Prostaglandin E$_2$ promotes hepatocellular carcinoma cell invasion through upregulation of YB-1 protein expression

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Abstract. Prostaglandin E$_2$ (PGE$_2$) has been implicated in hepatocellular carcinoma cell invasion. Recently, it was reported that Y box-binding protein 1 (YB-1) is closely correlated with malignancy. This study was designed to examine the mechanisms by which PGE$_2$ increases YB-1 expression and promotes HCC cell invasion. PGE$_2$ greatly enhanced HCC cell invasion through upregulation of the YB-1 protein, and the EP1 receptor is mainly responsible for this regulation. Src and EGFR were both activated by PGE$_2$, which in turn increased the phosphorylation levels of p44/42 MAPK. Src, EGFR and p44/42 MAPK were all involved in PGE$_2$-induced YB-1 expression. Chemical inhibitors and RNAi analysis all confirmed the role of mTOR complex 1 in YB-1 expression induced by PGE$_2$. Furthermore, YB-1 was able to regulate the expression of a series of EMT-associated genes, which indicated that YB-1 could have the potential to control the epithelial-mesenchymal transition process in HCC cells. These findings reveal that PGE$_2$ upregulated YB-1 expression through the EP1/Src/EGFR/p44/42 MAPK/mTOR pathway, which greatly enhanced HCC cell invasion. This study for the first time describes the mechanisms through which PGE$_2$ regulates YB-1 expression and promotes HCC cell invasion.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver and the third most common cause of cancer-related death worldwide (1). An increasing number of studies have indicated that there is close correlation between HCC and long-standing chronic inflam-

mation (2,3). However, the detailed molecular mechanisms linking chronic inflammation and malignant transformation remain to be further defined.

There is substantial evidence showing that inflammation mediators, such as cyclooxygenase (COX)-derived prostaglandins (PGs) may have a causally important role in hepatocellular carcinogenesis (4,5). Prostaglandin E$_2$ (PGE$_2$), which is one of the key products of cyclooxygenase-2, could greatly promote tumor cell proliferation, anti-apoptosis, angiogenesis and invasion. For example, PGE$_2$ significantly promoted hepatocellular carcinoma cells proliferation and survival by upregulating the expression of FUSE-binding protein 1 (6) and Survivin (7), in human glioma cells PGE$_2$ altered the proliferative, apoptotic and migratory properties (8), in cholangiocarcinoma cells PGE$_2$ was able to enhance the invasion by upregulating the MMP2 expression through the CREB pathway (9), and in a breast cancer model it was shown to control the tumor growth, angiogenesis, lymphangiogenesis and metastasis to the lungs and lymph nodes through the EP4 receptor (10). However, in hepatocellular carcinoma, the mechanisms for its ability to promote invasiveness are not clarified.

Although surgical resection and adjuvant therapy can cure well confined primary tumors, metastatic disease is largely incurable because of its systemic nature and the resistance of disseminated tumor cells to existing therapeutic agents, this also explains why >90% of mortality from cancer is attributable to metastases, not the primary tumors from which these malignant lesions arise (11). Hepatocellular carcinoma is a highly malignant disease, the recurrence and metastasis is quite common in patients, so it is imperative to fully understand the mechanisms underlying its invasion and metastasis.

YB-1 (Y-box-binding protein 1) encoded by the YBX1 gene, is a member of the cold-shock protein super family, all of which contain a highly conserved nucleic-acid-binding motif that binds to both DNA and RNA (12). It is a kind of nuclear-cytoplasm shuttle protein, and could function not only as a transcription factor to regulate genes transcription in the nuclear, but also control a subset of mRNA translational efficiency in the cytoplasm. Plethora of publications indicate that YB-1 can function as an oncoprotein, and is highly correlated with cancer progression and poor prognosis. For example, the expression level of cytoplasmic YB-1 has been

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shown to correlate with progression in breast cancers (13). The importance of the correlation between nuclear YB-1 levels with patient prognosis has been strengthened by similar observations for non-small cell lung carcinoma and prostate cancer (14,15). IHC and genomic studies have shown that YB-1 protein and mRNA levels are frequently elevated in advanced breast cancer, and are associated with poor patient outcome (16-19). Although YB-1 has been regarded as a useful biomarker of cancer progression, the detailed mechanisms for its tumor-promoting functions are not very clear, and whether the YB-1 could be viewed as a novel therapeutic target is still controversial (20).

Given our previous results showing that E Prostanoid 1 (EP1) receptor could enhance the invasion of HCC (21,22), and the involvement of YB-1 in the malignance of carcinoma, this study was designed to evaluate our hypothesis that PGE$_2$ may promote hepatocellular carcinoma cell invasive growth through upregulation of YB-1 via the EP1 receptor and its signaling pathway. Our data reveal that PGE$_2$ enhances HCC cell invasion through EP1 receptor-mediated upregulation of YB-1 and this process involves the mTOR pathway. When binding with PGE$_2$ or selective agonist, EP1 receptor activates Src and EGFR, which subsequently induces the phosphorylation of p-44/42 MAPK, another key serine and threonine protein kinase critical for HCC cell invasion. Furthermore, mTOR is activated via the above signal pathways upregulating the expression level of YB-1, which in turn regulates the expression of a series of proteins associated with the epithelial-mesenchymal transition. These findings reveal that PGE$_2$ could promote HCC cell invasion through upregulating YB-1 expression level via the EP1/Src/EGFR/Erk/mTOR pathway. This is the first study detailing the role of PGE$_2$-EP1 receptor signal pathway in YB-1 expression in human HCC cell lines.

Materials and methods

Cell culture. Human HCC cell lines Hep3B and Huh7 were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained at 37˚C in a humidified CO$_2$ incubator. Hep3B cells were cultured in minimum essential medium (MEM) and Huh7 cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco).

Western blotting. At the end of each treatment, the cells were washed twice with ice-cold phosphate-buffered saline and then sonicated on ice in a lysis buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, with the protease inhibitor tablets from Roche, or together with 50 mM sodium fluoride, 25 mM glycerophosphate, or 1 mM Na$_3$VO$_4$ for phosphorylation assay). Cell lysates were centrifuged at 12000 x g for 10 min at 4˚C, and the supernatants were collected for western blotting. Protein concentration was measured using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). After boiling for 5 min in the loading buffer with 10% 2-mercaptoethanol, the samples containing 30 μg protein were separated on 10% Tris-glycine gels; the separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad). Immunoblotting was performed using individual antibodies (including rabbit monoclonal anti-YB-1, phosphor-p44/42 MAPK, raptor, E-Cadherin, mouse monoclonal

anti-Snail, total-p44/42 MAPK from Cell Signaling (Boston, MA, USA); rabbit polyclonal anti-GAPDH, Vimentin from SAB Signalway Antibody (Nanjing, China).

Cell invasion assay. The cell invasion assay was performed in transwell chambers (Coster Corning, USA). The Matrigel (BD Biosciences Discovery Labware, Bedford, MA, USA) was diluted with serum-free medium (1:5 dilutions), and then 40 μl of diluted Matrigel was added to the upper chamber, and incubated in 37˚C for 5 h to make the gel solidified. Cells ($5x10^4$) in 100 μl serum-free medium in the presence or absence of PGE$_2$ or EP1 receptor agonist 17-P-T-PGE$_2$ (Cayman Chemical, Ann Arbor, MI, USA) were seeded in the upper chamber. Regular medium containing 10% FBS were added in the lower chamber as chemoattractants. To determine the role of YB-1 protein in PGE$_2$ induced Huh7 and Hep3B cell invasion, the cells transfected with the control siRNA or YB-1 siRNA ($5x10^4$) in 100 μl of serum-free medium in the presence or absence of PGE$_2$ or EP1 receptor agonist were seeded in the upper chamber; the regular medium containing 10% FBS was added in the lower chamber. After 24 h of incubation at 37˚C, the cells were fixed with ethanol and stained with 0.1% crystal violet for 30 min. After washing the cells with PBS, the cells on the upper surface of the filter were mechanically removed with a cotton swab. The invading cells on the lower surface were solubilized with 300 μl 10% acetic acid and the absorbance of which was measured at 570 nm. These experiments were repeated three times, and three wells were used for each treatment.

RNAi interference. The sequences of EP1 siRNA (siRNA ID: 194727), Raptor siRNA (siRNA ID: 33215) and YB-1 siRNA (siRNA ID: s9732) were from Ambon, Life Technology Co. HCC cells ($2.3x10^5$) were plated in 6-well plates for 24 h, resulting in a 30-50% confluent cell monolayer. The cells were then transfected with the target siRNA, or a non-silencing 21-nucleotide irrelevant RNA duplex as a negative control, using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). After 72 h, depletion of target protein was confirmed by immunoblotting and real-time PCR and subsequently used for further experiments.

RNA isolation and real-time PCR. Total RNA from the cultured cells was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was carried out with PrimeScript™ RT reagent kit (Takara Co., Japan) according to the standard protocol. The sequences of the primers for EP1 receptor are as follows: forward: 5'-AGCAGCACCTTCAACCCTCA-3'; reverse: 5'-TCCAGCAGATGCACGACAC-3'. The RNA level of EP1 receptor was determined by the real-time PCR analysis, using the Power SYBR Master Mix kit (ABI).

Statistical analysis. Results are expressed as the mean ± SD. Statistical analysis was performed with Student's t-test. p-values <0.05 were considered statistically significant.

Results

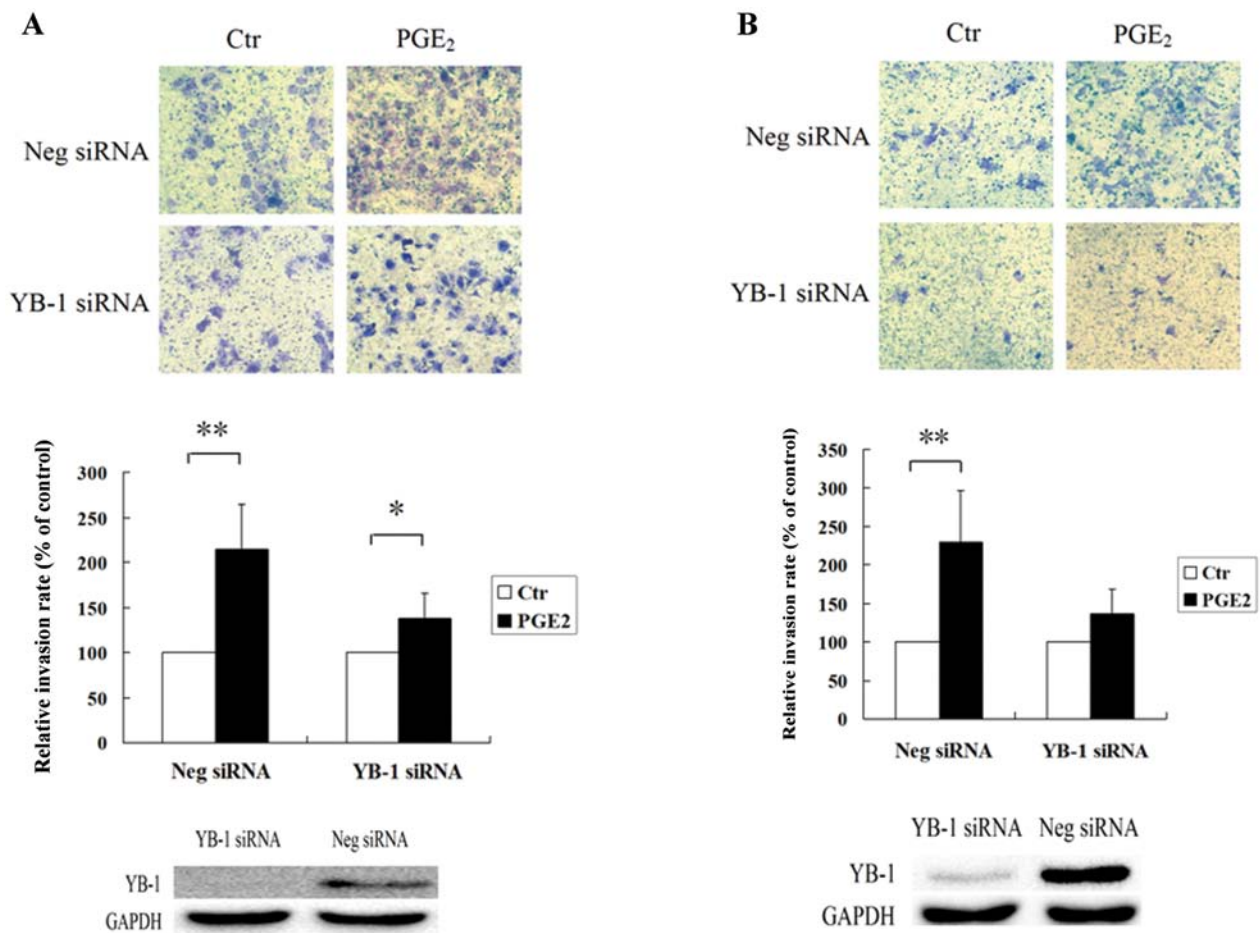*YB-1 protein plays a key role in PGE$_2$-induced invasion of hepatocellular carcinoma cells*. The cell invasion assay

Figure 1. The role of YB-1 in the invasion of HCC cells induced by PGE$_2$. (A) Effects of YB-1 on the invasiveness of Huh7 cell induced by PGE$_2$. The cell invasion assay was performed in Matrigel-coated transwell chamber. Huh7 cells cultured in regular medium were transfected overnight with YB-1 siRNA or negative siRNA. After 72 h of incubation, 100 μl serum-free medium containing Huh7 cells transfected with YB-1 siRNA or negative siRNA (5x10^4) were seeded in the upper chamber in the presence or absence of 5 μM PGE$_2$, the regular medium containing 10% FBS was added to the lower chamber. After 24 h, the cells were fixed with ethanol and stained with 0.1% crystal violet for 30 min. After washing the cells with PBS, the cells on the upper surface of the filter were mechanically removed with a cotton swab. The invading cells on the lower surface were solubilized with 300 μl 10% acetic acid and the absorbance of which was measured at 570 nm. These experiments were repeated three times, and three wells were used for each treatment. Representative images of invaded cells from independent groups are shown in the upper panels. Quantitative analysis of invaded cells is shown in the middle panels. RNAi suppression efficiency of YB-1 was confirmed by immunoblotting (lower panel). Data are presented as the mean ± SD of three independent experiments. (B) Similar results were also observed in Hep3B cells.
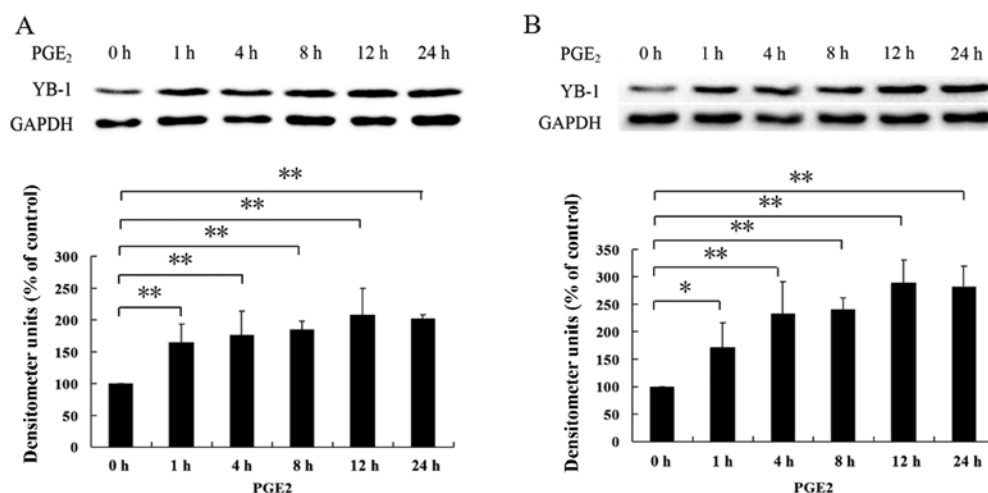

Figure 2. PGE$_2$ induces YB-1 expression in HCC cells. (A) Effects of PGE$_2$ on YB-1 expression. Huh-7 cells cultured in serum-free medium for 24 h were treated with PGE$_2$ at 5 μM for indicated times. The cell lysates were obtained. YB-1 expression level was determined by immunoblotting with anti-YB-1 antibody (upper panel); GAPDH as loading control was determined by immunoblotting with anti-GAPDH antibody (lower panel). Quantitative analysis of YB-1 expression level was carried out by calculating the ratio between YB-1 protein and GAPDH levels from three different experiments. $^*p<0.05$, $^{**}p<0.01$ compared with the control. Data are presented as the mean ± SD of three independent experiments. (B) Similar results were also obtained from Hep3B cell line.
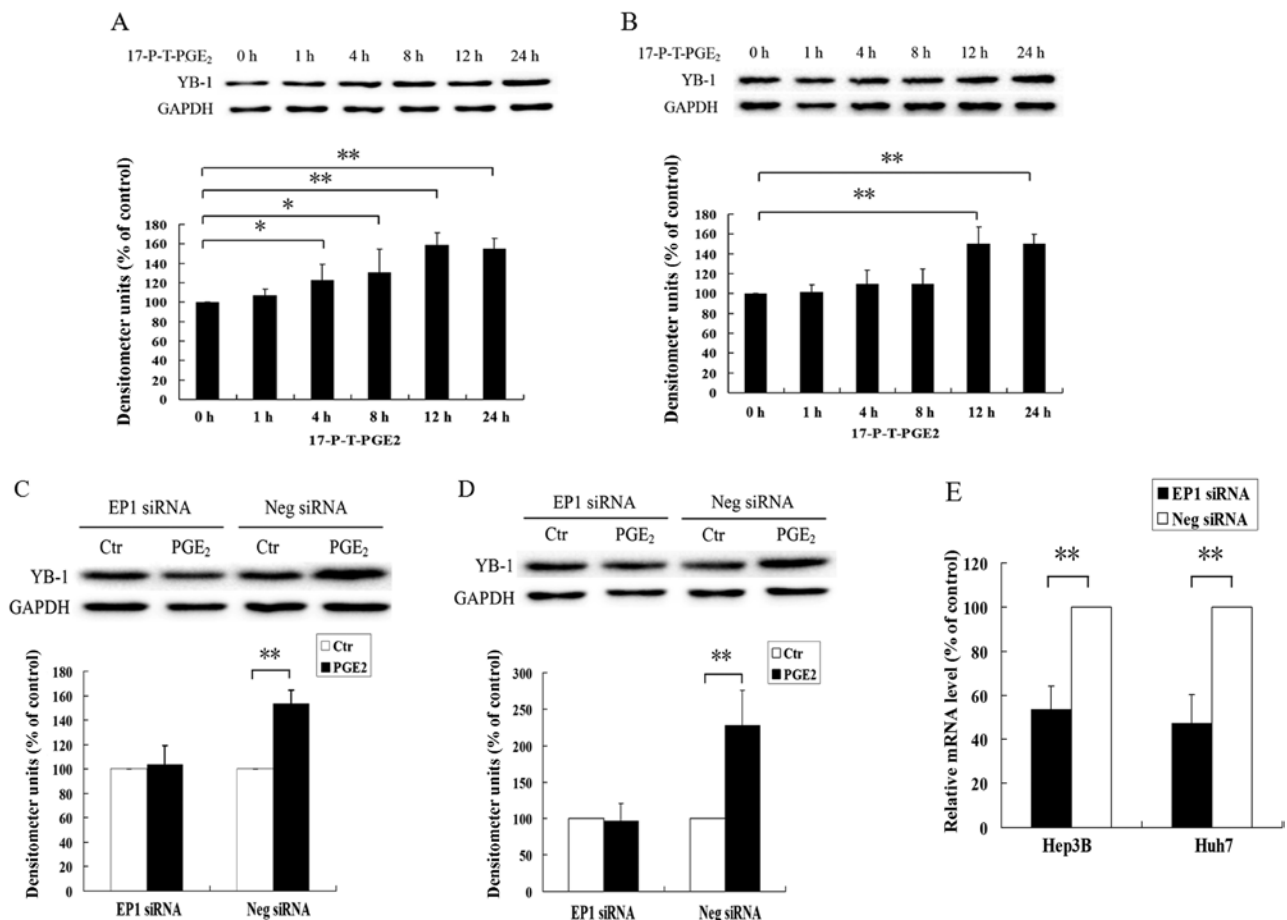
Figure 3. PGE$_2$ induces YB-1 expression via the EP1 receptor in HCC cell lines. (A) Effects of EP1 receptor agonist on YB-1 expression. Huh7 cells cultured in serum-free medium for 24 h were then treated with EP1 receptor agonist (17-P-T-PGE$_2$) at 5 μM for indicated times. The cell lysates were obtained. YB-1 expression level was determined by immunoblotting with anti-YB-1 antibody (upper panel); GAPDH as loading control was determined by immunoblotting with anti-GAPDH antibody (lower panel). Quantitative analysis of YB-1 expression level was carried out by calculating the ratio between YB-1 protein and GAPDH expression level from three different experiments. *p<0.05; **p<0.01 compared with the control. Data are presented as the mean ± SD of three independent experiments. (B) Similar results were also obtained from Hep3B cell line. (C) RNAi suppression of the EP1 receptor inhibits YB-1 expression induced by PGE$_2$. Huh7 cells were transfected overnight with the EP1 receptor siRNA or control siRNA in regular medium and then treated with PGE$_2$ at 5 μM for 12 h. The cell lysates were obtained. YB-1 expression level was determined by immunoblotting with anti-YB-1 antibody (upper panel); GAPDH as loading control was determined by immunoblotting with anti-GAPDH antibody (lower panel). Quantitative analysis of YB-1 expression level was carried out by calculating the ratio between YB-1 protein and GAPDH expression level from three different experiments. **p<0.01 compared with the control. Data are presented as the mean ± SD of three independent experiments. (D) Similar results were also observed in Hep3B cell line. (E) RNAi efficiency of EP1 receptor in Huh7 and Hep3B cells. Real-time PCR analysis showing the RNAi efficiency in Huh7 and Hep3B cells transfected with EP1 receptor siRNA (**p<0.01) compared with the cells transfected with negative control siRNA. Data are presented as the mean ± SD of three independent experiments.

was utilized to analyze the role of YB-1 protein in PGE$_2$-induced invasion of HCC cells. Huh7 and Hep3B cells were transfected with YB-1 siRNA or negative control siRNA, and the transfected cells were then analyzed for PGE$_2$-induced cell invasion. As shown in Fig. 1, PGE$_2$ greatly enhanced the invasion properties of Huh7 and Hep3B cells transfected with negative control siRNA, while in the cells transfected with YB-1 siRNA, the invasion ability induced by PGE$_2$ was significantly suppressed. Since YB-1 was able to regulate the cell proliferation, in order to rule out the suppressed invasion ability was caused by lower proliferating rate, we performed the WST analysis and found that RNAi suppression of YB-1 expression could not inhibit the cell proliferation rate (data not shown). These results indicated that the YB-1 protein may play an important role in PGE$_2$-induced HCC cell invasion.

PGE$_2$ induces YB-1 protein expression in hepatocellular carcinoma cells. RNAi suppression of YB-1 protein signifi-

cantly blocked the PGE$_2$-induced invasion of hepatocellular carcinoma cells suggesting a possible interconnection between PGE$_2$ and YB-1. Given that PGE$_2$ could greatly enhance the invasion of tumor cells (23-25) and that YB-1 has been reported to be necessary for maintaining the malignance of cancer (12,26,27), we postulated that PGE$_2$ could enhance HCC invasion through upregulation of YB-1 expression level. To evaluate this hypothesis, we treated Huh7 and Hep3B cells with PGE$_2$ for indicated times, as shown in Fig. 2, treatment of Huh7 and Hep3B cells with PGE$_2$ greatly increased the expression level of YB-1. These findings indicate that PGE$_2$ was able to upregulate YB-1 expression in hepatocellular carcinoma.

EP1 receptor-mediated upregulation of YB-1 enhances HCC cell invasion. The EP1 receptor plays an important role in promoting HCC cell invasion, although the detailed mechanisms are not very clear (28). RNAi suppression of
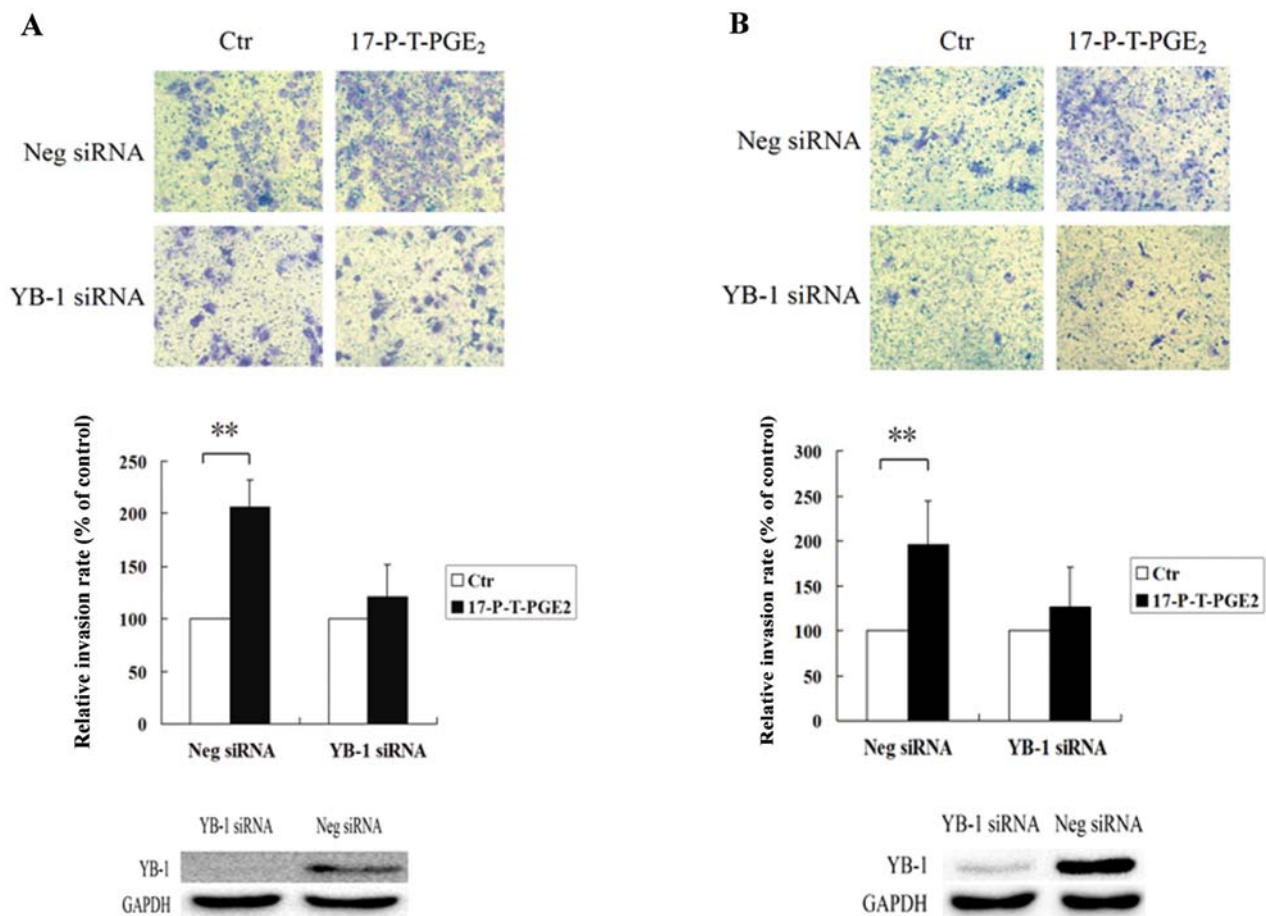
Figure 4. The role of YB-1 in the invasion of HCC cells induced by PGE_2 via EP1 receptor. (A) Effects of YB-1 on the invasiveness of Huh7 cells induced by PGE_2 via EP1 receptor. The cell invasion assay was performed in Matrigel-coated transwell chamber. Huh7 cells cultured in regular medium were transfected overnight with YB-1 siRNA or negative siRNA. After 72 h of incubation, 100 μl serum-free medium containing Huh7 cells transfected with YB-1 siRNA or negative siRNA ($5x10^4$) were seeded in the upper chamber in the presence or absence of 5 μM PGE_2 or 5 μM 17-P-T-PGE_2, the regular medium containing 10% FBS was added to the lower chamber. After 24 h, the cells were fixed with ethanol and stained with 0.1% crystal violet for 30 min. After washing the cells with PBS, the cells on the upper surface of the filter were mechanically removed with a cotton swab. The invading cells on the lower surface were solubilized with 300 μl 10% acetic acid and the absorbance of which was measured at 570 nm. These experiments were repeated three times, and three wells were used for each treatment. Representative images of invaded cells from independent groups are shown in the upper panels. Quantitative analysis of invaded cells is shown in the middle panels. RNAi suppression efficiency of YB-1 was confirmed by immunoblotting (lower panel). Data are presented as the mean ± SD of three independent experiments. (B) Similar results were also observed in Hep3B cells.

YB-1 protein also blocked PGE_2-induced cell invasion, which suggests that YB-1 is closely associated with HCC cell invasion. Therefore, we investigated whether EP1 receptor is involved in PGE_2-induced YB-1 expression. As shown in Fig. 3A and B, we found that treatment of HCC cells with 17-P-T-PGE_2 significantly increased the expression level of YB-1. In order to further confirm this result, we downregulated the EP1 receptor expression level, as shown in Fig. 3C and D, we found that when the EP1 receptor expression was suppressed, PGE_2-induced YB-1 expression was almost completely inhibited. These observations indicate that EP1 receptor plays an important role in PGE_2-induced YB-1 expression. Since YB-1 is critical to PGE_2-induced HCC cell invasion, further experiment was performed to investigate the role of YB-1 in 17-P-T-PGE_2-induced HCC cell invasion. As shown in Fig. 4, knockdown of YB-1 expression dramatically suppressed the HCC cell invasion induced by 17-P-T-PGE_2, whereas, the HCC cells transfected with negative control siRNA, when treated with EP1 receptor agonist, still exhibited greatly enhanced invasion ability.

Involvement of EGFR and Src in PGE_2-induced YB-1 expression. EGFR and Src have been reported to be downstream of the EP1 receptor, and are both important for cancer cell invasion (28,29). We further investigated whether EGFR and Src are also involved in the YB-1 expression induced by 17-P-T-PGE_2. As shown in Fig. 5, pretreatment of Huh7 and Hep3B cells with EGFR inhibitor AG1478 or Src inhibitor PP2 significantly suppressed the YB-1 expression induced by 17-P-T-PGE_2. These observations indicate that EGFR and Src both play important roles in YB-1 expression induced by 17-P-T-PGE_2.

Activation of p44/42 MAPK via EP1 receptor increases YB-1 expression. p44/42 MAPK is a kind of serine/threonine protein kinase, which is believed to be a critical factor associated with cancer cell invasion (30-32). Many studies pointed out that PGE_2 could activate p44/42 MAPK via EP1 receptor, so the potential involvement of p44/42 MAPK in upregulating YB-1 expression via EP1 receptor was determined by using the MEK inhibitor PD98059. As shown in
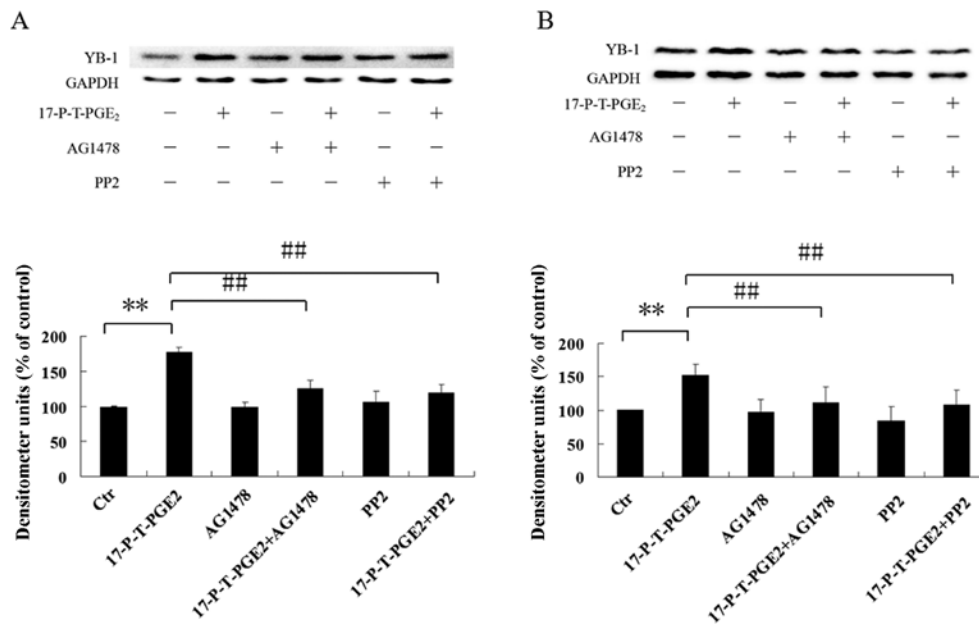
Figure 5. The role of EGFR and Src kinase in upregulating the YB-1 protein via EP1 receptor. (A) Effects of EGFR inhibitor AG1478 and Src inhibitor PP2 on YB-1 expression induced by 17-P-T-PGE$_2$. Huh7 cells cultured in serum-free medium were pretreated with 2 μM AG1478 or 10 μM PP2 for 1 h, then they were treated with 5 μM 17-P-T-PGE$_2$ for 12 h. The cell lysates were obtained. The YB-1 expression level was determined by immunoblotting with anti-YB-1 antibody (upper panel); GAPDH as loading control was determined by immunoblotting with anti-GAPDH antibody (lower panel). Quantitative analysis of the YB-1 expression level was carried out by calculating the ratio between YB-1 protein and GAPDH expression levels from three different experiments. **p<0.01 compared with the control; $^{##}$p<0.01 compared with the 17-P-T-PGE$_2$ treatment. Data are presented as the mean ± SD of three independent experiments. (B) Similar results were also obtained in the Hep3B cell line.

Fig. 6A and B, pretreatment of Huh7 and Hep3B cells with PD98059 could greatly inhibited the YB-1 expression induced by 17-P-T-PGE$_2$. Furthermore, we also found that the p44/42 MAPK could be activated by 17-P-T-PGE$_2$ in a time-dependent manner (Fig. 6C and D). These findings indicate that PGE$_2$-induced YB-1 expression via EP1 receptor is mediated through activation of p44/42 MAPK.

EGFR and Src are involved in the p44/42 MAPK activation induced by PGE$_2$. EGFR and Src are both involved in YB-1 expression induced by 17-P-T-PGE$_2$, while p44/42 MAPK, activated by PGE$_2$ via EP1 receptor, also plays a key role in regulating YB-1 expression. Therefore, we postulated that p44/42 MAPK activation induced by 17-P-T-PGE$_2$ is mediated, at least in part, through EGFR and Src. To evaluate this hypothesis, we pretreated Huh7 and Hep3B cells with EGFR inhibitor AG1478 or Src inhibitor PP2 for 1 h, then treated the cells with 17-P-T-PGE$_2$ for indicated times. As shown in Fig. 7, pretreatment of Huh7 cells with AG1478 or PP2 significantly suppressed the phosphorylation level of p44/42 MAPK induced by 17-P-T-PGE$_2$. In Hep3B cells, AG1478 or PP2 almost completely inhibited the phosphorylation of p44/42 MAPK. These results clearly indicate that EGFR and Src are both involved in p44/42 MAPK activation induced by 17-P-T-PGE$_2$.

mTOR regulates the YB-1 expression induced by PGE$_2$. The mechanistic target of rapamycin (mTOR) signaling pathway senses and integrates a variety of environmental cues to regulate organismal growth and homeostasis. This pathway regulates many major cellular processes and is implicated in an increasing number of pathological conditions, including cancer and others disease (33). Dysregulated mTOR pathway was able to influence many aspects of tumor formation, such as proliferation, anti-apoptosis, cell cycle activation, angiogenesis and metastasis, and p44/42 MAPK could activate mTOR pathway by inhibiting the activity of TSC1/TSC2. We have shown that PGE$_2$ activated p44/42 MAPK via EP1 receptor, thus, we speculated that mTOR pathway is involved in YB-1 expression induced by 17-P-T-PGE$_2$. Pretreatment of Huh7 and Hep3B cells with rapamycin or PP2, as shown in Fig. 8A and B, significantly suppressed the YB-1 expression level induced by 17-P-T-PGE$_2$. Since mTOR forms two complexes to show its functions, we investigated through which complex the 17-P-T-PGE$_2$ upregulated YB-1 expression. As shown in Fig. 8C and D, RNAi suppression of raptor expression, existing only in mTOR complex 1, functions as scaffold for assembling the complex and for binding substrates and regulators, almost completely blocked the YB-1 expression induced by 17-P-T-PGE$_2$. These observations suggest that 17-P-T-PGE$_2$ increased the expression level of YB-1 through the mTOR complex 1 pathway.

YB-1 regulates EMT-associated gene expression. Based on our results, we known that YB-1 is critical for hepatocellular carcinoma cell invasion induced by PGE$_2$. A recent study showed that in breast cancer cells, YB-1 is associated with the EMT process, and could regulate a series of EMT-associated gene expression (20). Thus, we further investigated whether in HCC cells, YB-1 could regulate EMT-associated gene expression. As shown in Fig. 9A, RNAi suppression of YB-1 in Huh7 significantly increased the expression level of E-Cadherin, an epithelial marker protein, and downregulated the expression level of vimentin, a mesenchymal marker
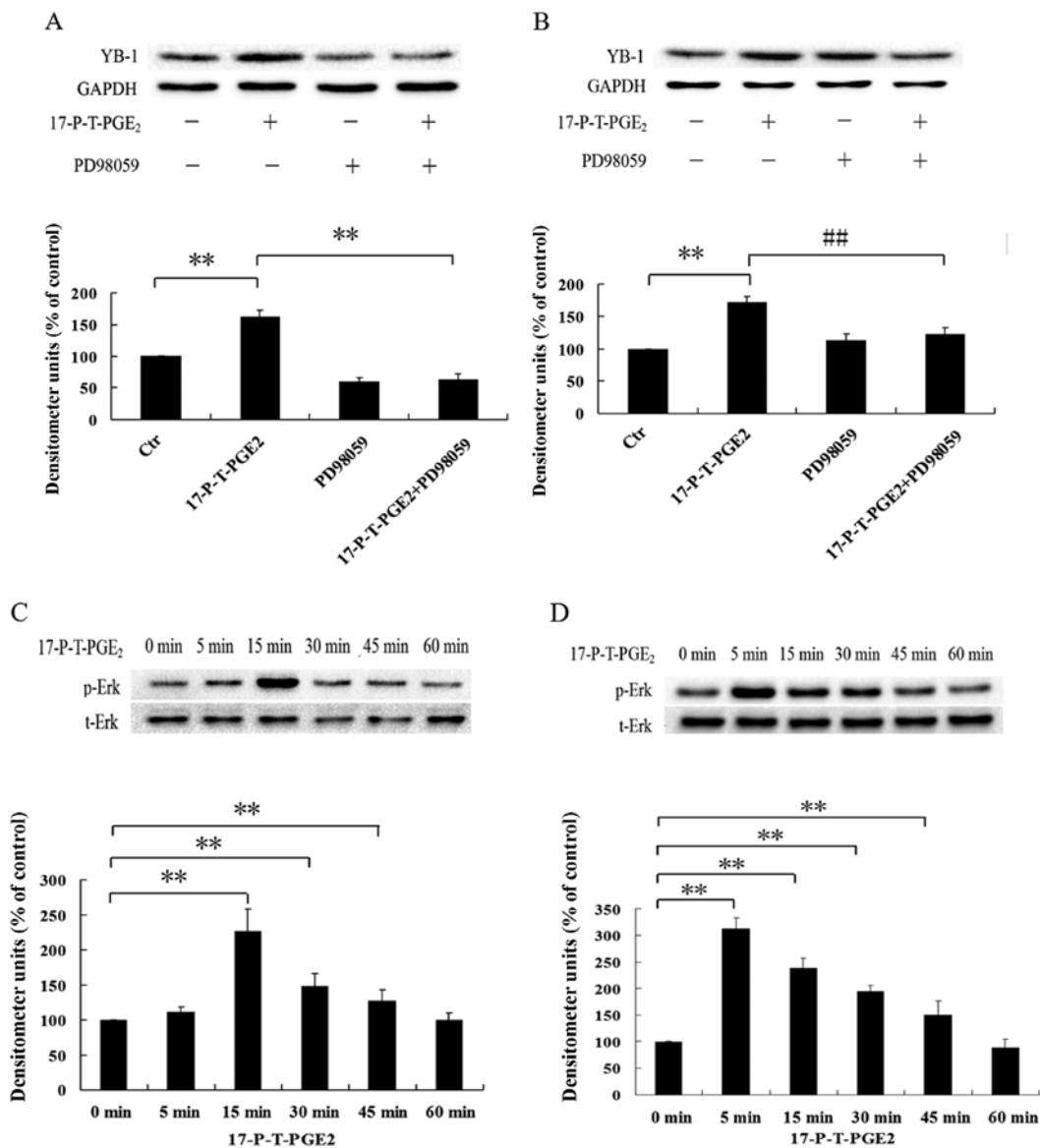
Figure 6. The role of p44/42 MAPK in upregulating the YB-1 protein via the EP1 receptor. (A) Effects of the MEK inhibitor PD98059 on YB-1 expression induced by 17-P-T-PGE$_2$. Huh7 cells cultured in serum-free medium were pretreated with MEK inhibitor PD98059 at 10 μM for 1 h, then they were treated with 5 μM 17-P-T-PGE$_2$ for 12 h. The cell lysates were obtained. YB-1 expression level was determined by immnuoblotting with anti-YB-1 antibody (upper panel); GAPDH as loading control was determined by immunoblotting with anti-GAPDH antibody (lower panel). Quantitative analysis of the YB-1 expression level was carried out by calculating the ratio between YB-1 protein and GAPDH expression levels from three different experiments. **p<0.01 compared with the control; ##p<0.01 compared with the 17-P-T-PGE$_2$ treatment. Data are presented as the mean ± SD of three independent experiments. (B) Similar results were also observed in the Hep3B cell line. (C) 17-P-T-PGE$_2$ activated p44/42 MAPK kinase in a time-dependent manner. Huh7 cells cultured in serum-free medium for 24 h were treated with 17-P-T-PGE$_2$ at 5 μM for indicated times, and the cell lysates were obtained. p44/42 MAPK phosphorylation was determined by immunoblotting with anti-phospho-p44/42 MAPK antibody (upper panel). Total p44/42 MAPK in the cell lysates was determined by re-probing the same blot with anti-p44/42 MAPK antibody (lower panel). Quantitative analysis of the p44/42 MAPK phosphorylation was carried out by calculating the ratio between p44/42 MAPK protein and phosphorylation level from three different experiments. **p<0.01 compared with the control. Data are presented as the mean ± SD of three independent experiments. (D) Similar results were also observed in the Hep3B cell line.

protein. In addition, the expression level of Snail protein, which is one of the key transcription factors controlling the EMT process, was also greatly suppressed. The expression level of total-p44/42 MAPK, which, as we discussed above, is a Serine/Threonine protein kinase associated with cancer cell invasion, was also dramatically decreased. Similar results were also observed in Hep3B cells (Fig. 9B), except vimentin expression, which we could not detect in Hep3B cells. These results indicate that YB-1 was able to regulate a series of EMT-associated genes expression in HCC cells, and have the potential to control EMT progression.

Discussion

PGE$_2$, an inflammation mediator, exerts its biological functions mainly through four G-protein-coupled receptors (GPCR) on the cell surface membrane, designated as EP1, EP2, EP3 and EP4, respectively. The EP1 receptor is coupled with G$_{\alpha q}$ protein and thus signals through phospholipase C and intracellular Ca^{2+}. The EP2 and EP4 receptors are coupled with G$_{\alpha s}$ protein, signaling through elevation of intracellular cAMP level and activation of protein kinase A (PKA), while the EP3 receptor is more complex, according to our previous
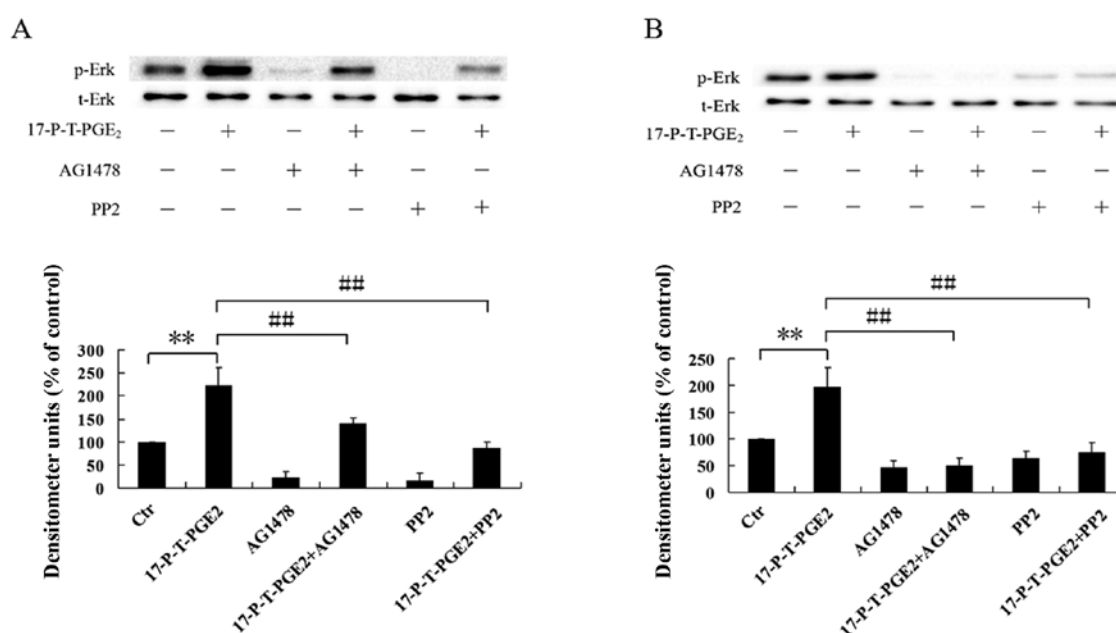
Figure 7. The role of EGFR and Src kinase in activating p44/42 MAPK induced via EP1 receptor. (A) Effects of EGFR inhibitor AG1478 and Src inhibitor PP2 on p44/42 MAPK phosphorylation induced by 17-P-T-PGE₂. Huh7 cells cultured in serum-free medium were pretreated with AG1478 at 2 μM, or PP2 at 10 μM for 1 h, then they were treated with 5 μM 17-P-T-PGE₂ for 15 min. The cell lysates were obtained and the p44/42 MAPK phosphorylation was determined by immunoblotting with anti-phospho-p44/42 MAPK antibody (upper panel), Total p44/42 MAPK in the cell lysates was determined by reprobing the same blot with anti-p44/42 MAPK antibody (lower panel). Quantitative analysis of the p44/42 MAPK phosphorylation was carried out by calculating the ratio between p44/42 MAPK protein and phosphorylation level from three different experiments. **p<0.01 compared with the control; ##p<0.01 compared with 17-P-T-PGE₂ treatment. Data are presented as the mean ± SD of three independent experiments. (B) Similar results were also observed in the Hep3B cell line.

results, it is believed to have multiple isoforms generated through alternative mRNA splicing in the carboxyl tail of the EP3 receptor (6). The different EP3 receptor splice variants, coupled with a different G protein, may have multiple signal transduction pathways in different tissues. The interaction between the four EP receptors subtypes and PGE₂ depends on the different expression level of an individual receptor on the cell membrane, the binding affinity to PGE₂, and the differential threshold value for activation. On one hand, each subtype of EP receptor could transmit different signals to the downstream pathway, and regulate the different aspects of cellular functions; on the other hand, the different signals from the four subtypes could be integrated as a whole to control the physiological or the pathological phenotypes of the cells.

The EP1 receptor has been reported to be closely associated with cancer cell migration and invasion. EP1 receptor enhanced the phosphorylation of FAK promoting the hepatocellular carcinoma cell migration and invasion (21,22). In cholangiocarcinoma cells, EP1 receptor has been shown to upregulate the MMP-2 expression through the CREB pathway. $\alpha 2\beta 1$ integrin is associated with cell migration, and EP1 receptor enhanced the cell migration through upregulating the expression of $\alpha 2\beta 1$ integrin (34). Some studies have indicated that EP1 receptor was able to transactivate the EGFR receptor and subsequently activate the Akt kinase, but the detailed mechanisms how the activated Akt kinase would promote cancer cell invasion are not clear (28,29).

Metastasis is responsible for as much as 90% of cancer-associated mortality, yet it remains the most pooly understood component of cancer pathogenesis. During metastatic

dissemination, a cancer cell from a primary tumor executes the following sequence of steps: it locally invades the surrounding tissue, enters the microvasculature of the lymph and blood systems (intravasation), survives and translocates largely through the bloodstream to microvessels of distant tissues, exits from the bloodstream (extravasation), survives in the microenvironment of distant tissues, and finally adapts to the foreign microenvironment of these tissues in ways that facilitate cell proliferation and the formation of a macroscopic secondary tumor (35).

Some researchers pointed out that the complex metastatic cascade can be conceptually organized and simplified into two major phases: i) physical translocation of a cancer cell from the primary tumor to the microenvironment of a distant tissue and ii) colonization (36). EMT (epithelial mesenchymal transition) has been implicated as a critical process that drives the epithelial derived tumor to gain the malignant properties. During the EMT process, the epithelial cells would lose the expression profile of epithelial cell marker proteins, and begin to express the mesenchymal cell marker proteins. This expression profile switch makes the epithelial cell to appear as mesenchymal phenotype. Thus, the EMT confers on epithelial cells precisely the set of traits that would empower them to disseminate from primary tumors and seed metastases (37). Long standing chronic inflammation is required to initiate the EMT process, and which is tightly controlled by EMT-inducing transcription factors (EMT-TFs). However, whether PGE₂, as a kind of inflammation mediator, could promote the tumor EMT process is still unclear and an associated study is undergoing in our laboratory.

Y box binding protein 1 (YB-1) belongs to the family of the cold-shock containing proteins which could not only func-
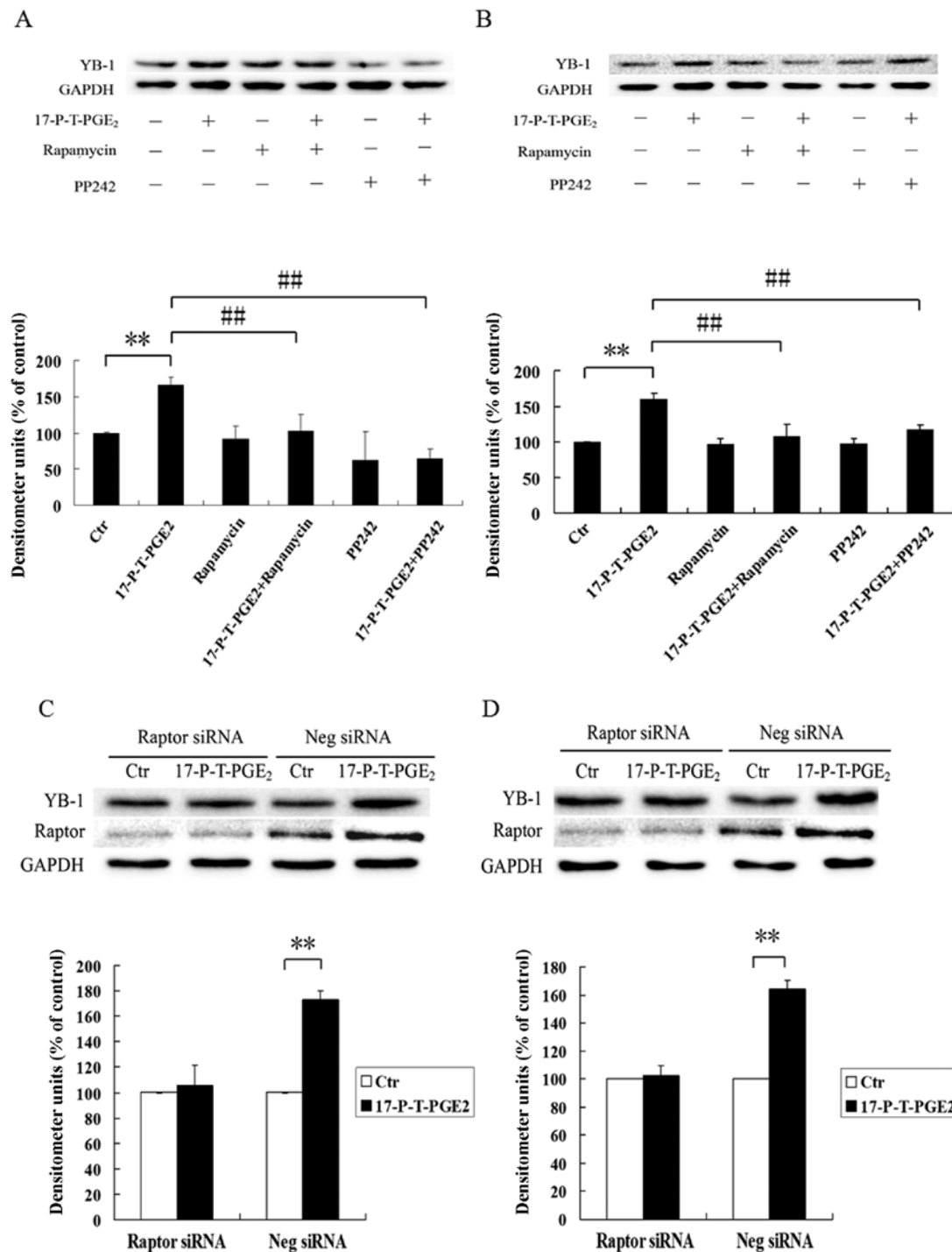
Figure 8. The role of mTOR in upregulating the YB-1 expression induced via EP1 receptor. (A) Effects of rapamycin and PP242 on YB-1 expression induced by 17-P-T-PGE$_2$. Huh7 cells cultured in serum-free medium were pretreated with 50 nM rapamycin or 5 μM PP242 for 1 h, and then they were treated with 17-P-T-PGE$_2$ for 12 h. The cell lysates were obtained, and the YB-1 expression level was determined by immunoblotting with anti-YB-1 antibody (upper panel), GAPDH as loading control was determined by immunoblotting with anti-GAPDH antibody (lower panel). Quantitative analysis of YB-1 expression level was carried out by calculating the ratio between YB-1 protein and GAPDH expression from three different experiments. **p<0.01 compared with the control; ##p<0.01 compared with the 17-P-T-PGE$_2$ treatment. Data are presented as the mean ± SD of three independent experiments. (B) Similar results were also observed in Hep3B cell line. (C) RNAi suppression of Raptor inhibits YB-1 expression induced by 17-P-T-PGE$_2$. Huh7 cells were transfected overnight with the Raptor siRNA or control siRNA in regular medium and then treated with 5 μM 17-P-T-PGE$_2$ for 12 h. The cell lysates were obtained and the YB-1 expression level was determined by immunoblotting with anti-YB-1 antibody (upper panel), RNAi suppression efficiency of Raptor was determined by immunoblotting with anti-Raptor antibody (middle panel), GAPDH as loading control was determined by immunoblotting with anti-GAPDH antibody (lower panel). Quantitative analysis of YB-1 expression level was carried out by calculating the ratio between YB-1 protein and GAPDH expression level from three different experiments. **p<0.01 compared with negative siRNA transfected Huh7 cells control group. Data are presented as the mean ± SD of three independent experiments. (D) Similar results were also observed in the Hep3B cell line.

tion as a transcription factor in nuclear (38,39), but also control subsets of mRNA translational efficiency in the cytoplasm (40).

YB-1 has been regarded as an oncoprotein and prognostic marker (12), as it can promote the cancer development and
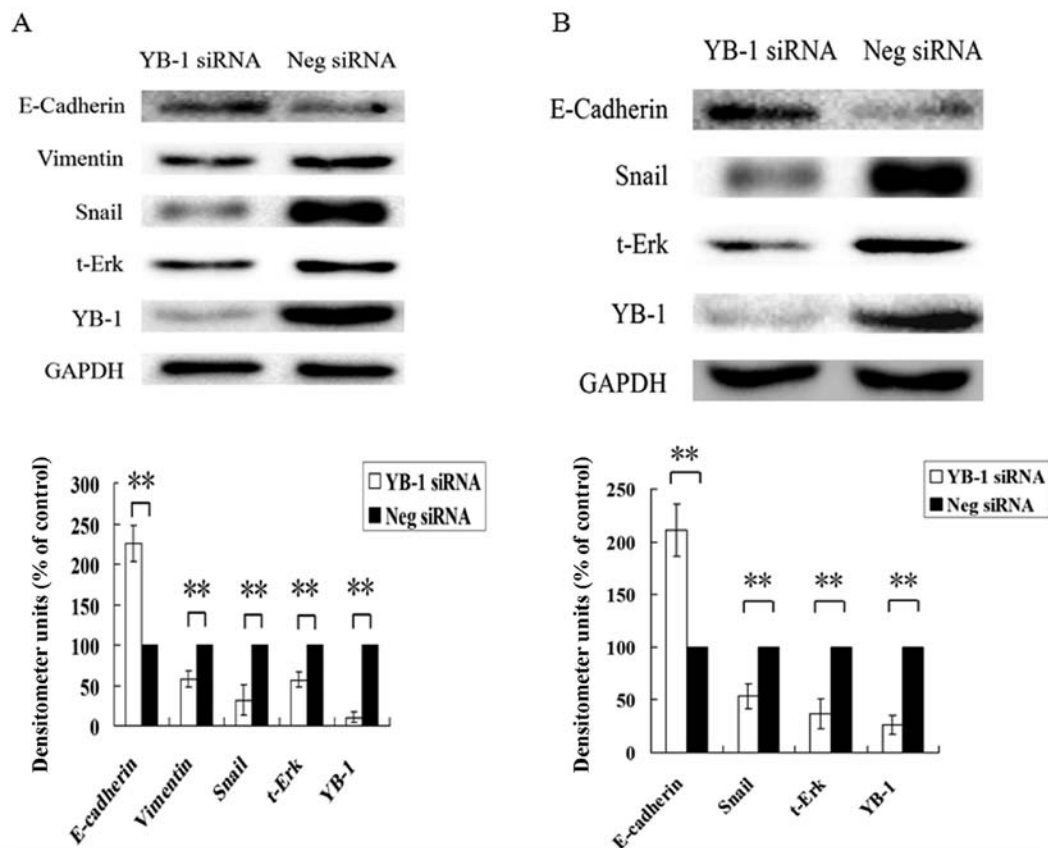
Figure 9. The role of YB-1 protein in regulating gene expression associated with EMT process. (A) Effects of YB-1 on regulating gene expression levels associated with EMT process. Huh7 cells were transfected overnight with YB-1 siRNA or control siRNA. After 72 h of incubation, the cell lysates were obtained and the expression levels of a series of genes related with EMT process were determined by immunoblotting with anti-E-Cadherin, anti-Vimentin, anti-Snail, anti-t-Erk antibody, the RNAi suppression efficiency of YB-1 was determined by immunoblotting with anti-YB-1 antibody, GAPDH as loading control was determined by immunoblotting with anti-GAPDH antibody. Quantitative analysis of target gene expression levels were carried out by calculating the ratio between target genes and GAPDH expression level from three different experiments. **p<0.01 compared with control siRNA transfected Huh7 cells. Data are presented as the mean ± SD of three independent experiments. (B) Similar results were also observed in the Hep3B cell line.

progression. In gastric cancer cells, RNAi knockdown YB-1 expression greatly inhibited cell migration (41). While in breast cancer, YB-1 was able to promote cancer cell invasion and metastasis by altering MT1-MMP trafficking (27). Recent work showed that YB-1 could regulate the expression of some EMT associated genes, such as Snail (20), and have an important role in controlling the EMT (epithelial-mesenchymal transition) and MET (mesenchymal-epithelial transition) processes. Therefore, we believe that YB-1 is a critical regulator to promote cancer cell invasion.

Since PGE$_2$ and YB-1 are both involved in cancer cell invasion, and have the potential to regulate the EMT process, the internal relationship between PGE$_2$ and YB-1 is of particular interest to us. In our study, we found that PGE$_2$ could significantly increase the invasion ability of HCC cells, and this effect is primarily mediated via EP1 receptor, which is consistent with previous results of others (28). In order to determine the role of YB-1 in PGE$_2$-induced HCC cell invasion, we downregulated the YB-1 expression level, and observed that knockdown YB-1 expression greatly suppressed the HCC cell invasion ability induced by PGE$_2$. All these results firmly confirm that YB-1 is a critical regulator in PGE$_2$-induced HCC cell invasion.

YB-1 could regulate Snail protein expression and promote EMT process in breast cancer (20), so we investigated whether

YB-1 would influence the expression of some EMT-associated genes in our HCC cells. According to our results, we found that knockdown of YB-1 expression dramatically suppressed Snail and vimentin expression, while increased E-Cadherin expression. These observations further confirm that YB-1 could regulate the cell invasion ability.

Since YB-1 is involved in PGE$_2$-induced HCC cell invasion, we next analyzed whether PGE$_2$ could directly regulate YB-1 expression. Our results show that PGE$_2$ could significantly increase YB-1 expression, and EP1 receptor is the primary receptor responsible for it. These results are also consistent with our previous cell invasion assay, indicating that EP1 receptor plays an important role in cell invasion induced by PGE$_2$.

An increasing number of evidence indicates that GPCR could transactivate the receptor-tyrosine kinase on the cell membrane (42). It has been reported that EP1 receptor, as a kind of GPCR located mainly on the cell surface membrane, could transactivate the EGFR by forming the complex with EGFR and Src (28,29). Therefore, EGFR and Src may be downstream proteins of EP1 receptor. We hypothesized that Src and EGFR are involved in YB-1 expression induced by PGE$_2$. The chemical inhibitor analysis confirmed our hypothesis. Src and EGFR are located upstream in the EP1 receptor mediated signal pathway, which would activate other effector

proteins to exert their functions. Akt and p44/42 MAPK are kinases downstream of EGFR. p44/42 MAPK regulates many cellular functions. Recently, it has been reported that p44/42 MAPK is mainly responsible for regulating cancer cell invasion (30,43,44). In our study, chemical inhibitor analysis showed that when the activity of p44/42 MAPK was inhibited, the PGE$_2$ induced YB-1 expression was suppressed. Further experiments confirmed that the phosphorylation level of p44/42 MAPK was increased when EP1 receptor was activated, and when the activities of EGFR or Src were inhibited, the phosphorylation level of p44/42 MAPK was also suppressed. These results altogether indicate that p44/42 MAPK is involved in YB-1 expression induced by PGE$_2$, and the activity of which is regulated, at least in part, through EGFR and Src kinase.

mTOR is an atypical serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and interacts with several proteins to form two distinct complexes the mTOR complex 1 (mTORC 1) and 2 (mTORC 2) (33). The mTOR pathway regulates many major cellular functions, such as cell growth, proliferation, metabolism and autophagy. Thus, dysregulated mTOR pathway has significant promoting effect on tumor progression. mTOR pathway is activated mainly by Akt and p44/42 MAPK. It is known that PGE$_2$ could activate p44/42 MAPK via EP1 receptor, so we hypothesized that mTOR participated in YB-1 expression induced by PGE$_2$. In our results, we found that two kinds of mTOR inhibitors could greatly suppress the YB-1 expression induced by 17-P-T-PGE$_2$, which suggests mTOR is involved in this process. However, mTOR exerts its functions mainly through two mTOR complexes, so further experiments were performed to analyze which complex is responsible for this regulation. RNAi suppression of raptor expression, which exists only in mTORC 1, almost completely blocked YB-1 expression induced by 17-P-T-PGE$_2$, which indicates that mTORC 1 is the primary complex involved in this regulation. All these results verify our hypothesis that PGE$_2$ increases YB-1 expression level through activating the mTOR pathway and the mTORC 1 plays the major role in this process.

It has been reported that YB-1 was able to promote cancer cell proliferation and RNAi suppression of YB-1 expression might inhibit cell proliferation (45). However, we found RNAi suppression of YB-1 expression did not significantly affect the cell proliferation rate in 17-P-T-PGE$_2$ treatment group compared with negative siRNA transfected HCC cells by WST analysis (data not shown). This finding suggests that PGE$_2$-induced YB-1 upregulation mainly involved cell invasion but not proliferation in HCC cells.

In summary, our study revealed a signal transduction pathway through which PGE$_2$ regulates YB-1 expression. PGE$_2$ increased the YB-1 expression through EP1 receptor; EGFR, Src and p44/42 MAPK are all involved in this process; p44/42 MAPK activated the mTOR pathway, which in turn upregulate the YB-1 expression, while YB-1 has significant role in promoting cancer cell invasion through regulating EMT-associated gene expression. To our knowledge, this is the first study detailing the role of PGE$_2$-EP1 receptor signal pathway in YB-1 expression in human hepatocellular carcinoma cells. Our findings reveal the new mechanisms through which PGE$_2$ enhances hepatocellular carcinoma cell invasion and may be helpful in finding a new therapeutic strategy to prevent and cure malignant diseases.

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