c-Jun and FOXO1 mediate the expression of oncogenic PKC-ι in human prostate cancer cells with an interplay between NF-κB, IL-8 and ICAM-1

WISHRAWANA S. RATNAYAKE, CHRISTOPHER A. APOSTOLATOS, SLOAN BREEDY and MILDRED ACEVEDO-DUNCAN

Department of Chemistry, University of South Florida, Tampa, FL 33620, USA

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Abstract. Aggressive and metastatic prostate cancers are among the leading causes of fatality in men. Prior observations by the authors regarding atypical protein kinase C isoforms (aPKCs) in relation to prostate cancers demonstrated elevated levels of PKC-iota (PKC-ι) in patient samples compared to non-malignant prostate tissues. This indicates that PKC-ι is a potential biomarker for initiating and maintaining prostate carcinogenesis. In addition, PKC-ι is an oncogene that encourages the activation of the nuclear factor (NF)-κB, assisting carcinogenesis. The specific inhibition of PKC-ι de-regulated the expression of both PKC-ι and its phosphorylation; thus, PKC-ι functionally controls its own expression in prostate carcinoma. The present study aimed to investigate the underlying mechanisms of PRKCI gene transcriptional regulation in prostate carcinoma cells. Forkhead box protein O1 (FOXO1) and c-Jun, along with several other transcription factors that exhibited potential to bind on or near the promoter region of the PRKCI were identified. Each transcription factor was systematically silenced. The decrease in the expression levels of FOXO1 and c-Jun significantly affected PKC-ι expression. The decrease in FOXO1 expression by siRNA enhanced PKC-ι expression by 33% ($P \leq 0.05$) and 9% ($P \leq 0.05$) in the PC-3 and DU-145 cells, respectively. The diminution of c-Jun expression by siRNA diminished PKC-ι expression by 42% ($P \leq 0.05$) and 24% ($P \leq 0.05$) in the PC-3 and DU-145 cells, respectively. According to the results of the present study, c-Jun and FOXO1 are the two major transcription factors involved in PKC-ι expression in prostate cells. PKC-ι and its phosphorylation improved due to FOXO1 diminution, and vice versa for c-Jun silencing, indicating that c-Jun upregulates PRKCI expression, while FOXO1 negatively affects its expression. This was mediated through signal transducer and activator of transcription (STAT)3/5 and NF-κB. An upregulation in the expression of intercellular adhesion molecule 1 (ICAM-1) and interleukin (IL)-8 was observed as a result of PKC-ι specific inhibition. PKC-ι inhibition thus promotes ICAM-1/FOXO1 signaling and downregulates IL-8/JNK/c-Jun signaling, indicating that PKC-ι inhibition subdues its production mechanism. Overall, an analysis of the results led us to suggest that PKC-ι inhibition downregulates its own oncogenic signaling, while the induction of anti-tumor signaling pathways strongly suggests that PKC-ι related molecular mechanisms provide a novel therapeutic route for mitigating prostate cancer.

Introduction

The National Cancer Institute of the United States estimated that approximately 175,000 new prostate cancer cases will be identified in 2020, while there will be a projected 32,000 cancer-related deaths among American males. Approximately 14% of men will acquire the disease throughout their lifetimes (1). To date, radiation therapy, chemotherapy and hormone therapy are the most common treatments for PC. Radiation therapy is not ideal as it destroys surrounding healthy cells and tissues, leading to a number of side-effects. Hormone treatment implies clinical castration and the usage of anti-androgens, that can have adverse effects and can affect the lifestyle of the recipient (2). Moreover, PC treatments involve the use of chemotherapeutic agents, such as docetaxel. Docetaxel only increases the survival time of the patient by approximately 100 days, primarily due to the emergence of resistance (3).

In previous studies by the authors, protein kinase C-ι (PKC-ι) was recognized as a major driving factor of prostate and melanoma carcinogenesis, and was therefore proposed as a potential and novel therapeutic target (4-10). PKC is a part of the protein kinase enzyme class that post-translationally modifies certain other proteins and participates in a variety of cellular signaling cascades. A total of 15 PKC isoforms are recognized in humans; these are further grouped as classical, novel and atypical PKCs (aPKCs). aPKCs comprise two structurally and functionally distinctive isoforms; PKC-ζ and

Correspondence to: Dr Mildred Acevedo-Duncan, Department of Chemistry, University of South Florida, 4202 E Fowler Ave., CHE 205, Tampa, FL 33620, USA
E-mail: macevedo@usf.edu

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PKC-ι (11-13). Other than PC and melanoma, PKC-ι has been found to function as an oncogene in several other cancer types, such as neuroblastoma, ovarian cancer and glioma, where the upregulation of PKC-ι expression has been shown to be associated with a low survival rate (10,11,14). In previous studies by the authors, it was also reported that higher levels of PKC-ι were observed in DU-145 and PC-3 cells compared to undetectable levels in normal tissues and the normal prostate epithelial cell line, RWPE-1 (7,10). In addition, it was demonstrated that PKC-ι phosphorylates to activate IκB kinase (IKKα/β). This p-IKKα/β activation triggers the dissociation of nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor (IκB) from the nuclear factor NF-κB complex, which leads to the ubiquitination of IκB. IκB releasing from NF-κB activates the translocation of NF-κB to the nucleus. Previous studies by the authors suggested that PKC-ι specific leads to the suppression of NF-κB nuclei translocation, thereby causing a downregulation of NF-κB activity (9,10). These findings indicated that the inhibition of PKC-ι not only impaired pathways regulated by PKC-ι, but also downgraded its protein expression (7-10). There are data to suggest that PKC-ι maintains a self-propagative mechanism, as observed in certain other cancer-related cycles, such as the transformation of the growth factor (TGF)-β and CD147 (15). Since transcription factors play a pivotal role in gene expression, the aim of the present study was to determine which transcription factors were key PKC-ι regulators, as well as which pathways were integral components for these transcription factors.

In the present study, the outcomes of the knockdown of the expression of c-Jun, FOXO1, PKC-ι and NF-κB are demonstrated, giving emphasis to pathways associated with PKC-ι. The findings suggested that c-Jun is a crucial transcriptional activator, while FOXO1 functions as a transcriptional suppressor of PRKCI expression. The roles performed by these transcription factors were determined in an inflammatory process that promotes PKC-ι and is dependent on PKC-ι for the continuation of the process. Moreover, the pathway through which cytokines stimulate PKC-ι expression to release activated NF-κB is demonstrated, which leads to the production of additional cytokines; this process is used in certain types of cancer as part of a loop through which to grow and propagate. In addition, IL-8 promotes c-Jun and NF-κB signaling to enhance PKC-ι production. On the other hand, PKC-ι inhibition induces the production of ICAM-1, which promotes FOXO1 to reduce PKC-ι production. In general, these findings indicate that with a dynamic and closely controlled expression profile, PKC-ι plays a central role in the development of prostate cancer. The specific inhibition of PKC-ι may interact with its own regulatory process, contributing to a distortion of its oncogenic function in prostate cancer.

Materials and methods

Materials and reagents. [4-(5-Amino-4-carbamoylimidazol-1-yl)-2,3-dihydroxycyclopentyl] methyl dihydrogen phosphate (ICA-1T) was purchased by Therachem and the NF-κB-specific inhibitor, 4-methyl-N1-(3-phenylpropyl)-1,2-benzenediamine (JSH-23, J4455), was purchased from Sigma-Aldrich; Merck KGaA. Sterile distilled water was used as the solvent for the inhibitors. The following materials were acquired: Antibodies against PKC-ι (610175, BD Biosciences); NF-κB p65 (sc-372-G, Santa Cruz Biotechnology, Inc.); p-PKC-ι (T555; ab5813, Abcam); FOXO1 (2880S), p-FOXO1 (T24; 9464S), c-Jun (9165S), p-c-Jun (S73; 3270S), mammalian target of rapamycin (mTOR; 2972S), p-AKT (S473; 4059S), signal transducer and activator of transcription (STAT)3 (9139S) and STAT5 (25656S) (all from Cell Signaling Technology, Inc.); and β-actin (A3854, Sigma-Aldrich; Merck KGaA); enhanced chemiluminescence solution (34080, Pierce; Thermo Fisher Scientific, Inc.); human small interfering RNA (siRNA) for PKC-ι (SR303741), c-Jun (SR302499), FOXO1 (SR301618), early growth response 1 (EGR1; SR301358), paired box gene 3 (PAX3; SR303360), interferon regulatory factor 9 (IRF9; SR307030), NF-κB p65 (SR321602) (all from Origene Technologies, Inc.); DPBS without magnesium and calcium ions (Dulbecco's phosphate-buffered saline, D8537), Trypsin-ethylenediaminetetraacetic acid (EDTA; T4049, Sigma-Aldrich; Merck KGaA); recombinant protein tumor necrosis factor (TNF)-α (human, 10602HNAE25, Thermo Fisher Scientific, Inc.).

Cells and cell culture. DU-145 (ATCC® HTB81™) and PC-3 (ATCC® CRL-1435™) cells were obtained from the American Type Tissue Culture Collection (ATCC). All cell lines were authenticated by ATCC using karyotyping, morphology and PCR-based approaches. Early passages of cells were cryo-preserved in liquid nitrogen and cells of early passages were resuscitated from liquid nitrogen for experiments. A temperature of 37˚C and 5% CO_2 were maintained as the cell culture conditions. EMEM (ATCC 30-2003) and RPMI-1640 media (ATCC 30-2001) were used with fetal bovine serum (FBS, 10% v/v) and penicillin (5 μg/ml) for the DU-145 and PC-3 cells, respectively.

Identification of c-Jun and FOXO1 as probable transcription factors to bind to the PRKCI promoter region. The gene sequence of PRKCI which is located on chromosome 3 between bp170222365-170305981 (3q26.2) was acquired from ensemble.org (ENSG00000163558) (16,17). The promoter sequence was identified by comparing the forward strand sequence with the Eukaryotic Promoter Database (EPD, https://epd.vital-it.ch/index.php). The sequence between bp170220768-170225128 was selected and which contained the promoter, promoter flank, enhancer and a motif feature. Transcription factors that exhibited a probability to bind with an accuracy >90% were selected using PROMO; a virtual laboratory for reviewing transcription factor binding sites in DNA selected sequences (http://alggen.lsi.upc.es/). The PROMO results were also compared with the Genomatix Matinspector results to confirm the accuracy.

Knockdown of c-Jun, FOXO1, PKC-ι and NF-κB gene expression by siRNA. The PC-3 and DU-145 ($1x10^5$) cells were seeded in T25 flasks and at 24 h post-seeding, siRNA (30 nM) transfections were conducted against scrambled siRNA for 2 days using 'siTran' siRNA transfection reagent (TT300002) from Origene Technologies, Inc. according to the manufacturer's recommended ratios. The cell pellets were collected at the end of the 48-h incubation period and cell lysates were prepared using cell lysis buffer (C7027, Thermo Fisher Scientific, Inc.).

Western blot and densitometric analyses were executed as previously described by Ratnayake *et al* (9,18).

Prostate cancer cellular cytokine expression analysis. The cytokine array kit (ARY005B, R&D Systems) contained with an enzyme-linked immunosorbent assay (ELISA) was used for the experiment. Approximately $1x10^5$ cells were cultured in T25 flasks (PC-3 and DU-145) and at 24 h post-plating, the cells were treated with a ICA-1T (2.5 μM) for 2 consecutive days at 24-h intervals. The cells were then collected and cell lysates were prepared and administered according to the manufacturer's instructions. The experiment was repeated and TNF-α (250 ng/ml) was added to the flasks 30 min prior to the harvesting point. Total protein (150 μg) was used from each sample and introduced to the immunoblots provided and chemiluminescence images were acquired using 'ECL Western Blotting Substrate' (Thermo Fischer Scientific, PI32106). These images were then analyzed as instructed in the cytokine array kit manual (ARY005B).

Immunopaired antibody detection assay. Approximately $1x10^5$ cells (PC-3 and DU-145) were cultured in T25 flasks and ICA-1T (2.5 μM) treatments were conducted as descirbed above. Cells were then collected and cell lysates was prepared to contain the final total protein concentration >2 μg/ml. Samples were sent to ActivSignal, LLC for analysis. The ActivSignal IPAD assay is a multiplex ELISA-based proprietary tool for evaluating multiple signaling cascades considering both upstream and downstream targets. In total, >20 signaling pathways were examined at once in a single well by assessing the expression or protein phosphorylation of 70 human proteins targets.

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was performed on RNA isolated from PC-3 and DU-145 cell lysates collected following ICA-1T (with or without TNF-α) or siRNA treatments (as described in above) against their respective controls. The detailed procedure was previously described by Ratnayake *et al* (18). Total RNA was isolated from the cell pellets using RNA lysis buffer (RNeasy mini kit, 74104) from Qiagen, Inc. RNA was reverse transcribed into cDNA with You-Prime First Strand Beads (27-9264-01) form GE Healthcare. qPCR was performed on cDNA using the QuantStudio3 Real-Time PCR system (Thermo Fisher Scientific, Inc.). The following primers were used: PKC-ι forward, TTGCAATGAGGTTCGAGACA and reverse, CTG AGATGATACTGTACACGGG; c-Jun forward, GTGCCG AAAAAGGAAGCTGG and reverse, CTGCGTTAGCAT GAGTTGGC; FOXO1 forward, ATGGCTTGGTGTCTTTCT TTTCT and reverse, TGTGGCTGACAAGACTTAACTCAA; IL-8 forward, CAGAGACAGCAGAGCACAC and reverse, ATCAGGAAGGCTGCCAAGAG; ICAM-1 forward, GGG AACAACCGGAAGGTGTA and reverse, CAGTTCCACCCG TTCTGGAG; and β-actin forward, AGAGCTACGAGCTGC CTGAC and reverse, AGCACTGTGTTGGCGTACAG which was used as an internal control. PCR reactions conditions were used as explained by Livak and Schmittgen (19). PCR reactions used SYBR-Green PCR Mix (Applied Biosystems). cDNA was denatured at 95˚C for 10 min, followed by 40 cycles of denaturing at 95˚C for 20 sec and an annealing stage of 65˚C for 40 sec. QuantStudio Software 2.0 was used to quantify gene expression by the $2^{-\Delta\Delta Cq}$ method (Thermo Fisher Scientific, Inc.).

Statistical analysis. All data are presented as the means ± SD. Statistical analysis was carried out using one or two-way ANOVA followed by Tukey's HSD test as multiple comparisons tests using the statistical research online tool 'VassarStats'. P-values ≤0.05 or ≤0.01 were considered to indicate statistically significant or highly statistically significant differences, respectively.

Results

The unique PRKCI sequence, carefully selected to contain the promoter, promoter flank, enhancer and a motif element, was 4,360 bp in length (chr3; bp170220768-170225128). The promoter allowed TFs to bind and start transcription, while the enhancer ensured a regulating area on the flank that promoted transcription factor binding. By having only transcription factors, which bind within a dissimilarity range of approximately 10%, potential hits were narrowed down to achieve a high specificity. After analyzing the results, approximately 70 transcription factor hits to the target were obtained. c-Jun, ISGF3, PAX3, EGR1 and FOXO1 were identified as the top 5 transcription factors with the greatest likelihood of binding to the PRKCI gene sequence.

c-Jun and FOXO1 are the two key TFs of PKC-ι expression in PC-3 and DU-145 cells. As presented in Fig. 1, the results of western blot analysis revealed that each siRNA transfection targeting FOXO1 and c-Jun markedly diminished the expression levels of those targets. siRNA against FOXO1 significantly knocked down FOXO1 by 64% (P≤0.05) and 27% (P≤0.05), while p-FOXO1 (T24) by 19% (P≤0.05) and 79% (P≤0.05) in the PC-3 and DU-145 cells, respectively. siRNA against c-Jun knocked down c-Jun by 48% (P≤0.05) and 73% (P≤0.05), while p-c-Jun (S73) by 26% (P≤0.05) and 43% (P≤0.05) in the PC-3 and DU-145 cells, respectively. These findings indicated that transfection with siRNA knocked down the respective target expression. Only the knockdown of c-Jun and FOXO1 in both cell lines was shown to have an affect on PKC-ι levels. The diminution of FOXO1 by siRNA increased total PKC-ι expression by 33% (P≤0.05) and 9% (P≤0.05) in the PC-3 and DU-145 cells, respectively. The diminution of c-Jun by siRNA diminished PKC-ι expression by 42% (P≤0.05) and 24% (P≤0.05) in the PC-3 and DU-145 cells, respectively. Similarly, lower levels of FOXO1 due to transfection with siRNA augmented p-PKC-ι (T555) expression by 18% (P≤0.05) and 22% (P≤0.05) in the PC-3 and DU-145 cells, respectively. Of note, c-Jun diminution decreased PKC-ι (T555) expression by 36% (P≤0.05) and 13% (P≤0.05) in the PC-3 and DU-145 cells, respectively. The knockdown of the expression of ISGF3, EGR1 and PAX3 did not exert notable effect on PKC-ι expression or on its phosphorylated protein levels; thus, these data were not included in this manuscript. Hence, FOXO1 and c-Jun were selected for use in the following experiments.

In addition to the total and p-PKC-ι levels, the levels of mTOR, STAT3, STAT5, NF-κB p65 and p-AKT (S473) were determined following transfection with c-Jun and FOXO1 siRNA. Transfection with FOXO1 siRNA increased the

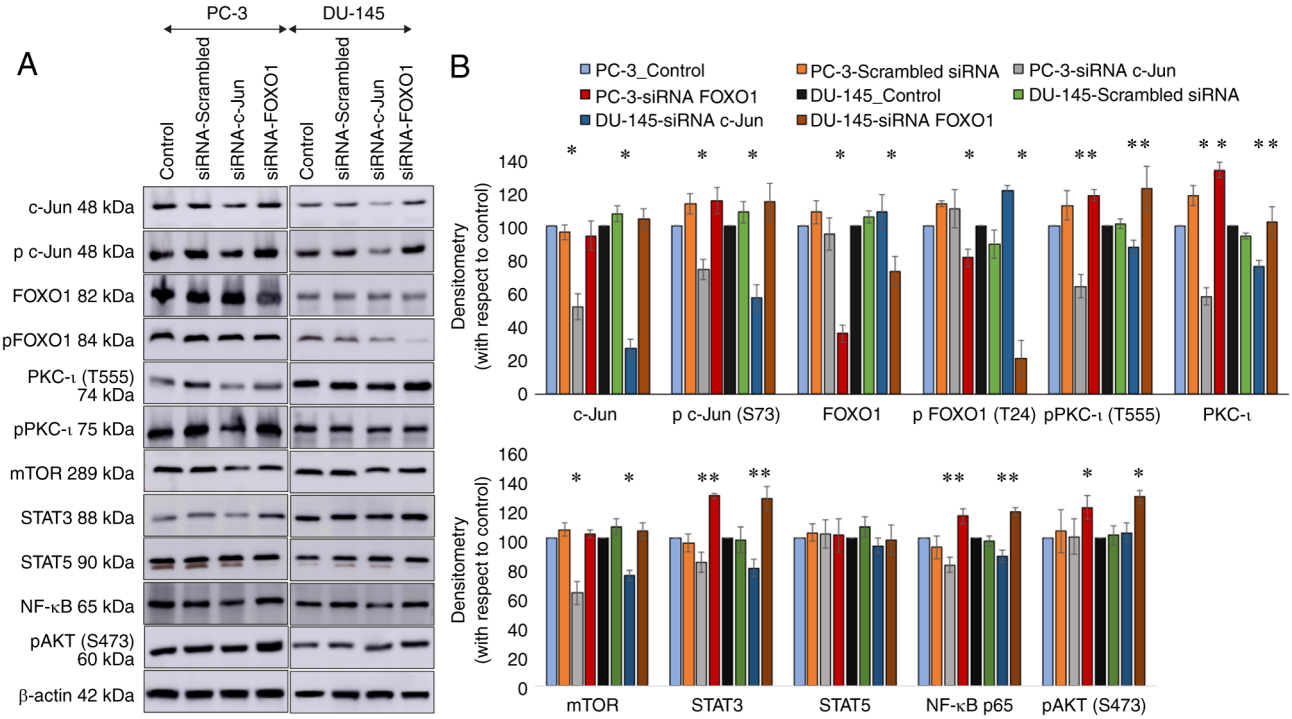

Figure 1. Effect of RNA interference (siRNA) of the transcription factors, c-Jun and FOXO1, in two prostate cancer cell lines (PC-3 and DU-145). (A) Expression of the protein levels of phosphor-PKC-ι (T555), total PKC-ι, c-Jun, phosphor-c-Jun (S73), FOXO1, phosphor-FOXO1 (T24), mTOR, STAT3, STAT5, NF-κB p65 and phosphor-AKT (S473) following the siRNA knockdown of the expression FOXO1 and c-Jun for PC-3 and DU-145 cell lines. Total protein (80 μg) was loaded into each well and β-actin was used as the internal control in each western blot. (B) Representative densitometry values for the western blots shown in (A) Experiments (n=3) were performed in each trial and representative bands are shown. Densitometry values are reported as the means ± SD. Statistical significance is indicated by an asterisk (*P≤0.05).

expression of STAT3 by 29% (P≤0.05) and 27% (P≤0.05) in the PC-3 and DU-145 cells, respectively. On the other hand, transfection with c-Jun siRNA reduced STAT3 expression by 16% (P≤0.05) and 20% (P≤0.05) in PC-3 and DU-145 cells, respectively. Of note, transfection with FOXO1 and c-Jun siRNA did not exert a significant effect on STAT5 expression. The knockdown of c-Jun by siRNA deceased mTOR expression by 37% (P≤0.05) and 25% (P≤0.05) in the PC-3 and DU-145 cells, respectively. FOXO1 diminution did not alter the protein levels of mTOR. Transfection with FOXO1 siRNA increased the expression of p-AKT (S473) by 21% (P≤0.05) and 28% (P≤0.05) in the PC-3 and DU-145 cells, respectively. Transfection with c-Jun siRNA did not exert a significant effect on the levels of p-AKT (S473). Transfection with FOXO1 siRNA increased the expression of NF-κB p65 by 15% (P≤0.05) and 18% (P≤0.05) in the PC-3 and DU-145 cells, respectively. On the other hand, the knockdown of c-Jun significantly deceased NF-κB p65 expression by 18% (P≤0.05) and 12% (P≤0.05) in the PC-3 and DU-145 cells, respectively (Fig. 1).

c-Jun and FOXO1 regulate atypical PKC-ι expression through NF-κB and STAT3 signaling in prostate cancer cells. As presented in Fig. 2, the findings of western blot analysis revealed that the knockdown of NF-κB expression by siRNA substantially increased the overall PKC-ι levels by 24 % (P≤0.05) and 17% (P≤0.05) in the PC-3 and DU-145 cells, respectively. The expression of p-PKC-ι (T555) was not significantly altered. Of note, the FOXO1 and p-FOXO1 levels were not affected as a result of NF-κB depletion. Notably, the p-c-Jun (S73) level was significantly decreased upon NF-κB depletion. The total c-Jun levels were not affected as a result of NF-κB depletion. Similar outcomes were acquired with JSH-23 (100 nM) treatments. JSH-23 is an established NF-κB specific inhibition available on the market.

In addition, Fig. 3 demonstrates the effects of PKC-ι knockdown or specific inhibition using ICA-1T on total PKC-ι, p-PKC-ι (T555), FOXO1, p-FOXO1, p-AKT (S473), STAT3, c-Jun and p-c-Jun (S73) expression. Transfection siRNA against PKC-ι and ICA-1T treatment yielded similar results. The total PKC-ι and p-PKC-ι levels decreased significantly (P≤0.05) with PKC-ι knockdown or inhibition. Of note, upon the depletion of PKC-ι, the total FOXO1 levels increased (P≤0.05), while the levels of p-FOXO1 decreased, indicating an upregulation of FOXO1 activity. Similarly, both the p-AKT (S473) and STAT3 levels significantly (P≤0.05) decreased owing to the decrease in PKC-ι expression. On the other hand, the levels of c-Jun or p-c-Jun were not substantially altered following the depletion of PKC-ι.

ELISA suggests the involvement of multiple pathways; JNK/c-Jun, NF-κB/AKT/FOXO1 and STAT3 for the regulation PKC-ι of expression. The specific inhibitor, ICA-1T, was to inhibit PKC-ι, permitting us to gain a clearer view of the mechanisms through which multiple cellular signaling pathways may affect PC-3 and DU-145 cells *in vitro* owing to PKC-ι regulation. The IPAD assay is an ELISA series, which enables several proteins to be identified simultaneously.

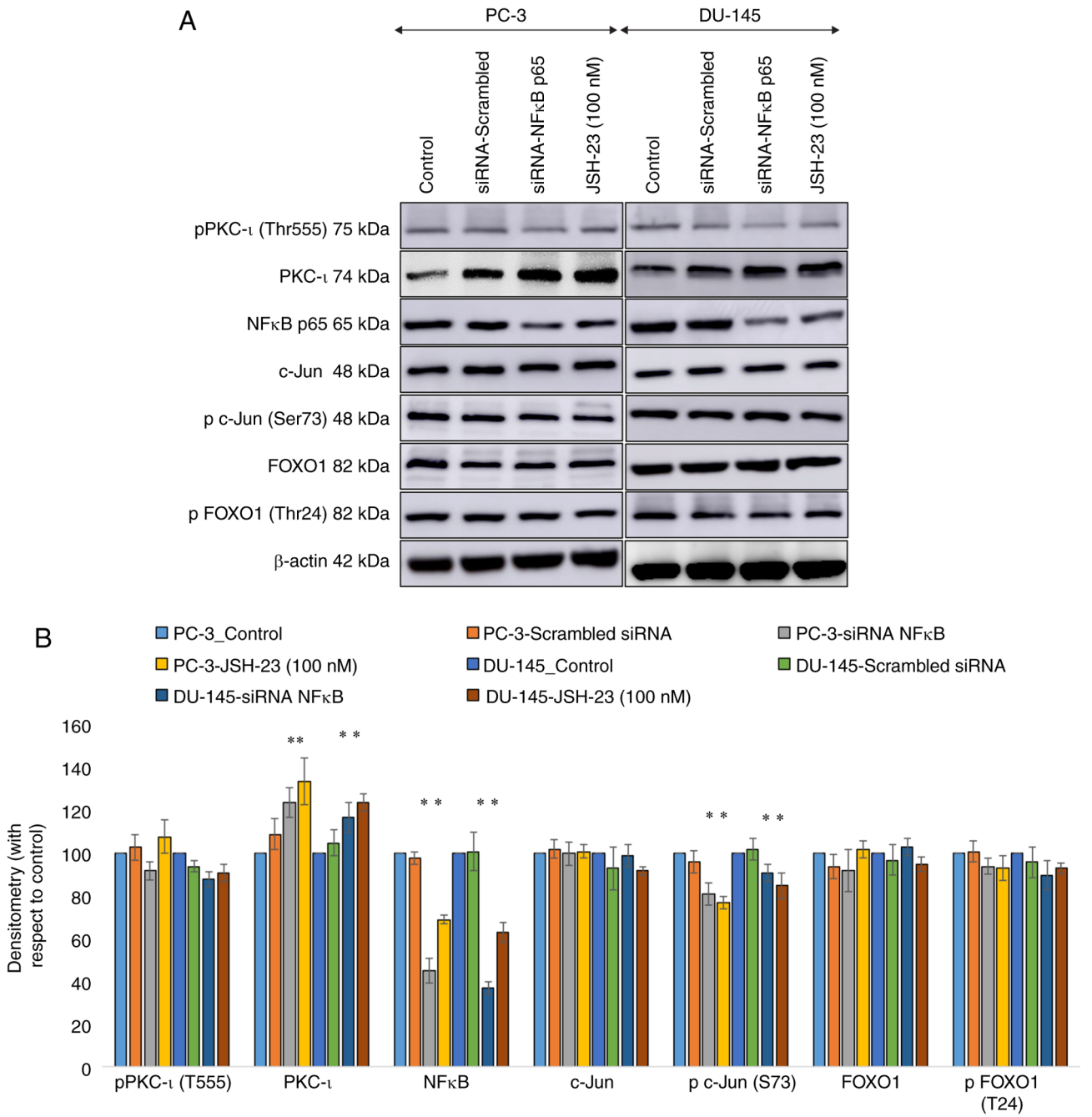

Figure 2. Effect of siRNA of the transcription factor, NF-κB, and the NF-κB inhibitor, JSH-23, on the expression of PKC-ι and targeted transcription factors in PC-3 and DU-145 cells. (A) Protein levels of phosphor-PKC-ι (T555), total PKC-ι, NF-κB p65, c-Jun, phosphor-c-Jun (S73), FOXO1 and phosphor-FOXO1 (T24) following the siRNA knockdown of the expression of NF-κB and JSH-23 treatments for PC-3 and DU-145 cell lines. Total protein (80 μg) was loaded into each well and β-actin was used as the internal control in each western blot. (B) Representative densitometry values for the western blots shown in (A) Experiments (n=3) were performed in each trial and representative bands are shown. Densitometry values are reported as the means ± SD. Statistical significance is indicated by an asterisk (*P≤0.05).

Fig. 4 demonstrates the changes in the expression of CD44, E-cadherin, caspase-3, H2AX, IκB and Myc, and the degree pf phosphorylation of p-4E-BP1 (T37/46), p-AKT (S473), p-β-catenin (S33/37), p-HER3 (Y1289), pIKKαβ (S176/180), p-JNK (T183), p-mTOR (S2448), p-NF-κB p65 (S536), p-Met1, p-STAT3 (Y705), p-STAT5 (Y694) and p-ZAP70 (Y493), as a result of ICA-1T inhibition against the respective control samples for both PC-3 and DU-145 cell lines. The present study observed that the levels of p-JNK (T183) p-mTOR (S2448) and p-ZAP70 (Y493) significantly increased in DU-145 cells following PKC-ι inhibition, while those of p-AKT (S473) and p-β-catenin (S33/37) significantly decreased in DU-145 and PC-3 cells, respectively.

IL-8/c-Jun and ICAM-1/FOXO1 affect PKC-ι regulation positively and negatively. As revealed in Fig. 5, immunoblot analysis of cytokines in the PC-3 and DU-145 cell lines demonstrated a significant increase in the levels of IL-8 and ICAM-1 in the cells treated with ICA-1T. IL-1α, IL-1β, IL-18, CXCL-1, CXCL-12, GM-SCF, MIF and Serpin E1 were also found at

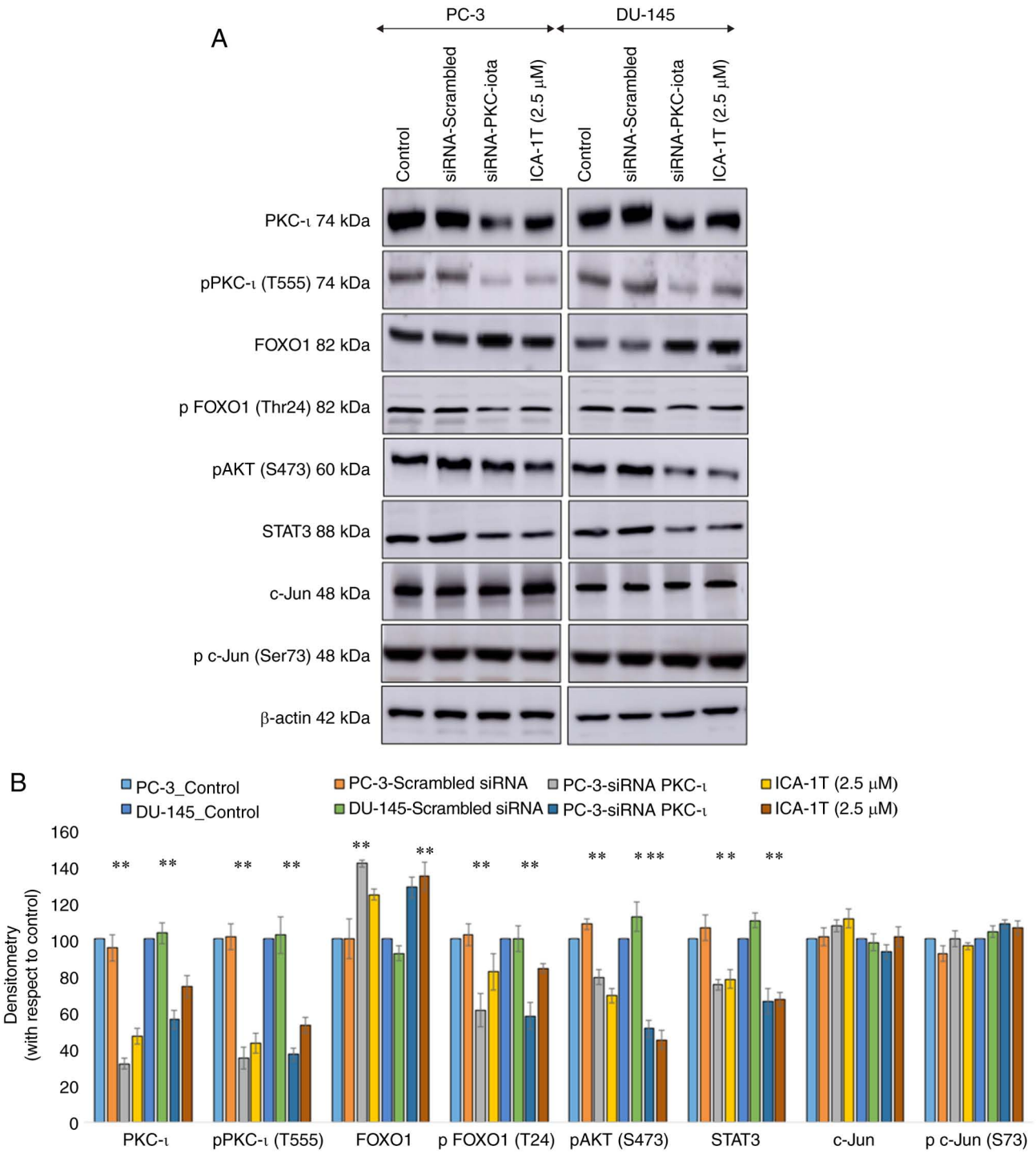

Figure 3. Effect of siRNA of PKC-ι and the PKC-ι specific inhibitor, ICA-1T, on the expression of PKC-ι and targeted proteins in PC-3 and DU-145 cells. (A) Protein levels of phosphor-PKC-ι (T555), total PKC-ι, c-Jun, phosphor-c-Jun (S73), FOXO1, phosphor-FOXO1 (T24), STAT3 and phosphor-AKT (S473) following the siRNA knockdown of the expression of PKC-ι and ICA-1T treatments for PC-3 and DU-145 cell lines. Total protein (80 μg) was loaded into each well and β-actin was used as the internal control in each western blot. (B) Representative densitometry values for the western blots in (A) Experiments (n=3) were performed in each trial and representative bands are shown. Densitometry values are reported as the means ± SD. Statistical significance is indicated by an asterisk (*P≤0.05).

detectable levels, although the levels of these cytokines were not altered substantially owing to PKC- ι inhibition, apart from CXCL-1, which exhibited a significant (P≤0.05) change in PC-3 cells. These results of PKC-ι inhibition by ICA-1T were compared to the samples treated with TNF-α prior to extraction. TNF-α, a cytokine known to upregulate NF-κB signaling, did lead to a significant change in the expression profiles with ICA-1T treatments. As shown in Fig. 6A, RT-qPCR analyses were also conducted for these samples for which the western blot data was presented in Fig. 5. As shown in Fig. 6A, the PKC-ι mRNA levels significantly decreased by 32% (P≤0.05) and 23% (P≤0.05) in the PC-3 and DU-145 cells treated with ICA-1T, respectively. Along with PKC-ι depletion, ICAM-1 expression increased significantly by 45% (P≤0.05) and

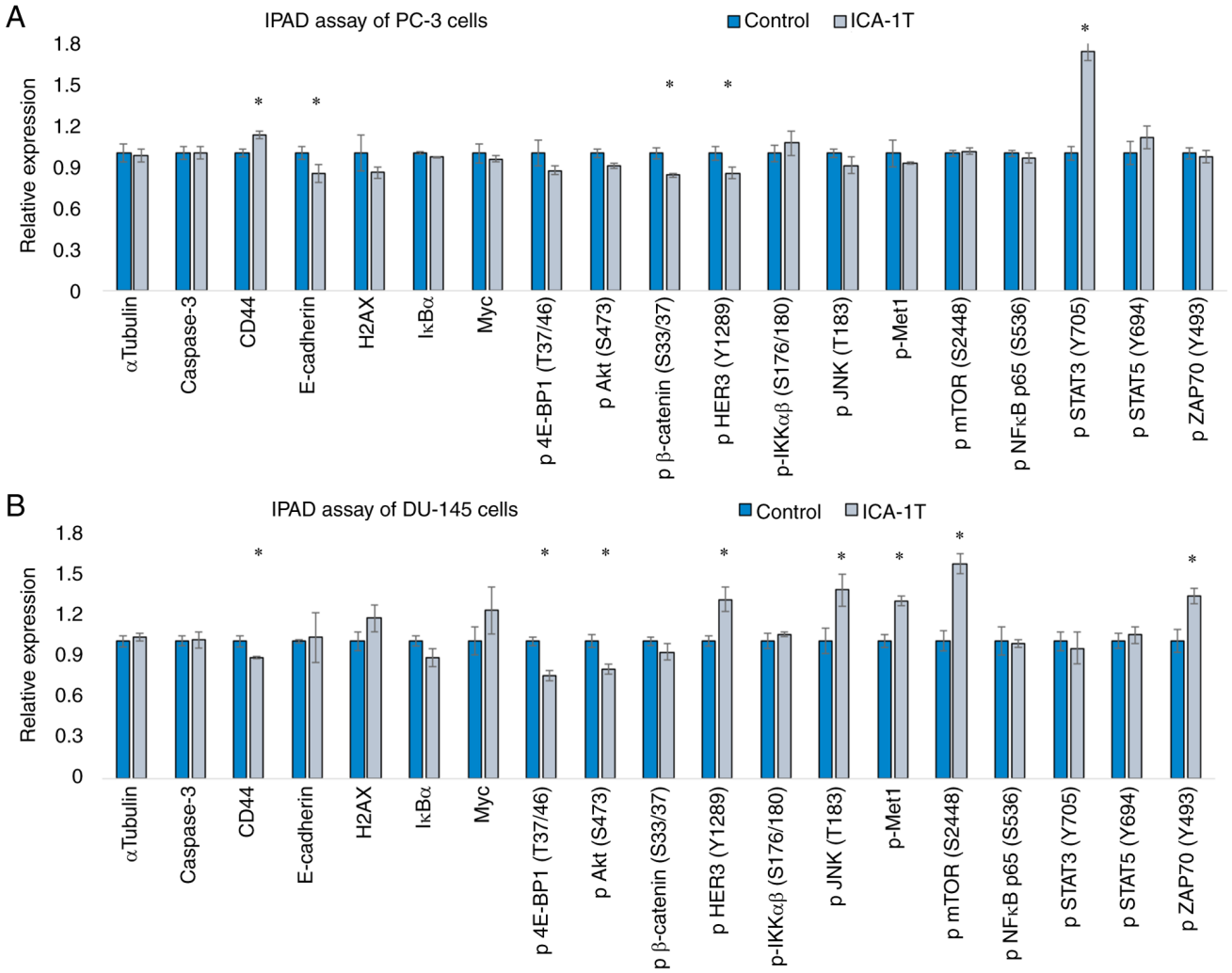

Figure 4. Immunopaired antibody detection assay (IPAD) for PC-3 and DU-145 cells. (A and B) expression of IPAD assay targets for PC-3 and DU-145 cell lines, respectively. Approximately 1x105 cells were cultured in T75 flasks and 24 h post-plating, fresh medium was supplied and the cells were treated with either volume of sterile water (control) or the IC50 concentration of ICA-1T (2.5 μM). Additional concentrations were supplied every 24 h during a 3-day incubation period. The cells were then lysed and prepared lysates with the final total protein concentration to be >2 μg/ml and then sent to ActivSignal, LLC facility to conduct the IPAD assay. IPAD platform is a proprietary multiplexed ELISA technology for analyzing the activity of multiple signaling pathways in one reaction. Activities of multiple signaling pathways were monitored simultaneously in a single well through assessing the expression or protein phosphorylation of 25 target human proteins, such as caspase-3, CD44, CHOP, E-cadherin, IκBα, Myc, NOTCH, p-4E-BP1, p-AKT (S473), p-β-catenin, p-HER3, p-IRS-1, p-JNK, p-MEK1, p-mTOR, p-NF-κB, p-NUMB, p-SMAD1, p-SMAD2, p-STAT3, p-STAT5, p-YAP1, p-ZAP70, p21 and PARP. α-tubulin and β-tubulin were used as internal controls in each trial. Experiments (n=3) were performed in each cell lines and the means ± SD are plotted. Statistical significance is indicated by an asterisk (*P≤0.05).

93% (P≤0.05) in the PC-3 and DU-145 cells, respectively. Additionally, the IL-8 levels also increased significantly by 123% (P≤0.05) and 50% (P≤0.05) in the PC-3 and DU-145 cells, respectively.

Fig. 6B demonstrates the mRNA levels of PKC-ι, c-Jun, FOXO1 and NF-κB in the cells subjected to the knockdown of FOXO1, c-Jun and NF-κB p65 by siRNA for both cell lines with respect to the controls. Fig. 6B demonstrates the results of mRNA expression analysis following transfection of the cells with siRNA against FOXO1, c-Jun and NF-κB in which the western blot analysis data are presented in Figs. 1-3. The mRNA analysis for these siRNA transfections confirmed the western blot analysis observations presented in Figs. 1-3. The diminution of FOXO1 led to an increase in PKC-ι expression by 134 and 68% (P≤0.05) in the PC-3 and DU-145 cells, respectively. Additionally, the diminution of c-Jun expression decreased PKC-ι expression by 38 and 18% (P≤0.05) in the PC-3 and DU-145 cells, respectively. These outcomes confirmed that FOXO1 functions as a transcriptional deactivator for expressing the PRKCI gene, while c-Jun functions as a transcriptional activator.

Fig. 7 presents a graphical overview of PKC-ι expression modulation in prostate cancer cells based on the present study current and on previous evidence (7,10,18). This illustration reveals the connections between multiple pathways of JNK, NF-κB, AKT/FOXO1 and STAT3 in relation to PKC-ι regulation. It indicates that PKC-ι plays a vital role in controlling its expression via the c-Jun and FOXO1 transcriptional activation/deactivation. Owing to c-Jun transcriptional function, PKC-ι is overexpressed with the aid of pro-survival, oncogenic STAT3, NF-κB/PI3K/AKT and signaling cascades. PKC-ι inhibition using ICA-1T pledges an interruption to PKC-ι expression

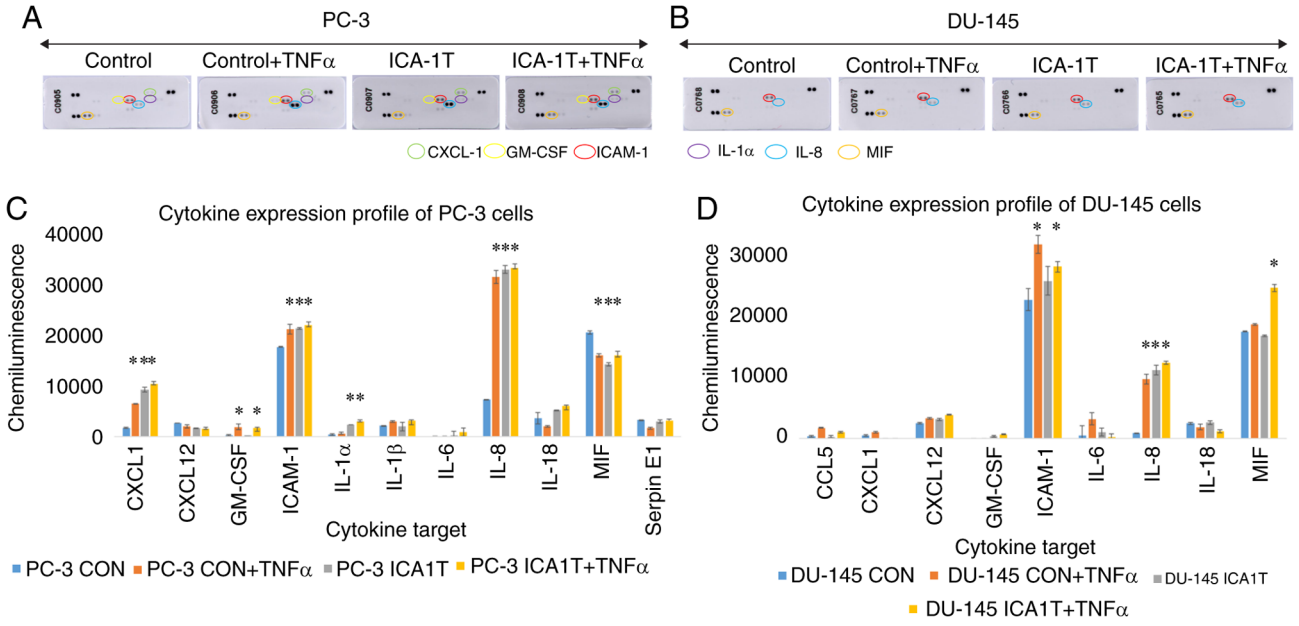

Figure 5. Cytokine expression analysis of prostate cancer cells upon PKC-ι inhibition using ICA-1T in the presence and absence of TNF-α. (A and B) Western blot array of the PKC-ι inhibition for PC-3 and DU-145 cells against the controls. (C and D) The quantified results of the western blots shown in (A and B) for the PC-3 and DU-145 cells, respectively. CXCL-1, CXCL-12, GM-SCF, ICAM-1, IL-1α, IL-1β, IL-6, IL-8, IL-18 and MIF were found in detectable levels in western blot analysis for PC-3 and DU-145 cell lysates. Experiments (n=3) were performed in each cell lines and the means ± SD are plotted. Statistical significance is indicated by an asterisk (*P≤0.05).

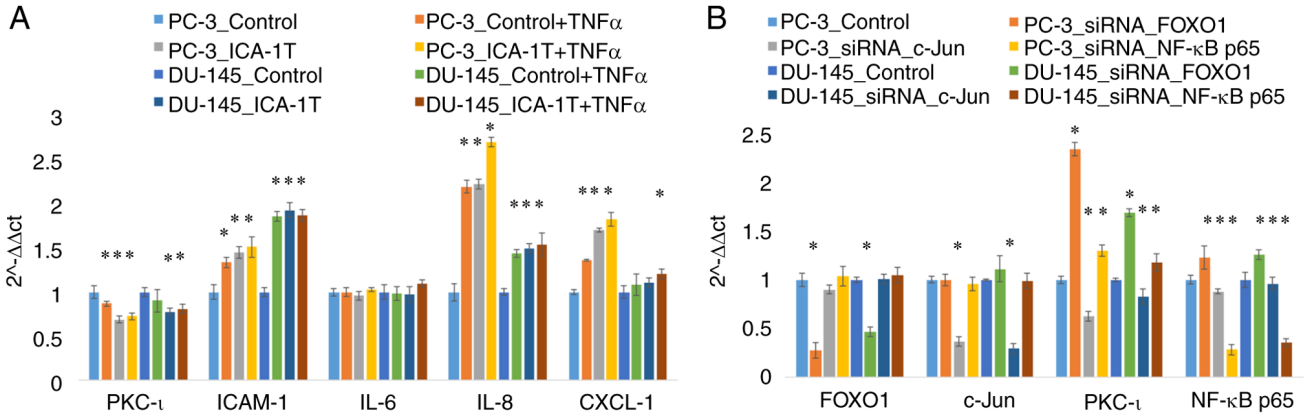

Figure 6. (A) RT-qPCR analysis of cytokines (ICAM-1, IL-6, IL-8 and CXCL-1) and PKC-ι for the PKC-ι specific inhibition using ICA-1T in the presence and absence of TNF-α for PC-3 and DU-145 cells respectively. (B) RT-qPCR analysis of FOXO1, c-Jun, PKC-ι and NF-κB following the siRNA knockdown of FOXO1, c-Jun and NF-κB for PC-3 and DU-145 cells. All targeted mRNA levels were plotted against β-actin as the internal control. Experiments (n=3) were performed in each cell line and the means ± SD are plotted. Statistical significance is indicated by an asterisk (*P≤0.05).

cycles through the downregulation of the NF-κB pathway by limiting IKKα/β due to the limitation of activated p-PKC-ι. It caused a suppression of NF-κB transcriptional activity and IL-8. Due to the lack of NF-κB stimulation, IL-8 accumulates in the cytosol and does not perform its intended paracrine further upregulation of PI3K/AKT signaling. AKT signaling decreases due to the lack of cytokine activation, such as IL-8, which ultimately contributes to FOXO1 upregulation. FOXO1 adversely governs the expression of PKC-ι and also decreases the function of JNKs to postpone its activation of c-Jun which upregulates the expression of PKC-ι. Moreover, FOXO1 downregulates STAT3 and NF-κB signaling. The cycle persists and contributes to the further downregulation of NF-κB and c-Jun, and the upregulation of FOXO1, decreasing PKC-ι expression. The whole process began upon the inhibition of PKC-ι. As a result of this signaling alteration, the total PKC-ι level decreases in the tested prostate cancer cells. The results of the present study closely reinforce the findings of our previous study, wherein precise inhibition utilizing PKC-ι inhibitors decreased overall PKC quantities (10).

Discussion

In our previous study, the selective binding of ICA-1T to an allosteric site located in the C-lobe of PKC-ι kinase domain was recognized. This binding led to the inhibition of PKC-ι activity (9). This consequentially leads to a reduction in cellular differentiation, proliferation, migration and invasion,

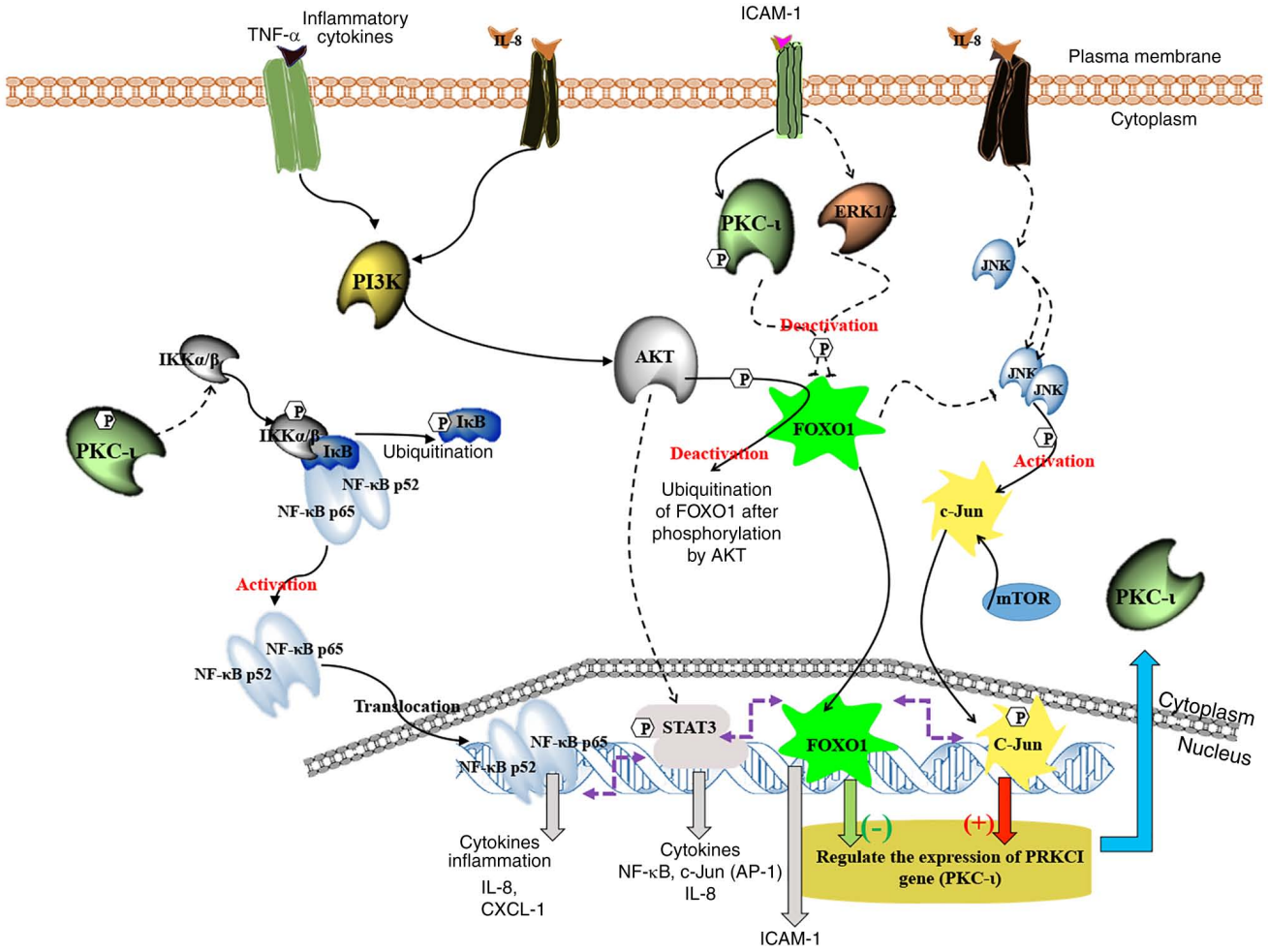

Figure 7. A schematic summary of the regulation of the expression of PKC-ι in PC-3 and DU-145 cell lines. This model depicts how the crosstalk occurs between the NF-κB, PI3K/AKT/FOXO1, JNK/c-Jun and STAT3/5 signaling pathways during the PKC-ι regulation. It is shown that PKC-ι plays a very important role in the regulation of its expression in a complex signaling network through the transcriptional activation/deactivation of c-Jun and FOXO1. The PKC-ι-specific inhibition by ICA-1T, downregulates the NF-κB, STAT3 and IL-8 activities. As a result, the activity of AKT decreases, which leads to the upregulation of FOXO1, which turns out to be the most important transcription factor regulating PKC-ι expression upon receiving stimulation from ICAM-1. FOXO1 downregulates the expression of PKC-ι, suppressing JNK activity to attenuate the activation of c-Jun. This reduces c-Jun expression. This whole process continues and leads to the further downregulation of NF-κB and c-Jun, while upregulating FOXO1, which leads to the continuation of the depletion of PKC-ι expression in the cell lines. PKC-ι inhibition leads to a decrease in its own production while enhancing multiple antitumor/pro-apoptotic signaling.

whilst simultaneously driving the apoptosis of prostate cancer cells via the diminution of the NF-κB pathway *in vitro*. Subsequently, PKC-ι was established as a key factor in the induction of cell growth, differentiation and survival (8,9,11). It was also recognized that PKC-ι undergoes self-regulation as a consequence of its inhibition and a decrease in its expression in the PC-3 and DU-145 cell lines. Thus, the aim of the present study was the identification of the underlying processes of PKC-ι regulation in the aforementioned cell lines *in vitro*.

In order to investigate PKC-ι regulation and expression, the roles of transcription factors which interacted with the PRKC1 promoter region were investigated. The gene which codes of PKC-ι is the PRKCI gene, which is positioned on chromosome 3 (3q26.2), which is an amplicon known to undergo replication events (20). In order to deduce key TFs in PRKCI regulation, a sequence encompassing the PRKCI promotor with a motif feature was selected, as well as a promoter flank and an enhancer. This was selected as it provides the ideal platform in which the TFs can bind to regulate transcription. Two systems, PROMO and Genomatix Matinspector, were utilized to predict probable transcription factor bindings. This led to the identification of 5 TFs two of which were FOXO1 and c-Jun. Subsequently, these TFs were silenced in order to analyze the downstream effect they would have on PKC-ι expression.

c-Jun was the first transcription factor found to be associated with numerous types of cancer, including metastatic breast cancer and non-small lung cancer (21). It functions through the formation of an early response complex containing AP-1 and c-Fos (22). The activation of c-Jun occurs via phosphorylation events by c-Jun N-terminal kinases (JNKs) on S63 and S73, and is regulated via multiple extracellular stimuli, i.e., cytokines (23). Upon phosphorylation at S63 and S73, not only is c-Jun activated, but it also leads to an increase in the transcription of c-Jun-targeted genes. Extracellular signal-regulated kinase (ERK) is also upregulated by activated c-Jun (24-26). c-Jun is also known to promote the oncogenic transformation of 'ras' and 'fos' in several cancer types (27,28). FOXO1 is known to play a role in regulating various metabolic pathways, such as gluconeogenesis, adipogenesis and insulin signaling.

Similar to c-Jun, phosphorylation plays a crucial role in FOXO1 function (29,30). FOXO1 is deactivated by AKT through phosphorylation on T24, leading to the induction of nuclear exclusion, which leads to ubiquitylation (31,32). Therefore, it is important to note that the phosphorylation of FOXO1 indicates its inactivation and the downregulation of FOXO1 signaling. In relation to cancer, FOXO1 is a well-established tumor suppressor (33-35). As such, there is a known association between FOXO dysregulation and cancer progression, as it is also plays a role in both intrinsic and extrinsic pathways of apoptosis (36,37). Experiments *in vitr*o and *in vivo* have confirmed that the overexpression of FOXO1 causes a reduction in cell migration, proliferation and tumorigenesis in cancer cells (38). Furthermore, ERK1/2, PKC-ι and AKT can downregulate FOXO1 (35). Thus, in the present study, it was demonstrated that through the specific inhibition of PKC-ι, the expression of active PKC-ι decreases, which renders it ineffective in its role to deactivate FOXO1 through phosphorylation events. This is a crucial indication of PKC-ι involvement in the regulation of its own expression, as PKC-ι inhibition leads to the continuous upregulation of FOXO1.

At the same time, previous data have demonstrated that the inhibition of PKC-ι causes the significant downregulation of the PI3K/AKT pathway and in particular, downregulates the activation of AKT (10). In the present study, as shown in Figs. 3 and 6, NF-κB downregulation led to elevated levels of active c-Jun (phospho c-Jun), which upregulated PKC-ι expression. These results validate our previous observation that PKC-ι inhibition, by which the phosphorylation of IKKα/β is reduced, inhibits NF-κB activation and translocation to the nucleus (10). Subsequently, NF-κB depletion induces an increase in c-Jun expression, which then attempts to increase the production of PKC-ι, which then needs to phosphorylate IKKα/β to restore NF-κB signaling. The tight regulation of PKC-ι expression through c-Jun may explain these results as it enhances PRKCI transcription. There was also no significant alteration in the levels of FOXO1 and phosphorylated FOXO1 resulting from NF-κB siRNA knockdown. This suggests that the downregulation of NF-κB does not disrupt PKC-ι expression through FOXO1, but rather that c-Jun provides cancer cells with resistance to apoptosis through interplay with NF-κB upon cytokine stimulation (21). In our previous study, it was demonstrated that in melanoma, TNF-α upregulates NF-κB, phosphor-AKT and PKC-ι expression (9). However, the results of the present study demonstrate that c-Jun 'switches on' PKC-ι expression and FOXO1 'switches off'.

Apart from identifying c-Jun and FOXO1 out of 5 TFs which could bind to the PKC-ι gene promoter region, other key molecular factors were also identified. Through the conduction of ELISA using IPAD assay and a cytokine array, crosstalks between multiple pathways were examined. The data indicated links between PKC-ι expression with cytokines IL-8 and ICAM-1, along with some other key cellular signaling points.

As shown in Fig. 4, the IPAD ELISA data revealed that there was a significant increase in the expression levels of p-STAT3 (Y705), p-JNK (T183) and p-mTOR, whilst displaying a significant decrease in p-AKT (S473), p-β-catenin and CD44 levels. Moreover, it has been demonstrated that irregular STAT3/5 is associated with the progression of various cancer types (39-44). Cell survival in multiple cancers has been shown to be induced by upregulated STAT signaling, which is often stimulated by the cytokines, IL-6 and IL-8 (39,40,45). STAT3 signaling enhances the production of c-Jun, thereby inducing c-Jun-targeted transcription (39,46). The IPAD data of the present study strongly suggested that STAT3 was upregulated due to PKC-ι inhibition, suggesting that the deprivation of PKC-ι tries to accelerate the production of c-Jun through the upregulation of STAT3, JNK and mTOR. Connections between the JNK pathway and FOXO1 have been explored in few studies (35,47,48). Hornsveld *et al* summarizes the tumor-suppressing features of FOXO1 resulting in a decreased JNK activity (47). Whilst JNK activates c-Jun, by contrast, PKC-ι inhibition renders it ineffective at increasing c-Jun or phospho-c-Jun levels, as can be seen in Fig. 3. Instead, the FOXO1 levels were increased, while the phosphor-FOXO1 levels along with the levels of phosphor-AKT and STAT3 were reduced in both cell lines. This demonstrates that the activation of FOXO1 leads to a reduction in c-Jun levels by blocking the activity of phosphor-JNK. Therefore, it was deduced that FOXO1 plays a major role in c-Jun regulation only upon PKC-ι inhibition. This process likely employs multiple mechanisms, such as JNK signaling inhibition, causing the further retardation of PKC-ι expression, which will eventually lead to cell cycle arrest. This is further corroborated by FOXO1 being established as being able to induce cell cycle arrest. It accomplishes this through the promotion of the transcription of cell cycle kinase inhibitors or cyclin-dependent kinase inhibitor (CKI). p21 and p27 are two of the most well-known FOXO-induced downstream CKIs (35,47). FOXO1 has also been shown to be associated with the induction of anoikis (apoptosis that occurs when cells detach from the extracellular matrix) (47). Once again, this displays another downstream effect of PKC-ι involvement, as the inhibition of its expression augments FOXO1 antitumor activity.

As shown in Fig. 7, it is summarized that the expression of PRKCI is negatively affected by FOXO1, whilst being positively affected by c-Jun. The inhibition of PKC-ι leads to the following downstream effects. The downregulation of NF-κB activity through the lack of phosphor-IKKα/β, decreases the levels of phosphor-AKT (S473), thereby diminishing AKT activity. Subsequently the low activity of AKT, along with PKC-ι, lead to the decreased phosphorylation of FOXO1, also leading to elevated levels of active unphosphorylated FOXO1. These elevated levels of activated FOXO1 lead to the further suppression of PRKCI gene expression. This acts as a 'switch off' effect on PRKCI expression. PKC-ι downregulation also leads to decreased STAT3, mTOR and JNK signaling. As a consequence, this reduces c-Jun activity, leading to the cancellation of the positive effects of c-Jun towards PKC-ι expression. Furthermore, STAT3 and STAT5 upregulate NF-κB transcription in addition to c-Jun (46,49). Due to this, it was deduced that PKC-ι inhibition causes the downregulation of NF-κB and STAT3, leading to a decrease in both the transcription and activation of c-Jun. Therefore, these data suggest that the PKC-ι levels were decreased when c-Jun expression was silenced by siRNA (Figs. 1 and 6B).

In the present study, further *in vitro* experiments (Figs. 5 and 6A) demonstrated the deviations in cytokine expression (IL-8 and ICAM-1) in the PC-3 and DU-145 cells upon PKC-ι knockdown. In both cell lines, the protein levels of IL-8

and ICAM-1 (as well as their mRNA levels) were shown to undergo a significant intensification following PKC-ι knockdown by siRNA, as proven by western blot and RT-qPCR analyses. These data suggest that PKC-ι self-regulation is involved in autocrine signaling. Tumor cellular environments, with prostate cancer in particular, are constantly exposed to a variety of immune cells and inflammatory factors. The effects of which function to either promote chronic inflammation or engage in antitumor activity (50). Examples of these inflammatory factors are cytokines; they play a crucial role in controlling the tumor microenvironments (51). To achieve their functions, cytokines utilize multiple signaling pathways. They can either act to promote or downregulate tumor progression and metastasis. Examples of tumor promoting cytokines are; as CXCL-1, CXCL-12, IL-18, CXCL-10, IL-6 and IL-8. CXCL1, also known as melanoma growth-stimulatory activity/growth-regulated protein α, functions in processes of wound healing, angiogenesis and inflammation after being secreted by cancer cells. It has also been linked to tumor formation (52). Metastatic regulation has also been linked to high levels of CXCL10/CXCR3, with CXCL10 playing an important role in the promotion of tumor growth and metastasis (52). Metastatic regulation has also been linked to high levels of CXCL10/CXCR3, with CXCL10 playing an important role in the promotion of tumor growth and metastasis (52,53). CXCL12 (stromal-derived factor-1) utilizes the receptors CXCR4 and CXCR7 and it has been linked to playing a role in the regulation of tumor metastasis. However, CXCL-10, CXCL-12 and IL-18 were not observed as being significantly altered as result of PKC-ι inhibition.

IL-6 has been linked to the stimulation of the degradation of IκB-α, which in turn results in the upregulation of NF-κB translocation. As previously demonstrated, PKC-ι stimulates NF-κB translocation through IκB-α degradation (9). Upon translocation to the nucleus, NF-κB induces cell survival via the transcription of multiple survival factors and cytokines (39,45,53), with IL-8 being one such cytokine. It plays a key role in the regulation of polymorphonuclear neutrophil mobilization. It is also associated with the extravasation in the steps of cancer metastasis. IL-8 has been shown through studies to be regulated by NF-κB in prostate cancer cells. As such, an increased IL-8 expression has been connected to the promotion of a favorable microenvironment for metastasis (54,55). Notably, the findings of the present study demonstrated that upon transfection with PKC-ι siRNA, the IL-8 expression levels increased. This may be a result of a backup mechanism in order to upregulate IL-8. IL-8 also plays an essential role in upregulating c-Jun through JNKs. As the inhibition of PKC-ι downregulates c-Jun, the cells may be attempting to reinstate these downregulated pathways by a higher IL-8 production. As shown in Fig. 7, the expression of IL-8 is regulated through both NF-κB and STATs. These results indicate that IL-8 is important in upregulating PKC-ι expression, activating c-Jun, while deactivating FOXO1. Though it would appear that due to the high activity of FOXO1, the effect of IL-8 are canceled out.

Through utilizing an immune response, some cytokines promote antitumor activity. One such cytokine is ICAM-1, which plays a role in the immune response, including antigen recognition and lymphocyte activation (56,57). As such, ICAM-1 has beeb linked to the inhibition of tumor progression via the inhibition of the PI3K/AKT pathway. In its role in inhibiting this pathway, ICAM-1 exposes tumor cells to attack and death through cytotoxic T-lymphocytes (57). ICAM-1 expression inhibition has also been shown in clinical research to be associated with an increased risk of metastasis within the first 5 years of ovarian cancer diagnosis (57). Of note, the results of the present study demonstrated that upon the silencing of PKC-ι by siRNA, the ICAM-1 levels increased. This confirmed that upon the knockdown of oncogenic PKC-ι, antitumor/pro-apoptotic signaling was upregulated through an autocrine manner via ICAM-1. Furthermore, these results demonstrate that ICAM-1 plays an important downregulatory role in the regulation of PKC-ι expression along with FOXO1, opposite to c-Jun and IL-8.

To conclude, the results of the present study illustrate that PKC-ι plays an imperative role in its own expression via an intricate signaling grid that involves the transcriptional activation/deactivation of c-Jun and FOXO1. The inhibition of PKC-ι activity, based on its specific inhibition, downregulates the NF-κB pathway along with the transcriptional activity of STAT3 and IL-8. The results in a decrease in AKT activity that leads to FOXO1 upregulation. FOXO1 was identified to be the most important transcription factor when it comes to regulating PKC-ι, along with ICAM-1 stimulation. FOXO1 negatively regulates PKC-ι expression, diminishing JNK activity and further suppressing the activation of c-Jun. The consequence of this process is that it leads to the downregulation of NF-κB and c-Jun, and further upregulates FOXO1. This continues to deplete the PKC-ι expression, subsequently leading to a decrease in the total PKC-ι levels in prostate cancer cells. The regulation of PKC-ι is complex, and PKC-ι itself plays a key role in that process. As such, when inhibited, it leads to a decrease in PKC-ι production, prompting multiple antitumor/pro-apoptotic signaling. PKC-ι is therefore a key factor to target when attempting to treat prostate cancer *in vitro*. Finally, the results of the present study demonstrate that PKC-ι is not only a novel biomarker to target for personal therapeutics for prostate cancer, but also that ICA-1T shows promise as one such therapy in relation to the proposed mechanism.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WSR and MAD were involved in the conceptualization of the study. WSR, CAA and MAD were involved in the data analysis. MAD and WSR were involved in the investigation. WSR and SB were involved in cell culturing. WSR was involved in western blot analysis. WSR and CAA were involved in the cytokine analysis, IPAD assay and RT-qPCR. WSR and SB were involved in the writing of the original draft. MAD was involved in reviewing the manuscript. WSR and CAA edited the manuscript. MAD were involved in obtaining resources and was also involved in the supervision of the study and funding acquisition. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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