

## Cyclooxygenase-Deficient Pancreatic Cancer Cells Use Exogenous Sources of Prostaglandins

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### Abstract

Genes that are differentially expressed in pancreatic cancers and under epigenetic regulation are of considerable biological and therapeutic interest. We used global gene expression profiling and epigenetic treatment of pancreatic cell lines including pancreatic cancer cell lines, pancreatic cancer-associated fibroblasts, and cell lines derived from nonneoplastic pancreata. We examined expression and epigenetic alterations of cyclooxygenase-1 (*COX-1*) and *COX-2* in pancreatic cancers and normal pancreas and performed proliferation, knockdown, and coculture experiments to understand the role of stromal sources of prostaglandins for pancreatic cancers. We identify *COX-1* as a gene under epigenetic regulation in pancreatic cancers. We find that *COX-1* expression is absent in many pancreatic cancer cells and some of these cancers also lack *COX-2* expression. Suspecting that such cancers must rely on exogenous sources of prostaglandins, we show that pancreatic cancer stromal cells, such as fibroblasts expressing *COX-1* and *COX-2*, are a likely source of prostaglandins for pancreatic cancer cells deficient in COX. Knocking down the prostaglandin transporter multidrug resistance-associated protein-4 in fibroblasts suppresses the proliferation of cocultured pancreatic cancer cells lacking COX. Pancreatic cancers that lack COX can use exogenous sources of prostaglandins. Blocking multidrug resistance-associated protein-4 may be a useful therapeutic strategy to deplete COX-deficient pancreatic cancers of prostaglandins. *Mol Cancer Res*; 8(6); 821–32. ©2010 AACR.

### Introduction

Pancreatic cancer remains the fourth leading cause of cancer-related death in the United States. There are only a few agents that have chemotherapeutic activity against pancreatic cancer cells. One therapeutic target investigated for treatment and prevention of pancreatic and other cancers is the cyclooxygenases (COX). COX are the rate-limiting step in prostaglandin synthesis and are encoded by the COX genes *COX-1* and *COX-2* [also known as prostaglandin H synthases (*PTGS*) or prostaglandin endoperoxide synthases; Fig. 1A]. *COX-1* has been generally considered the “constitutive” gene, expressed in most tissues under basal conditions, whereas *COX-2* is considered the “inducible” gene (1), undetectable in most normal tissues (2, 3) but highly expressed in a number of human cancers,

including pancreatic cancer and its precursors (4–6). Overexpression of *COX-2* results in excess prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production, which promotes cell survival and proliferation and angiogenesis (7). The importance of COX in cancer development and progression has led to many studies examining the role of nonsteroidal anti-inflammatory drugs (NSAID) in the prevention and treatment of cancers and precursor neoplasms. Thus, many observational studies have confirmed a 40% to 50% relative risk reduction of developing colorectal adenomas and cancer when comparing regular users of aspirin or NSAIDs to nonusers (8, 9). Furthermore, randomized, double-blinded, placebo-controlled trials of COX inhibition in patients with familial adenomatous polyposis have shown that these agents induce polyp regression (10–13).

Interestingly, epidemiologic studies do not support a role for NSAIDs in the prevention of pancreatic cancer (14, 15). In addition, although *in vitro* studies suggest that COX-2 inhibitors are effective against pancreatic cancer cells that express COX-2 (16), clinical trials have not found these agents to improve the treatment of patients with pancreatic cancer (17). The reasons for this apparent lack of benefit are not certain. NSAIDs also have COX-independent mechanisms, but these effects are not thought to explain the benefit of these agents in chemoprevention (10, 11, 18, 19, 20, 21). NSAID inhibition can shunt arachidonic acid metabolites down the 5-lipoxygenase pathway and 5-lipoxygenase is overexpressed in pancreatic cancers. Indeed, 5-lipoxygenase

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inhibitors have been evaluated as therapies for pancreatic cancer (22, 23). Pancreatic cancer precursors such as pancreatic intraepithelial neoplasias and intraductal papillary-mucinous neoplasms overexpress *COX-2* (24), and *COX-2* inhibitors reduce the development of pancreatic intraepithelial neoplasias in a mutant *Kras*-driven mouse model of pancreatic neoplasia (25). Polymorphisms in *COX-2* influence the level of *COX-2* transcripts (26) and may contribute to sensitivity to *COX-2* inhibitors and the development of intestinal polyps, although they do not seem to contribute to pancreatic cancer risk (ref. 27; Fig. 1A).

The putative housekeeping function of *COX-1* suggests an essential role of *COX* enzymes and prostaglandins for normal cell functions. Mice with knockout of either *COX-1* or *COX-2* display a variety of phenotypes (28). *COX* are also thought to be important in the development of pancreatitis. *COX-2* is overexpressed in chronic pancreatitis tissues, and mice lacking *COX-2* develop minimal pancreatitis whereas mice lacking *COX-1* develop severe pancreatitis (29, 30). Yet interestingly, recent studies indicate that *COX-1* expression is more restricted in normal tissues than previously appreciated (2, 3). Although many cancers overexpress *COX-2*, epigenetic silencing of *COX-2* occurs in some pancreatic and other cancers (16). During an investigation of genes silenced in pancreatic cancers, we identified cancers lacking *COX-1* expression as well as cancers lacking expression of both *COX-1* and *COX-2*. We find that *COX-1* is epigenetically silenced in many pancreatic cancers. We also find evidence that pancreatic cancers lacking *COX* enzymes can use stromal fibroblasts as a source of prostaglandins and show that targeting the prostaglandin transporter multidrug resistance-associated protein-4 (*Mrp4*) in fibroblasts can diminish the proliferation of pancreatic cancer cells.

## Materials and Methods

### Cell lines and tissue samples

Fourteen human pancreatic cancer cell lines were used (AsPC1, BxPC3, Capan1, Capan2, CFPAC1, MiaPaCa2, panc1, su86.86, panc215, A32-1, A38-5, panc2.5, panc2.8, and panc3.014). AsPC1, BxPC3, Capan1, Capan2, CFPAC1, MiaPaCa2, panc1, and su86.86 were obtained from the American Type Culture Collection. Panc215, A32-1, A38-5, panc2.5, panc2.8, and panc3.014 were obtained from the investigator who created them [Dr. James Eshleman (Johns Hopkins University, Baltimore, MD) for panc215, A32-1, and A38-5 and Dr. Elizabeth Jaffee (Department of Oncology, Johns Hopkins University, Baltimore, MD) for panc215, A32-1, and A38-5]. Cancer-associated fibroblasts (CAF) and immortalized normal fibroblasts (SC-2) were established previously in our laboratory (31, 32). Immortalized cell lines, nonneoplastic human pancreatic ductal epithelium (HPDE) and human pancreatic Nestin-expressing cells (HPNE), were generously provided by Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada) and Dr. Michel Ouellette (University of Nebraska Medical Center, Omaha, NE), respectively.

Discarded frozen normal and neoplastic tissues were obtained from patients who had undergone pancreatic resection for pancreatic adenocarcinoma or pancreatic neuroendocrine neoplasm at Johns Hopkins Hospital. We included 16 previously established pancreatic cancer xenografts as described (33). In addition, tissue microarrays (TMA) of formalin-fixed paraffin-embedded tissues were retrieved from 144 patients who underwent surgical resection at our institution. Specimens were collected and analyzed with the approval of the Johns Hopkins Committee for Clinical Investigation.

### Treatment with 5-aza-2'-deoxycytidine and trichostatin A

Cells were treated with 1  $\mu\text{mol/L}$  5-aza-2'-deoxycytidine (5-aza-dC; Sigma Chemical Co.) for 4 days and/or 1  $\mu\text{mol/L}$  trichostatin A (TSA) for 24 hours as previously described (34).

### RNA isolation

Total RNA from frozen tissues or cell lines was extracted using mirVana miRNA Isolation Kit (Ambion) following the manufacturer's protocol. Isolated total RNA was treated with DNA-free kit (Ambion) to eliminate possible DNA contamination.

### Affymetrix exon arrays

The Affymetrix Exon Array ST 1.0 (Affymetrix) was used to define gene expression profiles. Using the GeneChip Whole Transcript Sense Target Labeling Assay, labeling and hybridization were done following the manufacturer's recommendations. Data analysis was done using Partek Genomics Suite v6.3 beta (Partek, Inc.). We are in compliance with the Minimum Information about a Microarray Experiment (MIAME) guidelines and have submitted our microarray data set to the Gene Expression Omnibus repository (ref. no. GSE21163).

### Quantitative reverse transcriptase-PCR

Two micrograms of total RNA were reverse transcribed using Superscript III Reverse Transcriptase and random hexamers (Invitrogen Life Technologies) for quantitative reverse transcriptase-PCR (RT-PCR). *COX-1* and *COX-2* cDNAs were quantified using SYBR Green PCR Master Mix for SYBR Green I or TaqMan Universal PCR Master Mix (Applied Biosystems). PCR was done on an ABI 7300 real-time thermocycler. Primers and probes for *COX-1* and *COX-2* were as previously described (35). The housekeeping gene *GAPDH* or *PGKI* was used as a reference for SYBR Green and *18s-rRNA* (Applied Biosystems) was used for TaqMan (Supplementary Table S1).

### Bisulfite-modified sequencing and methylation-specific PCR

The methylation status of the 5' CpG sites of *COX-1* was determined by bisulfite-modified sequencing and methylation-specific PCR (MSP) as previously described (ref. 36; primer sequences are provided in Supplementary

Table S1). For MSP, *Sss*I methylase (SSSI)-treated DNA (New England Biochemicals) and whole-genome amplified DNA (Qiagen, Inc.) were used as controls for methylated and unmethylated DNA, respectively.

### Chromatin immunoprecipitation

Chromatin immunoprecipitation was done as previously described (37). Briefly, cells were treated with 1% (v/v) formaldehyde and cross-links were quenched with glycine. Cells were rinsed with ice-cold PBS with protease inhibitors (Roche Applied Science), scraped, and collected by centrifugation before being resuspended in lysis buffer plus protease inhibitors. Chromatin was sheared with the Bioruptor system (Diagenode). Antibodies to the repressive mark H3K27m3 and the active mark acetylated H3 or normal IgG as a control were used. DNA was PCR amplified and quantified using SYBR Green with COX-1 primers (fwd CACCAGGCATCAGAAACGTA, rev CT-CCCTCCAGCTGTACC). The percentage enrichment of immunoprecipitated DNA was calculated relative to input DNA (20 ng):  $dCt(H) = Ct(H) - Ct(i)$ ,  $dCt(IgG) = Ct(IgG) - Ct(IgG)$ , and  $ddCt = dCt(H) - ddCt(IgG)$ , where *i* is immunoprecipitation and *H* is the histone mark.

### Immunohistochemistry

Immunohistochemical labeling was done using HRP EnVision<sup>+</sup> System (DAKO Corp.) on TMA slides as previously described (38). Deparaffinized slides were subjected to heat-induced epitope retrieval using a steamer and DAKO Target retrieval solution (pH 6.0-6.2). Slides were incubated with rabbit polyclonal anti-ovine COX-1 (Cayman Chemical) diluted to 1:1,000 at 4°C overnight. Collecting ducts of kidney and glial cells express COX-1 and were used as positive controls for inter-slide normalization (3). The immunostaining area was categorized into four scores as follows: 0, 0% to 5% of labeled tumor cells; 1, 6% to 25%; 2, 26% to 50%; 3, 51% to 75%; and 4, above 76%. A score of 0 to 2 was attributed to the immunostaining intensity as follows: 0, no appreciable labeling; 1, mild; and 2, strong intensity. The scoring index was determined by multiplying the area score and the intensity score.

### Western blotting

Western blotting was done as previously described (39) using the primary antibodies rabbit polyclonal anti-ovine COX-1, diluted at 1:2,000, mouse monoclonal anti-human COX-2, diluted at 1:1,000 (Cayman Chemical), or rabbit polyclonal anti-GAPDH (Sigma).

### MTS assay

Aspirin was used as a COX inhibitor (0.4-10 μmol/L; Sigma) and nordihydroguaiaretic acid was used as a lipoxigenase inhibitor (Calbiochem). Cells were seeded (4,000 per well) onto 96-well plates, incubated overnight, washed twice with PBS, and serum starved for 24 hours before replacing with 1% FCS medium containing aspirin (0.4-10 μmol/L), nordihydroguaiaretic acid (1.01-100 μmol/L),

or DMSO for another 72 hours. Cell proliferation was quantified by adding 20 μL of CellTiter Aqueous One Solution (Promega) into each well containing 100 μL of culture medium and incubating for 2 h at 37°C. Absorbance at 490 nm was measured using a microplate reader (Perkin-Elmer). Dose-response graphs were performed in triplicate and linear regression lines were used to calculate IC<sub>50</sub> values.

### Measurement of PGE<sub>2</sub> levels

Cells ( $1 \times 10^4$  per 500 μL of 1% FCS-DMEM) were seeded into 24-well microplates and grown for 48 hours, and PGE<sub>2</sub> in the culture medium was determined (Prostaglandin E<sub>2</sub> Express EIA kit, Cayman Chemical) according to the manufacturer's instructions.

### PGE<sub>2</sub> treatment

Cells were seeded (10,000 per well) onto 96-well plates, incubated overnight, washed twice with PBS, and serum starved for 48 hours before replacing with medium (1% FCS) containing 1 or 10 μmol/L of PGE<sub>2</sub> (Sigma). Cell proliferation was quantified by MTS assay.

### Small interfering RNA transfection and pancreatic cancer/fibroblast coculture

A small interfering RNA (siRNA) targeting *MRP4* (SMARTpool, L-007313-00-0005) and a nontargeting control siRNA were obtained from Dharmacon. CAF19 fibroblasts were seeded ( $1 \times 10^5$  per well) in the lower wells of 24-well plates (BD Biosciences) and incubated for 24 hours. Cells were transfected with *MRP4* siRNA or control siRNA (100 nmol/L) using DharmaFECT4 transfection reagent, incubated for 24 hours, and subjected to coculture. RT-PCR primers used to amplify *MRP4* were F: CGAGTAGCCATGTGCCATATGA and R: TGACTATCTGGCCTGTGGTTGTCT.

Pancreatic cancer cells AsPC-1, MiaPaCa2, or BxPC3 were seeded separately ( $1 \times 10^4$ ) on polycarbonate membrane with 0.45-μm pores in the Transwell permeable support inserts (Corning, Inc.) and serum starved for 48 hours. Cells were cocultured with fibroblasts for 48 hours in 1% FCS-DMEM with or without PGE<sub>2</sub>. Culture medium PGE<sub>2</sub> was quantified as above and cell number quantified using the MTS assay.

### Statistical analysis

Values are reported as means ± SD. All data were normally distributed and underwent equal variance testing. Statistical analysis of gene expression array data was determined by Partek Genomics Suite v6.3B. Raw Affymetrix intensity measurements of all probe sets were background corrected and normalized by the Robust Multichip Average method. Sample relationships were examined using principal component analysis to reveal any technical effects that would encumber the subsequent analysis. Gene expression intensities were summarized by the one-step Tukey's biweight method. Two-way ANOVA analysis was done to identify significant expression changes between cancer cells and the

noncancer cell lines and between 5-aza-dC- and/or TSA-treated cancer cells and untreated cancer cells, based on a fold change criteria of  $\pm 5$ -fold and a  $P$  value of  $<0.001$ .

## Results

### Identification of epigenetically regulated genes

To identify genes that are silenced in pancreatic cancers and regulated by epigenetic mechanisms, we compared the gene expression profiles of six pancreatic cancer cell lines (panc215, A32-1, A38-5, panc2.5, panc2.8, and panc3.014) to the nonneoplastic pancreas cell line HPDE and to nine pancreatic fibroblast lines including seven pancreatic cancer-associated fibroblast lines and fibroblast lines derived from nonneoplastic pancreas (HPNE and SC2). We also compared the baseline gene expression of the pancreatic cancer cell lines to expression patterns after treatment with 5-aza-dC alone, TSA alone, and the combination of 5-aza-dC/TSA. Gene expression profiles were obtained using the Affymetrix Exon Array ST 1.0, which contains 1.4 million probe sets representing known full-length and alternate spliced mRNAs.

Data quality was visualized using principal component analysis using the distribution of probe intensities for all samples (see Materials and Methods). For example, the cancer cell lines robustly separated from CAFs, HPNE, and SC-2, but were relatively similar to the nonneoplastic epithelial cell line HPDE (Fig. 1B).

To search for epigenetically silenced genes in pancreatic cancer, we identified genes with reduced expression in the pancreatic cancer cell lines relative to the nonneoplastic cell lines, and then merged this list to the list of genes significantly induced by epigenetic drug treatment using Tukey's biweight method. As shown in the three-dimensional volcano plot (Supplementary Fig. S1), subsequent fold-change and ANOVA analyses showed that 702 of 21,980 (3.19%) genes were expressed at significantly lower levels ( $<5$ -fold lower and  $P < 0.001$  by ANOVA test) in the six pancreatic cancers compared with the nonneoplastic pancreatic samples. Among the 702 genes underexpressed in the pancreatic cancer cell lines, 10 genes were upregulated ( $>5$ -fold) in response to the combination epigenetic treatment with 5-aza-dC and the histone deacetylase inhibitor TSA in these pancreatic cancer cell lines. This criterion identified *TFPI-2*, a gene previously identified as epigenetically silenced in pancreatic and other cancers (ref. 40; Supplementary Fig. S1).

The most interesting of the 10 genes identified was *COX-1* [prostaglandin H synthase-1 (*PTGS-1*)]. *COX-1* expression was absent in most pancreatic cancers by microarray compared with modest expression in HPDE and higher levels in the fibroblast lines (Fig. 1C). By array, several pancreatic cancer cell lines showed reexpression by treatment either with the DNA methyltransferase inhibitor 5-aza-dC or with the histone deacetylase inhibitor TSA (Fig. 1C, red or blue dots, respectively, compared with purple dots). Treatment of cancer cell lines with the

combination of 5-aza-dC and TSA further increased the reexpression of *COX-1* in five of the six pancreatic cancer cell lines studied compared with 5-aza-dC or TSA treatment alone (Fig. 1C, green dots).

We also found that either 5-azaC or the combination of 5-aza and TSA increased *COX-2* mRNA expression in five of six pancreatic cancer cell lines (3.69-fold change,  $P = 0.0014$ ; Supplementary Fig. S2). As previous studies have examined the epigenetic regulation of *COX-2*, we focused our attention on *COX-1* expression.

We confirmed the differential expression of *COX-1* treatment in response to 5-aza-dC or TSA treatment by quantitative RT-PCR. Pancreatic cancer cell lines treated with these agents either alone or in combination led to robust induction of *COX-1* mRNA in several cancer lines after drug treatment, but not in the nonneoplastic pancreatic samples, HPDE and HPNE (Fig. 2A). The effect of epigenetic treatment on *COX-2* expression was also examined (see Supplementary Fig. S2).

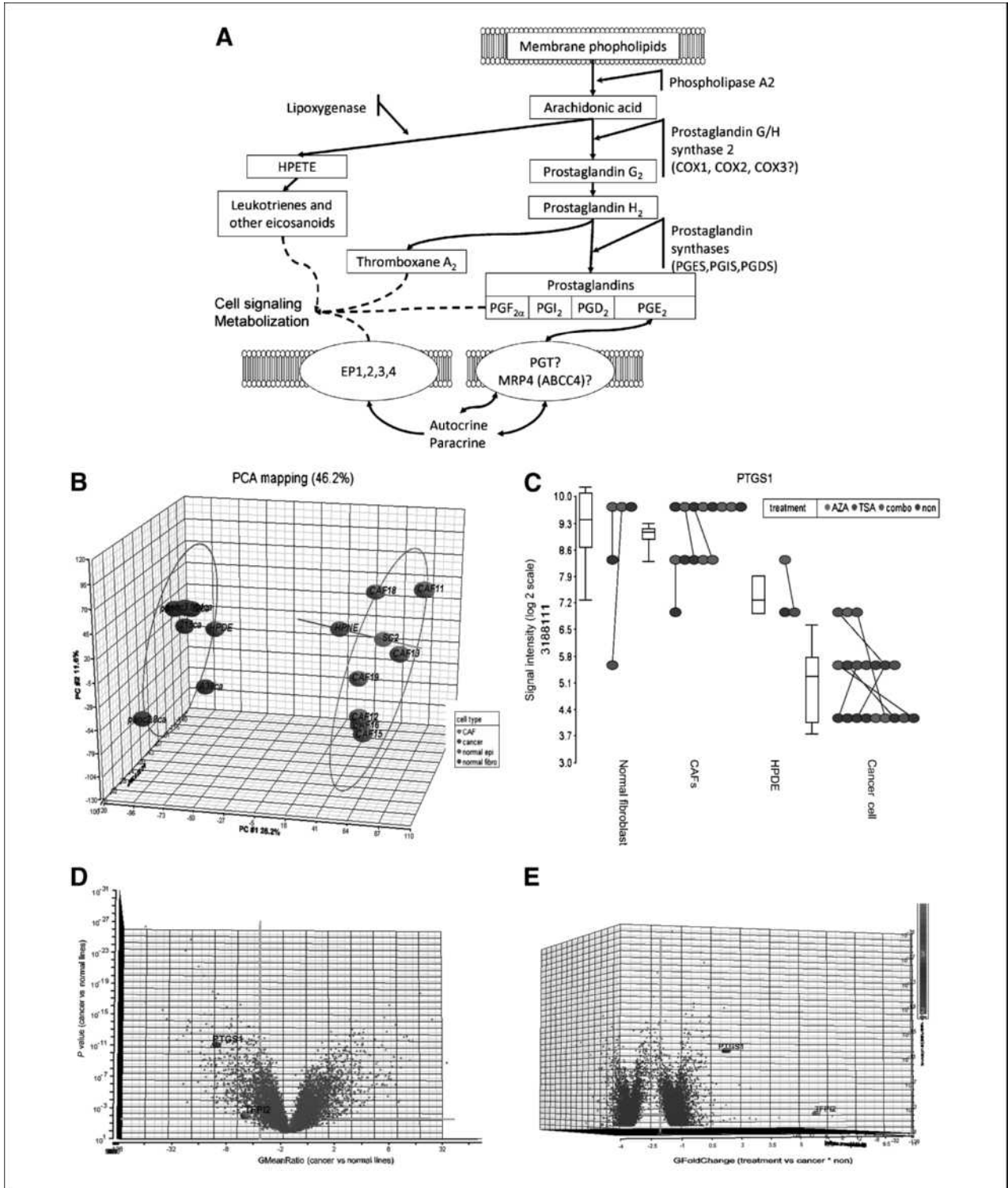
We then verified the DNA methylation of the *COX-1* promoter by bisulfite sequencing. The *COX-1* promoter has a CpG-rich region (length = 343, %GC = 62.8, observed CpG/expected CpG = 0.652) around the 5'-untranslated region. Sequencing of 12 CpG sites within the product 400-bp region ( $-188$  to  $+212$  of the transcription start site; Fig. 2B) revealed all CpG sites to be unmethylated (converted to T by bisulfite modification) in the nonneoplastic *COX-1*-expressing cell line HPDE, whereas the non-*COX-1*-expressing cell line MiaPaCa2 was highly methylated in all of the CpGs sequenced. Several other non-*COX-1*-expressing pancreatic cancer cell lines (e.g., panc215, panc2.8, and panc3.014) had unmethylated CpGs.

Further analysis of the *COX-1* promoter for aberrant methylation using MSP revealed that MiaPaCa2 was the only completely methylated pancreatic cancer cell line of the 11 lines examined, consistent with bisulfite sequencing data (representative data is shown in Fig. 2B). MSP analysis of *COX-1* in pancreatic cancer xenografts and normal pancreas tissues found 3 of the 16 cancers (18%), but 0 of 7 normal pancreata, to be methylated (data not shown).

We next performed quantitative chromatin immunoprecipitation-PCR of the *COX-1* promoter using antibodies to active and inactive chromatin marks. The repressive mark was more abundant by chromatin Immunoprecipitation-PCR in the pancreatic cancer cell lines panc215 and panc2.5 that lacked *COX-1* expression compared with the *COX-1*-expressing cell line HPDE (see below for expression). In contrast, there was no significant difference in the level of acetylated H3 in any of the three cell lines (see Fig. 2C).

### *COX-1* and *COX-2* mRNA expression in pancreatic cancer cells

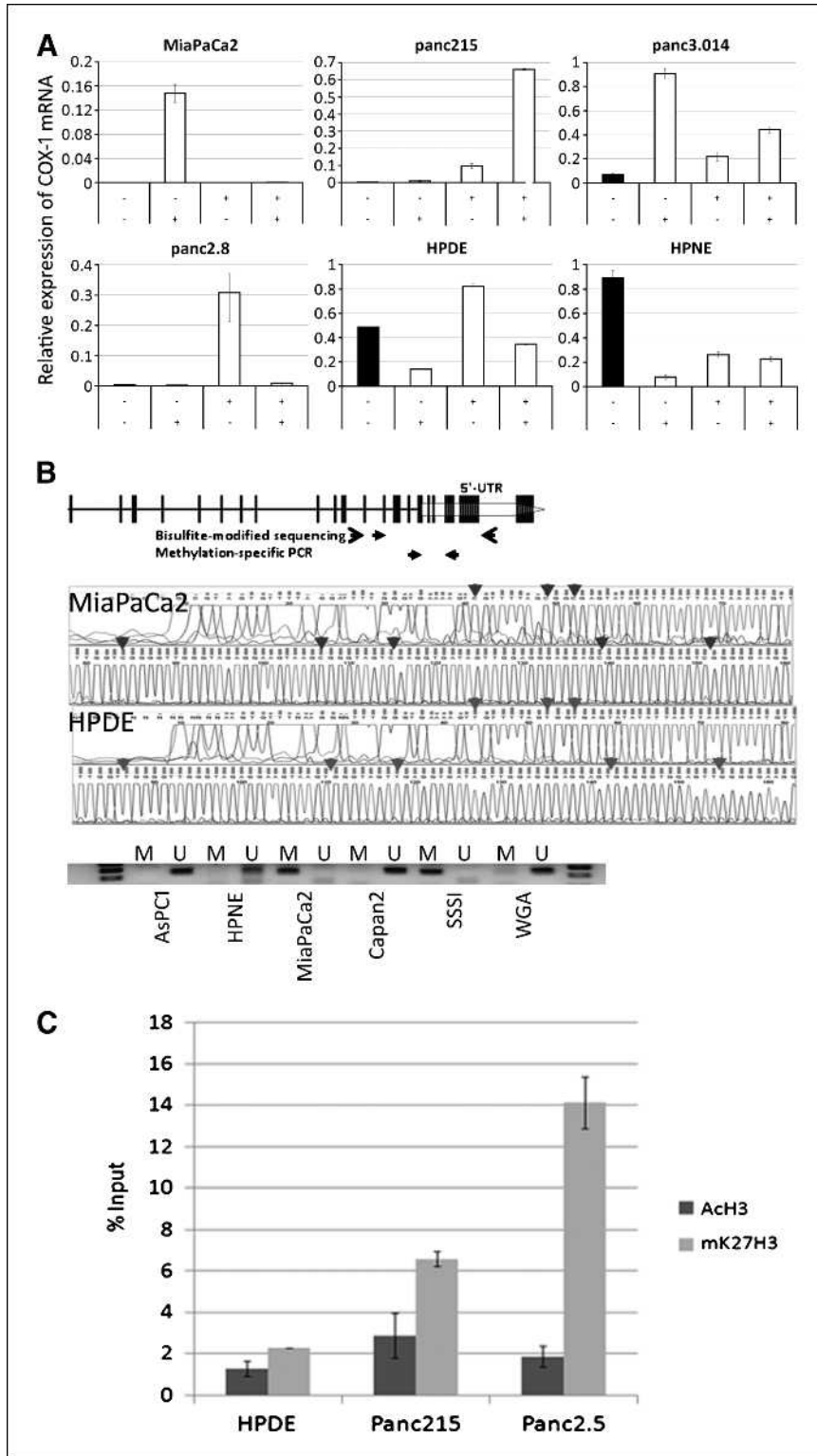
Because different pancreatic cancers are known to overexpress as well as silence *COX-2*, we further examined *COX-1* (Fig. 3A) and *COX-2* expression (Fig. 3B) in pancreatic cancer cell lines versus nonneoplastic pancreatic cells using



**FIGURE 1.** A, the arachidonic acid cascade. B, principal components analysis plots of Affymetrix exon array gene expression of pancreatic cancers and nonneoplastic pancreatic samples. Each data point represents one sample and the ellipse is drawn at 2 SD around the centroid of the samples; 6 pancreatic cancer lines (blue), 7 pancreatic cancer-associated fibroblasts (red), 1 nonneoplastic pancreatic duct line (green), and 2 nonneoplastic pancreatic fibroblasts (purple). The x-axis represents the first principle component (PC), the y-axis represents the second PC, and the z-axis represents the third PC (C) PTGS1 (COX-1) expression by Affymetrix exon array. Each cell line was treated with 5-aza-dC (red), TSA (blue), the combination (green), or mock treated (purple). Each dot represents one sample and each cell line is connected by a solid line. The box plot shows the variance among each type of cell lines. Signal intensity is plotted in log 2 scale.

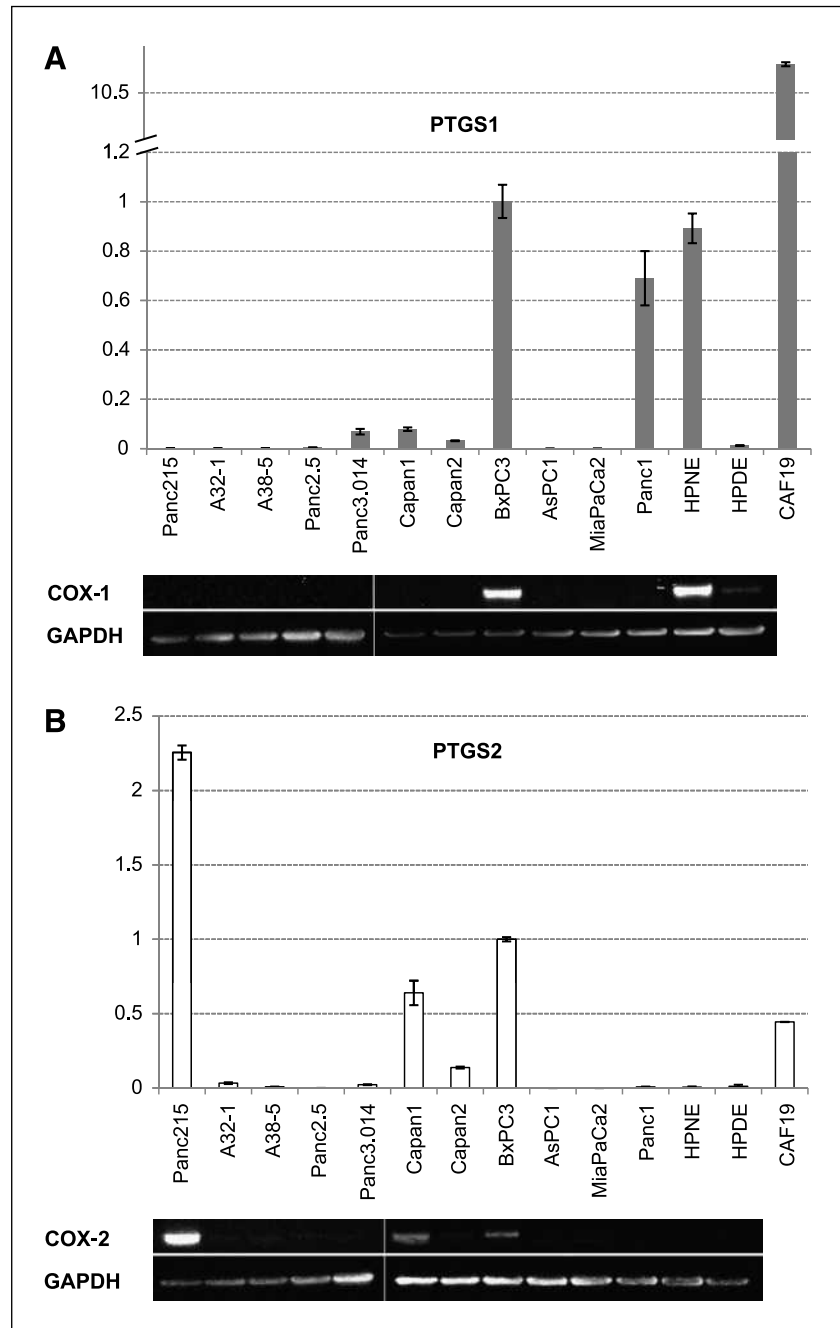
quantitative RT-PCR. Surprisingly, 5 of 11 pancreatic cancer cell lines examined lacked expression of both *COX-1* and *COX-2* RNA. All the nonneoplastic pancreatic fibroblast lines expressed *COX-1* and *COX-2*. We also

examined our Serial Analysis of Gene Expression (SAGE) data for *COX-1* and *COX-2* in normal pancreatic duct and 24 pancreatic cancers (41). There were no detectable SAGE tags for *COX-1* in primary pancreatic duct whereas



**FIGURE 2.** A, effect of epigenetic treatment with 5-aza-dC and/or TSA on pancreatic *COX-1* expression. *COX-1* mRNA was measured by quantitative RT-PCR. Ct values were normalized by *GAPDH* and calibrated by the *COX-1* level of BxPC3. The data are represented as means ( $n = 3$ )  $\pm$  SD. B, primers used in bisulfite sequencing and MSP. Broken arrow represents the 5'-untranslated region of *COX-1*. Vertical lines represent each CpG dinucleotide and black solid arrows indicate primer locations. Middle, a representative bisulfite sequencing chromatogram; bottom, MSP. After bisulfite treatment, methylated cytosines remain unmodified (C; represented by blue peaks and arrows), but unmethylated cytosines are converted to thymines (T; red peak and arrow). By MSP, the MiaPaCa2 and the SSSI-treated DNA are methylated (M) but the other samples are unmethylated (U) WGA, whole-genome amplified. C, quantitative chromatin immunoprecipitation-PCR analysis of the *COX-1* promoter using antibodies to acetylated H3 and mK27H3. Y-axis reveals the percentage enrichment of *COX-1* DNA with immunoprecipitation relative to input DNA.

**FIGURE 3.** A, expression level of *COX-1* was measured by quantitative RT-PCR [mean  $\pm$  SD ( $n = 3$ ); top] and Western blot (bottom). B, expression level of *COX-2* was measured by quantitative RT-PCR [mean  $\pm$  SD ( $n = 3$ ); top] and Western blot (bottom). For quantitative RT-PCR, Ct values were normalized by *GAPDH* and calibrated by BxPC3 expression. For protein expression analysis by Western blot, *GAPDH* (~36 kDa) was used as a reference protein. Note that due to insufficient sample, there are no protein data for CAF19.



*COX-2* tags were detectable (124 of ~1.5 million tags). Eleven pancreatic cancers did not have any *COX-1* SAGE tags (of >1 million tags sequenced per sample). Of the remaining 13 pancreatic cancers analyzed, only 9 had more than 5 *COX-1* tags. *COX-2* expression was more variable, with several pancreatic cancers expressing high levels of *COX-2* and 8 of 24 expressing little or no *COX-2* (<6 tags per sample).

Western blotting confirmed the RNA expression patterns (Fig. 3A and B, bottom). Bands of the expected size (~70 kDa) were consistent with quantitative RT-PCR

results, except that cell lines with low levels of RNA by quantitative RT-PCR did not have detectable protein by Western blot analysis.

#### Immunohistochemical analysis of pancreatic *COX-1* expression

To confirm the RNA expression patterns of *COX-1*, we performed immunohistochemical labeling of primary pancreatic adenocarcinomas and corresponding nonneoplastic pancreatic tissues on TMAs. Recent evidence suggests that *COX-1* is expressed in certain organs such as the collecting

ducts in the kidney, astrocytes in the central nervous system, and endocrine cells in crypts of intestines (3). We found these tissues (kidney, small intestine, and brain) to express COX-1 on our TMAs and served as an internal control. In pancreas tissues, positive labeling was clearly detectable in interstitial cells and in spindle-shaped cells in pancreatic duct, but expression was usually undetectable in other pancreatic duct epithelial cells (Fig. 4).

Of 140 primary pancreatic cancers evaluated, only 14 cases (10%) showed diffuse immunolabeling for COX-1 (labeling score  $\geq 4$ ; Fig. 4B and C), whereas expression was only focal or completely absent throughout the tumor in the remaining cases (score  $< 4$ ; Fig. 4A). In contrast, CAFs and stromal inflammatory cells displayed moderate to strong COX-1 labeling (Fig. 4A and B).

#### PGE<sub>2</sub> levels of pancreatic cell lines

Because COX expression is important for the generation of prostanoids, we examined PGE<sub>2</sub> levels in a panel of pancreatic cancer cell lines as well as control lines. As expected, pancreatic cancers lacking both COX-1 and COX-2 expression (e.g., A32-1, A38-5, panc2.5, AsPC1, and MiaPaca2) showed very low levels of PGE<sub>2</sub> in their culture media ( $< 100$  pg/mL; Fig. 5A). We suspect that these very low levels of PGE<sub>2</sub> detectable in cancer cell lines lacking both COX-1 and COX-2 expression are due to the 1% FCS required to grow the cells. In contrast, the COX-1- and COX-2-expressing cell line BxPC3 and the pancreatic cancer-associated fibroblast line CAF-19 had higher levels of PGE<sub>2</sub> ( $282.25 \pm 23.69$  and  $1,140.69 \pm 89.57$  pg/mL, respectively). Pancreatic cancer cell lines expressing either COX-1 or COX-2 or both

(e.g., panc215 and Capan1; Fig. 3) had higher levels of PGE<sub>2</sub> overall in their culture media than did the cell lines without COX expression (Fig. 5A).

#### Effects of COX and lipoxygenase inhibitors on pancreatic cancer cells

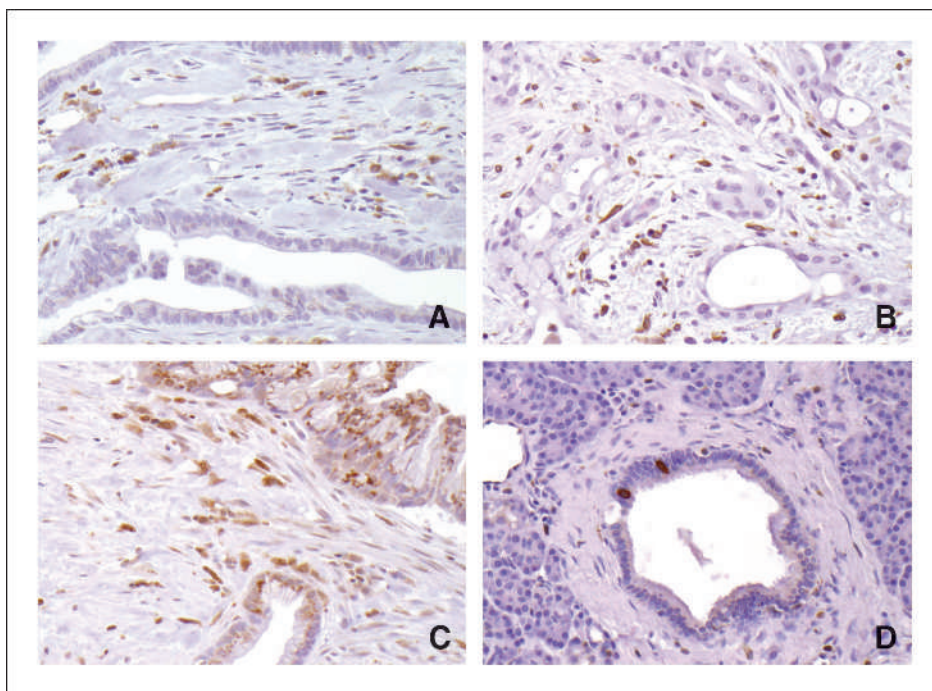
We considered that sensitivity to COX and lipoxygenase inhibitors might vary by their COX gene expression status, but found no evidence for differences among eight pancreatic cancer cell lines in response to COX and lipoxygenase inhibitors by their baseline COX expression as measured by IC<sub>50</sub> determined using MTS assay (Supplementary Fig. S3).

#### Effect of PGE<sub>2</sub> on pancreatic cancer cell lines

Pancreatic cancer cell lines without COX expression (AsPC1 and MiaPaCa2) treated with PGE<sub>2</sub> (1 and 10  $\mu$ mol/L) had significant increases in cell proliferation compared with untreated cells, whereas the COX-expressing line BxPC3 showed smaller but still significant increases in proliferation (Fig. 5B).

#### Knockdown of the prostaglandin transporter MRP4

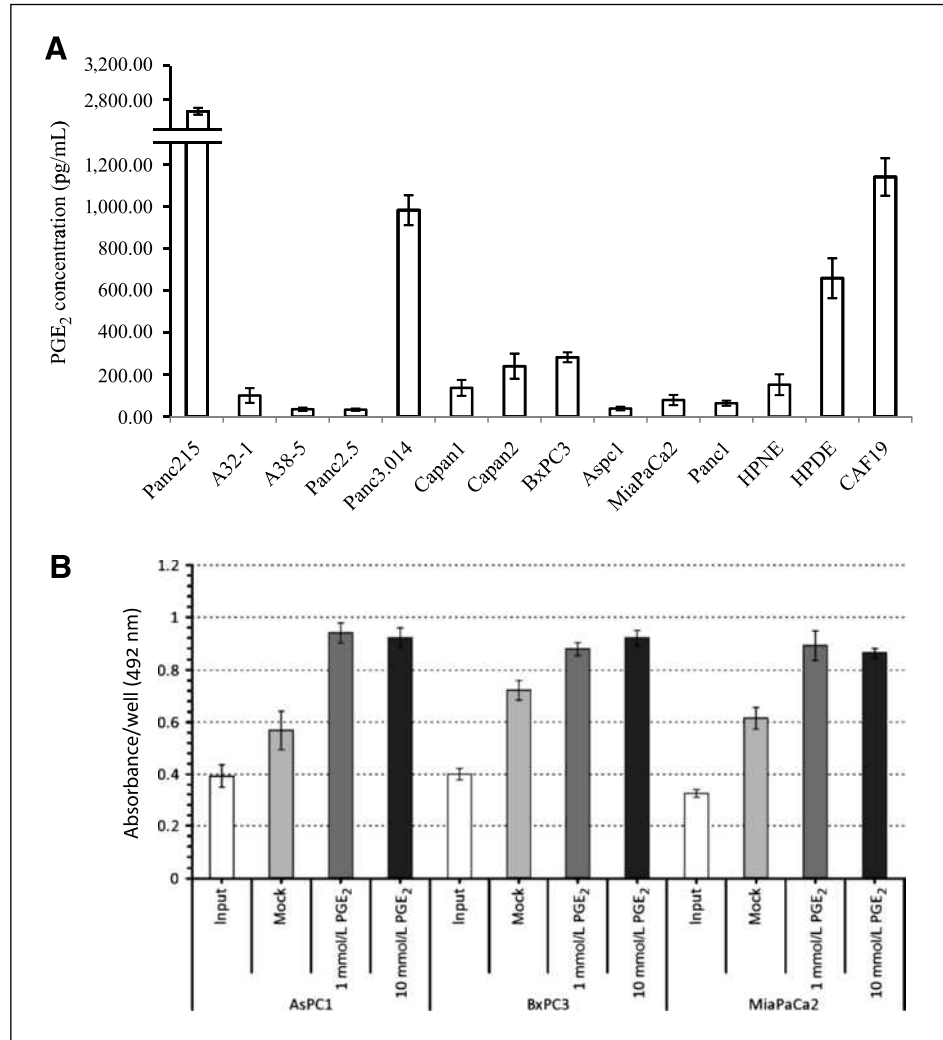
Because pancreatic cancer-associated fibroblasts, such as CAF19, produce and release PGE<sub>2</sub>, we hypothesized that blocking the release of PGE<sub>2</sub> from pancreatic cancer-associated fibroblasts could be a selective strategy to therapeutically target pancreatic cancer cells that fail to produce PGE<sub>2</sub> because of their lack of COX. The main prostaglandin transporter is MRP4 (42). We therefore knocked down MRP4 in CAF19 cells. Using a Dharmacon SMARTpool of MRP4 siRNAs, we were able to knock down MRP4 with 80% to 90% efficiency (Fig. 6A). Cell



**FIGURE 4.** COX-1 expression in primary pancreatic tissues by immunohistochemistry. A and B, cancer tissues lacking COX-1 labeling, with labeling in CAFs and lymphocytes. C, cancer tissue with COX-1 labeling both in cancer cells and in CAFs. D, Normal pancreatic ductal epithelial cells do not show labeling with COX-1 apart from a few strongly positive ductal endocrine cells ( $\times 20$  magnification).



**FIGURE 5.** A, PGE<sub>2</sub> concentrations in conditioned media from pancreatic cell lines. B, pancreatic cancer cell proliferation in response to PGE<sub>2</sub> by MTS colorimetric assay.



culture media from untreated CAF19 cells had higher PGE<sub>2</sub> levels than culture media from MRP4-knockdown fibroblasts (Fig. 6B). To determine if fibroblast PGE<sub>2</sub> contributed to cancer cell growth, we cocultured three pancreatic cancer cell lines (AsPC1, BxPC3, and MiaPaCa2) with CAFs with and without siRNA-mediated knockdown of *MRP4* (CAF19-KD and CAF19-control, respectively). Media from *MRP4*-knockdown CAFs had lower levels of PGE<sub>2</sub> when cocultured with pancreatic cancer cell lines lacking COX (AsPC1 and MiaPaCa2), but not with the COX-expressing line BxPC3 (Fig. 6C). Moreover, when cocultured with the knockdown CAFs, AsPC1 and MiaPaCa2 both showed a markedly slower growth (Fig. 6D, dark gray columns) that was overcome by the addition of exogenous PGE<sub>2</sub> in the culture media (Fig. 6D, light gray columns).

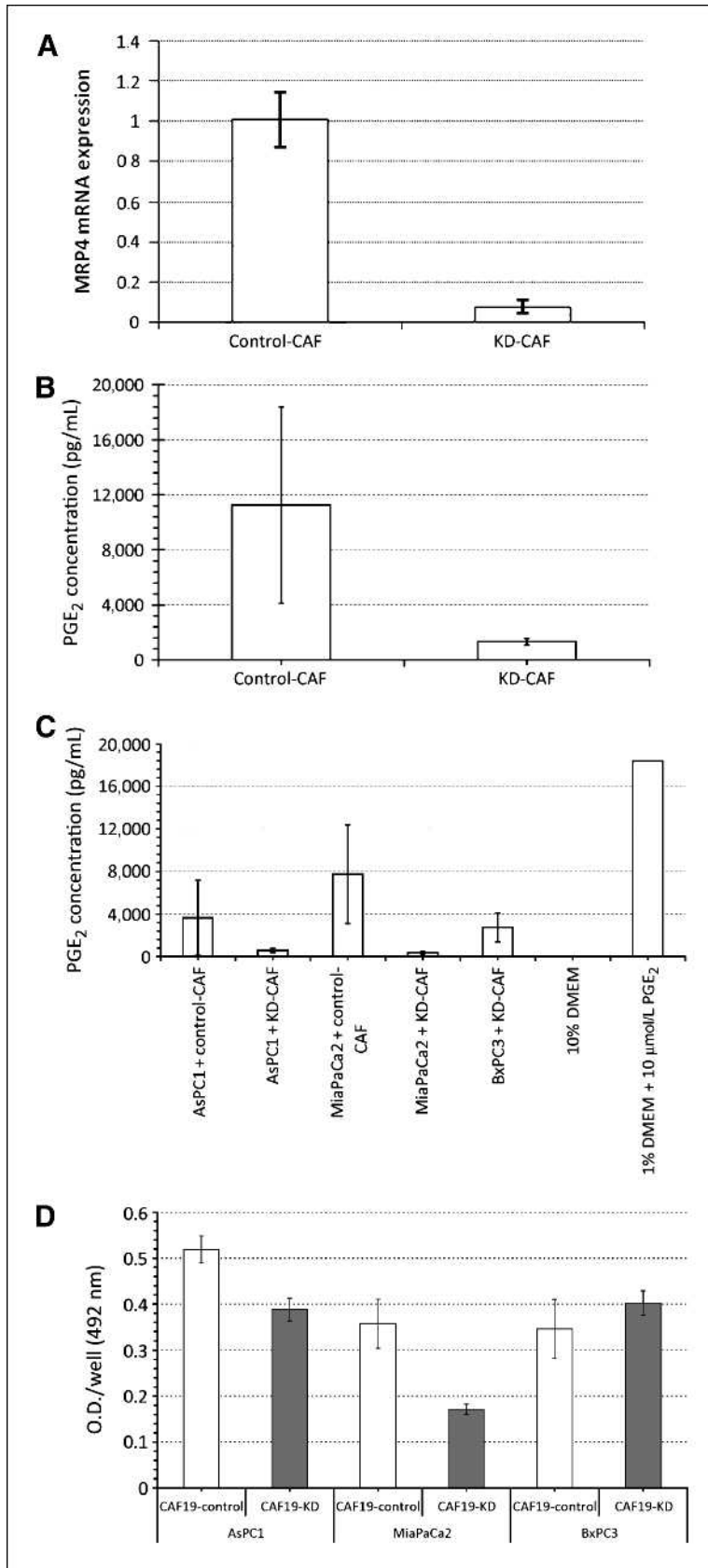
## Discussion

Using a global DNA methylation profiling strategy, we find that *COX-1* is epigenetically regulated in pancreatic cancers

and is not expressed in most normal pancreatic duct cells. Furthermore, as epigenetic silencing of *COX-2* can also occur during pancreatic cancer development (16), some pancreatic cancers evolve to lack both COX. Because stromal cells adjacent to infiltrating pancreatic cancers such as fibroblasts, endothelial cells (43), and inflammatory cells express high levels of *COX-1* and *COX-2*, these cells are a likely source of prostaglandins for pancreatic cancer cells deficient in COX. Although pancreatic cancer cells lacking COX do not produce PGE<sub>2</sub>, they still proliferate in response to PGE<sub>2</sub>. Previous studies have identified cancers lacking *COX-2* expression, but to our knowledge, no prior studies have recognized that cancer cells could be deficient in both *COX-1* and *COX-2*.

Until recently, *COX-1* had been reported to be the constitutive and *COX-2* the inducible COX. It is now clear that many tissues lack *COX-1* expression and based on our gene expression and immunohistochemical data, it seems that pancreatic duct cells rely on *COX-2* rather than *COX-1* to produce prostaglandins (6, 44).

The lack of expression of *COX-1* in pancreatic duct may help explain epidemiologic studies showing that aspirin,



**FIGURE 6.** A, effect of MRP4 siRNA on MRP4 mRNA expression in CAF19 cells. B, effect of MRP4 knockdown on PGE<sub>2</sub> concentrations. C, PGE<sub>2</sub> concentrations in pancreatic cancers and CAF cocultures. AsPC1 and MiaPaCa2 lack COX expression whereas BxPC3 expresses COX-1 and COX-2. KD, MRP4 knockdown by siRNA in CAFs. For positive control, 1% DMEM containing 10 μmol/L PGE<sub>2</sub> was used. D, pancreatic cancer cell proliferation in response to coculture with CAFs transfected or not with MRP4 siRNA.

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which is primarily a COX-1 inhibitor, does not prevent the development of pancreatic cancer, but reduces colon cancer incidence and mortality (16).

We hypothesized that the lack of COX expression in some pancreatic cancer cells may render them dependent on exogenous PGE<sub>2</sub>, such as from stromal fibroblasts. Prostaglandins are secreted by most cells and act as autocrine and paracrine signaling molecules requiring controlled release, uptake, and metabolism to initiate and terminate signaling (7). The main efflux transporter of PGE<sub>2</sub> is Mrp4, which exports PGE<sub>2</sub> to the extracellular milieu (45, 46). Exported PGE<sub>2</sub> can bind to transmembrane prostaglandin receptors. The other main transmembrane prostaglandin transporter (PGT) carries PGE<sub>2</sub> into the cytoplasm. Interestingly, Mrp4 is overexpressed in colorectal and other cancers (47). In model systems, MRP4 knockout results in a pronounced reduction in extracellular PGE<sub>2</sub>, and Mrp4 is inhibited by certain NSAIDs (45, 48). We find that stromal fibroblasts supply PGE<sub>2</sub> to pancreatic cancer cells, and blocking Mrp4-dependent PGE<sub>2</sub> excretion from fibroblasts reduces the proliferation of pancreatic cancer cells lacking COX. Thus, inhibition of Mrp4 may represent a useful treatment strategy for pancreatic cancer cells deficient in COX. Inhibiting Mrp4 could be a more effective strategy for targeting the COX pathway in pancreatic cancers because COX inhibitors will not have any direct effect on pancreatic cancer cells lacking COX expression. COX inhibitors could still target stromal fibroblast COX expression, but because fibroblasts express both COX-1 and COX-2, nonselective COX inhibitors would be required, with greater potential for systemic toxicity. The provision of prostaglandins by stromal fibroblasts may partly explain why

COX-2 inhibitors have not been shown to be effective in treating pancreatic cancers (49). It is not known if inhibiting PGE<sub>2</sub> production by blocking Mrp4 would be a more targeted and therapeutically safer approach to blocking PGE<sub>2</sub> effects. An alternative approach to inhibiting the cancer-promoting effects of PGE<sub>2</sub> would be to inhibit PGE<sub>2</sub> receptors (50-52), but because there are four PGE<sub>2</sub> receptors, EP1, EP2, EP3, and EP4, this might require blocking multiple receptors.

In summary, we find evidence for epigenetic regulation of *COX-1* in pancreatic cancers and show that most pancreatic cancers lack *COX-1* expression. Indeed, some pancreatic cancers are devoid of either *COX-1* or *COX-2* expression, rendering them dependent on exogenous sources of prostaglandins. Inhibiting the efflux of prostaglandins from stromal fibroblasts by blocking the prostaglandin transporter MRP4 inhibits the proliferation of pancreatic cancer cells lacking COX.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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