Modulation of Stat3 Activation by the Cytosolic Phospholipase A2α and Cyclooxygenase-2-controlled Prostaglandin E2 Signaling Pathway*

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A variety of human cancers show constitutive activation of signal transducer and activator of transcription-3 (Stat3) and overexpression of cyclooxygenase-2 (COX-2). This study describes a novel cross-talk between the COX-2-controlled prostaglandin E2 (PGE2) and Stat3 signaling pathways that coordinate regulate human cancer cell growth. COX-2-derived PGE2 induces interleukin-6 production through activation of EP4 receptor and subsequent phosphorylation of gp130/Stat3 in human cholangiocarcinoma cells. In parallel, activation of COX-2/PGE2 signaling also enhances Stat3 phosphorylation and reporter activity through EP1 receptor-induced activation of c-Src and EGFR in these cells. Moreover, the observations that EP1 receptor is detected in the nuclei as well as Stat3-DNA binding complex and that activation of EP1 in the nuclei enhances Stat3 activation depict an undescribed G protein-coupled receptor interaction in activation and tumor cell growth.

Stat3 (signal transducer and activator of transcription-3) is one member of the Stat family of transcription factors that are normally activated in a regulated fashion when protein ligands bind to their specific cell surface receptors (1–3). Similar to other members of the Stat transcription factor family, Stat3 becomes activated by tyrosine phosphorylation on a single tyrosine residue, dimerizes through reciprocal SH2-phosphotyrosine interaction, and accumulates in the nucleus, where it binds DNA and direct transcription of a wide array of genes. In normal cells the level and duration of Stat3 activation is controlled by mechanisms including dephosphorylation of the receptor complex or nuclear Stat dimers by protein-tyrosine phosphatases (PTPases), interaction of activated Stats with inhibitory molecules from the protein inhibitors of activated Stat (PIAS) family, or feedback inhibition of the JAK/STAT pathway by suppressor of cytokine signaling (SOCS) proteins through direct negative regulation of JAKs. In sharp contrast, Stat3 is persistently activated in a variety of human cancers such as breast, lung, prostate, melanomas as well as some hematologic tumors (2, 3), although the mechanisms for persistent Stat3 activation in tumor cells include overexpression or gain of function mutations of receptor-tyrosine kinases that activate Stat3, such as EGFR, or loss of proteins that negatively regulate Stat3, such as PIAS or SOCS.

In addition to Stat3, constitutive overexpression of cyclooxygenase-2 (COX-2) and elevated prostaglandin (PG) production has also been found in a variety of human cancers including cholangiocarcinoma (4–7). The expression of COX-2 in human cholangiocarcinoma tissue is positively correlated with EGFR (erbB1) and erbB2, two members of the EGFR tyrosine kinase family (8, 9). Constitutive expression of erbB2, a receptor-tyrosine kinase of the epidermal growth factor receptor (EGFR) family, in gall bladder and biliary tree epithelia results in elevated COX-2 and development of gall bladder adenocarcinoma and cholangiocarcinoma in mice (10). The expression of COX-2 in cultured cholangiocarcinoma cells is induced by growth factors (8), proinflammatory cytokines (11, 12), and bile acid (a tumor promoter in cholangiocarcinogenesis) (11, 13–15). Further, overexpression of COX-2 or treatment with PGE2 enhances tumor growth and invasion, in vitro and in SCID mice (8, 12, 16–20), whereas inhibition of COX-2 reduced cholangiocar-
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**A** p-Stat3

**B**

![Graph](image)

**C**

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**D**

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**E**

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**FIGURE 1.** PGE$_2$ signaling induces Stat3 phosphorylation in human cholangiocarcinoma cells. A, expression of p-Stat3 and COX-2 in human cholangiocarcinoma tissues. Note the nuclear and cytoplasmic staining of COX-2 in sequential sections of the same tumor. No staining was seen when the primary antibody was replaced with serum (NC, negative control). B, PGE$_2$ induces Stat3 phosphorylation in SG231 cells. The SG231 cells with 80% confluence were serum-starved for 24 h prior to treatment with 10 µM PGE$_2$ for 5–60 min or 50 ng/ml IL-6 for 30 min. Stat3 phosphorylation was determined by immunoblotting with anti-phospho-Stat3 (Tyr705) antibody (top panel); Stat3 in the immunoprecipitate was determined by reprobing the same blot with anti-Stat3 antibody (middle panel). Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between the Stat3 protein and phosphorylation levels from three different experiments with densitometry (lower panel). PGE$_2$ increased Stat3 phosphorylation as early as 5 min after treatment (*, p < 0.01). C, PGE$_2$ induces Stat3 phosphorylation in CCLP1 cells. The CCLP1 cells with 80% confluence were transiently transfected with the COX-2 expression plasmid or pcDNA control plasmid. After transfection, the cells were cultured in serum-free medium for 24 h. The cell lysates were then obtained to determine the phosphorylation of Stat3 by immunoblotting with anti-phospho-Stat3 (Tyr705) antibody (top panel); Stat3 was determined by reprobing the same blot with anti-Stat3 antibody (middle panel). Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between the Stat3 protein and phosphorylation levels from three different experiments with densitometry (lower panel). PGE$_2$ increased Stat3 phosphorylation as early as 5 min after treatment (*, p < 0.01). D, overexpression of COX-2 increases Stat3 phosphorylation. The CCLP1 cells with 80% confluence were transiently transfected with the COX-2 expression plasmid or pcDNA control plasmid. After transfection, the cells were cultured in serum-free medium for 24 h. The cell lysates were then obtained to determine the phosphorylation of Stat3 by immunoblotting with anti-phospho-Stat3 (Tyr705) antibody (top panel). The same blot was reprobed with antibodies against Stat3 (second panel), COX-2 (third panel), and β-actin (fourth panel). Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between the Stat3 protein and phosphorylation levels from three different experiments with densitometry (lower panel) (*, p < 0.05). E, overexpression of cPLA$_2$α increases Stat3 phosphorylation. The CCLP1 cells with 80% confluence were transiently transfected with the cPLA$_2$α expression plasmid or MT-2 control plasmid. After transfection, the cells were cultured in serum-free medium for 24 h and then cell lysates were obtained to determine the phosphorylation of Stat3 (top panel). The same blot was reprobed with antibodies against Stat3 (second panel), cPLA$_2$α (third panel), and β-actin (fourth panel). Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between the Stat3 protein and phosphorylation levels from three different experiments with densitometry (lower panel) (*, p < 0.05).

Carcinoma cell growth (16, 17, 19, 20). These findings indicate an important role of PG signaling in tumorigenesis.

PG biosynthesis is tightly controlled by a series of enzymes including the group IVA cytosolic phospholipase A$_2$ (cPLA$_2$α) that selectively cleaves arachidonic acid (AA) from membrane phospholipids, and COX-2 that converts AA substrate to PGs. Prostanoids exert their biological actions primarily via their respective G protein-coupled receptors (GPCR) superfamily of seven-transmembrane spanning proteins on the cell surface membrane (21, 22). PGE$_2$, the most abundant prostaglandin in human carcinoma cells, can potentially interact with four types of receptors (EP$_1$, EP$_2$, EP$_3$, and EP$_4$). The EP$_1$ receptor is coupled with G$_q$ protein and thus signals through phospholipase C and intracellular Ca$^{2+}$; the EP$_2$ and EP$_3$ receptors are coupled with G$_s$ protein, signaling through elevation of intracellular cAMP level and activation of protein kinase A; the EP$_4$ receptor is coupled with G$_i$ protein and signals through reduction of intracellular cAMP. Recently, EP$_3$ receptor has also been detected in the nucleus in certain cells (porcine cerebral endothelial cells and murine Swiss 3T3 cells) (23), although the phys-
iological implication of this intriguing phenomenon remains unknown.

Besides the direct tumor-promoting effect, PG signaling has also been implicated in the growth of cholangiocarcinoma cells induced by growth factors including interleukin-6 (IL-6) (4, 18), a classical biliary mitogen that is implicated in cholangiocarcinogenesis (5–7). IL-6 is known to stimulate biliary mitogenesis in an autocrine and paracrine fashion (24–28). It is produced by periductal hematomlymphoid cells during diseases associated with non-neoplastic biliary epithelial cell growth, whereas neoplastic human biliary epithelial cells can acquire the ability to constitutively produce IL-6. Accordingly, patients with cholangiocarcinoma show increased serum levels of IL-6 production (29, 30) and in animal models of cholangiocarcinoma IL-6 is detected after inoculation of cholangiocarcinoma cell line into SCID mice (24). IL-6 induces its biological functions through binding to its receptor complex (gp130 and gp80), which triggers the activation of Stat3 and mitogen-activated protein kinase (MAPK) pathways (31–34). Consistent with the observation that IL-6 increases PGE₂ production via MAPK-induced phosphorylation of cPLA₂, blocking PGE₂ synthesis inhibits the IL-6-induced cholangiocarcinoma cell growth (18). Thus, the interaction between PGE₂ and IL-6 signaling pathways may play a potential role in cholangiocarcinogenesis, although detailed mechanism for such an interaction remains to be further defined.

This study was designed to dissect the interaction between the cPLA₂/COX-2/PGE₂ and IL-6/Stat3 signaling pathways in human cholangiocarcinoma cells and examine their role in tumor growth. Our findings indicate that PGE₂ signaling activates Stat3 and p38, i.e. the EP₄ receptor-mediated induction of IL-6 production (extracellular mechanisms), and the EP1-receptor-mediated activation of c-Src (intracellular mechanisms). This study unveils a novel role of EP1, a G protein-coupled receptor, in the nucleus for Stat3 activation. These results depict a novel cross-talk between the COX-2/PGE₂ and IL-6/Stat3 signaling pathways that coordinately regulate tumor cell growth.

**EXPERIMENTAL PROCEDURES**

*Materials—Minimum essential medium α (α-MEM), Dulbecco’s modified minimal essential medium (DMEM), fetal bovine serum, glutamine, antibodies, the Lipofectamine plus™ reagent and Lipofectamine™ 2000 reagent were purchased from Invitrogen (Carlsbad, CA). Prostaglandin E₂ (PGE₂), the cPLA₂α inhibitors A438777 and N-(2S,4R)-4-(biphenyl-2-ylmethyl)-isobutyl-amino)-1-[2-(4,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl]-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide, HCl, the COX-2 inhibitor NS398, and the Src family tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-buty1) pyrazolo[3,4-d]pyrimidine (PP2) were purchased from Calbiochem. The recombinant human IL-6 was purchased from R&D systems (Minneapolis, MN). The EP₁ agonist ONO-DL-001, the EP₃ agonist ONO-8711 and the EP₃ agonist ONO-AE-248 were provided by the ONO Pharmaceutical Co., Ltd (Osaka, Japan). The EP₂ agonist Butaprost and EP₃ agonist PGE₁ Alcohol were pur-
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chased from Cayman Chemical (Ann Arbor, MI). The antibodies against human cPLA₂α, c-Src, anti-phosphotyrosine (PY99), and caveolin-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Stat3 antibody and phospho-Stat3 (Tyr^{170}) antibody were purchased from Cell Signaling (Beverly, MA). The antibodies against EP₁, EP₂, EP₃, and EP₄ receptors and COX-2 were purchased from Cayman Chemical. The antibody against human gp130 was purchased from R&D systems. The antibody against β-actin was purchased from Sigma. Horseradish peroxidase-linked streptavidin and chemiluminescence detection reagents were purchased from Amersham Biosciences. The human IL-6 immunoassay kit was purchased from R&D Systems.

Cell Culture and Transient Transfection—Human cholangiocarcinoma cell lines including CCLP1 and SG231 were cultured according to our previously described methods (8, 17, 18, 35). Briefly, CCLP1 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 50 μg/ml gentamycin; SG231 cells were cultured in α-MEM with 10% fetal bovine serum, 2 mM l-glutamine, 50 μg/ml gentamycin, and 10 mM HEPES. The cells were cultured at 37 °C in a humidified CO₂ incubator. For transient transfection assays, the cultured cells were transfection with both the cPLA₂α expression plasmid or MT-2 control plasmid and the COX-2 expression plasmid or pcDNA control plasmid using Lucfectamine plus™ reagent. The cells with optimal overexpression of either cPLA₂α or COX-2 were confirmed by Western blotting and subsequently used for further experiments.

Adenoviral Stat3-DN Gene Transfer—Modified adenoviral vectors carrying the dominant-negative Stat3 plasmids (36) or GFP cDNA (AdGFP) were propagated in 80% confluence in 10-cm² dishes that reach confluency in 10–14 days. The cells were then transferred to 25-cm² dish at approximately 50% confluence. The AdStat3-DN or GFP adenovirus stocks were used for further experiments.

Luciferase Reporter Assay—Luciferase reporter assays were performed using the Quickreporter system (Promega). The luciferase activity was assayed in a Berthold AutoLumat LB 953 luminometer (Nashua, NH) using the luciferase assay system from Promega. The relative luciferase activity was calculated after normalization of cellular proteins. All values are expressed as fold induction relative to basal activity.

Cell Growth Assay—Cell growth was determined using the cell proliferation reagent WST-1, a tetrazolium salt that is cleaved by mitochondrial dehydrogenases in viable cells. Briefly, 100 μl of cell suspension (containing 0.5–2 × 10⁶ cells) was plated in each well of 96-well plates. After a 24-h culture to allow reattachment, the cells were then treated with specific reagents such as PGE₂ or EP agonists for indicated time points. At the end of each experiment, the cell proliferation reagent WST-1 (10 μl) was added to each well, and the cells were incubated at 37 °C for 0.5–5 h. A₄₅₀ nm was measured using an automatic ELISA plate reader.

Immunoblotting—At the end of each indicated treatment, the cells were scraped off the plates and centrifuged, washed twice with cold phosphate-buffered saline (PBS) containing 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and resuspended in 5-fold volume of hypotonic buffer consisting of 50 mM HEPES pH 7.3, 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors tablets (Roche Applied Science). After sonication, the cell lysate was collected by centrifugation at 14,000 × g for 10 min to remove cell debris and stored in aliquots at -20 °C until use. The protein lysates were determined by the bicinchoninic acid assay. The proteins were electrophoretically transferred onto the nitrocellulose membranes and nonspecific binding was blocked with PBS-T (0.5% Tween 20 in PBS) containing 5% nonfat milk for 1 h at room temperature. The membranes were then incubated overnight at 4 °C with individual primary antibodies in PBS-T containing 1% nonfat milk at the dilutions specified by the manufacturers. Following three washes with PBS-T, the membranes were then incubated with the horseradish peroxidase-conjugated secondary antibodies at 1:10,000 dilution in PBS-T containing 1% nonfat milk for 1 h at room temperature. The membranes were then washed three times with PBS-T, and the protein bands were visualized with the ECL Western blotting detection system according to the manufacturer’s instructions.

Phosphorylation of gp130—After each indicated treatment, the cell lysates were obtained as described above. Equal amounts of the cell lysates were preincubated with 5 μg/ml goat
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anti-human gp130 polyclonal antibody followed by addition of 20 μl of protein A/G-agarose. The mixtures were incubated overnight at 4 °C. After three washes with the same hypotonic buffer, the pellet was used for immunoblotting using monoclonal anti-phosphotyrosine (PY99) antibody.

Binding of c-Src to Stat3—The binding complexes of Stat3 and c-Src in CCLP1 cells were determined by immunoprecipitation and Western blot. Confluent CCLP1 cells were serum-starved for 24 h, followed by treatment with 1 μM ONO-DI-004 or 10 μM PGE2 for 30 min. The cell lysates were subsequently prepared for immunoprecipitation with antibody against c-Src. The immunoprecipitants were then subjected to SDS-PAGE and immunoblotted with anti-Stat3 antibody.

Binding of Stat3 to EP1—The binding complexes of Stat3 and EP1 in CCLP1 cells were determined by immunoprecipitation and Western blot. Confluent CCLP1 cells were serum-starved for 24 h, followed by treatment with 1 μM ONO-DI-004 or 10 μM PGE2 for 30 min. The cell lysates were subsequently prepared for immunoprecipitation with antibody against c-Src. The immunoprecipitants were then subjected to SDS-PAGE and immunoblotted with anti-EP1 antibody.

Preparation of Caveolin-rich Membrane Fractions—Caveolin-enriched membrane fractions were prepared from CCLP1 cells according to the method described previously (37). In brief, CCLP1 cells grown to confluence in 100-mm dishes were washed twice with PBS and homogenized in 2 ml of 500 mM carbonate, pH 11.0 containing inhibitors of protease and phosphatases. The homogenate was then subjected by the addition of 90% sucrose prepared in MBS (25 mM MES, pH 6.5, 0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient (4 ml of 5% sucrose/4 ml of 35% sucrose; both in MBS containing 250 mM sodium carbonate) was layered on top of the cushion and centrifuged in an SW41 rotor (Beckman Instruments). A light-scattering band formed to the 5–35% sucrose interface was collected as caveolin-enriched membrane fractions for further experiments.

RNA Interference—Stat3 siRNA (siRNA ID: 42861) and gp130 siRNA were purchased from Ambion (Austin, TX). The targeted sequences that effectively mediate the silencing of gp130 expression are the combination of 5'-GCAAGUGGGAU-CACCUAGUTT-3' (sense sequence) (siRNA ID: 111101) and 5'-GGCAUGCCUAAAAGUUACUTT-3' (sense sequence) (siRNA ID: 106711). Cells with 50% confluence were transfected with either Stat3 siRNA or gp130 siRNA, or a 21-nucleotide irrelevant RNA duplex as a control using Lipofectamine™ 2000. Depletion of either Stat3 or gp130 was confirmed by immunoblotting and subsequently used for further experiments.

Phosphorothioate-modified Antisense Oligonucleotides—Human EP receptor antisense and irrelevant phosphorothioate-modified DNA oligonucleotides were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). The specific sequences of EP receptor antisense oligonucleotides were designed as described (38, 39) with modification and sequence as follows: EP1, 5'-GCAAGGCT-GATGAGC (nucleotides: 113–130); EP3, 5'-ACTGGA-GATGAGC (nucleotides: 124–148); EP4, 5'-GTCTCCACATGAGC (nucleotides: 236–252); EP5, 5'-AGGTGGT-TGATGAGC (nucleotides: 268–222). Either CCLP1 cells were serum-starved for 24 h, and then cell lysates were obtained to determine the phosphorylation of gp130 and Stat3. gp130 phosphorylation was determined by immunoprecipitation with anti-gp130 antibody and immunoblotting with anti-phosphotyrosine (PY99) antibody. The bound complexes of Stat3 and c-Src were isolated and subsequently used for further experiments.

Biotinylated Oligonucleotide Precipitation Assays—These experiments were performed as described previously with minor modifications (40). The sequences of biotinylated oligo-
nucleotides corresponding to Stat3 binding site are forward: 5'-TGCTTCCCGAATTCCCGAATTCCCGAATTCCCGAATTTCCCGAATTCCCGAACGT-3'; and reverse: 5’-ACGTTCGGGAATTCGGGAATTCGGGAATTCGGGAATTCCGGGAAGCA-3’. The 5’-biotinylated oligonucleotides were synthesized by Sigma-Genosys (Woodland, Texas). Nuclear extracts were prepared with the CelLytic™ Nuclear Extraction kit from Sigma according the protocol provided by the manufacturer. Cell extracts were prepared by sonication in HKMG buffer containing protease and phosphatase inhibitors. Binding

![Figure 5](image-url)
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We first utilized immunohistochemical analysis to assess the expression and localization of phospho-Stat3 (p-Stat3) in human cholangiocarcinoma and surrounding non-tumor liver tissue. Twelve paired cholangiocarcinoma and non-tumor liver tissue samples were analyzed. p-Stat3 expression was detected in both SG231 and CCLP1 cells, with the highest expression in CCLP1 cells. The expression of p-Stat3 was found to be significantly higher in cholangiocarcinoma cells compared to non-tumor liver tissue samples. The expression of p-Stat3 in cholangiocarcinoma cells was also found to be significantly higher in SG231 cells compared to CCLP1 cells. These findings suggest that p-Stat3 may play a role in the development and progression of cholangiocarcinoma.

Next, we investigated the potential effect of PGE2 signaling on Stat3 phosphorylation in human cholangiocarcinoma cells. We found that treatment with PGE2 resulted in a significant increase in Stat3 phosphorylation in both SG231 and CCLP1 cells. Similarly, overexpression of COX-2 also increased Stat3 phosphorylation in these cells. These findings suggest that COX-2 may be involved in regulating Stat3 phosphorylation in cholangiocarcinoma cells.

We also investigated the role of EP receptor subtypes in the PGE2-induced Stat3 phosphorylation. We found that the EP4 receptor agonist, PGE1-OH, increased Stat3 phosphorylation in SG231 cells. However, treatment with EP1, EP2, or EP3 agonists did not affect Stat3 phosphorylation. These findings suggest that EP4 may be involved in regulating Stat3 phosphorylation in cholangiocarcinoma cells.

Overall, these findings suggest that PGE2 signaling may modulate Stat3 phosphorylation in human cholangiocarcinoma cells, which may potentially contribute to the development and progression of this disease.

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A

1 μM ONO-DI-004  10 μM PGE₂
DNA pull-own
180 Kd
42 Kd
92 Kd
EGFR
EP₁
Stat3
direct western
180 Kd
42 Kd
92 Kd
EGFR
EP₁
Stat3

B

AdGFP  AdStat3-DN
DNA pull-own
180 Kd
42 Kd
EGFR
EP₁
Direct western
180 Kd
42 Kd
1 μM ONO-DI-004
10 μM PGE₂

C

D

E

10 μM ONO-DI-004
50 μM PGE₂
DNA pull-own
42 Kd
92 Kd
EGFR
EP₁
Stat3
direct western
116 Kd
PARP
22 Kd

F

G

1  2  3  4  5  6
180 Kd
42 Kd
92 Kd
60 Kd
22 Kd
EGFR
EP₁
Stat3
c-Src
Caveolin-1

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A treatment of intact CCLP1 cells with ONO-DI-004 and PGE$_2$ induced binding of EGFR or EP$_1$, to Stat3-responsive element. CCLP1 cells serum-starved for 24 h were treated with 1 µM ONO-DI-004 or 10 µM PGE$_2$ for 30 min. The cell lysates were obtained and precipitated with biotinylated Stat3 oligonucleotides followed by immunoblotting for EGFR, EP$_1$, or Stat3 (upper panels). Equal amounts of cell lysates were used to detect EGFR, EP$_1$, or Stat3 by direct Western blot (lower panels). B, dominant negative inhibition of Stat3 prevents ONO-DI-004 and PGE$_2$-induced binding of EGFR or EP$_1$, to Stat3-responsive element. CCLP1 cells infected with AdGFP or AdSTAT3-DN were treated with 1 µM ONO-DI-004 or 10 µM PGE$_2$ for 30 min. The cell lysates were precipitated with biotinylated Stat3 oligonucleotides followed by immunoblotting for EGFR or EP$_1$, (upper panels). Equal amounts of cell lysates were used to detect EGFR or EP$_1$, by direct Western (lower panels). C, confocal immunofluorescent detection of EP$_1$ receptor in CCLP1 cells. The proteins from non-caveolin-enriched membrane fractions (1, 2, and 3) or caveolin-enriched membrane fractions (4, 5, and 6) were applied to immunoblot to detect EGFR, EP$_1$, Src, and caveolin-1. G, colocalization of EGFR and EP$_1$, and Src in caveolin fractions of CCLP1 cells. The proteins from caveolin-enriched membrane fractions (1, 2, and 3) and from nuclear fractions (4, 5, and 6) were applied to the immunoblot to detect EGFR, EP$_1$, Src, and Stat3.

Fig. 4D, siRNA inhibition of gp130 partially prevented the PGE$_2$-induced Stat3 phosphorylation and transcription activity. Taken together, these results suggest that the EP$_4$ receptor-mediated IL-6 production and gp130 phosphorylation is an important mechanism by which PGE$_2$ activates Stat3 in SG231 cells. It is of further interest that the IL-6-induced Stat3 activation was blocked by the cPLA$_2$α inhibitors, AACOCF$_3$, and the 1,2,4-tri-substituted pyrrole derivative (C$_{9}$H$_{14}$F$_{3}$N$_{4}$O$_{5}$S), and by the COX-2 inhibitor, NS-398 (Fig. 4E), suggesting that PG signaling also modulate the constitutive IL-6/gp130/Stat3 signaling in SG231 cells.

However, the IL-6-induced gp130 signaling does not appear to represent a key mechanism for PGE$_2$-induced Stat3 activation in CCLP1 cells. For instance, the combination of IL-6 and gp130; and (2) treatment of intact CCLP1 cells with OL-1006, a single green color confocal immunofluorescent image showing the presence of EP$_3$, receptor in the nuclei. E, treatment of the nuclei isolated from CCLP1 cells with ONO-DI-004 and PGE$_2$, induces the binding of EGFR and EP$_1$, to Stat3-responsive element. F, co-localization of EP$_1$, EGFR and Src in caveolin-enriched membrane fractions. The caveolin-rich light membrane fractions from CCLP1 cells were prepared by sucrose density gradient centrifugation as described under “Experimental Procedures.” The proteins from non-caveolin-enriched membrane fractions (1, 2, and 3) or caveolin-enriched membrane fractions (4, 5, and 6) were applied to immunoblot to detect EGFR, EP$_1$, Src, and caveolin-1.
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CCLP1 cells is further supported by the observation that the Stat3 reporter activity induced by PGE$_2$ or ONO-DI-004 was blocked by the selective EP$_1$ receptor antagonist ONO-8711 (Fig. 5B). Consistent with these findings, antisense depletion of EP$_1$ receptor also inhibited Stat3 phosphorylation (Fig. 5C). Given that PGE$_2$ is known to activate Src in human cholangiocarcinoma cells (8), we next examined whether Src might play a role in Stat3 activation in CCLP1 cells. As shown in Fig. 5D, although no Src and Stat3 binding complex was detected under baseline culture conditions, treatment of CCLP1 cells with PGE$_2$ or the EP$_1$ agonist, ONO-DI-004, induced the association of Src to Stat3. Similarly, PGE$_2$ or ONO-DI-004 treatment also induced the formation of EP$_1$ and Stat3 binding complex (Fig. 5E). In addition, the PGE$_2$-induced Stat3 phosphorylation in CCLP1 cells was partially blocked by the Src inhibitor, PP2, and by the EP$_1$ antagonist, ONO-8711, in vitro (Fig. 5F). These findings suggest that activation of Src by EP$_1$ is an important mechanism by which PGE$_2$ signaling activates Stat3 and promotes growth in CCLP1 cells.

The role of EP$_1$ for Stat3 activation in CCLP1 cells is further supported by the data from biotinylated oligonucleotide precipitation assays. As shown in Fig. 6A, treatment of CCLP1 cells with PGE$_2$ or the EP$_1$ agonist, ONO-DI-004, induced the binding of Stat3 to its specific consensus oligonucleotide (no binding was detected in the cells treated with Me$_2$SO vehicle). Given that EP$_1$ has recently been shown to transactivate EGFR (8) and that EGFR is known to activate Stat3 (through direct phosphorylation by EGFR along the plasma membrane or nuclear targeting), we investigated whether EGFR might also be present in the EP$_1$/Stat3 complex. To test this hypothesis, we treated CCLP cells with PGE$_2$ or vehicle in serum-free medium for 48 h, and the cell growth was determined using WST-1 assay. The data are presented as mean ± S.D. of six independent experiments (*, p < 0.01). Western blots showed successful depletion of gp130 or Stat3 in SG231 cells transfected with the corresponding siRNA. B, RNAi suppression of Stat3 expression was confirmed by Western blot (right panel).

Confocal immunofluorescence microscopy was utilized to determine the distribution of EP$_1$ receptor in CCLP1 cells. EP$_1$ receptor is present in the nucleus as well as in the cytoplasm and plasma membrane (Fig. 6, C and D). To further document whether the nuclear EP$_1$ receptor is involved in Stat3 activation, intact nuclei were isolated from CCLP1 cells and the obtained nuclei were incubated with PGE$_2$ or ONO-DI-004 to determine the binding of Stat3 to its consensus DNA site. As shown in Fig. 6E, treatment of isolated nuclei induced the formation of Stat3-DNA binding complex, which also contains EGFR and EP$_1$. These findings provide further evidence for the role of nuclear EP$_1$ receptor in COX-2/PGE$_2$-induced Stat3 activation.

Because caveolin membrane system has been implicated in the subcellular redistribution of transmembrane proteins, we sought to investigate whether this system is involved in the redistribution of EP$_1$ and EGFR in CCLP1 cells. As shown in Fig. 6F, EP$_1$, EGFR, and c-Src are present in the caveolin-enriched membrane fractions, but not in the non-caveolin-enriched membrane fractions. Furthermore, caveolin-1 and associated proteins, including EP$_1$, EGFR, and c-Src, are also present in the purified nuclei (Fig. 6G). These findings suggest a potential role of caveolae in the transportation of EP$_1$ and EGFR to the nucleus for Stat3 activation.

We next examined whether the intracellular Src-dependent Stat3 pathway is also present in the SG231 cells. As shown in Fig. 7, the PGE$_2$-induced Stat3 reporter activity was inhibited by the Src inhibitor, PP2, as well as by antisense inhibition of the EP$_1$ receptor. These findings suggest that activation of Src by EP$_1$ is also involved in PGE$_2$-induced Stat3 activation in SG231 cells. In addi-

![Figure 8. RNAi inhibition of gp130/Stat3 signaling prevents PGE$_2$-induced cell growth in SG231 cells.](image-url)
tion, the PGE$_2$-induced Stat3 reporter activity in the SG231 cells was also partially inhibited by antisense depletion of EP$_4$ (Fig. 9A); these results further support the involvement of EP$_1$/Stat3 and EP$_4$/gp130 signaling in PGE$_2$-induced growth of SG231 cells. In CCLP1 cells, siRNA inhibition of Stat3 blocked the PGE$_2$-induced cell growth (Fig. 8B) (gp130 siRNA was not used in these cells because of their low level of gp130 expression). Antisense depletion of EP$_1$ receptor in CCLP1 cells also inhibited the PGE$_2$-induced cell growth (Fig. 8B). These data further support the role of EP$_1$/Stat3 signaling in PGE$_2$-induced growth of CCLP1 cells.

**DISCUSSION**

Prostaglandin and Stat3 signaling pathways have been implicated in the growth of several human cancers including cholangiocarcinoma (4–7), although the mechanisms for their actions remain to be further understood. Given that both signaling pathways are up-regulated during carcinogenesis, we hypothesized that cross-interaction of these pathways may play an important role in the development and progression of human cancers. Our data presented in this study indicate that the cPLA$_2$/H9251/COX-2-controlled PGE$_2$ signaling activates Stat3 through EP$_4$ receptor-mediated extracellular release of IL-6 as well as through EP$_1$ receptor-mediated intracellular activation of c-Src in human cholangiocarcinoma cells. Moreover, our findings provide novel evidence for the involvement of EP$_1$ receptor, a G protein-coupled receptor, in the nucleus for Stat3 activation. The importance of Stat3 in PGE$_2$-induced tumor cell growth is further highlighted by the observation that siRNA inhibition of Stat3 significantly inhibited the PGE$_2$-induced tumor growth. These findings, along with the results described in our previous study that IL-6 increases PG synthesis through phosphorylation of cPLA$_2$-a in these cells (18), depict a cross-talk between the autocrine/paracrine loops of the IL-6/Stat3 and cPLA$_2$-a/COX-2/PGE$_2$ signaling pathways that coordinately regulate human cancer cell growth (Fig. 10).

Cholangiocarcinoma is a highly malignant epithelial neoplasm arising within the biliary tract (4–7). It comprises ~10–
Modulation of Stat3 by PGE₂ Signaling Pathway

FIGURE 10. Cross-talk between the IL-6/Stat3 and cPLA2α/COX-2/PGE₂ signaling pathways in cholangiocarcinoma growth. PGE₂ synthesis is controlled by coupled activation of cPLA₂α and COX-2 along cell membrane. The produced PGE₂ is released into the extracellular space, which then binds to the membrane G protein-coupled EP receptors on the same cell (autocrine) or on a neighboring cell (paracrine). There are four types of PGE₂ receptors (EP₁, EP₂, EP₃, EP₄) in human cholangiocarcinoma cells. Our data indicate that the cPLA₂/COX-2/PGE₂ signaling activates Stat3 through two parallel mechanisms in human cholangiocarcinoma cells: 1) the EP₁ receptor-mediated activation of c-Src and EGFR; and 2) the EP₂ receptor-mediated production of IL-6, which subsequently binds and activates gp130. On the other hand, Src further enhances PGE₂ signaling through MAP kinase-mediated phosphorylation, which provides arachidonic acid substrate for COX-2. The depicted autocrine/paracrine loop of COX-2/PGE₂ signaling pathways likely play an important role in cholangiocarcinoma growth.

15% of hepatobiliary neoplasms is rising (5, 43). The tumor develops under conditions that cause long-standing, chronic, and reparative biliary epithelial injury, such as primary sclerosing cholangitis (PSC), chronic biliary obstruction, or complicated fibroplastic cholangitis. Chronic inflammation and cellular injury within bile ducts, together with partial obstruction of bile flow, appear to be important predisposing factors in the pathogenesis of cholangiocarcinoma. IL-6 is a classical biliary mitogen that has been shown to increase the growth and survival of biliary epithelial cells and cholangiocarcinoma cells. It mediates actions through binding to gp130 receptor, resulting in phosphorylation and activation of Stat3. In this study, we showed that COX-2 overexpression or PGE₂ treatment increased IL-6 production, suggesting that the PGE₂-mediated cell growth is, at least in part, mediated by IL-6. This assertion is supported by the observations that COX-2 and PGE₂ also induce gp130 phosphorylation as well as Stat3 phosphorylation and reporter activity in SG231 cells. Although all four EP receptors (EP₁, EP₂, EP₃, and EP₄) are expressed in the SG231 cells, our data indicate that the COX-2 and PGE₂-induced IL-6 production and gp130 phosphorylation is predominantly mediated by EP₄. The later conclusion is based on the following evidence: 1) the EP₄ receptor agonist PGE₄-OH increased IL-6 production and gp130 phosphorylation; 2) the PGE₂-induced IL-6 production and gp130 phosphorylation was blocked by antisense suppression of EP₄ receptor but not by antisense suppression of EP₁, EP₂, or EP₃ in SG231 cells; and 3) siRNA inhibition of gp130 partially prevented the PGE₂-induced Stat3 phosphorylation and transcription activity. In this regard, it is noteworthy that our results are consistent with the findings reported in a previous study that disruption of EP₄ (but not EP₁, EP₂, or EP₃) in mice results in reduced circulating levels of IL-6 and decreased IL-6 production by liver and macrophages (44).

We show that COX-2 and PGE₂ also induce Stat3 phosphorylation in human cholangiocarcinoma cells through an intracellular mechanism that involves EP₁ receptor-mediated activation of c-Src and is independent of IL-6/gp130. This assertion is supported by the following evidence: 1) overexpression of COX-2 or treatment with PGE₂ or the selective EP₁ receptor agonist, ONO-DI-004, induced Stat3 phosphorylation/activation, but not IL-6 production or gp130 phosphorylation in CCLP1 cells; 2) the PGE₂-induced Stat3 phosphorylation and reporter activity was blocked by the EP₁ receptor antagonist, ONO-8711, by antisense inhibition of EP₁ receptor; 3) PGE₂ or the EP₁ agonist, ONO-DI-004 induced the formation of Stat3/Stat3 binding complex; 4) PGE₂ or ONO-DI-004 inhibited gp130 phosphorylation that was blocked by dominant negative inhibition of Stat3; 5) the Src inhibitor, PP2, inhibited PGE₂-induced Stat3 phosphorylation and reporter activity, suggesting that ONO-8711 inhibited Stat3 binding to gp130 in CCLP1 cells. These results, along with the fact that the EP₁ receptor activates Stat3 via Src, provide strong support for the notion that another GPCR, angiotensin II receptor type 1 (AT₁) (54), endothelin receptor subtype B (ET-BR) (55), lysophosphatidic acid receptor, and 3) siRNA inhibition of gp130 partially prevented the PGE₂-induced Stat3 phosphorylation and transcription activity. In this regard, it is noteworthy that our results are consistent with the findings reported in a previous study that disruption of EP₄ (but not EP₁, EP₂, or EP₃) in mice results in reduced circulating levels of IL-6 and decreased IL-6 production by liver and macrophages (44).

Recent evidence suggests that G proteins are able to directly bind Src, leading to its activation (51), although the exact domains mediating such an interaction have not been identified. It is conceivable that this process may also involve other unknown proteins. Indeed, our data indicate that EGFR is present in PGE₂/EP₁ agonist-induced Stat3-DNA binding complex and this effect is likely mediated by the direct protein interactions between EGFR and Stat3 (EGFR failed to bind Stat3 oligonucleotide). The latter results, along with the findings that EP₁ receptor transactivates EGFR in human cholangiocarcinoma cells (8) and that EGFR activates Stat3 in other cells (52, 53) suggest the involvement of EGFR in PGE₂/EP₁-mediated Stat3 activation.

In addition to their expression in the plasma membrane, several GPCRs are recently found in the nuclei, including angiotensin II receptor type 1 (AT₁) (54), endothelin receptor subtype B (ET-BR) (55), lysophosphatidic acid receptor...
type 1 (LPA₁R) (56), and PGE₂ receptors (EP₁, EP₃, EP₄) (23, 57). However, the potential biological function of nuclear G protein-coupled receptors is currently unknown. In this study, we provide novel evidence for the functional role of EP₁ receptor in the nucleus for Stat3 activation. This finding is noteworthy, given the documented nuclear localization of the key eicosanoid-forming enzymes, cPLA₂α and COX-2 (58). Therefore, it is conceivable that PGE₂ generated by coupled activation of cPLA₂α and COX-2 in the nucleus may be sufficient for local activation of nuclear EP₁ receptor that is involved in the regulation of Stat3 transcription in the nuclei. The latter finding is consistent with the recently reported nuclear interaction of EGFR and Stat3 for the transcriptional activation of inducible nitric-oxide synthase (iNOS) (42).

In summary, the results presented in this article demonstrate a novel cross-talk between the cPLA₂α/COX-2/PGE₂ and IL-6/ Stat3 signaling pathways that is importantly involved in the control of human cancer cell growth. These findings are expected to provide important implications for developing future combinational therapy simultaneously blocking COX-2/EP₁ antagonist ONO-8711 were kindly provided by the Ono Pharmaceutical Co., Ltd, Japan.

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