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RESEARCH ARTICLE

Intracellular EP2 prostanoid receptor promotes cancer-related phenotypes in PC3 cells

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Abstract Prostaglandin E_2 (PGE_2) and hypoxia-inducible factor-1α (HIF-1α) affect many mechanisms that have been involved in the pathogenesis of prostate cancer (PC). HIF-1α, which is up-regulated by PGE_2 in LNCaP cells and PC3 cells, has been shown to contribute to metastasis and chemo-resistance of castrate-resistant PC (a lethal form of PC) and to promote in PC cells migration, invasion, angiogenesis and chemoresistance. The selective blockade of PGE_2-EP2 signaling pathway in PC3 cells results in inhibition of cancer cell proliferation and invasion. PGE_2 affects many mechanisms that have been shown to play a role in carcinogenesis such as proliferation, apoptosis, migration, invasion and angiogenesis. Recently, we have found in PC3 cells that most of these PGE_2-induced cancer-related features are due to intracellular PGE_2 ($iPGE_2$). Here, we aimed to study in PC3 cells the role of $iPGE_2$-intracellular EP2 (iEP2)-HIF-1α signaling in several events linked to PC progression using an experimental approach involving pharmacological inhibition of the prostaglandin uptake transporter and EGFR and pharmacological and genetic modulation of EP2 receptor and HIF-1α. We found that $iPGE_2$ increases HIF-1α expression through iEP2-dependent EGFR transactivation and that inhibition of any of the axis iEP2-EGFR-HIF-1α in cells treated with PGE_2 or EP2 agonist results in prevention of the increase in PC3 cell proliferation, adhesion, migration, invasion and angiogenesis in vitro. Of note, PGE_2 induced EP2 antagonist-sensitive DNA synthesis in nuclei isolated from PC3 cells, which indicates that they have functional EP2 receptors. These results suggest that PGE_2-EP2 dependent intracrine mechanisms involving EGFR and HIF-1α play a role in PC.

Keywords Intracellular prostaglandin E2 · Prostate cancer · EP2 receptor · PC3 cells · HIF-1α · EGFR

Abbreviations

BG	Bromocresol green
EGFR	Epidermal growth factor receptor
HIF-1α	Hypoxia-inducible factor-1α
PGE_2	Prostaglandin E_2
PGT	Prostaglandin transporter
VEGF-A	Vascular endothelial growth factor-A
HRE	HIF-responsive element
PC	Prostate cancer

Introduction

Prostaglandin E_2 (PGE_2) is the predominant tumor promoting prostaglandin, found at high levels in various tumor types including colon, lung, breast and prostate cancer [1, 2]. PGE_2 affects many mechanisms that have been shown to play a role in carcinogenesis such as proliferation, apoptosis, migration, invasion and angiogenesis [3]. These PGE_2 effects have been also particularly documented in prostate cancer (PC) cells [4–6]. Furthermore, inhibition of cyclooxygenase (and thereby of PGE_2 production) with NSAIDs in experimental PC inhibits cell proliferation, induce apoptosis, and decreases metastasis [7, 8].

Hypoxia-inducible factor-1α (HIF-1α) plays a relevant role among the mechanisms through which PGE_2

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contributes to the development of PC: HIF-1α has been shown to contribute to metastasis and chemo-resistance of castrate-resistant PC and to promote in PC cells migration, invasion, angiogenesis and chemoresistance [9, 10] and the expression and activity of HIF-1α increases upon treatment with PGE_2 in PC cells [11, 12]. Furthermore, treatment with NSAIDs reduces the levels of HIF-1α in PC cells [13]. As a result, there is a decrease in the levels of vascular endothelial growth factor (VEGF) [13], a HIF-regulated protein and the major growth factor that regulates angiogenesis and tumor growth [14].

PGE_2 mediates its effects via four distinct receptors EP1–EP4, which belong to the family of G-protein coupled receptors. EP receptors have been demonstrated to play a role in human PC as well as in the proliferation, migration and angiogenic ability of PC cells [4–6, 9, 10, 15–17]. The current view on prostanoid signaling is that prostanoids, once formed, are quickly released to the outside of cells—by simple diffusion or active transport by the multidrug resistance protein 4—and act as autocrine or paracrine mediators in the vicinity of their sites of production to maintain local homeostasis [18]. A prostaglandin uptake transporter (PGT) that mediates the influx of prostaglandins into the cells has been identified [19–22] and it has been proposed to be involved in signal termination and metabolic clearance of PGE_2 (and other prostaglandins) by its selective uptake across the plasma membranes followed by non-selective oxidation within the cell by 15-prostaglandin dehydrogenase (15-PGDH) [23]. However, recent studies [12, 24–29], have disclosed that intracellular PGE_2 has biological effects, so that PGT (or SLCO2A1) appears to provide at least one mechanism whereby PGE_2 and other prostaglandins can be delivered to an intracellular site of action. Accordingly, inhibition of PGT with bromocresol green (BG) or bromosulfophthalein results in prevention of PGE_2-mediated effects [12, 28, 30]. In fact, we have previously found that PGE_2-induced HIF-1α up-regulation—that is mediated by the transactivation of epidermal growth factor receptor (EGFR)—is prevented by BG. This allowed us to identify the role of intracellular PGE_2 and intracellular EP2 receptors in EGFR-dependent increase in HIF-1α expression [30, 31].

There are no studies on the role of intracellular PGE_2 in cancer, with the only exception of the reports of Lalier and colleagues [26, 27, 32] on the induction of apoptosis through activation of Bax in colon cancer cells and glioblastoma cells. In addition, we have recently shown in androgen-independent PC3 cells (a model of lethal castrate-resistant PC) that BG prevents the stimulatory effects of 16,16-PGE_2 (a PGE_2 analogue) on HIF-1α expression, in vitro angiogenesis and cell proliferation, migration and invasion [17]. This suggests that PGE_2 has to gain access to the intracellular compartment as a prerequisite for stimulating important PC-related features. Therefore, PGT might be an important player in PC and this view is reinforced by the presence of intracellular EP receptors in PC3 cells [17].

In the present work, we aimed to assess the role of intracellular EP2 receptor in the stimulatory effects of PGE_2 in cancer-related phenotypes in PC3 cells and the participation of EGFR and HIF-1α in these effects.

Materials and methods

Reagents and antibodies

PGE_2, BG, YC-1, AH6809, AG1478, PF-04419948 and butaprost were purchased from Sigma (St. Louis, MO). ONO-AE1-259-01 (prostanoid EP2 receptor agonist) was a generous gift of Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Antibodies anti-EP1-4 (epitope regions against C-terminal amino acids 380–402, 335–358, 308–327 and 459–488 for anti-EP1, anti-EP2, anti-EP3 and anti-EP4, respectively) were from Cayman Chemical (Ann Arbor, MI). The specificity of these antibodies has been previously described [15, 33, 34]. Anti-HIF-1α antibody, nucleoporin p62 and anti-BrdU were from BD Biosciences (Palo Alto, CA), anti-annexin II and anti-phospho EGFR was from Santa Cruz Biotechnology (Temecula, CA), anti-hsp60 was from Cell Signaling (Danvers, MA, USA), anti-β-actin and rabbit anti-mouse IgG peroxidase conjugate from Sigma (St. Louis, MO); α-mouse-Alexa-Fluor® 488 and α-rabbit-Alexa-Fluor®488 were from Invitrogen (Eugene, Oregon USA).

Cell culture

The human prostate cancer cell line PC3 was from purchased from American Type Culture Collection (Manassas, VA). Cells were grown in sterile conditions and maintained in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin/amphotericin B from Life Technologies (Barcelona, Spain). The culture was performed in a humidified 5 % CO_2 environment at 37 °C. After the cells reached 70–80 % confluence, they were washed with phosphate buffered saline (PBS), detached with 0.25 % trypsin/0.2 % EDTA (ethylenediaminetetraacetic acid), and seeded at 30,000–40,000 cells/cm^2. The culture medium was changed every 3 days. In all experiments, cells were plated at 70–90 % confluence and 24 h later they were treated with 0.5 μM EP_2 agonist for different periods of time, 10 μM AG1478, 1 μM AH6809, 1.9 μM PF-04419948, 10 μM YC-1 were added 1 h before the treatment. Samples were immediately analysed except samples for the determination

of the production of VEGF-A, which were stored at −80 °C until analysed.

MTT assay

Cell proliferation was determined using a colorimetric assay with MTT. The MTT assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of the intact mitochondria of living cells. Cells were plated in 96-well plates (10^4 cells/well) and maintained in serum free RPMI-1640 for 24 h before being treated as indicated in the "Results" section. Afterwards cell proliferation was evaluated by measuring the conversion of the tetrazolium salt MTT to formazan crystals. Briefly, 0.1 mg/ml MTT was added to the cells and incubated for 3 h at 37 °C. The medium was removed and 100 µl of dimethyl sulfoxide were added to dissolve the formazan precipitates. The amount of formazan crystals formed correlates directly with the number of viable cells. The reaction product was quantified by measuring the absorbance at 570 nm using an ELISA plate reader. Results were expressed as percentage of the control (control equals 100 %).

Western blot analysis

PC3 cells were split into six-well plates at a density of 3×10^5 cells/well and incubated for 24 h before treatment. Afterwards immunoblotting was performed essentially as described previously [12]. In short, cell lysates were prepared and measured for protein content using the Bradford assay. Approximately 30 µg of protein was electrophoresed 10 % SDS–polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4 °C with anti-HIF-1α antibody (1:1000 dilution) or antiphospho-EGFR (1:500) and then for 1 h at room temperature with the horseradish peroxidase-conjugated secondary antibody (1:4000 dilution). To ensure equal loading of proteins, the membranes were stripped and re-probed with anti-β-actin antibody. The signals were detected with enhanced chemiumines-cence reagent (Amersham Healthcare, Buckinghamshire, England).

HRE reporter gene assay

Cells were split into six-well plates at a density of 3×10^5 cells/well. 24 h before later, the cells were co-transfected with 1 µg of p9HIF1-Luc firefly luciferase reporter plasmid [it contains nine copies of the human HIF binding sequence located between positions −958 and −951 of the 5′ human VEGF gene promoter [35] and was generously gifted by Dr. Manuel Ortiz de Landázuri, (Service of Immunology, Hospital Princesa, Madrid, Spain)] and 1 µg renilla luciferase reporter as an internal control. Transfected cells were next incubated with complete growth medium for 24 h. Finally, firefly luciferase was measured with a Lumat LB9506 luminometer (Berthold Technologies, Herts, UK) and normalized against renilla luciferase activity by using the dual-luciferase reporter assay system (Promega, Madison, WI).

Determination of VEGF secretion

PC3 cells were seeded in 24 well plates (5×10^4 cells/well), grown for 24 h and treated as indicated in the "Results" section. Then, the medium containing the secreted VEGF was removed and kept at −80 °C until analyzed. Afterwards VEGF was measured essentially using the human VEGF DuoSet (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol.

Nuclear isolation

Cells were washed three times with ice-cold PBS, gently scraped, and pelleted at 500×*g* for 10 min. The cell pellet was resuspended in 300 µl lysis buffer [10 mM Tris–HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 100 µg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride (PMSF)], and homogenized (100 gentle strokes) with a potter tissue grinders and then, centrifuged at 600×*g* for 10 min at 4 °C. The pellet was resuspended in 300 µl lysis buffer with 0.1 % (v/v) NP-40, left on ice for 10 min and pelleted thereafter at 600×*g* for 10 min. Nuclear pellet was resuspended in 20 mM HEPES/Tris (pH 7.0) containing 10 nM $CaCl_2$ and 300 mM sucrose and immunofluorescence assay was performed. Validation of the purity of the nuclear fraction was determined by immunoblotting with anti-annexin II (plasma membrane-specific protein), anti-hsp60 (cytosol-specific protein) or anti-nucleoporin p62 (nuclear-specific protein).

Immunofluorescence analysis

PC3 cells or isolated nuclei were fixed with 2 % paraformaldehyde for 10 min, permeabilized with 0.1 % (v/v) Triton X-100 in PBS for 10 min, washed with PBS, blocked with 4 % bovine serum albumin (BSA) for 1 h at room temperature and incubated overnight at 4 °C with anti-EP1-4 (1:500 dilution) antibodies. Cells or nuclei were then incubated at 37 °C with α-rabbit-Alexa-Fluor® 488 (1:2000) for 1 h in the darkness. Coverslips were then washed and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen Eugene, Oregon). Detection was performed by confocal laser scan microscopy LEICA TCS-SL (Heidelberg, Germany).

Cell proliferation assay with 5′-Br-2′-deoxyuridine (BrdU)

PC3 cells were placed in 24-well plates (15×10^3 cells/well) and maintained in medium for 24 h. Cells were pretreated with AH6809, AG1478, YC-1 or BG for 1 h and then treated PGE_2 or EP2 agonist for 24 h. The cells were pulsed with 10 μM BrdU, (BD Bioscience) during the last 16 h of incubation. Afterwards, the cells were washed with PBS and fixed with 2 % paraformaldehyde for 15 min. Cells were washed with PBS and DNA was partially denatured by incubation with 2 M HCl for 20 min at room temperature and then, 2 min with 0.1 M $Na_2B_4O_7$. Cells were washed three times with PBS containing 0.05 % Tween-20 (pH 7.4) and 0.1 % BSA. Cells were incubated for 1 h with anti-BrdU (1:50) monoclonal antibody (BD Bioscience) and 1 h with α-mouse-Alexa-Fluor® 488 in the dark (1:400). Detection was performed by confocal laser scan microscopy LEICA TCS-SL (Heidelberg, Germany).

DNA synthesis reactions in vitro

Nuclei for replication reactions were supplemented as previously described [36]. Briefly, isolated nuclei were resuspended in 50 μl of a buffer which contained 40 mM K-HEPES (pH 7.8); 7 mM $MgCl_2$; 3 mM ATP; 0.1 mM each of GTP, CTP, UTP; 0.1 mM each of dATP, dGTP, and dCTP; 0.25 μM BrdU; 0.5 mM DTT; 40 mM creatine phosphate; and 5 μg phosphocreatine kinase. The mixes were pretreated with 10 μM AG1478, 1 μM AH6809, 10 μM YC-1 for 1 h and treated for 3 h with EP2 agonist or PGE_2 at 37 °C. The reaction was stopped with 500 μl PBS and centrifuged to $600 \times g$ for 5 min. After that, nuclei were seeded on polylysine-coated glass microscopy slides and fixed with paraformaldehyde for 15 min. Then BrdU incorporation into the nuclei was assessed as above for whole cells (see "Cell proliferation assay with 5′-Br-2′-deoxyuridine (BrdU)").

Cell adhesion assay

The cell adhesion to collagen was evaluated as follows: cultured cells were detached by trypsinization, resuspended in serum-free RPMI-1640 medium (2.5×10^5 cells/ml) and treated as stated in the "Results" section. Then cells were plated in 96-well plates pre-coated with 0.1 ml of 0.0167 g/100 ml type-I collagen (Sigma, St. Louis, MO) and incubated at 37 °C for up to 2 h. The non-adherent cells were washed out with PBS, and the number of cells that adhered to collagen was assessed by MTT assay. The independent experiments were run in three times.

Cell migration

Cell migration was determined using wound-healing assay. PC3 cells were seeded in 24-well plates until confluent. Cells were treated as indicated in the "Results" section. Confluent cells were carefully wounded using sterile pipette tips. Cell migration or wound repair was photographed (0, 4, 8 and 20 h) and measured vs time 0 h and the narrowing of the wound by migrating cells was monitored by measuring the width in microphotographs. Three representative fields of each monolayer wound were captured using a Nikon Diaphot 300 inverted microscopy camera (10× magnification) up to 20 h. Monolayer wound areas of untreated samples were averaged and assigned a value of 100.

Invasion assay

For the invasion assays, we used transwell polycarbonate filters (8-μm pore size, Corning Costar, Cambridge, UK), which were coated with 50 μl of Matrigel Basement Membrane Matrix (BD Biosciences Bedford, MA, 1:10 dilution with serum free media). Cells were harvested and resuspended in serum-free RPMI-1640 medium at a concentration of 5×10^5 cells/ml. Cells in suspension were treated as indicated in the "Results" section and then 0.2 ml of cell suspension and 0.6 ml medium were added to the upper and lower chamber, respectively. Cells were incubated for 24 h to allow them to colonize the lower chamber. Migrated cells were fixed with methanol, stained with Giemsa and counted in four different fields in a Nikon inverted microscope.

Tube formation of HUVEC on Matrigel

The ability of PC3 cells to promote angiogenesis in vitro was evaluated in a capillary tube formation assay using HUVECs cultured on a synthetic basement membrane matrix. PC3 cells were pre-treated with AH6809, AG1478, YC-1 or BG and incubated with PGE_2/EP2 agonist and after 24 h, the extracellular medium was removed and kept at −80 °C for tube formation assay.

The human umbilical vein endothelial cells (HUVECs) are capable of morphological differentiation into an extensive network of capillary-like structures composed of highly organized three-dimensional cords [37]. 96-well plates were coated with a 40 μl layer of the synthetic basement membrane substrate Matrigel (Collaborative Research, Bedford, MA) at 10 mg/ml concentration and were incubated at 37 °C for 2 h to promote gelling. HUVECs cells were purchased from ATCC and cultured in endothelial basal medium supplemented with VEGF, EGF, FGF basic, IGF-1,

L-glutamine, heparin sulfate, hydrocortisone and fetal bovine serum (ATCC); and cells between passages 3 and 8 were seeded (at 7.5×10^4 cells in 100 µl of extracellular PC3 medium) in each of the Matrigel-coated wells. Tubular structures were photographed 6 h later.

Statistical analysis

Each experiment was repeated at least three times. The results are expressed as the mean ± SD. They were subjected to one way analysis of variance (ANOVA) following by the Bonferroni's test for multiple comparisons. The level of significance was set at $P < 0.05$.

Results

Intracellular EP2 receptors mediate the increase in the expression of HIF-1α in PC3 cells upon treatment with PGE_2 through an EGFR-dependent mechanism: role of EP2 receptor in PGE_2-induced HIF-1α up-regulation

We have previously found that PGE_2-induced HIF-1α up-regulation in PC3 cells is prevented by EP2 antagonist AH6809 [17]. Therefore, we first asked whether HIF-1α is up-regulated by intracellular EP2 in PC3 cells. To this end, we studied (1) the effect on PGE_2-induced HIF-1α up-regulation of an EP2 antagonist (PF-04419948) and (2) the effect of an EP2 agonist (ONO-AE1-259-01) on HIF-1α expression, as well as its prevention by an EP2 antagonist (AH6809) or PGT inhibitor BG. Figure 1a shows that EP2 agonist increased the expression of HIF-1α in PC3 cells and that EP2 antagonist and BG prevented the increase. EP2 antagonist also prevented PGE_2-induced HIF-1α up-regulation. Taken together, the results shown in Fig. 1a indicate that EP2, and more specifically intracellular EP2, is the EP receptor that mediates the up-regulation of HIF-1α by PGE_2.

In PC3 cells, PGE_2 augments the phosphorylation of EGFR, which is prevented by EP2 receptor antagonist AH6809 [5]. EP2 also plays a relevant role in mediating the increase in the production of VEGF-A in PGE_2-treated PC3 cells [5]. Interestingly, we have previously found that PGE_2 increases the expression of HIF-1α in human proximal tubular HK-2 cells through EP-dependent transactivation of EGFR [30] and it has been reported that EGF up-regulates HIF-1α in PC3 cells. Taking into account this background, we sought to determine the role of EGFR in intracellular EP2-induced up-regulation of HIF-1α in PC3 cells, which was confirmed by our experiments: as indicated in Fig. 1b, treatment with EP2 agonist promoted the phosphorylation of EGFR (the hallmark of its activation) and EP2-induced up-regulation of HIF-1α in PC3 cells was prevented by the inhibitor of EGFR activation AG1478.

We finally studied the effect of EP2 agonist on the expression of EP2 receptor in PC3 cells. As shown in Fig. 1c, treatment with EP2 agonist determined a BG-sensitive increase in EP2 receptor expression. This suggests that EP2 agonist might amplify the responsiveness of PC3 cells through a positive feedback involving intracellular EP2-mediated up-regulation of EP2 receptors.

Taken together, the results shown in Fig. 1 are in good agreement with the view that intracellular EP2, acting through transactivation of EGFR, plays a relevant role in PGE_2-induced increase in HIF-1α expression.

Intracellular EP2 receptors mediate the increase in the proliferation of PC3 cells upon treatment with PGE_2 through an AG1478-sensitive, HIF-1α-dependent mechanism: role of nuclear EP receptors

Unchecked proliferation is a hallmark of cancer cells that commonly exhibit increased proliferation when compared to normal cells. EP2 receptors [4], and most likely intracellular EP2 receptors [17], have been found to mediate the enhancing effect of PGE_2 on PC3 growth which facilitates PC progression. Here, we first sought to confirm the role of EP2 receptor in our experimental setting. To this end, we measured the mitochondrial reduction of MTT in cells which were transfected with siRNA EP2 prior to being treated with 1 µM PGE_2 or in cells which were treated with EP2 agonists ONO-AE1-259-01 or butaprost. Our results (Fig. 2a, upper panel) indicated that PGE_2-induced PC3 cell proliferation was fully prevented by knocking-down EP2 receptor, whereas treatment with EP2 agonists resulted in increased cell proliferation. Finally, EP2 antagonist AH6809 and PGT inhibitor BG prevented ONO-AE1-259-01-induced PC3 cell proliferation (Fig. 2a, middle panel). These results were reproduced when cell proliferation was assessed through incorporation of BrdU into cellular DNA (Fig. 2a, lower panel) and they prove that intracellular EP2 receptors play a relevant role in PGE_2-induced PC3 cell proliferation.

The cell nucleus might participate in the signal transduction cascades triggered by intracellular PGE_2. We decided to investigate whether nuclei isolated from PC3 cells expressed EP receptors. Figure 2b, upper panel, shows that this was the case indeed. Therefore, we hypothesized that nuclear EP receptors might participate in the synthesis of DNA required for PGE_2-induced cell proliferation. We tested this hypothesis through studying the incorporation of BrdU to isolated nuclei which where pre-treated or not with EP2 inhibitor AH6809 before being treated with PGE_2. Our results (Fig. 2b, lower panel) indicated that the BrdU content increased in PGE_2-treated

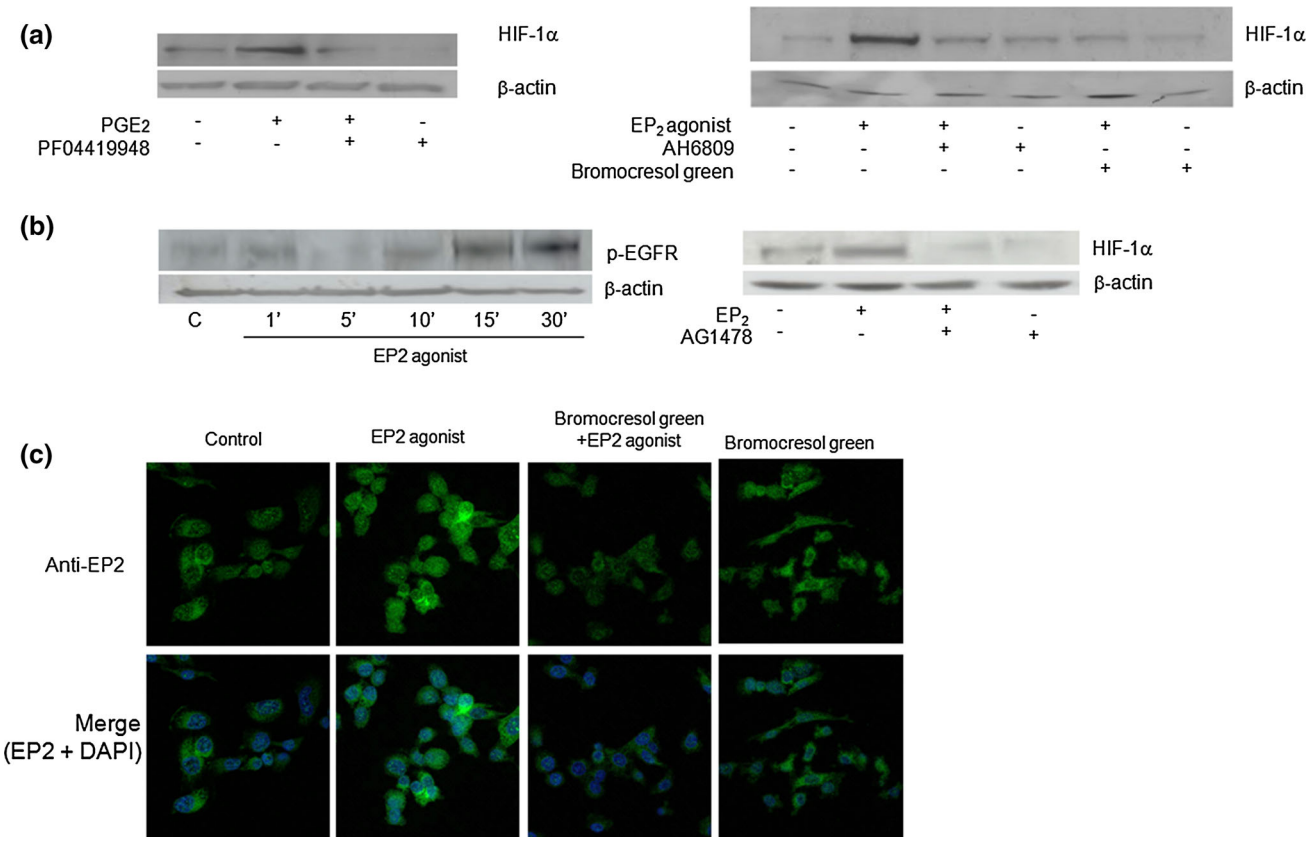


Fig. 1 Role of EP2 receptor in PGE_2-induced HIF-1α up-regulation. **a** *Left* treatment with PGE_2 results in increased HIF-1α expression and its prevention by 1.9 μM EP2 antagonist PF04419948. PC3 cells were pre-incubated for 1 h with 1 μM PF04419948 and then, treated for 8 h with 1 μM PGE_2. Afterwards, HIF-1α expression was assessed by Western blot. *Right* expression of HIF-1α expression increases upon treatment with EP2 receptor selective agonist ONO-AE1-259-01. Preventive effect of AH6809, a selective antagonist of EP2 receptors, and bromocresol green, an inhibitor of the PGT transporter. PC3 cells were pre-incubated for 1 h with 1 μM EP2 antagonist AH6809 or with 50 μM PGT inhibitor bromocresol green and then, incubated for 8 h with 0.5 μM EP2 agonist ONO-AE1-259-01. Afterwards, HIF-1α expression was assessed by Western blot. All experiments were repeated three times. **b** *Left panel* treatment with EP2 receptor agonist results in increased EGFR tyrosine phosphorylation. PC3 cells were incubated for up to 30 min with a selective agonist of EP2 receptor, 0.5 μM ONO-AE1-259-01 and tyrosine phosphorylation of EGFR was assessed by Western blot analysis. *Right panel* the inhibitor of the EGFR phosphorylation AG1478 prevents EP2 agonist-induced increase in EGFR tyrosine phosphorylation. PC3 cells were pre-treated with 1 μM AG1478 for 1 h and incubated with 0.5 μM EP2 agonist for 8 h and phosphorylation of EGFR was assessed by Western blot analysis. All experiments were repeated three times. **c** EP2 agonist determined a BG-sensitive increase in EP2 receptor expression. PC3 cells were pretreated with 50 μM bromocresol green for 1 h and the treated with 0.5 μM EP2 agonist for 8 h and the expression and location of EP2 receptor was assayed for immunocytochemistry

nuclei in an AH6809-sensitive manner, which suggested that nuclear EP receptors participate in mediating the effect of intracellular PGE_2 on PC3 cell proliferation.

In another set of experiments, we examined the role of EGFR transactivation and HIF-1α up-regulation in EP2 agonist-induced PC3 cell proliferation. As shown in Fig. 2c (upper panel), AG1478 and YC-1 (respective inhibitors of EGFR activation and HIF-1α activity), prevented the increase in cell proliferation induced by EP2 agonist (of note both agents diminished the incorporation of BrdU below control values and, particularly, AG1478 blunted it). The role of HIF-1α in EP2 agonist-induced PC3 cell proliferation was further confirmed in further studies in PC3 cells transfected with siRNA HIF-1α (Fig. 2c, lower panel). These results suggest that EGFR and HIF-1α, either as separate entities or (more likely) as linked components of the EGFR-dependent pathway leading to increased HIF-1α activity (Fig. 1b) upon activation of intracellular EP2 receptors, mediate EP2 agonist-induced PC3 cell proliferation.

Intracellular EP2 receptors mediate the loss in PC3 cell adhesion induced by PGE_2 through an AG1478-, YC-1-sensitive mechanism

Cancer cell detachment is the initial event in metastases formation of carcinomas. Tumor cells often show a decrease in cell–cell and/or cell–matrix adhesion in which

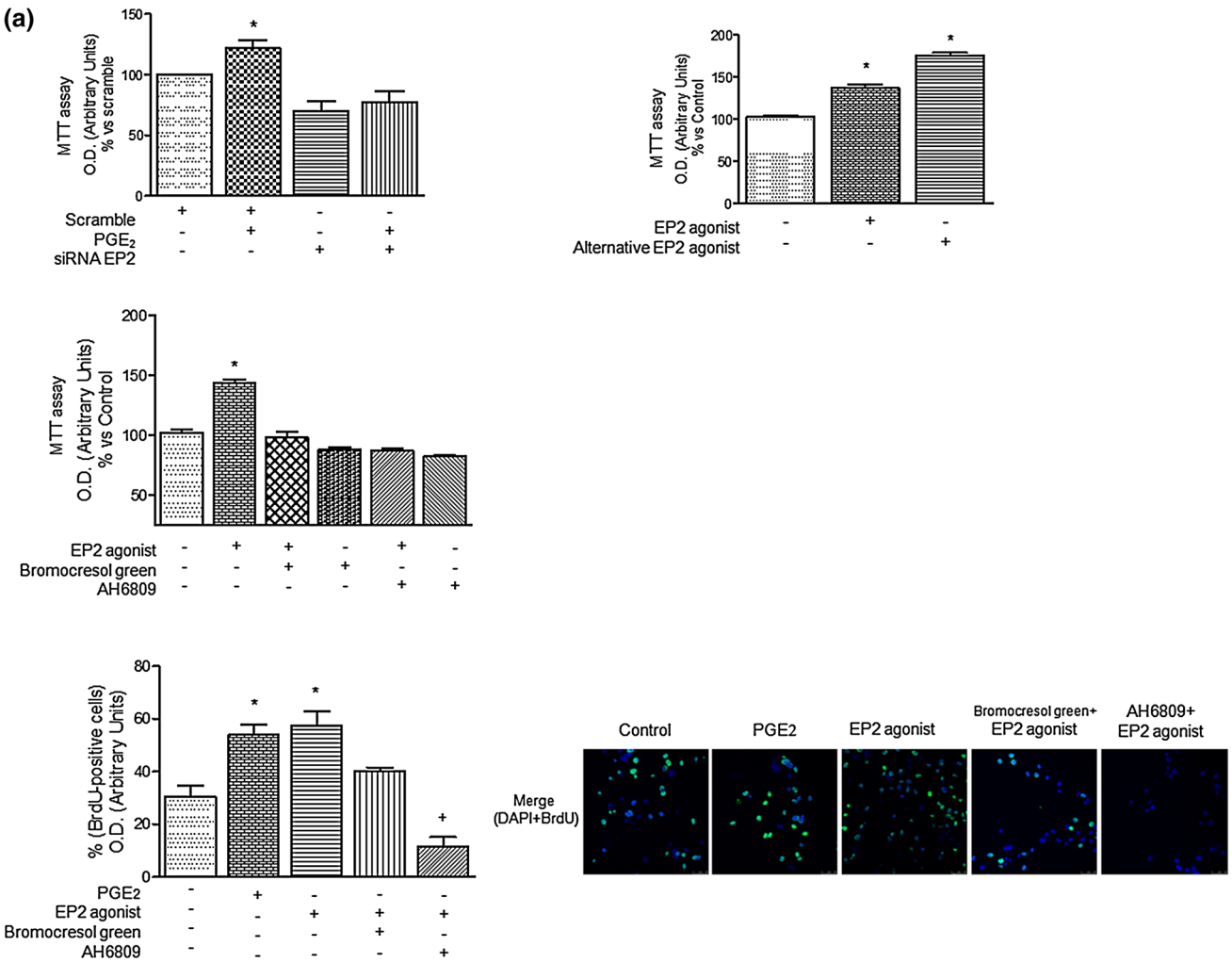


Fig. 2 Intracellular EP2 receptors, in an AG1478-, YC-1-sensitive manner, mediate the increase in the proliferation of PC3 cells upon treatment with PGE_2. **a** Intracellular EP2 receptors mediate in PGE_2-induced proliferation of PC3 cells. PC3 cells were transfected with EP2 siRNA or control siRNA (scramble) as indicated in section "Materials and methods" and were treated with 1 μM PGE_2 agonist for 24 h (*upper panel left*). Alternatively, PC3 cells were treated with 0.5 μM EP2 agonist ONO-AE1-259-01 or 1 μM alternative EP2 agonist butaprost for 24 h (*upper panel right*), and then cell proliferation was assessed by MTT assay as described in "Materials and methods". Other set of cells were pre-treated with 50 μM PGT inhibitor bromocresol green or 1 μM EP2 antagonist AH6809 and then cell proliferation was assessed by MTT assay (*middle panel*) or BrdU incorporation assay (*lower panel*). **b** Nuclear EP receptors participate in the synthesis of DNA required for PGE_2-induced cell proliferation. *Upper panel* nuclei of PC3 cells were isolated as indicated in "Materials and methods" section (the *inset* shows the purity of the nuclear fraction as assessed by immunoblotting with annexin II, hsp60 or nucleoporin p62; which are, respectively plasma membrane-, cytosol- or nucleus-specific proteins) and the expression of EP receptors was assayed by immunofluorescence assay. *Lower panel* PGE_2 increased the DNA synthesis in isolated nuclei of PC3 cells. PC3 nuclei were isolated and pre-incubated with 1 μM EP2 antagonist AH6809 for 1 h and then, incubated with 1 μM PGE_2 for 3 h. The incorporation of BrdU was analysed by immunofluorescence assay as indicated in section "Materials and methods". **c** EGFR transactivation and HIF-1α participated in mediating the effect of intracellular PGE_2 on PC3 cell proliferation. *Upper panel* cells were pretreated with 1 μM EGFR inhibitor AG1478 or 10 μM HIF-1α inhibitor YC-1 for 1 h and treated with 0.5 μM EP2 agonist ONO-AE1-259-01 for 24 h. Cell proliferation was assayed by MTT assay (*left panel*) or incorporation of BrdU (*right panel*). *Lower panel* PC3 cells were transfected with HIF-1α siRNA or control siRNA (scramble) as indicated in section "Materials and methods" and then, they were treated with 0.5 μM EP2 agonist ONO-AE1-259-01 for 24 h. Cell proliferation was assessed by MTT assay (*left panel*) or incorporation of BrdU (*right panel*). All experiments were repeated three times. Statistical analysis: $*P < 0.01$ vs all groups

HIF-1α may play a significant role. We have just shown above that transactivation of EGFR by iEP2 receptor determines the up-regulation of HIF-1α in PC3 cells (Fig. 1). We have also previously found [17] that PGE_2 induces loss of PC3 cell adhesion to collagen type I, which was most likely due to $iPGE_2$ because the loss of cell adhesion was prevented by PGT inhibitor BG. Therefore, we decided to study the effect of the pharmacological inhibition of EP2 receptors, EGFR, HIF-1α and PGT on the attachment of suspensions of PC3 cells over 120 min to an extracellular

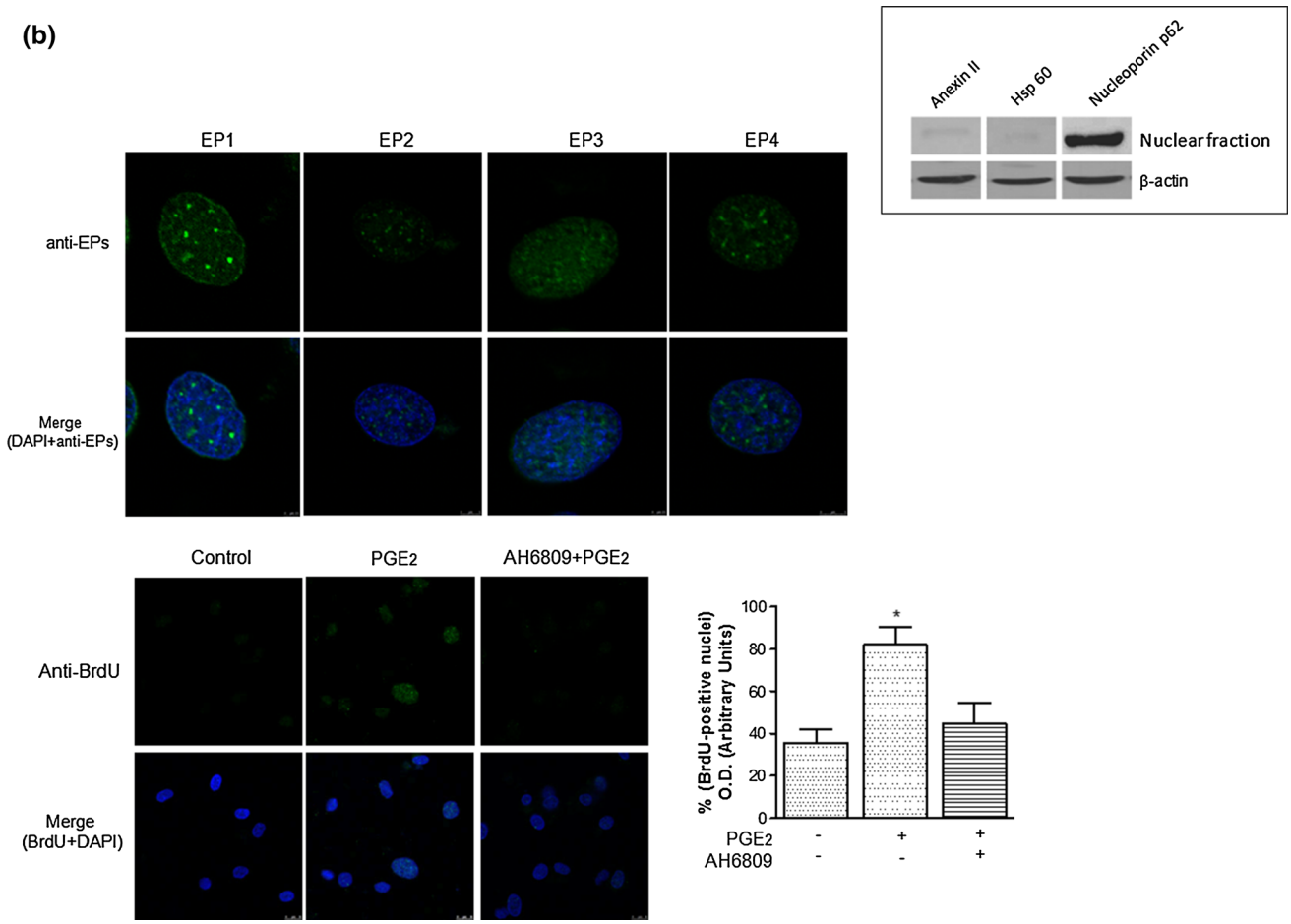


Fig. 2 continued

matrix of collagen type I upon treatment with EP2 agonist ONO-AE1-259-01. As shown in Fig. 3, the values for optical density in the MTT assay (adherent cells were assessed by the MTT assay) increased steadily over the time in control cells. Treatment with EP2 agonist resulted in loss of cell adhesion, which was prevented by all the pharmacological modulators used. These results indicated that activation of iEP2 receptors results in EGFR-, HIF-1α-dependent loss of PC3 cell adhesion. Worth to mentioning, this is the first time (to the best of our knowledge) that HIF-1α has been shown to have a critical role in cancer cell adhesion to an extracellular matrix protein.

Intracellular EP2 receptors mediate the increase in PC3 cell motility induced by PGE_2 through an AG1478-sensitive, HIF-1α-dependent mechanism

The results shown in Fig. 3 suggested that intracellular EP2 activation, through inhibiting PC3 cell adhesion to the extracellular matrix, might contribute to PC metastasis. The migratory activity of PC cells also contributes to metastasis and we have recently reported that intracellular PGE_2 promotes PC3 cell migration [17]. It has also been previously found that PGE_2-induced PC3 cell migration is prevented by EP2 receptor antagonist AH6809 [13]. Therefore, we studied the role of intracellular EP2 receptors in PGE_2-induced cell migration. To this end, we performed a scratch wound healing assay in PC3 cells (1) transfected with siRNA EP2 and then treated with PGE_2 or (2) pre-treated with BG and then treated with EP2 agonist ONO-AE1-259-01 (PGT inhibitor BG was used to explore the role of intracellular EP2 receptors). Figure 4a shows that PGE_2-induced PC3 cell migration was substantially reduced EP2 receptor knock-down and thatEP2 agonist increased the migration of PC3 cells, in a BG-sensitive manner, to a similar extent to that induced by PGE_2. The effect of ONO-AE1-259-01 was blunted by EP2 antagonist AH6809. These results indicated that PGE_2-induced tumour cell motility is dependent on intracellular EP2 receptors.

HIF-1α, which is up-regulated by $iPGE_2$ in PC3 cells in an EGFR-dependent manner (Fig. 1), has been previously

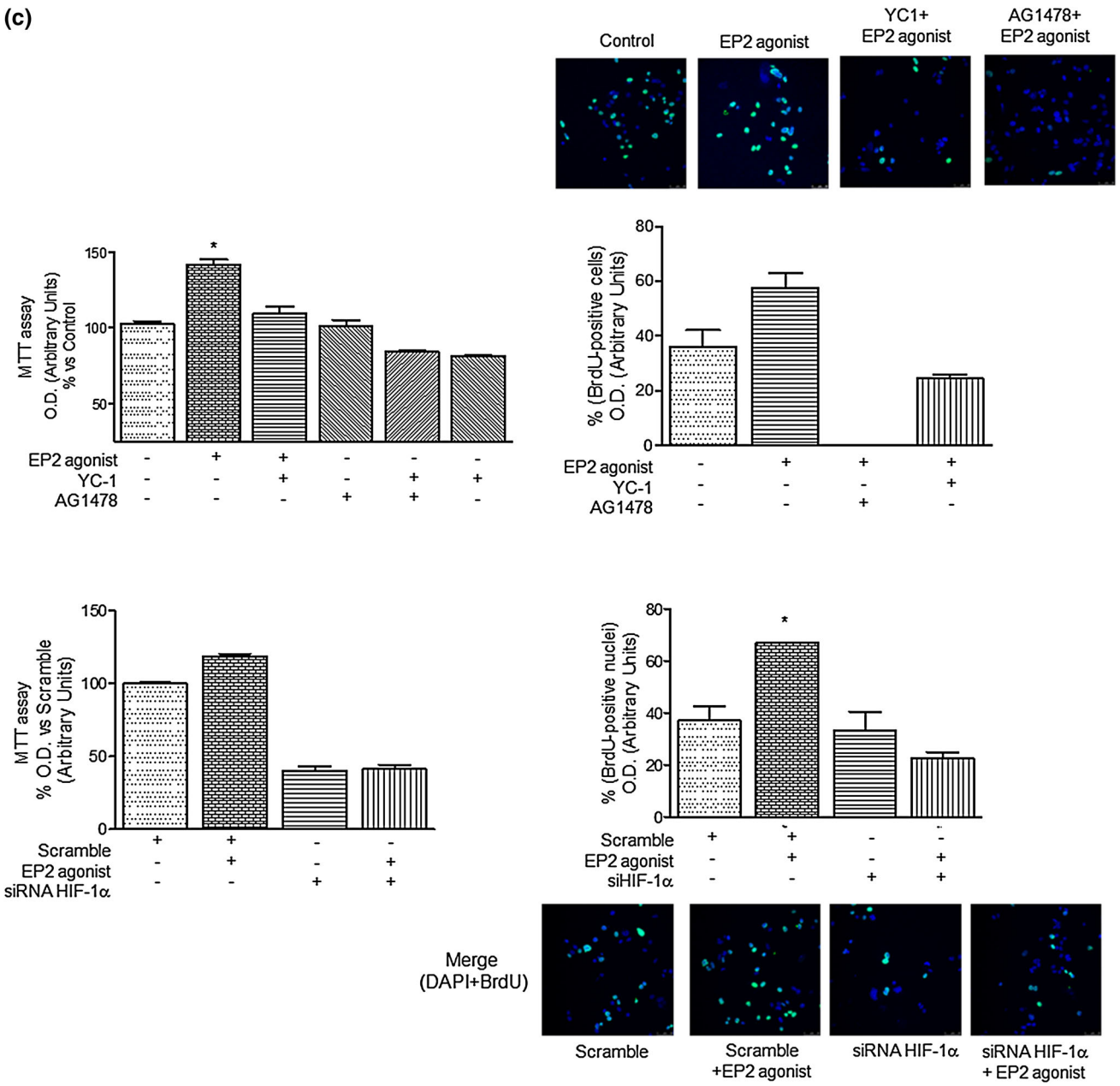


Fig. 2 continued

reported to be a positive regulator of their migratory and invasive activities [9, 10]. Therefore, HIF-1α might also play a role in EP2-dependent PC3 cell migration. In order to test this hypothesis, we first had to check that our experimental setting reproduces the regulation by HIF-1α of the increase in tumour cell motility induced by PGE_2. It was confirmed because scratch wound healing assay in HIF-1α knocked-down PC3 cells treated with PGE_2 showed no differences with cells transfected with scrambled RNA regarding the migratory activity (Fig. 4b). To confirm that HIF-1α mediates the EP2-dependent increase in PC3 cell motility, we studied here the effect of HIF-1α inhibitor YC-1. In addition, we also examined the effect of the inhibitor of EGFR activation AG1478 since it has been previously shown that EGF increases PC3 cell migration [6] and because the role of EGFR in iEP2-induced HIF-1α up-regulation (Fig. 1). As shown in Fig. 4b, both inhibitors prevented the increase in cell migration induced by EP2 agonist. These results suggest that EGFR and HIF-1α mediate EP2 agonist-induced increase PC3 cell motility. Worth to mentioning basal cell migration was substantially diminished by AG1478, siRNA HIF-1α and YC-1, which reflects the relevance of EGFR and HIF-1α in the basal migratory activity of PC3 cells.

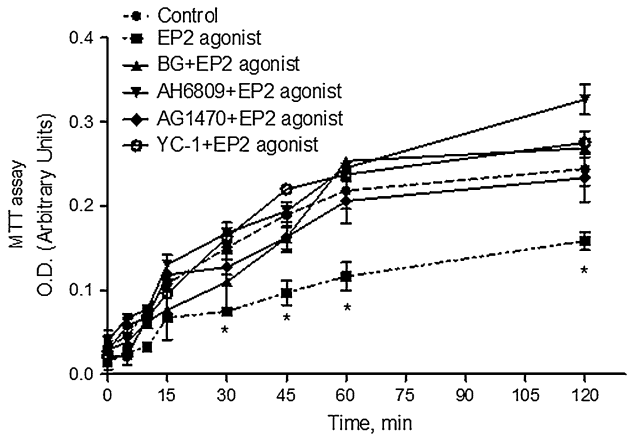


Fig. 3 Intracellular EP2 receptors, in an 1 μM EGFR inhibitor AG1478-, 10 μM HIF-1μ inhibitor YC-1-sensitive manner, promote PC3 cell adhesion. PC3 cells in suspension were pre-incubated for 1 h with 50 μM PGT inhibitor bromocresol green, 1 μM EGFR inhibitor AG1478, 1 μM EP2 antagonist AH6809 or 10 μM HIF-1α inhibitor YC-1 and treated with 0.5 μM EP2 agonist ONO-AE1-259-01. Then cells were plated in collagen coated 96-well plates (25×10^3 cells per well) and incubated for up to 120 min. The number of adherent cells was assessed by the MTT assay. All experiments were repeated three times. Statistical analysis: $^*P < 0.01$ vs all groups

In summary, the results shown in Fig. 4 indicate that PC3 cell migration induced by PGE_2 is mediated by intracellular EP2 receptors and is dependent on HIF-1α and EGFR.

Intracellular EP2 receptors mediate the increase in PC3 cell invasion induced by PGE_2 through an AG1478-sensitive, HIF-1α-dependent mechanism

The most threatening feature of malignancy in cancer is the potential for invasion and metastases. PGE_2 has been recently found to enhance cell invasion in PC cell lines [6] and our previous results have shown that intracellular PGE_2 promotes PC3 cell invasion [17]. In addition, EP2 receptor mediates PGE_2-induced PC3 cell invasion [4]. Given that our present data indicate that intracellular EP2 receptor mediates PGE_2-induced PC3 cell proliferation, adhesion and migration, we sought to explore the role of iEP2 in cell invasion in a transwell assay. To this end PC3 were first (1) transfected with siRNA EP2 and then treated with PGE_2 or (2) pre-treated with BG and then treated with EP2 agonist ONO-AE1-259-01 (PGT inhibitor BG was used to explore the role of iEP2 receptors). Afterwards, cells were incubated for 24 h to allow them to colonize the lower chamber of the transwell insert pre-loaded with Matrigel. Our results (Fig. 5a) indicated that knock-down of EP2 receptor abrogated PGE_2-induced PC3 cell invasion, that EP2 agonist increased the migration of PC3 cells (in an AH6809-sensitive manner) to a similar extent to that induced by PGE_2 and that PGT inhibitor BG prevented the effect of EP2 agonist. Taken together, these results indicated that iEP2 receptors mediate PGE_2-induced PC3 cell invasion.

Next, we studied the role of HIF-1α and EGFR in iEP2-dependent PC3 cell invasion following an experimental approach identical to that described above for PC3 cell motility. Accordingly, (1) we confirmed that our experimental setting reproduced the previously described [9, 10] regulation by HIF-1α of PGE_2-induced PC3 increase in cell invasion: transwell assay in HIF-1α knocked-down PC3 cells treated with PGE_2 showed no differences with cells transfected with scrambled RNA (Fig. 5b) and (2) we studied, and confirmed, the inhibitory effect YC-1 and AG1478 on EP2-increase in PC3 cell invasion (Fig. 5b) and also found that the basal invasive activity was substantially diminished by AG1478, siRNA HIF-1α and YC-1. These results indicate that HIF-1α and EGFR play a relevant role in both basal and EP2 agonist-induced increase PC3 cell invasion.

Intracellular EP2 receptors mediate the increase in VEGF-A production induced by PGE_2 in PC3 cells through AG1478-sensitive, HIF-1α-dependent activation of the HRE in the promoter of the VEGF-A gene

It has been previously shown in PC3 cells that PGE_2 induces the secretion of HIF-1α-regulated angiogenic factor VEGF through EP2 [5, 16] and our previous results have also shown that treatment of PC3 cells with 16,16-dimethyl-PGE_2 (a PGE_2 analogue) increases their production of VEGF-A through intracellular PGE_2 [17]. Taking into account this background, we asked whether intracellular EP2 receptors, through HIF-1α-dependent pathways, mediates the increase in the production of VEGF-A (as determined by ELISA) by PGE_2-treated PC3 cells.

We first studied its production in PC3 cells (1) transfected with siRNA EP2 and then treated with PGE_2 or (2) pre-treated with BG and then treated with EP2 agonist ONO-AE1-259-01 (PGT inhibitor BG was used to explore the role of intracellular EP2 receptors). Figure 6a shows that PGE_2-induced production of VEGF-A was substantially reduced EP2 receptor knock-down, that EP2 agonist increased the production of VEGF-A (in an AH6809-sensitive manner and that PGT inhibitor BG prevented the effect of EP2 agonist. Taken together, these results indicated that iEP2 receptors mediate the increase in the production of VEGF-A induced by PGE_2 in PC3 cells.

HIF-1α is up-regulated by $iPGE_2$ in PC3 cells through iEP2 receptors (Fig. 1) and might contribute to PGE_2-induced increase in VEGF-A production because expression of VEGF-A is regulated by HIF-1α [38]. We addressed this issue through knocking-down HIF-1α in PC3 cells. In these

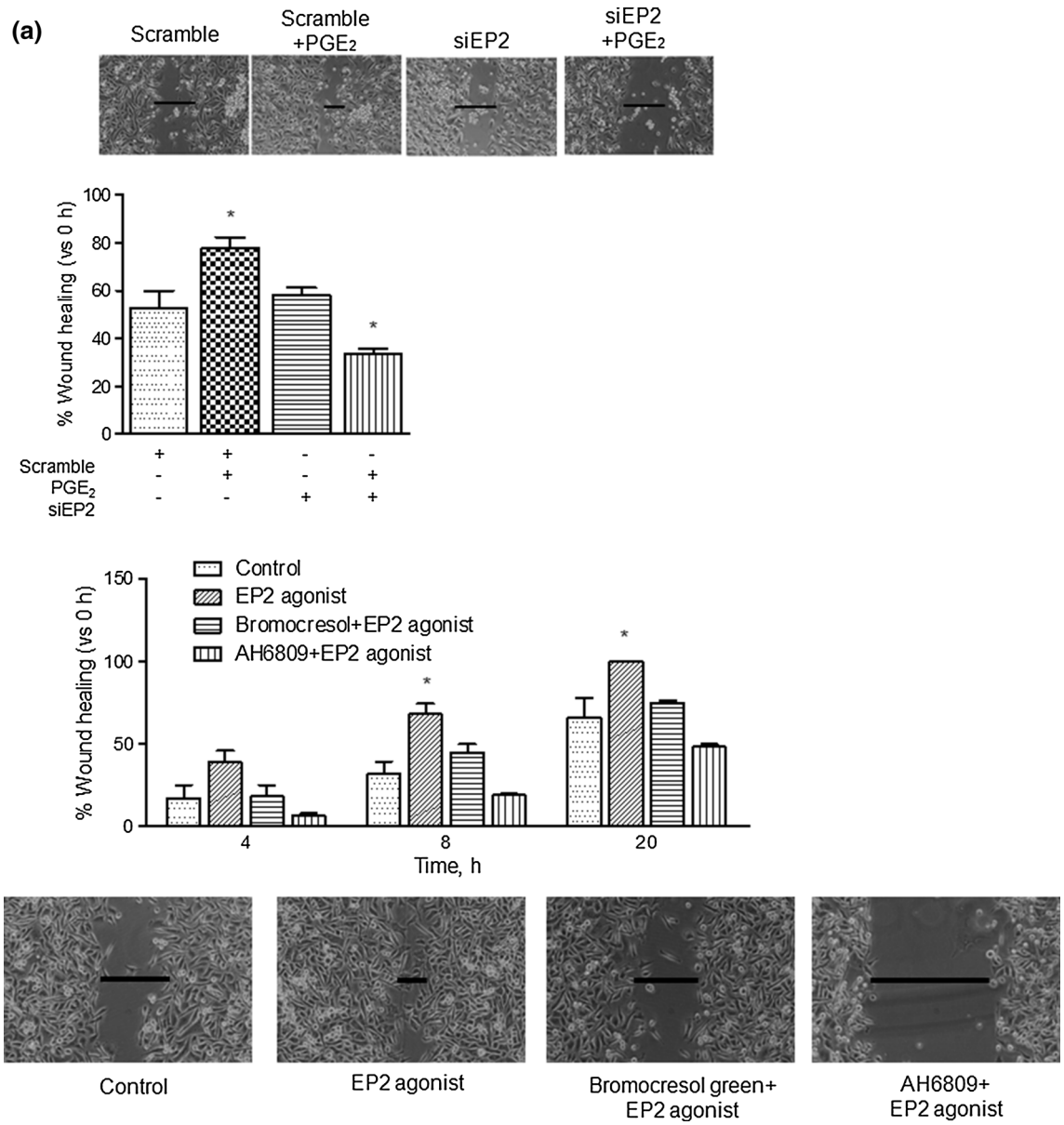


Fig. 4 Intracellular EP2 receptors, in an EGFR inhibitor AG1478-sensitive manner, promote HIF-1α-dependent PC3 cell motility. **a** PGE_2-induced tumour cell motility is dependent on intracellular EP2 receptors. PC3 cells were transfected with EP2 siRNA or control siRNA (scramble) as indicated in section "Materials and methods" and were seeded in 24-well plates until confluent (*upper panel*) and treated with or without 1 μM PGE_2 (*upper panel*). Other set of cells was cultured in 24-well plates until confluent and then were pre-treated with 50 μM PGT inhibitor bromocresol green or 1 μM EP2 antagonist AH6809. Afterwards, PC3 cells were incubated with or without a selective 0.5 μM EP2 agonist ONO-AE1-259-01 (*lower panel*). Confluent cells were carefully wounded using sterile pipette tips. Cell migration or wound repair was photographed (0, 4, 8 and 20 h) and measured vs time 0 h. All experiments were repeated three times and a representative photograph is shown. Statistical analysis: $*P < 0.01$ vs control. **b** EGFR and HIF-1α mediate EP2 agonist-induced increase PC3 cell motility. Cells were pretreated with 1 μM EGFR inhibitor AG1478 or 10 μM HIF-1α YC-1 for 1 h and treated with 0.5 μM EP2 ONO-AE1-259-01 agonist and cell motility was measured as **a**. *Inset* PC3 cells were transfected with HIF-1α siRNA or control siRNA (scramble), were seeded in 24-well plates until confluent and treated with or without 1 μM PGE_2. Cell motility was analysed as **a**

conditions, there was no increase in the production of VEGF-A in PGE_2-treated PC3 cells (Fig. 6b). The same was true when cells were pre-incubated with HIF-1α inhibitor YC-1 or with EGFR antagonist AG1478 (Fig. 6b), which inhibits the EP2-dependent increase in HIF-1α, as it was shown in Fig. 1b. Therefore, HIF-1α, in an AG1478-sensitive manner, mediates the iEP2-dependent increase in the production of VEGF-A upon treatment of PC3 cells with PGE_2.

HIF stimulates transcriptionally the expression of VEGF-A [38], through its binding to hypoxia-responsive

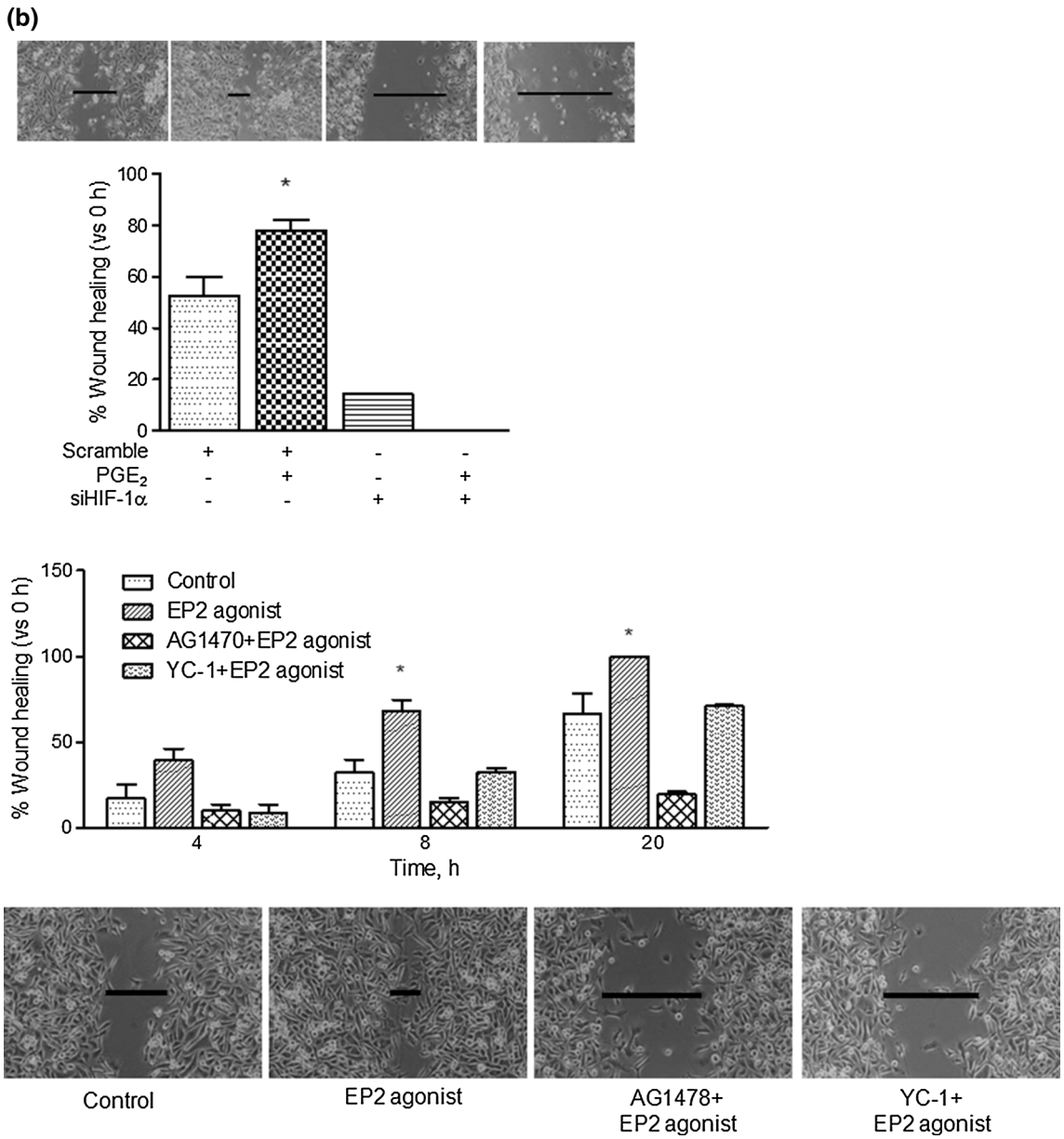


Fig. 4 continued

elements (HRE) in the VEGF-A gene [36]. Therefore, the mechanism involved in the increase of the production of VEGF-A by PGE_2-treated PC3 cells should also increase the activity of HRE. We sought to confirm this prediction in PC3 cells that were transiently transfected with p9HIF1-Luc (a plasmid which contained part of the human HIF binding sequence of the 5′ human VEGF gene promoter). As expected, treatment EP2 agonist resulted in a BG-, AH6809-, AG1478- and YC-1- sensitive increase in luciferase activity (Fig. 6c). PGE_2 also induced an AH6809-sensitive increase in luciferase activity (Fig. 6c, inset).

Taken together, the results shown in Fig. 6a–c, indicate that intracellular EP2 receptors mediate the increase in VEGF-A production induced by PGE_2 in PC3 cells. The mechanism most likely requires transactivation of EGFR by iEP2 leading to HIF-1α-dependent activation of the HRE in the promoter of the VEGF-A gene.

Intracellular EP2 receptors mediate the increase in vitro angiogenesis induced by PGE_2 in PC3 cells through an AG1478-, HIF-1α-dependent mechanism

It has been previously shown in PC3 cells that EP2 receptor is involved in matrigel angiogenesis induced by PC3 cells treated with PGE_2 [5]. Our previous results have also shown that treatment of PC3 cells with 16,16-dimethyl-

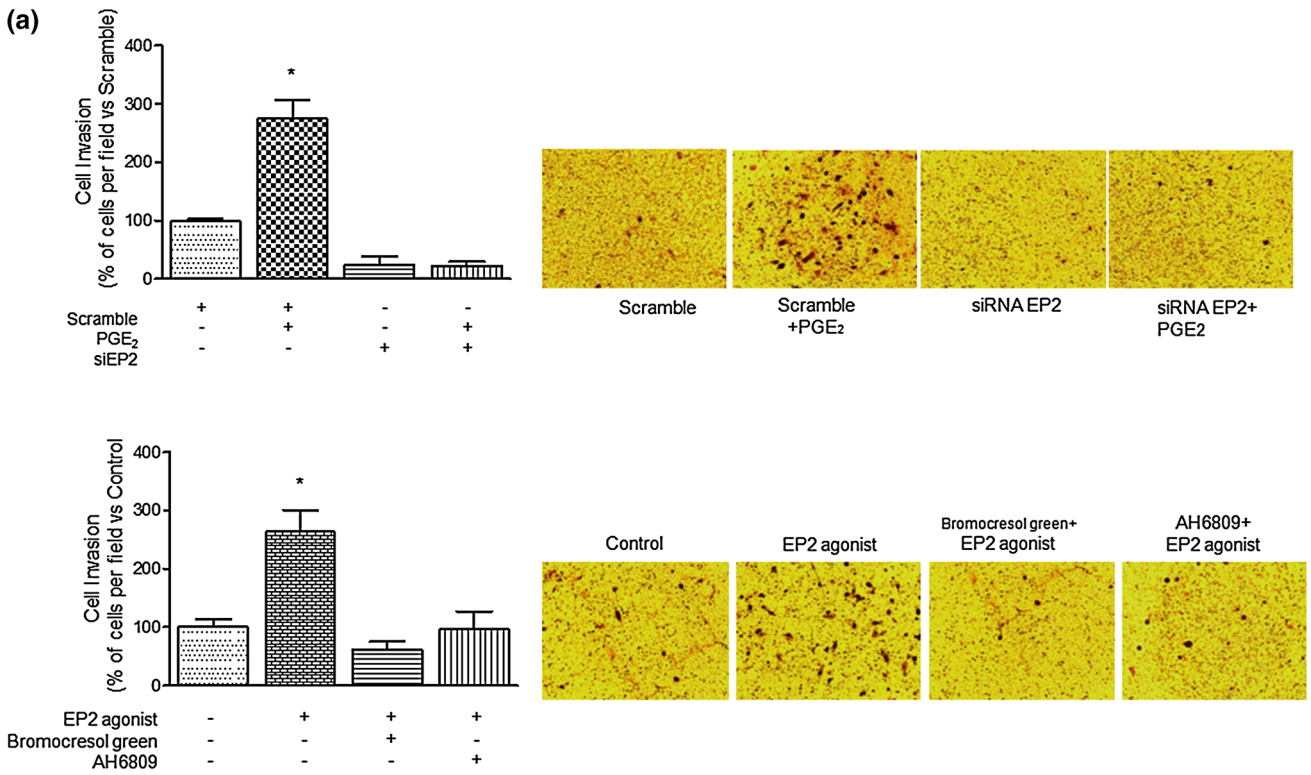


Fig. 5 Intracellular EP2 receptors, in an EGFR inhibitor AG1478-, HIF-1α inhibitor YC-1-sensitive manner, mediate the increase of PC3 cells invasion upon treatment with PGE_2. **a** Intracellular EP2 receptors mediate PGE_2-induced PC3 cell invasion. Cells in suspension (5×10^5 cells/ml) were transfected with EP2 siRNA or scramble and treated with 1 μM PGE_2 (*upper panel*). Other set of cells was treated for 1 h with 50 μM PGT inhibitor bromocresol green or 1 μM PGE_2 antagonist AH6809 and incubated with 0.5 μM EP2 agonist ONO-AE1-259-01 (*lower panel*). Then cells were seeded into transwell inserts which had been previously coated with Matrigel basement membrane matrix and invasive activity was assessed as indicated in "Materials and methods". All experiments were repeated three times and a representative photograph (×10 magnification) is shown. Statistical analysis: $*P < 0.01$ vs scramble/control. **b** HIF-1α and EGFR play a relevant role in both basal and EP2 agonist-induced increase PC3 cell invasion. Cells were pretreated with 1 μM EP2 antagonist AG1478 or 10 μM HIF-1α inhibitor YC-1 for 1 h and treated with 0.5 μM EP2 agonist ONO-AE1-259-01 and invasive activity was measured as **a**. *Inset* PC3 cells were transfected with HIF-1α siRNA or control siRNA (scramble) and treated with or without 1 μM PGE_2. Invasive activity was analysed as **a**

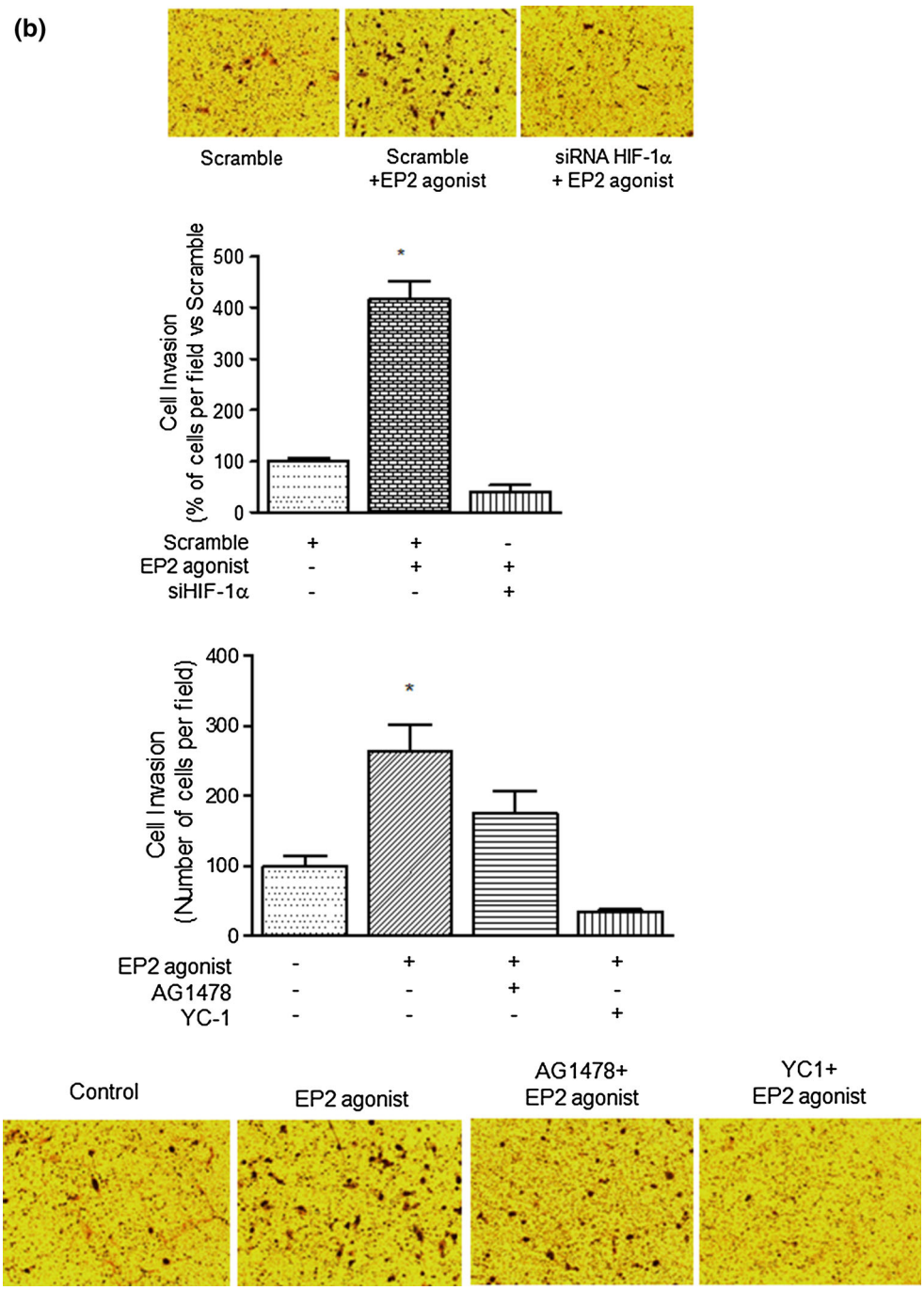
PGE_2 promotes endothelial tube formation (an assay for angiogenesis) in these cells through intracellular PGE_2 [17]. Given that our present data indicate that the iEP2/EGFR/HIF-1α pathway mediates PGE_2-induced PC3 cell proliferation, adhesion, migration and production of angiogenic factor VEGF-A, we sought to explore its role in angiogenesis. To this end PC3 cells were pre-treated with AH6809, AG1478, YC-1 or BG and incubated with EP2 agonist for 24 h. Then, extracellular medium was removed and used for capillary tube formation by HUVECs. Our results (Fig. 7) indicated that treatment of PC3 cells with EP2 agonist resulted in a BG-, AH6809-, AG1478- and YC-1-sensitive tube formation by HUVECs. PGE_2 also induced an AH6809-sensitive increase in capillary tube formation (Fig. 7 inset).

These results indicated that iEP2 receptors mediate PGE_2-induced in vitro angiogenesis and that HIF-1α and EGFR play a relevant role in this effect.

Discussion

Prostate cancer (PC) is the second most common cancer in men worldwide and continues to impose a significant disease burden and a growing healthcare problem. However, our understanding of the mechanisms that contribute to the development of PC is still limited [40]. Epidemiological, histopathological, and molecular pathological studies have been providing emerging evidence implicating inflammation in the pathogenesis of PC [41–45]. In the present study, we investigated the involvement of intracellular EP2 receptors (iEP2) in mediating the tumor-promoting effects of PGE_2 in androgen-independent PC3 cells. We found that iEP2 receptors—through EGFR transactivation- mediate important effects of PGE_2 on PC3 cells, such are the increase in cell proliferation, adhesion, migration, invasion and angiogenesis in vitro. These effects were absolutely dependent on HIF-1α, whose role in PC has been

Fig. 5 continued



previously recognized. Therefore, intracrine signaling through the iEP2/EGFR/HIF-1α axis provides a mechanism contributing to the pro-tumorigenic effects of PGE_2 and have important implications regarding PC progression and therapy.

The regulation of PGT in cancer has been scarcely studied. The current view on the biological role of PGT is that it mediates uptake of secreted prostaglandins, enabling their oxidative inactivation by cytoplasmic 15-PGDH. Therefore, the relevance of PGT, in the few studies aimed at elucidating its role in cancer, has been exclusively linked to the catabolism of prostaglandins: like 15-PGDH, down-regulation of PGT has been reported in both human colorectal tumours and in pre-malignant adenomas in $APC^{min/+}$ mice, and this was suggested to result, at least in part, from epigenetic silencing [46]. As a corollary, down-regulation or inhibition of PGT, in addition to 15-PGDH, would constitute a very effective means of increasing local levels of pro-tumorigenic PGE_2, as impaired PGT-mediated prostaglandin import would be expected to directly

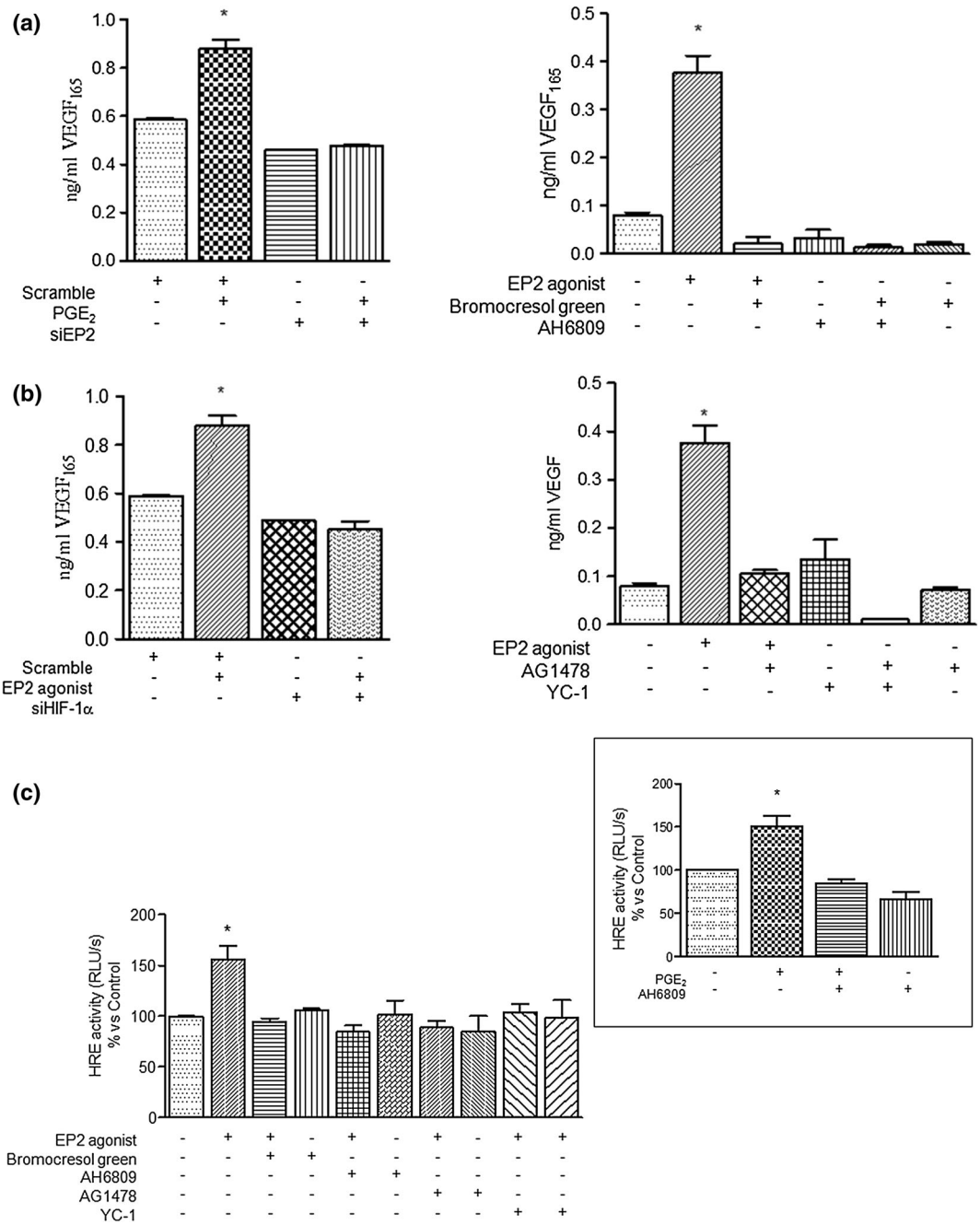


Fig. 6 Intracellular EP2 receptors mediate the increase in VEGF-A production induced by PGE_2 in PC3 cells through HIF-1α-dependent activation of the HRE in the promoter of the VEGF-A gene. **a** iEP2 receptors mediate the increase in the production of VEGF-A induced by PGE_2 in PC3 cells. Cells were transfected with EP2 siRNA or scramble and treated with 1 μM PGE_2 for 24 h (*left panel*). Other set of cells were treated for 1 h with 50 μM bromocresol green or 1 μM AH6809 and incubated with 0.5 mM EP2 agonist ONO-AE1-259-01 for 24 h (*right panel*). The extracellular medium was collected to analysed the VEGF-A levels by ELISA assay.*$P < 0.01$ vs all groups. **b** HIF-1α, in an EGFR inhibitor AG1478-sensitive manner, mediates the iEP2-dependent increase in the production of VEGF-A upon treatment of PC3 cells with PGE_2. Cells were pretreated with 1 μM EGFR inhibitor AG1478 or 10 μM HIF-1α inhibitor YC-1 for 1 h and treated with 0.5 μM EP2 agonist ONO-AE1-259-01 and VEGF production was measured as **a**. *Inset* PC3 cells were transfected with HIF-1α siRNA or control siRNA (scramble) and treated with or without 1 μM PGE_2. VEGF levels were analysed as **a**, **c** EP2 agonist-induced increased activity of a hypoxia-responsive element (HRE)-driven reporter construct is abolished by an inhibitor of the PGT transporter, an inhibitor of the EGFR phosphorylation and a selective antagonist of EP2 receptor. PC3 cells were transiently transfected with the plasmid as described in section "Materials and methods". Then, cells were pre-incubated with 50 μM PGT inhibitor bromocresol green, 1 μM EGFR inhibitor AG1478 or 1 μM EP2 antagonist AH6809 for 1 h and incubated with 1 μM PGE_2 (*left panel*) or 0.5 μM EP2 agonist ONO-AE1-259-01 (*center* and *right panel*) for 4 h and luciferase activity in cell lysates was measured and expressed as Relative Luminescence Units (RLU/s). All experiments were repeated three times. Statistical analysis: *$P < 0.01$ vs all groups

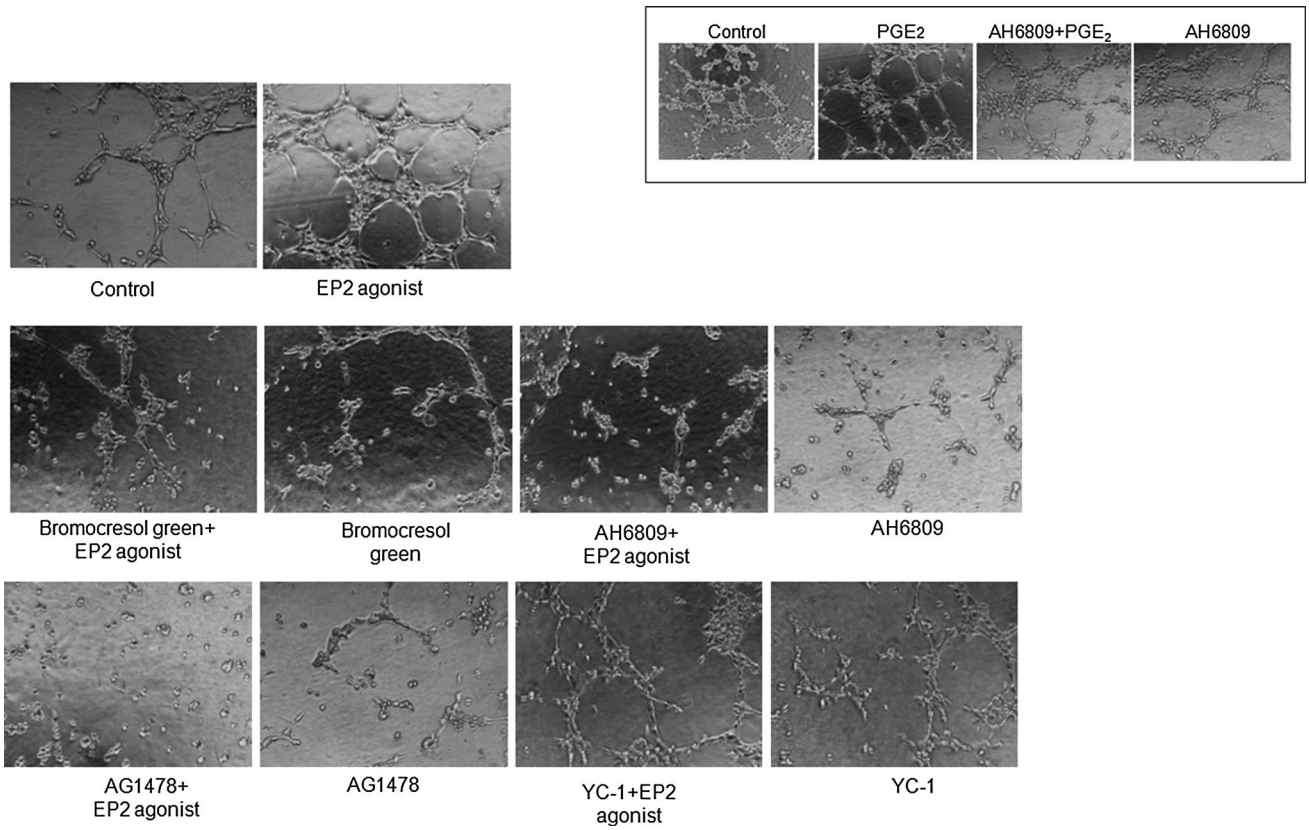


Fig. 7 Conditioned media collected from EP2 agonist treated PC3 cells increases tube formation by HUVECs in a bromocresol green-, AG1478-, AH6809- and YC-1-dependent manner. HUVECs cells were seeded on Matrigel-coated wells and cultured in conditioned media collected from PC3 cells after pre-treatment with 50 μM PGT inhibitor bromocresol green, 1 μM EGFR inhibitor AG1478, 1 μM EP2 antagonist AH6809 or 10 μM HIF-1α inhibitor YC-1, and incubated with 0.5 μM EP2 agonist ONO-AE1-259-01 for 24 h. Representative photographs of tube-like structures were showed

increase extracellular prostaglandin concentrations available to interact with cell surface receptors during [47]. While this hypothesis may be true in the context of colorectal tumours, our current results suggest that therapeutics targeting PGT in PC might result in diminished cancer progression.

It is assumed that prostaglandin E_2 (PGE_2) exerts its biological effects from the extracellular medium through activation of G-protein coupled receptors (GPCR) EP receptors located at the cell membrane [48]. Our current results challenge this view and open the possibility that, in PC, signal cascades that proceed entirely in the intracellular compartment might be responsible for several protumorigenic effects of PGE_2 that are assumed to be due to cell membrane EP receptors. In sharp contrast with cell membrane EP2 receptors, whose activation results in increased production of cAMP [49, 50], we have previously shown in proximal tubular HK-2 cells that intracellular EP2 signaling was dependent on EGFR (which agrees with our present results in PC3 cells), but independent of cAMP [51]. These results indicate that the signal cascades activated by intracellular EP receptors are not necessarily the same which are activated by plasma membrane EP receptors. In fact, it has been proposed that the distinct localization of EP receptors and other GPCR may dictate different signaling pathways for the same receptor [29].

Inhibitor of PGT bromocresol prevents the transport of PGE_2 to the inside of PC3 cells [17] and proximal tubular HK2 cells [12, 30, 51]. In addition, our current results in PC3 cells with BG and our previous results in HK2 cells [51] are in good agreement with the view that the EP2 agonists used in those studies are also substrates for PGT. The fact that EP2 receptor antagonist AH6809 prevents the BG-sensitive effects of EP2 agonists indicate that AH6809 reaches the intracellular compartment, thereby inhibiting iEP2-dependent effects. Therefore, AH6809 might also be substrate for PGT, although further experiment should be performed to confirm it. More importantly, our studies open the possibility that perhaps other EP agonists and antagonists may also be transported to the inside the cell and activate intracellular EP receptors. If the researchers are unaware of this possibility, they will probably assign to

cell membrane EPs the results of experiments with EP agonists and antagonists. Therefore, the actual extension to which intracellular EP receptors may have contributed to PGE_2 effects attributed to cell membrane EP receptors remains to be determined.

PGE_2 and EGF independently propagate, through their respective membrane receptors, signals regulating important cell functions. However, growing evidence indicates that the epithelial tumorigenic drive is largely sustained by the close interplay of the prostanoid with the EGF system, resulting in the activation of EGFR. In our study, the issue of PGE_2/EGFR transactivation (*i.e.* whether the prostanoid conveys its tumorigenic potential by promoting the phosphorylation of the EGFR) appears straightforward for PC3 cells: treatment with EP2 agonist promoted the phosphorylation of EGFR (the hallmark of its activation) and the blockade of EGFR phosphorylation by AG1478 obliterated the EP2-dependent up-regulation of HIF-1α expression and the increase in PC3 cell proliferation, adhesion, migration, invasion and angiogenesis in vitro. The phenomenon of EGFR tyrosine kinase transactivation by PGE_2-activated EP2 receptors in PC3 cells has also been previously described for transducing angiogenic signals in PC3 cells [5].

Transactivation of plasma membrane EGFR by PGE_2-activated EP receptors is a particular case of activation of EGFR by plasma membrane G-protein coupled receptors (GPCR) [52–55]. However, our results (Fig. 1) suggest that EGFR might be also transactivated by intracellular EP receptors. The cell nucleus is particularly important in this context: recent studies have disclosed that the nuclear envelope plays a major role in signal transduction cascades: functional EP receptors can be localized at the nuclear membranes of a variety of cell types and tissues [12, 24, 25, 28]. The same is true for phospholipase A_2, cyclooxygenases and prostaglandin E synthase [56]. Thus the cell nucleus has to be considered as a possible intracellular location for PGE_2 production and for the initiation of signal transduction cascades dependent on EP receptors. EGFR has been detected only in two subcellular locations, the nucleus and the mitochondrion [57] and we have recently described that isolated nuclei from proximal tubular cells express EGFR and EP receptors and that treatment with PGE_2 results in transactivation of EGFR by EP2 receptors [51]. Furthermore, the signaling pathway activated upon the transactivation of nuclear EGFR was the same which was found to be responsible for the increase in HIF-1α expression following intracellular EP2-dependent EGFR transactivation in whole proximal tubular cells [51]. Interestingly, we show here that EP2 receptors in nuclei isolated from PC3 cells mediate the effect of PGE_2 on the incorporation of BrdU (Fig. 2), which suggests that nuclear EP receptors may contribute to convey the effect of intracellular PGE_2 on PC3 cell proliferation. This does not exclude that non-nuclear iEP2 receptors may also mediate PGE_2-induced PC3 cell proliferation. Whether transactivation of EGFR by EP2 mediates the increase in the incorporation of BrdU to PGE_2-treated nuclei is an interesting possibility that remains to be investigated. It should be kept in mind that most iEP2 receptor immunofluorescence did not co-localize with DAPI nuclear staining in PC3 cells (Fig. 1c). Therefore, it is very likely the participation of non-nuclear iEP2 receptors in the EP2 agonist-induced cancer-related phenotypes in PC3 cells.

In a recent study, EP2 and EP4 were overexpressed in areas of PC in 85 % specimens analyzed, whereas EP3 expression was reduced in all specimens and EP1 showed no specific differential expression pattern [15]. In another study, expression of EP2 and EP4 in areas of PC was significantly higher than in non-tumour glands (although there was no correlation with the clinicopathological features) and expression of EP2 correlated positively with cancer cell proliferation [34]. The studies in cell lines derived from PC also have highlighted the pathogenic relevance of EP2 receptors: in PC3 cells and other PC cell lines, EP2 receptor activation has been found to mediate the enhancing effects of PGE_2 on the expression of HIF-1α [17], cell growth [4, 5, 15, 17, 58], migration [5], invasion [4, 15], production of angiogenic factor VEGF-A [5, 45] and angiogenesis [5]. These results are in good agreement with the important role of EP2 in tumour development, which have been recently reviewed [59]. However, it is likely that the contribution of EP receptors to the pathogenesis is not restricted to EP2 as suggested by several studies. For instance, immunopositivity for EP1 in PC tissue was significantly greater than in non-tumour glands and correlated positively with the Gleason score and cancer proliferation [34]. Another studies have suggested that EP3 might have tumor-suppressive properties [15] or have involved EP4 receptors in mediating the effects of PGE_2 on PC3 cell migration and PC3 cell-induced angiogenesis in vitro [5, 6]. Clearly, characterization of EP receptors in tumor cells is only at its beginning, and the precise role of each EP receptor in the pathogenesis of PC has yet to be elucidated. In this context, our present results in PC3 cells, which demonstrate that important PGE_2-induced cancer-related effects are mediated by iEP2, bring thereby an additional level of complexity in the highly complex role played by PGE_2 in PC.

In summary, the results shown here indicate that a number of pro-tumorigenic effects of PGE_2 in androgen-independent PC3 cells are mediated through iEP2 receptors, which requires the internalization of PGE_2 through PGT. Therefore, therapeutics targeting iEP2 and/or PGT might be promising against prostate cancers.

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Conflicts of interest There are no conflicts of interest to disclose.

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