

# FOXO1 regulates oncogenic PKC- $\iota$ expression in melanoma inversely to c-Jun in an autocrine manner via IL-17E and ICAM-1 activation

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**Abstract.** Regardless of abundant efforts to enhance primary prevention and early detection, the number of melanoma cases in the United States has increased steadily over the past 15 years, thus greatly affecting public health and the economy. In previous studies, we demonstrated protein kinase C- $\iota$  (PKC- $\iota$ ) to be an oncogene in melanoma, which promotes the activation of nuclear factor (NF)- $\kappa$ B, thereby supporting survival and progression. In addition, we demonstrated that PKC- $\iota$  induced the metastasis of melanoma cells by activating Vimentin, and PKC- $\iota$  inhibition downregulated epithelial-mesenchymal transition (EMT), while inducing apoptosis. Of note, PKC- $\iota$  specific inhibitors downregulated the expression of both PKC- $\iota$  and phosphorylated PKC- $\iota$ , suggesting that PKC- $\iota$  plays a role in regulating its own expression in melanoma. In this study, we report the underlying mechanisms of the transcriptional regulation of PKC- $\iota$  (PRKCI gene) expression in melanoma. c-Jun, interferon-stimulated gene factor 3 (ISGF3), paired box gene 3 (PAX3), early growth response protein 1 (EGR1) and Forkhead box protein O1 (FOXO1), which bind on or near the promoter sequence of the PRKCI gene, were analyzed for their role in PKC- $\iota$  regulation in SK-MEL-2 and MeWo cell lines. We silenced selected transcription factors using siRNA, and the results revealed that the silencing of c-Jun and FOXO1 significantly altered the expression of PRKCI. The levels of both phosphorylated and total PKC- $\iota$  increased upon FOXO1 silencing and decreased upon c-Jun silencing, suggesting that c-Jun acts as an upregulator, while FOXO1 acts as a down-regulator of PRKCI expression. We also used a multiplex

ELISA to analyze multiple pathways other than NF- $\kappa$ B that were affected by treatment with PKC- $\iota$  inhibitor. The silencing of NF- $\kappa$ B p65 and PKC- $\iota$  by siRNA suggested that the regulation of PKC- $\iota$  expression was strongly associated with FOXO1. In addition, we observed a significant decrease in the mRNA levels of both interleukin (IL)-6 and IL-8, with a significant increase in the levels of IL-17E and intercellular adhesion molecule 1 (ICAM-1) upon the knockdown of expression of PKC- $\iota$  in both cell lines. This suggested that PKC- $\iota$  expression was affected by these cytokines in an autocrine manner. Overall, the findings of this study suggest that PKC- $\iota$  inhibition suppresses its own expression, diminishing oncogenic signaling, while upregulating anti-tumor signaling, thus rendering it an effective novel biomarker for use in the design of novel targeted therapeutics for melanoma.

## Introduction

Melanoma is one of the most rapidly growing types of cancer worldwide in terms of incidence and holds 5.3% of all cancers reported in the United States in 2018 (1). The majority of U.S. Food and Drug Administration (FDA)-approved melanoma drugs target the BRAF (V600E) mutation, which occurs in >50% of melanoma cases; however, melanoma is associated with a poor prognosis and tumors attain resistance to BRAF mutation inhibition (2). Even though surgical resection, immunotherapy, radiation therapy, or chemotherapy can improve survival rates, current trends demand effective and personalized targeted therapeutics for melanoma.

We recently established protein kinase C- $\iota$  (PKC- $\iota$ ) as an oncogene and a prospective therapeutic target for metastatic melanoma *in vitro* (3-5). PKC belongs to the protein kinase enzyme family, which post-translationally modifies other proteins and is involved in a number of signal transduction cascades. In total, 15 PKC isozymes have been identified in humans; these are divided as classical, novel and atypical PKCs (aPKCs). aPKCs contain two structurally and functionally distinct isozymes: PKC- $\iota$  and PKC- $\zeta$  (6-8). PKC- $\iota$  is an oncogene that has been associated in several signaling pathways in ovarian, glioma and prostate carcinomas (9-11). Moreover, PKC- $\iota$  overexpression has been shown to be associated with a poor prognosis (11).

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We have previously demonstrated that PKC- $\iota$  is overexpressed in melanoma cell lines compared to undetectable levels in normal melanocytes using the PCS-200-013 and MEL-F-NEO cell lines (4). Our previous results confirmed that PKC- $\iota$  plays a vital role in inducing epithelial-mesenchymal transition (EMT), in melanoma by regulating Vimentin dynamics (3). We have also reported that PKC- $\iota$  is involved in the tumorigenesis, progression and survival of melanoma. PKC- $\iota$  inhibition using specific inhibitors or the knockdown of its expression using siRNA significantly induces apoptosis, and reduces the migration and invasion of melanoma (3). In our previous study, we demonstrated that PKC- $\iota$  was heavily involved in the phosphorylation of I $\kappa$ B kinase (IKK $\alpha/\beta$ ) at S176/180 to activate it. Activated phospho-IKK $\alpha/\beta$  (S176/180) then phosphorylates nuclear factor of  $\kappa$  light polypeptide gene enhancer in B-cells inhibitor (I $\kappa$ B), which causes I $\kappa$ B to dissociate from the nuclear factor (NF)- $\kappa$ B complex and undergo ubiquitination. This process releases NF- $\kappa$ B in the active form to translocate to the nucleus. Upon PKC- $\iota$  inhibition using two PKC- $\iota$  specific inhibitors, [4-(5-amino-4-carbamoylimidazol-1-yl)-2,3-dihydroxycyclopentyl] methyl dihydrogen phosphate (ICA-1T) and 5-amino-1-((1R,2S,3S,4R)-2,3-dihydroxy-4-methylcyclopentyl)-1H-imidazole-4-carboxamide (ICA-1S), we found that NF- $\kappa$ B nuclei translocation was blocked, causing NF- $\kappa$ B activity to downregulate (3). Our previous findings on both melanoma and prostate cancer cell lines demonstrated that PKC- $\iota$  inhibition not only affected the pathways regulated by PKC- $\iota$ , but also downregulated its protein expression (3,4,12). This suggests that PKC- $\iota$  plays a role in a self-propagating cycle, as observed in some other cycles related to cancer growth, such as transforming growth factor (TGF)- $\beta$  and CD147 (13). We therefore designed the current study to investigate the role of PKC- $\iota$  in its expression regulatory mechanisms.

Transcription factors play a key role in gene expression, controlling the rate of transcription of genetic information from DNA to messenger RNA by binding to a specific DNA sequence. Various cytokines often trigger such signaling. In this study, we sought to determine which transcription factors are the main regulators of PKC- $\iota$  expression in melanoma cells, giving emphasis to cytokine stimulation and expression.

In the present study, we report the effects of the systematic silencing of PKC- $\iota$ , NF- $\kappa$ B, c-Jun and Forkhead box protein O1 (FOXO1) on PKC- $\iota$  levels in relation to NF- $\kappa$ B, PI3K/AKT/FOXO1, JNK/c-Jun and signal transducer and activator of transcription (STAT) signaling, along with cytokine production, to establish the mechanism of PRKCI regulation. The levels of both phosphorylated and total PKC- $\iota$  increased upon FOXO1 knockdown by siRNA and decreased upon the knockdown of c-Jun by siRNA. The results confirmed that c-Jun acts as a transcriptional activator and that FOXO1 acts as a transcriptional suppressor of PRKCI expression. Going forward, we establish the roles that these transcription factors play in an inflammation cycle that governs PKC- $\iota$  expression and is dependent on PKC- $\iota$  for the cycle to continue. Furthermore, we establish that major signaling pathways such as PI3K/AKT/FOXO1 and JNK/c-Jun play a vital role in regulation of PKC- $\iota$  expression. In addition, the cytokines IL-6 and IL-8 promote PKC- $\iota$  expression, thereby enhancing

NF- $\kappa$ B activity, producing more cytokines as a part of a cycle such cancers use to develop and spread. IL-17E and ICAM-1 induce FOXO1 to subdue PKC- $\iota$  expression. Overall, these results suggest that PKC- $\iota$  plays a central role in melanoma progression with a complex and tightly regulated expression profile. The specific inhibition of PKC- $\iota$  can disrupt its own regulatory cycle, leading to the disruption of its oncogenic role in melanoma.

## Materials and methods

**Reagents and antibodies.** ICA-1 nucleotide (ICA-1T) and nucleoside (ICA-1S) were synthesized by Therachem (Jaipur, India). They were dissolved in sterile distilled water (vehicle) prior to use. Antibodies were purchased as follows: PKC- $\iota$  (610175) from BD Biosciences (San Jose, CA, USA) and NF- $\kappa$ B p65 (sc-372-G) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); phospho-PKC- $\iota$  (T555; 44-968G) from Thermo Fisher Scientific (Waltham, MA, USA); early growth response protein 1 (EGR1; 4153S), c-Jun (9165S), phospho-c-Jun (S73; 3270S), FOXO1 (2880S), phospho-FOXO1 (T24; 9464S) and phospho-AKT (S473; 4059S) from Cell Signaling Technology (Danvers, MA, USA); and  $\beta$ -actin peroxidase (A3854) from Sigma-Aldrich (St. Louis, MO, USA). Enhanced chemiluminescence solution (34080) was purchased from Pierce, Inc. (Rockford, IL, USA). Dulbecco's phosphate-buffered saline without Mg<sup>2+</sup> and Ca<sup>2+</sup> (D8537) and Trypsin-EDTA (ethylenediaminetetraacetic acid) solution (T4049) were purchased from Sigma-Aldrich. Human small interfering RNA (siRNA) for PKC- $\iota$  (SR303741), for EGR1 (SR301358), c-Jun (SR302499), FOXO1 (SR301618) paired box gene 3 (PAX3; SR303360), interferon regulatory factor 9 (IRF9; SR307030) and NF- $\kappa$ B p65 (SR321602) were purchased from Origene Technologies, Inc. (Rockville, MD, USA). The NF- $\kappa$ B specific inhibitor, 4-methyl-N1-(3-phenyl propyl)-1,2-benzenediamine (JSH-23) (J4455) was purchased from Sigma-Aldrich.

**Cells and cell culture.** The SK-MEL-2 (ATCC<sup>®</sup> HTB68<sup>™</sup>) and MeWo (ATCC<sup>®</sup> HTB65<sup>™</sup>) cell lines were purchased from the American Type Tissue Culture Collection (ATCC; Rockville, MD, USA) in November, 2015. All cells were frozen in liquid nitrogen immediately with early passages. The cells of passages 2 to 5 were resuscitated from liquid nitrogen and cultured for <3 months before re-initiating culture from the same passage for each tested experiment. The ATCC authenticated the cell lines using morphology, karyotyping and PCR-based approaches. The cells were cultured at 37°C and 5% CO<sub>2</sub>. Eagle's minimum essential medium (90% v/v) (ATCC 30-2003) with fetal bovine serum (10% v/v) and penicillin (5  $\mu$ g/ml) were used for SK-MEL-2 and MeWo cell culturing according to the ATCC guidelines. All cell lines were seeded and grown as monolayers in T25 or T75 flasks.

**Identification of possible transcription factors (TFs) which bind to the PRKCI gene.** The PRKCI gene sequence was obtained from ensemble.org (ENSG00000163558) which locates in chromosome 3 from bp170222365-170305981 (3q26.2) (14,15). The forward strand sequence was used and compared with EPD/Eukaryotic Promoter Database

(<https://epd.vital-it.ch/index.php>) for its promoter sequence. A specific sequence was then selected (chromosome 3; 170220768-170225128); the sequence contains the promoter, promoter flank, enhancer and a motif feature. This sequence predicted possible TFs that can bind within a dissimilarity margin  $\leq 10\%$  using PROMO, which is a virtual laboratory for studying transcription factor binding sites in DNA sequences (<http://algggen.lsi.upc.es/>). TF targets were then compared with the Genomatix Matinspector results to generate the final TF list for the following experiments.

*Knockdown of TFs, PKC- $\iota$  and NF- $\kappa$ B gene expression by siRNA.* Each siRNA contained a pool of three combined RNA sequences for the targeted gene and respective control siRNA contained a scrambled sequence, which did not lead to specific degradation of any known cellular messenger RNA and whose sequence is a proprietary of Origene Technologies, Inc. The experiments performed with siRNA are as follows: Approximately  $1 \times 10^5$  cells (SK-MEL-2 and MeWo) were cultured in T25 flasks and at 24 h post-plating, fresh medium was supplied and the cells were treated with a 20 nM concentration of one of the transcription factor siRNAs or scrambled siRNA as a control using 'siTran' siRNA transfection reagent (TT300002) from Origene Technologies, Inc. according to the manufacturer's recommended ratios. After 48 h of the post-treatment period, cells were subsequently lifted and cell lysates were collected with cell lysis buffer (C7027; Invitrogen/Thermo Fisher Scientific). Western blot analysis was performed as previously described in the study by Win and Acevedo-Duncan and samples were then fractionated by SDS-PAGE and immunoblotted (16).

*Western blot analysis.* The Bradford protein assay was used to measure the protein concentrations of extracted cell lysates in each siRNA experiment. Total protein (80  $\mu$ g) was loaded into each well, separated by 10% SDS-PAGE and electro-blotted onto supported nitrocellulose membranes. Each blot was blocked for 1 h with 5% bovine serum albumin (BP1600-100; Thermo Fischer Scientific) in TTBS solution (0.1% v/v Tween in 1X TBS) at room temperature (approximately 25°C). Protein bands were probed with each targeted primary antibody at 4°C overnight followed by horseradish-peroxidase-conjugate anti-mouse or anti-rabbit secondary antibody for 2 h at room temperature. Immuno-reactive bands were visualized with enhanced chemiluminescence solution (34080) according to the manufacturer's instructions (Pierce, Inc.). Goat anti-mouse IgG (170-6516) and goat anti-rabbit IgG (170-6515) secondary antibodies were used from Bio-Rad Laboratories (Hercules, CA, USA). Manufacturer's recommended concentrations were used for all tested primary and secondary antibodies.  $\beta$ -actin was used as the internal control in each western blot.

*Densitometry.* The intensity of western blot bands was measured using 'Image Studio Lite 5.x' software developed by LI-COR Biosciences (Lincoln, NE, USA) in which the background intensity was subtracted from the intensity of each band to obtain the corrected intensity of the proteins.

*Analysis of cytokine expression using enzyme-linked immunosorbent assay (ELISA).* An ELISA containing a cytokine

array (cat. no. ARY005B) was obtained from R&D Systems (Minneapolis, MN, USA). Approximately  $1 \times 10^5$  cells (SK-MEL-2 and MeWo) were cultured in T25 flasks and at 24 h post-plating, fresh medium was supplied and the cells were treated with a 20 nM concentration of PKC- $\iota$  siRNA or scrambled siRNA as a control. After 48 h of the post-treatment period, the cells were subsequently lifted, lysed, processed and analyzed according to manufacturer's instructions using cytokine array kit reagents. Total protein (100  $\mu$ g) from each sample was used to expose the membranes and chemoluminescence photographs as western blots were taken to analyze the cytokine expression profiles of the melanoma cells upon PKC- $\iota$  siRNA knockdown.

*Immunopaired antibody detection assay (IPAD).* Approximately  $1 \times 10^5$  cells were cultured in T25 flasks and at 24 h post-plating, fresh medium was supplied and the cells were treated with either volume of sterile water (control) or the IC<sub>50</sub> concentration of ICA-1T (1  $\mu$ M). Additional doses were supplied every 24 h during a 3-day incubation period at 37°C. The cells were then lysed and lysates were prepared with the final total protein concentration being  $>2 \mu$ g/ml and then delivered to ActivSignal, LLC (Natick, MA, USA) for further processing and analyzing. The ActivSignal IPAD platform is a multiplex ELISA-based proprietary technology for analyzing the activity of multiple signaling pathways in one reaction with high sensitivity and specificity. The activities of  $>20$  signaling pathways were monitored simultaneously in a single well through assessing the expression or protein phosphorylation of 70 target human proteins.

*Reverse transcription-quantitative PCR (RT-qPCR).* qPCR was performed on RNA isolated from SK-MEL-2 and MeWo cell lysates collected after siRNA treatments for PKC- $\iota$ , c-Jun and FOXO1 against scrambled siRNA as the control. Total RNA was isolated from the cell pellets using RNA lysis buffer which comes with the RNeasy mini kit (74104) from Qiagen (Germantown, MD, USA). RNA was reverse transcribed into cDNA with You-Prime First Strand Beads (27-9264-01) from GE Healthcare UK Ltd. (Buckinghamshire, UK). qPCR was performed on cDNA using the QuantStudio3 Real-Time PCR system (Thermo Fisher Scientific). Gene expression was observed for PKC- $\iota$  (primers: Forward, CACACTTCCAAGC CAAGCG and reverse, GGCGTCCAAGTCCCCATATT), c-Jun (primers: Forward, GTGCCGAAAAGGAAGCTGG and reverse, CTGCGTTAGCATGAGTTGGC), FOXO1 (primers: Forward, ATGGCTTGGTGTCTTTCTTTTCT and reverse, TGTGGCTGACAAGACTTAACTCAA), IL-17E (primers: Forward, GCCACCCTCTGTCTCTTCTC and reverse, CCAGGGCTCTTTCTTCTCC), IL-6 (primers: Forward, GCTCCCTACACATGCCTT and reverse, CCT TCCCTGTGCATGGTGAT), IL-8 (primers: Forward, CAG AGACAGCAGAGCACAC and reverse, ATCAGGAAGGCT GCCAAGAG) and ICAM-1 (primers: Forward, GGAACAA CCGGAAGGTGTA and reverse, CAGTTCCACCCGTTCTG GAG).  $\beta$ -actin (primers: Forward, AGAGCTACGAGCTGCCT GAC and reverse, AGCACTGTGTTGGCGTACAG) was used as an internal control. PCR reactions used SYBR-Green PCR Mix (Applied Biosystems, Foster City, CA, USA). cDNA was denatured at 95°C for 10 min, followed by 40 cycles of

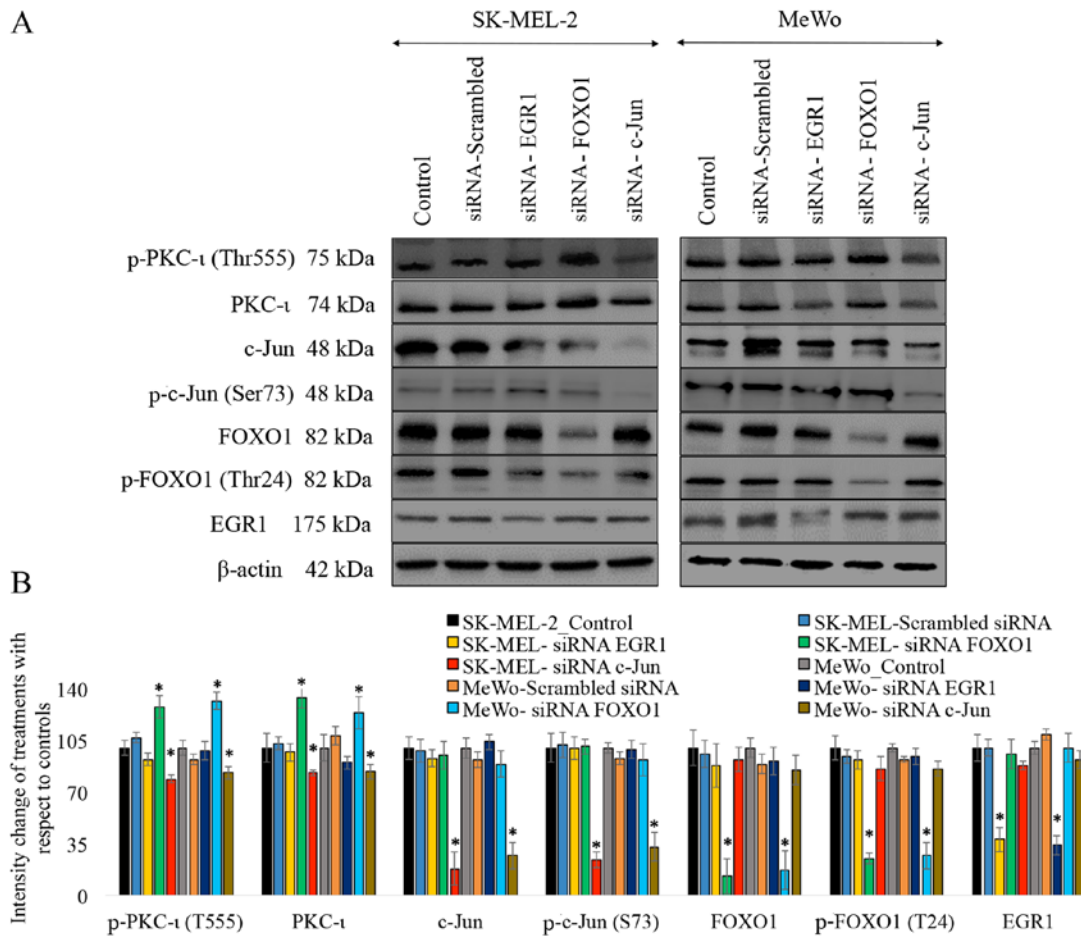


Figure 1. Effect of RNA interference (siRNA) of the transcription factors of EGR1, FOXO1 and c-Jun on the expression of PKC- $\iota$  and targeted transcription factors in melanoma cells (SK-MEL-2 and MeWo). (A) The expression of the protein levels of phosphorylated PKC- $\iota$  (T555), total PKC- $\iota$ , c-Jun, phosphorylated c-Jun (S73), FOXO1, phosphorylated FOXO1 (T24) and EGR1 for the siRNA knockdown of the expression of EGR1, FOXO1 and c-Jun (20 nM of each siRNA for 48 h) for SK-MEL-2 and MeWo cell lines. Total protein (80  $\mu$ g) was loaded into each well and  $\beta$ -actin was used as the internal control in each western blot. (B) Representative densitometry values for the western blots in (A). Experiments (n=4) were performed in each trial and representative bands are shown. Densitometry values are reported as the means  $\pm$  SD. Statistical significance is indicated by an asterisk (\* $P$ ≤0.05).

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 using  $2^{-\Delta\Delta CT}$  (Thermo Fisher Scientific) as explained by Livak and Schmittgen (17).

**Statistical analysis.** All data are presented as the means  $\pm$  SD. Statistical analysis was performed with one- or two-way ANOVA followed by Tukey's HSD test as a multiple comparisons test using the 'VassarStats' web tool for statistical analysis. P-values  $\leq 0.05$  or  $\leq 0.01$  were considered to indicate statistically significant differences.

## Results

The specific sequence of the PRKCI gene (chromosome 3; 170220768-170225128), which was selected to contain the promoter, promoter flank, enhancer and a motif feature, was 4,360 bp in length. The promoter allows TFs to bind and initiate transcription, and the enhancer is a regulatory region in the flank that facilitates TF binding. We narrowed down possible hits by allowing only TFs, which can bind within a dissimilarity margin  $\leq 10\%$ , thereby achieving high specificity. We obtained approximately 70 TF hits to the given target after

comparing the outcomes of PROMO analysis and Genomatix Matinspector. We selected c-Jun, ISGF3, PAX3, EGR1 and FOXO1 as the top 5 TFs with the highest binding probability to the selected sequence of the PRKCI gene.

*FOXO1 and c-Jun stand out as the main transcriptional regulators of PKC- $\iota$  expression in melanoma cells.* As shown in Fig. 1, the results of western blot analysis revealed that transfection with each siRNA for EGR1, FOXO1 and c-Jun significantly reduced expression levels of these proteins. The siRNA for EGR1 decreased the expression of EGR1 by 72% ( $P$ ≤0.05) and 76% ( $P$ ≤0.05) in the SK-MEL-2 and MeWo cells, respectively. The siRNA of FOXO1 decreased the expression of FOXO1 by 87% ( $P$ ≤0.05) and 83% ( $P$ ≤0.05), while the levels of phospho-FOXO1 (T24) decreased by 75% ( $P$ ≤0.05) and 73% ( $P$ ≤0.05) in the SK-MEL-2 and MeWo cells, respectively. The siRNA of c-Jun decreased the expression of c-Jun by 82% ( $P$ ≤0.05) and 73% ( $P$ ≤0.05), while the levels of phospho-c-Jun (S73) decreased by 76% ( $P$ ≤0.05) and 67% ( $P$ ≤0.05) in the SK-MEL-2 and MeWo cells, respectively. These results suggested that transfection with each of the siRNAs knocked down the expression of its respective target. Only the knockdown of FOXO1 and c-Jun was found

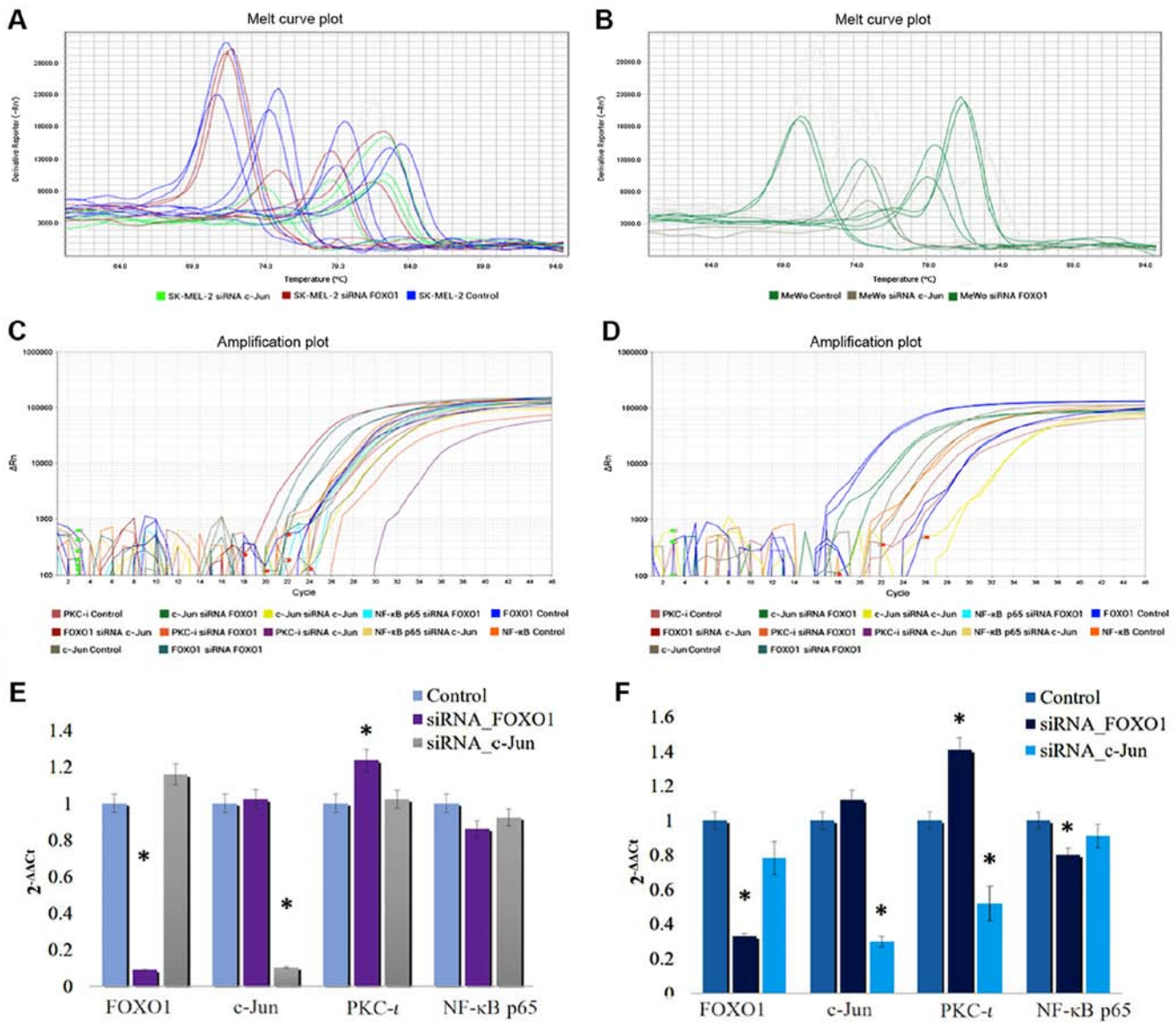


Figure 2. RT-qPCR analysis of siRNA knockdown of FOXO1 and c-Jun for SK-MEL-2 and MeWo cells. mRNA levels of FOXO1, c-Jun, PKC-ι, NF-κB p65 were plotted against β-actin as the internal control. (A and B) Melt curves of the RT-qPCR analysis for tested primers for SK-MEL-2 and MeWo cells, respectively. (C and D) Amplification plots of the said markers for SK-MEL-2 and MeWo cells, respectively. (E and F) Quantitative data for the amplification plots shown in (C and D) for the SK-MEL-2 and MeWo cells, respectively. 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).

to have an effect on the levels of total and phosphorylated PKC-ι (T555) in both cell lines. The knockdown of FOXO1 by siRNA increased the expression of total PKC-ι by 34% (P<0.05) and 24% (P<0.05), while it increased the level of phospho-PKC-ι (T555) by 28% (P<0.05) and 32% (P<0.05) in the SK-MEL-2 and MeWo cells, respectively. The knockdown of c-Jun by siRNA decreased the expression of total PKC-ι by 17% (P<0.05) and 16% (P<0.05), and it decreased the level of phospho-PKC-ι (T555) by 21% (P<0.05) and 17% (P<0.05) in the SK-MEL-2 and MeWo cells, respectively. The knockdown of the expression of the TFs, ISGF3, EGR1 and PAX3, by siRNA was not found to have a significant effect on the expression of total and phosphorylated PKC-ι (T555) in both cell lines. Therefore we decided to omit these 3 TFs, and only FOXO1 and c-Jun were selected for use in the subsequent experiments. As shown in Fig. 1, only the EGR1 negative results were included.

As shown in Fig. 2, the mRNA level of FOXO1 significantly decreased by 90.8 and 67.3% in the SK-MEL-2 and MeWo cells, respectively, following transfection with FOXO1 siRNA. The mRNA level of c-Jun significantly decreased by 89.7 and 30.15% in the SK-MEL-2 and MeWo cells, respectively, following transfection with c-Jun siRNA. These data confirmed the efficiencies and specificities of the applied siRNAs. The RT-qPCR data revealed that the PKC-ι mRNA levels decreased (by 47.2%) following transfection with siRNA for c-Jun in the MeWo cells, even though the PKC-ι levels in the SK-MEL-2 cells were not significantly altered. On the other hand, the PKC-ι mRNA levels were significantly increased by 23.5 and 42% in the SK-MEL-2 and MeWo cells transfected with FOXO1 siRNA, respectively. Therefore, the results of RT-qPCR results tally with the protein expression, which is shown in Fig. 1. These results indicate that the down-regulation of FOXO1 expression enhances PKC-ι expression,

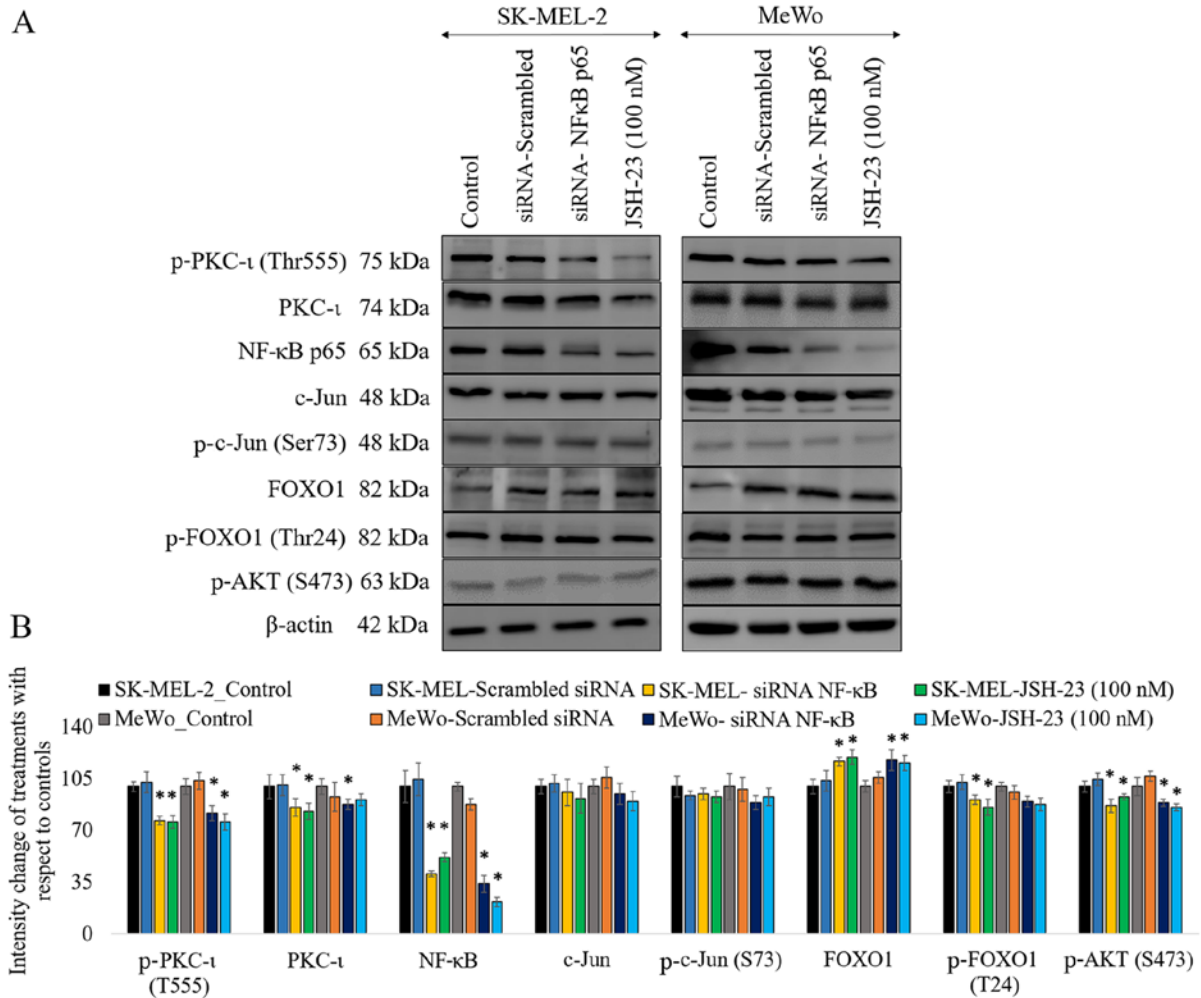


Figure 3. Effect of RNA interference (siRNA of the transcription factor NF- $\kappa$ B) and NF- $\kappa$ B inhibitor JSH-23 on the expression of PKC- $\iota$  and targeted transcription factors in melanoma cells (SK-MEL-2 and MeWo). (A) Protein levels of phosphorylated PKC- $\iota$  (T555), total PKC- $\iota$ , NF- $\kappa$ B p65, c-Jun, phosphorylated c-Jun (S73), FOXO1, phosphorylated FOXO1 (T24) and phosphorylated AKT (S473) for the siRNA knockdown of the expression of NF- $\kappa$ B (20 nM of each siRNA for 48 h) and JSH-23 treatments (100 nM) for SK-MEL-2 and MeWo cell lines. Total protein (80  $\mu$ g) was loaded into each well and  $\beta$ -actin was used as the internal control in each western blot. (B) Representative densitometry values for the western blots in (A). Experiments (n=4) were performed in each trial and representative bands are shown. Densitometry values are reported as the means  $\pm$  SD. Statistical significance is indicated by an asterisk (\* $P$ ≤0.05).

while the silencing of-Jun expression reduces PKC- $\iota$  expression. This indicates that FOXO1 downregulates the expression of PKC- $\iota$  and c-Jun upregulates PKC- $\iota$  expression in melanoma cells *in vitro*.

**FOXO1 holds the key to PKC- $\iota$  expression in melanoma cells.** As shown in Fig. 3, the results of western blot analysis revealed that the knockdown of the expression of NF- $\kappa$ B by siRNA significantly decreased the levels of phosphorylated PKC- $\iota$  by 23% ( $P$ ≤0.05) and 18% ( $P$ ≤0.05) in the SK-MEL-2 and MeWo cells, respectively. The levels of total PKC- $\iota$  significantly decreased by 14% ( $P$ ≤0.05) and 12% ( $P$ ≤0.05) in the SK-MEL-2 and MeWo cells, respectively. Of note, FOXO1 expression increased by 17% ( $P$ ≤0.05) and 18% ( $P$ ≤0.05) in the SK-MEL-2 and MeWo cells, whereas the levels of phosphorylated FOXO1 decreased in the cells transfected with NF- $\kappa$ B siRNA. As we have reported previously, the inhibition of PKC- $\iota$  significantly downregulated the PI3K/AKT pathway, thereby suppressing the activation of AKT (3). Phosphorylated AKT (S473) phosphorylates FOXO1 to cause nuclear exclusion and thereby the degradation of FOXO1. This explains the elevated

levels of active FOXO1 that are due to the downregulation of phospho-AKT upon the downregulation of NF- $\kappa$ B caused by PKC- $\iota$  knockdown by siRNA. In this study, the silencing of NF- $\kappa$ B decreased the levels of phosphorylated AKT (S473) by 13% ( $P$ ≤0.05) and 11% ( $P$ ≤0.05) in the SK-MEL-2 and MeWo cells, respectively. Notably, the levels of phosphorylated c-Jun (S73) and total c-Jun were not significantly altered upon NF- $\kappa$ B knockdown. Similar results were obtained with NF- $\kappa$ B inhibition using a well-known NF- $\kappa$ B inhibitor, JSH-23 (100 nM).

**Results of ELISA confirm an interplay between the PI3K/AKT, JNK, NF- $\kappa$ B and STAT signaling pathways upon PKC- $\iota$  inhibition to coordinate the regulation of its expression.** We used ICA-IT (with the tested IC<sub>50</sub> concentration of 1  $\mu$ M) to specifically inhibit PKC- $\iota$ , allowing us to obtain a broad picture of how multiple pathways may influence melanoma cells *in vitro* as a result of PKC- $\iota$  inhibition related to PKC- $\iota$  regulation. IPAD assay is an array-based ELISA allowing the simultaneous detection of multiple proteins. As shown in Fig. 4, the caspase-3, CD44, CHOP, E-cadherin, I $\kappa$ B $\alpha$ , Myc, NOTCH, p-4E-BP1 (T37/46), p-AKT (S473), p- $\beta$ -catenin (S33/37),

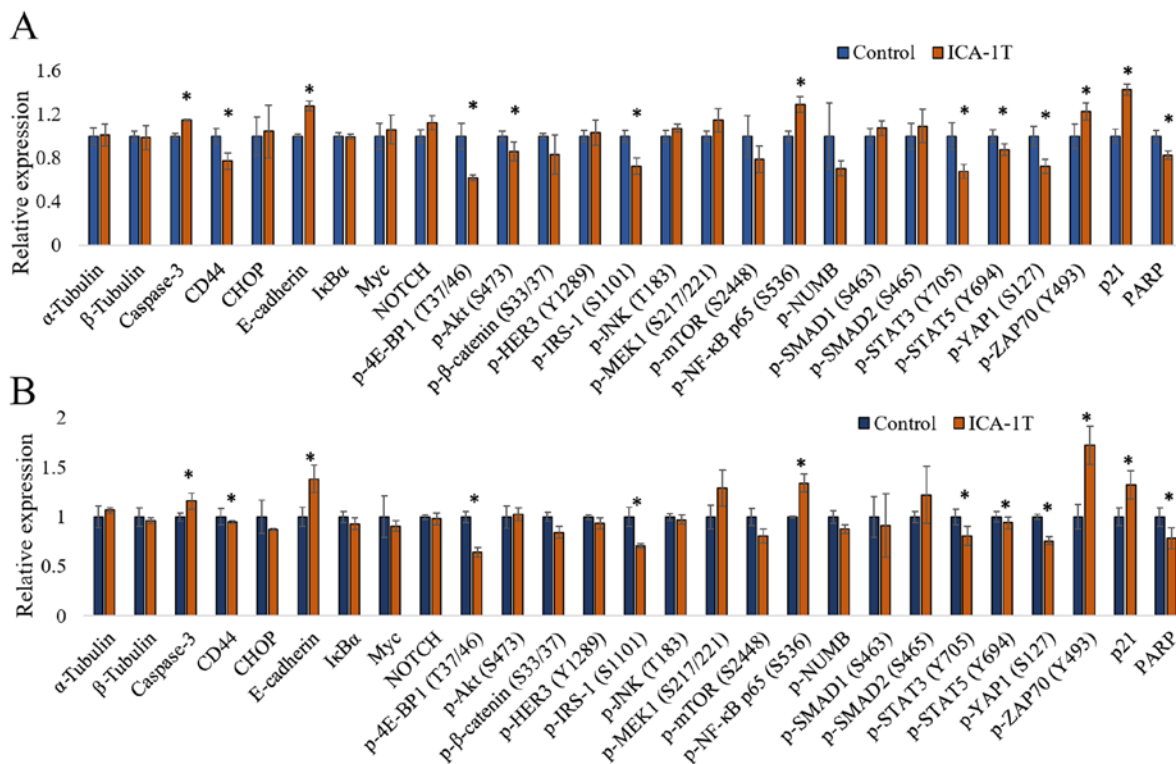


Figure 4. Immuno-paired antibody detection assay (IPAD) for melanoma cells (SK-MEL-2 and MeWo). (A and B) The expression of IPAD assay targets for SK-MEL-2 and MeWo cell lines, respectively. Approximately  $1 \times 10^5$  cells were cultured in T75 flasks and 24 h post-plating, fresh medium was supplied and the cells were treated with either volume of sterile water (control) or  $IC_{50}$  concentration of ICA-1T ( $1 \mu M$ ). Additional doses 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  $>2 \mu g/ml$  and then sent them 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, I $\kappa$ B $\alpha$ , Myc, NOTCH, p-4E-BP1 (T37/46), p-AKT (S473), p- $\beta$ -catenin, p-HER3, p-IRS-1, p-JNK, p-MEK1, p-mTOR, p-NF- $\kappa$ B, p-NUMB, p-SMAD1, p-SMAD2, p-STAT3, p-STAT5, p-YAP1, p-ZAP70, p21 and PARP.  $\alpha$ -tubulin and  $\beta$ -tubulin were used as internal controls in each trial. Experiments (n=3) were performed in each cell lines and the means  $\pm$  SD are plotted. Statistical significance is indicated by an asterisk (\* $P \leq 0.05$ ).

p-HER3 (Y1289), p-IRS-1 (S1101), p-JNK (T183), p-MEK1 (S217/221), p-mTOR (S2448), p-NF- $\kappa$ B p65 (S536), p-SMAD1 (S463), p-SMAD2 (S465/467), p-STAT3 (Y705), p-STAT5 (Y694), p-YAP1 (S127), p-ZAP70 (Y493), p21 and PARP levels were reported upon ICA-1T treatments against the controls for both cell lines. The caspase-3, E-cadherin, p-NF- $\kappa$ B p65 (S536), p-ZAP70 (Y493) and p21 levels significantly increased upon PKC- $\iota$  inhibition, while the CD44, p-4E-BP1 (T37/46), p-AKT (S473), p-IRS-1 (S1101), p-STAT3 (Y705), p-STAT5 (Y694), p-YAP1 (S127) and PARP levels significantly decreased.

*IL-6, IL-8, IL-17E and ICAM-1 may participate in PKC- $\iota$  regulation in an autocrine manner through transcriptional activation/deactivation.* As shown in Fig. 5, the western blot cytokine expression profile for the two melanoma cell lysates exhibited a significant decrease in the levels of IL-6 and IL-8, while the levels of IL-17E and ICAM-1 increased in the cells transfected with PKC- $\iota$  siRNA (siRNA for PKC- $\iota$ , 20 nM) compared to the control (scrambled siRNA, 20 nM). We also found CXCL-1, CXCL-12, GM-SCF, MIF and Serpin in detectable levels, although these were not significantly altered due to PKC- $\iota$  knockdown. The results of the RT-qPCR analysis of the same lysates are shown in Fig. 6. Since only IL-6, IL-8, IL-17E and ICAM-1 exhibited a significant change in expression upon PKC- $\iota$  silencing by siRNA, we only tested these for the

mRNA levels in the RT-qPCR experiments. These RT-qPCR data revealed how the mRNA levels of PKC- $\iota$  significantly decreased by 35.6 and 56.7% in the SK-MEL-2 and MeWo cells, respectively following transfection with PKC- $\iota$  siRNA. We observed a significant decrease in the mRNA levels of both IL-6 and IL-8, with a significant increase in the levels of IL-17E and ICAM-1 upon the knockdown of expression of PKC- $\iota$  in both cell lines. In addition, we found that the FOXO1 mRNA levels increased significantly by 36.1 and 21.5% in the SK-MEL-2 and MeWo cells, respectively; the c-Jun mRNA levels decreased by 8 and 15.5% in the SK-MEL-2 and MeWo cells, respectively. These data confirm the association between PKC- $\iota$ , FOXO1 and c-Jun presented in Figs. 1 and 2.

Fig. 7 shows a schematic summary of the regulation of the expression of PKC- $\iota$  in melanoma based on the current and previous data (3,4). This model depicting the interactions between NF- $\kappa$ B, PI3K/AKT/FOXO1, JNK/c-Jun and STAT3/5 signaling pathways during the PKC- $\iota$  regulation. It is shown that PKC- $\iota$  plays a very important role in the regulation of its expression through the transcriptional activation/deactivation of c-Jun and FOXO1. PKC- $\iota$  is overexpressed as a result of c-Jun transcriptional activity with the help of pro-survival, oncogenic PI3K/AKT, NF- $\kappa$ B, STAT3/5 signaling cascades in melanoma cells. Specific inhibitors of PKC- $\iota$  initiates a

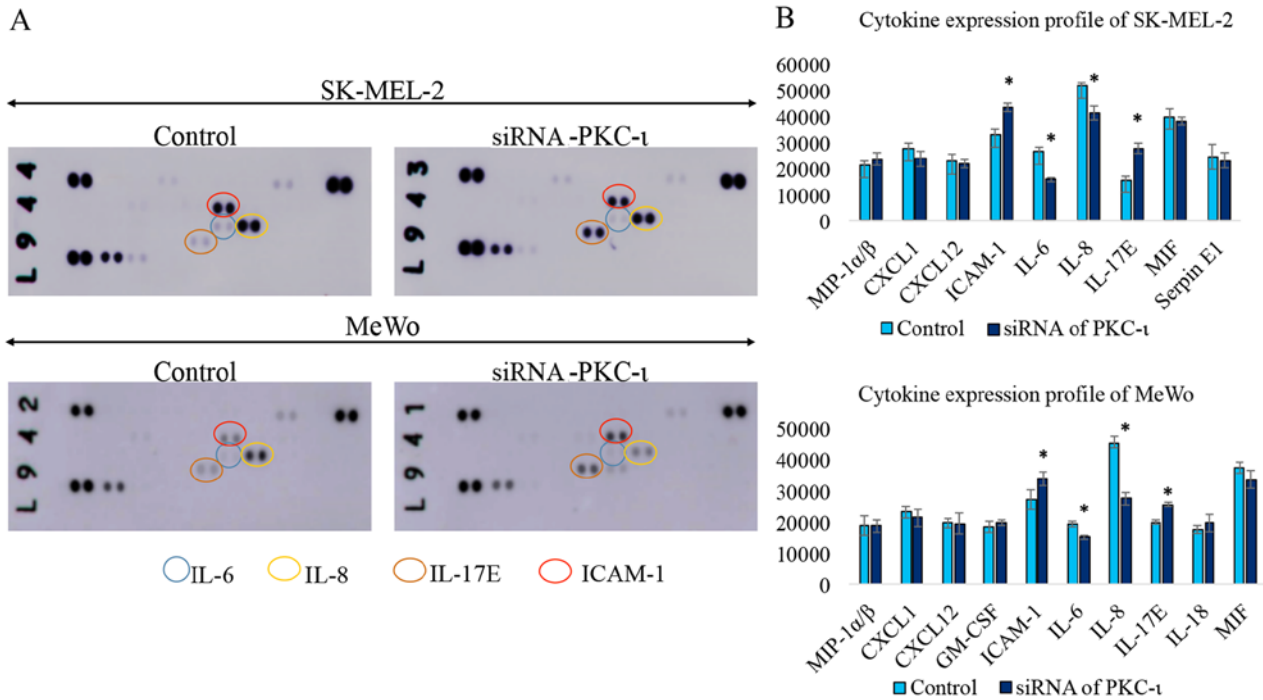


Figure 5. Cytokine expression analysis of melanoma cells upon PKC- $\iota$  knockdown of expression. (A) The Western blot array of the PKC- $\iota$  silenced SK-MEL-2 and MeWo cells against the controls. (B) The quantified results of the western blots shown in (A) for the SK-MEL-2 and MeWo cells, respectively. IL-6, IL-8, IL-17E, ICAM-1, CXCL-1, CXCL-12, GM-CSF, MIF and Serpin were found in detectable levels in western blot analysis for the two melanoma cell lysates. Experiments (n=3) were performed in each cell lines and the means  $\pm$  SD are plotted. Statistical significance is indicated by an asterisk (\* $P$ <0.05).

disruption to rapid PKC- $\iota$  expression cycle where the reduced activity of PKC- $\iota$  downregulates the NF- $\kappa$ B pathway and its transcriptional activity thereby decreases the expression of IL-6 and IL-8. The activity of AKT decreases as a result of lacking the stimulation from cytokines, such as IL-6, IL-8 and TNF- $\alpha$ , which leads to the upregulation of FOXO1, which turns out to be the most important TF regulating PKC- $\iota$  expression after the disruption initiated as a result of PKC- $\iota$  inhibition. FOXO1 negatively regulates the expression of PKC- $\iota$  and also diminishes the JNK activity to retard its activation of c-Jun. We found c-Jun as the transcription component which upregulates PKC- $\iota$  expression. This process continues and leads to the further downregulation of NF- $\kappa$ B, c-Jun and the upregulation of FOXO1, which leads to the continuation of the diminution of PKC- $\iota$  expression. As a result, the total PKC- $\iota$  level decreases in melanoma cells. These findings strongly support our previous data where a reduction in total PKC- $\iota$  levels was observed upon the specific inhibition using PKC- $\iota$  inhibitors, such as ICA-1T and ICA-1S (3,4).

## Discussion

In our previous study, we demonstrated that ICA-1T and ICA-1S inhibited PKC- $\iota$  by selectively binding to a druggable allosteric site within the C-lobe of PKC- $\iota$  kinase domain, thereby blocking the activity of PKC- $\iota$ . These inhibitors reduced cell proliferation, migration and invasion, while inducing apoptosis in melanoma *in vitro* via the downregulation of the NF- $\kappa$ B pathway. Therefore, we identified PKC- $\iota$  as a major component responsible for inducing cell growth, differentiation, survival and EMT promotion in melanoma, as a result of PKC- $\iota$  specific inhibitor applications (3,4). Apart

from these findings, we noted that the inhibition of PKC- $\iota$  leads to a decrease in its own expression. This indicates that PKC- $\iota$  plays a role in its expression in melanoma. The goal of the current study was therefore to identify the PKC- $\iota$  regulatory mechanisms in melanoma *in vitro*.

The PRKCI gene is located on chromosome 3 (3q26.2), a region identified as an amplicon with the tendency to undergo replication events (18). To identify TFs which regulate the PRKCI gene, we selected a specific sequence which includes the PRKCI promoter with a motif feature, promoter flank and an enhancer. This area provides the optimal platform for TFs to bind to regulate the transcription, so the selection of this sequence is highly justified. Possible TF bindings were predicted using two different systems: PROMO and Genomatix Matinspector. Through these, we identified 5 TFs, including FOXO1 and c-Jun. We systematically silenced these TFs to analyze the downstream effect on PKC- $\iota$  expression.

c-Jun is a transcription factor that combines with AP-1 and c-Fos to form an early response transcription factor complex (19). c-Jun is activated by phosphorylation at S63 and S73 by c-Jun N-terminal kinases (JNKs), and c-Jun expression is regulated by various extracellular stimuli, such as cytokines (20). Extracellular signal-regulated kinase (ERK) increases c-Jun transcription (21). c-Jun is the first discovered oncogenic TF that is associated with metastatic breast cancer, non-small cell lung cancer and several other types of cancer (22-24). Phosphorylation at S63 and S73 activates c-Jun, thereby increasing transcription of c-Jun targeted genes. c-Jun promotes the oncogenic transformation of 'ras' and 'fos' in several types of cancer (25,26). In addition, FOXO1 regulates gluconeogenesis, insulin signaling and adipogenesis. Phosphorylation plays a key role in the function of



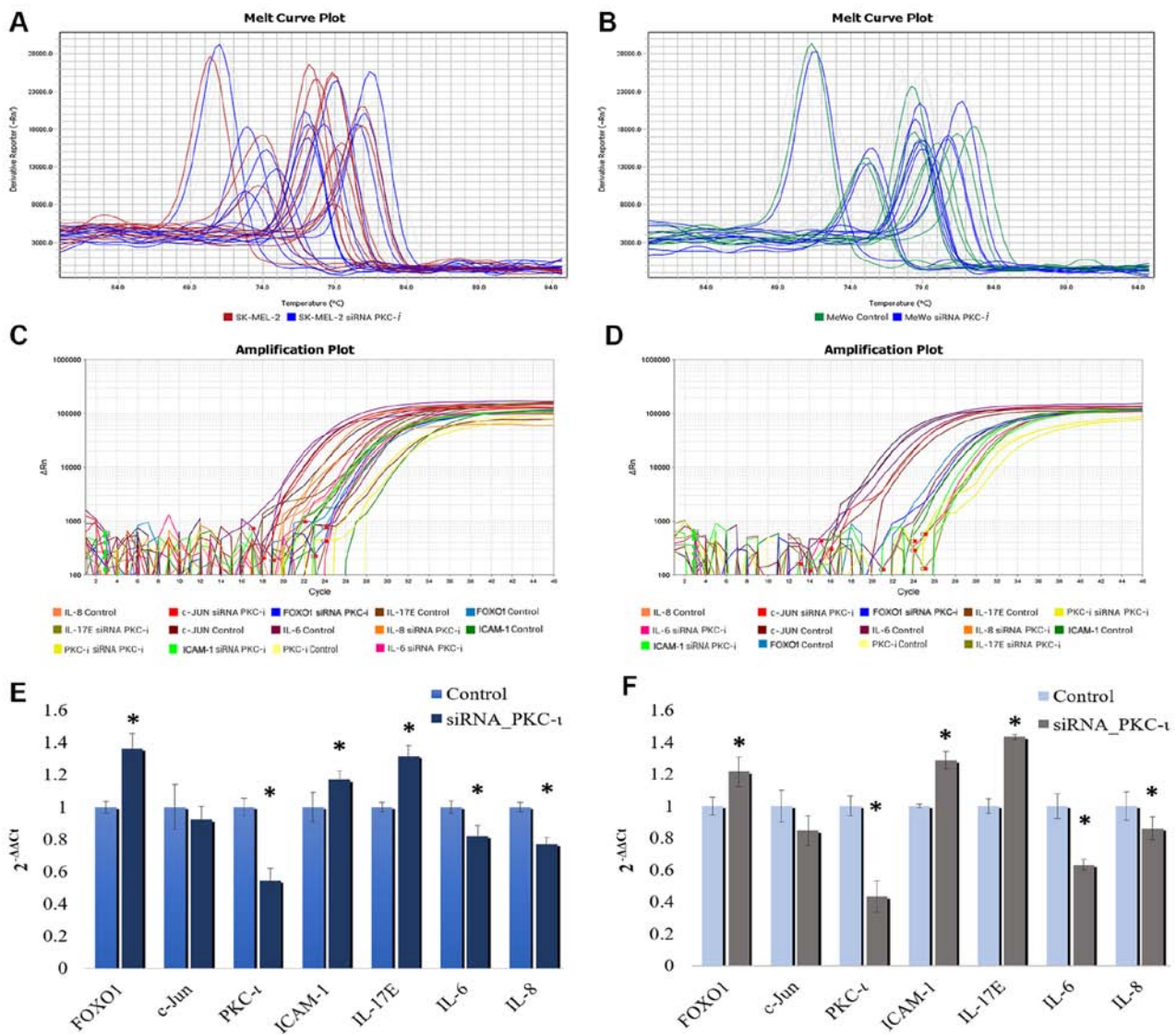


Figure 6. RT-qPCR analysis of cytokines (IL-6, IL-8, IL-17E and ICAM-1), FOXO1, c-Jun for PKC-ι siRNA knockdown in SK-MEL-2 and MeWo cells. (A and B) Melt curves of the RT-qPCR analysis for tested primers for SK-MEL-2 and MeWo cells, respectively. (C and D) Amplification plots of the said markers for SK-MEL-2 and MeWo cells, respectively. (E and F) Quantitative data for the amplification plots shown in (C and D) for the SK-MEL-2 and MeWo cells, respectively. 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).

FOXO1 (27,28). AKT phosphorylates FOXO1 at T24, which causes FOXO1 to drive nuclear exclusion, leading to ubiquitination (29,30). Therefore, the phosphorylation of FOXO1 is an indication of its downregulation. FOXO1 is a well-known, bona fide tumor suppressor (31-33). It plays a regulatory role in both the intrinsic and extrinsic pathways of apoptosis in many types of cancer, exhibiting an association between FOXO dysregulation and cancer progression (34,35). Furthermore, the overexpression of FOXO1 decreases cancer cell proliferation and inhibits migration and tumorigenesis. *In vitro* and *in vivo* experiments have proven this tumor suppressing activity (36). Importantly, FOXO1 can also be downregulated by ERK1/2 and PKC-ι, in addition to AKT (33). In the current study, we demonstrate that, due to PKC-ι inhibition, the availability of active PKC-ι decreases so that it becomes ineffective at deactivating FOXO1 through phosphorylation. Importantly, this is one of the direct involvements of PKC-ι in its own expression regulation and PKC-ι inhibition that leads to continuous upregulation of FOXO1.

On the other hand our previous data showed that PKC-ι inhibition significantly downregulated the PI3K/AKT pathway, thereby suppressing the activation of AKT (3,4). In this study, we provide additional data for the downregulation of NF-κB, which reduces the activity of AKT. This affects FOXO1, as shown by the significantly higher levels of total FOXO1, while a reduction in its phosphorylated levels was observed, suggesting that NF-κB downregulation upregulates FOXO1 activity. The elevated levels of FOXO1 negatively influenced PKC-ι expression and phosphorylation as shown in Fig. 3 as result of NF-κB depletion. This further confirms our previous observations with PKC-ι inhibition with ICA-1T and ICA-1S, where total PKC-ι, phosphorylated PKC-ι, NF-κB activation and activated AKT (S473) were significantly reduced (3). These results could be due to the tight regulation of PKC-ι expression by FOXO1, which retards PRKCI from transcription. Such results confirmed that FOXO1 is a major regulator which suppresses the expression of PKC-ι regulation. Of note, the c-Jun and phosphorylated c-Jun (S63) levels

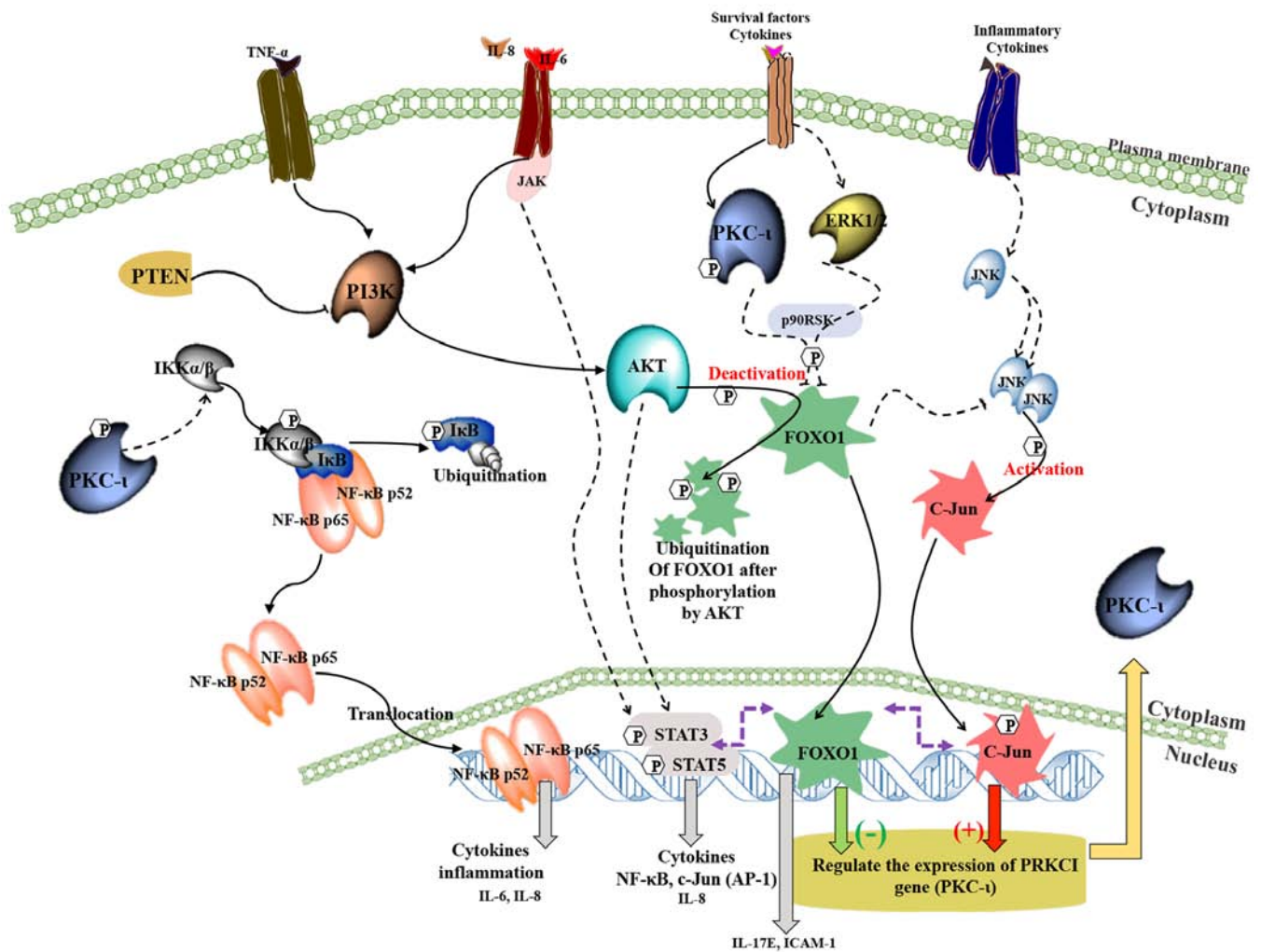


Figure 7. A schematic summary of the regulation of the expression of PKC- $\iota$  in melanoma. This model depicting how the crosstalk takes place between NF- $\kappa$ B, PI3K/AKT/FOXO1, JNK/c-Jun and STAT3/5 signaling pathways during the PKC- $\iota$  regulation. It is shown that PKC- $\iota$  plays 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. In melanoma cancer cells, PKC- $\iota$  is overexpressed as a result of c-Jun transcriptional activity with the help of pro-survival, oncogenic PI3K/AKT, NF- $\kappa$ B, STAT3/5 signaling cascades. The inhibition of PKC- $\iota$  initiates a disruption to rapid PKC- $\iota$  expression cycle where the reduced activity of PKC- $\iota$  downregulates the NF- $\kappa$ B pathway and its transcriptional activity, which in turn depletes the expression of IL-6 and IL-8. The activity of AKT decreases as a result of lacking the stimulation from cytokines, such as IL-6, IL-8 and TNF- $\alpha$ , which leads to the upregulation of FOXO1, which turns out to be the most important TF regulating PKC- $\iota$  expression after the disruption initiated as a result of PKC- $\iota$  inhibition. FOXO1 negatively regulates the expression of PKC- $\iota$  and also diminishes the JNK activity to retard its activation of c-Jun. We found c-Jun as the transcription component which upregulates PKC- $\iota$  expression. The downregulation of IL-6 and IL-8 expression leads to the impaired STAT3/5 signaling, which causes c-Jun transcriptional reduction. This whole process continues and leads to the further downregulation of NF- $\kappa$ B, c-Jun and the upregulation of FOXO1, which leads to the continuation of the diminution of PKC- $\iota$  expression. As a result, the total PKC- $\iota$  level decreases in melanoma cells. These findings strongly support our previous data where a reduction in total PKC- $\iota$  levels was observed upon the specific inhibition using PKC- $\iota$  inhibitors, such as ICA-1T and ICA-1S (3,4).

were not significantly altered as a result of NF- $\kappa$ B siRNA knockdown. This suggests that NF- $\kappa$ B downregulation does not affect PKC- $\iota$  expression through c-Jun. Instead, c-Jun protects cancer cells from apoptosis by cooperating with NF- $\kappa$ B to prevent apoptosis upon TNF- $\alpha$  stimulation (19). We have previously shown how TNF- $\alpha$  upregulates NF- $\kappa$ B, phospho-AKT and PKC- $\iota$  expression in these two melanoma cell lines (3). However, the data from the current study suggest that the TNF- $\alpha$  downstream target is mainly FOXO1, where it 'switches off' through the phosphorylation of elevated AKT. The inhibition of PKC- $\iota$  diminishes this AKT activation, thereby upregulating FOXO1 activity.

On the other hand, our systematic silencing of c-Jun, FOXO1 and EGR1 revealed that (Figs. 1 and 2) c-Jun also seemed to regulate PKC- $\iota$  expression, apart from FOXO1. To

explain the crosstalk between these cell signaling pathways in relation to PKC- $\iota$  regulation, we conducted two other *in vitro* experiments, ELISA using IPAD assay and a cytokine array. These findings demonstrated links between PKC- $\iota$  expression with the cytokines, IL-6, IL-8, IL-17E and ICAM-1, along with some other key cellular signaling points.

As shown in Fig. 4, the IPAD ELISA data revealed a significant increase in the levels of caspase-3, E-cadherin, p-NF- $\kappa$ B p65 (S536), p-ZAP70 (Y493) and p21 levels upon PKC- $\iota$  inhibition, while the levels of CD44, p-4E-BP1 (T37/46), p-AKT (S473), p-IRS-1 (S1101), p-STAT3 (Y705), p-STAT5 (Y694), p-YAP1 (S127) and PARP levels significantly decreased. We have already shown in our previous study, using various apoptotic markers, that PKC- $\iota$  inhibition induced the apoptosis of melanoma cells (3,4). This is again evident from increases in the

levels of caspase-3 and the cleavage of PARP in the IPAD assay. Additionally, PKC- $\iota$  inhibition delayed EMT in melanoma and we observed elevated levels of E-cadherin with downregulation of Vimentin and CD44 expression (3,4). Therefore, this IPAD assay provided additional data for the results we have shown in our previous studies. Phosphorylation at S536 on the NF- $\kappa$ B p65 transactivation domain is an indication of dimerization of NF- $\kappa$ B subunits. Since PKC- $\iota$  inhibition downregulates NF- $\kappa$ B translocation to the nucleus, phospho-NF- $\kappa$ B levels increase in order to diminish the effect of PKC- $\iota$  inhibition. However, elevated FOXO1 does not allow NF- $\kappa$ B to take over the control since it is missing the crucial assistance needed from PKC- $\iota$  due to its inhibition from ICA-1T and ICA-1S inhibitors. Aberrant STAT3/5 activity has been shown to be connected to multiple types of cancer (37-42). The cytokines, IL-6 and IL-5, are known to upregulate STAT signaling, which induces cell survival in many types of cancer (37,38,43). Upregulated STAT3 increases the transcription of c-Jun (37,44). Our IPAD results indicated that STAT3 and STAT5 activities were downregulated due to PKC- $\iota$  inhibition, suggesting that c-Jun expression can be retarded. Few studies, including the one by Hornsveld *et al*, have provided connections between the JNK pathway and FOXO1, elaborating its tumor suppressing features for weakening JNK activity (33,45,46). However, JNK activates c-Jun. The data from our western blot and RT-qPCR analysis demonstrated that c-Jun depletion diminished PKC- $\iota$  expression, which suggested that c-Jun acts as an activator of PKC- $\iota$  expression. It is therefore evident that both FOXO1 and c-Jun are involved in regulating PKC- $\iota$  expression. The results suggest that FOXO1 plays a major role over c-Jun only upon PKC- $\iota$  inhibition, possibly through multiple mechanisms, such as the reduction of JNK signaling, retarding PKC- $\iota$  expression and cell cycle arrest. Upregulated FOXO1 is well known to induce cell cycle arrest by promoting the transcription of cell cycle kinase inhibitors or cyclin-dependent kinase inhibitor (CKI). p21 and p27 are two of the most well-known downstream CKIs induced by FOXOs (33,45). Notably, FOXO1 is also believed to induce anoikis, which is apoptosis that occurs when cells detach from the extracellular matrix. Our IPAD-ELISA results revealed significantly elevated levels of p21 by approximately 25% in both cell lines, suggesting that the inhibition of PKC- $\iota$  induces cell cycle arrest through FOXO1. This is another indirect downstream effect of PKC- $\iota$  involvement in its expression where inhibition of PKC- $\iota$  enhances FOXO1 anti-tumor activity.

As shown in Fig. 7, our results summarize that PRKCI expression is negatively regulated by FOXO1 and positively regulated by c-Jun. When PKC- $\iota$  is inhibited, the following series of downstream effects take place. The downregulation of NF- $\kappa$ B activity decreases the levels of phospho-AKT (S473). As a result of a low activity of AKT along with diminished levels of PKC- $\iota$ , the phosphorylation of FOXO1 is reduced and therefore active FOXO1 (unphosphorylated FOXO1) levels are being elevated. Elevated FOXO1 suppresses PRKCI gene expression similar to 'switch off' effect. The downregulation of PKC- $\iota$  also diminishes STAT3/5 activity, as shown by the IPAD assay data. STAT3 and STAT5 are known to upregulate the transcription of c-Jun and NF- $\kappa$ B (44,47). Therefore, it is evident that PKC- $\iota$  inhibition induces the downregulation of STAT3/5, which decreases the transcription and activation

c-Jun. These data suggest that PKC- $\iota$  levels were decreased when c-Jun expression is silenced by siRNA (Fig. 1), and as shown by RT-qPCR in Fig. 6, the knockdown of PKC- $\iota$  decreased c-Jun mRNA expression, but not significantly.

As shown in Fig. 5, further *in vitro* experiments demonstrated changes in cytokine expression (IL-6, IL-8, IL-17E and ICAM-1) in melanoma cells upon PKC- $\iota$  knockdown. As shown by the results of both western blot and RT-qPCR analyses, the protein levels of IL-6 and IL-8 (as well as their mRNA levels) decreased, while the levels of IL-17E and ICAM-1 increased significantly in both cell lines upon PKC- $\iota$  knockdown by siRNA. This suggests that the PKC- $\iota$  self-regulated expression cycle is involved in autocrine signaling. The cellular environment of a tumor, and in particular melanoma, is frequently exposed to various inflammatory factors and immune cells. The effect of these factors function to either promote chronic inflammation or engage in anti-tumor activity (48). Cytokines are examples of these inflammatory factors; they play an essential role in regulating tumor microenvironments (49). Cytokines utilize several signaling pathways to carry out their functions. They act in order to promote or dysregulate tumor progression and metastasis. Cytokines, such as CXCL-1, CXCL-12, IL-18, CXCL-10, IL-6 and IL-8 promote cancer progression by facilitating metastasis. CXCL1, also known as melanoma growth-stimulatory activity/growth-regulated protein  $\alpha$ , is secreted by melanoma cells and is associated with roles in wound healing, angiogenesis and inflammation. In particular, it has been linked to tumor formation (50). High levels of CXCL10/CXCR3 expressed in melanoma have been linked to metastasis regulation (50). CXCL10 plays an important role in promoting tumor growth and metastasis (51). CXCL12, also known as stromal-derived factor-1, utilizes the receptors CXCR4 and CXCR7. CXCL12 and its receptors have been linked to roles in regulating tumor metastasis. CXCL-1, CXCL-10, CXCL-12 and IL-18 levels were not significantly altered due to PKC- $\iota$  depletion.

IL-6 is a very important cytokine and contributes to the degradation of I $\kappa$ B- $\alpha$ , leading to the upregulation of NF- $\kappa$ B translocation. We have previously shown that PKC- $\iota$  stimulates NF- $\kappa$ B translocation through I $\kappa$ B- $\alpha$  degradation (3). The translocation of NF- $\kappa$ B to the nucleus induces cell survival through the transcription of various survival factors as well as other cytokines (37,43,52). IL-8 is an example of such a cytokine. IL-8 plays a role in regulating polymorphonuclear neutrophil mobilization. In melanoma, IL-8 has been attributed to extravasation, a key step in metastasis. Studies have shown that the expression of IL-8 in melanoma is regulated via NF- $\kappa$ B. When NF- $\kappa$ B is translocated to the nucleus, IL-8 expression increases, leading to the promotion of a more favorable microenvironment for metastasis (53,54). The results of this study indicated that both IL-6 and IL-8 expression levels decrease upon transfection with PKC- $\iota$  siRNA. As is summarized by the diagram in Fig. 7, IL-6 expression is regulated by NF- $\kappa$ B, and IL-8 expression is regulated by both NF- $\kappa$ B and STATs. Our data justified how the PKC- $\iota$  inhibition/knockdown downregulates the NF- $\kappa$ B and STAT signaling pathways. The results suggested that IL-6 and IL-8 play an important role in upregulating PKC- $\iota$  expression, activating c-Jun, while deactivating FOXO1.

Some cytokines promote anti-tumor activity by utilizing an immune response. ICAM-1 plays a key role in the immune response, including antigen recognition and lymphocyte activation (55,56). ICAM-1 has been linked to the inhibition of tumor progression through the inhibition of the PI3K/AKT pathway. The inhibition of this pathway via ICAM-1 exposes tumor cells to death via cytotoxic T-lymphocytes (56). Clinical research has also proven that, within the first 5 years of ovarian cancer diagnosis, ICAM-1 expression inhibition is associated with an increased risk of metastasis (55,56). Another anti-tumor cytokine is IL-17E. IL-17E belongs to a family of cytokines known as IL-17. In various forms of cancer, including melanoma and pancreatic cancers, treatment with recombinant IL-17E has been shown to decrease tumor growth (57,58). The upregulation of IL-17E is linked to the increased expression of TH17 cells. T cells, such as TH17 have been implicated in the inhibition of tumor-infiltrating effector T cells. The exact mechanism of IL-17E function in the anti-tumor effect has not been thoroughly explored (59). Notably, the results of our western blot and RT-qPCR analyses indicated that ICAM-1 and IL-17E protein and mRNA expression increased upon the silencing of PKC- $\iota$  by siRNA. This confirms that anti-tumor/pro-apoptotic signaling is upregulated upon the knockdown of oncogenic PKC- $\iota$  via an autocrine manner through IL-17E and ICAM-1. Moreover, the results suggest that IL-17E and ICAM-1 play an important downregulatory role in the regulation of PKC- $\iota$  expression along with FOXO1, opposite to c-Jun, IL-6 and IL-8.

In conclusion, our overall results demonstrate PKC- $\iota$  itself to play an important role in its expression in a complex signaling network through the transcriptional activation/deactivation of c-Jun and FOXO1. The reduced activity of PKC- $\iota$  due to its specific inhibition, downregulates the NF- $\kappa$ B pathway and its transcriptional activity, which turns out to 'strike' the expression of IL-6 and IL-8. As a result, the activity of AKT decreases, which leads to the upregulation of FOXO1. FOXO1 is the most important TF regulating PKC- $\iota$  expression upon receiving stimulation from IL-17E and ICAM-1. FOXO1 negatively regulates the expression of PKC- $\iota$ , diminishing JNK activity to retard the activation of c-Jun. IL-6 and IL-8 expression are downregulated via PKC- $\iota$ -mediated NF- $\kappa$ B transcriptional activity reduction. This leads to STAT3/5 signaling downregulation, reducing c-Jun expression. This whole process continues and leads to the further downregulation of NF- $\kappa$ B, c-Jun and upregulation of FOXO1, which leads to the continuation of the depletion of PKC- $\iota$  expression. As a result of this sequence of events, the total PKC- $\iota$  level decreases in melanoma cells, which began as a result of PKC- $\iota$  inhibition. These results indicate that PKC- $\iota$  is being regulated in a rather complex manner, which involves itself as a key component. PKC- $\iota$  inhibition leads to a decrease in its own production, and during this process, PKC- $\iota$  inhibition also triggers multiple anti-tumor/pro-apoptotic signaling. This makes PKC- $\iota$  one of the central key point of interest to specifically target and diminish as a means of treating melanoma *in vitro*. Therefore, our overall results confirm that PKC- $\iota$  inhibition using specific and effective inhibitors, such as ICA-1T and ICA-1S, is significantly effective in the treatment of melanoma. The results also strongly suggest that PKC- $\iota$  is a prime novel biomarker that can be targeted to design and develop personalized and targeted therapeutics for melanoma.

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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

AHA, WSR and MAD were involved in the conceptualization of the study. WSR and CAA 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, SB and CAA were involved in the cytokine analysis and sample preparation for IPAD assay and RT-qPCR. CAA, SB and WSR were involved in the RT-qPCR analysis. WSR and SB were involved in the writing of the original draft. MAD was involved in the writing and reviewing of 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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