

Gene Expression Profiling of Tumor–Stromal Interactions between Pancreatic Cancer Cells and Stromal Fibroblasts

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ABSTRACT

The interactions between cancer cells and surrounding stroma play a critical role in tumor progression, but their molecular basis is largely unknown. Global gene expression profiling was performed using oligonucleotide microarrays to determine changes in the gene expression of pancreatic cancer cells (CFPAC1) and stromal fibroblasts induced by coculture. This analysis identified multiple genes as differentially expressed in pancreatic cancer cells and in fibroblasts as a consequence of their mutual interactions, including those that encode for proteins associated with tumor invasion, metastasis, and angiogenesis. Among the genes identified, the *cyclooxygenase-2 (COX-2)/PTGS2* gene was of particular interest because *COX-2* expression was markedly augmented in both cell types (cancer cells and fibroblasts) in response to coculture. Coculture with fibroblasts also induced *COX-2* expression in additional pancreatic cancer cells with an unmethylated *COX-2* promoter, but not in those with a methylated *COX-2* promoter. Using an *in vitro* invasion assay, we found an increase in the invasive potential of CFPAC1 cells when they were cocultured with fibroblasts, an effect blocked partially by the addition of a selective *COX-2* inhibitor, NS-398, or by *COX-2* knockdown with small interfering RNA. Thus, *COX-2* inhibitors can decrease the invasive properties of pancreatic cancer cells acquired through tumor–stromal interactions.

INTRODUCTION

There is growing evidence that interactions between tumor cells and surrounding stroma (notably fibroblasts) play a critical role in tumor growth, invasion, metastasis, angiogenesis, and chemoresistance (1–6). Histologically, pancreatic ductal adenocarcinoma is almost uniformly characterized by a prominent host desmoplastic response at the site of primary invasion, suggestive of the presence of extensive tumor–stromal interactions. The importance of tumor–stromal interactions in the aggressive behavior of pancreatic cancer is also supported by experimental evidence that the invasive potential of pancreatic cancer cells can be greatly enhanced by coculture with stromal fibroblasts (7). Despite the importance of tumor–stromal interactions in cancer progression, underlying molecular mechanisms have not been well characterized, partly because of their diversity and complexity. Several molecules have been identified that participate in tumor–stromal interactions, including hepatocyte growth factor (2), interleukin (IL)-8 (4), SPARC/osteonection (8), transforming growth factor β (5), and several matrix metalloproteinases (MMPs) (9, 10). The identification and characterization of genes/pathways involved in tumor–stromal interactions can identify targets for novel therapeutic strategies. For example, targeted blockade of hepatocyte growth factor, a pleiotrophic cytokine known to be produced primarily by

stromal fibroblasts, inhibits the growth, invasion, and metastasis of various cancers including pancreatic cancer (11, 12).

Recently, several investigators have used global gene expression profiling to identify genes associated with tumor–stromal interactions (13–15). Prior gene expression studies of the stromal compartment of pancreatic cancers have focused on the analysis of a limited number of genes (16). In the present study, we used a transwell coculture system and high-density oligonucleotide microarrays (GeneChip; Affymetrix Santa Clara, CA) to systematically analyze the gene expression changes induced by tumor–stromal interactions. Our analysis successfully identified a set of genes that are potentially involved in such interactions, many of which have been previously implicated in tumor invasion, metastasis, and angiogenesis. We also characterized the role of the *cyclooxygenase-2 (COX-2)/PTGS2*, a gene identified as markedly induced in both cancer cells and fibroblasts in our coculture system, in the pancreatic tumor–stromal interactions.

MATERIALS AND METHODS

Cells and Culture Conditions. Human pancreatic cancer cell lines (AsPC1, BxPC3, Capan1, CFPAC1, and MiaPaCa2) were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, streptomycin, and penicillin (complete medium) at 37°C in a humidified atmosphere containing 5% CO₂. Primary pancreatic fibroblasts were kindly provided by Prof. Masao Tanaka and Dr. Kazuhiro Mizumoto (Kyushu University, Fukuoka, Japan). These fibroblasts were originally outgrown from pancreatic adenocarcinoma tissues, isolated by serial passages, and evaluated carefully by light microscopy to exclude epithelial cell contamination.

Pancreatic Cancer/Fibroblast Coculture. Fibroblasts were seeded in the lower wells of a transwell cell culture system (6-well type, high-density membrane with 0.45-mm pores; BD Biosciences, San Jose, CA) and grown to 70–80% confluence for 24 to 72 hours. Pancreatic cancer cells ($\sim 1 \times 10^5$) were then seeded in the upper chambers (cell culture inserts) and cultured in complete medium. After a 48-hour incubation, pancreatic cancer cells and fibroblasts were harvested separately by a brief trypsinization or directly subjected to RNA extraction using RNeasy kit (Qiagen, Valencia, CA). For some experiments, pancreatic cancer cells were treated with 5-aza-2'-deoxycytidine (5Aza-dC; 1 μ mol/L) for 4 days or transfected with small interfering RNAs (siRNAs), and then they were cocultured with fibroblasts for 48 hours.

Coculture Invasion Assay. Cell culture inserts (24-well type, 8-mm pore size) were coated with 20 μ g of Matrigel (BD Biosciences). Fibroblasts were plated in the lower wells and grown to 80–90% confluence for 24 to 48 hours, and culture medium was replaced with RPMI 1640 with 1% fetal bovine serum immediately before coculture. Pancreatic cancer cells were suspended in serum-free medium (RPMI 1640 with 0.1% bovine serum albumin) and seeded into the upper chamber at a density of 5×10^4 cells/mL (500 μ L/well). After a 48-hour incubation, cells attached to the upper side of the filter were removed, and the filters were fixed and stained with hematoxylin and eosin. The number of cells that had invaded through Matrigel and migrated to the undersurface of the membrane was counted in randomly selected microscopic fields in each sample. Coculture invasion assays were also performed in the presence of a *COX-2* inhibitor, NS-398, which was added to the upper and lower chambers at the beginning of assay.

Transfection of Small Interfering RNAs. A siRNA targeting *COX-2* (SMARTpool, M-004557) and a nontargeting control siRNA (siCONTROL siRNA) were obtained from Dharmacon (Lafayette, CO). Cells were seeded in 6-well plates at a density of 1×10^5 cells/well. After overnight incubation,

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cells were transfected with *COX-2* siRNA or control siRNA at a concentration of 100 nmol/L using LipofectAMINE 2000 (Invitrogen).

Oligonucleotide Array Hybridization and Data Analysis. Global gene expression profiling was performed using oligonucleotide microarrays (Human Genome U133A chips; Affymetrix) as described previously (17). Signal intensity for each transcript (background subtracted and adjusted for noise) and detection call (present, absent, or marginal) were determined using Microarray Suite Software 5.0 (Affymetrix). Hierarchical cluster analysis was performed using dChip (DNA-Chip Analyzer) software⁴ after filtering genes with the greatest variation across all samples ($SD/mean > 1$). Scatter plot and fold change analyses were performed using Data Mining Tool (Affymetrix).

Reverse Transcription-Polymerase Chain Reaction. Complementary DNA was synthesized using Superscript II (Invitrogen). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using primer sets specific for nine genes (sequences are available on request) under the following conditions: 95°C for 5 minutes; 25 to 35 cycles of 95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 30 seconds; and a final extension for 4 minutes at 72°C. For semiquantitative analysis, the RT-PCR was performed with primers to amplify *GAPDH* (as an internal control) in duplex reactions.

Methylation-Specific Polymerase Chain Reaction. Methylation status of the *COX-2* CpG island was determined using methylation-specific polymerase chain reaction. Briefly, bisulfite-treated DNA (1 μ g) was amplified using primers specific for either the methylated or unmethylated DNA under the following conditions: 95°C for 5 minutes; 40 cycles of 95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 30 seconds; and a final extension for 4 minutes at 72°C. Primer sequences were GTAGTGAGTGTAGGAGTATG (forward) and ACAACAAAACACAAAACAAACATC (reverse) for unmethylated reactions (139 bp) and GTAGTGAGCGTTAGGAGTAC (forward) and CAAAACGCGAACGAACATCG (reverse) for methylated reactions (135 bp).

Statistical Analysis. Statistical analysis was performed using unpaired Student's *t* test (two-tailed). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Effects of Tumor-Stromal Coculture on the Invasion of Pancreatic Cancer Cells. Interactions between cancer cells and stromal cells have a significant impact on cancer progression, especially on cancer invasion. We first assessed the effect of tumor-stromal interactions on the invasive behavior of pancreatic cancer cells. Using an *in vitro* invasion assay, we determined the invasive ability of pancreatic cancer cells (CFPAC1) in the presence or absence of coculture with primary fibroblasts derived from pancreatic cancer tissue. A relatively small number [an average of 35 cells per microscopic field ($\times 200$)] of CFPAC1 cells invaded through Matrigel when they were cultured alone (Fig. 1A). By contrast, CFPAC1 cells cocultured with fibroblasts showed a marked increase in the number of invading cells (145 cells per field) of ~ 4 -fold (Fig. 1B). This finding led us to hypothesize that pancreatic cancer cells and stromal fibroblasts exchange signals that may result in alterations in their transcriptional programs, thereby accelerating tumor invasion.

Global Changes in Gene Expression Patterns Induced by Coculture. In an attempt to elucidate the molecular mechanism of tumor-stromal interactions, we explored the global changes in gene expression profiles in pancreatic cancer cells and stromal fibroblasts induced by coculturing them in a transwell system where the cells can communicate via soluble factors. We cultured CFPAC1 either alone (monoculture) or together with primary pancreatic fibroblasts (coculture) in a transwell system and harvested these cells separately after 48 hours of incubation for RNA extraction. We selected an incubation time point of 48 hours because the enhanced invasiveness in our coculture system was most prominent at this time point (data not shown). Expression was determined using oligonucleotide microarrays consisting of 18,462 probe sets (Human Genome U133A; Af-

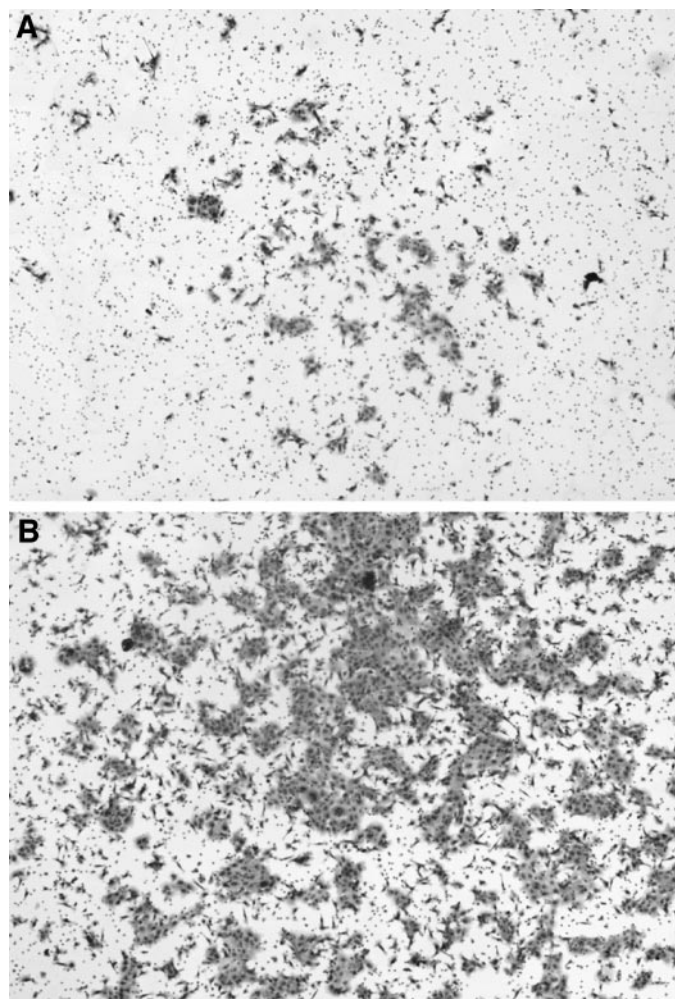


Fig. 1. Photomicrographs of *in vitro* invasion assay in CFPAC1 cells cultured alone (A) or cocultured with primary fibroblasts (B), showing a marked increase in their invasiveness as a consequence of tumor-stromal interactions.

fymetrix). Analysis of the global gene expression patterns in CFPAC1 (monoculture and coculture) and fibroblasts (monoculture and coculture) identified 718 transcripts that had the greatest variation ($SD/mean > 1$) among the four samples. Hierarchical cluster analysis with these 718 transcripts identified two major clusters that clearly discriminated between CFPAC1 cells and fibroblasts (Fig. 2A), perhaps reflecting their different cellular origins (epithelial *versus* stromal). Scatter plot and fold change analyses between monoculture and coculture conditions revealed a remarkable similarity in the expression profiles for each cell type, with only a small fraction of transcripts displaying differential expression (Fig. 2B–E). These findings imply that genes regulated through tumor-stromal interactions were likely represented by a small percentage of the total transcripts and that the minimal set of genes could be sufficient for the phenotypic changes (*i.e.*, increased invasiveness) observed in this cancer/fibroblast coculture system.

Identification of Genes Modulated by Coculture in Pancreatic Cancer Cells. To identify specific genes that are potentially regulated through tumor-stromal interactions, we screened for transcripts with at least 3-fold differences in signal intensity in coculture compared with monoculture conditions for both cell types. To minimize the detection of “falsely up-regulated or down-regulated genes,” we eliminated transcripts whose detection call was absent in both monoculture and coculture conditions. We first focused on genes modulated in

⁴ www.dChip.org.

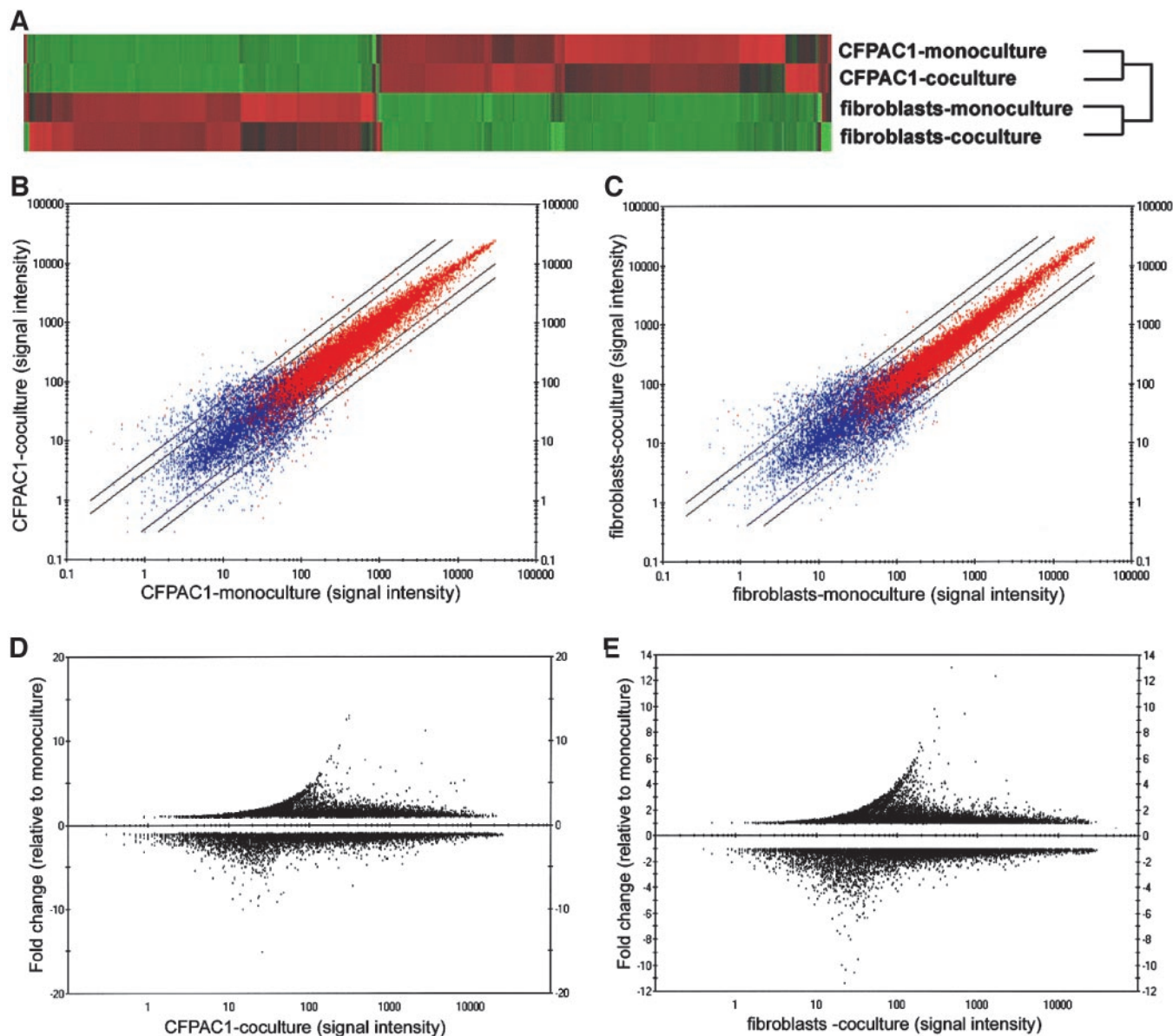


Fig. 2. Hierarchical clustering analysis of gene expression profiles in monoculture and coculture conditions of CFPAC1 and fibroblasts discriminates two different cell types (A). Scatter plot analysis between monoculture and coculture conditions of CFPAC1 (B) and fibroblasts (C) and fold change analysis between monoculture and coculture conditions of CFPAC1 (D) and fibroblasts (E) show a remarkable similarity in expression patterns for each cell type. Blue and red dots shown in the scatter plot graph (B and C) represent transcripts whose detection call was absent in both monoculture and coculture conditions and transcripts whose detection call was present (or marginal) in at least one of the monoculture and coculture conditions, respectively. The lines shown in the graph are fold change lines (3- and 5-fold cutoff).

CFPAC1 after coculture. Of the 18,462 transcripts analyzed, only 55 (0.30%) were expressed at levels more than 3-fold greater in coculture than in monoculture;⁵ conversely, only 88 (0.48%) transcripts were expressed at levels less than 3-fold lower in coculture system.⁵ A subset of five genes was selected for validation of mRNA expression using RT-PCR, and we were able to confirm the up-regulation of *COX-2*, *hyaluronan synthase 2 (HAS2)*, *MMP-1*, and *trefoil factor 1 (TFF1)* and the down-regulation of *gravin* (Fig. 3A). Among the genes identified, *MMP-1* and *MMP-2* have been shown to be induced in coculture between breast cancer cells and bone marrow fibroblasts (18), whereas the majority of genes identified as differentially expressed have not been reported previously in association with tumor-stromal interactions. Notably, several genes identified as induced by coculture have been closely related to increased properties for tumor

invasion by promoting cell motility [*TFF1*, *HAS2*, and *vasoactive intestinal peptide receptor 1 (VIPRI)*] and extracellular matrix proteolysis (*MMP-1* and *MMP-2*). In particular, TFF1, one of the trefoil factor family peptides, has been characterized as a potent mitogen and shown to stimulate migration of breast cancer cells (19) and promote invasiveness of kidney and colon epithelial cells (20). In addition, overexpression of *HAS2* leads to an increased hyaluronan synthesis, thereby stimulating cell migration (21). Furthermore, the binding of vasoactive intestinal peptide to its receptor, VIPRI, has been demonstrated to enhance growth and motility of prostate cancer cells (22). MMPs play a critical role in tumor invasion through proteolytic degradation of the extracellular matrix (23) and have been implicated in tumor-stromal interactions (24). Conversely, genes that have been suggested to counteract tumor invasion and metastasis [such as *gravin* and *soluble urokinase-type plasminogen activator receptor (SUPAR)*] were more likely to be down-regulated by coculture. For example,

⁵ <http://www.pathology2.jhu.edu/pancreas/coculture>.

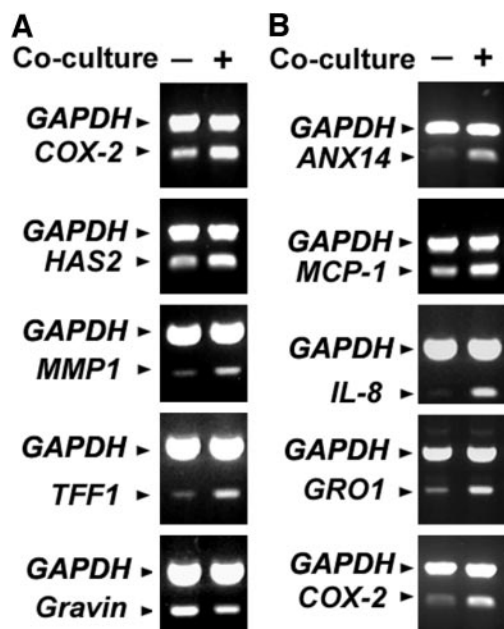


Fig. 3. RT-PCR validation of nine selected genes identified as differentially regulated in CFPAC1 cells (A) and fibroblasts (B) after coculture.

gravin/AKAP12/SSeCKS, a scaffolding protein for protein kinases A and C, has been shown to suppress lung metastasis of prostate cancer in mice (25). Recently, it has been demonstrated that SUPAR inhibits growth and invasion of breast cancer cells by blocking the signaling activity of membrane-anchored urokinase-type plasminogen activator receptor (26). Taken together, both the up-regulation of invasion-promoting genes and the down-regulation of invasion/metastasis suppressor genes may play a cooperative role in the enhanced invasion of CFPAC1 induced by coculture.

Identification of Genes in Fibroblasts Modulated by Coculture.

Next we determined the genes differentially regulated in fibroblasts by coculture. As compared with monoculture, 43 transcripts (0.23% of the 18,462 transcripts analyzed) showed a marked increase (>3-fold) in expression in coculture, whereas 31 (0.17%) were down-regulated more than 3-fold.⁵ Using RT-PCR, we validated the expression of a subset of 5 genes identified as up-regulated by coculture, including *annexin 14 (ANX14/ANXA10)*, *monocyte chemoattractant protein-1 (MCP-1)*, *IL-8*, *growth regulated oncogene 1 (GRO1)*, and *COX-2* (Fig. 3B). Of particular interest, among the most highly up-regulated genes identified were members of the CXC/CC chemokine subfamilies including *MCP-1 (CCL2)*, *IL-8 (CXCL8)*, *GRO1 (CXCL1)*, and *GRO2 (CXCL2)*. An essential role for these chemokines in human cancers has been well established. For example, MCP-1 is produced by a variety of cells including fibroblasts and has been implicated in macrophage infiltration, which is associated with advanced tumor stage, increased tumor invasion, and angiogenesis (27, 28). IL-8 has been characterized as a potent angiogenic factor (29) and is also known to promote tumor invasion through activation of MMP-2 (30). Of note, IL-8 expression has been previously implicated in tumor growth and metastasis of pancreatic cancer (31). Expression of GRO1, an autocrine growth factor for melanoma, has been associated with tumor growth, metastasis, and angiogenesis in different tumor models and been demonstrated to stimulate the growth of pancreatic cancer cells in an autocrine manner (32). Our present findings suggest that GRO1 produced by fibroblasts as a consequence of tumor-stromal interactions stimulates the growth of pancreatic cancer cells in a paracrine fashion. Thus, the up-regulation of these chemokines in fibroblasts is likely to modify the tumor microenvironment, which can

facilitate tumor growth, invasion, metastasis, and angiogenesis at the tumor-stromal interface.

Identification of COX-2 as a Mediator of Tumor-Stromal Interactions. Among the genes identified as differentially regulated by tumor-stromal interactions, the *COX-2/PTGS2* gene was markedly augmented in both CFPAC1 and fibroblasts after coculture. Specifically, the signal intensity value for *COX-2* in CFPAC1 was 246.5 (called present) in monoculture and increased dramatically to 2,764.3 (called present; detection $P = 0.0002$; 11.2-fold increase) in response to coculture, representing a gene with one of the highest expression values, the lowest detection P value, and the highest fold increase among all of the up-regulated genes identified. This finding is of particular importance because COX-2 is overexpressed in a wide spectrum of human cancers and plays an integral role in tumor growth, invasion, metastasis, and angiogenesis (33-36). Notably, it has been shown that in addition to expression of COX-2 in cancer cells, stromal-derived COX-2 promotes cancer progression *in vivo* (37), thus highlighting the importance of COX-2 expression in stromal fibroblasts. We therefore determined the role of *COX-2* induction in pancreatic tumor-stromal interactions and the invasive behavior of pancreatic cancer cells. We examined *COX-2* expression in cocultures between CFPAC1 and different isolates of primary pancreatic fibroblasts, and we found an increase in *COX-2* expression in both CFPAC1 and each of these fibroblast isolates after coculture (Fig. 4A), suggesting that this response is not unique to certain fibroblasts. We next assessed whether *COX-2* expression is up-regulated in other pancreatic cancer cell lines in response to fibroblast coculture. We

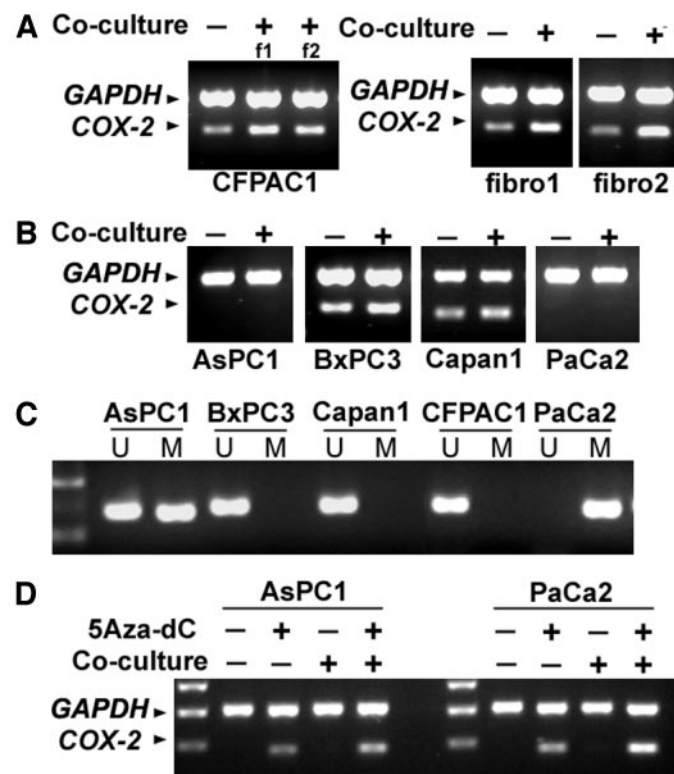


Fig. 4. A, RT-PCR analysis of *COX-2* mRNA expression in CFPAC1 and two different isolates of pancreatic fibroblasts (*fibro1* and *fibro2*) in response to cancer/fibroblast coculture. B, RT-PCR analysis of *COX-2* in four additional pancreatic cancer cell lines in response to fibroblast coculture. C, methylation-specific polymerase chain reaction analysis of *COX-2* promoter CpG island in five pancreatic cancer cell lines. The PCR products in Lanes U and M indicate the presence of unmethylated and methylated templates, respectively. D, effect of loss of methylation on *COX-2* expression in response to coculture in two pancreatic cancer cell lines with a methylated *COX-2* promoter. Cells were treated with 5Aza-dC (1 μ mol/L) for 4 days and then monocultured or cocultured with fibroblasts for 48 hours.

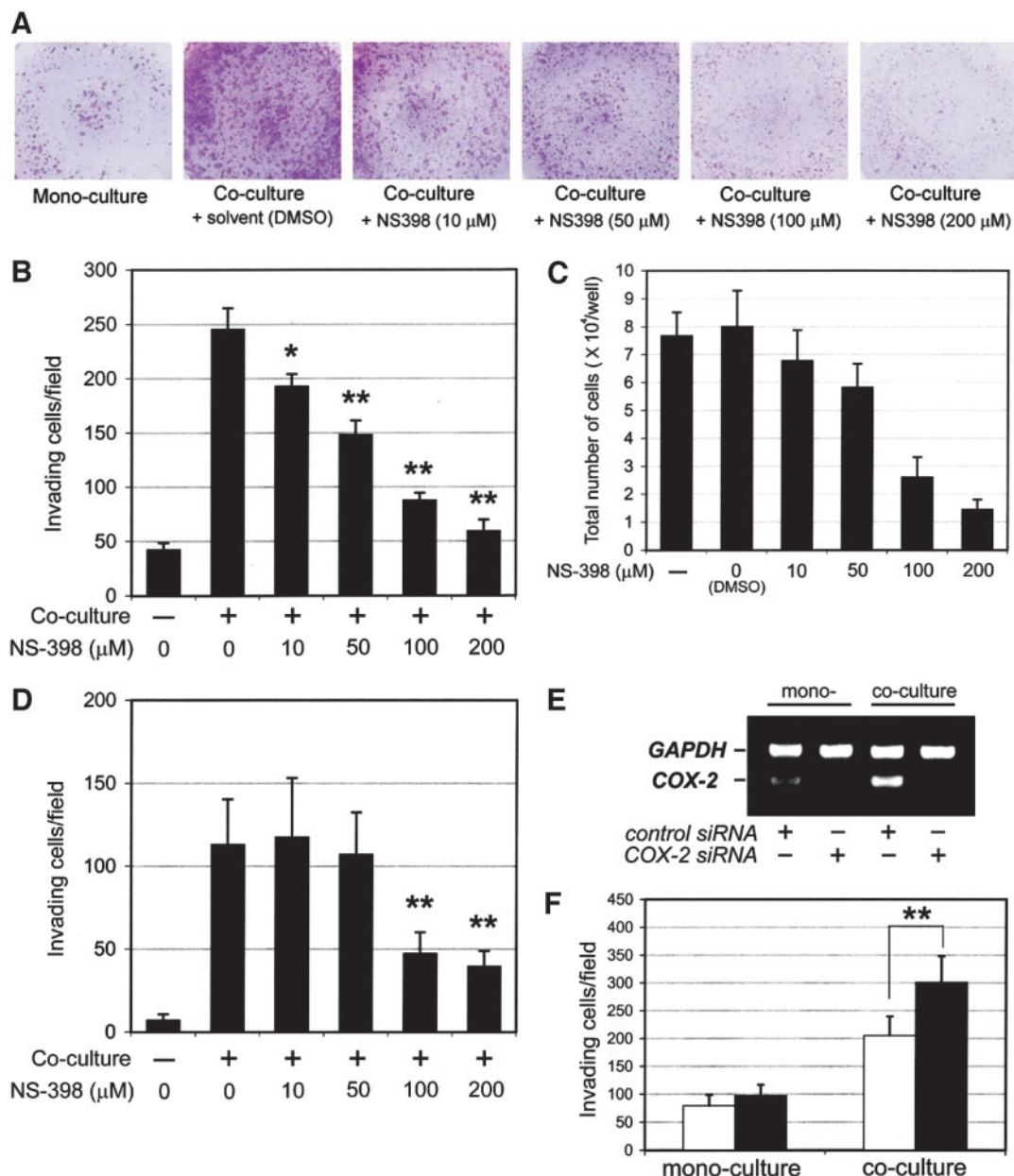


Fig. 5. A, a representative result of *in vitro* invasion assay in CFPAC1 cells either monocultured or cocultured with fibroblasts in the presence of different concentrations of NS-398. B, dose-dependent inhibition of the increased number of invading CFPAC1 cells in fibroblast coculture by NS-398. Data are the mean \pm SD of five fields from three independent wells. Results were significantly different from coculture-induced invasion without NS-398 treatment (*, $P < 0.001$; **, $P < 0.0001$). C, dose-dependent inhibition of cell proliferation of CFPAC1 by NS-398. Cells (seeded at 4×10^4 cells/well) were treated with various concentrations of NS-398 for 48 hours. Data are the mean \pm SD of three independent wells. D, effects of NS-398 on the increased invasion of AsPC1 cells in fibroblast coculture. Data are the mean \pm SD of five fields from three independent wells. Results were significantly different from coculture-induced invasion without NS-398 (**, $P < 0.0001$). E, COX-2 mRNA expression in CFPAC1 cells transfected with a siRNA targeting COX-2 or a nontargeting control siRNA, followed by monoculture or coculture with fibroblasts for 48 hours. F, effects of siRNA-mediated COX-2 knockdown on the enhanced invasion of CFPAC1 cells by fibroblast coculture. Cells transfected with COX-2 siRNA (\square) showed a significantly decreased number of invading cells after coculture compared with control siRNA-transfected counterparts (\blacksquare ; **, $P < 0.0001$).

tested four additional cell lines and found a modest increase in COX-2 mRNA expression after coculture in BxPC3 and Capan1, but not in AsPC1 and MiaPaCa2 (Fig. 4B). To elucidate the mechanism for the different responses of COX-2 induction in these cell lines to coculture, we investigated the methylation status of the COX-2 gene, which has been shown to be aberrantly methylated in various cancers (38, 39). Using methylation-specific PCR, we identified aberrant methylation of COX-2 in two cell lines (AsPC1 and MiaPaCa2) in which COX-2 induction was not observed after coculture (Fig. 4C). We then treated AsPC1 and MiaPaCa2 cells with DNA methyltransferase inhibitor 5Aza-dC to determine whether loss of methylation could restore

COX-2 expression in these cell lines after coculture. Although treatment with 5Aza-dC alone led to a slight induction of COX-2 expression, fibroblast coculture after 5Aza-dC treatment further increased the expression level in both cell lines (Fig. 4D). We also tested whether the invasive phenotype could be induced by fibroblast coculture in these cell lines with methylation-associated silent COX-2. Coculture with fibroblasts drastically increased the invasiveness of AsPC1 cells by ~ 16 -fold, but it did not affect the invasive behavior of MiaPaCa2 cells, suggesting that this enhanced invasion in AsPC1 is mediated through COX-2-independent pathways. Taken together, these results suggest that promoter hypermethylation is responsible

for loss of responsiveness of *COX-2* induction by coculture in a subset of pancreatic cancer cell lines and support *COX-2* as a general mediator of tumor-stromal interactions between pancreatic cancer cells and stromal fibroblasts. Overall, aberrant methylation of *COX-2* was observed in 3 of 23 (13%) of all of the pancreatic cancer cell lines tested and was also detected in 9 of 29 (31%) primary pancreatic adenocarcinomas and 4 of 24 (17%) intraductal papillary mucinous neoplasms of the pancreas. By contrast, *COX-2* was unmethylated in 10 normal pancreatic tissues, including 3 normal ductal epithelial samples selectively isolated by laser-capture microdissection.

Effects of a Selective *COX-2* Inhibitor or Small Interfering RNA Targeting *COX-2* on Pancreatic Cancer Invasiveness Induced by Fibroblast Coculture. The marked induction of *COX-2* observed in pancreatic cancer/fibroblast cocultures, along with previous evidence supporting a critical role of *COX-2* and subsequent prostaglandins in cancer invasion (34, 40, 41), prompted us to test the effect of a specific *COX-2* inhibitor (NS-398) on the enhanced invasiveness of pancreatic cancer cells by fibroblast coculture. We examined the invasive ability of CFPAC1 cells cocultured with stromal fibroblasts in the presence of various concentrations (10, 50, 100, and 200 $\mu\text{mol/L}$) of NS-398 or solvent (dimethyl sulfoxide) alone. Whereas coculture with fibroblasts resulted in a robust increase in the invasive potential of CFPAC1, addition of NS-398 blocked the increase in the number of invading cells in a dose-dependent manner (Fig. 5A and B). In a parallel experiment, we also determined the effects of NS-398 on the proliferation of CFPAC1 cells. Treatment with NS-398 (0–200 $\mu\text{mol/L}$) caused a dose-dependent inhibition of cell proliferation (Fig. 5C), whereas no apparent cell death was observed at any concentrations tested. It is unlikely that decreased invasiveness caused by NS-398 merely reflects the decreased cell number in the drug-treated cultures because the invasive behavior of cancer cells as determined by the *in vitro* invasion assay has been considered independent of their proliferative activity. In fact, we have demonstrated previously (42) that treatment with 5Aza-dC increases the invasive potential of pancreatic cancer cells despite the growth-inhibitory effects of 5Aza-dC. We then tested whether NS-398 exhibits a similar inhibitory effect on coculture-induced invasiveness in AsPC1 cells lacking *COX-2* induction. Treatment with NS-398 inhibited the enhanced invasiveness of AsPC1 by fibroblast coculture only at relatively higher concentrations (>100 $\mu\text{mol/L}$), suggesting a *COX-2*-independent mechanism of inhibition in this cell line (Fig. 5D).

Because *COX-2* inhibitors are known to exert antineoplastic effects through *COX-2*-independent and *COX-2*-dependent mechanisms (43, 44), we used *COX-2*-specific RNA interference to determine the direct role of coculture-induced *COX-2* expression in pancreatic cancer invasiveness. CFPAC1 cells were transfected with either a siRNA targeting *COX-2* or a nontargeting control siRNA and subjected to coculture invasion assay after 24 hours. Transfection with *COX-2* siRNA (100 nmol/L) resulted in ~85% inhibition of *COX-2* mRNA expression and a significant reduction in the number of invading cells after coculture as compared with control siRNA-transfected counterparts ($P < 0.0001$; Fig. 5E and F). Thus, these findings suggest that increased *COX-2* expression as a consequence of tumor-stromal interactions may contribute at least in part to the invasive phenotype of pancreatic cancer.

COX-2 is known to be induced by a variety of mitogenic and inflammatory stimuli, resulting in enhanced synthesis of prostaglandins in neoplastic and inflamed tissues, thereby promoting carcinogenesis through multiple mechanisms (45, 46). Several experimental studies have shown that treatment of certain pancreatic cancer cells with *COX-2* inhibitors suppresses cell proliferation, induces apoptosis, and inhibits both tumor invasion and tumor-induced angiogenesis

(36, 47–50). The efficacy of *COX-2* inhibitors for the treatment of pancreatic cancer is currently being investigated in clinical trials (51). In the present study, we demonstrate that, regardless of the mechanism for its action, NS-398 can decrease the invasive properties of pancreatic cancer cells acquired through tumor-stromal interactions. Our present findings provide an additional rationale for the use of *COX-2* inhibitors in the treatment of pancreatic cancer and also raise the possibility that the clinical benefit of *COX-2* inhibitors could be underestimated if only the standard measurements of clinical antitumor response (such as reduction in tumor size) are considered.

In summary, by analyzing gene expression alterations associated with tumor-stromal interactions, we have identified a number of genes that are likely to participate in tumor-stromal interactions.

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