

# Serine-Arginine Protein Kinase 1 Overexpression Is Associated with Tumorigenic Imbalance in Mitogen-Activated Protein Kinase Pathways in Breast, Colonic, and Pancreatic Carcinomas

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## Abstract

**Aberrant patterns of pre-mRNA processing are typical of human malignancies, yet the mechanisms responsible for these changes remain undefined. We have recently shown overexpression of a core splice regulatory protein, serine-arginine protein kinase 1 (SRPK1), in dysplastic and neoplastic pancreatic ductular cells. In the present study, we have established that SRPK1 levels are similarly up-regulated in breast and colonic tumors where its expression increases coordinately with tumor grade. Targeting SRPK1 for inhibition using small interfering RNA in breast and colonic tumor cell lines *in vitro* resulted in both increased apoptotic potential and enhanced cell killing after treatment with gemcitabine and cisplatin. Recent reports have described multifaceted interactions between the mitogen-activated protein kinase (MAPK) and AKT signaling networks and the splice regulatory machinery. Consequently, we have shown that targeted inhibition of SRPK1 in tumor cells results in reduced phosphorylation of MAPK3, MAPK1, and AKT. Alterations in the splice pattern and resulting expression of MAPK kinase are implicated in mediating the antitumoral effects resulting from SRPK1 down-regulation. The up-regulation of SRPK1 in multiple cancers and its ability to regulate multiple relevant signaling pathways provide support for developing agents to inhibit this kinase for possible broad application to treat epithelial cancers. [Cancer Res 2007;67(5):2072–80]**

## Introduction

Aberrant patterns of pre-mRNA splicing are typical of human malignancies, yet the mechanisms responsible for these alterations remain poorly understood. We recently reported the elevation of a kinase [serine-arginine protein kinase (SRPK) 1] in pancreatic carcinoma cells that is critical for the regulation of various mRNA splicing factors (1). In the current report, we have explored whether a similar abnormality in SRPK1 expression exists in other common epithelial malignancies (breast and colonic carcinomas) and have evaluated possible relevant signaling events. We have also examined the effect of selective inhibition of this kinase on cancer-relevant processes.

Proteins encoded by splice variants have been shown to affect a variety of tumor-associated processes. For example, BCL2L1 transcripts may undergo alternative splicing to produce either

the antiapoptotic long form or the proapoptotic short form. Therefore, a shift in the splicing pattern of these transcripts can have profound effects on the proliferative activity of cancer cells and on their response to proapoptotic therapies (2). The inappropriate retention of intron 4 during processing of cyclin D1 transcripts results in production of a variant protein termed cyclin D1b that serves as a nuclear oncogene (3), whereas expression of CD44 isoforms containing variable exon 6 increases metastatic propensity of pancreatic carcinoma cells (4, 5). These examples clearly show the potential functional effect of aberrant splicing on tumorigenesis.

Several specific alternative splicing events have been implicated in the progression of breast and colonic cancers. Alternatively spliced versions of androgen (6) and estrogen (7) receptors have been found in mammary carcinomas, and expression of the ESR1 $\Delta$ exon3 isoform has proven more potent in activating vascular endothelial growth factor than wild-type receptor (8), linking this variant to angiogenesis of breast tumors. A splice variant of the Rac1 small GTPase (Rac1b) has been shown in colonic tumors, where it has been shown to be active and to lead to anchorage-independent growth (9). Whereas the expression of the antiapoptotic protein survivin is grossly elevated in many cancers, the survivin2B splice variant has a growth-inhibitory effect and is lost in late-stage breast (10) and colonic cancers (11). Here, too, these examples show that changes in the splicing behavior of tumor cells can influence tumor progression.

The molecular alterations responsible for these tumor-related changes in pre-mRNA splicing remain poorly defined. In a murine breast tumor model, it has been shown that increases in splice factor levels correlate with the presence of alternatively spliced products common to many forms of malignancy (12). Serine-arginine proteins comprise a family of splice factors that are activated after phosphorylation by SRPKs in the cytoplasm. Phosphorylated serine-arginine proteins can be shuttled into the nucleus where they can exert influence over splice site usage by interacting with both *cis*-acting regulatory sequences present within a given pre-mRNA molecule and other splice regulatory proteins (13). We have recently reported the overexpression of a core splice regulatory protein, SRPK1, in dysplastic and neoplastic pancreatic epithelial cells (1). Targeting this protein for inhibition *in vitro* using small interfering RNA (siRNA) resulted in reduced proliferation of pancreatic tumor cells and altered expression of key apoptotic proteins, BAX and BCL2, to enhance apoptosis and responsiveness to cytotoxic agents.

In the present study, expression of SRPK1 was examined in breast and colon using immunohistochemistry, and SRPK1 was shown to be expressed predominantly in ductular epithelial cells of normal breast and colon. SRPK1 was found to be overexpressed in breast and colonic tumors when compared with adjacent normal epithelium, and levels of the kinase increased along with tumor

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grade. Strong expression of SRPK1 protein was also evident in the majority of breast and colonic tumor cell lines. Down-regulation of SRPK1 in breast and colonic tumor cell lines using siRNA increased the proportion of these cells undergoing apoptosis and sensitized these cells to chemotherapeutic intervention. Due to the emerging evidence of interactions between the splice regulatory machinery and activation of mitogen-activated protein kinase (MAPK) and AKT signaling pathways (14, 15), investigation was extended to examine expression and activation of proteins in these pathways in tumor cells expressing reduced levels of SRPK1 protein. Whereas total protein levels of MAPK3 [also known as extracellular signal-regulated kinase (ERK) 1], MAPK2 (ERK2), and AKT did not change in cells following reduction in SRPK1, decreases in phosphorylation of these three proteins were apparent in all the tumor lines studied. Decreasing expression of SRPK1 in these cells led to a reduction in phosphorylation of multiple serine-arginine proteins as well as alterations in the splice pattern of MAPK kinase (MAP2K) 2 [MAPK/ERK kinase (MEK) 2] transcripts. Together, these results suggest an important regulatory role for SRPK1 in MAPK and AKT pathways and support the development of drugs inhibiting SRPK1 for possible antitumor effects.

## Materials and Methods

**Immunohistochemistry.** Human breast and colonic cancer specimens were acquired for immunohistochemistry after approval by the Mayo Clinic Institutional Review Board. Tissue sections (6  $\mu$ m) were stained with a monoclonal antibody (mAb) against SRPK1 (BD Biosciences, San Jose, CA) as described previously (1). Sections were counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, CA), and photomicrographs were captured using the Zeiss AxioPlan2 imaging system (Zeiss, Thornwood, NY). Staining intensity was determined using a semiquantitative scale of 0 to 4, where 0 represents no detectable staining and 4 indicates strong staining of SRPK1 protein. Sections from multiple patients for each grade of breast and colonic carcinomas ( $n = 3$  for each grade of tumor) and normal controls were stained (total case number:  $n = 12$  for breast and  $n = 15$  for colon).

**Cell culture.** Breast (MCF10A, MCF7, MDA231, and MDA468), colonic (CaCO2, HT29, and LS174T), and pancreatic (MiaPaCa2 and Panc1) tumor cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured according to specifications. The nontransformed human pancreatic ductal cell line HPDE6 was supplied by Dr. M.S. Tsao (Ontario Cancer Institute, Toronto, Ontario, Canada) and cultured as described previously (16, 17). Pancreatic and breast cells were grown in 10-cm dishes to 70% to 80% confluency before transfection with 15  $\mu$ g of plasmid DNA using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). The colonic cell lines were harvested at 80% confluency and resuspended at  $10^7$ /mL in PBS from which 400  $\mu$ L aliquots were added to 2-mm cuvettes, incubated on ice with 15  $\mu$ g DNA, and electroporated at 300 V for 10 ms using a square wave electroporator (Bio-Rad Laboratories, Hercules, CA). Electroporated cells were immediately added to prewarmed medium and incubated under normal culture conditions. Efficiency of LipofectAMINE transfection using a green fluorescent protein-encoding reporter construct was determined to be at least 80% for the breast and pancreatic cells, whereas efficiency of electroporation was determined to be  $\sim 75\%$  for the colonic cells used in this study.

**Western blot analysis.** Cell lysates were prepared as described previously (1), and protein concentrations were determined using the Quick Start Bradford Protein Assay kit and SmartSpec spectrophotometer (both from Bio-Rad Laboratories). Lysates were diluted with an equal volume of  $2\times$  NuPAGE sample buffer (Invitrogen) containing 0.1 mol/L DTT. Protein (15  $\mu$ g) was loaded per well of 4% to 12% gradient NuPAGE gels, and resolved proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories) before being blocked in PBS containing 3% bovine serum albumin. Blots were incubated with primary antibody overnight at 4°C, washed in PBS-Tween 20, and then incubated with

peroxidase-conjugated secondary antibody for 1 h at room temperature. Antibodies at a concentration of 0.5 to 1  $\mu$ g/mL were incubated with blots. Following incubation, membranes were washed repeatedly and proteins were visualized using the SuperSignal enhanced chemiluminescence kit (Pierce, Rockford, IL) before digital photography of blots. Subsequent densitometry of digital images was done using ImageJ software (from NIH, Bethesda, MD). Antibodies for SRPK1 and SRPK2 were obtained from BD Biosciences, the anti-phosphorylated serine-arginine antibody (mAb 104; ref. 18) was obtained from ATCC, and all others were obtained from Cell Signaling (Danvers, MA; claimed specificity: phosphorylated MAPK family sampler, phosphorylated MAPK clone 28B10, phosphorylated AKT pathway sampler, and MAP2K2).

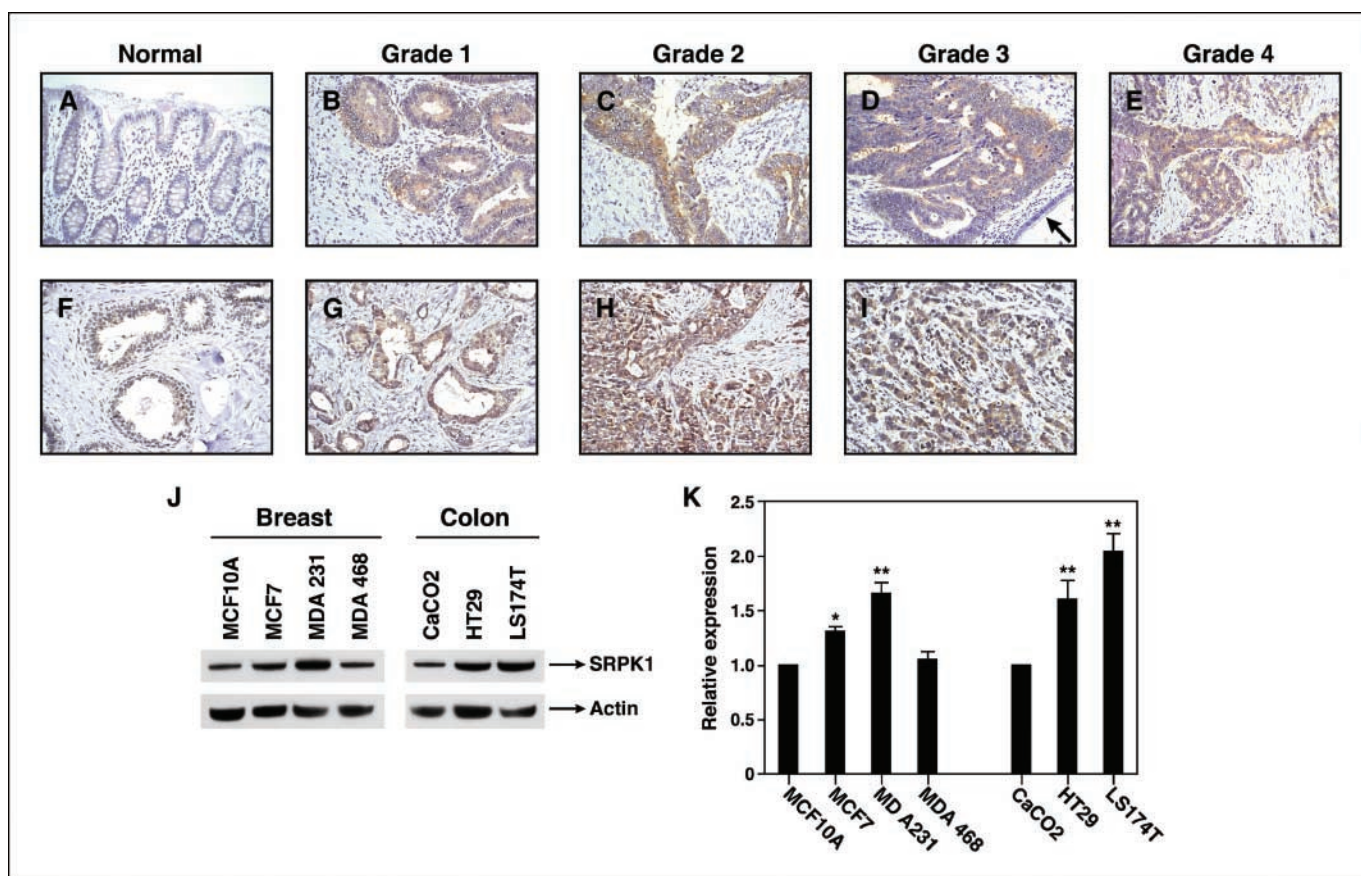
**siRNA-encoding short hairpin constructs.** Plasmids encoding siRNAs were generated as described previously (1). Briefly, sh1-SRPK1 encodes a siRNA targeting nucleotides 1,171 to 1,189 of the SRPK1 mRNA (GI:47419935). A plasmid encoding a scrambled version of this coding sequence was used as control. All sequences were aligned against the Genbank database to ensure specificity, and DNA constructs were purified using Qiagen MaxiPrep method (Qiagen, Inc., Valencia, CA).

**Cytotoxicity assays.** Transfected cells were harvested 24 h after transfection and replated at a concentration of  $2 \times 10^5$  viable cells per well into six-well plates. Cells were allowed to adhere overnight before addition of 100 nmol/L gemcitabine-HCl to selected wells (Gemzar; Eli Lilly and Co., Indianapolis, IN). Cisplatin (10  $\mu$ mol/L) was added the following day (Bedford Laboratories, Bedford, OH). This resulted in total incubation times of 48 h with gemcitabine and 24 h with cisplatin. After treatment, cells were harvested and stained with Annexin V-FITC and propidium iodide (BD Biosciences) per manufacturer's protocol and analyzed using flow cytometry on a Becton Dickinson (Franklin Lakes, NJ) FACScan instrument (19, 20).

**Reverse transcription-PCR analysis.** Transfected cells were harvested with cell dissociation solution and lysed in Trizol at a concentration of  $1 \times 10^7$ /mL (Invitrogen). RNA was purified from each extract using Qiagen RNeasy kit. Integrity of RNA samples was confirmed using electrophoresis and UV absorbance ratios. Approximately 0.5  $\mu$ g RNA was used to produce cDNA with Promega (Madison, WI) First-Strand Synthesis kit. Reverse transcription-PCRs (RT-PCR) were run using 25 ng of cDNA and amplifying product over 30 to 35 cycles. Oligonucleotide primers were designed against the 5'-end and 3'-end regions of MAP2K1 (MEK1; forward primer, 5'-CCAAAATGCCCAAGAAGAAGCCG-3'; reverse primer, 5'-CCAAACACT-TAGACGCCAGCAGC-3'), MAP2K2 (MEK2; forward primer, 5'-CACCATC-AACCTACCATCGCC-3'; reverse primer, 5'-CCACTTCTCCACCTCG-GACC-3'), and actin (forward primer, 5'-CCAGCTCACCATGGATGATGATATCG-3'; reverse primer, 5'-GGAGTTGAAGGTAGTTTCGTGGATGC-3'). PCR bands were subcloned and sequenced to confirm their identities.

## Results

**SRPK1 expression is elevated in tumors of the breast and colon.** We have previously reported that SRPK1 protein levels are increased in dysplastic and neoplastic human pancreatic ductular epithelial cells when compared with normal pancreatic ductular cells. To ascertain the precise localization of SRPK1 in normal breast and colon and to determine whether this protein might be overexpressed in breast and colonic tumors, SRPK1 immunohistochemistry was done (Fig. 1). Low levels of SRPK1 were detected in epithelial cells lining normal colonic ducts and crypts (Fig. 1A), whereas elevated expression of the kinase was found in colonic tumors of all grades (Fig. 1B-E). This relative increase in SRPK1 protein expression was clearly shown in sections that contained both normal and neoplastic ductular cells, where the level of staining was visibly increased in tumor cells compared with the adjacent normal epithelial cells (Fig. 1D, arrow). Expression of SRPK1 in the normal breast was similarly restricted primarily to epithelial cells lining the mammary ducts (Fig. 1F). Levels of SRPK1 were visibly greater in breast tumors of all grades than in normal



**Figure 1.** *In situ* expression of SRPK1 in normal and neoplastic human colon and breast. Immunohistochemistry was done on colon (A–E) and breast (F–I) tissue sections using an antibody against SRPK1. Tumors were graded on a scale of 1 to 4 for colonic tumors and on the Bloom-Richardson scale of 1 to 3 for breast tumors. Western blot analysis was done on a panel of four breast and three colonic cell lines (J) using mAbs against SRPK1 and actin. K, densitometry was done on multiple immunoblots. SRPK1 levels and expression were quantified relative to nontransformed MCF10A cells for breast or to CaCO2 cells for colon. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ;  $n = 3$ . Bars, SD.

epithelial cells (Fig. 1G–I). SRPK1 expression also seemed to increase coordinately with tumor grade, suggesting an association between SRPK1 levels and differentiation status of these epithelial tumors.

Expression of SRPK1 was examined by Western blot analysis using lysates derived from a panel of commonly studied breast and colonic tumor cell lines (Fig. 1J). In the breast cell lines, the lowest levels of SRPK1 protein were detected in the near-normal MCF10A cells compared with MCF7, MDA231, and MDA468. Densitometry was done on immunoblots, and the quantity of SRPK1 protein was found to be modestly, but significantly, increased in two of the three breast tumor cell lines relative to MCF10A cells (graphed in Fig. 1K). In the colonic cell lines examined, the best differentiated and closest to normal epithelial cells, the CaCO2 cells, expressed a lower amount of SRPK1 than either the HT29 or the LS174T cell lines (Fig. 1J), with this observation confirmed by quantitative densitometry (Fig. 1K). The detection of elevated levels of SRPK1 in breast and colonic tumors and in the majority of tumor cell lines, combined with data from our previous report (1), indicates that increased expression of SRPK1 is characteristic of diverse epithelial malignancies.

**Reducing the expression of SRPK1 in breast and colonic tumor cells increases apoptosis.** To determine whether drugs targeting and inhibiting SRPK1 may serve as a potential therapeutic option for breast and colonic malignancies, we did SRPK1

knockdown experiments using the previously described siRNA-encoding construct, sh1-SRPK1 (1). Transfection of this plasmid into MCF10A and MCF7 cells reduced expression of the SRPK1 protein, whereas transfection of a control plasmid encoding a scrambled version of the siRNA sequence did not affect SRPK1 expression (Fig. 2A). Levels of the related kinase, SRPK2, remained constant following transfection of either plasmid, supporting the specificity of this approach. SRPK1 protein levels were determined using densitometry and normalized to actin, and quantities were plotted relative to control-transfected cells. This showed a significant decrease in expression of SRPK1 after this treatment (Fig. 2A). Seventy-two hours after transfection, cells were harvested, stained with Annexin V, and analyzed using flow cytometry to determine the proportion of cells undergoing apoptosis, as we have described previously (Fig. 2B; ref. 1). Following transfection with the siRNA-encoding construct, there was reduced expression of SRPK1 in MCF7 tumor cells and a dramatic increase in the percentage of cells undergoing apoptosis, whereas there was no significant change in the proportion of nontransformed MCF10A cells undergoing apoptosis. Transfected MCF10A and MCF7 cells were also treated with gemcitabine and cisplatin to assess the potential of using SRPK1 knockdown in combination with conventional chemotherapeutic strategies (Fig. 2B). Addition of either gemcitabine, cisplatin, or a combination of the two drugs to MCF7 cells expressing reduced amounts of SRPK1 resulted in

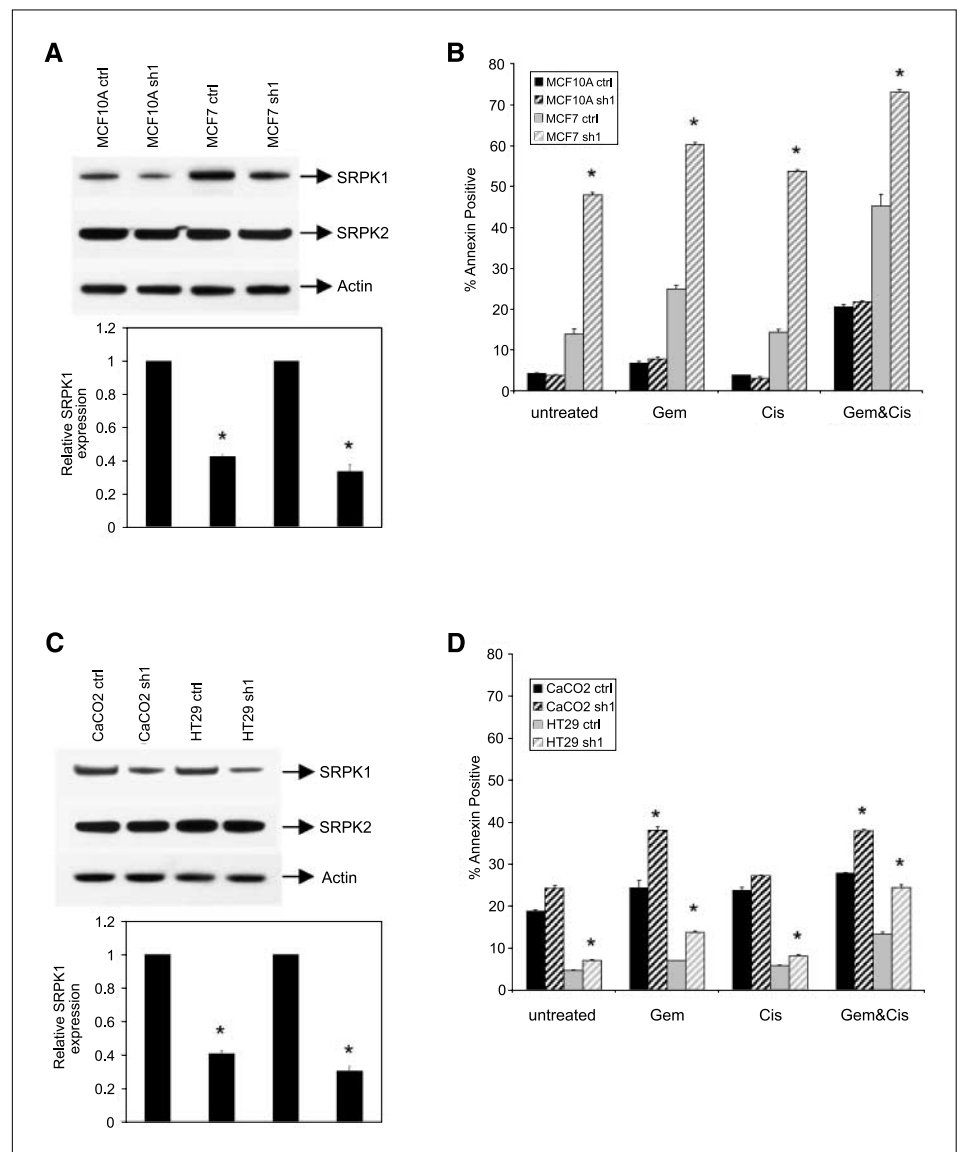
significantly higher levels of apoptosis when compared with MCF7 cells transfected with the control plasmid. Sensitivity of the non-transformed MCF10A cells to these agents remained unaffected by SRPK1 knockdown.

Parallel experiments were done on CaCO2 and HT29 cells to determine whether SRPK1 knockdown would also mediate an increase in apoptosis in colonic tumor cells. Electroporation of the sh1-SRPK1 construct decreased levels of SRPK1 protein in both cell lines, as determined by immunoblotting and densitometry, but had no effect on SRPK2 expression (Fig. 2C). Cells were subsequently incubated with Annexin V to determine the apoptotic proportion, and although the effect of reduced SRPK1 expression in these two cell lines was less dramatic than that observed in breast tumor cells, a significant increase in the proportion of HT29 cells undergoing apoptosis following this treatment was shown. Treatment of colonic tumor cells with gemcitabine or the combination of gemcitabine and cisplatin resulted in significant increases in apoptosis in both CaCO2 and HT29 tumor cell lines following SRPK1 reduction. These results show that targeting

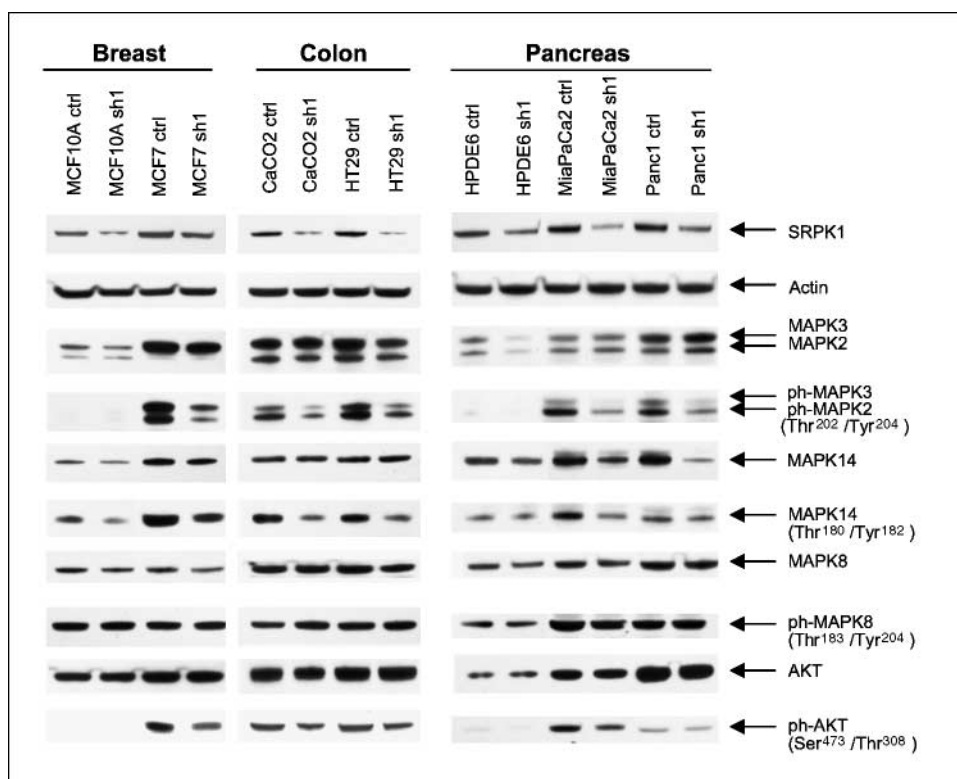
SRPK1 in both breast and colonic tumor cells can increase apoptosis and enhance cell death in response to chemotherapy.

**SRPK1 regulates the AKT and MAPK networks.** Excessive activation of MAPK and AKT signaling cascades has been well described for a broad range of malignancies (21, 22), including tumors of the breast and colon (23, 24). These pathways have also been shown to be relevant to the effect of chemotherapy (25). Recent advances have shown the capacity of these signaling cascades to regulate the activation and localization of splice factors resulting in changes in pre-mRNA processing (15, 26, 27). These studies have implicated critical interactions between the splice regulatory machinery and MAPK and AKT signaling networks. Thus, we postulated that a decrease in SRPK1 expression may affect apoptotic potential by altering signals transmitted by these networks. Both total protein expression and phosphorylation status of several well-characterized MAPK proteins and AKT were compared using Western blot analysis in the breast and colonic cell lines treated with either the siRNA-encoding sh1-SRPK1 construct or the control plasmid (Fig. 3). Each lysate (15  $\mu$ g) was

**Figure 2.** Reduced expression of SRPK1 in breast and colonic tumor cell lines increases apoptosis and response to gemcitabine and/or cisplatin. Breast and colonic cells were transfected with 10  $\mu$ g of either the siRNA-encoding sh1-SRPK1 plasmid or a plasmid encoding a scrambled version of the sequence as control, and Western blot analysis (A and C) was done to determine protein levels. Densitometry was done on blots from multiple experiments, and quantities of SRPK1 relative to control transfectants were graphed. B and D, the effect of SRPK1 expression on apoptosis after chemotherapy was assayed by treating transfected cells with 0.1  $\mu$ mol/L gemcitabine (*Gem*) and/or 10  $\mu$ mol/L cisplatin (*Cis*) before staining with Annexin V and propidium iodide and analysis by flow cytometry. \*,  $P < 0.01$ ;  $n = 3$ . Bars, SD.







**Figure 3.** SRPK1 expression regulates signaling through the MAPK and AKT pathways in breast, colonic, and pancreatic tumor cells. Western blot analysis was done on lysates derived from the indicated panel of cell lines transfected with 10  $\mu$ g of either the siRNA-encoding sh1-SRPK1 plasmid or a plasmid encoding a scrambled version of the sequence as control. MiaPaCa2 and Panc1 are pancreatic tumor cell lines and HPDE6 is a well-differentiated nontransformed pancreatic ductal epithelial cell line. Antibodies were used to detect total protein levels or phosphorylated versions of the indicated proteins (*ph*-).

loaded in each lane, and levels of each relevant protein were determined by Western blotting and densitometry. Expected changes in the levels of SRPK1 protein were confirmed using an antibody specific for this protein. Quantities of total MAPK3 and MAPK1 proteins remained stable following SRPK1 knockdown in each of the breast and colonic cell lines studied; however, the phosphorylated active forms of these proteins were reduced in the tumor cells in response to SRPK1 reduction. Phosphorylation of MAPK3 and MAPK1 in the nontransformed control MCF10A cells was barely detectable and remained unchanged after SRPK1 knockdown. Expression of MAPK14 (p38) decreased slightly or remained unaltered in the breast and colonic cell lines, respectively, after SRPK1 knockdown, whereas levels of phosphorylated MAPK14 were clearly reduced in all cells in response to reduced SRPK1. The quantity of total AKT protein (AKT isoforms 1, 2, and 3) also remained unaffected by knockdown of SRPK1 in these lines, whereas phosphorylated AKT was slightly reduced in the breast and colonic tumor lines in response to reduced levels of SRPK1. Similar to what was observed for phosphorylated MAPK3 and MAPK1, the levels of phosphorylated AKT protein in the nontransformed MCF10A breast cells were virtually undetectable compared with MCF7 breast tumor cells. This was consistent with earlier reports of increased activation of these proteins in tumors. Expression and phosphorylation status of MAPK8 (c-Jun NH<sub>2</sub>-terminal kinase) remained constant in each of the cell lines studied under these conditions, providing support that decreases in MAPK3, MAPK1, and AKT activation following SRPK1 knockdown were specific and were not due solely to an increase in the number of cells undergoing apoptosis.

Analogous experiments were done using lysates derived from control-transfected and sh1-SRPK1-transfected pancreatic cell lines that we have previously reported to respond similarly to the targeted disruption of SRPK1 expression (Fig. 3). The non-

transformed HPDE6 cell line represents a near-normal control (16, 17), whereas MiaPaCa2 and Panc1 cells are two commonly studied pancreatic tumor cell lines. Diminished levels of phosphorylated MAPK3, MAPK1, and MAPK14 were exhibited by pancreatic tumor cell lines following siRNA targeting of SRPK1, whereas levels of total and phosphorylated MAPK8 remained unchanged. Reduced amounts of phosphorylated AKT were also reproducibly observed in pancreatic tumor cell lines following SRPK1 reduction, although this reduction was quantitatively small. Relatively modest phosphorylated MAPK3, MAPK2, or AKT was detected in nontransformed HPDE6 cells regardless of level of SRPK1 expression, supporting the inappropriate activation of these proteins during pancreatic tumorigenesis. The observed reduction in total p38 levels in all of the pancreatic cell lines following SRPK1 knockdown may be responsible for the observed reduction in phosphorylated MAPK14. Collectively, these results implicate SRPK1 in regulating the expression or activation of multiple proteins in the MAPK and AKT networks, and because signaling through these cascades has been shown to enhance proliferation and survival of many types of tumors, targeting SRPK1 to inhibit these signals is believed to contribute to the proapoptotic effects of this treatment strategy.

**SRPK1 reduction alters splicing of MAP2K2.** Due to its role as a central regulator of the splicing process, down-regulation of SRPK1 is expected to have a widespread effect on the splicing and expression of a diverse set of genes. To elucidate the effect of decreased SRPK1 levels on splicing in breast and colonic cell lines, Western blots were done using an antibody specific to a phosphorylated epitope found in multiple serine-arginine protein splice factors (Fig. 4A; ref. 18). Disruption of SRPK1 expression in each of the cell lines resulted in the anticipated reduction in the phosphorylation status of multiple serine-arginine proteins, the conventional targets of this protein kinase. Reduced phosphorylation

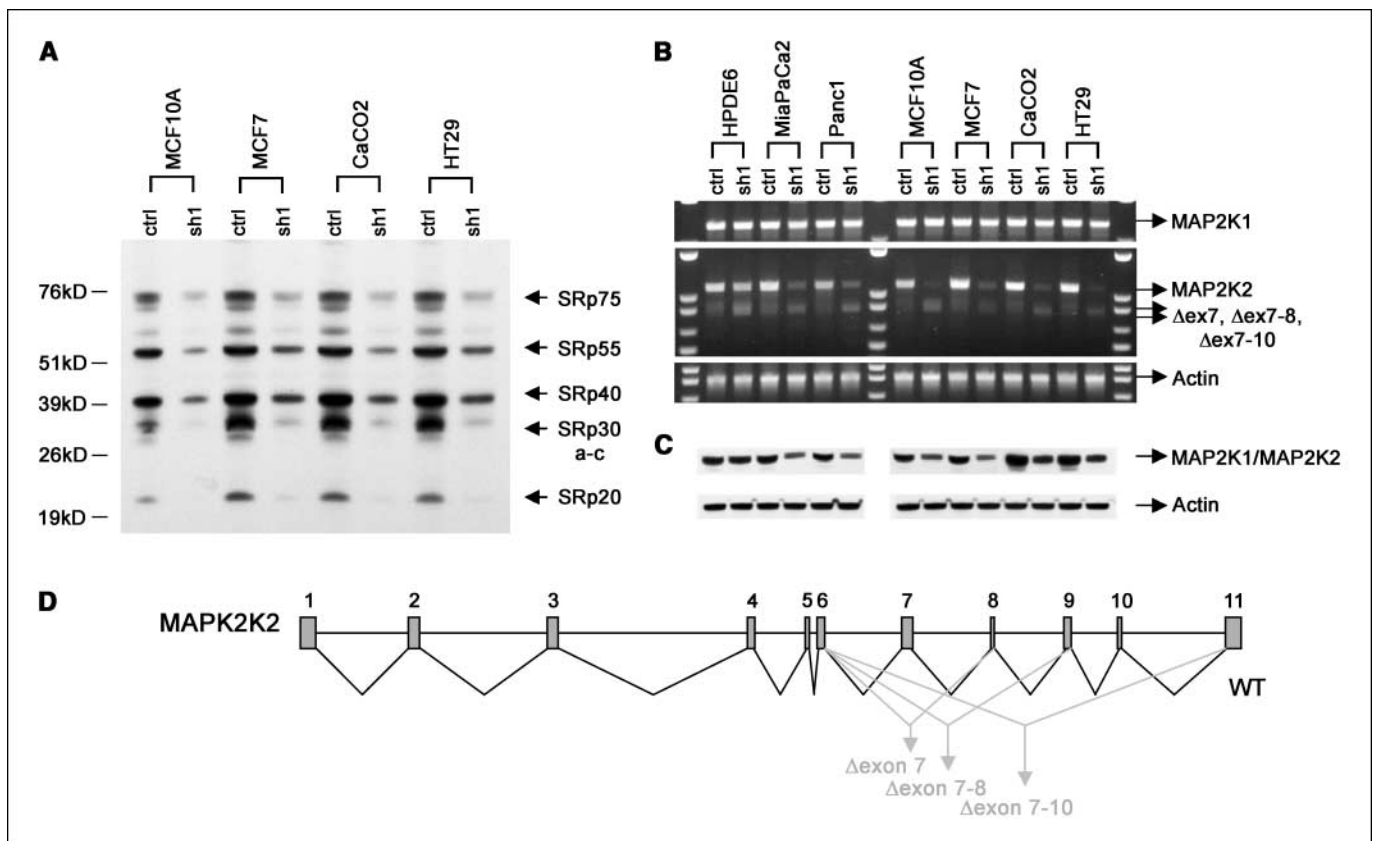
of the splice factors SRp20, 30a-c, 40, 55, and 75 following treatment was clearly shown by this method. This outcome is consistent with what we reported earlier in pancreatic tumor cell lines and suggests that, although multiple kinases have been described that are able to phosphorylate the serine-arginine family of proteins, the targeted reduction in SRPK1 activity is not fully compensated for by those other kinases.

Based on our observation that reduced SRPK1 expression in breast, colonic, and pancreatic tumor cells led to diminished activation of MAPK3 and MAPK1, we used RT-PCR to evaluate the effect of SRPK1 inhibition on the processing of the transcripts encoding two kinases responsible for their phosphorylation: MAP2K1 and MAP2K2 (Fig. 4B). SRPK1 knockdown in each of the tumor cell lines resulted in a dramatic shift in the mRNA processing of MAP2K2 transcripts that was less overt in the nontransformed and near-normal HPDE6 cell line. Sequencing of MAP2K2 PCR products revealed transcripts exhibiting deletions of exon 7, exons 7 and 8, or exons 7 to 10 (Fig. 4D) occurring in cells following inhibition of SRPK1. These previously unidentified MAP2K2 mRNAs are predicted both to result in either an in-frame coding deletion ( $\Delta$  exons 7–8) or loss-of-frame mutations ( $\Delta$  exon 7 and  $\Delta$  exons 7–10) during translation of the transcripts and to produce novel MAP2K2 isoforms whose functions remain to be determined. No significant alterations in MAP2K1 splice patterns

or expression levels were observed. Western blot analysis confirmed the reduction in MAP2K1/2 proteins following inhibition of SRPK1 (Fig. 4C). It is postulated that the change in splicing fidelity elicited by SRPK1 down-regulation in tumor cells leads to production of aberrant transcripts or protein products that may inhibit the expression or availability of these proteins to phosphorylate their intended targets, MAPK3 and MAPK1. Future experiments are planned to investigate further the specific roles these splicing alterations have on the activation and expression of MAP2K1/2 proteins.

## Discussion

SRPK1-encoding transcripts are ubiquitously expressed in many human tissues (28, 29); however, the precise cellular localization of this protein within breast and colon has not been described. Immunohistochemical analysis using a SRPK1-specific antibody showed that this protein is expressed almost exclusively within epithelial cells lining the ducts of normal breast and within epithelial cells along the colon. Importantly, elevated levels of SRPK1 were detected in breast and colonic tumors, where the quantity of SRPK1 seems to increase coordinately with increasing grade of malignancy. Substantial expression of this protein was also found across a panel of breast and colonic tumor cell lines. *In vitro*



**Figure 4.** Decreased SRPK1 expression in breast and colonic tumor cells affects splice factor activation, resulting in altered splicing of MAP2K2 transcripts. Western blot analysis (A) was done on lysates derived from breast and colonic cell lines transfected with 15  $\mu$ g of either the siRNA-encoding sh1-SRPK1 plasmid or a plasmid encoding a scrambled version of the sequence as control. Serine-arginine protein phosphorylation was detected using the mAb 104 that recognizes a phosphorylated serine-arginine epitope common to multiple activated splice factors (18). RT-PCR was done (B) using primers specific for MAP2K1 or MAP2K2, and cDNAs were derived from the control-transfected and sh1-SRPK1-transfected cell lines and resolved by agarose gel electrophoresis. C, total quantities of MAP2K1/2 and actin proteins were confirmed by Western blot analysis. D, genomic organization of MAP2K2 and the novel splice events identified using RT-PCR analysis are diagrammed.

down-regulation of SRPK1 using siRNA increased the proportion of breast and colonic tumor cells, but not nontransformed cells, undergoing apoptosis in addition to increasing their sensitivity to killing by two commonly used chemotherapeutic agents: gemcitabine and cisplatin. These results suggest that drugs inhibiting SRPK1 activity may be effective as stand-alone agents or in combination with conventional chemotherapeutic regimens.

We have further shown that decreased expression of SRPK1 inhibited signaling through MAPK3 and MAPK1 and that this may be due to the observed alterations in splicing of MAP2K2 transcripts that encode one of the two major kinases responsible for phosphorylation of MAPK3 and MAPK1 proteins. Reduction in SRPK1 protein levels was also found to slightly reduce signaling through AKT phosphorylation in the tumor cells, which could result in a loss of proliferation and survival signals normally induced by this cascade. The ability of SRPK1 to regulate multiple signaling pathways that are often exploited by tumor cells to enhance proliferation and survival points toward an opportunity to develop novel anticancer therapeutics targeting this protein.

The preponderance of missplicing mRNA in cancer cells has been well documented for a large number of malignancies; however, the changes in the splicing machinery that are responsible for this remain poorly defined. Although elevated levels of specific serine-arginine proteins have been correlated with the presence of alternatively spliced transcripts in breast (12) and lung tumors (30), the overlapping activity of splice factors and their restricted tissue distribution suggests that there may be a more central splicing protein that is responsible for the prevalence of altered splicing in tumors. Recently, overexpression of SRPK1 has been shown in both acute T cell (31) and chronic myelogenous leukemia (32), supporting an important role for SRPK1 in altered mRNA processing in leukemia. Our demonstration that SRPK1 is overexpressed in breast, colonic, and pancreatic tumors supports a contributory role for this protein in generating aberrant splice patterns across a broad and diverse group of malignancies.

Therapies targeting SRPK1 for inhibition would be anticipated to have a broad effect on the splicing and subsequent expression of a multitude of genes owing to the role of this kinase as a central splicing regulator. We have focused attention on the MAPK and AKT signaling pathways because activation of both has been implicated in the progression of a broad spectrum of human cancers (21, 22) as well as in mediating changes in the splice regulatory network (15, 26, 27). The MAPK cascade responds to mitogenic signals mediated by cell surface growth factor receptors, resulting in activation of the proto-oncogenes Ras and Raf. Signals through this hierarchical kinase pathway culminate in the phosphorylation of MAPK3 and MAPK1 that in turn activate a variety of transcription factors important to the expression of proteins necessary for cell proliferation and survival. It is remarkable that the MAPK3 and MAPK1 signaling cascade has been proven to be activated in most human tumors, where it has been shown not only to drive proliferation of tumor cells but also to mediate resistance to a variety of chemotherapeutic agents both *in vitro* and *in vivo*. Based on these epidemiologic and molecular findings, the MAPK network represents an area of intense interest in the development of anticancer drugs. Our results show that reduced SRPK1 expression in tumor cells modifies the splicing of transcripts encoding MAP2K2 to favor production of alternative mRNAs that do not produce wild-type proteins. The effect of decreased SRPK1 expression on MAP2K2 is likely responsible, at least in part, for the observed decrease in phosphorylation of

MAPK3 and MAPK1 and the antitumor effects observed. Prior reports have already established that inhibition of phosphorylation of MAPK3 and MAPK1 can reduce proliferation as well as increase apoptosis of tumor cells (33, 34). These studies lend support to our observations that the decrease in activation of these kinases may play a role in the antitumor effect of targeting SRPK1.

Two additional signaling pathways in the global MAPK network are the stress-activated protein kinase (MAPK8) and the MAPK14 (p38) kinase pathways that respond to a variety of cellular stress signals, including proinflammatory cytokines and environmental stress, leading to growth inhibition and apoptosis. Activation of the MAPK14 signaling cascade has been frequently reported to be an important regulator of apoptosis and may affect the sensitivity of tumor cells to various cytotoxic agents, including cisplatin and 5-fluorouracil (35). In the present study, a reduction in total MAPK14 expression and phosphorylation was observed in tumor cells with diminished SRPK1 levels, yet the percentage of cells able to undergo apoptosis was enhanced. This suggests that, although the quantity of phosphorylated MAPK14 is reduced, there may be sufficient activity remaining to induce apoptosis either alone or in combination with another proapoptotic signaling component. It is further inferred that the ability of the MAPK8 cascade to remain unaffected and provide stress-induced proapoptotic signals may be sufficient to increase the apoptotic potential caused by SRPK1 down-regulation in tumor cells.

The AKT cascade, another serine/threonine kinase signaling pathway, has also garnered much interest by cancer researchers, as signals transduced through this pathway may promote proliferation, growth, and survival (22). The siRNA-mediated reduction in SRPK1 protein levels resulted in a modest reduction in AKT phosphorylation that is believed to result in loss of prosurvival signals that, when coupled with loss of growth and survival signaling by MAPK3 and MAPK1, may act coordinately to facilitate tumor cell death. It is postulated that the increase in apoptotic potential of tumor cells with diminished SRPK1 expression may be due to an overall shift in these and possibly other yet unidentified signaling pathways to favor cell cycle arrest and apoptosis over growth. Although the antibodies used in this study detect all three AKT isoforms (1, 2, and 3), future investigation will need to discern whether SRPK1 reduction preferentially reduces phosphorylation of specific AKT isoforms because each has been shown to have unique effect on various tumor pathways (36–38). Together, the MAPK and AKT pathways offer great potential for cancer therapeutics to amend the altered signaling pathways exploited by tumor cells for growth and for resistance to current chemotherapeutic regimens. A variety of inhibitors targeting signaling through the MAPK pathway are currently being evaluated in clinical trials. Specific farnesyltransferase inhibitors designed to inhibit Ras have unfortunately been disappointing in early clinical trials, owing to the observation that higher concentrations of these inhibitors are required to inhibit oncogenic KRAS compared with wild-type protein (39). Inhibitors targeting MAP2K1 and MAP2K2 and those inhibiting Raf may offer more promise. We postulate that the development of inhibitors targeting SRPK1 will offer a means to inhibit the expression and/or activation of multiple signaling proteins that are overexpressed during tumorigenesis. It is also promising that targeting SRPK1 expression may prove therapeutically effective for a diverse range of malignancies exhibiting elevated expression of this protein.

The effect of SRPK1 on chemotherapeutic potential remains an area of controversy because expression of SRPK1 has been coupled

to both resistance and susceptibility of tumor cells to platinum-containing agents. Schenk et al. have shown an association between the resistance of testicular germ cell tumors and A2780 ovarian tumor cells to cisplatin and diminished SRPK1 expression (40, 41). In contrast, Plasencia et al. have shown that SRPK1 overexpression is associated with resistance of HT29 cells to oxaliplatin, and we have previously described the targeting of this kinase as a means of sensitizing pancreatic tumor cell lines to both gemcitabine and cisplatin (1, 42). These disparate effects of SRPK1 expression on tumor cell sensitivity to cytotoxic agents may be due to differential gene expression patterns exhibited by such cells. For example, it has already been shown that the primary substrate for SRPK1 in testicular germ cells is protamine 1, a protein required for chromatin condensation during spermatogenesis (28), whereas serine-arginine proteins are generally considered the predominant substrates for SRPK1 phosphorylation outside of the testicular compartment (29). Examination of the influence that SRPK1 expression may have on the splicing of specific genes or the activation of specific protein substrates differentially expressed in divergent tumor cell lines is ongoing and is expected to provide insights into the mechanisms responsible for chemotherapeutic sensitivity and resistance.

Whether alternative splicing is causative or a result of tumor formation remains an area of intense debate; however, the ability of aberrant splice products to affect the balance between cell

proliferation, differentiation, and apoptosis has emerged as a major facet in the tumorigenic process. It is intriguing that extensive alternative splicing is prevalent to both testicular germ cells and tumors that express a collection of proteins referred to as cancer/testes antigens (43–45). Previous reports have shown that SRPK1 transcripts are found ubiquitously across human tissues, but levels are highest in testicular germ cells (28, 29). The presence of high levels of SRPK1 in testes and its overexpression in tumors suggest that this protein follows a pattern of inappropriate regulation during tumorigenesis that could be similar to other cancer/testes antigens. It is further postulated that the high quantity of SRPK1 protein present in both germ cells and tumors is responsible for the extensive alternative splicing patterns prevalent to both types of cells, thus implicating this protein as a critical regulator of aberrant splicing in human cancers.

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## References

- Hayes GM, Carrigan PE, Beck AM, Miller LJ. Targeting the RNA splicing machinery as a novel treatment strategy for pancreatic carcinoma. *Cancer Res* 2006;66:3819–27.
- Mercatante DR, Bortner CD, Cidowski JA, Kole R. Modification of alternative splicing of Bcl-x pre-mRNA in prostate and breast cancer cells. Analysis of apoptosis and cell death. *J Biol Chem* 2001;276:16411–7.
- Lu F, Gladden AB, Diehl JA. An alternatively spliced cyclin D1 isoform, cyclin D1b, is a nuclear oncogene. *Cancer Res* 2003;63:7056–61.
- Gunthert U, Hofmann M, Rudy W, et al. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 1991;65:13–24.
- Rudy W, Hofmann M, Schwartz-Albiez R, et al. The two major CD44 proteins expressed on a metastatic rat tumor cell line are derived from different splice variants: each one individually suffices to confer metastatic behavior. *Cancer Res* 1993;53:1262–8.
- Zhu X, Daffada AA, Chan CM, Dowsett M. Identification of an exon 3 deletion splice variant androgen receptor mRNA in human breast cancer. *Int J Cancer* 1997;72:574–80.
- Gallacchi P, Schoumacher F, Eppenberger-Castori S, et al. Increased expression of estrogen-receptor exon-5-deletion variant in relapse tissues of human breast cancer. *Int J Cancer* 1998;79:44–8.
- Koduri S, Goldhar AS, Vonderhaar BK. Activation of vascular endothelial growth factor (VEGF) by the ER- $\alpha$  variant, ER $\Delta$ 3. *Breast Cancer Res Treat* 2006;95:37–43.
- Jordan P, Brazao R, Boavida MG, Gespach C, Chastre E. Cloning of a novel human Rac1b splice variant with increased expression in colorectal tumors. *Oncogene* 1999;18:6835–9.
- Ryan B, O'Donovan N, Browne B, et al. Expression of survivin and its splice variants survivin-2B and survivin- $\Delta$ Ex3 in breast cancer. *Br J Cancer* 2005;92:120–4.
- Suga K, Yamamoto T, Yamada Y, et al. Correlation between transcriptional expression of survivin isoforms and clinicopathological findings in human colorectal carcinomas. *Oncol Rep* 2005;13:891–7.
- Stickeler E, Kittrell F, Medina D, Berget SM. Stage-specific changes in SR splicing factors and alternative splicing in mammary tumorigenesis. *Oncogene* 1999;18:3574–82.
- Bourgeois CF, Lejeune F, Stevenin J. Broad specificity of SR (serine-arginine) proteins in the regulation of alternative splicing of pre-messenger RNA. *Prog Nucleic Acid Res Mol Biol* 2004;78:37–88.
- Matter N, Herrlich P, König H. Signal-dependent regulation of splicing via phosphorylation of Sam68. *Nature* 2002;420:691–5.
- Blaustein M, Pelisch F, Tanos T, et al. Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT. *Nat Struct Mol Biol* 2005;12:1037–44.
- Furukawa T, Duguid WP, Rosenberg L, Viallet J, Galloway DA, Tsao MS. Long-term culture and immortalization of epithelial cells from normal adult human pancreatic ducts transfected by the E6E7 gene of human papilloma virus 16. *Am J Pathol* 1996;148:1763–70.
- 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.
- Zahler AM, Lane WS, Stolk JA, Roth MB. SR proteins: a conserved family of pre-mRNA splicing factors. *Genes Dev* 1992;6:837–47.
- Moore A, Donahue CJ, Bauer KD, Mather JP. Simultaneous measurement of cell cycle and apoptotic cell death. *Methods Cell Biol* 1998;57:265–78.
- van England M, Nieland LJW, Ramaekers FCS, Schutte B, Reutelingsperger CPM. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 1998;31:1–9.
- Hoshino R, Chatani Y, Yamori T, et al. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* 1999;18:813–22.
- Yoeli-Lerner M, Tokar A. Akt/PKB signaling in cancer: a function in cell motility and invasion. *Cell Cycle* 2006;5:603–5.
- Sivaraman VS, Wang H, Nuovo GJ, Malbon CC. Hyperepression of mitogen-activated protein kinase in human breast cancer. *J Clin Invest* 1997;99:1478–83.
- Fang JY, Richardson BC. The MAPK signalling pathways and colorectal cancer. *Lancet Oncol* 2005;6:322–7.
- Sebolt-Leopold JS, Herrera R. Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat Rev Cancer* 2004;4:937–47.
- Pelisch F, Blaustein M, Kornbliht AR, Srebrow A. Cross-talk between signaling pathways regulates alternative splicing: a novel role for JNK. *J Biol Chem* 2005;280:25461–9.
- van der Houven van Oordt W, Diaz-Meco MT, Lozano J, Krainer AR, Moscat J, Caceres JF. The MKK3/6-p38-signaling cascade alters the subcellular distribution of hnRNP A1 and modulates alternative splicing regulation. *J Cell Biol* 2000;149:307–16.
- Papoutsopoulou S, Nikolakaki E, Chalepakis G, Kruff V, Chevallier P, Giannakouros T. SR protein-specific kinase 1 is highly expressed in testis and phosphorylates protamine 1. *Nucleic Acids Res* 1999;27:2972–80.
- Wang HY, Lin W, Dyck JA, et al. SRPK2: a differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. *J Cell Biol* 1998;140:737–50.
- Zerbe LK, Pino I, Pio R, et al. Relative amounts of antagonistic splicing factors, hnRNP A1 and ASF/SF2, change during neoplastic lung growth: implications for pre-mRNA processing. *Mol Carcinog* 2004;41:187–96.
- Hishiyama M, Imada K, Sakai T, Ueda M, Hori T, Uchiyama T. Serological identification of adult T-cell leukaemia-associated antigens. *Br J Haematol* 2005;130:382–90.
- Saleses S, Dylla SJ, Verfaillie CM. p210BCR/ABL-induced alteration of pre-mRNA splicing in primary human CD34<sup>+</sup> hematopoietic progenitor cells. *Leukemia* 2004;18:727–33.
- Ozaki K, Minoda A, Kishikawa F, Kohno M. Blockade of the ERK pathway markedly sensitizes tumor cells to HDAC inhibitor-induced cell death. *Biochem Biophys Res Commun* 2006;339:1171–7.
- Rice PL, Beard KS, Driggers LJ, Ahnen DJ. Inhibition of extracellular-signal regulated kinases 1/2 is required for apoptosis of human colon cancer cells *in vitro* by sulindac metabolites. *Cancer Res* 2004;64:8148–51.
- Olson JM, Hallahan AR. p38 MAP kinase: a convergence point in cancer therapy. *Trends Mol Med* 2004;10:125–9.



36. Zhou GL, Tucker DF, Bae SS, Bhatheja K, Birnbaum MJ, Field J. Opposing roles for Akt1 and Akt2 in Rac/Pak signaling and cell migration. *J Biol Chem* 2006;281:36443-53.
37. Meng Q, Xia C, Fang J, Rojanasakul Y, Jiang BH. Role of PI3K and AKT specific isoforms in ovarian cancer cell migration, invasion, and proliferation through the p70S6K1 pathway. *Cell Signal* 2006;18:2262-71.
38. Heron-Milhavet L, Franckhauser C, Rana V, et al. Only Akt1 is required for proliferation, while Akt2 Promotes cell cycle exit through p21 binding. *Mol Cell Biol* 2006;22:8267-80.
39. End DW, Smets G, Todd AV, et al. Characterization of the antitumor effects of the selective farnesyl protein transferase inhibitor R115777 *in vivo* and *in vitro*. *Cancer Res* 2001;61:131-7.
40. Schenk PW, Boersma AW, Brandsma JA, et al. SKY1 is involved in cisplatin-induced cell kill in *Saccharomyces cerevisiae*, and inactivation of its human homologue, SRPK1, induces cisplatin resistance in a human ovarian carcinoma cell line. *Cancer Res* 2001;61:6982-6.
41. Schenk PW, Stoop H, Bokemeyer C, et al. Resistance to platinum-containing chemotherapy in testicular germ cell tumors is associated with downregulation of the protein kinase SRPK1. *Neoplasia* 2004;6:297-301.
42. Plasencia C, Martinez-Balibrea E, Martinez-Cardus A, Quinn DI, Abad A, Neamati N. Expression analysis of genes involved in oxaliplatin response and development of oxaliplatin-resistant HT29 colon cancer cells. *Int J Oncol* 2006;29:225-35.
43. Venables JP. Alternative splicing in the testes. *Curr Opin Genet Dev* 2002;12:615-9.
44. Venables JP. Aberrant and alternative splicing in cancer. *Cancer Res* 2004;64:7647-54.
45. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis, and cancer. *Nat Rev Cancer* 2005;5:615-25.