Targeting the RNA Splicing Machinery as a Novel Treatment Strategy for Pancreatic Carcinoma

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Abstract

Aberrant patterns of pre-mRNA splicing have been established for many human malignancies, yet the mechanisms responsible for these tumor-specific changes remain undefined and represent a promising area for therapeutic intervention. Using immunohistochemistry, we have localized the expression of a central splicing regulator, serine-arginine protein kinase 1 (SRPK1), to the ductular epithelial cells within human pancreas and have further shown its increased expression in tumors of the pancreas, breast, and colon. Small interfering RNA–mediated down-regulation of SRPK1 in pancreatic tumor cell lines resulted in a dose-dependent decrease in proliferative capacity and increase in apoptotic potential. Coordinately, the disruption of SRPK1 expression resulted in enhanced sensitivity of tumor cells to killing by gemcitabine and/or cisplatin. A dose-dependent reduction in the phosphorylation status of specific SR proteins was detected following the down-regulation of SRPK1 and is likely responsible for the observed alterations in expression of proteins associated with apoptosis and multidrug resistance. These data support SRPK1 as a new, potential target for the treatment of pancreatic ductular cancer that at present remains largely unresponsive to conventional therapies. Furthermore, these results support the development of innovative therapies that target not only specific splice variants arising during tumorigenesis but also the splice regulatory machinery that itself may be abnormal in malignant cells. (Cancer Res 2006; 66(7): 3819-27)

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and lethal cancers with a median survival of ~6 months following detection and treatment with state-of-the-art diagnostic and therapeutic tools. Essentially, the only observed cures are in patients presenting with small tumors that are amenable to surgical resection (1). Current chemotherapeutic options have been largely palliative, approved based on prolonging survival by days or weeks (2). There is a clear need for new drugs that employ innovative mechanisms of action and are directed toward unique molecular targets.

Although a large series of mutations in oncogenes and proofreading mechanisms have now been described for pancreatic cancer (3), these have not provided broadly applicable nor effective therapeutic strategies for treating this tumor. In the current report, we focus on a distinct approach for the treatment of PDAC that may be similarly extended to other malignancies. This method involves the mRNA splicing machinery, thought to be abnormal in the vast majority, or even all, pancreatic carcinomas and malignant tumors in general.

Similar to other neoplasms, PDACs exhibit aberrant pre-mRNA splice patterns that differ markedly from that observed in their normal cellular counterparts. We have previously reported the presence of misspliced secretin and gastrin receptor transcripts, and their resulting protein isoforms, that are growth stimulatory in pancreatic tumors and cell lines (4, 5). Other groups have shown missplicing of Mucin type 4 (6), members of the fibroblast growth factor receptor family (7, 8), and CD44 in pancreatic neoplasms (9), yet the mechanisms responsible for these tumor-specific alterations remain unknown.

Important regulators of the splicing process include several serine-arginine protein kinases (SRPK) that serve to phosphorylate the serine-arginine (SR) domains found within a variety of splice factor proteins (10). *In vitro* experiments have shown that phosphorylation of these splice factors can facilitate both their transport into the nucleus and their release from nuclear storage sites (11–13). Once phosphorylated, SR proteins are believed to influence the selection of splice sites by mediating the interactions between nascent transcripts and components of the spliceosomal machinery (14). SRPK1 has been shown by Northern analysis to have a ubiquitous expression pattern among tissues, although the highest transcript levels exist in testis and pancreas (13, 15). Presently, the location and function of the SRPK1 protein within the pancreas remains unknown. Studies have shown a dual role for SRPK1 in testicular germ cells where, besides its role regulating splice factors, this kinase phosphorylates the SR domain-containing protein protamine 1 to effect a more compact condensation of chromatin during spermatogenesis (15).

Here, we provide the first description of pancreatic SRPK1 localization in the human pancreas, present predominantly in the ductular epithelial cells, and show that levels of SRPK1 are increased in ductular pancreatic carcinoma and tumors of the breast and colon. This has been further confirmed by showing high levels of SRPK1 protein in six of seven human pancreatic cancer cell lines. These observations, together with an understanding of the frequency of malignant missplicing and the functional ability of many protein variants to affect tumor behavior, suggest a possible role for this kinase during pancreatic tumorigenesis. Indeed, our *in vitro* experiments show that small interfering RNA (siRNA)–mediated down-regulation of SRPK1 elicits both a decrease in cell proliferation and an increase in apoptotic potential. Furthermore, down-regulation of SRPK1 facilitates killing of pancreatic tumor cells by gemcitabine and cisplatin. These results describe the novel targeting of a central splicing regulator to affect several mechanisms that typically promote tumor growth and provide an experimental basis to develop drugs targeting this protein in the treatment of pancreatic cancer and possibly other malignancies.

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Materials and Methods

Immunohistochemistry. Tissue sections were produced from paraffin-embedded blocks and stained with monoclonal antibodies against either SRPK1 (BD Biosciences, San Jose, CA) or the CA19-9 antigen (U.S. Biological, Swampscott, MA). Six-micrometer-thick paraffin sections were first submerged in xylene and then in decreasing concentrations (100-70%) of ethanol to depariffinize the samples. Subsequently, antigen retrieval was done by heating the sections in an unmasking solution per manufacturer's protocol (Vector Laboratories, Burlingame, CA), endogenous peroxidase inhibited by incubation in Immunopure Peroxidase Suppressor (Vector Laboratories), and blocked in goat serum for 30 minutes at room temperature. Approximately 0.5 mL of a 1:500 dilution for each primary antibody was then applied to serial sections and incubated for 1 hour before extensive washing in PBS. Each sample was then incubated with 0.5 mL of a biotinylated anti-mouse IgG secondary (1:1,000 dilution; Vector Laboratories) followed by washing in PBS. Sections were incubated a further 30 minutes in the Vectastain avidin-biotin complex solution before addition of 3,3′-diaminobenzidine substrate (Vector Laboratories). Counterstaining was done for 45 seconds using Hematoxylin QS (Vector Laboratories), dehydrated in increasing concentrations of ethanol, and mounted with Paramount (Sigma, Inc., St. Louis, MO). Slides were examined, and photographs were taken using the Ziess AxioPlan2 imaging system (Zeiss, Thornwood, NY).

Cell culture and transfection. The pancreatic tumor cell lines Capan-1, Capan-2, MiaPaCa2, Panc1, BxPC3, and Su86.86 were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA) and cultured according to ATCC specifications. The L3.6pl cell line was provided by Dr. Isaiah Fidler and grown in MEM supplemented with 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, L-glutamine, and vitamin solution (all from Invitrogen Life Sciences, Carlsbad, CA). The HPDE6 cell line was provided by Dr. M.S. Tsao (Ontario Cancer Institute, Canada) and cultured in Keratinocyte-SFM media supplemented with bovine pituitary extract (15 mg/500 ml) and EGF (100 ng/500 ml; all from Invitrogen). Both MiaPaCa2 and Panc1 cells were grown to ~80% to 90% confluency before transfection with the designated quantities of plasmid DNA (2-20 μg) purified via Qiagen's MaxiPrep procedure. Lipofectamine 2000 was used as the transfection reagent and transfections done as per manufacturer's protocol using Opti-MEM media (Invitrogen Life Sciences).

Western blot analysis. Adherent cell cultures were harvested by brief incubation in cell dissociation solution (Invitrogen Life Sciences), pelleted, and counted. Cells were lysed in PBS containing 1% (v/v) NP40, 1 mmol/L EDTA, 150 mmol/L NaCl, and 10 mmol/L Tris (pH 7.4) at a concentration of 2×10^7/mL lysis buffer. Samples were incubated on ice for 10 minutes, sonicated briefly, and centrifuged for 10 minutes at 13,000 rpm to remove the insoluble cellular debris. Supernatants were aliquoted and frozen at −80°C until needed, at which time one volume of 2× NuPage sample buffer (Invitrogen Life Sciences) containing 0.1 mol/L DTT was added to each, and samples were heated to 70°C for 10 minutes. Approximately 1.5×10^5 cell equivalents, or 15 μL, was loaded per well of 10% Bis-Tris NuPage gels (Invitrogen Life Sciences). Resolved proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad, Inc., Hercules, CA), blocked in PBS containing 5% skim milk powder, and then incubated for 1 hour with the primary antibody solutions. Detection of specific kinases was done using 1:1,000 dilutions of anti-SRPK1, anti-SRPK2, or anti-CLK1 antibodies (all from BD Biosciences). Detection of phosphorylated SR proteins was done using the previously described mAb104 specific for a phosphoepitope common to multiple SR proteins (16). Blots were washed extensively in PBS-Tween and then incubated for 1 hour with a 1:1,000 dilution of peroxidase-conjugated secondary antibody before being washed again. Proteins were subsequently visualized using the SuperSignal enhanced chemiluminescence kit (Pierce, Rockford, IL).

Creation of siRNA-encoding constructs. The coding sequence of SRPK1 was analyzed for sites of siRNA targeting using Promega's siRNA Target Designer (Promega, Madison, WI) and two potential sequences were selected. The first, sh1-SRPK1 (5′-GTGCAGCAGAAATTAATT-3′) corresponds to nucleotides 1171 to 1189 of the SRPK1 transcript (GI:47419935), whereas the second, sh4-SRPK1 (5′-GATCATCAAATCCAATTA-3′) targets nucleotides 647 to 665 of the mRNA. Short hairpin primers were designed around these sequences and annealed before their ligation into the *Pst*I site of the siSTRIKE U6 Hairpin Cloning System vector (Promega). In a similar fashion, control constructs were produced using scrambled versions of the sh1-SRPK1 and sh4-SRPK1 target sequences indicated above. Both the SRPK1 targeted and the control nucleotide sequences were confirmed against the Genbank database to prevent improper interaction upon other mRNA transcripts. Plasmids were produced via the Qiagen MaxiPrep protocol (Qiagen, Inc., Valencia, CA), and purity and concentration were confirmed by SmartSpec analysis (Bio-Rad).

Apoptotic assays. Twenty-four hours after transfection, MiaPaCa2 and Panc1 cells were harvested, counted, and then replated at 5×10^5 viable cells per well of six-well plates. Cells were incubated overnight at 37°C to allow them to adhere. The following day, medium was removed, and cells were washed once with PBS before the addition of fresh medium alone or medium containing 100 nmol/L gemcitabine-HCl (Gemzar; Eli Lilly Co., Indianapolis, IN). The next morning, 10 μmol/L cisplatin (Bedford Laboratories, Bedford, OH) was added to chosen wells and incubated for a further 24 hours. Total treatment periods were 24 hours for cisplatin and 48 hours for gemcitabine. Following treatment, the adherent and non-adherent cell populations were harvested and pooled and 1×10^5 cells stained with Annexin-FITC and propidium iodide (BD Biosciences) as per manufacturer's instructions. Cells were then analyzed via flow cytometry using Becton Dickinson's FACScan instrument and Cellquest software.

Proliferation assays. Twenty-four hours after transfection, MiaPaCa2 and Panc1 cells were harvested, counted, and replated into 96-well plates at 500 or 1,000 viable cells per well. The following day, bromodeoxyuridine (BrdUrd) was added to the culture medium and reincubated for a further 18 hours. Culture medium was subsequently removed, and cells were fixed and finally stained with an anti-BrdUrd-POD solution as per manufacturer's protocol (Roche, Indianapolis, IN). Cell proliferation was quantified using an ELISA plate reader at 370 nm, and an average reading of wells plated in triplicate was compared with wild-type, nontransfected MiaPaCa2 or Panc1 cells to determine their relative proliferation.

Reverse transcription-PCR and quantitative reverse transcription analysis. Cultured cells were harvested with cell dissociation solution (Invitrogen Life Sciences), pelleted, and lysed in Trizol at a concentration of 2×10^7/mL Trizol (Invitrogen Life Sciences). RNA was purified from 100 μL of each Trizol suspension using Qiagen's RNEasy columns per manufacturer's protocol. Purified RNA integrity was confirmed by gel electrophoresis before cDNA was produced with 0.5 μg of each RNA sample using Promega's First-Strand Synthesis kit. Subsequent quantitative reverse transcription-PCR (RT-PCR) reactions were run using 10 ng cDNA, 70 nmol/L of each primer, and 12.5 μL Absolute QPCR Sybr Green Mix (ABgene, Rochester, NY) in 25-μL reactions. Control actin reactions using the primers 5′-CTCTTCCAGCCTTCCTTCCTGGT-3′ and 5′-CGTACAGGTCTTTGCGGATGTCC-3′ were run for each cDNA in addition to the SRPK1 reactions using the primers 5′-CCGAAAGAAAAGGACCAAGGCC-3′ and 5′-CCTGCTCTGGTAGATCACTGTC-3′. Relative amounts of SRPK1 were determined via the Pfaffl method (Nucl Acids Res, 2001) described previously.

Standard RT-PCR reactions were used to examine the expression levels of Rpl17 (primers 5′-GGTGATCTGTGAAAATGGTTCGC-3′ and 5′-ATGCTGAATTTACTCCCGTGC-3′) and actin (primers 5′-CCAGCTCACCATGGATGATGATATCG-3′ and 5′-GGAGTTGAAGGTAGTTTCGTGGATGC-3′). Approximately 25 ng of cDNA template was used for each reaction under optimized conditions generally ranging from 30 to 35 cycles dependent upon the transcript being examined.

Results

SRPK1 expression in normal and neoplastic pancreas. Expression of SRPK1 transcripts in the intact pancreas has been described previously via Northern blot analysis (15, 17), yet the precise cellular and subcellular localizations of the resultant protein within this organ remain unspecified. To resolve this issue,

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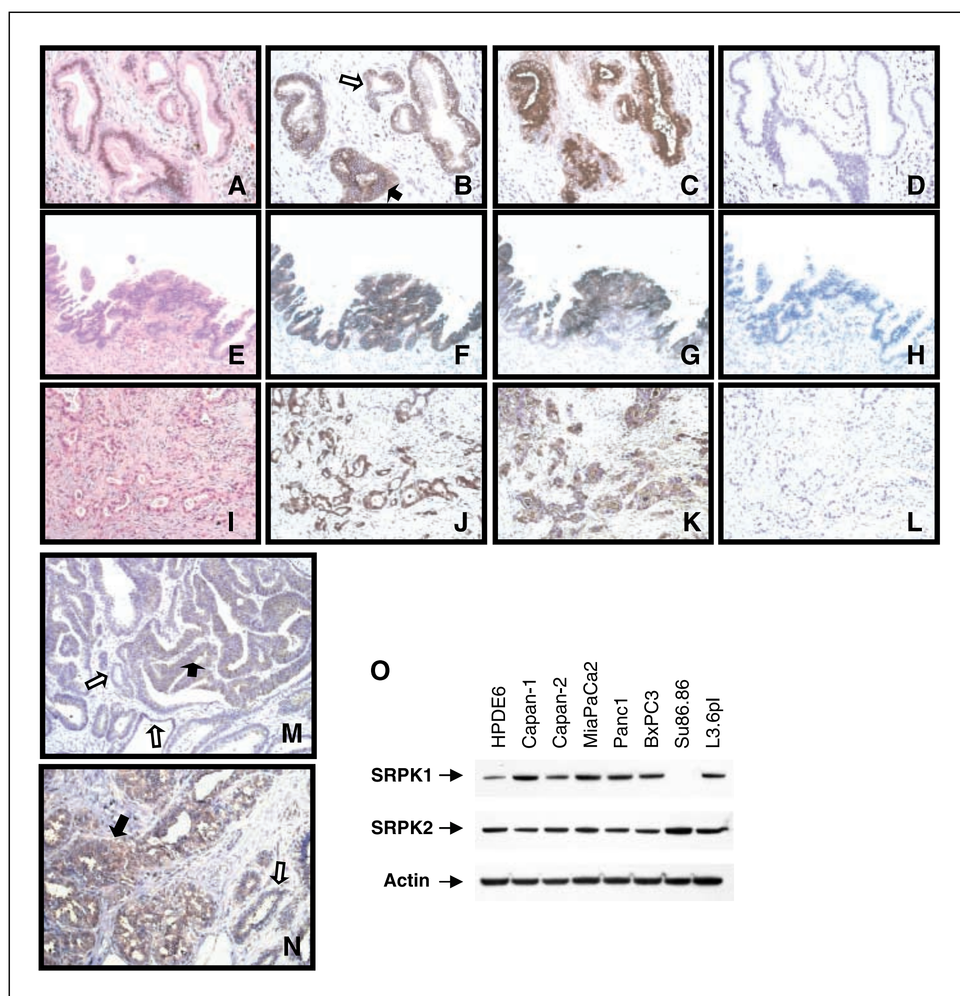

Figure 1. *In situ* expression of SRPK1 in normal human pancreas and epithelial tumors of the pancreas, breast, and colon. Immunohistochemistry was performed on tissue sections, including normal pancreatic ducts (*A-D*), dysplastic duct (*E-H*), and infiltrating pancreatic ductular carcinoma (*I-L*) using a monoclonal antibody against either SRPK1 (*B, F*, and *J*) or CA19-9 (*C, G*, and *K*). H&E-stained (*A, E*, and *I*) or secondary only control (*E, H*, and *L*) sections. Colon (*M*) and breast (*N*) tumor samples were similarly stained with the anti-SRPK1 antibody and benign epithelial regions (*open arrows*) or regions representing tumor (*solid arrows*). Western blot analysis was done on eight pancreatic tumor cell lines (*O*) to examine SRPK1 or SRPK2 expression using monoclonal antibodies against these proteins.

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we have done immunohistochemistry on pancreatic tissue sections using a monoclonal antibody specific for SRPK1 (Fig. 1). In the normal pancreas, SRPK1 was found predominantly in epithelial cells lining the ducts (Fig. 1*B*), whereas much lower amounts were detected in lymphatic germinal centers (data not shown). The localization of SRPK1 staining to duct cells was confirmed by staining analogous sections with a monoclonal antibody raised against the CA19-9 antigen known to be expressed by human pancreatic duct epithelial (HPDE) cells (Fig. 1*C*; ref. 18). Remarkably, in ducts exhibiting areas of hyperplasia (Fig. 1*B*, *solid arrow*), the level of SRPK1 staining was noticeably elevated in these regions compared with histologically normal duct cells (Fig. 1*B*, *hollow arrow*). SRPK1 staining was also noticeably increased in dysplastic pancreatic ducts characterized by the crowding of cells and a related loss of columnar phenotype and basal nuclei (Fig. 1*F*). Immunohistochemistry was similarly done on pancreatic tumor samples, and results revealed increased SRPK1 expression within malignant cells of exocrine origin (Fig. 1*J*) when compared with normal duct cells. In the majority of cells expressing SRPK1, the protein was distributed predominantly in the cytoplasm but was occasionally also detected in the nuclei, confirming previous reports of subcellular localization in HeLa cells and testicular tissue sections (13, 19). The expression of SRPK1 in pancreatic cell lines was investigated via Western blot analysis and is illustrated in Fig. 1*O*. Five of seven pancreatic tumor cell lines exhibited substantial levels of SRPK1 protein, whereas the near-normal human pancreatic duct epithelial cell line HPDE6 and the well-differentiated Capan-2 tumor line exhibited more moderate SRPK1 expression by this technique (20, 21).

Immunohistochemistry was done on breast and colon tumor samples to assess the prevalence of SRPK1 overexpression in other epithelial neoplasms. SRPK1 was found at relatively low levels in normal colonic epithelial cells (Fig. 1*M*, *hollow arrows*), but its expression increased considerably in regions of dysplasia and tumor growth (Fig. 1*M*, *solid arrows*). SRPK1 was similarly expressed by infiltrating breast carcinoma cells (Fig. 1*N*, *solid arrow*) and likewise was found in reduced amounts in cells lining normal breast ducts (Fig. 1*N*, *hollow arrow*). Collectively, these results suggest a possible role for SRPK1 in malignant epithelial neoplasms arising in the breast, colon, and pancreas.

SRPK1 expression impedes chemotherapeutic potential. To investigate the role elevated SRPK1 expression may play in the response of pancreatic malignancies to chemotherapeutic agents, we have designed small inhibitory RNAs against two distinct regions of the SRPK1 mRNA. The sh1-SRPK1 and sh4-SRPK1 constructs encode siRNAs targeting nucleotides 1171 to 1189 and 647 to 665 (GI:12345678), respectively, of the SRPK1 transcript and were created using Promega's siSTRIKE short hairpin system. Transfection of these constructs into either MiaPaCa2 or Panc1 cells substantially and specifically reduced SRPK1 expression as

confirmed by Western blot analyses, illustrated in Fig. 2*A*. No changes in SRPK1 levels were detected in cells transfected with control constructs encoding scrambled versions of the sh1 or sh4 sequences. Furthermore, there were no alterations in the expression of related serine-arginine protein kinases, SRPK2 or CLK1, in cells receiving any construct (Fig. 2*A*).

Treatment options for unresectable PDAC rely predominantly upon chemotherapeutic regimens, but unfortunately, they have had only slight success. Standard chemotherapy of this disease revolves around gemcitabine, whereas one variant of care adds cisplatin to this (22). Hence, we have investigated whether SRPK1 expression levels in pancreatic tumor cell lines have any effect upon such therapies. The proportion of MiaPaCa2 and Panc1 cells undergoing apoptosis following siRNA-mediated down-regulation of SRPK1 was assayed via annexin binding, and cells with reduced SRPK1 levels showed significantly higher levels of apoptosis (Fig. 2*B* and *C*, respectively). This increase in apoptotic fraction could not be attributed solely to the transfection procedure, because cells receiving equivalent quantities of either the sh1-control or sh4-control plasmids and Lipofectamine reagent exhibited only the basal proportion of apoptotic cells. Transfected cells were also treated with 0.1 μmol/L gemcitabine, 10 μmol/L cisplatin, or a combination of both agents. Doses were chosen based upon previous experimental and clinical reports and are considered to fall within the therapeutic dose ranges used clinically (23). Under these conditions, MiaPaCa2 and Panc1 cells exhibiting decreased SRPK1 levels were significantly and dramatically more sensitive to killing by gemcitabine and/or cisplatin compared with control cells (Fig. 2*B* and *C*) as determined by annexin binding. Photomicrographs of such cells are illustrated in Fig. 2*D* and confirm the apoptotic assay results by revealing the effect that decreased SRPK1 expression has upon cell growth both in the presence and

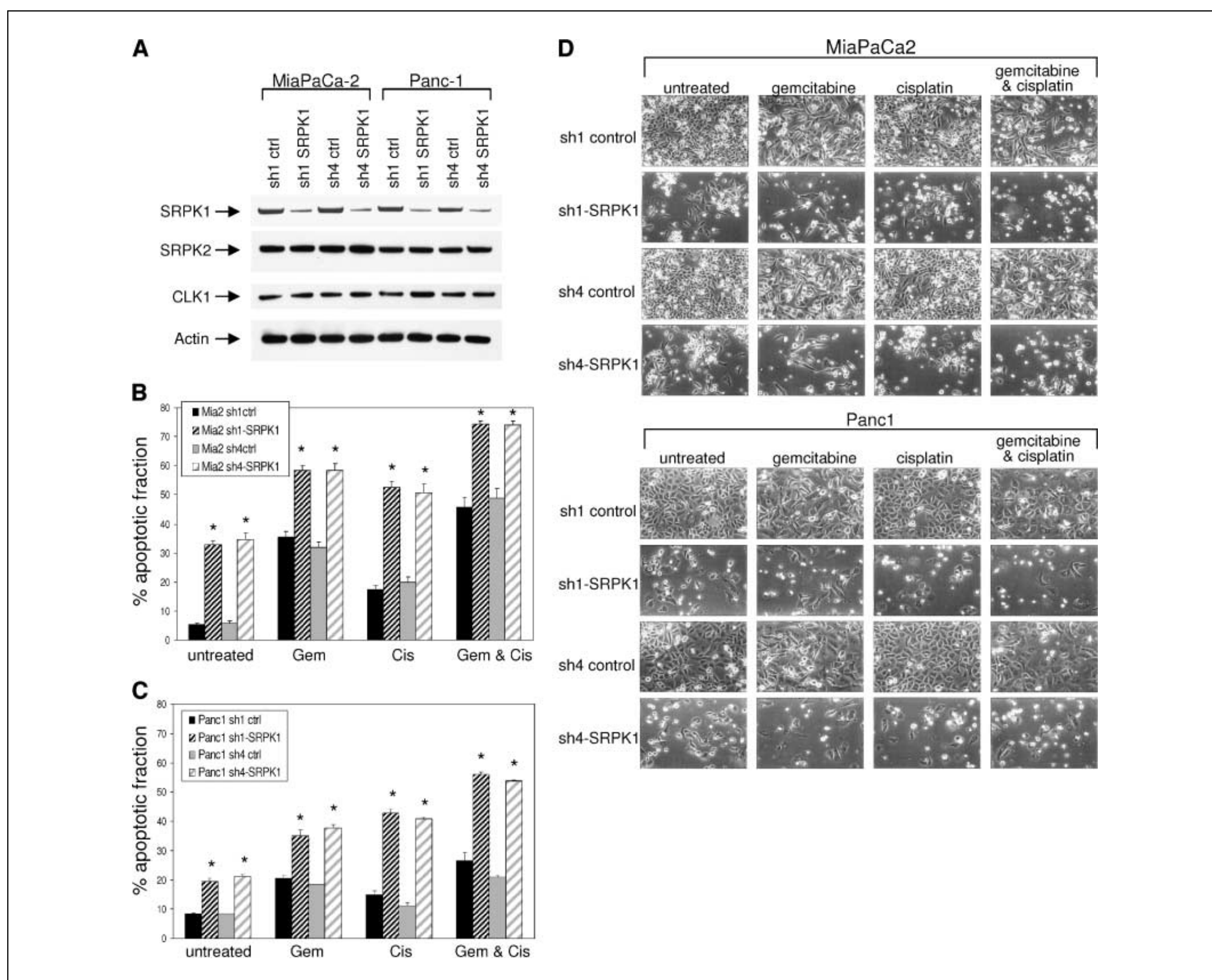

Figure 2. SRPK1 expression in pancreatic tumor cell lines affects growth potential and sensitivity to gemcitabine and/or cisplatin. MiaPaCa2 and Panc1 cells were transfected with 15 μg of either the sh1-SRPK1 or sh4-SRPK1 constructs that encode siRNAs targeting the SRPK1 transcript, and Western blot analysis was done on transfectants to determine their effects on the indicated proteins (*A*). Plasmids encoding scrambled target sequences were likewise transfected to serve as appropriate controls. The effect reduced SRPK1 levels have upon chemotherapy was assayed by treating transfected cell lines with 0.1 μmol/L gemcitabine for 48 hours and/or 10 μmol/L cisplatin for 24 hours. Photomicrographs of treated MiaPaCa2 or Panc1 (*D*) cells were taken before harvesting the cells for staining with annexin and propidium iodide to determine the apoptotic fractions (*B* and *C*, respectively) via flow analysis. *Columns,* means of multiple experiments ($n = 8$ for both MiaPaCa2 and Panc1); *bars,* SE. *, $P < 0.0001$, statistical significance was done via the Student's t test between analogously treated controls and short hairpin transfectants.

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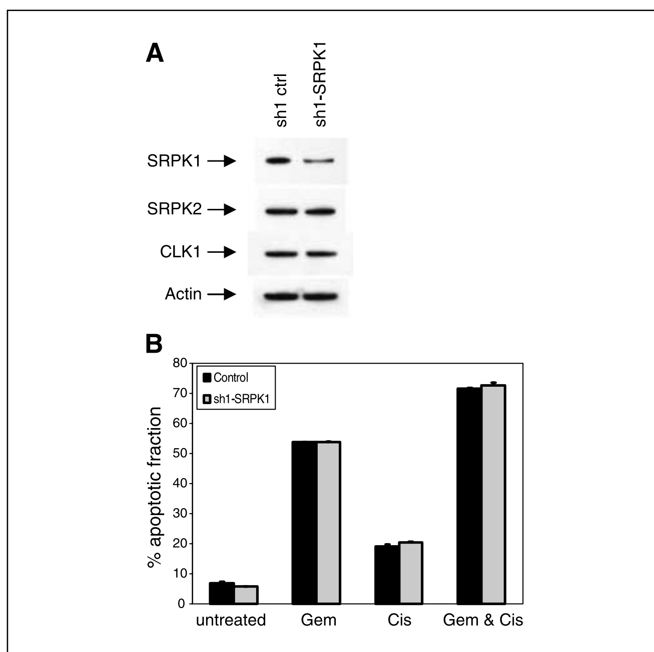

Figure 3. Decreased expression of SRPK1 in the near normal HPDE6 cell line does not affect apoptotic potential nor response to gemcitabine or cisplatin. HPDE6 cells were transfected with 15 μg of either the sh1-SRPK1 constructs or the scrambled plasmid control, and Western blot analysis was done on transfectants to determine their effects on the indicated proteins (*A*). The effect reduced SRPK1 levels has upon chemotherapy was assayed by treating transfected cells with 0.1 μmol/L gemcitabine for 48 hours and/or 10 μmol/L cisplatin for 24 hours before harvesting the cells and staining with annexin and propidium iodide to determine the apoptotic fractions (*B*) via flow analysis. *Columns,* means of multiple experiments ($n = 3$); *bars,* SE.

absence of these cytotoxic agents. In contrast, siRNA-mediated reduction of SRPK1 levels in the near normal HPDE6 cells (Fig. 3*A*) had no effect upon killing by either gemcitabine or cisplatin (Fig. 3*B*), suggesting that normal pancreatic epithelial cells may be more resistant to therapies targeting the SRPK1 protein.

Experiments were done to ascertain whether sensitivity to gemcitabine and/or cisplatin could be achieved in a dose-dependent manner relative to decreases in the SRPK1 protein. MiaPaCa2 and Panc1 cells were transfected with a range of 2 to 20 μg of sh1-SRPK1 plasmid, whereas control cells were transfected with the maximal dose (i.e., 20 μg) of the scrambled sh1-SRPK1 control plasmid. Western blot analysis, shown in Fig. 4*A*, illustrates the specific dose-dependent down-regulation of SRPK1 protein in these cell lines following transfection with increasing amounts of siRNA-encoding plasmid. As before, no change in SRPK2 expression levels was observed following transfection with any siRNA dose. Additionally, SRPK1 transcript levels were quantified using real-time PCR and confirmed the dose-dependent reduction in SRPK1 mRNAs following transfection (Fig. 4*B*). MiaPaCa2 and Panc1 cells were sensitized to killing by gemcitabine and/or cisplatin in a dose-responsive manner as assayed by apoptotic index, and these data are illustrated in Fig. 4*C*. Of note, even a relatively small reduction in SRPK1 transcript level and its resultant protein following transfection with 5 μg of siRNA-encoding plasmid was able to elicit a significant increase in sensitivity to both agents when used together. Finally, the effect of SRPK1 on cell proliferation was examined because reduced SRPK1 levels in tumor cells seemed to impede their growth rate in our initial experiments. For example, as illustrated in Fig. 2*D*, MiaPaCa2 and Panc1 cells exhibiting lower SRPK1 levels lagged in growth compared with cells receiving control plasmid. To confirm this observation, cells transfected with 2 to 20 μg of sh1-SRPK1 plasmid were also assayed for proliferative capacity via BrdUrd incorporation. Figure 4*D* shows that as SRPK1 expression decreased the cells' proliferation declined proportionately, supporting an effect of this kinase on tumor cell growth in addition to its effect on sensitivity to chemotherapeutic agents.

Decreased SRPK1 levels diminish SR protein phosphorylation and lead to alterations in apoptosis-associated protein expression. The quintessential targets of SRPK1's kinase activity are a family of SR proteins that function as splice factors during pre-mRNA processing (14, 24). To investigate the effect that reduced expression of SRPK1 has upon SR protein phosphorylation in pancreatic cancer cells, Western analysis was done on cell lysates produced from the sh1-SRPK1 and control transfectants using an antibody recognizing a phospho-specific epitope common to multiple SR proteins (16). siRNA-mediated decreases in SRPK1 expression resulted in inhibition of SR protein phosphorylation in a dose-dependent manner in both MiaPaCa2 and Panc1 cells, as illustrated in Fig. 5*A*. Interestingly, decreases in the phosphorylation status of SF2, SRp20, SRp30c, 9G8, SRp40, and SRp75 were clearly identified in cells transfected with higher quantities of sh1-SRPK1 plasmid, whereas there was no visible change in SRp55 phosphorylation. The consistent phosphorylation state of SRp55 in cells exhibiting reduced SRPK1 levels may be due to a preferential ability of the SRPK2 protein, expressed at strong levels in both cell lines and unaffected by transfection of these plasmids, to act upon this substrate. These results also show the inability of other SR protein kinases, such as SRPK2 and CLK1, to fully compensate for the loss of SRPK1 activity, suggesting there may be more specificity in substrate selection for these kinases *in vivo* than what has been previously reported by *in vitro* experimentation (13, 24).

Based on these results, it was postulated that alterations in SRPK1 expression and the resultant loss of SR protein phosphorylation could affect the expression of a variety of genes involved in the apoptotic pathway underlying the increased sensitivity to cytotoxic agents. To investigate this possibility, RT-PCR and Western blot analyses were done on the MiaPaCa2 and Panc1 sh1-SRPK1 or control transfectants to determine the expression of genes able to influence the apoptotic potential of these cells or their ability to resist killing by gemcitabine and/or cisplatin. Illustrated in Fig. 5*B* by Western blot analysis and band densitometry, as the levels of SRPK1 decreased, the ratio of Bax to Bcl2 proteins increased in a proportionate manner for both MiaPaCa2 and Panc1 cells. Increases in the proapoptotic protein Bax may be responsible for the cells' increased sensitivity to cytotoxic agents, suggesting a regulatory role for SRPK1 in mediating the cells' apoptotic network and potential for cell killing (25, 26). Furthermore, RT-PCR reactions were employed to examine the expression of the ribosomal protein L17 (Fig. 5*C*), a member of the ribosomal 60S subunit family that contains multiple proteins implicated in mediating multidrug resistance (27). The expression of RPL17-encoding mRNA decreased coordinately with SRPK1 protein levels, suggesting a role for SRPK1 in the expression of this gene. This may be responsible, at least in part, for the resistance of pancreatic tumor cells to these chemotherapeutic agents.

Discussion

The defined localization of SRPK1 in the human pancreas has, until now, remained elusive. Our immunohistochemistry results provided herein present the novel discovery that SRPK1 is located

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primarily in a minor cell population within normal pancreas, the ductular epithelial cells. Importantly, we have further shown that SRPK1 is expressed at elevated levels in malignancies arising not only in the pancreas but also in tumors of the breast and colon, thus signifying a possible role for SRPK1 in tumor development. Using pancreatic cell lines as a model *in vitro* system, our experiments revealed that down-regulation of this kinase via siRNA technology reduced tumor cell proliferation, increased the cells' apoptotic potential, and rendered tumor cells significantly more sensitive to the two chemotherapeutic agents gemcitabine and cisplatin. Knockdown of SRPK1 in the normal ductal line HPDE6, however, showed no significant alterations in apoptotic potential or sensitivity to killing by gemcitabine and/or cisplatin. Lastly, decreased SRPK1 expression was shown to increase the expression of the proapoptotic factor Bax and to decrease the ribosomal protein RPL17, a member of the 60S subunit whose proteins have been previously shown to be associated with multidrug resistance (27). Collectively, these results suggest a potential role for SRPK1 in regulating multiple tumor-associated processes and fundamentally introduce SRPK1 as a novel molecular target for treating PDAC and possibly other neoplasia.

Virtually, all human cancers exhibit aberrant patterns of pre-mRNA splicing; yet, the mechanisms responsible for such tumor-specific alterations remain poorly understood. The spliceosomal machinery that removes the noncoding intronic sequences from primary pre-mRNA transcripts is comprised of >300 distinct proteins in addition to a number of essential ribonucleotides (28). Upwards of 50 *trans*-acting factors, including members of the SR and heterogenous nuclear ribonucleoprotein families, collectively referred to as splice factors, are believed to bind *cis*-acting sequences present in the primary transcript and influence the use of specific splice sites, resulting in exon inclusion or exclusion from the processed transcript (29). The complexity of this process has made the characterization of its functional components difficult and the identification of those components that are altered and subsequently responsible for tumor-specific splicing changes especially challenging. Regardless, the expression of protein variants resulting from missplicing has been shown to effect a multitude of tumor-associated pathways, including regulation of the cell cycle, apoptosis, angiogenesis, and metastasis. For example, a misspliced version of cyclin D1, found in a variety human tumors, encodes a variant that is inappropriately stabilized in the nucleus and is

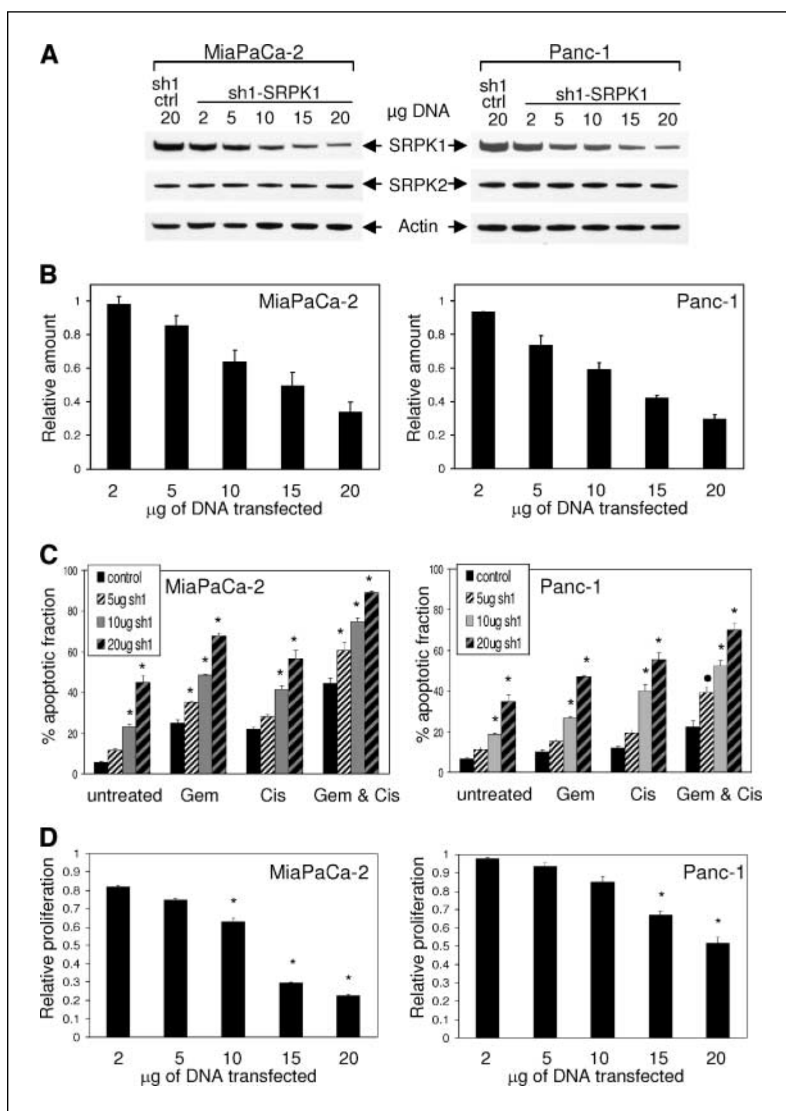

Figure 4. SRPK1 expression in pancreatic tumor cells affects therapeutic potential and proliferation in a dose-dependent manner. MiaPaCa2 and Panc1 cells were transfected with 2 to 20 µg of the sh1-SRPK1 plasmid or 20 µg of the scrambled plasmid control. Lysates were produced from the transfectants and analyzed via Western blot analysis for expression of SRPK1 or SRPK2 (*A*). Quantitative RT-PCR analysis was done on transfectants to confirm dose-dependent decreases in SRPK1 encoding mRNA. Sybr Green reactions for SRPK1 and an actin control were run in triplicate for each three experiments, and the mean quantities of SRPK1 transcripts relative to control cells determined (*B*). sh1-SRPK1 or control transfected cells were replated into six-well plates and treated with 0.1 µmol/L gemcitabine for 48 hours and/or 10 µmol/L cisplatin for 24 hours before harvesting and staining with annexin-FITC to determine the apoptotic fraction for MiaPaCa2 and Panc1 (*C*). *Columns,* means of multiple experiments (n = 7 for MiaPaCa2;n = 5 for Panc1); *bars,* SE. *, $P < 0.001$; ●, $P < 0.01$, statistical significance was done via the Student's t test between analogously treated controls and each of the sh1-SRPK1 transfected doses. Transfected cells were also seeded in triplicate into 96-well plates to determine proliferative capacity via BrdUrd incorporation for MiaPaCa2 or Panc1 (*D*) transfectants compared with wild-type cells. *Columns,* means of multiple experiments (n = 3 for both cell lines); *bars,* SE. *, $P < 0.001$, statistical analysis determined using the Student's t test.

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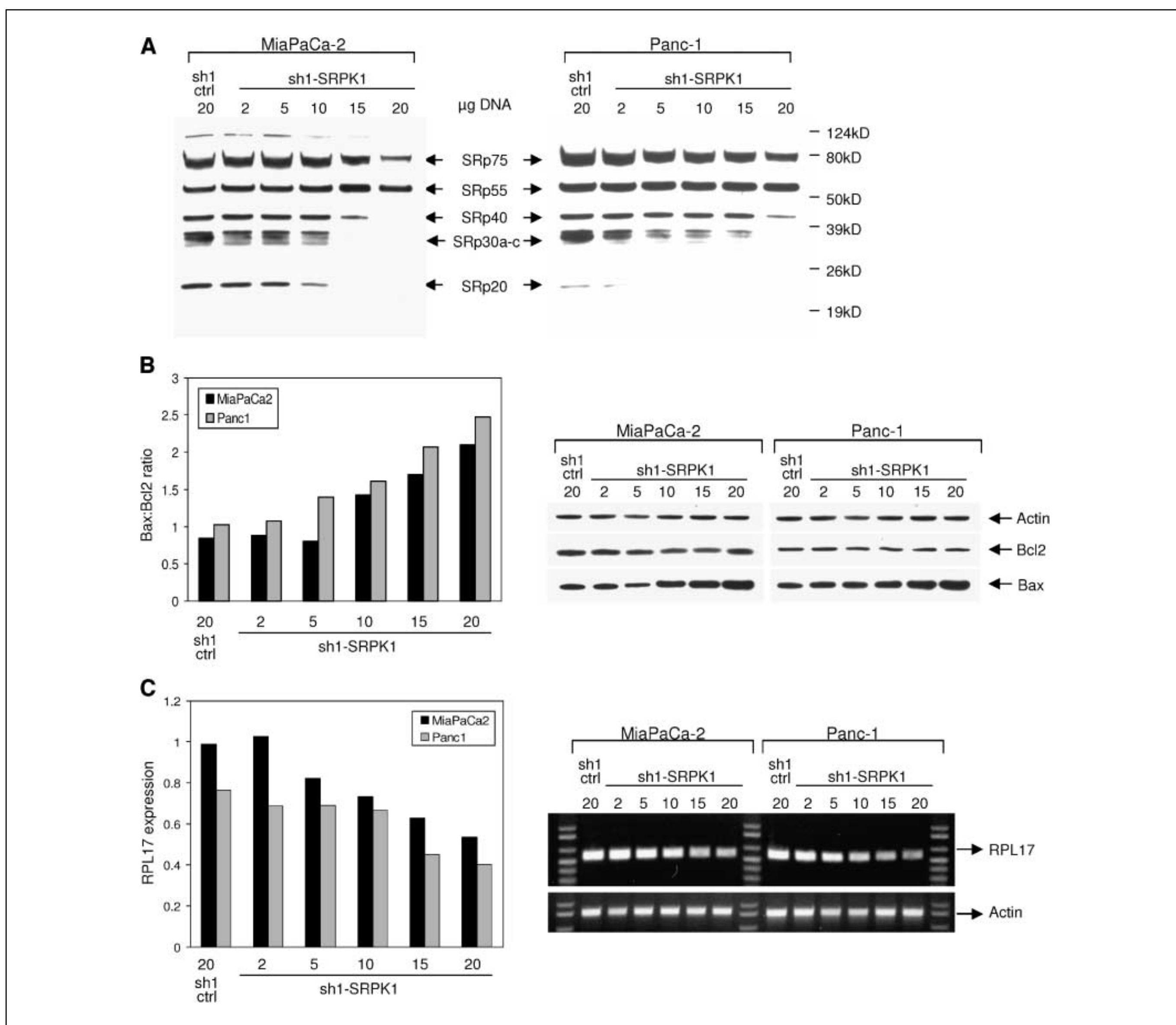

Figure 5. Decreased SRPK1 expression impacts SR protein phosphorylation and the expression of *Bax* and *RPL17* genes. Western blot analysis was done on lysates produced from the sh1-SRPK1 transfections (*top*) and the level of multiple SR-proteins' phosphorylation investigated using the mAb104 (*A*). Western analysis of these lysates also investigated Bcl2 and Bax levels using monoclonal antibodies specific to each protein (*B*). Band densitometry was done, Bax and Bcl2 levels were equilibrated to actin, and the ratio of Bax to Bcl2 was determined for each transfection. RT-PCR analysis was done to investigate the expression of ribosomal protein L17 and actin using cDNAs produced from each transfected cell population (*C*). Densitometry was used to quantitate RPL17 expression and was equilibrated to actin.

oncogenic in nature (30), whereas alternatively spliced forms of CD44 have been shown to confer metastatic propensity to tumor cell lines *in vivo* (31). These studies substantiate an important role for aberrant splicing among a wide range of tumors.

Recent reports have shown increased expression of certain SR proteins for tumors of the breast (32), lung (33), and ovary (34). Elevated levels of SR proteins were correlated with increases in alternatively spliced CD44 and multidrug resistance protein 1 variants. In combination with studies relying upon *in vitro* assays to determine the effects individual splice factors have in determining splice site utilization (29), these results provide a compelling argument that tumor-specific changes in splice factors and/or their level of activation lead to the production of variants known to be elevated in these neoplasms. However, due to the restricted tissue distribution of many splice factors as well as their shown abilities to overlap functionally, it is unlikely that a few splice factors will be responsible for the aberrant splicing associated with tumor progression. In contrast, the ubiquitous expression pattern of SRPK1, its ability to activate a diverse array of SR protein splice factors, and our demonstration that it is increased in tumors of different origin provide a persuasive hypothesis that it serves a more global role in the missplicing observed across tumor systems. This premise is supported by a recent report by Hishizawa et al. (35), showing the overexpression of SRPK1 in acute type adult T-cell leukemia (ATL) but not in chronic ATL or normal peripheral blood mononuclear cells, as well as the presence of autologous antibody to SRPK1 in the acute ATL patients.

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Chemotherapy of pancreatic ductal neoplasms has had only limited success. This illustrates the need for better understanding of the molecular nature of the disease and the need for introduction of new innovative treatment strategies. The *in vitro* experiments undertaken in this work provide a novel basis for sensitizing pancreatic tumor cells to two commonly used chemotherapeutic agents gemcitabine and cisplatin. Implicit reductions in SRPK1 expression in two commonly used pancreatic tumor cell lines, MiaPaCa2 and Panc1, significantly ($P < 0.0001$) sensitizes such cells to clinically relevant doses of both agents, whether used alone or in combination. Currently, there are no reports linking gemcitabine resistance to increased SRPK1 expression level; however, results presented here do counter two previous studies showing that decreased SRPK1 expression in testicular germ cell tumors (19) and the A2780 ovarian carcinoma cell line (36) correlates with cisplatin resistance rather than sensitivity. We have done similar experiments using the A2780 ovarian cell line, and our results confirm those reported earlier (data not shown) that decreased SRPK1 expression in these ovarian tumor cells leads to an increase in cisplatin resistance. It can be postulated that the divergence between these results is due to fundamental differences in the molecular characteristics of reproductive tumors versus PDAC. It has already been established that the primary substrate for SRPK1 activity in testicular germ cells is protamine 1, a protein that once phosphorylated and successively dephosphorylated allows for a more compact chromatin condensation necessary during spermatogenesis (15). It is likely, however, that there are still other unidentified proteins phosphorylated by SRPK1 in the ovarian and testicular compartments, but not in pancreas, that may differentially regulate the effect SRPK1 has upon the response to chemotherapy for such tumors.

Currently, clinical trials are investigating the use of agents that target tumor-specific splice variants, including CD44, as well as others, known to be up-regulated in various cancers (37). SRPK1 presents an exciting new target for therapeutic intervention in PDAC. A number of genetic lesions have been associated with the progression of normal pancreatic duct epithelial cells to a state of infiltrating adenocarcinoma. Notably, alterations of HER-2/*neu*, K-ras, p16, DPC4, and p53 seem to occur at specific stages during the progression of this disease (38). However, even with the identification of such mutations, effective therapies are still lacking for the treatment of this disease. The targeting of a central splicing regulator, such as SRPK1, represents an innovative approach for cancer therapeutics. It is fully anticipated that inhibition of normal SRPK1 expression and function will have a pleiotrophic effect and alter the splicing of numerous gene products. Our results show that even a slight decrease in SRPK1 expression is sufficient to elicit a significantly increased sensitivity to gemcitabine and cisplatin. This ability to enhance tumor cell killing via frequently applied chemotherapeutic agents may aid in the treatment of pancreatic tumors and provides a foundation for developing supplementary agents that specifically target the SRPK1 protein to be used in adjuvant therapies.

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