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# Prostaglandin E<sub>2</sub> stimulates $\beta$ 1-integrin expression in hepatocellular carcinoma through the EP1 receptor/PKC/NF- $\kappa$ B pathway

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Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) has been implicated in cell invasion in hepatocellular carcinoma (HCC), via increased  $\beta$ 1-integrin expression and cell migration; however, the mechanism remains unclear. PGE<sub>2</sub> exerts its effects via four subtypes of the E prostanoid receptor (EP receptor 1–4). The present study investigated the effect of EP1 receptor activation on  $\beta$ 1-integrin expression and cell migration in HCC. Cell migration increased by 60% in cells treated with 17-PT-PGE<sub>2</sub> (EP1 agonist), which was suppressed by pretreatment with a  $\beta$ 1-integrin polyclonal antibody. PGE<sub>2</sub> increased  $\beta$ 1-integrin expression by approximately 2-fold. EP1 receptor transfection or treatment with 17-PT-PGE<sub>2</sub> mimicked the effect of PGE<sub>2</sub> treatment. EP1 siRNA blocked PGE<sub>2</sub>-mediated  $\beta$ 1-integrin expression. 17-PT-PGE<sub>2</sub> treatment induced PKC and NF- $\kappa$ B activation; PKC and NF- $\kappa$ B inhibitors suppressed 17-PT-PGE<sub>2</sub>-mediated  $\beta$ 1-integrin expression. FoxC2, a  $\beta$ 1-integrin transcription factor, was also upregulated by 17-PT-PGE<sub>2</sub>. NF- $\kappa$ B inhibitor suppressed 17-PT-PGE<sub>2</sub>-mediated FoxC2 upregulation. Immunohistochemistry showed p65, FoxC2, EP1 receptor and  $\beta$ 1-integrin were all highly expressed in the HCC cases. This study suggested that PGE<sub>2</sub> upregulates  $\beta$ 1-integrin expression and cell migration in HCC cells by activating the PKC/NF- $\kappa$ B signaling pathway. Targeting PGE<sub>2</sub>/EP1/PKC/NF- $\kappa$ B/FoxC2/ $\beta$ 1-integrin pathway may represent a new therapeutic strategy for the prevention and treatment of this cancer.

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer death in the United States and worldwide, especially in males<sup>1,2</sup>. Recent cases of HCC are increasing in United States and Canada<sup>2</sup>. Although a combination of resection and chemotherapy can improve survival, HCC prognosis is still extremely poor, especially in advanced HCC, which is often associated with malignant migration and metastasis<sup>3</sup>.

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), one of most important products of cyclooxygenase-2 (COX-2), has been proposed as an important cellular factor associated with tumor development in many types of cancers<sup>4–7</sup>. Previous studies indicated that COX-2 expression was upregulated in many cancer tissues and that exogenous PGE<sub>2</sub> increased cancer cell growth, migration and invasion<sup>5–8</sup>. In hepatocellular carcinoma, PGE<sub>2</sub> was reported to activate Akt and FAK signaling pathways to promote cell proliferation and migration<sup>8,9</sup>, and to upregulate MMP-2 expression to promote cell invasion<sup>10</sup>. New targets aimed at cellular COX-2/PGE<sub>2</sub> signaling pathways have provided therapeutic strategies for the treatment of metastasis of HCC<sup>11</sup>.

Integrins are a family of transmembrane cellular receptors that mediate cell-cell and cell-matrix interactions. They are heterodimeric glycoproteins, serve as adhesion receptors for ECM proteins and also transduce biochemical signals into the cell. These receptors are composed of an  $\alpha$  and a  $\beta$  subunit. Integrins of the  $\beta$ 1-family mainly transduce signals from the extracellular matrix to modulate growth, differentiation, invasion or metastasis<sup>12</sup>.  $\beta$ 1-integrin has been implicated in cell proliferation, adhesion and metastasis in a wide variety of human cancers, including breast, colon and ovary<sup>13–16</sup>. In HCC,  $\beta$ 1-integrin is necessary for cell migration<sup>17</sup> and protects



tumor cells from chemotherapy-induced apoptosis<sup>18</sup>. Recently,  $\beta$ 1-integrin was identified as a suitable marker in HCC identification, classification, prevention and treatment<sup>19,20</sup>.

In Huh-7 cells, PGE<sub>2</sub> increased  $\beta$ 1-integrin expression and promoted cell adhesion and migration<sup>10</sup>. However, the exact mechanism remains largely unknown. PGE<sub>2</sub> regulates tumor development and progression by combining with E prostanoid receptors (EP receptors) on the surface of the cell membrane<sup>21</sup>. Our data showed that the EP1 receptor plays a major role in PGE<sub>2</sub>-mediated  $\beta$ 1-integrin expression. The current study suggested that PGE<sub>2</sub> regulates  $\beta$ 1-integrin expression and cell migration in HCC cells through the EP1 receptor, and the PKC/NF- $\kappa$ B/FoxC2 signaling pathway may be involved in EP1 receptor-mediated  $\beta$ 1-integrin upregulation.

## Results

**The EP1 receptor is involved in PGE<sub>2</sub>-mediated  $\beta$ 1-integrin expression and cell migration in HCC cells.** Huh-7 cells were treated with EP1, EP2, EP3 and EP4 receptor agonists. Fig. 1A showed that treatment with butaprost (EP2 agonist), sulprostone (EP3 agonist) and PGE<sub>1</sub> alcohol (EP4 agonist), respectively, had little or no effect on  $\beta$ 1-integrin expression. By contrast, treatment with 17-PT-PGE<sub>2</sub>, a specific agonist of EP1 receptor, significantly enhanced  $\beta$ 1-integrin expression. Pretreatment with antagonists of EP receptors in Huh-7 cells showed mild effects on PGE<sub>2</sub>-mediated  $\beta$ 1-integrin upregulation, except for treatment with sc-19220, a specific antagonist of the EP1 receptor, which markedly blocked PGE<sub>2</sub>-mediated  $\beta$ 1-integrin upregulation (Fig. 1B).

To corroborate the role of the EP1 receptor in the induction of  $\beta$ 1-integrin expression, HEK293 cells were transfected with the EP1R-pcDNA3. Fig. 1C showed that expression of the EP1 receptor did not alter the basal expression level of the  $\beta$ 1-integrin protein. However,  $\beta$ 1-integrin expression was significantly upregulated in the EP1R-transfected cells (compared with the control cells) when treated with PGE<sub>2</sub>. The PGE<sub>2</sub>-induced  $\beta$ 1-integrin expression was diminished by the addition of sc-19220 in EP1R-transfected cells.

To further study the specific role of EP1 in  $\beta$ 1-integrin expression, Huh-7 cells were transfected with an EP1R siRNA. As shown in Fig. 1D, depletion of the EP1 receptor greatly reduced the basal level of  $\beta$ 1-integrin protein. PGE<sub>2</sub> induced  $\beta$ 1-integrin expression was completely blocked in the EP1R siRNA-transfected cells.

To demonstrate if  $\beta$ 1-integrin was involved in EP1 receptor-mediated cell migration in HCC cells, Huh-7 cells were pretreated with a  $\beta$ 1-integrin polyclonal antibody (AB1952P) for 30 min, followed by the incubation with 17-PT-PGE<sub>2</sub>. Cell migration was increased by 60% when treated with 17-PT-PGE<sub>2</sub>. The pretreatment by the  $\beta$ 1-integrin polyclonal antibody (3  $\mu$ g/ml) significantly inhibited 17-PT-PGE<sub>2</sub>-mediated cell migration (Fig. 1E). These data indicated that  $\beta$ 1-integrin plays an important role in EP1 receptor-mediated cell migration in HCC cells, and PGE<sub>2</sub> increased  $\beta$ 1-integrin expression and promoted cell migration via the EP1 receptor in Huh-7 cells.

We went on to detect the correlation between the expression of the EP1 receptor and  $\beta$ 1-integrin in liver cancer tissues. By immunohistochemistry, all 24 samples showed positive EP1 receptor expression in the cytoplasm and membrane. Of the 20 HCC cases tested,  $\beta$ 1-integrin expression was mainly found in the membrane of the cancer cells. The samples expressing higher levels of EP1 receptor also displayed higher levels of  $\beta$ 1-integrin expression; the normal liver tissue samples with lower levels of EP1 receptor showed lower or even negative expression of  $\beta$ 1-integrin (Fig. 2).

### PKC is involved in EP1 receptor-mediated $\beta$ 1-integrin expression.

The relationship between PKC activation and  $\beta$ 1-integrin expression was examined in the present study. PKC activity in response to 17-PT-PGE<sub>2</sub> treatment was measured in Huh-7 cells. Treatment of Huh-7 cells with 17-PT-PGE<sub>2</sub> for 15 min resulted in an approximately 2-fold increase in PKC activity, reaching a maximal

response (approximately 6-fold) after 20 min of treatment (Fig. 3A). PKC activator phorbol-12-myristate-13-acetate (PMA) markedly increased  $\beta$ 1-integrin expression in Huh-7 cells (Fig. 3B). Pre-treatment of cells with the PKC inhibitor rottlerin significantly reduced the 17-PT-PGE<sub>2</sub>-mediated  $\beta$ 1-integrin expression (Fig. 3C). Similarly, pre-treatment of cells with rottlerin diminished 17-PT-PGE<sub>2</sub>-increased  $\beta$ 1-integrin expression in EP1R-transfected HEK293 cells (Fig. 3D). In addition, rottlerin inhibited the EP1 receptor-mediated cell migration (Fig. 3E) in Huh-7 cells.

### NF- $\kappa$ B is involved in EP1 receptor-mediated $\beta$ 1-integrin expression.

To examine whether NF- $\kappa$ B activation is involved in EP1-induced  $\beta$ 1-integrin expression in HCC cells, we detected the phosphorylation of p65 and its upstream molecules regarding EP1 receptor activation. Huh-7 cells were exposed to 17-PT-PGE<sub>2</sub> for different periods of time. As shown in Fig. 4A, an increase in p65 phosphorylation at the Ser536 site was detected after 17-PT-PGE<sub>2</sub> treatment, reaching its peak 120 min after treatment. For the upstream molecules of p65, I $\kappa$ B- $\alpha$  phosphorylation at the Ser32/36 site was upregulated 30 min after 17-PT-PGE<sub>2</sub> treatment. A similar response was found in EP1R-transfected HEK293 cells: in Fig. 4B, the increase in I $\kappa$ B- $\alpha$  and p65 phosphorylation were detected after PGE<sub>2</sub> treatment. Furthermore, p65 translocation was detected by immunofluorescence. As shown in Fig. 4C, Huh-7 cells were exposed to 17-PT-PGE<sub>2</sub> treatment for different periods of time. Normally, p65 was located in the cytoplasm; after 17-PT-PGE<sub>2</sub> treatment for 120 min, activated p65 was translocated into the nuclei. To clarify the role of the NF- $\kappa$ B pathway in EP1 receptor-mediated  $\beta$ 1-integrin expression in HCC cells, an NF- $\kappa$ B inhibitor, Ammonium pyrrolidinedithiocarbamate (PDTC), was added to Huh-7 cells. Pretreatment with PDTC inhibited the 17-PT-PGE<sub>2</sub>-mediated  $\beta$ 1-integrin expression (Fig. 4D) and cell migration (Fig. 4E) in Huh-7 cells.

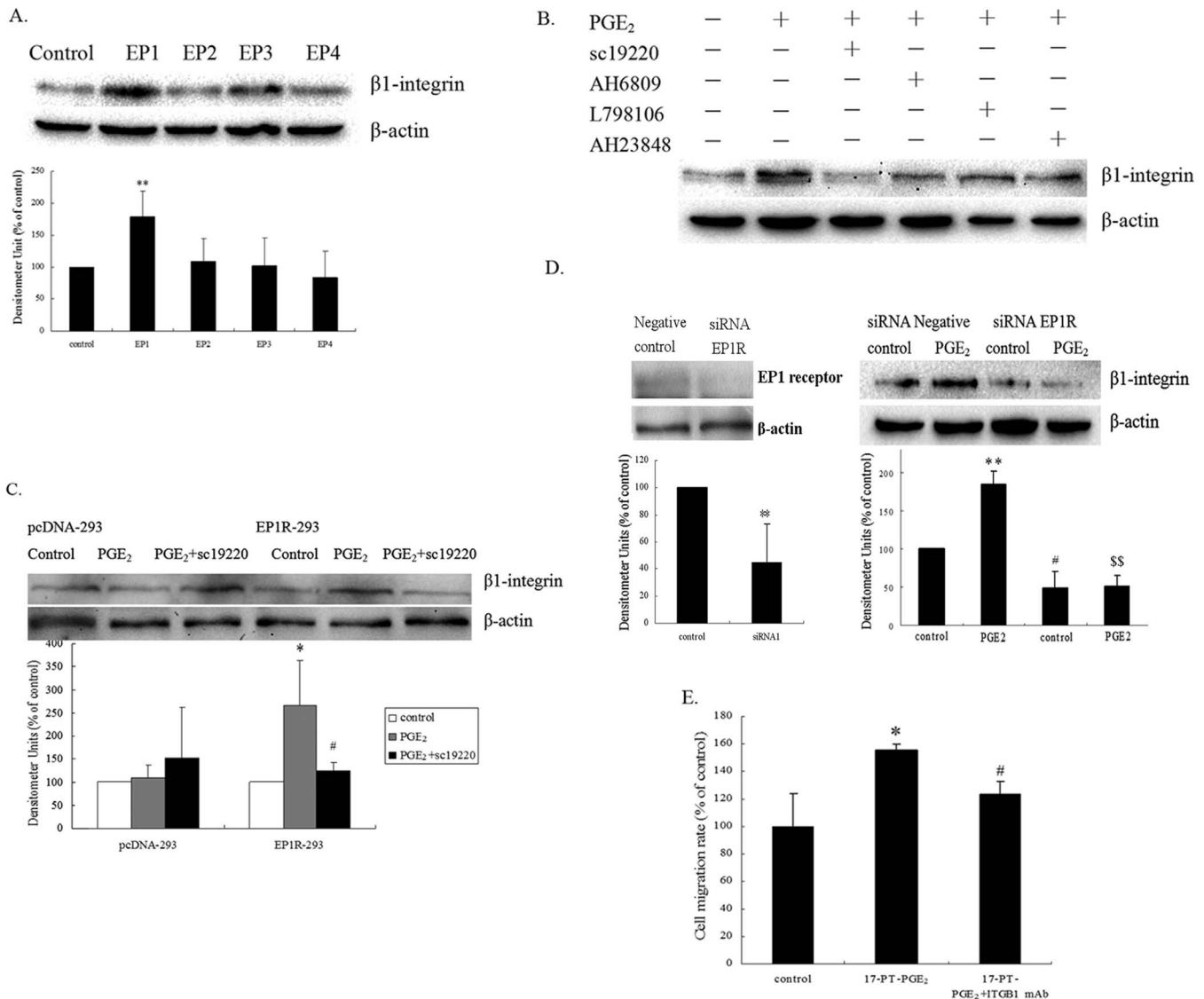
### FoxC2 is involved in the NF- $\kappa$ B signal pathway in EP1 receptor-mediated $\beta$ 1-integrin expression.

To investigate the direct involvement of the NF- $\kappa$ B pathway in the induction of  $\beta$ 1-integrin expression, we analyzed the sequences of human integrin  $\beta$ 1 promoter in detail. However, the 2.0-kb fragment upstream of the start codon does not contain any NF- $\kappa$ B transcription factor-binding elements (<http://genome.ucsc.edu/cgi-bin/hgTracks>). Thus, NF- $\kappa$ B regulation of  $\beta$ 1-integrin expression may not act by direct binding to its promoter. There must be another transcription factor that binds to the  $\beta$ 1-integrin promoter directly to upregulate its expression. Transcriptional regulatory elements are located in chr10:33,239,813–33,330,260. In addition, we identified many Fox-binding elements (FBEs) in the promoter of  $\beta$ 1-integrin. (<http://genome.ucsc.edu/cgi-bin/hgGateway>). Recently, the forkhead transcription factor FoxC2 was reported to upregulate  $\beta$ 1-integrin expression by directly binding FBEs in the integrin  $\beta$ 1 promoter<sup>22</sup>. We detected the role of FoxC2 in the EP1 receptor/NF- $\kappa$ B pathway in Huh-7 cells. 17-PT-PGE<sub>2</sub> treatment increased FoxC2 expression significantly, while PDTC inhibited 17-PT-PGE<sub>2</sub>-mediated FoxC2 upregulation completely (Fig. 5A). Thus, the EP1 receptor/NF- $\kappa$ B signal pathway may upregulate  $\beta$ 1-integrin expression by promoting FoxC2 expression.

To observe the effects of FoxC2 and NF- $\kappa$ B on EP1 receptor-mediated  $\beta$ 1-integrin expression in HCC tissues, the sections were incubated with anti-p65 and FoxC2 antibodies. By immunohistochemistry, all 20 HCC cases showed positive p65 expression, mainly in the cytoplasm. FoxC2 was expressed mainly in the cytoplasm and some in nuclei (arrow). Serial sections showed highly expression of both p65, FoxC2 and EP1 receptor (Fig. 5B).

## Discussion

COX-2-mediated production of PGE<sub>2</sub> is involved in cell growth and metastasis of various cancers<sup>4,8,23–25</sup>. Integrins are a family of cell



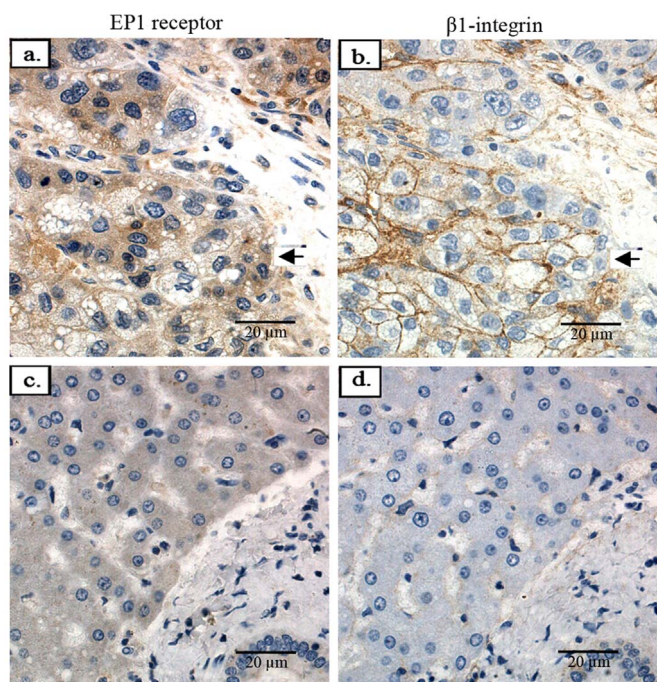
**Figure 1** | EP1 receptor activation promoted  $\beta 1$ -integrin expression in hepatocellular carcinoma cells. (A). Effects of EP agonists on  $\beta 1$ -integrin expression in Huh-7 cells. Huh-7 cells were exposed to 5  $\mu$ M EP1 agonist (17-PT-PGE<sub>2</sub>), EP2 agonist (butaprost), EP3 agonist (sulprostone) and EP4 agonist (PGE1 alcohol) for 24 h, respectively. The cropped gels are used and full-length gels are presented in Supplementary Figure S1 and S2. (B). Effects of EP antagonists on PGE<sub>2</sub>-mediated  $\beta 1$ -integrin expression in Huh-7 cells. Huh-7 cells were pretreated with various EP antagonists for 1 h, followed by PGE<sub>2</sub> for 24 h (EP1 antagonist sc19220, EP2 antagonist AH6809 and EP3 antagonist L-798106, EP4 antagonist AH23848). The cropped gels are used and full-length gels are presented in Supplementary Figure S3 and S4. (C). Effects of expression of the EP1 receptor on PGE<sub>2</sub>-mediated  $\beta 1$ -integrin regulation in HEK293 cells. HEK293 cells ( $3 \times 10^5$  cells) were transfected with EP1R-pcDNA3 plasmid or empty pcDNA3 plasmid as a control. After transfection, cells expressing the EP1 receptor were selected by G418. EP1 receptor-transfected HEK293 cells were exposed to PGE<sub>2</sub> for 24 h, with or without sc19220 pre-treatment. Results are presented as the mean  $\pm$  SD from three different experiments. \* $P < 0.05$ , compared to control cells; # $P < 0.05$ , compared with PGE<sub>2</sub>-treated cells. (D). RNA interference targeting the EP1 receptor suppressed PGE<sub>2</sub>-mediated  $\beta 1$ -integrin upregulation in Huh-7 cells. Huh-7 cells were transfected with an EP1R-siRNA. After 72 h, the cells were exposed to PGE<sub>2</sub> for 24 h. The cropped gels are used and full-length gels are presented in Supplementary Figure S5 and S6. Results are shown as the mean  $\pm$  SD from three different experiments. \*\* indicates a significant difference at  $P < 0.01$  compared with the cells without PGE<sub>2</sub> treatment; # indicates a significant difference at  $P < 0.05$  compared with the siRNA negative control cells. \$\$ indicates a significant difference at  $P < 0.01$  compared with the siRNA negative control cells after PGE<sub>2</sub> treatment. (E). Effect of anti- $\beta 1$ -integrin antibody on 17-PT-PGE<sub>2</sub>-mediated cell migration in Huh-7 cells. The cell migration assay was performed in a 12-well transwell. Huh-7 cells were pretreated with an anti- $\beta 1$ -integrin antibody for 30 min, followed by stimulation with PGE<sub>2</sub>. The in vitro migration activity was measured after 24 h. Results are presented as the mean  $\pm$  SD from three different experiments. \* $P < 0.05$ , compared with control cells; # $P < 0.05$ , compared with 17-PT-PGE<sub>2</sub>-treated group. The gels have been run under the same experimental conditions.

surface receptors for extracellular matrix (ECM) proteins<sup>26,27</sup>. Among them,  $\beta 1$ -integrin-mediated attachment to the ECM results in an activation of protein tyrosine kinases that protect cells from chemotherapy-induced apoptosis<sup>28</sup>.  $\beta 1$ -integrin was highly expressed in liver cancer tissue and mice model<sup>20,29</sup>. PGE<sub>2</sub> improves  $\beta 1$ -integrin expression in

many cells<sup>30–33</sup>. However, the mechanism of PGE<sub>2</sub>-mediated  $\beta 1$ -integrin expression remained unknown in HCC cells.

PGE<sub>2</sub> exerts its effects by coupling to four subtypes of the EP receptor<sup>21,34</sup>. Among the four receptor types, the EP1 receptor was shown to play an important role in the development of many cancer





**Figure 2 | The expression of EP1 receptor and  $\beta$ 1-integrin in liver cancer tissues.** (a). Representative immunohistochemical images of human hepatocellular carcinoma tissue stained with the anti-human EP1 receptor antibody. (b). Representative immunohistochemical images of human hepatocellular carcinoma tissue stained with the anti-human  $\beta$ 1-integrin antibody. (c). Representative immunohistochemical images of normal human liver tissue stained with the anti-EP1 receptor antibody. (d). Representative immunohistochemical images of normal human liver tissue stained with the anti- $\beta$ 1-integrin antibody. (Magnification:  $\times 400$ ).

types: EP1 receptor activated PKC/c-Src pathway in primary cultured rat osteoblasts<sup>35</sup>. The data from our previous studies showed that the EP1 receptor upregulated survivin expression and FAK phosphorylation to promote cell growth and migration in HCC cells<sup>36,37</sup>. PGE<sub>2</sub> promoted human cholangiocarcinoma cell growth and invasion through EP1 receptor-mediated activation of the EGFR, ERK, CREB and Akt<sup>38–40</sup>. However, until now, little was known about the association between the EP1 receptor and the integrin family.

In the current study, EP1 receptor activation enhanced  $\beta$ 1-integrin expression in Huh-7 cells. RNA interference of the EP1 receptor inhibited PGE<sub>2</sub>-mediated  $\beta$ 1-integrin upregulation. Pretreatment with a  $\beta$ 1-integrin antibody inhibited 17-PT-PGE<sub>2</sub>-mediated cell migration. Furthermore, immunohistochemical staining of human HCC tissues showed that EP1 receptor and  $\beta$ 1-integrin are both highly expressed in liver cancer tissues. These data suggested that EP1 receptor may promote cell migration by increasing  $\beta$ 1-integrin expression in HCC cells.

The PKC family was first identified as intracellular receptors for tumor promoting phorbol esters<sup>41</sup>. Recently, activation of PKC is thought to play a central role in the regulation of cellular responsiveness to external stimuli<sup>41,42</sup>. PKC is associated with the development of HCC. For example: PKC expression is significantly correlated with tumor size and the tumor/node/metastasis (TNM) stage<sup>43</sup>; Reduction of PKC expression by RNA interference or selective inhibitors greatly decreased cell proliferation, migration and invasion<sup>44</sup>. Our previous results indicated that PKC is involved in EP1 receptor-mediated cell adhesion and migration in HCC cells<sup>37</sup>. The present data showed that PKC activities were enhanced after 17-PT-PGE<sub>2</sub> treatment. The involvement of PKC in EP1 receptor-mediated  $\beta$ 1-integrin expression was further confirmed using PMA. In addition, pre-treatment

with the rottlerin diminished the 17-PT-PGE<sub>2</sub>-mediated  $\beta$ 1-integrin expression and cell migration.

Transcription factors of the nuclear factor  $\kappa$ B (NF- $\kappa$ B)/Rel family play a pivotal role in the inflammatory response and neoplastic development<sup>45,46</sup>. There are five family members in mammals: RelA (p65), c-Rel, RelB, NF- $\kappa$ B1 (p105/p50) and NF- $\kappa$ B2 (p100/p52). The RelA/p65 activating signaling pathway is a critical regulator for cell growth, differentiation, and tumorigenic transformation<sup>45</sup>. Indeed, p65 is constitutively activated at Ser536 in cancer cells, and is then translocated from the cytosol into the nucleus to regulate gene expression<sup>47,48</sup>. NF- $\kappa$ B activation is necessary for the cell migration and invasion<sup>49,50</sup>. Recently, NF- $\kappa$ B-p65 was found to be involved in progression and development of HCC<sup>51,52</sup>.

I $\kappa$ B proteins were phosphorylated at Ser32 and Ser36, releasing NF- $\kappa$ B to enter the nucleus where it regulates gene expression<sup>45,53</sup>. I $\kappa$ B phosphorylation is mediated by a high molecular weight signalosome complex comprising two I $\kappa$ B kinases (IKK $\alpha$  and IKK $\beta$ ). IKK induces I $\kappa$ B $\alpha$  phosphorylation and degradation, NF- $\kappa$ B nuclear translocation and NF- $\kappa$ B DNA binding activity<sup>54</sup>.

PKC isoforms play a key role in mediating the NF- $\kappa$ B signal pathway. PKC $\theta$  is essential for TCR-initiated NF- $\kappa$ B activation; PKC $\theta$  activates NF- $\kappa$ B through the selective induction of IKK<sup>55–57</sup>. Our data show that PKC is involved in EP1 receptor-mediated  $\beta$ 1-integrin upregulation; therefore, we hypothesize that NF- $\kappa$ B is also involved in this process. Our study showed that EP1 activation stimulated I $\kappa$ B $\alpha$  phosphorylation, followed by p65 activation and translocation from the cytosol into the nucleus. The NF- $\kappa$ B inhibitor PDTC diminished the 17-PT-PGE<sub>2</sub>-mediated  $\beta$ 1-integrin upregulation and cell migration.

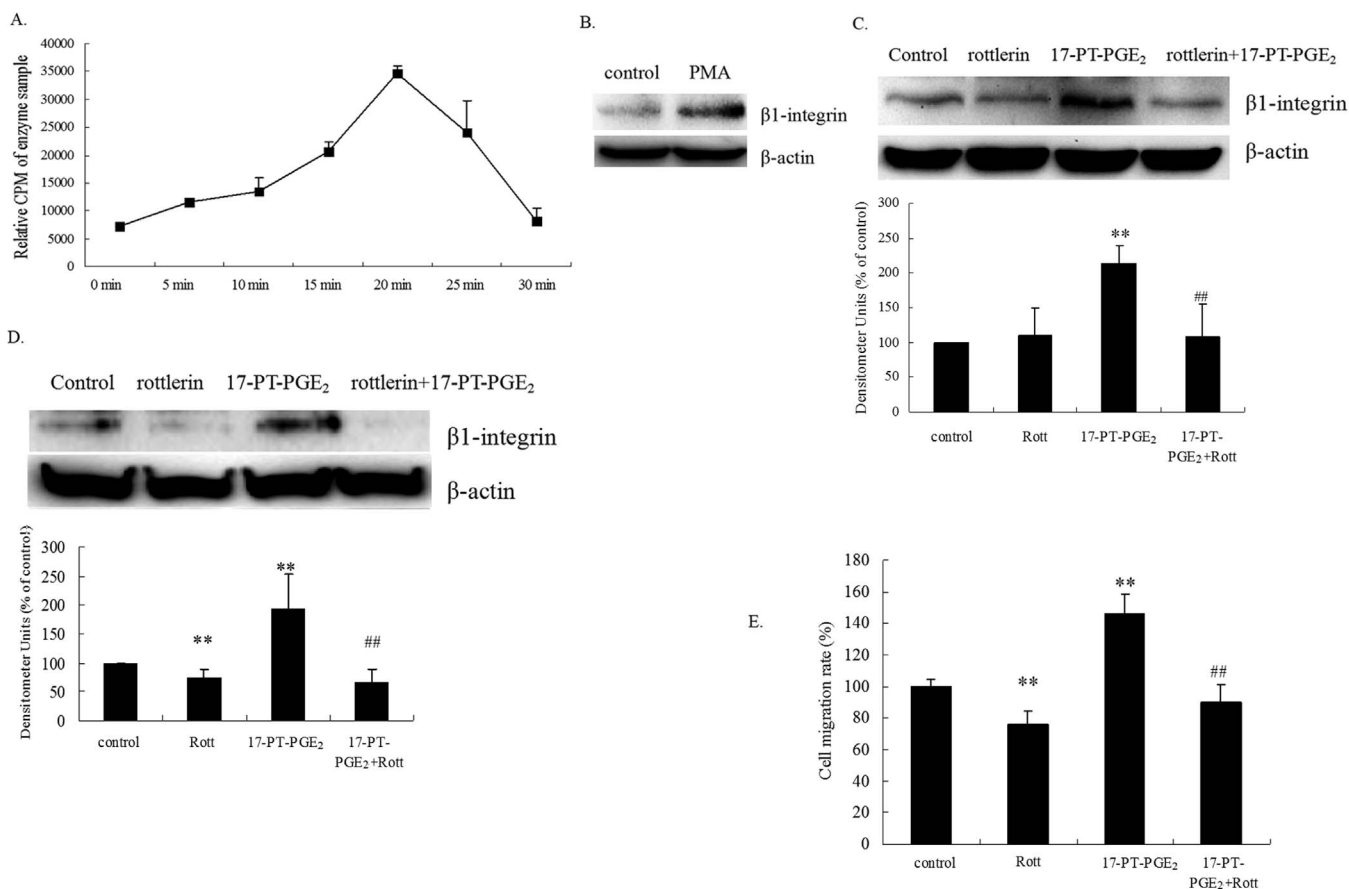
The involvement of NF- $\kappa$ B signal pathway in EP1 receptor-mediated  $\beta$ 1-integrin expression suggested the presence of an NF- $\kappa$ B response element in the promoter of  $\beta$ 1-integrin; however, we did not find one. Thus, other transcription factor(s) must bind to the  $\beta$ 1-integrin promoter directly and be regulated by NF- $\kappa$ B signal pathway. Interestingly, there are many FBEs in the promoter of  $\beta$ 1-integrin in liver cells. The forkhead transcription factor Foxc2 enhances the expression of  $\beta$ 1-integrin in osteoblast cells by direct binding to a FBE in its promoter<sup>22</sup>.

FoxC2 is a member of the family of winged helix/forkhead transcription factors. It is strongly expressed in the developing embryo and is required for various developmental processes<sup>58</sup>. In particular, FoxC2 is highly expressed in breast cancer, esophageal cancer and colon cancer, and increase the metastatic potential<sup>59</sup>. NF- $\kappa$ B upregulates FoxC2 expression<sup>60</sup>; therefore, we investigated the role of FoxC2 in EP1 receptor/NF- $\kappa$ B-mediated  $\beta$ 1-integrin expression. Little is known about the association between the EP receptors and FoxC2. Our results showed that EP1 receptor activation increased FoxC2 expression and PDTC pretreatment suppressed 17-PT-PGE<sub>2</sub>-mediated FoxC2 upregulation. Furthermore, immunohistochemistry showed EP1 receptor, p65, and FoxC2 were all highly expressed in HCC tissues. These data suggested that FoxC2 is also involved in EP1 receptor/NF- $\kappa$ B-mediated  $\beta$ 1-integrin expression (Fig. 6).

We demonstrated that the PGE<sub>2</sub> can upregulate  $\beta$ 1-integrin expression via the EP1 receptor to promote HCC cell migration. PKC and NF- $\kappa$ B signaling pathways are involved in EP1 receptor-mediated  $\beta$ 1-integrin expression. Our findings provide important new information regarding the putative role of the EP1 receptor in  $\beta$ 1-integrin expression in HCC cells and suggest that targeting the PGE<sub>2</sub>/EP1/PKC/NF- $\kappa$ B/FoxC2/ $\beta$ 1-integrin signal pathway may represent a new therapeutic strategy for the prevention and treatment of this malignant disease.

## Methods

**Materials.** The human HCC cell line, Huh-7, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) and Lipofectamine™ 2000 were from Invitrogen (Carlsbad, CA,



**Figure 3** | PKC is involved in EP1 receptor-mediated  $\beta 1$ -integrin upregulation in hepatocellular carcinoma cells. (A). PKC activity assay. Huh-7 cells were treated with 5  $\mu$ M 17-PT-PGE<sub>2</sub> for 0, 5, 10, 15, 20, 25 or 30 min. Equal amounts of total proteins (30  $\mu$ g) were added to microcentrifuge tubes and assayed for PKC levels using a direct human PKC enzyme activity assay kit. (B). Effect of a PKC activator on  $\beta 1$ -integrin expression in Huh-7 cells. Huh-7 cells were treated with 100 nM PMA for 24 h. Total protein was isolated and visualized with an anti- $\beta 1$ -integrin antibody. Levels of  $\beta$ -actin served as a loading control. (C). Effect of a PKC inhibitor on 17-PT-PGE<sub>2</sub>-mediated  $\beta 1$ -integrin expression in Huh-7 cells. Huh-7 cells were treated with 17-PT-PGE<sub>2</sub> for 24 h, with or without pre-treatment of 5  $\mu$ M rottlerin for 1 h. Total protein was isolated and visualized with an anti- $\beta 1$ -integrin antibody. Levels of  $\beta$ -actin served as a loading control. The cropped gels are used and full-length gels are presented in Supplementary Figure S7. Results are presented as the mean  $\pm$  SD from three different experiments. **\*\*** $P$  < 0.01, compared with control cells; **##** $P$  < 0.01, compared with 17-PT-PGE<sub>2</sub>-treated group. (D). Effect of a PKC inhibitor on 17-PT-PGE<sub>2</sub>-mediated  $\beta 1$ -integrin expression in EP1 receptor-expressed HEK293 cells. Stable EP1 receptor-expressed HEK293 cells were treated with 17-PT-PGE<sub>2</sub> for 24 h, with or without pre-treatment of rottlerin for 1 h. Total protein was isolated and visualized with an anti- $\beta 1$ -integrin antibody. Levels of  $\beta$ -actin served as a loading control. Results are presented as the mean  $\pm$  SD from three different experiments. **\*\*** $P$  < 0.01, compared with control cells; **##** $P$  < 0.01, compared with 17-PT-PGE<sub>2</sub>-treated group. (E). Effect of a PKC inhibitor on 17-PT-PGE<sub>2</sub>-mediated cell migration in Huh-7 cells. The cell migration assay was performed in a 12-well transwell plate. Huh-7 cells were treated with 17-PT-PGE<sub>2</sub> for 12 h, with or without pre-treatment of 5  $\mu$ M rottlerin for 1 h. Cells on the lower surface were stained with 0.1% crystal violet, solubilized with acetic acid solution and quantified by measuring their absorbance at 570 nm. Results are presented as the mean  $\pm$  SD from three different experiments. **\*\*** $P$  < 0.01, compared with control cells; **##** $P$  < 0.01, compared with 17-PT-PGE<sub>2</sub>-treated cells. The gels have been run under the same experimental conditions.

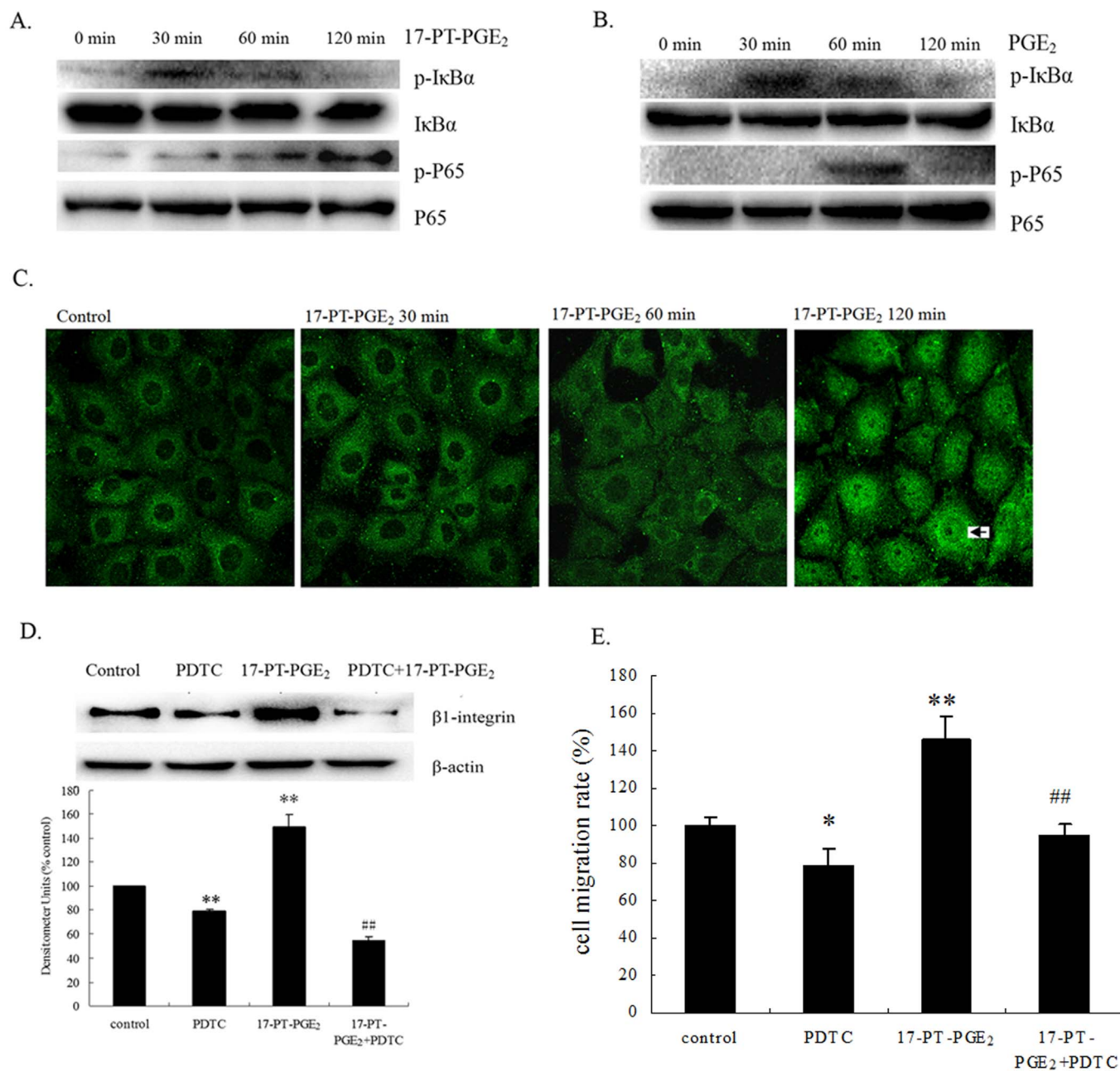
USA). PGE<sub>2</sub>, 17-phenyl trinor-PGE<sub>2</sub> (17-PT-PGE<sub>2</sub>), Butaprost, Sulprostone, PGE1 alcohol, sc19220, AH6809 and AH23848 were from Cayman Chemical Co (Ann Arbor, MI, USA). Ammonium pyrrolidinedithiocarbamate (PDTC) and L-798106 were from Sigma-Aldrich (St. Louis, MO, USA). Rottlerin (#557370) and phorbol-12-myristate-13-acetate (PMA, #524400) were obtained from Merck (Darmstadt, Germany). The protein assay was from Bio-Rad (Hercules, CA, USA). Electrochemiluminescence (ECL) reagents were from Amersham Biosciences (Piscataway, NJ, USA). The transwell unit (12-well) was from Costar Corning (USA). The PKC assay kit was from Millipore (Billerica, MA, USA). [ $\gamma$ -<sup>32</sup>P]ATP (#BLU002A) was from Perkin-Elmer (Waltham, MA, USA). G418 sulfate was from Amresco (Solon, OH, USA). The following were commercially obtained antibodies: the anti-EP1 receptor antibody was obtained from Cayman Chemical Co. (Ann Arbor, MI); the anti- $\beta 1$ -integrin antibodies were obtained from BD Bioscience (#610467, Becton Dickinson, USA) and Chemicon International (#AB1952P, USA); the anti-phosphorylated I $\kappa$ B $\alpha$  antibody (#9246s) and anti-phosphorylated p65 antibody at Ser536 (#3036s) were obtained from Cell Signaling Technology (Danvers, MA, USA); the anti-I $\kappa$ B $\alpha$  antibody (#ab7217) was obtained from Abcam plc (Cambridge, UK); the anti-p65 antibody (#sc-372) and the FITC-labeled secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the anti- $\beta$ -actin antibody was obtained from Sigma Chemical Co. (St. Louis, MO).

EnVision™ + single reagents (Mouse, Rabbit) were from DAKO (K4000, K4002, Glostrup, Denmark).

**Cell lines and culture.** Huh-7 cell line and HEK293 cells were cultured in DMEM with 10% fetal calf serum, 100 IU/ml penicillin and 100 g/ml streptomycin at 37°C with 5% CO<sub>2</sub>.

**Patients and specimens.** Primary surgical specimens were obtained from 20 patients (aged from 36 to 61; average is 48) who were clinically diagnosed for HCC, from the first affiliated hospital with Nanjing Medical University between Jan. 2013 and June 2013. Samples 5, 8, 18 have intrahepatic metastasis. None of them had distant metastasis. All of them were approached for participation in the project. There were four surgical specimens of normal liver tissue. All the specimens were collected following approval from the Human Ethics Committee of Nanjing Medical University. The Work conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Tokyo 2004). All the written informed consent from the donors were obtained for use of these samples in research. Resected specimens were fixed with 10% paraformaldehyde and embedded in paraffin blocks.

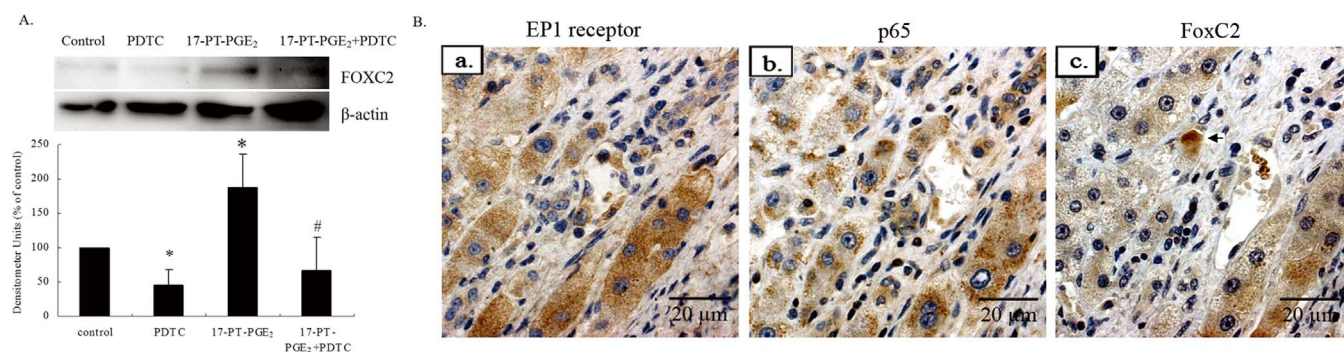




**Figure 4** | NF- $\kappa$ B is involved in EP1 receptor-mediated  $\beta$ 1-integrin upregulation in hepatocellular carcinoma cells. (A). Effects of 17-PT-PGE<sub>2</sub> on NF- $\kappa$ B and I $\kappa$ B phosphorylation in Huh-7 cells. Huh-7 cells were treated with 5  $\mu$ M 17-PT-PGE<sub>2</sub> for 0, 30, 60, 120 min. Equal amounts of total proteins were separated by SDS-PAGE. Relative levels of phosphorylated and total I $\kappa$ B $\alpha$  and total I $\kappa$ B $\beta$  or p65 were determined using specific antibodies. The cropped gels are used and full-length gels are presented in Supplementary Figure S8 and S9. (B). Effects of PGE<sub>2</sub> on NF- $\kappa$ B and I $\kappa$ B phosphorylation in EP1 receptor-expressed HEK293 cells. EP1 receptor-expressed HEK293 cells were treated with PGE<sub>2</sub> for 0, 30, 60, 120 min. (C). Effects of 17-PT-PGE<sub>2</sub> on NF- $\kappa$ B translocation in Huh-7 cells. Huh-7 cells were treated with 5  $\mu$ M 17-PT-PGE<sub>2</sub> for 0, 30, 60, 120 min; the cells were then fixed with ice-cold methanol. The p65 protein was detected by immunofluorescence and activated p65 was translocated into the nuclei (arrow). All pictures were taken at 400 $\times$  magnification. (D). Effect of NF- $\kappa$ B inhibitor on 17-PT-PGE<sub>2</sub>-mediated  $\beta$ 1-integrin expression in Huh-7 cells. Huh-7 cells were treated with 17-PT-PGE<sub>2</sub> for 24 h, with or without pre-treatment of PDTC for 24 h. Total protein was isolated and visualized with an anti- $\beta$ 1-integrin antibody. Levels of  $\beta$ -actin served as a loading control. The cropped gels are used and full-length gels are presented in Supplementary Figure S10 and S11. Densitometric quantitation of the above blots is shown. Results are presented as the mean  $\pm$  SD from three different experiments. \*\* $P < 0.01$ , compared with control cells; ## $P < 0.01$ , compared with 17-PT-PGE<sub>2</sub>-treated cells. (E). Effect of NF- $\kappa$ B inhibitor on 17-PT-PGE<sub>2</sub>-mediated cell migration in Huh-7 cells. The cell migration assay was performed in a 12-well transwell plate. Huh-7 cells were treated with 17-PT-PGE<sub>2</sub> for 12 h, with or without pre-treatment of 10  $\mu$ M PDTC for 24 h. Results are presented as the mean  $\pm$  SD from three different experiments. \*\* $P < 0.01$ , compared with control cells; ## $P < 0.01$ , compared with 17-PT-PGE<sub>2</sub>-treated cells. The gels have been run under the same experimental conditions.

**Immunohistochemical staining.** Sections (4  $\mu$ m) of 20 tumor blocks were used for immunohistochemical analysis. The slides were placed in boiling citric acid buffer (10 mM sodium citrate and citric acid) for 10 minutes. Sections were treated with primary antibodies  $\beta$ 1-integrin (#610467, BD Bioscience), EP1 receptor, p65, and

FoxC2, applied at a 1:100 or 1:200 dilution and incubated overnight at 4 $^{\circ}$ C. Bound antibody was detected using EnVision polymer technology. After a complete wash in PBS, the slides were developed in freshly prepared diaminobenzidine solution (DAB) for 8 min, and then counterstained with hematoxylin. PBS substituted for the



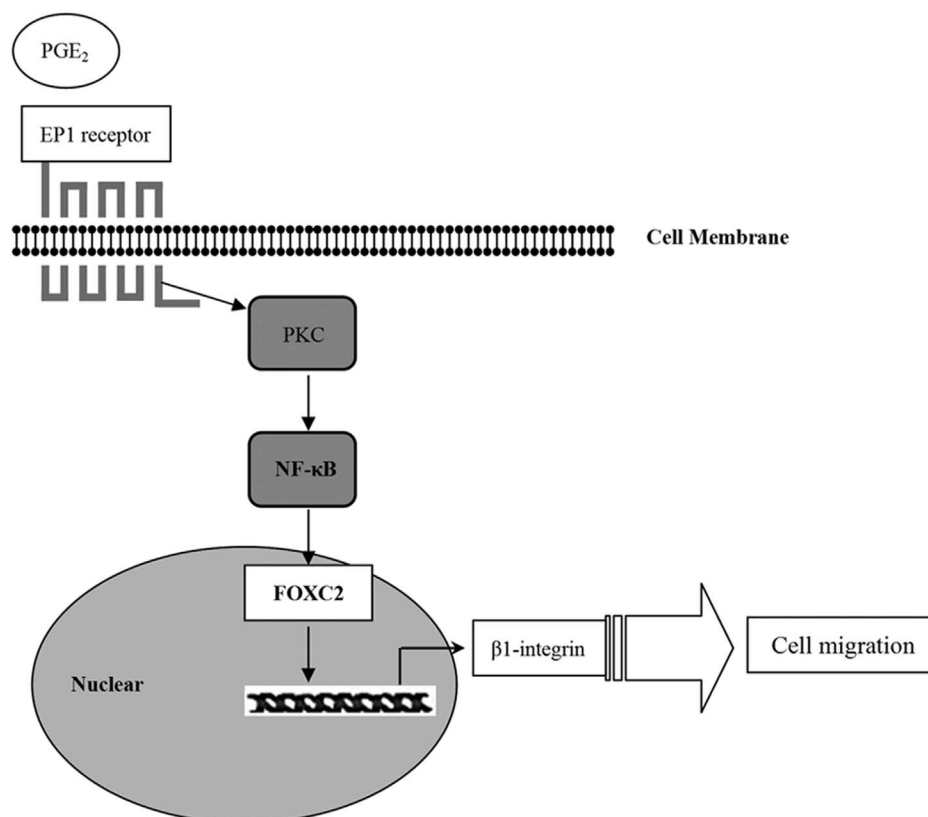
**Figure 5 | FoxC2 is involved in EP1 receptor/NF- $\kappa$ B-mediated  $\beta$ 1-integrin upregulation in hepatocellular carcinoma.** (A). Effect of NF- $\kappa$ B in EP1 receptor-mediated FoxC2 upregulation in hepatocellular carcinoma cells. Huh-7 cells were treated with 17-PT-PGE<sub>2</sub> for 24 h, with or without pre-treatment of PDTC for 24 h. Total protein was isolated and visualized with an anti-FoxC2 antibody. Levels of  $\beta$ -actin served as a loading control. The cropped gels are used and full-length gels are presented in Supplementary Figure S12. Densitometric quantitation of the above blots is shown. Results are presented as the mean  $\pm$  SD from three different experiments. \* $P < 0.05$ , compared with control cells; # $P < 0.05$ , compared with 17-PT-PGE<sub>2</sub>-treated cells. The gels have been run under the same experimental conditions. (B). Co-expression of EP1 receptor, p65 and FoxC2 in liver cancer tissues. (a). Representative immunohistochemical images of human hepatocellular carcinoma tissue stained with the anti-human EP1 receptor antibody. (b). Representative immunohistochemical images of hepatocellular carcinoma tissue stained with the anti-p65 antibody. (c). Representative immunohistochemical images of hepatocellular carcinoma tissue stained with the anti-FoxC2 antibody. (Magnification:  $\times 400$ ).

primary antibody as a negative control. The sections were photographed by Leica microscopy and Image analyse system. 4 low power views (400 $\times$ ) were randomly selected from each samples in a blind manner.

**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%). Huh-7 cells ( $5 \times 10^4$ ) were added to the upper chamber. Pharmacological agents were added at the indicated times. After incubation at 37 $^{\circ}$ C for 12 h, the cells were fixed with ethanol and then stained with 0.1% crystal violet. After washing with PBS, the cells were removed from the upper surface of the

membrane by wiping with moist cotton swabs. Cells migrated to the lower surface of the membrane were solubilized with 300  $\mu$ l of 10% acetic acid and quantified by measuring the absorbance at 570 nm.

**PKC measurements.** Cells were treated with pharmacological agents at 37 $^{\circ}$ 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  $\mu$ g/ml PMSF and aprotinin), and then cleared by centrifugation at 12,000  $\times$  g for 15 min at 4 $^{\circ}$ C. PKC levels were assayed using a direct human PKC enzyme activity assay kit, according to the manufacturer's instructions.



**Figure 6 | Proposed mechanisms for PGE<sub>2</sub>/EP1 receptor-mediated hepatocellular carcinoma cell migration.** Our data showed that the EP1 receptor played a key role in PGE<sub>2</sub>-mediated hepatocellular carcinoma cell migration. EP1 receptor may upregulate  $\beta$ 1-integrin expression to improve cell migration. PKC/NF- $\kappa$ B/FOXK2 signaling pathways were involved in EP1 receptor-mediated  $\beta$ 1-integrin expression.



Briefly, 10  $\mu$ l of substrate cocktail, PKA inhibitor cocktail, Assay Dilution Buffer II (ADBI), lipid activator and diluted [ $\gamma$ - $^{32}$ 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- $\mu$ l 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 ( $2 \times 10^5$ ) were seeded in 6-well culture plates and transfected with the EP1R-pcDNA3 plasmid or pcDNA3 empty vector control (2  $\mu$ g) using Lipofectamine 2000™ (5  $\mu$ l). The efficiency of transfection was assayed by flow cytometry. The G418 antibiotic was used to select for HEK293 cells stably expressing the EP1 receptor.

**Immunofluorescence assay.** Huh-7 cells ( $2 \times 10^5$ ) were cultured in 6-well plates for 24 h, followed by 17-PT-PGE<sub>2</sub> treatment for various period of time. The cells were fixed with ice-cold methanol, permeabilized with 0.1% Triton X-100 in PBS, and incubated at 4 °C overnight with anti-p65 receptor antibody (1 : 100 dilution). Antibody binding was localized using an FITC-labeled secondary antibody. The cells were visualized using a Confocal Microscope (400 $\times$ ).

**RNA interference.** The siRNAs targeting the EP1 receptor (EP1R-siRNA) (ID: s194727) were obtained from Ambion. The sequence of the siRNAs used was ACCUUCUAGCACAACAGAtt (sense sequence). Huh-7 cells ( $4 \times 10^4$ ) were plated in 12-well plates for 24 h, resulting in a 30–50% confluent cell monolayer. The cells were then transfected with the EP1R-siRNA, or a non-silencing 21-nucleotide irrelevant RNA duplex as a negative control, using Lipofectamine™ 2000. After 72 h, depletion of the EP1 receptor was confirmed by western blotting, and the cells were subsequently used for further experiments.

**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 and then cleared by centrifugation at 12,000  $\times$  g for 15 min at 4°C. Equal amounts of total proteins (40  $\mu$ g) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were probed with the appropriate antibodies at 4°C overnight. The immunoreactivity was detected by ECL and analyzed using Image lab 4.0 analysis software from Bio-Rad.

**Statistical analysis.** All data are presented as means  $\pm$  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  $P < 0.05$ .

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## Author contributions

X.B. participated to the design of the study, to performed and interpreted W.B. analysis on EP1/PKC/NF- $\kappa$ B/FoxC2/ $\beta$ 1-integrin pathway and drafted the manuscript. J.W. participated to the collection and interpreted W.B. analysis on EP1/NF- $\kappa$ B/ $\beta$ 1-integrin pathway. Y.G. and J.P. performed and interpreted immunohistochemical analyses of EP1 receptor,  $\beta$ 1-integrin, p65 and FoxC2. Q.Y. helped to the design of the study, collected and analysed data. M.Z. and H.L. collected samples and carried out immunohistochemical analyses of EP1 receptor and p65 in tumour tissue. L.Z. and J.M. coordinated and interpreted molecular studies and participated in drafting the manuscript. F.S. helped to the design and coordination of the study, and to draft the manuscript. W.S. and Y.W. helped to draft the manuscript. J.L. conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

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