Prostaglandin E_2 receptor EP1-mediated phosphorylation of focal adhesion kinase enhances cell adhesion and migration in hepatocellular carcinoma cells

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Received December 6, 2012; Accepted February 4, 2013

DOI: 10.3892/ijo.2013.1859

Abstract. The prostaglandin E_2 (PGE_2) EP1 receptor has been implicated in hepatocellular carcinoma (HCC) cell invasion. However, little is known about the mechanisms of EP1 receptor-mediated cell adhesion and migration. We previously showed that PGE_2 promotes cell adhesion and migration by activating focal adhesion kinase (FAK). The present study was designed to elucidate the association between the EP1 receptor and FAK activation in HCC cells and to investigate the related signaling pathways. The effects of PGE_2, EP1 agonist 17-phenyl trinor-PGE_2 (17-PT-PGE_2), PKC and EGFR inhibitors on FAK activation were investigated by treatment of Huh-7 cells. Phosphorylation of FAK Y397 and c-Src Y416 was investigated by western blotting. Cell adhesion and migration were analyzed by WST and transwell assays, respectively. Protein kinase C (PKC) activity was measured with a PKC assay kit. The results showed that 17-PT-PGE_2 (3 μM) increased FAK Y397 phosphorylation by more than 2-fold and promoted cell adhesion and migration in Huh-7 cells. In transfected 293 cells, expression of the EP1 receptor was confirmed to upregulate FAK phosphorylation, while the EP1 receptor antagonist sc-19220 decreased PGE_2-mediated FAK activation. PKC activity and c-Src Y416 phosphorylation were enhanced after 17-PT-PGE_2 treatment. Both PKC and c-Src inhibitor suppressed the 17-PT-PGE_2-upregulated FAK phosphorylation, as well as 17-PT-PGE_2-induced cell adhesion and migration. In addition, exogenous epidermal growth factor (EGF) treatment increased FAK phosphorylation. The EGF receptor (EGFR) inhibitor also suppressed 17-PT-PGE_2-upregulated FAK phosphorylation. Our study suggests that the PGE_2 EP1 receptor regulates FAK phosphorylation by activating the PKC/c-Src and EGFR signal pathways, which may coordinately regulate adhesion and migration in HCC.

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Key words: EP1 receptor, focal adhesion kinase, cell adhesion, migration, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer related mortality worldwide (1). Clinically, surgical resection remains the primary mode of therapy for advanced HCC. In the absence of an effective adjuvant therapy, liver cancer has a poor prognosis (2). HCC is a highly malignant tumor with a potent capacity to invade locally and metastasize distantly (2,3); therefore, an approach that decreases its ability to invade and metastasize may facilitate the development of an effective adjuvant therapy.

Focal adhesion kinase (FAK) is a non-receptor cytoplasmic tyrosine kinase that plays a key role in the regulation of cell proliferation and migration in both inflammation and neoplasia (4). FAK appears to be essential for the regulation of cell migration and invasion properties (5-7). It is associated with integrin receptors and integrates with other molecules to form a complex that transmits signals from the extracellular matrix to the cell cytoskeleton (8,9). In 2010, the expression and prognostic significance of FAK were reported in liver cancer and FAK was found to play an important role in invasion and metastasis of HCC (10). Therefore, FAK may be a promising therapeutic target for HCC (11). Our previous studies showed that prostaglandin E_2 (PGE_2) induces cell adhesion and migration by activating FAK in HCC cells (12). However, the specific mechanism of this activation was not investigated.

PGE_2 is one of the predominant metabolic products of arachidonic acid. In previous studies, PGE_2 was found to play a major role in promoting tumor cell growth, migration and invasion in many cancer types (13-16). PGE_2 has been shown to regulate tumor development and progression by combining

with E prostanoid receptors (EP receptors) on the surface of the cell membrane and activating their predominant signal transduction pathways (17).

There are four types of EP receptors expressed on the membrane surface of HCC cells (1). Among them, the EP1 receptor is accepted to be involved in cell growth, invasion and metastasis in many cancers, such as colon cancer (18), skin cancer (19) and cervical cancer (20). Recently, the EP1 receptor was reported to enhance cell invasion in HCC cells (21), although it is unclear whether FAK activation is involved. In the present study, PGE_2 was found to upregulate FAK phosphorylation via the EP1 receptor in HCC cells. Protein kinase C (PKC), c-Src and epidermal growth factor receptor (EGFR) were all involved in the EP1 receptor-mediated FAK phosphorylation and cell adhesion and migration. Our data generated from the present studies may provide significant insights into the mechanisms of EP1 receptor-mediated FAK activation and ultimately lead to the development of novel management strategies and therapeutics for cell invasion and metastasis in hepatocellular carcinoma.

Materials and methods

Materials. The human HCC cell line Huh-7 and human embryonic kidney (HEK) 293 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen (Carlsbad, CA, USA). PGE_2, 17-phenyl trinor-PGE_2 (17-PT-PGE_2) and sc-19220 were from Cayman Chemical Co. (Ann Arbor, MI, USA). AG1478 was from Sigma-Aldrich (St. Louis, MO, USA). Bisindolymaleimide I (#203290), phorbol-12-myristate-13-acetate (#524400) and PP2 (#529573) were obtained from Merck (Darmstadt, Germany). Recombinant human epidermal growth factor (EGF; #AF-100-15) was from PeproTech (Rocky Hill, NJ, USA). WST reagent from the Cell Counting Kit-8 (CCK-8) was from Dojindo Laboratories (Kumamoto, Japan). The protein assay was from Bio-Rad (Hercules, CA, USA). Electrochemiluminescence (ECL) reagents were from Amersham Biosciences (Piscataway, NJ, USA). The PKC assay kit was from Millipore (Billerica, MA, USA). [γ-^{32}P]ATP (#BLU002A) was from Perkin-Elmer (Waltham, MA, USA). G418 sulfate was from Amresco (Solon, OH, USA). The following were commerically obtained antibodies: anti-FAK antibody (sc-558; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phosphorylated FAK Y397 antibody (#611806, BD Biosciences, Franklin Lakes, NJ, USA); phosphorylated Src antibody (#2101, Cell Signaling Technology Danvers, MA, USA); total Src antibody (#21168, SAB, College Park, MD, USA); anti-β-actin antibody (Sigma).

Cell lines and culture. Huh-7 cells and HEK293 cells were cultured in DMEM with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37˚C with 5% CO_2.

Cell adhesion assays. Cell adhesion assays were performed in 96-well culture cell plates. Cells ($5x10^4$) were added to each well of the plates and pharmacological agents were added at the indicated time. The plates were incubated at 37˚C for 3 h and then washed three times with phosphate-buffered saline (PBS) to remove unattached cells. The attached cells were stained with WST at 37˚C for 2 h. Adherent cells were quantified by measuring the absorbance at 450 nm.

Cell migration assays. Cell migration assays were performed in 12-well transwell units. Before the experiment, the lower surfaces of the membranes were coated with gelatin (1%) diluted in PBS. Huh-7 cells ($5x10^4$) were added to the upper chamber and 1 ml complete DMEM to the lower chamber of the transwell. Pharmacological agents were added at the indicated time. After incubation at 37˚C for 12 h, the cells were fixed with ethanol and then stained with 0.1% crystal violet for 30 min at room temperature. After washing the wells with PBS, the cells were removed from the upper surface of the membrane by wiping with a moist cotton swab. The cells migrated to the lower surface of the membrane were solubilized with 300 μl of 10% acetic acid and quantified by measuring the absorbance at 570 nm.

PKC measurements. Cells were treated with pharmacological agents at 37˚C for various times, as indicated in the experiments. The cells were collected into lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 100 μg/ml PMSF and aprotinin) and placed on ice for 30 min. Cell lysates were sonicated on ice for at least 30 sec and then cleared by centrifugation at 120,000 x g for 30 min at 4˚C. Total protein (30 μg) (10 μl) for each sample was added to a microcentrifuge tube and assayed for PKC levels using a direct human PKC enzyme activity assay kit according to the manufacturer's instructions. Briefly, 10 μl of substrate cocktail, PKA inhibitor cocktail, Assay Dilution Buffer II (ADBII), lipid activator and diluted [γ-^{32}P]-ATP mixture were added to a microcentrifuge tube and then the mixture was incubated for 10 min at 30˚C with constant agitation. A 25-μl aliquot from each sample was transferred onto the center of a P81 phosphocellulose paper. The assay squares were washed with 0.75% phosphoric acid three times, followed by one wash with acetone. The assay squares were transferred to vials with a scintillation cocktail and read in a scintillation counter. The counts per minute (cpm) of the enzyme samples were compared to those of the control samples containing no enzyme.

Plasmid transfections. The pcDNA3-based plasmid encoding the human EP1 receptor (EP1R-pcDNA3) was a generous gift of Kathy Mccusker in 2007 (Merck Frosst Centre for Therapeutic Research, Canada). HEK293 cells ($2x10^5$) were seeded and grown in 6-well culture plates for 24 h before transfection with the EP1R-pcDNA3 plasmid or pcDNA3 empty vector control (2 μg) using Lipofectamine 2000™ (5 μl). After incubation for 5 h, the medium was changed to fresh normal growth DMEM. The efficiency of transfection was assayed by flow cytometry. The G418 antibiotic was used to select for HEK293 cells stably expressing the EP1 receptor.

Western blotting. Cells were treated with pharmacological agents at 37˚C for various times, as indicated in the experiments. The cells were collected into lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 100 μg/ml PMSF and aprotinin) and placed on ice for 30 min. Cell lysates were sonicated on ice

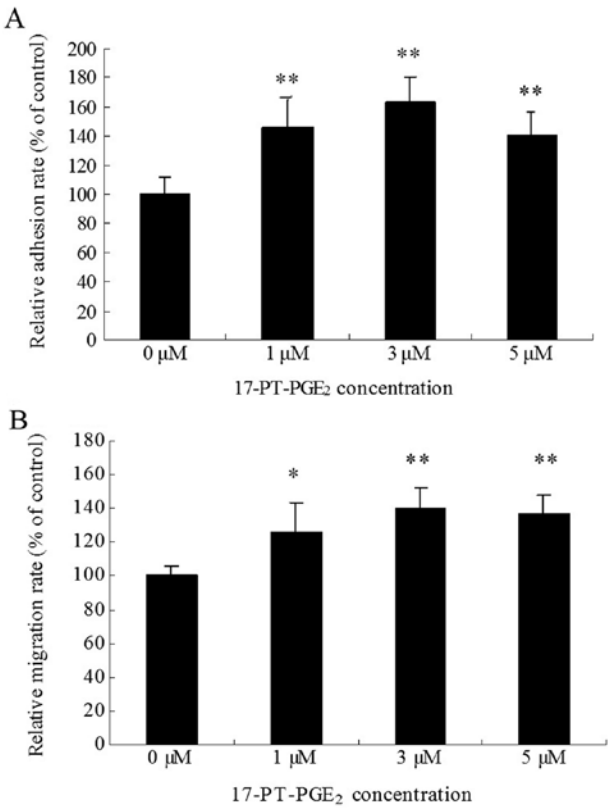

Figure 1. Effects of 17-PT-PGE_2 treatment on cell adhesion and migration in Huh-7 cells. (A) Cell adhesion assays were performed in 96-well plates by incubation for 3 h at 37˚C in serum-free DMEM supplemented with or without 17-PT-PGE_2. Attached cells were stained with WST and optical density (OD) was measured at 450 nm. (B) Cell migration assay. Huh-7 cells ($5x10^4$ cells) were placed in the upper chamber of 12-well transwells with or without 17-PT-PGE_2 in serum-free DMEM and allowed to migrate for 12 h. Cells on the lower surface were fixed with ethanol and stained with 0.1% crystal violet. Migrated cells were solubilized with 300 μl of a 10% acetic acid solution and quantified by measuring absorbance at 570 nm. Results are presented as means ± SD from three different experiments. $^*P<0.05$, compared with cells without treatment; $^{**}P<0.01$, compared to cells without treatment.

for ≥30 sec and then cleared by centrifugation at 120,000 x g for 30 min at 4˚C. Equal amounts of total proteins (40 μg) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were probed with the appropriate antibodies at 4˚C overnight with gentle shaking. The immunoreactivity was detected by ECL and analyzed using Image lab 4.0 analysis software from Bio-Rad.

Statistical analysis. Data are presented as means ± SD. P-values were calculated using the Student's t-test for unpaired samples with MS Excel software. The results were considered significantly different at $P<0.05$.

Results

EP1 receptor promotes FAK phosphorylation, cell adhesion and migration in Huh-7 cells. Huh-7 cells were treated with various concentrations of the EP1 receptor agonist 17-PT-PGE_2 for 3 h. The WST assay was used to detect the cell adhesion rate. The results showed that 17-PT-PGE_2 produced a 40-60% increase in cell adhesion in Huh-7 cells (Fig. 1A). In the transwell assay, cell migration was found to increase by 20-40% when the cells were treated with 17-PT-PGE_2 for 12 h (Fig. 1B). At the concentration of 3 μM, 17-PT-PGE_2 caused maximal responses both in cell adhesion and migration assays.

We previously found that PGE_2 stimulates cell adhesion and migration in HCC cells by increasing FAK Y397 phosphorylation (12). To determine whether the EP1 receptor is involved in PGE_2-mediated FAK phosphorylation, Huh-7 cells were exposed to different concentrations of exogenous 17-PT-PGE_2 for different periods of time. As shown in Fig. 2A, an increase in FAK phosphorylation at the Y397 site was detected 15 min after 17-PT-PGE_2 treatment and the maximal response (~2-fold induction) was reached at 30 min post-treatment with 3 μM 17-PT-PGE_2 (Fig. 2B). Based on these findings, treatment with 3 μM 17-PT-PGE_2 for 30 min was used for subsequent experiments in Huh-7 cells. As shown in Fig. 2C, pre-treatment with sc-19220, a specific antagonist of the EP1 receptor, suppressed PGE_2-mediated upregulation of FAK Y397 phosphorylation.

To confirm the role of the EP1 receptor in the induction of FAK phosporylation, HEK293 cells were transfected with the EP1 receptor expression plasmid (EP1R-pcDNA3) and selected with G418 to obtain a stable expression cell culture. As illustrated in Fig. 2D, expression of the EP1 receptor did not alter the basal phosphorylation level of FAK. However, FAK phosphorylation was significantly upregulated in the EP1R-transfected cells when treated with PGE_2, compared with control cells. At the same time, pre-treatment with sc-19220 significantly decreased PGE_2-mediated FAK Y397 phosphorylation in EP1R-transfected cells.

PKC/c-Src signaling is involved in EP1-mediated FAK phosphorylation, cell adhesion and migration in Huh-7 cells. The PKC/c-Src pathway is reportedly involved in EP1-mediated cell migration (22). Therefore, the relationship between PKC/c-Src activation and FAK phosphorylation was examined in the present study. PKC activity and c-Src phosphorylation in response to 17-PT-PGE_2 were directly measured in Huh-7 cells. Treatment of Huh-7 cells with 17-PT-PGE_2 upregulated PKC activity >2-fold after 15 min and then decreased after 30 min (Fig. 3A). Pre-treatment of cells with the PKC inhibitor bisindolymaleimide I (Bis) significantly reduced the 17-PT-PGE_2-mediated FAK phosphorylation (Fig. 3B). At the same time, 17-PT-PGE_2 induced the phosphorylation of c-Src at Y416, which could be detected at 15 min after treatment and then decreased after 60 min (Fig. 3C). In addition, pre-treatment of cells with a c-Src inhibitor (PP2) diminished 17-PT-PGE_2-increased FAK phosphorylation significantly (Fig. 3D). In addition, pre-treatment of Huh-7 cells with Bis and PP2 completely blocked 17-PT-PGE_2-mediated cell adhesion and migration (Fig. 3E and F).

In order to clarify whether c-Src functions downstream of the 17-PT-PGE_2-induced PKC pathway, effects of the PKC activator phorbol-12-myristate-13-acetate (PMA) and inhibitor Bis on c-Src phosphorylation were examined. PMA treatment increased c-Src phosphorylation, as well as the FAK phosphorylation (Fig. 4A), while Bis significantly suppressed the 17-PT-PGE_2-induced c-Src phosphorylation (Fig. 4B).

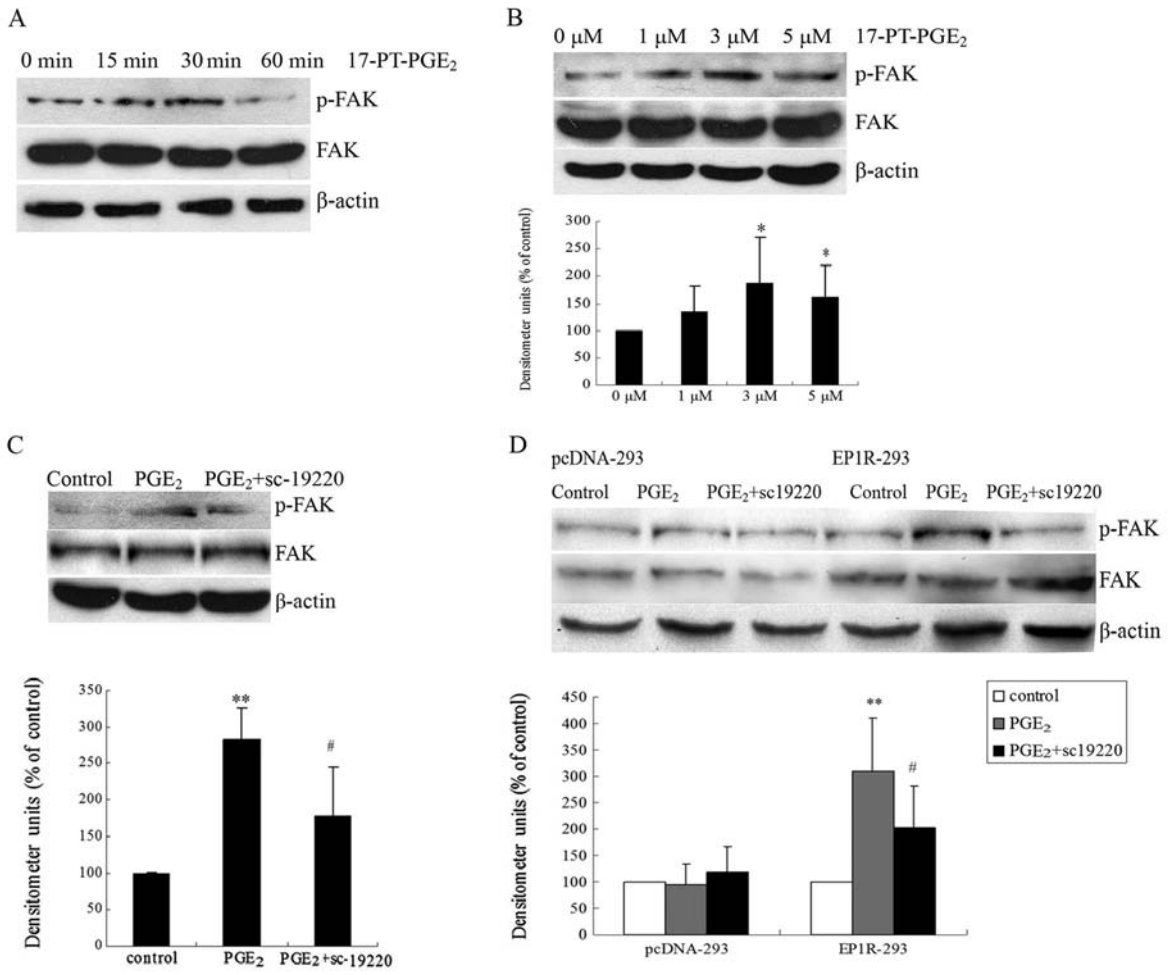

Figure 2. Effects of EP1 receptor on FAK Y397 phosphorylation in Huh-7 cells. (A) Effects of EP1 agonist on FAK phosphorylation at Y397 in Huh-7 cells cultured and treated with 3 μM exogenous 17-PT-PGE_2 for 0, 15, 30 and 60 min. (B) Effects of EP1 agonist 17-PT-PGE_2 treatment (0, 1, 3 or 5 μM for 30 min) on FAK Y397 phosphorylation in Huh-7 cells. FAK phosphorylation increased after stimulation with different concentrations of 17-PT-PGE_2 and the maximal response was reached at 30 min post-treatment with 3 μM 17-PT-PGE_2. (C) Effects of an EP1 antagonist on 17-PT-PGE_2-mediated FAK phosphorylation in Huh-7 cells. Cells were pre-treated with 10 μM sc-19220 for 30 min, followed by treatment with 3 μM 17-PT-PGE_2 for 30 min. (D) Effects of the EP1 receptor expression on 17-PT-PGE_2-mediated FAK phosphorylation in HEK293 cells. HEK293 cells ($3x10^5$ cells) were seeded in 6-well plates and cultured for 24 h before transfection with 2 μg of the EP1R-pcDNA3 plasmid or empty pcDNA3 plasmid as a control. After transfection, cells expressing the EP1 receptor were selected by 300 μg/ml G418. EP1 receptor-transfected HEK293 cells were exposed to 2 μM PGE_2 for 30 min, with or without sc-19220 pre-treatment. Equal amounts of total proteins were separated by SDS-PAGE and relative levels of phosphorylated FAK and total FAK expression were determined using anti-phospho-FAK and anti-FAK antibodies. β-actin was detected as a loading control. Densitometric quantitation of the above blots is shown. Results are presented as means ± SD from three different experiments. $^*P<0.05$, compared to control cells; $^{**}P<0.01$, compared to control cells; $^{\#}P<0.05$, compared to PGE_2-treated cells.

EGFR is involved in EP1-mediated FAK phosphorylation. In our previous studies, we found that the phosphorylation level of EGFR increases significantly after 17-PT-PGE_2 treatment in HCC cells (23). In the present study, FAK Y397 phosphorylation was found to increase after exposure of Huh-7 cells to exogenous EGF for 30 min (Fig. 5A). In addition, pre-treatment of cells with the EGFR inhibitor AG1478 significantly suppressed the 17-PT-PGE_2-induced FAK phosphorylation (Fig. 5B). As shown in Fig. 5C and D, pre-treatment with AG1478 mildly suppressed the 17-PT-PGE_2-mediated cell adhesion and completely blocked the 17-PT-PGE_2-mediated migration in Huh-7 cells. In order to clarify whether c-Src is involved in the 17-PT-PGE_2-induced EGFR/FAK pathway, the effect of AG1478 on c-Src phosphorylation was detected, but surprisingly it had no effect on 17-PT-PGE_2-mediated c-Src phosphorylation (Fig. 5E).

Discussion

Prostaglandin E_2 is one of the major products of cyclooxygenase-2 (COX-2), which has been shown to drive cancer cell growth and invasion in many cancer cells. Previous studies have indicated that COX-2 is overexpressed in many cancer tissues and that the level of PGE_2 is increased in COX-2 overexpressing cells (24-26). Endogenous and exogenous PGE_2 induce angiogenesis (27,28) and promote tumor cell growth, migration and invasion in colon cancer (13), renal cancer (16) and lung cancer (15). Selective COX-2 inhibitors were shown to suppress PGE_2 production (29,30) and reduce cell growth, migration and invasion (29-32).

PGE_2 exerts its functions through four subtypes of receptors expressed on the surface of tumor cells: EP1, EP2, EP3 and EP4 receptors (17). It is well accepted that the EP1

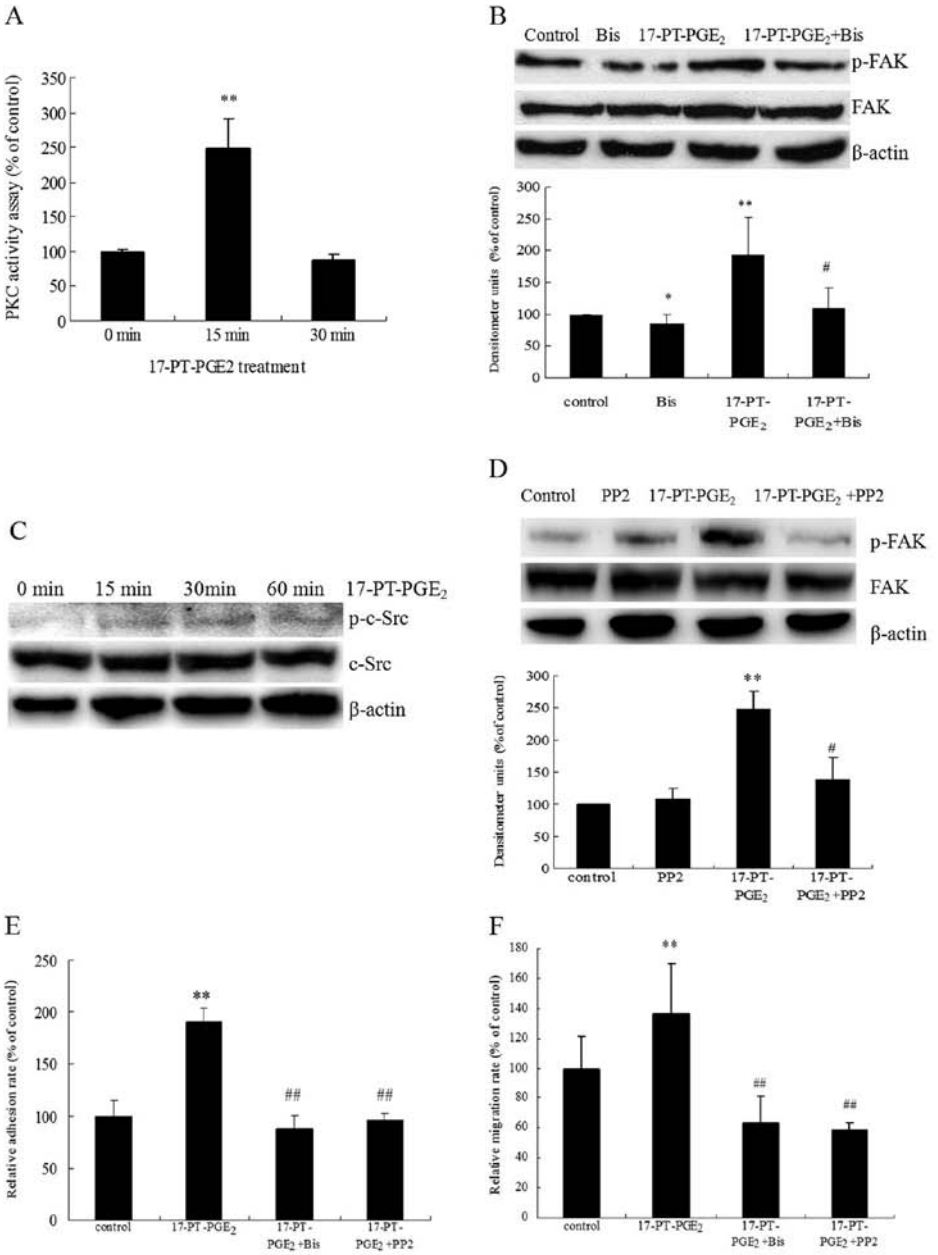

Figure 3. Roles of PKC and c-Src pathways in 17-PT-PGE$_2$-mediated FAK phosphorylation in Huh-7 cells. (A) PKC activity assay. Huh-7 cells were treated with 3 μM 17-P-T-PGE$_2$ for 0, 15 or 30 min. Equal amounts of total proteins (30 μg) were added to microcentrifuge tubes and assayed for PKC levels using a direct human PKC enzyme activity assay kit. (B) Effects of PKC inhibitor on 17-PT-PGE$_2$-mediated FAK phosphorylation in Huh-7 cells treated with 3 μM 17-PT-PGE$_2$ for 30 min, with or without pre-treatment of 5 μM Bis for 1 h. Equal amounts of total proteins were separated by SDS-PAGE. Relative levels of phosphorylated FAK and total FAK expression were determined using anti-phospho-FAK and anti-FAK antibodies. β-actin was detected as a loading control. Densitometric quantitation of the above blots is shown. (C) Effects of 17-PT-PGE$_2$ on c-Src phosphorylation at Y416 in Huh-7 cells treated with 3 μM exogenous 17-PT-PGE$_2$ for 0, 15, 30 or 60 min. Equal amounts of total proteins were separated by SDS-PAGE. Relative levels of phosphorylated c-Src and total c-Src expression were determined using anti-phospho-c-Src and anti-c-Src antibodies. β-actin was detected as a loading control. These experiments were performed three times with similar results. (D) Effects of c-Src inhibitor on 17-PT-PGE$_2$-mediated FAK phosphorylation in Huh-7 cells treated with 3 μM 17-P-T-PGE$_2$ for 30 min, with or without pre-treatment of 10 μM PP2 for 1 h. Equal amounts of total proteins were separated by SDS-PAGE. Relative levels of phosphorylated FAK and total FAK expression were determined using anti-phospho-FAK and anti-FAK antibodies. β-actin was detected as a loading control. Densitometric quantitation of the above blots is shown. (E) Effects of PKC or c-Src inhibitors on 17-PT-PGE$_2$-mediated cell adhesion in Huh-7 cells. The cell adhesion assay was performed in 96-well plates. Huh-7 cells were treated with 3 μM 17-P-T-PGE$_2$ for 3 h, with or without pre-treatment with 5 μM Bis or 10 μM PP2 for 1 h. The attached cells were stained with WST and quantified by reading absorbance at 450 nm. (F) Effects of PKC or c-Src inhibitors on 17-PT-PGE$_2$-mediated cell migration in Huh-7 cells. The cell migration assay was performed in 12-well transwells. Huh-7 cells were treated with 3 μM 17-PT-PGE$_2$ for 12 h, with or without pre-treatment of 5 μM Bis or 10 μM PP2 for 1 h. Cells on the lower surface were stained with 0.1% crystal violet, solubilized with acetic acid solution and quantified by measuring absorbance at 570 nm. Results are presented as means ± SD from three different experiments. *P<0.05, compared to control cells; **P<0.01, compared to control cells; $^{\#}$P<0.05, compared to cells with 17-PT-PGE$_2$ treatment; $^{\#\#}$P<0.01, compared to 17-PT-PGE$_2$-treated cells.

receptor promotes development and progression of many cancers (20). For example, expression of the EP1 receptor is frequently observed both in the cytoplasm and/or in the nuclear membrane in many cancer cells (21,33,34). BK5.EP1 transgenic mice produce more skin tumors than wild-type (WT) mice (35). Furthermore, the EP1 receptor level is associ-

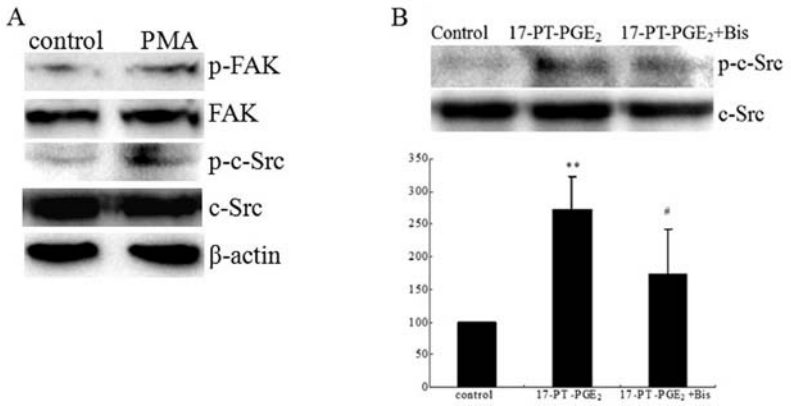

Figure 4. Role of PKC pathway in 17-PT-PGE_2-mediated c-Src phosphorylation in Huh-7 cells. (A) Effects of PKC activator on c-Src and FAK phosphorylation in Huh-7 cells treated with PMA (100 nm) for 30 min. Equal amounts of total proteins were separated by SDS-PAGE. Relative levels of phosphorylated and total c-Src or FAK were determined using specific antibodies. β-actin was detected as a loading control. These experiments were performed three times with similar results. (B) Effects of PKC inhibitor on 17-PT-PGE_2-mediated c-Src phosphorylation in Huh-7 cells treated with 3 μM 17-P-T-PGE_2 for 15 min, with or without pre-treatment with 5 μM Bis for 30 min. Equal amounts of total proteins were separated by SDS-PAGE. Relative levels of phosphorylated c-Src and total c-Src expression were determined using anti-phospho-c-Src and anti-c-Src antibodies. Results are presented as means ± SD from three different experiments. **P<0.01, compared to control cells; $^{\#}$P<0.05, compared with 17-PT-PGE_2-treated cells.

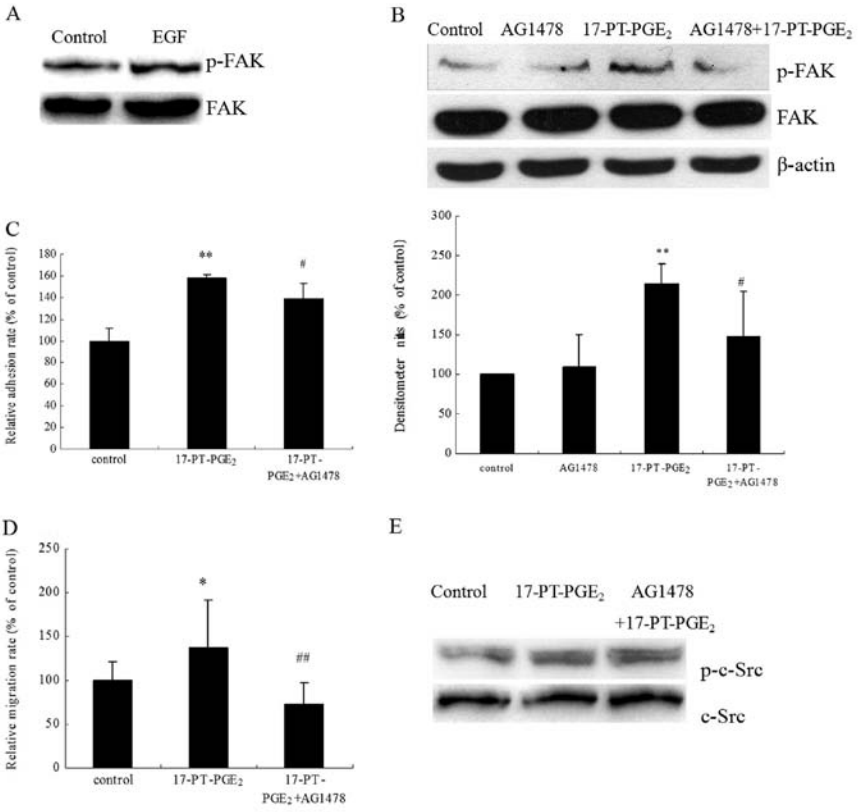

Figure 5. Role of EGFR pathway in 17-PT-PGE_2-mediated FAK phosphorylation in Huh-7 cells. (A) Effect of EGF on FAK phosphorylation in Huh-7 cells treated with recombinant human EGF (25 μg/ml) for 30 min. Equal amounts of total proteins were separated by SDS-PAGE. Relative levels of phosphorylated FAK and total FAK expression were determined using antiphospho-FAK and anti-FAK antibodies. These experiments were performed three times with similar results. (B) Effects of EGFR inhibitor on 17-PT-PGE_2-mediated FAK phosphorylation in Huh-7 cells treated with 3 μM 17-P-T-PGE_2 for 30 min, with or without pre-treatment of 2.5 μM AG1478 for 1 h. Equal amounts of total proteins were separated by SDS-PAGE. Relative levels of phosphorylated FAK and total FAK expression were determined using anti-phospho-FAK and anti-FAK antibodies. β-actin was detected as a loading control. Densitometric quantitation of the above blots is shown. (C) Effects of EGFR inhibitor on 17-PT-PGE_2-mediated cell adhesion in Huh-7 cells. The cell adhesion assay was performed in 96-well plates. Huh-7 cells were treated with 3 μM 17-PT-PGE_2 for 3 h, with or without pre-treatment with 2.5 μM AG1478 for 1 h. Attached cells were stained with WST and quantified by measuring absorbance at 450 nm. (D) Effects of EGFR inhibitor on 17-PT-PGE_2-mediated cell migration in Huh-7 cells. The cell migration assay was performed in 12-well transwells. Huh-7 cells were treated with 3 μM 17-PT-PGE_2 for 12 h, with or without pre-treatment with 2.5 μM AG1478 for 1 h. Cells on the lower surface were stained with 0.1% crystal violet, solubilized with an acetic acid solution and quantified by measuring the absorbance at 570 nm. (E) Effects of EGFR inhibitor on 17-PT-PGE_2-mediated c-Src phosphorylation in Huh-7 cells treated with 3 μM 17-PT-PGE_2 for 15 min, with or without pre-treatment with 2.5 μM AG1478 for 30 min. Equal amounts of total proteins were separated by SDS-PAGE. Relative levels of phosphorylated c-Src and total c-Src expression were determined using anti-phospho-c-Src and anti-c-Src antibodies. These experiments were performed three times with similar results. Results are presented as means ± SD from three different experiments. *P<0.05, compared to control cells; **P<0.01, compared to control cells; $^{\#}$P<0.05, compared with 17-PT-PGE_2-treated cells; $^{\#\#}$P<0.01, compared with 17-PT-PGE_2-treated cells.

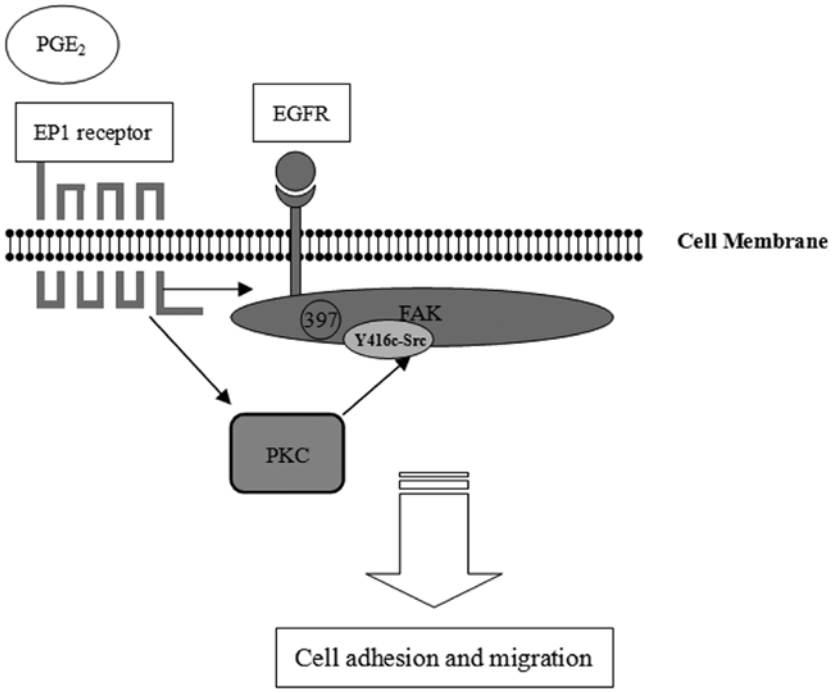

Figure 6. Proposed mechanisms for PGE_2/EP1 receptor-mediated hepatocellular carcinoma cell adhesion and migration. Our data showed that the EP1 receptor played a key role in PGE_2-mediated hepatocellular carcinoma cell adhesion and migration. EP1 receptor may upregulate FAK phosphorylation at Y397 to improve cell adhesion and migration. PKC/c-Src and EGFR signaling pathways were both involved in EP1 receptor-mediated FAK phosphorylation. The PGE_2/EP1/PKC/c-Src and PGE_2/EP1/EGFR signaling pathway may coordinately regulate FAK activation, cell adhesion and migration in HCC.

ated with cell migration or invasion in colon cancer cells (18), chondrosarcoma cells (22) and oral cancer cells (36).

FAK is a non-receptor, cytoplasmic protein tyrosine kinase that appears to play a central role in integrin-mediated signal transduction. Overexpression of FAK enhances cell proliferation (8,37); while FAK depletion impairs cell adhesion or migration in colon cancer (38), breast cancer cells (7) and melanoma cells (39). Recently, FAK mRNA and protein, as well as phosphorylated FAK Tyr397, were found to be overexpressed both in HCC clinical samples and HCC cell lines (10,11). Increased FAK and phosphorylated FAK Tyr397 expression levels have been correlated with tumor stage, vascular invasion and intrahepatic metastasis in HCC (11).

In our previous study, we found that PGE_2 improves cell adhesion, migration and invasion in HCC cells. FAK plays an important role in PGE_2-mediated cell adhesion and migration, as exogenous PGE_2 was shown to greatly increase phosphorylation of FAK at the Y397 site in a PGE_2 concentration-dependent manner. RNA interference targeting FAK can suppress PGE_2-mediated cell adhesion and migration significantly (12). In the present study, 17-PT-PGE_2 treatment mimicked the effects of PGE_2 and promoted cell adhesion and migration in HCC cells. Therefore, we hypothesize that PGE_2 upregulates FAK phosphorylation via the EP1 receptor in HCC cells.

Autophosphorylation at Y397 is required for FAK activation (40). To date, very little is known about the effects of EP1 receptor on FAK Y397 activation and molecular mechanisms mediating these effects. In our previous study, FAK phosphorylation and expression were found to be upregulated by PGE_2 in HCC cells (12). In the present study, 17-PT-PGE_2 treatment caused upregulation of FAK Y397 phosphorylation in Huh-7 cells. Expression of the EP1 receptor in HEK293 cells mimicked the effect of 17-PT-PGE_2 treatment. In addition, pre-treatment with sc-19220, the specific antagonist of the EP1 receptor, suppressed PGE_2-mediated upregulation of FAK Y397 phosphorylation. Our data suggest that the EP1 receptor is involved in PGE_2-mediated activation of FAK. Because FAK is increasingly recognized as a critical factor in the development of HCC (11), the present findings suggest that the EP1 receptor may possibly be explored as a drug target to prevent the phosphorylation of FAK Y397 in the prevention and treatment of liver cancer.

PKC has been reported to be involved in the EP1 receptor signaling pathway (17). The PKC family was first identified as intracellular receptors for the tumor promoting phorbol esters (41). Activation of PKC by calcium ions and the second messenger diacylglycerol is thought to play a central role in the induction of cellular responses to a variety of ligand-receptor systems and in the regulation of cellular responsiveness to external stimuli (41,42). PKC was found to be associated with the development of HCC. For example, mRNA levels of several PKC isoforms were shown to be significantly increased in HCC samples, as compared to the corresponding non-cancerous liver tissues. PKCα expression is also significantly correlated with tumor size and the tumor, node and metastasis (TNM) stage (43). Meanwhile, reduction of PKC expression by RNA interference or selective inhibitors greatly decreases cell proliferation, migration and invasion in HCC cells (44).

Our data show that PKC activities were enhanced after 17-PT-PGE_2 treatment for 15 min, while the selective PKC inhibitor, Bis, completely blocked 17-PT-PGE_2-mediated cell adhesion and migration. These results support that PKC is

involved in EP1 receptor-mediated cell adhesion and migration in HCC cells. The involvement of PKC in EP1 receptor-mediated FAK phosphorylation was further confirmed by use of PMA, a selective PKC activator, which promoted the phosphorylation of FAK at Y397 in Huh-7 cells. In addition, Bis pre-treatment diminished the 17-PT-PGE_2-mediated FAK phosphorylation.

Phosphorylation of FAK Y397 was determined to create a high-affinity binding site for SH2 domains of Src family kinases to form a FAK/Src complex. A major function of FAK is to recruit and activate Src at cell-extracellular matrix adhesion sites (40). However, FAK Y397 is not strictly an autophosphorylation site and signal amplification can also result from phosphorylation of this site by Src (45). Therefore, the effect of c-Src on EP1 receptor-induced FAK Y397 phosphorylation in Huh-7 cells was further evaluated in this study.

C-Src activity is regulated by tyrosine phosphorylation at two distinct sites, Y416 and Y527 and the active state of c-Src is p-Y416-c-Src (46). Our results showed that c-Src Y416 phosphorylation was enhanced after 17-PT-PGE_2 treatment for 15 min. The selective Src inhibitor PP2, caused a significant reduction in 17-PT-PGE_2-mediated FAK Y397 phosphorylation. PP2 pre-treatment completely blocked 17-PT-PGE_2-mediated cell adhesion and migration. Furthermore, PMA treatment increased c-Src phosphorylation, while Bis partly suppressed the 17-PT-PGE_2-induced c-Src phosphorylation. These data suggest that the PKC/c-Src signal pathway is involved in EP1 receptor-mediated FAK activation, cell adhesion and migration in HCC cells.

EGFR has been widely accepted to improve cell growth and invasion in many cancer cell types (47). Several studies in the last decade have shown that the EP receptor pathway also modulates activation of the EGFR (48). In 2006, Han *et al* revealed a novel crosstalk between the EP1 and EGFR signaling pathways that synergistically promote cancer cell growth and invasion. The association of EP1 with EGFR was found by immunoprecipitation after HCC cells were treated with PGE_2 or EP1 agonist (21). Of interest, EGFR activation was found to be sufficient to induce activation of the Src-FAK pathway in tumor cells (49,50). As the EGFR/Src/FAK pathway has been implicated in cell growth, migration and invasion in various cancer cells (49-51), the involvement of this signaling pathway in EP1 receptor-mediated cell adhesion and migration was explored in this study.

In our previous study, we found that the phosphorylation level of EGFR increases significantly after 17-PT-PGE_2 treatment in HCC cells (23). In the present study, exogenous EGF was observed to increase FAK Y397 phosphorylation. The selective EGFR inhibitor, AG1478, greatly suppressed 17-PT-PGE_2-induced FAK Y397 phosphorylation. At the same time, AG1478 pre-treatment mildly suppressed 17-PT-PGE_2-mediated cell adhesion and completely blocked 17-PT-PGE_2-mediated cell migration. Surprisingly, AG1478 had no effect on 17-PT-PGE_2-mediated c-Src Y416 phosphorylation. Our results suggest that EGFR is also associated with EP1 receptor-mediated FAK activation, while c-Src may be not involved in EGFR-induced FAK activation in HCC cells.

In conclusion, this study demonstrated that the PGE_2 EP1 receptor upregulates FAK phosphorylation at Y397 to improve cell adhesion and migration. PKC/c-Src and EGFR signaling pathways are both involved in EP1 receptor-mediated FAK phosphorylation. The PGE_2/EP1/PKC/c-Src and PGE_2/EP1/EGFR signaling pathway may coordinately regulate FAK activation, cell adhesion and migration in HCC (Fig. 6). In this regard, our present findings provide important new information regarding the putative role of the EP1 receptor in FAK phosphorylation in HCC cells and suggest that targeting the PGE_2/EP1/FAK signal pathway may provide new therapeutic strategies for both the prevention and treatment of this malignant disease.

Acknowledgements

We thank Dr Kathy Mccusker (Merck Frosst Centre for Therapeutic Research, Canada) for providing the pcDNA3 plasmid construct encoding the EP1 receptor. This study was supported in part by the National Natural Science Foundation of China (81101496, 81172003), Research Fund for the Doctoral Program of Higher Education of China (20113234120009) and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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