

Modulation of PGE₂-induced EP4 Expression on *Snail* Signaling and the Impact on Epithelial-Mesenchymal Transition: Significance of EP4 Antagonism

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Abstract. *Background:* Although significant accumulation of prostaglandin E₂ (PGE₂) in the human prostate cancer tissues has been reported, there is lack of substantial evidence regarding the key role of PGE₂-induced E-prostanoid-4 receptor (EP4) on *Snail*, a master regulator of epithelial mesenchymal transition (EMT). *In this study, we investigated a novel connection between PGE₂-induced EP4 and Snail (encodes DNA binding zinc finger protein that acts as transcriptional repressor) signaling in prostate cancer. Materials and Methods:* To investigate the key role of serum PGE₂, EP4, p-Akt and *Snail* in prostate cancer progression, we used prostate-specific phosphatase and tensin homolog (PTEN)-knockout (PTEN-KO) mice of different age groups from 4 to 28 weeks. To determine the EP4-specific interaction with *Snail* in prostate cancer, we used cell-based assays, including siRNA knockdown, and treatment with EP4 antagonist. *Results:* An interaction between EP4 with *Snail* was evident in prostate-specific PTEN-KO mice that showed an elevated level of PGE₂ in the serum and of EP4, p-Akt and *Snail* in the tissues. Prostate cancer cells transfected with EP4-siRNA and treatments with EP4 antagonist suggest a link between EP4, and *Snail* activation, potentially via p-Akt. Cells treated with EP4 antagonist exhibited a significant decrease in *Snail*, mesenchymal markers and cell migration, and cell cycle arrest with a gain in E-cadherin levels. *Conclusion:* Our findings provide key evidence that support there being a role of PGE₂/EP4/p-Akt in *Snail* signaling and conferring cell survival advantage. Cancer progression via EMT can be reversed by an EP4 antagonist in this model of prostate cancer.

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Among the prostanoids, prostaglandin E₂ (PGE₂), derived from arachidonic acid, is widely produced in the body and the receptors mediating prostanoid actions have been documented in earlier reports (1-4). Mechanistically, PGE₂-induced cell proliferation appears to be mediated by its overall effect on second messenger response, which in turn depends on receptor-ligand concentration and interactions (5). It has been established that PGE₂ signaling stimulates the E-prostanoid (EP) receptors, which activate cyclic adenosine monophosphate (cAMP)/phosphatidylinositol 3-kinases (PI3K)/AKT and the extracellular signal-regulated kinase (ERK1/2) pathways, as well as the glycogen synthase kinase (GSK-3 β) pathways in some cancer types (6). In previous studies, we found a significant association between EP4, Akt and Nuclear factor kappaB (NF- κ B) signaling and their modulation by cyclooxygenase 2 (COX2) inhibitors in preclinical models for prostate cancer (7, 8). Extensive analysis of the data gathered from a PubMed search on EP4 signaling clearly reveals a strong interaction of EP4 with receptor activator of NF- κ B ligand (RANKL) through cAMP (9-11), and early growth response protein-1 (Egr-1) regulation via EP4/Extracellular signal-regulated kinase (ERK) and cAMP Responsive Element Binding (CREB) pathway in colon cancer cells (12, 13). Recent studies in prostate cancer models (14, 15) indicate that *Snail* (encodes DNA binding zinc finger protein that acts as transcriptional repressor), a key member of the epithelial mesenchymal transition (EMT) and also the transcription factor that deregulates E-cadherin expression, promotes tumor invasiveness, tumorigenicity (16) and mediates EMT by binding to the E-box elements of E-cadherin promoter (17-20). Although a significant accumulation of PGE₂ in human prostate cancer tissues has been reported (21), there is lack of substantial evidence regarding the influence of PGE₂-induced EP4 on *Snail* activation in prostate cancer. In this study, we provide evidence for a key role of serum PGE₂, EP4, p-Akt and *Snail* in prostate cancer progression, using Phosphatase and tensin homolog (PTEN)-knockout (PTEN-KO) mice of different age groups from 4 to 28 weeks. To

dissect the mechanisms of EP4 specific interaction with Snail in prostate cancer, we used cell-based assays, including EP4-siRNA knockdown and migration studies.

Materials and Methods

Serum and prostate tissue samples. Prostate tumor progression associated with an increased expression of EP4, Akt and Snail was determined in genetically modified prostate-specific PTEN-KO mice. Briefly, PTEN-KO mice were developed by deleting the *Pten* tumor suppressor gene, specifically in the prostatic epithelium that results in the multistep tumorigenesis similar to that of human prostate cancer (22). In this model, conditional homozygous deletion of *Pten* in mouse prostate epithelium significantly shortens the latency of prostate intraepithelial neoplasia (mPIN) and promotes their progression to metastatic cancer. These conditional prostate-specific PTEN-KO mice were propagated in-house at the departmental animal core facility, by crossing *Pten^{loxP/loxP}* mice with the PB-Cre4 mice that carries the *Cre* gene under the control of a composite promoter, *ARR2PB* which is a derivative of the rat prostate-specific probasin (PB) promoter (22-25), according to the protocol described in our recent publication (26). Briefly, the male *ARR2PB-Cre4* transgenic mice obtained from the National Institute of Health (NIH)/National Cancer Institute (NCI) mouse repository, (mouse.ncifcrf.gov) were first crossed with the female B6.129S4-*Pten^{tm1Hwu}/J* mice purchased from JAX Mice Services (Bar Harbor, ME, USA). Subsequent cross breeding of the F1 generation offsprings resulted in homozygous deletion of *Pten* in the F2 generation. *Pten* deletion in these mice has been confirmed by PCR using tail DNA as described earlier (27). Animal were sacrificed at various time points (n=5) between the age 4 and 28 weeks by CO₂-asphyxiation and necropsied. At the time of sacrifice, blood was collected by cardiac puncture and serum was obtained by centrifugation at 14,000 rpm for PGE₂ analysis. The prostate was micro-dissected and snap-frozen for total RNA and protein extraction. A portion of the prostate tissue was fixed in 10% neutral-buffered formalin for histopathological evaluations and immunohistochemical (IHC) studies.

Histology and IHC detection. Prostate tissues of PTEN-KO mice age 4, 7, 17 and 28 weeks were used for histopathological evaluation of tumor progression. Histological evaluations indicative of tumor progression from mPIN to adenocarcinoma of the dorsolateral prostate were conducted as described in our earlier publication (26). Stromal and tumor regions were differentiated histologically using H&E stained sections in order to identify the regions that specifically stain Snail and/or E-cadherin. Here, prostate cancer tissues from 28-week-old PTEN-KO mice with adenocarcinoma were used to conduct IHC staining for Snail (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and E-cadherin at an antibody dilution of 1:200 in five paraffin-embedded sections each. Positively stained cells were identified using a light microscope under 10 high power fields

PGE₂ measurement. To measure PGE₂ levels in the serum, Correlate-EIA-prostaglandin (PG) E₂ enzyme immunoassay was performed following the manufacturer's protocol (Assay Designs, Ann Arbor, MI, USA), as described by us previously (7). Briefly, at the time of sacrifice, serum samples from 4 and 28-week-old PTEN-KO mice (n=5 each) were collected and stored at -80°C

until further analysis. PGE₂ standard 50,000 pg/ml was used for subsequent lower dilutions. The assay involves the use of a monoclonal antibody that binds in a competitive manner with PGE₂ in the sample or an alkaline phosphatase molecule, which has PGE₂ covalently attached. After a short incubation time, the enzyme reaction was stopped, and the yellow color generated was read on a microplate reader at 405 nm. The intensity of bound yellow color is inversely proportional to the concentration of PGE₂ in the sample. The results presented here are based on data from three independent assays.

Cell culture. The human prostate cancer cells (PC-3 and LNCaP) obtained from the American Type Culture Collection (Manassas, VA, USA), were grown in RPMI (Gibco Life Technologies, Inc., Gaithersburg, MD, USA) with 10% FBS in addition to adopting the standard cell culture conditions as described earlier (27). The mouse prostate cancer cells (CaP8) derived from the adenocarcinoma of the *Pten* knockout mice were kindly provided by Dr. Hong Wu, UCLA School of Medicine, CA, USA. The CaP8 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Atlanta biological, Lawrenceville, GA, USA), as well as 25 µg/ml bovine pituitary extract, 5 µg/ml bovine insulin, and 6 ng/ml recombinant human epidermal growth factor (BioWhittaker, Walkersville, MD, USA), as described earlier (28). Cells were stimulated with 0.5 µM of PGE₂ for 1 h or 5 ng transforming growth factor beta (TGF-β) for 24 h before treatment.

Transient transfections. Cells were transfected with two sets of siRNAs designed for EP4 and COX2 gene. Complex of siRNA duplexes and control siRNA along with HiPerFect transfection reagent purchased from Qiagen Inc (Valencia, CA, USA), was prepared as described by us earlier (27). Transfection efficiency with siRNA was assessed by immunofluorescence followed by western blot analysis after 48 h. Snail promoter activity was determined by transfecting PC-3, LNCaP and CaP8 cells plated in 12-well plates at a density of 1×10⁵ cells/well using *Snail* gene-specific proximal promoter (*Snail-1pl*) fragment plasmid or control plasmid (*Snail-0pl*) 4 µg each. Transient transfection assays were followed by treatment with EP2 (AH6809) or EP4 antagonist (AH23848) (Cayman, Ann Arbor, Michigan, USA) at a dose of 5 µM which was appropriately selected based on our dose-dependent assay. Luciferase activities were measured by a dual luciferase assay in which Renilla luciferase activity was used to normalize the activity of firefly luciferase using a Dual-Glo Luciferase Assay System (Promega, Madison WI, USA) and by using LMax-II³⁸⁴ luminometer (Molecular devices, Sunnyvale, CA, USA). All the above experiments were repeated at least three times.

RNA isolation and real-time PCR. Total RNA was extracted from transfected or treated cells using Trizol reagent (GibcoBRL) as described earlier by us (27). cDNA was made from total RNA using the superscript first-strand synthesis system with oligo dT primers (IDT, Coralville, IA, USA) made specifically for *Snail*, vimentin, fibronectin, E-cadherin, and Gapdh. The following primer sequences were used for amplification: Snail: forward GGG CAG GTA TGG AGA GGA AGA, reverse TTC TTC TGC GCT ACT GCT GCG; E-cadherin: forward CAG CAC GTA CAC AGC CCT AA, reverse GCT GGC TCA AGT CAA AGT CC; Vimentin: forward TCG TTT CGA GGT TTT CGC GTT AGA GAC, reverse CGA CTA AAA CTC GAC CGA CTC GCG. Fibronectin: forward-CCG CCG AAT

GTA GGA CAA GA, reverse TGC CAA CAG GAT GAC ATG AAA and GAPDH: forward CCT GGC CAA GGT CAT CCA TGA C, reverse CAT GTA GGC CAT GAG GTC CAC CAC. The amplification conditions were 94°C for 5 m, and 21 cycles for real-time quantitative PCR were performed in triplicate with a Smart Cycler (Cepheid, Sunnyvale, CA) and with SYBR-Green Real time PCR-Supremix (Bio-Rad, Hercules, CA, USA) as described earlier (27). Results were normalized to GAPDH levels. Fold change in the mRNA expression level was calculated as $2^{\Delta\Delta Ct}$.

Western blot analysis. Relative expression of the molecular targets at the protein level before and after transfection with siRNA or treatment with antagonist for EP4 was determined by western blot analysis using specific antibodies as described earlier (27). Briefly, total protein extracted from prostate cancer tissues or cancer cells were fractionated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). We used 50 µg of each sample protein after extraction with a buffer containing 150 mmol/l NaCl, 10 mmol/l Tris (pH 7.2), 5 mmol/l EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS in addition to a mixture of protease inhibitors (Boehringer Mannheim, Mannheim, Germany). Fractionated proteins were transferred onto polyvinyl derivative (PVD) membranes. Standard western blot procedure was carried out using specific monoclonal antibody (1:1000 dilution) to detect EP4, Snail, p-Akt (ser-473) p21 and cyclinD1 (Santa Cruz Biotechnology). The level of β-actin expression was used as reference for equal sample loading. Reactive protein bands were detected using the ECL kit (Amersham, Biosciences, Uppsala, Sweden). Densitometric analysis was performed to quantify the reactive protein bands.

Immunofluorescence detection. We performed immunofluorescence detection of Snail in CaP8, and PC-3 and LNCaP cells as described earlier (27). Cells grown in two- or four-well chamber slides were stimulated with 0.5 µM of PGE₂ for 1 h or 5 ng TGF-β for 24 h before treatment with AH23848 at a dose of 5 µM for 48 h. Detection of EP4 and Snail was performed by double staining with their specific antibodies (27). Green fluorescence signal (FITC) for Snail and red signal (Rhodamine) for EP4 or E-cadherin over 4'.6-diamidino-2-phenylindole (DAPI) for DNA was viewed at ×400 using an Olympus AX-70 epi-fluorescence microscope (Olympus America, Melville, NY USA).

Cell proliferation assay. Prostate cancer cells were grown in 96-well plates at a density of 1×10^4 per well (100 µl). Cells were stimulated with 0.5 µM of PGE₂ for 1 h, followed by treatment with different concentrations (0–40 µM) of AH23848 (this dose range was chosen based on earlier studies) (2). Cells treated with serum-free medium or DMSO served as the control. Standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell proliferation assay was performed following the manufacturer's protocol (Cayman, Ann Arbor, MI, USA). At the end of the assay, absorbance at 570 nm was detected using a microplate Reader (PerkinElmer, Boston, MA, USA). The assays were performed in triplicate.

Cell migration assay. A 24-well BD FluoroBlok Transwell Insert (BD Biosciences, Bedford, MA, USA) with 8 µm pore size was used to measure cell migration. Briefly, cells pretreated with the EP4 antagonist were seeded (200 µl) into the inserts with RPMI containing 0.25% serum, with the bottom well medium containing only RPMI with 10% FBS. After 48 h of incubation the bottom well

was filled with 500 µl of calcein fluorescent dye prepared according to the directions of the manufacturer (BD Biosciences, Bedford, MA, USA). The fluorescence intensity emitted by the migrated cells was measured at 540 nm using a microplate reader. The experiments were repeated three times.

Cell cycle analysis. Prostate cancer cells, PC-3 and CaP8 cells stimulated with 0.5 µM of PGE₂ for 1 h followed by treatment with AH23848 for 48 h were used to conduct cell cycle analysis as described earlier (29). Briefly, treated cells were harvested after 48 h by trypsinization and fixed in 10% neutral buffer formalin followed by fixing in 80% ethanol for 24 hours. The cells were then washed in phosphate buffered saline (PBS) and resuspended in 1 ml of 5 mg/ml of propidium iodide with 0.1% RNase A (Sigma, St. Louis, MO, USA) in PBS to measure DNA content. After 30 min incubation at room temperature in the dark, the cells were analyzed by flow cytometry with Coulter Epic Elite ESP (Beckman Coulter, Miami, FL, USA). Cell cycle analysis was performed using Multicycle analysis software (Phoenix Flow System, San Diego, CA, USA). To determine the significant difference in the DNA content between control and treatments, the analysis was repeated with triplicate samples.

Statistical analysis. Data from all the experiments are presented as the mean±SD, from at least three independent experiments. Measures of statistical difference were determined using two-way ANOVA followed by Tukey's multiple comparison procedure (30). Differences among the treatment and control groups were analyzed using Student's *t*-test. Statistical analyses were performed using GraphPad Prism 4 software (San Diego, CA, USA).

Results

In vivo evidence for an elevated level of serum PGE₂, EP4 and Snail expression in a progressive model for prostate cancer deleted for Pten. We measured serum-PGE₂, EP4 and Snail expression in samples obtained from prostate-specific PTEN-KO mice of different age groups, known to exhibit progressive changes in the prostate from mPIN to adenocarcinoma stages (22). Findings from ELISA assays showed >3-fold increase in the serum PGE₂ in mice with cancer at 28 weeks of age in contrast to the level in the 4-week-old mice (Figure 1A). However in the wild-type mice, no significant change in the PGE₂ levels was observed (Figure 1B). Consistent with this, western blot analysis for EP4 and Snail indicated a gradual increase from week 4 to 28 in PTEN-KO mice as compared to the wild-type mice (Figure 1C). Furthermore, the expression of Snail and E-cadherin in the stromal and tumor regions of the prostate were analyzed by IHC. Interestingly, we found detectable levels of Snail and E-cadherin in the non-tumor (stromal) regions of the prostate. However, our findings showed an elevated level of *Snail* (>4-fold, $p < 0.001$) associated with a significant decrease in staining for E-cadherin (Figure 1D and E) in the prostate tumors of 28 week old PTEN-KO mice, suggesting that the loss of E-cadherin in the tumor is associated with EP4 and *Snail* expression in the stroma.

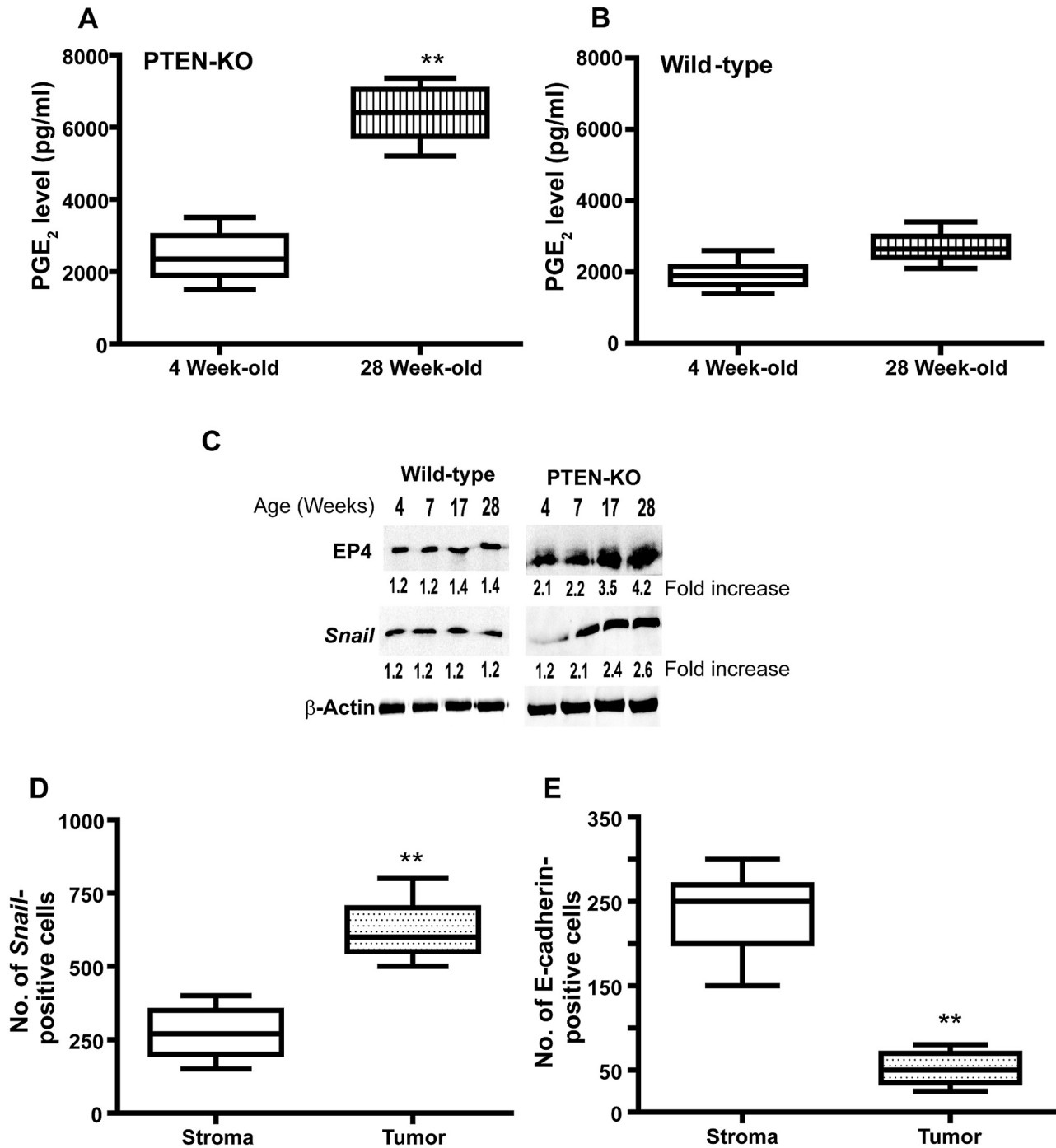


Figure 1. Serum prostaglandin E₂ (PGE₂), E-prostanoid-4 receptor (EP4) and Snail (transcriptional repressor) expression in the prostate tumor and stroma of PTEN-KO mice. A, B: PGE₂ was analyzed by ELISA assays using serum samples obtained from PTEN-KO and wild-type mice of 4 and 28 weeks of age (n=5). The assays were repeated three times. Box plots represent the mean±SD. **Significant difference at p<0.001. C: Tumor progression is associated with an increase in the protein for EP4 and Snail. Prostate tissues of PTEN-KO mice ages 4, 7, 17 and 28 weeks were used to isolate protein (50 µg/lane) for western blot analysis. The data presented for EP4 and Snail expression are representative of each experiment out of three. Fold increase in the reactive protein band was calculated by densitometric analysis. D, E: Snail and E-cadherin expression in the stroma vs. tumor regions. Prostate cancer tissues from 28-week-old PTEN-KO mice with advanced cancer stage was used to conduct IHC staining using Snail and E-cadherin antibody at a dilution of 1:200 in a minimum of 5 paraffin-embedded sections each. Positively stained cells were identified using a light microscope in 10 high-power fields. Stromal and tumor regions were differentiated histologically using H&E stained sections to identify the regions that specifically express Snail and/or E-cadherin. Box plots represent mean±SD. **Significant difference at p<0.001.

Based on the endogenous expression level of PGE₂, EP4, *Snail* and E-cadherin, we further investigated the mechanistic aspects of EP4 antagonism in EMT.

PGE₂-induced EP4 and Snail activation in AR-independent prostate cancer cells. It is known from previous studies that overexpression of COX2 is associated with an increase in the accumulation of PGE₂ in prostate cancer tissues (21, 31). To determine whether the PGE₂-mediated EP4 activation in adenocarcinoma results in a subsequent downstream effect on triggering EMT pathways specifically promoting Snail signaling, we used PC-3 cells that are depleted for COX2 or EP4 by specific siRNAs. Evidence from transient transfection assays followed by western blot analysis showed no change in the expression of either EP2 or EP4 in COX2 knock-down cells (Figure 2A). However, PC-3 cells transfected with EP4-siRNA exhibited a significant decrease in the expression (>5-fold) of both p-Akt and Snail (Figure 2B). These findings suggest a potential interaction between activated EP4, p-Akt, and Snail that may be independent of COX2 but may require EP4 and Akt for *Snail* expression.

Effect of EP4 antagonist on Snail promoter activity in AR-dependent and -independent prostate cancer cells. We determined the specific effect of EP4 antagonist on *Snail* promoter activity by transiently transfecting PC-3, LNCaP and CaP8 cells with *Snail* promoter-driven reporter construct. To determine the pharmacological effect of EP4, the transfected cells were exposed to an established antagonist for EP4 (AH23848) and/or EP2 (AH6809). We found a significant decrease in *Snail* promoter activity by EP4 antagonist in contrast to a weak effect induced by EP2 antagonist (Figure 3A).

Snail activation mediated by EP4 and/or TGF- β is suppressed by EP4 antagonist. Although the major cellular events of EMT are known to be attributed to the influence of TGF- β on Snail activity (19, 32), we extended our studies to determine whether EP4 agonist may synergistically influence Snail activity in the presence of TGF- β . To determine this, cells were stimulated with TGF- β (5 ng) for 24 h followed by incubation with an EP4 agonist PGE1-OH (1.0 μ mol/l) for 48 h and was examined for Snail expression at the mRNA level. While we observed a ~3-fold increase in Snail expression in the presence of TGF- β , this increase was significantly reduced in cells treated with EP4 antagonist. Subsequently, cells transfected with siRNA for EP4 for 48 h followed by incubation with TGF- β showed a similar trend in Snail expression, suggesting the requirement of EP4 for Snail transcription. These findings suggest a synergistic activation of *Snail* by EP4 under the conditions of an exogenous source of TGF- β (Figure 3B). These results were further confirmed in all three prostate cancer cells by

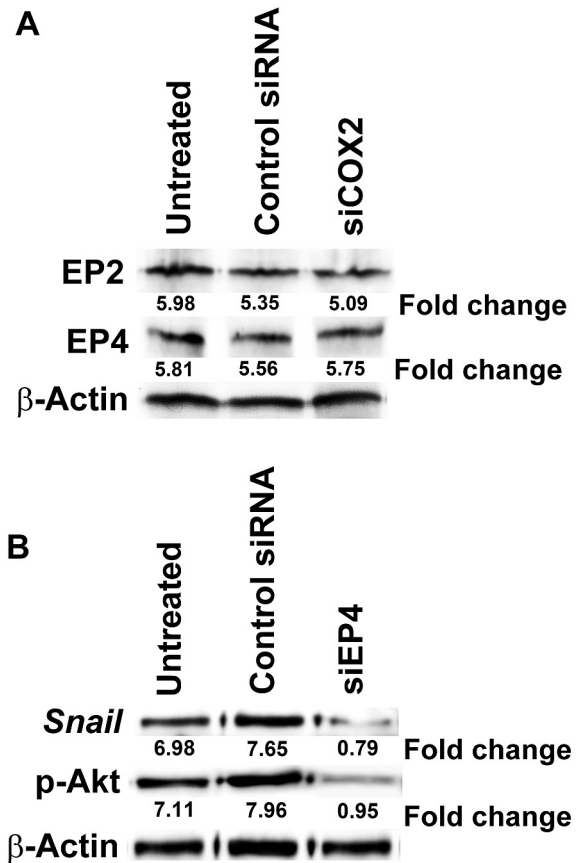


Figure 2. PGE₂-induced EP4, p-Akt and Snail expression. PC-3 cells were pre-treated with PGE₂ (0.5 μ M) for 1 h to stimulate EP4. Subsequently, cells were transiently transfected with 30 nmol of COX2 siRNA (A), or EP4 siRNA (B) for 24 h. For controls, cells were treated with DMSO (Untreated) and/or control siRNA (Scrambled). Protein lysates (10 μ g/lane) from transfected cells were used for Western blot analysis to detect EP2, EP4, p-Akt(ser-473) and Snail protein. Fold changes in the reactive protein bands were calculated based on pixel/densitometric analysis.

immunofluorescence detection of Snail. As shown in Figure 3C and D, there was a significant decrease in the nuclear signal for Snail in CaP8 cells treated with antagonist for EP4 compared to the control. A similar trend was observed in LNCaP and PC-3 cells by immunofluorescence detection.

Influence of EP4 on the transcription of EMT markers. To investigate the influence of EP4 signaling on the markers of EMT which may facilitate a more aggressive phenotype of prostate cancer, we measured the changes expression of vimentin, and fibronectin in PC-3 cells that are treated with EP4 antagonist but stimulated with PGE₂ as described earlier. Results from RT-PCR analysis showed >3-fold decrease in vimentin, fibronectin and Snail in comparison to

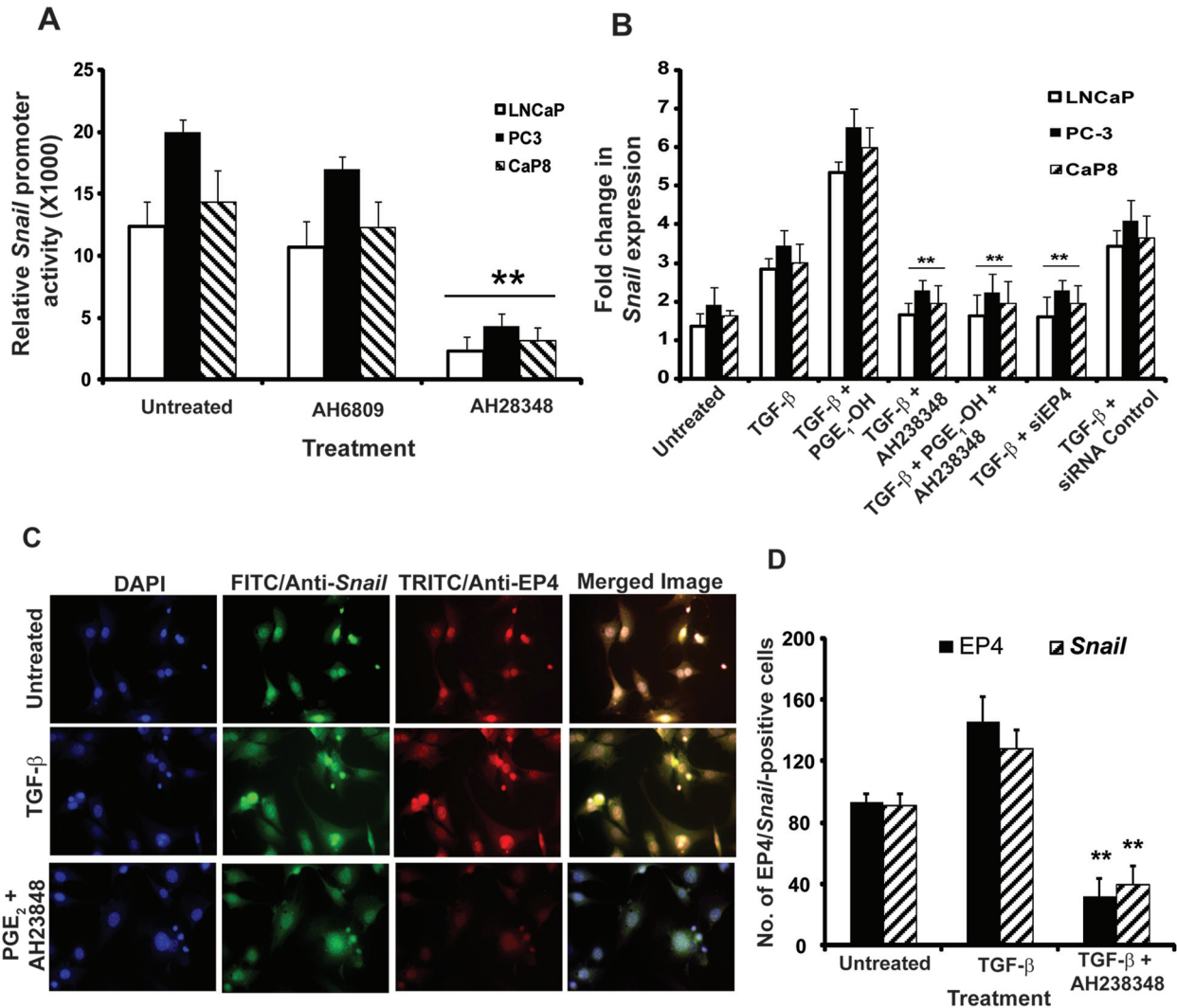


Figure 3. A: EP4 antagonist suppresses Snail promoter activity. Prostate cancer cells, PC-3 and CaP8 and LNCaP, seeded in 6-well plates were co-transfected with 1.5 μ g of Snail promoter construct ligated to luciferase. After transfection, cells were treated with DMSO or 5 μ M each of EP4 antagonist AH23848 or EP2 antagonist AH6809. Reporter activities were measured in the cellular extract after 6 h. The data reported are the relative luciferase activity from three independent experiments. Bar represents mean \pm SD. **Significant difference at $p < 0.01$. B: Synergy in the activation of Snail by EP4 and TGF- β . Human PC-3, LNCaP and CaP8 cells were stimulated with TGF- β (5 ng) for 24 h followed by incubation with an EP4 agonist PGE₁-OH for 48 h for stimulation. To determine the effect of EP4 antagonist AH23848 and/or siEP4 on Snail transcription, total RNA (5 μ g) isolated from the treated cells was used for qRT-PCR using specific primers for Snail. Fold change in Snail expression was determined as described in the Materials and Methods. Amplification of GAPDH was used as the internal control. Experiments were repeated three times. Bar represents mean \pm SD. **Significant difference at $p < 0.001$. C: Effect of EP4 antagonist on Snail expression determined by immunofluorescence. Prostate cancer CaP8 cells of PTEN-KO mice grown in 4-well chamber slides were pre-treated with TGF- β followed by treatment with AH23848 as described. Immunofluorescence detection was performed in CaP8 cells using tetramethyl rhodamine isothiocyanate (TRITC)-conjugated antibody for EP4 (Red) and fluorescein isothiocyanate (FITC)-conjugated antibody for Snail (Green) (1:200 dilution each). Co-staining with DAPI was used for nuclear localization. Specific inhibitory effect of EP4 antagonist on Snail at the cellular level is shown in the merged panel. A similar trend was observed in androgen-dependent LNCaP cells determined by immunofluorescence staining. D: Bar graph represents mean \pm SD. **Significant difference at $p < 0.01$.

the untreated control. Our findings, however, also revealed a significant increase in the E-cadherin signal. A similar trend was observed in LNCaP and CaP8 cells (Figure 4A). Interestingly, immunofluorescence detection of E-cadherin in

PC-3 showed a significant increase in the signal at the cell-cell contact regions (Figure 4B). The above findings suggest that loss of E-cadherin could be partially abrogated by treatment with EP4 antagonist.

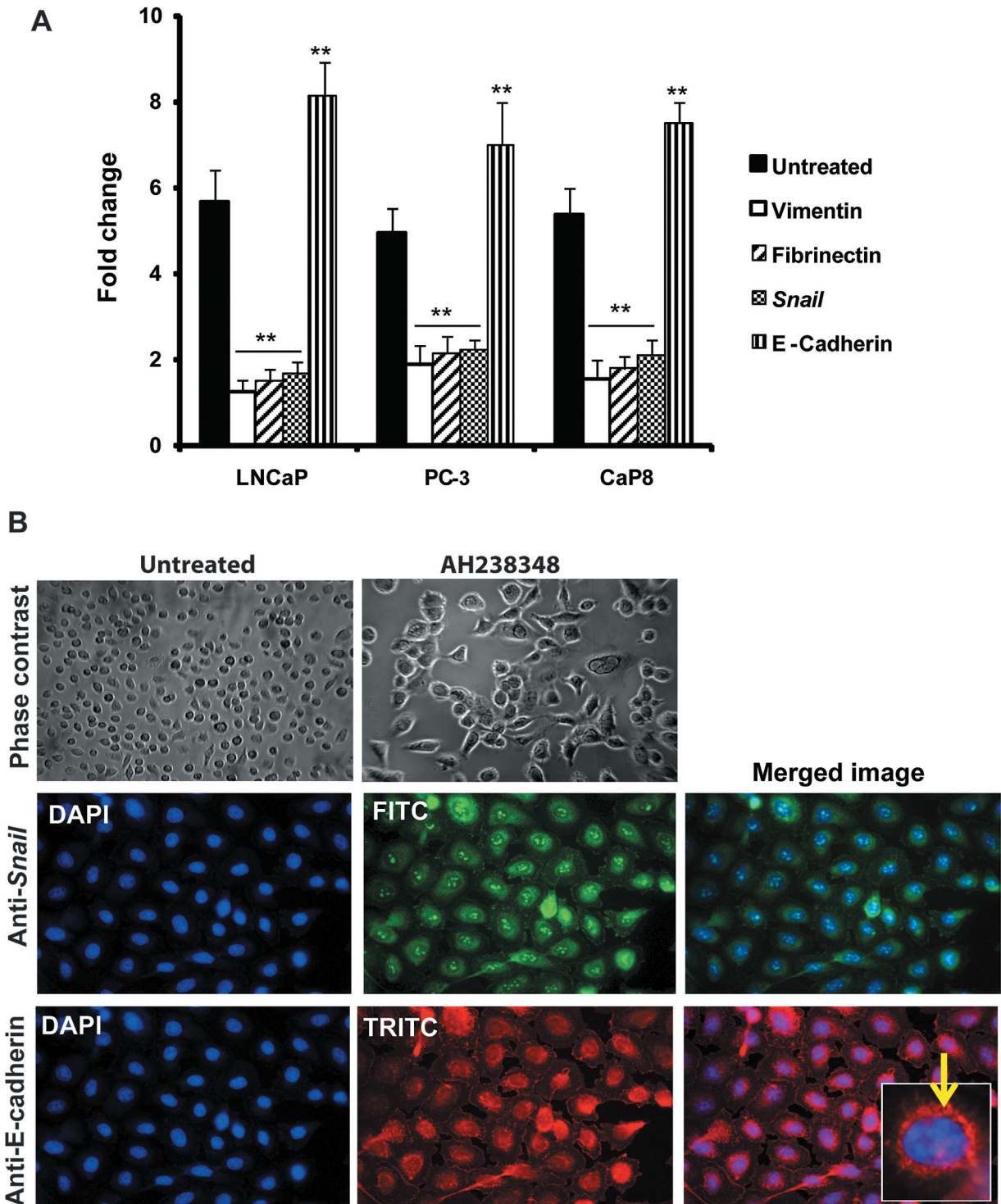


Figure 4. A: Effect of EP4 antagonist on epithelial and mesenchymal markers. PC-3 cells pretreated with PGE₂ were incubated with DMSO or EP4 antagonist AH23848 (5 μ M) for 48 h. Expression levels of vimentin, fibronectin, E-cadherin and Snail were determined by qRT-PCR analysis with specific primers using total RNA isolated from the treated cells. Amplification of Gapdh was used as the internal control. Fold changes in the expression level were calculated as described in the Materials and Methods. B: Gain of E-cadherin and loss of Snail signal in cells treated with EP4 antagonist. PC-3 cells grown in 4-well chamber slides were treated with or without AH23848 for 48 h and processed for immunofluorescence detection. E-cadherin and Snail were analyzed by double staining with TRITC (red) or FITC (Green) conjugated antibodies for Snail and E-cadherin respectively. Fluorescence signaling was captured under an Epi-fluorescence microscope at $\times 40$ magnification. Merged images indicate loss of Snail and an increase in the expression of E-cadherin at the cell-cell contact regions compared to a weak signal in the control (far-right panel; arrow: single cell magnified).

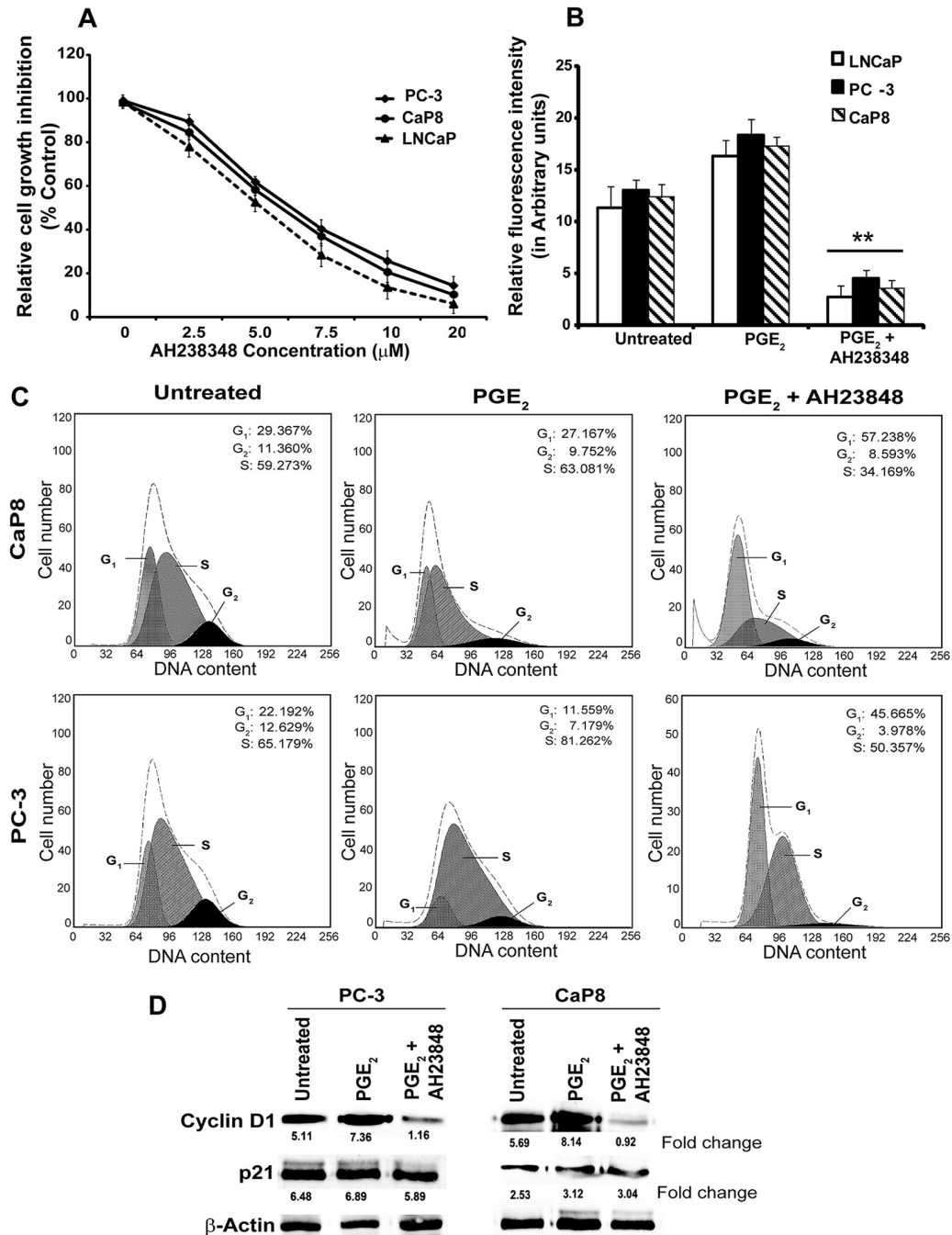


Figure 5. EP4 antagonist induces cell growth inhibition and cell-cycle arrest. A: Inhibitory effect of EP4 antagonist on human prostate cancer cell proliferation. Prostate cancer cells were grown in 96-well plates at a density of 1×10^4 per well. Cells were pre-treated with 0.5μ M of PGE₂ for 1 h, followed by treatment with AH23848 for 48 h. The data presented are from MTT assays conducted in triplicate as described in the Materials and Methods. B: Effect of EP4 antagonist on cell migration. This was determined by measuring calcein fluorescence (emission, 530 nm) in a BD Falcon FluorBlok-24-Multiwell Insert system. The rate of cell migration of PC-3 and CaP8 cell was calculated based on the total number of cells migrated under the microporous membrane specifically designed to absorb the fluorescence. The data presented are from three sets of assays including treated, control (DMSO) and blank (calcein) to determine the net fluorescence signal. Bar represents mean \pm SD. **Significant difference at $p < 0.01$. C: Effect of EP4 antagonist on the cell cycle. PC-3 and CaP8 cells were stimulated with PGE₂ followed by treatment with AH23848 for 48 h. Cell-cycle analysis for DNA content was performed as described in the Materials and Methods with PI staining for DNA content. D: Effect of EP4 antagonist on cell-cycle regulatory proteins. Protein lysate isolated from human PC-3 and murine CaP8 cells treated with PGE₂ plus 5μ M of EP4-specific antagonist AH23848 for 48 h was used for western blot analysis using specific antibodies for cyclin D1 and p21(Waf1/Cip1) as described in the Materials and Methods. Fold changes in the expression were calculated based on the densitometric analysis of the reactive protein bands.

Effects of EP4 antagonist AH23848 on prostate cancer cell growth and motility. To further investigate the influence of EP4 antagonist on prostate cancer cell proliferation, MTT assay was conducted of PC-3, LNCaP, and CaP8 cells that were stimulated with PGE₂ for 1 h before being treated with AH23848. As shown in Figure 5A, there was a dose-dependent inhibitory effect on the rate of cell proliferation by AH23848 treatment compared to the control. The rate of cell migration determined by measuring calcein fluorescence using Falcon FluoroBlok-24-Multiwell Insert system showed AH23848 to have a significant inhibitory effect on cell migration with stimulation by PGE₂ (Figure 5B).

EP4 antagonist induces cell cycle arrest in androgen-dependent and -independent prostate cancer cells. Our findings from cell cycle analysis for DNA content in PC-3, and CaP8 cells stimulated with PGE₂ followed by treatment with AH23848 for 48 h indicated cell-cycle arrest at the G₁/S transition phase (G₁: 57.23% and 45.66% respectively). This was associated with a decline in the percentage of cells in the S phase (S: 34.16% and 50.35% respectively) (Figure 5C). We also observed a similar trend in LNCaP cells, suggesting a potential impact of EP4 antagonism on DNA synthesis associated with cell-cycle arrest. To further examine the effect of EP4 antagonist on cell-cycle regulatory proteins, we examined the expression of proteins for cyclin D1 and the Cdk inhibitor p21 (Waf1/Cip1) that are associated with G₁ arrest. Western blot analysis showed a significant decrease in cyclin D1 expression, but there was no significant change in the expression of Cdk inhibitor p21 (Figure 5D). Protein lysate from LNCaP cells with similar treatments also showed a small increase in p21 expression, suggesting an impact on cell- cycle regulatory mechanisms.

Discussion

The zinc finger family of transcription repressors, including the Snail gene, is a master regulator of EMT in cancer progression and metastasis (17, 18), and is known to be activated by several factors including TGF- β and similar to mothers against decapentaplegic (SMAD) (32, 33). In spite of recent findings that support a key role for Snail in prostate cancer (34, 35), there is a lack of substantial evidence regarding the involvement of an inflammatory axis that could influence Snail through multiple mechanisms. Prostaglandin is one of the eicosanoids that are accumulated in human prostate cancer tissues, which induces a variety of inflammatory cellular responses (21). In this study, we addressed the question of whether PGE₂-induced EP4 receptor activation influences Snail activity and whether it could be altered by EP4-specific antagonists.

Recently, we have shown there to be an elevated level of p-Akt in prostate cancer tissues of PTEN-KO mice (26), which is consistent with our current observation; in addition we also observed an elevated level of serum PGE₂, EP4 and Snail protein. Together, these findings clearly suggest the influence of PGE₂/EP4 and p-Akt on Snail activation in prostate cancer partly due to loss of *PTEN*. Findings from cell-based assays with EP4-siRNAs suggest that Snail activation could occur *via* PGE₂/EP4, and independently of COX2. It is also evident that EP4 antagonist can suppress Snail signaling in prostate cancer cells both at the mRNA and protein level irrespective of their androgen receptor (AR) dependence. Our findings are consistent with a report by Terada *et al.* (36) in a castration resistant xenograft model for prostate cancer in that EP4 overexpression *via* AR activation could be one of the mechanisms in castration-resistant prostate cancer progression. Furthermore, our findings on the loss of E-cadherin and gain in Snail in the prostate tissue are consistent with an earlier observation in which *Snail* acts as survival factor and influences specific aspects of EMT in prostate cancer (34) *via* stromal - epithelial interactions mediated through PI3k/Akt axis in models that exhibit loss of *PTEN*. Since Akt is also one of the master regulators of prostate cancer progression and metastasis in *PTEN*-depleted tumors (37-39), our overall findings further support a close interaction between EP4, p-Akt and Snail activity. Although not much information is yet available on a synergistic activation of Snail by PGE₂/EP4 and TGF- β , our data reveal that specific inhibitor of EP4 was able to suppress Snail activated by both PGE₂/EP4 and TGF- β in prostate cancer cells. An increased rate of cell proliferation and migration stimulated with PGE₂ is consistent with a recent report of Snail-induced EMT conversion with enhanced migratory and proliferative potential (40). It is also evident that prostate cancer cells that express Snail protein are more motile than cells that overexpress E-cadherin, as reported in other studies (41). Thus, our findings on the pharmacological effect of EP4 antagonist on EMT markers, cell migration, cell cycle and its regulatory proteins support a potential role of EP4 as a therapeutic target (42), and thus metastatic prostate cancer progression might be prevented by blocking EMT processes mediated through the inflammatory axis involving PGE₂/EP4, p-Akt and Snail.

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