

# Regulation of Plasminogen Activator Inhibitor-1 Expression by Tumor Suppressor Protein p53\*

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H1299 lung carcinoma cells lacking p53 (p53<sup>-/-</sup>) express minimal amounts of plasminogen activator inhibitor-1 (PAI-1) protein as well as mRNA. p53<sup>-/-</sup> cells express highly unstable PAI-1 mRNA. Transfection of p53 in p53<sup>-/-</sup> cells enhanced PAI-1 expression and stabilized PAI-1 mRNA. On the contrary, inhibition of p53 expression by RNA silencing in non-malignant human lung epithelial (Beas2B) cells decreased basal as well as urokinase-type plasminogen activator-induced PAI-1 expression because of accelerated degradation of PAI-1 mRNA. Purified p53 protein specifically binds to the PAI-1 mRNA 3'-untranslated region (UTR), and endogenous PAI-1 mRNA forms an immune complex with p53. Treatment of purified p53 protein with anti-p53 antibody abolished p53 binding to the 3'-UTR of PAI-1 mRNA. The p53 binding region maps to a 70-nucleotide PAI-1 mRNA 3'-UTR sequence, and insertion of the p53-binding sequence into  $\beta$ -globin mRNA destabilized the chimeric transcript. Deletion experiments indicate that the carboxyl-terminal region (amino acid residues 296–393) of p53 protein interacts with PAI-1 mRNA. These observations demonstrate a novel role for p53 as an mRNA-binding protein that regulates increased PAI-1 expression and stabilization of PAI-1 mRNA in human lung epithelial and carcinoma cells.

Wild-type p53, a tumor suppressor protein, inhibits cellular growth and cell cycle progression at several checkpoints. Approximately 80% of tumor cells lack a p53 mutation but exhibit a 5–10-fold reduction of p53 protein levels compared with corresponding normal tissues (1). p53 is likewise present in non-malignant lung epithelial cells in which it contributes to the apoptotic response to injury.

Lung epithelial cells secrete the proenzyme single chain uPA,<sup>2</sup> which is activated by plasmin and other proteases. During the last decade, it has become increasingly clear that uPA-

mediated plasminogen activation (PA) is involved in remodeling of the extracellular matrix in acute and chronic lung injury, repair (2, 3), and neoplasia (4–6). uPA-mediated PA is tightly controlled by its high affinity receptor uPAR and two fast-acting specific inhibitors PAI-1 and PAI-2. PAI-1 is the primary inhibitor of uPA, and it regulates both the PA activity of uPA as well as the level of uPA·uPAR complex formation and recycling. Lung epithelial cells express PAI-1 (7), which may thereby contribute to the pathogenesis of diverse lung diseases such as acute respiratory distress syndrome or the interstitial lung diseases (3).

We recently found that uPA regulates lung epithelial cell apoptosis/growth in a biphasic, concentration-dependent manner through elaboration of p53 (8), demonstrating the first direct link between epithelial cell survival/cell cycle regulation and alveolar fibrinolysis. Increased p53 and PAI-1 expression is observed in bleomycin-induced lung injury (9, 10). Cigarette smoke decreased endothelium-derived fibrinolytic activity (11) and induced p53 expression by lung fibroblasts (12). Suppression of p53 function because of deletion or mutation (13–15) and increased PA activity occur in tumor cells, including lung carcinomas (16–18). Inhibition of tumor growth and invasion by expression of p53 and PAI-1 (8, 19, 20) as well as stabilization of PAI-1 mRNA by uPA (7) prompted us to test the possibility that p53 could regulate PAI-1 expression. We now describe a newly recognized uPA-p53 cross-talk that regulates PAI-1 expression and that influences uPA/uPAR-dependent pathophysiological activities of lung epithelial cells, including cellular fibrinolytic capacity and viability. Here, for the first time, we identify p53 as a sequence-specific mRNA-binding protein that regulates the stability of PAI-1 mRNA.

## EXPERIMENTAL PROCEDURES

**Cell Culture**—Human bronchial epithelial (Beas2B) and p53-deficient human lung non-small cell carcinoma (H1299) cells obtained from ATCC were maintained in LHC-9 medium containing 1% antibiotics and in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 1% glutamine, and 1% antibiotics (Invitrogen), respectively, as described previously (8, 21). Bleomycin was purchased from Cell Pharm GmbH Hannover, Germany. DeadEnd colorimetric TUNEL system was obtained from Promega Corp., Madison, WI.

**Total Cell Extraction and Western Blotting**—Beas2B or H1299 cells were treated with PBS or uPA in serum-free media.

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<sup>2</sup> The abbreviations used are: uPA, urokinase-type plasminogen activator; siRNA, small inhibitor RNA; PAI-1, plasminogen activator inhibitor-1; UTR, untranslated region; uPAR, uPA receptor; CM, conditioned medium; CL, cell lysate; nt, nucleotide; PBS, phosphate-buffered saline; RT, reverse transcription; TSE, tobacco smoke extract; TUNEL, terminal dUTP nick-end labeling; GST, glutathione S-transferase; Nsp, nonspecific; DRB, 5,6-di-

chloro-1- $\beta$ -D-ribofuranosylbenzimidazole; rp53, recombinant p53; mlgG, murine IgG; PA, plasminogen activation.

Conditioned medium (CM) and cell lysate (CL) were analyzed for PAI-1 expression by Western blotting using anti-PAI-1 monoclonal antibody (American Diagnostica, Greenwich, CT) (7). Membranes having proteins from the cell lysates were stripped and reprobed with anti-p53 and anti- $\beta$ -actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (8). The proteins were detected by ECL (PerkinElmer Life Sciences).

**Northern Blotting of PAI-1 or p53 mRNA**—Northern blotting assays were performed to assess the level of PAI-1 or p53 mRNA in Beas2B or H1299 cells as described previously (7, 8). PAI-1 mRNA stability was assessed by transcription chase experiments. In brief, cells stimulated with PBS or uPA for 6 h were then treated with 20  $\mu$ g/ml of DRB (Calbiochem) to inhibit ongoing transcription, after which total RNA was isolated at specific time points. PAI-1 mRNA was measured by Northern blotting.

**Transfection of p53-deficient H1299 Cells with p53 cDNA**—To confirm that p53 regulates PAI-1 expression, p53 cDNA was cloned into a eukaryotic expression vector pcDNA3.1. H1299 cells were transfected with vector cDNA or vector containing p53 cDNA, and stable cell lines were created as described earlier (22). The cells were treated with PBS or uPA, and the effect of p53 expression on basal and uPA-mediated p53, PAI-1 protein and mRNA levels was analyzed by Western or Northern blotting, respectively, as described above. The effect of p53 expression on basal and uPA-mediated PAI-1 mRNA synthesis was determined by run-on transcription assay, and PAI-1 mRNA stability was assessed by transcription chase experiments after inhibiting ongoing transcription as we previously described (7).

**Inhibition of p53 Expression by RNA Silencing in Beas2B Cells**—Beas2B cells grown to 70% confluence were treated with nonspecific siRNA or p53 specific siRNA (Santa Cruz Biotechnology) for 36 h. The cells treated with PBS or uPA were analyzed for the expression of p53 or PAI-1 protein and mRNA by Western or Northern blotting, respectively. The effect of p53 inhibition on basal and uPA-mediated PAI-1 mRNA synthesis or decay was determined as described above.

**Effect of Tobacco Smoke Extract (TSE) or Bleomycin on Beas2B Cell p53 and PAI-1 Expression**—To determine the effects of TSE on Beas2B cells *in vitro*, we prepared extracts of tobacco smoke by passing smoke from a burning cigarette through sterile PBS. An absorbance of 1.00 at 260 nm was designated as 100%. The extract was sterile-filtered before use, and fresh extract was used each time. Alternatively, we treated Beas2B cells with bleomycin (40  $\mu$ g/ml) for 24 h, and the CL and CM were analyzed for p53 and PAI-1 expression by Western blotting as described above.

**TUNEL Staining of Beas2B Cells**—TSE- and bleomycin-induced Beas2B cell apoptosis was assessed by DNA staining to determine the extent of DNA damage using DeadEnd colorimetric TUNEL system kit according to the manufacturer's protocol. Briefly, biotinylated nucleotide was incorporated at the 3'-OH DNA using the terminal deoxynucleotidyltransferase, recombinant enzyme. Horseradish peroxidase-labeled streptavidin was then bound to these biotinylated nucleotides, which were detected using the peroxidase substrate, hydrogen perox-

ide, and the stable chromogen diaminobenzidine. The apoptotic nuclei are stained dark brown using this procedure. Alternatively, we analyzed Beas2B cells treated with TSE or bleomycin by flow cytometry following annexin-V/propidium iodide staining to quantitate the apoptotic cell population, as described earlier (8).

**Molecular Cloning, Expression, and Purification of p53**—The coding sequences of p53 or various deletion fragments were PCR-amplified using a previously cloned full-length cDNA packaged in pcDNA 3.1 vector, in conjunction with sense and antisense oligonucleotide primers, and the design was based on the 5'- and 3'-regions of the open reading frame. The 1,182-bp full-length or different deletion PCR products were subcloned and transformed into *Escherichia coli*. GST-p53 fusion proteins expressed by these cells were purified according to the method described previously (23). Recombinant full-length p53 (rp53) proteins obtained by thrombin digestion or GST fusion proteins containing various p53 deletions were analyzed on a SDS-PAGE and Western blotting using anti-p53 antibody to check the purity.

**p53-PAI-1 mRNA Binding Assays**—Binding activities were tested by gel mobility shift assay using uniformly  $^{32}$ P-labeled transcripts corresponding to the PAI-1 coding (CDR) or 3'-untranslated (UTR) regions synthesized using T7 or SP6 polymerase (Ambion, TX) (21). Reactions were performed at 30 °C by incubating these transcripts (20,000 cpm) with either full-length or truncated rp53 protein as described earlier (21). Samples were then separated by electrophoresis on 5% native polyacrylamide gels with 0.25 $\times$  TBE running buffer. The gels were dried and autoradiographed at -70 °C using Kodak x-ray film. In a separate experiment, following heparin digestion, the reaction mixtures were UV-irradiated on ice, and the immobilized RNA-protein complex was separated on SDS-PAGE, dried, and autoradiographed. Alternatively, full-length p53 or various deletion fragments were separated on a SDS-PAGE, electroblotted onto nitrocellulose membrane, and subjected to Northwestern assay using  $^{32}$ P-labeled PAI-1 mRNA 3'-UTR transcript as described earlier (23). We also treated rp53 protein or preformed rp53-PAI-1 mRNA complex with varying amounts of monoclonal antibody against human p53 protein or nonspecific mouse IgG (mIgG) and subjected to gel mobility shift assay.

**Immunoprecipitation of RNA-Protein Complex**—To confirm the direct interaction of p53 protein with PAI-1 mRNA *in vivo*, we cross-linked Beas2B cells treated with PBS and uPA (50 ng/ml or 1  $\mu$ g/ml) with formalin as described before (24). The cytosolic extracts were immunoprecipitated using nonspecific mIgG followed by p53 monoclonal antibody in the presence of RNase inhibitor and ribosomal RNA for 1 h at room temperature. The immune complexes were later precipitated using protein A/G plus-agarose and the agarose beads were washed three times with lysis buffer. Total RNA was isolated from the immune complexes using total RNA isolation reagent and associated PAI-1 mRNA was amplified by RT-PCR using specific primers in the presence of  $^{32}$ P-labeled dCTP. The corresponding nonradioactive PCR products were later identified by nucleotide sequencing (23).

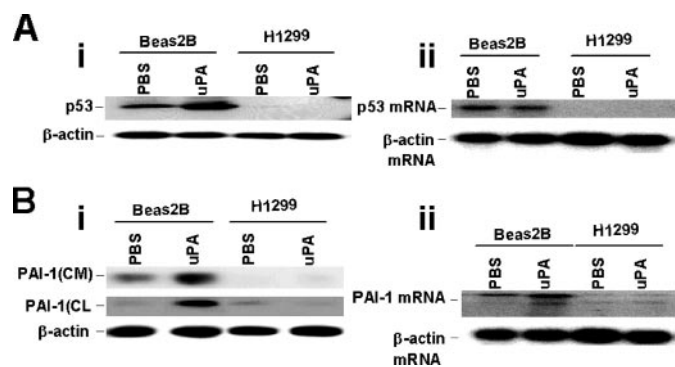
## Regulation of PAI-1 Expression by p53

**Competitive Inhibition by Sense and Antisense mRNA or Polyribonucleotides**—rp53 protein was incubated with various amounts (0–100-fold excess) of unlabeled PAI-1 sense or antisense 3'-UTR mRNA or 500 M excess of ribonucleotide poly(A), poly(C), poly(G), or poly(U) (Sigma) for 30 min at 30 °C prior to the <sup>32</sup>P-labeled PAI-1 mRNA and RNase T1 steps. In a separate experiment, rp53 was treated with SDS (0.1%) or proteinase K (2.5 mg/ml) for 30 min at 37 °C prior to addition of <sup>32</sup>P-labeled PAI-1 mRNA. The reaction mixtures were later subjected to gel mobility shift assay. Similarly, <sup>32</sup>P-labeled PAI-1 3'-UTR mRNA was pre-digested with RNase A and T1 (50 units) for 30 min at 37 °C before subjecting to gel mobility shift assay using rp53 protein.

**Determination of p53-binding Site on PAI-1 mRNA 3'-UTR**—PAI-1 3'-UTR cDNA fragments of differing size were synthesized by PCR amplification of a full-length PAI-1 cDNA template using different sense and antisense oligonucleotide primers that were designed based on 5' and 3' regions of the open reading frame. These deletion fragments were cloned into pcDNA 3.1 and transcribed *in vitro* in the presence of [<sup>32</sup>P]UTP. <sup>32</sup>P-labeled deletion transcripts were subsequently used as probes for gel mobility shift or UV cross-linking studies to localize the rp53 protein-binding sequence on PAI-1 3'-UTR mRNA (21).

**Construction of  $\beta$ -Globin/PAI-1 Chimeric Message**—The complete human  $\beta$ -globin cDNA in plasmid (pSP6 $\beta$ c) was excised and inserted into the HindIII-XbaI site of the plasmid pcDNA 3.1. Two 70-bp DNA fragments, one corresponding to the rp53-binding (C3) and the other corresponding to the control nonbinding sequence (C4), were prepared from PAI-1 cDNA. Each of these cDNA fragments was inserted into the 3'-UTR of  $\beta$ -globin cDNA. These chimeric cDNA constructs were then subcloned into a eukaryotic expression vector, pcDNA3.1. The orientations and sequences of  $\beta$ -globin/PAI-1 chimeric clones were verified by sequencing. Beas2B cells were transfected with the prepared chimeric plasmid constructs by lipofection. Total RNA was isolated at various time points after the inhibition of transcription by DRB. Chimeric  $\beta$ -globin/PAI-1 mRNA was then measured by Northern blotting using <sup>32</sup>P-labeled cDNA (25). The half-life of the mRNA at each interval was determined by densitometry, normalized to the  $\beta$ -actin control mRNA of the samples, and subsequently compared with the densitometric values of samples determined at the 0-h base line of each experiment.

**Effect of p53 Binding PAI-1 mRNA 3'-UTR Sequence on PAI-1 Expression**—Chimeric mRNAs were created using methods we reported previously (21, 22). H1299 cells expressing vector cDNA or p53 cDNA were transfected with chimeric  $\beta$ -globin/PAI-1 cDNA containing the 70-nt p53 protein-binding sequence of the PAI-1 mRNA 3'-UTR or control non-p53 binding CDR sequences as described above for 48 h to competitively inhibit p53 binding to endogenous PAI-1 mRNA. The expression of chimeric  $\beta$ -globin/PAI-1 mRNA containing 70-nt p53-binding or nonbinding control sequences was confirmed by RT-PCR using  $\beta$ -globin and PAI-1-specific sense and antisense sequences or by Northern blotting as described above. PAI-1 expression was determined by Western blotting.

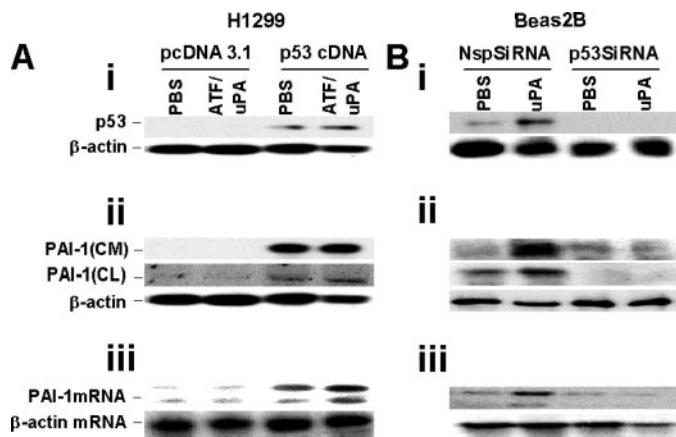


**FIGURE 1.** A, expression of p53 protein and mRNA by lung-derived cells. *Panel i*, lysates of Beas2B and p53-deficient non-small cell carcinoma (H1299) cells treated with PBS or uPA (50 ng/ml) for 24 h were subjected to Western blotting using anti-p53 antibody. The same membrane was stripped and developed with an anti- $\beta$ -actin antibody for equal loading. *Panel ii*, RNA isolated from Beas2B and H1299 cells treated with PBS or uPA (50 ng/ml) for 6 h was subjected to Northern blotting using <sup>32</sup>P-labeled p53 cDNA and <sup>32</sup>P-labeled  $\beta$ -actin cDNA for equal loading. B, involvement of p53 in expression of PAI-1 protein and mRNA by lung epithelial cells. *Panel i*, CM and total CL of Beas2B and H1299 cells treated with PBS or uPA (50 ng/ml) for 24 h were subjected to Western blotting using an anti-PAI-1 antibody. *Panel ii*, RNA isolated from Beas2B and H1299 cells treated with PBS or uPA (50 ng/ml) for 6 h were subjected to Northern blotting using <sup>32</sup>P-labeled PAI-1 and  $\beta$ -actin cDNAs. The data shown are representative of three repetitions.

**Statistical Analysis**—We tested the differences between Beas2B cells treated with p53 siRNA or PAI-1 siRNA and corresponding nonspecific control siRNA transfected cells exposed to TSE or bleomycin, respectively, by Student's *t* test.

## RESULTS

**Expression of PAI-1 Protein and mRNA by p53-deficient Lung Carcinoma Cells and Non-malignant Lung Epithelial Cells**—We recently reported that uPA induces PAI-1 expression through post-transcriptional stabilization of its mRNA (7) in lung epithelial cells. uPA (200 ng/ml or 4 nM) likewise induced PAI-1 expression in rat smooth muscle cells (26). At similar uPA concentrations (25–250 ng/ml), p53 expression is induced in lung epithelial cells (8). To determine whether p53 influences PAI-1 expression, we initially treated non-malignant Beas2B as well as malignant large cell lung carcinoma H1299 (p53<sup>-/-</sup>) cells with PBS or uPA (50 ng/ml) and assessed the induction of p53 expression by Western blotting. As shown in Fig. 1A, *panel i*, Beas2B cells express basal level of p53 and uPA-induced p53 protein, whereas H1299 cells neither express p53 nor respond to uPA stimulation. Northern blotting indicated that uPA failed to alter p53 mRNA in Beas2B cells, and p53 mRNA likewise was not detected in H1299 cells treated with or without uPA (Fig. 1A, *panel ii*). Therefore, the lysates of these two cell types were used for Western blotting to determine whether PAI-1 is differentially expressed because of the differences in their basal levels of p53. As shown in Fig. 1B, *panel i*, Beas2B cells express minimal PAI-1, and uPA (50 ng/ml) increased PAI-1 expression. This was consistent with a similar increase in expression of both 3.2- and 2.4-kb alternate spliced variants of PAI-1 mRNA in Beas2B cells stimulated with uPA (Fig. 1B, *panel ii*). Unlike Beas2B cells, p53-deficient cells expressed minimal amounts of PAI-1 protein as well mRNA and failed to respond to uPA treatment (Fig. 1B, *panels i* and *ii*). These observations led us to speculate that p53 is involved in uPA-mediated regu-



**FIGURE 2. p53 induces PAI-1 protein and mRNA expression.** *A, panel i*, expression of WT p53 in H1299 cells. H1299 cells were transfected with vector cDNA (pcDNA 3.1) or p53 cDNA (p53) in pcDNA 3.1. Stable cell lines expressing vector cDNA or p53 cDNA were treated with PBS or 50 ng/ml of the amino-terminal fragment (ATF) of uPA for 24 h. The cell lysates were analyzed for p53 expression by Western blotting using an anti-p53 antibody as described in Fig. 1A. *Panel ii*, induction of PAI-1 expression by WT p53. H1299 cells transfected with vector pcDNA3.1 or WT p53 cDNA were treated with PBS or uPA (50 ng/ml). The CM and CL were subjected to Western blotting using an anti-PAI-1 antibody. *Panel iii*, induction of PAI-1 mRNA expression by WT p53. RNA isolated from H1299 cells transfected with vector cDNA or p53 cDNA treated with PBS or uPA (50 ng/ml) for 6 h was subjected to Northern blotting using  $^{32}$ P-labeled PAI-1 and  $\beta$ -actin cDNAs. *B, panel i*, inhibition of p53 expression by siRNA in Beas2B cells. Beas2B cells transfected with control Nsp siRNA or p53 siRNA were treated with PBS or uPA (50 ng/ml) for 24 h. Cell lysates were subjected to Western blotting using anti-p53 and  $\beta$ -actin antibodies. *Panel ii*, Beas2B cells treated with control siRNA or p53 siRNA were treated with PBS or uPA (50 ng/ml) for 24 h. The CM and CL were subjected to Western blotting using anti-PAI-1 antibody. *Panel iii*, Beas2B cells transfected with control siRNA or p53 siRNA were treated with PBS or uPA (50 ng/ml) for 6 h. The total RNA isolated from these cells was subjected to Northern blotting using  $^{32}$ P-labeled PAI-1 and  $\beta$ -actin cDNAs. Data are representative of four independent replications.

lation of PAI-1 expression by lung epithelial and carcinoma cells.

**Effect of p53 on PAI-1 Protein and mRNA Expression**—To confirm that p53 is involved in lung epithelial cell PAI-1 expression mediated by uPA, p53-deficient H1299 cells were transfected with either vector (pcDNA 3.1) cDNA alone or p53 cDNA cloned in pcDNA3.1. The cells were analyzed for p53 expression. H1299 cells transfected with p53 cDNA expressed p53 protein, whereas empty vector cDNA transfected cells failed to express p53 protein (Fig. 2A, *panel i*). Treatment of p53 cDNA overexpressing cells with 50 ng/ml of either full-length uPA (data not shown) or its amino-terminal uPAR-binding fragment failed to further increase p53 expression. These cells were also analyzed for PAI-1 expression by Western blotting. As shown in Fig. 2A, *panel ii*, pcDNA 3.1 cDNA-transfected H1299 cells expressed minimal amounts of PAI-1, and uPA stimulation failed to increase the expression of PAI-1 in these cells. However, transfection of p53 in H1299 cells induced relatively large amounts of basal PAI-1 expression. Inhibition of uPA gene transactivation by p53 probably caused less PAI-1 to remain at the cell surface uPA-uPAR complex which may thereby contribute to the differences in PAI-1 antigenic signals between CL and CM. We later analyzed the effects of p53 restoration at the PAI-1 mRNA level by Northern blotting and found that p53 expression similarly increased PAI-1 mRNA expression compared with vector cDNA-transfected cells. These cells further failed to respond to uPA stimulation (Fig.

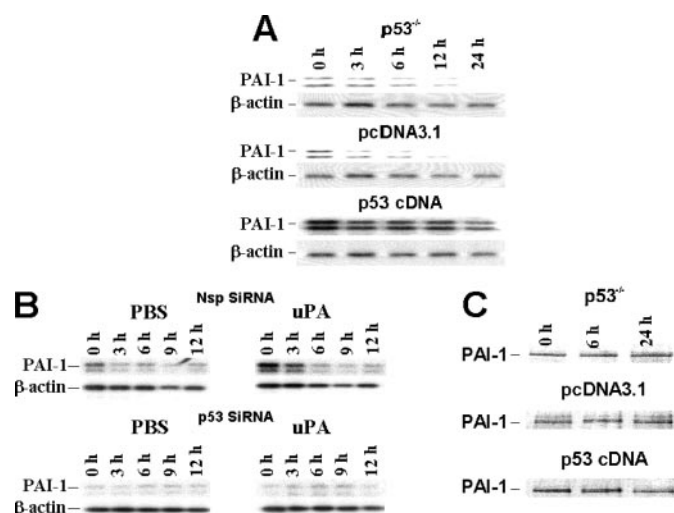
2A, *panel iii*). These results confirm that p53 plays a major role in PAI-1 expression by H1299 cells.

To extend these studies, we next inhibited p53 expression by RNA silencing in non-malignant Beas2B lung epithelial cells and analyzed p53 expression by Western blotting. We found that treatment of Beas2B cells with p53 siRNA inhibited both basal and uPA-mediated p53 expression by more than 95% compared with control nonspecific siRNA (Nsp siRNA)-treated cells (Fig. 2B, *panel i*). We next analyzed basal and uPA-mediated PAI-1 expression in siRNA-treated cells. As shown in Fig. 2B, *panel ii*, Beas2B cells treated with nonspecific siRNA expressed minimal basal levels of PAI-1, and uPA stimulation enhanced PAI-1 in the CM and CL by severalfold. However, inhibition of p53 expression by p53 siRNA suppressed both basal and uPA-mediated PAI-1 expression in these cells compared with control siRNA-treated cells. We also confirmed that blocking the expression of p53 inhibited basal PAI-1 mRNA and that uPA failed to increase PAI-1 mRNA compared with control siRNA-treated Beas2B cells (Fig. 2B, *panel iii*). These results demonstrate that p53 regulates PAI-1 expression in Beas2B cells.

**Effect of p53 Expression on PAI-1 mRNA Synthesis and Degradation**—p53 protein binds to the PAI-1 promoter sequence and induces PAI-1 mRNA transcription (27, 28). Therefore, we next tested the inference that p53 expression affects the rate of PAI-1 mRNA transcription in H1299 cells. Nuclear extracts from p53-deficient cells transfected with or without vector or p53 cDNA in pcDNA 3.1 were subjected to run-on transcription to determine the rate of PAI-1 mRNA synthesis. p53 expression failed to affect PAI-1 mRNA synthesis in H1299 cells (data not shown). Because uPA induces PAI-1 expression through post-transcriptional stabilization of PAI-1 mRNA (7), we inferred that p53 might control PAI-1 expression at the level of PAI-1 mRNA degradation. We therefore treated naive H1299 or H1299 cells transfected with vector cDNA or p53 cDNA with DRB to inhibit ongoing transcription, and the decay of PAI-1 mRNA was analyzed at varying time periods by Northern blotting. H1299 cells expressing p53 produced stable PAI-1 mRNA ( $t_{1/2} > 12$  h) when compared with untransfected ( $t_{1/2} < 3$  h) or vector cDNA ( $t_{1/2} < 3$  h) expressing p53-deficient cells (Fig. 3A). These results confirm that p53 regulates PAI-1 mRNA stability.

To independently confirm that p53 down-regulates PAI-1 mRNA degradation at the post-transcriptional level in non-malignant lung epithelial cells, Beas2B cells were treated with control siRNA and p53 siRNA to suppress basal p53 expression. Because Beas2B cells express low levels of basal PAI-1 protein and respond to uPA stimulation (Fig. 1B, *panel i*), both control and p53 siRNA transfected cells were treated with PBS or uPA (50 ng/ml) for 6 h to induce maximum PAI-1 mRNA (7). Ongoing transcription was inhibited, and the level of PAI-1 mRNA was determined by Northern blotting. Our results show that PAI-1 mRNA is relatively unstable ( $t_{1/2} < 3$  h) in control siRNA treated Beas2B cells, and uPA treatment stabilized PAI-1 mRNA in these cells. However, uPA also failed to slow the decay of PAI-1 mRNA ( $t_{1/2} < 3$  h) (Fig. 3B) in Beas2B cells in which p53 expression was inhibited by p53 siRNA. PAI-1 expression was relatively low but detectable in PBS-treated Beas2B cells, which

## Regulation of PAI-1 Expression by p53



**FIGURE 3. Effect of p53 on PAI-1 mRNA stability.** *A*, expression of WT p53 in non-small cell lung carcinoma cells lacking p53 ( $p53^{-/-}$ ) stabilizes PAI-1 mRNA. Naive  $p53^{-/-}$  cells ( $p53^{-/-}$ ) or stable  $p53^{-/-}$  cells over expressing vector cDNA (*pcDNA3.1*) or p53 cDNA were subjected to transcription chase experiment by treating with DRB (20  $\mu\text{g}/\text{ml}$ ). The level of PAI-1 mRNA was measured by Northern blotting at 0–24 h. Experiments were repeated five times with similar results. *B*, inhibition of p53 expression destabilizes PAI-1 mRNA. Beas2B cells transfected with control nonspecific (*Nsp*) siRNA or p53 siRNA were treated with PBS or uPA (50 ng/ml) for 6 h to induce maximum PAI-1 mRNA. Ongoing transcription was inhibited by treatment with DRB, and the decay of PAI-1 mRNA was analyzed for 0–12 h by Northern blotting.  $\beta$ -Actin mRNA expression is shown for equal loading. Representative figure of four replications is shown. *C*, effect of p53 on PAI-1 protein translation. H1299 cells ( $p53^{-/-}$ ) or H1299 cells transfected with vector cDNA (*pcDNA3.1*) or p53 cDNA were subjected to metabolic labeling using [ $^{35}\text{S}$ ]methionine and [ $^{35}\text{S}$ ]cysteine in the presence of proteasome inhibitor (MG-132) for varying time periods (0–24 h).  $^{35}\text{S}$ -Labeled PAI-1 proteins were immunoprecipitated from the CM using anti-PAI-1 antibody, separated on SDS-PAGE, and autoradiographed. The results represent two independent experiments.

were used to assess the effect of p53 inhibition on basal PAI-1 mRNA levels. However, the observed lack of PAI-1 mRNA stabilization in p53 siRNA-treated Beas2B cells following uPA treatment confirms that p53 contributes to the regulation of PAI-1 mRNA stability. We next tested if p53 likewise regulates PAI-1 translation by inhibiting protein degradation using MG-132 as described by Takagi *et al.* (29) and found that p53 expression in H1299 cells failed to enhance the rate of PAI-1 protein translation (Fig. 3C).

**Identification of p53 as a PAI-1 mRNA-binding Protein—**Post-transcriptional regulation of mRNA in general and PAI-1 mRNA in particular involves its interaction with specific mRNA-binding proteins. We therefore hypothesized that p53 binds to PAI-1 mRNA and regulates its degradation. To test this possibility, we expressed a recombinant p53-GST fusion protein (rp53) in a prokaryotic system and affinity-purified the rp53 protein by using glutathione-Sepharose column chromatography. Purification (>95%) of the rp53 protein was confirmed by Western blotting using an anti-p53 monoclonal antibody (30). Next, we tested the ability of rp53 to interact with the PAI-1 mRNA CDR and 3'-untranslated region (UTR) by gel mobility shift assay. As shown in Fig. 4A, rp53 protein failed to bind  $^{32}\text{P}$ -labeled PAI-1 mRNA CDR, whereas it formed a specific RNA-protein complex with the PAI-1 mRNA 3'-UTR. To confirm that p53 also binds to PAI-1 mRNA in cultured lung airway epithelial cells, we immunoprecipitated lysates from

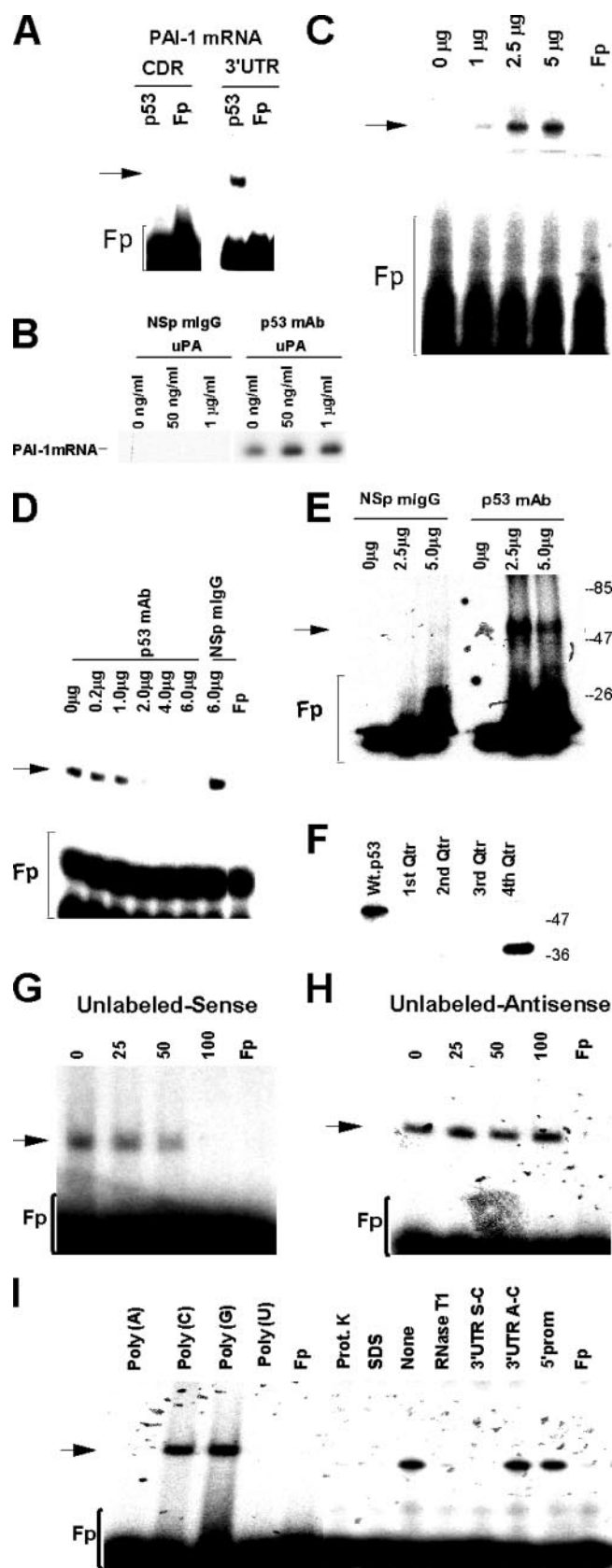
Beas2B cells treated with PBS or uPA (50 ng or 1  $\mu\text{g}/\text{ml}$ ) with anti-p53 antibody, and p53-associated PAI-1 mRNA was amplified with RT-PCR. As shown in Fig. 4B, the p53-PAI-1 mRNA interaction increased with uPA treatment indicating that the effect is concurrent with induction of PAI-1 mRNA expression mediated by uPA (7). UV cross-linking experiment showed a dose-dependent increase in p53 and PAI-1 mRNA 3'-UTR interaction (Fig. 4C).

Incubation of preformed rp53-PAI-1 mRNA 3'-UTR complex with anti-p53 antibody inhibited p53 binding activity in a concentration-dependent manner, whereas nonspecific mIgG failed to interfere (Fig. 4D). Exposure of rp53 protein to anti-p53 antibody likewise prevented it from binding to PAI-1 mRNA (data not shown). Anti-p53 antibody interferes with rp53-PAI-1 mRNA binding instead of shifting the preformed aggregate; hence the immobilized complex was immunoprecipitated using anti-p53 antibody to confirm the specificity of the p53 interaction. As shown in Fig. 4E, anti-p53 antibody precipitated mRNA and protein complex, whereas nonspecific mIgG failed. To identify the specific region of the p53 molecule responsible for binding to PAI-1 mRNA, we expressed p53 deletion fragments with amino acid residues 1–98, 99–196, 197–295, and 296–393 and subjected them to PAI-1 mRNA 3'-UTR binding by Northwestern assay. As shown in Fig. 4F, only the carboxyl-terminal region (residues 296–393, 4th quarter) of the p53 molecule bound to PAI-1 3'-UTR mRNA as much as that of full-length p53 protein.

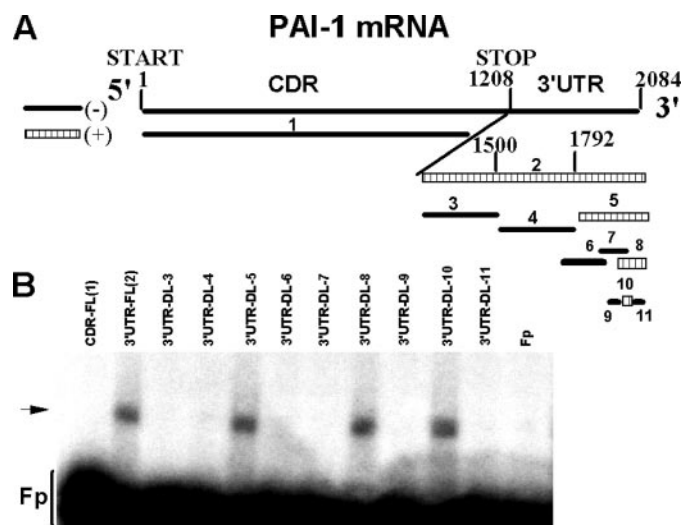
**Specificity of the p53-PAI-1 3'-UTR mRNA Interaction—**The specificity of p53-PAI-1 mRNA 3'-UTR interaction was next confirmed by a series of cold competition experiments. Preincubation of rp53 with a molar excess of unlabeled PAI-1 3'-UTR sense transcripts resulted in a dose-dependent inhibition (Fig. 4G), whereas addition of the same amount of unlabeled antisense transcript failed to interfere with p53 binding to the  $^{32}\text{P}$ -labeled PAI-1 mRNA 3'-UTR (Fig. 4H). Pretreatment of p53 protein with a molar excess of homopoly(G) or -(C) ribonucleotides likewise failed to affect the p53-PAI-1 3'-UTR mRNA interaction whereas molar excess of poly(A) or poly(U) totally abolished the binding (Fig. 4I) indicating that p53 binds to a unique A- or U-rich sequence of the PAI-1 mRNA 3'-UTR. Treatment of p53 protein with proteinase-K or SDS abolished its ability to interact with the PAI-1 mRNA. Finally, a molar excess of unlabeled p53 promoter binding DNA consensus sequence failed to attenuate rp53 binding to PAI-1 mRNA confirming that the p53 transactivation domain (amino acids 102–292) (31) is distinct from the PAI-1 mRNA binding region (amino acids 296–393) (Fig. 4F).

**Determination of the p53 Protein-binding Sequence on PAI-1 3'-UTR mRNA and Its Function in mRNA Degradation and PAI-1 Expression—**To identify the specific p53 protein-binding sequences on PAI-1 mRNA 3'-UTR, we made a series of  $^{32}\text{P}$ -labeled deletion transcripts by *in vitro* transcription. These transcripts were then individually tested for p53 binding by gel mobility shift assay. As shown in Fig. 5, p53 specifically bound to a 70-nt (nt 1958–2027) sequence present on the PAI-1 mRNA 3'-UTR.

To determine whether the p53-binding 70-nt PAI-1 3'-UTR sequence (nt 1958–2027) contains information for message



**FIGURE 4. p53 protein binds to PAI-1 3'-UTR mRNA.** *A*, recombinant p53 protein (rp53) expressed in prokaryotic system was incubated with the  $^{32}\text{P}$ -labeled PAI-1 mRNA coding (*CDR*) or 3'-untranslated region (3' *UTR*), and the mRNA-rp53 complexes were analyzed by gel mobility shift assay on 5% non-denaturing PAGE. PAI-1 mRNA was incubated with rp53 protein (*p53*) or buffer

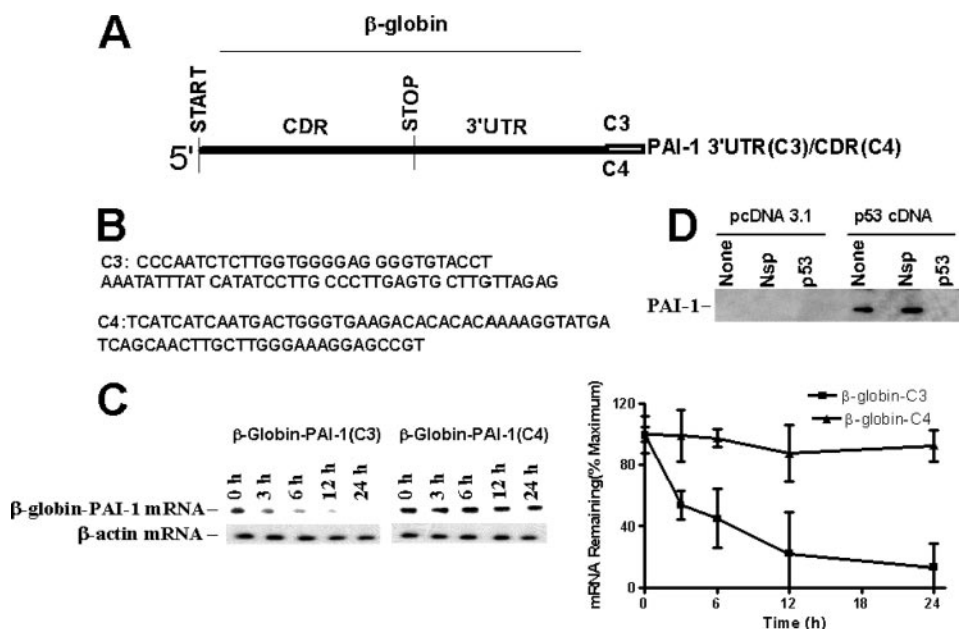


**FIGURE 5. Identification of the p53 protein-binding sequences on PAI-1 3'-UTR mRNA.** *A*, deletion map indicating the p53 protein-binding site on PAI-1 mRNA. *B*, rp53 protein was incubated with  $^{32}\text{P}$ -labeled PAI-1 mRNA full-length coding region (1) or full-length 3'-untranslated region (2) or 3'-UTR deletion transcripts (3'-UTR-DL-3-11). The RNA-protein complex was analyzed by gel mobility shift assay. Arrow indicates the PAI-1 mRNA-p53 protein complex. *Fp*,  $^{32}\text{P}$ -labeled PAI-1 mRNA probe is incubated with buffer alone. Data are representative of four independent analyses.

stability, we inserted the 70-bp sequence into the  $\beta$ -globin cDNA (Fig. 6A), and the chimeric  $\beta$ -globin-PAI-1 3'-UTR cDNA (Fig. 6, B, C3) was subcloned into the eukaryotic expression vector pcDNA3.1. We also prepared chimeric  $\beta$ -globin-PAI-1 cDNA containing a control 70-nt (nt 468-538) non-p53-

alone (*Fp*). Arrow indicates RNA-protein complex. *B*, Beas2B cells treated with varying amounts (0-1  $\mu\text{g}/\text{ml}$ ) of uPA for 24 h were lysed, and the lysates were immunoprecipitated using anti-p53 monoclonal antibody (*p53 mAb*) or mouse IgG (*Nsp mlgG*). The p53 protein-associated PAI-1 mRNA was detected by RT-PCR using  $^{32}\text{P}$ -labeled dCTP and verified by nucleotide sequencing of the corresponding nonradioactive PCR product. Experiments were repeated at least three times. *C*, rp53 protein (0-5  $\mu\text{g}/\text{lane}$ ) was subjected to PAI-1 mRNA 3'-UTR binding as described in *A*, and following heparin digestion the rp53-PAI-1 mRNA complex was exposed to UV light (2500  $\mu\text{J}$ ) for 10 min on ice, separated by 8% SDS-PAGE and autoradiographed. *D*, rp53-PAI-1 mRNA complex was treated with or without varying amounts (0-6  $\mu\text{g}/\text{lane}$ ) of anti-p53 antibody (*p53 mAb*) or 6  $\mu\text{g}/\text{lane}$  of nonspecific mlgG (*Nsp mlgG*). The reaction mixtures were subjected to gel mobility shift assay as described in *A*. *E*,  $^{32}\text{P}$ -labeled PAI-1 mRNA 3'-UTR-rp53 complexes were immunoprecipitated with either anti-p53 antibody or nonspecific mlgG. Immune complexes were then separated on SDS-PAGE and developed by autoradiography. *F*, identification of PAI-1 3'-UTR mRNA binding region on p53 molecule. Full-length rp53 protein (*Wt.p53*) obtained after thrombin digestion or GST-rp53 fusion deletion fragments corresponding to amino acid residues 1-98 (*1st Qtr*), 99-196 (*2nd Qtr*), 197-295 (*3rd Qtr*), and 296-393 (*4th Qtr*) were separated on SDS-PAGE and transferred to nitrocellulose membrane. The membrane was later subjected to Northwestern analyses using  $^{32}\text{P}$ -labeled PAI-1 mRNA 3'-UTR as a probe. The rp53-PAI-1 mRNA complexes were detected by autoradiography to determine the specificity of p53 protein-PAI-1 mRNA 3'-UTR interaction. Competitive inhibition of p53-PAI-1 mRNA binding using unlabeled PAI-1 mRNA sense (*G*) and antisense (*H*) transcripts is shown. rp53 proteins were incubated with  $^{32}\text{P}$ -labeled PAI-1 3'-UTR mRNA probe in the presence of 0-100-fold molar excess of unlabeled sense or antisense transcripts and analyzed by gel mobility shift assay. *I*, effects of polyribonucleotides, proteinase K (*Prot.K*), and SDS on p53 and PAI-1 mRNA binding. rp53 protein was incubated with a 500-fold excess of unlabeled poly(A), poly(C), poly(G), and poly(U) or a 100-fold molar excess of sense (3'-UTR S-C) or antisense (3'-UTR A-C) or p53 consensus sequence (5' *prom*), or proteinase K (2.5 mg/ml) and 0.1% SDS for 30 min at 30  $^{\circ}\text{C}$ .  $^{32}\text{P}$ -Labeled PAI-1 mRNA probe was added, and the mixture was digested with RNase T1 and analyzed by gel mobility shift assay.  $^{32}\text{P}$ -Labeled PAI-1 3'-UTR mRNA predigested with RNase T1 was also incubated with rp53 (*RNaseT1*). *Fp*, probe alone. Experiments are representative of four independent analyses.

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**FIGURE 6. Determining the destabilizing function of p53-binding PAI-1 3'-UTR mRNA sequence.** *A*, structure of  $\beta$ -globin/PAI-1 chimeric mRNA. The p53 protein-binding 70-nt 3'-UTR sequence corresponding to nt 1958–2027 (C3) and a nonbinding control sequence of similar size corresponding to the coding region from nt 468 to 538 (C4) of PAI-1 cDNA were inserted into the 3'-UTR of  $\beta$ -globin cDNA. The chimeric  $\beta$ -globin/PAI-1 cDNAs were subcloned to pcDNA 3.1. *B*, nucleotide sequence of the p53 binding region nt 1958–2027 (C3) or nonbinding control sequence 468–538 (C4). *C*, decay of  $\beta$ -globin/PAI-1 chimeric mRNA. Beas2B cells were transfected with the chimeric  $\beta$ -globin/PAI-1 3'-UTR gene containing the 70-nt (nt 1958–2027) p53-binding sequence ( $\beta$ -globin-PAI-1(C3)) or nonbinding control sequence of PAI-1 CDR (nt 468–538) ( $\beta$ -globin-PAI-1(C4)) in pcDNA 3.1. Total RNA was isolated at different time intervals after treatment with DRB as described above (Fig. 3B) and analyzed for the level of chimeric mRNA by Northern blotting. Densitometric scanning of individual bands from four experiments is shown as a line graph. *D*, effect of p53 binding PAI-1 mRNA 3'-UTR sequence on PAI-1 expression. H1299 cells expressing vector cDNA (pcDNA 3.1) or p53 cDNA were untreated (None) or transfected with chimeric  $\beta$ -globin/PAI-1 cDNA containing a non-p53-binding control (C4, Nsp) or p53-binding (C3, p53) PAI-1 3'-UTR sequence in pcDNA 3.1, as described in Fig. 6C. PAI-1 expression in the CM was determined by Western blotting using an anti-PAI-1 antibody. The data shown are representative of the findings of four independent analyses.

binding CDR sequence (Fig. 6, *B*, C4). Beas2B cells were then transfected with chimeric  $\beta$ -globin-PAI-1 cDNA constructs as well as  $\beta$ -globin cDNA alone. The decay of chimeric  $\beta$ -globin-PAI-1 mRNA was determined by Northern blotting after inhibiting ongoing transcription. Fig. 6C shows that insertion of the p53-binding 70-nt (C3) PAI-1 3'-UTR sequence destabilized otherwise stable  $\beta$ -globin mRNA ( $\beta$ -globin-PAI-1(C3)). However, insertion of a control non-p53-binding CDR sequence (C4) of similar size failed to alter the stability of  $\beta$ -globin mRNA ( $\beta$ -globin-PAI-1 (C4)), indicating that the p53-binding 70-nt PAI-1 3'-UTR sequence contains regulatory information for mRNA degradation. The presence of this destabilization determinant renders endogenous PAI-1 mRNA unstable in normal cells. However, binding of p53 to this sequence prevents its degradation and enhances PAI-1 mRNA stability as observed in Fig. 3.

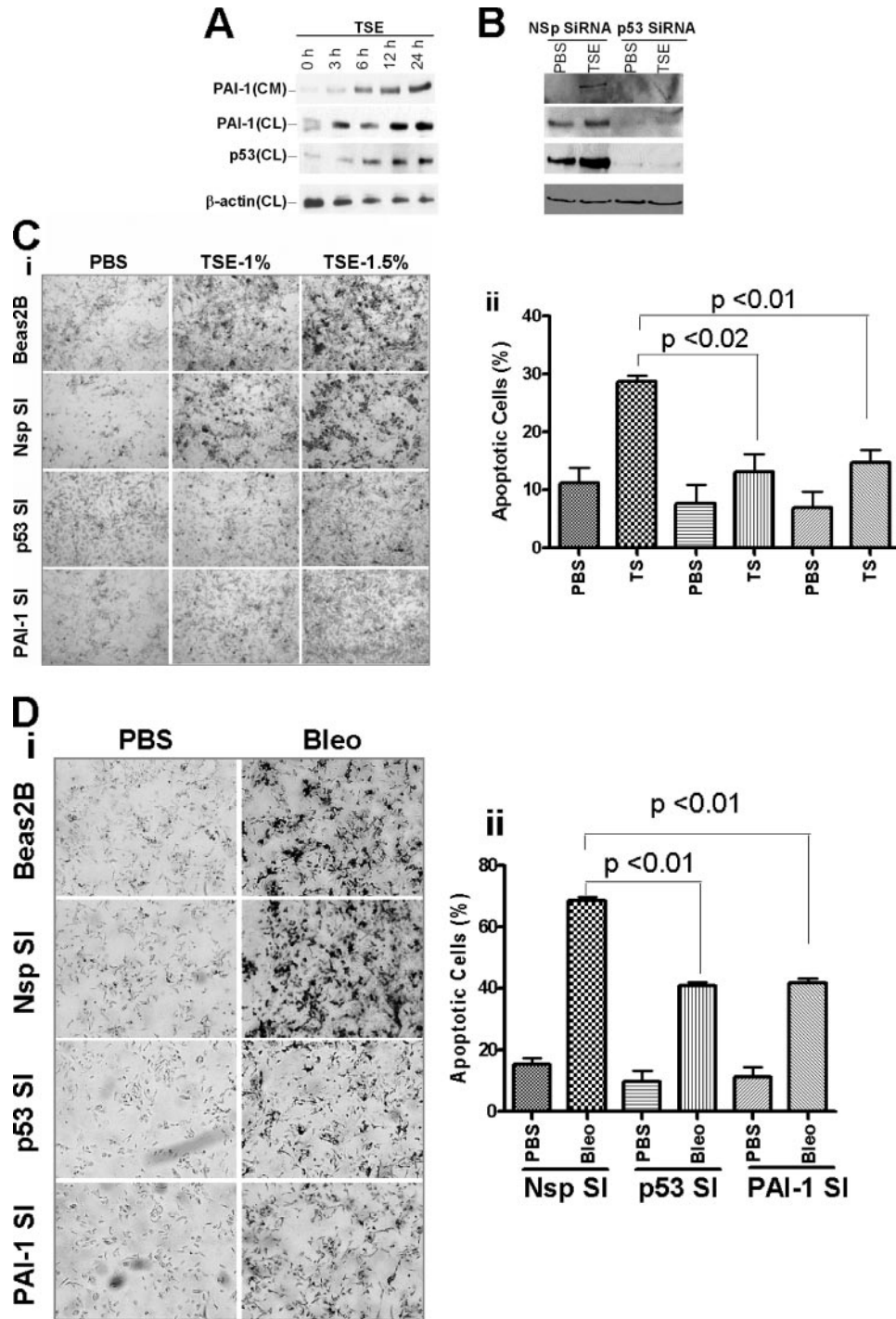
We next directly tested the effect of the p53 binding to the 70-nt (C3) PAI-1 mRNA 3'-UTR sequence on PAI-1 expression. H1299 cells transfected with vector cDNA or p53 cDNA were cotransfected with or without chimeric  $\beta$ -globin-PAI-1 cDNA containing the 70-nt (C3) p53-binding (p53) or a control 70-nt (C4) non-p53-binding (Nsp) sequence for 48 h to competitively inhibit p53 binding to endogenous PAI-1 mRNA, and CM were analyzed for PAI-1 expression. As shown in Fig. 6D, overexpression of p53 cDNA-transfected H1299 cells with the

chimeric  $\beta$ -globin-PAI-1 mRNA containing the 70-nt p53-binding but not the 70-nt control non-p53-binding sequence inhibited the stimulatory effect of p53 on PAI-1. However, the 70-nt p53-binding sequence had no effect on vector cDNA transfected H1299 cells. These results indicate that the interaction of p53 with the 70-nt PAI-1 mRNA (C3) 3'-UTR sequence up-regulates cellular PAI-1 expression.

**Induction of p53 and PAI-1 Expression by TSE in Lung Epithelial Cells**—Low concentrations (0–2 nM) of uPA released at the site of injury (9) promotes lung epithelial cell apoptosis, which is consistent with uPA-mediated p53 (8) and PAI-1 (7, 26) expression. uPA (>10 nM concentration) alternatively induces proliferation in a wide variety of cell types, including lung epithelial cells. At this uPA concentration, lung epithelial cell p53 expression is suppressed in a dose-dependent manner (8). A recent report indicates that p53 induced by TSE causes lung cell death (12). Patients with chronic obstructive pulmonary disease caused by chronic tobacco smoke exposure exhibit increased airway and alveo-

lar injury, which may involve PAI-1 overexpression as part of the inflammatory response. We therefore treated Beas2B cells with 1.5% TSE for 0–24 h and analyzed for changes in PAI-1 expression in CM and CL. TSE induced PAI-1 expression starting from 3 h after treatment, and the effect was maximal at 24 h. The membrane-containing proteins from CL were stripped and analyzed for p53 expression. As shown in Fig. 7A, TSE induced p53 expression, and the pattern is similar to that of PAI-1. CM and CL of bleomycin (40  $\mu$ g/ml)-treated Beas2B cells showed a similar increase in PAI-1 and p53 (data not shown). Because TSE and bleomycin induced both p53 and PAI-1 in Beas2B cells, we speculated p53 produced during TSE or bleomycin treatment caused PAI-1 expression. To confirm this possibility, we inhibited p53 expression in Beas2B cells by p53 siRNA. We also transfected Beas2B cells with nonspecific control siRNA. These cells were exposed to TSE or bleomycin and analyzed for the changes in PAI-1 expression by Western blotting. p53 siRNA-treated cells (p53Si RNA) did not respond to TSE, although Nsp siRNA-treated cells showed increased PAI-1 expression. We also made similar observations in which cells were treated with bleomycin (data not shown). The results indicate that p53 is involved in the regulation of PAI-1 expression induced in lung epithelial cells by TSE or bleomycin.

We next sought to determine whether TSE and bleomycin induce lung epithelial cell apoptosis. We found that TSE



**FIGURE 7. Regulation of TSE and bleomycin-induced lung epithelial cell apoptosis by p53-mediated and PAI-1 expression.** *A*, induction of PAI-1 and p53 expression by TSE. The CM and cell CL of Beas2B cells treated with TSE (1.5%) for 0–24 h were subjected to Western blotting using anti-PAI-1 antibody. The same CL were analyzed for expression of p53 and  $\beta$ -actin for equal loading using anti-p53 and anti- $\beta$ -actin antibodies. *B*, inhibition of p53 expression inhibits TSE-induced PAI-1 expression. The CM and CL of Beas2B cells expressing control nonspecific siRNA (*Nsp siRNA*) or p53 siRNA treated with PBS or TSE (1.5%) were analyzed for expression of PAI-1, p53, and  $\beta$ -actin by Western blotting as described in Fig. 7A. *C*, inhibition of p53 and PAI-1 expression protects lung epithelial cells from TSE-induced apoptosis. *Panel i*, untransfected Beas2B cells (*top row*) or Beas2B cells treated with nonspecific siRNA (*Nsp SI*), p53 siRNA, or PAI-1 siRNA (*subsequent rows*) were treated with PBS or 1 and 1.5% TSE for 24 h and were then subjected to TUNEL staining to assess apoptosis. The microscopic images were photographed at  $\times 20$  magnification. *Panel ii*, Beas2B cells treated with nonspecific siRNA (*Nsp SI*) or p53 or PAI-1 siRNA were treated with PBS or 1.5% TSE for 24 h. The cells were then detached and treated with anti-annexin-V antibody and propidium iodide. The apoptotic cells were analyzed by flow cytometry. The data are representative of three independent experiments. *D*, inhibition of p53 and PAI-1 expression protects lung epithelial cells from bleomycin (*Bleo*)-induced apoptosis. *Panel i*, untransfected Beas2B cells (*top row*) or Beas2B cells treated with nonspecific siRNA (*Nsp SI*) or p53 or PAI-1 siRNA (*subsequent rows*) were treated with PBS or bleomycin (40  $\mu$ g/ml) for 24 h, after which they were subjected to TUNEL staining to assess the apoptotic response, as described in Fig. 7C. Microscopic images were photographed at  $\times 20$  magnification. *Panel ii*, Beas2B cells treated with nonspecific siRNA (*Nsp SI*) or p53 or PAI-1 siRNA were treated with PBS or bleomycin (40  $\mu$ g/ml) for 24 h, after which they were subjected to flow cytometry as described in *C*, *panel ii*, to assess the apoptotic response.



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induced lung epithelial cell apoptosis in a dose-dependent manner. However, inhibition of either p53 or PAI-1 expression in lung epithelial cells by treating with specific siRNA-attenuated TSE induced Beas2B cell apoptosis as confirmed by TUNEL staining (Fig. 7C, panel i) and annexin-V/propidium iodide staining and flow cytometric analysis (Fig. 7C, panel ii). Treatment of Beas2B cell with control nonspecific siRNA failed to protect Beas2B cells from TSE-induced maximum damage. These results demonstrate a newly recognized, strong functional link exists between p53 and PAI-1 expression with TSE-induced apoptosis of lung epithelial cells. Inhibition of both p53 and PAI-1 expression likewise blocked lung epithelial cell apoptosis caused by bleomycin as confirmed by TUNEL staining (Fig. 7D, panel i) and flow cytometric analysis of annexin-V/propidium iodide-treated Beas2B cells (Fig. 7D, panel ii).

### DISCUSSION

Imbalances in regulation of PAI-1 and the expression of the tumor suppressor protein p53 have been independently implicated in the pathogenesis of acute and chronic lung inflammation and neoplasia (3, 32, 33). However, the molecular mechanisms linking these two important pathophysiologic processes remain unclear. PAI-1 is overexpressed in acute lung injury and potentiates the alveolar fibrinolytic defect that occurs in this setting (3, 34, 35). p53 regulates the viability or apoptosis of lung epithelial cells during lung injury and neoplasia (36). Suppression of PAI-1 protects human and mouse fibroblasts from replicative senescence irrespective of the presence of p53 suggesting that PAI-1 is a critical downstream target of p53 (37). Delineation of the mechanisms that govern expression of PAI-1 and p53 by lung epithelial cells are therefore germane to the better understanding of the pathogenesis of lung inflammation, its repair, and lung cancer.

It has been reported previously that p53 inhibits uPA-mediated cellular fibrinolysis via induction of PAI-1 expression through promoter transactivation. The PAI-1 promoter exhibits a p53-binding sequence (27). Adenoviral protein factor (E2F) negatively regulates PAI-1 promoter activity, and this process does not involve the retinoblastoma tumor suppressor gene Rb (38). We recently reported that uPA in association with uPAR induces both p53 and PAI-1 expression and that inhibition of p53 expression enhanced the proliferative response of lung alveolar epithelial cells (7, 8, 30).

We found that lung carcinoma cells lacking p53 expressed a minimal amount of PAI-1 and are highly resistant to apoptosis. The involvement of p53 in uPA-induced PAI-1 expression is further supported by our finding that restoration of p53 in p53<sup>-/-</sup> cells induced relatively large amounts of basal PAI-1. Interestingly, uPA failed to further induce either p53 or PAI-1 expression in these cells. These observations suggest that overexpression of p53 by cDNA transfection masks the uPA effect in these cells. Inhibition of basal PAI-1 expression and inability of uPA to induce PAI-1 in p53-inhibited (siRNA-treated) Beas2B cells further confirms the notion that p53 regulates cellular PAI-1 expression.

Our results show that reintroduction of p53 in p53<sup>-/-</sup> cells failed to induce PAI-1 mRNA synthesis. However, earlier

reports have suggested that the PAI-1 promoter has a p53-binding sequence and that p53 induces PAI-1 mRNA synthesis in other cell types (27). Nevertheless, expression of p53 stabilized PAI-1 mRNA in H1299 cells, suggesting the possibility that p53 plays a physiologic role in the induction of PAI-1 mRNA. The involvement of p53 in the post-transcriptional control of PAI-1 was supported by the observation that inhibition of p53 expression increased Beas2B cell PAI-1 mRNA degradation.

We therefore hypothesized that p53 directly interacts with PAI-1 mRNA to regulate its stability. We confirmed this hypothesis by using gel mobility shift and UV cross-linking assays in which we showed that rp53 protein directly binds to the PAI-1 mRNA 3'-UTR. Coprecipitation of PAI-1 mRNA with p53 protein from Beas2B cell lysates and immunoprecipitation of UV-irradiated rp53<sup>32P</sup>-labeled PAI-1 mRNA complex and inhibition of PAI-1 mRNA-rp53 protein binding by anti-p53 antibody confirm the interaction of p53 with PAI-1 mRNA. An antibody against p53 precipitates Beas2B cell PAI-1 mRNA complexed with p53. The anti-p53 antibody also inhibits binding and electrophoretic shift of the purified rp53 protein and PAI-1 mRNA complex *in vitro*. These differences may be attributable to differences in the *in vivo* and *in vitro* experimental procedures we used. Alternatively, the p53 antibody-p53-PAI-1 complex may be unstable under electrophoresis conditions. The p53-PAI-1 mRNA 3'-UTR complex was resistant to both RNase A and T1 digestion. The specificity of the p53 interaction with the PAI-1 mRNA 3'-UTR was assessed by self-competition experiments, where a labeled sense probe was successfully competed by its unlabeled analog. An unlabeled antisense probe failed to compete. Furthermore, addition of a molar excess of a homopolyribonucleotide poly(G) or poly(C) had no effect on p53 protein binding to the <sup>32</sup>P-labeled PAI-1 mRNA 3'-UTR. However, the presence of unlabeled poly(A) or poly(U) ribonucleotide inhibited p53-PAI-1 mRNA 3'-UTR binding, indicating that p53 binds to a specific AU-rich sequence. The specificity of p53 binding to the PAI-1 3'-UTR mRNA is also indicated by the findings that pretreatment of rp53 by proteinase K or SDS or pre-digestion of PAI-1 3'-UTR mRNA probe with RNase A and T1 totally abolished the p53-PAI-1 mRNA 3'-UTR complex. Inability of an unlabeled p53 binding DNA consensus element to inhibit p53-PAI-1 mRNA 3'-UTR interaction indicated that the PAI-1 mRNA 3'-UTR binding region of p53 is distinct from the transactivation domain. This was further confirmed by deletion experiments, where the PAI-1 3'-UTR mRNA binding region of p53 was restricted to carboxyl-terminal amino acid residues 296–393, whereas the core fragment (residues 102–292) that corresponds to the central portion of p53 molecule is the sequence-specific DNA binding domain (31).

p53 specifically binds to a 70-nt sequence on the PAI-1 mRNA 3'-UTR and augments PAI-1 expression through post-transcriptional PAI-1 mRNA stabilization. We recently reported that the same protein similarly binds to a 37-nt (TAAACCTGAAATCC-CCCTCTGCCCCTGGCTGGATCC) 3'-UTR sequence present on uPAR mRNA. Unlike the p53 interaction with the PAI-1 mRNA 3'-UTR, its binding to a 37-nt uPAR mRNA 3'-UTR sequence accelerates uPAR mRNA degradation and attenuates

its cell surface expression (30). Although p53 binding PAI-1 and uPAR mRNA sequences exhibit little homology, it is highly intriguing to note that the same molecule performs diverse functions by interacting with two nucleotide sequences on different genes. These observations concur with earlier findings that overexpression of PAI-1 or p53 protein inhibits proliferation of malignant tumor cells that often overexpress cell surface uPAR (15, 20).

Our study indicates that there is no other p53-binding sequence either in the PAI-1 CDR or 3'-UTR other than the sequence from nt 1958 to 2027 (39). Insertion of the PAI-1 3'-UTR-binding sequence into the  $\beta$ -globin mRNA destabilized the chimeric transcript indicating that the p53-binding sequence contains information for mRNA destabilization. Competitive inhibition of p53 binding to the endogenous PAI-1 mRNA transcript by transfection of the binding sequence also abolished PAI-1 expression in p53-expressing H1299 cells confirming that the p53 interaction with this sequence enhances PAI-1 mRNA stability. Increased expression of PAI-1 occurs in cells where expression of wild-type p53 is elevated, delineating a novel regulatory mechanism. In addition to its previously known roles in transcriptional (40–42) and translational (29, 43–46) regulation, we now show that p53 controls PAI-1 expression at the post-transcriptional level of mRNA stability.

Induction of p53 and PAI-1 expression by TSE and bleomycin in Beas2B cells and inhibition of bleomycin and TSE-mediated PAI-1 following suppression of p53 indicate that p53 contributes to this response. These findings are of particular importance to the pathogenesis of various forms of acute lung injury, where overexpression of both p53 and PAI-1 is commonly observed. Inability of TSE and bleomycin to induce apoptosis in Beas2B cells where p53 or PAI-1 expression is attenuated further demonstrates that p53-mediated apoptosis resulting from bleomycin or TSE-induced lung epithelial cell damage is affected downstream by PAI-1.

In summary, we confirmed that the p53-PAI-1 mRNA 3'-UTR interaction regulates PAI-1 mRNA stability in lung epithelial cells. To our knowledge, this newly identified pathway is the first description of the ability of p53 to bind PAI-1 mRNA in a sequence-specific manner and to induce PAI-1 expression through post-transcriptional mRNA stabilization. We believe this to represent the first report of the stabilization of any mRNA by p53. This pathway adds a new dimension to the current understanding of the p53 functional repertoire and represents a new link between viability of lung epithelial cells and functionality of the fibrinolytic system. Pathophysiologically relevant consequences of this highly coordinated pathway can be inferred from the *in vitro* experiments involving TSE and bleomycin-mediated lung injury. p53-mediated PAI-1 induction could be involved in a range of alternative situations likewise characterized by p53 and PAI-1 overexpression and epithelial apoptosis, which could include other forms of acute lung injury or chronic lung diseases like idiopathic pulmonary fibrosis that are characterized by pulmonary fibrosis. If operative *in vivo*, this pathway could also contribute to the relative overexpression

of PAI-1 and regulate the transformation, growth, and spread of lung neoplasms.

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## REFERENCES

- Bensaad, K., and Vousden, K. H. (2005) *Nat. Med.* **11**, 1278–1289
- Chapman, H. A. (2004) *J. Clin. Invest.* **113**, 148–157
- Shetty, S., and Idell, S. (2006) in *Encyclopedia of Respiratory Medicine* (Laurent, J., ed) pp. 205–210, Elsevier Publications, London
- Dvorak, H. F. (1986) *N. Engl. J. Med.* **315**, 1650–1659
- Dano, K., Behrendt, N., Brunner, N., Ellis, V., Ploug, M., and Pyke, C. (1994) *Fibrinolysis* **8**, 189–203
- Yanamandra, N., Konduri, S. D., Mohanam, S., Dinh, D. H., Olivero, W. C., Gujrati, M., Nicolson, G. L., Obeyesekke, M., and Rao J. S. (2005) *Clin. Exp. Metastasis* **18**, 611–615
- Shetty, S., Khalil, B., Cines, D. B., and Idell, S. (2003) *J. Biol. Chem.* **278**, 18124–18131
- Shetty, S., Gyetko, M., and Mazar, A. (2005) *J. Biol. Chem.* **280**, 28133–28141
- Ghosh, S., Mendonza, T., Ortiz, L. A., Gary, W. H., Cesar, D. F., Arnold, R. B., Mitchell, E., and Gilbert, F. M. (2002) *Am. J. Respir. Crit. Care Med.* **166**, 890–897
- Olman, M. A., Mackman, N., Gladson, C. L., Moser, K. M., and Loskutoff, D. J. (1995) *J. Clin. Invest.* 1621–1630
- Barua, R. S., Ambrose, J. A., Saha, D. C., and Reynold, L. J. (2002) *Circulation* **106**, 905–908
- Nyunoya, T., Monick, M. M., Klingelhutz, A., Yarvinsky, O. T., Cagely, J. R., and Hunnughake, G. W. (2006) *Am. J. Respir. Cell Mol. Biol.* **35**, 681–688
- Levine, A. J. (1997) *Cell* **88**, 323–331
- Vogelstein, B., Lane, D., and Levine, A. J. (2000) *Nature* **408**, 307–310
- Lane, D. (2004) *N. Engl. J. Med.* **350**, 2711–2712
- Shetty, S., and Idell, S. (1999) *Mol. Cell Biochem.* **199**, 189–200
- Tressler, R. J., Pitot, P. A., Stratton, J. R., Forrest, L. D., Zhuo, S., Drummond, R. J., Fong, S., Doyle, M. V., Doyle, L. V., Min, H. Y., and Rosenberg, S. (1999) *APMIS* **107**, 168–173
- Andreassen, P. A., Egelund, R., and Petersen, H. H. (2000) *Cell Mol. Life. Sc.* **57**, 25–40
- Haupt, Y., Rowan, S., Shaulian, E., Vousden, K. H., and Oren, M. (1995) *Genes Dev.* **9**, 2170–2183
- Inoue, M., Sawada, T., Uchima, Y., Kimura, K., Nishihara, T., Tanaka, H., Yashiro, M., Yamada, N., Ohira, M., and Hirakawa, K. (2005) *Oncol. Rep.* **14**, 1445–1451
- Shetty, S., Kumar, A., and Idell, S. (1997) *Mol. Cell Biol.* **17**, 1075–1083
- Shetty, S., and Idell, S. (2001) *J. Biol. Chem.* **276**, 24549–24556
- Shetty, S., Muniappa, H. B., Halady, P. K. S., and Idell, S. (2004) *Am. J. Respir. Cell Mol. Biol.* **31**, 100–106
- Niranjanakumari, S., Lasda, E., Brazas, R., and Garcia-Blanco, M. A. (2002) *Methods (Duluth)* **26**, 182–190
- Shetty, S., and Idell, S. (2000) *J. Biol. Chem.* **275**, 13771–13779
- Lau, H. K. F., and Ho, J. (2002) *Br. J. Haematol.* **117**, 151–158
- Kunz, C., Pebler, S., Otte, J., and Von Der Ahe, D. (1995) *Nucleic Acids Res.* **23**, 3710–3717
- Parra, M., Jardi, M., Koziczak, M., Nagamine, Y., and Munoz-Canoves, P. (2001) *J. Biol. Chem.* **276**, 36303–36310
- Takagi, M., Absalon, M. J., McLure, K. G., and Kastan, M. B. (2005) *Cell* **123**, 49–63
- Shetty, S., Velusamy, T., Idell, S., Shetty, P., Mazar, A. P., Bhandary, Y. P., and Shetty, R. S. (2007) *Mol. Cell Biol.* **27**, 5607–5618
- Pavletich, N. P., Chambers K. A., and Pabo, C. O. (1993) *Genes Dev.* **7**, 2556–2564
- Mazar, A. P., Henkin, J., and Goldfarb, R. H. (2000) *Angiogenesis* **3**, 15–32

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33. Mishra, A., Doyle, N. A., and Martin W. J., II (2000) *Am. J. Respir. Cell Mol. Biol.* **22**, 543–549
34. Sisson, T. H., Hattori, N., Xu, Y., and Simon, R. H. (1999) *Hum. Gene Ther.* **10**, 2315–2323
35. Swaisgood, C. M., French, E. L., Noga, C., Simon, R. H., and Ploplis, V. A. (2000) *Am. J. Pathol.* **157**, 77–187
36. Oren, M. (2003) *Cell Death Differ.* **10**, 431–442
37. Kortlever, R. M., Higgins, P. J., and Bernards, R. (2006) *Nat. Cell Biol.* **8**, 877–888
38. Koziczak, M., Krek, W., and Nagamine, Y. (2000) *Mol. Cell Biol.* **20**, 2014–2022
39. Strausberg, R. L., Feingold, E. A., Grouse, L. H., Derge, J. G., Klausner, R. D., Collins, F. S., Wagner, L., Shenmen, C. M., Schuler, G. D., and Altschul, S. F. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 16899–16903
40. Barak, Y., Juven, T., Haffner, R., and Oren, M. (1993) *EMBO J.* **12**, 461–468
41. Chen, C. Y., Oliner, J. D., Zhan, Q., Fornace, A. J., Jr., Vogelstein, B., and Kastan, M. B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 2684–2688
42. Shmueli, A., and Oren, M. (2004) *Mol. Cell* **13**, 4–5
43. Fu, L., and Benchimol, S. (1997) *EMBO J.* **16**, 4117–4125
44. Fu, L., Ma, W., and Benchimol, S. (1999) *Oncogene* **18**, 6419–6424
45. He, G., Siddik, Z. H., Huang, Z., Wang, R., Koomen, J., Kobayashi, R., Khokhar, A. R., and Kuang, J. (2005) *Oncogene* **24**, 2929–2943
46. Motti, M. L., Califano, D., Troncone, G., De Marco, C., Migliaccio, I., Palmieri, E., Pezzullo, L., Palombini, L., Fusco, A., and Viglietto, G. (2005) *Am. J. Pathol.* **166**, 737–749