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Loss of 15-Hydroxyprostaglandin Dehydrogenase Increases Prostaglandin E₂ in Pancreatic Tumors

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Abstract

Objectives—Prostaglandin E₂ (PGE₂) is a product of cyclooxygenase (COX) and prostaglandin E synthase (PGES) and deactivated by 15-hydroxyprostaglandin dehydrogenase (PGDH). Down-regulation of PGDH contributes to PGE₂ accumulation in lung and colon cancers but has not been identified in pancreatic cancer.

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Methods—Human pancreatic normal and tumor matched tissues as well as MiaPaCa-2 and BxPC-3 cell lines were assessed for COX-2, mPGES-1, PGDH, SNAI1 and SNAI2 expression by RT-PCR and Western blotting and PGE₂ by ELISA.

Results—Normal tissues exhibited low COX-2 mRNA and protein expression, high PGDH mRNA and protein expression and PGE₂ levels at 13 pg/mg protein. In contrast, tumor tissues exhibited high COX-2 mRNA and protein expression, low PGDH mRNA and protein expression and PGE₂ levels at 32 pg/mg protein. Tumor tissues exhibited significantly elevated expression of SNAI2 mRNA and protein but not SNAI1 as SNAI1 and SNAI2 reportedly down-regulate PGDH expression. COX-2-positive BxPC-3 but not COX-2-negative MiaPaCa-2 treated with 100 nM of PGE₂ induced pERK that was blocked by MEK inhibitor U0126, demonstrating the ability of PGE₂ to activate ERK.

Conclusions—These results suggest that enhanced PGE₂ production proceeds through the expression of COX-2 and mPGES-1 and down-regulation of PGDH by SNAI2 in pancreatic tumors.

Keywords

prostaglandin E₂; cyclooxygenase-2; 15-hydroxyprostaglandin dehydrogenase; microsomal prostaglandin E synthase; SNAI2

INTRODUCTION

Prostaglandin E₂ (PGE₂) exerts pleiotropic biological effects on a variety of cells and tissue.¹ Excess levels of PGE₂ are implicated in mediating several types of human malignancies, including pancreatic cancer.^{2,3} PGE₂ mediates angiogenesis in promoting endothelial cell migration, capillary morphogenesis and vascular endothelial growth factor expression in pancreatic adenocarcinoma cells as well as neovascularization in vivo.^{4,5} PGE₂ biosynthesis is controlled at cyclooxygenase (COX) conversion of arachidonic acid to PGH₂.⁶ COX encompasses a constitutive COX-1 and an inducible COX-2 isoform that are structurally similar but differ in their transcriptional regulation.⁷ COX-2 is an immediate-early response gene activated by growth factors, cytokines and other factors and is over-expressed in pancreatic cancers.⁸⁻⁹ Prostaglandin E synthase (PGES) converts PGH₂ to PGE₂ and consists of a constitutive cytosolic (cPGES) and an inducible membrane-associated (mPGES) isoforms.⁵ Interestingly, induction of COX-2 parallels the induction of mPGES-1, suggesting that COX-2 and mPGES-1 share similar regulatory mechanisms.¹⁰ Transcriptional regulation of COX-2 and mPGES-1 appear to involve mitogen-activated protein kinase (MAPK) signaling, resulting in COX-2 expression, mPGES-1 expression and elevated PGE₂.^{11,12}

Catabolism of PGE₂ proceeds with the initial oxidation by 15(S)-hydroxyprostaglandin dehydrogenase (PGDH) to a biologically inactive 15-keto PGE₂.¹³ Type I NAD⁺-dependent PGDH is considered the key enzyme for the inactivation of prostaglandins and its loss of expression has been identified in bladder, thyroid and colon cancer.¹⁴⁻¹⁶ PGDH is present in normal intestine and down-regulated in several carcinoma cell lines.^{17,18} Inhibition of MAPK signaling elevates PGDH expression that also parallels a decrease in transcriptional repressors ZEB1, SNAI1 and SNAI2.¹⁹⁻²¹ The regulation of PGDH in conjunction with COX-2/mPGES-1 production of PGE₂ is unclear but appears to converge on MAPK signaling, suggesting elevated PGE₂ may result from the coupled up-regulation of COX-2 and mPGES-1 with simultaneous or subsequent down-regulation of PGDH.

Thus, the aim of this report was to demonstrate that elevated PGE₂ levels stem not only from the presence of COX-2 over-expression but also, at least in part, from the absence of PGDH

expression in pancreatic cancer. Human pancreatic normal tissues exhibited decreased COX-2 expression with increased Type 1 PGDH expression that corresponded with low PGE₂ levels. In contrast, pair-matched tumor tissues exhibited increased COX-2 expression with decreased PGDH expression that corresponded with elevated PGE₂ levels. Similarly, human pancreatic cancer cells expressing COX-2 but not PGDH exhibited increased PGE₂. The human pancreatic tumors expressed significantly elevated SNAI2 but not SNAI1, although both these transcriptional repressors reportedly down-regulate PGDH expression. These results suggest accumulated levels of PGE₂ feed-forward inducing further COX-2-mediated PGE₂ production and down-regulating PGDH expression that appear to proceed through ERK activation and SNAI2 expression in pancreatic cancer.

MATERIALS AND METHODS

Materials

FBS, DMSO, chloroform and methanol were purchased from Sigma (St. Louis, MO). The selective COX-2 inhibitor N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398), non-selective COX inhibitor indomethacin, PGE₂, COX-2 antibody and PGDH antibody were purchased from Cayman Chemical (Ann Arbor, MI). SNAI1 and SNAI2 antibodies were purchased from Abgent (San Diego, CA). Phosphorylated ERK (p-ERK) and β -actin antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG and ECL reagents were obtained from Amersham-Pharmacia (Piscataway, NJ). The MEK specific inhibitor U0126 was obtained from Calbiochem (EMD Biosciences, San Diego, CA).

Human Pancreatic Tissues

Human pancreatic tumor tissues from surgical resections were frozen and stored in liquid nitrogen. Each donor tumor sample was matched with adjacent normal tissue collected during the process. All tumors were ductal adenocarcinoma. No IPMN (intraductal papillary mucinous neoplasm), cystic lesions or other. Pancreatic specimens were dissected macroscopically by trained pathologists. Tumor was sectioned as well as 'normal' pancreatic tissue from a region of pancreas included in the surgical specimen that was not grossly affected by tumor and was located as far away from the tumor mass as was feasible. Many slides of 'normal' pancreas included PanIN (pancreatic intraductal neoplasia) or chronic pancreatitis. These tissue samples were obtained from the UCLA Pancreas Tumor Bank supported by the Hirshberg Foundation for Pancreatic Cancer Research. These clinical specimens were reviewed and categorized by a pathologist before analyses. This human protocol was approved by the University of California at Los Angeles Internal Review Board.

Cell Culture

Human pancreatic cell lines MiaPaCa-2 (CRL 1420) and BxPC-3 (CRL 1687) were purchased from American Type Culture Collection (Rockville, MD). Stock cultures of MiaPaCa-2 were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose, supplemented with 10% fetal bovine serum (FBS) and PSG antibiotic mix (100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine; Invitrogen). Stock cultures of BxPC-3 were maintained in RPMI 1640 medium containing 2 mM L-glutamine, 2.0 g/L sodium bicarbonate, and 2.0 g/L glucose, supplemented with 10% fetal bovine serum (FBS) and PSG antibiotic mix (100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine; Invitrogen). MiaPaCa-2 cells, maintained in a humidified atmosphere with 10 % CO₂ at 37 °C, and BxPC-3 cells, maintained in a humidified atmosphere with 5 % CO₂ at 37 °C, were passaged weekly. For experiments, these cells were plated in 100-mm tissue culture dishes and allowed to achieve

confluence (5–7 days) before use. Media of confluent MiaPaCa-2 or BxPC-3 cells were then exchanged for serum-free DMEM medium or serum-free RPMI medium, respectively, overnight.

RNA Extraction

For tissue RNA extraction, human pancreatic tissues were frozen in liquid nitrogen, grounded to powder using a mortar and pestle and harvested with 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA) in 1.5 mL eppendorf tubes. For cell culture, cells were washed with 4 ml of Versene containing 0.48 mM EDTA in phosphate-buffered saline (Invitrogen) and harvested with 1 ml of Trizol reagent (Invitrogen). Total RNA was extracted with 0.2 ml of chloroform at $12,000 \times g$ for 15 min at 4 °C and precipitated with 0.5 ml of 2-propanol at $12,000 \times g$ for 10 minutes at 4 °C. The RNA pellet was washed with 75% ethanol at $7,500 \times g$ for 5 minutes at 4 °C, dissolved in 30 μ L of RNA Storage Solution with 1 mM sodium citrate, pH 6.4 (Ambion, Austin, TX) and stored at –20 °C for subsequent analysis. RNA concentration was quantified on a spectrophotometer (GeneQuant Pro, Amersham Biotechnology, Piscataway, NJ) reading dual wavelengths of 260 and 280 nm.

Tissue Protein Extraction

Human pancreatic tissue samples (0.1 g) were homogenized in 1 mL of PBS containing protease inhibitor cocktail (cat 11 697 498 001, Roche, Indianapolis, IN) carried out on ice. Tissue homogenates were sonicated for 20 seconds at 60 hz before centrifuging at $800 \times g$ for 10 minutes at 4 °C. The supernatants were filtered through 0.8 μ m filter and protein concentration was determined by absorbance at 595 nm using the Bradford assay (Bio-Rad, Hercules, CA).

Real Time PCR (RT-PCR)

Total RNA samples (25 ng) are reverse transcribed and cDNAs amplified using TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Transcripts encoding human COX-2, mPGES-1, PGDH, SNAIL1, SNAIL2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were quantified by real-time PCR analysis using an ABI Prism 7700 Sequence Detection System (PE Biosystems, Foster City, CA). The human primers used are as follows: COX-2 sense 5'-GGC TCA AAC ATG ATG TTT GCA-3', antisense 5'-CCT CGC TTA TGA TCT GTC TTG A-3' with corresponding probe 5'-TCT TTG CCC AGC ACT TCA CGC ATC AGT TT-3'; mPGES-1 sense 5'-AGA GAT GCC TGC CCA CAG-3', antisense 5'-CCA CGT ACA TCT TGA TGA CCA -3' with Universal Probe 9: 5'-TTG TGA TG-3' (Roche); SNAIL1 sense 5'-GCT GCA GGA CTC TAA TCC AGA-3', antisense 5'-ATC TCC GGA GGT GGG ATG-3' with corresponding Universal Probe 66: 5'-CAG CAG CC-3' (Roche); SNAIL2 sense 5'-TGG TTG CTT CAA GGA CAC AT-3', antisense 5'-GTT GCA GTG AGG GCA AGA A-3' with corresponding universal probe 7: 5'-GGG AGA G-3' and PGDH sense 5'-GAA GGC GGC ATC ATT ATC AA-3', antisense 5'-GCC ATG CTT TGA AGC ACA A-3' with corresponding Universal Probe 66: 5'-CAG CAG CC-3'. The human GAPDH primer and probe set was acquired from Applied Biosystems (Foster City, CA). Thermal cycling conditions for reverse transcription and amplification activation were set at 50 °C for 30 minutes and 95 °C for 10 minutes, respectively. PCR denaturing was set at 95 °C at 15 seconds and annealing/extending at 60 °C at 60 seconds for 40 cycles, according manufacturer's protocol (Brilliant II, Stratagene, La Jolla, CA).

Protein Expression

For tissue samples, protein extracts from tissue samples were with diluted 1:1 (vol/vol) with 2 $\times$ LDS buffer containing SDS (Invitrogen) and denatured at 95 °C for 10 minutes in a

water bath. For cell culture, confluent, serum-starved cells were washed with 1 ml of ice-cold phosphate buffered saline (PBS, Sigma), harvested in LDS loading buffer and denatured at 95 °C for 10 minutes in a water bath. These protein extracts were subjected to a variable 4–12% SDS-polyacrylamide gel electrophoresis (NuPAGE Novex Bis-Tris Gels, Invitrogen) for 45 minutes at 200 V, and transferred to a PVDF membrane (90 min at 30 V). The membrane was washed with Tris buffered saline (TBS, Sigma), blocked with 5% dried nonfat milk and 5% BSA in 1% tween-TBS and probed with antibody raised against either COX-2, PGDH, SNAI1, SNAI2 or p-ERK (1:1000) followed by antibody IgG linked to horseradish peroxidase conjugate (1:2500) according to the manufacturer's protocol (Cayman Chemicals). The blot was visualized by enhanced chemoluminescence (Amersham Biosciences). For internal standards, the blot was stripped with Restore Western Blot stripping buffer (Pierce, Rockford, IL), probed for β -actin (1:2500) as the internal standard and visualized by enhanced chemiluminescence.

Prostaglandin E₂ Levels

PGE₂ was quantified from serum-starved, confluent cells cultured in 24-well plates or from human tissue protein extracts according to EIA kit instructions (STAT-Prostaglandin E₂ EIA kit and STAT-Prostaglandin F_{1 α} EIA kit, Cayman Chemical, Ann Arbor, MI). PGE₂ determination was enhanced using total lipid extraction with chloroform/methanol (2:1, vol/vol).²² Absorbance readings were set between 405–420 nm on a spectrophotometer. PGE₂ levels were normalized to protein concentration measured by absorbance at 595 nm using the Bradford assay (Bio-Rad).

Statistical Analyses

Data are expressed as means $\pm$ SEM. Two-way ANOVA was performed using Sigma Plot (SPSS, Chicago, IL) and $P < 0.05$ was considered statistically significant.

RESULTS

Increased COX-2 and mPGES-1 mRNA Coupled with Decreased PGDH mRNA Expression in Pancreatic Tumors

Human pair-matched pancreatic normal and tumor samples were extracted for total RNA. These RNA extracts were reverse-transcribed for human COX-2, PGDH and GAPDH expression. Pooled Ct values of COX-2 expression from normal and tumor samples were normalized to the housekeeping gene GAPDH as shown in Fig. 1A. The pancreatic tumor samples expressed 11.0 ± 2.4 fold-increase in COX-2 mRNA expression compared to normal pancreatic tissue at 1.0 ± 0.2 fold COX-2 expression. In contrast, tumor tissue expressed only 0.04 ± 0.002 fold PGDH mRNA compared to normal pancreatic tissue at 1.0 ± 0.1 fold PGDH mRNA shown in Fig. 1B. Additionally, since COX-2 and mPGES-1 seemingly co-express to generate PGE₂, mPGES-1 mRNA expression was assessed by RT-PCR using the same RNA samples. Pooled pancreatic tumor samples expressed 9.0 ± 1.4 fold-increase in mPGES-1 mRNA expression compared to normal pancreatic tissue at 1.0 ± 0.1 fold mPGES-1 expression (Fig. 1C). These findings show an inverse relationship between the COX-2 and the degradative PGDH enzyme as well as an inverse relationship between mPGES-1 and PGDH at the mRNA expression level in pancreatic tissue. The marked fold difference in PGDH mRNA expression of tumor tissue compared to normal tissue suggests a more pronounced role for PGDH in maintaining PGE₂ levels.

Increased COX-2 Protein Expression Coupled with Decreased PGDH Protein Expression in Human Pancreatic Tumors

To confirm whether the COX-2 and PGDH mRNA expression also correlates with the COX-2 and PGDH protein expression, the human pair-matched normal and tumor tissues were homogenized and filtered for protein analysis as described in detail under the Methods section. The pair-matched samples were resolved by gel electrophoresis loaded at 45 µg per lane and transferred onto a PDVF membrane. The membranes were probed for COX-2 and PGDH with β-actin for visual loading control as shown in Fig. 2. Densitometric assessment (data not shown) of the blots showed that ten (83 %) of the twelve pancreatic tumor samples exhibited COX-2 expression (case 1, 2, 3, 4, 5, 7, 8, 9, 11 and 12) whereas six of the twelve (50 %) normal samples exhibited COX-2 expression (case 1, 2, 7, 9, 10 and 12). In contrast to the increased COX-2 expression exhibited by the tumor samples, PGDH expression was lost in most of the tumor samples. Of the twelve pancreatic tumor samples, only three tumor samples (25 %) exhibited PGDH expression (case 3, 8 and 11). The normal pancreatic tissue samples, however, expressed PDGH in ten (83 %) of the twelve samples (case 1, 2, 3, 4, 5, 6, 8, 9, 10 and 12).

Human Pancreatic Tumors Express Increased PGE₂

Based on the COX-2 and PGDH expression results, lysates from the human pair-matched pancreatic normal and tumorigenic tissues were lipid extracted to assay for PGE₂ levels. Fig. 3A shows elevated PGE₂ levels in the tumorigenic tissues at 31.3 ± 5.1 pg/mg in comparison to normal tissues at 13.3 ± 2.7 pg/mg protein. In the presence or absence of COX-2 and PGDH protein expression (Fig. 3B), samples that expressed only PGDH but not COX-2 protein exhibited the lowest PGE₂ level at 10.6 ± 0.9 pg/mg protein. Samples that expressed only COX-2 but not PGDH protein exhibited the highest PGE₂ level at 31.36 ± 2.9 pg/mg protein. Samples expressing both COX-2 and PGDH protein exhibited moderate PGE₂ levels at 17.1 ± 2.4 pg/mg protein that was significantly lower than COX-2 expressing samples alone and significantly higher than PGDH expressing samples alone. This finding demonstrates control of PGE₂ levels could be achieved through PGDH expression and activity and suggests that a combination of both COX-2 inhibition and PGDH induction could abolish PGE₂ production in carcinogenesis.

Slug Expression Is Increased in Human Pancreatic Tumors

To develop a possible explanation for the down-regulation of PGDH in the pancreatic tumors, the human pair-matched pancreatic normal and tumor samples were processed for mRNA and protein expression of SNAIL (Snail) and SNAI2 (Slug) as these nuclear transcription factors, particularly Slug, have been identified as a repressor of PGDH expression.^{21,23,24} Expression of SNAIL mRNA in normal tissue at 1.0 ± 0.2 fold induction was not significantly different from that of tumor tissue at 1.4 ± 0.3 fold induction, shown in Fig. 4A. However, SNAI2 (Slug) mRNA expression in tumor tissue at 5.8 ± 0.9 fold induction was significantly higher than normal tissue at 1.0 ± 0.3 (Fig. 4B). To determine if the mRNA expression profile paralleled with the protein profile, pair-matched human pancreatic normal and tumor tissue protein extracts were probed for SNAIL, SNAI2 and β-actin protein expression shown in Fig. 4C. The pair-matched sample 4N with 4T, 5N with 5T and 9N with 9T were used as 4N, 5N and 9N exhibited robust PGDH expression with minimal COX-2 expression (Fig. 2). Similarly, 4T, 5T and 9T tumor samples exhibited marked COX-2 expression with minimal PDGH expression (Fig. 2). Both the normal and tumor tissue samples (pair-matched samples 4, 5 and 9) expressed SNAIL (Fig. 4C). SNAI2, however, was markedly expressed in the tumor samples (4T, 5T and 9T) whereas SNAI2 was minimally expressed in the normal tissue samples (4N, 5N and 9N). Both SNAIL and SNAI2 mRNA expression profile paralleled the SNAIL and SNAI2 protein expression profiles. Interestingly, however, the increased SNAI2 expression in the tumor sample

paralleled increased COX-2 expression but correlated with low PGDH expression. The correlation of the increased Slug expression with decreased PGDH expression in the tumor samples suggests the possibility of Slug-induced down-regulation of PGDH expression as have been reported in other epithelial systems.^{24,25}

PGE₂ Stimulates ERK Phosphorylation in COX-2⁺ BxPC-3 Cells

To further demonstrate the reciprocal expression of COX-2 and PGDH, six human pancreatic carcinoma cell lines were assessed for COX-2 and PGDH expression. Fig. 5A shows COX-2 expression for AsPC, MiaPaCa-2, BxPC-3, Capan-2, HPAF-II and Panc-1 with β -actin as a visual loading control. None of the cell lines expressed PGDH. Based on this COX-2 profile in this report and previous report, MiaPaCa-2 as a COX-2 negative (COX-2⁻) and BxPC-3 as a COX-2 positive (COX-2⁺) cell lines were used to test their response to PGE₂.²⁶ The COX-2⁻ MiaPaCa-2 treated with PGE₂ did not induce phosphorylated ERK expression, suggesting that this particularly cell line may not express PGE₂ receptors or expresses limited PGE₂ receptors to elicit detectable signaling differences. Only COX-2⁺ BxPC-3 responded to a physiological dosage of 100 nM of PGE₂ as indicated in Fig. 5B. Induction of ERK phosphorylation peaked at 15 minutes and decreased at 20 and 30 minutes. Pre-treatment with 100 nM of MEK1/2 inhibitor U0126 for 1 hour followed subsequently with 100 nM of PGE₂ challenge to COX-2⁺ BxPC-3 cells abolished the ERK phosphorylation (Fig. 5C) that was observed without U0126. These findings suggests a possible mechanism by which elevated PGE₂ levels continue to accumulate by activating the MAPK cascade to down-regulate PGDH expression through the induction of SNAI2 expression.

DISCUSSION

Overall, this study shows that elevated PGE₂ levels in human pancreatic cancer tissue result from a seemingly coupled increase in COX-2 and mPGES-1 expression that is further maximized by a decrease in PGDH expression. Elevated PGE₂ levels correlated with increased COX-2 mRNA and protein expression in cancer tissue, consistent with prior reports of increased PGE₂ in pancreatic cancers.^{4,26} Over-expression of COX-2 has been documented in human pancreatic carcinomas and adenocarcinomas.^{9,28,29} COX-2 over-expression appears to extend to human pancreatic cancer cell lines as well.^{9,26,30} Indeed, the identification of COX-2 over-expression in pancreatic cancers has prompted several studies inhibiting COX-2 and production of PGE₂ as a therapeutic adjuvant in combination with gemcitabine standard for pancreatic cancer treatment.^{31–33} Additionally, the increased PGE₂ levels have been linked directly to COX-2 expression that paralleled similar increased mPGES-1 and -2 expression in pancreatic cancer tissue and cells.^{26,27} Maximal PGE₂ levels were observed in pancreatic tissue expressing only COX-2 and not PGDH as shown in Fig. 3, suggesting the importance of down-regulating PGDH in pancreatic tumors resulting in the accumulated PGE₂ levels.

In the context of enhanced PGE₂ production in pancreatic tumorigenesis, the contribution and regulation of PGDH has not been fully investigated. Studies on the regulation of PGDH have been limited to lung and colon cancer, but have contributed in understanding the mechanism.^{16,24} These reports on the regulation of PGDH have identified several transcriptional repressors, including SNAI1 (Snail) and SNAI2 (Slug), that interact with the E-box element of the PGDH promoter.¹⁷ This study also confirms the presence of Snail and Slug repressors in pancreatic tissue. Moreover, the findings showed that Snail mRNA expression was not significantly different in pancreatic normal and matched tumor samples but that pancreatic tumor samples exhibited significantly robust expression of Slug mRNA (Fig. 4A and 4B). Determination of Snail and Slug protein expression in three human pair-matched normal and tumor samples revealed similar pattern observed with the mRNA

expression. Snail expression was not significantly different between normal and tumor but Slug expression was significantly augmented in the tumor samples that also exhibited pronounced COX-2 expression (Fig. 4C). This finding was consistent with a report demonstrating Slug expression and binding to the PGDH promoter in lung carcinoma cells¹⁹ but contrasted the report of enhanced expression of Snail exhibited by human colon cancer cell,²⁵ suggesting that PGDH regulation could be mediated by cell or tissue-specific mechanism utilizing specific transcriptional repressors.

Both transcriptional repressors Snail and Slug expression are regulated through the MEK/ERK pathway, prompting the question whether excess PGE₂ might activate MEK/ERK in expressing Slug expression in the pancreatic cancer. Targeting of EGFR signaling through MAPK using the EGFR tyrosine kinase inhibitor erlotinib or the MEK-specific small molecule inhibitor U0126 elevates PGDH expression in human lung cancer cells.¹⁹ Inhibition of MAPK suppresses transcriptional repressors ZEB1, SNAIL1 and SNAIL2, with only SNAIL2 (Slug) shown to bind to an E-box element upstream of the TATA box and start site of PGDH.^{19–21} In addressing a possible mechanism for the down-regulation of PGDH in the pancreatic cancer, two human pancreatic cell lines the COX-2⁻ MiaPaCa-2 and the COX-2⁺ BxPC-3 were exposed to excess PGE₂, mimicking the tumor environment observed with the poorly-differentiated exocrine and ductal pancreatic tumors.^{34–36} PGE₂ serves as a ligand for four EP receptor subtypes EP1 to EP4 that are coupled to distinct G proteins.^{37–39} Activation of EP4 receptor correlates with an activation of MAPK signaling but has not been demonstrated directly.³⁸ Our finding shows BxPC-3 treated with PGE₂ increases phosphorylated ERK expression that was blocked when treated with U0126, indirectly demonstrating the involvement of MAPK signaling. Transcriptional regulation of COX-2 and mPGES-1 appear to involve mitogen-activated protein kinase (MAPK) signaling as MAPK has been reported to activate Egr-1 in the transcriptional activation of mPGES-1 as well as the induction of COX-2 expression, resulting in elevated PGE₂.^{11,12} Additionally, MAPK signaling is capable of mediating COX-2 expression at the transcriptional level through ERK and CREB^{40–42} and post-transcriptional through mRNA message stability,^{43–45} as well as mediating mPGES-1 expression through Egr-1.^{10,11} The regulation of PGDH in conjunction with COX-2/mPGES-1 production of PGE₂ is unclear but appears to converge on MAPK signaling, suggesting elevated PGE₂ may result from the coupled up-regulation of COX-2 and mPGES-1 with simultaneous or subsequent down-regulation of PGDH. These results suggest that enhanced PGE₂ production proceeds through the seemingly coupled expression of COX-2 and mPGES-1 and down-regulation of PGDH by SNAIL2 in pancreatic tumors.

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Abbreviations

COX-2	cyclooxygenase-2
MEK	mitogen-activated protein kinase kinase
MAPK	mitogen-activated protein kinase
ERK	extracellular signal-related kinase
EGF	epidermal growth factor

PKC	protein kinase C
PG	prostaglandin
PGDH	15-hydroxyprostaglandin dehydrogenase
mPGES	microsomal prostaglandin E synthase

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Figure 1. COX-2 and mPGES-1 mRNA expression is increased in human pancreatic tumors
Human pair-matched normal and tumor samples were extracted for total RNA using Trizol reagent as detailed in the Methods section. RNA samples (25 ng per sample) were reverse-transcribed and probed for human (A) COX-2, (B) PGDH, (C) mPGES-1 and GAPDH as an internal standard. Raw Ct values were normalized to GAPDH and data represented as mean fold induction $\pm$ SEM.

Figure 2. Increased COX-2 is coupled with decreased PGDH expression in human pancreatic tumors

Pair-matched human pancreatic normal (N) and tumor (T) samples were processed for protein expression as detailed under Methods section. Protein samples loaded at 45 μ g of sample/lane were resolved by PAGE, Western blotted for COX-2 (1:1000) and PGDH (1:1000) with β -actin (1:2500) as visual loading control, visualized by chemiluminescence (ECL) and recorded using a luminescent image analyzer.

Figure 3. Prostaglandin E₂ levels are elevated in human pancreatic tumors

Human pair-matched normal and tumor samples were homogenized, sonicated, centrifuged and filtered yielding protein lysates as detailed in the Methods section. These lysates were lipid extracted and assayed for PGE₂ by EIA as outlined in the manufacturer's protocol. (A) Comparison of mean PGE₂ levels from normal and tumor tissues are standardized to protein concentration as determined by the Bradford assay. (B) Mean PGE₂ levels from only COX-2-expressing, only PGDH-expressing or both COX-2/PGDH-expressing human samples are standardized to protein concentration.

Figure 4. SNAI2 (Slug) mRNA and protein expression is increased in human pancreatic tumors
Human pair-matched normal and tumor samples were extracted for total RNA using Trizol reagent detailed in the Methods section. RNA samples were reverse-transcribed and probed for human (A) SNAI1 (Snail), (B) SNAI2 (Slug) and GAPDH as an internal standard. Raw Ct values were standardized to GAPDH and data represented as mean fold induction $\pm$ SEM. Pair-matched human pancreatic normal (N) and tumor (T) samples were processed for protein expression as detailed under Methods section. (C) Protein from samples 4N, 4T, 5N, 5T, 9N and 9T were loaded at 45 μ g/lane per sample, resolved by PAGE, Western blotted for SNAI1 (1:1000) and SNAI2 (Slug) (1:1000) with β -actin (1:2500) as visual loading

control, visualized by chemiluminescence (ECL) and recorded using a luminescent image analyzer.

Figure 5. Human pancreatic cell lines express COX-2 but not PGDH

(A) Subconfluent human pancreatic cell lines AsPC, MiaPaCa-2, BxPC-3, Capan-2, HPAF-II and Panc-1 were processed for protein expression as detailed under Methods section. Protein samples from cell extracts loaded at 45 μ g of sample/lane were resolved by PAGE, Western blotted for COX-2 (1:1000) and PGDH (1:1000) with β -actin (1:2500) as visual loading control, visualized by chemiluminescence (ECL) and recorded using a luminescent image analyzer. (B) COX-2 positive BxPC-3 cells treated with 100 nM of PGE₂ for 0, 5, 10, 15, 20, or 30 min were harvested for protein expression. Protein extracts loaded at 45 μ g of sample/lane resolved by PAGE, Western blotted for phosphorylated ERK (p-ERK) (1:1000) with β -actin (1:2500) as visual loading control, visualized by ECL and recorded using a

luminescent image analyzer. (C) COX-2 positive BxPC-3 cells were incubated with MEK inhibitor U0126 (100 nM) for 1 hr. BxPC-3 cells were then treated with 100 nM of PGE₂ for 0, 5, 10, 15, 20, or 30 minutes and harvested for protein expression. Protein extracts loaded at 45 µg of sample/lane were resolved by PAGE, Western blotted for phosphorylated ERK (p-ERK) (1:1000) with β-actin (1:2500) as visual loading control, visualized by ECL and recorded using a luminescent image analyzer.