Prostaglandin E_2 Stimulates Human Lung Carcinoma Cell Growth through Induction of Integrin-Linked Kinase: The Involvement of EP4 and Sp1

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Abstract

Cyclooxygenase-2–derived prostaglandin E_2 (PGE_2) stimulates tumor cell growth and progression. However, the mechanisms by which PGE_2 increases tumor growth remain incompletely understood. In studies performed in non–small cell lung carcinoma (NSCLC) cells, we found that PGE_2 stimulates the expression of integrin-linked kinase (ILK). ILK small interfering RNA (siRNA) inhibited the mitogenic effects of PGE_2. In view of its perceived importance, we turned our attention to the mechanisms involved in PGE_2-induced ILK expression and found that this effect was blocked by an antagonist of the PGE_2 receptor subtype EP4 and by EP4 siRNA. Furthermore, we showed that PGE_2 induction of ILK was associated with phosphorylation of extracellular signal-regulated kinase and phosphatidylinositol 3-kinase/Akt, which were abrogated by ILK siRNA. Transient transfection, gel mobility shift assays, and chromatin immunoprecipitation experiments showed that PGE_2 induced ILK promoter activity and increased Sp1, although it had no effect on nuclear factor-κB and AP-2 DNA-binding activity. Blockade of Sp1 abrogated the effect of PGE_2 on expression of ILK and promoter activity and on cell growth. In summary, our observations show that PGE_2 increases NSCLC cell growth through increased ILK expression, which is dependent on EP4 signaling and on induction of Sp1 protein and Sp1 DNA-binding activity in the ILK promoter. These studies suggest a novel molecular mechanism by which PGE_2 stimulates NSCLC cell growth and unveils a new molecular target for the development of therapies against NSCLC. [Cancer Res 2009;69(3):896–904]

Introduction

Lung cancer remains the leading cause of cancer-related mortality in the United States, and 30% to 40% of newly diagnosed patients with non–small cell lung cancer (NSCLC) present with regionally advanced and unresectable stage III disease (1). Overexpression of cyclooxygenase-2 (COX-2) is frequently observed in several human cancers, including lung cancer. Elevated tumor COX-2 expression is associated with increased angiogenesis, tumor invasion, and suppression of host immunity and promotes tumor cell resistance to apoptosis (2). One of the bioactive products of COX-2, prostaglandin E_2 (PGE_2), serves as an autocrine and paracrine mediator and is involved in a variety of biological processes, including immunoregulation and cell survival (3). PGE_2 stimulates cancer cell growth and invasion, whereas inhibition of PGE_2 synthesis blocks growth of cancer cells (3, 4). The effect of PGE_2 has been attributed to its known capacity to bind to one or more of its four G protein–coupled receptors, designated EP1, EP2, EP3, and EP4 (5). These receptors have been implicated in cancer cell growth and progression (5, 6).

Integrin-linked kinase (ILK) is a serine-threonine protein kinase that mediates diverse cellular functions. ILK activity is stimulated by cellular adhesion to the extracellular matrix and by growth factors in a phosphatidylinositol 3-kinase (PI3K)–dependent manner, and the activation of ILK promotes cell survival and protects against apoptosis (7–9). ILK is a PI3K-dependent effector of integrin-mediated cell adhesion as well as growth factors and is an upstream regulator of Akt. Overexpression of ILK leads to anchorage-independent cell growth and tumorigenicity in nude mice (8). Furthermore, elevated ILK expression and activity have been shown in several types of cancers, including lung cancer (10, 11). Hence, ILK seems to be important in carcinogenesis.

Here, we report that PGE_2 stimulates human lung carcinoma cell growth through induction of ILK and activation of its downstream signals, including extracellular signal-regulated kinases (ERK) and PI3K/Akt. To our knowledge, this is the first demonstration of a link between PGE_2 and ILK in human lung carcinoma.

Materials and Methods

Culture and chemicals. The human NSCLC cell lines H1838 and H1792 were obtained from the American Type Culture Collection and grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, HEPES buffer, 50 IU/mL penicillin/streptomycin, and 1 μg amphotericin (complete medium) as previously described (12). Oligofectamine 2000 reagent was purchased from Invitrogen. The CellTiter-Glo Luminescent Cell Viability Assay kit was purchased from Promega. [Methyl-^{3}H]thymidine was purchased from Amersham Biosciences. Polyclonal antibodies specific for ILK, Akt, phosphorylated Akt (Ser^{473}), ERK1/2, and phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}) were purchased from Cell Signaling. Polyclonal antibody against Sp1 and Sp2 was purchased from Santa Cruz Biotechnology, Inc. 16,16-Dimethyl-PGE_2 (dmPGE_2), AH6809, NS398, polyclonal antibodies against EP4, and PGE_2 EIA kit were obtained from Cayman Chemical Co. AH23848, Sp1 inhibitor, mithramycin A, and all other chemicals were purchased from Sigma unless otherwise indicated.

Reverse transcription-PCR. Total RNA was prepared from human lung carcinoma cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. To amplify the 190-bp ILK and 200-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragments, the sequences of PCR primers (Sigma Genosys) were 5′-AGGGGGACGATCATGG-3′ (forward) and 5′-ATGTCTGCCTTGTACTGC-3′ (reverse) for ILK and 5′-CCATGGAGAAGGCTGGGG-3′ (sense) and 5′-CAAAGTTGTCATGGATGACC-3′ (antisense) for GAPDH, according to their sequences and

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published data (12, 13). The reverse transcription-PCR (RT-PCR) was carried out as previously described (12). The samples were first denatured at 95°C for 30 s, followed by 32 PCR cycles, each with temperature variations as follows: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The last cycle was followed by an additional extension incubation of 7 min at 72°C. Analysis of amplicons was accomplished on 1% agarose gel containing 0.2 μg/μL ethidium bromide and visualized under UV transilluminator.

Western blot analysis. The procedure was performed as previously described (12). Protein concentrations were determined by the Bio-Rad protein assay. Equal amounts of protein from whole-cell lysates were solubilized in 2× SDS sample buffer and separated on SDS (8–12%) polyacrylamide gels. Blots were incubated with antibodies against EP4, ILK, Sp1, Sp2, ERK1/2, Akt, and their phosphorylated forms (1:1,000). After washing, the blots were washed and followed by incubation with a secondary goat antibody raised against rabbit IgG conjugated to horseradish peroxidase (1:2,000; Cell Signaling). The blots were washed, transferred to freshly made enhanced chemiluminescence solution (Amersham) for 1 min, and exposed to X-ray film. In controls, the primary antibodies were omitted or replaced with a control rabbit IgG.

Treatment with EP4, ILK, and Sp1 small interfering RNA. The ILK small interfering RNA (siRNA) was purchased from Cell Signaling. Sp1 siRNA was purchased from Santa Cruz Biotechnology. The EP4 siRNA and control nonspecific siRNA oligonucleotides were purchased from Dharmacon, Inc. as described previously (13). For the transfection procedure, cells were grown to 60% confluence, and ILK, EP4, and Sp1 siRNAs and control siRNA were transfected using the Oligofectamine reagent according to the manufacturer's instructions. Briefly, Oligofectamine reagent was incubated with serum-free medium for 10 min. Subsequently, a mixture of respective siRNA was added. After incubation for 15 min at room temperature, the mixture was diluted with medium and added to each well. The final concentration of siRNAs in each well was 100 nmol/L. After culturing for 30 h, cells were washed and resuspended in new culture medium in the presence or absence of $dmPGE_2$ for an additional 24 h for Western blot and cell growth and gel mobility shift assays.

Site-directed mutagenesis. To prepare site-directed mutants of the promoter, the following oligonucleotides were synthesized: mutated Sp1 (+78–90 bp), 5′ GGCCCCCACGGGGCttGGC; mutated nuclear factor-κB (NF-κB; +121–136 bp), 5′ GACGGcAGTTCCCCGG. The lower case letters indicate mutations. The ILK plasmid constructs containing site-directed mutations of Sp1 and NF-κB *cis*-acting elements were generated by oligonucleotide-directed mutation using the GeneEditor *in vitro* site-directed mutagenesis system according to recommendations by the manufacturer (Promega). Briefly, double-stranded ILK promoter plasmid was alkaline denatured, precipitated, washed, and resuspended in Tris-EDTA buffer. Mutated Sp1 and NF-κB oligonucleotides and selection oligonucleotides were annealed; mutant strands were synthesized, ligated, and transformed into BMH 71-18 mutS-competent cells. The mutated Sp1 and NF-κB ILK plasmids were isolated and transformed into JM109-competent cells. Colonies (10–15) were selected and screened for mutants by sequencing using an Applied Biosystems ABI Prism 377 DNA sequencer.

Transient transfection assay. The human ILK promoter constructs (pILK-Pr) ligated to the luciferase reporter gene were a gift from Drs. Michalik and Desvergne (Center for Integrative Genomics, University of Lausanne, Lausanne, Switzerland) and have been reported previously (14). The ILK promoter construct contains ~730 bp of the 5′ flanking region of the human *ILK* gene connected to the pGL3 Basic Luciferase reporter vector (Promega). The inactive (ILK-S343A) and superactive ILK cDNA (ILK-S343D) in pUSEamp vector under the control of the cytomegalovirus promoter were purchased from Upstate Biotechnology. Briefly, NSCLC cells were seeded at a density of 5×10^5 per well in six-well dishes and grown to 50% to 60% confluence. For each well, 2 μg of the above ILK plasmid DNA constructs, with or without 0.2 μg of the internal control phRL-TK Synthetic *Renilla* Luciferase Reporter Vector, were cotransfected into the cells using FUGENE 6 lipofection reagent (for ILK promoters; Roche Molecular Biochemicals) as described in our earlier work (13). In a separate experiment, cells were transfected with the inactive (ILK-S343A) and superactive ILK cDNA (ILK-S343D) using the Oligofectamine reagent according to the manufacturer's instructions. After 24 h of incubation, cells were treated with or without $dmPGE_2$ or with mithramycin A (100 nmol/L) for 2 h before exposure of the cells to PGE_2 for an additional 24 h. The preparation of cell extracts and measurement of luciferase activities were carried out using the Dual-Luciferase Reporter kit according to recommendations by the manufacturer (Promega). The assays for firefly luciferase activity and *Renilla* luciferase activity were performed sequentially in a Labsystems Luminoskan Ascent luminometer equipped with dual injectors. Changes in firefly luciferase activity were calculated and plotted after normalization with changes in *Renilla* luciferase activity within the same sample.

Enzyme immunoassay for PGE_2. H1838 cells (1×10^4) were treated with NS398 (10 μmol/L) for 48 h or transfected with control or ILK siRNA for 40 h. Afterwards, PGE_2 levels that secreted into culture medium were determined by enzyme immunoassay according to the instructions of the manufacturer (Cayman Chemical). The concentrations of PGE_2 were determined using a microplate reader (model 450, Bio-Rad). The detection limit for PGE_2 was 15 pg/mL with <0.01% cross-reactivity for other prostaglandins.

[Methyl-^{3}H]thymidine incorporation assay. This method was described previously (12). Briefly, human NSCLC cells were treated with increasing concentrations of $dmPGE_2$ for the indicated time period or were transfected with ILK siRNA for 30 h or with inactive (ILK-S343A) and superactive ILK cDNA (ILK-S343D) using the Oligofectamine reagent according to the manufacturer's instructions for 24 h before incubating with 1 μCi/mL [methyl-^{3}H]thymidine (specific activity, 250 Ci/mmol; Amersham) and exposing the cells to PGE_2 ($dmPGE_2$) for an additional 24 h. Afterwards, medium was removed and attached cells were washed with 1× PBS. Attached cells were treated with ice-cold 6% trichloroacetic acid (TCA) at 4°C for 20 min and washed once with 6% TCA. Cells were solubilized with 0.1 N NaOH and counted in a liquid scintillation counter in 4 mL of scintillation fluid.

Cell viability assay. NSCLC cells (10^5 per well) were treated with increasing concentrations of $dmPGE_2$ for the indicated time period or were transfected with ILK siRNA for 30 h before exposing the cells to $dmPGE_2$ (0.1 μmol/L) for an additional 48 h in 96-well plates. In a separate experiment, cells were transfected with inactive (ILK-S343A) and superactive ILK (ILK-S343D) cDNA using the Oligofectamine reagent according to the manufacturer's instructions. After 24 h of incubation, cells were treated with or without $dmPGE_2$ (0.1 μmol/L) or with mithramycin A (100 nmol/L) for 2 h. Afterwards, the numbers of viable cells in culture were determined using the CellTiter-Glo Luminescent Cell Viability Assay kit, which evaluates the presence of ATP, an indicator of metabolically active cells, according to the manufacturer's instructions (Promega).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) experiments were performed as described before (12). The oligonucleotides used as probes were wild-type Sp1 (5′-GGCCCCCA-CGGGCGGG-3′), mutant Sp1 (5′-GGCCCCCACGGttGGG-3′), wild-type NF-κB (5′-ACGGGAGTTCCCCG-3′), mutant NF-κB (5′-ACGttAGTTCCCCG-3′), wild-type AP-2 (5′-TCCTCCCCGCCTCCGC-3′), and mutant AP-2 (5′-TCCTC-tttGCCTCCGC-3′), which are based on the ILK promoter sequences (15) and consensus Sp1-binding motifs (5′-ATTCGATCGGGGCGGGGCGAGC-3′). The complimentary sequences were annealed and purified following the manufacturer's protocol. The Sp1, NF-κB, and AP-2 oligonucleotides were end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase as recommended by the manufacturer. Nuclear proteins (5 μg) were first incubated under binding conditions [10 mmol/L HEPES, Tris-HCl (pH 7.9), 50 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, 12% (v/v) glycerol, and 2 μg poly(deoxyinosinic-deoxycytidylic acid)] for 10 min, and then we added [γ-^{32}P]ATP probe for another 20 min at room temperature in a final volume of 20 μL in the presence or absence of Sp1 and Sp2 antibodies (2 μg/μL each). For cold competition, a 100-fold excess of the respective unlabeled consensus oligonucleotides was incubated for 15 min before adding the probe. The same amount of mutated oligonucleotides mixed with the probe was used as another control. All of these experiments were performed in the same binding conditions as described before. Afterwards, the protein-DNA complexes were electrophoresed on a native 4.5% polyacrylamide gel at

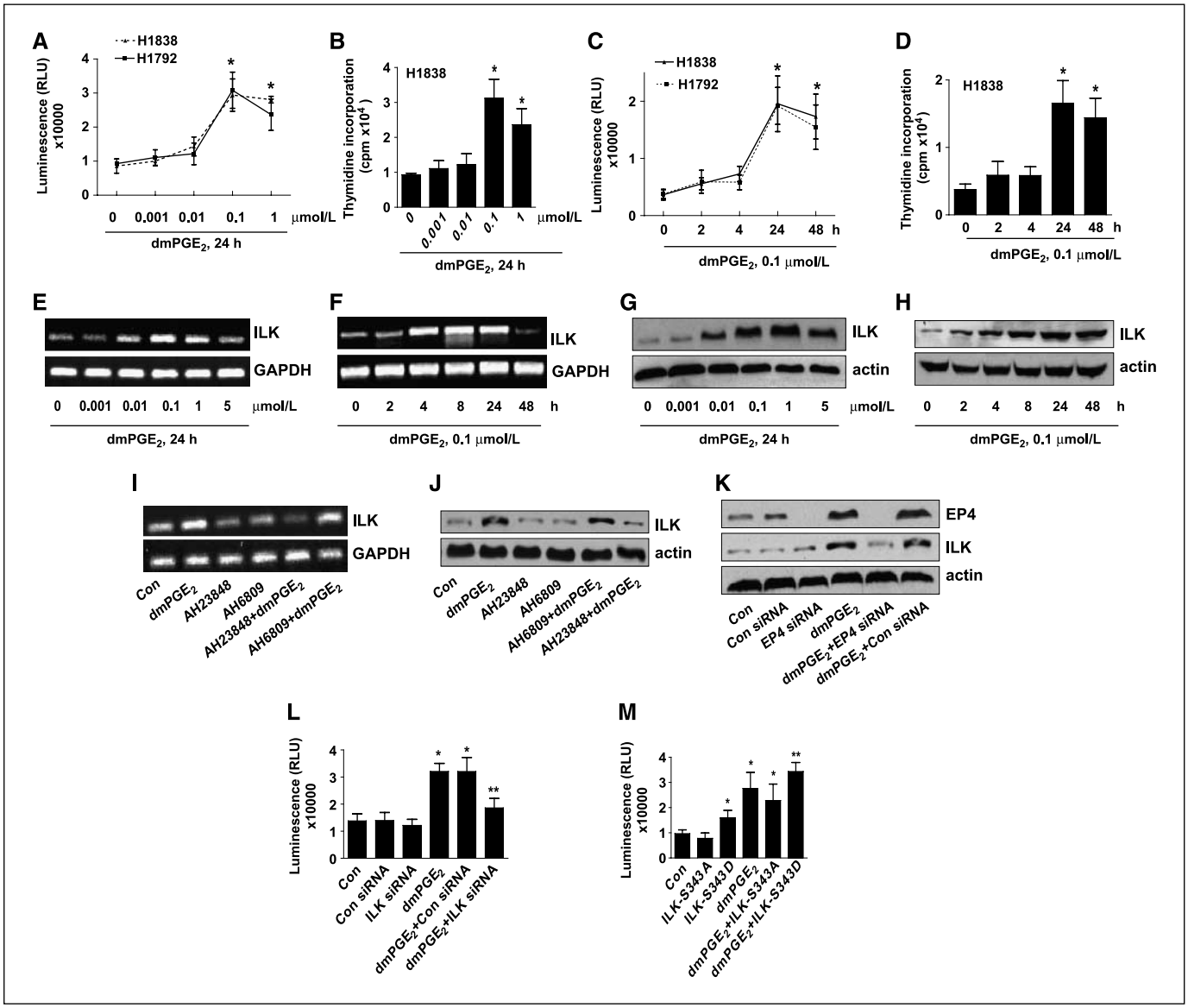

Figure 1. PGE_2 increases cell proliferation and ILK protein expression, and silencing of ILK blocks PGE_2-induced human lung cancer cell growth. *A* and *B,* H1838 and H1792 cells were cultured with increasing doses of $dmPGE_2$ for 24 h. Afterwards, viable cells were determined by the CellTiter-Glo Luminescent Cell Viability Assay (*A*) or by incubation with 1 μCi/mL [methyl-^{3}H]thymidine for 24 h (*B*). Data are expressed as mean ± SD of at least three independent experiments. *C* and *D,* H1838 and H1792 cells were cultured with $dmPGE_2$ (0.1 μmol/L) for the indicated time period. Afterwards, viable cell numbers were determined by the CellTiter-Glo Luminescent Cell Viability Assay (*C*) or by incubation with 1 μCi/mL [methyl-^{3}H]thymidine for up to 48 h (*D*). All data are presented as mean ± SD. *, significant difference from untreated control or zero time point. *E,* total RNA was isolated from H1838 cells treated with increasing concentrations of $dmPGE_2$ as indicated for up to 24 h followed by RT-PCR for ILK mRNA. *F,* total RNA was isolated from H1838 cells treated with $dmPGE_2$ (0.1 μmol/L) for the indicated time period. Afterwards, RT-PCR was performed using primer against ILK. GAPDH was used as loading control for normalization purposes. *G,* cellular proteins were isolated from H1838 cells treated with increasing concentrations of $dmPGE_2$ as indicated for up to 24 h followed by Western blot for ILK protein. *H,* cellular proteins were isolated from H1838 cells treated with $dmPGE_2$ (0.1 μmol/L) for the indicated time period. Afterwards, Western blot analyses were performed using a polyclonal antibody against ILK. *I,* total RNA was isolated from H1838 cells cultured for up to 2 h in the presence or absence of AH23848 (5 μmol/L) or AH6809 (1 μmol/L) before exposure of cells to $dmPGE_2$ (0.1 μmol/L) for an additional 24 h and then subjected to RT-PCR analysis for ILK mRNA. GAPDH was used as loading control for normalization purposes. *Con,* untreated control cells. *J,* cellular protein was isolated from H1838 cells cultured for up to 2 h in the presence or absence of AH23848 (5 μmol/L) or AH6809 (1 μmol/L) before exposure of cells to $dmPGE_2$ (0.1 μmol/L) for an additional 24 h and then subjected to Western blot analysis for ILK protein. *K,* cellular protein was isolated from H1838 cells cultured for 30 h in the presence or absence of control or EP4 siRNA (100 nmol/L) before exposure of cells to $dmPGE_2$ for an additional 24 h and then subjected to Western blot analysis for EP4 and ILK. *L,* H1838 cells were transfected with control or ILK siRNA (100 nmol/L) for 30 h before exposure of the cells to $dmPGE_2$ (0.1 μmol/L) for up to 48 h. Afterwards, cell numbers were determined by the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega). *M,* H1838 cells were transfected with inactive (*ILK-S343A*) and superactive ILK (*ILK-S343D*) cDNA in pUSEamp vector under the control of the cytomegalovirus promoter (2 μg/μL) as described in Materials and Methods for 24 h before exposure of the cells to $dmPGE_2$ (0.1 μmol/L). Afterwards, viable cells were determined by the CellTiter-Glo Luminescent Cell Viability Assay. *, significant difference from control (*Con*); **, significance of combination treatment compared with $dmPGE_2$ alone ($P < 0.05$).

150 V using 1× Tris-glycine buffer [250 mmol/L Tris base (pH 8.5), 1.9 mol/L glycine, 10.5 mmol/L EDTA]. Each gel was then dried and subjected to autoradiography at −80°C.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed in NSCLC cells following the protocol provided by the manufacturer (Upstate Biotechnology). Briefly, after cross-linking with formaldehyde at 1% final concentration for 10 min at 37°C and subsequent quenching with 125 mmol/L glycine, cells were lysed in SDS buffer in the presence of protease inhibitor cocktail (Roche) and 0.5 mmol/L phenylmethylsulfonyl fluorides. Samples were sonicated and

supernatants containing fragmented chromatin were precleared by adding salmon sperm-DNA protein A-agarose beads. A small portion of the supernatants was kept as "input" material. The remaining cleared chromatin was incubated overnight with 5 μg of the anti-Sp1 antibody to study Sp1-DNA interactions, or normal human IgG (Upstate Biotechnology) as negative control. Finally, an overnight incubation with no antibody was also done. Five percent of the sample (~10 μg of DNA) from each immunoprecipitation was reserved for input controls. DNA was purified with QIAquick PCR purification columns according to the manufacturer's instructions (Qiagen Sciences). Genomic sequences of interest were amplified by PCR using primers (+14/+33 bp) 5′-TCGGGCTCTAATATCCGCC-3′ (forward) and (+172/+191 bp) 5′-CCTCTCGTCGCACTGAAAC-3′ (reverse). A total of 1% to 2.5% of each immunoprecipitate was assayed by PCR using primers specific for the region of interest. For semiquantitative PCR, the sample signal was calculated by comparison with titrated inputs, separated on an agarose gel, and stained with ethidium bromide. A 100-bp ladder (Invitrogen) was used as a size standard.

Statistical analysis. All experiments were repeated a minimum of three times. All data from Western blot analysis, PCR, luciferase assays, gel shift assays, and cell growth assays were expressed as mean ± SD. The data presented in some figures are from a representative experiment, which was qualitatively similar in the replicate experiments. Statistical significance was determined with Student's *t* test (two tailed) comparison between two groups of data set. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition ($P < 0.05$, see figure legends).

Results

PGE_2 increases human lung cancer cell proliferation through induction of LK. We first examined the effect of exogenous PGE_2 on NSCLC cell growth. Using two NSCLC cell lines (H1838 and H1792), we showed that PGE_2 induced NSCLC cell proliferation in a dose- and time-dependent manner with maximal effect at a concentration of 0.1 μmol/L at 24 hours as determined by Luminescent Cell Viability Assay (Fig. 1*A* and *C*). This was confirmed by [^{3}H]thymidine incorporation assay (Fig. 1*B* and *D*). Because ILK has been shown to promote tumor cell survival, we examined if PGE_2 also affected the expression of ILK. We found that $dmPGE_2$ enhanced both mRNA (Fig. 1*E* and *F*) and protein levels (Fig. 1*G* and *H*) of ILK in a time- and dose-dependent manner as determined by RT-PCR and Western blot analysis, respectively.

To test whether PGE_2 affects ILK through its EP4 receptor, we blocked EP4 function using a selective EP4 antagonist, AH23848. We showed that AH23848 abrogated the effect of PGE_2 on induction of ILK mRNA and protein expression (Fig. 1*I* and *J*). In contrast, an EP2 antagonist, AH6809, had no effect (Fig. 1*I* and *J*). This was further confirmed by using EP4 siRNA approach (Fig. 1*K*). Silencing EP4 expression blocked the effect of PGE_2 on induction of ILK protein expression, whereas the control siRNA had no effect (Fig. 1*K*).

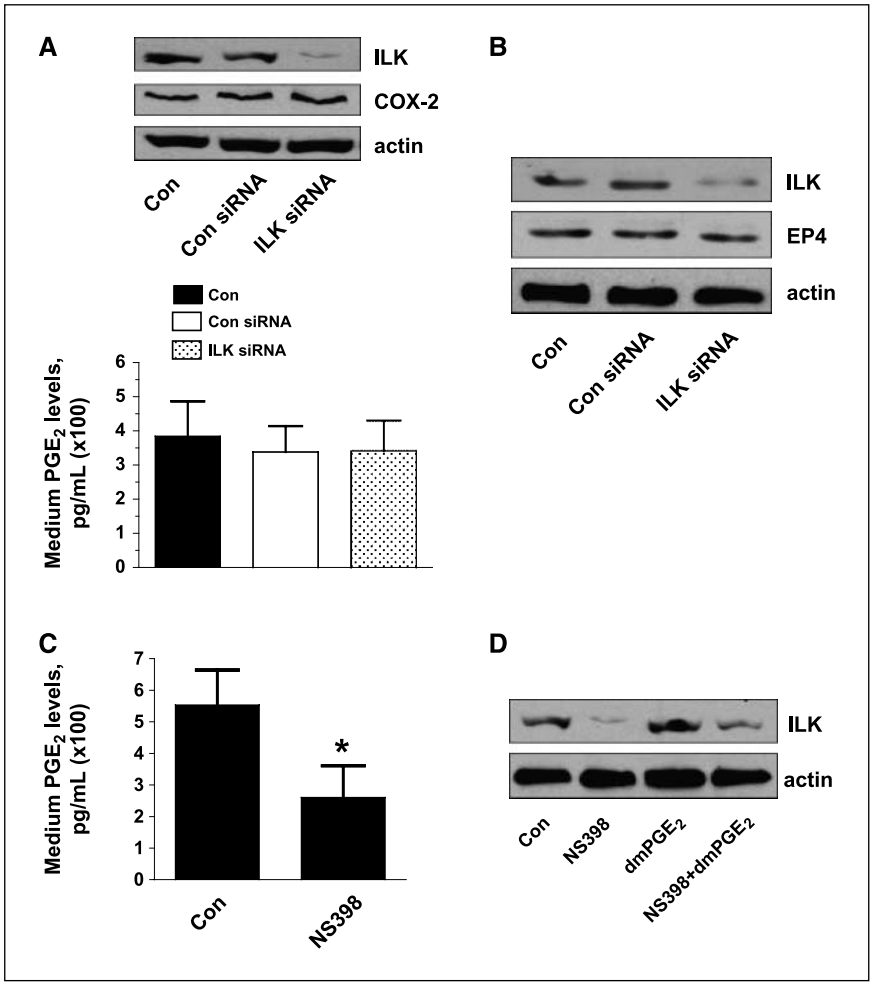

Figure 2. Silencing of ILK had no effect on PGE_2 secretion or COX-2 and EP4 expression. *A, top,* cellular protein was isolated from H1838 cells in the presence or absence of control or ILK siRNA (100 nmol/L) for 30 h and then subjected to Western blot analysis for COX-2 and ILK; *bottom,* H1838 cells (1×10^4) were transfected with control or ILK siRNA (100 nmol/L) for 30 h followed by measurement of medium PGE_2 levels using a PGE_2 enzyme immunoassay kit according to the instruction of the manufacturer (Cayman Chemical). *B,* cellular protein was isolated from H1838 cells in the presence or absence of control or ILK siRNA (100 nmol/L) for 30 h and then subjected to Western blot analysis for EP4 and ILK. *C,* H1838 cells (1×10^4) were treated with NS398 (10 μmol/L) for 48 h. PGE_2 levels secreted into culture medium were determined by a PGE_2 enzyme immunoassay kit according to the instruction of the manufacturer (Cayman Chemical). *D,* cellular protein was isolated from H1838 cells treated with NS398 (10 μmol/L) for up to 24 h in the presence or absence of $dmPGE_2$ (0.1 μmol/L) and then subjected to Western blot analysis for ILK protein.

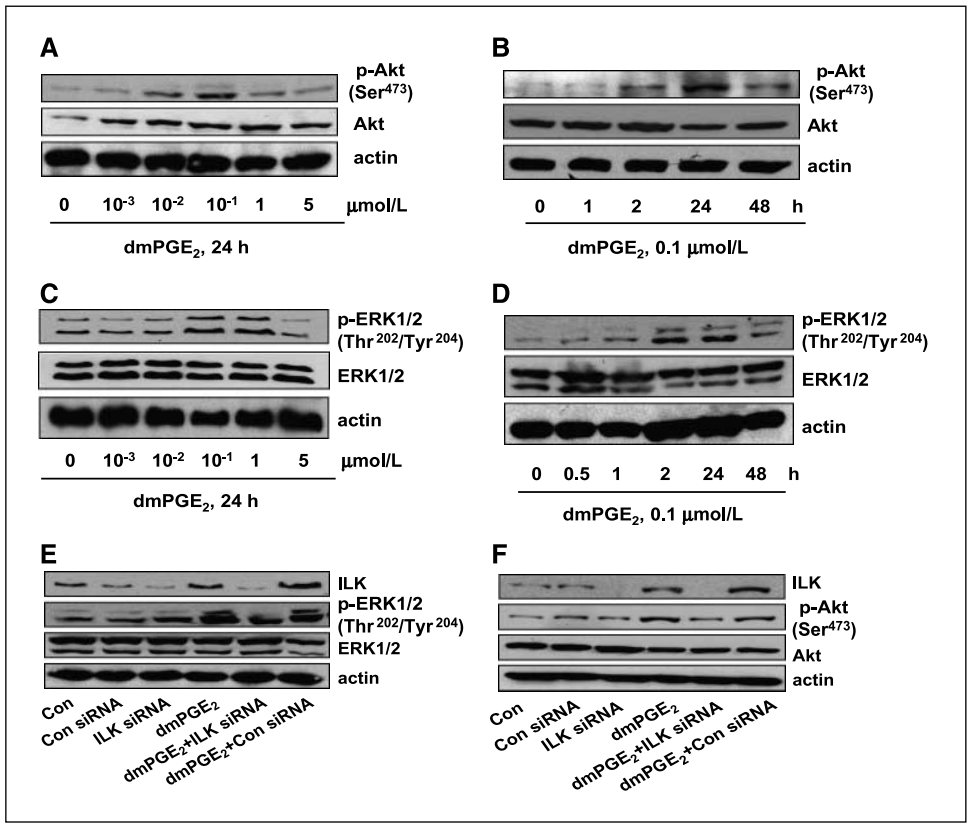

Figure 3. ILK siRNA inhibits PGE_2-induced phosphorylation of ERK and Akt. *A,* cellular protein was isolated from H1838 cells that were cultured with increasing concentrations of $dmPGE_2$ for 24 h followed by Western blot analysis with antibodies against total Akt and phosphorylated Akt (*p-Akt*). *B,* cellular protein was isolated from H1838 cells that were cultured with 0.1 μmol/L $dmPGE_2$ for up to 48 h followed by Western blot analysis with antibodies against total Akt and phosphorylated Akt. *C,* cellular protein was isolated from H1838 cells that were cultured with increasing concentrations of $dmPGE_2$ for 24 h followed by Western blot analysis with antibodies against total ERK1/2 and phosphorylated ERK1/2 (*p-ERK1/2*). *D,* cellular protein was isolated from H1838 cells that were cultured with 0.1 μmol/L $dmPGE_2$ for up to 48 h followed by Western blot analysis with antibodies against total ERK1/2 and phosphorylated ERK1/2. *E,* cellular protein was isolated from H1838 cells cultured for 30 h in the presence or absence of control or ILK siRNA (100 nmol/L) before exposure of cells to $dmPGE_2$ for an additional 2 h and then subjected to Western blot analysis for ILK, ERK1/2, and phosphorylated ERK1/2. *F,* cellular protein was isolated from H1838 cells cultured for 30 h in the presence or absence of control or ILK siRNA (100 nmol/L) before exposure of cells to $dmPGE_2$ for an additional 2 h and then subjected to Western blot analysis for ILK, Akt, and phosphorylated Akt. Actin served as internal control for normalization purposes.

We then tested whether ILK mediated PGE_2-induced cell growth and found that the ILK siRNA inhibited cell growth induced by PGE_2 as determined by Luminescent Cell Viability assays (Fig. 1*L*). To further evaluate the role of ILK in mediating the effect of PGE_2 on cell growth, cells were transfected with overexpression and inactive ILK plasmids. We showed that whereas the inactive ILK plasmid (ILK-S343A) transfected into cells significantly diminished the PGE_2-induced cell growth, cells transfected with hyperactive ILK vector (ILK-S343D) showed an induction in cell growth, and this was further enhanced in the presence of PGE_2 as determined by Luminescent Cell Viability assays (Fig. 1*M*). Similar results were obtained with H1792 cells (data not shown).

Silencing of ILK had no effect on PGE_2 secretion and COX-2/EP4 expression. We also tested whether expression of ILK affects COX-2, PGE_2, and EP4 levels. We showed that silencing of ILK had no effect on expression of COX-2 protein (Fig. 2*A, top*), PGE_2 production (Fig. 2*A, bottom*), and expression of EP4 protein (Fig. 2*B*). Others have reported that blockade of COX-2 inhibited ILK (16). Consistent with this, we found that NS398, a COX-2 inhibitor, reduced PGE_2 secretion (Fig. 2*C*). It also inhibited the expression of ILK protein, which was partially restored by administration of exogenous PGE_2 ($dmPGE_2$; Fig. 2*D*).

PGE_2 increases phosphorylation of ERK1/2 and Akt through ILK. The PI3K/Akt and ERK1/2 signaling pathways are involved in the control of cell cycle progression and cell proliferation. A link between PGE_2 and activation of PI3K and ERK has been reported in other studies (2, 13, 17), but their role in NSCLC remains unclear. Here, we report that $dmPGE_2$ increases the phosphorylation of Akt, a downstream target of PI3K, in a time- and dose-dependent manner with maximal stimulation in 24 hours at 0.1 μmol/L concentration (Fig. 3*A* and *B*). Total Akt protein was not affected. PGE_2 also stimulated phosphorylation of ERK in a similar time- and dose-dependent fashion (Fig. 3*C* and *D*). Similar results were obtained with H1792 cells (data not shown).

ILK is a downstream substrate of PI3K and an important upstream kinase for the regulation of protein kinase B/Akt (18). Therefore, we next examined if knockdown of ILK could block the effect of PGE_2 on its downstream proteins ERK and Akt. We found that ILK siRNA eliminated the stimulatory effects of PGE_2 on phosphorylation of ERK1/2 (Fig. 3*E*) and of Akt (Fig. 3*F*), whereas the ILK siRNA alone or a control siRNA had no effect. Similar results were obtained with H1792 cells (data not shown).

PGE_2 increased ILK promoter activity. Having established a role for ILK in PGE_2-stimulated NSCLC cell proliferation, we next examined whether the effects of PGE_2 on ILK expression occur at the transcriptional level. The ILK promoter contains multiple transcription factor-binding sites, including AP-2, NF-κB, and Sp1 (Fig. 4*A*). We found that H1838 and H1792 cells transfected with the wild-type ILK promoter (−267/+157 bp) luciferase reporter construct and exposed to PGE_2 showed increased promoter activity (Fig. 4*B*). Furthermore, we showed that PGE_2-induced ILK promoter activity was eliminated in ILK promoter reporter constructs in which one Sp1 site (+78–90 bp), but not one NF-κB site, was mutated (Fig. 4*C*).

To further explore the role of PGE_2 in regulation of ILK promoter activity, EMSAs were performed to identify the transcription factors involved. We found that H1838 cells treated with PGE_2 showed a significant increase in Sp1 (Fig. 4*D*), but small or no effects were observed for NF-κB (Fig. 4*E*) and AP-2 (Fig. 4*F*) nuclear

protein-binding activity compared with solvent controls. The addition of a Sp1, but not a Sp2, antibody induced a supershift band (Fig. 4*G*). The specific bands for Sp1, NF-κB, and AP-2 were attenuated by a 100-fold molar excess of unlabeled wild-type oligonucleotides but were not inhibited by the mutated unlabeled oligonucleotides. Oligonucleotides containing a mutated Sp1 or NF-κB or AP-2 sites were end labeled with [γ-^{32}P]ATP and used as another control to confirm binding specificity.

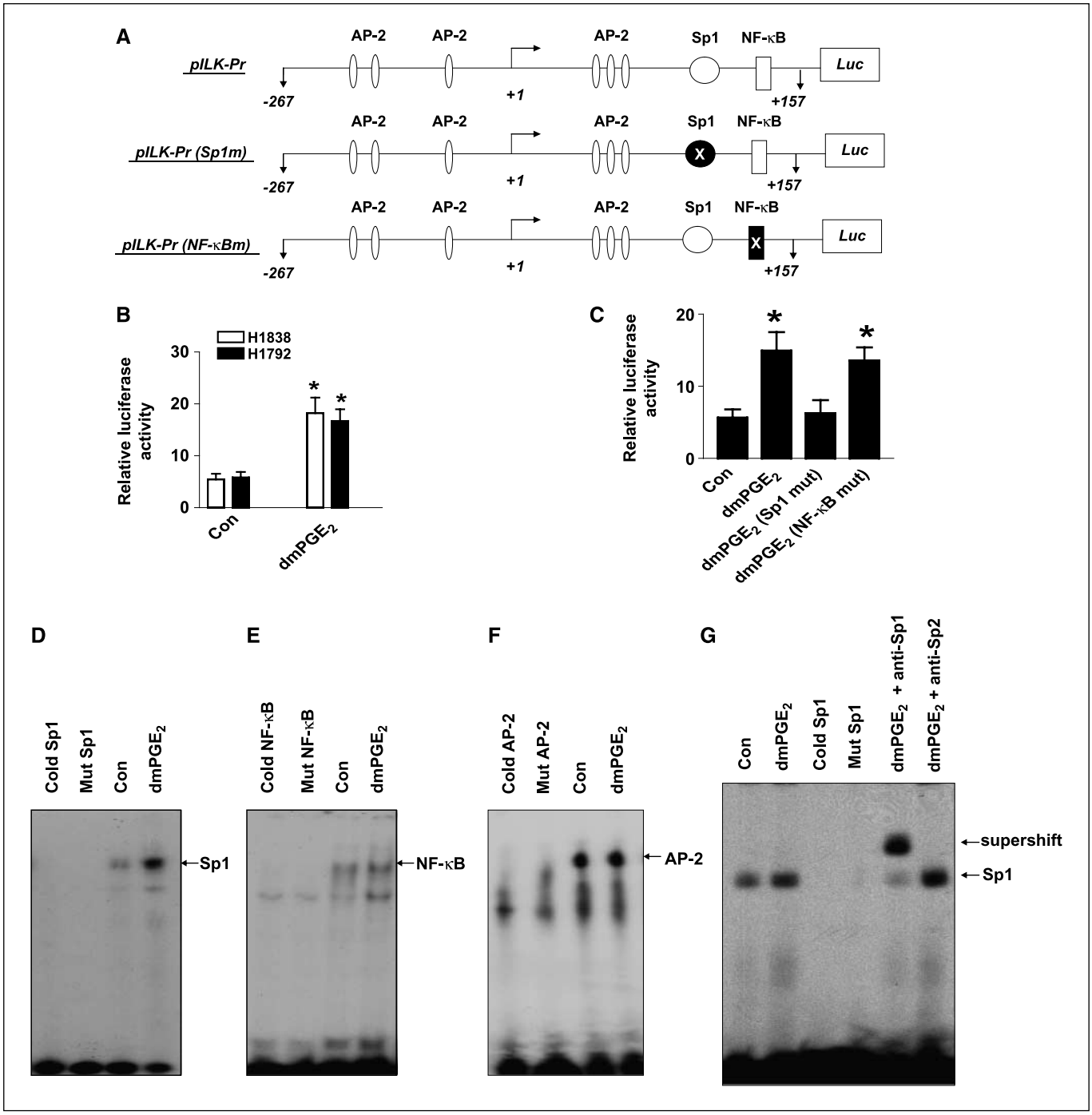

Figure 4. PGE_2 increased ILK promoter activity via induction of Sp1 in human lung carcinoma cells. *A,* the human ILK wild-type and mutation promoter construct schematics are presented. These regions contain several transcription factor binding sites, including AP-2, Sp1, and NF-κB. *B* and *C,* H1838 cells (1×10^5) were transfected with a wild-type or two human ILK promoter mutation reporter constructs (shown in *A*) ligated to luciferase reporter gene and an internal control phRL-TK Synthetic *Renilla* Luciferase Reporter Vector as described in Materials and Methods for 24 h and then treated as indicated with vehicle control (*Con*) and $dmPGE_2$ (0.1 μmol/L) for an additional 24 h. The ratio of firefly luciferase to *Renilla* luciferase activity was quantified as described in Materials and Methods. *Columns,* mean of at least four independent experiments for each condition; *bars,* SD. *, significant increase of activity compared with controls. *D* to *F,* oligonucleotides containing the Sp1 (*D*), NF-κB (*E*), and AP-2 (*F*) sites were end labeled with [γ-^{32}P]ATP and incubated with nuclear extracts (5 μg) from H1838 cells treated with 0.1 μmol/L $dmPGE_2$ for an additional 24 h. *G,* oligonucleotides containing Sp1 sites were end labeled with [γ-^{32}P]ATP and incubated with nuclear extracts (5 μg) and Sp1 and Sp2 antibodies (2 μg/μL each) for 24 h. For competition assays, a molar excess (×100) of consensus Sp1 (*Cold Sp1*) or NF-κB (*Cold NF-κB*) or AP-2 (*Cold AP-2*) oligonucleotide was added to the binding reaction. Oligonucleotides containing a mutated Sp1 (*Mut Sp1*) or NF-κB (*Mut NF-κB*) or AP-2 (*Mut AP-2*) site that were end labeled with [γ-^{32}P]ATP were used to confirm the binding specificity.

The role of transcription factor Sp1 in PGE_2 induction of ILK and cell growth. We further tested the role of Sp1 in mediating PGE_2-induced ILK expression in human lung carcinoma cells. PGE_2 induced the expression of Sp1 protein in a time-dependent manner (Fig. 5*A*). Mithramycin A, an inhibitor of Sp1, abrogated PGE_2-induced ILK protein expression (Fig. 5*B*). Consistent with these findings, we found that treatment with mithramycin A resulted in inhibition of PGE_2-induced ILK promoter activity (Fig. 5*C*). Mithramycin A also reduced the stimulatory effect of PGE_2 on NSCLC cell growth (Fig. 5*D*). This was mimicked by knockdown of the *Sp1* gene because Sp1 siRNA abrogated the expression of Sp1 protein (Fig. 5*E, top*) and antagonized the stimulatory effect of PGE_2 on cell growth as determined by cell viability assays (Fig. 5*E*). Note that the control siRNA had no effect.

To determine if Sp1 complex bound to the endogenous Sp1 site of the ILK promoter region, which is critical for PGE_2-mediated effect on ILK expression, we performed ChIP assays on NSCLC cells. Sp1 occupancy of the Sp1 (+87) site on the ILK promoter was enhanced by exposure of cells to PGE_2. Sp1 was immunoprecipitated from H1838 cell nuclear extracts and the associated DNA was assessed by PCR amplification. The DNA sequence, including the Sp1 site, was specifically immunoprecipitated with anti-Sp1 but not control IgG (Fig. 5*F*). This suggests that these sequences are bona fide Sp1-binding sites, and PGE_2 induces ILK expression by stimulating Sp1 binding to the *ILK* gene promoter in NSCLC cells. Similar results were obtained with H1792 cells (data not shown).

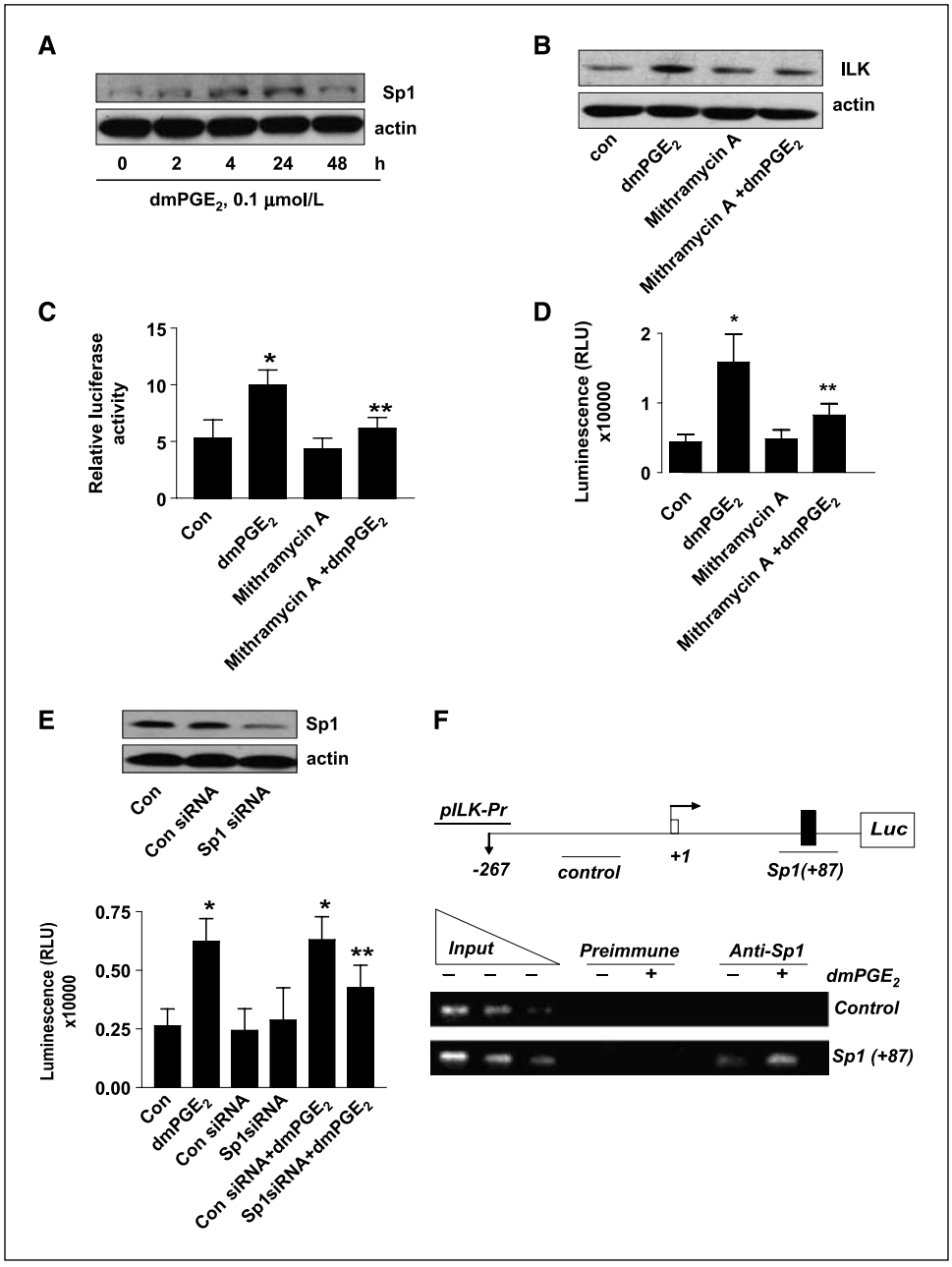

Figure 5. The role of transcription factor Sp1 in PGE_2 induction of ILK. *A,* cellular proteins were isolated from H1838 cells treated with $dmPGE_2$ (0.1 μmol/L) for the indicated time period. Afterwards, Western blot analyses were performed using a polyclonal antibody against Sp1. *B,* cellular proteins were isolated from H1838 cells treated with mithramycin A (100 nmol/L) for 2 h before exposure of the cells to $dmPGE_2$ (0.1 μmol/L) for an additional 24 h. Afterwards, Western blot analysis was performed to examine for ILK protein. *C,* H1838 cells were transfected with a wild-type ILK promoter constructs (−267/+157 bp) together with the mithramycin A (100 nmol/L) for 24 h followed by exposure of the cells to $dmPGE_2$ (0.1 μmol/L) for an additional 24 h. The ratio of firefly luciferase to *Renilla* luciferase activity was quantified as described in Materials and Methods. *Columns,* mean of at least four independent experiments for each condition; *bars,* SD. *, significant increase of activity compared with controls. *D,* H1838 cells were treated with mithramycin A (100 nmol/L) for 2 h before exposure of the cells to $dmPGE_2$ (0.1 μmol/L) and incubation with 1 μCi/mL [methyl-^{3}H]thymidine for an additional 24 h. *Columns,* mean of at least four independent experiments for each condition; *bars,* SD. *E,* H1838 cells were transfected with control or Sp1 siRNA (100 nmol/L) for 30 h followed by Western blot analysis for Sp1 protein. *Bottom,* H1838 cells were transfected with control or Sp1 siRNA (100 nmol/L) for 30 h before exposure of the cells to $dmPGE_2$ (0.1 μmol/L) and cell number was determined by the CellTiter-Glo Luminescent Cell Viability Assay according to the instruction from the manufacturer. *Columns,* mean of at least four independent experiments for each condition; *bars,* SD. *F,* H1838 cells were lysed after exposure of $dmPGE_2$ (0.1 μmol/L) for 24 h, and nuclei were isolated and then sonicated. Chromatin from H1838 cells was immunoprecipitated using antibodies against Sp1 protein (*Anti-Sp1*) or preimmune serum (*Preimmune*). PCR analysis using primers surrounding the Sp1 sites shows that this DNA sequence (+87) is specifically immunoprecipitated, indicating that Sp1 binds to endogenous Sp1 site in the ILK promoter. A non-Sp1 sequence was used as control. Aliquots of the chromatin were also analyzed before immunoprecipitation (*Input*).

Discussion

PGE_2 plays important roles in proliferation, differentiation, and inflammation (19). PGE_2 has been shown to enhance tumor growth and to increase vascularization in several systems (19, 20). We and others reported that PGE_2 induces NSCLC cell growth (3, 4). The cellular mechanisms by which PGE_2 promotes tumor growth, especially NSCLC cell growth, have not been fully elucidated. Herein, we report that PGE_2 increases the expression of ILK. In turn, activation of ILK promotes cell growth (this report) and protects against apoptosis (7–9).

The concentrations of PGE_2 used in this study were based on our previous work showing that fibronectin, a matrix glycoprotein, induced PGE_2 production in NSCLC cells *in vitro* (12). Similar or even higher doses of exogenous PGE_2 have been shown to suppress both Th1- and Th2-polarized antigen-specific human T-cell responses and reduce radiation-induced apoptosis in human colon cancer cells (21, 22). The effect of PGE_2 has been attributed to its known capacity to bind to its four G protein–coupled receptors (5, 6). Among them is EP4, a PGE_2 receptor subtype that has been implicated in tumor cell growth and progression (23). In this study, blockade of EP4 by antagonists and by EP4 siRNA abrogated the effect of PGE_2 on ILK protein expression, suggesting that EP4 mediates the effect of PGE_2 on ILK expression.

ILK has been implicated in the regulation of anchorage-dependent cell growth/survival, cell cycle progression, and cell invasion and migration. Furthermore, blockade of ILK has been shown to inhibit tumor cell growth in several studies (8, 9, 24). One study showed that an ILK inhibitor, QLT-0267, induced apoptosis in human breast cancer cells, although it had no effect on Akt Ser^{473} phosphorylation and apoptosis in normal human breast epithelial or vascular smooth muscle cells (25). Interestingly, overexpression of ILK has been found in human NSCLC cells (11, 26). We found that exogenous overexpression of ILK enhanced NSCLC cell proliferation, whereas cells transfected with ILK inactive cDNA showed inhibition of growth in the setting of PGE_2 stimulation. The ILK inactive cDNA used acts as a dominant negative and has been shown to inhibit tumor cell growth in other study (7). These findings unveil a correlation between the expression of ILK and NSCLC cell growth and suggest that ILK plays significant roles in the aggressive growth properties of NSCLC.

Interestingly, we showed that silencing of ILK had no effect on COX-2 and EP4 expression and did not affect PGE_2 production in our system, suggesting that ILK plays no role in controlling COX-2/PGE_2 and EP4 functions and, therefore, that ILK is not upstream of COX-2/PGE_2 and EP4. Instead, ILK is downstream of COX-2/PGE_2, and consistent with a report by others (16), the COX-2 inhibitor NS398 reduced the secretion of PGE_2 and the expression of ILK, which was partially restored by $dmPGE_2$.

ILK is an important upstream kinase for ERK and Akt and their downstream signals (9, 27). PGE_2 has been shown to stimulate tumor cell growth through activation of ERK and PI3K/Akt signal pathways in several studies (28, 29). Consistent with reports by others (30), our results suggest that ILK is an upstream regulator of ERK1/2 and PI3K/Akt signal cascades in human lung carcinoma cells. Similarly, others found that QLT-0267, an inhibitor of ILK, blocked the phosphorylation of Akt and downstream targets in glioma cells (31).

Having shown the important role of ILK in PGE_2-related NSCLC proliferation, we further investigated whether the PGE_2-mediated up-regulation of ILK reflected transactivation of the gene. To this end, we performed transient transcription experiments using human ILK promoter-reporter constructs connected to a luciferase reporter gene and found that PGE_2 increased ILK promoter activity. The induction of ILK promoter activity by PGE_2 was abrogated when Sp1, but not NF-κB, sites were mutated in the ILK promoter, suggesting a role for Sp1 in mediating the effect of PGE_2 on ILK promoter activity. Several transcription factor-binding sites within regions of the ILK promoter have been characterized, including regulatory elements for AP-2, NF-κB, and Sp1 (15). We evaluated the possibility that these sites might play a role in PGE_2-induced ILK expression. We showed that treatment of H1838 cells with PGE_2 significantly increased protein-binding activity of Sp1 in the *ILK* gene promoter, although it had little effects on AP-2 and NF-κB. This, together with the supershift assay results, indicates that Sp1 binding to the Sp1 site is necessary for the up-regulation of ILK gene transcription in response to PGE_2. We also found that PGE_2 induced expression of Sp1; this led to enhanced ILK signal. By mutating the Sp1 site, the effect of PGE_2 on ILK promoter activity was abolished, further suggesting a key role for Sp1 in mediating the effect of PGE_2.

To our knowledge, a role for the Sp1 site in regulation of ILK expression has not been reported. Sp1 is a member of a family of transcription factors, which bind GC/GT-rich promoter elements through three C_2H_2-type zinc fingers, and plays an important role in tumor growth and metastasis by regulating the expression of cell cycle genes and vascular endothelial growth factor because Sp1/KLF proteins are also potential targets for cancer chemotherapy (32). Sp1 regulates the activation of many genes involved in tumor growth, apoptosis, and angiogenesis. Down-regulation of Sp1 activity may inhibit urokinase-type plasminogen activator receptor expression and may reduce the migration ability of breast cancer cells, indicating its potential therapeutic use to prevent tumor dissemination (33). Overexpression of Sp1 is thought to be important in the malignant transformation of human fibroblasts (34). Mithramycin A, a DNA-binding antibiotic that inhibits Sp1 activity and Sp1 binding to DNA, has been shown to block gene promoter functions and to inhibit promoter-dependent transcription in several cells studied (35, 36). Consistent with these observations, we found that cells exposed to mithramycin A showed inhibition of PGE_2-induced ILK protein expression and promoter activity. In addition, silencing of Sp1 using siRNA approaches also overcame the stimulatory effect of PGE_2 on cell growth. Whereas the ILK inactive expression vector caused inhibition of ILK promoter activity and cell growth, cells overexpressing ILK overcame the inhibitory effect of the Sp1 inhibitor on ILK promoter activity and on cell proliferation. This, together with the results of ChIP assays, highlights the critical role of Sp1 transactivation of the *ILK* gene promoter.

In summary, we showed that PGE_2 stimulates NSCLC cell proliferation via EP4-mediated signals that activate ILK through increased Sp1 expression and Sp1 DNA-binding activity. In turn, ILK stimulates ERK and PI3K/Akt signaling pathways culminating in cell proliferation. To our knowledge, this represents the first demonstration of a direct link between PGE_2 and ILK. Furthermore, this work unveils a novel molecular mechanism by which PGE_2 stimulates NSCLC cell growth, which might lead to new therapeutic strategies for NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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