Research Article

Prostaglandin E_2 Regulates Tumor Angiogenesis in Prostate Cancer

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Abstract

In cancer management, the cyclooxygenase (COX)–targeted approach has shown great promise in anticancer therapeutics. However, the use of COX-2 inhibitors has side effects and health hazards; thus, targeting its major metabolite prostaglandin E_2 (PGE_2)–mediated signaling pathway might be a rational approach for the next generation of cancer management. Recent studies on several *in vitro* and *in vivo* models have revealed that elevated expression of COX-2 correlates with prostate tumor growth and angiogenesis. In this study, we have shown the in-depth molecular mechanism and the PGE_2 activation of the epidermal growth factor receptor and β3 integrin through E prostanoid 2 (EP2)–mediated and EP4-mediated pathways, which lead to activator protein-1 (AP-1) activation. Moreover, PGE_2 also induces activating transcription factor-4 (ATF-4) activation and stimulates cross-talk between ATF-4 and AP-1, which is unidirectional toward AP-1, which leads to the increased expressions of urokinase-type plasminogen activator and vascular endothelial growth factor and, eventually, regulates prostate tumor cell motility. *In vivo* Matrigel angiogenesis assay data revealed that PGE_2 induces angiogenesis through EP2 and EP4. Human prostate cancer specimen analysis also supported our *in vitro* and *in vivo* studies. Our data suggest that targeting PGE_2 signaling pathway (i.e., blocking EP2 and EP4 receptors) might be a rational therapeutic approach for overcoming the side effects of COX-2 inhibitors and that this might be a novel strategy for the next generation of prostate cancer management. [Cancer Res 2008;68(19):7750–9]

Introduction

Treatment of cancer by chemotherapeutic agents is considered one of the most effective approaches in cancer management in recent times. Earlier reports have depicted that reduced apoptosis, increased neovascularization, and immunosuppression are some of the known consequences of cyclooxygenase-2 (COX-2) overexpression, and each effect could have an important role in tumor progression and angiogenesis (1). Several selective and nonselective COX-2 inhibitors have been in use for the treatment of different cancers, but many questions have arisen regarding their side effects (2). Various studies have shown the correlation between COX-2 overexpression and enhanced production of prostaglandin E_2 (PGE_2) by cancer cells (3). It has been reported that the rate of PGE_2 conversion from arachidonic acid is almost 10-fold higher in malignant prostatic tissues than in benign prostatic tissues (4). Thus, the concerns regarding the safety of these COX-2 inhibitors, as well as the identification of the more effective therapeutic agents, prompted us to understand the downstream signaling events regulated by PGE_2 in prostate cancer, which might help to develop new therapeutic approach in the treatment of prostate cancer.

PGE_2 interacts with the E prostanoid (EP) family of receptors, which consist four different subtypes (EP1–EP4). The enhanced expressions of EP2 and EP4 receptors have been shown in prostate cancer, as well as in endothelial cells (5, 6). In this study, we have examined the role of PGE_2-mediated signaling during prostate cancer progression and suggested that blocking the interaction between PGE_2 and its receptors, rather than global prostaglandin synthesis by using specific COX-2 inhibitors, might circumvent some of the adverse side effects. Recently, we have shown that the chemokine-like protein, osteopontin, induces COX-2–dependent PGE_2-mediated prostate cancer progression (7). However, the molecular mechanism by which PGE_2 directly regulates prostate cancer progression and angiogenesis is not well defined.

Previous studies have shown that PGE_2 augments cyclic AMP (cAMP) production (8), increases cellular growth, and regulates differentiated cell functions by promoting the activation of cAMP-dependent protein kinase A (PKA). The PKA-mediated phosphorylation of cAMP-responsive element binding protein (CREB) and regulation of transcription via interaction between cAMP-response elements with CREB are considered as the major pathways that alter gene expression in cancer cells (9, 10). Earlier studies have revealed that activating transcription factor 4 (ATF-4; also called CREB-2) regulates the expression of genes involved in oxidative stress, amino acid synthesis, differentiation, metastasis, and angiogenesis (11). It has been reported that the expression of ATF-4 is induced by various external stimuli in cancer microenvironment and regulates various processes that control cancer progression (11), but the function of ATF-4 in prostate cancer progression remains unknown.

The overexpression of proteases often correlates with the enhanced tumor cell invasion and metastasis by virtue of degradation of extracellular matrix and basement membranes in almost all malignancies, including prostate cancer (12, 13). Urokinase-type plasminogen activator (uPA), a protease, plays an important role in tumor cell invasion and metastasis (14). Increased expressions of uPA and vascular endothelial growth factor (VEGF) have been reported in malignancies of various organs including prostate (14, 15), and the increased expression of these molecules is associated with an enhanced metastatic and angiogenic potential and poor survival of patients (16). Earlier data have shown that the response elements for activator protein 1 (AP-1) and ATF-4 are present in the promoter region of uPA and VEGF (17–20). Although it has been reported that PGE_2 plays a crucial role in VEGF production in prostate cancer cells (21), the molecular mechanism by which PGE_2 regulates ATF-4/AP-1–mediated uPA and VEGF expressions, which lead to prostate tumor cell motility and *in vivo* angiogenesis, remains unknown.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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In this study, we have shown that PGE_2 triggers mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK1/2 signaling through activation of epidermal growth factor receptor (EGFR) and augments expression and activation of β3 integrin in prostate cancer cells. Moreover, we have shown that PGE_2 induces the activation of ATF-4 and AP-1 via EGFR-MEK-ERK1/2 or β3 integrin–mediated pathway, which ultimately leads to the increased expressions of uPA and VEGF. Furthermore, we have observed that PGE_2 regulates endothelial cell motility and *in vivo* angiogenesis. Analysis of human prostate clinical samples showed that the expression profiles of EP2 and EP4 receptors correlated with levels of AP-1, ATF-4, uPA, and VEGF. These data suggested that, at least in part, PGE_2 plays a crucial role in the oncogenesis and angiogenesis of prostate cancer. Thus, targeting PGE_2 receptor–mediated signaling might be a potential approach for the improved prostate cancer therapeutics.

Materials and Methods

Cell culture and transfection. Human prostate cancer cell lines (PC-3, DU-145, and LNCaP) were obtained from American Type Culture Collection. Human umbilical vein endothelial cell (HUVEC) was purchased from Cambrex. COX-2 cDNA (Dr. Stephen Prescott, University of Utah), wild-type (wt) and dominant-negative (dn) ATF-4 (wt, pEF/mATF-4; dn, pEF/mATF-4M; Dr. Javed Alam, Yale University School of Medicine), and A Fos (Dr. Charles Vinson, National Cancer Institute) were transfected in PC-3 cells using Lipofectamine 2000.

Small interfering RNA. PC-3 cells were transfected with small interfering RNA (siRNA) that specifically targets COX-2 (COX-2 siRNA, Santa Cruz Biotechnology), EP2 (ON-TARGET plus SMARTpool PTGER2; L-005712-00), EP4 (siGENOME SMARTpool PTGER4; M-005714-00), human integrin β3 (siGENOME SMARTpool ITGB3; M-004124-02), and control siRNA (siGENOME nontargeting siRNA; D-001206-14-05 and ON-TARGET plus nontargeting pool; D-001810-10-05; Dharmacon) according to the manufacturer's instructions.

Western blot and EMSA. The Western blot and EMSA experiments were performed as described earlier (7, 22).

Immunofluorescence. Immunofluorescence studies were performed using specific antibodies as described earlier (22, 23).

Flow cytometry. Flow cytometry experiments were performed as described (24).

Reverse transcription–PCR. Total RNA was isolated from PC-3 cells and analyzed by reverse transcription–PCR (RT-PCR). The following primers were used: uPA sense, 5′-CAC GCA AGG GGA GAT GAA-3′; uPA antisense, 5′-ACA GCA TTT TGG TGG TGA CTT-3′; VEGF sense, 5′-CCT CCG AAA CCA TGA ACT TT-3′; VEGF antisense, 5′-AGA GAT CTG GTT CCC GAA AC-3′; β-actin sense, 5′-GGC ATC CTC ACC CTG AAG TA-3′; β-actin antisense, 5′-GGG GTG TTG AAG GTC TCA AA-3′. The amplified cDNA fragments were analyzed by 1.5% agarose gel electrophoresis.

Cell migration and comigration assay. The migration and comigration experiments were performed as described (22). Briefly, PC-3 cells, either alone or individually transfected with dn ATF-4, dn c-Jun, and A-Fos or pretreated with PKA inhibitor peptide, were added to the upper chamber of the Boyden chamber. PGE_2 was added in the upper chamber. For comigration assay, PC-3 cells, either alone or transfected with COX-2 cDNA or COX-2 siRNA, were used in the lower chamber. Endothelial (HUVEC) cells, either alone or pretreated with EP2 (AH6809, Sigma) or EP4 (AH23848, Sigma) receptor antagonist, were used in the upper chamber. The migrated endothelial cells to the reverse side of the upper chamber were fixed and stained with Giemsa and counted in three high-power fields under an inverted microscope (Nikon). Data are represented as the average of three counts ± SE.

Wound assay. Wound assays were performed using PC-3 and endothelial cells as described earlier (7). Wounds with a constant diameter were made. PC-3 cells were treated with PGE_2 alone or pretreated with EP2 or EP4 receptor antagonist or PKA inhibitor peptide, or transfected with dn ATF-4, dn c-Jun, A-Fos and then treated with PGE_2. In separate experiments, endothelial cells were treated with PGE_2 alone or pretreated with EP2 or EP4 receptor antagonist and then treated with PGE_2. After 12 h, wound photographs were taken through a microscope (Nikon).

***In vivo* Matrigel plug assay.** *In vivo* Matrigel plug angiogenesis assay was carried out, as described previously (22). Briefly, Matrigel, either alone or along with PGE_2, was injected s.c. into the ventral groin region of male athymic NMRI (nu/nu) mice. In separate experiments, PGE_2 containing Matrigel was mixed with EP2 or EP4 antagonist (30 μmol/L) and injected into the mice. In another experiment, conditioned medium of PC-3 cells, either nontransfected or transfected with COX-2 cDNA, was mixed with Matrigel and injected into the mice. After 21 d, mice were sacrificed, dissected out, and photographed. The Matrigel plugs were excised and used for immunohistochemistry. The paraffin sections were immunostained with anti-vWF (Chemicon), anti-CD31 (Chemicon), anti–phosphorylated p65, nuclear factor-κB (NF-κB; Cell Signaling Technology), and anti–phosphorylated Akt (Santa Cruz) antibodies and visualized under confocal microscope (Ziess).

Human prostate cancer specimen analysis. Specimens of different Gleason grades and normal tissues of prostate were collected from a local hospital with informed consent and analyzed by immunohistochemistry as described (7). The expression profiles of EP2, EP4, ATF-4, c-Jun, c-Fos, uPA, and VEGF were detected by immunohistochemistry using their specific antibodies. Five specimens from each group [normal, prostatic intraepithelial neoplasia (PIN), and malignant] were analyzed.

Statistical analysis. The data reported in cell migration, comigration, *in vivo* Matrigel plug angiogenesis, and the clinical specimen analysis are expressed as mean ± SE. Statistical differences were determined by Student's t test. A P value of <0.05 was considered significant. All bands were analyzed densitometrically (Kodak Digital Science), and fold changes were calculated. The *in vivo* angiogenesis and clinical specimen data were quantified using the Image Pro Plus 6.0 Software (Nikon).

Results

PGE_2 augments EP2/EP4-mediated EGFR/MAPK and β3 integrin activation in prostate cancer cells. To examine the effect of PGE_2 on EGFR, MEK, ERK1/2, and β3 integrin phosphorylation, serum-starved PC-3 cells were treated with PGE_2 in a dose (0–1.0 μmol/L)–dependent and time (0–60 minutes)–dependent manner. The levels of phosphorylation of these signaling molecules were analyzed by Western blot using their phosphorylated-specific antibodies. The data indicated that PGE_2 induces phosphorylation of EGFR, MEK, ERK1/2, and β3 integrin, and maximum phosphorylations were observed between 10 and 15 minutes (Fig. 1*A*) with 0.5 μmol/L of PGE_2 (Supplementary Fig. S1*A*). Moreover, the effect of PGE_2 on the activation of these signaling molecules (EGFR, MEK, ERK, and β3 integrin) was examined in other prostate cancer (DU-145 and LNCaP) cells. The data showed significant phosphorylations of these molecules in DU-145 compared with LNCaP cells in response to PGE_2 (Supplementary Fig. S1*B*). Previous reports have shown that PC-3 cells express higher levels of EP2 and EP4 receptors (21). Therefore, to examine the involvement of EP2 and EP4 receptors in PGE_2-induced EGFR and β3 integrin phosphorylation, PC-3 cells were pretreated with EP2 (AH6809) or EP4 (AH23848) receptor antagonist in a dose-dependent manner (0–30 μmol/L) for 1 hour and then treated with PGE_2, and the levels of phosphorylated EGFR and phosphorylated β3 integrin were analyzed by Western blot. AH6809 or AH23848 at 30 μmol/L concentration showed maximum inhibition of PGE_2-induced EGFR and β3 integrin phosphorylation (Fig. 1*B, I* and *II*). To examine whether EP2 and EP4 receptor

agonists mimic the effect of PGE_2 and regulate the downstream molecular events, PC-3 cells were treated with butaprost (EP2 agonist) and PGE_1 alcohol (EP4 agonist) and phosphorylated EGFR and phosphorylated β3 integrin were analyzed. The data showed that both the agonists induce the phosphorylation of EGFR and β3 integrin (Supplementary Fig. S2*A* and *B*). These data revealed that PGE_2 induces the phosphorylation of EGFR and β3 integrin through EP2 and EP4 receptors–mediated process. Recently, it has been reported that PGE_2 induces β1 integrin expression in hepatocellular carcinoma cells (25). To determine whether PGE_2 regulates the expression of β3 integrin in prostate cancer cells, PC-3 cells were treated with PGE_2 for 12 hours and the expression of β3 integrin was analyzed by flow cytometry (Fig. 1*C*). To determine the roles of EP2 and EP4 receptors in PGE_2-induced β3 integrin expression, PC-3 cells were pretreated with AH6809 or AH23848 and then treated with PGE_2, and expression of β3 integrin was analyzed by immunofluorescence. The data showed that AH6809 and AH23848 suppressed the PGE_2-induced β3 integrin expression, indicating that EP2 and EP4 receptors play crucial roles in regulating this process (Fig. 1*D*). These data suggested that PGE_2 does not only stimulate EGFR and β3 integrin phosphorylation but also induces the expression of β3 integrin via EP2/EP4 receptor–mediated pathway.

EGFR and β3 integrin play crucial roles in PGE_2-induced AP-1 activation. Earlier studies have shown the role of AP-1 in prostate cancer progression (26, 27). Activation of AP-1 involves the increased expression or activation of Jun and Fos proteins (28–30). To examine the effect of PGE_2 on c-Fos and c-Jun expression/activation, PC-3 cells were treated with PGE_2, and expressions of c-Fos and phosphorylation of c-Jun were analyzed by Western blot and immunofluorescence, whereas AP-1–DNA binding was performed by EMSA. The results revealed that PGE_2 does not only augment the expression of c-Fos and phosphorylation of c-Jun (Fig. 2*A* and Supplementary Fig. S3*A*) but also stimulates the AP-1–DNA binding (Supplementary Fig. S3*B*). Furthermore, to study the role of EGFR/MAPK or β3 integrin on PGE_2-induced AP-1

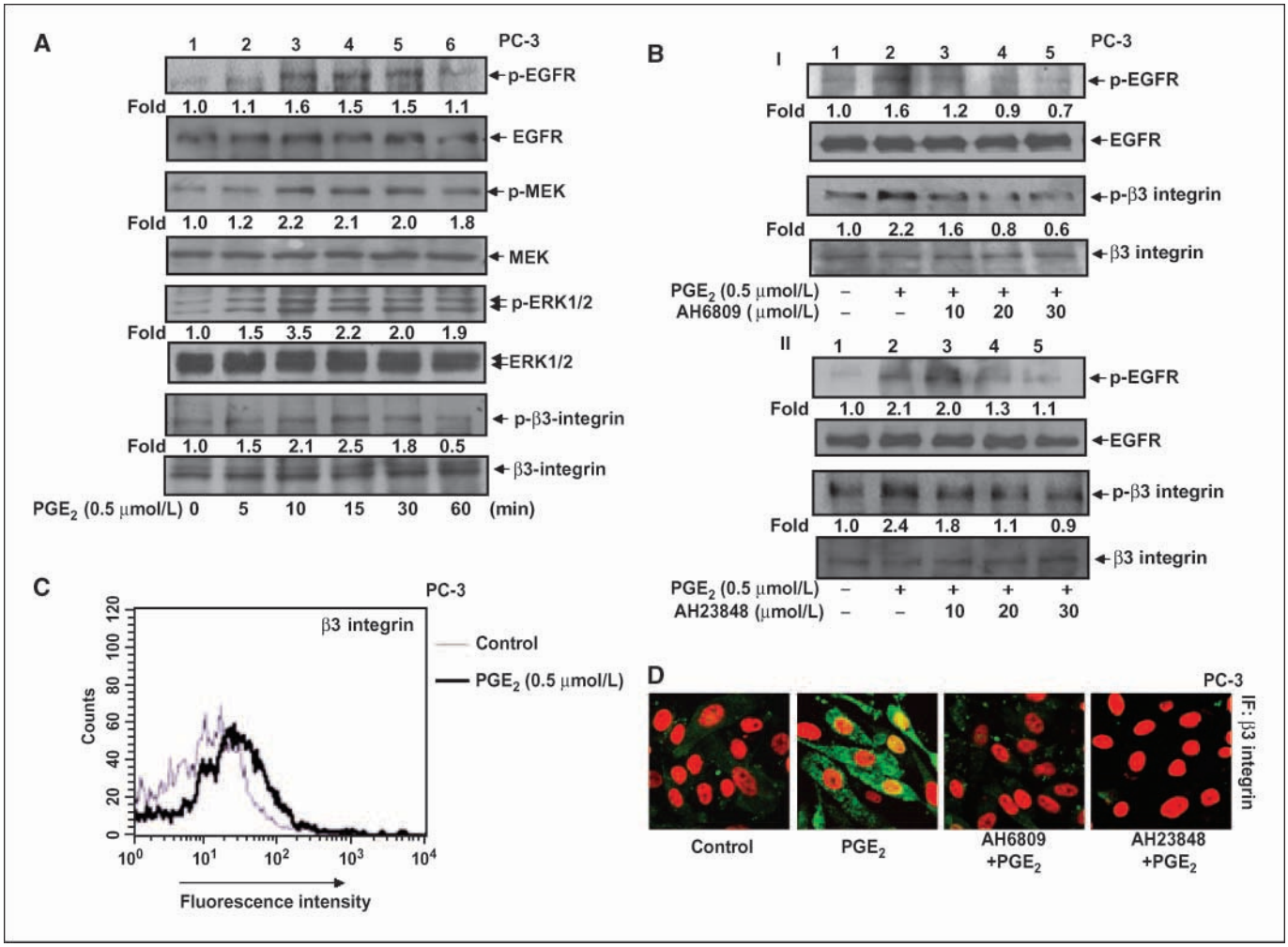

Figure 1. PGE_2 augments phosphorylation of EGFR, MEK, ERK, and β3 integrin in PC-3 cells. *A,* PC-3 cells were incubated with 0.5 μmol/L PGE_2 for 0 to 60 min, and the levels of p-EGFR, p-MEK, p-ERK, and p-β3 integrin were analyzed by Western blot using their specific antibodies. Total EGFR, MEK, ERK, and β3 integrin expressions in the cells were used as loading controls. *B,* roles of EP2 and EP4 receptors in PGE_2-induced phosphorylations of EGFR and β3 integrin. PC-3 cells were pretreated with either EP2 receptor antagonist (AH6809) or EP4 receptor antagonist (AH23848) in a dose-dependent manner (0–30 μmol/L) for 1 h and then treated with PGE_2, and the levels of phosphorylated EGFR and phosphorylated β3 integrin were analyzed by Western blot (*I* and *II*). *C,* PC-3 cells were treated with PGE_2, and β3 integrin expression was analyzed by flow cytometry using anti–β3 integrin antibody. *D,* PC-3 cells were pretreated with AH6809 or AH23848 and then treated with PGE_2, and the level of β3 integrin was analyzed by immunofluorescence using anti-β3 integrin antibody followed by staining with Cy2-conjugated IgG (*green*). Nuclei were stained with propidium iodide (*PI, red*). All figures are representation of three experiments. Fold changes were calculated.

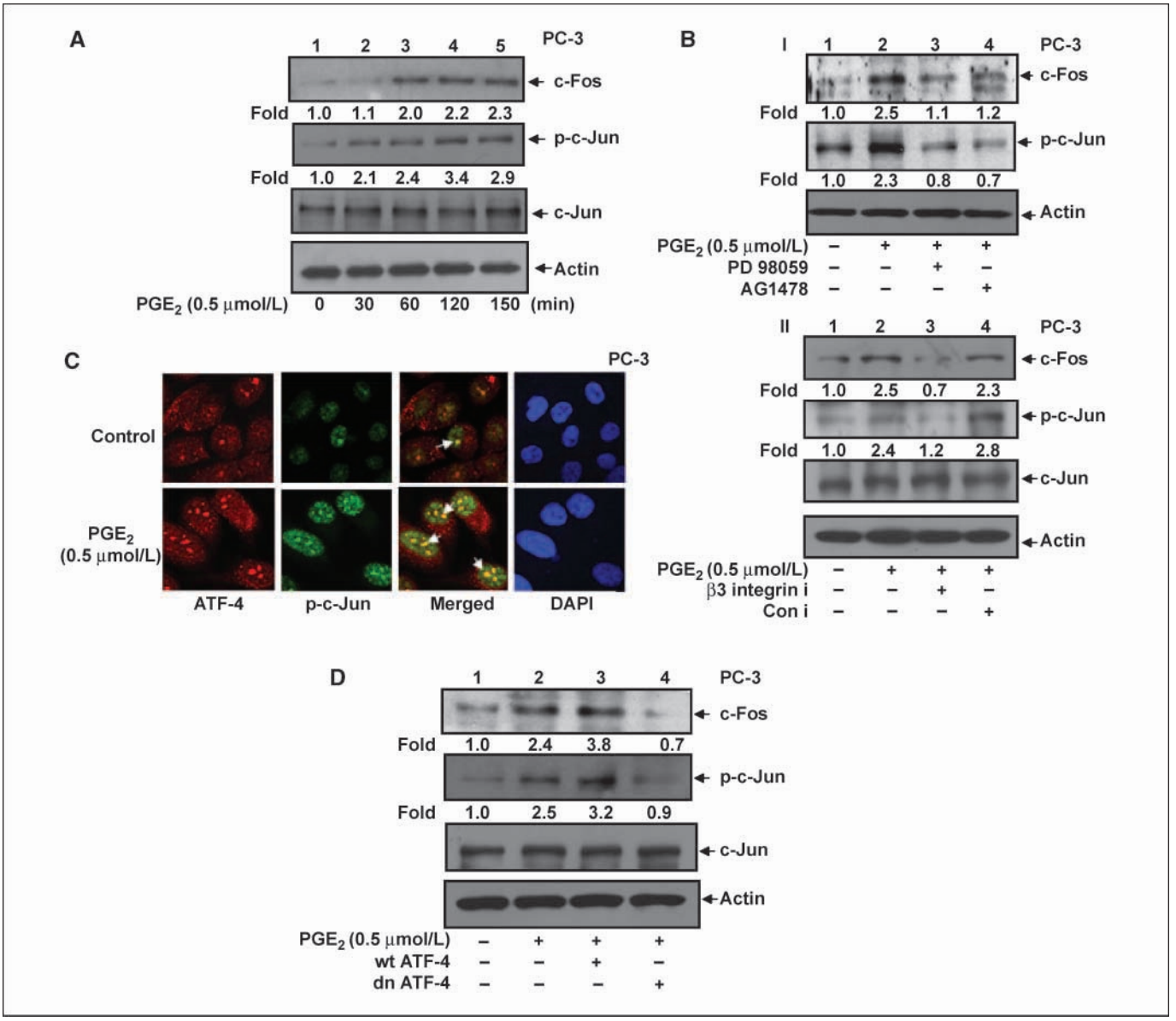

Figure 2. PGE_2 induces EP2/EP4-mediated EGFR/MAPK or β3 integrin–dependent c-Fos expression and c-Jun phosphorylation and enhances colocalization of ATF-4 with phosphorylated c-Jun. *A,* PC-3 cells were treated with 0.5 μmol/L of PGE_2 for 0 to 150 min, and the levels of c-Fos and phosphorylated c-Jun were detected by Western blot. Total c-Jun and actin were used as loading controls. *B,* PC-3 cells were pretreated with PD98059 (MEK inhibitor; 40 μmol/L) or AG1478 (EGFR inhibitor; 500 nmol/L) for 1 h and then treated with PGE_2. c-Fos and phosphorylated c-Jun were analyzed by Western blot (*I*). PC-3 cells were individually transfected with β3 siRNA or control siRNA and then treated with PGE_2, and the levels of c-Fos and phosphorylated c-Jun were analyzed (*II*). *C,* PC-3 cells were treated with PGE_2, and the colocalization of phosphorylated c-Jun (stained with Cy2; *green*) and ATF-4 (stained with Cy3; *red*) was analyzed by immunofluorescence. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*blue*). *D,* ATF-4 regulates PGE_2-induced expression of c-Fos and phosphorylation of c-Jun. PC-3 cells were transfected with wt and dn ATF-4 and then treated with PGE_2, and the levels of c-Fos, phosphorylated c-Jun, and c-Jun were analyzed by Western blot. All figures are representation of three experiments. Fold changes were calculated.

activation, PC-3 cells were pretreated with PD98059 (MEK inhibitor) or AG1478 (EGFR inhibitor) or transfected with β3 integrin siRNA and expression of c-Fos and levels of the phosphorylated c-Jun were analyzed by Western blot. The data showed that inhibition of EGFR-MAPK pathway or down-regulation of β3 integrin suppressed PGE_2-induced c-Fos expression and c-Jun phosphorylation, indicating that PGE_2 triggers EGFR-MAPK and β3 integrin–mediated AP-1 activation (Fig. 2*B, I* and *II*). Altogether, these results suggested that EGFR and β3 integrin play crucial roles in PGE_2-induced AP-1 activation in PC-3 cells.

PGE_2 stimulates ATF-4–dependent AP-1 activation. Elevated expression of ATF-4 has been observed in various cancers associated with enhanced malignancy (11). Recent findings have shown that ATF-4 is also involved in the regulation of expression of various oncogenic molecules and plays a crucial role in cancer progression (11). Therefore, we have examined the expression of ATF-4 in PC-3, DU-145, and LNCaP cells by immunofluorescence. The results showed the significant level of ATF-4 expression, particularly in PC-3 and DU-145 cells (data not shown). To investigate the role of PGE_2 on ATF-4 activation, PC-3 cells were treated with PGE_2 and ATF-4 nuclear localization and DNA binding

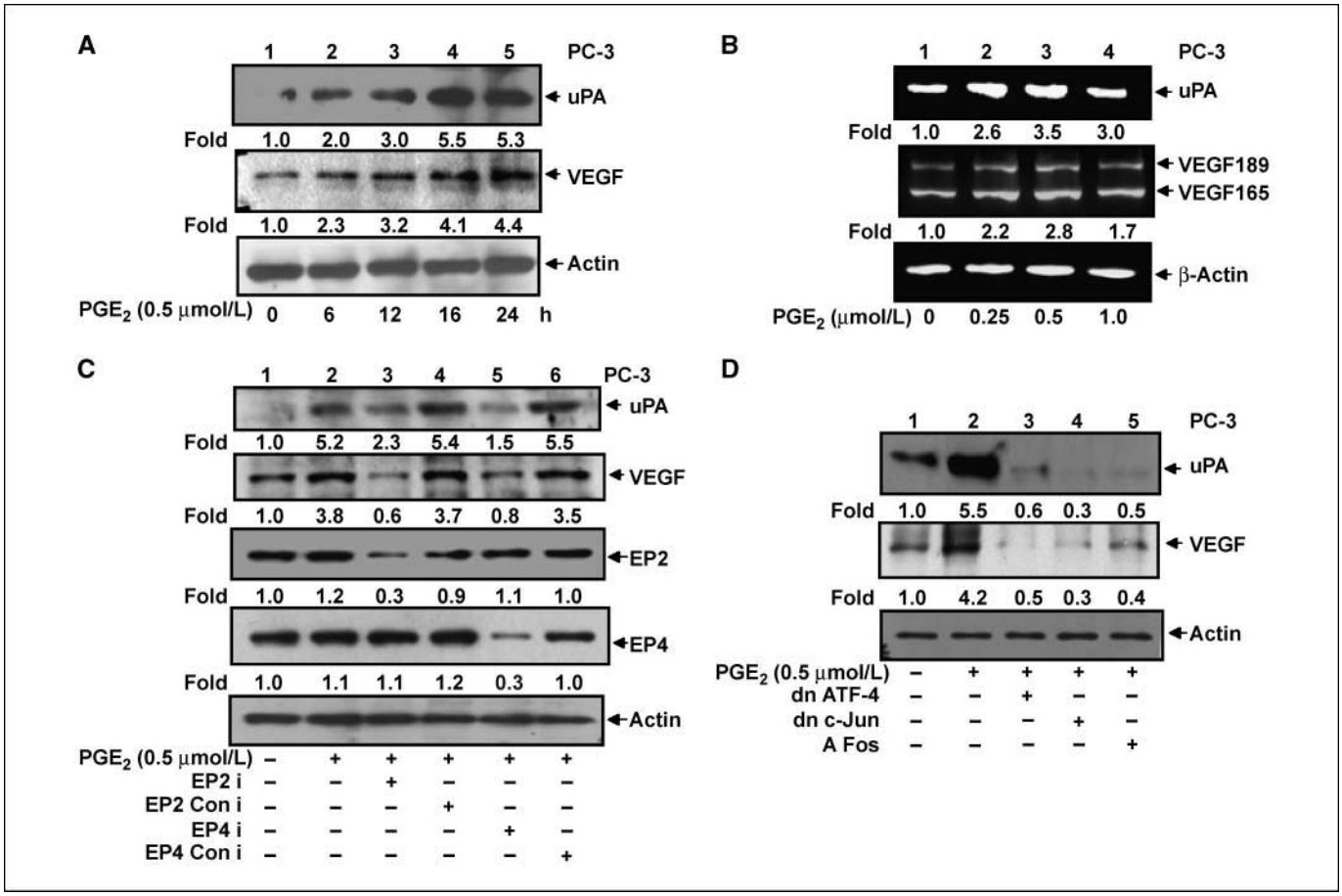

Figure 3. PGE_2 augments EP2/EP4 receptor–mediated ATF-4/AP-1–dependent uPA and VEGF expression in PC-3 cells. *A,* PC-3 cells were treated with 0.5 μmol/L PGE_2 for 0 to 24 h, and the levels of uPA and VEGF were detected by Western blot using their specific antibodies. Actin was used as loading control. *B,* cells were treated with PGE_2 in a dose (0–1.0 μmol/L)–dependent manner, total RNA was isolated, and the levels of uPA and VEGF mRNA were detected by semiquantitative RT-PCR. β-Actin was used as internal control. *C,* PC-3 cells were transfected with EP2 or EP4 specific siRNA (EP2 i or EP4 i) and then treated with PGE_2, and the levels of uPA, VEGF, EP2, and EP4 were analyzed by Western blot. Actin was used as loading control. *D,* roles of ATF-4 and AP-1 in PGE_2-induced uPA and VEGF expression. Cells were individually transfected with dn ATF-4, dn c-Jun, and A-Fos followed by treatment with PGE_2, and the levels of uPA and VEGF were analyzed by Western blot. All figures are representation of three experiments. Fold changes were calculated.

were determined by immunofluorescence and EMSA. The data indicated that PGE_2 induces nuclear localization and DNA binding of ATF-4 (Supplementary Fig. S4*A* and *B*). Moreover, we have observed the enhanced nuclear colocalization of ATF-4 with phosphorylated c-Jun in response to PGE_2 (Fig. 2*C*). Furthermore, to explore the cross-talk between ATF-4 and AP-1, PC-3 cells were individually transfected with wt or dn ATF-4, followed by treatment with PGE_2. The levels of c-Fos and phosphorylated c-Jun expressions were analyzed by Western blot. The data indicated that wt ATF-4 enhances, whereas dn ATF-4 suppresses, PGE_2-induced c-Fos and phosphorylated c-Jun expression (Fig. 2*D*). EMSA data further confirmed that ATF-4 regulates AP-1–DNA binding in response to PGE_2 (Supplementary Fig. S4*C*). However, wt and dn c-Jun or A-Fos had no effect on PGE_2-induced ATF-4–DNA binding (data not shown), which further suggested that PGE_2-regulated cross-talk between ATF-4 and AP-1 is unidirectional toward AP-1.

PGE_2 induces EP2/EP4-mediated uPA and VEGF expressions in prostate cancer cells. To examine the role of PGE_2 on uPA and VEGF expressions, PC-3 cells were treated with PGE_2 in a time-dependent (0–24 h) and dose-dependent (0–1.0 μmol/L) manner. The levels of uPA and VEGF were analyzed by Western blot. The results indicated that PGE_2 with 0.5 μmol/L concentration induced maximum expressions of uPA and VEGF at ~16 hours (Fig. 3*A* and Supplementary Fig. S5*A*). Similarly, PGE_2 at 0.5 μmol/L concentration stimulated maximum uPA and VEGF expressions at mRNA levels (Fig. 3*B*). The PGE_2-induced uPA and VEGF expressions were also detected in DU-145 and LNCaP cells (Supplementary Fig. S5*B*). The data indicated that PGE_2 up-regulates uPA and VEGF expressions, both at transcriptional and protein levels. Earlier reports have indicated that COX-2 regulates PGE_2 production in prostate tumor cells (3). Therefore, to determine the role of tumor-derived PGE_2 on uPA and VEGF expressions, PC-3 cells were transfected with COX-2 cDNA or COX-2 siRNA (COX-2i) and expressions of uPA and VEGF were detected by Western blot. The data showed that overexpression of COX-2 enhances, whereas silencing of COX-2 suppresses, uPA and VEGF expressions (Supplementary Fig. S5*C*), which further suggested that tumor-derived PGE_2 is also involved in the regulation of uPA and VEGF expressions in these cells. To delineate the roles of EP2 and EP4 receptors in PGE_2-induced uPA and VEGF expressions, PC-3 cells were transfected with EP2 or EP4 siRNA (EP2i or EP4i) and then

treated with PGE_2. The levels of uPA, VEGF, EP2, and EP4 were analyzed by Western blot. The data indicated that silencing of EP2 or EP4 receptor suppresses PGE_2-induced uPA and VEGF expressions (Fig. 3*C*). Moreover, to examine the roles of ATF-4 and AP-1 on PGE_2-induced uPA and VEGF expressions, PC-3 cells were transfected with dn ATF-4 or dn c-Jun or A-Fos cDNA construct and then treated with PGE_2, and the levels of uPA and VEGF were analyzed by Western blot. The data revealed that dn ATF-4, dn c-Jun, or A-Fos suppressed PGE_2-induced uPA and VEGF expressions (Fig. 3*D*), demonstrating the roles of AP-1 and ATF-4 in PGE_2-induced uPA and VEGF expressions. Taken together, these data indicated that both exogenous and tumor-derived PGE_2 induced uPA and VEGF expressions via EP2 and EP4 receptors–mediated ATF-4–dependent and AP-1–dependent pathway.

ATF-4 and AP-1 regulate PGE_2-induced prostate tumor cell motility. It has been reported that nonsteroidal antiinflammatory drugs and selective COX-2 inhibitors suppress invasiveness of human prostate cancer cell lines, PC-3 and DU-145, and this effect can be reversed by the addition of PGE_2 (31). Although it has been proposed that overexpression of COX-2/PGE_2 may enhance the invasive properties of tumors (3), leading to increase in tumor cell migration, the molecular mechanism underlying this process is not well defined. Therefore, to delineate the molecular mechanism of PGE_2-regulated tumor cell migration, PC-3 cells were either individually transfected with dn ATF-4, dn c-Jun, and A-Fos or pretreated with AH6809, AH23848, and PKA inhibitor peptide and then treated with PGE_2, and wound migration assay was performed. These data showed that antagonists of EP2 and EP4 receptors, PKA inhibitor peptide, dn ATF-4, dn c-Jun, and A Fos significantly suppressed PGE_2-induced tumor cell migration (Fig. 4*A, I* and *II*). The roles of these molecules in PGE_2-mediated PC-3 cell migration were further confirmed by migration assay

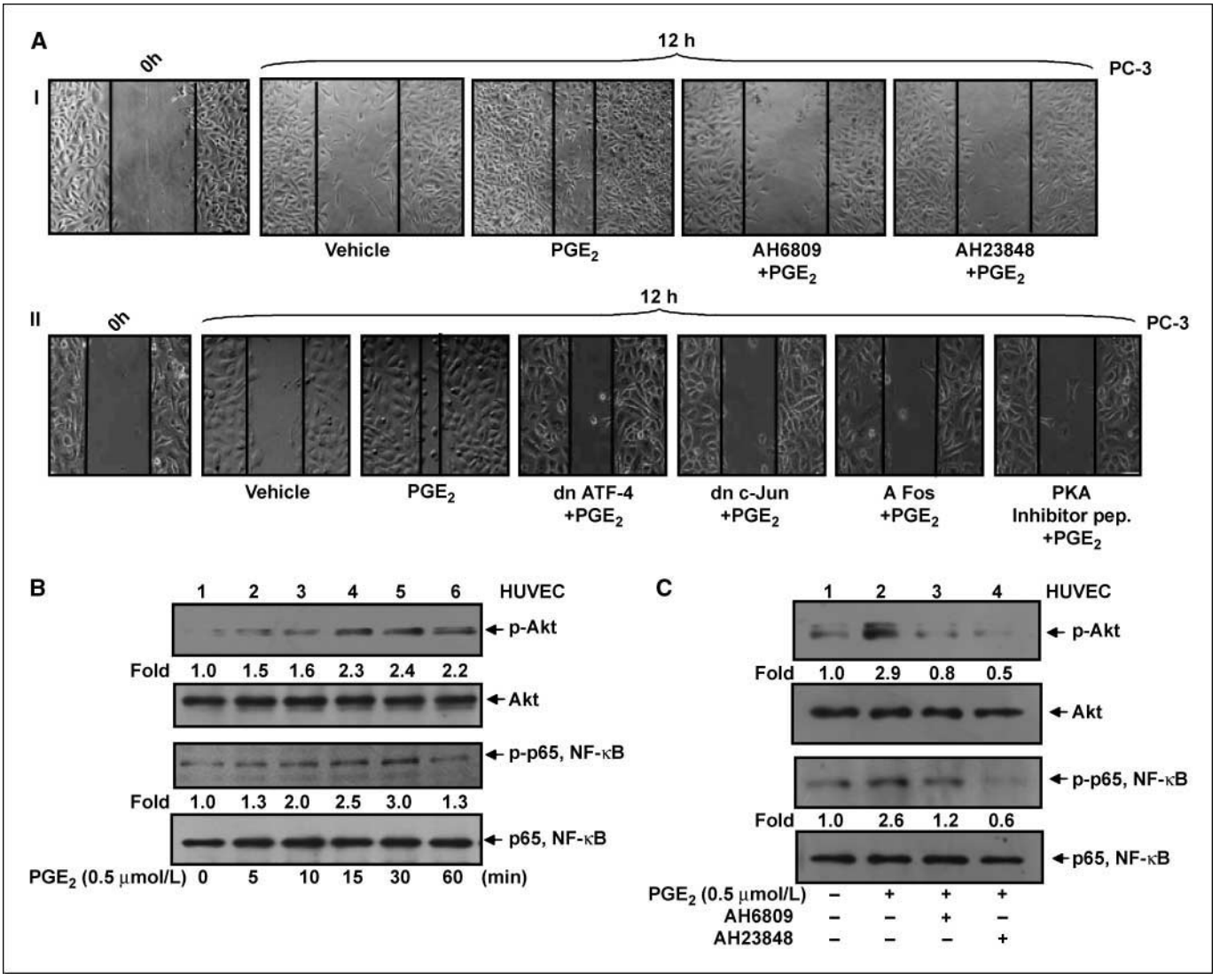

Figure 4. *A,* EP2 and EP4 receptors play crucial roles in PGE_2-induced tumor cell motility. PC-3 cells were either individually transfected with dn ATF-4, dn c-Jun, and A-Fos or pretreated with AH6809 or AH23848 or PKA inhibitor peptide and then treated with PGE_2, and wound migration assay was performed. Wound photographs were taken at 0 and 12 h (*I* and *II*). *B,* PGE_2 controls Akt and p65, NF-κB phosphorylation in endothelial (HUVEC) cells. HUVEC were treated with PGE_2 for 0 to 60 min, and the levels of phosphorylated Akt and phosphorylated p65 were detected by Western blot. Total Akt and p65 were used as control. *C,* HUVEC were pretreated with AH6809 or AH23848 and then treated with PGE_2. The levels of phosphorylated Akt and phosphorylated p65 were analyzed by Western blot. Total Akt and p65 were used as control. Data represent three experiments exhibiting similar results. Fold changes were calculated.

using Boyden chamber (Supplementary Fig. S6*A*). Taken together, the results showed that PGE_2 regulates ATF-4/AP-1–dependent prostate tumor cell motility through interaction with EP2 and EP4 receptors.

PGE_2 induces EP2/EP4-mediated Akt/NF-κB activation in endothelial cells, tumor-endothelial cell interaction, and angiogenesis. NF-κB regulates the expression of various factors that control endothelial and tumor cell motility and invasion (32). The serine/threonine protein kinase Akt is an important component in the migratory and prosurvival signaling pathways (33). Therefore, to examine the effect of PGE_2 on the activation of NF-κB and Akt, endothelial cells (HUVEC) were treated with PGE_2 in a time-dependent manner and the levels of phosphorylated Akt and phosphorylated p65 and NF-κB were analyzed by Western blot. The data showed that PGE_2 induces the phosphorylations of Akt and p65 in these cells (Fig. 4*B*). To examine the role of EP2 or EP4 receptor on PGE_2-induced phosphorylation of Akt and p65, HUVEC were pretreated with AH6809 and AH23848 and then treated with PGE_2, and the levels of phosphorylated Akt and phosphorylated p65 were analyzed. The data showed that EP2 and EP4 receptor antagonists suppressed PGE_2-induced phosphorylation of Akt and p65, suggesting that EP2 and EP4 play crucial roles in this process (Fig. 4*C*). To examine the roles of EP2 and EP4 on PGE_2-mediated endothelial cell motility, wound migration assay was performed. The data indicated that EP2, as well as EP4 receptor antagonists, suppressed PGE_2-induced endothelial cell motility (Supplementary Fig. S6*B*).

Various studies have indicated that overexpression of COX-2 and PGE_2 correlates with tumor angiogenesis (3, 7, 34). To determine the role of tumor-derived PGE_2 on endothelial cell motility, direct comigration assay was performed. PC-3 cells, either alone or transfected with wt COX-2 cDNA or COX-2 siRNA, were used in the lower chamber, whereas HUVEC, either alone or pretreated with EP2 or EP4 receptor antagonist, were used in the upper chamber. In separate experiments, PGE_2 was used in the lower chamber as positive control. The endothelial cells migrated toward the reverse side of the upper chamber were stained with Giemsa, photographed, counted, and represented in the form of a bar graph (Fig. 5*A*). The data revealed that overexpression of COX-2 significantly enhanced, whereas silencing COX-2 or antagonists of EP2 or EP4 receptor drastically suppressed, endothelial cell motility toward tumor cells, suggesting that tumor-derived PGE_2 plays a crucial role in this process (Fig. 5*A*).

To examine the effect of PGE_2 on *in vivo* tumor angiogenesis, Matrigel plug angiogenesis assay was performed. Accordingly, PGE_2 was mixed with growth factor depleted Matrigel alone or along with EP2 or EP4 receptor antagonists and Matrigel and injected into the nude mice. After the termination of the experiments, Matrigel plugs were photographed and analyzed by immunohistochemistry using anti-CD31, anti-vWF, anti–phosphorylated p65, and anti–phosphorylated Akt antibodies. The results showed that PGE_2-induced angiogenesis was inhibited by EP2 or EP4 receptor antagonist (Fig. 5*B*). The expressions of vWF and CD31 (endothelial cell markers) and phosphorylations of p65, NF-κB, and Akt were higher in PGE_2-treated plugs compared with the plugs developed with EP2 and EP4 receptor antagonists (Fig. 5*B*). In other experiments, conditioned medium of PC-3 cells, either nontransfected or transfected with COX-2 cDNA, was mixed with Matrigel and injected into the mice. The Matrigel plugs generated from the conditioned medium of COX-2 overexpressing PC-3 cells showed enhanced tumor angiogenesis compared with the conditioned medium of PC-3 cells alone, suggesting that tumor-derived PGE_2 plays a crucial role in regulating tumor angiogenesis (data not shown). The PGE_2-induced angiogenesis (vWF positivity) was analyzed and represented in the form of a bar graph (Fig. 5*B*). These data showed that PGE_2-induced EP2 and EP4 receptors mediated angiogenesis via NF-κB and Akt-dependent pathway and further suggested that both EP2 and EP4 receptors might play important roles in regulating PGE_2-induced tumor angiogenesis.

Correlation between expression profiles of EP2 and EP4 with ATF-4, c-Jun, c-Fos, uPA, and VEGF and their significance in prostate tumor progression. Our *in vitro* and *in vivo* data prompted us to examine the expression profiles of various oncogenic and angiogenic molecules in human prostate cancer specimens by immunohistochemistry. The results showed that the expression levels of ATF-4, c-Jun, c-Fos, uPA, and VEGF were higher in malignant tumors compared with normal and PIN specimens, which further correlated with the enhanced expressions of EP2 and EP4 in human prostate cancer specimens (Fig. 6*A*). The expressions of these molecules were quantified and represented in the form of a bar graph (Fig. 6*A*).

Discussion

Prostate cancer is considered as one of the most lethal disease for men in the United States and other parts of the world. To date, treatments like androgen deprivation therapy and chemotherapy are two of the major approaches known to increase survival of patients with metastatic prostate cancer; however, some side effects have been observed in patients undergoing these therapies (2, 35). Therefore, identification of novel prognostic marker and development of new therapeutic strategies could be the most promising approaches in the next generation of prostate cancer management.

Recently, several studies have shown the correlation between overexpression of COX-2 with prostate tumorigenesis; however, the molecular mechanism underlying COX-2–induced prostate cancer progression and angiogenesis is still not well understood. Although various reports have revealed the relationship between the elevated levels of PGE_2 with malignant cancers (3), the molecular mechanism underlying PGE_2-mediated prostate tumor progression is still the subject of intense investigation. In this study, we have shown the in-depth molecular mechanism underlying PGE_2-induced tumor cell motility and angiogenesis in prostate cancer via EP2 and EP4 receptor–dependent pathway.

EGFR and MAPK-mediated activation of transcription factor AP-1 has been reported as one of the crucial signaling cascade that affects tumor cell motility in various cancers, including the malignancies in prostate (36). Elevated expression of EGFR has been observed in higher grades of prostate cancer, which further correlate with poor clinical prognosis (37, 38). Earlier studies reported that activation of EGFR in response to PGE_2 leads to the phosphorylation of ERK, which, in turn, regulates downstream signaling events (36, 39). Moreover, it has also been observed that PGE_2 transactivates EGFR, which ultimately influences cell migration, proliferation, and invasiveness in different cancer models (36, 39, 40). In this paper, we have shown that PGE_2 induces the activation of EGFR and MAPK signaling cascade in prostate cancer cells. Recently, Wang and colleagues have shown that among different PGE_2 receptors, EP2 and EP4 predominantly express PC-3 cells in androgen-independent prostate cancer (21).

Here, we have shown that blocking of both PGE_2 receptors (EP2 and EP4) by their specific antagonists curbs the PGE_2-mediated activation of EGFR and downstreams signaling cascades, which ultimately suppress the prostate tumor cell motility.

Integrin β3 has been shown to play critical roles in several distinct processes, such as tumor growth, metastasis, and angiogenesis in various cancers, including prostate cancer (41–43). Phosphorylation of β3 integrin is essential for the activation of small GTP-binding proteins (Rho family), and activation of Rho is necessary for invasion and migration in a wide variety of cell types (44). Previous studies have indicated that integrins control activation of AP-1 in prostate cancer cells (26). Furthermore, earlier reports have shown that overexpression of β3 integrin correlates with enhanced metastatic phenotype in LNCaP cells (42). Moreover, it has been observed that stromal cell derived factor-1 transiently increased the expression and activation of β3 integrin in prostate cancer cells, which in turn augmented the aggressiveness of prostate cancer (43). In this study, we have reported that PGE_2 induces the expression and phosphorylation of β3 integrin in PC-3 cells. The EP2 and EP4 receptor antagonists suppressed PGE_2-induced expression and phosphorylation of β3 integrin, which further showed the involvement of both these receptors in this process. Silencing of β3 integrin expression by its specific siRNA suppresses PGE_2-induced AP-1 activation, suggesting that PGE_2 via EP2/EP4 controls AP-1 activation through β3 integrin–mediated pathway. Chen and colleagues have shown that arachidonic acid regulates PGE_2-mediated PKA-dependent expression of *c-fos* in PC-3 cells (5). In this study, we have shown the molecular mechanism, at least in part, whereby PGE_2 via EGFR-ERK or β3 integrin–mediated pathway augments the expression of c-Fos and phosphorylation of c-Jun, which ultimately regulates AP-1 activation in prostate cancer cells.

Previous data showed that ATF-4 forms heterodimers with either c-Fos or c-Jun subunit of AP-1 and regulates the activation of AP-1 (45). In this study, we have shown that PGE_2 augments ATF-4 activation, controls the interaction between ATF-4 and phosphor-

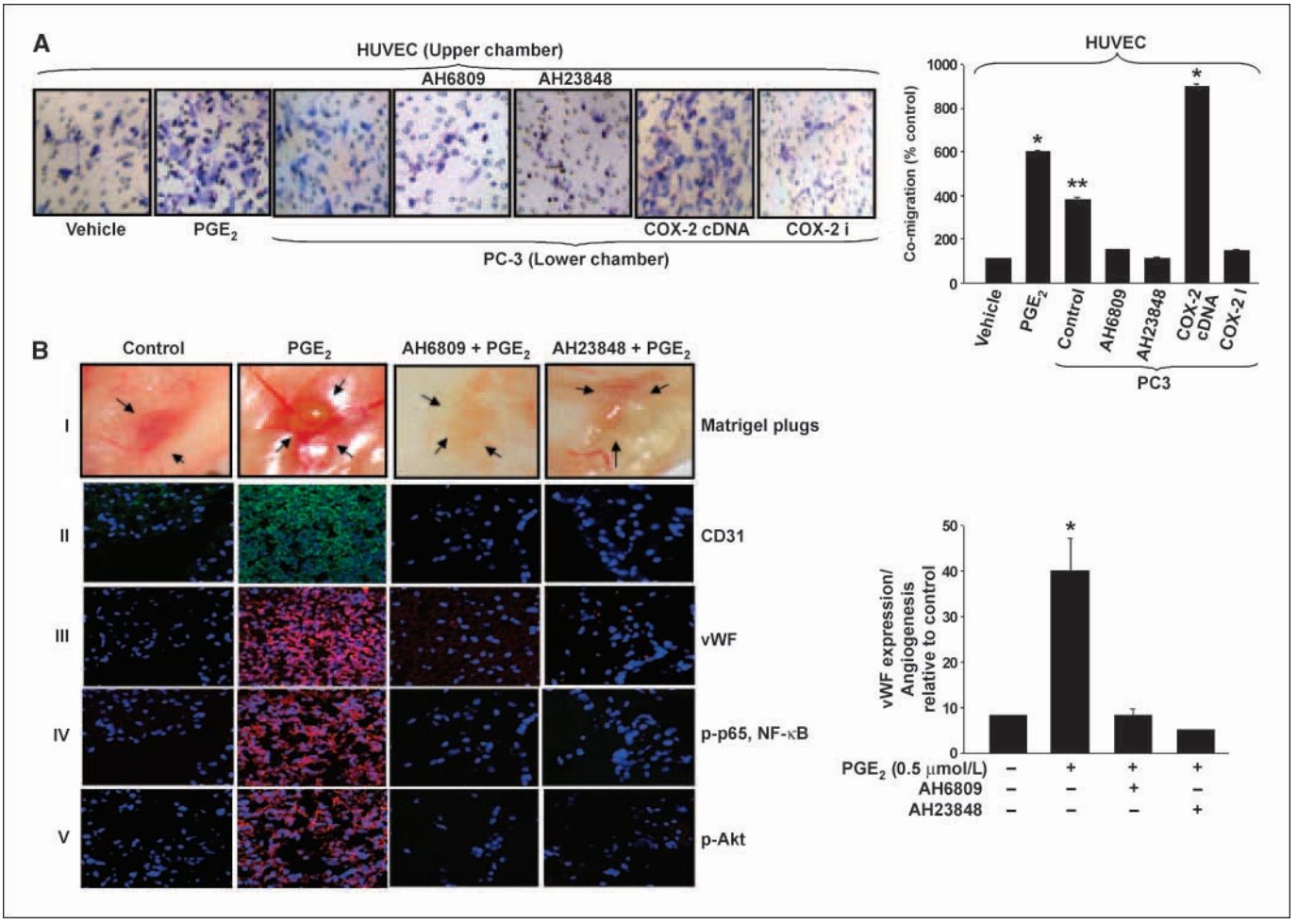

Figure 5. *A,* PGE_2 regulates migration of endothelial cells toward the tumor cells. HUVEC, either alone or pretreated with EP2 or EP4 receptor antagonist, were used in the upper chamber, whereas PC-3 cells, either alone or transfected with wt COX-2 cDNA or COX-2 siRNA, were used in the lower chamber. In separate experiments, PGE_2 was used in the lower chamber as positive control. The endothelial cells migrated toward the reverse side of the upper chamber were stained with Giemsa, photographed, counted in 3 hpf, and represented in the form of a bar graph (*, $P < 0.004$; **, $P < 0.015$). *B,* PGE_2 regulates EP2/EP4 receptor–dependent angiogenesis in Matrigel plug assay. PGE_2 alone or along with AH6809 or AH23848 was mixed with Matrigel and injected s.c. in male athymic nude mice (NMRI, nu/nu). After 21 d, mice were sacrificed and Matrigel plugs were photographed and analyzed by immunohistochemistry using anti-CD31, anti-vWF, anti–phosphorylated p65, and anti–phosphorylated Akt antibodies. CD31 was stained with Cy2-conjugated IgG (*green*), whereas vWF, phosphorylated p65, and phosphorylated Akt were stained with Cy3-conjugated IgG (*red*). Nuclei were stained with DAPI (*blue*). The vWF-positive staining was quantified by Image Pro Plus 6.0 Software and represented graphically. *Columns,* means of three determinations; *bars,* SE (*, $P = 0.036$).

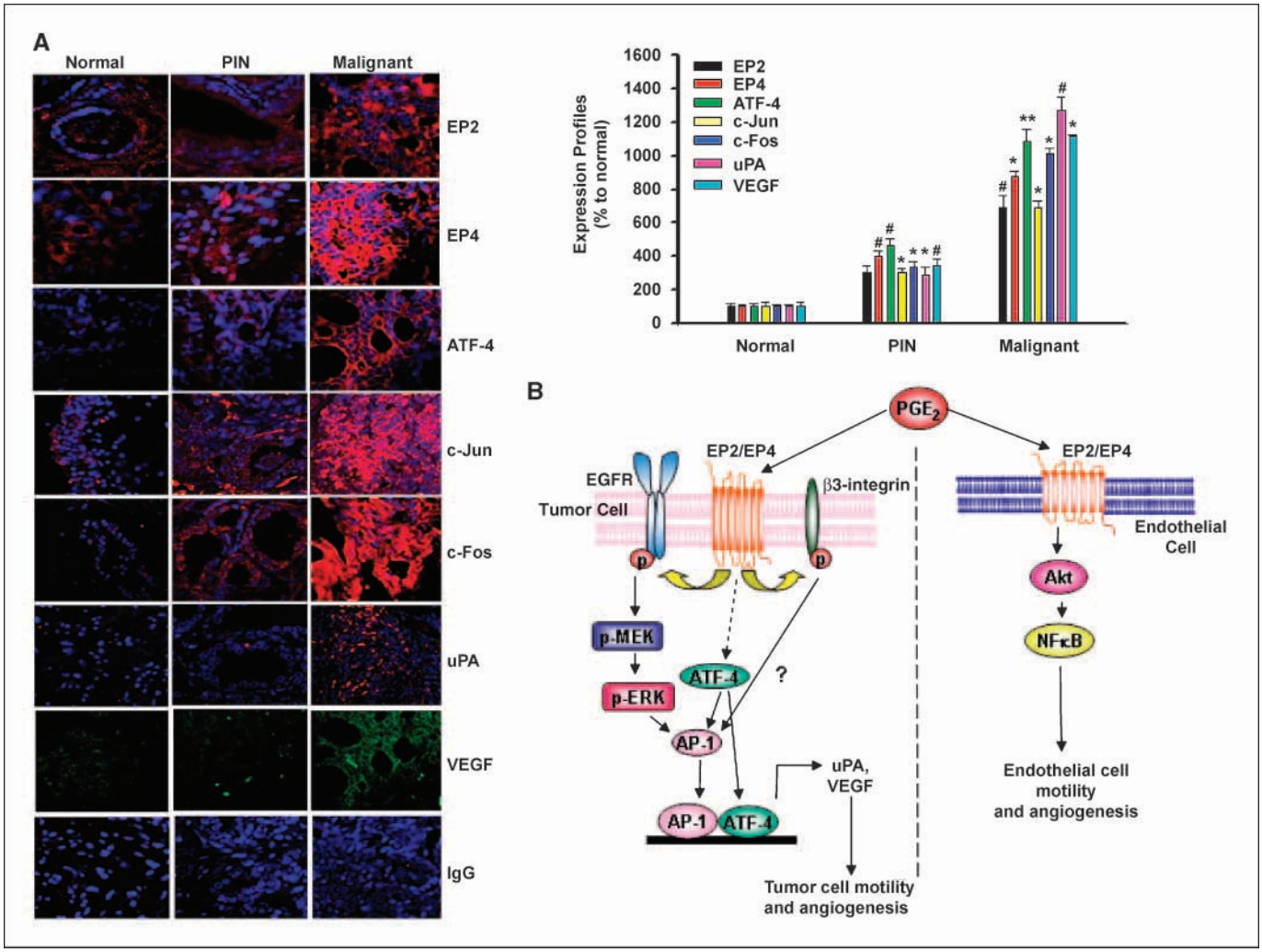

Figure 6. Expression profiles of EP2, EP4, ATF-4, c-Jun, c-Fos, uPA, and VEGF in human prostate cancer specimens and their correlation with human prostate cancer progression in different pathologic grades. *A,* the levels of EP2, EP4, ATF-4, c-Jun, c-Fos, uPA, and VEGF were detected by immunohistochemical studies using their specific antibodies. EP2, EP4, ATF-4, c-Jun, c-Fos, and uPA were stained with Cy3-conjugated IgG (*red*). VEGF was stained with Cy2-conjugated IgG (*green*). Sections stained with anti-rabbit IgG were used as control. Nuclei were stained with DAPI (*blue*). The expression profiles were quantified by Image Pro Plus 6.0 Software and represented in the form of a bar graph (*, $P < 0.003$; **, $P < 0.006$; #, $P < 0.02$). *B,* schematic representation of PGE_2-induced EP2/EP4-mediated EGFR/MAPK or β3 integrin–dependent ATF-4/AP-1 activation leading to enhanced uPA and VEGF expression, which in turn controls prostate tumor cell motility and angiogenesis. In endothelial cells, PGE_2 through EP2/EP4 receptor stimulates Akt and NF-κB activation leading to enhanced endothelial cell motility and angiogenesis.

ylated c-Jun, and regulates ATF-4–dependent AP-1 activation in prostate cancer cells.

uPA and its receptor uPAR-mediated signaling has been implicated in tumor cell invasion, survival, and metastasis in a variety of cancers including prostate (20). Both uPA and uPAR are expressed at higher levels in malignant prostate tissues than in benign and normal prostate tissues (20). VEGF acts as one of the key proangiogenic factor responsible for neovascularization in cancer cells (46). Previous studies have indicated that inhibition of VEGF expression is a critical step in arresting prostate tumor growth and progression (47, 48). In this study, we have shown in-depth molecular mechanism underlying PGE_2-induced EP2/EP4-mediated expression of uPA and VEGF via activation of AP-1 and ATF-4, which eventually affects tumor cell motility and angiogenesis. Moreover, our data revealed that inhibition of tumor-derived PGE_2 by COX-2 siRNA suppressed uPA and VEGF expression, suggesting that PGE_2, both tumor-derived and exogenous, regulates this process in prostate cancer cells.

Angiogenesis is one of the most crucial steps in the development of tumor. The proliferation and migration of endothelial cells play crucial roles in the regulation of tumor-associated angiogenesis (49). We have shown that PGE_2 induces the phosphorylation of Akt and NF-κB, p65 in endothelial cells. Moreover, both exogenous and tumor-derived PGE_2 augments endothelial cell motility, and blocking of endothelial EP2 and EP4 receptors by their antagonists suppresses endothelial cell motility toward tumor cells. *In vivo* Matrigel plug angiogenesis assay showed that PGE_2 induces angiogenesis whereas blocking EP2 and EP4 receptors suppressed this effect, indicating that PGE_2 augments EP2/EP4-mediated *in vivo* angiogenesis. Our data also revealed that tumor-derived PGE_2 induces tumor angiogenesis. Taken together, these results showed that PGE_2 via EP2/EP4 receptor promotes *in vivo* angiogenesis through activation of Akt and NF-κB, suggesting that PGE_2 plays an important role in the regulation of tumor angiogenesis. Furthermore, prostate tumor clinical specimen analysis data also

corroborated with *in vitro* and *in vivo* findings, indicating higher levels of uPA and VEGF expression in malignant prostate tumors compared with normal and PIN tissues, which further correlated with the enhanced expression levels of ATF-4, c-Fos, c-Jun, EP2, and EP4. These data provide, at least in part, the molecular basis by which PGE_2 controls downstream signaling cascades and leads to the expression of various oncogenic and angiogenic molecules that ultimately regulate the prostate cancer progression and angiogenesis (Fig. 6*B*). Thus, targeting PGE_2 by interfering its interaction with EP2 and EP4 receptors might be an alternative therapeutics that may aid in the rational design of therapeutic strategy for the next generation of prostate cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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