPAR/PAI-1 chimeric p53-binding sequence *in vivo* effectively suppresses the fibrogenic response to BLM exposure. Therefore, we sought to determine whether uPA expression was important for abrogation of ATII cell apoptosis and development of pulmonary fibrosis in mice expressing the p53-binding chimeric sequences in ATII cells. Mice deficient in uPA expression that received lentivirus expressing p53 binding or non-binding sequences were exposed to BLM for 3 days, and ATII cells, lung homogenates, and lung sections were analyzed for apoptosis and fibrosis. Results showed that the p53-binding sequence reduced PAI-1 protein and mRNA and cleaved caspase-3 levels (Figure 9, A and B). Consistent with these data, it also reduced ATII cell apoptosis as determined via TUNEL and immunofluorescent staining for SP-C and active caspase-3 (Figure 9, C and D) and subsequent development of pulmonary fibrosis (Figure 9, E and F). These results are similar to the outcomes found in WT mice, indicating that uPA is not important for the protective effects of the p53-binding sequence.

Consistent with our earlier reports,[1] mice deficient in PAI-1 or p53 exposed to BLM resisted ATII cell apoptosis. Quantification of apoptotic cells showed that there were no significant differences in the numbers of apoptotic cells between mice treated with saline or BLM. p53-binding or control sequences showed no further protection against BLM-induced acute lung injury or pulmonary fibrosis in mice deficient in PAI-1 or p53 (Figure 10).

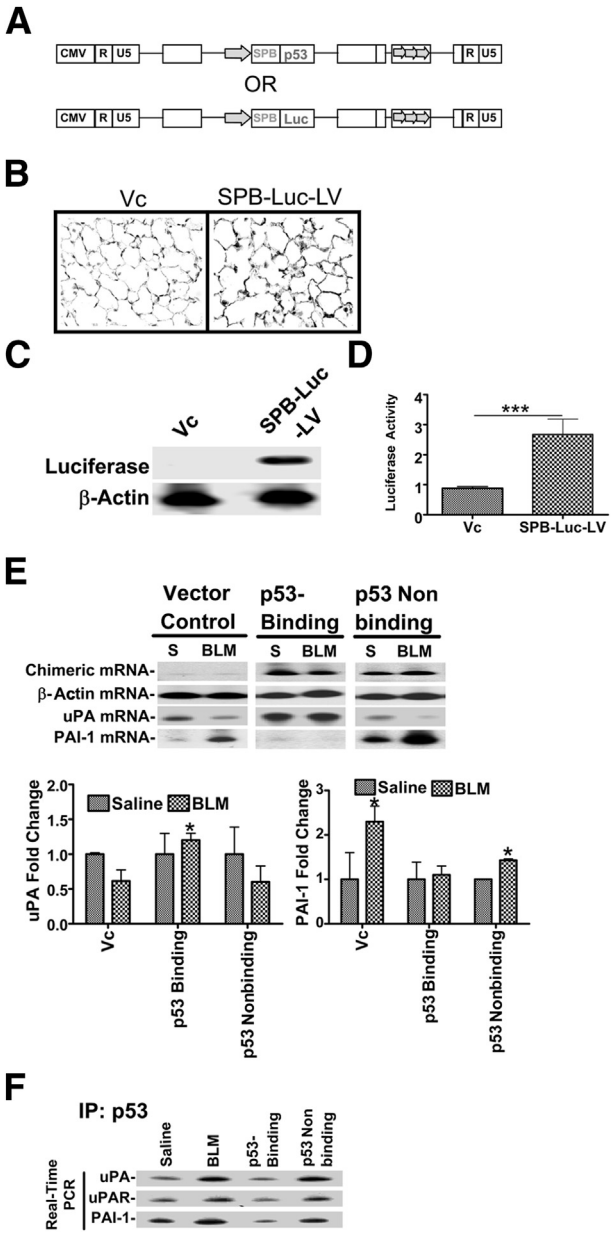

Figure 6 Expression of p53-binding uPA/uPAR/PAI-1 3′ UTR sequences or luciferase gene in mouse lung ATII cells. **A:** Schematic diagrams show lentivirus vector harboring *SP-B* promoter expressing p53-binding chimeric uPA/uPAR/PAI-1 3′ UTR sequence or luciferase (Luc) gene. CMV = cytomegalovirus; R = R sequence; U5 = U5 region. Lentivirus expressing luciferase under *SP-B* promoter control were injected into mice via orbital plexus, and luciferase expression in lung sections and ATII cell lysates at 72 hours after transduction were analyzed via immunohistochemical staining (**B**), Western blotting (**C**), or chemiluminescent assay (**D**). ***$P < 0.005$ compared with cells from mice injected with nonrecombinant lentivirus. **E:** Mice were administered lentivirus vector harboring *SP-B* promoter expressing p53 binding or non–p53-binding control chimeric uPA/uPAR/PAI-1 3′ UTR mRNA sequences via orbital plexus and after 24 hours were exposed to saline solution or BLM. Mice were sacrificed at 72 hours after BLM treatment. Total RNA isolated from ATII cells was analyzed for uPA and PAI-1 mRNAs via reverse transcription-PCR. Data are given as means ± SD ($n = 3$ mice per group). The differences between treatments are statistically significant. *$P < 0.05$. **F:** Expression of p53-binding uPA/uPAR/PAI-1 3′ UTR mRNA in ATII cells of mice with BLM-induced acute lung injury inhibits the p53 interaction with endogenous uPA/uPAR/PAI-1 mRNAs. ATII cell lysates from mice injected with lentivirus expressing p53-binding or non–p53 binding control sequences and treated with BLM for 72 hours were immunoprecipitated (IP) with anti-p53 antibody. Total RNA from p53 immune complexes were analyzed for uPA/uPAR/PAI-1 mRNAs via reverse transcription-PCR using ^{32}P–deoxycytidine triphosphate. PCR products were resolved on urea or polyacrylamide gel and exposed to X-ray film.

Discussion

ATII cell apoptosis, inflammation, abnormalities in fibrinolysis, and consequent fibroblast overgrowth and provisional matrix deposition typifies progressive pulmonary

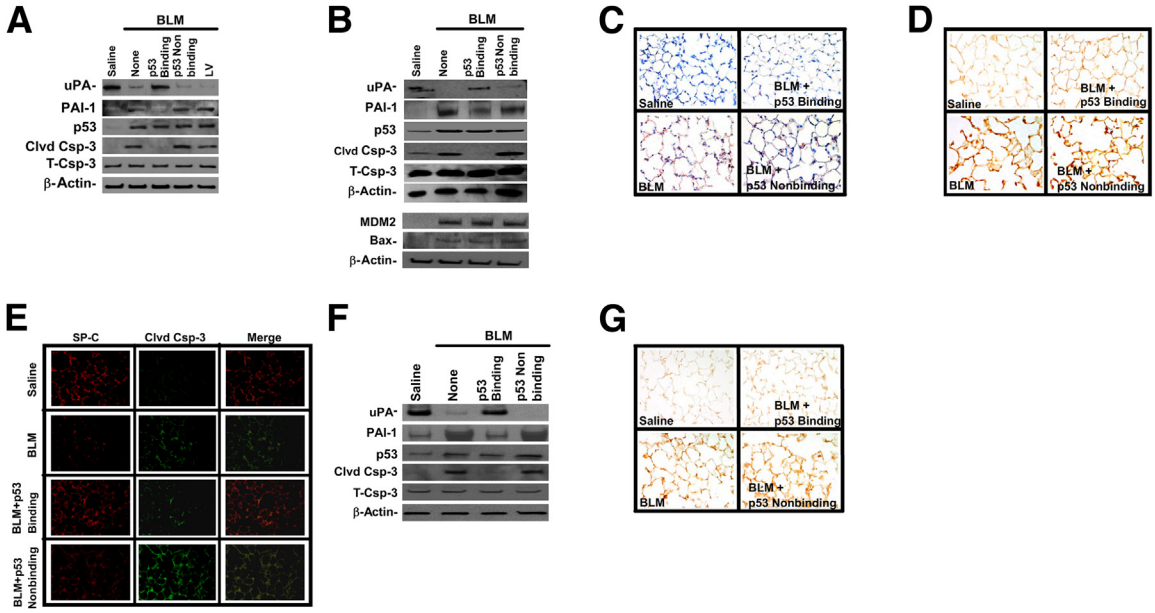

Figure 7 p53-binding chimeric sequence reverses BLM-induced changes in ATII cell uPA, p53, and PAI-1 expression and apoptosis *in vivo*. **A**: H441 cells transduced with lentiviral vector containing p53-binding or non—p53-binding sequences were treated with BLM for 24 hours, and p53, uPA, PAI-1, and cleaved and total caspase-3 levels (Clvd Csp-3 and T-Csp-3, respectively) were determined via Western blotting. β-Actin levels in cell lysates were determined to assess for loading differences. **B**: Mice were injected with lentivirus (LV) expressing p53 binding or non—p53 binding control chimeric uPA/uPAR/PAI-1 3′ UTR mRNA sequences via orbital plexus, exposed to saline solution or BLM after 24 hours, and sacrificed after 72 hours. ATII cell lysates were analyzed for p53, uPA, PAI-1, cleaved and total caspase-3, and β-actin via Western blotting. MDM2, Bax, and β-actin levels were analyzed to evaluate whether expression of either p53-binding or control non-binding sequences affect p53 transcriptional activity. Lung sections from mice injected with lentivirus were subjected to immunohistochemical staining for PAI-1 (**C**), TUNEL staining (**D**), immunofluorescence staining for cleaved caspase-3, and SP-C for assessment of ATII cell apoptosis (**E**). **F**: Mice were subjected to i.t. injection of lentivirus expressing p53-binding or non-binding control chimeric uPA/uPAR/PAI-1 3′ UTR mRNA sequences and after 24 hours were exposed to saline solution or BLM. Mice were sacrificed at 72 hours after BLM injury. Lysates from isolated ATII cells were analyzed for p53, uPA, PAI-1, cleaved and total caspase-3, and β-actin via Western blotting. **G**: Lung sections from mice described in Figure 7F were subjected to TUNEL staining. **C**, **D**, and **G**: ×400 magnification.

fibrosis. Mitigation of BLM-induced acute lung injury and pulmonary fibrosis after transplantation of healthy ATII cells[23] suggests that ATII cell apoptosis is a major contributor to fibrosing acute lung injury and development of pulmonary fibrosis. This strongly supports that preservation of ATII cell viability, either by inhibiting apoptosis or inducing cell proliferation, could mitigate development of pulmonary fibrosis. uPA, PAI-1, and p53 proteins elaborated by ATII cells influence a broad range of biological processes that are germane to forms of acute lung injury and repair including regulation of lung epithelial cell viability.[1,2,24–26]

Induction of epithelial cell proliferation by uPA is dose-dependent in lungs and other organs.[4–6,27–35] We have previously shown that uPA inhibits p53 expression while inducing tyrosine phosphorylation of Stat3. This process involves the interaction of uPA with its receptor, uPAR.[36–38] Cells lacking p53 showed elevated uPA and uPAR and low PAI-1 expression and increased proliferation.[4–6,39,40] However, reintroduction of p53 in p53-deficient (H1299) cells inhibits both uPA and uPAR and induces PAI-1 expression. Restoration of p53 in these cells resulted in less lung epithelial cell proliferation and greater apoptosis. These findings were later confirmed by other groups, who reported that suppression of uPAR augmented p53,[41,42] indicating intricate links between p53, the uPA fibrinolytic system, and ATII cell viability.

p53 is increased in ATII cells in mice after BLM treatment or lung injury induced by cigarette smoke or particulate matter.[1–3,42,43] This finding has been further supported by the recent observations using a BLM model of acute lung injury in which p53-mediated increase in PAI-1 and concurrent reductions in uPA and uPAR promoted ATII cell apoptosis and development of pulmonary fibrosis.[1] Mice deficient in p53 or PAI-1 resist BLM-induced ATII cell apoptosis and pulmonary fibrosis, whereas those deficient in uPA remain susceptible to both conditions.[1] Because p53 targets multiple downstream proapototic and antiapoptotic genes,[44,45] we initially used WT, p53-deficient, and transgenic mice expressing DNp53 to assess ATII cell apoptosis. We found that WT mice showed increased ATII cell apoptosis, whereas those deficient in p53 expression resisted BLM-induced apoptosis. Previous studies have indicated that p53-deficient mice that received BLM (2 to 4 U/kg) via i.v. or s.c. injection resisted alveolar epithelial cell apoptosis,[46,47] whereas those exposed to 50 U/kg BLM via endotracheal instillation showed pronounced apoptosis of alveolar macrophages and lung epithelial cells,[48] which

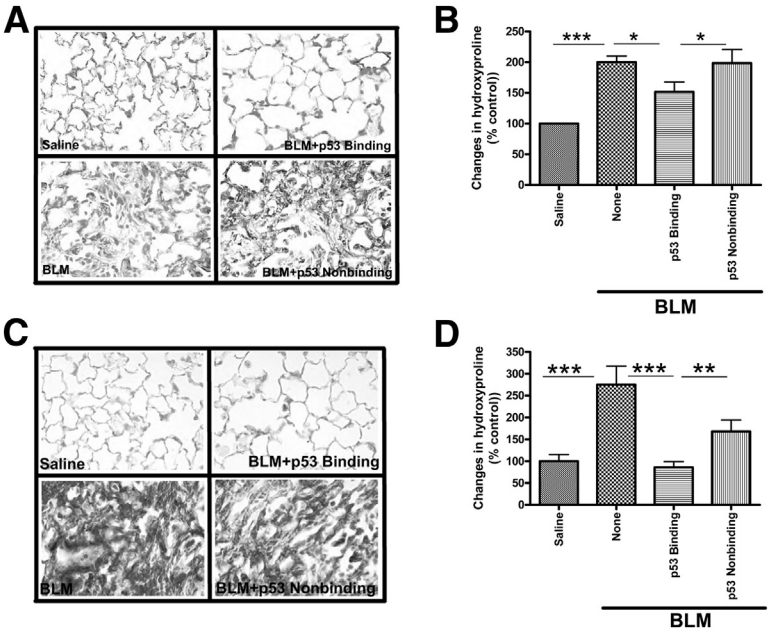

Figure 8 Inhibition of BLM-induced lung fibrosis in mice via administration of the chimeric p53-binding 3′ UTR sequences. **A**: Mice were injected with lentivirus expressing p53 binding or nonbinding control chimeric uPA/uPAR/PAI-1 3′ UTR mRNA sequences via orbital plexus and 24 hours later were treated with saline solution or BLM. Lung sections from mice were subjected to trichrome staining. Blue stain indicates deposition of collagen, fibronectin, and other matrix proteins. **Panel A** (×400 magnification) is representative of nine fields per mouse ($n = 3$ mice per group). **B**: Lung homogenates from mice exposed to saline solution or BLM were analyzed for hydroxyproline content. Data are given as means ± SD of at least three repetitions ($n = 3$ mice per group). Differences between treatments are statistically significant ($^*P < 0.05$, $^{***}P < 0.005$). **C**: Mice were exposed to BLM for 72 hours to induce lung injury and later were injected with lentivirus expressing p53-binding or non-binding control chimeric uPA/uPAR/PAI-1 3′ UTR mRNA sequences via orbital plexus. Mice were sacrificed on day 21, and lung sections were subjected to trichrome staining. **Panel C** (×400 magnification) is representative of nine fields per mouse ($n = 3$ mice per group). **D**: Lung homogenates from mice exposed to BLM and p53-binding or control non-binding 3′ UTR sequences as described in Figure 8C were analyzed for hydroxyproline content. Shaded columns represent means ± SD of at least three repetitions ($n = 3$ mice per group). Differences between treatments are statistically significant ($^{**}P < 0.05$, $^{***}P < 0.005$).

could be attributable to the unusually high dose (50 U/kg) of BLM used in the study reported by Davis et al[48] compared with 0.4 to 20 U/kg used to induce pulmonary fibrosis in other studies.[15,47,49,50] In addition, their results were derived from semiquantitative morphometric analysis of two-color TUNEL plus cytokeratin staining of lung tissues and not isolated ATII cells. As acknowledged by Davis et al,[48] accumulation of apoptotic cell debris due to reduction in phagocytosis of apoptotic cells as a consequence of suppression of thrombospondin, a p53 target gene, could have contributed to increased TUNEL staining in lung sections.

Consistent with previous reports,[15] compared with non-transgenic mice, transgenic mice expressing DNp53 showed higher ATII cell apoptosis and pulmonary fibrosis after BLM exposure despite lower expression of p21/WAF1 mRNA in SP-C−DNp53 mice. These reports question the importance of p53-mediated transcriptional mechanisms in control of apoptosis and fibrogenesis during BLM lung injury.[1,51] Increased p53 expression by ATII cells surrounding fibrotic foci in human lungs with idiopathic pulmonary fibrosis[51] and in a mouse model of BLM-induced lung injury,[1] and mitigation of pulmonary fibrosis after inhibition of epithelial cell apoptosis by caspase inhibitors in mouse and rat models of BLM injury[52,53] underscore the importance of p53-mediated ATII cell apoptosis in the development of fibrosis.

Transgenic SP-C−DNp53 mice express p53 with compromised transcriptional activity but with an intact C-terminal domain.[15] ATII cells of SP-C−DNp53 mice carry additional copies of the C-terminal domain of p53 due to expression of DNp53 and endogenous p53.[15] Therefore, increased ATII cell apoptosis and pulmonary fibrosis in SP-C−DNp53 mice exposed to BLM could be attributed to increased interactions of WT and mutant p53 proteins through their C-terminal domain with uPA, uPAR, and PAI-1 mRNAs to alter their expression. p53 targets the fibrinolytic system in a coordinated manner[4–6,54,55] through interactions with uPA, uPAR, and PAI-1 mRNA 3′ UTR. The importance of p53 posttranscriptional regulation in the control of fibrosis is further supported by a recent report that pifithrin-α, an inhibitor of p53 transcription, does not improve the outcome of renal fibrosis.[56] We found that pifithrin-α failed to reverse BLM-induced changes in ATII cell uPA or PAI-1 levels *in vitro*. Furthermore, inability of chimeric p53-binding uPA, uPAR, and PAI-1 3′ UTR sequences to inhibit p53 protein interaction with a consensus DNA binding site or transcription activation clearly suggests the involvement of p53 in posttranscriptional regulation of uPA, uPAR, and PAI-1 expression. This is further supported by attenuation of BLM-induced apoptosis and development of fibrosis through competitive inhibition of p53 binding to uPA, uPAR, and PAI-1 mRNAs. Although the present study has focused on p53 interactions with 3′ UTRs of uPA, PAI-1, and uPAR, the involvement of other genes is to be investigated. Nevertheless, our studies have indicated that concurrently targeting p53 binding to 3′ UTRs of uPA, PAI-1, and uPAR mRNA decrease ATII cell apoptosis and prevent lung fibrosis due to BLM exposure.

Lack of ATII cell apoptosis in PAI-1− and p53−deficient mice and increased ATII cell p53 and PAI-1 expression, apoptosis, and pulmonary fibrosis in uPA-deficient mice

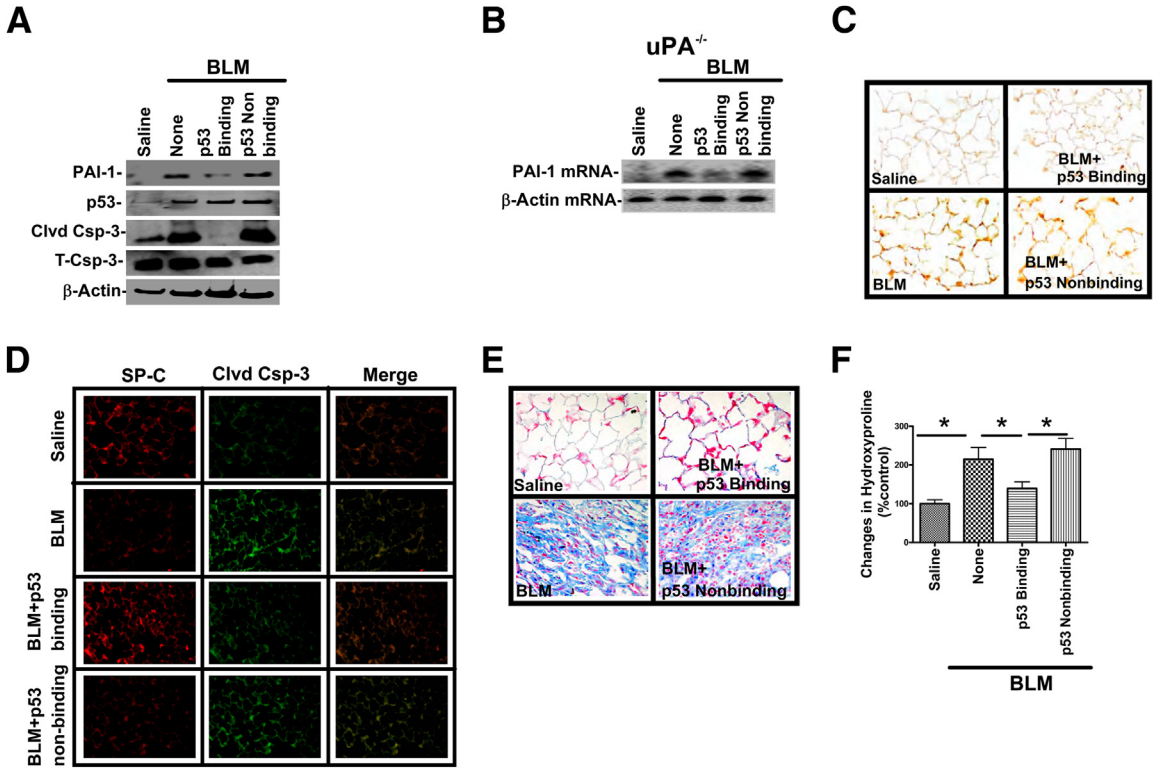

Figure 9 Protection against BLM-induced ATII cell apoptosis and pulmonary fibrosis by the chimeric p53 binding sequences does not require uPA expression. **A**: uPA-deficient mice were i.v. injected with lentivirus expressing p53-binding or non-binding control chimeric uPA/uPAR/PAI-1 3′ UTR mRNA sequences and after 24 hours were treated with saline solution or BLM, and ATII cells were isolated at 72 hours after BLM treatment. Levels of cleaved and total caspase-3 (Clvd Csp-3 and T-Csp-3, respectively), PAI-1, p53, and β-actin were determined via Western blotting. **B**: Levels of PAI-1 and β-actin mRNAs in ATII cells were determined via reverse transcription-PCR. Lung sections of uPA-deficient mice exposed to BLM were subjected to TUNEL (**C**) and immunofluorescence staining (**D**) for cleaved caspase-3 (green) and SP-C (red) to assess ATII cell apoptosis. Representative sections from three mice are shown at ×400 magnification. **E**: Mice were i.v. injected with lentivirus expressing p53-binding or non—p53-binding control chimeric uPA/uPAR/PAI-1 3′ UTR mRNA sequences and after 24 hours were treated with BLM. Mice were sacrificed at 21 days after treatment with BLM. Lung sections were subjected to trichrome staining. Panels are representative of nine fields per mouse ($n = 3$ mice per group) and shown at ×400 magnification. **F**: Mouse lung homogenates were analyzed for changes in hydroxyproline content. Data are shown as means ± SD of at least three repetitions ($n = 3$ mice per group). The differences between treatments are statistically significant ($^*P < 0.05$).

after BLM injury[1] indicates that ATII cell viability and protection against development of pulmonary fibrosis are coordinately regulated by uPA, PAI-1, and p53. This conclusion is further supported by protection against BLM injury in uPA-deficient mice transduced with the p53-binding chimeric sequence (present study) or lung-specific expression of uPA,[57] indicating that the protection is dependent on uPA-mediated inhibition of p53 expression. Unlike in WT mice with BLM-induced lung injury, mice deficient in uPA resist caveolin-1 scaffolding domain peptide—mediated inhibition of p53 and downstream PAI-1 expression and ATII cell apoptosis.[1] However, p53-binding chimeric 3′ UTR sequence inhibits PAI-1 expression and ATII cell apoptosis in both WT and uPA-deficient mice without suppressing BLM-induced p53 expression. The disparate response suggests that caveolin-1 scaffolding domain peptide attenuates ATII cell apoptosis through uPA-mediated suppression of BLM-induced p53, leading to inhibition of downstream PAI-1 expression, whereas p53-binding 3′ UTR sequence mitigates p53-mediated induction of PAI-1 expression and ATII cell apoptosis by interfering with p53 and PAI-1 mRNA interaction without affecting BLM-induced ATII cell p53 expression.

In summary, our results demonstrate for the first time that p53 cross talk with the fibrinolytic system contributes to the

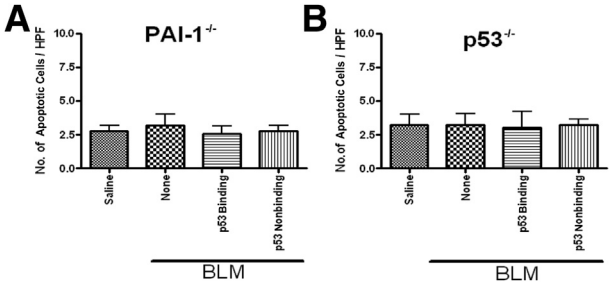

Figure 10 PAI-1— and p53-deficient mice resist ATII cell apoptosis. Lung sections of PAI-1— and p53-deficient mice expressing p53-binding or non-binding control chimeric uPA/uPAR/PAI-1 3′ UTR sequences were treated with BLM for 72 hours and subjected to TUNEL staining. TUNEL-positive cells were counted in high-power field (HPF) to assess apoptosis ($n = 3$ mice per group). Mice exposed to saline solution served as controls.

pathogenesis of acute lung injury and pulmonary fibrosis. Targeting the coordinate interactions of p53 with uPA, uPAR, and PAI-1 mRNA represents a potentially paradigm shifting approach to reverse acute lung injury and pulmonary fibrosis. Although interplay between the transcriptional and posttranscriptional functions of p53 in the regulation of gene expression contributes to alveolar epithelial injury and fibrogenic response, our data indicate that post-transcriptional regulation of the fibrinolytic system by p53 has a major role in the progression of BLM-induced pulmonary fibrosis. The salutary responses to the p53 decoy mRNA binding sequence indicate that p53-mediated regulation of uPA and PAI-1 mRNA stability in fibrosing lung injury assumes potential importance in a novel therapeutic context.

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