PTEN Has Tumor-Promoting Properties in the Setting of Gain-of-Function p53 Mutations

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Abstract

We show, for the first time, that the tumor suppressor PTEN can have tumor-promoting properties. We show that PTEN acquires these unexpected properties by enhancing gain-of-function mutant p53 (mut-p53) protein levels. We find that PTEN restoration to cells harboring mut-p53 leads to induction of G_1-S cell cycle progression and cell proliferation and to inhibition of cell death. Conversely, PTEN inhibition in cells expressing wild-type PTEN and mut-p53 leads to inhibition of cell proliferation and inhibition of *in vivo* tumor growth. We show the dependency of the tumor-promoting effects of PTEN on mut-p53 by showing that knockdown of mut-p53 expression inhibits or reverses the tumor-promoting effects of PTEN. Mechanistically, we show that PTEN expression enhances mut-p53 protein levels via inhibition of mut-p53 degradation by Mdm2 and possibly also via direct protein binding. These findings describe a novel function of PTEN and have important implications for experimental and therapeutic strategies that aim at manipulating PTEN or p53 in human tumors. They suggest that the mutational status of PTEN and p53 should be considered to achieve favorable therapeutic outcomes. The findings also provide an explanation for the low frequency of simultaneous mutations of PTEN and p53 in human cancer. [Cancer Res 2008;68(6):1723–31]

Introduction

PTEN and p53 are the two most frequently mutated tumor suppressors in human cancer, including gliomas (1–3). Most malignant gliomas harbor PTEN or p53 mutations, which are considered critical events in the development and growth of these tumors (4–7). Until recently, PTEN and p53 were regarded as autonomous anticancer units that functioned independently of each other. However, recent evidence points to a multilevel and complex cooperation between these tumor suppressors (2). PTEN and wild-type p53 (wt-p53) can enhance each other's tumor-suppressive functions. wt-p53 enhances *PTEN* gene transcription by binding to and activating the PTEN promoter (8). PTEN can protect wt-p53 from degradation through its ability to antagonize phosphatidylinositol 3-kinase (PI3K). This leads to inactivation of Mdm2, which in turn leads to inhibition of wt-p53 degradation (9, 10). PTEN also directly binds to wt-p53, leading to protein stabilization and induction of wt-p53 transcriptional activity (11). PTEN can also inhibit Mdm2 transcription by binding to the inhibitory P1 binding site (12). Recently, PTEN was found to autoregulate its expression by stabilizing wt-p53 in a phosphatase-independent manner and wt-p53 down-regulated PTEN protein stability through caspase-mediated degradation in cells with proteasome dysfunction (13).

In the present study, we show, for the first time, that PTEN can have oncogenic properties by enhancing the stability of gain-of-function p53 mutants (mut-p53). Gain-of-function mut-p53 possesses tumor-promoting functions, such as the transcriptional activation of genes that promote various malignancy variables (14–17). We found that PTEN restoration to glioblastoma cells harboring gain-of-function p53 mutations leads to induction of cell proliferation and inhibition of cell death possibly via inhibition of mut-p53 degradation by Mdm2 and direct stabilization of mut-p53 protein. Conversely, inhibition of endogenous PTEN in glioma cells expressing mut-p53 leads to inhibition of cell proliferation and inhibition of *in vivo* tumor growth. This discovery describes a novel unexpected tumor-promoting function of PTEN and has important implications for therapeutic strategies that aim at manipulating PTEN or p53 expression or function in human tumors. The findings also provide a potential explanation for the low frequency of simultaneous mutations of PTEN and p53 in human cancer.

Materials and Methods

Cell culture and reagents. U373 glioma cells were grown in DMEM (1 g/L glucose with L-glutamine) supplemented with HEPES buffer and 10% fetal bovine serum (FBS). SNB19 glioma cells were grown in DMEM/F12 supplemented with 10% FBS. U87 glioma cells were grown in MEM supplemented with sodium pyruvate, sodium bicarbonate, and 10% FBS. A172 glioma cells were grown in DMEM (4.5 g/L glucose with L-glutamine) and 10% FBS. U1242 glioma cells were grown in α-MEM with 10% FBS. The PI3K inhibitors wortmannin and LY294002 (Calbiochem) were added 1 h before the cells were transfected with PTEN. Camptothecin and trypan blue were purchased from Sigma, and propidium iodide, Annexin V-PE, Annexin V-FITC, and bromodeoxyuridine (BrdUrd) were from BD PharMingen.

Vectors and transfections. Plasmids encoding wt-p53, R273H mut-p53, and Mdm2 were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). PTEN, lipid phosphatase–dead (G129E), and lipid-dead and protein phosphatase–dead (C124A) PTEN mutants were a kind gift from Dr. Kenneth Yamada (NIH, Bethesda, MD; ref. 18). Adenoviruses encoding wt-PTEN (Ad-PTEN), G129E (Ad-G129E), C124A (Ad-C124A), and control (Ad-control) were constructed by us according to He et al. (19). Adenoviruses were amplified, purified, and titered by the

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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University of Pittsburgh Vector Core Facility. Viral titers were $\sim 10^{10}$ plaque-forming units. Plasmids were transfected with Fugene 6 transfection reagent (Roche). Adenovirus infections were conducted at a multiplicity of infection of 10.

Small interfering RNA/short hairpin RNA experiments. PTEN small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology. p53 siRNAs were designed following the guidelines of Ambion, Inc. and synthesized using the Silencer siRNA starter kit (Ambion). Random scrambled siRNAs were used as control. pSilencer plasmid vectors (Ambion) were constructed for stable expression of mut-p53 short hairpin RNAs (shRNA), PTEN shRNA, or control shRNA. shRNAs were designed, constructed, and inserted in pSilencer plasmids according to the guidelines of Ambion. Two different siRNAs and shRNAs were used in all experiments. The targeted sequences for p53 were 5′-AAACATTTTCAGACCTATGGA-3′ and 5′-AAACTACTTCCTGAAAACAAC-3′. The targeted sequences for PTEN were 5′-AAAGAGATCGTTAGCAGAAAC-3′ and 5′-AAACATTATTGCTATGGGATT-3′. To exclude nonspecific antiviral effects, lysates of cells transfected with siRNAs/shRNAs were immunoblotted for the IFN-induced 2′-5′-oligoadenylate synthetase 3, a general marker of antiviral responses (20). No significant antiviral response to siRNA was detected. Naked siRNAs (2 nmol/L) were transfected using siPORT transfection reagent (Ambion). pSilencer plasmids expressing mut-p53, PTEN, or control shRNAs (5 μg) were transfected with Fugene 6, and stable knockdown clones were selected for puromycin resistance and screened for mut-p53 or PTEN protein levels with immunoblotting as previously described (21).

Propidium iodide flow cytometry. The cell cycle status was analyzed using propidium iodide flow cytometry as previously described (22). To assess the effects of PTEN on the cell cycle, the cells were infected with Ad-PTEN, Ad-G129E, Ad-C124A, or Ad-control for 48 h. Alternatively, the cells were transfected with plasmids expressing PTEN, G129E, C124A, or control together with a plasmid expressing green fluorescent protein (GFP) at a DNA ratio of 3:1. To analyze the dependency of the cell cycle effects on mut-p53 expression, the cells were transfected with siRNA 6 h before infection with adenoviruses as described above. All adenovirus-infected cells but only GFP-positive (transfected) plasmid-transfected cells were analyzed on a FACScan (Becton Dickinson).

BrdUrd incorporation. BrdUrd incorporation was used to determine if PTEN restoration induces an increase in active S phase in mut-p53 cells. U373 and U87 (control) cells were infected with Ad-PTEN or Ad-control for 36 h before treatment with 10 μmol/L BrdUrd for 2 h. The treated cells were collected and DNA synthesis was assessed by BrdUrd incorporation using a PE-anti-BrdUrd antibody kit following the manufacturer's instructions (BD Biosciences) and 7-aminoactinomycin D (7AAD) staining for DNA content. The cell cycle was analyzed on a FACScan.

Trypan blue and propidium iodide staining. The effects of PTEN on cell death were analyzed by propidium iodide and trypan blue staining. Cells were infected with Ad-PTEN or Ad-control for 24 h as described above and subsequently treated with camptothecin (200 μmol/L) or γ-radiation (40 Gy) for 48 h. One of the latter two modalities was selected for each individual cell line to induce moderate cell death. The cells were then stained with trypan blue. The number of dead cells stained with trypan blue was determined by counting with a hemocytometer. To obtain morphologic evidence of cell death, the cells were stained with a solution of propidium iodide in PBS (10 μg/mL). Cell suspensions were mounted on slide glasses and red (dead) cells were identified by fluorescence microscopy.

Annexin V-PE and 7AAD flow cytometry. The dependency of PTEN-mediated cell death/survival on mut-p53 expression was analyzed using Annexin V-PE and 7AAD flow cytometry. Cells (1×10^6) were transfected with p53 siRNA for 6 h before transfection with plasmids expressing PTEN or control together with a plasmid expressing GFP at a DNA ratio of 3:1 for 24 h. Some cells were also subsequently treated with camptothecin (300 μmol/L) for 48 h before evaluation of cell death. The fraction of the GFP-positive (transfected) cells was determined by flow cytometry and analyzed for cell death (Annexin V-PE/7AAD–positive cells) on a FACScan.

Growth curves. For cell proliferation experiments, 30,000 cells per well were seeded in medium containing 1% FBS. To determine the effects of PTEN on cell proliferation, the cells were infected with Ad-PTEN or Ad-control before cell counting. For assessing the dependency of the PTEN cell proliferative effects on mut-p53 expression, the cells were transfected with p53 siRNA 6 h before being infected with Ad-PTEN or Ad-control and subsequently counted. The cells were harvested by trypsinization every day for 5 d and counted with a hemocytometer and growth curves were established.

Immunoblotting and immunoprecipitation. Immunoblotting was performed as previously described using antibodies specific for PTEN, wt-p53, mut-p53, cyclin-dependent kinase 2 (cdk2), cyclin E, E2F1, β-actin, α-tubulin, nucleolin (Santa Cruz Biotechnology), p27 (BD Biosciences), and Mdm2 (Calbiochem; ref. 22). Immunoprecipitations of PTEN and p53 were performed as previously described (21). Cells were infected with plasmids expressing either PTEN, wt-p53, R273H mut-p53, or GFP (control). After 24 h, lysates were immunoprecipitated with PTEN- or p53-conjugated agarose beads (Santa Cruz Biotechnology). The beads were collected by centrifugation, washed, heated to 100°C for 5 min in Laemmli buffer, and subjected to immunoblotting for p53 or PTEN as described above.

Cycloheximide chase experiments. Cycloheximide chase experiments were used to assess mut-p53 degradation in response to PTEN restoration to mut-p53 cells. U373 cells were infected with Ad-PTEN or Ad-control for 24 h. The cells were then treated with 100 μg/mL cycloheximide. Cell lysates were collected at various time points (0–7 h) after cycloheximide treatment and analyzed for mut-p53 protein levels by immunoblotting as described above. Band intensities were quantified by densitometry and protein half-life was calculated.

Northern blot analysis. Northern blot analysis was performed to determine the effects of PTEN on mut-p53 mRNA. Total RNA was isolated using the RNeasy Mini kit (Qiagen). Northern blot analysis was performed as previously reported (23). Blots were hybridized with a full-length cDNA probe for R273H mut-p53 (a kind gift from Dr. Bert Vogelstein) and labeled with [^{32}P]dCTP using a random priming kit (Boehringer Mannheim). The blots were stripped and then rehybridized with cDNA specific for glyceraldehyde-3-phosphate dehydrogenase. The signal was visualized by blot exposure to film.

***In vivo* xenograft experiments.** U1242 cells expressing PTEN and mut-p53 were stably transfected with pSilencer vectors encoding PTEN shRNA or scrambled control shRNA. Clones were selected in puromycin and assessed for PTEN and mut-p53 protein expression levels with immunoblotting. Two control (U1242-control) and two PTEN knockdown clones (U1242-PTEN-KD) were selected for *in vivo* implantation. The cells (3×10^5) were stereotactically implanted in the striatum of immunodeficient mice ($n = 10$). The animals were euthanized 5 wk after tumor implantation, when three of the control animals showed signs of tumor-associated morbidity. The brains were removed and tumor maximal cross-sectional areas were measured using computer-assisted image analysis as previously described (22).

Statistics. All experiments were performed at least in triplicates. Numerical data were expressed as mean ± SD. Two group comparisons were analyzed by two-sided Student's t test. Multiple group comparisons were analyzed with Bonferroni/Dunn multiple comparisons tests. P values were calculated and $P < 0.05$ was considered significant.

Results

PTEN induces cell cycle progression and cell proliferation in U373 and SNB19 but inhibits cell cycle progression and cell proliferation in U87 and A172 glioblastoma cell lines. While performing PTEN gene restoration experiments to PTEN-null glioblastoma cells with the aim of inhibiting various malignancy variables, we were surprised to find that PTEN expression consistently led to cell cycle progression in two of four cell lines (U373 and SNB19) as measured by propidium iodide flow cytometry. PTEN restoration led to cell cycle arrest in the two other cell lines (U87 and A172) as expected from a tumor suppressor (Fig. 1*A*). PTEN expression reduced the G_1-G_0 fraction from 68.0 ± 2.4% to 53.8 ± 1.1% ($n = 8$; $P < 0.05$) in U373 and from 50.7 ± 1.1% to 42.6 ± 1.3%

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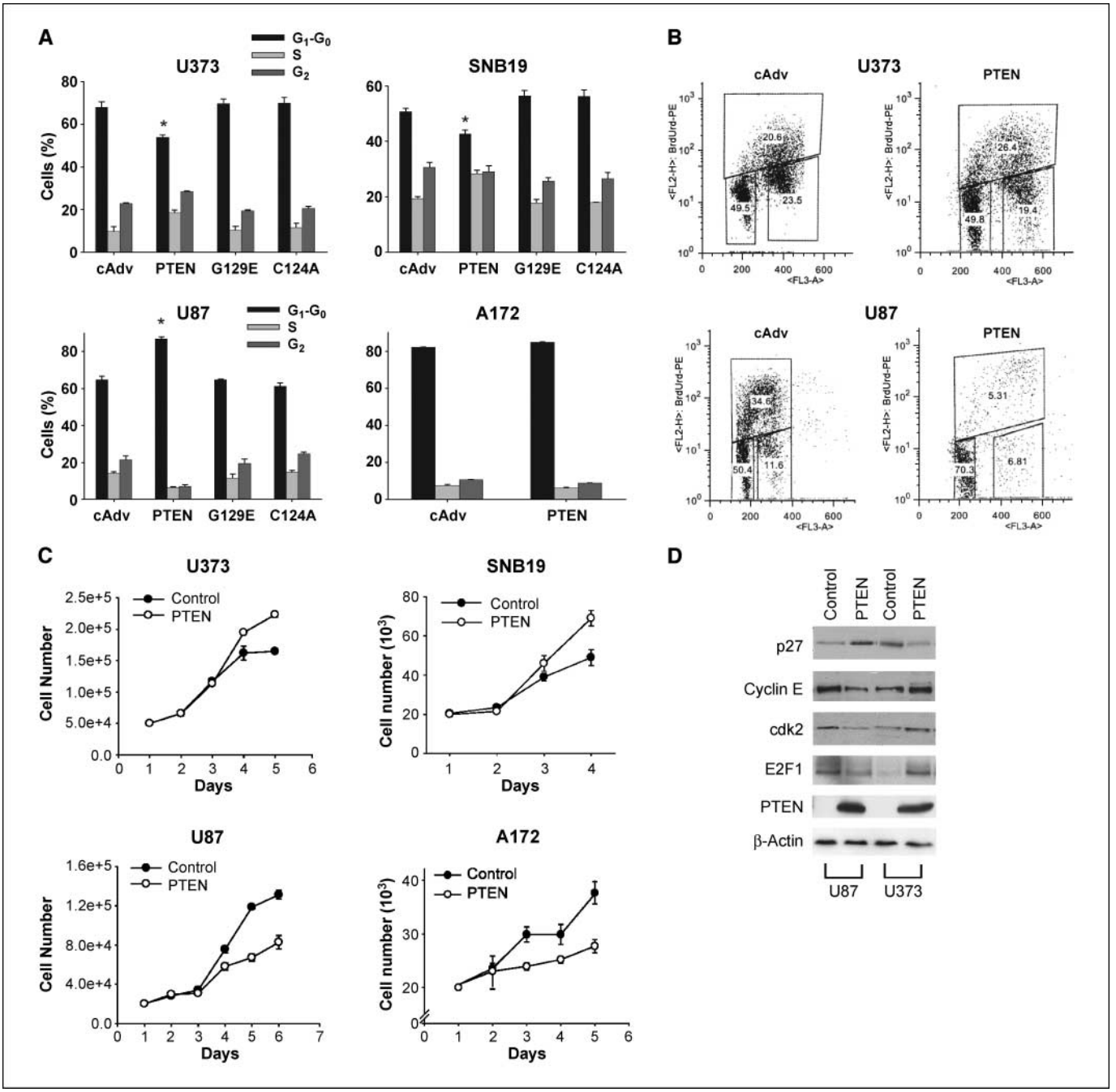

Figure 1. PTEN induces cell cycle progression and cell proliferation in U373 and SNB19 but inhibits cell cycle progression and cell proliferation in U87 and A172 glioma cell lines. PTEN was transfected into four PTEN-null glioma cell lines via adenovirus infections and the cells were subsequently tested for G_1-S cell cycle transition by propidium iodide flow cytometry, for active S-phase changes by BrdUrd incorporation, and for cell proliferation by cell counting. The effects of PTEN on cell cycle regulatory proteins were also assessed by immunoblotting. The results show the following: PTEN induces cell cycle progression in two cell lines (U373 and SNB19) and inhibits cell cycle progression in the two other cell lines (U87 and A172; *A*); PTEN induces active S-phase increase in U373 and decrease in U87 (*B*); similarly, PTEN induces cell proliferation in U373 and SNB19 cells and inhibits cell proliferation in U87 and A172 cells (*C*); and PTEN has opposite effects on the levels of cell cycle regulatory proteins in U373 and U87 cells that are consistent with its opposite effects on cell cycle and cell proliferation in these cell lines (*D*). *cAdv,* control; *G129E,* lipid phosphatase–dead PTEN mutant; *C124A,* phosphatase-dead PTEN mutant. *, $P < 0.05$, relative to control.

($n = 5$; $P < 0.05$) in SNB19 cells. PTEN expression increased the G_0-G_1 fraction from 64.7 ± 1.8% to 86.7 ± 1.2% ($n = 8$; $P < 0.05$) and from 82.2 ± 0.3% to 85.0 ± 0.2% ($n = 4$) in U87 and A172 cells, respectively. Using flow cytometry–based BrdUrd incorporation experiments, we confirmed that PTEN restoration induces an increase in active S phase in U373 cells and a decrease in active S phase in U87 cells (Fig. 1*B*). The cell cycle experiments were repeated multiple times using adenoviruses as well as plasmids to express PTEN cDNA of two different origins and consistently yielded the same results. PTEN expression in the cells was confirmed by immunoblotting and levels were comparable with those found in normal human astrocytes (data not shown). PTEN lipid phosphatase function was verified by testing the ability of PTEN to inhibit the phosphorylation of Akt in all four cell lines (data not

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shown). Expression of PTEN lipid phosphatase mutant (G129E) and phosphatase-dead mutant (C124A) did not change the cell cycle status in U373 or SNB19, indicating lipid phosphatase involvement. However, the PTEN effect on cell cycle progression could not be mimicked by treatment of the cells with the PI3K inhibitors LY294002 and wortmannin, which did not affect the cell cycle status of U373 and SNB19 (data not shown). This indicates that the effects of PTEN on the cell cycle also require a component that is independent of PTEN lipid phosphatase function. The cell cycle changes described above were consistent with PTEN-induced changes in cell proliferation. PTEN significantly induced cell proliferation in U373 and SNB19 cells and inhibited proliferation in U87 and A172 cells (Fig. 1*C*). We also examined the effects of PTEN restoration on the cell cycle regulatory proteins p27, cdk2, cyclin E, and E2F1 in U373 and U87 cells. Consistent with its effects on the cell cycle, PTEN restoration inhibited p27 and induced cdk2, cyclin E, and E2F1 in U373 but induced p27 and inhibited cdk2, cyclin E, and E2F1 in U87 (Fig. 1*D*). Therefore, PTEN restoration oppositely affected cell cycle regulatory proteins in U373 and U87 cells consistent with the opposite effects of PTEN on cell cycle and cell proliferation in these cells.

PTEN inhibits cell death in U373 and SNB19 but induces cell death in U87 and A172 glioblastoma cell lines. Similar to the results obtained for cell proliferation, PTEN expression led to unexpected effects on tumor cell death and apoptosis in two of four glioblastoma cell lines. PTEN restoration led to inhibition of radiation-induced or chemotherapy (camptothecin)-induced cell death and apoptosis in U373 and SNB19 cells but to the induction of these same variables in U87 and A172 cells. PTEN reduced the dead cell fraction from 27.5 $\pm$ 3.4% to 8.7 $\pm$ 3.4% ($n = 3$; $P < 0.05$) in U373 cells and from 56.3 $\pm$ 1% to 23.3 $\pm$ 4% ($n = 3$; $P < 0.05$) in SNB19 cells and increased the dead cell fraction from 35.0 $\pm$ 0.3% to 43.5 $\pm$ 1.3% ($n = 3$; $P < 0.05$) in U87 cells and from 42.2 $\pm$ 3% to 53.3 $\pm$ 7% ($n = 3$; $P < 0.05$) in A172 cells as assessed by trypan blue staining and confirmed by propidium iodide staining (Fig. 2*A* and *B*).

Altogether, the above results show that PTEN consistently exhibits unexpected tumor-promoting characteristics in two of four glioblastoma cell lines.

PTEN increases the levels of mut-p53 via inhibition of Mdm2-mediated degradation and possibly also via direct binding. We examined the genetic background of the four cell lines that were used in the above described experiments and noticed that U373 and SNB19 were mutated on both alleles for *p53* at amino acid 273 (R273H), whereas U87 and A172 were wild-type for *p53*. These observations were based on published literature and confirmed by us through Taqman allelic discrimination (Supplementary data; refs. 24–27). We hypothesized that, similar to what was previously reported for wt-p53, PTEN might stabilize gain-of-function mut-p53, to which the R273H mutation belongs, and thereby enhance their tumor-promoting function (28–30).

To test this hypothesis, we first assessed the effects of PTEN restoration on mut-p53 and wt-p53 protein in U373, SNB19, and U87 cells. We found that PTEN restoration leads to an increase in wt-p53 protein levels in U87 cells and to an increase in mut-p53 protein levels in U373 and SNB19 (84% increase per densitometry on film) as assessed by immunoblotting (Fig. 3*A*). To determine if the PTEN-induced increase in mut-p53 protein is due to inhibition of protein degradation, we performed a cycloheximide chase of mut-p53 protein in U373 cells and calculated PTEN protein half-life. We found that PTEN restoration to U373 cells prolongs mut-p53 half-life by $\sim$40% (from 3.7 to 5.2 h; Fig. 3*B*). To determine if the PTEN-induced increase in mut-p53 protein also has a transcriptional component, we analyzed the effects of PTEN restoration on mut-p53 mRNA in U373 cells by Northern blotting. We found that PTEN expression did not significantly change mut-p53 mRNA in U373 cells (Fig. 3*B*). Therefore, PTEN expression post-transcriptionally induces mut-p53 protein levels by inhibiting its degradation.

We then asked if PTEN-induced changes of mut-p53 levels are mediated by changes in the levels and cellular localization of Mdm2 and/or by direct binding and stabilization of the mutants by PTEN protein. To assess the involvement of Mdm2 in PTEN-induced mut-p53 protein levels, we first studied the effect of PTEN expression on the cytoplasmic and nuclear levels of Mdm2. PTEN restoration to mut-p53 U373 and SNB19 cells led to a reduction of the levels of nuclear Mdm2 as assessed by immunoblotting after cell fractionation (Fig. 3*C, left*). This shows that PTEN regulates the levels and distribution of Mdm2 in these cells. We then studied the effects of Mdm2 on mut-p53 levels by transfecting U373 and SNB19 cells with an Mdm2 cDNA expression vector and analyzing mut-p53 levels by immunoblotting. We found that Mdm2 expression leads to the decrease of nuclear mut-p53 levels in U373 and SNB19 cells (Fig. 3*C, right*). This shows that mut-p53 is regulated by Mdm2 in these cells. Together, the results described above show that PTEN expression could increase the levels of mut-p53 via inactivation of Mdm2. To determine if PTEN can also regulate mut-p53 levels by direct binding, we transfected U373 cells with PTEN before immunoprecipitation of either PTEN or mut-p53. Immunoprecipitates were immunoblotted for mut-p53 (PTEN) or PTEN (mut-p53). We found that PTEN protein binds to mut-p53 protein in the cells (Fig. 3*D*). Therefore, similar to what has been recently shown for wt-p53, PTEN protein might also increase the levels of mut-p53 via protein binding and stabilization.

PTEN-induced cell cycle progression, cell proliferation, and cell survival are dependent on mut-p53 expression. We then asked if PTEN-induced cell cycle progression, cell proliferation, and cell survival are dependent on mut-p53 expression. To answer this question, we inhibited mut-p53 expression in U373 with p53 siRNA and studied the effects of PTEN restoration on cell cycle, cell proliferation, and apoptosis in this setting. R273H mut-p53 was inhibited by either transient expression of siRNA or stable expression of shRNA encoded in pSilencer plasmids. p53 siRNA/shRNA expression in U373 cells led to substantial inhibition of mut-p53 protein as assessed by immunoblotting (Fig. 4*A*). U373 cells transfected with siRNA/shRNA were then infected with Ad-PTEN or Ad-control. The cell cycle status and apoptosis were determined by flow cytometry as described above and cell proliferation was determined by cell counting.

Inhibition of basal levels of R273H mut-p53 in U373 cells led to cell cycle arrest with the G_0-G_1 fraction increasing from 57.4 $\pm$ 8.8% in control cells to 82.4 $\pm$ 0.1% ($n = 5$; $P < 0.05$) in mut-p53–inhibited cells (Fig. 4*A*). This shows that R273H is a gain-of-function mut-p53 that induces cell cycle progression. PTEN expression reduced the G_0-G_1 fraction by 50.5% in control cells ($P < 0.05$) and by a statistically insignificant ($P > 0.05$) 32% in mut-p53–inhibited cells (Fig. 4*A*). PTEN induction of cell cycle progression was therefore inhibited by 45% in the setting of partially inhibited mut-p53 expression.

Similarly, inhibition of mut-p53 expression in U373 cells led to complete inhibition of PTEN-induced cell proliferation. Cells

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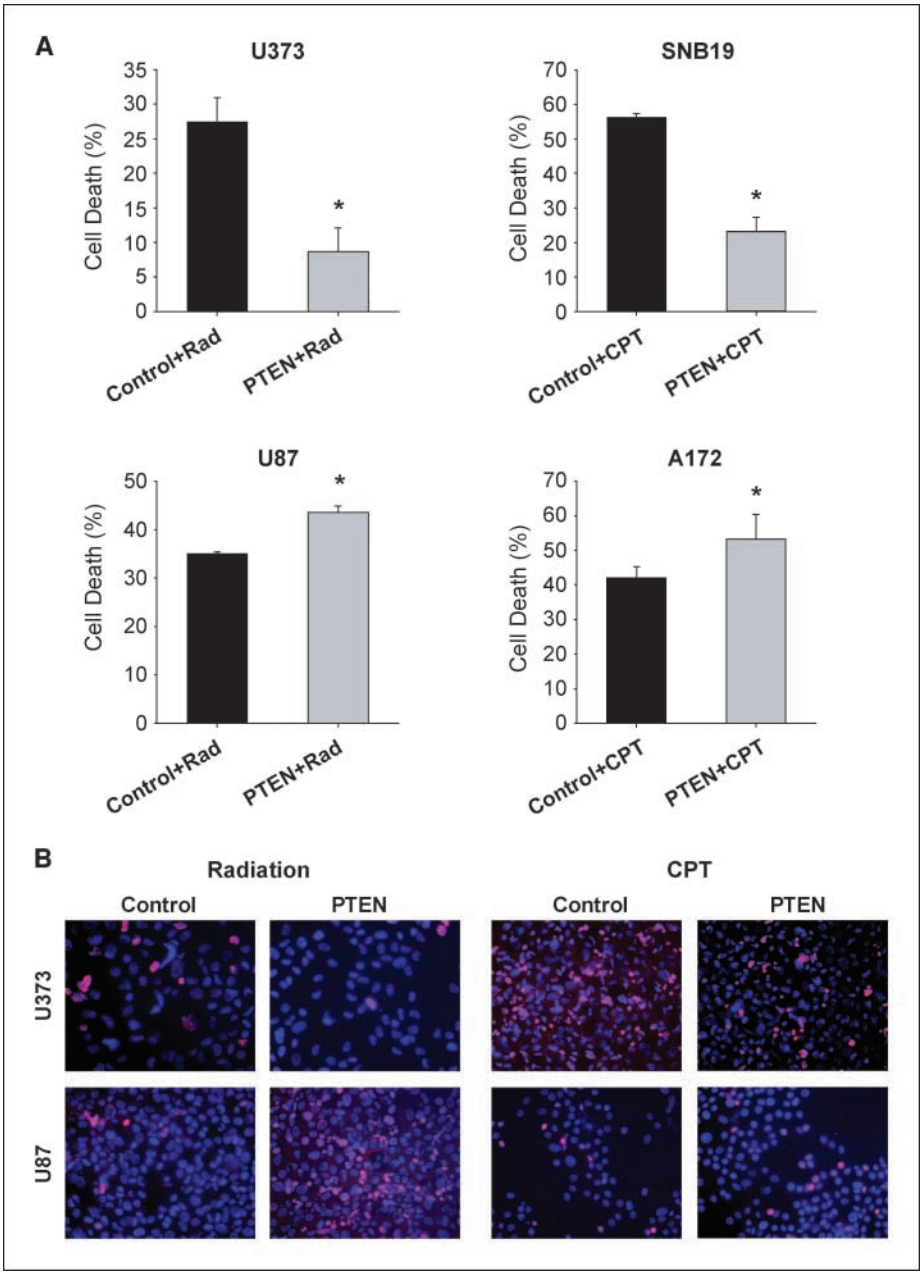

Figure 2. PTEN inhibits cell death in U373 and SNB19 but induces cell death in U87 and A172 glioma cell lines. PTEN was transfected into four PTEN-null glioma cell lines via adenovirus infections and the cells were subsequently tested for cell death by trypan blue staining (*A*) and propidium iodide staining (*B*). *A* and *B,* the results show that PTEN inhibits cell death in U373 and SNB19 cells and induces cell death in U87 and A172 cells. *Rad,* radiation; *CPT,* camptothecin. *, $P < 0.05$, relative to control.

transfected with control siRNA and Ad-control grew ~3-fold, whereas cells transfected with control siRNA and Ad-PTEN grew ~5-fold in 5 days ($n = 6$; $P < 0.01$). Cells transfected with p53 siRNA and Ad-control grew only ~2-fold, confirming that mut-p53 in U373 is a gain of function. Cells transfected with mut-p53 siRNA and Ad-PTEN grew <2-fold after 5 days ($n = 6$; $P > 0.05$, relative to control; Fig. 4*B*). Therefore, inhibition of mut-p53 expression completely abrogated the cell proliferative effect of PTEN.

Inhibition of mut-p53 expression also led to inhibition of PTEN-induced cytoprotection as measured by Annexin V flow cytometry. In U373 cells transfected with control siRNA, PTEN reduced cell death from 5.8 ± 0.1% to 2.9 ± 0.2% ($n = 3$; $P < 0.01$). Inhibition of mut-p53 expression with siRNA led to induction of cell death from 5.8 ± 0.1% to 11.4 ± 2.1% ($P < 0.01$; Fig. 4*C*). This further shows that R273H is a gain-of-function mut-p53 that elicits cytoprotective effects. In the setting of siRNA-inhibited mut-p53, PTEN increased cell death from 11.4 ± 2.1% to 22.8 ± 0.1% ($P < 0.01$; Fig. 4*C*). Therefore, inhibition of mut-p53 led to a complete reversal of the effects of PTEN on cell death. These results show that the PTEN-induced cytoprotective effects are dependent on gain-of-function mut-p53 expression.

Altogether, the above experiments consistently show that the tumor-promoting properties of PTEN are dependent on mut-p53 expression.

Inhibition of endogenous PTEN in wt-PTEN/mut-p53 U1242 glioma cells leads to inhibition of cell proliferation. To ascertain the tumor-promoting effects of endogenous PTEN in the setting of gain-of-function mut-p53, we inhibited endogenous PTEN expression in cells that express PTEN and gain-of-function mut-p53 and studied the effects of this inhibition on tumor cell proliferation. We used U1242 glioblastoma cell lines that express PTEN and have gain-of-function mutations of *p53* at codon 175 (R175H;

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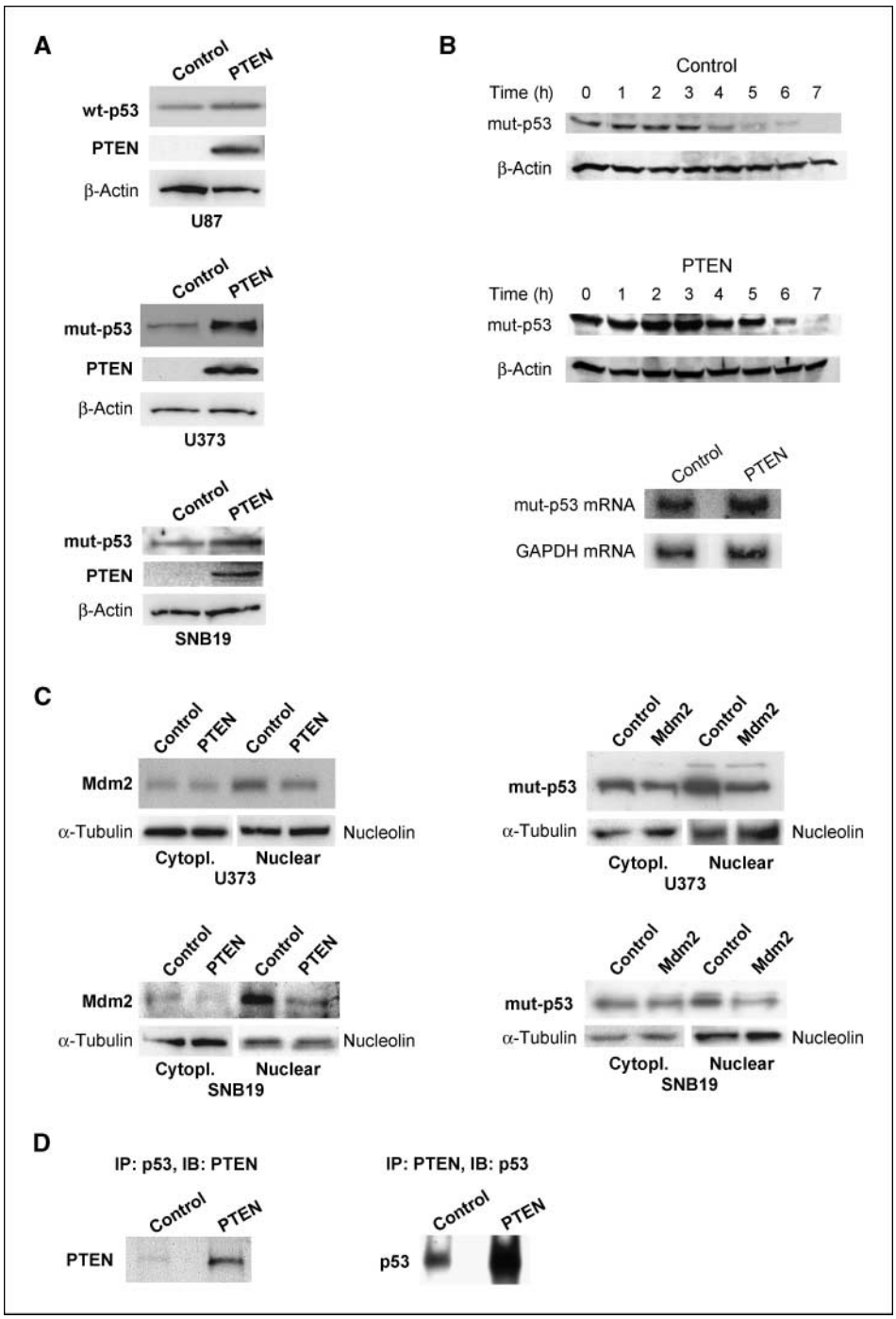

Figure 3. PTEN increases the levels of mut-p53 via inhibition of Mdm2-mediated degradation and possibly also via direct binding. *A,* PTEN was transfected into PTEN-null glioma cells and protein levels of wt-p53 (U87) and mut-p53 (U373 and SNB19) cells were assessed by immunoblotting. The results show that PTEN expression induces wt-p53 and mut-p53 protein levels. *B, top,* PTEN was transfected into PTEN-null U373 cells and a cycloheximide chase was performed. The results show that PTEN inhibits mut-p53 protein degradation. *Bottom,* PTEN was transfected into PTEN-null U373 cells and mut-p53 mRNA levels were assessed by Northern blotting. The results show that PTEN does not significantly change mut-p53 mRNA levels. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase. *C,* PTEN was transfected into PTEN-null/mut-p53 U373 and SNB19 cells. The cells were subjected to cytoplasmic and nuclear fractionation and subsequently analyzed for Mdm2 protein levels by immunoblotting. *Left,* the results show that PTEN expression inhibits Mdm2 protein levels. Mdm2 was transfected into mut-p53 U373 and SNB19 cells and the cells were assessed for mut-p53 levels by immunoblotting. *Right,* the results show that Mdm2 inhibits mut-p53 levels. Together, the above shows that PTEN can induce mut-p53 levels by inhibiting its degradation by Mdm2. *D,* PTEN was transfected into U373 cells. The cells were subsequently immunoprecipitated (*IP*) for PTEN and immunoblotted (*IB*) for mut-p53 or immunoprecipitated for mut-p53 and immunoblotted for PTEN. The results show that PTEN coimmunoprecipitates with mut-p53.

refs. 27, 30, 31). We used siRNA to inhibit PTEN expression in these cells and analyzed them for proliferation by cell counting. Consistent with the results described in the previous sections, inhibition of PTEN expression in these mut-p53 cells led to inhibition of cell proliferation. Whereas control siRNA–transfected U1242 glioma cells grew ~30-fold in 5 days, PTEN siRNA–transfected cells grew only ~10-fold (Fig. 5*A*). This provides evidence that endogenous PTEN has tumor-promoting effects in the setting of gain-of-function p53 mutations.

Inhibition of PTEN in wt-PTEN/mut-p53 U1242 cells leads to inhibition of *in vivo* tumor growth. To determine if the tumor-promoting characteristics of PTEN lead to *in vivo* tumor growth, we inhibited PTEN expression in wt-PTEN/mut-p53 U1242 cells and assessed the effect of this inhibition on *in vivo* tumor growth. These cells were also chosen because they are very tumorigenic *in vivo* as opposed to U373 cells that do not form tumors in animals. We generated PTEN knockdown U1242 clones by stable transfection with plasmids encoding PTEN shRNA and control clones by transfection with plasmids encoding scrambled shRNA. We verified PTEN knockdown as well as the ensuing mut-p53 inhibition in the clones by immunoblotting (Fig. 5*B, right*). We implanted two PTEN knockdown clones and two control clones in the brains of immunodeficient mice, euthanized the mice after 5 weeks, and measured tumor sizes. We found that inhibition

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of PTEN expression in U1242 cells that harbor a gain-of-function mutation of *p53* at codon 175 leads to a significant inhibition of *in vivo* xenograft growth. Whereas control clones had a cross-sectional area of $155.6 \times 10^3 \pm 6.9 \times 10^3$ μm^2, PTEN knockdown clones had a cross-sectional area of $43.9 \times 10^3 \pm 11.1 \times 10^3$ μm^2 ($n = 10$; $P < 0.01$; Fig. 5*B*). Therefore, inhibition of PTEN expression in glioblastoma cells harboring gain-of-function mut-p53 leads to inhibition of *in vivo* tumor growth, indicating that PTEN has tumor-promoting properties *in vivo* in the setting of mut-p53.

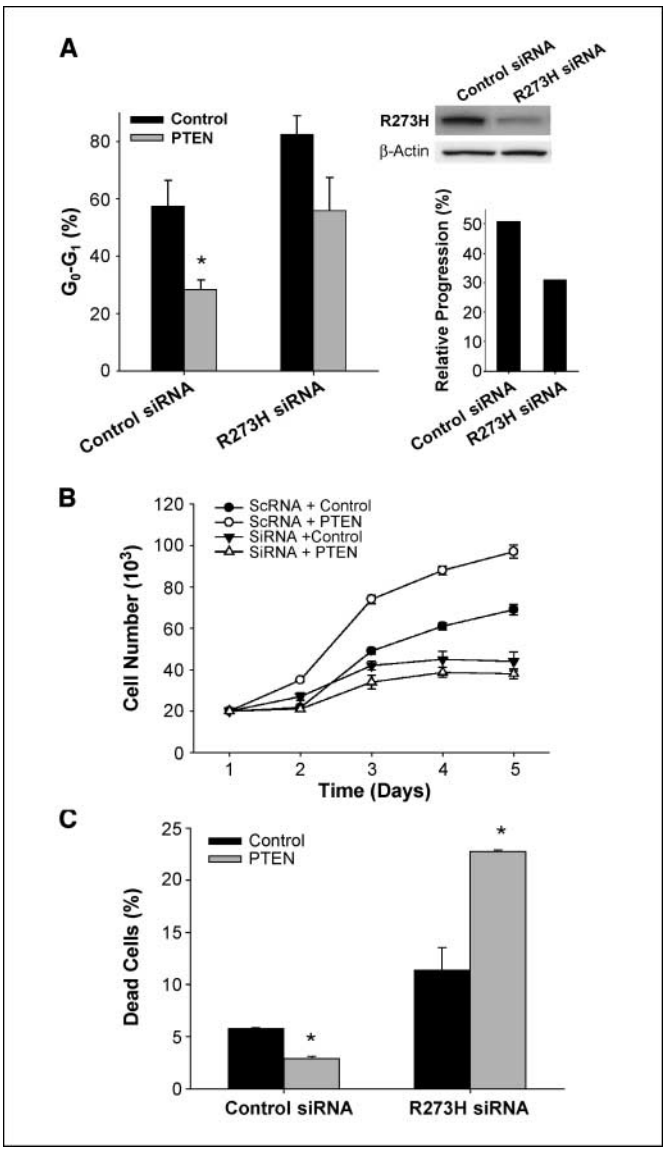

Figure 4. PTEN-induced cell cycle progression, cell proliferation, and cytoprotection are dependent on gain-of-function mut-p53 expression. R273H mut-p53 expression was inhibited in U373 cells by transfection with siRNA or shRNA before restoration of PTEN. Control cells were transfected with scrambled siRNA/shRNA (*scRNA*). *Top right,* expression inhibition was confirmed by immunoblotting. The effects of PTEN on cell cycle progression (*A*), cell proliferation (*B*), and cytoprotection (*C*) were subsequently assessed in this setting using propidium iodide flow cytometry, cell counting, or Annexin V flow cytometry, respectively. The results show that inhibition of mut-p53 expression leads to partial inhibition of PTEN-mediated induction of cell cycle progression, complete inhibition of PTEN-induced cell proliferation, and complete reversal of PTEN-induced cytoprotection. *, $P < 0.05$, relative to control.

Discussion

We showed, for the first time, that PTEN can have tumor-promoting properties in cells that harbor gain-of-function p53 mutations. We found that PTEN induces cell cycle progression, cell proliferation, cell survival, and *in vivo* tumor growth in mut-p53 glioma cells but exerts the opposite effects in wt-p53 cells. PTEN increased the levels of mut-p53 protein by inhibiting its degradation possibly via inhibition of PI3K/Mdm2 and physical binding. We showed that the unexpected effects of PTEN are dependent on mut-p53 expression.

We first found that PTEN induces cell proliferation and cell survival of glioma cells that have gain-of-function p53 mutations. Because these findings were unexpected and seemingly contrary to established knowledge, we carefully and thoroughly excluded potential procedural and other nonspecific errors. The following steps were taken. (*a*) To exclude adenovirus-induced nonspecific effects, all experiments showing that PTEN has tumor-promoting effects were repeated using plasmid-based transfections coupled with GFP selection. This approach yielded similar results to adenovirus-based experiments. (*b*) To ensure the integrity and functionality of expressed PTEN protein, the ability of PTEN to dephosphorylate Akt was shown. Additionally, PTEN cDNA of two different independent sources was used and yielded the same results. (*c*) Complementary techniques were used to assess cell proliferation (propidium iodide flow cytometry, BrdUrd incorporation, and growth curves) and cell death (trypan blue staining, propidium iodide staining, and Annexin V flow cytometry). (*d*) To avoid unphysiologically high expression levels of restored PTEN protein, adenoviral titers used were adjusted to yield PTEN expression levels comparable with the ones in primary normal human astrocytes. Additionally, proliferation assays and *in vivo* tumor growth experiments were conducted after inhibition of endogenous PTEN in cells with mut-p53 and yielded results consistent with the ones obtained in PTEN restoration experiments. (*e*) All experiments were repeated multiple times by at least two independent investigators and consistently yielded comparable results. In addition, the fact that PTEN consistently behaved as a tumor suppressor in U87 and A172 wt-p53 cells and as a tumor promoter in U373, SNB19, and U1242 mut-p53 cells under the same experimental conditions is an additional indication that the findings are real pathophysiologic events.

We then showed that PTEN expression regulates the levels of mut-p53 protein. PTEN seemed to regulate gain-of-function mut-p53 levels via PI3K-dependent and PI3K-independent mechanisms as indicated by the fact that phosphatase-dead PTEN mutants have no effect on cell proliferation and cell death in mut-p53 cells combined with the fact that the PTEN effect cannot be mimicked by pharmacologic inhibition of PI3K. We found that PTEN enhances mut-p53 levels at least partly through its effects on PI3K-regulated Mdm2. We showed the involvement of Mdm2 by showing that PTEN regulates nuclear Mdm2 levels in mut-p53 cells and that Mdm2 regulates mut-p53 levels in these same cells. We also examined the dependency of mut-p53 induction by PTEN on Mdm2. We inhibited Mdm2 binding to p53 with Nutlin-3 and assessed the effects of PTEN on mut-p53 protein in this setting. We found that Nutlin-3 and PTEN additively increase mut-p53 protein levels (see Supplementary Data). These additive effects are probably due to incomplete inhibition of Mdm2 by Nutlin-3 as has been reported in mut-p53 cells not subjected to chemotherapy and/or to partial dependence of

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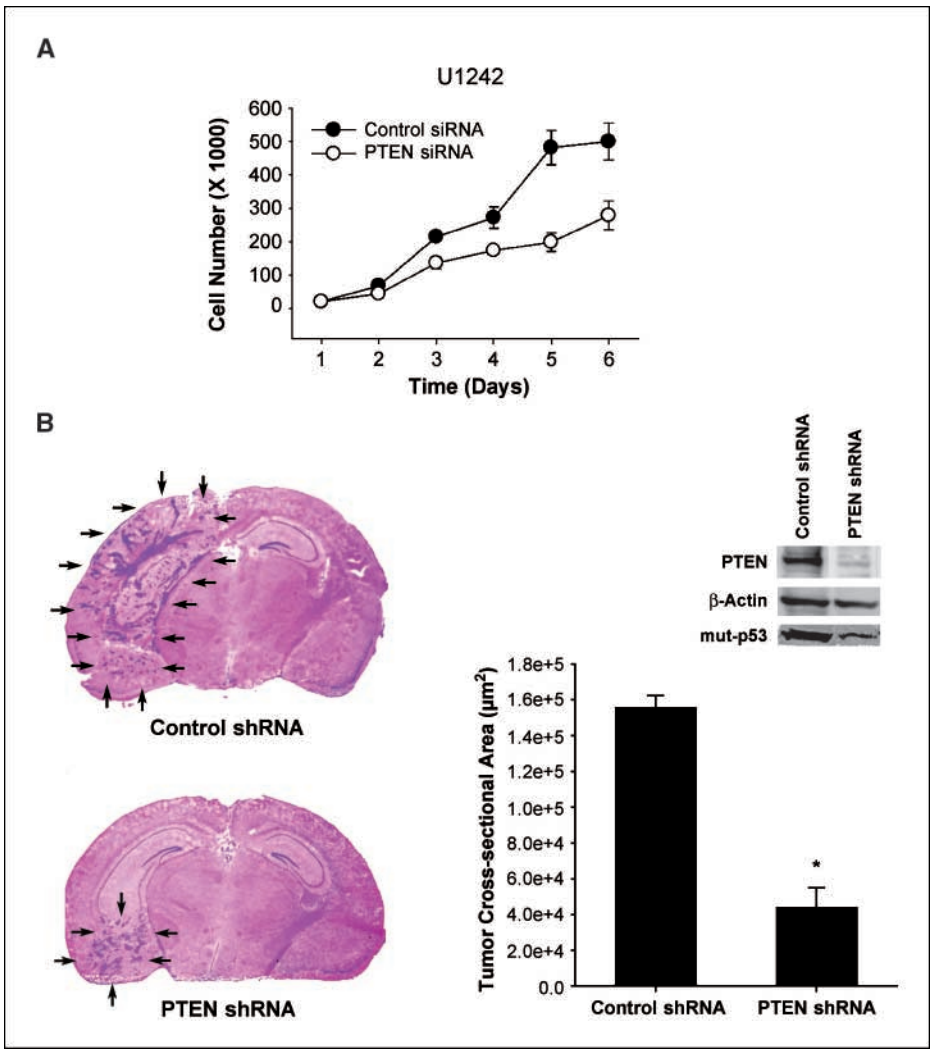

Figure 5. Inhibition of endogenous PTEN expression in wt-PTEN/mut-p53 glioma cells leads to inhibition of cell proliferation and *in vivo* tumor growth. *A,* endogenous PTEN was inhibited in wt-PTEN/mut-p53 U1242 cells by siRNA transfection and the cells were assessed for proliferation by cell counting. The results show that inhibition of PTEN expression in these mut-p53 cells leads to inhibition of cell proliferation. *B,* PTEN was inhibited in U1242 cells by stable transfection with pSilencer plasmids encoding PTEN shRNA. Control U1242 cells were transfected with pSilencer plasmids encoding scrambled control shRNA. *Right,* clones were selected for puromycin resistance and assessed for PTEN and mut-p53 protein levels by immunoblotting. Two PTEN knockdown and two control clones were selected and implanted intracranially (3×10^5 cells) in immunodeficient mice ($n = 10$ for each clone). The mice were sacrificed 5 wk after implantation and the brains were cryosectioned and H&E stained. Tumor maximal cross-sectional areas were measured with computer-assisted image analysis. The results show that endogenous PTEN inhibition in mut-p53 U1242 cells leads to inhibition of tumor growth. *Left,* representative tumors; *middle,* quantification of tumor sizes. *, $P < 0.05$, relative to control.

PTEN-induced mut-p53 on Mdm2 inhibition (32). The latter is consistent with our data, suggesting that PTEN might stabilize mut-p53 protein through physical binding. These findings are altogether consistent with what was reported on the regulation of wt-p53 by PTEN (9–11).

We next showed that the tumor-promoting effects of PTEN are dependent on mut-p53 expression. We found that siRNA-mediated down-regulation of mut-p53 expression partially inhibits the PTEN effects on cell cycle progression, completely inhibits the effects of PTEN on cell proliferation, and completely reverses the PTEN effects on cytoprotection. The *p53* mutations R273H and R175H present in the cells that were used in the present study have been previously characterized as gain-of-function mutations (28–30, 33, 34). About 80% of *p53* gene mutations are missense mutations that occur within the DNA-binding region of the protein (35). Some of these mutants acquire oncogenic activities per se (16, 17). These mutants function by activating the transcription of oncogenes such as c-myc, c-fos, EGFR, IGF1-R, and NF-κB (15, 36). By increasing the protein levels of these mutants, PTEN could acquire tumor-promoting functions. Although PTEN can acquire tumor-promoting properties in the setting of gain-of-function p53 mutations, PTEN probably still exerts tumor-suppressive effects in mut-p53 cells through p53-independent mechanisms. The overall effects of PTEN could therefore result from the balance of mut-p53–mediated tumor-promoting effects and mut-p53–independent tumor-suppressive effects.

We describe a novel function for PTEN and provide new insights into the interactions between PTEN and p53. Our findings provide a potential explanation for the low frequency of simultaneous occurrence of PTEN and p53 mutations in human cancer (4, 37, 38). Loss of PTEN in cells harboring gain-of-function p53 mutations would confer growth disadvantage to these cells compared with wt-PTEN/mut-p53 cells. Our findings also have implications for therapeutic approaches that aim at manipulating PTEN and p53 expression or function in tumors. These approaches would have to take into consideration the mutational status of PTEN and p53 to avoid unwanted harmful effects.

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PTEN Has Tumor-Promoting Properties in the Setting of Gain-of-Function p53 Mutations

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