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Prostaglandin E_2 Enhances Pancreatic Cancer Invasiveness through an Ets-1–Dependent Induction of Matrix Metalloproteinase-2

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ABSTRACT

Accumulating evidence suggests an important role for cyclooxygenase-2 (COX-2) in the pathogenesis of a wide range of malignancies. Here we tested the hypothesis that the COX-2 product prostaglandin E_2 (PGE_2) increases cellular invasive potential by inducing matrix metalloproteinase-2 (MMP-2) expression and activity through an extracellular signal-regulated kinase (ERK)/Ets-1–dependent mechanism in pancreatic cancer. PANC-1 and MIAPaCa-2 pancreatic cancer cells were treated with PGE_2 or rofecoxib, a selective COX-2 inhibitor. MMP-2 expression and activity were assayed using Western blot analysis and zymography, respectively. MMP-2 promoter activity was analyzed with a luciferase-based assay. Ets-1 activity was analyzed using gel shift assay. Ets-1 expression was specifically silenced using RNA interference. Cellular invasive and migratory potentials were determined using a Boyden chamber assay with or without Matrigel, respectively. Exogenous PGE_2 induced MMP-2 expression and activity and increased ERK1/2 phosphorylation, Ets-1 binding activity, and MMP-2 promoter activity. PGE_2 also increased cellular migratory and invasive potentials. The mitogen-activated protein kinase kinase inhibitor PD98059 and Ets-1 silencing each abolished PGE_2-induced increases in MMP-2 expression. PD98059 and Ets-1 silencing each abrogated the effect of PGE_2 on cellular invasive potential but not on cellular migratory potential. Rofecoxib suppressed MMP-2 expression and activity, Ets-1 binding activity, MMP-2 promoter activity, and cellular migratory and invasive potentials. These results suggest that PGE_2 mediates pancreatic cancer cellular invasiveness through an ERK/Ets-1–dependent induction of MMP-2 expression and activity. They also suggest that COX-2 inhibition may represent a strategy to inhibit invasive potential in pancreatic cancer.

INTRODUCTION

Cyclooxygenase-2 (COX-2) overexpression has been reported to be present in 74% to 100% of human pancreatic cancer specimens (1–3). COX-2 is the inducible isoform of COX, the enzyme catalyzing the rate-limiting step in the metabolic pathway that transforms arachidonic acid into the prostaglandins (PGs) and thromboxanes, whereas COX-1 is constitutively expressed in most tissues (4). Among the products of COX-2, prostaglandin E_2 (PGE_2), in particular, may play a biologically important role in cancer pathogenesis. Many cancers that overexpress COX-2 have high intratumoral levels of PGE_2 (5–7), and PGE_2 recently was shown to up-regulate the invasive potential of colorectal carcinoma cells (8).

We have reported previously that matrix metalloproteinase-2 (MMP-2) activity is an important determinant of pancreatic cancer cellular invasive potential (9). Ets-1 is a member of the Ets family of transcription factors that share a conserved Ets domain (10). It has been reported that Ets-1 is overexpressed in a variety of human malignancies, including cancers of the ovary (11), breast (12), colon and rectum (13), and pancreas (14). Because of its roles in the transcriptional regulation of MMPs, including MMP-2 (15, 16), Ets-1 is a candidate mediator of cancer invasion and metastasis.

In this study, we tested the hypothesis that PGE_2 increases pancreatic cancer cellular invasive potential through an Ets-1–dependent induction of MMP-2 expression and activity. We also tested the efficacy of COX-2 inhibition in inhibiting Ets-1–dependent MMP-2 induction and cellular invasive potential.

MATERIALS AND METHODS

Materials. Culture media and fetal bovine serum (FBS) were obtained from Life Technologies, Inc. (Rockville, MD). PGE_2, rofecoxib (RFX), and PD98059 were obtained from Sigma (St. Louis, MO), Merck (Montreal, Canada), and Calbiochem (La Jolla, CA), respectively. Anti–MMP-2 and antiactin monoclonal antibodies were obtained from NeoMarkers, Inc. (Fremont, CA). Anti–COX-2, anti–Ets-1, anti–Oct-1, anti–extracellular signal-regulated kinase (ERK) 1/2, and anti–phospho-ERK1/2 monoclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The BCA assay kit and the DAB liquid substrate kit were obtained from Sigma, and the Vecstain ABC protein detection system kit was obtained from Vector Laboratory (Burlingame, CA). All of the other reagents were purchased from Sigma unless otherwise specified.

Cell Culture. Human pancreatic cancer cells (PANC-1, MIAPaCa-2) were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM containing 10% FBS in 75-cm^2 culture flasks kept in a humidified (37°C, 5% CO_2) chamber. For the experiments described below, cells were trypsinized and harvested on reaching 80% to 90% confluency.

Treatment of Cells with PGE_2 or Rofecoxib. Cells (2×10^6) were seeded onto wells of six-well tissue culture dishes and allowed to adhere overnight. Either PGE_2 (10 μmol/L) or RFX (5 μmol/L) subsequently was added. Cells were harvested for the assays described below after incubation periods ranging from 15 minutes to 24 hours. Neither PGE_2 nor RFX at the concentrations used affected cellular viability, as determined by trypan blue exclusion tests performed on cells following 24 hours of incubation with these agents.

PGE_2 ELISA. Cells (1×10^5) in 0.5-mL culture media were seeded onto 24-well plates. At 24 hours after cell seeding, conditioned media were harvested, and PGE_2 concentrations were determined using an ELISA assay kit according to instructions supplied by the manufacturer (Cayman Chemical, Ann Arbor, MI). Samples were assayed in triplicate in each of three independent experiments.

Western Blot Analysis. Cells (2×10^6) were harvested and rinsed with PBS. Cell extracts were prepared using lysing buffer [20 mmol/L Tris-HCl (pH 7.5), 0.1% Triton X, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin] and centrifugation at 12,000 $\times$ g and 4°C. Total protein concentration was measured using the BCA assay. Cellular extracts containing 50-μg total protein were subjected to 10% SDS-PAGE, and the proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA). After blocking with PBS containing 0.2% casein for 1 hour at room temperature, membranes were incubated with 1 μg/mL antibody in PBS containing 0.2% Tween-20 overnight at 4°C. The Vecstain ABC kit and DAB liquid substrate were used for chromogenic detection, according to the manufacturer's instructions. Band intensities were quantified using Image Pro Plus 4.0 (Media Cybernetics, Silver Spring, MD) and normalized to those of actin. Blots were performed triplicate. Mean densitometric values ($\pm$SD) are given.

Zymography. Cells (1×10^5) were seeded onto wells of 24-well plates and allowed to adhere in the presence of serum. Media subsequently were replaced by 0.5 mL of serum-free medium per well. After 24 hours of incubation, the

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conditioned media were harvested for zymography. Zymography was carried out as described previously (17). In brief, 25 μL of the conditioned medium for each sample were subjected to 10% SDS/PAGE with 1 mg/mL gelatin incorporated into the gel mixture. Following the electrophoresis, the gels were soaked in 2.5% Triton X to remove SDS, rinsed with 10 mmol/L Tris (pH 8.0), and transferred to a bath containing 50 mmol/L Tris (pH 8.0), 5 mmol/L $CaCl_2$, and 2 μmol/L $ZnCl_2$ at 37°C for 17 hours. The gels then were stained with 0.1% Coomassie blue in 45% methanol and 10% acetic acid. Experiments were repeated three times.

Cloning of MMP-2 Promoter. The human MMP-2 promoter was cloned from human placental DNA by PCR using primers (5′-CACACCCACCAGACAAGCCT-3′ and 5′-AAGCCCCAGATGCGCAGCCT-3′) as described previously (18). The PCR product (the 1716-bp DNA fragment covering positions −1659 to +57 relative to the MMP-2 transcription initiation site) was cloned into the pGL3 vector (Promega, Madison, WI). A deletion mutant MMP-2 promoter (1081-bp fragment covering positions −1024 to +57 relative to the MMP-2 transcription initiation site) was made from the full-length MMP-2 promoter; it was digested by *Eco*RI, filled with Klenow fragment of DNA polymerase I, and religated. The vectors containing the inserts are designated pGL3-MMP2 (containing full-length promoter) and pGL3-MMP2Δ (containing deletion mutant promoter); sequencing was performed to confirm correct insert orientation.

Luciferase Assay. The luciferase assay was conducted as described previously (19). Empty pGL3 vector (pGL3e) was used for control transfections. Cells (4×10^5 cells/well) were plated onto wells of 12-well cell culture dishes and cultured for 12 hours in serum-containing medium. One microgram of pGL3-MMP2, pGL3-MMP2Δ, or pGL3e was cotransfected with 0.1 μg of pRL-CMV vector (Promega), which contains a cytomegalovirus promoter upstream of a renilla luciferase gene. Immediately afterward, cells were treated with either PGE_2 (10 μmol/L) or RFX (5 μmol/L). After 24 hours, luciferase activities in lysates of the transfected cells were measured, according to the manufacturer's recommended protocol (Dual-Luciferase Reporter Assay System; Promega). The activities of renilla luciferase were used to normalize any variations in transfection efficiency. The promoter activities of each plasmid construct were calculated as the firefly-renilla luciferase activity ratios. Measurements were performed three times per sample, and results are shown as mean ± SD of three independent experiments.

Nuclear Extract Preparation. Nuclear extracts were obtained using a modification of a previously described method (20). Harvested cells were lysed with buffer A (10 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L EDTA, 1.5 mmol/L $MgCl_2$, 0.2% NP40, 1 mmol/L DTT, and 0.5 mmol/L phenylmethylsulfonyl fluoride), followed by vortexing to shear the cytoplasmic membranes. Nuclei were pelleted by centrifugation at 3000 rpm for 30 seconds at 4°C in a microcentrifuge. Nuclear proteins were extracted with high-salt buffer C (20 mmol/L HEPES, 25% glycerol, 1.5 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 420 mmol/L NaCl, 1 mmol/L DTT, and 0.5 mmol/L phenylmethylsulfonyl fluoride). Total nuclear protein was determined by BCA assay.

Electrophoretic Mobility Shift Analysis. Ets-1 and Oct-1 transcription factor activities were assessed by electrophoretic mobility shift analysis (EMSA) using double-stranded oligonucleotides corresponding to the Ets-1 or Oct-1 consensus sequences (Ets-1, 5′-GATCTCGAGCAGGAAGTTCGA-3′; Oct-1, 5′-TGTCGAATGCAAATCACTAGAA-3′; Santa Cruz Biotechnology), respectively. Probes were end-labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Labeled probes were purified by Nick column (Amersham Biosciences, Piscataway, NJ). Nuclear protein (5 μg) was incubated with labeled probe in a binding buffer containing 2 μg of poly(dI·dC) (Amersham Biosciences), 20 mmol/L HEPES (pH 7.9), 10% glycerol, 1 mmol/L EDTA, 5 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride, and 105 mmol/L NaCl for 20 minutes at room temperature. Protein-DNA complexes were resolved by electrophoresis through a 6% polyacrylamide gel and were electrophoretically transferred to nylon membranes (Invitrogen). The membranes then were subjected to autoradiography. Band specificities were determined in competition experiments using a 100-fold molar excess of unlabeled nucleotide containing either the Ets-1 consensus sequence or a mutated sequence (Ets-1, 5′-GATCTCGAGCAAGAAGTTCGA-3′; Santa Cruz Biotechnology) that was added to nuclear extracts for 10 minutes before addition of radiolabeled probe. Ets-1 supershift was performed using an anti–Ets-1 monoclonal antibody. The antibody (1 μg/mL final concentration) was added after the oligonucleotide had reacted for 20 minutes with the nuclear extract and allowed to react for 45 minutes at room temperature before electrophoresis.

Small Interfering RNA Synthesis. Small interfering RNAs (siRNAs) were synthesized and high-performance purified (Qiagen, Valencia, CA). Ets-1 sense (ACUUGCUACCAUCCCGUAC-dTT) and antisense (GUACGGGAUGGUAGCAAGU-dTT) siRNAs and control siRNAs (sense, AGGAGAUAUUUCGAGGCUU-dTT; antisense, AAGCUCGAAAUAUCUCCU-dTT), bearing no homology with any known human genes, were dissolved in buffer [100 mmol/L potassium acetate, 30 mmol/L HEPES-potassium hydroxide, and 2 mmol/L magnesium acetate (pH 7.4)] to a final concentration of 20 μmol/L, heated to 90°C for 60 seconds, and incubated at 37°C for 60 minutes before use.

Small Interfering RNA Transfection. Cells (5×10^5) were plated onto wells of six-well cell culture plates and allowed to adhere for 24 hours. Eight microliters siPORT *Amine* transfection reagent (Ambion Inc., Austin, TX) per well were added to serum-free DMEM for a final complexing volume of 200 μL, vortexed, and incubated at room temperature for 20 minutes. Two microliters of siRNA solution were added to the diluted siPORT *Amine* transfection reagent, gently mixed, and incubated at room temperature for 15 minutes. The transfection agent/siRNA complex was added into the wells containing 800 μL DMEM with 10% FBS and incubated in normal cell culture conditions for 6 hours, after which time 1 mL DMEM with 10% FBS was added. All of the assays were performed after 48 hours.

Invasion and Migration Assays. The BD Biocoat Matrigel invasion chamber (BD Bioscience, San Jose, CA) was used according to the manufacturer's instructions. In brief, 2.5×10^4 cells in serum-free media were seeded onto the Matrigel-coated filters, and in the lower wells 5% FBS was added as the chemoattractant. After 24 hours incubation, the filters were stained with Diff-Quik kit (BD Bioscience), and the number of cells that had penetrated through the filter was counted under magnification (randomly selected high-

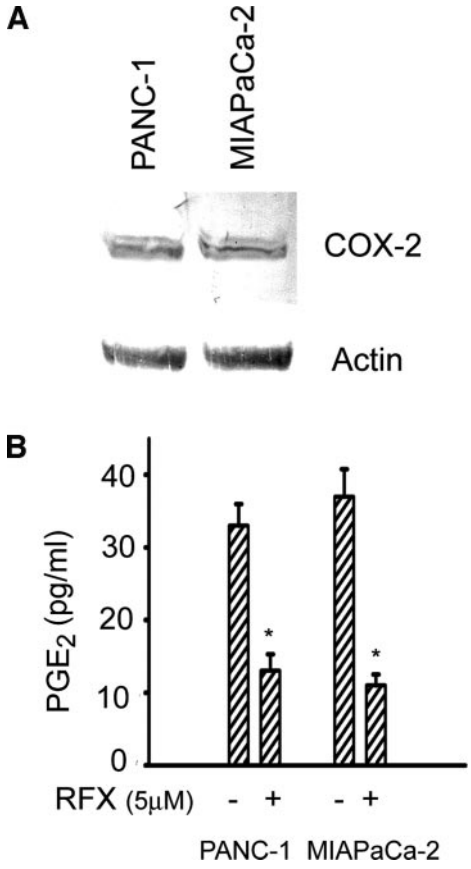

Fig. 1. COX-2 expression and the effect of RFX on PGE_2 production. *A*, COX-2 expression. COX-2 expression in PANC-1 and MIAPaCa-2 cells was confirmed using Western blot analysis. Actin was used as a loading control. *B*, the effect of RFX on PGE_2 production. Cells were treated with RFX (5 mmol/L), and the concentration of PGE_2 in the conditioned media was analyzed using ELISA. RFX reduced PGE_2 production by 61% and 70% in PANC-1 and MIAPaCa-2, respectively ($*P < 0.05$).

power fields). The counting was performed for three fields in each sample. In additional invasion experiments, anti–MMP-2 antibody (100 μg/mL or 400 μg/mL) was added to neutralize MMP-2 activity as described previously (21). To assess cellular migratory potential, the protocol described above was used, except that Matrigel was omitted. Experiments were performed in triplicate, and the results were shown as mean ± SD of three independent experiments.

Statistical Analysis. Differences between groups were analyzed using two-sided *t* test and ANOVA with $P < 0.05$ considered statistically significant. In cases in which averages were normalized to controls, the SDs of each nominator and denominator were taken into account in calculating the final SD.

RESULTS

COX-2 Expression and the Effect of Rofecoxib on PGE_2 Synthesis. First, we examined COX-2 expression and endogenous PGE_2 production in the pancreatic cancer cell lines using Western blot analysis and PGE_2 ELISA, respectively. For PANC-1 and MIAPaCa-2 cells, COX-2 expression was confirmed; the selective COX-2 inhibitor RFX (5 μmol/L) reduced PGE_2 production by 61% and 70%, respectively, in these cell lines ($P < 0.05$; Fig. 1).

Effects of PGE_2 and Rofecoxib on MMP-2 Expression and Activity. To determine the effects of PGE_2 on MMP-2 expression and activity, cells were administered exogenous PGE_2 (10 μmol/L). In parallel experiments, RFX (5 μmol/L) was administered to suppress endogenous PGE_2 production. As shown in Fig. 2*A*, exogenous PGE_2 increased MMP-2 protein expression by 85% and 122% ($P < 0.05$) in PANC-1 and MIAPaCa-2 cells, respectively. In contrast, RFX reduced MMP-2 expression by 48% and 55% ($P < 0.05$) in PANC-1 and MIAPaCa-2 cells, respectively. The effects of PGE_2 and RFX on MMP-2 gelatinolytic activity paralleled those on MMP-2 expression in PANC-1 and MIAPaCa-2 cells, as shown on zymography (Fig. 2*B*).

Effects of PGE_2 and Rofecoxib on MMP-2 Transcriptional Activity. Because of the observed alterations in MMP-2 expression induced by PGE_2 and by RFX, we next sought to determine the effects of PGE_2 and RFX on MMP-2 transcriptional activity using a luciferase-based reporter assay. The full-length MMP-2 promoter was incorporated into the pGL3 luciferase vector and transfected into the cells together with the pRL-CMV plasmid. The resulting luciferase activity was measured at 24 hours. As shown in Fig. 3*A*, PGE_2 increased MMP-2 promoter activities by 81% and 124% ($P < 0.05$) in PANC-1 and MIAPaCa-2 cells, respectively, whereas RFX reduced MMP-2 promoter activities by 41% and 46% ($P < 0.05$), respectively, in PANC-1 and MIAPaCa-2 cells. To localize the responsive element in the MMP-2 promoter sequence, the truncated MMP-2 promoter with deletion of the region spanning positions −1659 to −1025 relative to the MMP-2 transcription initiation site was constructed, and the effect of this deletion on PGE_2-induced luciferase activity was analyzed. As shown in Fig. 3*B*, deletion of aforementioned region reduced baseline promoter activities 39% and 34% in PANC-1 and MIAPaCa-2, respectively ($P < 0.05$), and abolished the effect of PGE_2 on promoter activity. This result implicates the region spanning positions −1659 to −1025 of the MMP-2 promoter in PGE_2-mediated induction of MMP-2 transcription.

Effect of PGE_2 on Transcription Factor Ets-1 Binding Activity. Ets-1 is a known transcriptional regulator of MMP-2 expression (15, 22), which binds to the region spanning −1255 to −1248 of the

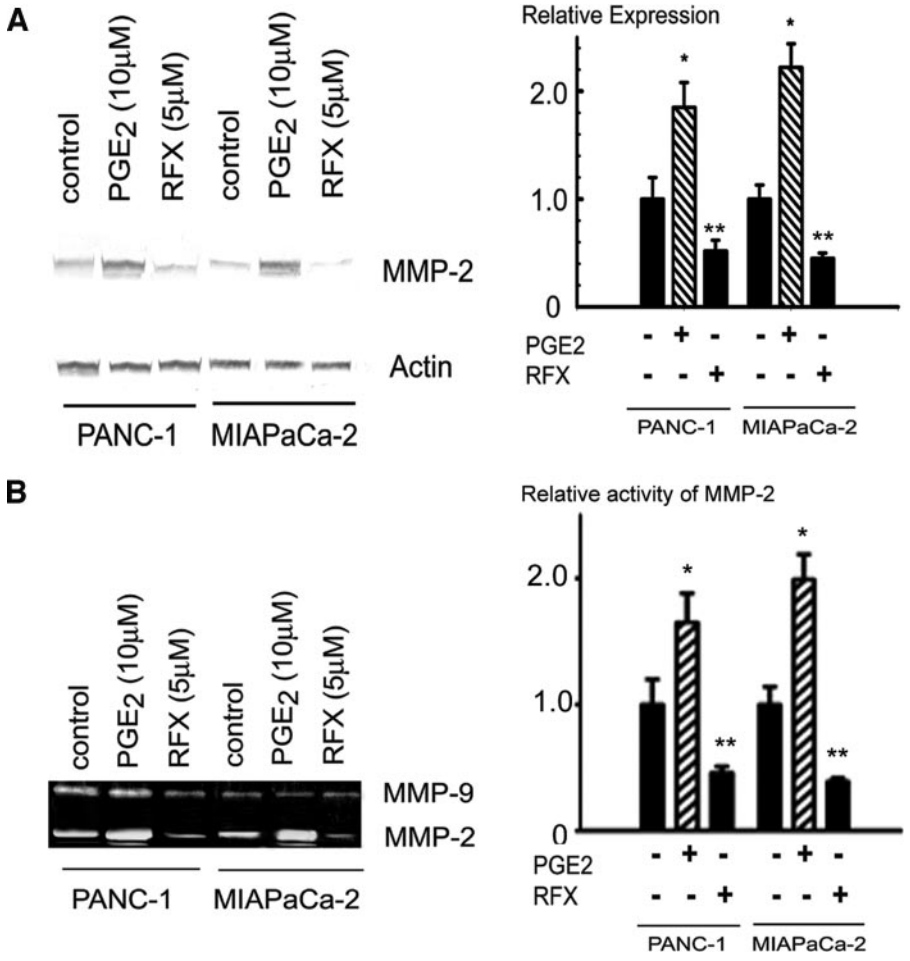

Fig. 2. The effects of PGE_2 and RFX on MMP-2 expression and activity. *A*, MMP-2 expression. Cells were treated with PGE_2 (10 μmol/L) or RFX (5 μmol/L) for 24 hours, and MMP-2 in cell lysates was detected using Western blot analysis. PGE_2 increased MMP-2 expression in PANC-1 and MIAPaCa-2 cells by 85% and 122%, respectively ($*P < 0.05$), whereas RFX reduced MMP-2 expression by 48% and 55%, respectively, relative to those in untreated control cells ($**P < 0.05$). The graph depicts intensity of bands representing MMP-2 normalized to the intensity of bands representing actin. *B*, MMP-2 activity. Cells were treated with PGE_2 (10 μmol/L) or RFX (5 μmol/L) for 24 hours, and MMP-2 activity in the conditioned medium was assayed using zymography. The effects of PGE_2 and RFX on MMP-2 activity paralleled those on MMP-2 expression. PGE_2 increased MMP-2 activity in PANC-1 and MIAPaCa-2 cells by 65% and 99%, respectively ($*P < 0.05$), whereas RFX reduced MMP-2 activity by 54% and 61%, respectively, relative to those in untreated control cells ($**P < 0.05$). The graph depicts intensity of bands representing of MMP-2 activities.

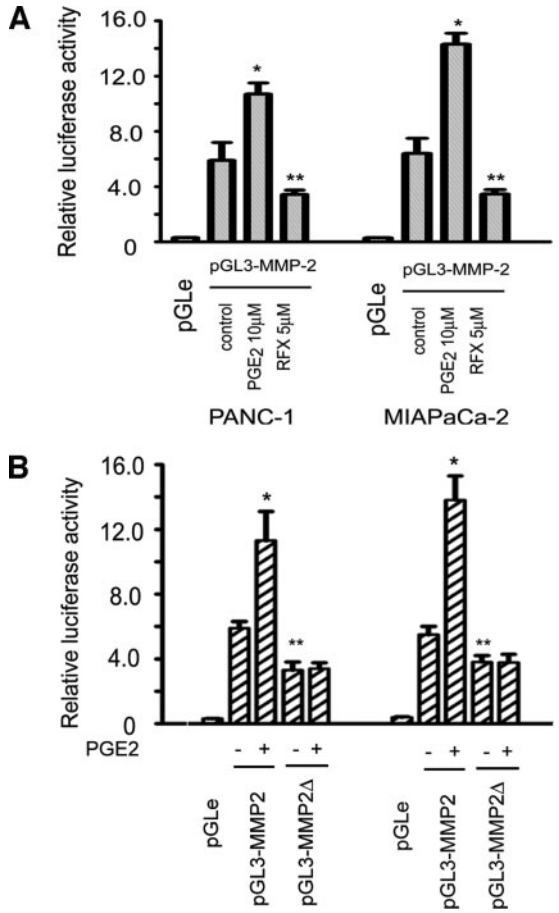

Fig. 3. The effects of PGE_2 and RFX on MMP-2 promoter activity. *A.* Cells were transfected with pGL3e (empty firefly luciferase vector) or pGL3-MMP2, containing the full sequence MMP-2 promoter, along with pRL containing the renilla luciferase gene. Immediately after transfection, cells were treated with PGE_2 (10 μmol/L) or RFX (5 μmol/L), and luciferase activity in cell lysates was assayed at 24 hours subsequently. The induced firefly luciferase activity was normalized to the renilla luciferase activity. PGE_2 increased MMP-2 promoter activities by 81% and 124% ($*P < 0.05$ *versus* untreated control) in PANC-1 and MIAPaCa-2 cells, respectively, whereas RFX suppressed MMP-2 promoter activities by 41% and 46%, respectively. ($**P < 0.05$ *versus* untreated control). *B.* Cells were transfected with pGL3-MMP2Δ, containing a deletion mutant of the MMP-2 promoter (deletion of the region spanning positions −1659 to −1025 relative to the MMP-2 transcription initiation site), and the luciferase activity in cell lysates was assayed at 24 hours. Deletion of the aforementioned region resulted in reduction of baseline promoter activity by 39% and 34% in PANC-1 and MIAPaCa-2, respectively ($*P < 0.05$) and abolished the effect of PGE_2 on promoter activity.

MMP-2 promoter (18). Because of our observation that PGE_2 up-regulates MMP-2 promoter activity, we sought to determine whether PGE_2 up-regulates Ets-1 binding activity. As shown in Fig. 4*A*, PGE_2 increased Ets-1 binding activities in PANC-1 and MIAPaCa-2 cells. Oct-1 binding was used as a loading control for nuclear extracts. In contrast, RFX reduced Ets-1 binding activities at 24 hours in PANC-1 and MIAPaCa-2, as shown in Fig. 4*B*. Band specificities were confirmed in competition and supershift experiments (Fig. 4*C*). These findings suggest that Ets-1 may play a role in the PGE_2-mediated increase in MMP-2 transcription.

Effects of *Ets-1* Gene Silencing on PGE_2-Induced Increase in MMP-2 Expression. To determine whether PGE_2-mediated MMP-2 induction is Ets-1 dependent, we suppressed Ets-1 expression using RNA interference. At 48 hours after transfection of siRNA, Ets-1 expression was determined using Western blot analysis. As shown Fig. 5*A*, Ets-1 expression was suppressed by 79% and 83% in PANC-1 and MIAPaCa-2 cells, respectively, as compared with their untransfected counterparts. There were no significant differences in Ets-1 expression between untransfected cells and cells transfected with control siRNA bearing no homology with any human genes. Forty-eight hours after the transfection, we treated cells with PGE_2 and assayed MMP-2 expression 24 hours subsequently. Ets-1 silencing reduced baseline MMP-2 expression by 63% and 66% ($P < 0.05$) in PANC-1 and MIAPaCa-2 cells, respectively, and abolished PGE_2-mediated MMP-2 induction in PANC-1 and MIAPaCa-2 cells (Fig. 5*B*). This finding suggests that Ets-1 is critical for PGE_2-mediated MMP-2 expression.

Role of ERK Signaling in PGE_2-Mediated Ets-1 Binding Activity. PGE_2 has been reported to activate ERK signaling (23). Therefore, we next tested the role of ERK activation in mediating PGE_2-induced Ets-1 activation. Cells were treated with PGE_2 (10 μmol/L) in the presence or absence of the mitogen-activated protein kinase (MAPK) kinase inhibitor PD98059, and ERK1/2 phosphorylation subsequently was analyzed. PGE_2 induced ERK1/2 phosphorylation within 30 minutes after its administration (Fig. 6*A*); PD98059 inhibited PGE_2-mediated ERK1/2 phosphorylation (Fig. 6*B*). We then determined the effect of inhibiting ERK1/2 activation on the PGE_2-induced increase in Ets-1 binding activity using EMSA. The induction of Ets-1 binding by PGE_2 was abolished by PD98059, as shown in Fig. 6*C*. These findings suggest that PGE_2-induced Ets-1 binding activity is mediated by ERK signaling.

Effects of PGE_2 on Cellular Invasion and Migration. We previously have shown that MMP-2 activity is associated with pancreatic cancer invasive potential (9, 24). Here we tested the effects of PGE_2 and RFX on cellular invasive and migratory potentials. PGE_2 induced 2.8-fold and 2.9-fold increases in PANC-1 and MIAPaCa-2 cellular invasiveness through Matrigel, respectively, relative to untreated cells ($P < 0.05$). In contrast, RFX induced 63% and 66% reductions in cellular invasiveness in MIAPaCa-2 and PANC-1, respectively, relative to untreated controls ($P < 0.05$). PGE_2 induced 3.2-fold and 3.7-fold increases in PANC-1 and MIAPaCa-2 cellular migration, respectively ($P < 0.05$); RFX induced 55% and 58% reductions in cellular migration, respectively (Fig. 7*A*; $P < 0.05$). Ets-1 silencing reduced PANC-1 and MIAPaCa-2 cellular invasiveness by 30% and 34%, respectively, and abrogated PGE_2-mediated increases in cellular invasiveness by 65% and 63%, respectively, relative to PGE_2-treated control cells ($P < 0.05$). Interestingly, Ets-1 silencing altered neither baseline cellular migration nor PGE_2-induced increase in cellular migration in PANC-1 and MIAPaCa-2 cells (Fig. 7*B*). The effects of MAPK kinase inhibition on cellular invasiveness and migration were similar to those of *Ets-1* gene silencing. PD98059 (40 μmol/L) reduced baseline PANC-1 and MIAPaCa-2 cellular invasiveness by 40% and 44%, respectively ($P < 0.05$), and abrogated PGE_2-induced increases in cellular invasiveness by 63% and 74%, respectively ($P < 0.05$), relative to PGE_2-treated control cells. There was no significant effect of PD98059 on cellular migration (Fig. 7*C*).

To examine the relationship between observed alterations in invasiveness and in MMP-2 expression, Matrigel invasion assays were conducted in the presence of MMP-2 neutralizing antibody. PGE_2-mediated increases in PANC-1 and MIAPaCa-2 cellular invasiveness through Matrigel were abrogated by anti–MMP-2 antibody but not by control IgG (Fig. 7*D*). These findings suggest that PGE_2-mediated effects on pancreatic cancer cellular invasiveness through Matrigel are at least in part MMP-2 dependent.

DISCUSSION

There is much evidence that implicates important roles for COX-2 and PGE_2 in carcinogenesis and progression for a wide range of malignancies (3, 25–29), including pancreatic cancer (2, 3). However, there are few data on the downstream targets by which COX-2 and PGE_2 mediate these processes. In this study, we

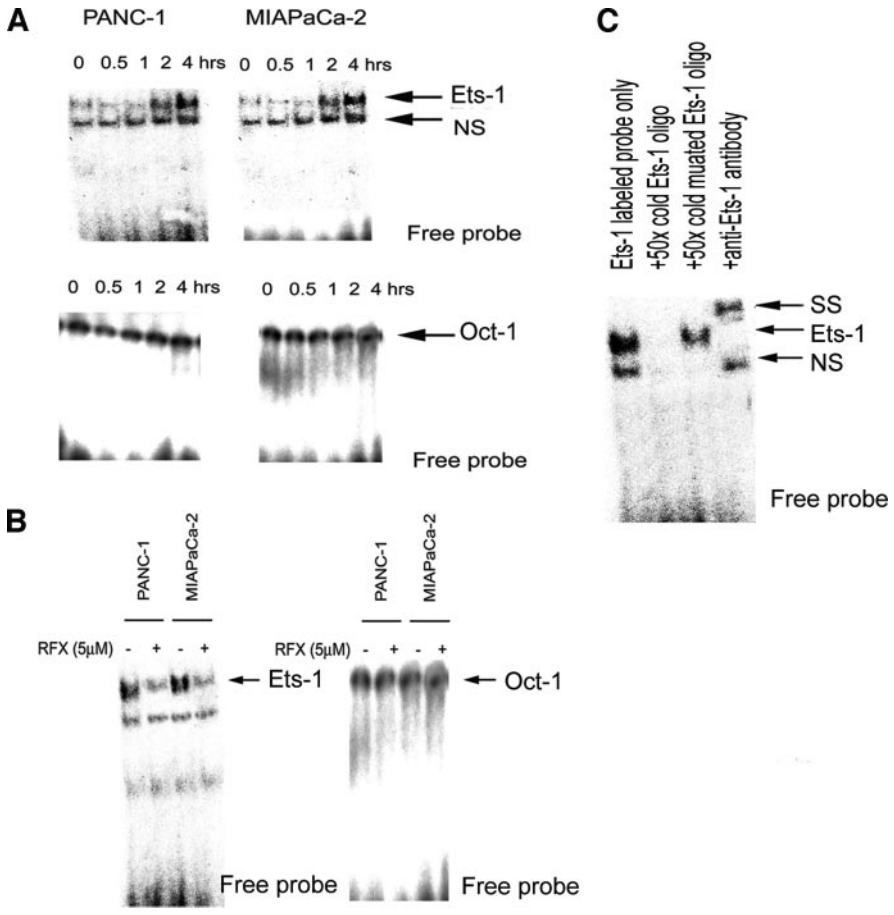

Fig. 4. The effects of PGE_2 and RFX on Ets-1 binding activity. *A,* the effect of PGE_2. Cells were serum starved overnight and then treated with PGE_2 (10 μmol/L). Extracted nuclear protein was subjected to EMSA with an oligonucleotide probe containing an Ets-1 binding consensus sequence. Binding to an Oct-1 probe was used as a control for quality and quantity of nuclear extract. The binding activity of Ets-1 was induced within 4 hours after PGE_2 treatment in PANC-1 and MIAPaCa-2 cells. NS indicates nonspecific band. *B,* the effect of RFX. Cells were treated with RFX (5 μmol/L) for 24 hours in the presence of serum. The binding activity of Ets-1 was attenuated with RFX treatment compared with untreated control. *C,* specificity of Ets-1 DNA binding activity. EMSA was carried out in the presence or absence of a molar excess of nonradioactive oligonucleotide containing the Ets-1 binding consensus sequence. The nonradioactive oligonucleotide competed against radioactive probe, whereas oligonucleotide containing a mutated consensus sequence did not. Band supershift (indicated as SS) was induced by anti–Ets-1 monoclonal antibody.

identified Ets-1 as an important mediator of PGE_2-induced induction of MMP-2 expression. Our study is the first to establish the association between PGE_2 and Ets-1 in cancer; it also provides evidence that COX-2 may mediate cancer invasion by inducing MMP-2.

The potentially important roles of COX-2 and PGE_2 in cancer invasion are supported not only by the observed relationship between COX-2 overexpression and cancer invasion or metastasis but also by other *in vitro* data. Tsujii *et al.* (30) showed that forcibly overexpressing COX-2 increases MMP-2 expression/activation and cellular invasiveness in colon cancer cells. Administration of COX-2 inhibitors also has been reported to suppress MMP-2 expression and invasiveness in cells derived from a range of cancers, including those of the colon and rectum (31, 32), breast (33), lung (34), and pancreas (35).

Our study builds on these previously reported findings by revealing a potential mechanism by which COX-2–derived PGE_2 mediates MMP-2 expression and cellular invasiveness. We hypothesized that Ets-1 might play a role in PGE_2-mediated MMP-2 induction. Ets-1 has been reported to be overexpressed in variety of malignancies, including pancreatic cancers (14), and to regulate the transcription of many MMPs, including MMP-2 (15, 22, 36, 37). A recently reported study localized an Ets-1 binding site to the MMP-2 promoter (18). Our findings that PGE_2 induces Ets-1

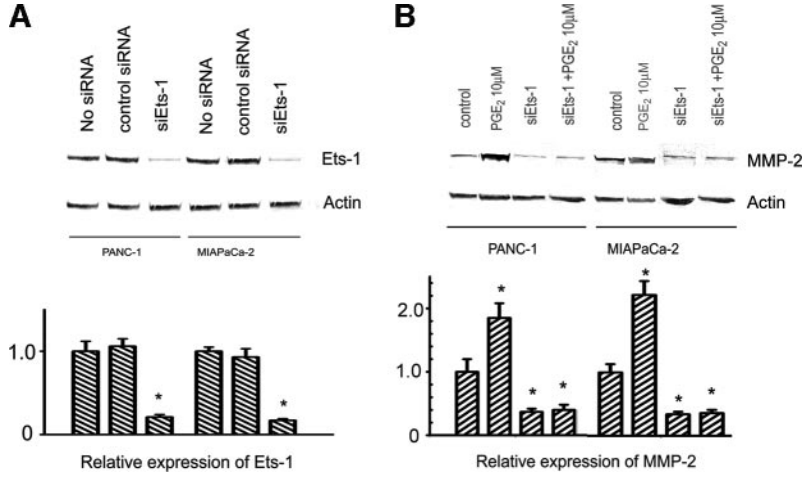

Fig. 5. The effect of *Ets-1* gene silencing on PGE_2-mediated MMP-2 expression. *A, Ets-1* gene silencing. Cells were transfected with Ets-1–specific siRNA (siEts-1), and at 48 hours, Ets-1 expression was assayed using Western blot analysis. Ets-1–specific siRNA reduced Ets-1 expression significantly in PANC-1 and MIAPaCa-2 cells [$*P < 0.05$, *versus* corresponding control (no siRNA)], whereas control siRNA containing no sequence homology to any human genes did not affect Ets-1 expression. The graph depicts the intensity of bands representing Ets-1 normalized to the intensity of those representing actin. *B,* MMP-2 expression. Forty-eight hours after transfection with siEts-1, the cells were treated with PGE_2 (10 μmol/L), and MMP-2 expression was assayed at 24 hours using Western blot analysis. *Ets-1* gene silencing reduced baseline MMP-2 expression by 63% and 66% in PANC-1 and MIAPaCa-2 cells, respectively ($*P < 0.05$, *versus* corresponding control), and abolished PGE_2-induced increases in MMP-2 expression. The graph depicts the intensity of bands representing MMP-2 normalized to the intensity of those representing actin.

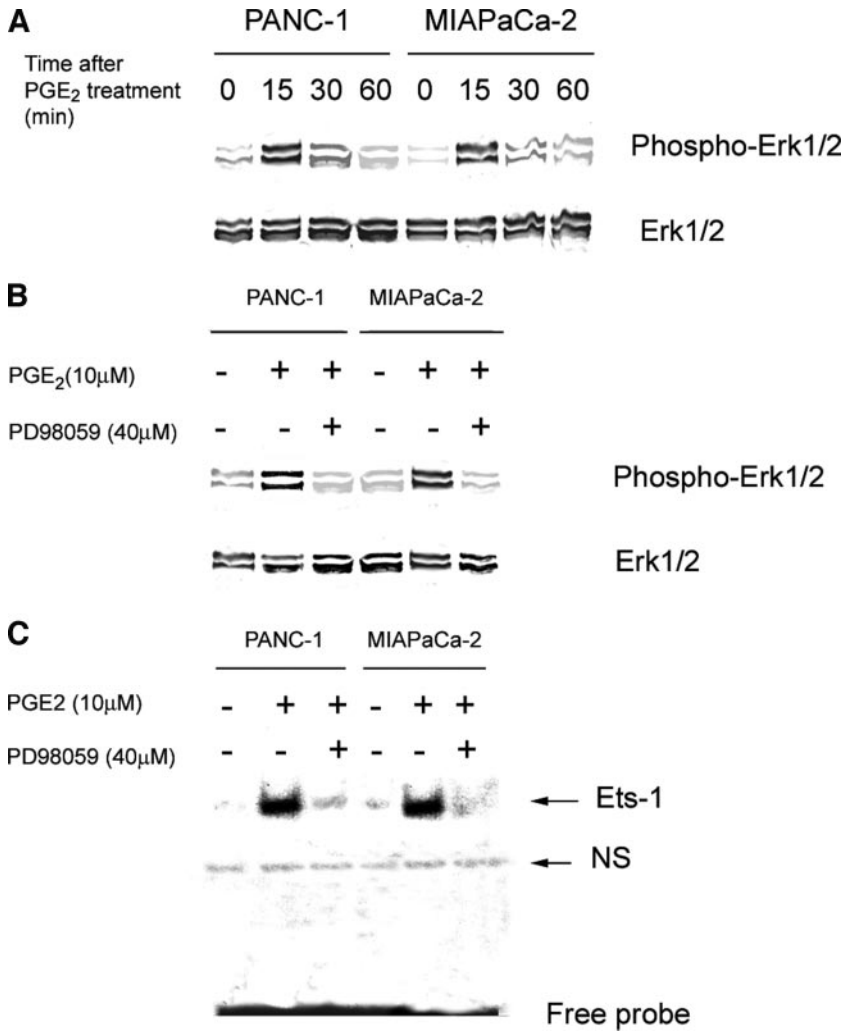

Fig. 6. The effect of PGE_2 on ERK1/2 phosphorylation and the effect of PD98059 on Ets-1 binding activity. *A*, ERK1/2 phosphorylation. Cells were serum starved overnight and then treated with PGE_2 (10 μmol/L). ERK1/2 phosphorylation was assayed using Western blot analysis with anti–phosphorylated-ERK1/2 antibody. Equal loading of proteins in each lane was confirmed using anti-ERK1/2 antibody. ERK1/2 phosphorylation was induced by PGE_2 within 15 minutes of administration in PANC-1 and MIAPaCa-2 cells. *B*, the effect of PD98059 on PGE_2-induced ERK1/2 phosphorylation. Cells were treated with PGE_2 (10 μmol/L) in the presence or absence of PD98059 (40 μmol/L) for 30 minutes, after which ERK1/2 phosphorylation was assayed. PD98059 abolished the effect of PGE_2 on ERK phosphorylation in PANC-1 and MIAPaCa-2 cells. *C*, the effect of PD98059 on Ets-1 binding activity. Cells were treated with PGE_2 (10 μmol/L) in the presence or absence of PD98059 (40 μmol/L) for 4 hours, after which Ets-1 binding activity was assayed using EMSA. PGE_2-induced increases in Ets-1 binding activity were decreased with PD98059 in PANC-1 and MIAPaCa-2 cells.

binding activity and MMP-2 promoter activity and that ETS-1 silencing abolishes the effect of PGE_2 on MMP-2 expression clearly support our hypothesis.

We further explored up-stream regulation of Ets-1 binding activity. PGE_2 previously has been reported to activate MAPK signaling in cancer cell lines (8, 23). Nonsteroidal anti-inflammatory drugs also are reported to suppress ERK1/2 phosphorylation (38). MAPK signaling is reported to enhance Ets-1/Ets-2 binding activity (39). These data, taken together with ours, suggest that Ets-1 binding activity is modified by MAPK signaling provoked by PGE_2. The precise mechanisms by which ERK regulates Ets-1 DNA binding affinity remain to be defined. Some studies suggest that Ets-1 protein is induced by MAPK signaling (40, 41). In our study, although Ets-1 binding activity peaked at 4 hours after administration of PGE_2, no changes in Ets-1 protein expression levels were observed to occur during this period (data not shown). This finding suggests the presence of other mechanisms regulating Ets-1 binding activity. Ets-1 is known to interact with other transcription factors such as nuclear factor κB (42, 43), activator protein 1 (44), and Sp1 (45), each of which can modulate its DNA binding activity. Further studies are required to determine the role of interaction between Ets-1 and these other transcription factors in the context of ERK/Ets-1 signaling.

Our data show that PGE_2 increases not only MMP-2–dependent cellular invasiveness through Matrigel but also cellular migratory potential. This finding is consistent with the study reported by Sheng *et al.*, in which they showed that PGE_2 increases colorectal cancer motility through phosphatidylinositol 3′-kinase/Akt signaling (8). In our study, neither *Ets-1* gene silencing nor MAPK kinase inhibition affected PGE_2-mediated cellular migration. This finding suggests that the effects of PGE_2 on MMP-2 expression and on cellular migration are regulated through separate signaling pathways. They further suggest that PGE_2 may activate multiple signaling pathways that have synergetic effects on cancer invasion.

The relationship between COX-2 expression and cancer invasion has been observed in several human malignancies (25, 46), including pancreatic cancer (1, 2). Our findings provide a mechanistic explanation for these observations. Detailed information on the molecular signaling pathways driving cancer invasion is important for two reasons: First, key components of these signaling pathways represent candidate biomarkers that may predict patient outcomes more reliably than traditional morphologic criteria; and second, understanding these pathways may suggest potential therapeutic targets for inhibiting invasion, a process that is a major contributor to death among patients with cancers such as those of the pancreas.

In summary, our finding indicates that COX-2–derived PGE_2 promotes pancreatic cancer cellular invasive potential through an ERK/Ets-1–dependent induction of MMP-2. This information provides a mechanistic rationale for the observed relationship between COX-2 overexpression and pancreatic cancer progression; targeting this pathway may be a potential therapeutic strategy to inhibit the progression of this highly invasive cancer.

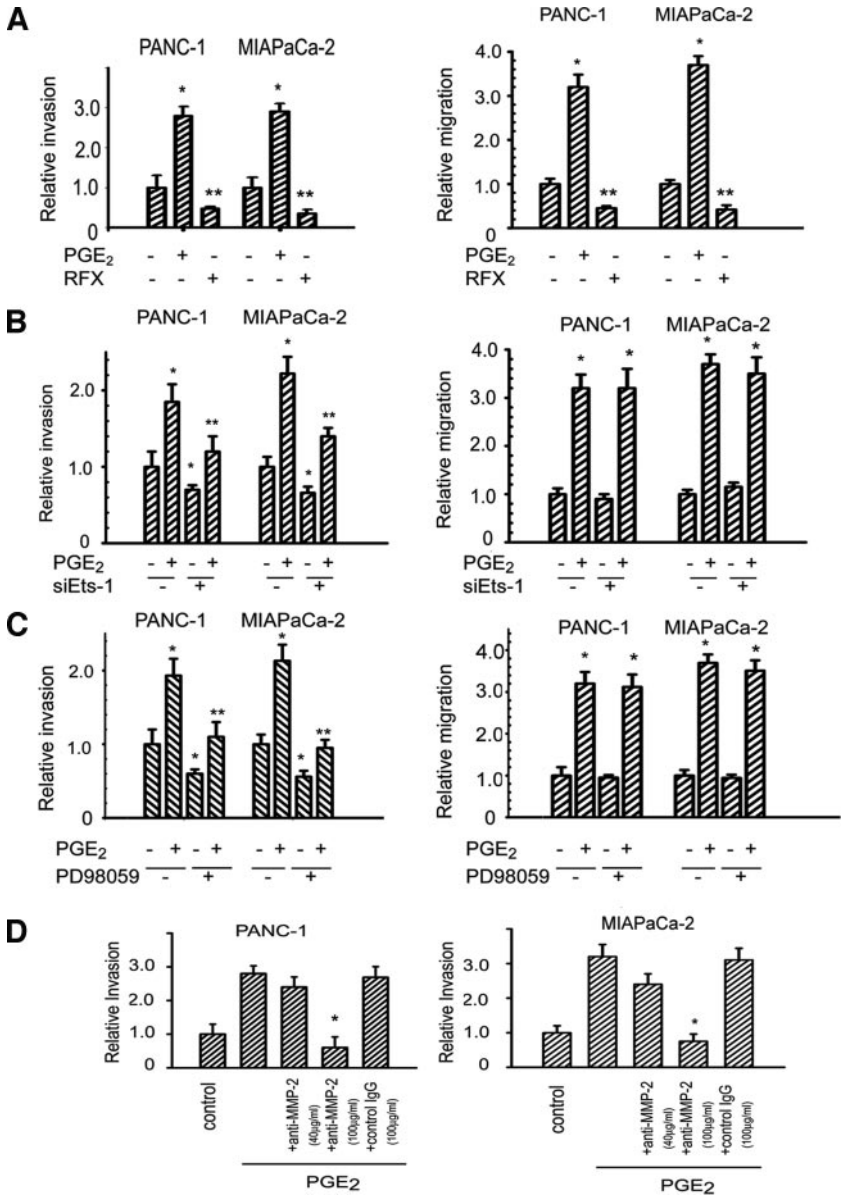

Fig. 7. The effects of PGE_2 and RFX on cellular invasiveness and migration. *A*, the effect of PGE_2 and RFX. Cellular invasiveness and migration were assayed using Boyden chambers with or without Matrigel, respectively, as described in the Materials and Methods section. *Left*, PGE_2 (10 μmol/L) increased cellular invasiveness through Matrigel by 178% and 191% in PANC-1 and MIAPaCa-2 cells, respectively ($*P < 0.05$, *versus* control), and RFX (5 μmol/L) reduced cellular invasiveness by 63% and 66% ($**P < 0.05$, *versus* control), respectively. *Right*, PGE_2 increased cellular migration by 224% and 268% in PANC-1 and MIAPaCa-2 cells, respectively ($*P < 0.05$, *versus* control), and RFX reduced cellular migration by 55% and 58%, respectively ($**P < 0.05$, *versus* control). *B*, the effect of *Ets-1* gene silencing. *Left*, *Ets-1* gene silencing reduced PANC-1 and MIAPaCa-2 cellular invasiveness by 30% and 34%, respectively ($*P < 0.05$, *versus* untransfected control) and abrogated PGE_2-induced increases in invasion by 65% and 63%, respectively ($**P < 0.05$, *versus* PGE_2-treated untransfected control cells). *Right*, *Ets-1* gene silencing had no effect on either PANC-1 and MIAPaCa-2 baseline cellular migration or PGE_2-induced increases in cellular migration ($*P < 0.05$, *versus* corresponding control cells that received no PGE_2). *C*, the effect of PD98059. *Left*, PD98059 (40 μmol/L) reduced PANC-1 and MIAPaCa-2 cellular invasiveness by 40% and 44%, respectively ($*P < 0.05$, *versus* untreated control), and abrogated PGE_2-induced increases in invasion by 63% and 74%, respectively ($**P < 0.05$, *versus* PGE_2-treated controls). *Right*, PD98059 had no effect on either PANC-1 or MIAPaCa-2 baseline cellular migration or PGE_2-induced increases in cellular migration ($*P < 0.05$, *versus* corresponding control cells that received no PGE_2). *D*, the effect of MMP-2 neutralizing antibody on cellular invasiveness. Cellular invasiveness was assayed in the presence of anti–MMP-2 antibody or control IgG. PGE_2-induced increases in cellular invasiveness were abrogated by anti–MMP-2 antibody but not by control IgG ($*P < 0.05$, *versus* PGE_2-treated control).

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