

# PTK6 Potentiates Gemcitabine-Induced Apoptosis by Prolonging S-phase and Enhancing DNA Damage in Pancreatic Cancer

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## Abstract

Protein Tyrosine Kinase 6 (PTK6) is a non-receptor-type tyrosine kinase known to be expressed in various cancers, including pancreatic cancer. The role of PTK6 in cancer chemoresistance remains unclear. Therefore, it was hypothesized that PTK6 mechanistically regulates gemcitabine resistance in pancreatic cancer. Gemcitabine treatment stimulated endogenous PTK6 overexpression in MIAPaCa2 and Panc1 cells. PTK6 gene silencing increased cell survival after gemcitabine treatment and decreased apoptosis, whereas PTK6 overexpression decreased cell survival and increased apoptosis. Selection for gemcitabine resistance revealed substantially lower PTK6 expression in the gemcitabine-resistant subclones compared with the parental lines, while restoring PTK6 rescued gemcitabine sensitivity. Gemcitabine induced phosphorylation of H2AX ( $\gamma$ -H2AX) and ataxia-telangiectasia mutated kinase (pATM), specific markers for DNA double-strand breaks.

Both gemcitabine-induced phosphorylation of H2AX and ATM were reduced by PTK6 knockdown and increased by PTK6 overexpression. PTK6 overexpression also increased the S-phase fraction 48 hours after gemcitabine treatment. Although gemcitabine activated both caspase-8 (CASP8) and caspase-9 (CASP9), the effect of PTK6 on gemcitabine-induced apoptosis required CASP8 but not CASP9. In mouse xenografts, PTK6 overexpression in subcutaneous tumors attenuated tumor growth after gemcitabine treatment. In conclusion, PTK6 prolongs S-phase and increases the ability of gemcitabine to cause DNA damage *in vitro* and *in vivo*.

**Implications:** PTK6 affects cell cycle and DNA damage, thus making it an important therapeutic target to improve the outcomes of patients with pancreatic cancer. *Mol Cancer Res*; 13(8); 1174–84. ©2015 AACR.

## Introduction

Pancreatic cancer is the fourth leading cause of cancer mortality in the United States, with 5-year overall survival rates even among patients having undergone complete surgical resection averaging only 15% to 25% (1, 2). Although the nucleotide analogue gemcitabine (2', 2'-difluoro-2'-deoxycytidine, dFdCyd) is used as a first-line chemotherapeutic agent for the treatment of pancreatic cancer, its clinical impact remains modest due to inherited and acquired resistance and most patients develop recurrence even after complete resection followed by adjuvant chemotherapy (3). Thus, there exists an urgent need to understand the molecular mechanisms of drug resistance against gemcitabine in pancreatic cancer in order to improve the outcomes of patients with pancreatic cancer.

Protein Tyrosine Kinase 6 (PTK6) is a non-receptor-type tyrosine kinase that is aberrantly expressed in various types of cancers (4–12). Although PTK6 is known to affect various aspects of cancer biology, including proliferation, cell migration, and invasion (10, 13, 14), its role in chemoresistance is unknown. As a

previous study suggested that PTK6 may be involved in DNA damage-induced apoptosis in nonmalignant murine intestinal epithelial cells (15), we hypothesized that PTK6 would regulate the apoptosis induced by DNA-targeting chemotherapy in cancer cells. In this study, we examined the role of PTK6 in gemcitabine-induced apoptosis in pancreatic cancer and elucidated the mechanism by which PTK6 regulates apoptosis. Our new findings may identify a novel target to improve the efficacy of chemotherapy for patients with pancreatic cancer.

## Materials and Methods

### Materials

Culture media, FBS, and penicillin/streptomycin (P/S) were obtained from Gibco. Anti-PTK6 antibody (C-18; sc-1188) and anti-CyclinE antibody (HE12; sc-247) were obtained from Santa Cruz Biochemistry, anti-Caspase3 (8G10; #9662), anti-cleaved Caspase3 (5A1E; #9664), anti-cleaved PARP (D64E10; #5625), anti-Caspase8 (1C12; #9746), anti-Caspase9 (#9502), anti-RIP1 (D94C12; #3493), anti- $\gamma$ -H2AX (20E3; #9718), and anti-phosphor-ATM (D6H9; #5883) were obtained from Cell Signaling Technology. Anti-ATM antibody (Ab-3; PC116) was obtained from Calbiochem, and anti- $\beta$ -actin antibody (AC-15; A5441) was obtained from Sigma-Aldrich. Gemcitabine hydrochloride was obtained from Sigma-Aldrich or Santa Cruz Biochemistry.

### Cell cultures

The human pancreatic cancer MIAPaCa2 and Panc1 cell lines were obtained from the American Type Culture Collection (ATCC) on January 2012. These cancer cell lines were authenticated by the ATCC with DNA profiling using STR analysis before purchase.

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**Note:** Supplementary data for this article are available at Molecular Cancer Research Online (<http://mcr.aacrjournals.org/>).

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These cells were maintained in DMEM medium containing 10% FBS and 1% P/S in a humidified 37°C, 5% CO<sub>2</sub> chamber.

#### Establishment of gemcitabine-resistant subclones from pancreatic cancer cell lines

Gemcitabine-resistant pancreatic cancer cell subclones were newly developed using MIA PaCa2 and Panc1 cells in our laboratory by the method others have previously described (16, 17). In brief, MIA PaCa2 and Panc1 cells were serially cultured by exposure to incrementally increasing gemcitabine concentrations for 2 months. The concentration of gemcitabine treatment began at 10 nmol/L and was increased up to 100 nmol/L for both MIA PaCa2 and Panc1 cells.

#### Western blot analysis

Cells were lysed in cell lysis buffer (Cell Signaling Technology) with 1 mmol/L Phenylmethanesulfonyl Fluoride (Cell Signaling Technology). The protein concentration of each cell lysate was estimated by BCA assay (Pierce Chemical). Cell lysates containing a total of 10 to 20 µg of protein were applied to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Invitrogen). After being blocked with Tris buffered saline with 0.2% Tween-20 (TBST) containing 5% BSA or skim milk for 4 hours at room temperature, membranes were incubated with antibody in appropriate dilutions at 4°C overnight. Horseradish peroxidase-conjugated donkey anti-rabbit IgG or sheep anti-mouse IgG were used as secondary antibodies and incubated with the membranes at a 1/3,000 dilution for 1 hour (GE Healthcare). Protein bands were visualized and their intensities were quantified using Odyssey Fc Imaging System (LI-COR Biosciences). β-Actin was used as a loading control marker for normalization of each lane. All exposures for densitometry were within the linear range. Western blotting was repeated at least three times, and representative blots were presented.

#### Gene silencing by small interfering RNA

Loss-of-function analysis was performed using siRNAs targeting PTK6 (s11487; Ambion: sense 5'-CAUCCAUGGUUAAGU-CAUAtt-3', antisense 5'-UAUGACUUAACCAUGGAUGaa-3'). Silencer Select Negative Control #2 siRNA (Ambion) served as a negative control. An alternative sequence of siRNA targeting PTK6 (Invitrogen: Stealth RNAi-PTK6HSS183907 sense 5'-CAGG-CUGUGCGGCACUACAAGAUCU-3', antisense 5'-AGAUCUUGUAGUGCCGCACGCCUG-3') was used to rule out off target effects of the siRNA and yielded similar results. Each siRNA (20 nmol/L) was transfected into pancreatic cancer cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The knockdown of each target gene was confirmed by Western blotting.

#### Overexpression of PTK6 by stable transfection

Human PTK6 cDNA (cloneID: 5746034) was purchased from Open Biosystems and amplified by PCR using primers (5'-CCCAAGCTTATGGTGTCCCGGGACCAGGC, and 3'-CGGG-ATCCTCAGGTCGGGTTCTCGTAGC). The PCR product was inserted into expression vector pcDNA3.1-myc/His B (Invitrogen) according to the manufacturer's protocol. The established PTK6 expression vector was subjected to DNA sequencing to confirm correct insertion of full-length PTK6 cDNA. The PTK6 expression vector or the corresponding empty vector was transfected into pancreatic cancer cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 72 hours of

transfection, cells were incubated in culture medium containing appropriate concentrations of G418. By culturing the cells in selection medium containing G418 over 2 weeks, stable transfectant clones were established. The overexpression of PTK6 protein was subsequently confirmed by Western blotting.

#### Cell viability assay

Cell viability was measured by an assay based on a colorimetric water-soluble tetrazolium salt, WST-8 (Cell Counting Kit-8; Dojindo Molecular Technologies). The cells ( $1 \times 10^3$  per well) were seeded in 96-well plates and incubated overnight at 37°C. Following 96 hours of gemcitabine treatment, the cell viability assay was performed according to the manufacturer's instructions. The absorbance of each well was measured at 450 nm using a µQuant Microplate Spectrophotometer (BioTek Instruments) and was within the linear range of the assay.

#### Fluorescent-immunocytochemistry

Cells were fixed in 4% formaldehyde diluted in phosphate-buffered saline for 15 minutes, blocked with blocking buffer (1× PBS/1% BSA/0.3% Triton X-100) for 60 minutes, and then incubated overnight with the primary antibody at 4°C [Alexa Fluor 555 Conjugated γ-H2AX (20E3; #8228) from Cell Signaling Technology].

#### Cell cycle analysis

Cells were fixed in 70% ethanol at -20°C for overnight. To determine the DNA content, cells were stained with 50 µg/mL propidium iodide (PI; Sigma Aldrich) with triton X-100 and RNAase. Then, the samples were subjected to flow cytometric analysis using LSRII (BD Bioscience). At least 10,000 events for each sample were acquired and analyzed with ModFit LT software (Verify Software House).

#### Mouse xenograft model

Five-week-old nude male mice were purchased from Charles River breeding laboratories. Mice were inoculated with PTK6-overexpressing transfectants (left) or corresponding empty vector transfectant controls (right) subcutaneously at their bilateral flanks (MiaPaCa-2,  $7.5 \times 10^6$ ; Panc-1,  $1 \times 10^7$ ). After 10 to 14 days, treatment commenced when tumors reached approximately 5 mm. Mice bearing tumors were treated with gemcitabine (150 mg/kg) by twice-weekly i.p. injection for 5 weeks for MIA PaCa2 cells or 8 weeks for Panc1 cells. Tumor volumes were calculated using the following formula: Volume =  $\frac{1}{2} a \times b^2$ , where a and b represent the larger and smaller tumor diameters, respectively

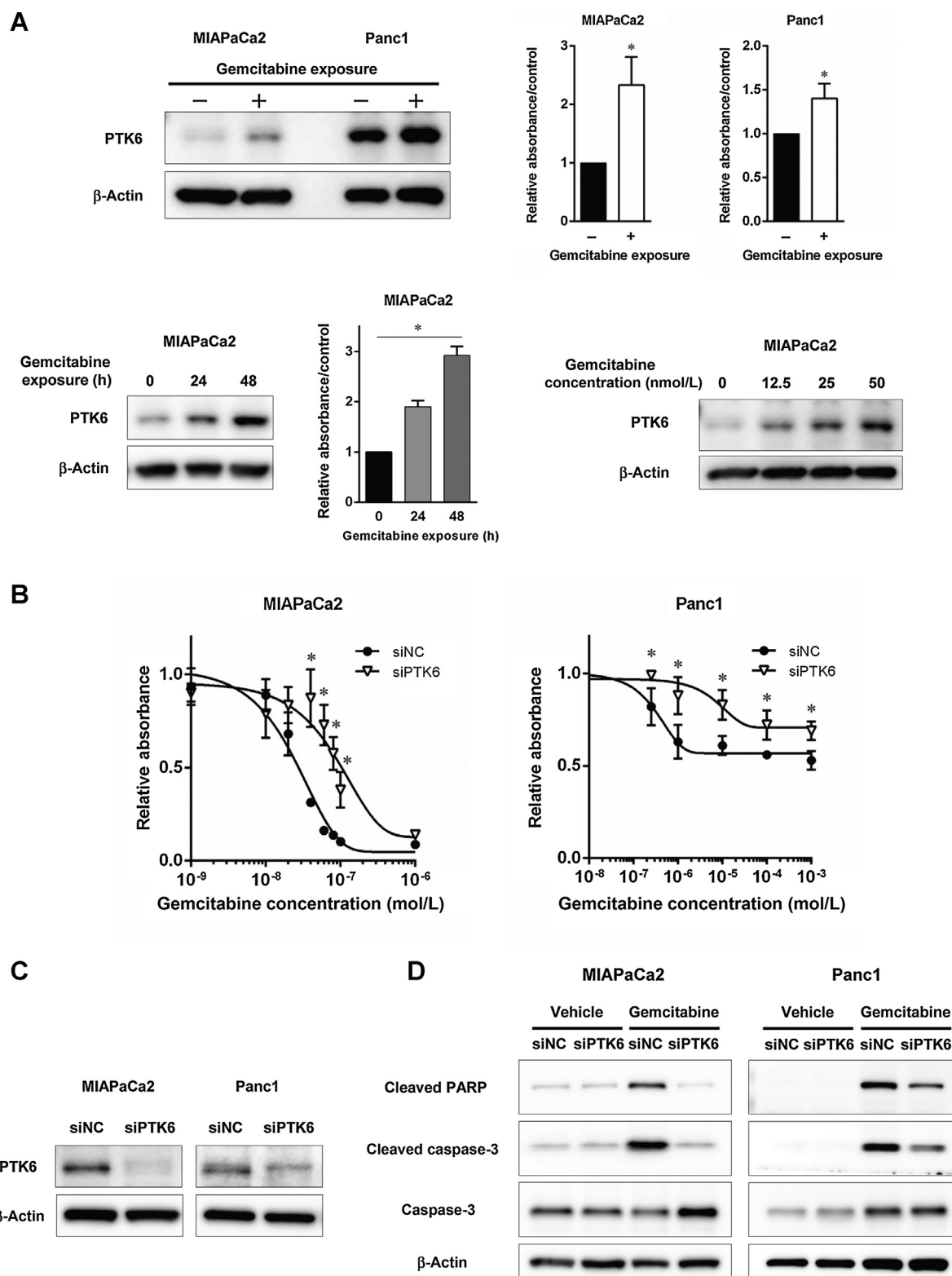
#### Statistical analysis

Drawing figures and fitting curves, calculating IC<sub>50</sub>, and statistical analyses were performed with GraphPad Prism6 software (GraphPad Software Inc.). Experiments were conducted in triplicate in independent settings, and the values were presented as their averages, and compared using the Student *t* test for single comparison or ANOVA with a *post hoc* Bonferroni correction for multiple comparisons as appropriate. *P* values <0.05 were considered as significant.

## Results

### PTK6 expression is increased after gemcitabine exposure in pancreatic cancer cells

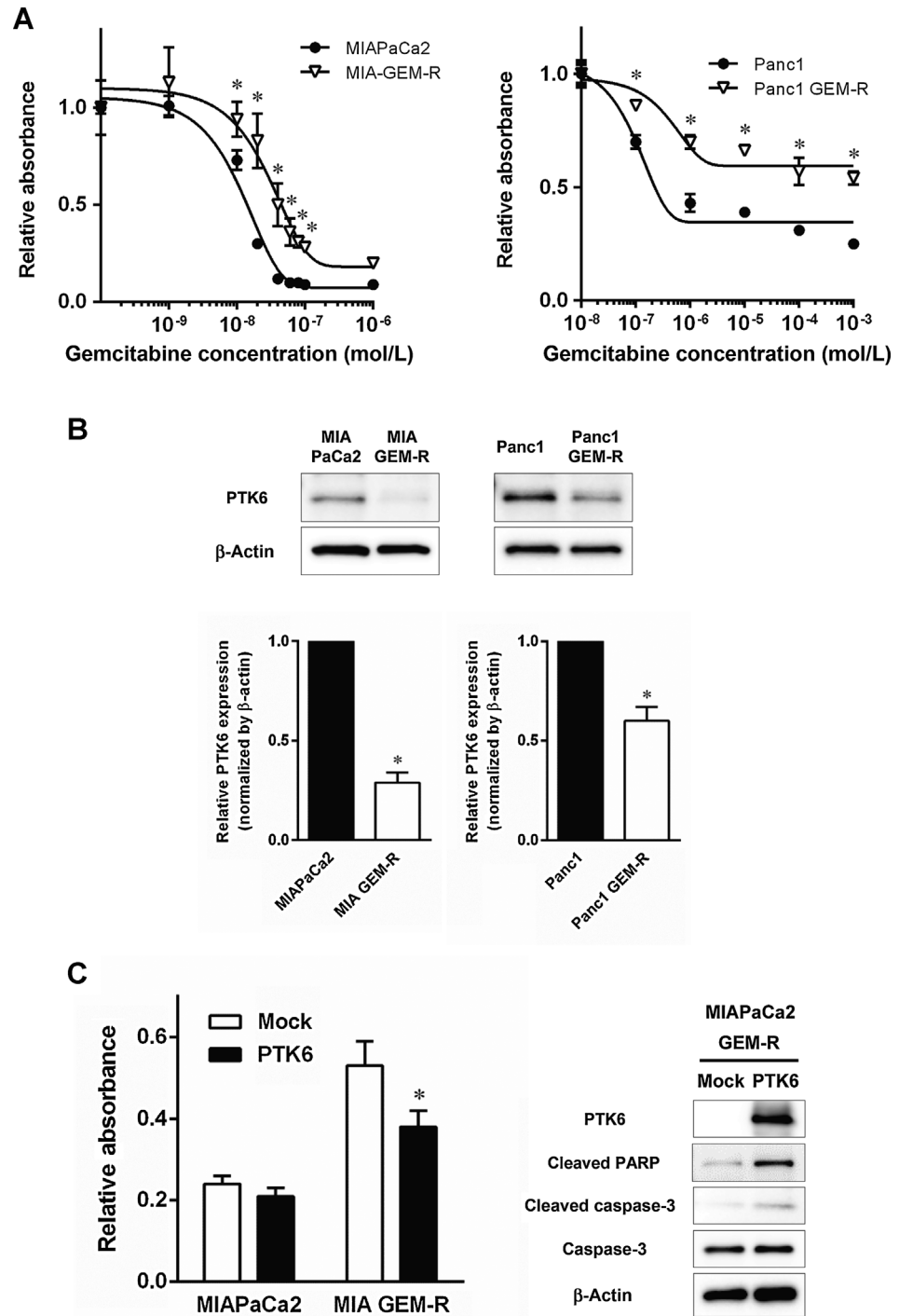
PTK6 has previously been shown to be induced in response to γ-radiation in murine nonmalignant intestinal epithelial



**Figure 1.** A, endogenous induction of PTK6 in pancreatic cancer cells in response to gemcitabine. MIAPaCa2 and Panc1 pancreatic cancer cells were treated with gemcitabine (50 nmol/L for MIPaCa2, 10  $\mu$ mol/L for Panc1, respectively) for 24 hours, and then cell lysates were subjected to Western blotting. PTK6 expression was significantly increased by gemcitabine treatment at 24 hours in both MIAPaCa2 and Panc1 cells (left, representative Western blotting for MIAPaCa2 and Panc1; right, the graph depicts averaged intensity of bands representing PTK6 normalized to the intensity of bands representing  $\beta$ -actin; \*,  $P < 0.05$  vs. control by t-test). The Western blotting assay was performed in triplicate. Furthermore, PTK6 induction by gemcitabine exposure was increased over exposure time and dose of gemcitabine (\*,  $P < 0.05$ ). For multiple group comparisons, ANOVA with a *post hoc* Bonferroni correction was used. (Continued on the following page.)

**Figure 2.**

A, cell viability dose-response curve over gemcitabine treatment for resistant subclones derived from MIAPaCa2 and Panc1 (MIA<sub>GEM-R</sub> and Panc1<sub>GEM-R</sub>). MIA<sub>GEM-R</sub> and Panc1<sub>GEM-R</sub> demonstrated stable resistance significantly against gemcitabine compared with their parental clones (\*,  $P < 0.05$  vs. control by ANOVA). The IC<sub>50</sub> against gemcitabine were 13.2 nmol/L for MIAPaCa2 and 31.2 nmol/L for MIA-GEM-R and 94.1 nmol/L for Panc1 and 278.6 nmol/L for Panc1 GEM-R, respectively. B, PTK6 expression in gemcitabine-resistant subclones MIA<sub>GEM-R</sub> and Panc1<sub>GEM-R</sub>. Basal PTK6 expression was significantly suppressed in gemcitabine-resistant subclones compared with their parental cells (\*,  $P < 0.05$  by t-test). Top, representative Western blotting; bottom, the graph depicts averaged intensity of bands representing PTK6 normalized to the intensity of bands representing  $\beta$ -actin. The Western blotting assay was performed in triplicate. C, the effect of restoration of PTK6 in MIA<sub>GEM-R</sub> on sensitivity to gemcitabine. PTK6 expression was restored in MIA<sub>GEM-R</sub> by transient overexpression vector and cell viability and apoptosis was assayed at 96 hours with gemcitabine treatment (50 nmol/L). Left, the graph depicts cell viability after gemcitabine treatment. The cell viability was significantly decreased with restoration of PTK6 in MIA<sub>GEM-R</sub> (\*,  $P < 0.05$  by t-test). Right, the apoptotic markers cleaved Caspase3 and PARP induced by gemcitabine were increased by restoration of PTK6 in MIA<sub>GEM-R</sub>.



cells (15). Therefore, we first examined whether PTK6 expression level is altered by gemcitabine exposure in human pancreatic cancer. Two established human pancreatic cancer cell

lines, MIAPaCa2 and Panc1 cells, were treated with gemcitabine at the appropriate concentrations to induce apoptosis in each cell line (50 nmol/L for MIAPaCa2, 10  $\mu$ mol/L for Panc1,

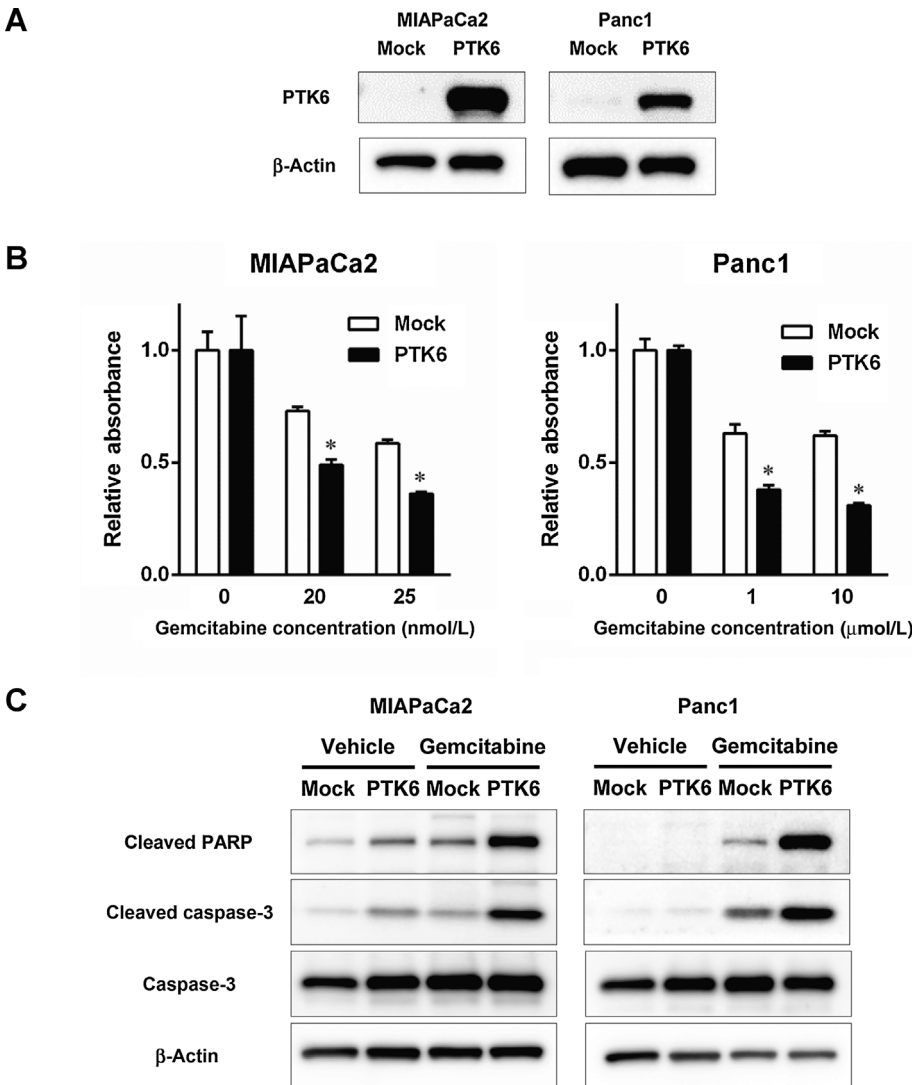
(Continued.) B, effect of PTK6 gene silencing on cytotoxicity of gemcitabine in pancreatic cancer cell lines. Cells were transfected with PTK6-specific siRNA (siPTK6) or negative control-siRNA (siNC) for 48 hours prior to gemcitabine treatment, and the cell viability was assayed at 96 hours. PTK6 gene silencing made the pancreatic cancer cells more resistant to gemcitabine (\*,  $P < 0.05$  vs. control by ANOVA). C, gene silencing of PTK6 by siRNA. After 48-hour transfection for PTK6-specific siRNA (siPTK6) or negative control-siRNA (siNC), the efficacy of gene silencing was evaluated by Western blot. Gene silencing of PTK6 expression was confirmed in 2 pancreatic cancer cell lines. D, effect of PTK6 gene silencing on gemcitabine-induced apoptosis in pancreatic cancers. MIAPaCa2 and Panc1 cells were treated for 96 hours with gemcitabine at 50 nmol/L and 10  $\mu$ mol/L, respectively. The apoptotic markers induced by gemcitabine, cleaved Caspase3, and PARP were reduced with gene silencing of PTK6.

respectively), and PTK6 protein levels were assessed by Western blotting at 24 hours. As shown in Fig. 1A, PTK6 expression increased significantly after gemcitabine treatment in MIAPaCa2 cells (2.3-fold increase in MIAPaCa2 at 24 hours,  $P < 0.05$ ). The increase of PTK6 expression in Panc1 cells was statistically significant but more modest in magnitude (1.4-fold increase in Panc1 at 24 hours,  $P < 0.05$ ), whereas the basal expression of PTK6 in Panc1 cells was much robust than that in MIAPaCa2 cells. Of note, MIAPaCa2 cells are more sensitive to gemcitabine than Panc1 cells. Furthermore, this induction of PTK6 gene expression by gemcitabine exposure was increased over the exposure time (1.9-fold increase at 24 hours and 2.9-fold increase at 48 hours, respectively,  $P < 0.05$  by ANOVA) and the dose of gemcitabine (Fig. 1A).

**Gene silencing of PTK6 impairs gemcitabine-induced apoptosis in pancreatic cancer cells**

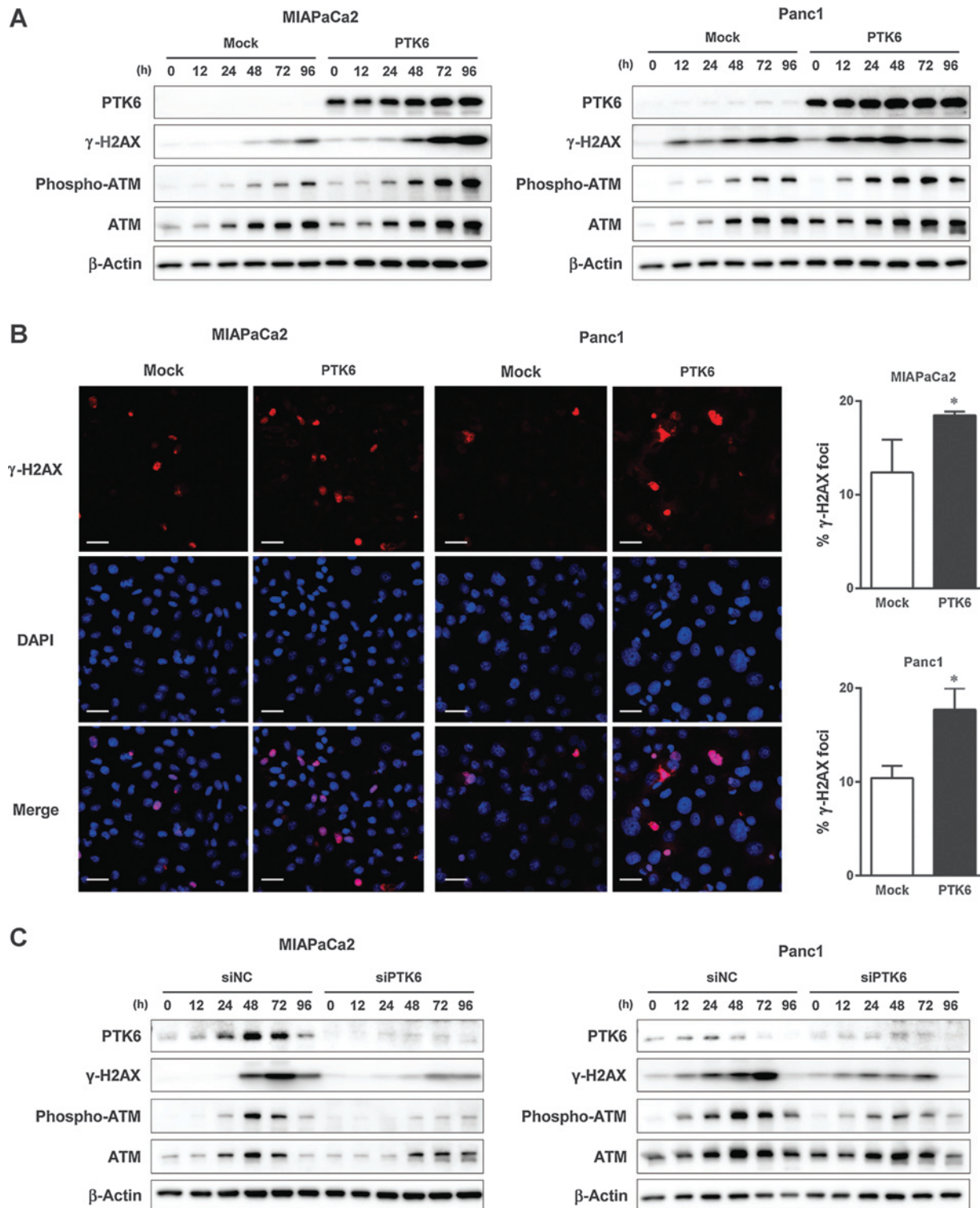
To explore the association between PTK6 expression and apoptosis of pancreatic cancer cells further, we next tested the effect of PTK6 gene silencing in pancreatic cancer cells on gemcitabine

cytotoxicity. MIAPaCa2 and Panc1 cells were treated with gemcitabine at various doses, and cellular viability was assessed using the WST-8 assay at 96 hours. Either siRNA targeting PTK6 or nonspecific siRNA was administered 48 hours prior to gemcitabine treatment. The effectiveness of PTK6 gene silencing by the PTK6-specific siRNA was confirmed by Western blotting (Fig. 1C). As shown in Fig. 1B, the cell viability curves over increasing doses of gemcitabine were shifted to the right for both pancreatic cancer cell lines by gene silencing of PTK6, indicating that PTK6 gene silencing made the pancreatic cancer cells more resistant to gemcitabine ( $P < 0.05$  by ANOVA, respectively). Similar effect of PTK6 gene silencing on the cytotoxicity of gemcitabine was also observed when an alternative siRNA targeting a different sequence of PTK6 was used (Supplementary Fig. S1). Furthermore, cellular markers of apoptosis in response to gemcitabine treatment, including cleaved Caspase-3 and PARP, were reduced by PTK6 gene silencing compared with the control counterparts transfected with nonspecific nontargeting siRNA (Fig. 1D). These findings suggest that PTK6 is critical for gemcitabine-induced apoptosis in pancreatic cancer cells.

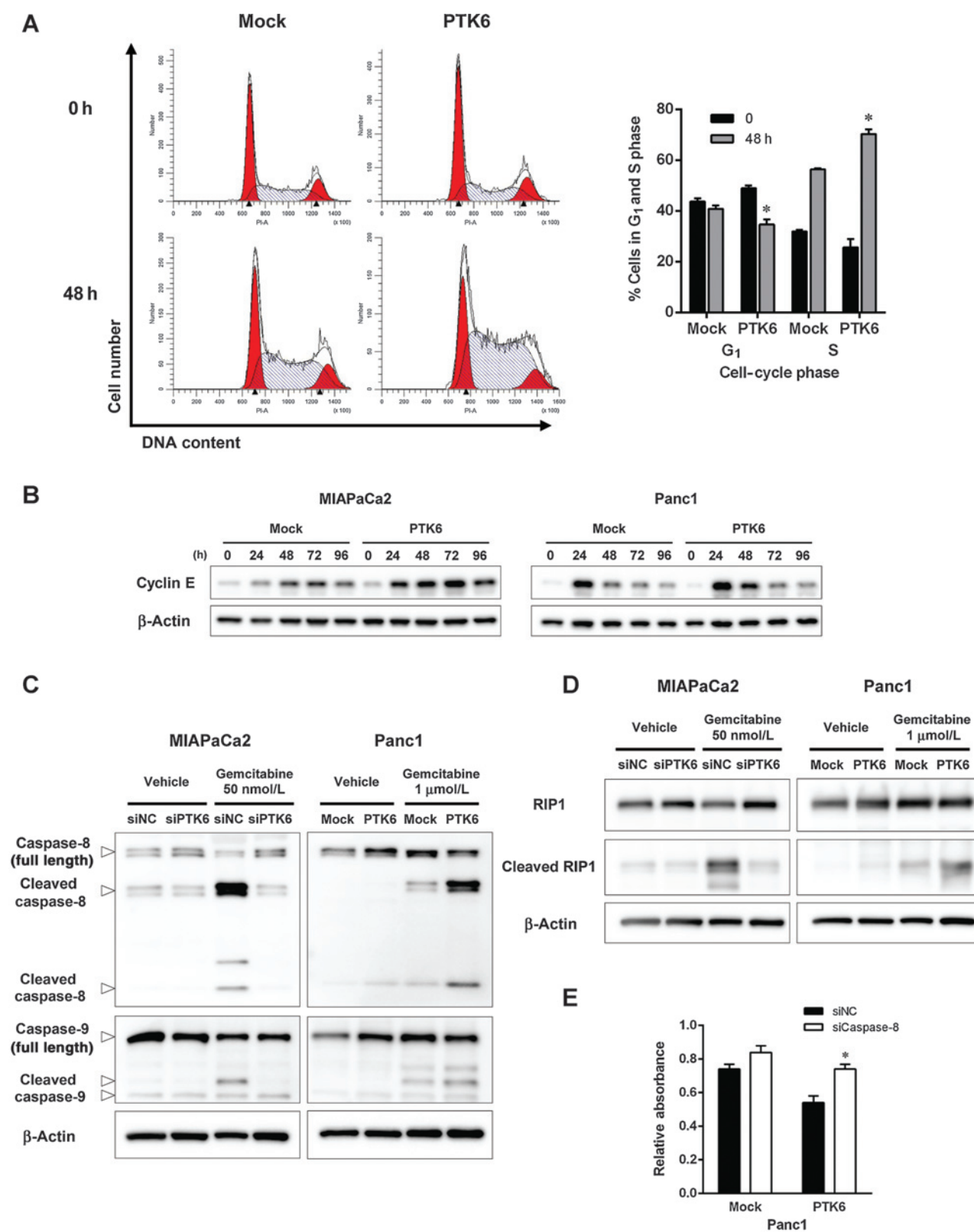


**Figure 3.** A, stable overexpression of PTK6 in MIAPaCa2<sup>PTK6</sup> and Panc1<sup>PTK6</sup>. Stable transfectants of PTK6 overexpression vector were established from MIAPaCa2 and Panc1 cells. B, effect of PTK6 overexpression on cytotoxicity of gemcitabine in MIAPaCa2 and Panc1 cells. The graph depicts the cellular viability after gemcitabine treatment of various doses at 96 hours. In contrast with the effect of PTK6 gene silencing on the cytotoxicity of gemcitabine, PTK6 overexpression made MIAPaCa2 and Panc1 cells more sensitive to gemcitabine (\*,  $P < 0.05$  vs. control by ANOVA). C, effect of PTK6 overexpression on gemcitabine-induced apoptosis in pancreatic cancers. Pancreatic cancer cells were treated with gemcitabine at 10 nmol/L for MIAPaCa2<sup>PTK6</sup> and 1  $\mu$ mol/L for Panc1<sup>PTK6</sup>, respectively, for 96 hours. Gemcitabine-induced cleaved Caspase3 and PARP were increased compared with their control counterparts, indicating increased apoptosis.

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**Figure 4.** A, effects of PTK6 overexpression on gemcitabine-induced activation of H2AX and ATM. MIAPaCa2 and Panc1 cells were treated with gemcitabine at 10 nmol/L and 1 μmol/L for 24 hours, respectively. Gemcitabine-induced γ-H2AX and phosphorylated ATM were significantly increased in MIAPaCa2<sup>PTK6</sup> and Panc1<sup>PTK6</sup>, compared with their respective controls, MIAPaCa2<sup>mock</sup> and Panc1<sup>mock</sup>. B, effect of PTK6 overexpression on gemcitabine-induced γ-H2AX foci in pancreatic cancer cells on fluorescent-immunohistochemistry. Left, representative images of immunofluorescent staining detecting γ-H2AX in pancreatic cancer cells. Right, the graphs depicted the average number of cells with positive γ-H2AX foci in the nucleus per the number of all cells observed in each high power field (HPF) under microscopic examination. The positive γ-H2AX foci were counted in five random fields. The cells with positive γ-H2AX foci after gemcitabine treatment were significantly increased in MIAPaCa2<sup>PTK6</sup> and Panc1<sup>PTK6</sup>, compared with their respective control counterparts (\*,  $P < 0.05$  by t-test). Bar, 50 μm. C, effects of gene silencing of PTK6 on activation of H2AX and ATM. MIAPaCa2 and Panc1 cells were transfected either with siRNA targeting to PTK6 or nonspecific control siRNA for 48 hours prior to treatment with gemcitabine. Then cells were treated with gemcitabine at 10 nmol/L for MIAPaCa2 and 1 μmol/L for Panc1. GEM-induced activation of H2AX and ATM were suppressed by gene silencing of PTK6 in MIAPaCa2 and Panc1 cells.



**Figure 5.** A, effect of PTK6 overexpression on cell cycle distribution after gemcitabine treatment. Cell cycle distribution in MIAPaCa2 cells was analyzed by flow cytometry at 0 and 48 hours after gemcitabine treatment (20 nmol/L). DNA histogram (left) and quantified cell cycle distributions (right) in MIAPaCa2<sup>mock</sup> and MIAPaCa2<sup>PTK6</sup> after gemcitabine treatment were shown. (Continued on the following page.)

### Decreased PTK6 expression is associated with gemcitabine resistance in pancreatic cancer cells

We next examined the association between PTK6 expression and acquired resistance against gemcitabine treatment in pancreatic cancer cells. Gemcitabine-resistant cell subclones (MIA<sub>GEM-R</sub> and Panc1<sub>GEM-R</sub>) were isolated from parental MIA PaCa2 and Panc1 cell lines by serial subculture through incrementally increasing gemcitabine concentrations over a 2-month period. These newly established cell lines demonstrated stable gemcitabine resistance compared with their parental cell lines (Fig. 2A). Furthermore, these cell lines with greater gemcitabine resistance also demonstrated significantly decreased PTK6 expression compared with their parental counterparts (71% decrease in MIA PaCa2 and 40% decrease in Panc1, respectively,  $P < 0.05$  by t-test; Fig. 2B). Moreover, when PTK6 expression was restored using a PTK6 overexpression vector in MIA<sub>GEM-R</sub> cells, cell survival after 50 nmol/L gemcitabine treatment over 96 hours was significantly decreased with increased apoptosis (23% decrease in survival compared with the control-resistant clone MIA<sub>GEM-R</sub>,  $P < 0.05$  by t-test; Fig. 2C). We used 50 nmol/L gemcitabine in this experiment in order to achieve sufficient gemcitabine toxicity in these gemcitabine-resistant cells. These findings indicate that PTK6 downregulation is associated with, at least in part, acquired resistance to gemcitabine in pancreatic cancer cells.

### PTK6 overexpression sensitizes pancreatic cancer cells to gemcitabine by enhancing the DNA damage response

Next, we evaluated the effect of constitutive PTK6 overexpression in pancreatic cancer cells on gemcitabine-induced apoptosis and DNA damage. An expression vector encoding full-length PTK6 cDNA was transfected into MIA PaCa2 and Panc1 cells, and stable transfectants overexpressing PTK6 were established for each cell line (MIA PaCa2<sup>PTK6</sup>, Panc1<sup>PTK6</sup> as shown in Fig. 3A). These stable transfectants were more sensitive to gemcitabine than their parental counterparts. When treated with several doses of gemcitabine (20 nmol/L to 25 nmol/L for MIA PaCa2, 1  $\mu$ mol/L to 10  $\mu$ mol/L for Panc1) over 96 hours, the survival of each PTK6-overexpressing cell line was lower than that of the corresponding control cell line ( $P < 0.05$  by ANOVA), with increased apoptosis demonstrated by increased cleaved Caspase-3 and PARP (Fig. 3B and C).

To further explore the mechanism by which PTK6 regulates the cytotoxic effect of gemcitabine on pancreatic cancers, we next examined the effect of altered PTK6 expression on DNA damage caused by gemcitabine. Phosphorylated H2AX ( $\gamma$ -H2AX) is a well-known marker for DNA double-strand breaks (DSB; refs. 18, 19). Gemcitabine (10 nmol/L for MIA PaCa2 and 1  $\mu$ mol/L for Panc1 for 24 hours) induced  $\gamma$ -H2AX in MIA PaCa2 and Panc1 cells as assessed by Western blotting and fluorescent-immunohistochemistry, indicating that gemcitabine induces DNA damage. When

PTK6 was overexpressed in these cells, the induced  $\gamma$ -H2AX protein expression was increased and the number of cells with  $\gamma$ -H2AX foci in the nucleus was increased 1.5-fold for MIA PaCa2 and 1.7-fold for Panc1, respectively ( $P < 0.05$  by t-test, Fig. 4A and B). Conversely, gemcitabine-induced  $\gamma$ -H2AX protein expression was decreased when PTK6 expression was reduced by siRNA (Fig. 4C). Furthermore, gemcitabine-induced activation of ataxia-telangiectasia mutated (ATM) protein kinase, a central initiator of key signals response to DNA damage (20, 21), was enhanced when PTK6 was forcefully overexpressed in MIA PaCa2 and Panc1 cells (Fig. 4A), while ATM activation was suppressed when PTK6 was gene-silenced (Fig. 4C). Because gemcitabine is considered to be effective to damage DNA only for the cells in S phase, where the cancer cells actively synthesize DNA (22), we investigated the effect of PTK6 overexpression on cell cycle distribution after gemcitabine treatment. In PTK6-overexpressing MIA PaCa2 cells, the fraction of cells in S phase was much increased 48 hours after gemcitabine treatment compared with the S phase fraction in control cells that did not overexpress PTK6 (Fig. 5A). As cyclin E expression is strictly regulated in the cell cycle and can be used as a marker for G<sub>1</sub>-S transition in cell cycles (23–25), we examined expression of cyclin E in pancreatic cancer cells after gemcitabine. As shown in Fig. 5B, cyclin E expression was greater at 24 to 48 hours after gemcitabine treatment in MIA PaCa2<sup>PTK6</sup> and Panc1<sup>PTK6</sup> cells, compared with cyclin E expression in their control counterparts ( $P < 0.05$  by t-test, respectively). Taken together, these findings suggest that PTK6 enhances the cytotoxicity of gemcitabine by increasing the S phase fraction to thereby facilitate DNA damage.

### The effect of PTK6 on gemcitabine-induced apoptosis is dependent on caspase-8 activation

We then evaluated which initial caspases might trigger PTK6-regulated apoptosis as result of the accumulation of DNA damage. We tested two initial caspases, Caspase-8 and Caspase-9. Both Caspase-8 and Caspase-9 were activated by gemcitabine treatment after 96 hours in MIA PaCa2 and Panc1 cells. However, gene silencing of PTK6 and overexpression of PTK6 altered the activation of Caspase-8 more robustly than that of Caspase-9 (Fig. 5C). Of note, Caspase-8 initiates the "extrinsic" apoptotic pathway and Caspase-9 initiates the "intrinsic" pathway. The apparent enhancement of the "extrinsic" pathway by PTK6 overexpression and its suppression by PTK6 gene silencing were confirmed by demonstrating parallel changes in the cleavage of RIP1, a direct substrate of activated Caspase-8 (26), as shown in Fig. 5D. When Caspase-8 was gene-silenced, the effect of PTK6 overexpression on gemcitabine-induced apoptosis was abolished (Fig. 5E and Supplementary Fig. S2). These findings indicated that the effect of PTK6 on gemcitabine-induced apoptosis requires Caspase-8.

(Continued.) DNA histogram depicts a representative graph, and quantification was based on the independent experiments performed duplicate (\*,  $P < 0.05$  by t-test). PTK6 overexpression increased the fraction of cells in S phase, whereas it decreased the fraction of cells in G<sub>0</sub>-G<sub>1</sub> phase at 48 hours. B, effects of PTK6 overexpression on Cyclin E expression. MIA PaCa2 and Panc1 cells were treated with gemcitabine at 20 nmol/L and 1  $\mu$ mol/L, respectively. Gemcitabine-induced Cyclin E expression was enhanced in MIA PaCa2<sup>PTK6</sup> and Panc1<sup>PTK6</sup>, compared with their respective controls, MIA PaCa2<sup>mock</sup> and Panc1<sup>mock</sup>. C, effect of PTK6 alteration on Caspase-8 and Caspase-9 activation after gemcitabine treatment (50 nmol/L for MIA PaCa2 and 1  $\mu$ mol/L for Panc1, respectively). PTK6 overexpression enhances Caspase-8 activation dominantly, and PTK6 depletion inhibited Caspase-8 activation. D, effect of PTK6 alteration on RIP1 activation after gemcitabine treatment. RIP1 is a direct substrate of Caspase-8 and it was activated after gemcitabine treatment. The gene silencing of PTK6 in MIA PaCa2 decreased RIP1 activation, and its overexpression in Panc1 increased RIP1 activation. E, effect of Caspase-8 inhibition on PTK6-mediated apoptosis. Panc1<sup>PTK6</sup> cells and Panc1<sup>mock</sup> cells were treated with/without Caspase-8 targeting siRNA and gemcitabine, and the cell viability was evaluated at 96 hours. PTK6-mediated apoptosis was suppressed by Caspase-8 gene silencing.



Taking all these *in vitro* findings together, we concluded that PTK6 enhances DNA damage accumulation and enhances the activation of a Caspase-8-dependent apoptotic pathway in pancreatic cancer cells treated with gemcitabine.

#### PTK6 overexpression sensitized pancreatic cancer xenografts to gemcitabine

Finally, we validated our *in vitro* observations by examining the effect of PTK6 overexpression on the efficacy of gemcitabine treatment of pancreatic cancer xenografts *in vivo*. Mice implanted with either MIAPaCa2 or Panc1 stable transfectants of PTK6 overexpression vectors, MIAPaCa2<sup>PTK6</sup> or Panc1<sup>PTK6</sup> or transfectants of the control empty vectors MIAPaCa2<sup>mock</sup> or Panc1<sup>mock</sup> were treated with gemcitabine over 8 to 10 weeks. The tumors of mice implanted with MIAPaCa2<sup>PTK6</sup> or Panc1<sup>PTK6</sup> grew significantly more slowly during gemcitabine treatment than tumors derived from the corresponding control cells (Fig. 6A). When the mice were sacrificed (at 8 weeks for the mice implanted with MIAPaCa2, and at 10 weeks for those with Panc1), tumors of MIAPaCa2<sup>PTK6</sup> and Panc1<sup>PTK6</sup> origin were much smaller than those derived from MIAPaCa2<sup>mock</sup> and Panc1<sup>mock</sup> (1,606.3 mm<sup>3</sup> vs. 252.4 mm<sup>3</sup> for tumors of MIAPaCa2, 435.1 mm<sup>3</sup> vs. 106.8 mm<sup>3</sup> for tumors of Panc1 in volume, respectively,  $P < 0.01$  by ANOVA, 608.8 mg vs. 128 mg for MIAPaCa2, 144 mg vs. 21.8 mg for Panc1 in weight, respectively,  $P < 0.01$  by ANOVA; Fig. 6B and C). The stability of PTK6 overexpression in these mouse xenograft tumors was confirmed by immunohistochemistry (Fig. 6D). Growth rates of MIAPaCa2 and Panc1 cells *in vitro* in the absence of gemcitabine were not changed by PTK6 overexpression (Supplementary Fig. S3). The lack of effect of PTK6 overexpression on basal proliferation in the absence of gemcitabine is also consistent with our *in vivo* observations in the xenograft model. After subcutaneous injection of equal numbers of PTK6 overexpressing or control cells, we obtained equivalent tumor sizes over the same times prior to gemcitabine treatment independently of whether the cells overexpressed PTK6. Therefore, the subsequent slower growth of the implanted tumors with PTK6 overexpression after gemcitabine treatment seemed more likely to reflect the increased cytotoxicity of gemcitabine against pancreatic cancer cells.

## Discussion

The dismal outcomes of patients with pancreatic cancer are primarily caused by inherited and acquired resistance of cancer cells to chemotherapy. Although gemcitabine has been used as the standard chemotherapeutic agent for pancreatic cancer, its efficacy in pancreatic cancer is limited in this disease (1, 3). Gemcitabine is a nucleotide analogue; once it enters into cancer cells, it is phosphorylated to triphosphate form and incorporated into DNA, and then it induces cell death (22). While the pharmacologic mechanism of action by which gemcitabine causes toxicity against cancer cells has been well studied, the molecular mechanism of the resistance against gemcitabine in pancreatic cancer is yet to be determined. In this study, we have shown that PTK6 plays an important role in regulating gemcitabine-induced DNA damage and resultant apoptosis in pancreatic cancer. Depleted PTK6 correlates with acquired resistance against gemcitabine in pancreatic cancer cells, and forceful overexpression of PTK6 increases the therapeutic efficacy of gemcitabine against pancreatic cancer xenografts *in vivo*. PTK6 expression varies substantially among

human pancreatic cancers (10), although the correlation of PTK6 expression with clinical human pancreatic cancer chemosensitivity and survival awaits study.

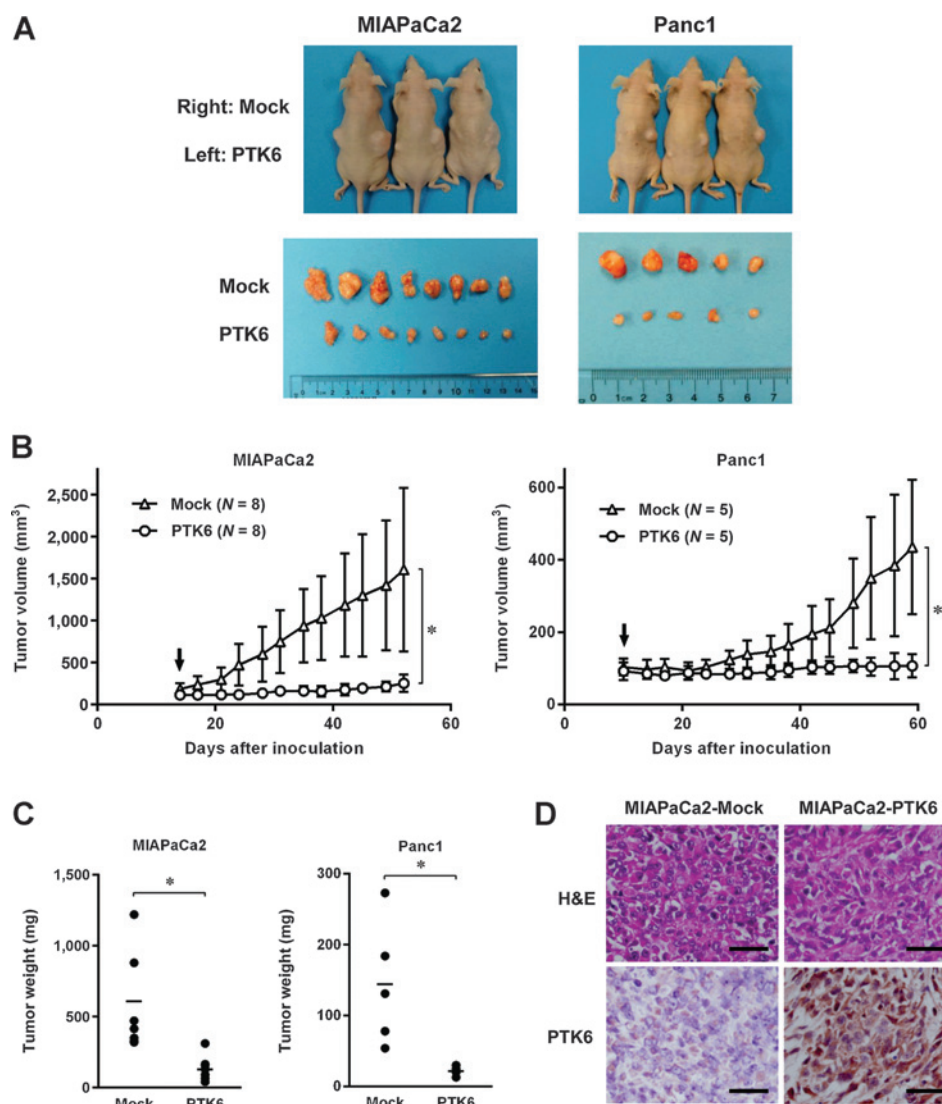
Previous studies of the role of PTK6 in mediating DNA damage in nonmalignant cells (15, 27, 28) and in other malignancies (29) have yielded conflicting results that likely reflect differences in the genetic background of host cells. Gierut and colleagues emphasized in their report that PTK6 signaling outcomes in cellular models are context-dependent and demonstrated the effect of PTK6 knockdown on radiation-induced apoptosis was different depending on the p53 gene mutation in their colon cancer cell line model (28, 29). In addition, the localization of PTK6 may be important in the role of overexpressed PTK6 in pancreatic cancer. Brauer and colleagues also described completely opposite effects of PTK6 overexpression on the cell phenotype depending on the intracellular localization of PTK6; in prostate cancer cell models, they demonstrated that overexpressing PTK6 in the cytoplasm increased proliferation, while localizing PTK6 in the nucleus suppressed proliferation (30). In pancreatic adenocarcinoma, the localization of PTK6 in both the cytoplasm and the nucleus has been observed by immunohistochemical staining (10), and in our animal model with xenotransplanted tumor with PTK6 overexpression, PTK6 was observed in both the cytoplasm and the nucleus as well (Fig. 6D). This study demonstrates that PTK6 is upregulated by gemcitabine and then contributes to DNA damage and apoptosis in pancreatic cancer cells treated with gemcitabine, the major current chemotherapeutic agent used clinically for this disease. Our *in vivo* observations suggest that this difference in DNA damage is likely to be clinically significant since it contributed to slower tumor growth in our murine model.

One of our key findings in this study is that PTK6 overexpression enhanced gemcitabine-induced DNA damage in pancreatic cancer cells. We assessed DNA damage by  $\gamma$ -H2AX and ATM phosphorylation. These molecules have been considered to be the early responders to the DSB of the cancer genome caused by DNA-damaging stimuli, including radiation or chemotherapeutic agents. Once ATM is activated upon DNA damage, it subsequently initiates diverse signals, regulating cell cycle check points, apoptosis, and DNA repair (20). The histone protein H2AX located at the DSB is then phosphorylated by ATM and mediates the relevant downstream signal(s) (18). Because constitutive overexpression of PTK6 did not alter the activity of H2AX and ATM without gemcitabine exposure in pancreatic cancer cells, it is unlikely that PTK6 directly activates these molecules. Rather, the increased gemcitabine-induced activation of H2AX and ATM by PTK6 overexpression more likely reflects increased DSBs in the genome of pancreatic cancer cells. As gemcitabine incorporation into DNA is critical for its cytotoxicity, and thus the gemcitabine toxicity is specific to the cells in S phase (22), we speculated that the effect of PTK6 overexpression on cell cycle arrest in pancreatic cancer may be a potential mechanism to enhance gemcitabine toxicity. Gemcitabine-induced cell cycle arrest at S phase in pancreatic cancers has been described in previous studies in which gemcitabine cytotoxicity was enhanced by increased S phase arrest with checkpoint kinase inhibitors (31, 32). Taken together with these previous reports, our observation that PTK6 overexpression increased S phase fraction after gemcitabine treatment along with increased DNA damage supports our hypothesis.

The mechanism by which gemcitabine-induced DNA damage induces apoptosis in pancreatic cancer is not fully elucidated. In

**Figure 6.**

Effect of PTK6 overexpression on treatment efficacy of gemcitabine against xenotransplanted pancreatic tumors in nude mice. Mice were inoculated with either MIAPaCa2<sup>PTK6</sup> or Panc1<sup>PTK6</sup> (right) and MIAPaCa2<sup>mock</sup> or Panc1<sup>mock</sup> (left) in their bilateral flanks by subcutaneous injection. Once tumors reached measurable size (approximately 50 mm<sup>3</sup>), treatment with gemcitabine (150 mg/kg) twice-weekly by i.p. injection commenced. Overexpression of PTK6 in pancreatic cancer increased gemcitabine chemosensitivity in this mouse xenograft model. A, representative images of xenotransplanted tumors in mice at the time of necropsy (top) and images of dissected tumors from mice after necropsy (bottom). B and C, the volume of the tumors in mice was recorded over the 8 to 10 weeks of gemcitabine treatment (B), and after necropsy, the tumors were extracted from mice and their weight was measured (C). PTK6 overexpression significantly suppressed the growth of the tumor during gemcitabine treatment. D, representative images of tumor sections from MIAPaCa2<sup>PTK6</sup> and MIAPaCa2<sup>mock</sup>. Stable overexpression of PTK6 in the xenografted tumor from MIAPaCa2<sup>PTK6</sup> was confirmed. Bar, 50  $\mu$ m.



cells with wild-type p53, p53 regulates an apoptotic signal pathway with bcl-2 and Caspase-9 ("intrinsic" pathway) response to DNA damage (33). However, many cancer cells including the pancreatic cancer cells we tested here have p53 mutations or deletions and we suspected that an alternative apoptotic signal pathway, the "extrinsic" pathway, initiated by Caspase-8 activation might regulate gemcitabine-induced apoptosis in pancreatic cancer. Pauwels demonstrated that gene expression patterns in apoptotic signaling changed similarly in pancreatic cancer cell lines exposed to gemcitabine and radiation to the changes observed after treatment with TRAIL, which activates the "extrinsic" apoptotic pathway (34). Our study clearly indicated that PTK6-regulated gemcitabine-induced apoptosis requires Caspase-8 and suggests the importance of the extrinsic apoptotic pathway in pancreatic cancer. The resistant subclones of pancreatic cancer cell lines selected from the parental cells by prolonged gemcitabine exposure expressed substantially less PTK6 than the parental cells. Although our study was not designed to prove the association between the

baseline PTK6 expression level and resistance in pancreatic cancer overall, our findings in this study raise the possibility that PTK6 expression may be of prognostic value for the long-term outcomes of patients with pancreatic cancer. We previously reported that PTK6 expression varies among human pancreatic adenocarcinoma tissues (10), and further study of the potential prognostic and predictive importance of PTK6 on patient outcomes after gemcitabine treatment of pancreatic cancer is warranted.

In summary, PTK6 expression is endogenously induced by gemcitabine in pancreatic cancer cells, and gene silencing of PTK6 causes resistance to gemcitabine. Conversely, overexpression of PTK6 enhances gemcitabine-induced DNA damage and increases subsequent apoptosis in pancreatic cancer cells as well as in pancreatic tumors in the mouse model. This is important because PTK6 levels vary profoundly among human pancreatic cancers (10). Tumors that express high levels of PTK6 may be more sensitive to gemcitabine. Thus, future studies of gemcitabine-based chemotherapy in pancreatic cancer might benefit from

stratification for PTK6 levels. Moreover, targeting PTK6 may improve the efficacy of gemcitabine-based chemotherapy against pancreatic cancer.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

**Conception and design:** H. Ono, M.D. Basson, H. Ito

**Development of methodology:** H. Ono, M.D. Basson, H. Ito

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** H. Ono, H. Ito

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** H. Ono, M.D. Basson, H. Ito

**Writing, review, and/or revision of the manuscript:** H. Ono, M.D. Basson, H. Ito

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** M.D. Basson, H. Ito

**Study supervision:** M.D. Basson, H. Ito

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