Loss of Tumor Suppressor p53 Decreases PTEN Expression and Enhances Signaling Pathways Leading to Activation of Activator Protein 1 and Nuclear Factor κB Induced by UV Radiation

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Abstract

Transcription factor p53 and phosphatase PTEN are two tumor suppressors that play essential roles in suppression of carcinogenesis. However, the mechanisms by which p53 mediates anticancer activity and the relationship between p53 and PTEN are not well understood. In the present study, we found that pretreatment of mouse epidermal Cl41 cells with pifithrin-α, an inhibitor for p53-dependent transcriptional activation, resulted in a marked increase in UV-induced activation of activator protein 1 (AP-1) and nuclear factor κB (NF-κB). Consistent with activation of AP-1 and NF-κB, pifithrin-α was also able to enhance the UV-induced phosphorylation of c-Jun-NH_2-kinases (JNK) and p38 kinase, whereas it did not show any effect on phosphorylation of extracellular signal-regulated kinases. Furthermore, the UV-induced signal activation, including phosphorylation of JNK, p38 kinase, Akt, and $p70^{S6K}$, was significantly enhanced in p53-deficient cells (p53−/−), which can be reversed by p53 reconstitution. In addition, knockdown of p53 expression by its small interfering RNA also caused the elevation of AP-1 activation and Akt phosphorylation induced by UV radiation. These results show that p53 has a suppressive activity on the cell signaling pathways leading to activation of AP-1 and NF-κB in cell response to UV radiation. More importantly, deficiency of p53 expression resulted in a decrease in PTEN protein expression, suggesting that p53 plays a critical role in the regulation of PTEN expression. In addition, overexpression of wild-type PTEN resulted in inhibition of UV-induced AP-1 activity. Because PTEN is a well-known phosphatase involved in the regulation of phosphatidylinositol 3-kinase (PI-3K)/Akt signaling pathway, taken together with the evidence that PI-3K/Akt plays an important role in the activation of AP-1 and NF-κB during tumor development, we anticipate that inhibition of AP-1 and NF-κB by tumor suppressor p53 seems to be mediated via PTEN, which may be a novel mechanism involved in anticancer activity of p53 protein. (Cancer Res 2005; 65(15): 6601-11)

Introduction

It is well-known that UV radiation acts as both a tumor initiator and a tumor promoter, and plays major roles in the development of human nonmelanoma skin cancer (1). *p53* is the most frequently mutated gene in human cancers and >90% of human nonmelanoma skin cancers harbor p53 mutation (2). During the process of photocarcinogenesis, UV-caused specific p53 mutations occur early in the keratinocytes (2). These mutations may result in the loss of the wild-type p53 function, and further UV exposure may lead to clonal expansion of p53-mutated keratinocytes and promotion of skin tumors (2). Therefore, p53 plays a critical role in the control of the immediate and adaptive responses to UV radiation and the onset of nonmelanoma skin cancer (2). Previous studies have also shown that p53 heterozygous mice show greatly increased susceptibility to skin cancer induction, and homozygous p53 knockout mice were even more susceptible (3). Accelerated tumor development in the heterozygotes was not associated with loss of the remaining wild-type allele of p53, as reported for tumors induced by some other carcinogens, but in many cases was associated with UV-induced mutations of p53 (3). All these reports support the idea that p53 plays a role in preventing the development of skin cancer induced by UV radiation.

The p53 tumor suppressor protein is a transcription factor that enhances the transcriptional rate of several genes involved in transducing signals from DNA damage (4–6). It is elevated in response to genotoxic agents, such as UV light, ionizing radiation, or certain chemicals (6). The activation of p53 has been implicated in cell cycle control, DNA repair, and apoptosis (7–9). The function of p53 is regulated at the levels of transcription, translation, protein modification and turnover, and cellular compartmentalization, as well as association with other proteins (10). However, the mechanisms by which p53 mediates anticancer activity is not completely understood although its activities in regulation of cell cycle and DNA repair, as well as apoptosis induction, are thought to participate in its anticancer activity (11–13). In the present study, we found that UV-induced signaling pathways related to activation of activator protein 1 (AP-1) and nuclear factor κB (NF-κB) were significantly increased in either the cells pretreated with a p53 inhibitor, pifithrin-α, or in the cells with p53 deficiency, suggesting that basal level of p53 normally has an inhibitory effect on signaling pathways leading to activation of AP-1 and NF-κB in cell response to UV radiation.

PTEN is a tumor suppressor that is a negative regulator of PI-3K/Akt pathway by dephosphorylation of the lipid second

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message, PI(3,4,5)P3, a product of phosphatidylinositol 3-kinase (PI-3K; ref. 14). PTEN has been shown to be involved in regulation of the cell apoptosis (15), cell cycle entry (16), cell proliferation (17), and cell adhesion (18). Although PTEN protein appears to be phosphoprotein in cells, its phosphorylation level is not regulated in cell response to extracellular stimuli (19). Thus, investigation of mechanisms involved in regulation of PTEN expression is a key step for understanding its tumor suppression function. In the studies presented here, we found that there is decreased PTEN protein expression in p53-deficient cells compared with that in p53 normally expressed cells. This may be the molecular basis by which p53 down-regulates signaling pathway leading to activation of AP-1 and NF-κB in cell response to UV radiation.

Materials and Methods

Reagents and plasmids. The p53-specific inhibitor pifithrin-α, p38 kinase inhibitor SB202190, and c-Jun-NH_2-kinase (JNK) inhibitor II were purchased from Calbiochem (La Jolla, CA); luciferase assay substrate was obtained from Promega (Madison, WI); fetal bovine serum (FBS), Eagle's MEM, and DMEM were purchased from BioWhittaker (Walkersville, MD). Antibodies specifically targeting phospho- and total-PTEN, p53, Akt, $p70^{S6k}$, and mitogen-activated protein kinase (MAPK) family members, including extracellular signal-regulated kinases (ERK), JNKs, and p38 kinase, were purchased from Cell Signaling Technology (Beverly, MA). Antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Abcam (Cambridge, MA). AP-1–luciferase reporter (AP-1-Luc) containing seven tandem AP-1 binding sites (TGACTAA) and NF-κB–luciferase reporter (NF-κB-Luc) containing five tandem NF-κB binding sites (TGGGGACTTTCCGC) were purchased from Stratagene (La Jolla, CA). p53-luciferase is pG13 containing 13 tandem p53 binding sites as described previously (20, 21). Mouse p53 small interfering RNA (siRNA) construct was a generous gift from Dr. Michelle Craig Barton (Department of Biochemistry and Molecular Biology, University of Texas MD Anderson Cancer Center, Houston, TX) and described in his previously published studies (22). Enhanced green fluorescent protein (EGFP)-PTEN fusion protein expression plasmid was constructed as described previously (23) and was a gift from Dr. Xia Zhang (Neuropsychiatry Research Unit, University of Saskatchewan, Saskatoon, Saskatchewan, Canada).

Cell culture. The JB6 P^+ mouse epidermal cell line Cl41 and its stable transfectants with various luciferase reporters (20, 21, 24, 25) were cultured in monolayers at 37°C under 5% CO_2 using MEM containing 5% FBS, 2 mmol/L L-glutamine, and 25 μg/mL gentamicin. Normal embryonic fibroblasts (p53+/+) or p53-deficient embryonic fibroblasts (p53−/−), as well as their transfectants, were cultured in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, and 25 μg/ml gentamicin (21, 26). The cultures were dissociated with trypsin and transferred to new 75 cm^2 culture flasks (Fisher, Pittsburgh, PA) from once to thrice per week.

Transfection. Cl41 cells were transfected with pAP-1-Luc plasmid alone or in combination with mouse p53 siRNA construct according to the LipofectAMINE 2000 reagent manual. Briefly, Cl41 cells were cultured in a six-well plate to 85% to 90% confluence. Five micrograms of plasmid DNA in combination with cytomegalovirus-neo vector for cotransfection, mixed with 10 μL of LipofectAMINE 2000 reagent, were used to transfect each well in the absence of serum. After 6 hours, the medium was replaced with 5% FBS MEM. Approximately 30 to 36 hours after the beginning of the transfection, the cells were digested with 0.033% trypsin and cell suspensions were plated onto 75 mL culture flasks and cultured for 24 to 28 days with G418 selection (400 μg/mL). Stable transfectant was established and cultured in G418-free DMEM for at least two passages before each experiment.

p53−/− fibroblasts were transiently transfected with AP-1-Luc, NF-κB-Luc alone, or in combination with either murine p53 expression plasmid or EGFP-PTEN fusion protein expression plasmid. The transfected cells were exposed to UV radiation for luciferase assay, Western Blot assay, or flow cytometry analysis 72 hours after the beginning of the transfection as described in the figure captions.

Activator protein 1 and nuclear factor-κB activity assay. Confluent monolayers of AP-1-Luc– or NF-κB-Luc–transfected cells were trypsinized, and 8×10^3 viable cells were added to each well of 96-well plates. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. After the cell density reached 80% to 90%, cells were treated as indicated in the figure captions. At different time periods after treatment, the cells were extracted with lysis buffer (Promega), and their luciferase activity was determined by the luciferase assay using a luminometer (Wallac 1420 Victor 2 multipliable counter system) after the addition of 50 μL of lysis buffer for 30 minutes at 4°C. The results are expressed as AP-1 or NF-κB activity relative to control medium (relative AP-1 or NF-κB activity; refs. 21, 26).

Assay for p53 activity. Confluent monolayers of Cl41 p53 mass1 cells were trypsinized, and 8×10^3 viable cells suspended in 100 μL of 5% FBS MEM were added to each well of 96-well plates. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. After the cell density reached 80% to 90%, cells were pretreated with pifithrin-α for 30 minutes at the concentrations indicated in the figure captions. The cells were then exposed to UVB or UVC radiation at doses indicated in the figure captions. At different time periods after treatment, the cells were extracted with lysis buffer (Promega) and their luciferase activity was determined by the luciferase assay using a luminometer (Wallac 1420 Victor 2 multipliable counter system) after the addition of 50 μL of lysis buffer for 30 minutes at 4°C. The results are expressed as p53 activity relative to control medium (relative p53 activity; refs. 20, 21).

Mitogen-activated protein kinase phosphorylation assay. Cl41 cells, normal embryonic fibroblasts (p53+/+), or p53-deficient embryonic fibroblasts (p53−/−), as well as their transfectants, were cultured in monolayers in six-well plates (26–28). After the cell density reached 70% to 80%, the cell culture medium was replaced with the medium supplemented with 0.1% FBS, 2 mmol/L L-glutamine, and 25 μg/ml of gentamicin per milliliter and cultured for 33 hours. Cells were incubated in serum-free medium for 3 to 4 hours at 37°C. Cells were exposed to UVB or UVC radiation at doses indicated in the figure captions. Cells were then extracted with Tris-glycine SDS sample buffer (Invitrogen, Carlsbad, CA). Western blots were done with either phosphospecific antibodies or pan antibodies against various kinases, including ERKs, JNKs, and p38 kinases. The protein band specifically bound to the primary antibody was detected using an anti-rabbit IgG-AP–linked and ECF Western blotting system (Amersham Biosciences, Piscataway, NJ; ref. 29).

Phosphorylation assay for Akt and $p70^{S6k}$. Thirty thousand Cl41 cells, normal embryonic fibroblasts (p53+/+), or p53-deficient embryonic fibroblasts (p53−/−), as well as their transfectants, were cultured in each well of six-well plates to 70% to 80% confluence with normal culture medium (26–28). The cell culture medium was replaced with 0.1% FBS medium with 2 mmol/L L-glutamine and 25 μg/ml of gentamicin and cultured for 33 hours. Cells were incubated in serum-free medium for 3 to 4 hours at 37°C. Cells were exposed to UVB or UVC radiation at doses indicated in the figure captions. Cells were washed once with ice-cold PBS and extracted with SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with one of the antibodies, including rabbit phosphospecific Akt (Thr^{308}) antibody, phosphospecific Akt (Ser^{473}) antibody, pan Akt antibody, phosphospecific $p70^{S6k}$ (Thr^{389}), phosphospecific $p70^{S6k}$ (Ser^{421}/Ser^{424}), and pan $p70^{S6k}$ antibody. The Akt and $p70^{S6k}$ protein bands specifically bound to primary antibodies were detected using an anti-rabbit IgG-AP–linked and ECF Western blotting system (30).

PTEN expression assay. Thirty thousand normal embryonic fibroblasts (p53+/+) or p53-deficient embryonic fibroblasts (p53−/−) were cultured in each well of six-well plates to 90% confluence with normal culture medium (27, 28). The cell culture medium was replaced with 0.1% FBS DMEM with 2 mmol/L L-glutamine and 25 μg/ml gentamicin and cultured for 33 hours. Cells were incubated in serum-free MEM for 3 to 4 hours at 37°C. Then, cells were exposed to UVB or UVC radiation at doses indicated in the figure captions. Cells were washed once with ice-cold PBS and extracted with SDS

sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with one of the antibodies, including rabbit phosphospecific PTEN (Ser^{380}) antibody and PTEN antibody. The PTEN protein bands specifically bound to primary antibodies were detected using an anti-rabbit IgG-AP–linked and ECF Western blotting system.

Statistical analysis. The significance of the difference between treated and untreated groups were determined with the Student's *t* test. The results are expressed as mean ± SE.

Results

Treatment of cells with pifithrin-α resulted in activation of activator protein 1 and nuclear factor-κB. AP-1 and NF-κB have served to detect one of the decisive DNA-binding motifs required for gene regulation by tumor promoters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and UV irradiation (31). Previous studies suggest that AP-1 plays a crucial role in tumor promoter–induced cell transformation (24). To investigate the potential role of p53 activation in the regulation of signaling pathways leading to activation of AP-1 and NF-κB in Cl41 cells, we first measured the effects of pifithrin-α, a p53 inhibitor that was discovered by Komarov et al. (32) and is now widely used as the inhibitor in p53 studies (33–35), on activation of AP-1 and NF-κB in mouse JB6 epidermal C141 cells. The results showed that pifithrin-α treatment alone was able to induce activation of AP-1 and NF-κB in Cl41 cells (Fig. 1*A* and *B*). The induction of AP-1 and NF-κB by pifithrin-α was observed in all time points tested (Fig. 1*C*). These results indicated that inhibition of p53 by pifithrin-α could result in activation of AP-1 and NF-κB in Cl41 cells, suggesting that normal p53 protein expression may function as an inhibitor in the regulation of basal level of AP-1 and NF-κB activity.

Inhibition of p53 led to increase in UV-induced activator protein 1 activities. Results from above studies suggested that p53 may provide an inhibitory signaling for AP-1 activation. If this is the case, the inhibition of AP-1 activation by p53 may be more significant in the Cl41 cells response to UV radiation. To carry out this investigation, both p53-luciferase reporter stable transfectant, Cl41 p53 mass1, and AP-1 luciferase reporter stable transfectant, P^+1-1, were pretreated with pifithrin-α for 30 minutes and then exposed to UV radiation. Pretreatment of cells with pifithrin-α led to dramatic inhibition of UV-induced p53-dependent transactivation (Fig. 2*A* and *B*), whereas it markedly increased UV-induced AP-1 transactivation (Fig. 2*B*). Moreover, the inhibition of p53-dependent transactivation occurred before the increase in AP-1 transactivation (Fig. 2*C*). Because it has been reported that pifithrin-α is also able to inhibit heat shock and glucocorticoid receptor signaling (35), p53 siRNA was further used to address the causality between loss of p53 function and elevation of AP-1 activation. The results indicated that p53 siRNA can inhibit the expression of p53, which subsequently leads to the decrease in phosphorylation of p53 at Ser^{15} induced by UV radiation (Fig. 2*D* and *E*). More importantly, knockdown of p53 expression in Cl41 cells by its specific siRNA caused the elevation of UV-induced AP-1 activation (Fig. 2*F*). These results, together with the induction of AP-1 activity by pifithrin-α alone, indicate that p53 is able to not only inhibit basal level of AP-1 activity, but also exhibits a strong inhibition of UV-induced AP-1 activation, which may participate in the anticancer activity of p53 in cells exposed to carcinogenic factors, such as UV radiation.

p53-modulated, UV-induced activator protein 1 activation through upstream p38 kinase and c-Jun-NH_2-kinases. Because our results indicated that inhibition of p53 transactivation by

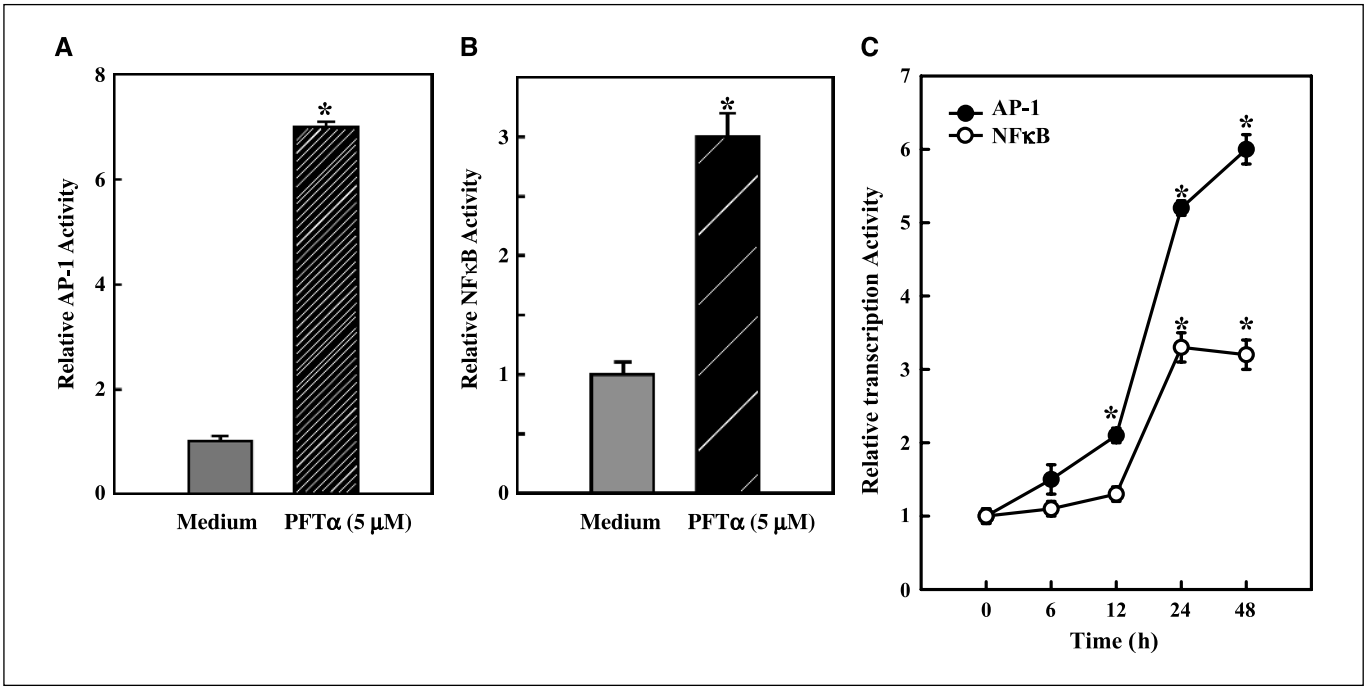

Figure 1. Activation of AP-1 and NF-κB by pifithrin-α (*PFTα*) in Cl41 cells. Eight thousand P^+1-1 or Cl41 NF-κB mass1 cells were seeded into each well of 96-well plates, and cultured in 5% FBS MEM at 37°C. After the cell density reached 80% to 90%, the cells were treated with 5 μmol/L pifithrin-α. After incubation for 24 hours (*A* and *B*) or at various time points as indicated (*C*), cells were extracted with lysis buffer, and luciferase activity was measured using Promega luciferase assay reagent with a luminometer after addition of 50 μL of lysis buffer for 30 minutes at 4°C. The results are expressed as AP-1 or NF-κB induction relative to control medium. *Columns,* mean of triplicate assay wells; *bars,* SD. *, significant increase from medium control ($P < 0.05$).

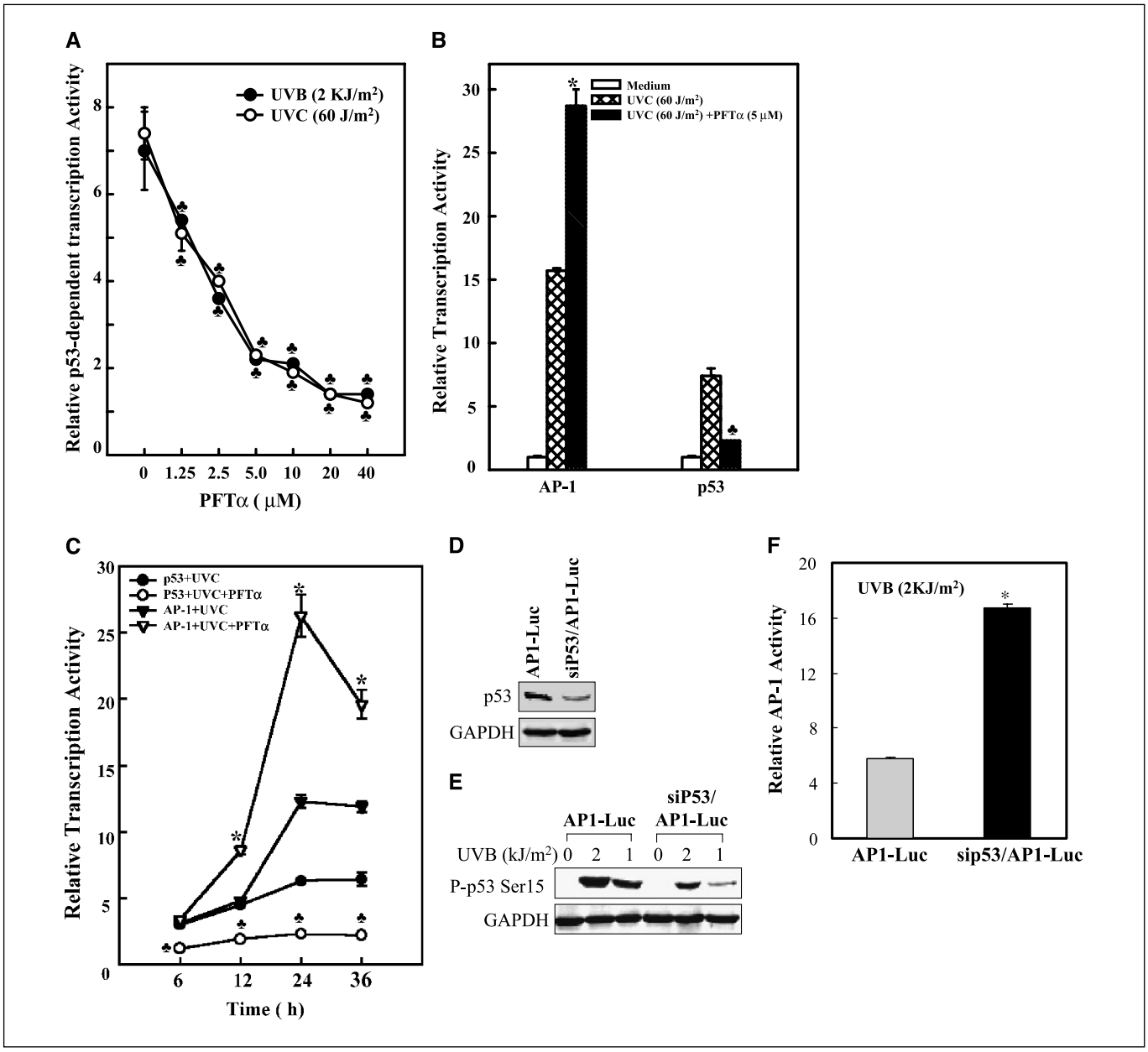

Figure 2. Inhibition of p53-dependent transactivation by pifithrin-α or its siRNA led to increase in UV-induced AP-1 activity. Eight thousand C141 p53 mass1 or P⁺1-1 cells were seeded into each well of 96-well plates and cultured in 5% FBS MEM at 37°C. After the cell density reached 80% to 90%, the cells were first pretreated with various concentrations of pifithrin-α for 30 minutes, then exposed to UVB (2 kJ/m²) or UVC (60 J/m²) for p53 induction (*A*); the cells were first pretreated with 5 μmol/L pifithrin-α for 30 minutes, then exposed to UVC (60 J/m²) for AP-1 or p53 induction (*B*). After a 24-hour incubation, cells were extracted with lysis buffer, and luciferase activity was measured using Promega luciferase assay reagent with a luminometer after addition of 50 μL lysis buffer for 30 minutes at 4°C. *C,* the cells were treated as described in (*B*) and then extracted for luciferase assay at different time points as indicated. The results are expressed as p53 or AP-1 induction relative to control medium. *Points,* mean of triplicate assay wells; *bars,* SD. *, significant increase from UV radiation ($P < 0.05$); ♣, significant decrease from UV radiation ($P < 0.05$). *D* and *E,* Cl41 p53 siRNA transfectant (Cl41 sip53/AP-1-Luc) and its vector control transfectant (Cl41 AP-1-Luc) were exposed to UVB radiation at doses as indicated. After a 12-hour incubation, the cells were washed once with ice-cold PBS and extracted with the SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with antibody against p53 (*D*) or phosphorylated p53 (Ser[15]; *E*). The protein band specifically bound with primary antibodies was detected by using anti-rabbit IgG-AP–linked and ECF Western blotting system. *F,* Cl41 AP-1-Luc and Cl41 sip53/AP-1-Luc cells were exposed to UVB (2.0 kJ/m²) and then extracted with lysis buffer 24 hours after the UVB exposure. The luciferase activity was measured as described in Fig. 1. The results are expressed as AP-1 induction relative to control medium. *, significant increase from vector control transfection ($P < 0.05$).

either pifithrin-α increased UV-induced AP-1 activation, it was important to know whether the increased AP-1 induction was due to p53 direct effect on AP-1 complex or AP-1 upstream kinase. Hence, we observed the effects of pifithrin-α on UV-induced activation of MAPKs family, including ERKs, JNKs, and p38 kinase, in C141 cells. As shown in Fig. 3, UVC radiation alone resulted in phosphorylation of JNKs and p38 kinase (Fig. 3), and pifithrin-α treatment resulted in marked enhancement of UV-induced phosphorylation of JNKs and p38 kinase, whereas it did not show any increased activation of ERKs and protein expression of these kinases (Fig. 3). Moreover, the elevation of AP-1 activity in p53 siRNA-transfected Cl41 cells was reversed by

the treatment of cells with JNKs and p38 inhibitors (Fig. 3*B*). These results indicate that p53 might play an important role in the regulation of AP-1 upstream kinases, p38 kinase, and JNKs.

Loss of p53 increased the phosphorylation of p38 kinase and c-Jun-NH_2-kinase, but not extracellular signal-regulated kinases, in UV responses. To obtain direct evidence for the involvement of p53 in UV-induced AP-1 activation, we then compared UV-induced phosphorylation of MAPK family members, including JNKs, p38 kinase, and ERKs, between wild-type p53 (p53+/+) and p53-deficient (p53−/−) fibroblast cell lines derived from mouse embryos (27, 28). Very interestingly, the results from studies of UV-induced MAPK activation showed that UV-induced phosphorylation of JNKs and p38 kinase were increased significantly in p53−/− cells compared with those in p53+/+ cells (Figs. 4*A* and *B*), whereas it did not show any effect on phosphorylation of ERKs (Fig. 4*C*). These data suggest that p53 deficiency specifically leads to increase in activation of p38 kinase and JNKs, but not ERKs, compared with p53 wild-type cells in UV responses.

Loss of p53 function increased the phosphorylation of Akt and $p70^{S6k}$ induced by UV radiation. Because our recently published data show that UV radiation is able to induce phosphorylation of Akt and $p70^{S6K}$ in Cl41 cells (36) and Akt phosphorylation is a mediator for activation of AP-1 and JNKs in Cl41 cells response to benzo(*a*)pyrene diol epoxide [B(*a*)PDE] exposure (30), we further investigated the effects of pifithrin-α on UV-induced phosphorylation of Akt and $p70^{S6k}$ in Cl41 cells. The results showed that pretreatment of cells with pifithrin-α led to a significant increase in UV-induced phosphorylation of Akt at Ser^{473} and Thr^{308} and $p70^{S6k}$ at Thr^{389} and Thr^{421}/Ser^{424} (Fig. 5*A*), indicating that an inhibition of p53 may lead to increase in the phosphorylation of Akt and $p70^{S6k}$ in mouse epidermal Cl41 cell response to UV radiation. To obtain a direct evidence for involvement of p53 in the regulation of Akt and $p70^{S6k}$, we further compared UV-induced phosphorylation of Akt and $p70^{S6k}$ between wild-type p53 (p53+/+) and p53-deficient (p53−/−) cells (27, 28). As shown in Fig. 5*B* and *C*, deficiency of p53 function by knockout of *p53* gene (p53−/−) significantly increased the phosphorylation of Akt at Ser^{473} and Thr^{308} and $p70^{S6k}$ at Thr^{389} and Thr^{421}/Ser^{424} in cell response to UV radiation compared with those in wild-type p53 (p53+/+) cells. Furthermore, we also observed that inhibition of p53 with its specific siRNA enhanced UV-induced phosphorylation of Akt in Cl41 cells (Fig. 5*D*). Taken together with the evidence that activation of Akt and $p70^{S6k}$ plays an important role in tumor development (37), these results suggest that p53 protein expression may function as an inhibitor of signaling pathways involved in UV-induced carcinogenesis.

p53 reconstitution down-regulated UV-induced activator protein 1, nuclear factor-κB, Akt, and mitogen-activated protein kinase activation. To further confirm that the elevation of AP-1, NF-κB, Akt, and MAPK activation in p53−/− cells in UV response directly resulted from the deficiency of p53, we transfected wild-type mouse p53 back into p53−/− cells and then compared the activation of these signal pathways between p53−/− and p53−/− with wild-type p53 reconstitution cells after UV radiation. The results showed that reconstitution of p53 led to obvious decreases in AP-1 and NF-κB transcriptional activation, phosphorylation of Akt, JNKs, and p38 kinase (Fig. 6*A-E*). It may be notable that the difference in JNK phosphorylation between p53−/− and p53−/− with wild-type p53 reconstitution cells is not as obvious as that between p53−/− and wild-type fibroblasts. This may be explained by the fact that the reconstituted p53 expression in p53−/− cells is a constitutive overexpression, which acts in a different manner from that in wild-type cells. Overall, these data provide reliable evidence to support our notion that p53 deficiency could cause activation of Akt, p38 kinase, and JNKs.

p53 deficiency led to reduction of PTEN expression. Because previous studies indicated that PTEN was able to effectively antagonize PI-3K/Akt signaling pathway, it is reasonable for us to investigate the role of p53 in the regulation of PTEN expression

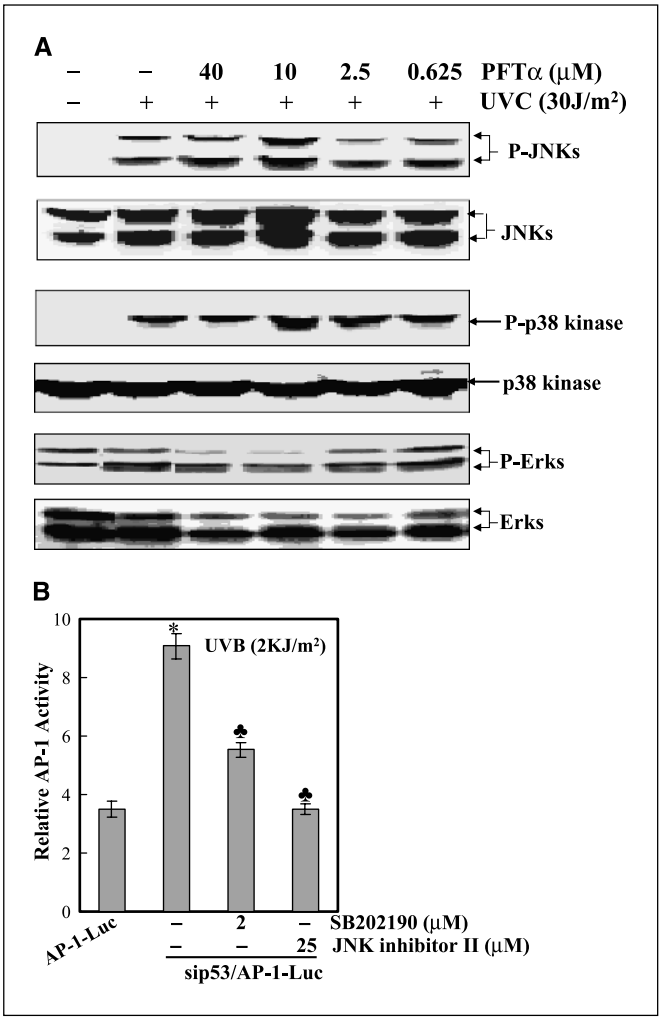

Figure 3. Inhibition of p53 by pifithrin-α resulted in increased UV-induced p38 kinases and JNKs, but not ERKs. Thirty thousand Cl41 cells were seeded into each well of six-well plates, and cultured in 5% FBS MEM at 37°C. After the cell density reached 70% to 80%, the cell culture medium was replaced with 0.1% FBS MEM. Thirty-three hours later, cells were incubated in serum-free MEM for 3 to 4 hours at 37°C. Cells were first pretreated with various concentrations of pifithrin-α for 30 minutes and then exposed to UVC (30 J/m^2). After a 60-minute incubation, cells were washed once with ice-cold PBS and extracted with a SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with either phosphospecific antibodies or pan antibodies against JNKs, p38 kinases, and ERKs. The protein band specifically bound with primary antibodies was detected by using anti-rabbit IgG-AP–linked and ECF Western blotting system. *B,* Cl41-sip53/AP-1-Luc cells were pretreated with 2 μmol/L SB202190 or 25 μmol/L JNK inhibitor II for 30 minutes. Then, Cl41-AP-1-Luc and the pretreated Cl41-sip53/AP-1-Luc cells were exposed to UVB (2 kJ/m^2) for AP-1 induction. After a 12-hour incubation, cells were extracted with lysis buffer, and luciferase activity was measured as described in Fig. 1. The results are expressed as AP-1 induction relative to control medium. *, significant increase from vector control transfection ($P < 0.05$); ♣, significant decrease from medium control ($P < 0.05$).

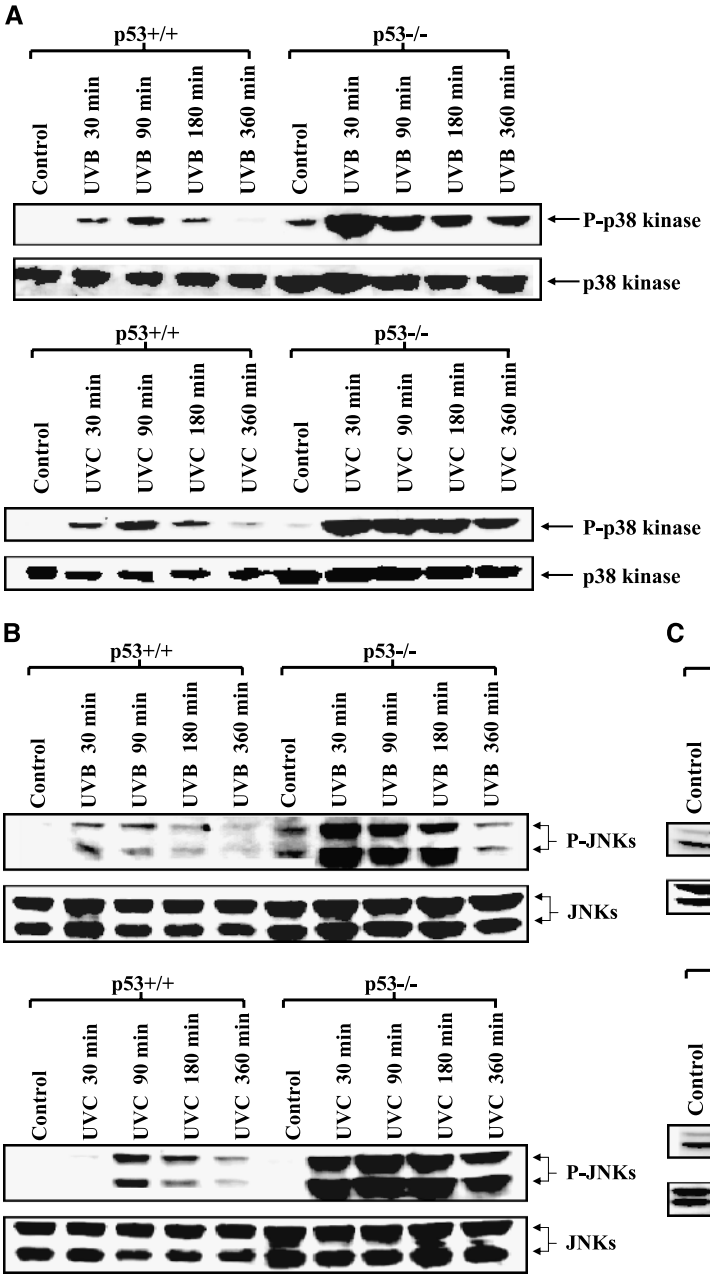

Figure 4. Loss of p53 protein expression led to increase in UV-induced MAPK activation. Thirty thousand p53+/+ or p53−/− embryonic fibroblasts were seeded into each well of six-well plates and cultured in 10% FBS DMEM at 37°C. After the cell density reached 70% to 80%, the cell culture medium was replaced with 0.1% FBS DMEM. Thirty-three hours later, cells were incubated in serum-free DMEM for 3 to 4 hours at 37°C, and then exposed to UVB (2 kJ/m^2) and UVC (30 J/m^2) radiation. After incubation at various time points as indicated, cells were washed once with ice-cold PBS and extracted with a SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with either phosphospecific antibodies or pan antibodies against p38 kinases (*A*), JNKs (*B*), and ERKs (*C*). The protein band specifically bound with primary antibodies was detected by using anti-rabbit IgG-AP–linked and ECF Western blotting system.

in wild-type p53 (p53+/+) and p53-deficient (p53−/−) cell lines. As shown in Fig. 7*A*, UVB and UVC radiation resulted in no alteration of expression and phosphorylation of PTEN in p53 wild-type (p53+/+) cells. However, deficiency of p53 expression by p53 knockout significantly decreased the phosphorylated and total PTEN protein expression compared with those in p53+/+ cells (Fig. 7*B*), which is consistent with previous findings that p53 is an activator of PTEN transcription through a p53-responsive element within the PTEN promoter (38). Moreover, transfection of PTEN into p53−/− cells (Fig. 7*C*) also resulted in a decrease in AP-1 transactivation (Fig. 7*D*). Taken together with the above results that p53 deficiency increased the phosphorylation of Akt and p70^{S6k} in the same cell culture models, and in view of the inhibitory effect of PTEN on phosphorylation of Akt and p70^{S6k} (14), we anticipate that basal level of p53 expression may inhibit UV-induced activation of Akt and p70^{S6k} by regulating the expression of PTEN protein.

Discussion

Transcription factor *p53* is one of the classic tumor suppressor genes that interfere with cancer development (39). Although molecular mechanisms involved in anticancer effects of p53 have attracted a great deal of attention in the last decades, many questions, specifically for the role of p53 in down-regulation of tumor promotion–related signaling pathways, are not well explored

(40). The present studies show that the p53 protein functions as a down-regulator in both basal level of AP-1 activities and UV-induced level of AP-1 activities. This conclusion was based on the findings that the inhibition of normal p53 function by either p53 chemical inhibitor, knockdown of p53 expression with its specific siRNA, or knockout of *p53* gene resulted in AP-1 activation and increased UV-induced AP-1 activities. We also found that inhibitory effects of p53 on AP-1 activation are due to its specific inhibition of the p38 kinase and JNK pathways, but not the ERK pathway. In addition, activation of Akt/p70^{S6k}, an important signaling pathway

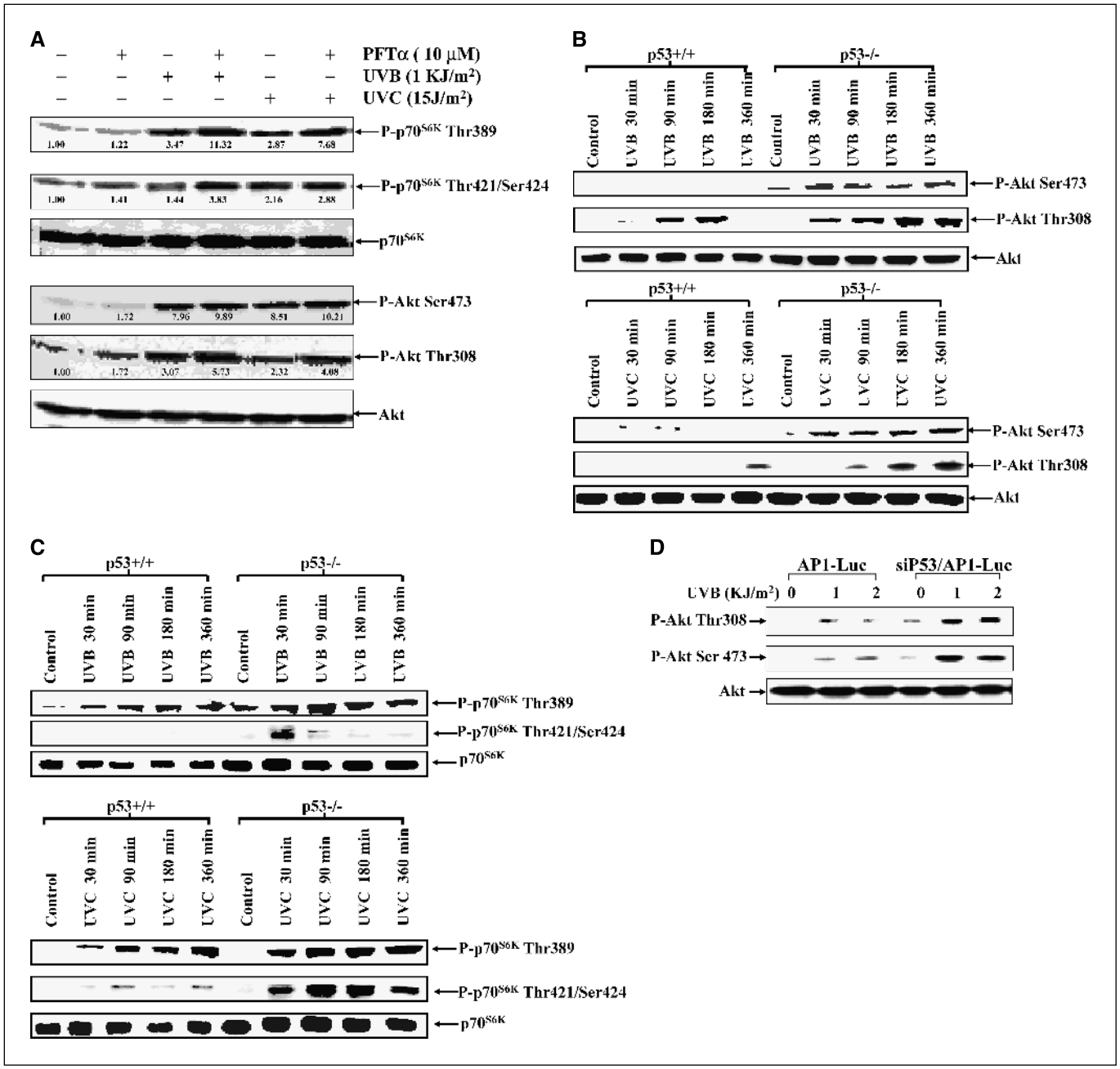

Figure 5. Effects of p53 on UV-induced phosphorylation of Akt and p70^{S6k}. *A,* 3×10^4 Cl41 cells were seeded into each well of six-well plates, and cultured in 5% FBS MEM at 37°C. After the cell density reached 70% to 80%, the cell culture medium was replaced with 0.1% FBS MEM. Thirty-three hours later, cells were incubated in serum-free MEM for 3 to 4 hours at 37°C. Cells were first pretreated with pifithrin-α for 30 minutes and then exposed to UVB (1 kJ/m^2) or UVC (15 J/m^2). After a 60-minute incubation, cells were washed once with ice-cold PBS and extracted with a SDS sample buffer. Western blot was done with either phosphospecific antibodies or pan antibodies against Akt and p70^{S6k}. The number below the bands indicates its relative quantity. *B* and *C,* 3×10^4 p53+/+ or p53−/− embryonic fibroblasts were seeded into each well of six-well plates, and cultured in 10% FBS DMEM at 37°C. After the cell density reached 70% to 80%, the cell culture medium was replaced with 0.1% FBS DMEM. Thirty-three hours later, cells were incubated in serum-free DMEM for 3 to 4 hours at 37°C, and then exposed to UVB (2 kJ/m^2) and UVC (30 J/m^2) radiation. After incubation at various time points as indicated, cells were washed once with ice-cold PBS and extracted with a SDS sample buffer. The cell extracts were used for Western blot and probed with either phosphospecific antibodies or pan antibodies against Akt and p70^{S6k}. The protein band specifically bound with primary antibodies was detected by using anti-rabbit IgG-AP–linked and ECF Western blotting system. The number below the bands in (*B*) indicates its quantity relative to the medium control. *D,* Cl41 AP-1-Luc and Cl41 sip53/AP-1-Luc cells were seeded and treated as described above. Ninety minutes after exposure to UVB radiation, the cells were extracted and analyzed by Western blot with antibodies against Akt or phosphorylated Akt.

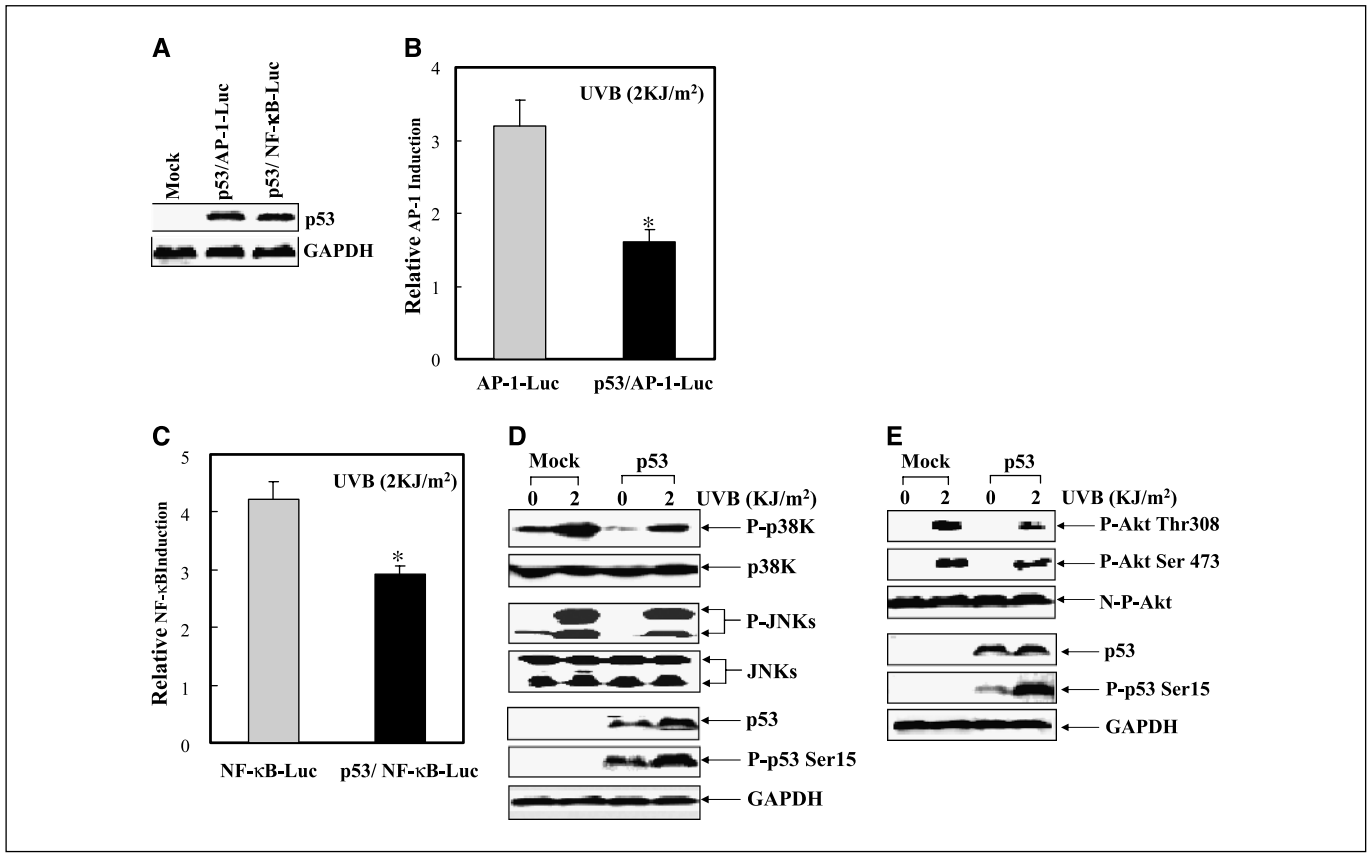

Figure 6. Reconstitution of p53 down-regulated AP-1 and NF-κB transactivation and phosphorylation of p38 kinase, JNK, and Akt. p53−/− embryonic fibroblasts were transiently transfected with pAP-1-Luc and pNF-κB-Luc alone or in combination with mouse wild-type p53 expression vector as described in Materials and Methods. *A,* the transfected cells were extracted with the SDS sample buffer, and p53 expression was identified by Western blot with anti-p53 antibody. *B* and *C,* 1×10^4 transfected cells were seeded into each well of 48-well plates, and cultured in 10% FBS DMEM at 37°C. After the cell density reached 80% to 90%, the cells were exposed to UVB (2 kJ/m^2) radiation. After incubation for 12 hours, cells were extracted with lysis buffer, and luciferase activity was measured as described in Fig. 1. The results are expressed as AP-1 and NF-κB induction relative to control medium. *Columns,* mean of triplicate assay wells; *bars,* SD. *, significant decrease from mock transfections ($P < 0.05$). *D* and *E,* 3×10^4 p53−/− embryonic fibroblasts transiently transfected with mock vector or mouse p53 were seeded into each well of six-well plates and cultured in 10% FBS DMEM at 37°C. After the cell density reached 70% to 80%, the cell culture medium was replaced with 0.1% FBS DMEM. Thirty-three hours later, cells were incubated in serum-free DMEM for 3 hours at 37°C and then exposed to UVB (2 kJ/m^2) radiation. After incubation for 60 minutes, cells were extracted with the SDS sample buffer and analyzed by Western blot with antibodies as indicated. GAPDH was used as a protein loading control.

required for tumor promotion, was also down-regulated by p53 because inhibition or knockout of p53 resulted in significant increases in phosphorylation of Akt at Ser^{473} and Thr^{308}, and p70^{S6k} at Thr^{389} and Thr^{421}/Ser^{424}. Furthermore, we found that the expression of PTEN, a tumor suppressor that is a negative regulator of PI-3K/Akt pathway, was decreased in p53-deficient embryonic fibroblast cell lines. All these data showed that anticancer activity of p53 is not only mediated by its regulation of DNA damage repair, growth arrest, and apoptosis, but also by its down-regulation of cancer promotion-associated signaling pathways.

p53 was first described as a cellular phosphorprotein that coprecipitated with the large T antigen of SV40, whose synthesis was enhanced in chemically transformed tumors (41). In the last 20 years, its role and the molecular mechanisms involved in p53 tumor suppression have attracted great attention. However, most of those studies focused on its regulation of DNA damage and its repair, cell cycle arrest, and apoptosis. It is known that p53 controls an essential growth checkpoint that protects against both genomic rearrangement and the accumulation of mutations caused by exposure of carcinogens (42). For example, UV radiation is able to activate p53 by induction of its protein expression, phosphorylation, and acetylation (42, 43). Once p53 is activated, it can lead to cell cycle arrest or apoptosis (i.e., damaged cells undergo DNA repair or severely damaged cells are discarded; ref. 42). The exact outcome of p53 induction may depend on the cell types and strength of the UV exposure. Because p53 is the most commonly (over 50%) mutated gene associated with human tumors, it seems to be localized at a central place that can receive signals via various pathways and control numerous downstream pathways (40, 44). Therefore, in addition to its important role in the control of cell cycle, apoptosis, and DNA repair, p53 may also affect the signaling pathways that are directly involved in cell growth and transformation. This hypothesis is indirectly supported by the previous findings that p53 can suppress cell transformation caused by oncogene activation (45). To address this issue, the present studies directly investigated the potential effects of p53 on basal level and UV-induced level of AP-1 and NF-κB activities. Our data shown here indicate that p53 inhibition leads to the induction of AP-1 and NF-κB, as well as the increase in UV-induced AP-1 activities. Because AP-1 and NF-κB are two key transcription factors that are required for tumor development, which has been well shown both *in vitro* and *in vivo* in previous studies (46, 47), we anticipate that p53 normally functions as an inhibitor of AP-1 and NF-κB signaling pathways to exert its anticancer activity.

Substantial contributions have been made to elucidate the signal transduction pathways involved in UV-induced activation of AP-1 and NF-κB. Of particular interest are the MAPK signal transduction pathways, including the ERKs, JNKs, and the p38 kinase, which control the activities of various transcription factors, including AP-1 and NF-κB (48). ERKs are activated and play a critical role in transmitting signals initiated by TPA and growth factors, such as epidermal growth factor and platelet-derived growth factor (48). JNKs and p38 kinases are potently activated by various forms of inflammatory signals or stress, including UV radiation (48). Recent studies indicate that wild-type p53 inhibits the activation of transcription factor Net, an effector of the Ras oncogene/MAPK pathway (49). Loss of p53 *in vivo* leads to an increase of Net phosphorylation in skin response to UV radiation (49). However, the exact effect of p53 on MAPK activation induced by UV radiation remains unclear. In this study, we found that inhibition or knockout of p53 resulted in an increase in the phosphorylation of JNKs and p38 kinases, but not ERKs, induced by UV radiation. Taken together with the evidence that MAPK signaling pathways play important roles in UV-induced AP-1 activation (50–52) and p53 functions as a main tumor suppressor, these results indicate that inhibitory effect of p53 on AP-1 activation may be due to its specific inhibition of the p38 kinase and JNK pathways, but not the ERK pathway.

The activation of Akt depends on phosphorylation of four sites, including Ser^{124}, Thr^{450}, Thr^{308}, and Ser^{473} (53). Mutagenesis studies have suggested that phosphorylation of Thr^{308} and Ser^{473} is required for Akt activity, whereas Ser^{124} and Thr^{450} seem to be basally phosphorylated (53). The activation of $p70^{S6k}$ is attributable to phosphorylation of Ser/Thr residues on multiple sites, such as Thr^{389}, Ser^{424}, and Thr^{421} (54). Upon activation, $p70^{S6k}$ phosphorylates the S6 protein of the 40S ribosomal subunit, resulting in increase of the production of translational machinery components, such as ribosomal proteins and elongation factors (54). Previous studies have shown that the activation of PI-3K/Akt/$p70^{S6k}$ pathways is required for AP-1 activation in cell responses to growth factors and oxidative stress (36, 55). In the present study, we found that inhibition or knockout of p53 resulted in a dramatic increase in the UV-induced phosphorylation of Akt at Ser^{473} and Thr^{308}, and $p70^{S6k}$ at Thr^{389} and Thr^{421}/Ser^{424}, suggesting that suppression of AP-1 activation by normal p53 could be mediated by targeting Akt/$p70^{S6k}$ pathway.

PTEN is an important tumor suppressor frequently mutated in human cancers and is a negative regulator of PI-3K/Akt–dependent cell survival (14, 56). Despite a relatively good understanding of the

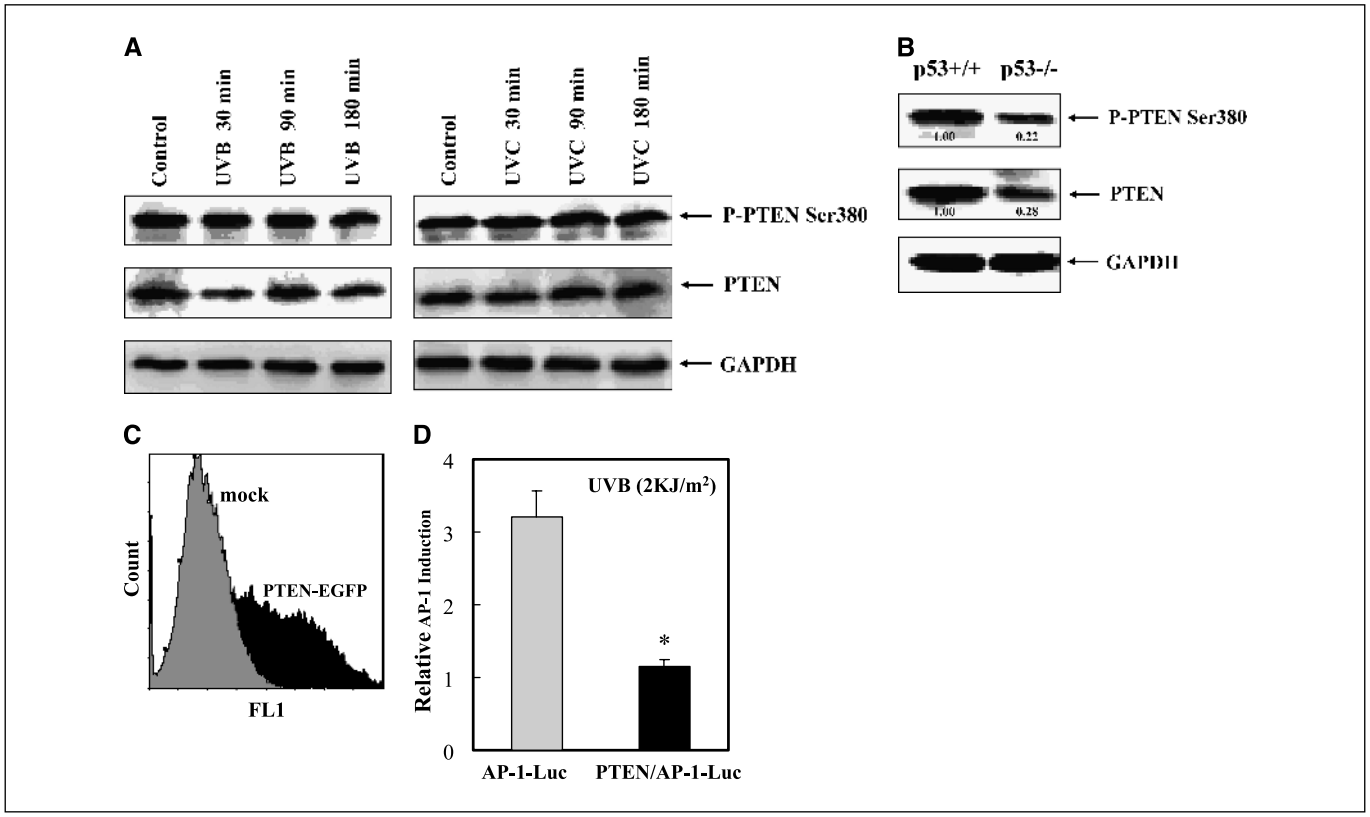

Figure 7. Loss of p53 caused the decrease in PTEN expression in embryonic fibroblast cells. Thirty thousand p53+/+ or p53−/− embryonic fibroblasts were seeded into each well of six-well plates, and cultured in 10% FBS DMEM at 37°C. After the cell density reached 70% to 80%, the cell culture medium was replaced with 0.1% FBS DMEM. Thirty-three hours later, cells were incubated in serum-free DMEM for 3 to 4 hours at 37°C, then exposed to UVB (2 kJ/m^2) and UVC (30 J/m^2) radiation (*A*), or without UV exposure (*B*). After incubation at various time points as indicated, cells were extracted with a SDS sample buffer. The cell extracts were used to carry out Western blot and probed with either phosphospecific or total antibodies against PTEN. The protein band specifically bound with primary antibodies was detected by using anti-rabbit IgG-AP–linked and ECF Western blotting system. *C* and *D,* p53−/− embryonic fibroblasts were transiently transfected with pAP-1-Luc alone or in combination with EGFP-PTEN expression vector as described in Materials and Methods. *C,* the expression of EGFP-PTEN was identified by FCM assay. *Gray histograms,* mock vector–transfected cells; *black histograms,* EGFP-PTEN–transfected cells. *D,* 2×10^4 transfected cells were seeded into each well of 48-well plates and cultured in 10% FBS DMEM at 37°C. After the cell density reached 80% to 90%, the cells were exposed to UVB (2 kJ/m^2) radiation. After incubation for 12 hours, the cells were extracted with lysis buffer, and luciferase activity was measured as described in Fig. 1. The results are expressed as AP-1 induction relative to control medium. *Columns,* mean of triplicate assay wells; *bars,* SD. *, significant decrease from vector control transfection ($P < 0.05$).

molecular roles of PTEN in the control of cellular functions, little is known about mechanisms of PTEN regulation. Recently, a functional p53 binding site was identified within the pten promoter (38). This DNA sequence was required for p53-mediated inducible PTEN expression, whereas basal levels of PTEN transcription are controlled by the element outside of the p53-responsive region within the PTEN promoter (38). Furthermore, p53 induction in DP16 erythroleukemia cells resulted in reduction of Akt phosphorylation (38), revealing a mechanism for the direct involvement of p53 in negative regulation of cellular survival via activation of PTEN transcription. In this study, we found that p53 deficiency resulted in a dramatic decrease in PTEN expression in embryonic fibroblast cells. Considering that p53 deficiency also enhanced activation of Akt and $p70^{S6k}$ in the same cells, and PTEN functions as an inhibitor of PI-3K/Akt pathway, we anticipate that p53 may inhibit UV-induced activation of Akt and $p70^{S6k}$ by down-regulating the PTEN expression. In addition, our previous studies showed that Akt can act as the upstream signal of MAPKs in B(*a*)PDE- and 5-methylacrysene-1,2-diol-3,4-epoxide–triggered signal cascades (30, 57). Here, we found that both overexpression of PTEN and MAPK inhibitors can inhibit p53-deficiency–induced elevation of AP-1 activation, suggesting that Akt may also be the upstream of MAPKs in UV response.

In summary, the present study indicated that p53 has an inhibitory effect on basal level and UV-induced level of AP-1 and NF-κB activities through specifically suppressing the p38 kinase and JNK pathways, but not the ERK pathway. The UV-induced activation of Akt and $p70^{S6k}$ could also be inhibited by normal p53 function. These findings show that p53 has a suppressive function in the cellular signaling pathways, which are thought to be essential for UV-induced carcinogenesis. Because p53 deficiency resulted in a dramatic decrease in expression of PTEN, a tumor suppressor and negative regulator of PI-3K/Akt pathway, taken together with the evidence that PI-3K/Akt participates in the activation of AP-1 and NF-κB, we anticipate that normal p53 may up-regulate PTEN protein expression, and PTEN expression leads to inhibition of PI-3K/Akt pathway, which may subsequently result in inhibition of AP-1 and NF-κB. This seems to be a potential novel mechanism involved in anticancer activity of p53 protein.

Acknowledgments

Received 11/22/2004; revised 4/14/2005; accepted 5/27/2005.

Grant support: NIH/National Cancer Institute grant R01 CA094964 and R01 CA112557; NIH/National Institute of Environmental Health Sciences grant R01 ES012451; and The Special Funds for Major State Basic Research Program of China, National Natural Science Foundation of China, and the Commission of Science and Technology, Shanghai Municipality (L. Wei).

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We thank Dr. Deepti S. Wilkinson (Department of Biochemistry and Molecular Biology, University of Texas, MD Anderson Cancer Center, Houston, TX) and Dr. Michelle Craig Barton for the gift of p53 siRNA construct and Dr. Xia Zhang for the gift of EGFP-PTEN fusion protein expression vector.

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