THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 11, pp. 7961–7972, March 16, 2007
Printed in the U.S.A.

Extracellular Matrix Fibronectin Increases Prostaglandin E_2 Receptor Subtype EP4 in Lung Carcinoma Cells through Multiple Signaling Pathways

*THE ROLE OF AP-2**

Received for publication, November 3, 2006, and in revised form, January 4, 2007 Published, JBC Papers in Press, January 19, 2007, DOI 10.1074/jbc.M610308200

ShouWei Han[‡1], Jeffrey D. Ritzenthaler[‡], Byron Wingerd[§], Hilda N. Rivera[¶], and Jesse Roman[‡¶]

From the [‡]Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia 30322, the [§]Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824, and the [¶]Atlanta Veterans Affairs Medical Center, Atlanta, Georgia 30033

We have previously demonstrated that fibronectin (Fn) stimulates the proliferation of non-small cell lung carcinoma (NSCLC) cell growth through the induction of cyclooxygenase-2 (COX-2) and prostaglandin E_2 secretion. Here, we demonstrate that NSCLC cells express mRNA and protein for the prostaglandin E_2 receptor EP4 and that Fn enhances its stimulatory effect by inducing the expression of EP4, but not of EP1, EP and EP3 receptor subtypes. The effect of Fn on EP4 wa ited by an antibody against $\alpha5\beta1$ integrin and by phosphoinositide 3-kinase (wortmannin) and nal-regulated kinase (PD98095), but not kinase C (calphostin C), of protein malian target of rapamycin (r fering RNA was also inhi binding activity in the sense oligonucleotides Using full-length and m found that Fn stimulation when one AP-2 site (−10 nuclear AP-2α protein expre pathways. Our results indicate t liferation is mediated through EP4 Fn induces EP4 expression through de- pendent signals that include induct signal- regulated kinase and phosphoinositi pathways as well as expression of COX-2. These even d to activation of the transcription factor AP-2α, which interacts with specific regions in the *EP4* gene promoter, leading to transcription of the *EP4* gene.

Extracellular matrix proteins are considered to play roles in the migration and differentiation of various cells, including carcinoma cells. Fibronecti),[2] a matrix glycoprotein highly expressed in tobac ng diseases, has been shown to stimulate car , including lung carcinoma (1–3). W Fn stimulated human lung car ction of cyclooxygenase-2 duction of prostaglandin nsidered important in hibition of PGE_2 syn- The effect of PGE_2 bind to its recep- These receptors d progression es to tumor growth lorectal carcinoma (7, 8). mice suggested a role for EP4 (7). PGE_2 and its signaling through been shown to mediate non-small cell (NSCLC) invasiveness (10). Taken together, ations suggest that manipulation of prostaglandin lism downstream from COX-2 produces more pro- und effects on carcinoma reduction than COX-2 inhibition alone and could be the basis for new approaches for the prevention of carcinoma.

At present, the mechanisms that link Fn and *EP4* gene expression and how they might relate to lung carcinoma are unknown. Herein, we explore the relationship between these molecules and their role in lung carcinoma cell growth. Our results show that Fn stimulates lung carcinoma cell growth and that this inductive effect is partly dependent upon stimulation of PGE_2 production and induced PGE_2 receptor subtype *EP4* gene expression, which is mediated through integrin-dependent signals, including the activation of phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) signaling pathways.

* This work was supported by American Lung Association Grant RG-10215N (to S. W. H.) and by a Merit Review Grant from the Department of Veterans Affairs (to J. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom all correspondence and reprint requests should be addressed: Division of Pulmonary, Allergy and Critical Care Medicine, Emory University School of Medicine, Whitehead Bioresearch Bldg., 615 Michael St., Suite 205-M, Atlanta, GA 30322. Tel.: 4047122661; Fax: 4047122151; E-mail: shan2@emory.edu.

[2] The abbreviations used are: Fn, fibronectin; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; NSCLC, non-small cell lung carcinoma; PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase; $dmPGE_2$, 16,16-dimethylprostaglandin E_2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; siRNA, small interfering RNA; ODN, oligodeoxynucleotide(s); C/EBP, CCAAT/enhancer-binding protein; mTOR, mammalian target of rapamycin; PKA, cAMP-dependent protein kinase; PKC, protein kinase C.

EXPERIMENTAL PROCEDURES

Culture and Chemicals—NSCLC cell lines H1838 and H2106 were obtained from the American Type Culture Collection (Manassas, VA), and were grown in RPMI 1640 medium (H1838) supplemented with 10% heat-inactivated fetal bovine serum, HEPES buffer, 50 IU/ml penicillin/streptomycin, and 1 μg of amphotericin (complete medium) or in Dulbecco's modified Eagle's medium/F-12 medium (H2106) supplemented with 10% heat-inactivated fetal bovine serum, 0.005 mg/ml insulin, 0.01 mg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β-estradiol, 10 mM HEPES, as described previously (4). Afterward, cells were harvested and replaced in serum-free medium on Fn- or collagen type 1-coated culture plates for all experiments described later (the plates were coated with the matrix components diluted in buffer containing bovine serum albumin overnight at 4 °C. Afterward, the supernatants were removed, and the dishes were washed with phosphate-buffered saline three times before experiments were initiated). Mouse anti-human integrin $\alpha5\beta1$ (MAB1969) and anti-integrin $\alpha2\beta1$ antibodies (MAB1967) were purchased from Chemicon International Inc. (Temecula, CA). 16,16-dimethylprostaglandin E_2 (dmPGE$_2$), Polyclonal antibodies against COX-2, EP1, EP2, EP3, and EP4 were obtained from Caymen Chemical Co. [*methyl*-^{3}H]Thymidine was purcha… from Amersham Biosciences. The [γ-^{32}P]ATP was pu… from PerkinElmer Life Sciences, Inc. Mammali… rapamycin (mTOR) inhibitor, rapamycin, a… bodies specific for Akt, ERK1, ERK2, a… forms ((AktS473) (ERK Thr^{421}/S… Cell Signaling Inc. The ERK1/… kinase C (PKC) inhibit… (PKA) inhibitor H89, … clonal antibody against … biochem. The CellTiter-… kit, gel shift assay system, a… kit were obtained from Pro… DNA Master SYBR Green 1… Applied Science. Antibodies a… (H-87), and AP-2δ (H77) were pu… technology, Inc. All RT-PCR kit c… from PerkinElmer Life Sciences. H… …ed from human fibroblasts), collagen type 1, a… …ier chemicals were purchased from Sigma unless othe… …se indicated.

Reverse Transcriptase PCR—Total RNA was prepared from human lung carcinoma cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To amplify 465-bp EP4 and 200-bp GAPDH cDNA fragments, the sequences of PCR primers (Sigma) were 5′-TCGCGCAAGGAGCAGAAGGAGAC-3′ (for EP4 sense), 5′-GACGGTGGCGAGAATGAGGAAGGA-3′ (for EP4 antisense), 5′-CCATGGAGAAGGCTGGGG-3′ (for GAPDH sense), and 5′-CAAAGTTGTCATGGATGACC-3′ (for GAPDH antisense) according to published data (11, 12). The 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. The densitometric analysis of PCR products was performed by computer software (Bio-Rad Quantity One) and a GS-800 Imaging Densitometer (Bio-Rad) and standardized to the GAPDH product. EP4/GAPDH density bands in control groups were considered as 100%. Values of treatment group EP4/GAPDH ratios are given as percentage of controls. A 100-base pair ladder (Invitrogen) was used as a size standard.

Real Time RT-PCR—This procedure, which is based on the time point during cycling when amplification of the PCR product is first detected, rather than on the amount of PCR product accumulated after a fixed number of cycles, was described previously (12). Final results, which were expressed as *n*-fold differences in *EP4* gene expression relative to the *GAPDH* gene, were calculated using a formula based on a doubling of the product after each cycle (1… The procedures for treatment and total RNA preparati… dentical to those described for RT-PCR. All PC… htCycler-FastStart DNA Master SYBR G… med in the Cepheid Smart-Cycle… ale, CA). The cycling con… ation at 95 °C for 10 min, …0 °C for 10 s, and 72 °C …riplicate for each data …ut templates were …

…g RNA (siRNA) …ioate oligode… …n this study were …was 5′-GCCGCCGCC… …RK2), and the corresponding …-ATGGCGGCGGCGGCGGC-3′ …P-2 antisense was 5′-CGTCAATTTC… …CATGGATCGG-3′ (−13 to +18 of the AP-2 …d the control oligonucleotide, 5′-CAAAGTCT… …TATTCGGTCATAATGGCC-3′, consisted of similar …leotides with scrambled sequences. They were synthesized by Sigma (The Woodlands, TX) according to published data (13, 14). The COX-2 siRNA (catalog ID number 116912) was purchased from Ambion. EP4 siRNA (catalog number M-005714-00) and control nonspecific siRNA oligonucleotides (catalog number D-001206-13-05) were purchased from Dharmacon, Inc. (Lafayette, CO), as described previously (15). For the transfection of ERK and AP-2 ODN, cells were grown to 70% confluence, and a 1 μM concentration of ERK and 4 μM AP-2 phosphorothioate ODN mixed with FuGENE 6 transfection reagent per well of serum-free medium was added to the cells for 24 h at 37 °C. COX-2 and EP4 siRNA or control siRNA were transfected using the oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Briefly, oligofectamine reagent was incubated with serum-free medium for 10 min. Subsequently, a mixture of respective antisense or sense ODN or 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 nM. After culturing for 30 h, cells were washed and resuspended in new culture medium in the presence

or absence of Fn for an additional 24 h for Western blot analysis, cell growth, and gel mobility shift assays.

Western Blot Analysis—The procedure was performed as previously described (16). 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 (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 5–10% 2-mercaptoethanol, and 0.004% bromphenol blue) and separated on SDS-8–10% polyacrylamide gels. The separated proteins were transferred onto nitrocellulose using a Bio-Rad Trans Blot semidry transfer apparatus for 1 h at 25 V, blocked with Blotto (1× TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl)) with or without 5% bovine serum albumin, 5% nonfat dry milk, and 0.1% Tween 20 overnight at 4 °C, and washed twice for 5 min with wash buffer (1× TBS and 0.1% Tween 20). Blots were incubated with polyclonal antibodies against COX-2, EP1, EP2, EP3, EP4, Akt, ERK1, ERK2, and their phosphorylated forms and for AP-2, AP-2α, AP-2β, and AP-2δ (1:1000) overnight at 37 °C, washed three times for 5 min with wash buffer, and incubated with a secondary antibody raised against rabbit IgG conjugated to horseradish peroxidase (1:1000; Sigma) for 1 h at room temperature. The blots were washed four times in wash buffer, transferred to freshly made ECL solution (Amersham Biosciences) for 1 min, and exposed to x-ray film. Protein bands were quantified by densitometric scanning using a Bio GS-800 calibrated densitometer. In controls, antibo omitted or replaced by serum IgG.

Cell Viability Assay—NSCLC cells (1 transfected with EP4 siRNA for 30 h b Fn (20 μg/ml)-coated culture pla additional 48 h in 96-well pla cells in culture were d nescent cell viability as ATP, an indicator of me manufacturer's instructio

[methyl-^{3}H]Thymidine I was described previously (1 were transfected with EP4 or c AP-2 antisense or sense ODN fo μCi/ml [*methyl*-^{3}H]thymidine (sp Amersham Biosciences) and expo (20 μg/ml)-coated culture plates or dmP an additional 24 h. Medium was removed, a ned cells were washed with 1× PBS. Afterward, the attached cells were treated with ice-cold 6% trichloroacetic acid at 4 °C for 20 min and washed once with 6% trichloroacetic acid. Cells were solubilized with 0.1 N NaOH and counted in a liquid scintillation counter in 4 ml of scintillation fluid.

Site-directed Mutagenesis—To prepare site-directed mutants of the promoter, the following oligonucleotides were synthesized: mutated AP-2 (−1529 bp), 5′-GGTTTTAATTG-CCCttTGGTGTTTTCCGATC; mutated AP-2 (−1133 bp), 5′-GCTCGCCTTCCTCCttGCCTCCGCTTTGG; mutated AP-2 (−1000 bp), 5′-GCCTCTGCCAAGTCttACCCGGAGCTC-TCG. The lowercase letters indicate mutation, and the underlined letters indicate the AP-2 binding site. The EP_4 plasmid constructs containing site-directed mutations of AP-2 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 *EP4* promoter plasmid was alkaline-denatured, precipitated, washed, and resuspended in Tris-EDTA buffer. Mutated AP-2 oligonucleotides and selection oligonucleotides were annealed; mutant strands were synthesized, ligated, and transformed into BMH 71-18 *mut*S competent cells. The mutated AP-2 EP_4 plasmid was 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 *EP4* wild-type and deletion promoter constructs (pGlep4-1 to -5) ligated to the luciferase reporter gene have been reported previously (18). The EP_4 promoter construct contains ~4200 bp of the 5′-flanking region of the mouse *EP4* receptor gene connected to the pGL3 basic luciferase reporter vector (Promega). NSCLC cells were seeded at a density of 5×10^5 cells/well in 6-well dishes and grown to 50–60% nce. For each well, 2 μg of the above plasmid D nd 0.2 μg of the internal control phRL-T ciferase reporter vector were cotran UGENE 6 lipofection rea- g ribed in our earlier work treated with or without) for 1 h before expos- μg/ml each)-coated cts and measure- t using the dual dations by the uciferase activ- ormed sequentially luminometer equipped irefly luciferase activity were normalization with changes in ity within the same sample.

Mobility Shift Assay—Electrophoretic mobil- y experiments were performed as described before e oligonucleotides used as probes were as follows: wild pe Sp1 (5′-CTCCCCGCCCAAGCCTGG-3′), mutant Sp1 (5′-CTCCCttCCCAAGCCTGG-3′), wild type C/EBP (5′-GATAATTAAGAAATGAT-3′), mutant C/EBP (5′-GATcc-TTAAGAAATGA-3′); wild-type AP-2 (5′-TCCTCCCCGCC-TCCGC-3′), and mutant AP-2 (5′-TCCTCtttGCCTCCGC-3′), which is based on the *EP4* promoter sequences (18) and consensus AP-2α binding motif (5′-GATCGAACTGACCGC-CCGCGGCCCGT-3′). The complementary oligonucleotides were annealed and purified following the manufacturer's protocol. The Sp1, C/EBP, 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 mM HEPES, 10 mM Tris-HCl (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 12% (v/v) glycerol, and 2 μg of poly(dI-dC)) for 10 min, followed by the addition of [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 AP-2 antibodies (2 μg/μl). For cold competition, a 100-fold excess of the respective unlabeled consensus oligonucleotides was incubated for 15 min before adding the

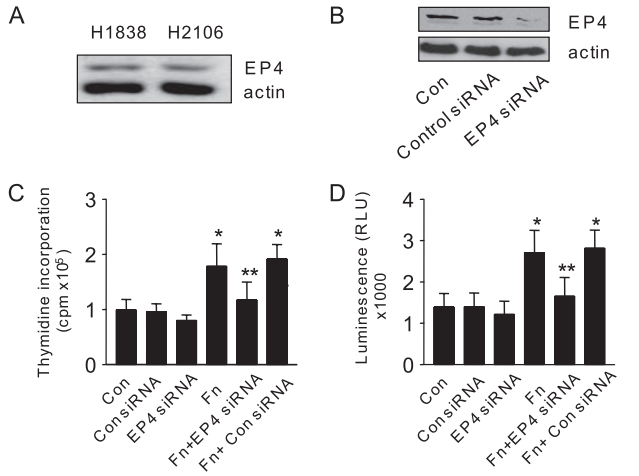


FIGURE 1. **EP4 siRNA blocks Fn-induced lung carcinoma cell growth.** *A*, expression of EP4 in NSCLC cells. Cellular proteins were isolated from H1838 and H2106 cells, followed by Western blot analysis for EP4 protein. *B*, blockade of EP4 production by EP4 siRNA. Cellular protein was isolated from H1838 cells transfected with control or EP4 siRNA pool (100 nM each) for 30 h and then subjected to Western blot analysis for EP4 protein. C, effect of EP4 siRNA on Fn-induced NSCLC cell growth. H1838 cells were transfected with control or EP4 siRNA pool for 30 h before exposing the cells to culture plates coated with Fn (20 μg/ml) and incubation with 1 μCi/ml [*methyl*-^{3}H]thymidine for an additional 24 h as indicated. Data are expressed as mean ± S.D. of at least three independent experiments. *D*, effect of EP4 siRNA on Fn-induced NSCLC cell growth. H1838 cells were transfected with contr... or EP4 siRNA pool for 30 h before exposing the cells to culture plates c... with Fn (20 μg/ml) for an additional 48 h, Afterward, viable cell nu... determined by the CellTiter-Glo Luminescent cell viability as... presented as means ± S.D. *, significant difference from... cance of combination treatment as compared with F... indicates untreated control cells.

probe. The same amount... with the probe was us... the same binding con... the protein-DNA compl... 4.5% polyacrylamide gel a... (10× Tris/glycine: Tris-bas... (3.92 g), and H_2O added up to... dried and subjected to autorad...

Statistical Analysis—All exper... mum of three times. All data from... assays, luciferase activity assays, RT-... PCR, and Western blot analysis were expre... S.D. The data presented in some figures are from... sentative experiment, which was qualitatively similar in... replicate experiments. Statistical significance was determined with Student's *t* test (two-tailed) comparison between two groups of data sets. The *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

Effect of Fn on EP4 Gene Expression in Human Lung Carcinoma Cells—We previously showed that mRNAs encoding for the four PGE_2 receptor subtypes are present in human NSCLC cells (17). Consistent with this, we show that the PGE_2 receptor subtype EP4 protein is expressed in the two NSCLC cell lines studied (Fig. 1*A*). We also demonstrated that Fn stimulated NSCLC cell growth (4). Here, we examined whether blockade of EP4 could influence the effects of Fn on cell growth. We first depleted EP4 from cells in culture using siRNA approaches. Treatment of H1838 cells with EP4 siRNA blocked EP4 production. Levels of EP4 were unchanged in cells transfected with control siRNA oligonucleotides (Fig. 1*B*). To determine if EP4 siRNA can block Fn-induced lung carcinoma cell growth in our system, H1838 cells were transfected with EP4 siRNA duplexes. Afterward, the cells were plated onto Fn-coated culture plates for an additional 24 h. As shown in Fig. 1*C*, the EP4 siRNA duplexes inhibited Fn-induced H1838 cell proliferation, whereas the control siRNA had no effects as determined by the [^{3}H]thymidine incorporation assay. Similar results were also found by cell viability assays (Fig. 1*D*).

Since EP4 has been shown to be involved in human lung carcinoma biology, we tested if Fn can affect its expression. H1838 cells exposed to Fn showed increased EP4 protein levels in a time- and dose-dependent manner with maximal increases noted in 24 h at concentrations of 20 μg/ml (Fig. 2, *A* and *C*). Similar results were al... in an additional NSCLC cell line (H2106) (Fig. 1, *B*... o significantly stimulated EP4 mRNA leve... ent manner, with maximal increas... of 20 μg/ml Fn as deter-mi... collagen type 1, another ...g. 2*F*). This result was ...Fig. 2*G*). Of note, cells ... plates adhered well ...). In order to deter-... P4, we examined ...ypes. We found ...t on other EP ...or did silencing *EP4* ...n these EP receptors (Fig. ... with H2106 cells (not shown).

... and COX-2, but Not PKC and PKA ...n Fn-induced EP4 Expression—We then ... effect of Fn was mediated by its integrin ...β1. To this end, we treated H1838 cells with or ... anti-integrin α5β1 (MAB1969) or anti-integrin α2β1 ...ibodies (MAB1967; 25 μg/ml each) for 2 h before exposing the cells to culture plates coated with Fn. We found that Fn-induced EP4 protein was eliminated in the presence of anti-α5β1 antibodies, whereas the anti-α2β1 antibodies had no effect (Fig. 3*A*). This suggests that α5β1 integrin mediates Fn-induced EP4 expression.

Fn has been shown to affect kinase signaling pathways in several studies (21–23). We previously demonstrated that Fn activated ERK and PI3K/Akt signaling pathways in NSCLC cells (4, 23, 24). Here, we examined if inhibition of these kinase signal pathways diminished or abrogated the effects of Fn on EP4. The specific inhibitors of ERK (PD98095 (25 μM)) and of PI3K (wortmannin (100 nM)) significantly blocked Fn-induced EP4 protein levels in H1838 cells (Fig. 3*B*). We previously demonstrated that mTOR signals were mediating some of the effects of Fn on NSCLC cell growth (23). However, we found that rapamycin, an inhibitor of mTOR, had no effect on inhibition of Fn-induced expression of EP4 protein (Fig. 3*C*). Also, the inhibitor of PKA (H89 (10 μM)) or of PKC (calphostin C (*Cal*; 0.5 μM)) had no effects (Fig. 3, *D* and *E*). In addition, we showed

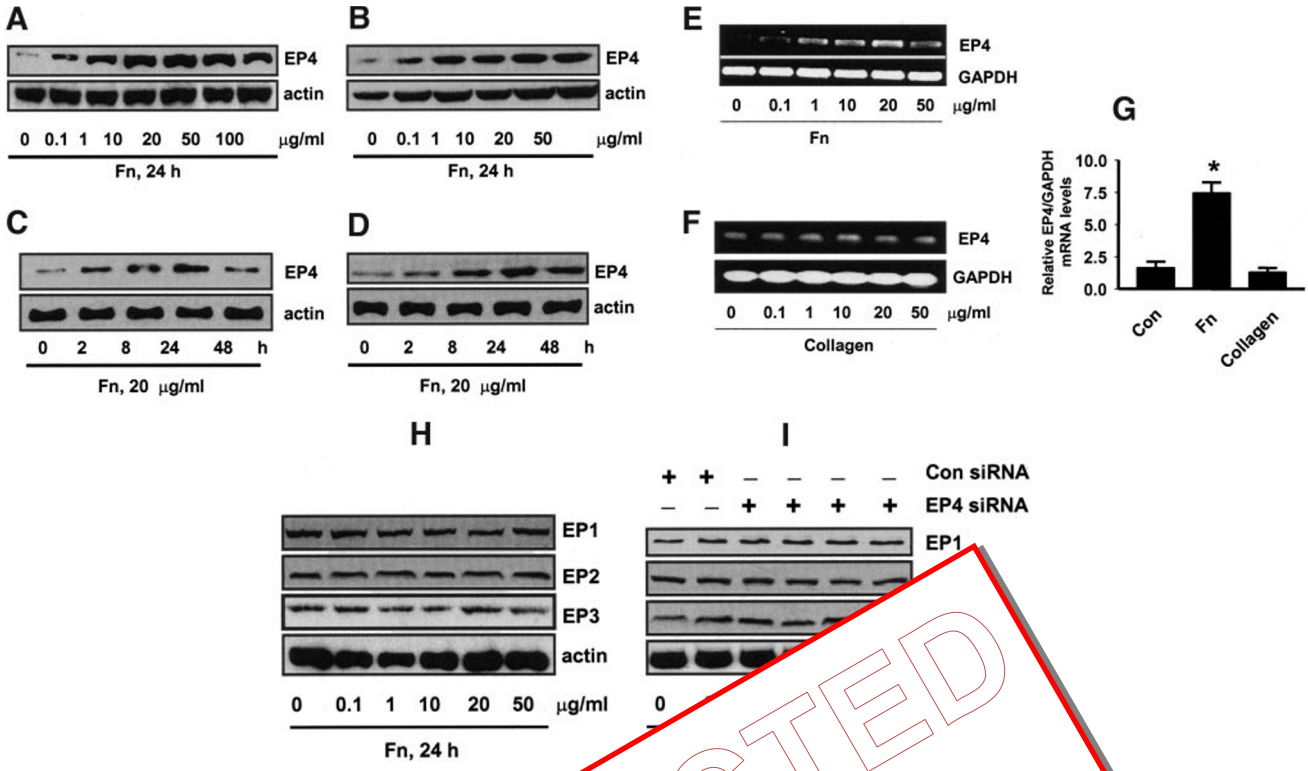


FIGURE 2. **The effect of Fn on *EP4* gene expression in human lung** pression. Cellular proteins were isolated from H1838 (*A*) and H2106 (*B*) cells cultured on pl by Western blot for EP4. *C* and *D*, time-dependent effect of Fn on EP4 expression. *C* Fn (20 μg/ml)-coated plates for the indicated time period. Afterward, Weste malization purposes. *E* and *F*, dose-dependent effect of Fn and collage ated with increased concentrations of Fn or collagen and then s f Fn on EP4 mRNA expression as determined by real time R type 1 (20 μg/ml each), followed by real time PCR an ontrol ($p < 0.05$). *Con*, indicates untreated control cell in H1838 cells cultured on plates coated with increas time-dependent effect of Fn on EP1, EP2, and EP3 expressi or 30 h before exposing the cells to culture plates coated with F ct EP1, EP2, and EP3 protein levels. Actin served as internal control for

that the inhibitor of PI3K, w hways were involved in EP4 transcriptional on the phosphorylation of E y Fn and found that, as expected, the inhibitors of of ERK, PD98095, had no effe RK or PI3K partially reduced Fn-induced *EP4* promoter tion of Akt (Fig. 3*G*). This sugge tivities (Fig. 4*C*). Similar results were obtained with H2106 the PI3K/Akt pathway. Similar cells (not shown).

Fn Increased EP4 Promoter Activ examined whether the effects of Fn on EP4 expre ur at the transcriptional level. The *EP4* promoter co tains multiple transcription factor binding sites, including NF-κB, NF-IL6 (C/EBP), Sp1, and AP-2, among others (Fig. 4*A*). These sites have been shown to be differentially responsive to various stimuli (15, 18, 25, 26). We found that H1838 cells, transfected with the full-length wild-type *EP4* promoter (−4200/−116 bp) luciferase reporter construct, exposed to Fn showed increased promoter activity. Collagen type 1 had no effect on the wild-type promoter (Fig. 4*B*). The Fn-induced *EP4* promoter activity was slightly reduced in one EP4 deletion reporter construct (−1555/−116 bp). There was no response to Fn with another EP4 deletion reporter construct (−992/−116 bp) (Fig. 4*B*), indicating that the region between −1555 and −992 bp in the *EP4* promoter played an important role in stimulation of *EP4* gene expression in response to Fn. We also tested whether

Regulation of AP-2, Sp1, and C/EBP in the EP4 Promoter by Fn in Human Lung Carcinoma Cells—To further explore the role of Fn in regulation of *EP4* promoter activity, electrophoretic mobility shift assays were performed to identify the transcription factors involved. We found that H1838 cells treated with Fn for 24 h showed a significant increase in AP-2 (Fig. 5*C*), but we observed no effect in C/EBP (Fig. 5*A*) and a slight increase in Sp1 (Fig. 5*B*) nuclear protein binding activities as compared with solvent controls. In contrast, collagen type 1 (20 μg/ml) had no effect. We also tested if the activation of ERK signals by Fn was involved in the induction of AP-2 binding activity. ERK1/2 antisense oligonucleotide (1 μM) completely blocked ERK1 and reduced ERK2 production. The levels of ERK1, ERK2, and actin were unaffected in untransfected cells and in cells treated with ERK sense oligonucleotides (Fig. 5*D*, *top*). The antisense ERK ODN prevented the Fn-induced AP-2 binding activity (Fig. 5*D*, *bottom*). Similarly, the inhibitors of ERK (PD98095) and of PI3K (wortmannin) also blocked Fn-

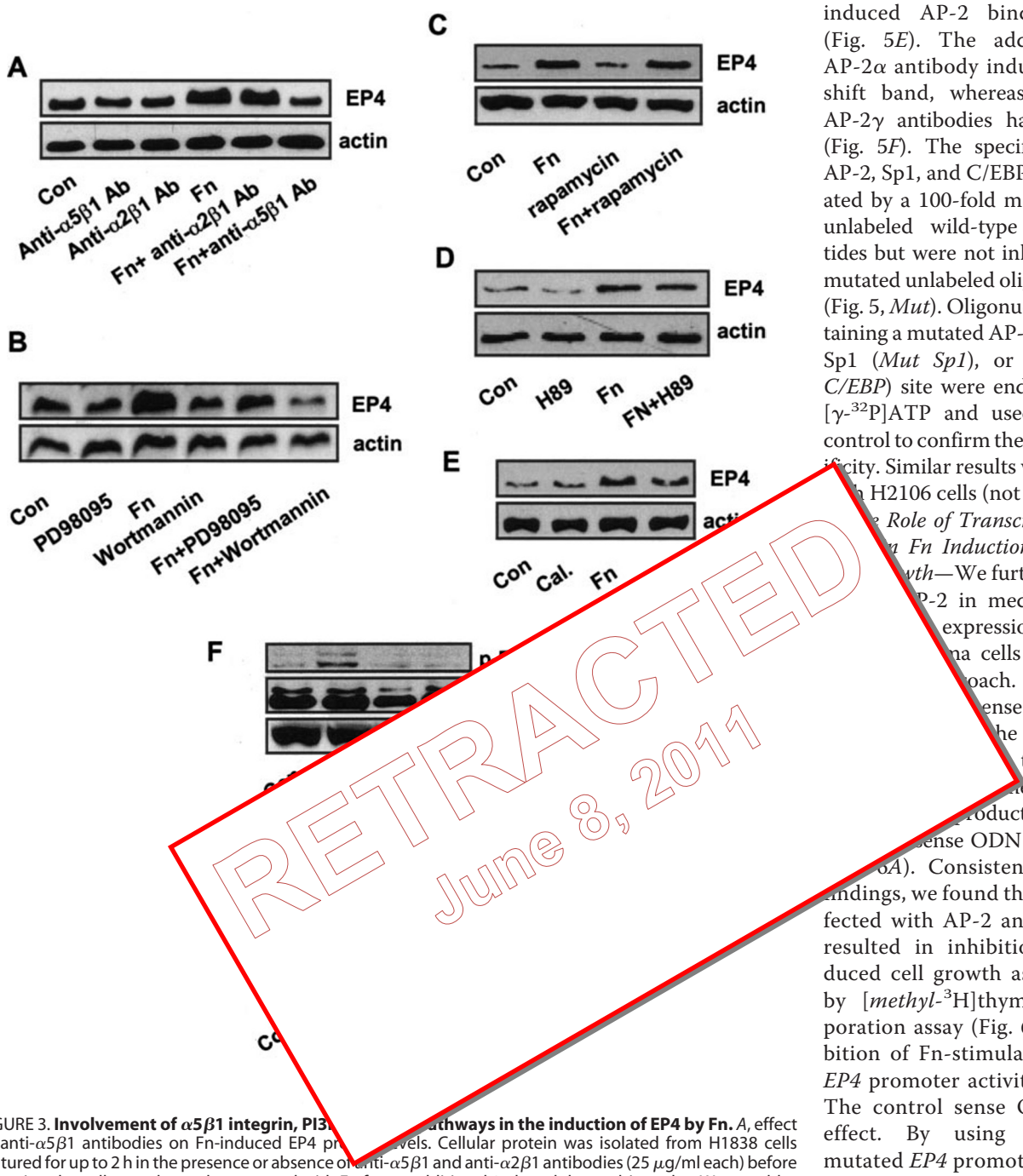


FIGURE 3. **Involvement of $\alpha5\beta1$ integrin, PI3K, and ERK pathways in the induction of EP4 by Fn.** *A*, effect of anti-$\alpha5\beta1$ antibodies on Fn-induced EP4 protein levels. Cellular protein was isolated from H1838 cells cultured for up to 2 h in the presence or absence of anti-$\alpha5\beta1$ and anti-$\alpha2\beta1$ antibodies (25 μg/ml each) before exposing the cells to culture plates coated with Fn for an additional 24 h and then subjected to Western blot analysis for EP4. *B*, effect of inhibitors of ERK1/2 and PI3K on Fn-induced EP4 protein levels. Cellular protein was isolated from H1838 cells cultured for up to 2 h in the presence or absence of PD98095 (25 μM) or wortmannin (100 nM) before exposing the cells to Fn-coated culture plates for an additional 24 h and then subjected to Western blot analysis for EP4. *C*, effect of mTOR inhibitor on Fn-induced EP4 protein levels. Cellular protein was isolated from H1838 cells cultured for up to 4 h in the presence or absence of rapamycin (10 nM) before exposing the cells to Fn-coated culture plates for an additional 24 h and then subjected to Western blot analysis for EP4 protein. *D*, effect of PKA inhibitors on Fn-induced EP4 protein levels. Cellular protein was isolated from H1838 cells cultured for up to 2 h in the presence or absence of H89 (10 μM) before exposing the cells to Fn-coated culture plates for an additional 24 h and then subjected to Western blot analysis. *E*, effect of PKC inhibitors on Fn-induced EP4 protein levels. Cellular protein was isolated from H1838 cells cultured for up to 2 h in the presence or absence of calphostin C (0.5 μM) before exposing the cells to Fn-coated culture plates for an additional 24 h, then subjected to Western blot analysis. *F*, effect of PI3K inhibitor on ERK1/2. Cellular protein was isolated from H1838 cells cultured for 1h in the presence or absence of wortmannin (100 nM) before exposing the cells to Fn (20 μg/ml) coated onto the culture plates for an additional 1 h. Afterward, Western blot was performed to detect phosphorylated ERK1/2 and total ERK1 and ERK2 proteins. *G*, effect of ERK inhibitor on Akt. Cellular protein was isolated from H1838 cells cultured for 1 h in the presence or absence of PD98095 (25 μM) before exposing the cells to Fn (20 μg/ml) for an additional 1 h. Afterward, Western blot was performed to detect phosphorylated Akt and total Akt protein. Actin served as internal control for normalization purposes. *Con*, untreated control cells.

induced AP-2 binding activity (Fig. 5*E*). The addition of an AP-2α antibody induced a supershift band, whereas AP-2β and AP-2γ antibodies had no effects (Fig. 5*F*). The specific bands for AP-2, Sp1, and C/EBP were attenuated by a 100-fold molar excess of unlabeled wild-type oligonucleotides but were not inhibited by the mutated unlabeled oligonucleotides (Fig. 5, *Mut*). Oligonucleotides containing a mutated AP-2 (*Mut AP-2*), Sp1 (*Mut Sp1*), or C/EBP (*Mut C/EBP*) site were end-labeled with [γ-^{32}P]ATP and used as another control to confirm the binding specificity. Similar results were obtained [illegible] H2106 cells (not shown).

[illegible] Role of Transcription Factor [illegible] Fn Induction of EP4 and [illegible]wth—We further tested the [illegible]P-2 in mediating Fn-in[illegible] expression in human [illegible]na cells by using the [illegible]oach. We showed [illegible]ense ODN com[illegible]he production of [illegible] this antisense [illegible]e Fn-induced [illegible]roduction, whereas [illegible]sense ODN had no effect [illegible]6*A*). Consistent with these findings, we found that cells transfected with AP-2 antisense ODN resulted in inhibition of Fn-induced cell growth as determined by [*methyl*-^{3}H]thymidine incorporation assay (Fig. 6*B*) and inhibition of Fn-stimulated effect on *EP4* promoter activities (Fig. 6*C*). The control sense ODN had no effect. By using site-directed mutated *EP4* promoter constructs in which each of three AP-2 binding sites were mutated (−1529, −1133, and −1000 bp), we found that the stimulatory effect of Fn on *EP4* promoter activity was lost with *EP4* promoter constructs in which one AP-2 site was mutated (−1000 bp) (Fig. 6*D*).

PI3K, ERK, and α5β1 Integrin Signaling Are Involved in Fn-induced AP-2 Protein Expression—We also examined the effects of Fn on AP-2 protein expression, and, consistent with the gel shift experiment results,

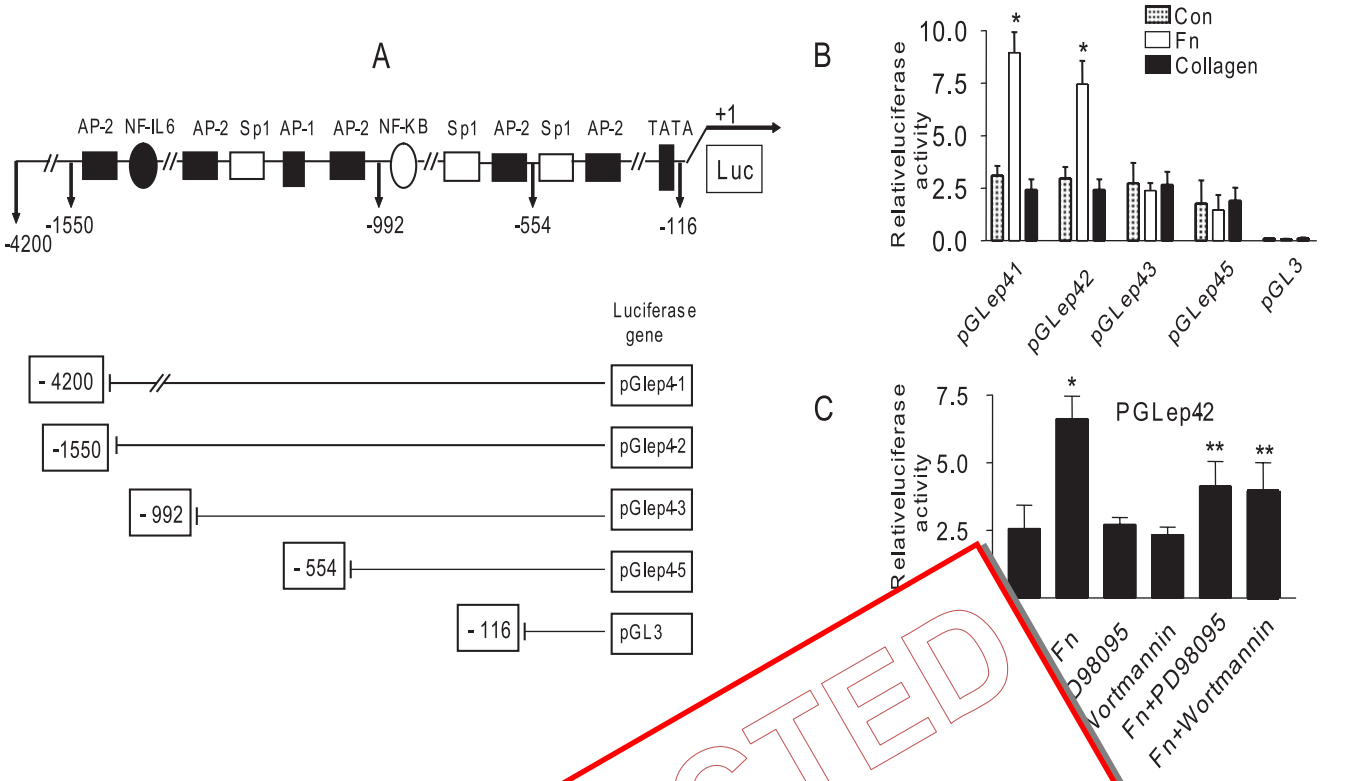


FIGURE 4. **Fn stimulates *EP4* promoter activity.** *A*, promoter ma… schematics are presented. These regions contain several tra… promoter activity. H1838 lung cancer cells (1 × 10^5 cells/… shown in *A* ligated to the luciferase reporter gene an… Procedures" for 24 h and then treated as indicat… 24 h. *C*, effects of inhibitors of ERK1/2 or PI… treated with PD98095 (25 μM) and wor… ratio of firefly luciferase to *Renilla*… least four independent experi… as compared with Fn alon… eted promoter constructs …6. *B*, effects of Fn on *EP4* …er reporter constructs …under "Experimental …es for an additional … for 24 h and then …additional 24 h. The …n the mean ± S.D. of at …e of combination treatment

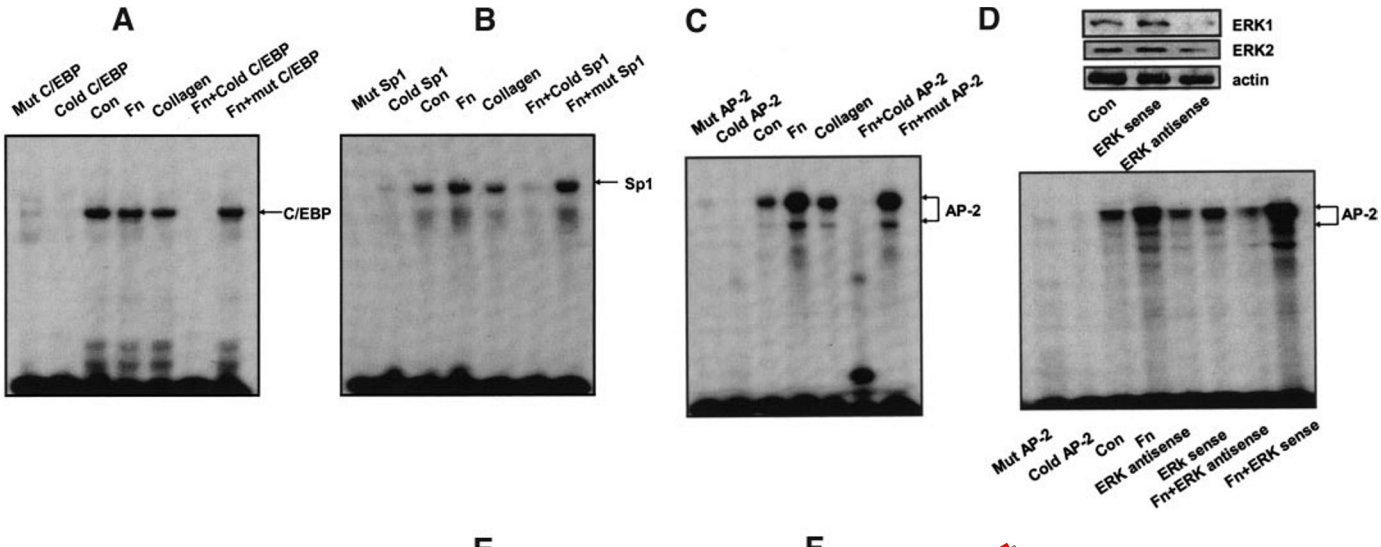
we found that Fn induc… and AP-2δ protein produ… of ERK (PD98095 (Fig. 7*C*)) … and the anti-$\alpha5\beta1$ antibodi… AP-2α protein expression. T… effect. Similar results were ob… shown).

COX-2 Signaling Is Involved in … EP4 Protein Expression—Having establis… stimulates NSCLC cell proliferation through CO… d via EP4 (this study), we explored whether COX-2 sig…als could also affect EP4 expression. We found that COX-2 siRNA blocks endogenous COX-2 protein production; no changes were noted in cells transfected with control siRNA (Fig. 8*A*). COX-2 siRNA significantly abrogated Fn-induced EP4 expression; the control siRNA had no effect (Fig. 8*A*). We also found that, under the same conditions, COX-2 siRNA abrogated the effect of Fn on AP-2α protein expression (Fig. 8*B*), whereas the control siRNA had no effect. These studies suggest that COX-2 activation not only promotes PGE_2 secretion but may also amplify its effects by stimulating EP4 expression. Therefore, we tested whether PGE_2 can stimulate EP4 expression. We observed that $dmPGE_2$ (0.1 μM) also enhanced AP-2α and EP4 protein expression, with greater stimulation seen when Fn and $dmPGE_2$ were used concomitantly (Fig. 8, *C* and *D*). Finally, similar to the effect of Fn

…1, *C* and *D*, the stimulatory effect of … cell proliferation was diminished in the … siRNA, whereas the control siRNA had no …ermined by the [^{3}H]thymidine incorporation assay …*E*) and by cell viability assays (Fig. 8*F*). Similar results …ere obtained with H2106 cells (not shown).

DISCUSSION

Fn is a heterodimeric extracellular matrix glycoprotein implicated in a number of physiological events during embryogenesis, angiogenesis, thrombosis, and inflammation (27–29). Fn expression is increased in lung carcinomas, particularly in non-small cell lung carcinoma (3, 29–31). Also, the adhesion of lung carcinoma cells to Fn enhances tumorigenicity and confers resistance to apoptosis induced by standard chemotherapeutic agents (32). Previously, we found that Fn stimulates human lung carcinoma cell growth *in vitro* by increasing expression of COX-2 and PGE_2 biosynthesis (4). We also demonstrated that all four PGE_2 receptors are expressed in NSCLC cells studied (17). Based on these data, we predicted that Fn stimulated NSCLC growth through one or more of these four EP receptors capable of recognizing PGE_2. The development of aberrant crypt foci and putative preneoplastic lesions in the colon was decreased in the EP4 knock-out mice (7). Blockade of EP4 production also mediated inhibition of NSCLC cell inva-



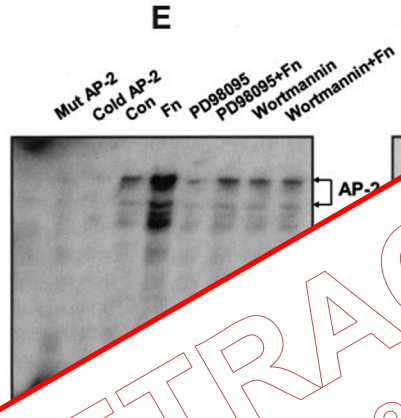


FIGURE 5. **Electrophoretic … romoter in response to Fn.** *A–C*, effect of Fn on C/EB… 2 (*C*) sites were end-labeled with [γ-^{32}P]ATP and incubated … each) for an additional 24 h. *D*, ERK … cted with control oligonucleotide or ERK antisense oligonucleotide (1 μ… protein. *Bottom*, oligonucleotides containing AP-2 sites were end-labeled wi… ells transfected with 1 μM ERK antisense or sense oligonucleotide for 24 h before ex… onal 24 h. *E*, the inhibitors of ERK1/2 and PI3K abrogated the Fn-induced AP-2 binding activi… with [γ-^{32}P]ATP and incubated with nuclear extracts (5 μg) from H1838 cells treated with PD98… posing the cells to culture plates coated with Fn (20 μg/ml) for an additional 24 h. *F*, anti-AP-2α antibo… sites were end-labeled with [γ-^{32}P]ATP and incubated with nuclear extracts (5 μg) and AP-2α, -β, and -δ anti… on assays, a molar excess (100×) of consensus Sp1 (*Cold Sp1*) or C/EBP (*Cold C/EBP*) or AP-2 (*Cold AP-2*) oligonucleotid… Oligonucleotides containing a mutated Sp1 (*Mut Sp1*) or C/EBP (*Mut C/EBP*) or AP-2 (*Mut AP-2*) site that were end-labeled… onfirm the binding specificity. *Con*, untreated control cells.

siveness (10). Here, we reported tha… y induced the expression of EP4, whereas it had no effe… on other EP receptors. A similar lack of changes in the other EP receptors was observed when EP4 was knocked down by EP4 siRNA. This finding indicates that Fn selectively targets EP4 receptor subtypes in NSCLC cells. In view of the above, we focused on EP4 and explored the mechanisms involved.

First, we demonstrated that EP4 siRNA antagonized Fn-induced lung carcinoma cell growth, suggesting a direct role for EP4 in mediating this process in our system. This is consistent with data from others who reported that EP4 antisense oligonucleotides diminished EP4 protein expression and abolished the PGE_2-stimulated production of cAMP and blocked the ability of PGE_2 to augment release of immunoreactive substance P and calcitonin gene-related peptide in sensory neurons (33). The EP4 antagonist, ONO-AE3-208, and the EP4 siRNA have been shown to inhibit extracellular matrix-induced metalloproteinase-9 expression in macrophages (34). More recently, EP4 antagonists inhibited breast cancer cell growth and reduced breast, lung, and colon cancer metastasis, suggesting that blockade of EP4 may be an alternative approach to the use of COX-2 inhibitors to prevent tumor metastasis (35, 36).

In this study, we confirm that Fn increased *EP4* gene expression in NSCLC cells, whereas collagen type 1 had no effect. This suggested that Fn may induce NSCLC growth not only by stimulation of PGE_2 but may also enhance this process by inducing the expression of EP4. The connection of Fn and EP4 expression has never been reported in lung cancer cells, although an antagonist of the PGE_2 receptor EP1 has been shown to decrease Fn synthesis in a rat diabetic nephropathy model (37). Also, PGE_2-accelerated ProNectin F(TM) (a proteolytic fragment of Fn)-dependent adhesion was mediated through coop-

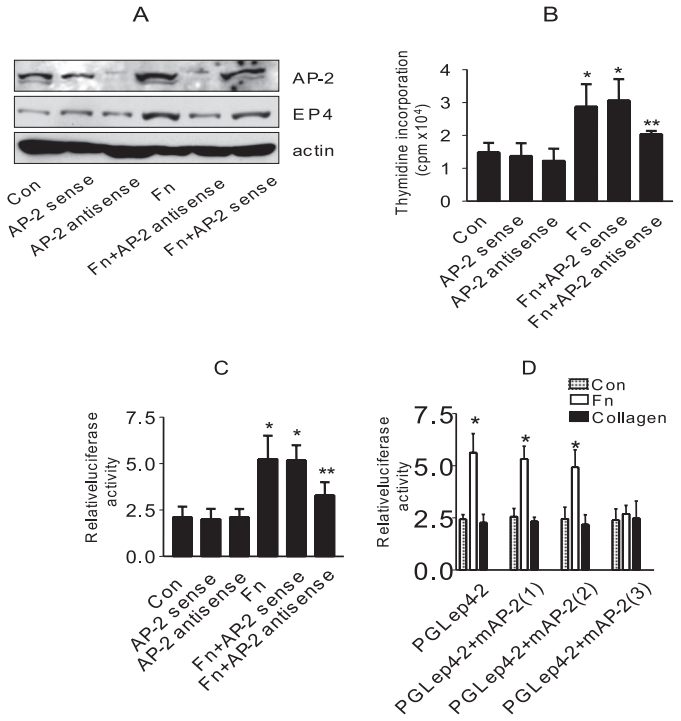


FIGURE 6. **The role of transcription factor AP-2 in Fn induction of EP4.** *A*, AP-2 antisense blocked Fn-induced EP4 protein expression. Cellular protein was isolated from H1838 cells transfected with AP-2 antisense ODN (4 μM) for 30 h before exposing the cells to the plate Fn for an additional 24 h. Afterward, Western blot analysis examine for AP-2 and EP4 protein. *B*, AP-2 antisense oli Fn-induced cell proliferation. H1838 cells transfe antisense ODN (4 μM) for 30 h before exposin with 20 μg/ml Fn and incubation with 1 additional 24 h. The *bars* represent t ent experiments for each condi moter activity. H1838 cell −116) together with the A then the cells were exposed an additional 24 h. *D*, effects moter activities. H1838 cells w structs (−1529, −1133, and −10 then treated with 20 μg/ml Fn or C of firefly luciferase to *Renilla* lucife under "Experimental Procedures." T least four independent experiments compared with controls. **, significanc pared with Fn alone ($p < 0.05$). *Con*, untre

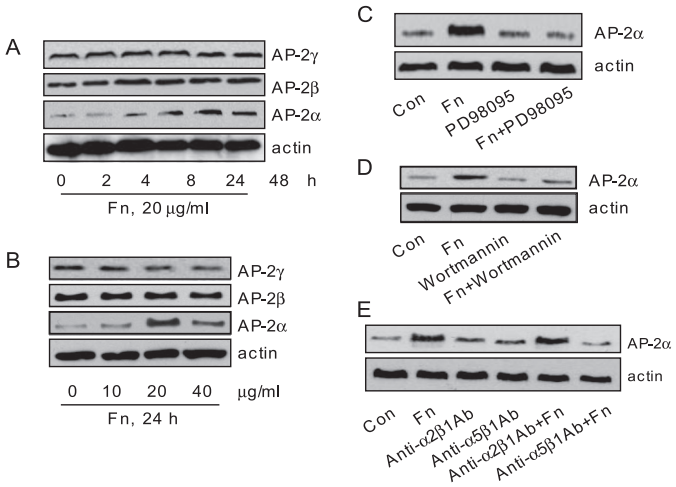


FIGURE 7. **The role of PI3K, ERK, and $\alpha5\beta1$ integrin in Fn-induced AP-2α expression.** *A*, time-dependent effect of Fn on AP-2 protein expression. Cellular protein was isolated from H1838 cells exposed to culture plates coated with Fn (20 μg/ml) at the i time periods. Afterward, Western blot analysis was performed nal antibodies against AP-2α, AP-2β, and AP-2δ. *B*, dose- Fn on AP-2 protein expression. Cellular proteins we lls exposed to different concentrations of Fn lot analysis was performed using polycl and AP-2δ. *C*, effect of ERK inhibit Cellular protein was isolated) for 2 h before exposing the ml) for an additional 24 h. rotein expression. Cellular wortmannin (0.1 μM) for with Fn (20 μg/ml) for an t of Fn on AP-2α pro- 8 cells treated with efore exposing the tional 24 h. After- P-2α protein levels. purposes. *Con*, untreated

erative activation of EP3 and EP4 in m oma P-815 cells (38).

We previously demonstrated that Fn stimulated lung carcinoma cell growth through its receptor $\alpha5\beta1$, since anti-$\alpha5\beta1$ antibodies eliminated the mitogenic response (4). Here, the Fn effect on EP4 protein levels was blocked by anti-$\alpha5\beta1$ antibodies but not by anti-$\alpha2\beta1$ antibodies, indicating that the integrin receptor $\alpha5\beta1$ mediated this regulation. In order to elucidate the mechanism(s) involved in Fn induction of EP4, we attempted to delineate the signaling pathways involved in induction of EP4 expression in lung carcinoma cells in response to Fn treatment. Data from our laboratory (4, 21, 24, 39, 40) and data of others (3, 41) have demonstrated that adhesion to Fn activates several kinase signaling pathways, including ERK, PI3K, PKC, and PKA. We found that inhibitors of the PI3K kinase and ERK prevented Fn-induced EP4 protein expression, suggesting that the activation of these dual kinase signaling

-induced EP4 expression. We pre- hat Fn up-regulated COX-2 expression of the ERK signal pathway and that blockade abrogated Fn-induced COX-2 expression (4). ogether suggested a strong connection between COX-2, 4, and ERK signals. However, ERK played no role in the increase of EP4 expression induced by peroxisome proliferator-activated receptor β/δ activation (15), suggesting the existence of independent pathways that differ according to the stimulus. Fn activates MMP-9 via the ERK and PI3K/Akt signaling pathways in NSCLC and ovarian cancer cells (24, 42, 43). The inhibitor of PI3K, wortmannin, blocked the effect of Fn on stimulation of ERK phosphorylation, indicating cross-talk between the PI3K and ERK1/2 pathways in NSCLC cells. The cross-talk between these kinases has been reported in other cell systems as well (44, 45). In contrast, our data indicated that mTOR, PKC, and PKA signaling pathways are not involved in the up-regulation of the *EP4* gene induced by Fn, although the latter two kinases were involved in prostaglandin E receptor expression in other studies (46, 47). This might suggest distinct effects, depending on the stimulant and cells studied.

EP4 has been shown to be regulated at the level of gene transcription in different cell types (21, 47). We found that Fn, not collagen type 1, increased *EP4* promoter activity. Furthermore, the region between −1555 and −992 was demonstrated to play

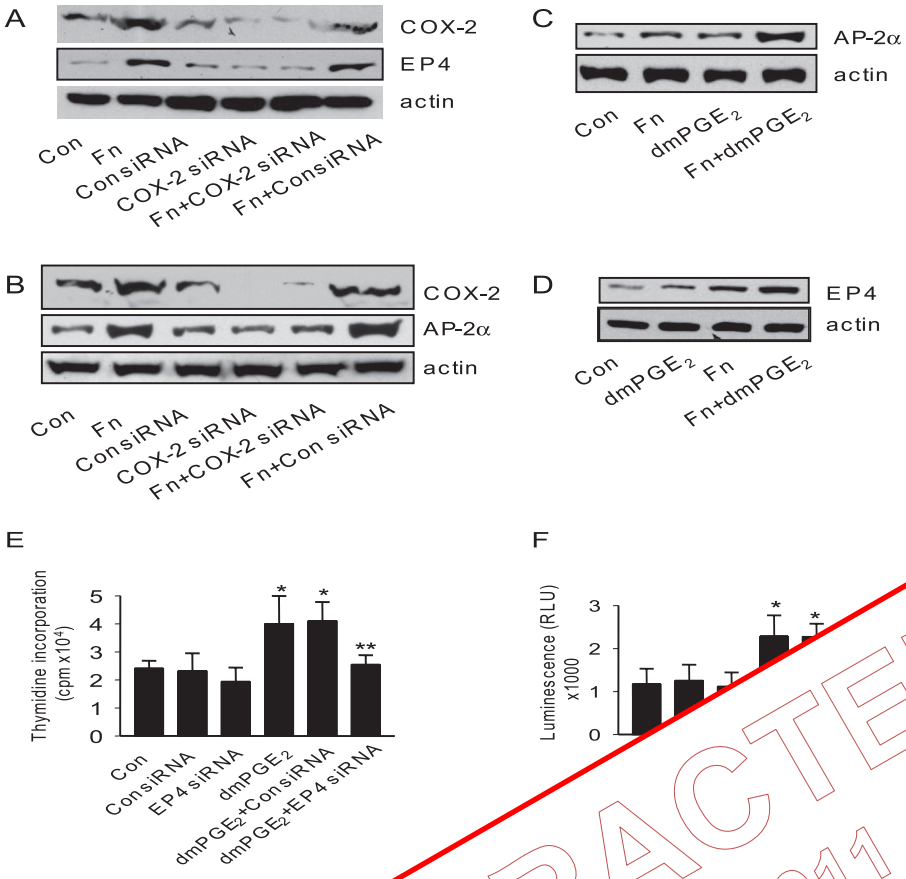


FIGURE 8. **The role of COX-2 signaling in me… and inducing lung carcinoma cell gro…** expression. Cellular protein was isol… each) for 30 h before exposing … subjected to Western blot a… Fn-induced AP-2α protein… or COX-2 siRNA (100 nM eac… an additional 24 h and then s… *C*, effect of PGE_2 on AP-2α pro… without $dmPGE_2$ (1 μM) for 4 h … additional 24 h. Afterward, Weste… PGE_2 on EP4 protein expression. Ce… (1 μM) for 4 h before exposing the … Afterward, Western blot analysis was p… normalization purposes. *E* and *F*, effect… *E*, H1838 cells were transfected with cont… μM) and incubation with 1 μCi/ml [methyl-… as mean ± S.D. of at least three independen… siRNA pool for 30 h before exposing the cells … for an additional 48 h, Afterward, viable cell numbers were determined by the CellTiter-G… cell viability assay. All data are presented as means ± S.D. *, significant difference from contro… gnificance of combination treatment as compared with $dmPGE_2$ alone ($p < 0.05$). *Con*, untreated control cells.

binding sites in this region. By mutations of each of these sites, we found that only one site (−1000 bp) was involved in the Fn-induced *EP4* promoter activity. To our knowledge, a role for the AP-2 site in regulation of EP4 expression has never been reported. The transcription factor AP-2 regulates genes involved in a spectrum of important biological functions. Data obtained from different experimental models *in vitro* and *in vivo* indicate that AP-2 proteins function as important regulators of c-Myc targets in cell cycle progression and apoptosis (48). AP-2α overexpression leads to …chorage-independent growth and …ignant transformation *in vitro* …t has therefore been suggested …P-2 is involved in malignant …mation of breast cancer …unohistochemical anal… …P-2α- and AP-2γ-spe… confirmed up-regu… proteins in human …(50). Other data …P-2α might be …roliferation by …inducing ter… …iation, apoptosis, …wth retardation (51). …are a few studies that explore the transcriptional regulation of EP4 and transcription factor interactions in its promoter region. Studies show that a GC-rich/Sp1 binding site located within the first 80 bases of the transcription start site in the *EP4* promoter region is important in transcription initiation of the *EP4* gene (24), and several negative, positive and lipopolysaccharide/serum-responsive regions are located at different areas in the mouse *EP4* promoter (16). We confirmed that AP-2 sites were involved in Fn-induced *EP4* gene expression using mutated EP4 constructs.

a major role. The results showing that both PD98095 and wortmannin partially prevented Fn-stimulated *EP4* promoter activity further suggested a role for PI3K and ERK kinase signaling pathways in mediating Fn up-regulation of *EP4* gene expression. Several transcription factor binding sites within regions of the *EP4* promoter have been characterized, including regulatory elements for AP-2, C/EBP, Sp1, and others (15, 18, 25). We showed that treatment of H1838 cells with Fn significantly increased protein binding activities of AP-2 in the *EP4* promoter, whereas it had little effect on C/EBP and Sp1. This, together with the supershift assay results, indicated that AP-2α binding to the AP-2 site was necessary for the up-regulation of *EP4* gene transcription in response to Fn. There are three AP-2

Fn increased nuclear AP-2α protein levels, and inhibition of ERK signals prevented Fn-induced AP-2 expression. The connection between the AP-2 and ERK signal has been reported in other studies. For example, the ability of estradiol to increase AP-2 protein expression and AP-2 DNA binding activity was reversed by PD98059 (52). ERK activation is necessary for induction of the binding activities of AP-2 in T cells (53). Also, increased ERK signal has been reported in the absence of AP-2α in mouse epidermis (54). In addition, we found that blockade of AP-2 by AP-2 antisense approaches had no effect on ERK activ-

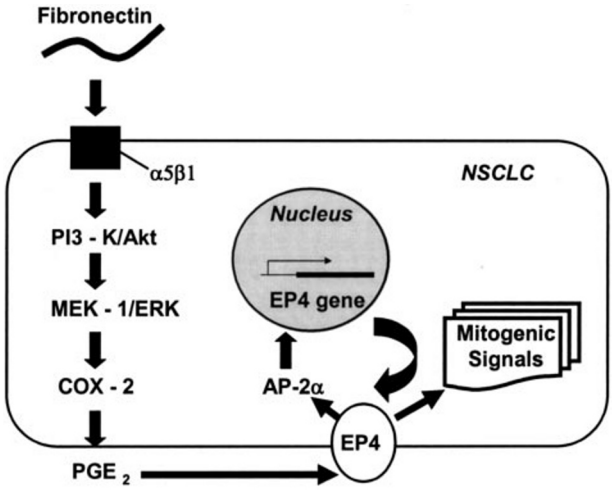


FIGURE 9. **Schematic representation of signal pathways triggered in NSCLC in response to Fn.** By binding to its $\alpha5\beta1$ integrin receptor, Fn stimulates PI3K/Akt, followed by the induction of MEK-1/ERK and COX-2 signal pathways. These events stimulate the production of PGE_2 and expression of the PGE_2 subtype EP4 receptor gene through activation of the transcription factor AP-2a. These two events, induction of PGE_2 and EP4, serve to amplify the mitogenic effects of Fn on lung carcinoma cells.

ity in the presence or absence of PGE_2 (not shown), suggesting that ERK was not a downstream signal of AP-2. Furthermore, we showed that inhibition of PI3K and blockade of $\alpha5\beta1$ integrin signals abrogated Fn-induced AP-2α protein e… indicating that multiple signals were involved in … showing that AP-2 antisense oligonucleoti… duced cell growth, our data suggest th… Fn is at least partly responsib… proliferation.

We previously dem… expression and stimula… siRNA abrogated Fn-in… indicating that COX-2 sig… expression. We showed tha… NSCLC cells (15). Since COX… production, the effect of Fn o… through stimulation of PGE_2 pro… literature contains data that link ex… signaling (55, 56). PGE_2 not only s… but also enhanced Fn-induced AP-2α pr… and cell proliferation, suggesting that stimulati… E_2 by Fn might account, at least partially, for the prod…tion of AP-2α that resulted in EP4 expression and, subsequently, increased cell proliferation. PGE_2 has been shown to enhance the binding activity of AP-2 in different gene promoters in several studies (57, 58). $dmPGE_2$ up-regulated expression of the receptor subtype EP4, suggesting that PGE_2 might combine with EP4 receptors in autocrine or paracrine modes. Thus, blockade of the *EP4* gene by siRNA approaches diminished the stimulatory effect of PGE_2 on lung carcinoma cell growth (this study). The dose of PGE_2 used in this study was based on our previous results showing Fn-induced PGE_2 production in NSCLC cells *in vitro* (4). 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 to reduce radiation-induced apoptosis in human colon cancer cells without toxicity (59, 60). Previously, we reported that PGE_2 enhanced NSCLC cell growth in the presence of the EP2 agonist, Butaprost, suggesting that EP2 also plays a role in mediating NSCLC cell growth (17). Whether EP2 is directly involved in the effect of PGE_2 on NSCLC cell proliferation and how this pathway is affected by Fn needs to be determined.

In summary, our studies show that Fn stimulates human lung carcinoma cell proliferation through the PGE_2 receptor subtype EP4. This effect is enhanced by Fn-induced EP4 expression. Control of *EP4* gene expression by Fn is dependent on $\alpha5\beta1$ integrin-mediated signals that include activation of PI3K/Akt, ERK, and COX-2. These signals stimulate PGE_2 production, which induces the transcription factor AP-2α and stimulates AP-2α interactions with critical DNA regions within the *EP4* gene promoter (−1555 to −992 bp). Thus, Fn, by increased PGE_2 production and *EP4* gene expression, induces mitogenic signals in NSCLC cells (Fig. 9). This study reveals a novel molecular mechanism for Fn regulation of human lung carcinoma cell growth and provi…s further evidence for the role of E prostanoid receptors i… …arcinoma biology.

Acknowledg… …illiam L. Smith (University of Michig… …constructs.

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