This article has been withdrawn by the authors. In Fig. 1A, the first two lanes of the 18S rRNA panel were reused in the last two lanes. In Fig. 1D, the last two lanes of the Id-1 panel were reused as 18S rRNA. Also, the first two lanes of the Id-1 panel in Fig. 1D were reused in Fig. 3B. A portion of the actin panel from Fig. 1D was reused in Fig. 4E. In Fig. 1E, lanes 2 and 3 of the 18S rRNA panel are the same. The 18S rRNA panel from this figure panel was also reused in Fig. 5C as actin. In Fig. 3B, there are no data in the IgG panel. Portions of the Egr-1 panel in Fig. 3C were reused in Fig. 5E as Id-1 and in Fig. 6C as Id-1. Portions of the actin panel in Fig. 3C were reused in Fig. 4G as actin, in Fig. 5E as 18S rRNA, and in Fig. 6C as actin. The 18S rRNA from the EP4 panel in Fig. 4A was reused in Fig. 7C and in Kekatpure, V. D., et al. (2009) J. Biol. Chem. 284, 7436-7445. The first lane of the EP3 panel in Fig. 4A was reused as 18S rRNA in the EP3 panel and as actin in Fig. 8B. The Id-1 panel was reused in Fig. 4D was reused in Subbaramaiah, K., et al. (2008) J. Biol. Chem. 283, 3433-3444. The actin panel in Fig. 4E and a portion of the 18S rRNA panel in Fig. 4F were reused in Kekatpure, V. D., et al. (2009) J. Biol. Chem. 284, 7436-7445. A portion of the pEGFR panel in Fig. 5A was reused in Fig. 8B as COX-2. A portion of the Egr-1 panel in Fig. 5E was reused in Fig. 4G as Id-1 and in Fig. 6F as pERK1/2. Also in Fig. 5E, the second lane of the Id-1 panel was reused in the last lane. The 18S rRNA panel in Fig. 5E was reused in Subbaramaiah, K., et al. (2008) J. Biol. Chem. 283, 3433-3444. The first and last lanes of the Id-1 panel in Fig. 6B are the same and were reused in Fig. 8C. The actin panel in Fig. 6C was reused in Subbaramaiah, K., et al. (2008) J. Biol. Chem. 283, 3433-3444. In Fig. 6E, lanes 2 and 3 of the actin panel are the same. In Fig. 6F, lanes 3 and 5 of the pERK1/2 panel are the same. A portion of the Id-1 panel in Fig. 7C was reused in Kekatpure, V. D., et al. (2009) J. Biol. Chem. 284, 7436-7445. The actin panel in Fig. 7D was reused as 18S rRNA in the same figure panel. The second lane of the COX-2 panel in Fig. 8B was reused as Id-1 in Fig. 8C. In Fig. 9A, lane 2 of the EP1 panel was reused in lane 2 of the EP3 panel.

Cyclooxygenase-2-derived Prostaglandin E2 Stimulates Id-1 Transcription

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Cyclooxygenase-2 (COX-2) catalyzes the first step in the synthesis of prostaglandins (PGs). Inhibitors of COX-2 have been shown to reduce breast cancer incidence and metastases. However, COX-2 is not inducible by a subset of procarcinogenic stimuli, including activation of the EGFR signaling pathway. We therefore tested the hypothesis that COX-2-derived PGs can stimulate cell proliferation, invasion, and migration of mammary epithelial cells. In MDA-MB-231 cells, treatment with COX-2-specific inhibitors reduced cell proliferation while increasing Id-1 expression, contributing to carcinogenesis by inhibiting cell differentiation, stimulating cell proliferation, preventing cellular senescence, and facilitating tumor angiogenesis.

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cancer cells. Notably, PGE\(_2\) via EP\(_4\) activated a signal transduction pathway comprised of EGFR \(\rightarrow\) ERK1/2 \(\rightarrow\) Egr-1 resulting in enhanced Id-1 gene expression. The increase in Id-1 expression mediated by PGE\(_2\) led in turn to increased invasiveness of breast cancer cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Leibovitz’s L-15 medium, Dulbecco’s modified Eagle’s medium F-12, and Lipofectamine were from Invitrogen. Indomethacin, poly(dIdC), \(\beta\)-actin antiserum, and normal mouse IgG and \(\alpha\)-nitrophenyl-\(\beta\)-D-galactopyranoside were from Sigma-Aldrich. 2‘-amino-3’-methoxyflavone (PD 98059) and AG1478 were from EMD Biosciences Inc. (San Diego, CA). PGE\(_2\), PGE1 alcohol, GW 627368X, and enzyme immunoassay reagents to quantify PGE\(_2\) were from Cayman Chemical Co. (Ann Arbor, MI). \[^{32}\text{P}]\text{CTP and }[^{32}\text{P}]\text{ATP were from PerkinElmer Life Sciences. Random priming kits were from Roche Applied Science. Nitrocellulose membranes were from Schleicher & Schuell. Reagents for the luciferase assay were from Analytical Luminescence (San Diego, CA). The mouse and human 18 S rRNA, \(\beta\)-actin cDNAs were from Ambion, Inc. (Austin, TX). Anti-COX-2, Egr-1, and Id-1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to ERK1/2, phospho-ERK1/2, EGFR, and phospho-EGFR were from Cell Signaling Technology (Beverly, MA). Western blotting detection reagents (ECL) were from Amersham Biosciences. Neutralizing antibodies to EGFR and amphiregulin and an enzyme immunoassay kit for amphiregulin were purchased from R&D Systems, Inc. (Minneapolis, MN). Plasmid DNA preparation kits and pSV\(_{\beta}\text{gal were obtained from Promega Corp. (Madison, WI). Celecoxib was purchased from LKT Laboratories Inc. (St. Paul, MN). Oligonucleotides were synthesized by Sigma-Genosys (The Woodlands, TX). The expression vector for a dominant negative form of ERK1, control vector, and ChIP assay kits were from Upstate Biotechnology, Inc. (Lake Placid, NY). siRNAs to Id-1, COX-2, GFP, Egr-1, and EP\(_{1-4}\) (murine, human) were obtained from Dharmacon Inc. (Lafayette, CO). Expression vectors for Id-1 and COX-2 were from Open Biosystems, Inc. (Huntsville, AL). Id-1 promoter deletion and
mutant constructs have been described previously (43). Human and murine cDNAs for EP1-4 were from the University of Missouri-Rolla Resource Center (Rolla, MO). Invasion assay kits were from Chemicon unit of Millipore (Temecula, CA).

**Cell Lines**—MDA-MB-231 human breast cancer cells (American Type Culture Collection; Manassas, VA) were maintained in Leibovitz’s L-15 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% sodium pyruvate. SCp2 and SCg6 murine mammary epithelial cell lines, obtained from Dr. Mina Bissell (Lawrence Berkeley National Laboratory, Berkeley, CA), were grown in Dulbecco’s modified Eagle’s medium F-12 medium supplemented with 10% Leibovitz medium. All cells were grown to 60% confluence in a 5% CO2, water-saturated incubator at 37°C before being placed in serum-free medium for 24 h. Subsequently, treatments were carried out in serum-free medium.

**PGE2 Production**—Cells were plated in 6-well dishes and grown to 60% confluence. The amount of PGE2 released by cells was measured by enzyme immunoassay. Production of PGE2 was normalized to protein concentrations.

**Western Blotting**—Cell lysates were prepared by treating cells with lysis buffer (150 mM NaCl, 100 mM Tris (pH 8.0), 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin). Lysates were separated by sonicating cells for 20 s on ice and centrifuged at 10,000 x g for 10 min to sediment the particulate protein concentration of the supernatant was measured by method of Lowry et al. (44). SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (45). The resolved proteins on nitrocellulose sheets were then probed with nitrocellulose membranes containing antibodies. Secondary antibody to IgG conjugated to horseradish peroxidase was used. The bands were visualized with ECL Western blot detection system according to the manufacturer’s instructions.

**Northern Blotting**—Total cellular RNA was isolated from cell monolayers using an RNA isolation kit from Qiagen Inc. 10 μg of total cellular RNA per lane were electrophoresed in a formaldehyde-containing 1.2% agarose gel and transferred to nylon-supported membranes. After baking, membranes were hybridized overnight in a solution containing 50% formamide, 5× sodium chloride/sodium phosphate/EDTA buffer (SSPE), 5× Denhardt’s solution, 0.1% SDS, and 100 μg/ml single-stranded salmon sperm DNA and then hybridized for 12 h at 42°C with radiolabeled cDNA probes. Probes were labeled with [32P]CTP by random priming. After hybridization, membranes were washed twice for 20 min at room temperature in 2× SSPE, 0.1% SDS, twice for 20 min in the same solution at 55°C, and twice for 20 min in 0.1× SSPE, 0.1% SDS at 55°C. Washed membranes were then subjected to autoradiography.

**Transient Transfections**—Cells were seeded at a density of 5 x 104 cells/well in 6-well dishes and grown to 50–60% confluence. For each well, 2 μg of plasmid DNA were introduced into cells using 8 μg of Lipofectamine as per the manufacturer’s instructions. After 7 h of incubation, the medium was replaced with fresh medium. The activities of luciferase and β-galactosidase were measured in cellular extracts. Transfection of siRNAs was carried out with a similar methodology.

**Oligonucleotide-electrophoretic mobility shift assay (EMSA)**—Cells were harvested, and cell pellets were solubilized by using 5 μg of nuclear protein in 20 mM HEPES (pH 7.9), 10% glycerol, 300 μg/ml bovine serum albumin, and 1 μg of poly (dl-ctc) in a final volume of 10 μl for 10 min at 25°C. The labeled oligonucleotide was added to the reaction mixture and allowed to incubate for an additional 20 min at 25°C. The samples were electrophoresed on a 4% nondenaturing polyacrylamide gel. The gel was then dried and subjected to autoradiography at −80°C.
Chromatin Immunoprecipitation (ChIP) Assay—ChiP assay was performed with a kit (Upstate Biotechnology) according to the manufacturer’s instructions. 10^6 cells were cross-linked in a 1% formaldehyde solution for 10 min at 37 °C. Cells were then lysed in 200 μl of SDS buffer and sonicated to generate 200–1000-bp DNA fragments. After centrifugation, the clarified supernatant was diluted 10-fold with ChIP buffer and incubated with 1.5 μg of the indicated antibody at 4 °C. Immune complexes were precipitated, washed, and eluted as recommended. DNA-protein cross-links were reversed by heating at 65 °C for 4 h, and the DNA fragments were purified and dissolved in 50 μl of water. 10 μl of each sample were used as a template for PCR amplification. Id-1 oligonucleotide sequences for PCR primers were 5’-AGCGGAGAATGCTCCAGCCCCAGTTGGCTCTCACA-TGGCGAC- and 5’-AGGCCTCCGAGCTAGCTCTCCCTGGAAGGTTAGCCGATCAGCTGCCAGCTGCTAAGGGGCTGCTGACG- (forward) and 5’-AGGCCTCCGAGCTAGCTCTCCCTGGAAGGTTAGCCGATCAGCTGCCAGCTGCTAAGGGGCTGCTGACG- (reverse). This primer set encompasses the Id-1 promoter segment from nucleotide 932 to 1156 bp, which includes the Egr-1 and cAMP-response element (CRE) binding sites. PCR was performed at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s for 30 cycles. The PCR products generated from the ChIP template were sequenced, and the identity of the Id-1 promoter was confirmed.

Cell Invasion Assay—Invasion assays were carried out in a 24-well modified Boyden chamber with 8.0-μm pore polycarbonate membrane inserts. Inserts were coated with extracellular matrix (ECM) that was provided by the manufacturer. Cells

**FIGURE 2.** Egr-1 binding site is important for PGE2-mediated stimulation of Id-1 promoter activity. A, represents the different Id-1 promoter deletions used for transfection analyses. B, MDA-MB-231 cells were transfected with 1.8 μg of a series of human Id-1 promoter-luciferase deletion constructs (−1.5 BV, 5’del1, 5’del2, 5’del3, 5’del4, 5’del5, 5’del6, and 5’del7) and 0.2 μg of pSVβgal. C, nucleotide sequence of human Id-1 promoter. D, cells were transfected with 1.8 μg of human Id-1 promoter-luciferase (−1147/−937) or the −1147/−937bp Id-1 promoter construct in which the cAMP-response element (mCREB), Ebox (mEbox), or Egr-1 (mEgr-1) sites were mutagenized. Cells also received 0.2 μg of pSVβgal. In B and D, 24 h after transfection cells were treated with vehicle (Control) or 0.5 μM PGE2 for 24 h. Reporter activities were then measured. Luciferase activity represents data that have been normalized to β-galactosidase activity. Columns, means; bars, S.D.; n = 6.
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**FIGURE 3.** Increased binding of Egr-1 to the Id-1 promoter is detected in cells treated with PGE₂. A, nuclear protein (5 μg) was isolated from MDA-MB-231 cells treated with 0, 0.1, 0.25, and 0.5 μM PGE₂ for 3 h and then incubated with a 32P-labeled oligonucleotide containing the Egr-1 site of the Id-1 promoter. Cold-chase experiments were performed by incubating nuclear protein (5 μg) from cells treated with 0.5 μM PGE₂ with a 32P-labeled oligonucleotide containing the Egr-1 site of the Id-1 promoter and a 100-, 75-, or 50-fold excess of unlabeled Egr-1 oligonucleotide, 100-fold excess of CREB oligonucleotide, or 100-fold excess of E box oligonucleotide. The protein-DNA complexes that formed were separated on a 4% polyacrylamide gel. B, ChIP assays were performed. MDA-MB-231 cells were treated with vehicle or 0.5 μM PGE₂, for 3 h. Chromatin fragments were immunoprecipitated with Egr-1 antibody, and the Id-1 promoter region was amplified by PCR. DNA sequencing was carried out, and the PCR product was confirmed to be the Id-1 promoter construct and corresponding empty expression vector. 24 h after transfection the cells were treated with vehicle (Control) or 0.5 μM PGE₂, 24 h post-treatment cellular lysates were isolated and subjected to either Western blotting (top) or measurements of reporter activities (bottom). In the top panel the blot was probed with antibodies to Egr-1 and β-actin, respectively. In the bottom panel luciferase activity represents data that have been normalized to β-galactosidase activity. Columns, means; bars, S.D.; n = 6. **, p < 0.001 compared with cells transfected with GFP siRNA.

**RESULTS**

COX-2-derived PGE₂ Induces the Expression of Id-1—Initially, we investigated the effects of exogenous PGE₂ on Id-1 levels in a human breast cancer cell line, MDA-MB-231. Treatment with PGE₂ led to a concentration-dependent increase in Id-1 protein, mRNA (Fig. 1A), nuclear factor kappa B (NFκB) (Fig. 1B), and promoter activity (Fig. 1B). The suppressive effects of celecoxib and indomethacin on Id-1 promoter activity were reversed by the addition of exogenous PGE₂ (Fig. 1F). Consistent with these pharmacological findings, treatment with siRNA to COX-2 suppressed Id-1 promoter activity, an effect that was reversed by the addition of PGE₂ (Fig. 1G).

**The Egr-1 Site Is Necessary for the Induction of Id-1 by PGE₂.—**We next were interested in identifying the region of the Id-1 promoter that was important for mediating the induc-
ment of MDA-MB-231 cells with PGE$_2$ led to a severalfold increase in Id-1 promoter activity when the $-1575$ bp deletion construct (1.5 BV) was used (Fig. 2B). The magnitude of PGE$_2$-mediated induction of Id-1 promoter activity remained essentially constant until the $-927$ bp deletion construct ($S'$del3) was used. The $-927$ bp Id-1 promoter construct was not stim-
ulated by PGE$_2$. This result implies that one or more promoter elements located between $-927$ bp and $-1147$ bp is necessary for PGE$_2$-mediated induction of Id-1 promoter activity. CREB, E-box, and Egr-1 sites are found within this region of the Id-1 promoter (Fig. 2C). To determine which promoter element(s) was important for mediating the induc-

**WITHDRAWN**

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The effects of PGE$_2$, transient transfections were performed utilizing Id-1 promoter constructs in which these enhancer elements were mutagenized. As shown in Fig. 2D, the induction of Id-1 promoter activity by PGE$_2$ was abrogated by mutagenizing the Egr-1 site. By contrast, mutagenizing the CREB and E-box sites had no effect on PGE$_2$-mediated stimulation of Id-1 promoter activity.

Electrophoretic mobility shift assays were performed to identify the transcription factor that contributed to the induction of Id-1 by PGE$_2$ (Fig. 3A). Increased binding of nuclear proteins to the Egr-1 site of the Id-1 promoter was detected after treatment with PGE$_2$. Binding was prevented when nuclear extract was incubated with an excess of Egr-1 cold probe. By contrast, binding was unaffected when nuclear extract was incubated with an excess of CREB or E-box containing cold probes. ChIP assays were also done. Protein-DNA complexes were immunoprecipitated with an antibody to Egr-1, and bound DNA fragments were recovered and subjected to semiquantitative PCR with oligonucleotides specific for the Id-1 promoter. The binding of Egr-1 to the Id-1 promoter was enhanced by incubation with PGE$_2$ (Fig. 3B). Transient transfections were carried out to further evaluate the functional importance of Egr-1 in mediating the activation of the Id-1 promoter in response to treatment with PGE$_2$. siRNA to Egr-1 suppressed PGE$_2$-mediated induction of Egr-1 protein and blocked PGE$_2$-mediated induction of Id-1 promoter activity (Fig. 3C).

Defining the Signaling Mechanism by which PGE2 Induces Id-1—We next attempted to define the signal transduction pathway by which PGE2 induces Id-1. PGE2 exerts its effects by binding to G protein-coupled receptors known as EP receptors. An siRNA was used successfully to knockdown each of the four EP receptors. The induction of Id-1 mRNA was blocked by siRNA to EP4 receptors (Fig. 4A). Importantly, treatment with PGE1 alcohol, an EP4 agonist, reversed the inhibition of Id-1 promoter activity that resulted from silencing of COX-2 or treatment with indomethacin (Fig. 4C). Treatment with PGE1 alcohol induced Id-1 mRNA (Fig. 4D) and protein (Fig. 4E), thereby mimicking the effects of PGE$_2$. Moreover, GW627368X, an EP$_4$ antagonist, suppressed PGE$_2$-mediated induction of Id-1 mRNA (Fig. 4F) and protein (Fig. 4G). Cross-talk between EP receptors and EGFR signaling occurs (47). Previously, stimulation of ERK1/2 MAPK, a downstream component of the EGFR signaling cascade, was found to induce Egr-1 (48). Hence, it was logical to determine whether PGE$_2$-mediated induction of Id-1 was a consequence of activation of the EGFR → ERK1/2 MAPK → Egr-1 pathway. As shown in Fig. 5A, treatment with PGE$_2$ led to increased phosphorylation of EGFR. Experiments were next done to determine whether EGFR activation was causally linked to the induction of Id-1. AG1478, a small molecule inhibitor EGFR tyrosine kinase, was used. Treatment with AG1478 blocked PGE$_2$-mediated induction of both Id-1 and Egr-1 (Figs. 5, B and C). Because EGFR can be activated by either extracellular or intracellular mechanisms, we next investigated whether an antibody to the EGFR ligand binding site suppressed PGE$_2$-mediated induction of Id-1 and Egr-1. As shown in Fig. 5D, the induction of Id-1 and Egr-1 by PGE$_2$ was suppressed by this neutralizing antibody (Fig. 5E). Importantly, control IgG did not suppress the induction of Egr-1. To further investigate whether Id-1 induction was causally related to Egr-1, a neutralizing antibody to Egr-1 was used. As shown in Fig. 5E, an antibody to Egr-1 suppressed the induction of Egr-1 and Id-1.

Pharmacological and genetic approaches were next used to determine the link between ERK1/2 MAPK activity and induction of Id-1 by PGE$_2$. In the first experiment, we found that treatment with PGE$_2$ stimulated ERK1/2 MAPK activity (Fig. 6A). Next we utilized PD98059, a specific inhibitor of MAPK kinase, which prevents activation of ERK1/2. Treatment with PD98059 suppressed PGE$_2$-mediated induction of Id-1 mRNA (Fig. 6B) and protein (Fig. 6C). To further investigate the importance of MAPK in mediating the induction of Id-1, transient transfections were performed. Consistent with the pharmacological findings, overexpressing a dominant-negative for ERK1 suppressed PGE$_2$-mediated activation of the Id-1 promoter (Fig. 6D). PD98059 also suppressed PGE$_2$-mediated induction of Egr-1 (Fig. 6E). To evaluate whether PGE$_2$-mediated activation of EGFR led to increased ERK1/2 activity,
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FIGURE 5. PGE₂-mediated induction of Id-1 is dependent on EGFR activation. A, MDA-MB-231 cells were treated with indicated concentrations of PGE₂ for 1 h. Cell lysate protein (100 µg) was subjected to Western blotting. The blots were probed with antibodies to phospho-EGFR (pEGFR) or EGFR. B, cells were treated with vehicle, 0.5 µM PGE₂, or PGE₂ plus the indicated concentration of AG1478 for 24 h. Total cellular RNA was isolated from cells. 10 µg of RNA was added to each lane. The blot was hybridized with probes that recognized Id-1 mRNA and 18 S rRNA. C, cells were treated with vehicle, 0.5 µM PGE₂, or PGE₂ plus IgG, or 0.5 µM PGE₂ plus neutralizing antibody (Ab) to EGFR for 24 h. D, total cellular RNA was isolated from cells. 10 µg of RNA was added to each lane. The blots were hybridized with probes that recognized Egr-1, Id-1, and 18 S rRNA. Data shown are representative of three independent experiments.

AG1478 was utilized (Fig. 6f). The induction of ERK1/2 activity by PGE₂ was attenuated in cells treated with the inhibitor of EGFR-tyrosine kinase. Treatment with AG1478 also inhibited PGE₁ alcohol-mediated induction of ERK1/2 activity.

Evidence That COX-2-derived PGE₂ Stimulates Cell Invasiveness by an Id-1-dependent Mechanism—SCp2 cells are a phenotypically normal murine mammary epithelial cell line that becomes invasive when Id-1 is overexpressed (49, 50). SCg6 cells share a common lineage with SCp2 cells but are transformed and exhibit invasive behavior (51). Cell transformation stimulates COX-2 transcription and PGE₂ production (8). Hence, we next evaluated the role of the COX-2 and PGE₂ as determinants of Id-1 expression and cell invasiveness in SCg6 and SCp2 cells. Interestingly, tumorigenic SCg6 cells expressed higher levels of COX-2 (Fig. 7A) and produced more PGE₂ (Fig. 7B) than nontumorigenic SCp2 cells. A corresponding increase in Id-1 levels was found in SCg6 cells (Fig. 7A). To determine whether COX-2-derived PGE₂ contributed to the higher levels of Id-1 in SCg6 cells, additional experiments were performed. Similar to what was found in MDA-MB-231 cells (Fig. 1), treatment with celecoxib or indomethacin led to a concentration-dependent decrease in PGE₂ production (data not shown) and reduced Id-1 (Fig. 7C). Moreover, treatment with exogenous PGE₂ increased amounts of Id-1 in SCg6 cells. This is consistent with the findings in nontumorigenic SCp2 cells. Treatment with PGE₁ alcohol or treatment with celecoxib caused more than a 50% reduction in PGE₂ production (p < 0.01) and a corresponding decrease in the migration of cells through ECM (Fig. 8A). Notably, silencing of Id-1 led to a similar reduction in the ability of these cells to traverse the ECM-coated inserts (Fig. 8A). By contrast, treatment with 0.5 µM PGE₂ enhanced the migration of cells through ECM (Fig. 8A). In nontumorigenic SCp2 cells (Fig. 8B), COX-2 overexpression led to more than a 2-fold increase in PGE₂ production (p < 0.01) and a severalfold increase in migration of cells through ECM. Treatment with PGE₂ also enhanced the migration of cells through ECM. Importantly, silencing of Id-1 attenuated the increased in cell invasion mediated by overexpressing COX-2 or treatment with exogenous PGE₂. Because COX-2-derived PGE₂ induced Id-1 transcription in MDA-MB-231 cells, we also evaluated cell invasion. Silencing of COX-2 or treatment with celecoxib caused more than a 50% decrease in PGE₂ production (p < 0.01) and a marked reduction in the ability of cells to migrate through ECM (Fig. 8C). Silencing of Id-1 mimicked this inhibitory effect, whereas treatment with PGE₂ enhanced cell invasion. Taken together, these results suggest that COX-2-derived PGE₂ induces Id-1, which contributes in turn to enhanced cell invasiveness.
Because the EP<sub>4</sub> receptor is responsible for the induction of Id-1 by PGE<sub>2</sub>, we next evaluated the role of this receptor in mediating cell invasiveness. Small inhibitory RNA was used to knockdown each of the four EP receptors in SCp2 cells (Fig. 9A). Next we investigated the effect of silencing these receptors on PGE<sub>2</sub>-mediated induction of cell invasion. Treatment with PGE<sub>2</sub> enhanced the migration of SCp2 cells through ECM (Fig. 9B), an effect that was suppressed by silencing EP<sub>4</sub> or treatment with GW627368X, an EP<sub>4</sub> antagonist. In contrast, silencing EP<sub>1–3</sub> did not affect the increase in cell invasiveness mediated by PGE<sub>2</sub> (Fig. 9B). Consistent with these findings, treatment with PGE1 alcohol, an EP<sub>4</sub> agonist, also stimulated the migration of SCp2 cells through ECM (Fig. 9C). To complement the above studies, we investigated whether PGE1 alcohol, the EP<sub>4</sub> receptor agonist, could reverse the suppression of cell invasion mediated by genetic or pharmacological inhibi-
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FIGURE 7. COX-2-derived PGE₂ contributes to the increased levels of Id-1 found in SCg6 cells. A, total RNA was isolated from SCP2 and SCg6 cells. 10 μg of RNA was added to each lane. The blots were hybridized with probes that recognized COX-2, Id-1, and β-actin. B, medium was collected from SCP2 and SCg6 cells, and amounts of PGE₂ were measured by enzyme immunoassay. Columns, means; bars, S.D.; n = 6. C, SCg6 cells were treated with vehicle or the indicated concentrations of celecoxib or indomethacin for 24 h. Total cellular RNA was isolated. 10 μg of RNA was added to each lane. The blot was hybridized with probes that recognized Id-1 mRNA and 18 S rRNA. D, SCP2 cells were treated with vehicle or the indicated concentrations of PGE₁ alcohol for 24 h. The blot was probed with antibodies to Id-1 and β-actin. In the bottom panel total RNA was isolated from cells. 10 μg of RNA was added to each lane. The blot was hybridized with probes that recognized Id-1 mRNA and 18 S rRNA. E, SCP2 cells were treated with vehicle or the indicated concentrations of PGE₁ alcohol for 24 h. Total cellular RNA was isolated from cells. 10 μg of RNA was added to each lane. The blot was hybridized with probes that recognized Id-1 mRNA and 18 S rRNA.

In the current study we show that COX-2-derived PGE₂ induced Id-1 gene expression and cell invasion. PGE₂ via EP₄ activated the EGFR → ERK1/2 → Egr-1 pathway leading to enhanced Id-1 transcription. Several observations support a critical role for EP₄ in PGE₂-mediated induction of Id-1. First, an agonist of EP₄ mimicked PGE₂ by inducing Id-1. Moreover, an EP₄ receptor antagonist or silencing of EP₄ suppressed the inductive effects of PGE₂. The EGFR also played a central role in mediating the inductive effects of PGE₂. Treatment with PGE₂ stimulated the phosphorylation of EGFR. Additionally, we found that AG1478, an EGFR tyrosine kinase inhibitor, or antibody blockade of the ligand binding site of EGFR abrogated PGE₂-mediated induction of Id-1. EGFR can be activated via either intracellular or extracellular mechanisms (52–54). The fact that a neutralizing antibody blocked the induction of Id-1 by PGE₂ is consistent with prior evidence that EGFR can regulate gene expression and mediates cell invasion. Notably, these findings are consistent with growing evidence that cross-talk between EP receptors and EGFR is likely to be important for regulating a number of cellular functions that are relevant in carcinogenesis (47, 53–55). Additional experiments were carried out to define the signal transduction pathway downstream of EGFR that mediated the induction of Id-1. The data suggest an important role for ERK1/2 in mediating the induction of Id-1 by PGE₂. First, the activity of ERK1/2 was increased by treatment with PGE₂. Second, an inhibitor of MAPK kinase blocked the induction of Id-1 in PGE₂-treated cells. Third, overexpression of dominant negative ERK1 suppressed the activation of Id-1 promoter activity by PGE₂.

Id-1 gene expression can potentially be regulated by either Egr-1 or CREB (43, 56). Previous studies have shown that PGE₂ can regulate gene expression by either Egr-1 or CREB-dependent mechanisms (48, 57, 58). We report that induction of Id-1 promoter activity by PGE₂ was mediated through an Egr-1 binding site. Several findings support a role for Egr-1 in mediating the induction of Id-1 in cells treated with PGE₂. First, the activity of ERK1/2 was increased by treatment with PGE₂. Second, an inhibitor of MAPK kinase blocked the induction of Id-1 in PGE₂-treated cells. Third, overexpression of dominant negative ERK1 suppressed the activation of Id-1 promoter activity by PGE₂.

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results suggest a mechanism whereby COX-2-derived PGE₂ stimulated *Id-1* transcription via the EP₄ → EGFR → ERK1/2 → Egr-1 pathway.

Both COX-2/PGE₂ and *Id-1* have been suggested to play a role in tumor metastases (9, 31, 32, 41, 42, 50). Our results suggest that COX-2/PGE₂-mediated induction of *Id-1* contrib-
FIGURE 9. EP4 is important for PGE2-mediated induction of cell invasion. A, SCp2 cells were transfected with 2 μg of siRNAs to GFP or murine EP1–4 and allowed to grow for 36 h before analysis. Total RNA was prepared from cells and subjected to northern blotting (10 μg/lane). The blots were hybridized sequentially with the indicated probes. In panels B–E (SCp2; B, C, and D), (MDA-MB-231; E), (SCg6; D), cells were aliquoted into inserts containing ECM-coated filters. Cells were transfected as indicated 36 h before being aliquoted into inserts. The inserts including the indicated treatments were then placed into wells containing conditioned medium and incubated at 37 °C for 20 h. The cells that migrated into the lower well were counted after being fixed and stained. In panels B and C, the labeling of bars is as follows: Control, cells treated with vehicle; PGE2, cells treated with 0.5 μM PGE2; PGE1 alc., cells treated with 0.2 μM PGE1 alcohol; PGE2 + GFP siRNA, cells transfected with 2 μg of GFP siRNA were treated with 0.5 μM PGE2; PGE2 + EP1 siRNA, cells transfected with 2 μg of EP1 siRNAs were treated with 0.5 μM PGE2. In C, PGE2 + EP1-4 siRNA2 represents cells that were transfected with a second siRNA to EP4; PGE2 + 100 nM or 250 nM GW627368X represents cells that were co-treated with 0.5 μM PGE2 and 100 nM or 250 nM GW627368X, respectively. In panels D and E, the labeling of bars is as follows: Control, cells treated with vehicle; GFP siRNA, cells received 2 μg of GFP siRNA; COX-2 siRNA, cells received 2 μg of COX-2 siRNA; Cel, cells treated with 1 μM celecoxib; Indo, cells treated with 1 μM indomethacin; COX-2 siRNA + PGE1 alc., cells transfected with COX-2 siRNA were treated with 0.2 μM PGE1 alcohol; Cel + PGE1 alc. represents cells co-treated with 1 μM celecoxib and 0.2 μM PGE1 alcohol; Indo + PGE1 alc., cells were co-treated with 1 μM indomethacin and 0.2 μM of PGE1 alcohol. The data shown are representative of three independent experiments. Columns, means; bars, S.D.; n = 6. In B and C, **, p < 0.001 versus PGE2 + GFP siRNA-treated cells or PGE2-treated cells, respectively. In D and E, *, p < 0.01 versus COX-2 siRNA-treated cells; **, p < 0.001 versus celecoxib- and indomethacin-treated cells, respectively.
utes to cell invasion through ECM. Several findings support this conclusion. In the non-transformed mammary epithelial cell line SCp2, overexpression of COX-2 or treatment with PGE2, an inducer of Id-1, stimulated cell invasiveness through ECM. These effects were attenuated by silencing Id-1. In transfected SCG6 cells, inhibition of COX-2 activity led to a reduction in both amounts of Id-1 and cell invasiveness; silencing of Id-1 led to a comparable decrease in cell invasion. A similar cascade was observed in MDA-MB-231 cells in which high levels of endogenously expressed COX-2-mediated an Id-1-dependent line SCp2, overexpression of COX-2 or treatment with PGE2, a...
