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Original article

Relationship between Protein kinase C isoforms, Telomerase and Alpha-fetoprotein through PI3K/AKT/mTOR pathway in Hepatocellular carcinoma

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Abstract: Protein kinase C (PKC) family has been an alluring objective for new cancer drug discovery. It has been reported to regulate telomerase in several cancer types. Our team had previously used telomerase to elucidate alpha-fetoprotein (AFP) modulation in hepatocellular carcinoma (HCC). The aim of this study was to investigate the interrelationships among PKC isoforms, telomerase and AFP in HCC. PKCα and PKCδ were the most expressed isoforms in HepG2/C3A, PLC/PRF/5, SNU-387 and SKOV-3 cells. Following the upregulation of AFP using pCMV3-AFP and the human telomerase reverse transcriptase (hTERT) using a construct expressing a wild-type hTERT, and after their inhibition with all-trans retinoic acid and hTERT siRNA each respectively, we found that the expression of PKCα, PKCβI, PKCβII and PKCδ was affected by the variation of AFP and hTERT mRNA levels. An increase in AFP expression and secretion was observed after gene silencing of PKCα, PKCβ, PKCδ, and PKCε in HepG2/C3A. A similar pattern was observed in transfected PLC/PRF/5 cells, however PKCδ isoform silencing decreased AFP expression. Furthermore, telomerase activity was quantified using quantitative telomeric repeat amplification protocol. The variations in hTERT expression and telomerase activity were similar to those of AFP. Further investigation showed that PKC isoforms regulate AFP and hTERT expression levels through PI3K/AKT/mTOR pathway in HepG2/C3A and PLC/PRF/5 cells. Thus, these results show for the first time a possible interrelationship that links PKC isoforms to both AFP and hTERT via PI3K/AKT/mTOR pathway in HCC.

Keywords: Alpha-fetoprotein; Protein kinase C; Telomerase.

1. INTRODUCTION

Hepatocellular carcinoma (HCC) has become the most prevalent of all hepatic malignancies worldwide [1], and ranks as the second cause of cancer-related death in men and the sixth in women [2]. Alpha-fetoprotein (AFP) has been most widely used as a serum diagnostic marker for HCC, and was added into the international guidelines for HCC surveillance [3,4]. AFP is a glycoprotein, that is highly expressed during fetal development and decreases progressively after birth [5]. In HCC patients, levels of AFP >400 ng/mL are considered reliable for confirming an HCC diagnosis [6]. In addition to being a diagnostic indicator, AFP has been identified as a type of growth regulator during tumor progression and oncogenic growth [7]. Several studies have reported that AFP is involved in pleiotropic activities that influence tumorigenesis, cell proliferation and differentiation [8,9]. Moreover, it has been indicated that cytoplasmic AFP induces HCC cell

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proliferation by stimulating the expression of Src and c-myc [10], while extracellular AFP can affect the proliferation and expression of HCC cells through AFP receptors on the cell membrane [11]. Several studies have indicated that the expression of AFP receptors correlates with AFP expression in the cells [12].

AFP/AFP receptors generate calcium ion (Ca^{2+}) influx, after which cyclic adenosine monophosphate (cAMP) increases protease A activity, causing DNA synthesis, which, in turn, leads to tumor cell proliferation [13]. Moreover, recent studies have shown that P21Ras is promoted through cAMP-PKA, Ca2+, tyrosine protein kinase-Ras-mitogen-activated protein kinase, and several signaling pathways [14]. In addition, the phosphatidylinositol 3-kinase (PI3K) catalytic subunit can bind directly to Ras, and activate the PI3K/AKT signaling pathway [15]. The latter plays an important role in inhibiting apoptotic cells and enhancing proliferation by activating multiple downstream effector molecules [16]. Also, AFP can activate PI3K/AKT signaling pathway by inhibiting the function of phosphatase and tensin homolog. On the other hand, it can block the retinoic acid-RAR signaling pathway to promote tumor cell growth [17,18].

Furthermore, several mechanisms have been investigated that are involved in the development of HCC in patients, and accelerating cancer formation. These mechanisms include modifications in the macro- and microenvironments that stimulate cellular proliferation and telomere dysfunction [4]. At the end of eukaryotic chromosomes, telomeres play a crucial role in cellular survival, by maintaining genomic integrity in normal cells [20]. Chromosomal instability increases as telomeres progressively shorten during successive cell divisions. Their integrity is controlled by upregulation and reactivation of the catalytic subunit of the telomerase, the human telomerase reverse transcriptase (hTERT), in 90% of cancer cells [21]. In addition, this enzymatic protein complex, with its reverse transcriptase role, comprises an RNA component known as human telomerase RNA that acts as a template for DNA telomere synthesis, and plays a role in the localization, catalysis, and assembly of the telomerase holoenzyme [22]. In addition to its canonical role, expression regulation, apoptosis, cell proliferation, WNT/β-catenin signaling, PI3K/AKT/mTOR signaling, cell migration, and cell adhesion, also involve telomerase [23]. Several studies have shown that telomerase activity and hTERT expression are regulated by several transcriptional activators and repressors such as c-myc and Sp1 and Mad1 respectively [24], as well as by posttranscriptional regulators such as AKT, c-Abl, and protein kinase C (PKC) by kinase phosphorylation [25].

PKC, a class of serine/threonine kinases with diverse biological functions, plays a crucial role in transmembrane signal induction and transduction of many cellular responses (gene expression, proliferation, differentiation, survival, apoptosis and motility) [26]. Pharmaceutical companies and academic laboratories have recently reported an association between PKCs cancer cell metabolism [27]. Eminent levels of PKC can be found in urinary bladder, prostate, breast and lung cancers[28–32]. PKCs are classified into three groups. "classical" or "conventional" (α, βI, βII and γ), "novel" (δ, ε, η and θ) and "atypical" (ζ, ι and λ) [26]. Unlike other kinases that are implicated in cancer, such as Erk, AKT and JNK, PKC phosphorylation does not correlate mainly with activation status [33]. In fact, several studies have suggested that when the expression of PKC family members is altered, they may function as either oncogenes or tumor suppressors. Although specific carcinogens might induce alterations and phosphorylation in the protein kinase expression [34], the role of PKC in HCC remains unclear. New studies reversed the paradigm and identified PKC as a tumor suppressor [35,36]. Hence, new therapeutic targets for treating human diseases can be identified by understanding the molecular mechanisms through which a particular PKC isoform monitors cellular signaling pathways [37].

2. MATERIALS AND METHOD

Cell lines

Four cancer cell lines were purchased from American Type Cell Culture (Manassas, VA, USA). HepG2/C3A, PLC/PRF/5**,** SNU-387, and SK-OV-3 (or SKOV-3). C3A is a clonal derivative of HepG2 selected for its high production of alpha-fetoprotein (AFP) as well as for its ability to grow in glucose-deficient medium. This cell line strongly expresses telomerase, however, there is no evidence of a hepatitis B virus genome. PLC/PRF/5 are hepatic, adherent epithelial cells that secrete AFP, express telomerase and produce hepatitis B surface antigen (HbAg). The SNU-387 cell line is isolated from a primary hepatocellular carcinoma expressing telomerase but not AFP. In addition, they contain the hepatitis B virus since the DNA of the virus was detected by Southern blot, however, the genomic RNA of the HBV is not expressed. HePG2/C3A, PLC/PRF/5, and SNU-387 were cultured in Dulbecco's Modified Eagle's Medium (DMEM), minimum essential medium eagle (Sigma-Aldrich, Munich, Germany), and DMEM Nutrient Mixture F-12 (*DMEM*/F-12), respectively. SKOV-3 (hTERT negative [-/-]), an ovarian cancer cell line, expressing neither telomerase nor AFP, was cultured in *DMEM*/F-12 (Sigma-Aldrich). All media were supplemented with 10% fetal bovine serum, and 1% antibiotics (penicillin/streptomycin) (Sigma Chemical Co., St. Louis, MO, USA); 1% nonessential amino acids (Sigma, USA) was supplemented for only HepG2/C3A, and were incubated at 37°C in 5% CO_2.

RNA extraction and reverse-transcription polymerase chain reaction

RNA was extracted using the NucleoSpin® RNA extraction kit (Macherey-Nagel, Düren, Germany) from the different cell lines, according to the manufacturer's instructions. The RNA quality and yields were analyzed using the Nanodrop Spectrophotometer. Complementary DNA (cDNA) was then synthetized by reverse transcription of 100ng of total RNA in a 20µl total volume; using the iScript cDNA Synthesis Kit (Bio-Rad, Laboratories,CA, USA).

Semi quantitative reverse transcription polymerase chain reaction (RT-PCR) was conducted, and the genes of interest were amplified in triplicate using 5X FIREPol® Master Mix (Solis BioDyne, Tartu, Estonia). The sequences of the PCR primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PKCα, PKCβI, PKCβII, PKCδ, PKCε, and PKCγ are shown in Table 1.

Reactions were initiated at 95°C for 3 min, followed by 55°C (for PKC isoforms) and 60°C (for GAPDH) for 30 s, and

by 72°C for 1min (for PKC isoforms) or 70°C for 30 s (for GAPDH), and 35 cycles for PKC and GAPDH, followed by a final elongation step at 72°C for 5 min. The amplified DNA was run on 2% agarose gel and visualized using SYBR® Safe DNA Gel Stain (Invitrogen, Life Technologies, USA) using the UVP BioDoc system (Analytik Jena US LLC., Upland, CA, USA). Data analysis and gel image evaluation were conducted using the GelAnalyzer software and GAPDH was used as internal control gene to normalize PKC isoforms' levels.

Table 1. Primers sequences

GAPDH	Reverse: 3'-ACTGGCGTCTTCACCACCATGG-5'
	Forward: 5'-TGGGATGGACTGTGGTCATGAG-3'
PKCα	Reverse: 3'-CCTGTCGGCAAGCATCACCTTT-5'
	Forward: 5'-CGAGGAAGGAAACATGGAACTCAG-3'
PKCβI	Reverse: 3'-AGAGACAAGAGAGACACCTCCAAC-5'
	Forward: 5'-AGAGACAAGAGAGACACCTCCAAC-3'
PKCβII	Reverse: 3'-TCTCATCAGAAAATCAAACATGGATGCAACTTGGC-5'
	Forward: 5'-TGTGGGCGAAATGCTGAAAACTTCGACCGA-3'
PKCδ	Reverse: 3'-ACACACCCACGGTCACCTCAGA-5'
	Forward: 5'-GCATCGCCTTCAACTCCTATGAGCT-3'
PKCε	Reverse: 3'-CCTGAGAGATCGATGATCACATAC-5'
	Forward: 5'-TCAATGGCCTTCTTAAGATCAAAA-3'
PKCγ	Reverse: 3'-CTCATACAATTCCAGGGGGTAGTT-5'
	Forward: 5'-TGACGAAACAGAAGACCCGAAC-3'

Treatment with PKC, AFP and P3IK-AKT pathway inhibitors

Cells were treated with a 5 μM *pan-PKC inhibitor* Go 6983, 10μM PI3K inhibitor PI 828, 100nM AKT inhibitor GSK 690693, 200 nM mTOR inhibitor Rapamycin from Tocris (Tocris Bioscience,Bristol, UK), and 10 and 20 μM AFP inhibitor all-trans retinoic acid (ATRA) purchased from (Millipore Sigma, Burlington, MA, USA).

PKC isoforms and hTERT knockdown

Table 2. siRNA sequences

PKCα	Sense	: 5'-CCAUCCGCUCCACACUAAATT-3'
	Antisense	: 5' -UUUAGUGUGGAGCGGAUGGTT- 3'
PKCβ	Sense	: 5' -CAAGAGCTAAGTAGATGTGTA- 3'
	Antisense	: 5' -AGAGCUAAGUAGAUGUGUATT- 3'
PKCδ	Sense	: 5' -CUCUACCGUGCCACGUUUUTT- 3'
	Antisense	: 5'- AAAACGUGGCACGGUAGAGTT- 3'
PKCε	Sense	: 5' -CGGAAACACCCGUACCUUATT- 3'
	Antisense	: 5' -UAAGGUACGGGUGUUUCCGTG- 3'
PKCγ	Sense	: 5' -ACGCCAUCAAGAUCUUGAATT- 3'
	Antisense	: 5' -UUCAAGAUCUUGAUGGCGUAG- 3'
hTERT	Sense	: 5 '-GGAGCAAGUUGCAAAGCAUTT- 3'
	Antisense	: 5' -AUGCUUUGCAACUUGCUCCAG- 3'

The small interfering RNAs (siRNAs) were used for PKCα, PKCβ, PKCδ, PKCε, PKCγ and hTERT. Cell death siRNA was used as a positive control for transfection and nonsilencing siRNA was used as negative control. These were purchased from Qiagen (Valencia, CA, USA), and the transfection was conducted according to the manufacturer's instructions. Briefly, 10nM siRNA diluted with serum-free medium and Hi-perfect (Qiagen) were added to the wells of 24-well and 6-well plates and incubated at room temperature. After 15 min, HepG2/C3A and PLC/PRF/5 cell were seeded, and incubated for 72 h. PKC isoforms and hTERT siRNA sequences are shown in Table 2. Specific downregulation of PKCα, PKCβ, PKCδ, PKCε, PKCγ and hTERT was confirmed by qRT-PCR (S2_Fig).

AFP quantification

The levels of AFP secreted into the cell media of the different cell lines were measured using the sandwich enzyme-linked immunosorbent assay (ELISA) technique (AFP ELISA kit from (Human Biochemica und diagnostic GmbH Germany) according to the manufacturer's instructions. Briefly, after each treatment the supernatant was collected, diluted 1/50 with serum-free media for HepG2/C3A cells only, and assayed. The optical density was measured at 450nm using an ELISA reader (Thermo Fisher Scientific, Inc).

Quantitative real-time PCR

To evaluate the expression of each isoform, and the effect of the isoform silencing on AFP and hTERT expressions, qRT-PCR was conducted. The QuantiFast SYBR Green PCR Kit (Qiagen) was used to amplify cDNA samples. The primers sequences are shown in Table 3.

Table 3. Primers sequences

PKCα	Reverse primer: 5'TTGTGGTCCTTCACCTCGTG 3'
	Forward primer: 5'ACCATGGCTGACGTTTTCCC 3'
PKCβI	Reverse primer: 5'TCTCTTGTCTCTAGCTTTTGGCT 3'
	Forward primer: 5'GACCTGAAGGCGAACGTGAT 3'
PKCβII	Reverse primer: 5'CTGATGACTTCCTGGTCGGG 3'
	Forward primer: 5'AAAGCTTGTGGGCGAAATGC 3'
PKCδ	Reverse primer: 5'CCTCCTTCATCTTCACGGCA 3'
	Forward primer: 5'CCGTTTCTCCTGGTGGTTGG 3'
PKCε	Reverse primer: 5'GGGCAGGAATGAAGAACCGA 3'
	Forward primer: 5'CGGCGAGGAAATACATGCAC 3'
PKCγ	Reverse primer: 5'CTGGGGTGCAGGATATGACG 3'
	Forward primer: 5'TACGTGAACCCCGACTTCGT 3'
AFP	Reverse primer: 5'GCAGCGCTACACCCTGAGCT 3'
	Forward primer: 5'ACCCTGGTGTTGGCCAGTGC 3'
hTERT	Reverse primer: 5'CTCCCACGACGTAGTCCATG 3'
	Forward primer: 5'CGGAAGAGTGTCTGGAGCAA 3'
GAPDH	Reverse primer: 5'TCCGACGCCTGCTTCACCAC 3'
	Forward primer: 5' TCATCATCTCTGCCCCCTCT 3'

Rotor-Gene Q PCR cycler (Qiagen) was used to amplify cDNA through a PCR program of 35 cycles, with denaturation at 95°C for 10s, followed by annealing at 60°C for 30s, and elongation at 72°C for 10 s and then assessing the melting curve from 55°C to 95°C.

The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative fold gene expression level. PKC isoform, AFP and hTERT expression levels were normalized to the expression of GAPDH serving as internal control gene. All samples were analyzed in at least three independent experiments.

AFP and hTERT expression constructs

AFP expression plasmid pCMV3-AFP was purchased from Sino Biological Inc. and the wild-type (WT) hTERT expression plasmid Pbabe-neo-hTERT and the scramble vector Pbabe-neo were purchased from Addgene (Addgene plasmid #1774, #1775, and #1767 respectively).

After being transformed using the heat shock technique, the *Escherichia coli* DH5α strain was spread using a sterile loop onto a prepared lysogeny broth (LB) agar plate containing ampicillin to isolate individual colonies of bacteria carrying the plasmids cited above and incubated overnight at 37°C. After 24h, one colony was transferred into LB media + ampicillin and incubated at 37°C for 24h while shaking. After incubation, bacterial growth was characterized by a cloudy haze in the media. The plasmids were extracted and purified

from the transformed and proliferated *E. coli* DH5α using the GenElute HP Plasmid Maxiprep kit (Sigma-Aldrich).

The cells were then transfected using the fast-forward protocol with Attractene Transfection Reagent (Qiagen) following the manufacturer's instructions. After 24 and 48 h of transfection, RNA was extracted as previously described. AFP, hTERT, and PKC isoform levels were quantified using qRT-PCR as described above.

Assay of telomerase activity and quantification of proteins

Cells were extracted for use in assessing telomerase activity using the following methods. First the cells were trypsinized and centrifuged at 2000rpm for 5min; washed three times with PBS, centrifuged and resuspended at 10^6 cells/200µl lysis buffer (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate, 10 mM Tris pH 8.0). Second, the lysate was incubated on ice for 30 min, and centrifuged at 12000g at 4°C for 20min. Finally, aliquots of the supernatant were collected. BCA Protein Assay (BioRad) was used to determine the protein concentration in the extracts.

For the real-time quantitative telomeric repeat amplification protocol, 1µL cell lysate (0.5µg/µL), telomerase primer TS (5'-AATCCGTCGAGCAGAGTT-3') and reverse primer ACX (5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′) were used. Samples were incubated at 37°C for 30 min, at 95°C for 10min and then amplified for 40 cycles (5sec at 95°C and 60 sec at 60°C).

Data were analyzed using Rotor-Gene Q (Qiagen) which integrates the RT-PCR effectiveness that was calculated by the successive dilution of the most active sample.

Cell proliferation analysis

Cell proliferation was determined using the colorimetric method based on the oxidation of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium and monosodium salt (WST-8) (Sigma-Aldrich) in the cell mitochondria. HePG2/C3A and PLC/PRF/5 cells (10^5 cells) were transfected according to the manufacturer's protocol in a 96-well culture plate with 10nM siRNA using HiPerfect Transfection Reagent (Qiagen). After 72h, 10µL tetrazolium salt was added to each well. The added product was cleaved into formazan using an enzyme—succinate-tetrazolium reductase—that exists only in the mitochondrial respiratory chain. The number of living cells in the culture was proportional to the formazan produced and were evaluated using an ELISA reader at 450nm.

Statistical analysis

The experimental data are expressed as the mean ± SD from at least three experiments. The analysis of variance and unpaired two-tailed Student's *t*-test were conducted to determine significant differences among more than two groups or between only two groups, respectively. $P \leq 0.05$ was considered significant.

3. RESULTS

Identification of PKC isoforms in HepG2/C3A, PLC/PRF/5, SNU-387, and SK-OV-3

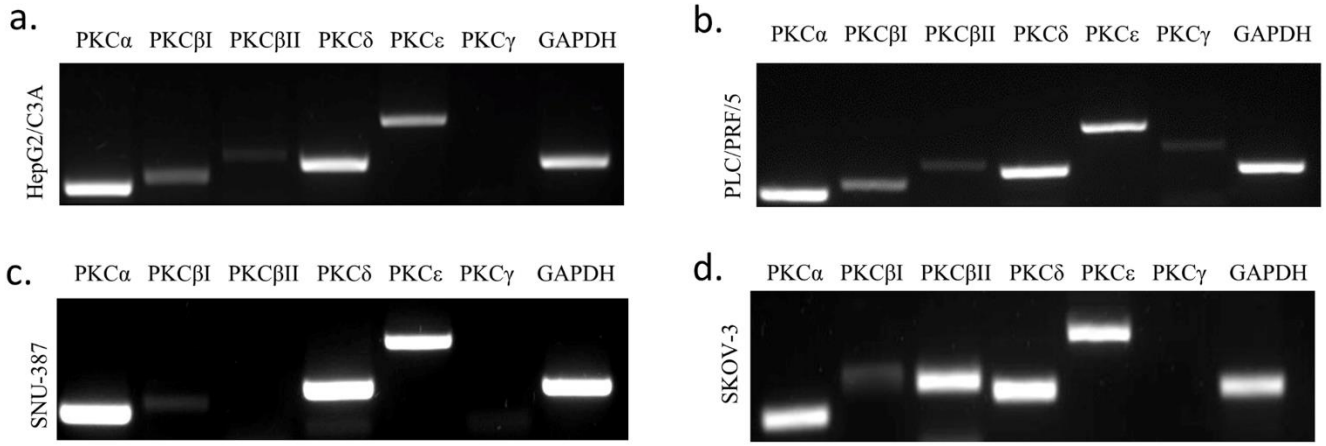

Figure 1. PKC isoforms identification in HepG2/C3A, PLC/PRF/5, SNU-387 and SKOV-3. Semi-quantitative RT-PCR analysis of PKC isoforms expression from HepG2/C3A (a), PLC/PRF/5 (b), SNU-387 (c) and SK-OV-3 (d) cells. The four cell lines were seeded in Petri Dish, each in 10mL Media (DMEM1g/L for HepG2/C3A, αMEM for PLC/PRF/5, and in DMEM/F-12 for SNU-387 and SKOV-3). RNA was extracted after 80% confluence, then a RT-PCR was performed. The BioDoc UVP system was used to visualize the amplified DNA. The GelAnalyzer-2010 software was used to obtain the relative expression of mRNA of PKC's isoforms relatively to GAPDH. PKC: protein kinase C; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

We investigated the expression of each PKC isoform in three different HCC cell lines HePG2/C3A, PLC/PRF/5**,** and SNU-387. Their expression was also studied in an ovarian cancer cell line SK-OV-3 (hTERT -/-) (Fig 1). Cells were cultured as previously described and then PKC isoforms expression was assessed using RT-PCR. We observed that PKCα and PKCδ were highly expressed in the four studied cell lines, and their expression levels were more significant HepG2/C3A and PLC/PRF/5 (AFP +/+). Moreover, we observed that PKCδ's expression level was more pronounced in HePG2/C3A, the cell line with the higher level of AFP expression.. PKCβI was observed to be lightly expressed in

the four studied cell lines; however this expression was more pronounced in the AFP secreting cell lines. PKCβII was highly expressed exclusively in the hTERT and AFP-negative cell line, SKOV-3.. However, of the two remaining isoforms normally expressed in HCC, PKCε was observed to be significantly expressed in all four cell lines, whereas PKCγ was slightly and exclusively expressed in PLC/PRF/5, with no significant association with either the presence or absence of AFP or hTERT (S1_Fig).

AFP increased PKCα, PKC βI, PKC βII and PKCδ expression in SNU-387 and SKOV-3 cell lines

To ensure whether AFP's presence had an effect on the expression of the different isoforms, the expression and secretion of AFP was studied in two AFP-negative cell lines, SNU-387(hTERT+) and SK-OV-3(hTERT-) (Figs 2a-b-c-d-e). We transfected these cells using two different constructs an empty vector as a control and an AFP construct (pCMV3-AFP). Interestingly, our results showed that pCMV3-AFP significantly increased AFP expression 24h post-transfection (p=0.0004 for SNU-387(p= 0.0004 for SNU-387 and p= 0.0058 for SKOV-3) (Fig 2a). In conformity with the post-transfection levels of AFP mRNA, SNU-387and SKOV-3 transfection with (pCMV3-AFP) increased PKCα mRNA levels 0.4-fold (p=0.0021) and 0.5-fold (p=0.02) respectively (Fig 2-b). PKCβI levels increased 0.5-fold (p=0.01214) in SNU-387 and 2.8-fold (p=0.003) in SKOV-3 (Fig 2-c). Moreover, PKCδ expression levels also increased 0.2-fold (p=0.0093) and 0.95-fold (p=0.0189) in SNU-387 and SKOV-3 cells respectively (Fig 2-e).

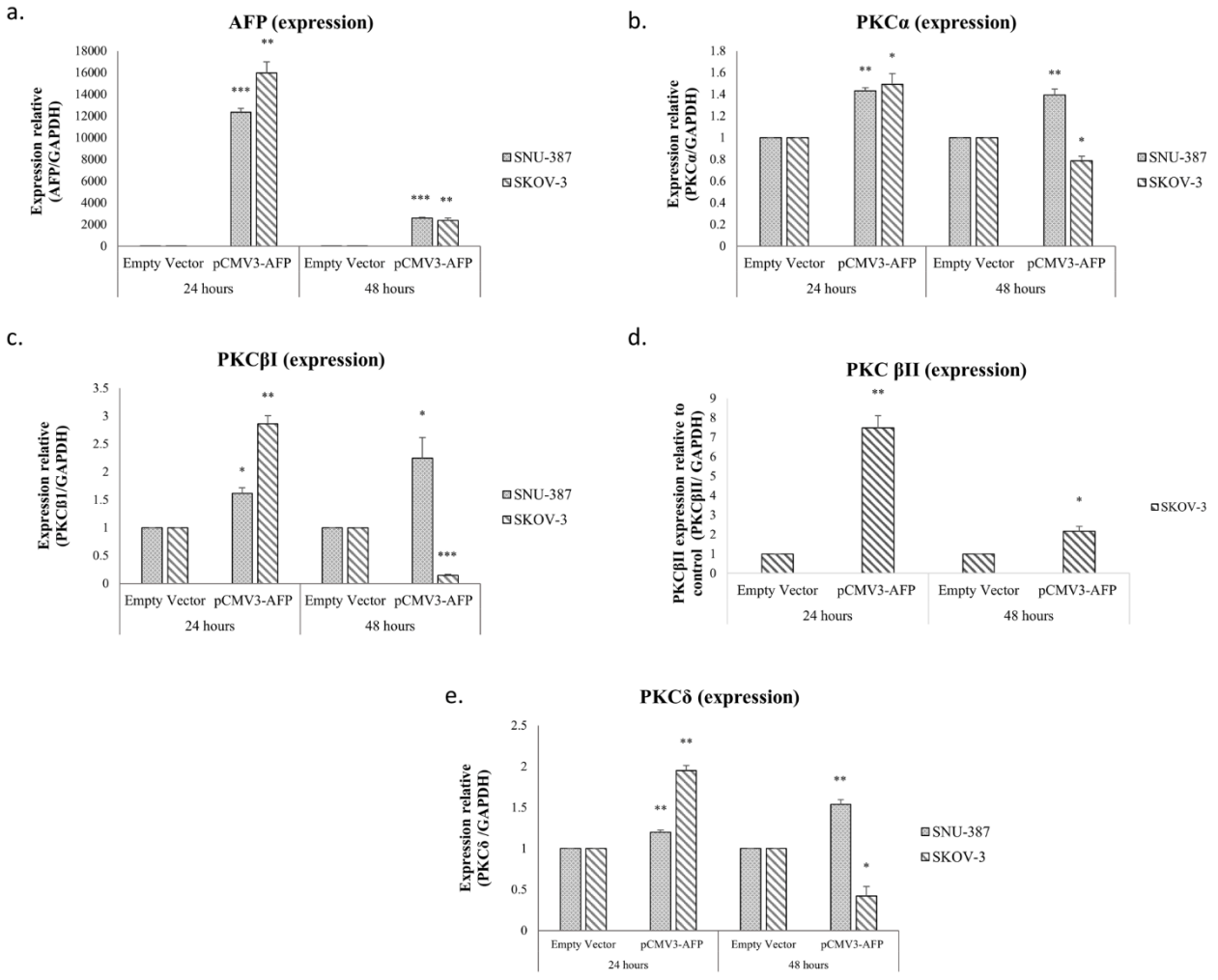

Figure 2. Effect of AFP on PKCα, PKC βI, PKC βII and PKCδ expression. SNU-387 and SK-OV-3 (a, b, c, d, e) were transiently transfected with an empty vector and pCMV3-AFP for 24hours and 48hours in six-well plates. Total RNAs were isolated from the treated cells and AFP (a), PKCα (b), PKCβ1 (c) and PKCδ (d) expression was then quantified by real-time PCR with GAPDH as the internal control. Results were expressed as the mean ±SD from a minimum of three experiments. (*P < 0.05, **P < 0.01, ***P < 0.001). AFP: Alpha-fetoprotein; PKC: Protein kinase C; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; CTR: Control.

We also studied the effect of pCMV3-AFP on the expression level of PKCβII in SKOV-3, the cell line most expressing this isoform. Our results shows that the expression level of PKCβII increased significantly after 24 hours (p=0.0046) (Fig 2-d). After 48h, the increase in expression levels remained steady in SNU-387 but decreased in SKOV-3 by 0.5, 2, 5 and 1.5-fold for PKCα, PKCβI, PKCβII and PKCδ respectively (Fig 2-b-c-d-e).

Effect of all-trans retinoic acid ATRA treatment on PKCα, PKCβI, PKCβII and PKCδ expression in HepG2/C3A and PLC/PRF/5 cells

To better understand the effect of AFP on PKCα, PKCβI, PKCβII and PKCδ expressions, we investigated whether the inhibition of AFP could also be implicated in regulating isoform expressions in the studied cell lines. To determine this, HepG2/C3A and PLC/PRF/5 cells were treated with 10 µM ATRA. After 48h, total RNA was extracted and PKC isoforms' expression levels were assessed using RT-PCR. Our results showed that 10 µM ATRA significantly decreased the expression levels of PKCα, PKCβI, PKCβII and PKCδ in HepG2/C3A cells (p=0.0154, p=0.0062, p=0.0062 and p=0.0001, respectively) (Fig 3-a). Interestingly, in PLC/PRF/5, PKCα, PKC βI and PKC βII expression levels significantly decreased after ATRA treatment (p<0.05), whereas PKCδ expression significantly increased (p<0.01) (Fig 3-b).

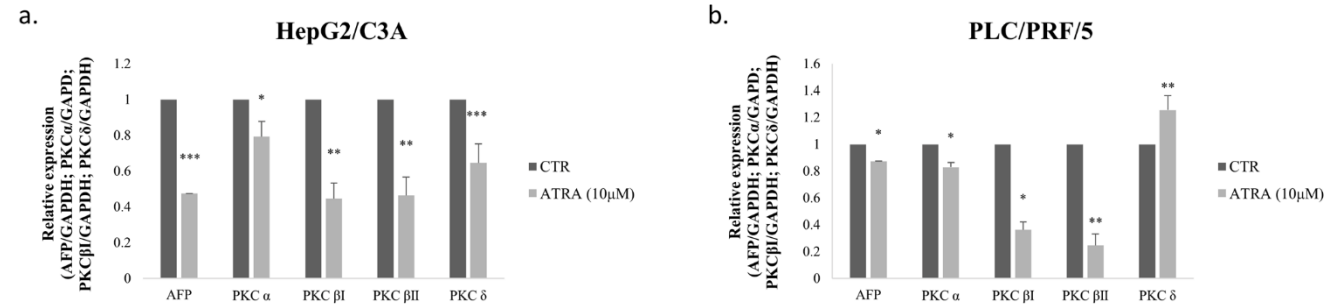

Figure 3. Effect of AFP on PKCα, PKC βI, PKC βII and PKCδ expression. HepG2/C3A (a) and PLC/PRF/5 (b) cells were treated for 48 hours with ATRA at 10 µM in six-well plates. Total RNAs were isolated from the treated cells and AFP, PKCα, PKCβI, PKCβII and PKCδ expression was then quantified by real-time PCR with GAPDH as the internal control. Results were expressed as the mean ±SD from a minimum of three experiments. (*P < 0.05, **P < 0.01, ***P < 0.001). AFP: Alpha-fetoprotein; PKC: Protein kinase C; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; CTR: Control; ATRA: All-trans retinoic acid

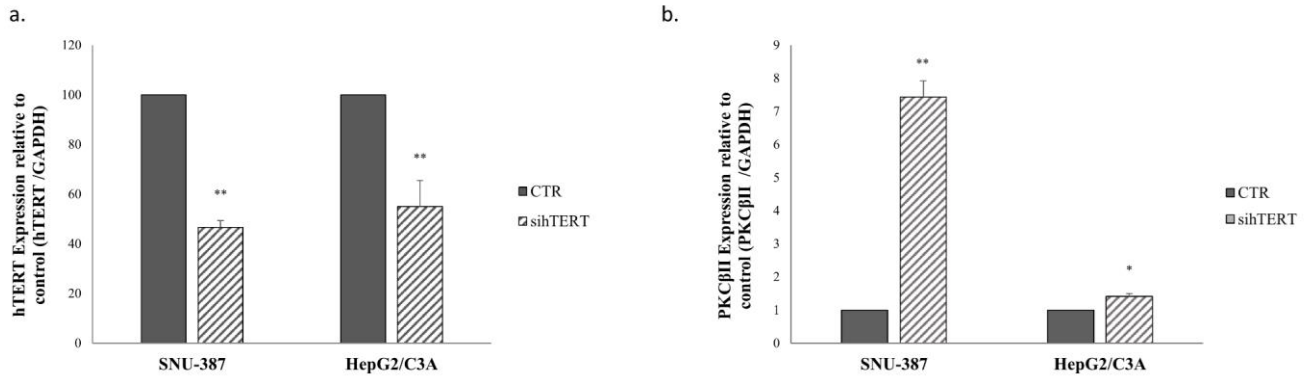

Figure 4. Telomerase inhibtion regulates PKC βII expression levels. SNU-387 and HepG2/C3A cells were treated with control siRNA or hTERT siRNA for 72 hours. Then the expression of hTERT (a) and PKCβII (b) were quantified by real-time PCR with GAPDH as the internal control. Results were expressed as the mean ±SD from a minimum of three experiments. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)4

hTERT regulated PKCβII expression levels

Our results showed that PKC βII is expressed more in the AFP (-/-) and hTERT (-/-) cell line; SKOV-3. To confirm that hTERT plays a role in regulating PKC isoform expression, hTERT was knocked down in SNU-387(AFP (-/-) and hTERT (+/+)) and in HepG2/C3A (AFP (+/+) and hTERT (+/+)) cells. Interestingly, our results showed that while hTERT expression decreased 72hours after knockdown (Fig 4a), PKCβII expression levels significantly increased by 6.4-fold in SNU-387 and by 0.5-fold in HepG2/C3A (Fig 4b).

To further evaluate the effect of hTERT, we overexpressed hTERT in SKOV-3, SNU-387 and HepG2/C3A, by transfecting these cell lines with two different constructs an empty vector as a control and hTERT-WT construct. The efficiency of the transfection was then evaluated by assessing hTERT levels using RT-PCR. Our results showed that hTERT expression significantly increased 24h post-transfection and then decreased after 48h following cells transfection with hTERT-WT (Figs 5a-c-e). Interestingly, relative to hTERT expression levels, SKOV-3, SNU-387 and HepG2/C3A transfection with hTERT-WT decreased PKCβII expression levels 0.4-fold (p=0.001), 0.3-fold (p=0.033) and 0.36-fold (p=0.011) (Figs 5-b-d-f) respectively.

PKC isoforms expression regulated AFP secretion and expression in HepG2/C3A and PLC/PRF/5 cells

To investigate the effect of PKC isoform inhibition on AFP secretion, we treated HepG2/C3A cells with PKCα, PKCβ, PKCδ and PCKε siRNAs and PLC/PRF/5 cells with PKCα, PKCβ, PKCδ, PCKε and PKCγ siRNAs that were constructed and used to abolish the expression of the cited isoforms in the two cell lines. It is worth mentioning that these isoforms are the five that are the most expressed in HCC and, especially, in the cell lines used in this study. After

knockdown of the different PKC isoforms expression, secreted AFP levels were determined using ELISA.

The evaluation of AFP concentration levels after isoforms' knockdown, showed that PKCα knockdown (Fig 6-a) increased AFP secretion by 37% (p=0.0011), and that PKCβ, PKCδ and PCKε knockdown by siRNA significantly increased AFP secretion by 15% (p=0.0011), 27% (p=0.0074), 19% (p=0.038), respectively in the media of HepG2/C3A cells.

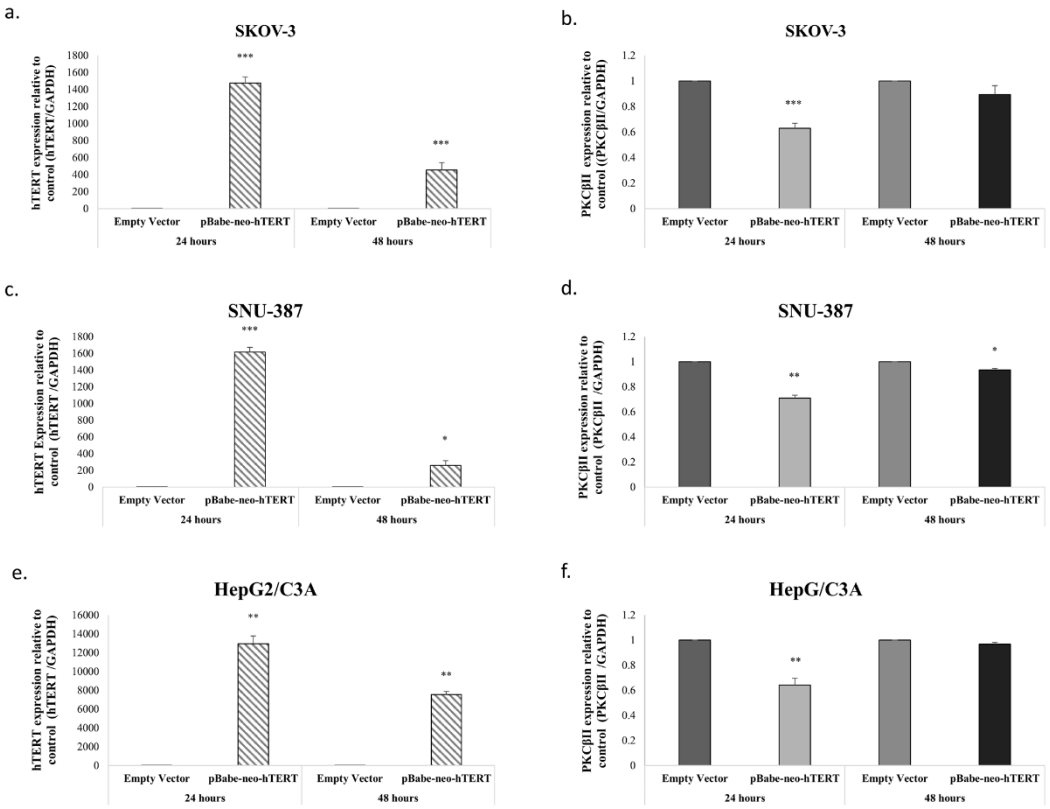

Figure 5. Effect of telomerase on PKC βII expression level. SKOV-3 (a, b), SNU-387 (c, d) and HepG2/C3A (e, f) cells were transiently transfected with an empty vector and with hTERT-WT. hTERT (a, c, e) and PKCβII (b, d, f) expression were quantified by real-time PCR. Results were expressed as the mean ±SD from a minimum of three experiments. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

A similar pattern was observed in transfected PLC/PRF/5 cells (Fig 6-c); however, silencing of PKCδ decreased AFP secretion by 50% in the cellular medium.

Moreover, cells treated with *pan-PKC inhibitor (*Go 6983) and with a pool of siRNAs (PKCα, PKCβ, PKCδ, PCKε and PKCγ) led to a significant decrease in AFP secretion in HepG2/C3A cells ($p \leq 0.001$) and PLC/PRF/5 cells ($p < 0.05$) (Figs 6-a-c).

Furthermore, the effect of PKC isoform inhibition was also assessed based on AFP mRNA expression. Knockdown of PKCα, PKCβ, PKCδ, and PCKε in HepG2/C3A cells increased AFP mRNA levels 2-fold (p=0.0061), 2.1-fold (p=0.045), 1.7-fold (p=0.0011), and 1.5-fold (p=0.0279) respectively (Fig 6-b).

PKCα, PKCβ, PCKε, and PKCγ siRNAs increased the expression levels of AFP mRNA in PLC/PRF/5 cells by 2-fold (p=0.005), 1.5-fold (p=0.023), 2-fold (p=0.0045), and 1.5-fold (p=0.0056), respectively; whereas PKCδ siRNAs increased AFP mRNA expression level (p=0.0004) (Fig 6-d).

On the other hand, our results show that treatment with Go6983 and the pool of siRNAs significantly decreased AFP mRNA expression levels ($p < 0.001$) in both cell lines (Figs 6-b-d).

PKC isoforms regulated hTERT expression and telomerase activity in HepG2/C3A and PLC/PRF/5 cells

Next, we studied the effect of the PKC isoform knockdown on telomerase expression and activity. Remarkably, in HepG2/C3A cells, hTERT mRNA significantly increased 0.64-fold when treated with PKCα siRNA (p=0.0475), 0.32-fold with PKCβ siRNA (p=0.0324), 0.6-fold with PKCδ siRNA (p=0.0158) and 0.63-fold with PCKε siRNA (p=0.0296); however, after treating with Go 6983, hTERT mRNA decreased 0.7-fold (p=0.0021). A decrease of 0.3-fold in hTERT mRNA levels was also observed after treating with the pool of siRNA (p=0.0039) (Fig 7-a).

The results in the PLC/PRF/5 cells, were similar to those in the HepG2/C3A cells, as shown in Figure 7-b, except that the knockdown of PKCδ inhibited the expression of hTERT mRNA in the PLC/PRF/5 cells 0.2-fold (p=0.0269), but induced its expression in HepG2/C3A cells (Fig 7-a-b). When treated with Go 6983 and the pool of siRNAs, the expression levels of hTERT mRNA also decreased 0.2-fold (p=0.0042) and 0.3-fold (p=0.05) respectively in the two cell lines.

Because our results have shown that PKCα and PKCδ are the two most expressed isoforms in HepG2/C3A and PLC/PRF/5 cell lines, and given that they have interestingly

different effects on AFP and hTERT expression, we have investigated their effect on telomerase activity and on PI3K/AKT/mTOR signaling pathway, known to be implicated in cell proliferation, migration, invasion, and apoptosis.

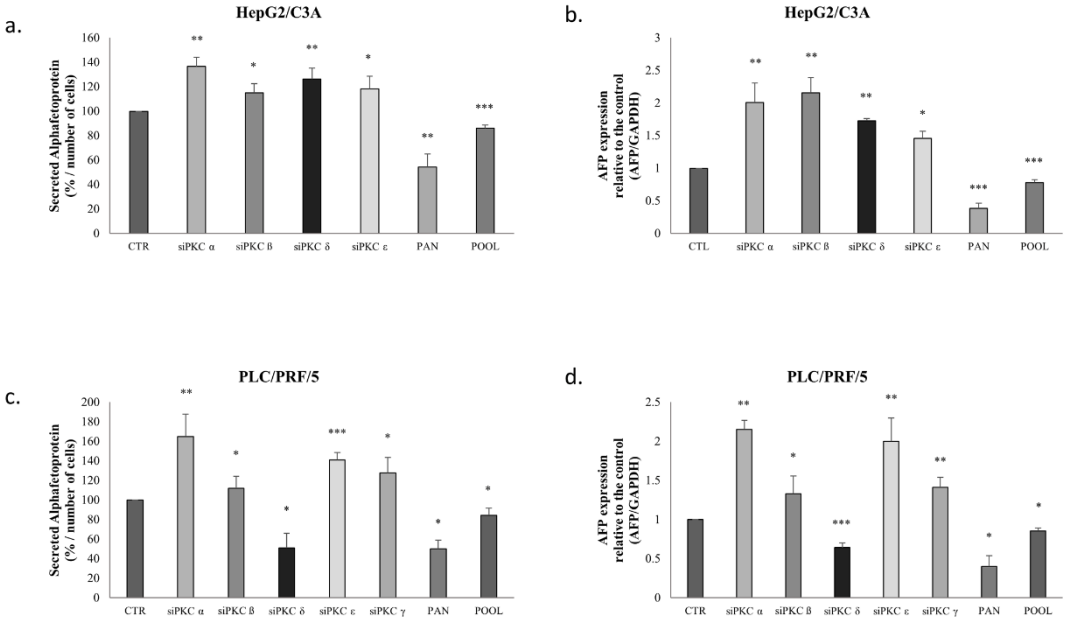

Figure 6. PKC isoforms' expression in HepG2/C3A and PLC/PRF/5 cells regulates AFP secretion and expression. AFP secretion (a, c) was assessed by ELISA following a 72-h treatment with 5µM Go6983 and after PKCα, PKCβ, PKCδ, PKCε, and a pool of PKC isoforms knockdown with siRNAs in HepG2/C3A (a) and with 5µM Go6983, PKCα, PKCβ, PKCδ, PKCε, PKCγ and a pool of PKC isoforms knockdown with siRNAs in PLC/PRF/5 (c). Total RNAs were isolated from the cells and AFP expression was analysed by qRT-PCR with GAPDH as the internal control (b, d). Results were expressed as the mean ±SD from a minimum of three experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). AFP: Alpha-fetoprotein; PKC: Protein kinase C; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; CTR: Control

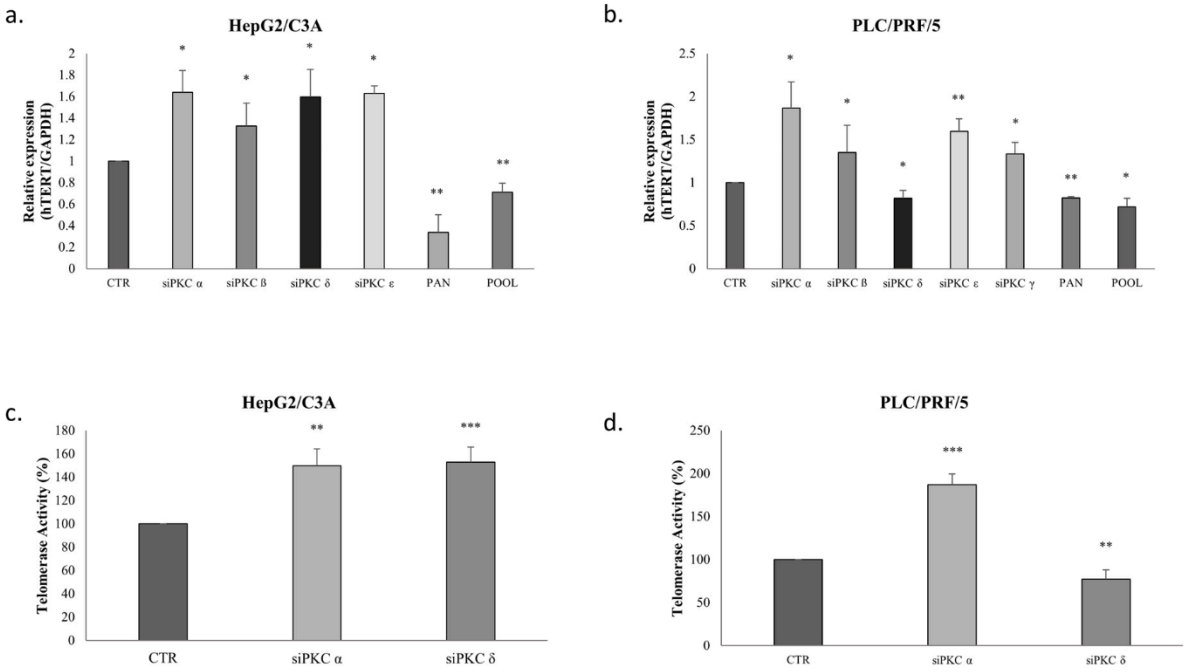

Figure 7. PKC isoforms' expression regulates hTERT expression and telomerase activity. HepG2/C3A (a) and PLC/PRF/5 (b) cells were treated with Go6983, PKC α, PKCβ, PKCδ, PKCε, PKCγ and a pool of PKC isoforms siRNAs. Total RNAs were isolated from the treated cells and hTERT expression was analysed by qRT-PCR with GAPDH as an internal control. (c, d) Telomerase activity was detected in HepG2/C3A (c) and PLC/PRF/5 (d) treated with PKCα and PKCδ siRNAs. Results were expressed as the mean ± SD from three experiments. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). PKC: Protein kinase C; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; CTR: Control

The inhibition of PKCα and PKCδ by siRNAs as shown in figure 7-c, increased telomerase activity of HepG2/C3A cells by 50% (p=0.0011) and 52% (p=0.0002), respectively; whereas in PLC/PRF/5, PKCα siRNA increased telomerase activity by 86% (p=0.0003), while PKCδ siRNA significantly inhibited telomerase activity by 28% (p=0.0019) (Fig 7-d).

Implication of PI3K, AKT, mTOR and hTERT in the regulation of AFP by PKCα and PKCδ

Considering that PKCα and PKCδ knockdown modulated AFP expression levels and secretion, and, in parallel, regulated hTERT expression and activity in the studied cancer cell lines, we investigated whether this connection was ensured through regulation of hTERT or through PI3K/AKT pathway, one of the most activated signaling pathways in cancer cells, to elucidate the relationship that both PKCα and PKCδ might have with AFP.

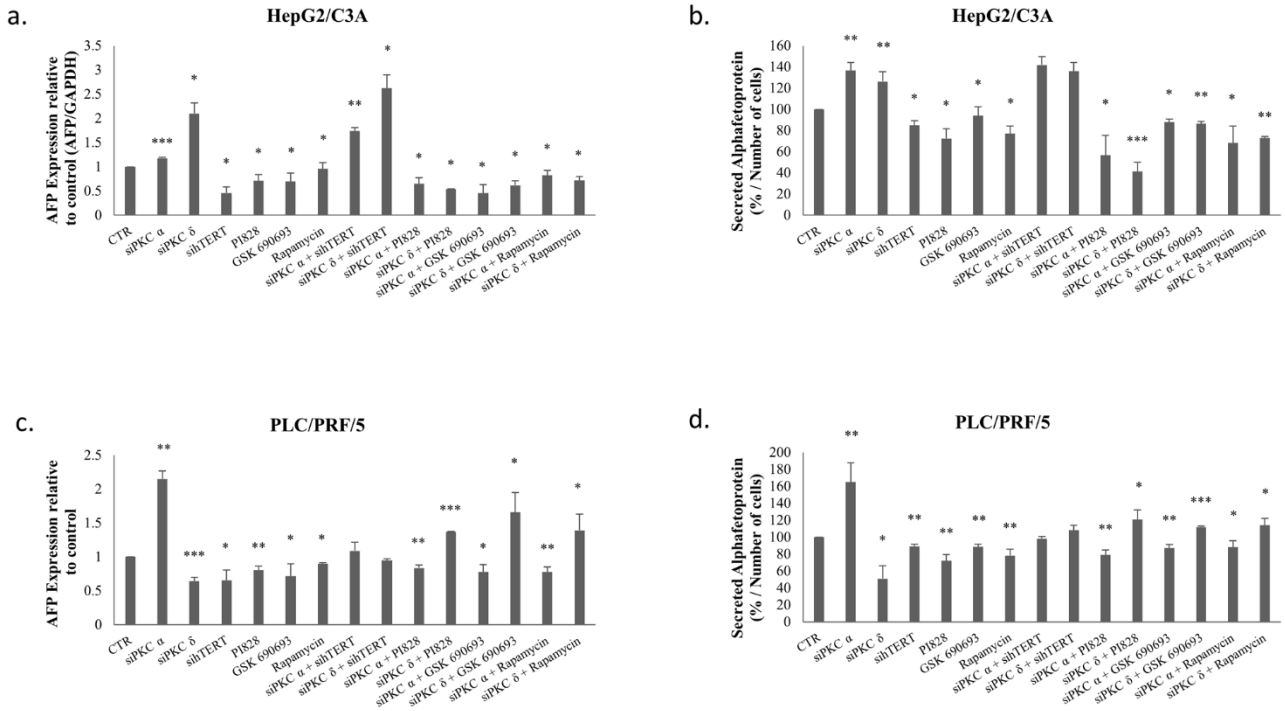

Figure 8. PKCα and PKCδ knockdown regulates AFP expression via PI3K, AKT, mTOR and hTERT. HepG2/C3A (a, b) and PLC/PRF/5 (c, d) were treated with PKCα, PKCδ, hTERT siRNAs for 72h, and with 10μM PI828, 100nM GSK-690693, and 200nM rapamycin for 48h. Then treated with PKCα and PKCδ siRNAs combined with hTERT siRNA, PI828, GSK-690693, and rapamycin. AFP secretion (b, d) was quantified using ELISA. Total RNAs from the treated cells were collected and AFP expression level was quantified by qRT-PCR (a, c). Results were expressed as the mean ± SD from three experiments. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). CTR: Control; AFP: Alphafetoprotein; PKC:Protein Kinase C

To investigate the implication of PI3K/AKT/mTOR pathway in the correlation between PKC and AFP, AFP mRNA levels were evaluated following the inhibition of hTERT, PI3K, AKT, and mTOR using hTERT siRNA, 10μM PI828, 100nM GSK-690693, and 200nM rapamycin, respectively, on HepG2/C3A and PLC/PRF/5 cells.

Figures 8- a and c, clearly demonstrate a significant decrease in AFP expression levels (p≤0.05) for HepG2/C3A after these inhibitions, as well as a significant decrease in AFP expression levels (p=0.0176 (hTERT siRNA), p=0.0041 (PI828), p=0.05 (GSK-690693), p=0.01 (rapamycin)) for PLC/PRF/5 cells.

Figure 8 also shows a significant decrease in AFP secretion (p≤0.05) for HepG2/C3A (Fig 8-b) and (p<0.01) for PLC/PRF/5 (Fig 8-d) following these inhibitions.

Interestingly, the combination of PKC isoform knockdown using PI3K/AKT pathway inhibitors significantly abolishes AFP upregulation by PKCα and PKCδ siRNAs in HepG2/C3A cells (Figs 8-a-b). Whereas, in PLC/PRF/5, a combined treatment using PKCα and PI3K/AKT/mTOR inhibitors, significantly abrogates AFP upregulation, while it significantly potentiate the effect with PKCδ (Figs 8-c-d). This led us to evaluate the reason behind this enhancement.

Therefore we evaluated the effect of this combinatory treatment after 24 and 48 hours in PLC/PRF/5. The results showed a highly significant reduction in the expression level of AFP during the first 24 hours of treatment with siPKCδ and PI3K inhibitors (77% (p = 0.001)), AKT (68% (p = 0.0004)) and mTOR (65% (p = 0.0022). However, after 48 hours of treatment, we note that the expression level of AFP began to increase until reaching 79% (p = 0.049) for siPKC δ + PI828, 80% (p = 0.044) for siPKC δ + GSK 690693 and 86% (p = 0.032) for siPKC δ + Rapamycin, and continues to increase after 72 hours to reach 125% (p = 0.0004), 147% (p = 0.0166) and 129% (p = 0.049) for siPKC δ + PI828, siPKC δ + GSK 690693 and siPKC δ + Rapamycin respectively (Fig 9).

On the other hand, Figure 8 showed that a combined inhibition of hTERT, PKCα and PKCδ by siRNAs enhances AFP expression and secretion in HepG2/C3A cells (Figs 8-a-b), but had no significant effect on AFP expression in PLC/PRF/5 cells (Figs 8-c-d). The reason that led us to investigate the effect of the siPKCα, siPKCδ with sihTERT in

the first 24 and 48 hours in HepG2/C3A cells. No significant effect was observed on the expression level of AFP after 24 and 48 hours of combinatory treatment.

In order to confirm the implication of PI3K/AKT/mTOR signaling pathway, we further evaluated the effect of PKCα and PKCδ knockdown on PI3K, AKT, and mTOR levels. As shown in Figure 10, the inhibition of PKC isoforms significantly increased PI3K, AKT, and mTOR expressions in HepG2/C3A cells (Fig 10-a). In PLC/PRF/5 cells, PKCα knockdown had the same effect as in HepG2/C3A, however, PKCδ knockdown decreased the expression level of PI3K, AKT, and mTOR (Fig 10-b).

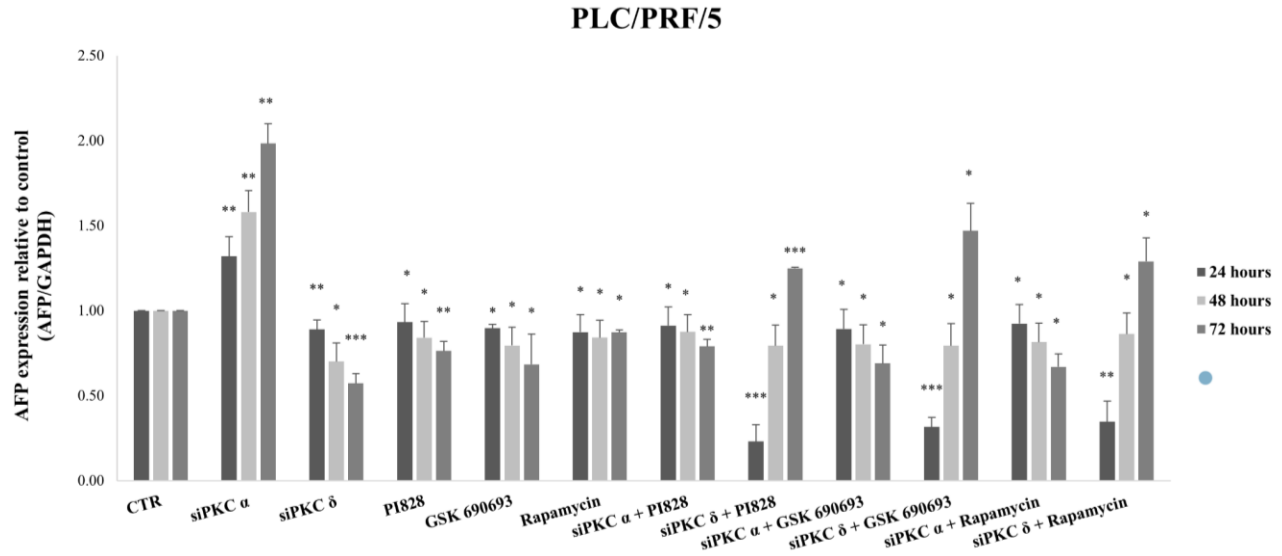

Figure 9. Effect of the combinatory treatment on AFP expression after 24, 48 and 72 hours. PLC/PRF/5 cells were treated with PKCα and PKCδ siRNAs and with 10µM PI828, 100nM GSK-690693, and 200nM rapamycin. Then treated with PKCα and PKCδ siRNAs combined with PI828, GSK-690693, and rapamycin. Total RNAs from the treated cells were collected after 24, 48 and 72 hours, and AFP expression level was quantified by qRT-PCR. Results were expressed as the mean ± SD from three experiments. (*P < 0.05, **P < 0.01, ***P < 0.001). CTR: Control; AFP: Alphafetoprotein; PKC: Protein Kinase C

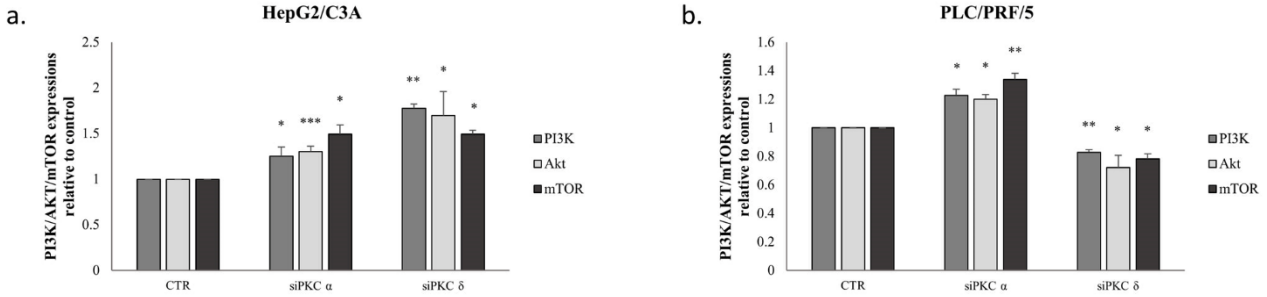

Figure 10. PKCα and PKCδ knockdown regulates PI3K, AKT, mTOR expression. HepG2/C3A (a) and PLC/PRF/5 (b) were treated with control siRNA, PKCα or PKCδ siRNAs for 72h. Total RNAs were collected from treated cells, and PI3K, AKT, and mTOR expression was then quantified with qRT-PCR with GAPDH as an internal control. Results were expressed as the mean ± SD from three experiments. (*P < 0.05, **P < 0.01, ***P < 0.001). CTR: Control; PKC: Protein kinase C; PI3K: Phosphoinositide *3*-kinase

Combined inhibition of PKCα and PKCδ with PI3K and AKT inhibitors reduced hTERT expression in HepG2/C3A and PLC/PRF/5 cell

Because we observed that PKCα and PKCδ knockdown modified the expression and activity levels of hTERT in HepG2/C3A and in PLC/PRF/5 cell, we investigated whether the PI3K/AKT signaling pathway might be involved in this mechanism of action. As shown in Figure 11, inhibition of PI3K, AKT, and mTOR, significantly decreased hTERT mRNA levels in the two cell lines (p=0.037, p=0.0006 and p=0.0334 respectively for HepG2/C3A and p<0.01 for PLC/PRF/5).

Moreover, the combined inhibition of PKCα and PKCδ using PI3K and AKT inhibitors abrogates the upregulation of hTERT by PKCα and PKCδ siRNAs in HepG2/C3A cells with siPKCα and siPKCδ for PI828 (p = 0.0268 and p = 0.05 respectively) and for GSK-690693 (p = 0.0083 and p = 0.0196 respectively) (Fig 11-a). Similarly, in PLC/PRF/5 cells, the knockdown of PKCα in the presence of PI828 and GSK- 690693 significantly inhibits the stimulation of expression of hTERT (p=0.0001 and p=0.0055 respectively). Therefore the combinatorial treatment of siPKCδ with PI3K and AKT inhibitors significantly abrogates the downregulation of hTERT (p = 0.0454 and p = 0.0.05 respectively) (Fig 11-b).

Moreover, the combined inhibition of PKC isoforms andmTOR had no significant effect on hTERT expression levels in both cell line (Figs 11-a-b).

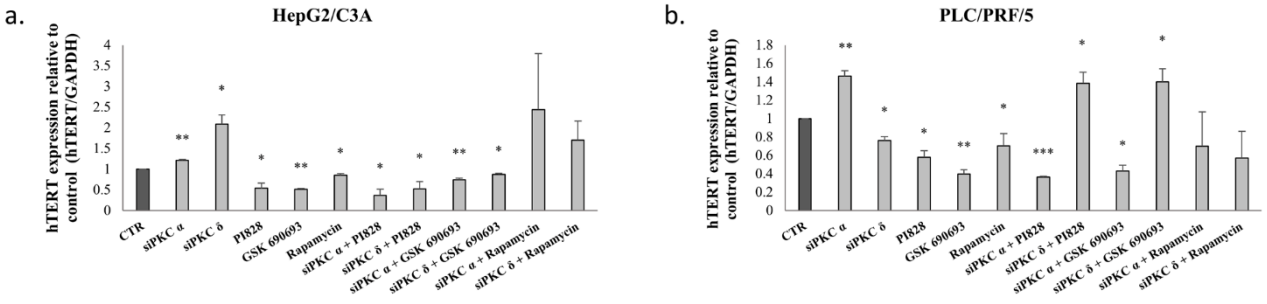

Figure 11. A combined inhibition of PKCα and PKCδ with PI3K and AKT inhibitors reduces hTERT expression in HepG2/C3A and PLC/PRF/5 cell lines. HepG2/C3A (a) and PLC/PRF/5 (b) were treated with PKCα and PKCδ siRNAs for 72h, and with PI828, GSK-690693, and Rapamycin for 48h. Then treated with PKCα and PKCδ siRNAs combined with PI3K/AKT/mTOR inhibitors. hTERT transcript amount in HepG2/C3A (a) and PLC/PRF/5 (b) was then quantified by qRT-PCR. Results were expressed as the mean ± SD from three experiments. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). CTR: Control; PKC: Protein kinase C

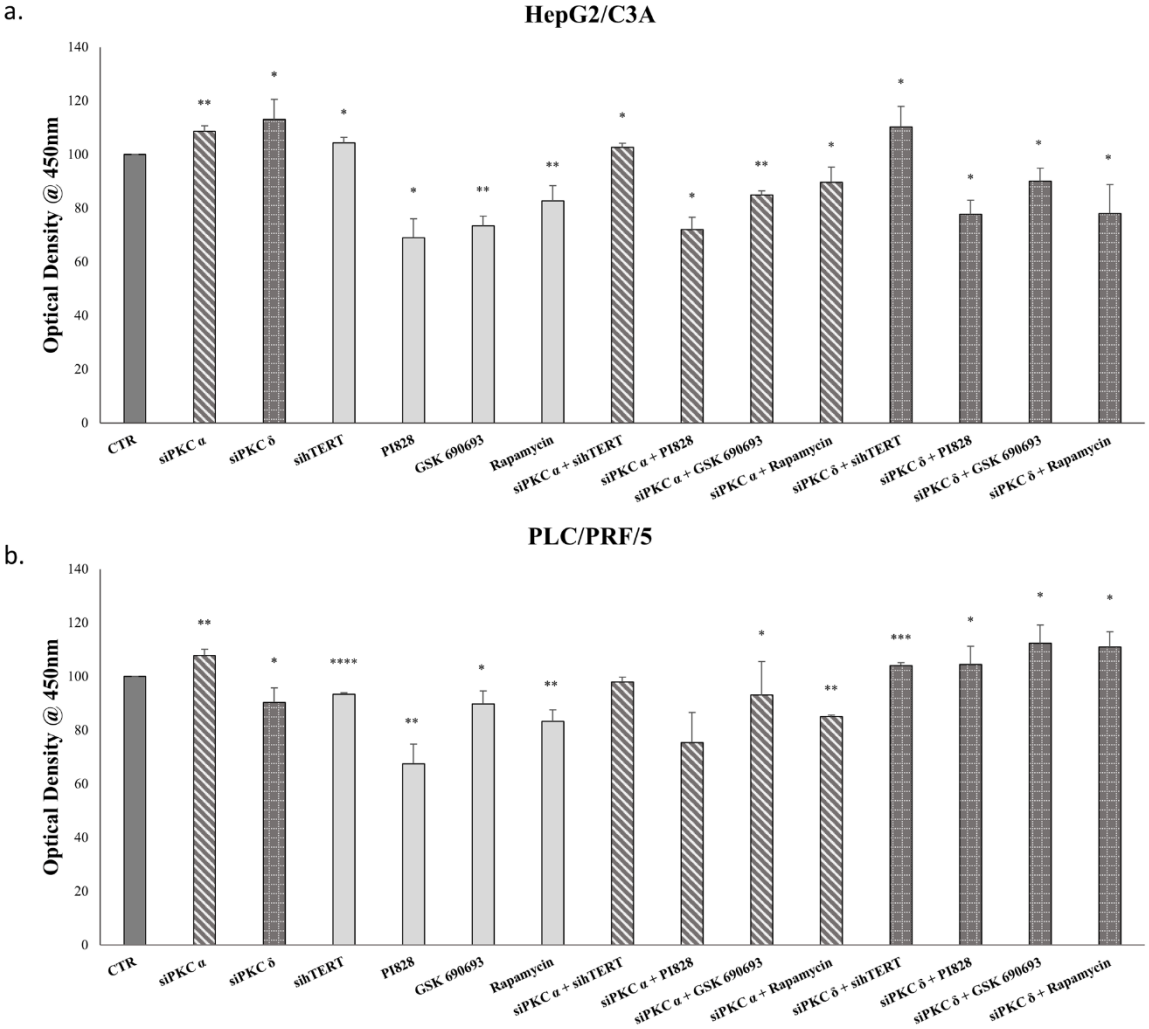

Figure 12. PKC alpha and delta inhibition regulates the proliferation of HepG2/C3A and PLC/PRF/5 cell lines. HepG2/C3A (a) and PLC/PRF/5 (b) were cultured in 96-well plates and treated with PKCα, PKCδ, and hTERT siRNAs for 72h, and with PI828, GSK-690693, and rapamycin for 48h. Then treated with PKCα and PKCδ siRNA combined with hTERT siRNA, PI828 (10μM), GSK-690693 (100nM) and rapamycin (200nM). After treatment, 10μL of Tetrazolium salt was added to 100μL of the culture media in each well and the mixture was incubated 45 minutes. Following formazan formation, variations in absorbance was detected using an ELISA reader at 450nm. Each value represents the mean of three assays. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). CTR: Control; PKC: Protein kinase C

PKCα and PKCδ inhibition regulated the proliferation of HepG2/C3A and PLC/PRF/5 cell

Because the PI3K/AKT pathway is known to be highly implicated in the regulation of cancer cell proliferation, and considering that our results suggest that this pathway might be implicated in the relationship among PKC isoforms, hTERT, and AFP, we studied the effect of PKCα and PKCδ, hTERT, PI3K, AKT, and mTOR inhibition on HepG2/C3A and PLC/PRF/5 proliferation.

Our results showed that PKCα knockdown increased the proliferation of both cell lines ($p<0.01$) (Figs 12-a-b); however, PKCδ knockdown increased the proliferation of HepG2/C3A cells ($p=0.041$) (Fig 9a), and decreased PLC/PRF/5s' proliferation ($p=0.0372$) (Fig 12-b). Moreover, the inhibition of hTERT, PI3K, AKT, and mTOR significantly decreased cell proliferation (Figs 12-a-b); however, the combined knockdown of PKC isoforms and inhibition of the PI3K/AKT pathway, annulled any increase in the proliferation rate of HepG2/C3A cells induced by the knockdown of PKCα and PKCδ alone and had the same effect for PLC/PRF/5 with siRNA of PKCα; but PKCδ knockdown combined with PI3K/AKT signaling pathway inhibitors, significantly enhanced PLC/PRF/5 proliferation (Fig 12).

4. DISCUSSION

HCC is one of the most common of all cancers worldwide and is known in many countries to have high mortality and incidence rates. Previous studies have shown a possible relationship between the progression, invasion, metastasis, and proliferation of HCC with AFP [38]. AFP is considered not only to be a marker of a greater pathological grade, TNM stage, and larger tumors [9,39], but to be a possible target in the treatment of HCC patients [40]; however, the pathophysiological and biological roles of AFP, that are associated with the high mortality and occurrence rates of HCC are still being studied [41].

Several Studies have explored the role of AFP in the prediction of treatment outcome and clinical follow-up; a strong positive correlation between the concentration of AFP in the Serum of a patient and the aggressiveness of HCC has been found [42–44]. Moreover, Zhou et al., have shown that when using a standard AFP level of 400 µg/l, the expression of hTERT in the AFP positive HCC tissues was significantly higher than the one in AFP negative HCC tissues [45]. These studies, alongside with the fact that cell immortalization is controlled in more than 85% of cancers by the telomerase, the enzyme linked to the development, occurrence, and prognosis of HCC [46], had led a team in our laboratories to investigate a possible interaction between AFP and telomerase in HCC. The inhibition of hTERT, the catalytic subunit of this enzyme, decreased AFP expression and secretion in HCC; however, the signaling pathway through which the latter effect was expressed was not clear [47].

Li et al. [48] have shown that PKC mediates the phosphorylation of hTERT and produces a marked increase in telomerase activity. On the other hand, PKC inhibitors induce a significant decrease in telomerase activity in the treated cells[49]. Within the cell, PKC isoforms mediate several functions, such as proliferation, apoptosis, gene expression, and regulation of the mitogenesis cell cycle [50]. Several studies have assessed the expression of PKC isoforms and their roles in the progression and development of HCC. A study by Tsai et al. has demonstrated that the development of human liver cancer might be related to a decrease [34]. The ultimate aim of our study was to investigate a possible relationship among AFP, telomerase, and PKC isoforms in HCC cell lines and whether the communication among them involves the PI3K/AKT signaling pathway.

We first identified PKC isoforms in three HCC cell lines—HepG2/C3A, PLC/PRF/5 and SNU-387—and the ovarian cancer cell line SK-OV-3 used as a control in our study. In agreement with other studies [51,52], our results showed that PKCα, PKCδ, and PKCε isoforms were highly expressed in the four cell lines, especially PKCα and PKCδ in the AFP-producing cell lines HepG2/C3A and PLC/PRF/5. Moreover, PKCδ was even more expressed in HepG2/C3A. PKCβI was slightly expressed in the four cell lines but was found more pronounced in the AFP-producing cell lines; therefore, we suggest that the higher the expression of AFP, the higher the expression of PKCα, PKCδ, and PKCβI. Because PKCβII was only slightly expressed in the PLC/PRF/5 cells, we suggest that HBV infection might influence those expressions, especially given that PKCβII plays a role in the first phase of oncogenesis [50]. We also observed that PKCβII was more expressed in the hTERT- and AFP-negative cell line SK-OV-3. After analyzing the different profiles of each cell line, we found that PKCγ was slightly expressed in the PLC/PRF/5 cell line independent of the expression of AFP and hTERT.

A limited number of studies have examined the effect of AFP expression on PKC isoform expression in HCC cell lines. Interestingly, we observed that AFP overexpression in AFP-negative cells SNU-387 (hTERT +) and SKOV-3 (hTERT -) increased the expression of PKCα, PKCβI, and PKCδ. According to Li et al., AFP colocalizes and interacts in the cytoplasm, with retinoic acid receptors β, and play a role in inhibiting the translocation of the receptors into the nucleus by competitively binding to RAR-β with ATRA[10]. Accordingly, treatment with ATRA inhibited AFP expression and thus significantly decreased the expression of the three isoforms in the HepG2/C3A cells; however, in PLC/PRF/5, PKCα and PKCβI expressions decreased, while PKCδ expression significantly increased. This difference might be correlated with the grade of both HCC and HBV infection. In fact, Lu et al have suggested that HBV infection might influence the expression of PKCβ isoform in HCC in the early stages of oncogenesis [51].

Most research has focused on the regulation of telomerase activity and hTERT expression by the PKC isoforms[25]. Our preliminary results showed that SKOV-3, an AFP- and hTERT-negative cell line, expresses PKCβII more highly than the other studied cell lines. These results, together with a lack of studies in this area, prompted us to investigate the effect of the presence or absence of hTERT on the expression of PKCβII. Following inhibition of hTERT by siRNA in SNU-387, an AFP-negative cell line, and in HepG2/C3A, an AFP highly secretory cell line, PKC βII expression levels significantly increased in both cell lines. Moreover, we observed that the overexpression of hTERT significantly decreased the expression of PKCβII in HepG2/C3A and in SNU-387 cells. Hence, PKCβII is regulated by hTERT expression independent of AFP expression.

Furthermore, we examined whether the presence of AFP affects PKCβII expression in the same manner by which it affected the other isoforms. By inhibiting AFP with ATRA in the HepG2/C3A cells and overexpressing it in SKOV-3 cells, PKCβII expression decreased and increased, respectively. Hence, we demonstrated that PKCβII is independently regulated by both hTERT and AFP expression.

As mentioned, AFP correlates with the truculence of HCC, and studies have suggested a relationship among tumor size, degree of etiology, and differentiation with AFP serum levels[53]. Taking these observations together with the knowledge that PKC isoforms are responsible for cell proliferation, regulation of mitogenesis, and other functions in several cancer types, we investigated the possible relationship between the most expressed PKC isoforms and AFP expression and secretion in the HCC AFP-positive cell lines used in this study. We observed some unexpected results. In the HepG2/C3A cells, the knockdown of PKCα, PKCβ, PKCδ, PCKε, and PKCγ by siRNAs increased AFP expression and secretion. The same pattern was observed in PLC/PRF/5 cells, except that PKCδ siRNA had the opposite effect on AFP expression and secretion. We suggest that this might be the result of the unique and, at times, opposing roles of PKC isoforms in both the normal and diseased states[54]. In fact, even the same isoform within the same cell can play contrasting roles depending on the stimuli[55]; therefore, we studied the effect of Go6983, a pan-PKC inhibitor, against PKCα, PKCβ, PKCγ, and PKCδ, as well as the effect of a pool of siRNAs (PKCα, PKCβ, PKCδ, PKCε, and PKCγ) on AFP expression and secretion. We found that a wide-range inhibition of PKC isozymes decreased AFP expression and secretion in both HepG2/C3A and PLC/PRF/5 cells.

Further investigation showed that PKC isoforms regulated hTERT expression. Indeed, a number of studies have reported that PKC is involved in the regulation of telomerase activity coincident with the expression of hTERT in the treated cells[49]. The present study demonstrates the gene-silencing effect of PKCα, PKCβ, PKCδ, PKCε, and PKCγ on the regulation of hTERT expression. Our results showed an increase in hTERT expression in the HepG2/C3A cell line, and the same pattern was observed in the transfected PLC/PRF/5 cell line; however, silencing PKCδ decreased hTERT expression. Both Go6983 and the siRNA pool of PKC isoforms decreased hTERT expression in the two studied cell lines; therefore, separately inhibiting each isoform mostly enhanced AFP and hTERT expressions, while inhibiting several isoforms together repressed AFP and hTERT expression.

Because the two most expressed isoforms in HepG2/C3A and PLC/PRF/5 cells—PKCα and PKCδ—have different effects on AFP and hTERT expression, and because several studies have shown that these isoforms can promote or suppress tumors and play different roles in tumor growth and proliferation [56], we were impelled to further investigate their roles in regulating telomerase activity in HCC cells. The inhibition of their expressions increased telomerase activity in HepG2/C3A cells, and PKCα siRNA increased telomerase activity in PLC/PRF/5 cells, while PKCδ decreased it concurrent with hTERT expression. Taken together, our results suggest that AFP expression and secretion, as well as telomerase expression and activity, are distinctively modulated by PKC isoform expression, and show for the first time a possible relationship of PKC isoforms to both AFP and hTERT in HCC.

Several growth factors rouse cell growth and proliferation are induced by receptors and ligands to activate the PI3K/AKT pathway in different types of cancer [57]. PI3K/AKT has been shown to be a critical pathway in the development of liver carcinogenesis and for HCC cell survival and growth. As mentioned, PKC isoforms are implicated in many cellular processes, and their regulation is linked with the prognosis and progression of HCC [29]. Several reports have shown that PKCα plays a crucial role in cell proliferation, differentiation and survival [27]. Upstream modulators of PI3K pathway, like scaffolding proteins, and growth factor receptors can be deactivated by PKCα [58]; however, it can also directly inhibit PI3K activity [59]. Moreover, PKCδ also plays opposite roles in regulating the PI3K/AKT pathway. It can both endorse apoptosis by interfering with Akt [60], and intervene in an anti-apoptotic signaling cascade via PI3K mediated survival pathway [61]. In our study, we investigated whether the relationship between PKCα, PKCδ and AFP is involved in the PI3K/AKT signaling pathway or hTERT regulations.

Intriguingly, we observed that PKCα and PKCδ siRNAs increased the expression of PI3K, AKT, and mTOR in the HepG2/C3A cells. The same pattern was observed in PLC/PRF/5 cells; however, the inhibition of PKCδ decreased PI3K, AKT, and mTOR expression. Thus, PKCα and PKCδ regulate the expression of the PI3K/AKT pathway in the HCC cell lines, but specifically in the HepG2/C3A and PLC/PRF/5 cells.

We further investigated the effect of the inhibition of PKC isoforms on AFP expression and secretion by combining siRNAs with PI3K, AKT, and mTOR inhibitors. We first demonstrated that the effect of each individual inhibitor decreased AFP expression and secretion, and then observed that in the HepG2/C3A cells, treatment with both PKCα and PKCδ siRNAs repealed AFP upregulation; however, in PLC/PRF/5 cells, combined treatment of PKCα and PI3K/AKT/mTOR inhibitors annulled AFP regulation, while the combined treatment of PKCδ significantly decreased AFP expression in the first 24 hours, but because of a retro-control mechanism this expression has enhanced 48 and 72 hours after treatment.

Nevertheless, the effect of the combined treatment of inhibiting hTERT and PKCα and PKCδ using siRNAs had no relevant effect on AFP expression and secretion, except to note that AFP expression and secretion decreased after hTERT gene silencing with siRNAs. Hence, we suggest the regulation of AFP expression by PKCα and PKCδ through the PI3K/AKT pathway.

The results of the current study, as well as those previously conducted in our laboratories, have shown that telomerase is involved in AFP regulation; therefore, it is important to identify the locus of hTERT within the signaling cascade. The inhibition of PI3K, AKT, and mTOR in the two studied HCC cell lines decreased hTERT expression; however, treatment with PI3K and AKT inhibitors combined with PKCα and PKCδ repeals hTERT expression, while mTOR inhibitors combined with PKCα and PKCδ had no effect on hTERT upregulation. Furthermore, HepG2/C3A and PLC/PRF/5

proliferation was correspondingly regulated in proportion to AFP up- or downregulation following the individual and combined treatments with PKCα, PKCδ, and hTERT siRNAs and PI3K, AKT, and mTOR inhibitors.

Conclusion

We documented here for the first time the effect of PKC isoforms on AFP expression and secretion and the effect of the presence or absence of AFP and hTERT on the expression of PKC isoforms in different HCC cell lines (S3_Fig).

From our results, we suggest that PKCα and PKCδ inhibit AFP expression and cellular proliferation by blocking the PI3K/AKT/mTOR signaling pathway in HepG2/C3A cells; however, although PKCα exhibits the same pattern in PLC/PRF/5 cells, PKCδ increases both AFP expression and cellular proliferation by activating the PI3K/AKT/mTOR pathway. In addition, hTERT is regulated by PI3K and AKT in both cell lines; consequently, it regulates AFP expression; however, additional studies should be conducted to assess the effect of a wide range of inhibitors on AFP through the PI3K/AKT/mTOR pathway and on cell proliferation, and to confirm the *in vitro* observations using *in vivo* experiments.

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REFERENCES

1. Byam J, Renz J, Millis JM. Liver transplantation for hepatocellular carcinoma. Hepatobiliary Surg Nutr. 2013 Feb;2(1):22–30.
2. Cancer today [Internet]. [cited 2019 Jan 24]. Available from: http://gco.iarc.fr/today/home
3. Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology. 2005;42(5):1208–36.
4. Bruix J, Sherman M. Management of hepatocellular carcinoma: An update. Hepatology. 2011 Mar 1;53(3):1020–2.
5. Gao F, Zhu H-K, Zhu Y-B, Shan Q-N, Ling Q, Wei X-Y, Xie H-Y, Zhou L, Xu X, Zheng S-S. Predictive value of tumor markers in patients with recurrent hepatocellular carcinoma in different vascular invasion pattern. HBPD INT. 2016 Aug;15(4):371–7.
6. Bai D-S, Zhang C, Chen P, Jin S-J, Jiang G-Q. The prognostic correlation of AFP level at diagnosis with pathological grade, progression, and survival of patients with hepatocellular carcinoma. Scientific Reports. 2017 Oct 9;7(1):12870.
7. Mizejewski GJ, MacColl R. Alpha-fetoprotein growth inhibitory peptides: potential leads for cancer therapeutics. Mol Cancer Ther. 2003 Nov;2(11):1243–55.
8. Vakharia D, Mizejewski GJ. Human alpha-fetoprotein peptides bind estrogen receptor and estradiol, and suppress breast cancer. Breast Cancer Res Treat. 2000 Sep;63(1):41–52.
9. Muehlemann M, Miller KD, Dauphinee M, Mizejewski GJ. Review of Growth Inhibitory Peptide as a biotherapeutic agent for tumor growth, adhesion, and metastasis. Cancer Metastasis Rev. 2005 Sep;24(3):441–67.
10. Li M, Li H, Li C, Guo L, Liu H, Zhou S, Liu X, Chen Z, Shi S, Wei J, McNutt MA, Li G. Cytoplasmic alpha-fetoprotein functions as a co-repressor in RA-RAR signaling to promote the growth of human hepatoma Bel 7402 cells. Cancer Letters. 2009 Nov 28;285(2):190–9.
11. Schmelzer E, Zhang L, Bruce A, Wauthier E, Ludlow J, Yao H, Moss N, Melhem A, McClelland R, Turner W, Kulik M, Sherwood S, Tallheden T, Cheng N, Furth ME, Reid LM. Human hepatic stem cells from fetal and postnatal donors. J Exp Med. 2007 Aug 6;204(8):1973–87.
12. Li P, Wang S-S, Liu H, Li N, McNutt MA, Li G, Ding H-G. Elevated serum alpha fetoprotein levels promote pathological progression of hepatocellular carcinoma. World Journal of Gastroenterology. 2011 Nov 7;17(41):4563–71.
13. Wang X, Wang Q. Alpha-Fetoprotein and Hepatocellular Carcinoma Immunity. Can J Gastroenterol Hepatol [Internet]. 2018 Apr 1 [cited 2019 Jan 15];2018. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5899840/
14. Li M-S, Li P-F, Chen Q, Du G-G, Li G. Alpha-fetoprotein stimulated the expression of some oncogenes in human hepatocellular carcinoma Bel 7402 cells. World J Gastroenterol. 2004 Mar 15;10(6):819–24.
15. Zhang XL, Shi JQ, Zuo SH. [A comparative study of the expression of ras oncogenic protein P21 and oncofetal protein AFP, CEA in human hepatocellular carcinoma (HCC)]. Zhonghua Bing Li Xue Za Zhi. 1994;23(3):155–8.
16. Zheng L, Gong W, Liang P, Huang X, You N, Han KQ, Li YM, Li J. Effects of AFP-activated PI3K/Akt signaling pathway on cell proliferation of liver cancer. Tumor Biol. 2014 May 1;35(5):4095–9.
17. Li M, Li H, Li C, Wang S, Jiang W, Liu Z, Zhou S, Liu X, McNutt MA, Li G. Alpha-fetoprotein: a new member of intracellular signal molecules in regulation of the PI3K/AKT signaling in human hepatoma cell lines. Int J Cancer. 2011 Feb 1;128(3):524–32.
18. Li M, Li H, Li C, Zhou S, Guo L, Liu H, Jiang W, Liu X, Li P, McNutt MA, Li G. Alpha fetoprotein is a novel protein-binding partner for caspase-3 and blocks the apoptotic signaling pathway in human hepatoma cells. Int J Cancer. 2009 Jun 15;124(12):2845–54.
19. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology. 2007 Jun;132(7):2557–76.
20. Blackburn EH, Epel ES, Lin J. Human telomere biology: A contributory and interactive factor in aging, disease risks, and protection. Science. 2015 Dec 4;350(6265):1193–8.
21. Heidenreich B, Kumar R. TERT promoter mutations in telomere biology. Mutat Res. 2017 Mar;771:15–31.
22. Zhang Q, Kim N-K, Feigon J. Architecture of human telomerase RNA. Proc Natl Acad Sci USA. 2011 Dec 20;108(51):20325–32.
23. Méndez-Pertuz M, Martínez P, Blanco-Aparicio C, Gómez-Casero E, Belen García A, Martínez-Torrecuadrada J, Palafox M, Cortés J, Serra V, Pastor J, Blasco MA. Modulation of telomere protection by the PI3K/AKT pathway. Nat Commun [Internet]. 2017 Nov 2 [cited 2019 Jan 26];8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5668434/
24. Kyo S, Takakura M, Taira T, Kanaya T, Itoh H, Yutsudo M, Ariga H, Inoue M. Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). Nucleic Acids Res. 2000 Feb 1;28(3):669–77.
25. Chang JT, Lu Y-C, Chen Y-J, Tseng C-P, Chen Y-L, Fang C-W, Cheng A-J. hTERT phosphorylation by PKC is essential for telomerase holoprotein integrity and enzyme activity in head neck cancer cells. Br J Cancer. 2006 Mar 27;94(6):870–8.
26. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. Nat Rev Cancer. 2007 Apr;7(4):281–94.
27. Garg R, Benedetti LG, Abera MB, Wang H, Abba M, Kazanietz MG. Protein kinase C and cancer: what we know and what we do not. Oncogene. 2014 Nov 6;33(45):5225.
28. al VA et. Tumor grade-dependent alterations in the protein kinase C isoform pattern in urinary bladder carcinomas. - PubMed - NCBI [Internet]. [cited 2019 Feb 11]. Available from: https://www.ncbi.nlm.nih.gov/pubmed/15363561
29. al OC et. Elevated protein kinase C expression in human breast tumor biopsies relative to normal breast tissue. - PubMed - NCBI [Internet]. [cited 2019 Feb 11]. Available from: https://www.ncbi.nlm.nih.gov/pubmed/2720675
30. al LM et. Expression levels of protein kinase C-alpha in non-small-cell lung cancer. - PubMed - NCBI [Internet]. [cited 2019 Feb 11]. Available from: https://www.ncbi.nlm.nih.gov/pubmed/15555220
31. al KJ et. Protein kinase C alpha/beta inhibitor Go6976 promotes formation of cell junctions and inhibits invasion of urinary bladder carcinoma cells. - PubMed - NCBI [Internet]. [cited 2019 Feb 11]. Available from: https://www.ncbi.nlm.nih.gov/pubmed/15313909

32. Koren R, Ben Meir D, Langzam L, Dekel Y, Konichezky M, Baniel J, Livne PM, Gal R, Sampson SR. Expression of protein kinase C isoenzymes in benign hyperplasia and carcinoma of prostate. Oncol Rep. 2004 Feb;11(2):321–6.
33. Wu-Zhang AX, Newton AC. Protein kinase C pharmacology: refining the toolbox. Biochem J. 2013 Jun 1;452(2):195–209.
34. Tsai JH, Hsieh YS, Kuo SJ, Chen ST, Yu SY, Huang CY, Chang AC, Wang YW, Tsai MT, Liu JY. Alteration in the expression of protein kinase C isoforms in human hepatocellular carcinoma. Cancer Lett. 2000 Dec 20;161(2):171–5.
35. Newton AC. Protein kinase C as a tumor suppressor. Semin Cancer Biol. 2018;48:18–26.
36. Newton AC, Brognard J. Reversing the Paradigm: Protein Kinase C as a Tumor Suppressor. Trends Pharmacol Sci. 2017 May;38(5):438–47.
37. Reyland ME. Protein kinase C isoforms: Multi-functional regulators of cell life and death. Front Biosci (Landmark Ed). 2009 Jan 1;14:2386–99.
38. Lu Y, Zhu M, Li W, Lin B, Dong X, Chen Y, Xie X, Guo J, Li M. Alpha fetoprotein plays a critical role in promoting metastasis of hepatocellular carcinoma cells. J Cell Mol Med. 2016 Mar;20(3):549–58.
39. Wang Q, Fiel MI, Blank S, Luan W, Kadri H, Kim KW, Manizate F, Rosenblatt AG, Labow DM, Schwartz ME, Hiotis SP. Impact of liver fibrosis on prognosis following liver resection for hepatitis B-associated hepatocellular carcinoma. Br J Cancer. 2013 Aug 6;109(3):573–81.
40. Li M, Zhou S, Liu X, Li P, McNutt MA, Li G. alpha-Fetoprotein shields hepatocellular carcinoma cells from apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand. Cancer Lett. 2007 May 8;249(2):227–34.
41. Mizejewskia GJ, Butterstein G. Survey of functional activities of alpha-fetoprotein derived growth inhibitory peptides: review and prospects. Curr Protein Pept Sci. 2006 Feb;7(1):73–100.
42. Vora SR, Zheng H, Stadler ZK, Fuchs CS, Zhu AX. Serum alpha-fetoprotein response as a surrogate for clinical outcome in patients receiving systemic therapy for advanced hepatocellular carcinoma. Oncologist. 2009 Jul;14(7):717–25.
43. Alpha-fetoprotein response after locoregional therapy for hepatocellular carcinoma: oncologic marker of radiologic response, progression, and survival - PubMed [Internet]. [cited 2021 Jan 7]. Available from: https://pubmed.ncbi.nlm.nih.gov/19805671/
44. Chen L-T, Shiah H-S, Chao Y, Chang J-Y, Cheng L-T, Whang-Peng J. Alpha-fetoprotein response in advanced hepatocellular carcinoma receiving cytostatic agent. J Clin Oncol. 2009 Dec 20;27(36):e271; author reply e272.
45. Zhou X, Lu J, Zhu H. Correlation between the expression of hTERT gene and the clinicopathological characteristics of hepatocellular carcinoma. Oncol Lett. 2016 Jan;11(1):111–5.
46. Miura N, Shiota G, Nakagawa T, Maeda Y, Sano A, Marumoto A, Kishimoto Y, Murawaki Y, Hasegawa J. Sensitive detection of human telomerase reverse transcriptase mRNA in the serum of patients with hepatocellular carcinoma. Oncology. 2003;64(4):430–4.
47. Tahtouh R, Azzi A-S, Alaaeddine N, Chamat S, Bouharoun-Tayoun H, Wardi L, Raad I, Sarkis R, Antoun NA, Hilal G. Telomerase Inhibition Decreases Alpha-Fetoprotein Expression and Secretion by Hepatocellular Carcinoma Cell Lines: In Vitro and In Vivo Study. PLOS ONE. 2015 Mar 30;10(3):e0119512.
48. Li H, Zhao L, Yang Z, Funder JW, Liu JP. Telomerase is controlled by protein kinase Calpha in human breast cancer cells. J Biol Chem. 1998 Dec 11;273(50):33436–42.
49. Ku WC, Cheng AJ, Wang TC. Inhibition of telomerase activity by PKC inhibitors in human nasopharyngeal cancer cells in culture. Biochem Biophys Res Commun. 1997 Dec 29;241(3):730–6.
50. Lu H-C, Chou F-P, Yeh K-T, Chang Y-S, Hsu NC, Chang J-G. Expression of protein kinase C family in human hepatocellular carcinoma. Pathol Oncol Res. 2010 Sep;16(3):385–91.
51. Kang J-H. Protein Kinase C (PKC) Isozymes and Cancer [Internet]. New Journal of Science. 2014 [cited 2019 Feb 19]. Available from: https://www.hindawi.com/journals/njos/2014/231418/
52. Tepekoy F, Ustunel I, Akkoyunlu G. Protein kinase C isoforms α, δ and ε are differentially expressed in mouse ovaries at different stages of postnatal development. J Ovarian Res [Internet]. 2014 Dec 10 [cited 2019 Feb 19];7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4271327/
53. Kashkoush S, Saleh S, Elmoghazy W. Serum Alpha-Fetoprotein as a Biomarker in Liver Transplantation. In: Patel VB, Preedy VR, editors. Biomarkers in Liver Disease [Internet]. Dordrecht: Springer Netherlands; 2017 [cited 2019 Mar 1]. p. 837–70. (Biomarkers in Disease: Methods, Discoveries and Applications). Available from: https://doi.org/10.1007/978-94-007-7675-3_34
54. Murriel CL, Mochly-Rosen D. Opposing roles of delta and epsilonPKC in cardiac ischemia and reperfusion: targeting the apoptotic machinery. Arch Biochem Biophys. 2003 Dec 15;420(2):246–54.
55. Basu A, Pal D. Two faces of protein kinase Cδ: the contrasting roles of PKCδ in cell survival and cell death. ScientificWorldJournal. 2010 Nov 16;10:2272–84.
56. Irie K, Yanagita RC, Nakagawa Y. Challenges to the development of bryostatin-type anticancer drugs based on the activation mechanism of protein kinase Cδ. Med Res Rev. 2012 May;32(3):518–35.
57. Jackson RJ, Hellen CUT, Pestova TV. The mechanism of eukaryotic translation initiation and principles of its regulation. Nat Rev Mol Cell Biol. 2010 Feb;11(2):113–27.
58. Koese M, Rentero C, Kota BP, Hoque M, Cairns R, Wood P, Vilà de Muga S, Reverter M, Alvarez-Guaita A, Monastyrskaya K, Hughes WE, Swarbrick A, Tebar F, Daly RJ, Enrich C, Grewal T. Annexin A6 is a scaffold for PKCα to promote EGFR inactivation. Oncogene. 2013 Jun 6;32(23):2858–72.
59. Hoshino D, Jourquin J, Emmons SW, Miller T, Goldgof M, Costello K, Tyson DR, Brown B, Lu Y, Prasad NK, Zhang B, Mills GB, Yarbrough WG, Quaranta V, Seiki M, Weaver AM. Network analysis of the focal adhesion to invadopodia transition identifies a PI3K-PKCα invasive signaling axis. Sci Signal. 2012 Sep 11;5(241):ra66.
60. Li L, Sampat K, Hu N, Zakari J, Yuspa SH. Protein Kinase C Negatively Regulates Akt Activity and Modifies UVC-induced Apoptosis in Mouse Keratinocytes. J Biol Chem. 2006 Feb 10;281(6):3237–43.
61. Xia S, Forman LW, Faller DV. Protein kinase C delta is required for survival of cells expressing activated p21RAS. J Biol Chem. 2007 May 4;282(18):13199–210.

SUPPLEMENTARY MATERIAL

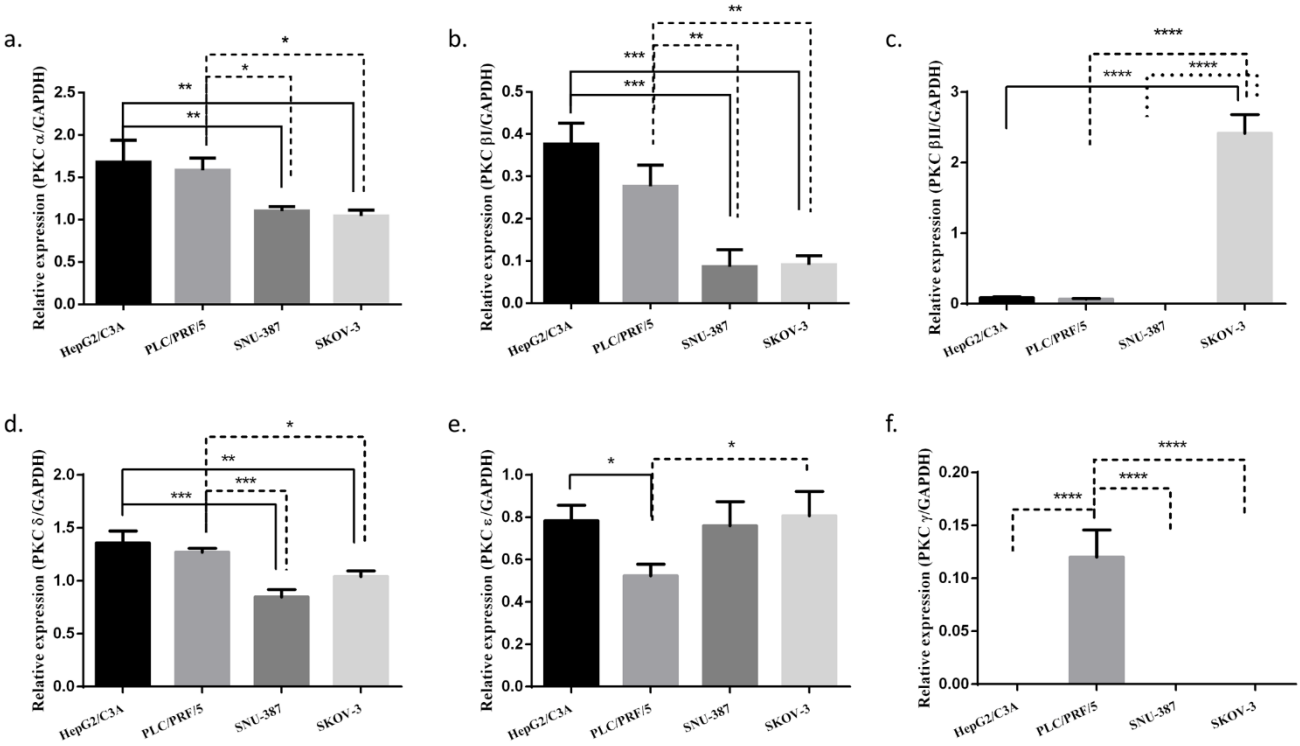

Figure S1. Semi-quantitative RT-PCR data analysis of the expression of PKC isoforms. The GelAnalyzer-2010 software was used to obtain the relative expression of mRNA of PKC isoforms (PKCα (a), PKCβI (b), PKC βII (c), PKCδ (d), PKCε (e), PKCγ (f) in HepG2/C3A, PLC/PRF/5, SNU-387, and SKOV-3) relatively with GAPDH. Results were expressed as the mean ±SD from a minimum of three lots. * P<0.05, ** P<0.01 and *** P<0.001 (comparing each isoform in different cell lines)

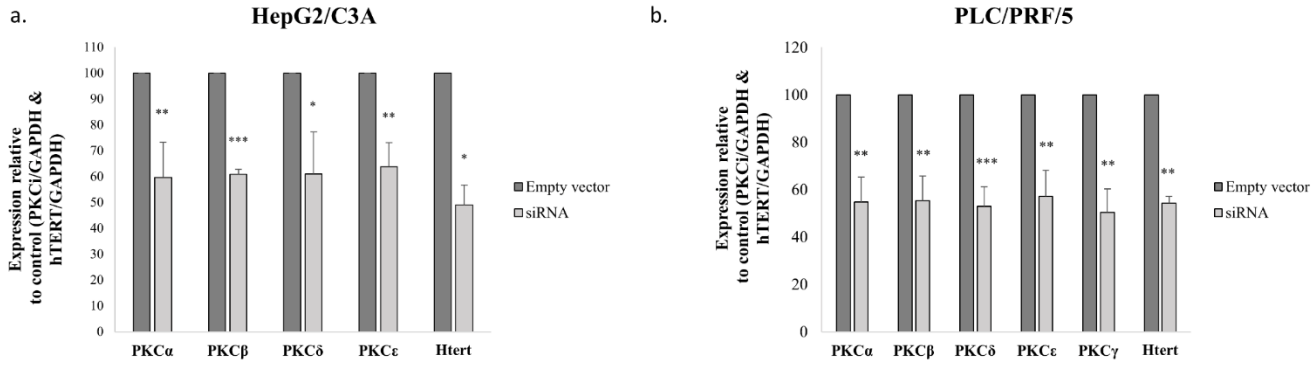

Figure S2. Effect of siRNA treatment on hTERT and PKC isoforms in HepG2/C3A and in PLC/PRF/5. For each siRNA we evaluated the expression of the inhibited gene. HepG2/C3A cells (a) were treated with siRNA of PKCα, PKCβ, PKCδ, PKCε, and hTERT and PLC/PRF/5 cells (b) were treated with siRNA of PKCα, PKCβ, PKCδ, PKCε, PKCγ and hTERT. The expression of each gene was then analyzed by qRT-PCR with GAPDH as the internal control. Results were expressed as the mean ±SD from a minimum of three experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

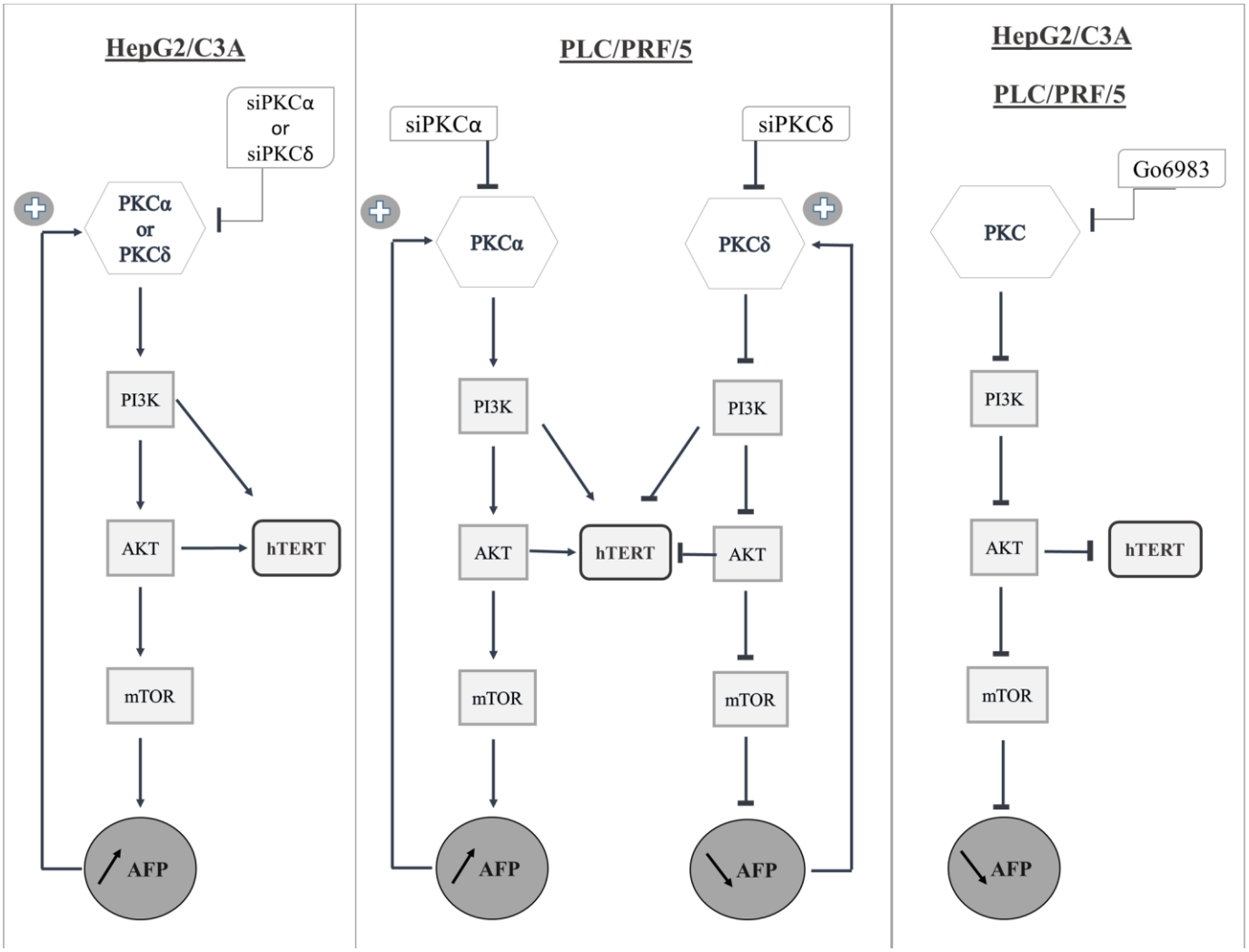

Figure S3. Summary Diagram. Our study demonstrates that the inhibition of PKCα and PKCδ by siRNA leads to an increase in AFP expression and secretion and hTERT expression in HepG2/C3A cells in a mechanism that implicates the PI3K/AKT/mTOR pathway. The same pattern was observed in PLC/PRF/5, however PKCδ inhibition by siRNA decreases AFP and hTERT expression. This modulation also involves PI3K/AKT/mTOR pathway. On the other hand, the inhibition of PKC isoform with *Gö6983*, a broad spectrum *PKC* inhibitor, leads to a significant decrease i*n* AFP expression and secretion and hTERT expression also through the PI3K/AKT/mTOR pathway in HepG2/C3A and PLC/PRF/5 cells. Our study also demonstrates an inter-relation occurs when the expression of AFP affects PKC isoforms expression in both cell lines