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# Signaling Pathways Involved in Cyclooxygenase-2 Induction by Hepatocyte Growth Factor in Non–Small-Cell Lung Cancer[S]

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## ABSTRACT

Many studies have suggested a role for the hepatocyte growth factor (HGF)/c-Met pathway in tumorigenesis. Some actions of HGF are believed to be mediated by cyclooxygenase-2 (COX-2), resulting in the production of prostaglandin E2 ($PGE_2$). We examined four c-Met-positive non–small-cell lung cancer (NSCLC) cell lines for effects of HGF on COX-2. HGF increased COX-2 protein expression 3-fold over basal levels. Induction of COX-2 occurred through both the extracellular signal-regulated kinase 1/2 and p38 pathways. HGF treatment caused activation of the activator protein-1, CCAAT/enhancer-binding protein, and cAMP response element-binding protein transcription factors, and COX-2 induction was blocked by actinomycin D. The half-life of COX-2 mRNA was also increased by HGF. HGF stimulation resulted in a 4-fold increase in $PGE_2$ secretion, and treatment of NSCLC cells with exogenous $PGE_2$ significantly increased cell proliferation. The addition of $PGE_2$ to NSCLC cells also led to rapid phosphorylation of c-Met in the absence of HGF, which was blocked by epidermal growth factor receptor (EGFR) inhibition. EGFR ligands were released in response to $PGE_2$. This suggests that secretion of $PGE_2$ induced by HGF/c-Met pathway activation can further activate the c-Met pathway via EGFR in a reinforcing loop that is independent of HGF. HGF and $PGE_2$ each significantly stimulated invasion in NSCLC cells. Cells transiently transfected with c-Met antisense plasmid showed a significant decrease in HGF- or $PGE_2$-induced invasion. $PGE_2$-induced invasion was EGFR-dependent, confirming a link between $PGE_2$, EGFR, and c-Met. Targeting of both the HGF/c-Met and $PGE_2$ pathways with a neutralizing antibody to HGF and celecoxib resulted in enhanced anti-invasion effects in response to HGF.

Lung cancer is the number one cause of death from cancer in men and women in the United States and worldwide is also a major cause of cancer deaths. The 5-year survival rate for lung cancer has shown little improvement over the past 20 years. New therapies that target specific lung cancer-related growth pathways are needed to make an impact on the course of this disease, which is still often diagnosed at late stages that do not respond well to current therapies. The recent experience with the inhibitor of EGFR, gefitinib, which showed limited therapeutic efficacy (Twombly, 2005), suggests that the success of targeted therapies in clinical use may require different approaches, such as use in selected sensitive patients, combination therapy against several targets, or use of drugs intermittently at high doses to induce apoptosis rather than continuously at low doses to impair cell division.

Growth factors and their receptors are attractive targets for therapy because these signaling pathways control cell division and cell survival, two processes that are in imbalance in malignant cells. The literature has repeatedly demonstrated that many growth factor and oncogene signaling pathways overlap and interact with each other. This suggests that not only is there redundancy in how signaling is carried out by cancer cells but also that strategies to interrupt the cross-signaling may have additive or synergistic effects on

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**ABBREVIATIONS:** EGFR, epidermal growth factor receptor; COX-2, cyclooxygenase-2; HGF, hepatocyte growth factor; NSCLC, non–small-cell lung cancer; ELISA, enzyme-linked immunosorbent assay; $PGE_2$, prostaglandin E2; TGF$\alpha$, transforming growth factor $\alpha$; AS, antisense; NA, neutralizing antibody; AP-1, activator protein-1; CREB, cAMP response element-binding protein; MEK, mitogen-activated protein kinase kinase; PI3K, phosphoinositide-3 kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; MAPK, mitogen-activated protein kinase; C/EBP, CCAAT/enhancer-binding protein; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole.

tumor inhibition (Thomas et al., 2005). In clinical use, doses of targeted agents to the tumor that produce 50% or greater inhibition of any one target are difficult to achieve. Partially reducing two intersecting signaling components or two segments of the same signaling pathway may therefore improve therapeutic efficacy.

Hepatocyte growth factor (HGF) was first discovered as a serum component released immediately after liver injury and is important in fetal development (Sonnenberg et al., 1993; Schmidt et al., 1995). HGF was found to be the ligand for the c-Met protein, a tyrosine kinase receptor that is found in most epithelial cells and in endothelial cells (Bottaro et al., 1991). The c-Met protein can be constitutively activated by mutations (Schmidt et al., 1997), which are sometimes found in small cell and non–small-cell lung cancer (Ma, 2004); The c-Met receptor and/or its ligand HGF is often overexpressed in tumors (Di Renzo et al., 1995; Furukawa et al., 1995). Both c-Met and HGF overexpression by non–small-cell lung cancer (NSCLC) cells is associated with poor prognosis (Ichimura et al., 1996; Siegfried et al., 1997). Elevated HGF content in stage I lung adenocarcinoma was strongly associated with reduced time to recurrence (Siegfried et al., 2004). The c-Met protein couples to a number of signaling molecules that are initiated by adaptor proteins, including Gab-1, Grb2, and Shc, leading to downstream activation of several signaling pathways (Christensen et al., 2005). Activation of c-Met initiates cell movement, cell growth, invasion, and angiogenesis (Nakanishi et al., 1999; Furge et al., 2000). Because HGF/c-Met signaling is primarily involved in development and is relatively silent in the adult except for wound healing and tissue regeneration, it may prove to be an attractive therapeutic target. Its inhibition is not expected to have severe effects on organ function unless injury is present.

COX-2, an inducible form of cyclooxygenase, is the rate-limiting step in the production of prostaglandins, which are believed to be involved in long-term inflammation and growth promotion of cancer (Dubois et al., 1998; Shiff et al., 2003). Induction of COX-2 protein should result in increased prostaglandin production and secretion, which in turn should activate growth pathways and inhibit apoptotic pathways. $PGE_2$ mediates a number of effects, including stimulation of invasion and motility, stimulation of angiogenesis, and protection from apoptosis (Kamiyama et al., 2006). All of these are also effects linked to HGF, suggesting that COX-2 is an important downstream mediator of HGF effects. Increased $PGE_2$ may result in several tumor-promoting stimuli, such as enhanced secretion of interleukin-8, resistance to apoptotic stimuli such as radiation or cisplatin, and increased survivin levels, an antiapoptotic protein (Konturek et al., 2003). $PGE_2$ has been reported to cause phosphorylation of c-Met that is independent of HGF but dependent on EGFR in hepatocellular carcinoma (Han et al., 2006). Inhibitors of COX-2 such as celecoxib and sulindac are currently being tested as therapeutic agents against cancer.

We demonstrate here that induction of COX-2 is a common downstream event following the activation of c-Met by HGF in lung tumor cells and that release of $PGE_2$, the product of COX-2 activity, can lead to independent phosphorylation of c-Met in the absence of HGF. We also examine the molecular pathways that lead to COX-2 induction by HGF, and the combined effect of targeting both c-Met activation and COX-2 action in inhibiting invasion, a process known to be enhanced by HGF and $PGE_2$.

## Materials and Methods

**Cell Lines.** H23 cells were obtained from the American Type Culture Collection (Manassas, VA). Cell lines 201T, 273T, and 784T were produced previously from primary NSCLC tumors as described previously (Siegfried et al., 1999). Cell lines 201T, 273T, and 784T were maintained in basal medium Eagle's with 10% fetal calf serum, and H23 cells were maintained in RPMI medium with 10% fetal calf serum. All cultures were mycoplasma-free.

**Protein Extraction and Western Analysis.** Lung cancer cells were grown to 85% confluence in T75 flasks. Protein extracts were prepared using ice-cold radioimmunoprecipitation buffer as described previously (Stabile et al., 2005), and 25 $\mu$g of total protein was separated by size on a 10% SDS-Tricine gel. Nonspecific binding sites were blocked by incubation in 1× Tris-buffered saline/Tween 20/5% milk followed by incubation overnight at 4°C with anti-COX-2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI) at a 1:1000 dilution. The blots were washed in 1× Tris-buffered saline/Tween 20 and then incubated with horseradish peroxidase-conjugated anti-mouse IgG. Immune complexes were detected using SuperSignal West Pico Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL). The blots were stripped using Restore Western Blot Stripping Buffer (Pierce) and reprobed with an anti-c-Met antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilution. The blots were stripped and reprobed with anti-actin rabbit polyclonal antibody (Abcam, Cambridge, MA) at 1:10,000 dilution. Blots were quantified by densitometry and ImageQuant analysis (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

For induction of COX-2 protein, lung cancer cells were grown to 75% confluence and were serum-deprived for 48 h. Cells were then treated with 50 ng/ml HGF (R&D Systems, Minneapolis, MN) for the indicated times. In all experiments, the HGF used was the recombinant human protein. For inhibitor experiments, NSCLC cells were serum-deprived for 48 h followed by inhibitor treatment (2 T25 flasks per experimental treatment). Inhibitors used were the following: 10 $\mu$M U0126 (MEK1/2 inhibitor; Cell Signaling Technology, Danvers, MA) for 2 h; 100 $\mu$M LY294002 (PI3K inhibitor; Cell Signaling Technology) for 2 h; and 10 $\mu$M SB202190 (p38 inhibitor; Tocris Bioscience, Ellisville, MO) for 90 min. HGF was added after the inhibitor pretreatment. Protein extracts were prepared and analyzed as described above.

For phospho-c-Met detection, equal amounts of protein were separated by size on a 6% SDS-Tris-glycine gel and transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked the same as for c-Met described above. The primary antibody was a 1:1000 dilution of phospho-c-Met (Tyr1234/1235) rabbit monoclonal antibody (Cell Signaling Technology) and secondary was 1:1000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG. Washes and detection were performed as described above. Blots were stripped and reprobed for total c-Met protein detection. All phospho-c-Met results were also confirmed by immunoprecipitation using phospho-c-Met antibody for immunoprecipitation and total c-Met antibody for blotting (data not shown). For phospho-EGFR detection, immunoprecipitation followed by immunoblotting was carried out as described in Zhang et al. (2006).

**Nuclear Extracts and Electrophoretic Mobility Shift Assay.** Nuclear extracts were prepared from NSCLC cell lines treated with or without 50 ng/ml HGF for 1 h in the presence or absence of various inhibitors as indicated in the figure using the Nuclear Extraction Kit (Panomics, Redwood City, CA) according to the manufacturer's instructions. A 5-$\mu$g sample of each nuclear extract was incubated with poly-D(I-C) for 5 min before the addition of biotin-labeled or unlabeled activator protein-1 (AP-1), cAMP response element-binding protein (CREB), or CCAAT/enhancer binding protein (C/EBP)-specific oligonucleotide probes (Panomics). Binding reactions were sep-

arated on a 6% native polyacrylamide gel in 0.5× Tris borate-EDTA buffer to separate bound and unbound probe. Protein/DNA complexes were transferred to nylon membrane, immobilized to the membrane by 1-h incubation at 80°C, and cross-linked using a Stratalinker UV illuminator (Stratagene, La Jolla, CA) for 3 min. The membrane was blocked, and detection of protein/DNA complexes were visualized using chemiluminescent detection agents provided in an electrophoretic mobility shift assay gel shift kit (Panomics) and exposed to autoradiography film. Results were quantitated by densitometry and ImageQuant analysis.

**Dependence of COX-2 Induction on RNA Transcription.** NSCLC cells were serum-deprived for 48 h followed by a 1-h treatment of 1 $\mu$g/ml actinomycin D (Sigma-Aldrich, St. Louis, MO). HGF (50 ng/ml) was then added and incubated for 4 or 8 h. Protein extracts were prepared, and Western blotting for COX-2 and actin proteins proceeded as described above using 25 $\mu$g of total protein.

**RNA Isolation and Northern Analysis.** RNA was isolated from NSCLC treated with HGF and/or actinomycin D for the indicated times by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Total RNA (10 $\mu$g/lane) was separated by size in a 1% denaturing agarose gel (0.66 M formaldehyde) and transferred to GeneScreen membrane (PerkinElmer Life and Analytical Sciences, Waltham, MA) using capillary transfer. The transferred RNA was UV cross-linked to the membrane by two cycles on "autocrosslink" (120,000 $\mu$J/cm$^2$) using a Stratalinker UV illuminator and hybridized with a COX-2 cDNA (Cayman Chemical) random-prime $^{32}$P- labeled probe using the Prime-It II labeling kit (Stratagene) according to the manufacturer's instructions. Hybridization and posthybridization washes were as described previously (Stabile et al., 2006). Radiographic signals were exposed to autoradiography film. Blots were stripped and reprobed with a GAPDH probe using the GAPDH-mouse DECA probe template (Ambion, Austin, TX). Quantitation was performed using densitometry and ImageQuant analysis. The mRNA half-life was calculated using log regression.

**$PGE_2$ ELISA.** NSCLC cells were serum-deprived for 48 h followed by 50 ng/ml treatment (3 wells per experimental treatment) of HGF for the indicated times. Cell culture media were collected and analyzed in duplicate by $PGE_2$-specific enzyme-linked immunosorbent assay (ELISA; Cayman Chemical) according to the manufacturer's instructions. In brief, cell culture media and $PGE_2$ standards were placed in the provided antibody-coated 96-well plates. Anti-$PGE_2$ antibody and $PGE_2$ tracer were added and incubated overnight at 4°C. The unbound components were washed off, whereas bound $PGE_2$ was determined by inverse proportion to $PGE_2$ tracer as detected by colorimetric quantitation.

**$PGE_2$ Proliferative Effects.** NSCLC cells were plated in 96-well plates and allowed to attach overnight. The cells were serum-deprived for 48 h followed by treatment with $PGE_2$ (0–1000 nM) for 48 h. Cell proliferation was measured using the fluorometric DHL Cell Viability and Proliferation Assay kit (AnaSpec, San Jose, CA). Cell viability was compared with control, untreated wells.

**TGF$\alpha$ and HGF ELISA.** The same cells used for phospho-c-Met induction by $PGE_2$ were used to analyze TGF$\alpha$ and HGF in the cell culture media. Cells were serum-deprived for 48 h followed by treatment with 25 $\mu$M $PGE_2$. Media were collected at the indicated times and concentrated to 300 $\mu$l using Amicon Ultra-4 centrifugal filter devices and tested by TGF$\alpha$-specific or HGF-specific ELISA (R&D Systems) in duplicate following the manufacturer's instructions.

**Effects of Inhibition of c-Met and COX-2 on NSCLC Invasion.** Serum-deprived NSCLC cells or cells that have been transiently transfected with c-Met antisense (AS) plasmid as described previously (Stabile et al., 2004) were plated at a density of $1 \times 10^4$ cells/well in a 24-well BD Biocoat Matrigel invasion chamber (BD Biosciences, San Jose, CA) and control wells. HGF (50 ng/ml) or $PGE_2$ (10 $\mu$M) was added to the media in the lower chamber as indicated in the figures and incubated for 48 h. In some wells, PP2 (10 $\mu$M), 300 ng/ml neutralizing HGF antibody (R&D Systems), gefitinib (10 $\mu$M), or celecoxib (75 $\mu$M) was added to the top and lower chambers. Noninvading cells were removed, and invading cells were fixed and stained with the Diff-Quik (VWR International, West Chester, PA) staining kit. The membranes were placed on microscope slides, and the number of invading cells were scored on a microscope by counting five fields per membrane at 40× magnification. Invasion is expressed as the mean number of cells invading through the Matrigel matrix membrane relative to invasion through the control membrane × 100.

**Statistical Analysis.** All values are expressed as the mean ± S.E.M. Student's $t$ test was used for all statistical analyses. Significance tests were performed with a two-sided significance level of 0.05.

## Results

**COX-2 Induction Mediated by HGF.** NSCLC cell lines were uniformly positive for c-Met expression, suggesting that most NSCLC cells will respond to HGF (Fig. 1). Relative expression normalized for $\beta$-actin varied 3-fold among cell lines. The highest relative expression was found in the adenocarcinoma cell line 201T, with lower relative expression in the squamous cell carcinoma cell lines 273T and 784T, and the H23 adenocarcinoma cell line. Basal COX-2 protein levels varied 6-fold among these cell lines and did not correlate with c-Met protein levels. All cell lines showed detectable basal COX-2 expression. This analysis was performed three times in each cell line with similar results. Figure 1B shows the densitometry results (mean and S.E.) of replicate experiments.

We have documented previously HGF-induced phosphorylation of c-Met and activation of phospho-MAPK in these NSCLC cells; this occurs rapidly (with 5 min using 50 ng/ml HGF; Stabile et al., 2004). To examine biological effects that may be downstream of this activation, we examined the

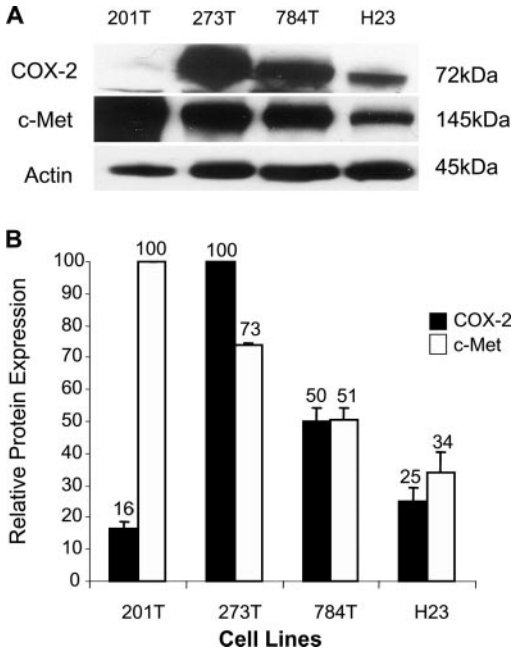


**Fig. 1.** A, representative immunoblots of whole-cell lysates from lung tumor cell lines 201T, 273T, 784T, and H23. Cell extracts were prepared, and equal amounts (25 $\mu$g) of each sample were analyzed by Western blotting as described under *Materials and Methods*. B, blots were quantified by densitometry and ImageQuant analysis, and relative protein levels were normalized for actin protein expression. Relative expression values are shown above each bar for the mean ± S.E. of replicate immunoblots. ■, COX-2 protein levels. □, c-Met protein levels.

extent of COX-2 protein expression. COX-2 protein was induced in all cell lines by HGF. Changes in COX-2 protein were detectable in as little as 30 to 60 min (data not shown). COX-2 protein was highly induced by 2 h, was maintained for 12 h (Fig. 2A), and in general returned to basal levels by 19 h (data not shown). Induction was reproduced in at least three separate experiments and was statistically significant; mean protein levels detected over time in replicate experiments is shown in Fig. 2B. 273T and H23 cells demonstrated the strongest induction of COX-2 protein (2.2- and 3.3-fold) when normalized for $\beta$-actin expression (Fig. 2B). Comparison of results in Fig. 2 with the basal levels of COX-2 and c-Met shown in Fig. 1 revealed that neither low basal levels of COX-2 protein nor high c-Met expression was required for effective COX-2 induction by HGF. This suggests that COX-2 induction through the HGF/c-Met pathway is a common event in NSCLC.

**Downstream Effectors of COX-2 Induction by HGF.** We investigated several signaling pathways known to be activated by c-Met in NSCLC for association with COX-2 induction by HGF. H23 cells were treated with recombinant human HGF for 4 or 6 h in the presence or absence of selective signaling inhibitors, followed by immunoblotting for COX-2 protein levels. Data for the 4-h HGF exposure are shown in Fig. 3; similar results were seen at 6 h. Inhibition of the MEK1/2 pathway with U0126 resulted in effective abrogation of HGF-induced COX-2 induction (HGF versus control, $P < 0.05$; HGF plus U0126 versus U0126, $P = 0.71$), whereas treatment with the PI3K inhibitor LY294002 resulted in no inhibition of HGF action (HGF plus LY294002 versus LY294002, $P < 0.05$; Fig. 3A). The p38 inhibitor SB202190 was also effective in blocking COX-2 induction after HGF treatment (HGF versus control, $P < 0.005$, SB202190 versus HGF plus SB202190, $P = 0.73$; Fig. 3B). These observations suggest that in NSCLC, both MEK1/2 and p38 pathways participate in HGF induction of COX-2, whereas blockade of either pathway is sufficient to inhibit COX-2 induction.

**HGF Induces Transcription Factor Activation.** Activation of both the MEK1/2 and p38 pathways results in phosphorylation of transcription factors that can modulate RNA synthesis of genes involved in growth and invasion. To identify transcription factors involved in the HGF-induced COX-2 activation, we first examined the activation of a panel of transcription factors using the TranSignal Protein/DNA Array I (Panomics), which allowed us to profile the activities of 54 common transcription factors simultaneously. H23 cells were treated with HGF for 0, 15, 30, 60, and 120 min followed by isolation of nuclear extracts. Consistently, an average 2.2-, 1.7-, and 3.3-fold increase in activity was observed for AP-1, C/EBP, and CREB, respectively, in response to 60-min HGF treatment (data not shown). The COX-2 gene promoter is known to contain recognition sequences for all three of these transcription factors (Tazawa et al., 1994). There was little or no difference in the activity of the other 51 transcription factors examined after HGF treatment (data not shown). Confirmation that these three transcription factors are activated by HGF was demonstrated using electrophoretic mobility shift assays with specific biotin-labeled transcription factor probes (Fig. 4). As evidenced by the presence of shifted bands, nuclear extracts from HGF-treated cells were found to bind to oligonucleotide probes for AP-1, C/EBP, and CREB. The transcription factor C/EBP showed evidence of basal

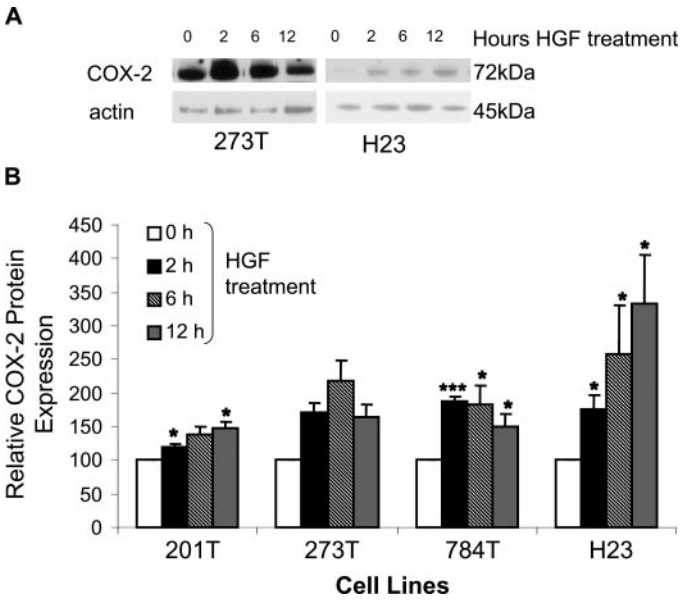


**Fig. 2.** Induction of COX-2 protein by HGF in NSCLC cells. A, representative immunoblots from 273T and H23 cells. NSCLC cells were grown to 75% confluence and serum-deprived for 48 h. Cells were then treated with 50 ng/ml HGF for 0 to 12 h and harvested for immunoblotting as described under *Materials and Methods*. B, blots were quantified by densitometry and ImageQuant analysis. COX-2 protein levels in four cell lines were expressed relative to actin. No treatment was set to 100 for each cell line. Mean of four to eight samples per treatment. Bars, S.E.; *, $P < 0.05$; ***, $P < 0.0005$, unpaired Student's $t$ test compared with 0 h for each cell line.

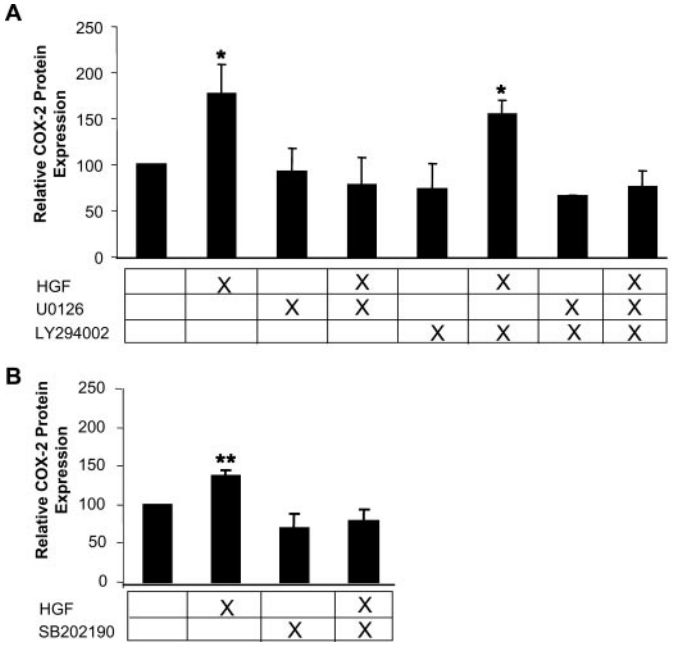


**Fig. 3.** Effect of signaling inhibitors on COX-2 induction in NSCLC cells. H23 cells were serum-deprived for 48 h followed by treatment with signaling inhibitors. After inhibitor pretreatment, HGF (50 ng/ml) was added for an additional 4 h. Cells were harvested for immunoblotting as described under *Materials and Methods*. Mean (4–8 samples per treatment group) relative COX-2 protein expression in the presence of 10 $\mu$M U0126 (MEK1/2 inhibitor) and/or 100 $\mu$M LY294002 (PI3K inhibitor), 2-h pretreatment (A), or 10 $\mu$M SB202190 (p38 inhibitor), 90-min pretreatment (B).*, $P < 0.05$; **, $P < 0.005$, unpaired Student's $t$ test compared with no treatment control. Bars, S.E. No treatment set to 100.

activation, but further activation was evidenced by a supershifted band in response to HGF. The -fold induction with HGF compared with control was 4.0-fold for AP-1, 2.4-fold for C/EBP, and 6.4-fold for CREB. The effect of HGF was verified four times. Results in Fig. 4 are for H23 cells; activation of the same three transcription factors was also observed in 273T cells (data not shown). Competition reactions using unlabeled probe supplied by Panomics inhibited the HGF response, demonstrating specificity of the observed bands. The activation of AP-1 and CREB transcription factors was completely inhibited by blocking either the MEK1/2 pathway using U0126 or the p38 pathway using SB202190 (Fig. 4), consistent with our previous results measuring COX-2 induction. Binding of C/EBP in response to HGF was partially inhibited by U0126 (1.7-fold over control) and SB202190 (1.4-fold over control). U0126 and SB202190 alone had no effect on transcription factor binding. Effect of each inhibitor was performed twice. These results suggest that multiple phosphorylation sites are probably involved in the activation of these factors by HGF, mediated by MEK1/2 and p38, and that transcription factor binding to each respective DNA element requires the action of both kinases.

**COX-2 Induction by HGF Involves Gene Transcription and mRNA Stabilization.** Both transcription of new mRNA and increased mRNA stability have been reported to be involved in the induction of COX-2 (Dixon, 2004). To determine whether gene transcription was required for COX-2 induction by HGF, NSCLC were pretreated with 1 $\mu$g/ml actinomycin D for 1 h before the addition of HGF (50 ng/ml) without removal of actinomycin D. Actinomycin D treatment partially abolished the induction of COX-2 protein at 4 h (Fig. 5A). HGF induction was 2.2-fold in 273T cells ($P < 0.05$); this was inhibited 45% by actinomycin D treatment at 4 h. A similar partial inhibition was observed in H23 cells at this time point. At 8 h, COX-2 induction was completely abolished in both cell lines (Fig. 5A). HGF produced a significant COX-2 induction in both cell lines (1.8-fold, $P < 0.05$ in 273T, $< 0.005$ in H23), which was reduced to baseline by actinomycin D treatment. Relative stability of mRNA was also measured by calculating the half-life of COX-2 mRNA in the presence of HGF. HGF treatment led to a prolongation of decay of detectable COX-2 mRNA in Northern blots (Fig. 5B). The average calculated half-life of COX-2 mRNA was 2.5 h in the absence of HGF and 5.9 h in the presence of HGF, an increase of more than 2-fold. These data suggest that c-Met signaling can both stabilize already transcribed COX-2 mRNA and activate COX-2 gene transcription. The partial inhibition by actinomycin D on COX-2 protein expression at 4 h probably reflects the ability of stabilized pretranscribed COX-2 mRNA to serve as a template for additional protein synthesis.

**Induction of $PGE_2$ Release by HGF in NSCLC.** If COX-2 protein is elevated by HGF, an accompanying increase in $PGE_2$ release should occur. ELISA was used to detect the release of $PGE_2$ from NSCLC cells after application of recombinant human HGF. In H23 cells, which showed strong COX-2 induction, $PGE_2$ release was increased almost 16-fold by 24 h ($P < 0.0005$; Fig. 6A). Some increase in $PGE_2$ was observed in as little as 5 to 15 min (1.3- to 1.8-fold), probably reflecting stabilized COX-2 mRNA. In 201T cells, which showed a more modest COX-2 induction by HGF, secreted $PGE_2$ levels were increased 4-fold by 24 h ($P < 0.0005$) but were much lower relative to those observed in H23 cells (Fig. 6B). Again, a trend of increased $PGE_2$ secretion was apparent in 5 to 15 min. The concentration of $PGE_2$ found in culture medium after HGF treatment, adjusting for observed 50% recovery from the medium, was approximately 6 nM for H23 cells and 0.3 nM for 201T cells. $PGE_2$ was a

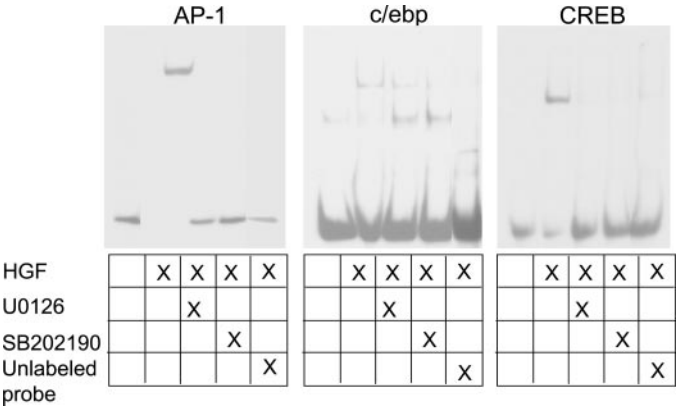


**Fig. 4.** HGF can induce activation of transcription factors. Nuclear extracts were prepared from H23 cells pretreated for 1 h with either 10 $\mu$M U0126, 10 $\mu$M SB202190, or no pretreatment followed by the addition of 50 ng/ml HGF for 1 h. Electrophoretic mobility shift assays were performed by incubating biotin-labeled probes specific for AP-1, C/EBP, or CREB transcription factors with 5 $\mu$g of each nuclear extract. Unlabeled probe competition reactions are shown in the last lane for each transcription factor. Electrophoresis was performed as described. Representative blots are shown from H23 cells.

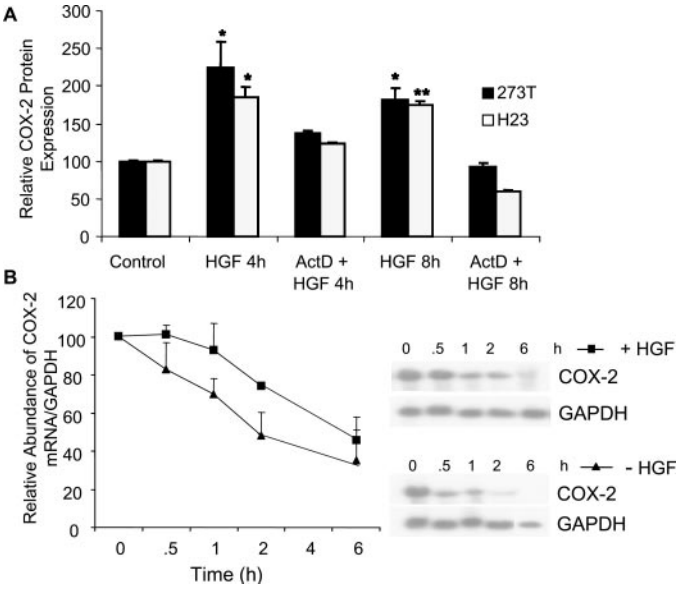


**Fig. 5.** Effect of actinomycin D pretreatment on COX-2 induction by HGF. A, H23 and 273T cells were serum-deprived for 48 h followed by a 1-h treatment of 1 $\mu$g/ml actinomycin D. HGF (50 ng/ml) was then added and incubated for 0 to 8 h. Protein extracts were prepared and immunoblotting performed. COX-2 protein was expressed relative to actin. Results are the mean of three samples per treatment group. No treatment control was set to 100. Bars, S.E.; *, $P < 0.05$; **, $P < 0.005$, unpaired Student's $t$ test compared with control for each cell line. B, characterization of mRNA stability in HGF-treated and untreated cells after the addition of actinomycin D. 273T cells were treated with or without 50 ng/ml HGF for 30 min. Actinomycin D (1 $\mu$g/ml) was added, and the cells were harvested for RNA at 0.5, 1, 2, and 6 h after the addition. Results represent the mean $\pm$ S.E. Representative Northern blot is shown. Relative level of COX-2 message after normalization with GAPDH level in each RNA sample was plotted against the time of actinomycin D treatment. ■, samples with HGF treatment; ▲, samples without HGF treatment.

strong signal for proliferation in NSCLC cells (Fig. 6C). A 1.7-fold induction of cell proliferation was found in 201T cells at 48 h after application of 0.1 nM $PGE_2$ ($P < 0.001$). Similar results were obtained in 273T cells but required much higher concentrations of $PGE_2$ (10 $\mu$M) to override the endogenous $PGE_2$ production (data not shown). This suggests that the extent of $PGE_2$-mediated autocrine NSCLC proliferation achieved via COX-2 induction by HGF may be dependent on basal COX-2 levels. In vivo, however, secreted $PGE_2$ may also have paracrine effects on stromal, inflammatory, or endothelial cells, which would contribute to a protumorigenic effect.

**$PGE_2$ Induces Phosphorylation of c-Met in the Absence of HGF.** The receptors for $PGE_2$ are G-protein-coupled receptors that have been reported to cross-activate the receptor tyrosine kinase EGFR (Han et al., 2006); EP3 and EP4, two of the known $PGE_2$ receptor isoforms, are commonly expressed by NSCLC cell lines (Krysan et al., 2005). EGFR-c-Met cross-activation has also been reported in hepatocellular carcinoma (Jo et al., 2000). We examined the ability of $PGE_2$ to cause phosphorylation of c-Met through EGFR pathway activation in NSCLC cells. We have published previously that in the cell lines used in these experiments, autocrine HGF production is not found; neither mRNA nor protein for HGF can be detected (Singh-Kaw et al., 1995). To check whether $PGE_2$ could induce HGF production in NSCLC cells, we measured HGF by ELISA assay in the culture medium after $PGE_2$ exposure compared with known positive (lung fibroblast conditioned medium and medium spiked with HGF) and negative (medium unexposed to cells) control medium. With a limit of detection of 50 pg/ml, we found no detectable HGF in culture medium of NSCLC cells after exposure to $PGE_2$ (see Supplemental Data).

In both 201T cells and 273T cells, phospho-c-Met was detected within 5 min of exposure to PGE2 (Fig. 7, A and B). The extent of c-Met phosphorylation continued to increase over time, with maximal phosphorylation of c-Met occurring within 60 to 120 min (3.3-fold). These experiments were performed three times, and representative immunoblots are shown for 201T (A) and 273T (B) cells. In comparing the ability of HGF to induce c-Met phosphorylation in these cells with that of $PGE_2$, a 7.4-fold induction of phospho-c-Met (Fig. 7C) was observed in 201T cells (that show the highest expression of c-Met shown in Fig. 1) at 50 ng/ml HGF (the concentration of HGF we have found previously to give maximal effects) (Stabile et al., 2004). This stimulation occurred at 2 min and declined thereafter to baseline by 30 min (data not shown). Although the peak induction of c-Met phosphorylation seen with $PGE_2$ was approximately 40% of that of the maximal HGF response, the response to $PGE_2$ is sustained over a much longer period of time. This suggests the c-Met-mediated biological response over time to $PGE_2$ could have been just as high or higher than that in response to HGF, and the difference in time course suggests a different mechanism for the induction of phospho-c-Met.

Phosphorylation of c-Met by EGFR has been reported (Han et al., 2006), and so we the examined release of EGFR ligands in response to $PGE_2$. $PGE_2$ caused secretion of TGF$\alpha$ by NSCLC cells (Fig. 7D); a 2- to 4-fold induction of TGF$\alpha$ present in culture medium was detected ($P < 0.05$), beginning within 5 min and peaking at 15 to 30 min, before the peak in c-Met phosphorylation. Analysis of EGF secretion showed low to undetectable EGF at all time points. Analysis of secretion of amphiregulin, another EGFR ligand, also showed 1.5- to 2-fold induction by HGF (data not shown). $PGE_2$ treatment resulted in phosphorylation of EGFR (Fig. 7E, lane 2, top), showing that the released EGFR ligands activate their receptor. This was accompanied by the induction of c-Met phosphorylation in response to $PGE_2$ (Fig. 7E, lane 2, bottom). Both EGFR and c-Met phosphorylation in response to $PGE_2$ was blocked by the EGFR tyrosine kinase inhibitor gefitinib (Fig. 7E, lane 4), demonstrating that the phosphorylation of c-Met is mediated by EGFR. There was no difference between basal and $PGE_2$-treated EGFR or c-Met phosphorylation levels in the presence of the src inhibitor

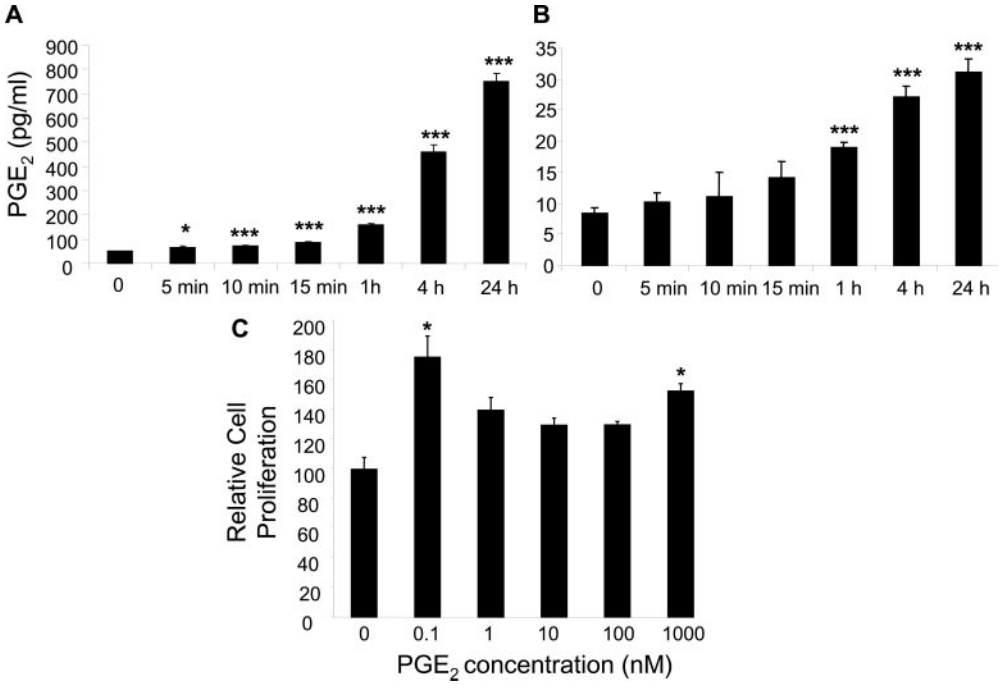


**Fig. 6.** Secretion of $PGE_2$ after HGF treatment. H23 cells (A) and 201T cells (B) were serum-deprived for 48 h followed by 50 ng/ml HGF treatment for 0 to 24 h. Media were collected and analyzed by $PGE_2$ ELISA. Graph represents the mean of three to six samples per treatment. Bars, S.E.; *, $P < 0.05$; ***, $P < 0.0005$, unpaired Student's $t$ test. C, proliferation of 201T cells in response to $PGE_2$ treatment. 201T cells were plated in 96-well plates and allowed to attach overnight. The cells were serum-deprived for 48 h followed by treatment with $PGE_2$ (0–1000 nM) for 48 h. Cell proliferation assays were performed, and the mean of six samples per treatment group is expressed compared with control, untreated wells. Bars, S.E.; *, $P < 0.05$, unpaired Student's $t$ test.

PP2 (Fig. 7E, lanes 5 and 6), suggesting that the release of EGFR ligands induced by $PGE_2$ that we observed may be Src-dependent. This will require confirmation with other selective Src family kinase inhibitors.

**Both $PGE_2$ and HGF Induce Invasion in NSCLC Cells.** Increased invasion has been reported in response to both HGF and $PGE_2$ in colon cancer (Pai et al., 2003), and we hypothesized that invasion induced by $PGE_2$ was dependent on its activation of c-Met. We used movement through an artificial extracellular matrix as a measure of relative invasive ability of NSCLC cells (H23, with a high basal level of $PGE_2$; see Fig. 6) in the presence of $PGE_2$ or HGF. As seen in Fig. 8A, $PGE_2$-induced invasion required c-Met. $PGE_2$ induced a significant increase ($P < 0.005$) in the relative number of cells invading the matrix by 48 h (Fig. 8A). This was significantly blocked ($P < 0.05$) by inhibiting c-Met expression with c-Met AS plasmid. We have shown previously that the c-Met AS construct specifically down-regulates c-Met and blocks c-Met signaling by approximately 70% and that a control sense construct had no effect on c-Met (Stabile et al., 2004). Invasion induced by $PGE_2$ was also blunted by Src inhibitor PP2 or gefitinib ($P < 0.001$), suggesting that an Src-EGFR-c-Met axis may be involved in invasion induced by $PGE_2$. Src has been shown by us to be activated by $PGE_2$ and to induce EGFR ligand release in head and neck cancer cells (Thomas et al., 2006).

We next examined the combined effect of blocking both HGF-dependent and HGF-independent activity of c-Met in H23 cells. For these experiments, we used either the c-Met AS construct or an HGF neutralizing antibody (NA) to block signaling either at the level of the receptor or at the level of ligand. The HGF NA used (R&D Systems) was found to specifically block HGF-induced signaling, whereas a control goat IgG was completely inactive in an assay of MAPK activation (see Supplementary Data). We also used celecoxib to inhibit COX-2 activity and prevent release of $PGE_2$. The celecoxib concentration chosen was the $IC_{50}$ value for inhibition of cell proliferation in H23 cells, 75 $\mu$M. We confirmed that this concentration does not inhibit MEK1/2 by measuring phospho-MAPK in H23 cells (see Supplementary Data). HGF induced a significant increase ($P < 0.05$) in invasion, which was blocked 63% by HGF NA (Fig. 8B; $P = 0.065$); celecoxib alone blocked invasion induced by HGF by 95% ($P < 0.001$). The effect of celecoxib is due in part to inhibiting basal $PGE_2$ release, because celecoxib in the absence of HGF reduces invasion below baseline. Celecoxib in combination with neutralizing antibody to HGF resulted in a reduction of relative invasion of less than baseline (120% reduction, $P <$

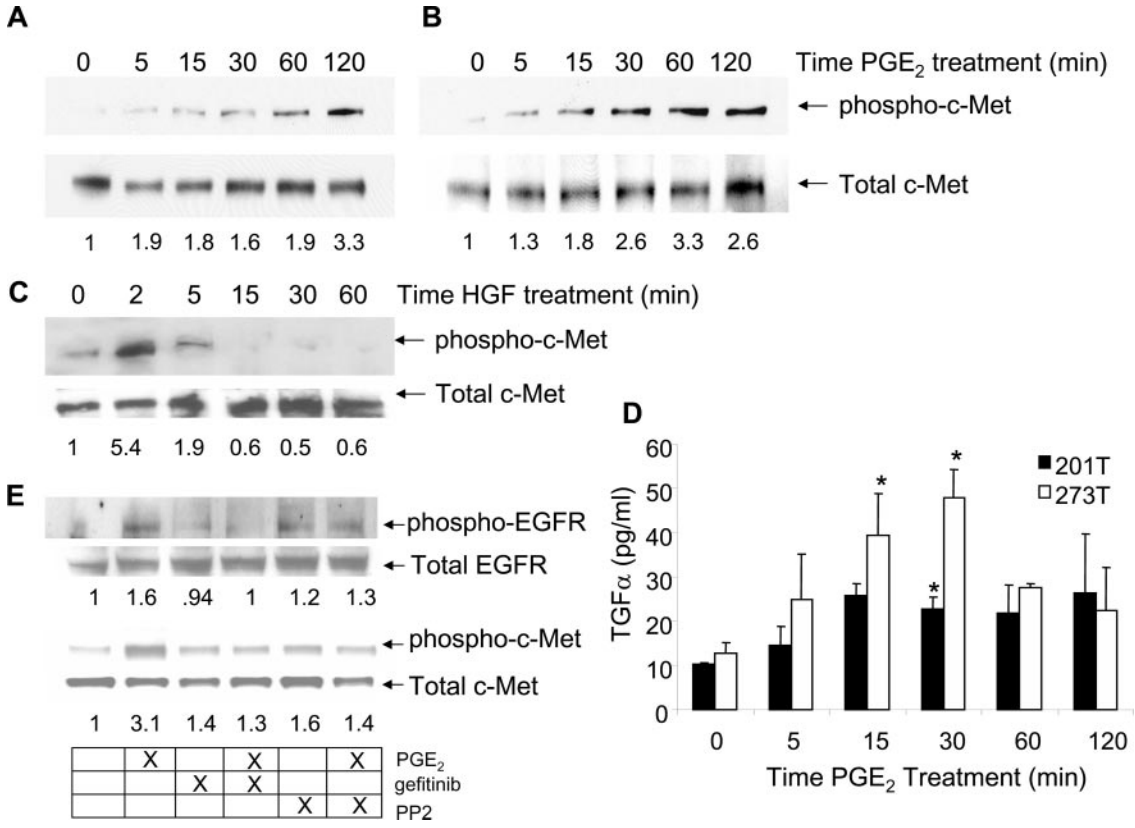


**Fig. 7.** $PGE_2$ can phosphorylate c-Met. Cells were serum-deprived for 48 h followed by the addition of $PGE_2$ (25 $\mu$M) for 0 to 120 min. Cell lysates were prepared and analyzed for phospho-c-Met expression. Representative immunoblots from 201T cells (A) and 273T cells (B) are shown with relative densitometry quantitation shown beneath each lane. No treatment was set to 1 for each cell line. C, 201T cells were treated with HGF (50 ng/ml) for 0 to 60 min to compare the extent and time course of c-Met phosphorylation with that shown in A with $PGE_2$. Cells were serum-deprived, and cells lysates were made and analyzed as described in A. D, cell culture media were collected from 201T and 273T cells treated with $PGE_2$ for 0 to 120 min, concentrated, and analyzed via TGF$\alpha$ ELISA. Graph represents the mean ± S.E. of at least three samples per treatment. *, $P < 0.05$, unpaired Student's $t$ test compared with control for each cell line. E, $PGE_2$-induced phospho-c-Met is EGFR and src-dependent. Representative experiment using 273T cells that were serum-starved for 48 h followed by 1-h pretreatment with either gefitinib (10 $\mu$M) or PP2 (10 $\mu$M). $PGE_2$ (25 $\mu$M) was then added for 1 h. Cell lysates were prepared, and 25 $\mu$g from each sample was used in phospho-EGFR and total EGFR immunoprecipitation and phospho-c-Met and total c-Met immunoblotting. Relative densitometry quantitation is shown beneath each lane with no treatment set to 1.

0.0001). The combination treatment was superior to either HGF NA alone or celecoxib alone in blocking HGF-induced invasion ($P < 0.02$, 0.004, respectively). This suggests that blocking the prolonged activation of c-Met by $PGE_2$ is important in inhibiting invasion.

HGF-induced invasion was also completely blocked by c-Met AS plasmid treatment. In support of our two-part hypothesis of a loop activating $PGE_2$ by HGF, and $PGE_2$ in turn inducing invasion via prolonged activation of c-Met, we found that celecoxib could inhibit HGF-induced invasion in H23 cells but not as well as c-Met AS. Effects of celecoxib on basal $PGE_2$ levels are contributing to the overall invasion, as shown with celecoxib alone in the absence of HGF, which reduced relative invasion 45% ($P < 0.0001$). There was no further effect of celecoxib in the presence of HGF when c-Met was down-regulated by the AS construct, as expected if $PGE_2$-mediated invasion requires c-Met. HGF NA alone had no effect on invasion, as expected, because the lung cancer cell lines studied produce less than 70 pg/ml (approximately 8 pM) HGF in the presence or absence of $PGE_2$ (see Supplemental Data). Changes in signaling have not been observed at HGF concentrations lower than 50 pM. The combination of celecoxib and HGF NA was similar to celecoxib alone ($P = 0.151$), also as expected in the absence of HGF.

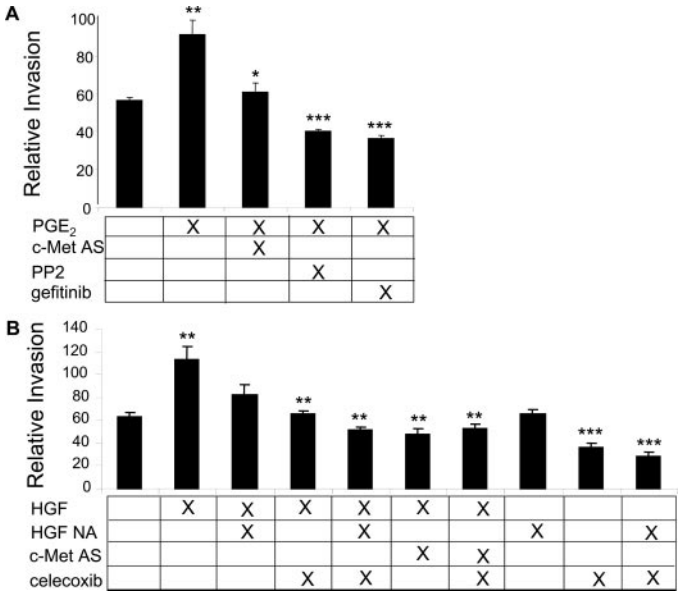


**Fig. 8.** Inhibition of $PGE_2$-induced cell invasion. H23 cells were serum-deprived for 48 h before plating in invasion chamber wells. A, cells were treated with $PGE_2$ (10 $\mu$M), PP2 (10 $\mu$M), gefitinib (10 $\mu$M), or transiently transfected with a c-Met AS expression plasmid as indicated. B, cells were treated with HGF (50 ng/ml), HGF NA (300 ng/ml), celecoxib (75 $\mu$M), and/or transiently transfected with c-Met AS expression plasmid as indicated. Forty-eight hours after treatment in all cases, cells were fixed and stained. Relative cell invasion was determined as the number of invading cells through the Matrigel matrix versus the number of cells invading through control membrane $\times$ 100. Positively stained cells in five high-powered fields per slide were counted at 40$\times$ magnification. Mean of 10 samples per treatment group. Bars, S.E.; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$, unpaired Student's $t$ test. All comparisons were to respective controls: HGF or $PGE_2$ versus no treatment; c-Met AS, PP2, HGF NA, gefitinib, or celecoxib in presence of ligand versus HGF or $PGE_2$; and HGF NA or celecoxib in the absence of ligand versus no treatment.

## Discussion

NSCLC cell lines commonly express the c-Met protein and reproducibly demonstrate induction of COX-2 protein by treatment with HGF at a concentration shown previously by us to be maximal for induction of cell proliferation in NSCLC cell cultures (Singh-Kaw et al., 1995). Induction of COX-2 by HGF has also been demonstrated in gastric cancer (Jones et al., 1999; Chen et al., 2006), and COX-2 is believed to play an important role in these tumor types and in lung cancer (Krysan et al., 2006). Targeting COX-2 is under investigation for both cancer prevention and treatment. Inhibition of COX-2 limits the production of prostaglandins that are known to stimulate cell proliferation, induce invasiveness, and mediate angiogenesis (Martey et al., 2004; Yoshimoto et al., 2005).

COX-2 expression induced by HGF in NSCLC cells examined here seems highly dependent on the activation of both the MEK1/2 and p38 pathways. We have shown previously that Erk1/2 is activated within 5 min of HGF treatment in NSCLC (Stabile et al., 2004), whereas p38 was shown to be activated within 5 to 10 min (data not shown) Although PI3K is known to be activated by HGF (Segarra et al., 2006), it does not seem to play a role in COX-2 induction in these NSCLC cells. In cortical neurons, c-Jun $NH_2$-terminal kinase was not found to be involved in HGF induction of neuron migration, whereas Erk1/2, p38, and PI3K were required (Segarra et al., 2006). The activity of Erk1/2 and p38 in NSCLC cells after HGF treatment is linked to downstream activation of three transcription factors that are known to recognize COX-2 promoter elements: AP-1, C/EBP, and CREB (Tazawa et al., 1994), and both Erk1/2 and p38 seem to participate in and be necessary for effective transcription factor activation. One or more of these three transcription factors are probably involved in increased COX-2 gene transcription after HGF treatment. Transcription factors are activated by specific phosphorylation on serine residues that increase their nuclear localization, modulate protein-protein interactions in the transcription complex, and regulate affinity for DNA recognition elements (Gardner and Montminy, 2005). Various kinases have been identified as upstream of transcription factor activation. AP-1 activation, for example, is associated with increased phosphorylation of Erk1/2, c-Jun $NH_2$-terminal kinase, and p38 (Khan et al., 2006). Activation of CREB is also carried out by numerous kinases, and whereas serine 133 is recognized as a major site of phosphorylation in response to cytokines and growth factors, full activation of CREB requires multiple phosphorylation events at several serines and involves multiple kinases, including Erk1/2 and p38 (Johannessen and Moens, 2007).

COX-2 induction in NSCLC cells also involves stabilization of COX-2 mRNA in addition to stimulation of new gene transcription. This is most likely mediated by p38, which has been shown to stabilize COX-2 mRNA in several systems, such as in response to aspirin or interleukin-1 in human intestinal myofibroblasts (Mifflin et al., 2002, 2004) Post-translational modification of COX-2 mRNA is recognized as an important mechanism regulating the amount of mRNA available for protein synthesis (Subbaramaiah and Dannenberg, 2003). The mechanism of stabilization involves an AU-rich element in the 3′-untranslated region of COX-2 mRNA; p38 activation results in increased recruitment of the mRNA

stabilization factor HuR to this element (Subbaramaiah et al., 2003). HuR seems to act by decreasing mRNA degradation (Dixon, 2004).

Increased COX-2 protein expression in NSCLC was accompanied by elevated release of $PGE_2$, the downstream product of COX-2 activity, and this occurred rapidly, in agreement with our observation that COX-2 mRNA stabilization occurs in addition to new mRNA transcription. $PGE_2$ was found to increase cell proliferation in these cells as well. This observation suggests that cell proliferation induced by HGF in NSCLC is at least partly mediated by an increased production of $PGE_2$. Chen et al. (2006) also found increased secretion of $PGE_2$ in response to HGF in gastric cancer cells. Because we also found release of EGFR ligands in response to $PGE_2$, cell proliferation in response to HGF may be partially attributed to activation of the EGFR pathway. The effect of released $PGE_2$ could also be a paracrine one in vivo, because receptors for prostaglandins are expressed on endothelial cells and inflammatory cells.

Cross-activation of c-Met by a $PGE_2$-related mechanism was also observed. Phosphorylation of c-Met after exposure to $PGE_2$ may be dependent on Src, which we have documented previously to cause the activation of proteases that release EGFR ligands from the cell surface (Thomas et al., 2006; Zhang et al., 2006). The use of additional Src inhibitors and a dominant-negative Src construct will be required to confirm this. EGFR ligands were detected in cell medium after exposure of NSCLC to $PGE_2$, and c-Met phosphorylation induced by $PGE_2$ was also dependent on an active EGFR. These observations suggest that in addition to activating c-Met in a ligand-dependent manner, HGF induces downstream signaling that leads to the release of a factor ($PGE_2$) that can induce further ligand-independent activation of c-Met. The prolonged time course of c-Met phosphorylation by $PGE_2$ also suggests that c-Met internalization and downregulation may be reduced when activated by $PGE_2$ compared with HGF. We found no evidence that $PGE_2$ could induce HGF release in NSCLC cells; in general, the amount of HGF detected in culture medium from these cell lines is too low to have biological function. The proposed reinforcing loop of c-Met-$PGE_2$-EGFR-c-Met activation (as depicted in Fig. 9) might extend c-Met signaling or amplify it later, after the initial activation by HGF. This is suggested by the prolonged time course of c-Met phosphorylation by $PGE_2$. We observed that invasion induced by $PGE_2$ is dependent on c-Met, suggesting that the $PGE_2$-EGFR-c-Met axis has biological significance. The combination of an antibody targeting HGF and a drug that prevents $PGE_2$ production showed an increased ability to inhibit invasion induced by HGF compared with either compound alone.

Because of the strong evidence for involvement of the HGF/c-Met pathway in the pathogenesis of human cancers, therapeutic inhibitors targeting this pathway are in development. Inhibitors of the HGF/c-Met signaling pathway under development include the following: 1) small-molecule c-Met tyrosine kinase inhibitors [ATP competitive kinase inhibitors (Christensen et al., 2003; Ma, 2005)]; 2) c-Met biologic inhibitors [ribozymes (Abounader et al., 1999, 2002), c-Met antisense vectors (Stabile et al., 2004), and dominant-negative receptors (Webb et al., 1998; Kaplan et al., 2000)]; decoy receptors (Michieli et al., 2004); and peptide antagonists (Bardelli et al., 1998; Atabey et al., 2001); 3) HGF and c-Met neutralizing antibodies (Burgess et al., 2006; Kim et al., 2006); and 4) truncated HGF antagonists (Date et al., 1997; Jiang et al., 1999; Hiscox et al., 2000; Parr et al., 2000). HGF-neutralizing antibodies have shown effects in phase I clinical trials (available at http://wwwext.amgen.com/science/pipe_AMG102.html).

The coupling of COX-2 induction with c-Met stimulation indicates that both pathways could be targeted for inhibition of lung cancer growth, especially if a ligand-independent reinforcing activation of the c-Met pathway occurs via $PGE_2$ induced by HGF/c-Met signaling. It has been suggested that c-Met tumor levels and HGF circulating levels be used as a criteria for COX-2 inhibitor use in gastric cancer (Chen et al., 2006). Although our data suggested that if c-Met down-regulated the presence of celecoxib had no additional effect in the invasion assay, this in vitro result cannot be directly extrapolated to the in vivo or clinical situation. First, paracrine actions of $PGE_2$ on endothelial cells, inflammatory cells, and stromal cells would also be inhibited by a COX-2 inhibitor that would not depend on an intact c-Met. Second, in animals or patients, it is unlikely that sufficient drug could be delivered safely to cause enough c-Met inhibition that would completely prevent its signaling over time. Thus, the dual inhibition strategy could also result in additive effects because of pharmacodynamic considerations. One of the limitations to targeted therapies used clinically to date has been delivery of a nontoxic level of drug that is still effective at inhibiting the targeted pathway at the tumor cell. Given that near 100% inhibition of any target with clinically deliverable doses is unlikely, partially reducing two segments of the same signaling pathway may improve therapeutic effects. Here, we used an invasion assay to demonstrate the additive inhibitory effects by blocking HGF action directly with a neutralizing antibody and indirectly by preventing ligand-independent c-Met activation through celecoxib, which blocks $PGE_2$ synthesis in response to HGF. Such combination strategies may prove effective for lung cancer therapy.

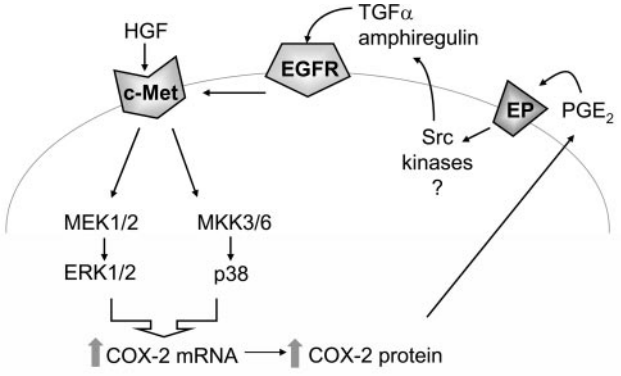


**Fig. 9.** Proposed model for ligand-dependent and ligand-independent activation of c-Met. HGF can bind to and activate its receptor, c-Met, which leads to the subsequent stimulation of COX-2 mRNA and protein production through both the ERK1/2 and p38 signaling pathways. HGF activation of c-Met also leads to the secretion of $PGE_2$. $PGE_2$, either produced through HGF stimulation or endogenous $PGE_2$, can then bind to $PGE_2$ receptors (EP) and release the EGFR ligands, TGF$\alpha$ and amphiregulin, which leads to EGFR autophosphorylation and phosphorylation of c-Met. The release of EGFR ligands may be carried out by an src family kinase.

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