S100A4 Contributes to the Suppression of BNIP3 Expression, Chemoresistance, and Inhibition of Apoptosis in Pancreatic Cancer

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Abstract
Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease that is characterized by a particularly marked resistance to chemotherapy. We previously showed an association between decreased expression of BNIP3 and chemoresistance in PDAC cell lines. To further explore the molecular basis of chemoresistance in PDAC, we analyzed microarray data obtained from normal pancreas and PDAC tumor samples to identify genes exhibiting a negative correlation with the expression profile of BNIP3. This analysis identified several S100 family proteins, of which two, S100A2 and S100A4, showed in vitro the ability to repress exogenous BNIP3 promoter activity. We subsequently showed that RNA interference–mediated S100A4 knockdown resulted in an elevated expression of BNIP3 in PDAC cell lines that possess an unmethylated BNIP3 promoter, suggesting that, in addition to hypermethylation, S100A4 overexpression may represent an alternative mechanism for inhibiting BNIP3 function in PDAC. S100A4 knockdown also resulted in an increased sensitivity of PDAC cell lines to gemcitabine treatment, which was coupled with an increase in apoptosis and cell cycle arrest. To investigate the underlying mechanisms mediating these effects, we studied the effect of silencing the expression of S100A4 on the induction of apoptosis, cell cycle arrest, and the activation of apoptotic mediators. Knockdown of S100A4 clearly induced apoptosis with increased fragmentation of DNA and phosphatidylinositol serine externalization; activation of caspase-3, caspase-9, and poly(ADP-ribose) polymerase; and release of cytochrome c into the cytosol. These findings provide evidence that supports a novel role for S100A4 as a prosurvival factor in pancreatic cancer. [Cancer Res 2007;67(14):6786–95]

Introduction
Pancreatic ductal adenocarcinoma (PDAC) represents the eighth most common cause of cancer-related death worldwide (1), with a 5-year survival rate of between 1% to 5%, and an overall median survival of <6 months (2). This dismal prognosis is due to a combination of late diagnosis, broad resistance to conventional therapies, and rapid tumor spread.

Deregulation of apoptotic signaling pathways in PDAC contributes to both intrinsic and acquired chemoresistance in PDAC; indeed, deregulated expression of the Bcl-2 family of apoptosis-regulating proteins, such as Bcl-2, Bax, and Bak, is a common feature of PDAC (3).

BNIP3 is a proapoptotic member of the Bcl-2 family that has been shown to induce a necrotic-like cell death through the opening of the mitochondrial permeability transition pore without the release of cytochrome c or caspase activation (4). Interestingly, in colorectal, pancreatic, and hematologic cancers, BNIP3 expression is down-regulated, and this down-regulation has been associated with a chemoresistant phenotype (5–8). Furthermore, BNIP3 can be induced under hypoxic conditions in a HIF-1–dependent manner and has been shown to be involved in the hypoxia-induced cell death of carcinoma, endothelial, and cardiomyocyte cell lines (9–11).

We have previously reported an association between the loss of BNIP3 expression and chemoresistance in PDAC cell lines (8); therefore, we wished to investigate the mechanism(s) by which PDAC cells achieve suppression of BNIP3 expression. To address this question, we used a bioinformatic approach to identify putative negative regulators of BNIP3 expression by analyzing microarray data obtained from normal pancreas and pancreatic cancer specimens to determine genes that exhibit an inversely correlating expression profile with respect to BNIP3. This analysis identified a number of S100 family proteins, of which S100A4 and S100A2 were shown to have the ability to repress BNIP3 promoter-mediated reporter gene expression. We subsequently confirmed this analysis by demonstrating a predominantly negative correlation between BNIP3 and S100A4 expression levels in a panel of 11 PDAC cell lines. Furthermore, knockdown of S100A4 was shown to result in an elevated expression of BNIP3 in PDAC cell lines in which the BNIP3 promoter is unmethylated, whereas no effect was seen in cell lines with a hypermethylated BNIP3 promoter. We subsequently showed that knockdown of S100A4 resulted in an increase in the sensitivity of PDAC cell lines to gemcitabine as well as an increase in the number of apoptotic cells. Interestingly, we found that knockdown of S100A4 alone stimulated apoptosis through the activation of proapoptotic signaling proteins, including caspase-3, caspase-9, and poly(ADP-ribose) polymerase, as well as release of cytochrome c from mitochondria. Additionally, the combined effect of S100A4 knockdown and treatment with gemcitabine resulted in a clear augmentation in the activation of the above apoptotic mediator proteins compared with control cells.

This study provides evidence that suggests that S100A4 contributes to the suppression of BNIP3 expression, chemoresistance,
and inhibition of apoptosis in PDAC cells. This represents a novel function for S100A4 in cancer and suggests that, in addition to the established role of S100A4 in the promotion of metastasis, it may also function as a prosurvival factor in pancreatic cancer.

Materials and Methods

**Tissues and cell lines.** All of the human PDAC lines used in this study were obtained from Cancer Research UK Cell Services (Middlesex, United Kingdom) and grown in E4 medium supplemented with 10% heat-inactivated FCS, penicillin (0.1 μg/mL), and streptomycin (100 units/mL). Cells were maintained at 37°C in a 5% CO₂/95% air incubator. The human pancreatic ductal epithelial cell line (HPDE) was a kind gift from Dr. Ming-Sound Tsao (University of Toronto, Toronto, ON, Canada) and was grown in keratinocyte medium as previously described. 293 Phoenix cells were obtained from Dr. S. Basu (Centre for Molecular Oncology Unit, Institute of Cancer and the Cancer Research UK Clinical Centre, London, United Kingdom) and were grown in complete E4 medium.

Paraffin-embedded pancreatic tissues were obtained from the Human Biomaterials Resource Centre, Charing Cross Hospital, London, United Kingdom with full ethical approval of the host institution.

**In situ RNA hybridization and immunohistochemical analysis.** The procedure used for the *in situ* hybridization and immunohistochemical analysis was done as previously described (12). The primers used for the preparation of the BNP3 riboprobes were as follows: forward 5'-GCTCTCGGTTGAGCAGCTGC-3' and reverse 5'-GGCTGGCTTTTTGAA-3'. The 234 bp amplification product was subcloned into the pCR4-TOPO vector (Invitrogen) for the synthesis of the antisense and sense riboprobe.

The immunohistochemical analysis was conducted using the anti-human BNP3 monoclonal antibody (clone Ana40, Sigma) at the final dilution of 1:50.

**Statistical analysis.** Five primary PDAC and four normal pancreas specimens were profiled using Affymetrix HGU133A GeneChips. Data were analyzed within the open source R statistical environment using bioconductor packages. Background correction was achieved through robust multiarray analysis and normalization by the quantiles method. The Pearson product-moment coefficient was used to estimate the extent of correlation between the expression patterns of the 22,283 probe sets present in the HG-U133A array data set with respect to BNP3. A cutoff —0.75 was used to select the negatively correlating genes.

**Plasmids.** TOPO-BNP3 was constructed by ligating the full-length BNP3 cDNA clone, obtained by EcoRI/XhoI restriction of IMAGE clone 4066092 (MRC Gene Service, Babraham, Cambridgeshire, United Kingdom), into the pTOPO2.1 plasmid (Invitrogen), by TA cloning. The BNP3-luciferase plasmid (a kind gift from Professor Xuming Zhang, University of Arkansas, Little Rock, AR) consists of 588 nucleotides of BNIP3 promoter inserted upstream of a minimal promoter fused to the firefly luciferase cDNA (kindly provided by Dr. Luis Del Peso, Universidad Autónoma de Madrid, Madrid, Spain).

pSUPER-A4 was generated by annealing the following oligonucleotides: 5'-GATCCCAAGGAGAAGATCGGATCCTGAAGACAGCTT-CATCCTGCTTTTTTTGGAA-3' and 5'-AGCTTCTTCGAAAAAAGGACAGA-TGAAGCTGCTTCTTGAAAGCAGCTTCATCTGTCCTTGGG-3' and ligating the resulting duplex into the pBII/HindIII sites of pSUPER-RETRO-puro (Oligoengine). Clones were screen by restriction analysis, followed by conformational sequencing.

**Reverse transcription-PCR.** Reverse transcription-PCR (RT-PCR) was done as previously described (13). The primers used for amplifying the S100A4 transcript were as follows: forward 5’-ATCCTGGCTCTCGGAA-GAA-3’ and reverse 5’-TCATTCTCTCTCGGCTCT-3’, producing a 320 bp product. The β-actin gene primers were from Invitrogen and were used as an internal control to normalize the amount of mRNA loaded in all RT-PCR reactions.

**Immunoblot analysis.** Cells were lysed on ice in prechilled lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 0.1% SDS, 20 mmol/L sodium fluoride, 2 mmol/L sodium orthovanadate, 20 mmol/L sodium PPI] containing a cocktail of protease inhibitors. Cell extracts containing 40 μg of total protein were fractionated by electrophoresis on 4% to 12% SDS-PAGE gradient gels (Invitrogen), transferred onto a polyvinylidene difluoride membrane (Millipore UK Limited), and subjected to immunoblot assay using antibodies against BNP3 (CloneAna40, Sigma-Aldrich), actin (C-11, Santa Cruz Biotechnology), Phospho(Ser32)-IκB-α (Cell Signalling Technology), cytochrome c (clone THB2C12, BD PharMingen), caspase-3 (clone 31A1067, Alexis Biochemicals), caspase-8 (clone T-16, Santa Cruz Biotechnology), caspase-9 (clone H-170, Santa Cruz Biotechnology), poly(ADP-ribose) polymerase (clone C20, BioMol International), and Ku70 (clone C-19, Santa Cruz Biotechnology). The blots shown are examples of typical observations representative of at least three independent experiments.

**Transfection assays.** Panc-1 cells were seeded onto 24-well plates at 4 × 10³ per well. The following day, the cells were transfected with plasmid DNAs using Fugene-6 as recommended in the manufacturer's protocol (Roche). After 8 h, the medium in each well was replaced, and the cells were incubated for a further 16 h. Cells were lysed, and the luciferase activities were determined by use of the Dual-Luciferase Reporter Assay System (Promega UK) as recommended in the manufacturer's protocol. Each experiment was done at least thrice and in triplicate.

**Generation of recombinant retroviruses and stable cell lines.** 293 Phoenix retroviral packaging cells were seeded in 10-cm dishes at 60% confluence followed by transfection with 5 μg of either pSUPER-RETRO-puro or pSUPER-A4 plasmid using the Fugene-6 transfection agent (Roche Diagnostics). Following an incubation period of 24 h, the cells were then selected with 2 μg/mL puromycin for a period of 7 to 10 days to increase the viral titer. Sixteen hours before the infection of target cell lines, the medium in each dish was replaced with fresh antibiotic-free medium. The target PDAC cell lines grown to 60% confluence in 10-cm dishes were infected with 5 μL of 0.45-μm-filtered retrovirus-containing medium containing 4 μg/mL polybrene (Sigma). The following day, the cells were selected with 0.8 to 2.0 μg/mL puromycin over a period of between 7 and 10 days.

**Cell viability and proliferation assays.** To quantify the effect of S100A4 silencing on the sensitivity of PDAC cell lines to gemcitabine, 2 × 10⁵ cells were plated in triplicate onto 96-well plates for 24 h before the addition of gemcitabine (Lilly Oncology) at different concentrations (10 ng/mL–1 μg/mL). Three days later, the number of viable cells in each well was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (8). The absorbance was read at 570 nm and experiments conducted at least thrice. The results were expressed as a percentage of the control where the absorbance value of the untreated cells was normalized to 100%. A similar approach was used to assess the effect of S100A4 silencing on the proliferation rate of PDAC cells in vitro. Forty-eight hours after transfection with the indicated small interfering RNA (siRNA) oligonucleotides, 5 × 10³ cells were plated in triplicate onto 96-well plates in complete medium. Then, over an 8-day period, the number of cells in each well was quantified every 2 days using the MTT assay described above. The

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experiments were repeated at least thrice and expressed as percentage of the control.

**RNA interference.** The siRNA oligonucleotides used in this study were purchased from Dharmacon and were used according to the manufacturer's instructions. Briefly, cells were seeded in six-well plates at a confluence of 30% and were subsequently transfected with either 50 mmol/L siRNA Smart pool Human S100A4 or 50 mmol/L nontargeting control pool siRNA in the presence of 2 μL of transfection reagent 2 (Dharmacon). Three days later, the cells were trypsinized, seeded in appropriate culture plates, and used for the cell growth inhibition assay or for investigating the apoptosis of tumor cells after treatment with gemcitabine.

**Cell death analysis by flow cytometry.** The propidium iodine and Annexin V double staining was done as previously described (12). To quantify the number of cells in sub-G1 phase, cells were trypsinized at the indicated time, washed in PBS, and fixed in cold 70% ethanol for at least 30 min on ice. After two washes with phosphate-citrate buffer, the cells were treated with 100 μL of RNase A (100 μg/μL) for 15 min and incubated with 200 μL of propidium iodide (50 μg/μL) in the dark. A total of $2 \times 10^5$ events were acquired on a FACSCalibur cytometer and analyzed using Cellquest software (BD Biosciences). Each experiment was done at least thrice.

**Mitochondrial fractionation.** Cells were collected, washed twice in PBS, and resuspended in chilled isotonic buffer [200 mmol/L sucrose, 10 mmol/L Tris-HCl (pH 7.8), 1 mmol/L MgCl$_2$] before lysing the cells on ice using a Dounce homogenizer (10 strokes at maximum speed). The cell lysates were then centrifuged at 4°C for 15 min at 5000 rpm to remove large debris, followed by a further centrifugation at 4°C for 30 min at 12,500 rpm to pellet the mitochondria. The supernatant (cytosolic fraction) was then removed and the mitochondria-containing pellet resuspended in radio-immunoprecipitation assay buffer (RIPA).

**Caspase activity assay.** Caspase-3/7 activity was determined using the CaspaseGlo 3/7 activity assay (Promega) according to the manufacturer's instructions. Briefly, cells were treated as indicated before resuspension in RIPA lysis buffer. One volume of luminescent caspase-3/7 Glo substrate buffer (CaspaseGlo 3/7 assay, Promega UK) was then added to aliquots of protein lysate containing 30 μg of protein in a 96-well plate followed by gentle agitation for 1 h. The caspase activity in each sample was quantified by luminometry and the data obtained were expressed as relative luminescence units. The data presented represent the mean of three independent experiments.

**Results**

**Suppression of BNIP3 expression in PDAC.** We investigated BNIP3 protein expression levels in 20 PDAC and 3 normal pancreas specimens by immunohistochemistry (Fig. 1A). As can be seen from the table in Fig. 1, and in agreement with previous reports (6, 14), both the incidence and intensity of BNIP3 expression were found to be significantly reduced in PDAC cells compared with the normal pancreas specimens, although a few cases (4 of 20) did exhibit clear expression of BNIP3. BNIP3 expression was readily detectable in both the acinar (Fig. 1A, i, arrowhead) and ductal (Fig. 1A, i, arrow) cells of the normal pancreas; however, BNIP3 expression was barely detectable in the majority of PDAC samples analyzed (Fig. 1A, ii–iv, arrows).

We then did *in situ* hybridization to determine BNIP3 transcript levels in normal pancreas and PDAC samples. The pattern of mRNA expression was very similar to that of the protein with evident expression of BNIP3 mRNA in acinar cells (Fig. 2C, i, arrowhead) and weaker, but detectable, expression in the ductal cells of the normal pancreas (Fig. 2C, i, arrow). In contrast, BNIP3 mRNA expression was not detected in the PDAC specimens (Fig. 2C, ii–iv, arrows). Figure 2C(ii) illustrates the contrast between BNIP3 expression levels in the PDAC cells (arrows) versus an adjacent region of “normal” acinar cells (arrowheads).

**Identification of candidate BNIP3 suppressors.** To identify genes that may be acting directly or indirectly to suppress the expression of BNIP3 in PDAC, we analyzed our data from gene expression analysis derived from four normal pancreas and five PDAC specimens to determine which genes show a negative correlation in their expression profiles with respect to the expression profile of BNIP3, using a stringent cutoff value of −0.75 for the correlation coefficient.

A number of selected genes from this analysis are shown in Fig. 2A. To select genes that might be involved in the suppression of BNIP3 expression, we identified genes that are involved in either transcriptional repression or cell survival (Fig. 2A, Other genes). However, a striking feature of this list of genes was the presence of a number of members of the S100 protein family with some of the strongest negative correlation coefficients. We therefore decided to investigate whether this family of proteins might be involved in the repression of BNIP3 in PDAC. To test this hypothesis, we constructed a number of expression vectors encoding cDNAs for members of the S100 protein family that were identified by the above analysis. A series of reporter assays were then done to determine whether exogenous expression of these S100 proteins could achieve repression of BNIP3 promoter activity (Fig. 2C).

This analysis showed that S100A11, S100A6, and S100P did not significantly alter BNIP3 promoter-mediated reporter gene activity, but both S100A2 and S100A4 could achieve an ~2-fold repression. As the magnitude and prevalence of S100A4 overexpression in PDAC is considerably greater than that of S100A2, we therefore focused on S100A4 in our subsequent analysis. The negative correlation between the expression profiles of BNIP3 and S100A4 in the normal and PDAC samples is illustrated in Fig. 2B.

**S100A4 inhibits BNIP3 expression in PDAC cell lines.** We analyzed S100A4 mRNA levels by RT-PCR and BNIP3 protein levels by Western blotting in a panel of PDAC cell lines (Fig. 3A). This analysis showed that BNIP3 levels were down-regulated in 7 of the 11 PDAC cell lines analyzed compared with the immortalized pancreatic ductal cell line HPDE. Furthermore, there was a marked negative correlation between BNIP3 protein and S100A4 mRNA levels.

Knockdown of S100A4 expression in target PDAC cell lines was achieved by transduction with either a control (Ctl shRNA) retrovirus or an S100A4 shRNA-expressing retrovirus (S100A4 ShRNA), followed by selection of stably expressing cells. As expected, no effect of S100A4 knockdown on BNIP3 levels was observed in two cell lines, AsPc1 and MiaPaca-2 (Fig. 3B), which do not express detectable levels of BNIP3 as a result of hypermethylation of the BNIP3 promoter, as previously shown (6). However, in cell lines that possess unmethylated BNIP3 promoters and detectable basal BNIP3 expression levels (CFPac1, Suit-2, and Panc-1), we observed a clear induction of BNIP3 upon S100A4 knockdown, suggesting that partial suppression of BNIP3 expression is occurring in these cell lines as a result of S100A4 overexpression (Fig. 3C; ref. 14). To explore the possible signaling pathways that link S100A4 expression with repression of the BNIP3 promoter, we investigated whether nuclear factor-κB (NF-κB) could be involved in this process. NF-κB is activated by S100A4 and can repress the BNIP3 promoter activity in ventricular myocytes (15–17). Knockdown of S1004 resulted in reduced phospho-IκB levels in two of the four cell lines (Suit-2 and MiaPaca-2) examined; however, the CFPac1 cell line did not express detectable levels of phospho-IκB (Fig. 3D).

**S100A4 expression contributes to the chemoresistance of PDAC cell lines.** As we have previously reported a correlation between BNIP3 expression and chemosensitivity in PDAC cell lines, analysis of S100A4 expression and chemosensitivity in the above PDAC cell lines was performed (Fig. 3E). As can be seen from the table in Fig. 3, a strong negative correlation was observed between S100A4 expression and chemosensitivity in PDAC cell lines, as previously shown (6). However, in cell lines that possess unmethylated BNIP3 promoters and detectable basal BNIP3 expression levels (CFPac1, Suit-2, and Panc-1), we observed a clear induction of BNIP3 upon S100A4 knockdown, suggesting that partial suppression of BNIP3 expression is occurring in these cell lines as a result of S100A4 overexpression (Fig. 3C; ref. 14). To explore the possible signaling pathways that link S100A4 expression with repression of the BNIP3 promoter, we investigated whether nuclear factor-κB (NF-κB) could be involved in this process. NF-κB is activated by S100A4 and can repress the BNIP3 promoter activity in ventricular myocytes (15–17). Knockdown of S1004 resulted in reduced phospho-IκB levels in two of the four cell lines (Suit-2 and MiaPaca-2) examined; however, the CFPac1 cell line did not express detectable levels of phospho-IκB (Fig. 3D).
(8), we decided to investigate whether S100A4 could be a factor that contributes to this phenotype. CFPac1, Panc-1, and MiaPaca-2 cell lines were stably transduced with either the control or S100A4 shRNA-expressing retroviruses and seeded into 96-well plates and treated with a range of concentrations (0–1,500 ng/mL) of gemcitabine for 72 h. Cell viability was then determined using the MTT assay. For each cell line, knockdown of S100A4 resulted in a decrease in the IC50 value for gemcitabine treatment (Fig. 4A).

Figure 4A illustrates the change in cell viability of control CFPac1 (Ctl shRNA) and S100A4 shRNA-expressing CFPac1 cells (S100A4 shRNA), resulting from treatment with increasing concentrations of gemcitabine. S100A4 knockdown also resulted in a greater degree of chromosomal fragmentation after gemcitabine treatment as assessed by the difference in the percentage of cells contained within the sub-G1 peak (38% versus 26.5%) in S100A4 shRNA-expressing CFPac1 cells compared with control cells after gemcitabine treatment (200 ng/mL for 48 h; Fig. 4C). Furthermore, this analysis showed an accumulation of cells in the G2-M and to a lesser extent S phases in the S100A4 shRNA–expressing cells compared with the control shRNA cells (59.9% versus 48.6%), suggesting that S100A4 knockdown increased the effect of gemcitabine in blocking DNA replication during the S and G2 phases using Annexin V/propidium iodide staining (Fig. 4D); a significant increase in the number of apoptotic cells was observed in the gemcitabine-treated (200 ng/mL for 48 h) S100A4 shRNA–expressing cells (28.1%) versus the control cells (18.7%).

**S100A4 knockdown stimulates apoptosis.** We investigated whether S100A4 knockdown could directly influence cell viability in the absence of gemcitabine treatment by transfecting CFPac1 cells with either S100A4 siRNA or control siRNA, and assessing cell viability 48 h posttransfection using the MTT assay. A clear decrease in the number of viable cells was observed in response to S100A4 knockdown over a period of 8 days compared with the control siRNA-treated cells (Fig. 5A). The inset in Fig. 5A shows an almost complete knockdown of S100A4 mRNA in CFPac1 cells after treatment with the S100A4 siRNA.

**Figure 1.** Analysis of BNIP3 protein and mRNA expression in PDAC and normal pancreas specimens. A, analysis of BNIP3 expression in 20 different PDAC cases by immunohistochemistry. Staining prevalence was scored as follows: —, no staining; +, weak staining; ++, moderate staining; ++++, strong staining. Staining incidence was evaluated in terms of the average percentage of cells with observable staining in a given field, as follows: 0, no staining; 1, 1% to 25%; 2, 25% to 50%; 3, 50% to 75%; and 4, 75% to 100%. B, in the normal pancreas (i), strong staining was observed in the acinar cells (arrowhead) and ductal cells (arrow). In the three representative PDAC cases shown (ii–iv), weak staining can be observed in the PDAC cells (arrows) with an absence of observable staining in the stromal component. Negative controls consisting of either the omission of primary antibody or incubation with mouse IgG instead of primary antibody showed no staining (data not shown). Magnification (all images), ×200. C, in situ hybridization analysis of the BNIP3 transcript in PDAC and normal pancreas specimens. i, in normal pancreas, a strong signal is present in the acinar (arrow) cells with a weaker but clearly detectable signal present in the ductal cells. ii to iv, three representative PDAC cases (of a total of eight cases analyzed) show no detectable staining in PDAC cells (arrows). Negative controls consisting of tissues hybridized with sense riboprobes showed no staining (data not shown). i to iii, magnification, ×200. D, magnification, ×100.
Analysis of the cell cycle distribution of CFPac1 cells transfected with either control or S100A4 siRNA by flow cytometry (Fig. 5B) did not show any discernible effect of S100A4 knockdown on cell cycle progression. However, silencing of S100A4 did result in an increase in the sub-G₁ population and an increase in the number of Annexin V/propidium iodide–stained cells (Fig. 5D) compared with the cells that were transfected with the control siRNA oligonucleotides (12.62% versus 5.62%), demonstrating that S100A4 silencing alone resulted in an induction of apoptotic cell death (Fig. 5D).

As BNIP3-mediated cell death has been reported to occur via integration into the outer mitochondrial membrane followed by opening of the mitochondrial permeability transition pore, we determined whether the elevated levels of BNIP3 that result from S100A4 knockdown are associated with mitochondria (4). Knockdown of S100A4 resulted in a significant increase in the level of BNIP3 in the mitochondrial fraction compared with controls cells, with no expression observed in the cytoplasmic fraction (Fig. 5C).

S100A4 knockdown induces caspase activation. Immunoblot analysis of a range of apoptosis markers was done using protein lysates prepared from CFPac1 cells transfected with either control or S100A4 siRNA in the presence or absence of gemcitabine (100 and 400 ng/mL) for a period of 48 h. Both S100A4 knockdown alone and gemcitabine treatment resulted in an increase in the cleavage products of caspases-3 and poly(ADP-ribose) polymerase, as well as an increase in the translocation of cytochrome c from the mitochondria to the cytosol (Fig. 6A). We also observed a slight increase in the proteolytic processing of caspase-9, but no change in the processing of caspase-8, indicating that the observed cell death was not mediated by the extrinsic apoptotic pathway. Furthermore, the above effects were clearly augmented by the combination of S100A4 knockdown and treatment with gemcitabine.

As a marked increase in the cleavage of caspase-3 resulted from S100A4 knockdown, we sought to confirm this striking observation. As can be seen from Fig. 6B, a marked increase in the level of active caspase-3/7 was detected in cells transfected with the S100A4 siRNA compared with control siRNA. Again, this effect was significantly potentiated after gemcitabine treatment. To show that the increase in apoptosis resulting from S100A4 knockdown was dependent on caspase-3, we evaluated the effect of the irreversible caspase-3 inhibitor Ac-DEVD-CHO on cell viability after treatment with either control or S100A4 siRNA oligonucleotides and followed by incubation in the presence of gemcitabine for 96 h (Fig. 6C). S100A4 knockdown clearly decreased the number of viable cells; however, this effect was completely abolished in the presence of the caspase-3 inhibitor.
Discussion

We previously showed a significant correlation between BNIP3 expression levels and gemcitabine resistance in a panel of PDAC cell lines (8) and subsequently showed that BNIP3 expression is widely down-regulated in PDAC specimens at the protein and mRNA level (Fig. 1). We postulated that the identification of upstream factors that are responsible for the suppression of BNIP3 expression in PDAC may further illuminate our understanding of the molecular mechanisms underlying chemoresistance in pancreatic cancer.

Previous reports have shown a correlation between loss of BNIP3 expression in pancreatic cancer and methylation of the BNIP3 promoter (6, 14); however, it has been reported that up to 50% of PDAC cell lines exhibit detectable BNIP3 expression, suggesting that alternative mechanisms may exist to inhibit the function of this protein in these cell lines (14). To identify candidate BNIP3 suppressors, we analyzed microarray data obtained from normal pancreas and PDAC specimens to identify genes that show a negatively correlating expression profile with respect to the BNIP3 expression profile in these samples. This analysis identified a number of S100 family proteins with a strong negative correlation to the BNIP3 expression profile (correlation coefficient >-0.75; Fig. 2A). We found that both S100A2 and S100A4 were capable of repressing BNIP3 promoter-driven luciferase activity ~2-fold compared with the empty vector control. As the prevalence and magnitude of S100A4 overexpression in pancreatic cancer is significantly greater than that of S100A2, and S100A4 expression has been clinically correlated with a poor outcome for pancreatic cancer patients, we focused on this protein in subsequent studies (18–20).

We confirmed the negative correlation between S100A4 and BNIP3 in PDAC cell lines (Fig. 3A) and, using pancreatic cells lines stably expressing an S100A4 shRNA, we showed that knockdown of S100A4 resulted in an elevated expression of the BNIP3 protein in cell lines with an unmethylated BNIP3 promoter. The observed repression of BNIP3 expression in these cell lines by S100A4 may represent an alternative mechanism for the inhibition of BNIP3 function in pancreatic cancer. These observations are consistent with the many examples of genes that are hypermethylated in cancer for which alternative mechanisms of transcriptional repression have also been observed, as in the case of p21 and p14/ADP ribosylation factor (21–23).

To investigate the possible signaling pathways that link S100A4 expression with repression of the BNIP3 promoter, we identified NF-κB as a potential intermediary factor as it is activated by S100A4 and can repress the BNIP3 promoter activity in ventricular myocytes (15–17). Knockdown of S1004 resulted in reduced phospho-IκB levels in two of the four cell lines examined, with CFPac-1 showing no phospho-IκB expression; therefore, this mechanism may play a role in this process in some cell lines, but clearly not in others. As S100A4 has also been shown to bind to and modulate the transactivation activity of p53 (15), we investigated whether the repression of BNIP3 expression by S100A4

Figure 3. Effect of S100A4 knockdown on BNIP3 protein expression in PDAC cell lines. A, analysis of BNIP3 protein and S100A4 mRNA expression in a panel of 11 PDAC cell lines and HPDE cells. Immunoblot analysis of (B) BNIP3 and (C) phospho-IκB expression protein expression in PDAC cells lines that have been stably transduced with either parental retrovirus (Ctl shRNA) or recombinant retroviral vector encoding a shRNA targeting the S100A4 transcript (S100A4 shRNA). S100A4 mRNA expression levels in each cell line were determined by RT-PCR.
is mediated by p53. We transfected parental and p53−/− HCT116 cells with the BNIP3 promoter construct and determined the effect of exogenous S100A4 expression on BNIP3 promoter–driven luciferase activity. A similar level of repression was observed in both cell lines, indicating that p53 was not required for the S100A4-mediated repression of BNIP3 expression (data not shown).

The effect of S100A4 knockdown on gemcitabine sensitivity in these cell lines seemed to depend on the degree of intrinsic chemoresistance exhibited by each cell line, indicating that p53 was not required for the S100A4-mediated repression of BNIP3 expression (data not shown).

To investigate the molecular mechanism by which S100A4 contributes to chemoresistance, we studied the effect of S100A4 knockdown on cell viability. We used siRNAs for this analysis as we could achieve almost complete knockdown of the S100A4 transcript by this method as opposed to retrovirus-mediated shRNA expression that allowed us to transduce a wide range of PDAC cell lines but with a lower knockdown efficiency. This analysis showed that S100A4 knockdown resulted in a reduction in cell viability over time and increased DNA fragmentation, without any significant change in cell cycle progression in CFPac1 cells (Fig. 5B). These data were further clarified by the observation that S100A4 knockdown in CFPac1 significantly increased the number of apoptotic cells by Annexin V/propidium iodide staining (Fig. 5D). Our observation that the increased pool of BNIP3 resulting from S100A4 knockdown in CFPac1 cells is almost exclusively present in the mitochondrial fraction indicates that BNIP3 may be contributing to this effect (Fig. 5C).

These data were further supported by the observation that S100A4 knockdown in CFPac1 cells resulted in an increase in the proteolytic processing of caspase-3 and poly(ADP-ribose) polymerase and, to a lesser extent, caspase-9 (Fig. 6A). We also detected an increase in cytosolic cytochrome c levels after S100A4 knockdown. The activation of caspase-3/7 after S100A4 knockdown was
confirmed by a caspase activity assay and pharmacologic inhibition (Fig. 6B).

A role for S100A4 in the promotion of metastasis in a number of cancers, including pancreatic cancer, has been repeatedly shown; however, little has been reported on the role of S100A4 in cell survival (24). S100A4 has been shown to protect neuronal cells from proapoptotic stimuli, whereas another report showed that knockdown of S100A4 in OHS cells enhanced the sensitivity of these cells to IFN-γ–mediated apoptosis (25, 26). Additionally, S100A4 has been shown to modulate the transactivation of both proapoptotic and antiapoptotic p53 target genes (15).

Here, we clearly describe an antiapoptotic role for S100A4 in pancreatic cancer. It is interesting to speculate which signaling pathways/factors might mediate the prosurvival function of S100A4. We investigated two potential mechanisms, NF-κB activation and modulation of p53 activity, but we could not obtain convincing evidence to support the involvement of either of these factors. Alternative mechanisms might be through the inhibition of PKC-mediated phosphorylation of target proteins, as this activity has been ascribed to S100A4 in the case of p53 (27) and the heavy chain of non–muscle myosin (28). Another possibility is via the activation of AKT, as S100A4 has been shown to induce WISP1 and EZrin, both activators of AKT (28).

Moreover, it is possible that the secreted form of S100A4 may further promote cell survival in vivo. For example, the extracellular form of S100A4 has been shown to stimulate angiogenesis, which was associated with its ability to induce the expression of several matrix metalloproteinases as well as urokinase-type plasminogen activator (uPA; ref. 29). The induction of uPA expression by S100A4 in particular may contribute both to the inhibition of apoptosis and hence chemoresistance through the liberation of matrix-associated growth factors that could stimulate cell survival signaling pathways (30).

In summary, the data presented in this article describe a novel role for S100A4 in enhancing chemoresistance in pancreatic cancer through the suppression of apoptosis. This is an important finding in the context of pancreatic cancer for which all chemotherapeutic

Figure 5. Effect of siRNA-mediated knockdown of S100A4 expression on cell viability, cell cycle progression, and apoptosis in CFPac1 cells. A, CFPac1 cells were transfected with either Ctl siRNA or S100A4 siRNA and 48 h after the number of viable cells was quantified using the MTT assay over a period of 8 d. Points, mean based on three independent experiments; bars, SD. B, sub-G1 analysis of CFPac1 cells transfected with the indicated siRNA oligonucleotides. M1, sub-G1 region; M2, G phase; M3, S phase; M4, G2-M phase. C, S100A4 shRNA and control shRNA–expressing CFPac1 cells were subjected to cellular fractionation to obtain cytoplasmic (Cyt) and mitochondrial (Mito) fractions. An equal amount of protein (30 μg) from each fraction was subjected to immunoblot analysis using antibodies against either BNIP3 and β-actin. D, analysis of Annexin V/propidium iodide staining on CFPac-1 cells transfected with the indicated siRNA oligonucleotides.
strategies have provided limited benefit. In addition to its well-established role in the promotion of metastasis, we provide evidence that S100A4 also contributes to another crucial process in cancer progression, that of cell survival. Further studies are now required to establish whether S100A4 can function as a survival factor in other cancer types as well as delineating the precise molecular mechanisms that mediate this activity.

Figure 6. Suppression of caspase activation by S100A4 in CFPac1 cells. A, CFPac1 cells were transfected with either Ctl siRNA or S100A4 siRNA and incubated for 72 h followed by either the preparation of whole-cell lysates or an additional incubation in the presence of either 100 or 400 ng/mL gemcitabine for 48 h before cell lysis. The lysates obtained were then subjected to immunoblot analysis using antibodies against the indicated proteins. The same procedure was followed for the cytochrome c immunoblot analysis except that a cytosolic fraction was prepared as described in Materials and Methods. B, quantification of caspase-3/7 activity in whole-cell lysate samples. CFPac1 cells were treated as indicated for 48 h before resuspension in RIPAbuffer. One volume of luminiscent CaspaseGlo 3/7 substrate buffer was added to aliquots of precleared lysate containing 30 µg of protein followed by gentle agitation for 1 h. The caspase activity in each sample was then monitored using a luminometer and the data obtained are expressed as relative luminescence units. Columns, mean based on three independent experiments; bars, SD. C, effect of caspase-3 inhibitor on the viability of cells treated with gemcitabine. CFPac1 cells transfected with the indicated siRNA oligonucleotides in a 96-well plate were incubated under normal culture conditions for 1 h with either 100 µmol/L of the caspase-3 inhibitor Ac-DEVD-CHO (+) or 100 µmol/L DMSO vehicle control solution (−) before the addition of gemcitabine at a final concentration of 100 ng/mL. Cell viability was quantified 72 h later using the MTT assay.

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