Silencing of the Hypoxia-Inducible Cell Death Protein BNIP3 in Pancreatic Cancer

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ABSTRACT
Hypoxic conditions exist within pancreatic adenocarcinoma, yet pancreatic cancer cells survive and replicate within this environment. To understand the mechanisms involved in pancreatic cancer adaptation to hypoxia, we analyzed expression of a regulator of hypoxia-induced cell death, Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3). We found that BNIP3 was down-regulated in nine of nine pancreatic adenocarcinomas compared with normal pancreas despite the up-regulation of other hypoxia-inducible genes, including glucose transporter-1 and insulin-like growth factor-binding protein 3. Also, BNIP3 expression was undetectable even after hypoxia treatment in six of seven pancreatic cancer cell lines. The BNIP3 promoter, which was remarkably activated by hypoxia, is located within a CpG island. The methylation status of CpG dinucleotides within the BNIP3 promoter was analyzed after bisulfite treatment by sequencing and methylation-specific PCR. Hypermethylation of the BNIP3 promoter was observed in all BNIP3-negative pancreatic cancer cell lines and eight of 10 pancreatic adenocarcinoma samples. Treatment of BNIP3-negative pancreatic cancer cell lines with a DNA methylation inhibitor, 5-aza-2’-deoxycytidine, restored hypoxia-induced BNIP3 expression. BNIP3 expression was also restored by introduction of a construct consisting of a full-length BNIP3 cDNA regulated by a cloned BNIP3 promoter. Restoration of BNIP3 expression rendered the pancreatic cancer cells notably more sensitive to hypoxia-induced cell death. In conclusion, down-regulation of BNIP3 by CpG methylation likely contributes to resistance to hypoxia-induced cell death in pancreatic cancer.

INTRODUCTION
Pancreatic adenocarcinoma is the fourth leading cause of cancer death in the United States, and the occurrence of this disease ranks 10th among all cancers (1). Currently, the only curative treatment for pancreatic cancer is surgical resection, but only ~10–20% of patients are candidates for surgery at the time of diagnosis, and of this group, only ~20% of patients who undergo a curative operation are alive after 5 years (2). Pancreatic cancer tends to rapidly invade surrounding structures and undergo early metastatic spreading. In addition, pancreatic cancer is highly resistant to chemotherapy and radiation therapy, even though some new anticancer drugs and combinations of drugs with radiotherapy have been recently introduced for treatment of this disease (3).

Pancreatic cancer is pathologically characterized as nests of neoplastic cells within an abundant fibrotic stroma. These tumors are observed as a lower density area on contrast-enhanced computed tomography scan, which suggests that they have a reduced blood oxygen supply compared with normal tissue (4). Direct evidence that pancreatic cancer cells exist within a hypoxic environment has come from measurements of O2 tension that are significantly decreased in pancreatic cancer when compared with adjacent normal pancreas (5).

The ability of cancer cells to adapt to hypoxic environment is increasingly recognized as an important mechanism promoting tumor growth (6). In general, it is thought that tumor cells become resistant to hypoxia during the progression of the disease by alterations in a variety of cellular mechanisms (7, 8).

In the current study, we analyzed the levels and role of a hypoxia-inducible pro-apoptotic molecule, Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), in pancreatic cancer. BNIP3 was originally isolated through its interaction with anti-apoptotic proteins such as adenovirus E1B 19K and cellular Bcl-2 (9). BNIP3 belongs to the Bcl-2 family and the Bcl-2 homology domain-3-only subfamily (10).

BNIP3 expression is increased under hypoxic conditions by the actions of the transcription factor, hypoxia-inducible factor 1 (HIF-1; Refs. 11–13). Forced expression of BNIP3 has been shown to lead to cell death in cardiac myocytes and other cultured cell lines (14, 15). Thus, BNIP3 is considered to be a key regulator of hypoxia-induced cell death.

We observed that BNIP3 expression was decreased in pancreatic cancer compared with normal pancreas. This was not due to a general decrease in hypoxia gene induction in pancreatic cancer, because we observed increased levels of hypoxia-responsive genes including glucose transporter-1 (GLUT1) and insulin-like growth factor-binding protein 3 (IGFBP3). BNIP3 expression was also absent and could not be induced by hypoxic treatment in several pancreatic cancer cell lines. As an explanation for the low levels of BNIP3 expression, we explored the possibility that the gene might be hypermethylated. We found that the BNIP3 promoter was located within a CpG island in which numerous CpG dinucleotides were methylated in almost all pancreatic cancer cell lines and tumor samples but not in normal pancreas. Moreover, pharmacological inhibition of methylation restored expression and hypoxia induction of BNIP3 in pancreatic cancer cell lines. We were also able to restore hypoxia-induced BNIP3 expression by transfection with a construct containing the BNIP3 cDNA regulated by 754 bp of the BNIP3 promoter. Restoration of hypoxia-inducible BNIP3 expression increased the sensitivity of pancreatic cancer cells to hypoxia-induced cell death. Taken together, these data suggest that down-regulation of the hypoxia-inducible gene BNIP3 by methylation is an important adaptive response, allowing pancreatic cancer cell growth in a hypoxic environment.

MATERIALS AND METHODS
Pancreatic Tissues and Cell Lines. Pancreatic tissue specimens analyzed in this study were collected from the University of Michigan Health System with approval of the University of Michigan Institutional Review Board. After pathological examination, tissue samples were embedded in OCT-freezing medium (Miles Scientific, Naperville, IL) and cryotome sectioned (5 μm), and areas of relatively pure tumor or normal pancreas were microdissected and kept in TRIzol (Invitrogen, Carlsbad, CA) until RNA and DNA isolation.

Pancreatic cancer cell lines BxPC-3, Mia PaCa-2, HPAC, MPanc-96, AsPC-1, PSN-1, and PANC-1 were obtained from the American Type Culture Collection (Manassas, VA) or Japanese Cancer Research Resources Bank (Tokyo, Japan). These cells were cultured in high-glucose DMEM (Invitrogen).
with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with an atmosphere of 5% CO₂ and 95% air.

Reverse Transcription-PCR. Total RNA extraction was performed from microdissected tissue samples or cultured cells using RNA easy kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol, and purified RNA was quantitated and assessed for purity by UV spectrophotometry. cDNA was generated from 1 μg of RNA with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). The amplification of each specific RNA was performed in a 25-μl reaction mixture containing 2 μl of cDNA template, 1× PCR master mix, and 0.5 μmol of primers. The PCR primers used for detection of BNIP3 were: forward, 5'-GGCACCCTGCCTGCAGACAC-3'; and reverse, 5'-CAATCCGATGGCCAGAATGAGA-3'. The amplified product was 585 bp, and the PCR conditions were as follows: one cycle of denaturing at 95°C for 10 min, followed by 24 cycles of 94°C for 30 s, 60°C for 50 s, and 72°C for 1 min before a final extension at 72°C for 10 min. The PCR products were loaded onto 2% agarose gels and visualized with ethidium bromide under UV light. As a control for cDNA synthesis, reverse transcription-PCR was also performed using primers specific for β-actin gene (16).

Quantitative (Q)-Reverse Transcription-PCR. Q-reverse transcription-PCR for BNIP3, GLUT1, IGBP3, and GLUT1 were as described previously (17). The PCR conditions for BNIP3, GLUT1, IGBP3, and β-actin were as follows: one cycle of denaturing at 95°C for 10 min, followed by 30 cycles of 94°C for 30 s, 60°C for 50 s, and 72°C for 1 min. Each reaction included a negative control and serial 10-fold dilutions from 10⁻¹ to 10⁻³ of cDNA of positive control, and the experiment was done in triplicate. These serial-diluted positive controls were used for standards to confirm the linearity between the amount of target cDNA in the sample and the intensity of fluorescent signal. For comparisons between samples, the mRNA expression of the target genes was normalized to β-actin mRNA expression. Product specificity was controlled by melting curve analysis and migration on a 2% agarose gel.

Cell Protein Preparation, SDS-PAGE, and Western Blotting. Cells grown in 100-mm dishes were washed twice with cold PBS, harvested in 0.5 ml of lysis buffer (25 mM Tris-buffered saline (pH 7.4), 50 mM NaCl, 0.5% NP40, 0.5% sodium deoxycholate, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 250 μg/ml sodium vanadate for BNIP3 detection or 7 M urea, 10% glycerol, 10 mM Tris-buffered saline (pH 6.8), 1% SDS, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 250 μg/ml sodium vanadate for HIF-1α detection). After removal of cell debris by centrifugation, the protein concentrations in cell lysates were determined, and 30 μg of protein were added to loading buffer with 5% β-mercaptoethanol and heated for 10 min at 100°C. Samples were separated at 10% SDS-PAGE and transferred to nitrocellulose membranes by semidry blotting. Membranes were incubated in blocking buffer (1× Tris-buffered saline, 0.1% Tween-20, and 5% nonfat dry milk) for 1 h at room temperature and probed with anti-BNIP3 antibody (1000× diluted; Sigma, St. Louis, MO), anti-HIF-1α antibody (250× diluted; BD, Franklin Lakes, NJ), or anti-actin antibody (1000× diluted; Sigma) overnight at 4°C, followed by hybridization with a horseradish peroxidase-conjugated secondary antibody mouse IgG (1:3000) (Amersham Biosciences, Piscataway, NJ). Signals were detected by chemiluminescence using the ECL detection system (Amersham Biosciences). Genomic DNA was isolated from microdissected tissue samples and cultured cells using Wizard Genomic DNA purification kit (Promega) based on the manufacturer’s protocol. To differentiate methylated CpGs from unmethylated CpGs, 1 μg of genomic DNA was treated with sodium bisulfite at 50°C for 20 h using a CpGenome DNA Modification kit (Sorolologies Corp., Norcross, GA) according to the instructions of the manufacturer and finally resuspended in 50 μl of 10 mM Tris (pH 8)-1 mM EDTA buffer. After this treatment, unmethylated cytosine is converted to uracil, whereas methylated cytosine remains cytosine.

Amplification, Cloning, and Sequencing of Bisulfite-Treated DNA. By comparing the cDNA sequence of the BNIP3 gene (GenBank accession no. NM-004052) against a genomic DNA sequence of chromosome 10q26.3 (GenBank accession no. AL162274), a transcription start site of BNIP3 gene was determined. A region from 385–21 bp upstream of the transcription start site, which contains a CpG-rich fragment, was amplified from one-twentieth of the modified DNA by PCR using HotStartTag Taq DNA polymerase (Qiagen). PCR primers used in this reaction were designed to amplify the coding strand of bisulfite-treated DNA as follows: GTGGTAGTTAGTTGTAGAGAG (sense) and ACTCCAAAAAC AAAACRAAAAC (antisense; where R = G or A). The PCR conditions were as follows: one cycle of denaturing at 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 60°C for 50 s, and 72°C for 1 min before a final extension at 72°C for 10 min. The PCR product was cloned into pGEM-T Easy Vector (Promega), and 10 clones for each sample were sequenced using T7 or SP6 primer at the Sequencing Core Facility of the University of Michigan. Cytosines in CpG dinucleotides that remained unconverted after bisulfite treatment were considered to be methylated.

Methylation-Specific PCR. Methylation-specific PCR was performed according to the previously described principles (18). To detect the sequence differences between methylated and unmethylated DNA as a result of bisulfite treatment, each primer is designed to contain four or five CpG dinucleotides. Primer sequences for unmethylated reaction were 5'-TAGGTTTGTGGTTTGTTGTAATG-3' (sense) and 5'-ACCACACTACCACATTAAACCACA-3' (antisense), and for methylated reaction were 5'-TAGGTTGTTGTTGGTTTACCAC-3' (sense) and 5'-ACCGGTCGTCCTTAAACCACG-3' (antisense; the bold nucleotides represent the putative methylation sites), and amplified products were 94 bp for both reactions. The PCR conditions were as follows: one cycle of denaturing at 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 64°C (for methylated reaction) or 58°C (for unmethylated reaction) for 50 s, and 72°C for 1 min before a final extension at 72°C for 10 min.

5-Aza-2'-Deoxycytidine (5aza-dC) Treatment and Hypoxic Exposure. The pancreatic cancer cell lines Mia Paca-2, HPAC, BxPC-3, and PM Panc-96 were seeded at a density of 1 × 10⁴ cells/100-mm dish in culture medium and allowed to attach over a 24-h period. 5aza-dC (Sigma) was then added to a final concentration of 1 μM, and the cells were allowed to grow for 6 days. The medium with or without 5aza-dC was changed every other day. At the end of the treatment, the medium was removed; and the RNA, DNA, and protein were extracted for reverse transcription-PCR, methylation analysis, and protein analysis. Hypoxic conditions were achieved with an anaerobic chamber (Sugiyaman, Tokyo, Japan) and AnoroPack for Cell Gas generating system (Mitsubishi Gas Chemical, Tokyo, Japan), which catalytically reduced oxygen levels to less than 1% within 30 min (19).

Subcloning of BNIP3 Gene and Plasmids. Full-length cDNA for BNIP3 was amplified by PCR and subcloned into pcDNA 3.1 (+) vector (Invitrogen). The primer sets were as follows: 5'-GGATCCCCGCGCTGCTGCAGACG-3' and 5'-GGAAACCGCACTTTACAGAAC-3'. Plasmids were recovered, purified, and sequenced.

Colony Formation Assay. Cells were plated subconfluently in culture flask for 24 h before transient transfection with either pcDNA3.1 (+)-BNIP3 or pcDNA3.1 (+)-empty vector using Lipofectin (Invitrogen) according to the manufacturer’s protocol. Transfected cells were trypsinized and plated on 10-cm dishes 24 h after transfection and cultured in the presence of G418 (1000 μg/ml). After 10–14 days, colonies were fixed with methanol and stained with 0.1% crystal violet.

Cell Death Detection Assay. Full-length BNIP3 cDNA was inserted into the pAdTrack-CMV vector, which contained dual independent cytomegalovirus promoters, one expressing BNIP3 and the other green fluorescence protein (GFP). Ad-Track-BNIP3 vector or Ad-Track-empty vector was transiently transfected into pancreatic cancer cells. At the indicated times after transfection, cells were washed with PBS twice, scraped, and lysed with PBS containing 0.2% Triton X-100. GFP intensity of cell lysates was measured by a spectrophotometer (Perkin-Elmer, Wellesley, MA) with excitation at 488 nm and emission at 510 nm. That the intensity of GFP of cell lysates linearly correlated with the number of GFP-positive cells was confirmed by direct cell counting (data not shown).

Construction of Human BNIP3 Reporter Plasmid. Human BNIP3 5'-flanking sequence (GenBank accession no. AL162274) was amplified by PCR using genomic DNA isolated from human normal pancreas as the template. The PCR product was digested with BgIII and NciI and cloned into the pGL3-Basic vector (Promega). The primer sets were as follows: 5'-AGATCTCCGCCTGCGGCCGCAGAAGA-3' and 5'-CCATGGGCGCCAGAAGAAGA-3'. Transfected cells were washed with PBS twice, scraped, and lysed with PBS containing 0.2% Triton X-100, 100 μg/ml of chloramphenicol, and 0.1% sodium dodecyl sulfate. Plasmids were recovered, purified, and sequenced.

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GAGG-GCAACTGCG-3’. Plasmids were recovered, purified, and sequenced.

**Luciferase Assays.** Cells were seeded in a 6-well plate and grown to 90% confluence. For each well, human BNIP3 reporter construct was cotransfected with β-gal reporter vector into HEK293 or Mia PaCa-2 cells. Cells were harvested, and reporter activity was measured using the Dual Luciferase Assay (Promega) according to the manufacturer’s instructions. Transfection efficiency was normalized on the basis of β-galactosidase activity.

**Statistical Analysis.** Data are presented as mean ± SE. Statistically significant differences were determined by unpaired t test and were defined as P < 0.05.

**RESULTS**

**BNIP3 Expression in Pancreatic Tissues and Cells.** The expression of BNIP3 mRNA was examined by reverse transcription-PCR (Fig. 1A) and Q-reverse transcription-PCR (Fig. 1B) in 17 pancreatic tissues, 8 samples of normal pancreas, and 9 samples of pancreatic adenocarcinoma. In standard reverse transcription-PCR, obvious bands appeared in all eight normal samples, whereas faint bands were visible in two of nine tumor samples. Q-reverse transcription-PCR revealed a 56-fold (P < 0.01) difference of BNIP3 expression between normal and tumor tissues. BNIP3 mRNA expression was also undetectable by reverse transcription-PCR in five of six pancreatic cancer cell lines (Mia PaCa-2, HPAC, BxPC-3, MPanc-96, PANC-1, and AsPC-1) but was obvious in the PSN-1 cell line (Fig. 1C). Thus, all pancreatic tumors and all but one cancer cell line displayed no or extremely low expression of BNIP3.

To explore the effects of hypoxia on BNIP3 expression in pancreatic cancer, the cell lines were cultured under conditions of hypoxia. Hypoxia treatment (O₂ < 1%) was unable to induce BNIP3 mRNA in the same six pancreatic cancer cell lines that showed no expression

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**Fig. 1. BNIP3 expression in pancreatic cancer tissue, cell lines, and normal pancreas.** A, BNIP3 mRNA expression in normal pancreas and pancreatic cancer was examined by reverse transcription-PCR. Representative examples are shown. The fragment of human BNIP3 cDNA amplified is 585 bp. The β-actin gene was used as a control for RNA quality and loading. B, expression of BNIP3 mRNA was quantitated by Q-reverse transcription-PCR in the same samples as shown in A. The relative expression of BNIP3 and β-actin mRNA were determined and the BNIP3/β-actin ratio was calculated for each sample. The mean value of nine tumor tissues was given a value of 1, and the mean value of the eight normal samples was then expressed relative to the tumor values. Values are mean ± SE. *, P < 0.01. C, BNIP3 mRNA levels under normoxic (CO₂ = 5% and room air) conditions (N) and under hypoxic (O₂ < 1% for 24 h) conditions (H) in seven pancreatic cancer cell lines were examined by reverse transcription-PCR. D, BNIP3 protein levels under normoxic and hypoxic cell culture conditions in four pancreatic cancer cell lines were examined by Western blotting using a monoclonal anti-BNIP3 antibody. Actin is shown as a control for protein loading.
under normoxic conditions whether the cells were treated for 24 h (Fig. 1C) or even 48 h (data not shown). In contrast, the PSN-1 cell line, which expressed BNIP3 under normoxic conditions, displayed levels of BNIP3 mRNA that were 47-fold higher after hypoxia. BNIP3 protein expression was consistent with the mRNA expression data such that BNIP3 protein was not detected in Mia Paca-2, BxPC-3, or HPAC cells under either normoxic or hypoxic condition, whereas the PSN-1 cell showed BNIP3 protein expression after exposure to hypoxia (Fig. 1D).

**Induction of Hypoxia-Inducible Genes in Pancreatic Cancer.** To determine whether the lack of BNIP3 induction in the majority of pancreatic cancer cell lines was due to a general inability to respond to hypoxia, we evaluated the effect of hypoxia on the transcription factor HIF-1α protein expression by Western blotting. HIF-1α is a major regulator of hypoxia-induced gene expression, and its targets include BNIP3. HIF-1α protein levels were significantly increased in pancreatic cancer cell lines after incubation under hypoxic conditions for 24 h (Fig. 2A). In addition, mRNA levels of two other genes that are known to be induced by hypoxia (20), GLUT1 and IGFBP3, were examined by Q-reverse transcription-PCR in 3 pancreatic cancer cell lines, 17 pancreatic tissues, 8 normal pancreas, and 9 pancreatic cancer. mRNA expressions of GLUT1 and IGFBP3 were obviously induced after hypoxic conditions in all three pancreatic cancer cell lines (Fig. 2B). They were also expressed at significantly higher levels in pancreatic cancer compared with normal pancreas: 8.6-fold (P < 0.05) and 7.9-fold (P < 0.01), respectively (Fig. 2C).

**CpG Methylation in the Promoter Region of the BNIP3 Gene in Pancreatic Cancer Cells and Tissues.** To identify the promoter region of the BNIP3 gene, we cloned a 754-bp fragment of the BNIP3-flanking sequence into a reporter plasmid and examined hypoxia inducibility of this region using luciferase assay. We transiently transfected the BNIP3 reporter plasmid into HEK293 cells and Mia Paca-2 cells and exposed cells to hypoxic conditions or regular culture conditions for 24 h. The luciferase activity was markedly increased in hypoxic cells compared with the cells incubated in a normal culture condition in HEK293 cells and Mia Paca-2 cells: 12.3- and 8.8-fold in average, respectively (Fig. 3). This result demonstrated that this 754-bp BNIP3 5′-flanking sequence mediates transcriptional responses of BNIP3 to hypoxia.

Reviewing the sequence of the gene, we found that the BNIP3 promoter is located within a 1700-bp CpG island, which spans from −1162 to +538 bp of the transcription start site and contains the first exon of BNIP3 gene. This promoter also contains a consensus hypoxia-responsive element (HRE) motif (-CACGTG-) at −125 to −120 bp as previously reported (Fig. 4; Ref. 21). The BNIP3 promoter has a G and C content of 78.5% with 116 CpG dinucleotides existing in this region. Therefore, we hypothesized that the loss of expression of BNIP3 in pancreatic cancer might be due to hypermethylation of CpG dinucleotides in the promoter region.

To analyze the methylation status of the BNIP3 promoter, we performed sequence analysis and methylation-specific PCR after

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**Fig. 2. Hypoxic induction of HIF-1α and hypoxia-inducible genes in pancreatic cancer.** A, HIF-1α protein expression under normoxic conditions (N) and under hypoxic conditions (H) in four pancreatic cancer cell lines were examined by Western blotting using a monoclonal anti-HIF-1α antibody. Actin is shown as a control for protein loading. B, expression of GLUT1 mRNA and IGFBP3 mRNA was quantitated by Q-reverse transcription-PCR under normoxic (CO2 5% and room air) or hypoxic ( ) conditions for 24 h. The BNIP3 promoter activity is determined by luciferase assay. Transfection efficiency was normalized on the basis of β-galactosidase activity. The promoter activity in normoxic conditions was given a value of 1, and the data represent a mean of three experiments.

**Fig. 3. BNIP3 promoter activity in HEK 293 cells and Mia PaCa-2 cells.** After transfection of the BNIP3 reporter plasmid, cells were incubated in normoxic (square) or hypoxic (triangle) conditions for 24 h. The BNIP3 promoter activity is determined by luciferase assay. The promoter activity in normoxic conditions was given a value of 1, and the data represent a mean of three experiments.
bisulfite treatment of genomic DNA isolated from pancreatic cancer cell lines and pancreatic tissues. Among the CpG sites in the promoter region, we focused on those located around the HRE because the HRE plays an important role for hypoxia induction of BNIP3 expression (Ref. 21; Fig. 4). First, we analyzed the methylation status of three representative samples by sequencing: normal pancreatic tissue; the BNIP3-positive PSN-1 cell line; and the BNIP3-negative Mia PaCa-2 cell line (Fig. 5A). We found that most of 58 CpG dinucleotides in this region were methylated in Mia PaCa-2 cell, whereas few of them were methylated in normal pancreas and the PSN-1 cell, respectively. To investigate the methylation status in a larger series of pancreatic tissues and cell lines, we carried out methylation-specific PCR. Among 10 cancer cell lines, BNIP3 gene methylation was detected in nine cell lines: Mia PaCa-2; BxPC-3; MPanc-96; SU86.86; AsPC-1; CAPAN; HPAF-II; HPAC; and PANC-1 (Fig. 5B). Importantly, the methylation status of BNIP3 gene in these cell lines correlated with its loss of expression and its lack of induction by hypoxia. In all normal pancreatic tissues that we examined, the signal for unmethylated BNIP3 was detected, and no signal for methylation was observed (results from representative samples are shown in Fig. 5C). In contrast, the signal for methylated BNIP3 appeared in eight of 10 pancreatic cancer tissues. The unmethylated signals detected in cancer...
tissues were likely due to the presence of normal cells within the tumors such as stroma cells or to heterogeneity of cancer cells. These results were summarized in Table 1.

Restoration of BNIP3 Expression in Pancreatic Cancer Cells by Inhibition of Gene Methylation. To further investigate whether or not the expression of BNIP3 mRNA is inactivated by methylation, we treated four pancreatic cancer cells that displayed a constitutively methylated BNIP3 gene with the demethylating agent, 5aza-dC. We confirmed that treatment with 5aza-dC successfully blocked BNIP3 gene methylation, because the signal for unmethylated BNIP3 alleles appeared in Mia PaCa-2 and HPAC cells after, but not before, methylation inhibition (Fig. 6). Next, we examined the level of BNIP3 mRNA after 5aza-dC treatment under normoxic and hypoxic culture conditions in four pancreatic cancer cell lines with methylated BNIP3 gene, Mia PaCa-2, HPAC, BxPC-3, and MPanc-96 by reverse transcription-PCR (Fig. 7). No BNIP3 expression was detected in any of the four cell lines before the 5aza-dC treatment in either normoxic control conditions or hypoxic conditions. After a 6-day treatment with 5aza-dC, faint bands were detected in normoxic conditions in Mia PaCa-2, BxPC-3, and MPanc-96. When the cells were further exposed to hypoxic conditions for 24 h after the 5aza-dC treatment, all four cell lines displayed higher expressions of BNIP3. BNIP3 induction by hypoxia was 19-, 70-, 19-, and 40-fold, in Mia PaCa-2, HPAC, BxPC-3, and MPanc-96, respectively, as indicated by Q-reverse transcription-PCR (Fig. 7B). These results indicated that expression of BNIP3 under normoxic conditions and after induction by hypoxia were related, at least in part, to the methylation status of the gene.

BNIP3 Reintroduction Causes Cell Death in Pancreatic Cancer Cells. To evaluate the function of BNIP3 in pancreatic cancer, we transiently transfected a full-length cDNA for BNIP3 into PANC-1 and Mia PaCa-2 pancreatic cancer cell lines. Transfection with this construct, but not an empty vector, led to BNIP3 protein expression within 24 h after transfection (Fig. 8A). After transfection, we determined the efficiency of colony formation under selection with G418. Forced expression of BNIP3 reduced the number of colonies formed in both cell lines (Fig. 8B). We were unable to make stable cell lines that constitutively express BNIP3. To test whether BNIP3 caused cell death, we transfected a plasmid bearing BNIP3 and GFP expressed from separate cytomegalovirus promoters or a control vector bearing only GFP into PANC-1 cells and Mia PaCa-2 cells. The number of BNIP3-positive cells was evaluated by the intensity of GFP in cell lysates. The GFP intensity of cell lysates decreased after transfection with BNIP3 in a time-dependent manner, such that 75% of BNIP3-positive PANC-1 cells and 46% of BNIP3-positive Mia PaCa-2 cells were killed within 24 h after transfection (Fig. 8C). These data were confirmed by directly counting the GFP-positive cells using FACS analysis (data not shown). Thus, BNIP3 expression leads to pancreatic cancer cell death.

Restoration of Hypoxia-Inducible BNIP3 Expression Makes Pancreatic Cancer Cells More Sensitive to Hypoxia. Following the results that forced expression of BNIP3 causes pancreatic cancer cell death, we wished to determine whether the restoration of hypoxia-inducible BNIP3 expression makes pancreatic cancer cells more sensitive to hypoxia. To achieve the hypoxic induction of BNIP3 in pancreatic cancer cells that possess methylated BNIP3 genes, we made a vector (Pr-FI-BNIP3 vector) containing a full-length BNIP3 cDNA regulated by the BNIP3 promoter. This construct was trans-
fected into Mia PaCa-2 cells along with a GFP expression vector as a marker, and we then examined BNIP3 protein expression and cell death after hypoxic exposure. As expected from our reporter assays, whereas BNIP3 expression was undetectable in mock-transfected Mia PaCa-2 cells in normoxic and hypoxic conditions, its expression was clearly induced after hypoxic treatment (Fig. 9A). Furthermore, cell death analysis showed that GFP intensity of Pr-Fl-BNIP3-transfected Mia PaCa-2 cells decreased by 40% after hypoxia, whereas mock-transfected cells displayed only a slight decrease of GFP intensity. This result indicated that a greater number of Pr-Fl-BNIP3-transfected Mia PaCa-2 cells were killed by hypoxia than mock-transfected cells. Thus, restoration of hypoxia-inducible BNIP3 rendered Mia PaCa-2 cells more susceptible to hypoxic injury.

**DISCUSSION**

In the current study, we have identified BNIP3 as a tumor suppressor in pancreatic cancer, and our data suggest that silencing of BNIP3 is a molecular mechanism by which pancreatic cancer cells adapt to the hypoxic conditions that exist within these solid tumors. We have demonstrated (a) that BNIP3 is down-regulated in pancreatic cancer; (b) that BNIP3 induction in hypoxic conditions is not detectable in nearly all pancreatic cancer cell lines; (c) that CpG dinucleotides near the transcription start site of the *BNIP3* gene are methylated in pancreatic adenocarcinomas and BNIP3-negative pancreatic cancer cell lines; (d) that inhibition of DNA methylation restores BNIP3 expression under normoxic conditions and the ability of hypoxia to induce expression of BNIP3 in pancreatic cancer cell lines; (e) that reintroduction of BNIP3 by transient transfection into pancreatic cancer cells causes cell death; and (f) that restoration of hypoxia-inducible BNIP3 makes pancreatic cancer cells more sensitive to hypoxia. These observations provide important new insights into the functioning of pancreatic cancer cells.

We observed that BNIP3 mRNA expression is markedly down-regulated in pancreatic cancer tissue and cancer cell lines compared with normal pancreas. The presence of BNIP3 mRNA in normal pancreas supports the previous report that several normal human
Down-regulation of BNIP3 in pancreatic cancer

Biologic pathways, as well as several growth factors are induced by cellular viability under limited oxygen availability (25). For example, the majority of hypoxia-inducible genes contribute to maintaining hypoxia-inducible genes when exposed to low oxygen concentrations. Cells display a variety of physiological responses through hypoxia-inducible genes whose protein products play key roles in angiogenesis, vascular reactivity and remodeling, glucose and energy metabolism, and cell proliferation and survival (20). Thus, not surprisingly, expression of HIF-1α has been linked to increased tumorigenesis and tumor invasiveness (33, 34). Importantly, HIF-1α has previously been demonstrated to be overexpressed in primary and metastatic human cancers including pancreatic cancer by immunohistochemistry (35).

In our effort to elucidate the mechanisms by which BNIP3 expression is down-regulated in pancreatic cancer tissues including pancreas, heart, and liver express BNIP3 mRNA (14, 22). In contrast to our observation of BNIP3 down-regulation in pancreatic cancer, in breast cancer tissues, BNIP3 is expressed at higher levels than normal breast tissue (12). This indicates that down-regulation of BNIP3 expression is not a universal phenomenon that occurs during cancer development and progression but may be tissue specific.

Forced expression of BNIP3 killed 46 and 75% of transfected pancreatic cancer cells Mia PaCa-2 and PANC-1, respectively, within 24 h. This was not unexpected, because BNIP3 is a pro-apoptotic member of the BCL-2 family that has been shown to lead to cell death in a wide range of cultured cells (14, 15, 23). BNIP3-mediated death has been reported to occur by a pathway independent of caspase activation and cytochrome c release that is characterized by early plasma membrane and mitochondrial damage, before the appearance of chromatin condensation or DNA fragmentation (24). We did not investigate the mechanisms of cell killing by BNIP3 in the current study, but instead focused on the regulation of BNIP3 expression in pancreatic cancer.

BNIP3 belongs to the category of genes induced during hypoxia (11–13). Cells display a variety of physiological responses through hypoxia-inducible genes when exposed to low oxygen concentrations. The majority of hypoxia-inducible genes contribute to maintaining cellular viability under limited oxygen availability (25). For example, genes involved in angiogenesis, glucose uptake, and anaerobic metabolic pathways, as well as several growth factors are induced by hypoxia. Furthermore, these same pathways are often activated in cancer, suggesting that tumors take advantage of these responses to survive in the hypoxic environment that generally exists within tumors (6, 8). However, in response to hypoxia, normal cells may also diminish their proliferative rate, undergo cell cycle arrest, or even undergo apoptosis (26, 27). These growth inhibitory events can also be justified as a physiological adaptation to hypoxia. For example, cell cycle arrest might be important for cells to escape from harmful effects caused by hypoxia-induced genetic instability (28). Furthermore, hypoxia-induced cell death might help maintain tissue homeostasis by removing cells that are irreversibly damaged during oxygen deprivation. However, hypoxia-related growth inhibitory mechanisms are often missing in cancer cells (7), and our data suggest that this is the case for BNIP3 expression in pancreatic cancer.

BNIP3 induction by hypoxia is mediated by the transcription factor HIF-1 (11, 12). HIF-1 is composed of two subunits, HIF-1α and HIF-1β (29). HIF-1α is the oxygen-regulated component that determines HIF-1 activity (30). During hypoxia, accumulation of HIF-1α protein occurs because its constitutive proteolytic degradation through the ubiquitin proteasome pathway is inhibited (31, 32). Increased levels of HIF-1 activity lead to the transactivation of a number of genes whose protein products play key roles in angiogenesis, vascular reactivity and remodeling, glucose and energy metabolism, and cell proliferation and survival (20). Thus, not surprisingly, expression of HIF-1α has been linked to increased tumorigenesis and tumor invasiveness (33, 34). Importantly, HIF-1α has previously been demonstrated to be overexpressed in primary and metastatic human cancers including pancreatic cancer by immunohistochemistry (35).

In the current study, we demonstrated that HIF-1α protein expression was significantly induced after hypoxic treatment in all pancreatic cancer cell lines examined. Likewise, we observed increased expression of the HIF-1-regulated genes GLUT1 and IGFBP3 (36, 37). These data suggest that the hypoxia-HIF-1 pathway is intact and that the BNIP3 pathway is disrupted at some point after HIF-1 activation in pancreatic cancer cells. Thus, when pancreatic cancer cells are exposed to hypoxia, they may exhibit increased invasiveness and promote neovascularization, anaerobic metabolism, and proliferation through HIF-1-mediated genes; whereas they may evade hypoxia-induced cell death at least in part by lacking BNIP3 induction.

By allowing pancreatic cancer cells to survive during prolonged exposure to hypoxic stress, BNIP3 silencing may contribute to the progression of pancreatic cancer. Tumor hypoxia is thought to be associated with a malignant phenotype of tumors. Hypoxia increases the mutation rate of tumor cells, promotes metastatic potential and dedifferentiation, and decreases sensitivity to chemotherapy and radiation therapy (38–40). Moreover, it has been shown that the hypoxic status of tumors correlates with increased recurrence and a shorter survival (41–43). Therefore, it is strongly suggested that BNIP3 silencing contributes to aggressive biological nature of pancreatic cancer.

In our effort to elucidate the mechanisms by which BNIP3 expression is down-regulated in pancreatic cancer tissues and cell lines, we found that CpG dinucleotides in the CpG island of the BNIP3 promoter are densely methylated. Hypermethylation in CpG-rich regions is found in many tumors including pancreatic cancer, and this event is associated with the inactivation of cancer-related genes such as p16, E-cadherin, and hMLH1 (44, 45). In the current study, methylation of the BNIP3 gene occurred in nine of 10 pancreatic cancer cell lines and eight of 10 human pancreatic cancer tissues. Although the number of samples was relatively small, our findings suggest that BNIP3 methylation is a frequent event in pancreatic cancer. Of note, PSN-1 cells, which showed BNIP3 expression in normoxic culture conditions and prominent induction by hypoxia, displayed almost totally unmethyl-
ated BNIP3 gene by sequencing, whereas all five BNIP3-negative cells had methylated BNIP3 genes. That BNIP3 silencing was due to gene methylation was confirmed using a DNA methylation inhibitor. After DNA demethylation, BNIP3 expression was elevated under normoxic cell culture conditions, and BNIP3 induction by hypoxia was also restored. These results indicate that methylation of the BNIP3 gene plays a key role in down-regulation of BNIP3. This is the first report describing methylation of BNIP3 gene in cancer, although a recent microarray study suggested that BNIP3 expression is induced after treatment of pancreatic cancer cells with a DNA methylation inhibitor (46).

Based on the known pro-apoptotic role of BNIP3 in other cells and our data that BNIP3 was silenced in pancreatic cancer cells, we hypothesized that restoration of BNIP3 expression would increase the sensitivity of the cancer cells to apoptosis. We initially considered restoring hypoxia-inducible BNIP3 expression by using the demethylating agent. However, treatment with this agent is known to affect a large number of methylated genes including those involved in cell survival. Therefore, to more specifically restore BNIP3 expression, we generated a vector containing a full length of BNIP3 cDNA downstream of the BNIP3 promoter and transfected this construct into MiaPaCa-2 cells. Wild-type Mia PaCa-2 cells have a methylated BNIP3 gene and do not express BNIP3 under normoxic or hypoxic conditions. Transfection with the BNIP3 promoter construct restored hypoxia-inducible BNIP3 expression, and after hypoxia, a greater number of cells were killed compared with mock-transfected cells. These results indicate that the restoration of hypoxia-inducible BNIP3 expression renders pancreatic cancer cells more susceptible to hypoxia-induced cell death. In another words, the loss of BNIP3 induction by hypoxia due to gene methylation allows pancreatic cancer cells to avoid hypoxia-induced cell death.

In summary, the frequent inactivation of BNIP3 in pancreatic cancer and its role as an inducer of apoptosis support the role of this molecule as a tumor suppressor. The observation that BNIP3 expression is silenced while other hypoxia-regulated genes are induced suggests that BNIP3 silencing contributes to the survival and progression of pancreatic cancer in a hypoxic environment. Taken together, these observations suggest that BNIP3 reactivation might be a novel target for treatment of this disease.

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Silencing of the Hypoxia-Inducible Cell Death Protein BNIP3 in Pancreatic Cancer

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