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## Cyclooxygenase-2 Confers Growth Advantage to Syngeneic Pancreatic Cancer Cells

Hiroki Takahashi, MD, PhD<sup>1</sup>, Aihua Li, PhD<sup>1</sup>, David W. Dawson, MD, PhD<sup>2</sup>, O. Joe Hines, MD<sup>1</sup>, Howard A. Reber, MD<sup>1</sup>, and Guido Eibl, MD<sup>1</sup>

<sup>1</sup> Hirshberg Laboratories for Pancreatic Cancer Research, Department of Surgery, UCLA Center for Excellence in Pancreatic Diseases, David Geffen School of Medicine at UCLA, Los Angeles, California

<sup>2</sup> Department of Pathology and Laboratory Medicine, UCLA Center for Excellence in Pancreatic Diseases, David Geffen School of Medicine at UCLA, Los Angeles, California

### Abstract

**Objectives**—Targeting the COX-2/prostanoid pathway is considered an intriguing approach for therapy and prevention of several cancers. However, the molecular mechanisms that underlie the pro-tumorigenic properties of COX-2 in pancreatic cancer (PaCa) are still poorly understood. The purpose of the present study was to characterize the phenotype of COX-2 expressing syngeneic PaCa cells.

**Methods**—COX-2 negative MIA PaCa-2 cells were stably transduced with COX-2 or control viruses (MP2<sup>+COX-2</sup> and MP2<sup>-COX-2</sup>). Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production was measured by liquid chromatography and tandem mass spectrometry. Anchorage-dependent and -independent cell growth was analyzed by cell count and 3D collagen cell culture system, respectively. Changes in apoptotic gene expression were measured by a PCR array. The growth of tumors *in vivo* was evaluated in a xenograft animal model.

**Results**—Stable expression of COX-2 increased anchorage-dependent and -independent cell growth, which was accompanied by elevated PGE<sub>2</sub> production. Several significant differences in apoptotic gene expression were detected between MP2<sup>+COX-2</sup> and MP2<sup>-COX-2</sup> cells. Furthermore, MP2<sup>+COX-2</sup> cells grew faster than MP2<sup>-COX-2</sup> cells in a xenograft animal model.

**Conclusions**—Our results will provide the basis for more mechanistic studies on the role of COX-2 in PaCa and may help to develop novel therapeutic strategies aiming at the COX-2/prostanoid pathway.

### Keywords

Cyclooxygenase-2; prostaglandin E<sub>2</sub>; pancreatic cancer; proliferation; arachidonic acid

### Introduction

Of all gastrointestinal carcinomas, pancreatic cancer (PaCa) has the most unfavorable prognosis. Despite some progress made in other epithelial tumors, pancreatic cancer still remains an almost universally fatal disease, with mortality rates approaching the number of

Correspondence: Guido Eibl, Department of Surgery, David Geffen School of Medicine at UCLA, 675 Charles E. Young Drive South, MRL 2535, Los Angeles, California 90095, USA. Phone: (310) 794-9577; Fax: (310) 825-8975, Geibl@mednet.ucla.edu.

**Note:** H. Takahashi and A. Li contributed equally to this work.

newly diagnosed cases. An estimated 21,050 men and 21,420 women will be diagnosed with pancreatic cancer in the year 2009 in the United States, and the majority of these patients will die within 6 months (1). For the past decade, gemcitabine has been considered the standard chemotherapeutic agent in the treatment of pancreatic cancer. However, studies using gemcitabine either as monotherapy or as combination therapy with cytotoxic or molecular targeted agents revealed only a marginal overall survival benefit of 1 to 2 months at best (2).

Cyclooxygenase-2 (COX-2), the rate-limiting enzyme for prostanoid production, is over-expressed in many types of cancer, including PaCa (3–8). There is substantial evidence that COX-2 contributes to the phenotype of human malignancies. The pro-tumorigenic properties of COX-2 are thereby generally thought to be mediated by COX-2 generated prostanoids, of which prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is the most abundant prostanoid species in many tumors (9,10). PGE<sub>2</sub> has been shown to promote tumor growth and spread by multiple mechanisms, including stimulation of cellular proliferation, invasion, and angiogenesis, inhibition of apoptotic cell death, and modulation of immune response (11–14). In pancreatic cancers, COX-2 and COX-2 derived PGE<sub>2</sub> have been found to stimulate growth, invasion, and angiogenesis (15–17). We have previously shown that enzymes involved in PGE<sub>2</sub> biosynthesis, including cytoplasmic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), COX-2, microsomal and cytoplasmic prostaglandin E synthases (mPGES-1 and -2, cPGES), are variably over-expressed in the majority of human pancreatic cancers (18). In addition, we have previously demonstrated that selective COX-2 inhibitors attenuate the growth PaCa and delay the progression of PaCa precursor lesions in mouse models (19,20), indicating that the COX-2/prostanoid pathway is an intriguing target for PaCa therapy and prevention. However, the precise molecular signals that underlie the pro-tumorigenic properties of COX-2 in PaCa are still poorly described. The aim of the present study was to characterize the phenotype of COX-2 expressing syngeneic human PaCa cells to clearly delineate the phenotypic changes brought upon pancreatic cancer cells by expressing COX-2. Our data provide strong evidence that COX-2 confers profound growth advantage to human pancreatic cancer cells *in vitro* and *in vivo*.

## Materials and Methods

### Reagents

The following antibodies were used: mPGES-1, mPGES-2, cPGES, COX-2 (Cayman Chemical Company, Ann Arbor, MI); cPLA<sub>2</sub>, GAPDH (Cell Signaling Technology, Inc., Danvers, MS); β-actin (Sigma Chemical, St Louis, MO). COX-2 electrophoresis standard was purchased from Cayman Chemical Company. Arachidonic acid was obtained from Sigma Chemical). Other reagents were purchased from common commercial sources.

### Cell culture

The undifferentiated human pancreatic cancer cell line MIA PaCa-2 (COX-1 positive, COX-2 negative) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured and maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 µg/ml) at 37 °C in a 10% CO<sub>2</sub>.

### Creation of COX-2 expressing stable cell lines

The full length human COX-2 cDNA was cloned into a third generation, self-inactivating pRRL-sin-cPPT-hCMV-MCS-IRES-GFP lentiviral vector. COX-2 negative MIA PaCa-2 cells were transduced with COX-2/GFP (MP2<sup>+COX-2</sup>) or GFP alone (MP2<sup>-COX-2</sup>) encoding

lentiviruses. Transduced MIA PaCa-2 cells were subsequently sorted by FACS to yield >95% pure MP2<sup>+</sup>COX-2 and MP2<sup>-</sup>COX-2 cell clones.

### Western blot analysis

Total cell lysates from cultures were prepared using ice-cold modified radioimmunoprecipitation assay (RIPA) buffer. Equal amounts of protein were fractionated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Proteins were detected using specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL). Protein-antibody complexes were visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

### Immunofluorescent staining

Cells were seeded into a Lab-Tek®II Chamber Slide™ System (Nalge Nunc International, Naperville, IL). At subconfluence, cells were fixed in 4% paraformaldehyde at room temperature for 15 minutes. Cells were then washed three times and incubated with 5% BSA for 30 minutes to block nonspecific binding. Slides were incubated with monoclonal anti-COX-2 antibodies for 1 hour. After three washes with PBS, slides were incubated with secondary antibody conjugates in the dark for 1 hour. After three washes with PBS, slides were mounted with VECTASHIELD containing DAPI (Vector Laboratories Inc., Burlingame, CA). Fixed cells were observed under a fluorescence microscope.

### Liquid chromatography and tandem mass spectrometry

Prostaglandin levels in the culture medium were determined by liquid chromatography and tandem mass spectrometry (LC-MS/MS). Prostaglandins were extracted using a solid-phase method. A solution of 10% butylated hydroxytoluene and deuterated prostaglandins as internal standards were added to the culture medium. The solution was then applied to a Sep-Pak C18 cartridge (Waters Corp, Milford, MA) that had been preconditioned with methanol and water. Prostaglandins were eluted with methanol, and the eluate was evaporated under a stream of nitrogen. The residue was dissolved in methanol/10mM ammonium acetate buffer (70:30, v/v, pH 8.5). The extracted prostaglandins were quantified by LC-MS/MS using a Quattro Ultima MS/MS (Micromass, Beverly, MA) equipped with an Agilent HP 1100 binary pump high-performance liquid chromatography inlet. Prostaglandins were separated using a Luna 3K Phenyl-Hexyl 2- × 150-mm liquid chromatography column (Phenomenex, Torrance, CA). Prostaglandins were detected using electrospray negative ionization and multiple-reaction monitoring of the transition ions for the metabolites and their internal standards as described previously (21).

### Cell growth assay

Anchorage-dependent cell growth of MP2<sup>+</sup>COX-2 and MP2<sup>-</sup>COX-2 cells treated with arachidonic acid (AA) (10 μM) was analyzed by cell count. Briefly, cells were seeded on 24-well plates and incubated overnight. The medium was replaced with serum free medium, and the COX-2 substrate AA was added to the cultures once daily. Cell number was determined by counting the cells under a light microscope.

### Colony formation assay

Anchorage-independent cell growth was assessed by a 3D Collagen Cell Culture System (Chemicon, Temecula, CA). 20,000 cells were seeded on matrigel-coated 24-well plates and were treated with AA (10 μM). AA was added to the Matrigel and the culture medium containing 0.5% FBS and 0.1% BSA. The medium was changed every other day. After one week, colonies were counted and photographed under a light microscope.

## RNA isolation and apoptosis gene array

Total RNA was extracted using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA). RNA quality and quantity were measured using the Experion Automated Electrophoresis System (Bio-Rad). RNA concentrations were additionally confirmed spectrophotometrically. RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). The expression of 84 genes involved in apoptosis was analyzed by the human apoptosis RT<sup>2</sup> Profiler™ PCR array according to the manufacturers (SABiosciences, Frederick, MD) on an iQ5 Real-Time PCR Detection System (Bio-Rad).

## Animal model

Animal studies were approved by the Chancellor's Animal Research Committee of the University of California, Los Angeles, in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. MP2<sup>+COX-2</sup> and MP2<sup>-COX-2</sup> pancreatic cancer cells ( $2 \times 10^6$ ) were injected subcutaneously into the flank of nude mice ( $n = 5$  each). After 2 weeks, animals were sacrificed and the tumor volume was assessed using the formula for a hemi-ellipsoid ( $2/3 \cdot \pi \cdot r_1 \cdot r_2 \cdot r_3$ ) as described previously (20).

## COX-2 immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were sectioned (4  $\mu$ m), deparaffinized in xylene, and rehydrated in a graded ethanol series. Sections were subjected to heat-induced epitope retrieval using boiling citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched by 0.3% H<sub>2</sub>O<sub>2</sub> and 5% bovine serum albumin was applied for 10 minutes to block nonspecific binding. Sections were then incubated with anti-COX-2 antibody. Immunoreactivity was detected by the UltraVision Detection System using 3-amino-9-ethylcarbazol as the chromogen according to the manufacturer's instructions (Lab Vision, Fremont, CA).

## Western blot analysis of tumor homogenates

Tumor extracts were prepared by homogenizing and sonicating non-necrotic areas of pancreatic cancers in anti-protease lysis buffer (Roche Applied Science, Indianapolis, IN). Tumor homogenates were cleared by centrifugation. COX-2 protein expression was determined by sodium dodecyl sulfate-gel electrophoresis and immunoblotting as described above.

## Statistical analysis

Data are presented as means  $\pm$  SD, and statistical comparisons were made using the Student's *t* test for paired observations. Comparisons of more than two groups were made by a one-way ANOVA with post hoc Holm-Sidak analysis for pairwise comparisons and comparisons versus control. An alpha value of 0.05 was used to determine significant differences. All statistics were done in SigmaStat 3.1 (Systat Software, Inc.).

## Results

### Generation of MP2<sup>+COX2</sup> and MP2<sup>-COX-2</sup> cells

To clearly delineate the role of COX-2 on the malignant phenotype of human pancreatic cancer cells, MIA PaCa-2 cells were stably transduced with lentiviruses encoding for the full length human COX-2. Previous studies have demonstrated that MIA PaCa-2 cells express the COX-1 isoform but not COX-2 (17,22). The lentiviruses also contained the sequence for the green fluorescent protein (GFP). After transduction, COX-2 expressing MIA PaCa-2 cells (MP2<sup>+COX2</sup>) and control cells (MP2<sup>-COX2</sup>) were sorted to yield >95% pure cell populations. Expression of COX-2 in MP2<sup>+COX2</sup> and absence of COX-2 in MP2<sup>-COX2</sup> was

confirmed by immunofluorescence (Fig. 1A) and Western blotting (Fig. 1B). To confirm stable expression of COX-2 over several passages, MP2<sup>+COX2</sup> cells were cultured over 6 weeks. As shown in Fig. 1C, COX-2 expression was stable in MP2<sup>+COX2</sup> cells over 6 weeks.

### Characterization of PGE<sub>2</sub> production in MP2<sup>+COX2</sup> and MP2<sup>-COX2</sup> cells

There is strong evidence that the pro-tumorigenic effects of COX-2 are mediated largely by the generation of prostaglandins (9,10). Among the various prostaglandin species, PGE<sub>2</sub> is commonly found to be the most abundant in human cancers (9,10). We have previously demonstrated that PGE<sub>2</sub> is generated by COX-2 in COX-2 positive human pancreatic cancer cells and stimulates growth and angiogenesis in pancreatic cancer cells (17,22). Having generated MP2<sup>+COX2</sup> cells we first confirmed production of PGE<sub>2</sub> in these cells. PGE<sub>2</sub> levels in the culture medium of MP2<sup>+COX2</sup> and MP2<sup>-COX2</sup> cells under serum free conditions were measured by LC-MS/MS. In the absence of AA, the substrate for COX-2, PGE<sub>2</sub> production was not detected in either cell line. However, exposure of MP2<sup>+COX2</sup> cells to AA (10 μM) dramatically increased PGE<sub>2</sub> production. AA-induced PGE<sub>2</sub> production was elevated within minutes in MP2<sup>+COX2</sup>, reached its maximum at ~30 min, and was still enhanced after 6 hours. In contrast, only a slight increase in PGE<sub>2</sub> production was found in MP2<sup>-COX2</sup> cells after stimulation with AA (Fig. 2). The maximal AA-stimulated PGE<sub>2</sub> production in MP2<sup>-COX2</sup> cells was >2-fold lower than those in MP2<sup>+COX2</sup> cells. These results are completely consistent with our previous data that AA robustly stimulated PGE<sub>2</sub> production in COX-2 positive cell lines (BxPC-3, Capan-2, and HPAF-II), but only slightly in COX-2 negative cell lines (MIA PaCa-2 and Panc-1) (17).

The biosynthesis of PGE<sub>2</sub> is initiated by the release of the polyunsaturated fatty acid AA from membrane phospholipids by cytoplasmic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), and proceeds through cyclooxygenases and prostaglandin E synthases (23,24). There are three different prostaglandin E synthases, namely cytoplasmic prostaglandin E synthase (cPGES), microsomal prostaglandin E synthase-1, and -2 (mPGES1, mPGES2). We have previously demonstrated that the aforementioned enzymes are variably expressed in human pancreatic cancers (18). To evaluate whether forced expression of COX-2 in MP2<sup>+COX2</sup> cells alters expression of these enzymes, which could affect PGE<sub>2</sub> synthesis, we measured protein expression of cPLA<sub>2</sub>, COX-2, cPGES, mPGES1, and mPGES2 by Western blotting. We found no detectable changes in protein expression of any enzyme in MP2<sup>+COX2</sup> and MP2<sup>-COX2</sup> cells with and without AA (Fig. 3). Notably, there was no expression of cPLA<sub>2</sub> in these cells, underscoring the necessity of adding AA to the cell cultures to stimulate PGE<sub>2</sub> production.

### Overexpression of COX-2 enhances growth of MIA PaCa-2 cells *in vitro*

We have previously showed that exogenous AA stimulated growth of COX-2 positive PaCa cell lines, but not of COX-2 negative PaCa cell lines (22). To clearly delineate whether COX-2 is responsible for the more robust growth-stimulating effect of AA in human pancreatic cancer cells, MP2<sup>+COX2</sup> and MP2<sup>-COX2</sup> cells were exposed to AA and anchorage-dependent and -independent cell growth was measured. In the absence of exogenous AA there was no difference in the anchorage-dependent cell growth between MP2<sup>+COX2</sup> and MP2<sup>-COX2</sup> cells (data not shown). However, exposure of MP2<sup>+COX2</sup> cells to AA (10 μM) resulted in a significant increase in anchorage-dependent cell growth compared to MP2<sup>-COX2</sup> cells treated with AA (Fig. 4A). The differences in the number of MP2<sup>+COX2</sup> and MP2<sup>-COX2</sup> cells were significant from day 4. In addition, using an anchorage-independent 3D Collagen Cell Culture System, AA increased the number of MP2<sup>+COX2</sup> colonies after 7 days by 170% while it increased the number of colonies of MP2<sup>-COX2</sup> cells only by 35% (Fig. 4B and C).



### Expression of COX-2 alters apoptotic gene expression in MIA PaCa-2 cells

It has been reported that pharmacological inhibition or genetic deletion of COX-2 overcomes the resistance to apoptotic cell death in several cancer cell lines, including PaCa (25–29), suggesting that COX-2 and COX-2 derived prostaglandins protect cancer cells from apoptosis. To gain first insight whether the increase in cell growth in MP2<sup>+</sup>COX2 cells was mediated by inhibition of apoptotic cell death, we evaluated changes in apoptotic gene expression in MP2<sup>+</sup>COX2 and MP2<sup>-</sup>COX2 cells. MP2<sup>+</sup>COX2 and MP2<sup>-</sup>COX2 cells were treated with AA (10 μM), after which total RNA was extracted and changes in apoptotic gene expression was measured using the human apoptosis RT<sup>2</sup> Profiler™ PCR array kit. As shown in Figure 5, several apoptosis-related genes were significantly differentially expressed in MP2<sup>+</sup>COX2 and MP2<sup>-</sup>COX2 cells. Those differences (MP2<sup>+</sup>COX2 relative to MP2<sup>-</sup>COX2 cells) included up-regulation of genes of the TNF ligand family (TNFSF10), TNF receptor family (TNFRSF21), Bcl-2 family (BNIP1 and HRK), and CIDE domain family (CIDEA), as well as down-regulation of other genes in the TNF ligand family (FASLG), TNF receptor family (TNFRSF9, TNFRSF11B), and caspase family (CASP1 and CASP5).

### Expression of COX-2 confers growth advantage of MIA PaCa-2 cells *in vivo*

Having demonstrated that COX-2 expression stimulated growth of MP2<sup>+</sup>COX2 cells *in vitro*, we sought to determine whether a similar observation could be made in a xenograft animal model, in which MP2<sup>+</sup>COX2 and MP2<sup>-</sup>COX2 cells were injected subcutaneously into nude mice. After 2 weeks, MP2<sup>+</sup>COX2 tumors were significantly larger than MP2<sup>-</sup>COX2 tumors (294 ± 135 mm<sup>3</sup> vs. 122 ± 48 mm<sup>3</sup>, p<0.05) (Fig. 6A). Immunohistochemistry and Western blot confirmed COX-2 protein expression in MP2<sup>+</sup>COX2 tumors but not in MP2<sup>-</sup>COX2 tumors (Fig. 6B and C). Further histo-pathological examination revealed that MP2<sup>-</sup>COX2 tumors were comprised of variably-sized solid nests of tumor cells that were circumscribed by thin fibrous or fibrovascular septa with accompanying mild acute and chronic inflammatory cells. The solid tumor nests also had a delicate fibrovascular framework and were associated with scattered neutrophilic inflammation. In addition, there was prominent central necrosis (up to 30–40% of the tumor) and additional scattered smaller necrotic abscesses with associated neutrophilic inflammation. In striking contrast, MP2<sup>+</sup>COX2 tumors were comprised of densely packed sheets of tumor cells, lacking the nested arrangement and discrete fibrous septa of the MP2<sup>-</sup>COX2 tumors. In addition, the extent of central necrosis was significantly less prominent in MP2<sup>+</sup>COX2 tumors (Fig. 6D).

## Discussion

There is substantial evidence that COX-2, the rate-limiting enzyme in the biosynthesis of prostanoids, is critical for tumor development and growth (3–8,30). Tissue-specific expression of COX-2 in genetically-engineered mouse models increased the risk of developing certain types of cancer (31), suggesting a role in early tumor development. In this regard, we have previously shown that oral administration of a selective COX-2 inhibitor delayed the progression of pancreatic cancer precursor lesions in a conditional Kras mouse model of pancreatic cancer (19). In addition, genetic ablation of COX-2 or pharmacologic inhibition of its activity commonly results in decreased tumor growth in a variety of animal models (32–35), demonstrating the potential of COX-2 as a therapeutic target. The pro-tumorigenic properties of COX-2 are thereby believed to be mediated by the generation of prostanoids, of which PGE<sub>2</sub> is commonly the most abundant species in human cancers (9,10). The majority of human pancreatic cancers express COX-2 but a substantial proportion is considered COX-2 negative. To clearly delineate the role of COX-2 to the malignant phenotype of human pancreatic cancer cells, we generated syngeneic cell lines with and without COX-2 expression. This model allows a better understanding of the role of

COX-2 in pancreatic cancer biology than using different cell lines with varying COX-2 expression. Precise knowledge about the pro-tumorigenic mechanisms of COX-2 is necessary to understand and predict the efficacy of targeted therapies aimed at inhibiting the COX-2 enzyme.

Our studies demonstrated that in the absence of the COX-2 substrate, arachidonic acid, MP2<sup>+COX2</sup> cells grew in a similar fashion than MP2<sup>-COX2</sup> cells. This could be explained by the lack of detectable expression of cPLA<sub>2</sub>, the enzyme that releases AA from membrane phospholipids. In accordance to that finding was the observation that PGE<sub>2</sub> production was very low and virtually identical between MP2<sup>+COX2</sup> and MP2<sup>-COX2</sup> cells in the absence of AA. However, addition of AA to the cultures robustly enhanced the growth of MP2<sup>+COX2</sup> cells, which was significantly more pronounced than the effect of AA in MP2<sup>-COX2</sup> cells. This was accompanied by a significantly higher production of PGE<sub>2</sub> in MP2<sup>+COX2</sup> cells compared to MP2<sup>-COX2</sup> cells. There was a slight increase in AA-induced PGE<sub>2</sub> production in MP2<sup>-COX2</sup> cells, which may have been caused by constitutively expressed COX-1 in MIA PaCa-2 cells. Since there were no differences among all other enzymes involved in PGE<sub>2</sub> biosynthesis between MP2<sup>+COX2</sup> and MP2<sup>-COX2</sup> cells, the increased production of PGE<sub>2</sub> in MP2<sup>+COX2</sup> cells was clearly caused by COX-2. The lack of cPLA<sub>2</sub> expression in MIA PaCa-2 cells thereby provides a unique opportunity to functionally “turn on” or “turn off” COX-2 activity.

An increase in cellular growth can be generally accomplished by stimulation of cell division (proliferative effect) or inhibition of cell death (survival signal). In order to gain first insight into the mechanisms of the growth-promoting effects of COX-2 expression in MIA PaCa-2 cells, we employed an apoptosis array that covers 84 apoptosis-related genes. We found several significantly up- and down-regulated genes in MP2<sup>+COX2</sup> compared to MP2<sup>-COX2</sup> cells, indicating that COX-2 expression alters the apoptotic/survival pathways in pancreatic cancer cells. The importance of COX-2 and COX-2 derived prostanoids in cell death and survival have been demonstrated in many reports. Tjiu and colleagues revealed that COX-2 induces anti-apoptotic activity in basal cell carcinoma by up-regulation of Bcl-2 and Mcl-1. (36) Singh and coworkers also reported that COX-2 transfected MCF7 cells produced a significantly higher expression level of Bcl-2 than the parental cells, and that COX-2 expression correlated with the increased resistance to doxorubicin. (37) Having demonstrated here that COX-2 changes the expression of several apoptosis-related genes, further studies are clearly necessary to delineate the precise signaling network that is affected by COX-2.

Similar to our *in vitro* studies, the growth-promoting effect of COX-2 could also be observed in a xenograft mouse model. MP2<sup>+COX2</sup> tumors contained significantly higher levels of PGE<sub>2</sub> than MP2<sup>-COX2</sup> tumors (data not shown), suggesting a pivotal role of COX-2 derived PGE<sub>2</sub> in tumor growth; a notion which has been corroborated by many reports (11,15,32,38–40). In this context, our previous data demonstrated that inhibition of PGE<sub>2</sub> production by a selective COX-2 inhibitor decreased tumor angiogenesis (17). As a source of exogenous AA in our animal study, the animal diet contains significant amounts of linoleic acid, which can be converted *in vivo* to AA. In our current study MP2<sup>+COX2</sup> tumors lacked the prominent central necrosis that was present in MP2<sup>-COX2</sup> tumors, suggesting sufficient blood supply (and hence tumor angiogenesis) in MP2<sup>+COX2</sup> cancers. In addition, MP2<sup>+COX2</sup> tumors lacked the strong presence of inflammatory infiltrates and fibrous septae that were seen in MP2<sup>-COX2</sup>. The inflammatory infiltration and fibrous septae may be reactions to the central necrosis. However, the lack of both in MP2<sup>+COX2</sup> tumors could also indicate a direct immune-suppressive effect of COX-2, as has been described in other reports (41,42). In summary, we present strong evidence that expression of COX-2 in pancreatic cancer cells confers a distinct growth advantage *in vitro* and *in vivo*. This effect

may be mediated by modulating the apoptotic/survival machinery, by increasing tumor angiogenesis, and by immunosuppression. The syngeneic cell lines generated in our studies will provide an ideal basis for detailed mechanistic studies deciphering the precise role of COX-2 in pancreatic cancer pathobiology.

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The stable cell lines were generated at the UCLA Vector Core (Kasahara N.). Prostaglandins were measured by Peiyang Yang (M.D. Anderson Cancer Center, Houston, TX).

## Abbreviations

|                         |  |
|-------------------------|--|
| <b>COX-2</b>            | Cyclooxygenase-2                                   |
| <b>PGE<sub>2</sub></b>  | prostaglandin E <sub>2</sub>                       |
| <b>PaCa</b>             | pancreatic cancer                                  |
| <b>LC-MS/MS</b>         | liquid chromatography and tandem mass spectrometry |
| <b>AA</b>               | arachidonic acid                                   |
| <b>cPLA<sub>2</sub></b> | cytoplasmic phospholipase A <sub>2</sub>           |
| <b>mPGES</b>            | microsomal prostaglandin E synthases               |
| <b>cPGES</b>            | cytoplasmic prostaglandin E synthases              |

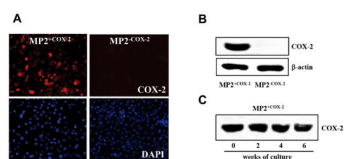
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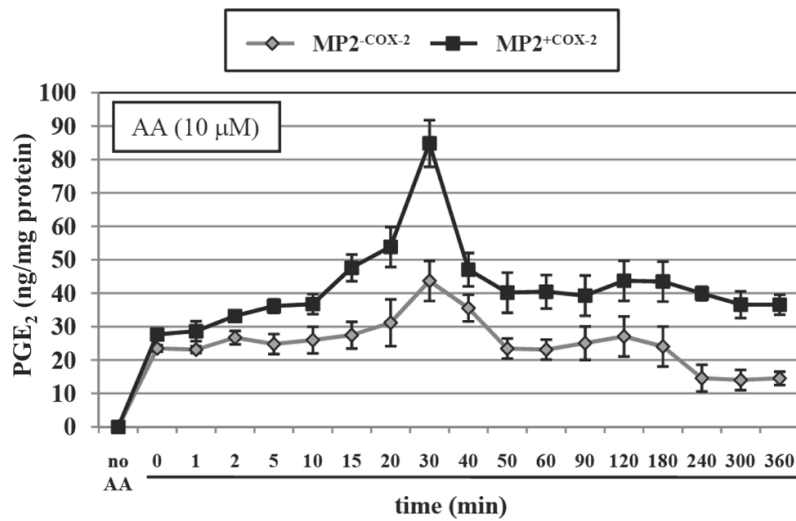
**Figure 1. Generation of MP2<sup>+</sup>COX2 and MP2<sup>-</sup>COX-2 cells**

A, COX-2 protein expression was detected by immunofluorescence staining (*top panel*).

Nuclei were stained with DAPI (*bottom panel*).

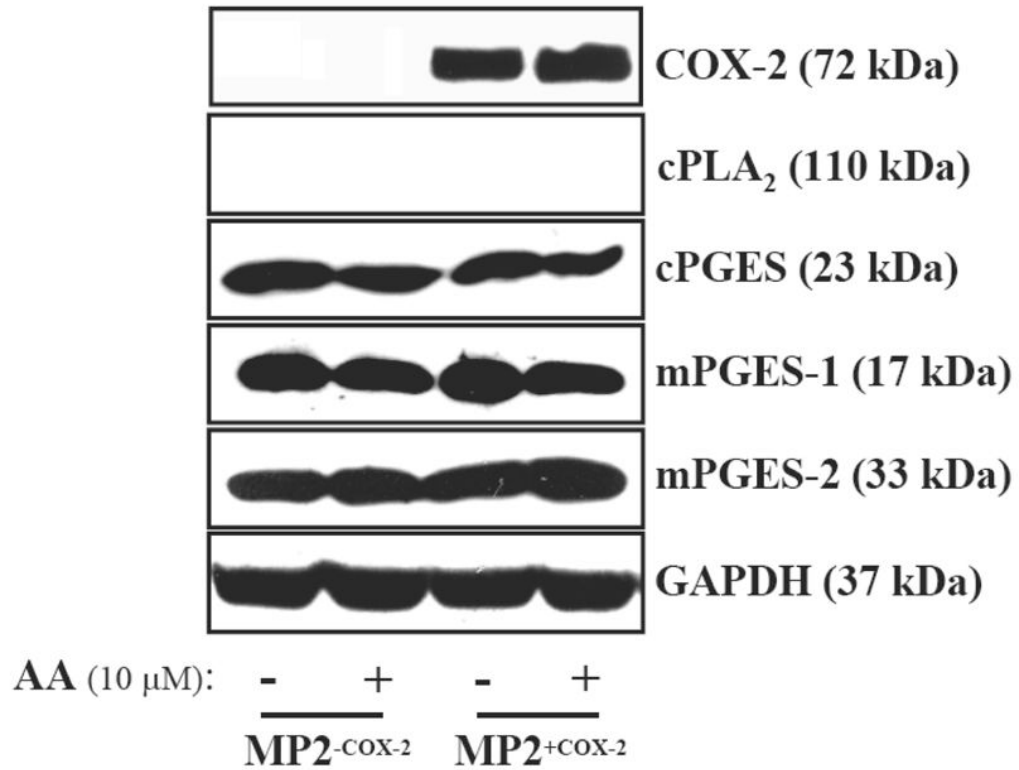
B, The expression of COX-2 in MP2<sup>+</sup>COX2 and the absence of COX-2 in MP2<sup>-</sup>COX-2 was confirmed by western blotting. Equal protein loading was confirmed by β-actin.

C, Successful stable expression of COX-2 over 6 weeks in MP2<sup>+</sup>COX2 cells was confirmed by western blot.



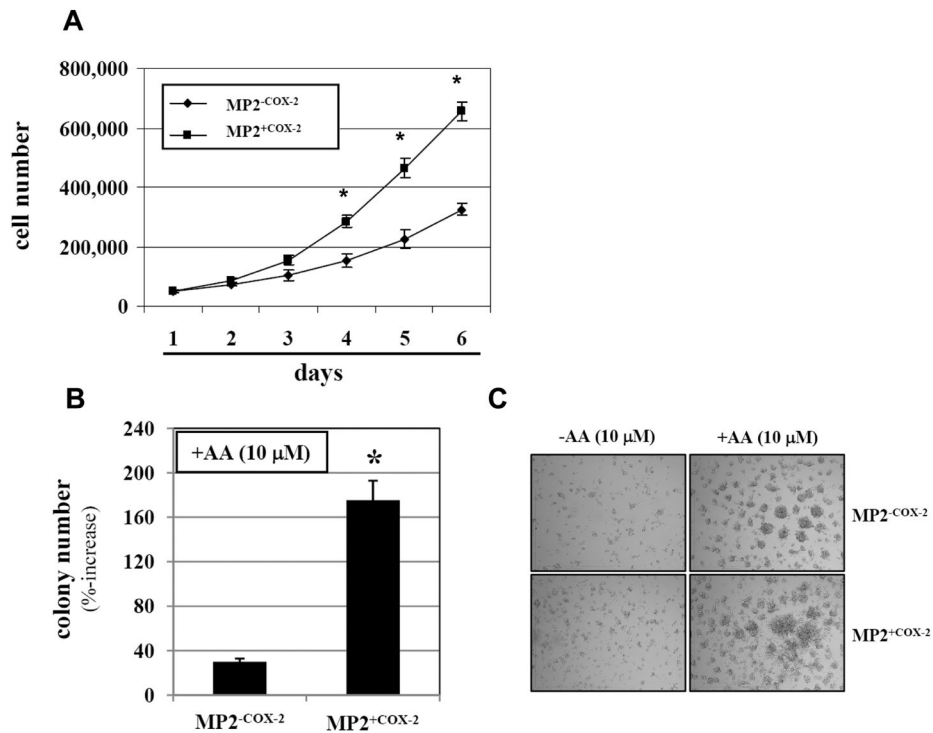
**Figure 2. PGE<sub>2</sub> production in MP2<sup>+</sup>COX2 and MP2<sup>-</sup>COX-2 cells**

The level of PGE<sub>2</sub> in the cell culture supernatant was measured by LC-MS/MS and expressed as ng/mg protein. Treatment of MP2<sup>+</sup>COX2 cells with arachidonic acid (10 μM) greatly increased PGE<sub>2</sub> production in a time-dependent manner, while only a slight increase was seen in the MP2<sup>-</sup>COX2 cells. Results are representative of three independent experiments and are presented as means ± SD (*bars*).



**Figure 3. Expression of proteins involved in PGE<sub>2</sub> biosynthesis**  
 Western blot analysis of COX-2, cPLA<sub>2</sub>, mPGES-1, mPGES-2, and cPGES were performed in MP2<sup>+COX2</sup> and MP2<sup>-COX2</sup> cells. GAPDH expression was used as a loading control. Results are representative of three independent experiments.

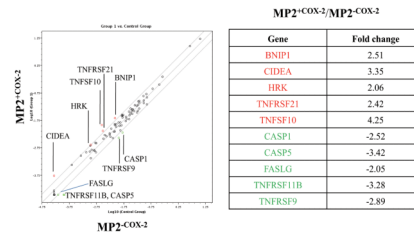




**Figure 4. Anchorage-dependent and -independent growth of MP2<sup>+</sup>COX<sup>2</sup> and MP2<sup>-</sup>COX<sup>2</sup> cells**  
 A, MP2<sup>+</sup>COX<sup>2</sup> and MP2<sup>-</sup>COX<sup>2</sup> cells were treated with 10 μM arachidonic acid for 6 days, and anchorage-dependent cell proliferation were measured by cell count. Results are representative of three independent experiments and are presented as mean±SD (bars). \**p*<0.01 vs. MP2<sup>-</sup>COX<sup>2</sup>.

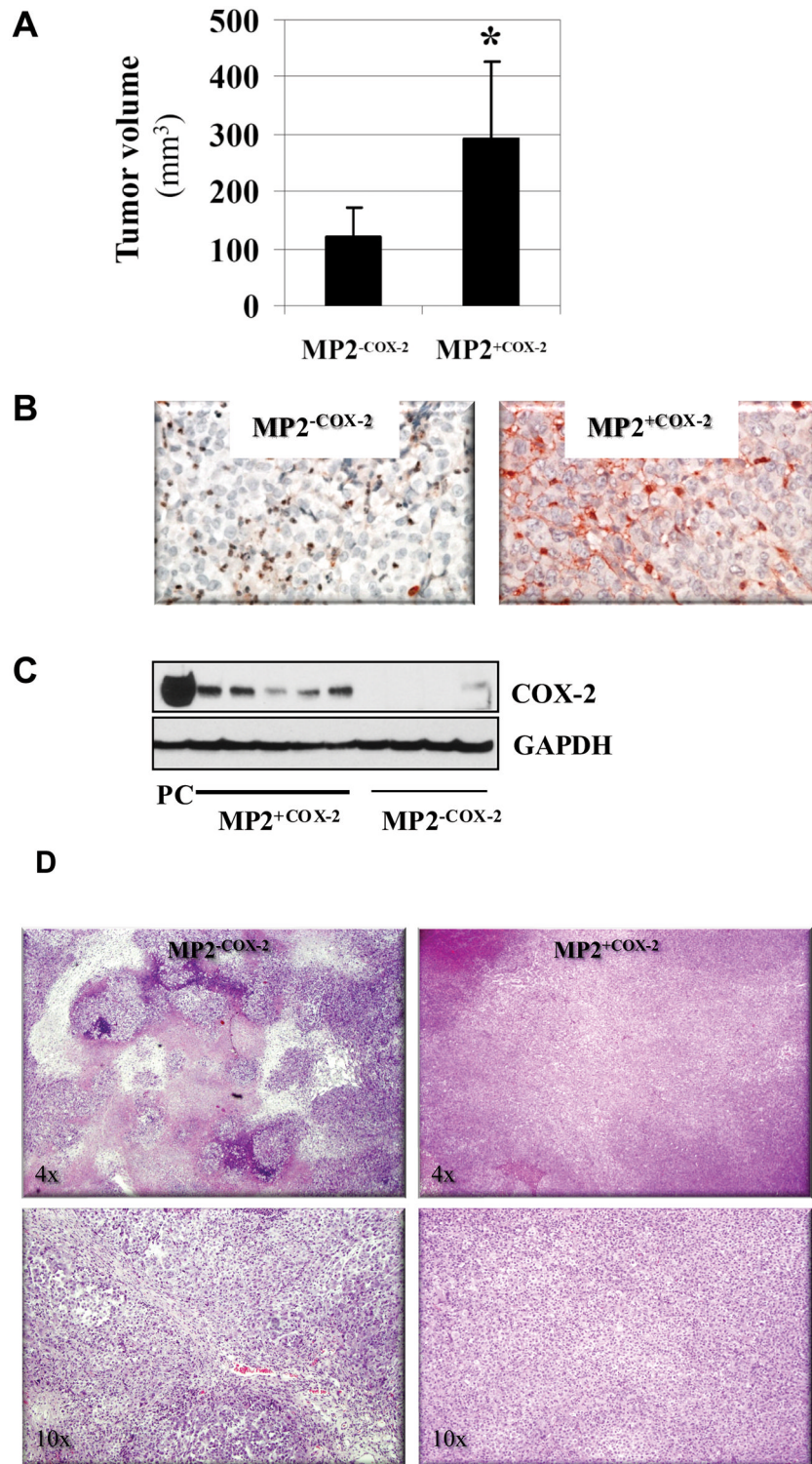
B, Anchorage-independent cell growth of MP2<sup>+</sup>COX<sup>2</sup> and MP2<sup>-</sup>COX<sup>2</sup> cells was measured by in 3D collagen cell culture system. Results are representative of three independent experiments and are presented as means ± SD (bars). \**p*<0.01 vs. MP2<sup>-</sup>COX<sup>2</sup>.

C, Representative images of colony formation. *Top panel*, MP2<sup>-</sup>COX<sup>2</sup> cells; *bottom panel*, MP2<sup>+</sup>COX<sup>2</sup> cells. There were no differences between MP2<sup>+</sup>COX<sup>2</sup> and MP2<sup>-</sup>COX<sup>2</sup> cells without AA (*left panel*).



**Figure 5. Apoptosis PCR array**

Total RNA was isolated from MP2<sup>+</sup>COX2 and MP2<sup>-</sup>COX2 cells, and the relative expression of 84 cell death-related genes was measured by Human Apoptosis PCR Array. Several significant differences (>2 fold increase (red) or decrease (green) in gene expression) were detected between MP2<sup>+</sup>COX2 and MP2<sup>-</sup>COX2 cells. Results are representative of three independent experiments.



**Figure 6. COX-2 confers growth advantage in an animal model**

A, Tumor volume of subcutaneous MP2<sup>+</sup>COX<sup>2</sup> and MP2<sup>-</sup>COX<sup>2</sup> tumors in nude mice. Data are presented as the means  $\pm$  SD of 5 MP2<sup>+</sup>COX<sup>2</sup> tumors and of 4 MP2<sup>-</sup>COX<sup>2</sup> tumors.

B, Immunohistochemistry of COX-2 in representative MP2<sup>+</sup>COX<sup>2</sup> and MP2<sup>-</sup>COX<sup>2</sup> tumors.

C, Analysis of COX-2 protein expression in MP2<sup>+COX2</sup> and MP2<sup>-COX2</sup> tumors by Western blot. For positive control (PC), cell lysates of BxPC-3 cells were used. GAPDH expression was used as a loading control.

D, Representative H.E. staining. *Left panel*, MP2<sup>-COX2</sup> tumors; *right panel*, MP2<sup>+COX2</sup> tumors. In MP2<sup>-COX2</sup> tumors, there is prominent central necrosis and additional scattered smaller necrotic abscesses with associated neutrophilic inflammation.