*Human Cancer Biology*

# Validation of TPX2 as a Potential Therapeutic Target in Pancreatic Cancer Cells

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**Abstract**

**Purpose:** The targeting protein for Xklp2 (*TPX2*) has recently gained attention as a putative oncogene possibly amplified in several human malignancies, including pancreatic adenocarcinoma. In this work, we sought to evaluate the copy number and expression of *TPX2* in pancreatic cancer cell lines and tumor tissues and to further explore the potential of TPX2 as a therapeutic target.

**Experimental Design:** The DNA copy number and expression of the *TPX2* gene were surveyed in pancreatic cancer cell lines and tumor tissues and compared with those of immortalized normal pancreatic ductal cells and normal pancreatic tissues. The cellular effects of TPX2 knockdown using small interfering RNA oligonucleotides in pancreatic cancer cells, such as growth in tissue culture, in soft agar, and in nude mice; apoptosis; and sensitivity to paclitaxel, were also investigated using various assays.

**Results:** Low-copy-number *TPX2* amplification was found in pancreatic cancer cell lines and low-passage pancreatic cancer tumor xenografts. TPX2 expression was upregulated in pancreatic cancer cell lines at both the mRNA and protein levels relative to the immortalized pancreatic ductal epithelial cell line HPDE6. Immunohistochemical staining of a tissue microarray showed that TPX2 expression was higher in pancreatic tumors compared with their normal counterparts. Treatment with TPX2 targeting small interfering RNAs effectively reduced pancreatic cancer cell growth in tissue culture, induced apoptosis, and inhibited growth in soft agar and in nude mice. Knockdown of TPX2 also sensitized pancreatic cancer cells to paclitaxel treatment.

**Conclusions:** Our results suggest that TPX2 might be an attractive target for pancreatic cancer therapy. (Clin Cancer Res 2009;15(21):6519–28)

Pancreatic ductal adenocarcinoma (PDAC), with a 5-year survival rate of ~5% for all stages combined in the United States, is among the most lethal of human cancers. In fact, the number of people estimated to die of pancreatic cancer (34,290 for 2008 in the United States) nearly matches the estimated number of individuals (37,680) who will be diagnosed with it (1). Currently, surgical resection is the only therapy that is considered to offer a cure; however, pancreatic adenocarcinoma is typically diagnosed as advanced inoperable disease characterized by resistance to current therapeutics. Therefore, new treatments as well as a better understanding of pancreatic cancer biology are urgently needed.

Genomic instability is thought to drive cancer, as regions with gains often harbor oncogenes and regions with losses commonly harbor tumor suppressor genes. PDAC harbors complicated aberrations of chromosomal alleles, with numerous specific gains and losses reported (2–5). Chromosomal gains of 20q are found in various types of adenocarcinoma and are also prominent in pancreatic cancer (6). Recently, *TPX2* was identified as a candidate oncogene from the amplicon on 20q11.2 showing copy number–driven overexpression in non–small-cell lung cancer and PDAC (7, 8). However, the frequency and the level of *TPX2* amplification in PDAC have not been reported. Additionally, it has been reported that the region containing *TPX2* is amplified in more than 50% of patients afflicted with giant-cell tumor of the bone. Additionally, high levels of *TPX2* mRNA and protein were detected in a high percentage of squamous cell carcinoma of the lung tumor samples, with the expression correlating to tumor grade, stage, and nodal status (9, 10).

TPX2 is a microtubule-associated protein downstream of Ran-GTP that plays a central role in mitotic spindle formation

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## Translational Relevance

With a 5-year survival rate of less than 5%, pancreatic cancer is among the most lethal types of human cancers. Current therapies are mostly ineffective and new therapies are desperately needed. This article describes the validation of TPX2 as a potential therapeutic target for pancreatic cancer. We present direct evidence that the *TPX2* gene is amplified and overexpressed in pancreatic tumor tissues; disruption of TPX2 function induces apoptosis and causes death of pancreatic cancer cells. This work is highly translational because further investigation of TPX2 as a therapeutic target and subsequent development of agents that target TPX2 may result in new and improved treatment for patients with pancreatic cancer.

and therefore proper segregation of chromosomes during cell division (11). Its expression has been associated with highly proliferative tissues. Throughout interphase, TPX2 is sequestered in the cell nucleus by interaction with the nuclear pore proteins importin $\alpha/\beta$, but is released at the early stages of mitosis in a RanGTP-dependent manner (12). During mitosis, TPX2 is able to interact with downstream partners, which includes the Aurora A kinase resulting in the localization of Aurora A to the microtubules of the mitotic spindle (13). Furthermore, TPX2 activates the kinase activity of Aurora A by locking it in an active conformation (14). Therefore, TPX2 exerts two levels of regulation on Aurora A kinase signaling (localization and enzymatic activity). Considering the potential upregulation of TPX2 in pancreatic cancer as well as its association with a signaling pathway involving oncogenic Aurora A, we hypothesize that TPX2 is a co-conspirator in driving pancreatic tumor development. In this work, we set out to further characterize *TPX2* amplification and evaluate TPX2 expression in pancreatic cancer cell lines and patient tumors. Furthermore, we analyzed the biological consequences of small interfering RNA (siRNA)–mediated knockdown of TPX2 expression in cultured pancreatic cancer cells.

## Materials and Methods

*Cell culture.* The cell line HPDE6 (an immortalized but nontransformed human pancreatic epithelial cell line) was obtained from Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada; ref. 15) and maintained in keratinocyte serum-free medium supplemented with 0.2 ng/mL epidermal growth factor and 30 µg/mL bovine pituitary extract (Invitrogen). Pancreatic cancer cell lines were purchased from the American Type Culture Collection and the European Collection of Cell Cultures. MUTJ cells were obtained from the University of Arizona Cancer Center. The cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 µg/mL). To preserve integrity, all cell lines were expanded and frozen down into a large number of cryogenic vials upon receipt from the sources. Cells were passaged every 3 to 5 d and discarded after 8 to 10 passages. If additional cells were needed, a new vial from the original cryogenic stock was then thawed and used. Cell lines were therefore passaged less than 6 mo in culture after receipt from the original sources. All cells were grown in a humidified incubator at 37°C and 5% $CO_2$. Cells were harvested with trypsin at 80% to 90% confluency. Cell counting was done using trypan blue staining on a hemacytometer.

*Gene copy number analysis.* Genomic DNA was isolated from cell lines and low-passaged pancreatic tumor xenografts using the DNeasy Tissue kit (Qiagen). Gene copy number was analyzed by quantitative PCR using an iCycler (Bio-Rad). Reactions were carried out in 20 µL reactions with 200 nmol/L of each primer, iQ SYBR Green Supermix (Bio-Rad), and 10 ng gDNA. Two-step amplification (95°C for 15 s and 56°C for 15 s) was repeated for 40 cycles. Following the PCR reaction, a melting curve analysis was done to determine PCR efficiency and purity of the amplified product. Data were provided as a threshold cycle value (Ct) for each sample, indicating the cycle at which a statistically significant increase in fluorescence was first detected. These data were then normalized to $\beta$-actin, which served as a reference gene. Primer sequences for *TPX2* were forward 5′-AGGGGCCCTTTGAACTCTTA-3′ and reverse 5′-TGCTCTAAACAAGCCCCATT-3′. Primer sequences used for $\beta$-actin were 5′-CTGGAACGGTGAAGGTGACA-3′ and 5′-AAGG-GACTTCCTGTAACAACGCA-3′.

*Quantitative reverse transcriptase-PCR.* Total RNA from cell pellets was isolated using the NucleoSpin RNA II isolation kit (BD Biosciences). One microgram of total RNA was used for reverse transcriptase (RT) reactions (20 µL total volume), which was carried out using the iScript cDNA Synthesis kit (Bio-Rad). An iCycler (Bio-Rad) was used to perform real-time fluorescence detection PCR. Reactions were carried out in 20 µL reactions with 200 nmol/L of each primer, iQ SYBR Green Supermix (Bio-Rad), and 1 µL cDNA. Primer sequences for *TPX2* were 5′-CGAAAGCATCCTTCATCTCC-3′ and 5′-TCCTTGGGACAGGTT-GAAAG-3′. Primer sequences used for $\beta$-actin were 5′-CTGGAACGGT-GAAGGTGACA-3′ and 5′-AAGGGACTTCCTGTAACAACGCA-3′. Relative expression was calculated using the $\Delta$-$\Delta$ Ct method (16) with $\beta$-actin serving as a reference gene for normalization.

*Western blot analysis.* Western blots were done as previously described (17) using 50 µg of nuclear lysate per sample and NuPAGE 4% to 12% Bis-Tris gels (Invitrogen). Membranes were probed with a mouse monoclonal antibody (clone18D5) against TPX2 (BioLegend) at a 1:5,000 dilution or a rabbit monoclonal antibody against $\alpha$-tubulin (Cell Signaling) at a 1:5,000 dilution. The membrane was washed and probed with anti-mouse or anti-rabbit horseradish peroxidase–linked secondary antibodies (Cell Signaling) and visualized with a chemiluminescence kit (Cell Signaling) and X-ray film. The film was scanned and quantified using the ImageQuant software (GE Healthcare).

*Tissue microarray construction and immunohistochemical analysis.* Needle cores of 1.0 mm in diameter were extracted from regions of interest from de-identified pancreatic tumor tissue blocks as well as normal pancreatic samples and arrayed in precise orientation in a composite paraffin block. The tissue microarray (TMA) master block was serially sectioned at 5-µm intervals and transferred onto standard charged glass by the water floatation method. The TMA slides were dipped in paraffin for uniform epitope preservation. Dewaxing and antigen retrieval were carried out with a Bond-MaX autostainer (Leica Microsystems, Inc.) using the accompanying Bond Refine Polymer Detection Kit. TPX2 antibody (mouse monoclonal clone 3164C6a, GenWay) was used at a dilution of 1:50, with an incubation time of 20 min. Staining (relative to background) received an intensity score on a 0 to 3 scale with 0 for absence of staining, 1 to indicate mild staining, 2 to indicate moderate staining, or 3 to indicate strong staining. A prevalence score was recorded based on the percent of tumor cells positive for the recorded intensity score with 1 representing <10% staining, 2 representing 10% to 40% staining, and 3 representing >40% staining. If the tissue in a core had multiple intensity scores, the highest intensity and its accompanying prevalence score was chosen. The intensity and prevalence were scored by a board-certified pathologist (G.H.). The overall staining scores were then computed by multiplying the intensity and prevalence scores for a composite range immunohistochemical (IHC) score of 0 to 9.

*siRNA treatment.* TPX2-s1 (targeting AAGAATGGAACTG-GAGGGCTT) and TPX2-s2 (targeting ATGAAAGTTTCTAACAACAAA of exon 6-7), the AllStars Negative Control siRNAs (nonsilencing siRNA), and the Ubiquitin B siRNA oligonucleotides were obtained

through Qiagen. Cells were transiently transfected using RNAiMAX (Invitrogen) according to the manufacturer's recommendations.

*Cell proliferation assay.* At 0, 24, 48, 72, and 96 h post siRNA transfection, cells were fixed with 10% trichloroacetic acid for 1 h at 4°C. Following fixation, cells were washed with water, then stained with a 0.04% sulforhodamine B (SRB) solution for 1 h. Cells were then washed with a 1% acetic acid solution. The plates were sat at room temperature until dry. Tris/HCl (50 mmol/L) was then added to each well and incubated for 15 min. Absorbance at 570 nm was quantified using a plate reader (BioTek). Four biological replicates (each done in triplicate) were done.

*Cell cycle analysis using flow cytometry.* Cells were treated with TPX2 siRNA oligonucleotides as described above for 48 h and harvested by trypsinization. The cells were resuspended and stained with propidium iodide (Sigma) in a modified Krishan buffer (18) for 1 h at 4°C. The propidium iodide–stained samples were then analyzed with a FACScan flow cytometer (BD Immunocytometry Systems). Histograms were analyzed for cell cycle compartments and the percentage of cells at each phase of the cell cycle was calculated using the CellQuest (BD Immunocytometry systems) analysis software.

*Apoptosis assay.* Cells were treated with TPX2 siRNA oligonucleotides as described above and harvested by trypsinization. Cell pellets were washed once with PBS buffer. The caspase-3 activity analysis was done by following the manufacturer's protocol (Clontech, BD Biosciences). Briefly, cell pellets were resuspended in 100 μL of chilled cell lysis buffer and incubated on ice for 10 min. Cell lysates were centrifuged in a microcentrifuge at $10,000 \times g$ for 10 min at 4°C and then the supernatants were transferred into new microcentrifuge tubes. The concentration of total protein of each sample was determined by the BCA protein assay (Pierce). Twenty-five micrograms of total protein of each sample were used for the analysis of caspase-3 activity.

*Cell death ELISA assay.* To further confirm the apoptosis-induced TPX2 siRNA oligonucleotides, we performed a TPX2 siRNA concentration–dependent treatment of the MIA PaCa-2 cells and quantified the induction of apoptosis using a second apoptosis assay, the Cell Death ELISA[PLUS] Kit (Roche Applied Science). The experimental protocol recommended by the kit manufacturer was followed. Briefly, cells were treated with a serial dilution of TPX2 siRNA oligonucleotides for 48 h as described above in a 96-well microplate. The microplate was then centrifuged at $200 \times g$ for 10 min and the supernatant was discarded. The cells were incubated with lysis buffer for 30 min. After centrifugation at $200 \times g$ for 10 min, a 20 μL aliquot of the supernatant in each well was transferred to a streptavidin-coated microplate. Eight microliters of the immunoreagents containing the biotin-conjugated anti-histone and anti-DNA antibodies were added to each well and incubated for 2 h at room temperature. The wells were then washed thrice with 300 μL of the incubation solution followed by the addition of 100 μL of the ABTS substrate solution. After incubating for 15 min at room temperature, 100 μL of ABTS stop solution were added to each well. The photometric signal intensities of the wells were finally measured by a microplate reader (BioTek) at 405 nmol/L.

*Soft-agar colony formation assay.* Cells were treated with siRNA for 24 h, trypsinized, mixed with Difco agar (final concentration 0.26%; BD Biosciences) and RPMI medium containing 10% fetal bovine serum, and overlaid onto an underlayer of 0.45% Difco agar containing the same medium in a 35-mm gridded Petri dish. Cells (2,000 per Petri dish) were seeded and allowed to grow for 14 d (MIA PaCa-2) or 21 d (PANC-1) before counting the number of colonies (defined as ≥50 cells).

*Xenograft tumor formation in nude mice.* MIA PaCa-2 cells were treated with the TPX2-targeting siRNA olionucleotides (TPX2-s1 and TPX2-s2) for 48 h as described above and harvested by trypsinization. Ten male athymic nude mice (*CrTac:NCR-Foxn1^nu*; Taconic) for each treatment group were inoculated s.c. in the right flank with 0.1 mL of a 50% RPMI/50% Matrigel (BD Biosciences) mixture containing a suspension of the siRNA- or vehicle-treated MIA PaCa-2 cells ($1.0 \times 10^7$/mouse). Starting from the day after the inoculation, tumors were mea-

sured twice weekly using calipers and tumor weights were calculated using the following formula: tumor weight (mg) = $(a \times b^2/2)$, where $b$ is the smallest diameter and $a$ is the largest diameter. Body weights were taken twice weekly thereafter in conjunction with tumor measurements. When the individual tumor weight of each mouse reached an approximate end point of 2,000 mg, mice were sacrificed with regulated $CO_2$. The mice were housed in microisolator cages (Lab Products) and maintained under specific pathogen-free conditions. All procedures were carried out under the institutional guidelines of TGen Drug Development Services Institutional Animal Care and Use Committee (protocol 06001, approved January 2006). All tumor growth data were collected using the animal study management software, Study Director V.1.6.58 (Study Log).

*Statistical analysis.* The $\chi^2$ test with Yates' correction (two-tailed) was used to analyze the difference in TPX2 IHC staining between the pancreatic tumors and the normal adjacent tissue. ANOVA with Tukey's multiple comparison test was used to compare the growth curves of the different siRNA treatment groups. Other $P$ values indicated in the figure legends were calculated using Student's $t$ tests (two-tailed). A $P$ value of <0.05 was considered statistically significant.

## Results

*Amplifications at the TPX2 locus in pancreatic cancer.* As previously mentioned, increased copy number of *TPX2* in pancreatic cancer cell lines and tumor samples by array comparative genomic hybridization (aCGH) has been reported (7, 8). To compare and verify *TPX2* amplification, we used quantitative PCR to examine the *TPX2* copy number in pancreatic cancer cell lines and low-passage xenograft tumors derived from PDAC tissues (19). Of the 17 cell lines tested, 7 did not contain extra copies of *TPX2*, 7 cell lines contained one extra copy, and 3 cell lines had 2 extra copies (Table 1). This low-level amplification is in agreement with what has previously been reported (7, 8). For the low-passage tumor xenografts, 13 of 20 samples exhibited at least 1 extra copy of *TPX2* in our analysis (10 samples have 3 copies, 1 sample has 4 copies, and 2 samples have 6 copies).

*TPX2 expression in PDAC cell lines and tumors.* *TPX2* mRNA levels in pancreatic cancer cell lines relative to the immortalized HPDE6 cell line were determined by real-time RT-PCR. The average of four independent RT-PCR measurements showed that *TPX2* mRNA expression was elevated in cancer cell lines compared with HPDE6 but varied widely by cell line with an ~10-fold difference in expression between MIA PaCa-2 (lowest) and CFPAC-1 (highest; Fig. 1A). Protein expression was also evaluated by Western blotting for the cell lines. Data from three independent Western blots (from three separate nuclear lysate

**Table 1.** *TPX2* copy numbers in pancreatic cancer cell lines

|  | **TPX2 copy number** | | |
|---|---|---|---|
|  | **2** | **3** | **4** |
| Cell lines | AsPC-1 | HPAC | BxPC-3 |
|  | Capan-1 | HPAF-II | Hs766T |
|  | Capan-2 | Hs700T | PANC-1 |
|  | CFPAC-1 | HUP-T4 |  |
|  | MIA PaCa-2 | MUTJ |  |
|  | PANC.02.03 | PSN-1 |  |
|  | PL-45 | SU.86.86 |  |
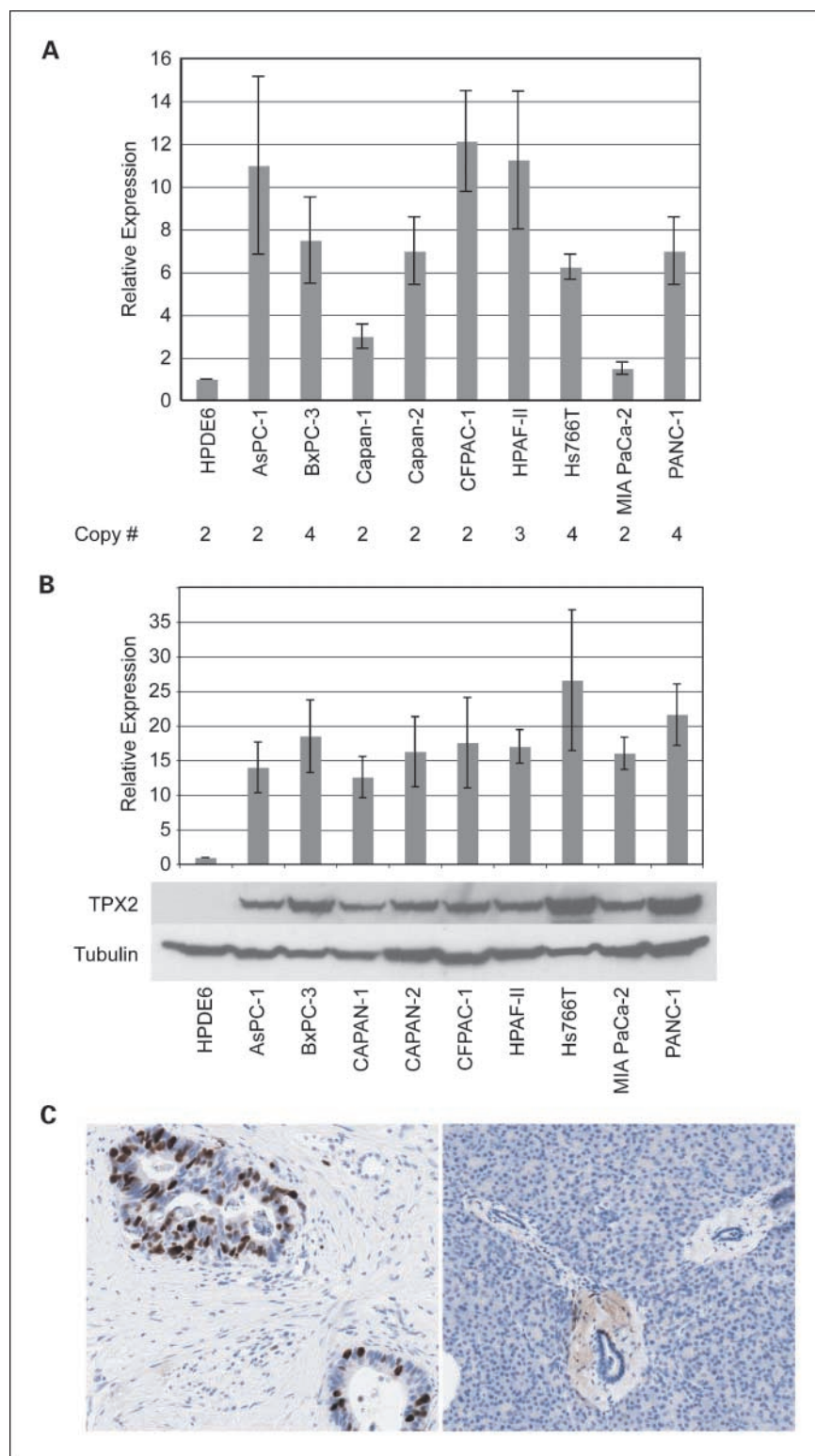
**Fig. 1.** TPX2 expression in pancreatic cancer cell lines. *A, TPX2* expression levels determined by real-time RT-PCR. The expression levels were expressed as a ratio to that in HPDE6. *B,* Western blotting analysis of TPX2 protein expression in pancreatic cancer cell lines. Top, relative TPX2 protein levels as determined by densitometry quantification of Western blots (four replicates). Bottom, representative image of a Western blot showing the TPX2 protein levels. *C,* IHC staining of TPX2 in a pancreatic cancer tissue microarray. Nuclear TPX2 staining was observed primarily in invasive adenocarcinoma (*left*) and not in normal pancreatic ducts (*right*).

preparations) showed that *TPX2* was expressed in all cell lines tested (Fig. 1B). Protein levels were especially high in Hs766t and PANC1 cells. The TPX2 protein was barely detectable in the HPDE6 cell line (Fig. 1B).

To evaluate protein levels of TPX2 between pancreatic tumors and normal pancreatic tissues, immunostaining was done on a

pancreatic tissue microarray (Fig. 1C). As described in Materials and Methods, for any given TMA core, a 0 to 3 score for staining intensity was multiplied by a 0 to 3 prevalence score of tumor cell staining to obtain an overall score that ranged from 0 to 9. As summarized in Table 2, staining was observed primarily in the invasive adenocarcinoma cells and not in the normal pancreatic

ductal cells. Of the 40 evaluable PDAC tissue samples, 29 (88%) stained positive (overall staining score of 2 or higher) for TPX2. In contrast, only 4 of 31 (12%) normal samples stained positive (Table 2). Of the PDAC cases, 22 cases had adjacent normal tissue with evaluable staining. Of these, 18 exhibited negative staining for the normal counterpart. Considering just the matched cases with negative TPX2 staining for the normal tissue, 16 of 18 (88%) tumors stained positive, similar to the overall findings when all evaluable samples were considered.

*siRNA-mediated knockdown of TPX2 inhibits pancreatic cancer cell proliferation.* Two siRNAs effective at knocking down the expression of TPX2 were identified from four different siRNA sequences. Real-time quantitative PCR analysis verified gene knockdown at >95% at the mRNA level for both siRNAs up to 96 hours posttransfection (data not shown).

We determined the effects of the TPX2 siRNAs on the viability of a panel of 10 pancreatic cancer cell lines. The cell viability was determined 96 hours after transfection by a SRB colorimetric assay. Cell viability was decreased in all 10 cell lines, but to varying degrees. Five of the pancreatic cancer cell lines experienced a >50% decrease in cell viability. Three cell lines showed about a 50% decrease in viability and the remaining two lines showed only modest decreases (Supplementary Fig. S1). The Hs766T cell line was one of the most resistant lines to decrease in cell viability; however, this lack of activity is partially due to an observed general toxicity of the nonsilencing siRNA on this cell line. The nonsilencing siRNA alone caused a 32% decrease in Hs766T cell viability relative to the untreated control. Therefore, if the TPX2-targeting siRNAs were evaluated relative to the untreated control, the results would indicate an ~45% decrease in cell viability rather than the ~20% reported on Supplementary Fig. S1. In our subsequent functional studies, we chose PANC-1 and MIA PaCa-2 cell lines because they both have a high level of TPX2 protein but showed minimal nonspecific toxicity to the nonsilencing siRNA treatment. Both TPX2-targeting siRNAs gave very similar results in all 10 cell lines, lending support to the conclusion that the observed decreases in cell proliferation were due to the disruption of TPX2 function and not some off-target effect. To further investigate the effects of TPX2 knockdown on cell viability and more specifically on cell growth, two of the cancer cell lines (MIA PaCa-2 and PANC-1) were treated as above, but monitored daily for 96 hours by SRB staining. This generated growth curves for the two cell lines and the effects of the TPX2 siRNAs on each cell line were consistent with the single time point assay done previously (Fig. 2A). The nonsilencing siRNA had little to no toxicity on the pancreatic cancer cell lines. As a comparison, we also treated the immor-

talized normal pancreatic ductal cell line HPDE6 with the TPX2 siRNAs. As shown in Supplementary Fig. S2, TPX2 had little effect on the growth of the cells. This result is consistent with the fact that HPDE6 does not express the TPX2 protein (Fig. 1B).

To better understand the effects of TPX2 inhibition on pancreatic cancer cell proliferation, we subjected siRNA-treated cells to DNA content analysis by flow cytometry to observe disruptions in cell cycle progression. TPX2 siRNA–treated MIA PaCa-2 and PANC-1 cells showed a dramatic increase in the $G_2$-M fraction, from <20% in the control samples (untreated or nontargeting siRNA) to >50% for TPX2-s1 siRNA and >60% for TPX2-s2 siRNA in both cell lines (Fig. 2B). Such increase in the $G_2$-M fraction concurs with a decrease in the $G_1$ population compared with the nontargeting control siRNA-treated sample (data not shown). We also observed a significant increase in the sub-$G_1$ peak in the DNA content histograms after 48 hours in the cells. Consistent with the known biological functions of TPX2, TPX2 knockdown by siRNA led to the failure of pancreatic cancer cells to progress through mitosis, and the appearance of the sub-$G_1$ peak suggests that apoptosis is a potential result following TPX2 inhibition.

*TPX2 knockdown induces apoptosis in pancreatic cancer cells.* To further explore the potential of TPX2 inhibition to induce apoptosis, we evaluated the activity of caspase-3 in siRNA-treated cells using a fluorescence-based assay. The caspase-3 activities were similar between untreated and nontargeting siRNA-treated cells, indicating minimal to no general toxicity from the siRNA transfections. However, there was a 7-fold increase in caspase-3 activity following 48 hours of treatment with TPX2-s1 in both PANC-1 and MIA PaCA-2 cells (Fig. 2C). Similarly, the siRNA TPX2-s2 caused an 8-fold (PANC-1) and 10-fold (MIA PaCa-2) increase in caspase-3 activity relative to the nonsilencing siRNA (Fig. 2C).

We also detected the apoptosis-inducing effects of TPX2 knockdown by evaluating cytoplasmic histone-DNA adducts using a cell death ELISA assay. For these experiments, MIA PaCa-2 cells were treated with the TPX2-s1 siRNAs at various concentrations between 20 and 0.027 nmol/L. To evaluate the knockdown of TPX2 expression, we also performed RT-PCR detection of TPX2 mRNA in the samples treated with the serial dilutions of TPX2 siRNAs. As shown in Fig. 2D, apoptosis as indicated by the signal of cell death ELISA was induced in a dose-dependent manner, which correlated well with percent knockdown of the TPX2 gene expression. The concentration at which 50% of the maximal apoptotic effect was reached ($EC_{50}$) was 1.6 nmol/L for TPX2-s1, and the $EC_{50}$ for TPX2 knockdown was 0.30 nmol/L for TPX2-s1.

**Table 2.** TPX2 expression levels in pancreatic cancer tissues detected by IHC

|  | IHC score | No. of tumor cases | No. of normal cases |
|---|---|---|---|
| Negative staining | 0 | 6 | 26 |
|  | 1 | 5 | 1 |
| Total (negative) |  | 11 (29%) | 27 (61%) |
| Positive staining | 2 | 11 | 2 |
|  | 3 | 5 | 1 |
|  | 4 | 3 | 0 |
|  | 6 | 6 | 1 |
|  | 9 | 4 | 0 |
| Total (positive) |  | 29 (88%) | 4 (12%) |
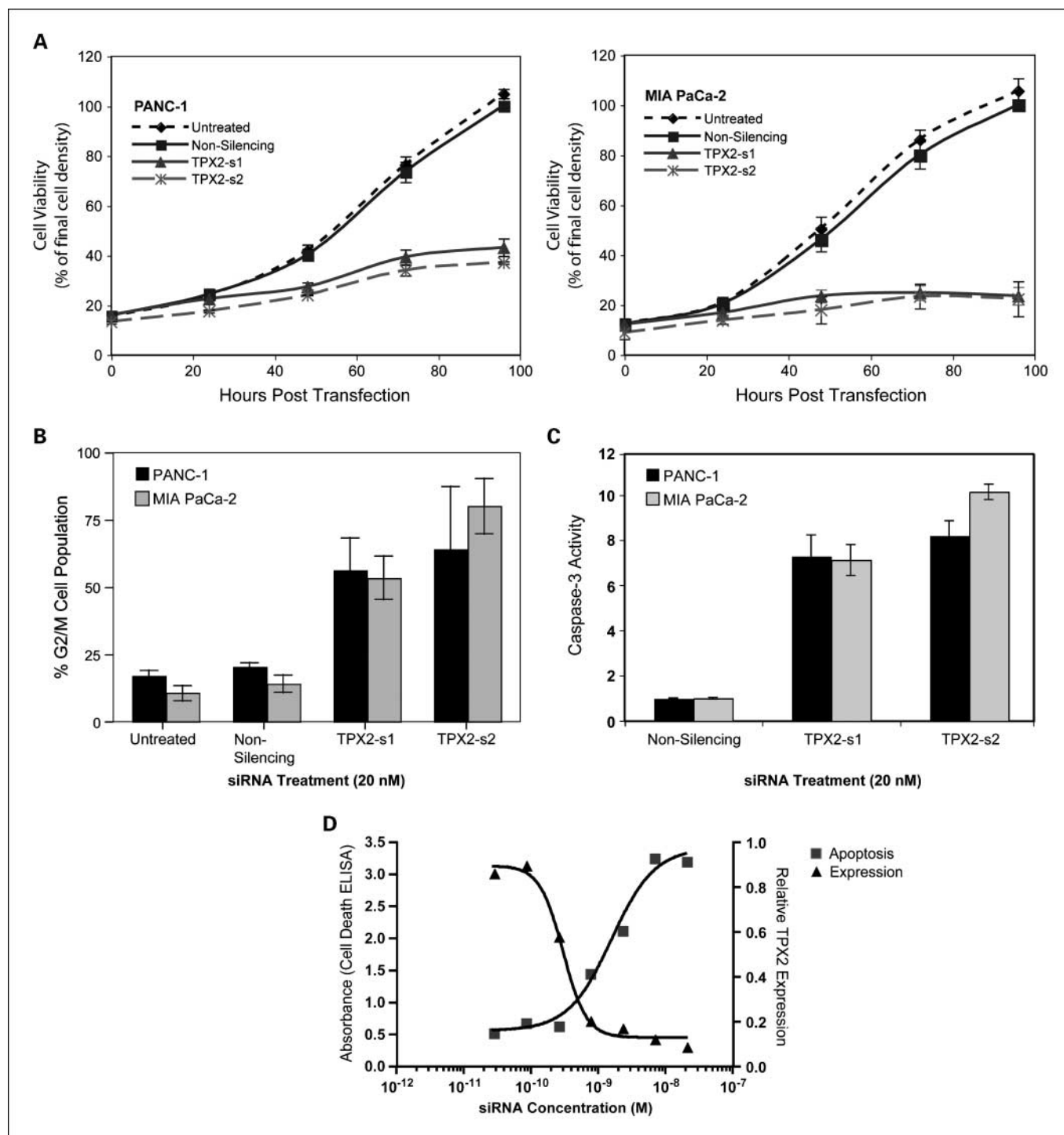
**Fig. 2.** TPX2 targeting siRNAs influence the proliferation, cell cycle progression, and apoptosis induction of pancreatic cancer cells. *A,* effects of TPX2 siRNAs on the growth of PANC-1 and MIA PaCa-2 cells over a 96-h period. *B,* induction of $G_2$-M arrest by TPX2 siRNAs in PANC-1 and MIA PaCa-2 cells. *C,* induction of caspase-3 activity by TPX2 siRNAs in PANC-1 and MIA PaCa-2 cells. *D,* concentration-dependent induction of apoptosis by the TPX2-s1 siRNA as measured by the Cell Death ELISA assay and the corresponding knockdown level of TPX2 mRNA expression as measured by RT-PCR.

***TPX2 is required for clonogenicity in soft agar.*** Additionally, we investigated the consequences of TPX2 knockdown by siRNA in MIA PaCa-2 and PANC-1 cells grown in soft agar. As shown in Fig. 3A, the number of colonies was significantly decreased in the cells treated with either TPX2 siRNA when compared with nonsilencing siRNA–treated cells. In

fact, colony formation was almost completely inhibited by TPX2 siRNA treatment, suggesting that TPX2 plays important roles in self-renewal and in the clonogenicity of pancreatic cancer cells.

***TPX2 is required for tumorigenicity of pancreatic cells in nude mice.*** We also examined the effect of TPX2 knockdown by

siRNA on the tumorigenicity of pancreatic cancer cells in nude mice. As shown in Fig. 3B, MIA PaCa-2 cells treated with either TPX2-s1 or TPX-s2 siRNA showed a dramatic reduction in the tumor growth compared with those treated with vehicle control or nonsilencing siRNA ($P < 0.001$). The tumor growth between the two control groups (vehicle control and nonsilencing siRNA) and between the two siRNA treatment groups (TPX2-s1 and TPX2-s2) was not significantly different ($P > 0.05$). These results indicate that TPX2 overexpression is required for aggressive tumor growth of MIA PaCa-2 cells in nude mice.

*TPX2 knockdown sensitizes pancreatic cancer cells to other mitosis-targeting agents.* It is known that inhibition of some mitotic regulators, such as Aurora A, sensitizes cancer cells to the treatment of taxanes (20, 21). The rationale for the combination of these agents comes from the notion that due to the action of the taxane, cells will accumulate in the phase of the cell cycle ($G_2$-M) where the mitotic regulator plays an essential role. To evaluate whether this rationale expanded to TPX2,

which plays an important role in the Aurora A signaling pathway, we observed the effects of TPX2 knockdown on the cytotoxicity of paclitaxel using a similar approach to the one describing the ability of Aurora A to sensitize cells (20). We first did a TPX2 siRNA dose-dependent treatment of the MIA PaCa-2 and PANC-1 cells and measured the cell growth using SRB assays. As shown in Fig. 4A, the two TPX2 siRNA oligonucleotides showed a dose-dependent growth inhibition in both cell lines. We found that the highest concentration at which the TPX2-targeting siRNAs had no significant effect on growth and viability of PANC-1 and MIA PaCa-2 cells was 0.1 nmol/L. Dose-dependent treatment of the two cell lines with paclitaxel found that the highest concentration at which paclitaxel does not significantly affect the growth of the cells was 10 nmol/L (Supplementary Fig. S3). Using these low doses of siRNA and paclitaxel, we transfected the cells with the TPX2-targeting siRNAs followed by the addition of paclitaxel 6 hours later. Cell viability was determined using a SRB assay after 96 hours of incubation. As expected, TPX2 siRNA or paclitaxel alone had no significant



**Fig. 3.** TPX2 targeting siRNAs influence the tumorigenicity of pancreatic cancer cells. *A,* TPX2 targeting siRNA treatment significantly reduces the colony formation of MIA PACa-2 and PANC-1 cells on soft agar. *B,* TPX2 targeting siRNA treatment significantly inhibits the xenograft tumor growth of MIA PaCa-2 cells in nude mice.
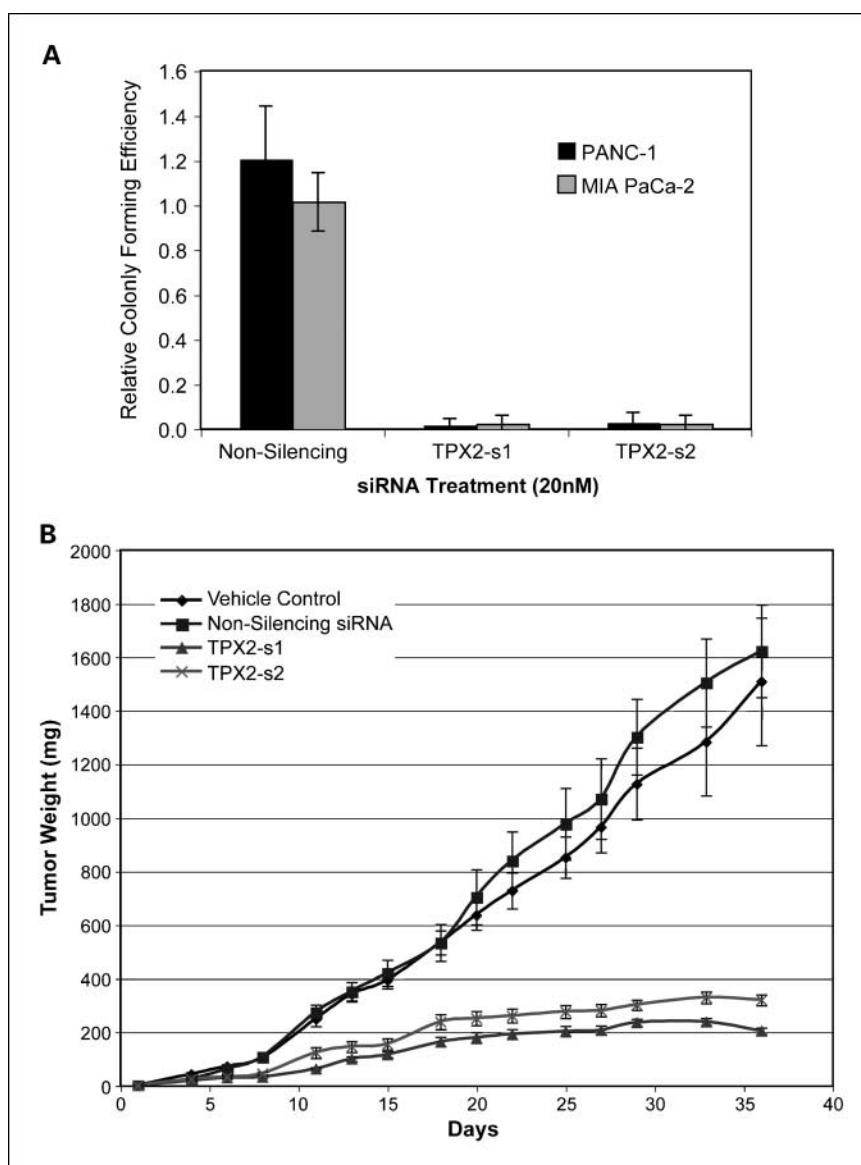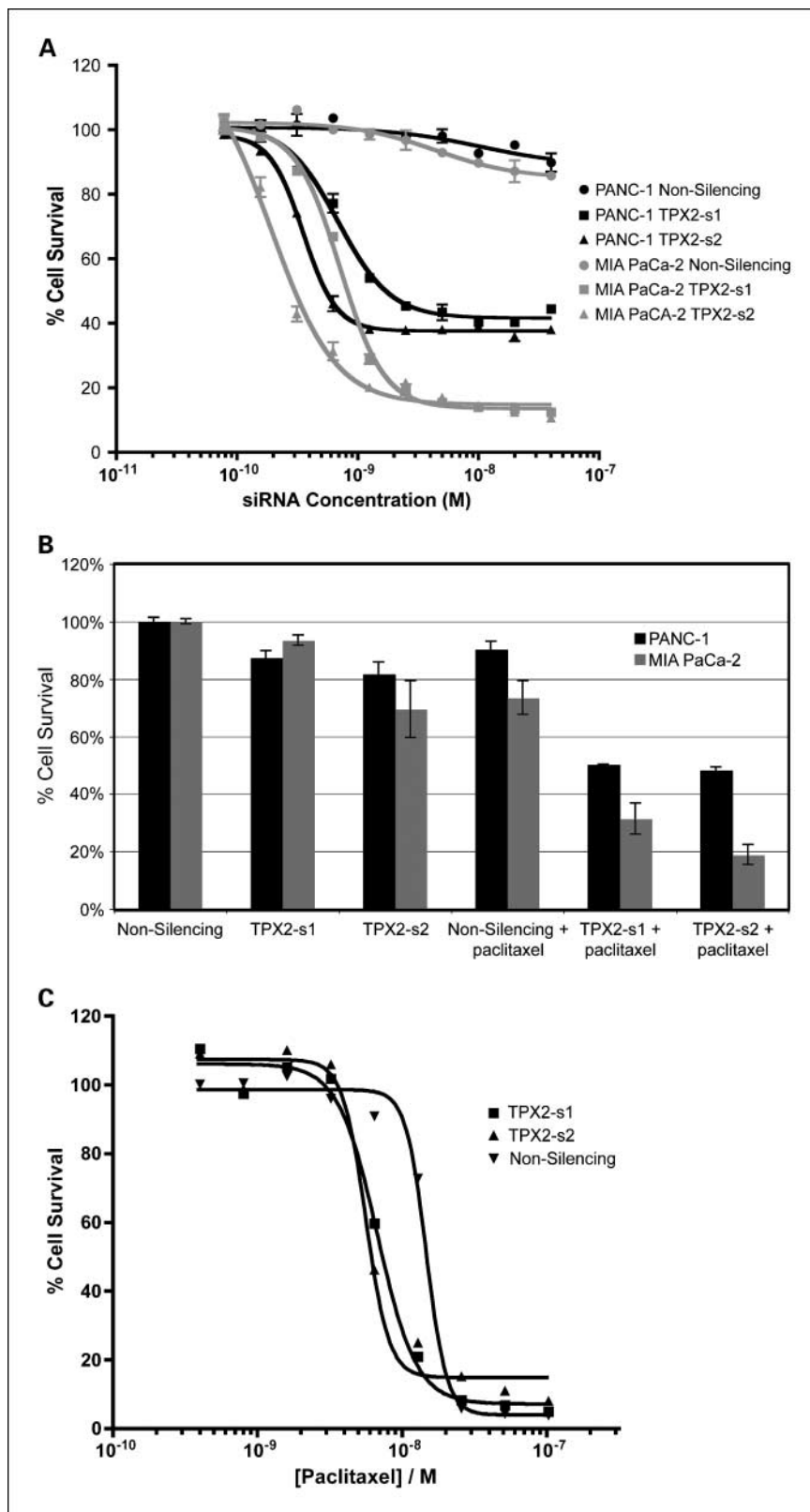
**Fig. 4.** TPX2 siRNAs sensitize pancreatic cancer cells to paclitaxel. *A,* concentration-dependent inhibition of pancreatic cancer cell growth by TPX2 siRNAs. Both TPX2 siRNA oligonucleotides showed concentration-dependent growth inhibition of PANC-1 (*black curves*) and MIA PaCa-2 (*gray curve*), whereas the nonsilencing siRNA showed little effect. *B,* combination treatment of pancreatic cancer cells by TPX2 siRNA oligonucleotides and paclitaxel. The *X* axis indicates the agents that were applied to each treatment group (nonsilencing siRNA, TPX2 siRNA, or paclitaxel only or in combination). At low concentrations of TPX2 siRNA (0.1 nmol/L) and paclitaxel (10 nmol/L), either agent alone showed no significant effect on the growth of PANC-1 and MIA PaCa-2 cells. When the cells were treated with both TPX2 siRNA and paclitaxel at the same low concentrations, their growth inhibition was significantly inhibited. *C,* TPX2 siRNA treatment causes a left shift of the paclitaxel concentration-dependent growth inhibition curves of MIA PaCa-2 cells. The TPX2 siRNA (0.1 nmol/L)–only treatment did not show any significant effect on MIA PaCa-2 cell growth as shown in *B*.

effect on cell viability at these concentrations; however, when combined, the TPX2 siRNA and paclitaxel reduced cell viability by ~50% (Fig. 4B). These results are further supported by experiments generating dose-response curves to paclitaxel in the presence of low-dose TPX2-targeting siRNAs or a nonsilencing siRNA (Fig. 4). The paclitaxel-dose response curves reveal a shift to the left when combined with the TPX2 siRNAs, indicating that TPX2 knockdown sensitizes cells to paclitaxel treatment.

Similar experiments with gemcitabine (a nucleoside analogue and the standard first-line therapy for pancreatic cancer) in combination with TPX2 siRNA did not show any significant synergistic effect (Supplementary Fig. S4).

## Discussion

TPX2 is a microtubule-associated protein that is tightly cell cycle regulated. Abnormally expressed TPX2 has been reported in various malignancies. TPX2 was found to be upregulated in squamous cell carcinoma of the lung with the expression correlating to tumor grade, stage, and nodal status (9). However, little work has been done to explore TPX2 protein levels in pancreatic cancer cell lines and tumor samples. In the present study, we show that TPX2 is expressed at high levels in pancreatic cancer cell lines and that in some cases amplification of the *TPX2* locus might be responsible for the increased expression. Immunohistochemical staining of a pancreatic cancer tissue microarray also shows that TPX2 is highly and extensively expressed in pancreatic tumor tissues taken directly from patients with 88% of the tumor cases expressing TPX2 compared with 12% of normal tissue found adjacent to the tumor.

About 60% of the pancreatic cancer cell lines and xenograft tumors we tested had low-copy-number amplification of the *TPX2* gene (three to six copies). The *TPX2* gene localizes to chromosome 20q11. The amplification of this chromosome region has been reported previously in pancreatic cancer (6). Using comparative genomic hybridization (CGH) array and fluorescence *in situ* hybridization, Fukushige and colleagues found that ~80% of the pancreatic cancer cell lines and primary tumors they evaluated had gains in 20q. The copy number increased in this study was also not very high (four to eight copies per cell; ref. 6). Interestingly, these gains were observed at the same frequency in early and advanced stages, suggesting that genes in this region might play an important role in the relatively early stage of pancreatic carcinogenesis. Due to the role of TPX2 in activating the Aurora A enzymatic activity and in promoting the progression of mitosis, the amplification of *TPX2* that we observed could confer a proliferation and growth advantage to pancreatic cancer cells compared with surrounding tissue. Furthermore, because Aurora A kinase has been shown to activate the Akt pathway (22), overexpression of TPX2 may also induce cell survival in cancer cells (23). Using TPX2 targeting siRNAs, we have shown that inhibition of TPX2 expression resulted in cell cycle arrest and apoptosis in cancer cell lines. Recently, Morgan-Lappe and coworkers identified *TPX2* as one of the three genes that significantly reduced the survival of multiple human tumor cell lines in a siRNA library–based screening using an *in vitro* cytotoxicity assay. It was further shown that TPX2 siRNA selectively reduced the survival of activated K-Ras–transformed cells compared with their normal isogenic counterparts (23). Given the high percentage of pancreatic tumors with activated K-ras, it is possible that knockdown of TPX2 would selectively kill cancer cells.

Furthermore, combination therapies of newly developed targeted agents combined with standard chemotherapy drugs are increasingly common in the clinic. Our findings suggest that a TPX2-targeted agent could synergistically combine with antimitotic agents, such as the taxanes. Our results show that exposure of pancreatic cancer cells to TPX2 siRNAs plus paclitaxel results in a synergistic decrease in cell viability, presumably through a profound mitotic arrest followed by extensive cell death. It is likely that this finding can be applied to additional agents that target mitosis, as it has been reported that *TPX2* (and Aurora A) amplification is associated with resistance to Eg5/KSP inhibitors (24). We postulate that targeting TPX2 in these cancers will sensitize them to Eg5/KSP inhibition.

As mentioned above, TPX2 is a binding partner of Aurora A, which functions as an activator of its kinase activity. TPX2 accomplishes this by binding to an allosteric site on Aurora A and increasing its binding affinity to ATP and substrate (25). Typically, Aurora small-molecule inhibitors were discovered and optimized in Aurora A kinase assays without TPX2. A recent report shows decreased inhibitory activity of many Aurora inhibitors against Aurora A when the kinase assays are done in the presence of TPX2, which would presumably model the activity of Aurora A *in vivo* (26). Therefore, TPX2 may serve as a valuable target given its direct link in the Aurora A activation pathway that has been shown to be critical in pancreatic cancer. Provided that TPX2 is amenable to small-molecule inhibitors, targeting TPX2 over Aurora A may have its advantages given that blocking TPX2 binding, and thus activation of Aurora A kinase, provides a higher specificity that may not be achievable with conventional kinase inhibitors (23).

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

## References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.

2. Nowak NJ, Gaile D, Conroy JM, et al. Genome-wide aberrations in pancreatic adenocarcinoma. Cancer Genet Cytogenet 2005;161:36–50.

3. Furukawa T, Kanai N, Shiwaku HO, Soga N, Uehara A, Horii A. AURKA is one of the downstream targets of MAPK1/ERK2 in pancreatic cancer. Oncogene 2006;25:4831–9.

4. Harada T, Baril P, Gangeswaran R, et al. Identification of genetic alterations in pancreatic cancer by the combined use of tissue microdissection and array-based comparative genomic hybridisation. Br J Cancer 2007;96:373–82.

5. Harada T, Chelala C, Bhakta V, et al. Genome-wide DNA copy number analysis in pancreatic cancer using high-density single nucleotide polymorphism arrays. Oncogene 2007.

6. Fukushige S, Waldman FM, Kimura M, et al. Frequent gain of copy number on the long arm of chromosome 20 in human pancreatic adenocarcinoma. Genes Chromosomes Cancer 1997; 19:161–9.

7. Aguirre AJ, Brennan C, Bailey G, et al. High-resolution characterization of the pancreatic adenocarcinoma genome. Proc Natl Acad Sci U S A 2004;101:9067–72.

8. Tonon G, Wong KK, Maulik G, et al. High-resolution genomic profiles of human lung cancer. Proc Natl Acad Sci U S A 2005; 102:9625–30.

9. Lin DM, Ma Y, Xiao T, et al. [TPX2 expression and its significance in squamous cell carcinoma of lung]. Zhonghua Bing Li Xue Za Zhi 2006;35:540–4.

10. Smith LT, Mayerson J, Nowak NJ, et al. 20q11.1 amplification in giant-cell tumor of bone: array CGH, FISH, and association with

outcome. Genes Chromosomes Cancer 2006; 45:957–66.

**11.** Gruss OJ, Vernos I. The mechanism of spindle assembly: functions of Ran and its target TPX2. J Cell Biol 2004;166:949–55.

**12.** Gruss OJ, Carazo-Salas RE, Schatz CA, et al. Ran induces spindle assembly by reversing the inhibitory effect of importin α on TPX2 activity. Cell 2001;104:83–93.

**13.** Kufer TA, Sillje HH, Korner R, Gruss OJ, Meraldi P, Nigg EA. Human TPX2 is required for targeting Aurora-A kinase to the spindle. J Cell Biol 2002;158:617–23.

**14.** Bayliss R, Sardon T, Ebert J, Lindner D, Vernos I, Conti E. Determinants for Aurora-A activation and Aurora-B discrimination by TPX2. Cell Cycle 2004;3:404–7.

**15.** Ouyang H, Mou L, Luk C, et al. Immortal human pancreatic duct epithelial cell lines with near normal genotype and phenotype. Am J Pathol 2000;157:1623–31.

**16.** Freeman WM, Walker SJ, Vrana KE. Quantita-tive RT-PCR: pitfalls and potential. Biotechniques 1999;26:112–22, 24–5.

**17.** Gray PJ, Jr., Bearss DJ, Han H, et al. Identifica-tion of human polo-like kinase 1 as a potential therapeutic target in pancreatic cancer. Mol Can-cer Ther 2004;3:641–6.

**18.** Krishan A. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol 1975;66:188–93.

**19.** Rubio-Viqueira B, Jimeno A, Cusatis G, et al. An *in vivo* platform for translational drug devel-opment in pancreatic cancer. Clin Cancer Res 2006;12:4652–61.

**20.** Hata T, Furukawa T, Sunamura M, et al. RNA interference targeting aurora kinase a sup-presses tumor growth and enhances the taxane chemosensitivity in human pancreatic cancer cells. Cancer Res 2005;65:2899–905.

**21.** Scharer CD, Laycock N, Osunkoya AO, et al. Aurora kinase inhibitors synergize with paclitaxel to induce apoptosis in ovarian cancer cells. J Transl Med 2008;6:79.

**22.** Yang H, He L, Kruk P, Nicosia SV, Cheng JQ. Aurora-A induces cell survival and chemo-resistance by activation of Akt through a p53-dependent manner in ovarian cancer cells. Int J Cancer 2006;119:2304–12.

**23.** Morgan-Lappe SE, Tucker LA, Huang X, et al. Identification of Ras-related nuclear protein, targeting protein for xenopus kinesin-like pro-tein 2, and stearoyl-CoA desaturase 1 as promising cancer targets from an RNAi-based screen. Cancer Res 2007;67:4390–8.

**24.** Jackson AL, Mao M, Kobayashi S, et al. Chro-mosome 20q amplification regulates *in vitro* re-sponse to Kinesin-5 inhibitor. Cancer Inform 2008;6:147–64.

**25.** Eyers PA, Erikson E, Chen LG, Maller JL. A novel mechanism for activation of the protein kinase Aurora A. Curr Biol 2003;13:691–7.

**26.** Anderson K, Yang J, Koretke K, et al. Bind-ing of TPX2 to Aurora A alters substrate and inhibitor interactions. Biochemistry 2007;46: 10287–95.

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## Validation of TPX2 as a Potential Therapeutic Target in Pancreatic Cancer Cells

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