Modulation of Protein Phosphatase 2A Activity Alters Androgen-Independent Growth of Prostate Cancer Cells: Therapeutic Implications

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Abstract
Earlier we identified PPP2CA, which encodes for the α-isoform of protein phosphatase 2A (PP2A) catalytic subunit, as one of the downregulated genes in androgen-independent prostate cancer. PP2A is a serine/threonine phosphatase and a potent tumor suppressor involved in broad cellular functions; however, its role in prostate cancer has not yet been determined. Here, we have investigated the effect of PP2A activity modulation on the androgen-independent growth of prostate cancer cells. Our data show that the PPP2CA expression and PP2A activity is downregulated in androgen-independent (C4-2) prostate cancer cells as compared with androgen-dependent (LNCaP) cells. Downregulation of PP2A activity by pharmacologic inhibition or short interfering RNA-mediated PPP2CA silencing sustains the growth of LNCaP cells under an androgen-deprived condition by relieving the androgen deprivation–induced cell-cycle arrest and preventing apoptosis. Immunoblot analyses reveal enhanced phosphorylation of Akt, extracellular signal–regulated kinase (ERK), BAD, increased expression of cyclins (A1/D1), and decreased expression of cyclin inhibitor (p27) on PP2A downregulation. Furthermore, our data show that androgen receptor (AR) signaling is partially maintained in PP2A-inhibited cells through increased AR expression and ligand-independent phosphorylation. Pharmacologic inhibition of Akt, ERK, and AR suggest a role of these signaling pathways in facilitating the androgen-independent growth of LNCaP cells. These observations are supported by the effect of ceramide, a PP2A activator, on androgen-independent C4-2 cells. Ceramide inhibited the growth of C4-2 cells on androgen deprivation, an effect that could be abrogated by PP2A downregulation. Altogether, our findings suggest that modulation of PP2A activity may represent an alternative therapeutic approach for the treatment of advanced androgen-independent prostate cancer.

Introduction
Prostate cancer is the most common malignancy in men and the second leading cause of male cancer deaths in the United States (1). According to the estimate by the American Cancer Society, nearly 192,280 patients were diagnosed with prostate cancer and approximately 27,360 died due to this malignancy in the year 2009 (2). Considering the central role of androgen receptor (AR) signaling in prostate cancer, surgical or medical castration [referred as androgen deprivation therapy (ADT)] is the first line of treatment for the advanced disease. Most patients treated with ADT initially exhibit a dramatic regression of the androgen-dependent cancer cells; however, the tumors eventually progress to an androgen-independent stage, resulting in a poor prognosis (1). The molecular mechanisms responsible for the failure of ADT are not yet clearly understood. It is believed that AR abnormalities, altered expression of AR coregulators, and dysregulation of non-AR-signaling cascades may be associated with the acquisition of hormone refractory phenotype (3–5). A cross-talk of AR with other cell signaling pathways has also been shown, which leads to its aberrant activation and thus compensate for androgen ablation (6, 7). Once the prostate cancer has recurred, it progresses to a highly aggressive disease with frequent metastasis and poses an increased risk of morbidity and death (1). Importantly, this relapsed disease (androgen-independent prostate cancer), unlike other cancers, also does not respond well to alternative approaches such as chemotherapy and radiotherapy (8–10). Therefore, high rate of mortality from prostate cancer is linked with its progression to hormone refractory phenotype and a lack of effective alternative therapeutic approaches.

In an earlier study, we characterized the transcriptomic variation associated with androgen-sensitive and androgen-refractory phenotypes though a genome-wide
expression profiling and identified many differentially expressed genes (11). PPP2CA, which encodes the catalytic subunit (α-isoform) of the protein phosphatase 2A (PP2Aα), was one of the genes of interest that exhibited a downregulated expression in androgen-independent prostate cancer cells. The level of PP2ACα was decreased in majority of androgen-independent prostate cancer cell lines and in cancer lesions as compared with the adjacent normal/benign tumor tissues. Interestingly, our study also showed an inverse correlation of PP2ACα expression with stage (early vs. late) and Gleason grade (low vs. high; ref. 11). In another study, the downregulated expression of β-isoform of PP2A catalytic subunit (PP2ACβ) in prostate cancer has also been reported (12). PP2ACα and PP2ACβ share 97% identity and are ubiquitously expressed; however, PP2ACα is about 10 times more abundant than PP2ACβ (13). PP2ACα/β is a well-conserved subunit of PP2A serine/threonine phosphatases, and the in vivo activity of PP2A is provided by related complexes that exist either as heterodimers or heterotrimers with scaffold (A) and regulatory (B) subunits (14).

PP2A does broad cellular functions and the functional diversity of PP2A is determined by different scaffold and regulatory subunits. In fact, PP2A has been shown to interact with a wide range of proteins via its 3 subunits (14). These interactions facilitate the cross-talk of PP2A with multiple cell signaling pathways including mitogen-activated protein kinase (MAPK), Akt/PKB, PKC, and IκB kinases (15–17). Most common role of PP2A catalytic activity in different organisms is in cell survival (18–20). More recently, important roles of PP2A in stem cell pluripotency, cell migration and invasion, DNA repair, translation, and stress response have been implicated (14, 21, 22). In the present study, we have investigated the functional significance of downregulated PPP2CA expression in androgen-independent growth of prostate cancer cells. Using lineage-associated androgen-dependent (LNCaP) and androgen-independent (C4-2) prostate cancer cell lines, we show that decreased PP2A activity is associated with enhanced potential to sustain under androgen-deprived condition. Specifically, our data reveal that the androgen-independent growth of prostate cancer cells on PP2A inhibition is sustained through a concerted action of Akt, extracellular signal–regulated kinase (ERK), and AR signaling pathways.

Materials and Methods

Reagents

RPMI 1640 media, penicillin, streptomycin, and Vybrant MTT cell proliferation assay kit were from Invitrogen. FBS was from Atlanta Biologicals. FuGENE transfection reagent and phosphatase/protease inhibitors cocktail were from Roche Diagnostics. PP2A immuno-precipitation phosphatase assay kit was from Upstate Biotechnology. Human PPP2CA-specific short interfering RNAs (siRNA; catalogue no. L-003598-01), nontarget siRNAs (catalogue no. D-001810-10), and DharmaFECT transfection reagent were from Dharmacon. Charcoal/dextran-stripped serum (CSS) was from Gemini Bio-Products. Propidium iodide (PI)/RNase staining buffer was from BD Bioscience. Fostriecin was from Enzo Life Science. Phosphatidylinositol 3-kinase (PI3K)/Akt inhibitor (LY294002) and ERK inhibitor (PD98059) and antibodies against ERK1/2 (rabbit monoclonal), pERK1/2 (mouse monoclonal), BAD (rabbit monoclonal), pBAD (rabbit polyclonal), Bcl-xL (rabbit monoclonal), and Bax (rabbit polyclonal) were from Cell Signaling Technology. Antibodies (rabbit monoclonal) against PP2Aα, Akt, p-Akt, AR, and prostate-specific antigen (PSA) were from Epitomics. Anti–phospho-AR (Ser81, rabbit polyclonal) and (Ser213/210, mouse monoclonal) antibodies were from Millipore and Imgenex, respectively. Antibodies against p21 (mouse monoclonal), p27, cyclin A1, cyclin D1 (rabbit polyclonal), and horseradish peroxidase–conjugated secondary antibodies were from Santa Cruz Biotechnology. Dihydrotestosterone (DHT), antienandrogen bicalutamide (Casodex), and C2 dihydroceramide were from Sigma-Aldrich. CaspACE FITC-VAD-FMK and Dual-Luciferase Assay System kit were from Promega. VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole was from Vector Laboratories Inc. ECL plus Western Blotting Detection Kit was from Thermo Scientific. Cignal AR Androgen Receptor Assay Kit was purchased from SA Biosciences.

Cell culture

Adherent monolayer cultures of androgen-dependent LNCaP (American Type Culture Collection) and AI C4-2 (UroCor Inc.) human prostate cancer cell lines were maintained in RPMI 1640 medium supplemented with 5.0% FBS and 100 μmol/L each of penicillin and streptomycin. Cells were grown at 37°C with 5% CO2 in humidified atmosphere, and media was replaced every third day. Cells were split (1:3), when they reached near confluence. To authenticate the cell lines, we carried out short tandem repeats genotyping. Furthermore, their response to androgens for growth and AR activity was also monitored intermittently during the study.

Treatments and transfections

For various treatments, cells were cultured either in 10-cm petri dishes or 6/24/96-well plates to about 60% to 80% confluence as specified above. Thereafter, media was replaced with steroid-reduced CSS-containing media and cells were treated with (i) DHT, (ii) fostriecin, (iii) LY294002, (iv) PD98059, (v) bicalutamide/Casodex, and (vi) ceramide alone or in combination at doses and times specified in figure legends. For the knockdown of PPP2CA, cells were cultured in 6/96-well plates to about 50% to 70% confluence and transiently transfected with 0.05 mmol/L of human PPP2CA-specific or nontarget control siRNAs using DharmaFECT (Dharmacon) as per the manufacturer’s protocol. Following 24 hours after transfection, cells were treated as described earlier.
**Western blot analysis**

Cells were processed for protein extraction and Western blotting as described earlier (23). Briefly, the cells were washed twice with PBS and cell lysates were prepared in NP-40 lysis buffer (150 mmol/L NaCl, 1% NP-40, 50 mmol/L Tris-Cl, pH 7.4, and 5 mmol/L EDTA) containing protease and phosphatase inhibitors. Cell lysates were passed through a needle syringe to facilitate the disruption of the cell membranes and centrifuged at 14,000 rpm for 20 minutes at 4°C and supernatants were collected. Protein lysates (10–60 μg) were resolved by electrophoresis on 10% SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membrane, and subjected to standard immunodetection procedure using specific antibodies: PP2Ac, Akt, pAkt, ERK1/2, pERK1/2, BAD, pBAD, AR, pAR (Ser81), Bel-xl, Bax (1:1,000), pAR (Ser213/210), PSA (1:2,500), p21, p27, cyclin A1, cyclin D1 (1:200), and β-actin (1:20,000). All secondary antibodies were used at 1:2,500 dilutions. Blots were processed with ECL Plus Western Blotting Detection Kit and the signal detected using an LAS-3000 image analyzer (Fuji Photo Film Co.).

**PP2A activity assay in vitro**

PP2A activity was determined using PP2A immunoprecipitation phosphatase assay kit according to the manufacturer’s instructions. Briefly, PP2Acα was immunoprecipitated with anti-PP2Acα monoclonal antibody and Protein A Agarose beads. PP2Acα-bound beads were collected by the centrifugation and washed with serine/threonine assay buffer. Thereafter, phosphopeptide (K-R-pT-I-R-R) was added to the washed beads (at final concentration 250 μmol/L), followed by incubation at 30°C for 15 minutes. After centrifugation, 25 μL of supernatant was transferred to an assay plate; 100 μL of Malachite Green phosphate detection solution was added and incubated at 30°C for 15 minutes for the color development. The relative absorbance was measured at 630 nm in a microplate reader (BioTek).

**Cell growth assay**

Cells were seeded at a density of 5 × 10³ cells per well in 96-well plate. After various treatments, cell viability was determined by using Vybrant MTT cell proliferation assay kit. Growth was calculated as percent = [(A/B) – 1] × 100, where A and B are the absorbance of treatment and control cells, respectively.

**Cell-cycle analysis**

Following various treatments, cells were trypsinized and washed twice in PBS. Subsequently, 70% ethanol was added and cells were fixed overnight at 4°C. Fixed cells were washed with PBS and stained with PI using PI/RNase staining buffer for 1 hour at 37°C. Stained cells were analyzed by flow cytometry on a BD FACS Calibur™ II (Becton Dickinson) and percentage of cell population in various phases of cell cycle was calculated using ModFit LT software (Verity Software House).

**Aptosis assay**

Cells cultured on glass bottom FluoroDish (World Precision Instruments) were subjected to various treatments as described in figure legend. Apoptosis was detected by staining the cells with CaspACE FITC-VD-FMK solution in PBS for 2 hours at 37°C. CaspACE FITC-VD-FMK In Situ Marker is a fluorescent analogue of the pan-caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethyl-ketone), which irreversibly binds to activated caspases and is a surrogate for caspase activity in situ. Following staining, cells were fixed with 4% paraformaldehyde at room temperature, washed with PBS, and mounted with Vectashield. The bound fluorescent marker was detected under a Nikon Eclipse TE2000-U fluorescent microscope (Nikon Instruments Inc.). The number of apoptotic cells per field (×100) was counted and results expressed as the mean ± SD of apoptotic cells in 10 random viewfields.

**AR transcriptional activity assay**

AR transcriptional activity was determined by Cignal AR Androgen Receptor Assay Kit according to the manufacturer’s protocol. Briefly, cells were grown in 24-well plate to about 50% to 60% confluency and thereafter, transiently transfected with AR reporter, negative control, and positive control plasmids using FuGENE transfection reagent as per manufacturer’s instructions. After 24 hours of transfection, cells were treated as described in figure legend for next 24 hours and total protein was isolated in passive lysis buffer. Firefly (for AR activity) and Renilla (for internal normalization) luciferase activities were measured using a Dual-Luciferase Assay System kit. All experiments were done in triplicate and relative luciferase units (RLU) were reported as mean ± SD from triplicates.

**Statistical analysis**

Each experiment was carried out at least 3 times and all the values were expressed as mean ± SD. The differences between the groups were compared using Student’s t tests. A value of P ≤ 0.05 was considered statistically significant.

**Results**

**Inhibition of PP2A enables androgen-dependent prostate cancer cells to grow under steroid-depleted condition**

Previously, we have reported the downregulated expression of PP2Acα in androgen-independent prostate cancer cells as compared with the androgen-dependent prostate cancer cells (11). Here, we examined the expression and activity of PP2Acα in 2 AR-expressing, lineage-associated human prostate cancer cell lines, LNCaP (androgen dependent) and C4-2 (androgen independent) under regular or steroid-reduced conditions. Our immunoblot and in vitro phosphatase activity data show that both the expression and activity of PP2Acα is significantly downregulated in C4-2 (androgen independent) cells as...
compared with LNCaP (androgen dependent) cells, and there is no significant change in the expression or activity of PP2AC on steroid depletion (Fig. 1A). Next, we examined the effect of fostriecin (a potent inhibitor of PP2A) and siRNA-mediated silencing of PPP2CA on the activity of PP2A in LNCaP cells. Our data showed that PP2A activity was decreased following treatment with fostriecin (~77.27% and 89.32% at 50 and 100 nmol/L, respectively) and knockdown of PP2AC with specific siRNA (~74%). C, to investigate the effect of PP2A inhibition on androgen-independent growth, LNCaP cells were incubated in steroid-reduced (CSS) media and treated with DHT (1.0 nmol/L), Fos (100 nmol/L), and PPP2CA-specific or scrambled siRNAs. Cell growth was assessed by MTT assay after 96 hours of treatment. Numbers below the bands represent the fold ratio of densitometric quantification relative to corresponding control. Bars represent the means ± SD (n = 3); *, statistically significant (P < 0.05). D, chemical structures of fostriecin (i) and DHT (ii).

Figure 1. PP2A activity is downregulated in AI prostate cancer cells and its inhibition sustains the growth of AD prostate cancer cells under steroid-depleted condition. A, total protein from LNCaP (androgen dependent) and C4-2 (androgen independent) prostate cancer cells was resolved and immunoblotted for PP2AC and β-actin (internal control). PP2A activity was determined by malachite green–based phosphatase assay. PP2A was expressed at low level in androgen-independent prostate cancer (C4-2) cells in comparison with androgen-dependent prostate cancer (LNCaP) cells and correlated with decreased activity (~70%) under both steroid-supplemented and -reduced conditions. B, androgen-dependent prostate cancer (LNCaP) cells were treated with different doses (50 and 100 nmol/L) of fostriecin (Fos) in steroid-reduced (CSS) media for 72 hours. In parallel, PPP2CA expression was silenced by transient transfection of LNCaP cells with PPP2CA-specific siRNA for 72 hours. Cells were also transfected with nontargeted scrambled siRNAs to serve as control. Activity of PP2A was decreased in LNCaP cells after treatment with Fos (~77.27% and 89.32% at 50 and 100 nmol/L, respectively) and knockdown of PP2AC with specific siRNA (~74%). C, to investigate the effect of PP2A inhibition on androgen-independent growth, LNCaP cells were incubated in steroid-reduced (CSS) media and treated with DHT (1.0 nmol/L), Fos (100 nmol/L), and PPP2CA-specific or scrambled siRNAs. Cell growth was assessed by MTT assay after 96 hours of treatment. Numbers below the bands represent the fold ratio of densitometric quantification relative to corresponding control. Bars represent the means ± SD (n = 3); *, statistically significant (P < 0.05). D, chemical structures of fostriecin (i) and DHT (ii).
suggest that the downmodulation of PP2A enables androgen-dependent prostate cancer cells to grow under steroid deprivation and thus may have an important role in androgen-independent growth of prostate cancer.

**Downregulation of PP2A sustains growth of LNCaP cells by preventing steroid depletion–induced cell-cycle arrest and apoptosis**

Earlier, it has been shown that steroid depletion induces arrest of cell cycle and apoptosis in androgen-dependent LNCaP cells, which leads to overall deceased growth (24–26). Therefore, we examined the effect of PP2A inhibition on cell-cycle progression and apoptosis under steroid-depleted (CSS) condition. The proliferation index was determined by DHT or fostriecin treatment of synchronized LNCaP cells followed by PI staining and flow cytometry (Fig. 2A). In accordance with previously published reports (24, 25), our data showed arrest of LNCaP cells in G0-G1 phase of cell cycle under steroid-reduced condition, an effect that was abrogated on treatment with DHT (1 nmol/L; Fig. 2A). Furthermore, we observed that the inhibition of PP2A by either fostriecin

![Figure 2. PP2A inhibition relieves hormone deprivation–induced G0-G1 arrest and suppresses apoptosis. A, LNCaP cells were synchronized by serum starvation and treated with DHT (1 nmol/L) or fostriecin (Fos; 100 nmol/L) for 24 hours in steroid-reduced (CSS) media. After treatments, distribution of cells in different phases of cell cycle was analyzed by PI staining followed by flow cytometry. Cell-cycle analysis was also carried out on control and PPP2CA-silenced LNCaP cells incubated in steroid-reduced media. Both the treatment with DHT or PP2A inhibition relieved the androgen deprivation–induced G0-G1 cell-cycle arrest, although the effect was more prominent with DHT. B, to determine the effect of PP2A inhibition on apoptosis, subconfluent cultures of LNCaP cells were treated with DHT (1 nmol/L), Fos (100 nmol/L), and PPP2CA-specific or scrambled (Scr) siRNAs under steroid-reduced condition for 96 hours. Apoptosis was detected by staining the cells with CaspACE FITC-VAD-FMK solution in PBS for 2 hours at 37 °C. Following fixation, bound marker was visualized by fluorescent detection under a Nikon microscope. Inhibition of PP2A suppressed the apoptosis as evident by the decreased fluorescence intensity and number of positively (dark green fluorescent) stained cells. Representative picture is from one of the random fields of view. Bars represent the means ± SD of apoptotic cells in 10 random viewfields; *, statistically significant (P < 0.05).**
or siRNA-mediated silencing of PPP2CA also led to the release of steroid depletion–induced cell-cycle arrest of LNCaP cells. The total percentage of LNCaP cells that entered S phase and then progressed to G2-M phase was 27.78% on fostriecin treatment as compared with 18.22% in CSS-only treated LNCaP cells. Similarly, 20.96% of PPP2CA-silenced LNCaP cells were in S and G2-M phases as compared with 15.0% in scrambled siRNA transfected cells (Fig. 2A). To analyze the apoptotic index, we stained the cells with CaspACE FITC-VAD-FMK, a fluorescent analogue of a pan-caspase inhibitor that binds to the active caspases. As a measure of activity of caspases or apoptosis, we counted the fluorescently stained LNCaP transfected cells (Fig. 2B). Our data showed that steroid depletion led to enhanced apoptosis of LNCaP cells (3.4-fold), which could be suppressed up to 1.67- and 2.35-folds by treatment with DHT and fostriecin, respectively. Similarly, PPP2CA silencing also led to the reduction of apoptosis (2.1-fold) under steroid-deprived condition as compared with the scrambled siRNA–transfected cells. Altogether, our data show that PP2A inhibition supports the growth of LNCaP cells under androgen-depleted condition by preventing cell-cycle arrest and apoptosis.

**Downregulation of PP2A leads to the activation of survival signaling and alters the expression of cell-cycle–associated proteins**

PP2A impacts multiple cell signaling pathways by causing dephosphorylation of the signaling proteins (14). Akt and ERK are among the most significant signaling proteins that are regulated by PP2A and have also been shown to be involved in androgen-independent growth of human prostate cancer cells (15, 27, 28). To determine if the sustained growth of LNCaP cells under steroid-depleted condition was due to the activation of Akt and ERK, we monitored the change in their phosphorylation on PP2A inhibition. Our immunoblot data with total and phospho-form–specific antibodies (Fig. 3) showed an increased phosphorylation of both Akt and ERK. Similarily, silencing of PPP2CA also resulted in an increased Akt and ERK phosphorylation. Furthermore, we observed that PP2A inhibition induced the phosphorylation of BAD protein, which causes the loss of its proapoptotic effect. Interestingly, treatment with DHT led to a decrease in Akt, ERK, and BAD phosphorylation, whereas both DHT and fostriecin induced the expression of antiapoptotic Bcl-xL and suppressed the expression of proapoptotic Bax. Effect of DHT on Akt is in corroboration with earlier studies (28, 29); however, DHT has also been shown to cause nongenomic activation of PI3K/Akt in AR (ectopic)-expressing PC3 prostate cancer cells (30). Therefore, it will be of interest to investigate these observations further to identify the underlying molecular mechanism(s). Nonetheless, our findings suggest that a balance of pro- and antiapoptotic signaling during steroid deprivation determines the overall effect of DHT or PP2A inhibition in potentiating the survival of prostate cancer cells. Cell cycle is controlled by actions of various cyclins and their inhibitors. As we observed the effect of steroid deprivation and PP2A inhibition on cell-cycle arrest in G0-G1 phase, we examined the expression of cyclins (D1 and A1) and their inhibitors (p27 and p21), which are involved during G1-S transition. Our data showed that androgen deprivation led to the downregulation of both cyclin D1 and A1 expression in LNCaP cells, whereas the treatment with DHT or PP2A inhibition (by fostriecin or silencing of PPP2CA) caused their induction (Fig. 3). Furthermore, the expression of p27, inhibitor of cyclin D1, was upregulated on androgen deprivation and downregulated on treatment with DHT or PP2A inhibition. Interestingly, our data showed that the expression of p21 was changed in an opposite manner (Fig. 3). The functional significance of such observation is not clear; however, these data are consistent with a previous
finding (28). Altogether, our data suggest that PP2A inhibition potentiates proliferation and survival signaling and thus maintains AI growth of prostate cancer cells.

**PP2A inhibition upregulates the expression of AR and partially sustains its transcriptional activity**

AR plays important roles in both androgen-dependent and -independent growth of prostate cancer cells (1). It has been established that AR can maintain its transcriptional activity even under androgen-deprived condition through ligand-independent activation (28). Notably, it has been shown earlier that both Akt and ERK can induce phosphorylation of AR at serine residues leading to its activation (28, 31). Therefore, we examined the effect of PP2A inhibition on the phosphorylation of AR in LNCaP cells under steroid-depleted condition (Fig. 4A). We observed that the inhibition of PP2A either by fostriecin or siRNA led to an increased phosphorylation of AR at serine-81 residue, whereas no change was detected at the serine-213. In contrast, stimulation with DHT induced phosphorylation at both the serines (81 and 213). Our immunoblotting data also showed an induced expression of AR and its target gene, PSA/KLK3 on treatment with DHT or PP2A inhibition (Fig. 4A). To substantiate the activation of AR pathway, we conducted promoter reporter assay to measure the transcription activity of an AR responsive promoter. LNCaP cells were transfected with promoter reporter and control plasmids (negative and positive) and, 24 hours posttransfection, treated with either DHT or fostriecin under steroid-depleted condition for next 24 hours. In parallel, cells also cotransfected with scrambled or PPP2CA-specific siRNAs for 48 hours. Transcriptional activity of AR is presented as the RLUs, which is the ratio between firefly (for AR activity) and Renilla (transfection efficiency control) luciferase activity (Fig. 4B). Our data show a limited induction of AR activity in LNCaP cells treated with fostriecin (1.57-fold) or silenced for PPP2CA expression (1.64-fold) under steroid-depleted condition as compared with the cells grown in normal FBS (2.02-fold) or cells treated with DHT (2.2-fold). Altogether, our findings suggest that the inhibition of PP2A partially sustains AR activity by inducing AR expression and ligand-independent phosphorylation.

**AR activity is regulated by both Akt and ERK and their concerted action supports the AI growth of prostate cancer cells**

Having evaluated the impact of PP2A inhibition on Akt, ERK, and AR signaling pathways, we next evaluated the cross-talk of these signaling nodes and their involvement in AI growth of LNCaP cells. To examine this, we used pharmacologic inhibitors of Akt (LY294002) and ERK (PD98059) and antiandrogen (Casodex) to obstruct their activation before PP2A inhibition under steroid-deprived condition. The blockade of Akt, ERK, and AR activation was confirmed by monitoring their phosphorylation and PSA expression by immunoblotting (Fig. 5A). Our data indicated that the induced expression of AR on PP2A inhibition involves activation of Akt, whereas its phosphorylation at serine-81 is associated with ERK activation. Furthermore, inhibition of both Akt and ERK led to the reduced expression of PSA, thus indicating a role of these signaling pathways in ligand-independent activation of AR. Evaluation of LNCaP cell growth on repression of Akt, ERK, and AR before PP2A inhibition suggested a major role of Akt and ERK signaling pathways in supporting the androgen-independent growth of LNCaP cells (Fig. 5B). Nonetheless, downregulation of AR also had a significant negative impact on the fostriecin-induced growth of LNCaP cells under...
androgen-deprived condition. These findings suggest that the inhibition of PP2A leads to the activation of Akt and ERK, which supports androgen-independent growth of LNCaP cells in AR-dependent (through partial activation) and AR-independent manners.

Activation of PP2A suppresses the androgen-independent growth of C4-2 prostate cancer cells

As C4-2 cells are androgen independent and possess low PP2A activity, we examined whether the activation of PP2A would diminish their growth under steroid-deprived condition. For this, we treated the C4-2 cells with ceramide, which is known to activate PP2A (32, 33) and observed its effect on their growth. Our data showed that ceramide treatment led to an increase (2.0-fold) in the activity of PP2A in C4-2 cells under both FBS and CSS conditions. Furthermore, we observed that the pretreatment of cells with fostriecin could arrest the ceramide-induced PP2A activity (Fig. 6A). Treatment of C4-2 cells with ceramide decreased their growth (∼34%) in regular media, whereas in steroid-deprived media, ceramide treatment showed even more potent effect (∼71% decrease in growth; Fig. 6B). To confirm that the effect of ceramide on cellular growth was mediated through PP2A, we inhibited PP2A activity by pretreating the C4-2 cells with fostriecin. Our data showed that the inhibition of PP2A significantly attenuated ceramide-induced growth inhibition of C4-2 cells under steroid-depleted condition (Fig. 6B). Our signaling data showed that ceramide treatment decreased the phosphorylation of Akt and ERK, which could be reversed by pretreatment with fostriecin (Fig. 6C). It was also observed that the expression of cyclins (D1 and A1), AR, pAR(Ser81), and PSA was downregulated, whereas the expression of p27 was upregulated on treatment of C4-2 cells with ceramide. Downregulation of PP2A with fostriecin abrogated ceramide-induced effect on cyclin A1, D1, p27, AR, and PSA (Fig. 6C). Altogether, these findings provide additional support for a role of PP2A in modulating AI growth of prostate cancer cells.

Discussion

Protein phosphorylation plays an important role in various biological processes and is regulated by a dynamic equilibrium between the protein kinases and phosphatases. Disruption of this balance often leads to various pathologic conditions including malignant transformation. Our earlier studies indicated that the down regulation of PP2A, a serine/threonine phosphatase,
might be of clinical relevance in prostate cancer (11). Moreover, a recent phase I dose-escalation study of sodium selenate (an activator of PP2A) in patients with castration-resistant prostate cancer suggested that targeting of PP2A in combination with cytotoxic drug could be an effective therapeutic approach (34). In this study, our data show the functional role of PP2A in facilitating the androgen-independent growth of prostate tumor cells. Our data show that PP2A inhibition causes the release of steroid depletion–induced cell-cycle arrest and prevents apoptosis. It has been reported earlier that androgen withdrawal leads to cell-cycle arrest, and prostate cancer cells are able to bypass this checkpoint during the androgen-independent progression (26, 35). Furthermore, it has been shown that prostate cancer cells over-express survival proteins, such as Bcl-2, or have deletion of tumor suppressor genes, such as PTEN, which enable them to resist apoptosis, and thus have a growth advantage under adverse conditions (36, 37). Therefore, our data are significant in explaining another possible mechanism by which prostate cancer cells gain apoptotic resistance and escape cell-cycle arrest under androgen deprivation.

Substantial body of evidence suggests that PP2A can impact cellular homeostasis by interacting with multiple signaling cascades (14). Many of these signaling pathways (Akt, MAPK, etc.) have functionally been implicated in the pathogenesis and androgen-independent nature of prostate cancer cells (15, 17, 28). We have observed that down-modulation of PP2A results in the activation of Akt and ERK, inactivation of BAD, and induction of cell-cycle–associated proteins in LNCaP cells. Akt is a downstream effector of PI3K and has often been implicated in androgen-independent progression of prostate cancer (28, 38, 39). PI3K is upregulated in LNCaP cells due to the deletion of PTEN resulting in the hyperactivation of Akt (37). As the activity of Akt can also be controlled through PP2A-mediated dephosphorylation (40), our data indicate that the loss of this regulatory checkpoint further promotes Akt activation. PP2A has also been shown to...
importance of these signaling pathways in sustaining mechanistic significance. Our data also highlighted the role of PP2A under steroid-depleted condition, and thus holds significance in accordance with an earlier report, where activation of AR signaling was found to be important in Akt- or ERK-induced AI growth of prostate cancer cells (42).

In summary, our data provide first experimental evidence to support the functional significance of PP2A downregulation in androgen-independent progression of prostate cancer. Our findings show that PP2A is upregulated in LNCaP (androgen dependent) cells as compared with C4-2 (androgen independent) prostate cancer cells, and the blockade of its activity sustains the growth of LNCaP cells under steroid-depleted condition. Our data clearly indicate that PP2A inhibition rescues LNCaP cells from steroid deprivation–induced cell-cycle arrest and apoptosis. Mechanistic studies show that both Akt and ERK get activated on PP2A inhibition and support the androgen-independent growth of LNCaP cells in AR-dependent and AR-independent manners. Our data reveal that the AR signaling is partially sustained on PP2A downregulation in LNCaP cells, in part, through induced expression of AR and its ligand-independent activation. These findings are further supported by our observations in androgen-independent C4-2 cells where activation of PP2A is shown to cause suppression of their growth under steroid-reduced condition. Altogether, these findings may aid in the development of novel therapeutic strategies targeting the PP2A signaling network and/or better treatment planning against androgen-independent prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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