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14. ABSTRACT Advanced prostate cancers (PCa) treated with first line androgen-deprivation therapy (ADT) eventually relapse in a hormone refractory or castration-resistant (CR) form. Relapsed disease is highly aggressive and poses an increased risk of morbidity and death. Previously, we demonstrated that *PPP2CA*, which encodes the catalytic-subunit (alpha-isoform) of the protein phosphatase 2A (PP2ACα), is downregulated in CR PCa. The level of PP2ACα was decreased in majority of CR PCa cell lines and cancer lesions as compared to the adjacent normal/benign tumor tissues. Under this project, we have utilized multiple approaches to demonstrate a functional role of PP2A in human prostate cancer progression. We have shown that PP2A downregulation promotes growth, androgen depletion-resistance and aggressive behavior of prostate cancer cells. We have also developed *in vivo* experimental support for a suppressor role of PP2A in prostate cancer progression using orthotopic mouse model. Furthermore, we delineated the molecular mechanisms involved in the PP2A-mediated growth and aggressive phenotypes of PCa cells. We observed PP2A downregulation facilitates castration-resistant growth of PCa cells in both androgen receptor (AR)- dependent and –independent manners in AR expressing (LNCaP and C4-2) cells. Moreover, we identified that *PPP2CA* downregulation favors EMT, migration and invasion of PCa cells through Akt-dependent activation of β-catenin and NF-κB pathway. Our data strongly suggest that downregulation of PP2A is associated with human prostate cancer progression and restoration of PP2A activity may be an effective approach for the treatment of the advanced disease.			
15. SUBJECT TERMS **PPP2CA, protein phosphatase 2A, castration-resistance, malignant behavior, prostate cancer**			

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Table of Contents

INTRODUCTION:

First line of therapy for advanced prostate cancer (PCa) is androgen-deprivation therapy (ADT) through surgical or chemical castration; however, in majority of cases, tumors relapse in a hormone refractory or castration-resistant (CR) form (1). Once the PCa has recurred in CR form, it progresses to a highly aggressive disease with frequent metastasis and poses an increased risk of morbidity and death (1). Previously, we demonstrated that *PPP2CA*, which encodes the catalytic-subunit (alpha-isoform) of the protein phosphatase 2A (PP2ACα), is downregulated in CR PCa (2). The level of PP2ACα was decreased in majority of CR PCa cell lines and cancer lesions as compared to the adjacent normal/benign tumor tissues (2). Another study also reported the downregulated expression of β-isoform of PP2A catalytic subunit ($PP2A_{C\beta}$) in PCa (3). $PP2A_{C\alpha}$ and $PP2A_{C\beta}$ share 97% identity and are ubiquitously expressed; however, $PP2A_{C\alpha}$ is about 10 times more abundant than $PP2A_{C\beta}$ (4). $PP2A_{C\alpha/\beta}$ 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 hetero-dimers or hetero-trimers with scaffold (A) and regulatory (B) subunits (5).

Based on these supporting data, we hypothesized that *dysregulation of PP2A plays an important role in the progression of prostate cancer*.

To test our hypothesis, we proposed three specific aims:

Aim 1: Examine the biological role of PP2Ac in androgen-independent growth and malignant properties of the prostate cancer cells.

Aim 2: Define the molecular pathways that are responsive for the changes in PP2A signaling and establish their association with observed phenotype.

Aim 3: Establish the clinical significance of the experimental findings.

BODY:

Task 1: To develop stable transfectants from the prostate cancer cell lines with knockdown or exogenous expression of PP2Acα.

We are working with three prostate cancer cell lines: LNCaP (castration- sensitive; AR positive; high PP2Acα expression), C4-2 (castration-resistant; AR positive; low PP2Acα expression), and PC3 (castration-resistant, AR negative; low PP2Acα expression). To investigate the role of PPP2CA downregulation in the castration-resistance and aggressive malignant characteristics of PCa, C4-2 and PC3 (low PPP2CA expressing) and LNCaP (high PPP2CA-expressing) PCa cells were stably transfected to generate their respective PPP2CA-overexpressing and -knockdown sublines (from pooled PPP2CA-overexpressing and PPP2CA-

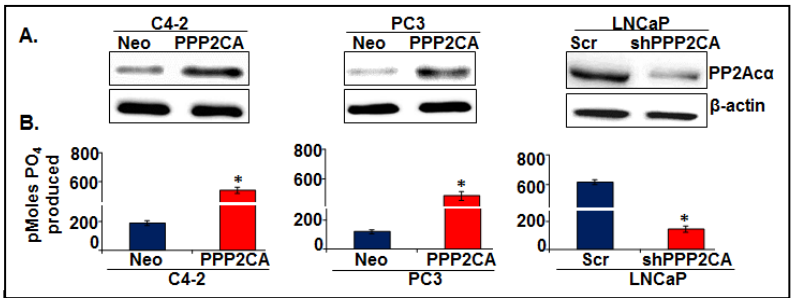

Fig. 1: Stable overexpression and silencing of PPP2CA in PCa cells. (A) Immunoblot analysis, **(B)** Assay of PP2A activity. Data shown as mean ± S.D. (n=3); * p< 0.05

kowckdown clones, respectively) along with their respective control transfectants. Later, these cells were characterized for the PP2Acα expression and activity by immunoblot and malachite green based assay, respectively. Data demonstrate that expression and activity of PP2Acα both are upregulated in C4-2-PPP2CA and PC3-PPP2CA cells, whereas decreased in case of LNCaP-shPPP2CA cells as compared to their respective controls (Figure 1 A and B).

Task 2: To examine the effect of *PPP2CA* overexpression /silencing on prostate cancer cell phenotype.

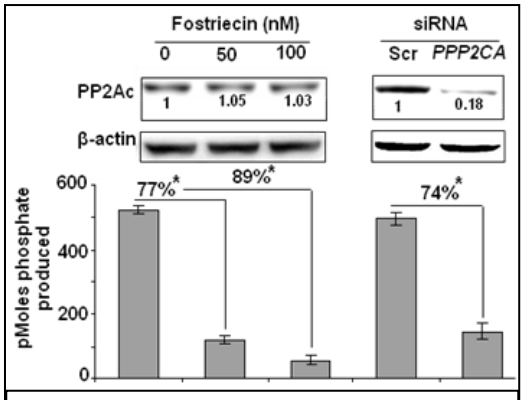

Fig. 2: Downregulation of PP2A activity by Fostriecin (pharmacological inhibitor) and siRNA-mediated *PPP2CA* silencing.

We have employed pharmacological and siRNA-mediated approaches to manipulate *PPP2CA* expression in *PPP2CA*-overexpressing LNCaP cells. Our data demonstrate that PP2A activity is decreased following treatment with fostriecin (~77.27% and 89.32% at 50nM and 100nM, respectively) or transfection with *PPP2CA*-specific siRNA (~74%) that resulted in over 80% reduction in gene expression (Figure 2). In next set of experiments, we analyzed the effect of PP2A inhibition on the growth of LNCaP cells under steroid-depleted condition. LNCaP cells were treated with fostriecin (100 nM) or DHT (1 nM) under steroid-reduced condition. Alternatively, following transfection with scrambled- or *PPP2CA*-specific siRNAs for 24 h, LNCaP cells were placed in steroid-reduced growth media. Growth of the LNCaP cells was analyzed by MTT assay after 96 h of treatments (Figure 3). We observed that LNCaP cells under steroid-depleted condition had ~4.3 fold decreased cell growth as compared to the cells grown in regular-media. The treatment with either DHT or fostreicin had a rescue effect exhibiting ~3.83 fold and ~3.06 fold growth induction, respectively. Similarly, siRNA-mediated silencing of *PPP2CA* also resulted in increased growth (~2.85 fold) as compared to the scrambled-siRNA transfected control cells under steroid-depleted condition (Figure 3). These findings suggest that the down-modulation 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.

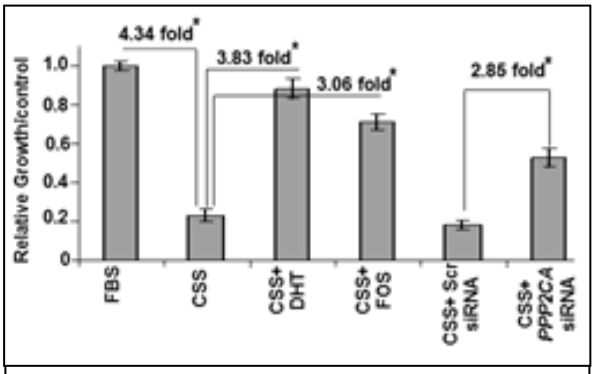

Fig. 3: **Effect of PP2A inhibition on androgen-independent growth of LNCaP cells**. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydro-testerone, Fos: fostriecin, Scr: scrambled sequence

Our subsequent studies provided evidence that PP2A inhibition sustains growth of LNCaP cells under androgen-deprived condition by preventing steroid-depletion induced cell cycle arrest and apoptosis. The proliferation index was determined by DHT or fostriecin treatments of synchronized LNCaP cells followed by propidium-iodide staining and flow cytometry (Figure 4, next page). In accordance with previously published reports (6;7), our data showed arrest of LNCaP cells in G_0/G_1 phase of cell cycle under steroid-reduced condition, an effect that was abrogated upon treatment with DHT (1 nM) (Figure 4, next page). Furthermore, we observed that the inhibition of PP2A by either fostriecin 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 G_2/M phase was 27.78% upon fostriecin treatment as compared to 18.22% in CSS-only treated LNCaP cells. Similarly, 20.96 % of *PPP2CA*-silenced LNCaP cells were in S and G_2/M phases as compared to 15.0% in scrambled-siRNA transfected cells (Figure 4). To analyze the apoptotic index, we stained the cells with CaspACE FITC-VAD-FMK, a fluorescent analog 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 cells in 10 random fields of view under a fluorescence microscope (Figure 5). Our data showed that steroid-depletion led to enhanced apoptosis of LNCaP cells (3.34 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-depriviation. Our data thus demonstrate that PP2A inhibition supports the growth of LNCaP cells under androgen-depleted condition by preventing cell cycle arrest and apoptosis.

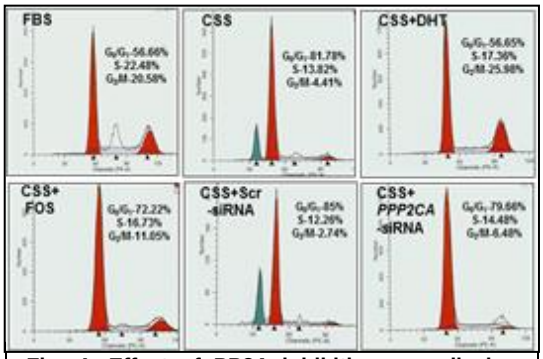

Fig. 4: Effect of PP2A inhibition on relieving steroid-deprivation-induced cell cycle arrest. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydrotesterone, Fos: fostriecin, Scr: scrambled sequence.

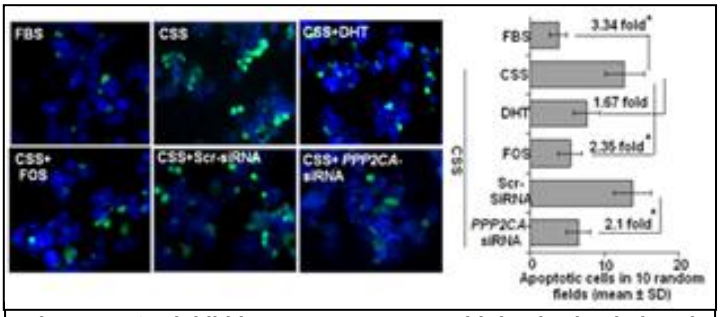

Fig. 5: PP2A inhibition suppresses steroid-deprivation-induced apoptosis. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydro-testerone, Fos: fostriecin, Scr: scrambled sequence.

As C4-2 cells are androgen-independent and possess low PP2A activity, we examined if 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 (8;9) 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 (Figure 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) (Figure 6B). To confirm that the effect of ceramide on cellular growth was mediated through PP2A, we inhibited PP2A activity by pre-treating the C4-2 cells with fostriecin. Our data demonstrated that the inhibition of PP2A significantly attenuated ceramide-induced growth inhibition of C4-2 cells under steroid-depleted condition.

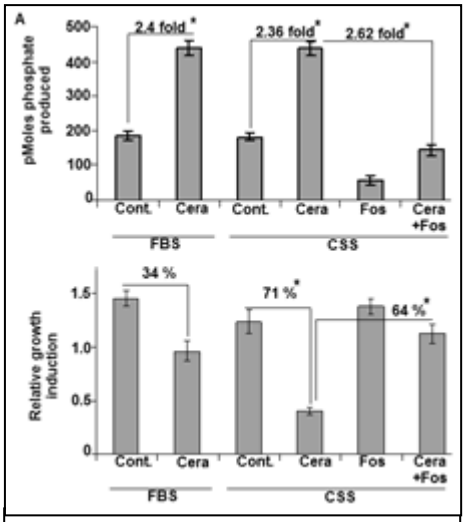

Fig. 6: A. Ceramide induces PP2A activity in C4-2 cells, which could be effectively suppressed by fostricin. B. Ceramide-induced growth suppression of C4-2 cells and rescue effect of fostriecin.

We also phenotypically characterized stable PCa sublines that are either overexpressed (C4-2 or PC-3) or are silenced (LNCaP) for *PPP2CA* expression. For, growth kinetics, cells ($1x10^4$) were seeded in 6-well plates and growth was monitored by counting the cell number up to 8 days. Our data demonstrate that over-expression of *PPP2CA* in C4-2 and PC3 cells significantly decrease their growth rate, whereas *PPP2CA*-silenced LNCaP cells exhibit increased growth as compared to their respective controls (Figure 7A). The total number of LNCaP-shPPP2CA cells on 8th day of culture indicate 31.6% increase in growth as compared to LNCaP-Scr cells, whereas 34.1% and 35.2% decrease is observed in the *PPP2CA*-overexpressing cells (C4-2-PPP2CA and PC3-PPP2CA, respectively) relative to their respective controls (Figure 7A). Growth analyses during exponential phase suggest a decrease in population doubling time of LNCaP-shPPP2CA (35.2 h) cells as compared with LNCaP-Scr (48.1 h) cells, whereas C4-2-PPP2CA and PC3-PPP2CA cells exhibited an increase in doubling time (34.7 and 38.9 h, respectively) compared with controls [C4-2-Neo (27.2 h) and PC3-Neo (29.1 h)] cells, respectively (Figure 7B). Altogether, our findings demonstrate that PP2A-downregulation potentiates growth of prostate cancer cells.

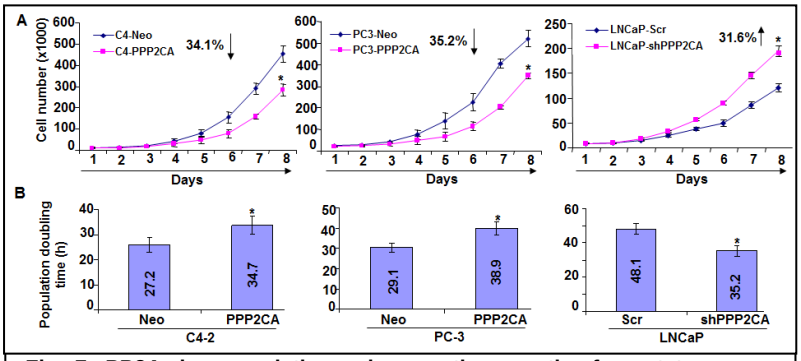

Fig. 7: PP2A downregulation enhances the growth of prostate cancer cells. (A) Cells ($1x10^4$) were seeded in 6-well plates and growth was monitored by counting the cell number upto 8 days. **(B)** Doubling time (h) was calculated during exponential phase (96–144 h). Data shown as mean ± S.D. (n=3); *, p< 0.05.

Above, we showed that downregulation of PP2A (by transient silencing or pharmological inhibition) in castration-sensitive LNCaP prostate cancer cells promotes their growth under androgen-deprived condition. Next, we examined the effect of *PPP2CA*-overexpression on the growth of C4-2 and PC3 cells under androgen-depleted condition. For this, we performed plating efficiency assay, an ideal test to monitor growth in long-term, under steroid-supplemented and –reduced conditions. Cells were seeded at low density ($1x10^3$ cells/well) in steroid-supplemented (FBS) and -reduced (CSS) media. After 2 weeks, colonies were stained with crystal violet, visualized, photographed, and counted using Image analysis software (Gene Tools, Syngene, Frederick, MD). Our data demonstrate that plating efficiency of *PPP2CA*-overexpressing C4-2 and PC-3 cells is decreased (53.4% and 43.3%, respectively), as compared to their respective controls under steroid-supplemented condition (Figure 8). Interestingly, plating efficiency is decreased further (~72.3% and 59.8% in C4-2-PPP2CA and PC-3-PPP2CA, respectively) under steroid-deprived condition (Figure 8). Thus, our data provide

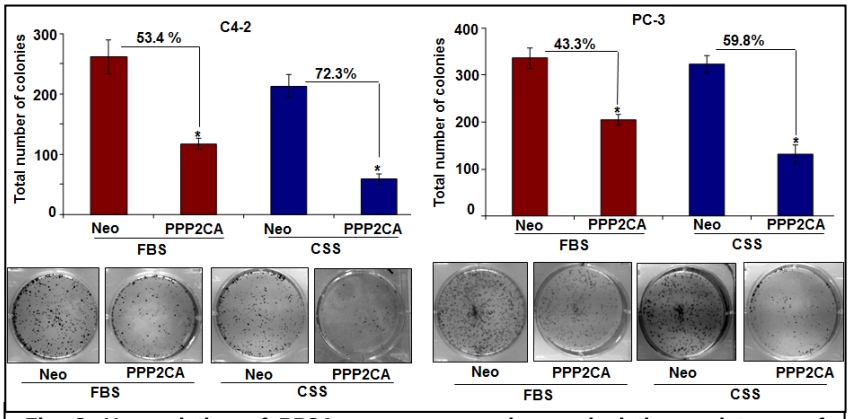

Fig. 8. Upregulation of PP2A suppresses androgen-depletion resistance of prostate cancer cells. Cells were seeded at low density ($1x10^3$ cells/well) in steroid-supplemented (FBS) and -reduced (CSS) media. After 2 weeks, colonies were stained with crystal violet, and visualized and photographed using imaging system. Bars represent mean ± S.D; n=3; *, p< 0.05.

additional *in vitro* support for an inhibitory role of PP2A in castration-resistant growth of prostate cancer cells.

Since castration-resistant stage of PCa is associated with increased aggressiveness (10), we next investigated the association of PP2A downregulation with malignant behavior of prostate cancer cells. We first examined the effect of PP2A activity modulation on cell migration (by trans-well chamber assays) and invasion (migration through a Matrigel-coated porous membrane). Data show that number of migrating cells are decreased in *PPP2CA*-overexpressing C4-2 (2.3 fold) and PC-3 (2.2 fold) cells as compared to their respective controls, whereas a 2.4 fold increase is observed in *PPP2CA*-knockdown LNCaP cells (Figure 9). Similarly, we observe a decrease in invasiveness of *PPP2CA* overexpressing C4-2 (2.7 fold) and PC-3 (2.8 fold) cells as compared to their respective control cells, whereas it is increased (3.0 fold) in *PPP2CA* silenced LNCaP cells (Figure 9). Another behavioral property associated with tumor cells is decreased cell-cell adhesion that is required to facilitate its dissemination. Therefore, we next examined the effect of *PPP2CA*-overexpression on homotypic interaction of prostate cancer cells in a cell aggregation assay. Our data show an increased cell-cell interaction in *PPP2CA* overexpressing C4-2 and PC-3 cells as compared to their respective controls (Figure 10). Likewise, we also observe decreased cell-cell interaction in *PPP2CA* silenced LNCaP cells as compared to the control cells (Figure 10). Altogether, our data indicate that PP2A downregulation is associated with aggressive behavior of the prostate cancer cells.

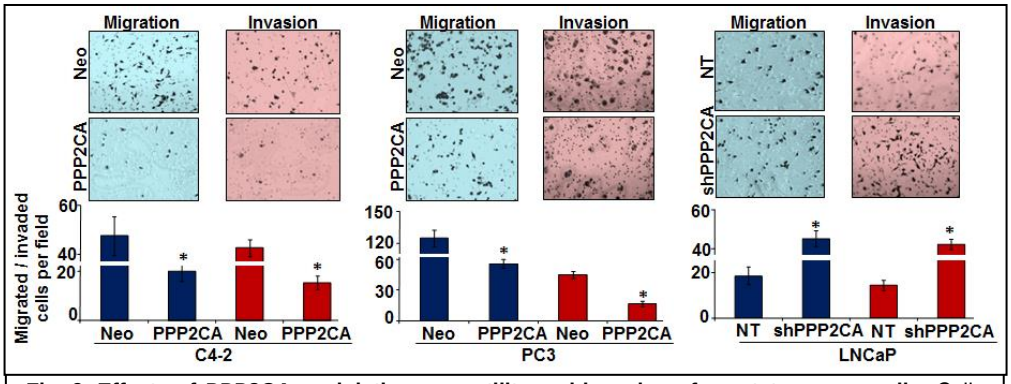

Fig. 9. Effects of *PPP2CA* modulation on motility and invasion of prostate cancer cells: Cells were seeded in a transwell chamber (8μ pore size, non-coated or Matrigel-coated) and allowed to migrate or invade through Matrigel under chemotactic drive for overnight. Next day, the cells that did not migrate or invade through were removed and migrated cells were stained using a commercial kit. Images were taken in 10 random fields (magnificationx100) and cell number counted. The data is presented as the mean ± S.D, (n=3). *, p<0.05.

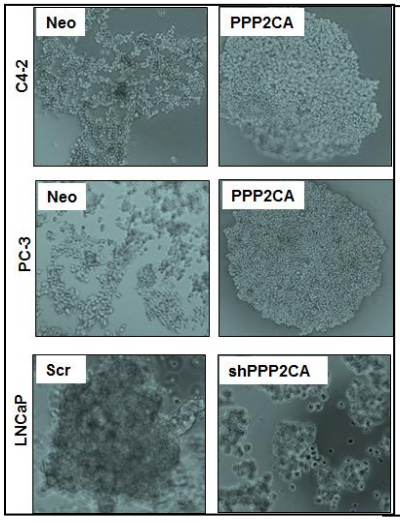

Fig. 10. Effect of PP2A modulation of cell- cell interaction: Cells ($20x10^3$) in a 20μL media were dropped on to inner surface of Petri-dish lid and allowed to hang overnight. Next morning, lid was open and cells were observed under micro-scope and picture taken (magnification x 100). Data show that less cell-cell interaction in PP2Ac-down-regulating PCa cells as compared to their respective controls.

Several lines of evidence indicate that increased malignant potential of cancer cells is associated with their transition from epithelial to mesenchymal phenotype, a process referred as epithelial-to-mesenchymal transition (EMT) (11). Thus, to investigate whether these effects are associated with PPP2CA downregulation-induced EMT, we examined actin-organization, a critical determinant of mesenchymal transition (12), in PPP2CA-overexpressing or -knockdown PCa cells. Staining of filamentous-actin with FITC–conjugated phalloidin revealed the presence

of many filopodial structures in PPP2CA-knowckdown (C4-2-, PC3-Neo and LNCaP-shPPP2CA) cells, while they were absent or less obvious in the low PPP2CA-expressing (C4-2-, PC3-PPP2CA and LNCaP-Scr) cells (Figure 11). We next examined the expression of markers specifically associated with epithelial (E-cadherin and cytokeratin-18) and mesenchymal (N-cadherin, Vimentin, Twist and Slug) phenotypes of a cell by immunoblot and real-time qRT-PCR assays at protein and transcriptional levels, respectively. Our data show an increased expression of epithelial and decreased expression of mesenchymal markers at protein as well as transcriptional level (Figure 12A and B, respectively) in PPP2CA-overexpressing C4-2 and PC3 cells as compared to respective controls and vice versa observed upon silencing of PPP2CA in LNCaP cells (Figure 12A and B).

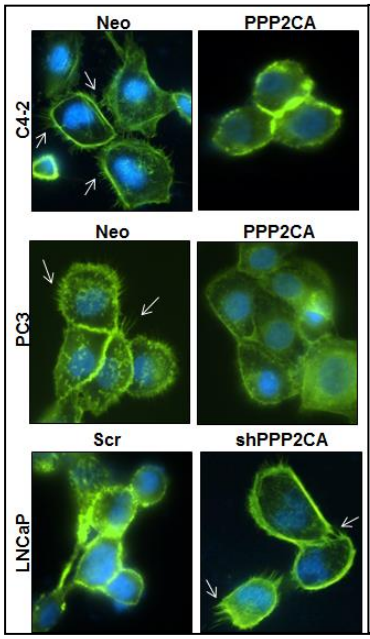

Fig. 11: Effect of PPP2CA on actin organization in PC cells: PPP2CA-overexpressing or -silenced prostate cancer cells were grown on fluorodish, fixed and stained with Alexa Fluor 488–conjugated phalloidin. Thereafter, effect of the modulation of PP2A activity on actin organization was analyzed and photographed using confocal microscope. Data show that low PPP2CA-expressing (C4-2 and PC3 -Neo and LNCaP-shPPP2CA) cells exhibited several filopodial and lamellipodia-like projections (white arrows) as compared with PPP2CA-overexpressing (PC3 and C4-2 -PPP2CA and LNCaP-Scr) cells.

In next set of experiments, we examined the role of PP2A downregulation on the tumorigenesis and metastatic property of prostate cancer (PC3) cells in an orthotopic mouse model of prostate cancer. For this, PPP2CA overexpressing (PC3-PPP2CA) or control (PC3-Neo) cells were injected into the dorsal prostatic lobe of immunodeficient male mice (4 to 6-week old). Our data demonstrated 100% tumor incidence in the mice of both the groups, however, tumors form PC3-PPP2CA group are significantly smaller as compared to mice group injected with the control (PC3-Neo) cells (Figure 13 A, next page). Average volume and weight of tumors in PC3-PPP2CA group were 317.1 mm3 (range from 171.5 to 490.8 mm3) and 0.31 g (range from 0.13 to 0.46 g), respectively, as compared to 1803.98 mm3 (range from 1369.9 to 2254.0 mm3) and 1.56 g; range from 1.1 to 2.11 g in PC3–Neo group (Figure 13 B and C, next page). Next, we per-formed immunohistochemistry (IHC) analysis on paraffin-embedded tissue sections to examine PP2Acα expression and proliferative and apoptotic markers. Our IHC data show intense PP2Ac staining in tissue section of PC3-

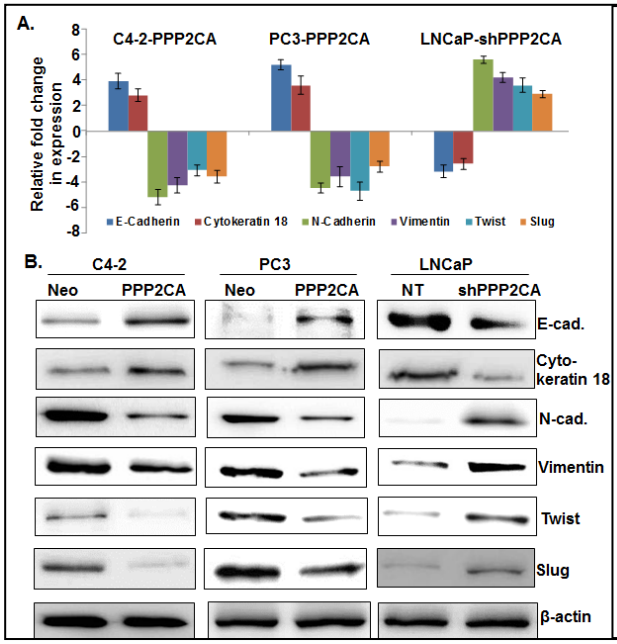

Fig. 12: Loss of PPP2CA favors epithelial to mesenchymal transition in prostate cancer cells. Expression of various epithelial (E-cadherin and cytokeratin-18) and mesenchymal (N-cadherin, Vimentin, Slug and Twist) markers at **(A)** protein and **(B)** transcript level was examined by immunoblot and real-time qRT-PCR assay, respectively. Data presented as fold change in expression in *PPP2CA*-overexpressing (C4-2- and PC3 -PPP2CA) and silenced (LNCaP-shMyb) cells as compared with their respective controls. Bars represents mean ±S.D, (n=3). Data show that downregulation of PPP2CA was associated with loss of epithelial and gain of mesenchymal markers, indicating its role in EMT.

PPP2CA group, while very low staining is observed in control group (Figure 14). Moreover, our data show that the average number of proliferating cells i.e. Ki67 positive-cells were decreased (>40%) in tumors generated from PC3-PPP2CA cells as compared with tumors of PC3-Neo cells (Figure 14 B). For the apoptosis index analysis, TUNEL assay was performed. Tumors from PC3-PPP2CA cells had significantly more TUNEL-positive cells (> 50%) as compared with control tumors (Figure 14C).

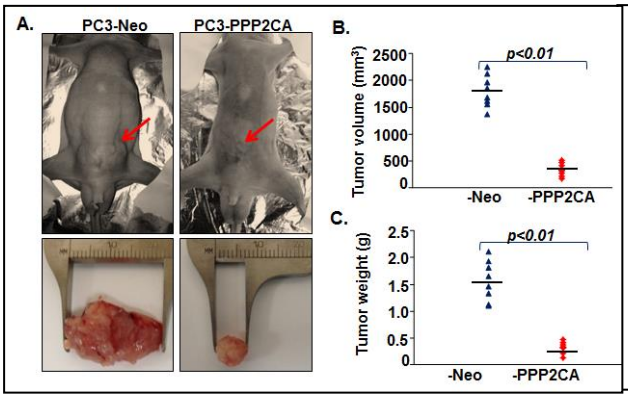

Fig. 13: *PPP2CA* overexpression decreases tumorigenicity of prostate cancer cells. PC3 cells (*PPP2CA*-overexpressing and control) were injected in mouse prostate and their growth in vivo was monitored. **(A)** Mouse carrying tumors generated from control (PC3-Neo) or *PPP2CA*-overexpressing PC3 cells (upper panel) and representative excised tumors (lower panel). **(B)** Average volume and **(C)** weight of the tumors from control and PP2A overexpressing group. Data is presented as mean±SD (n=7).

To analyze the effects of PPP2CA downregulation on prostate cancer metastasis, distinct organs (liver, lungs, lumber lymph nodes and bone) from both the groups were collected and fixed in Bouin's solution. Thereafter, visible metastatic nodules were quantified. Data demonstrate high metastases in lungs and livers (evident by the presence of multiple large metastatic nodules) and in lymph nodes (evident by large and indurate lymph nodes), in case of PC3-Neo mice, whereas no metastasis was observed in PC3-PPP2CA group (Figure 15). Metastasis in distinct organs was further confirmed by the presence of tumor-cell nests in the specific tissue sections (Figure 16, next page). Altogether, our data provide strong evidence of role of PP2A downregulation in the progression and metastasis of prostate cancer.

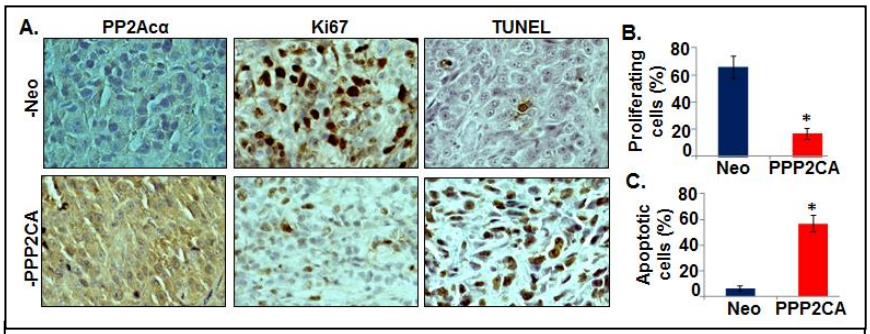

Fig. 14: PPP2CA overexpression is associated with decreased proliferation and induction of apoptosis in prostate cancer cells: (A) Immunohistological analysis of paraffin embedded prostate tumors for PP2Acα expression (left panel), Ki67 expression (a proliferative marker; middle panel) and Tunnel assay (right panel). **(B)** Number of proliferating and **(C)** apoptotic cells were counted in 10 random view fields (magnification 200x) in a double-blinded manner and expressed as mean ± SD (n=3), per field view. *, p < 0.01, significantly different from control group.

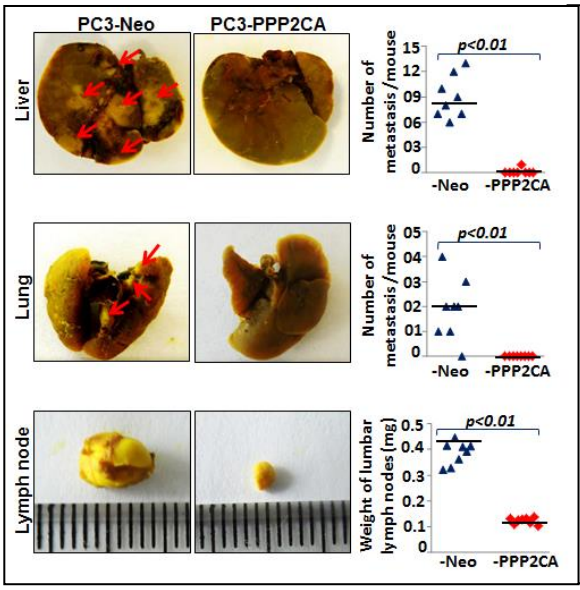

Fig. 15: Enforced *PPP2CA* expression decreases incidence of metastasis. Liver (upper), lungs (middle) and lumber lymph nodes (lower) from mice were excised out and fixed in Bouin`s solution and number of metastatic colonies were counted (in case of liver and lungs) and total mass of all the lumber lymph nodes was taken. Data presented as number of metastatic colonies per mouse in case of liver and lungs and, total weight of the lumber lymph nodes per mouse. Dark line indicates the median value of the group.

Task 3: To investigate the effect of PP2A on androgen receptor (AR)-dependent and –independent signaling pathways.

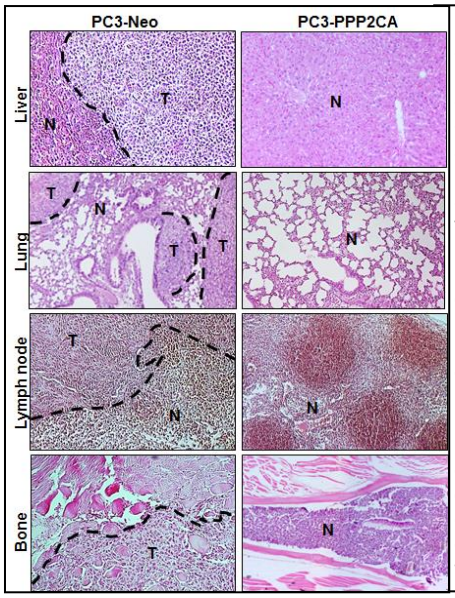

Fig. 16: Histological examination of metastatic tumor cells in different organs. H&E staining of liver, lungs, lymph nodes and bone section from control and PPP2CA overexpressing group. Mice group injected with PC3-Neo cells show metastatic deposits of cancer cells in liver, lungs and lymph nodes, while PPP2CA-overexpressing group had no gross evidence of deposits of cancer cells. T= tumor; N= normal.

PP2A impacts multiple cell signaling pathways by causing dephosphorylation of the signaling proteins (5). 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 (13-15). To determine if the sustained growth of PCa cells under steroid-depleted condition was due to the activation of Akt and ERK, we monitored the change in their phosphorylation upon PP2A inhibition. Our immunoblot data with total and phospho-form-specific antibodies (Figure 17) showed an increased phosphorylation of both Akt and ERK. Similarly, 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 pro-apoptotic effect. Similarly, we examined the activation of ERK and Akt in our stable transfectants. As expected, our data showed a decreased phosphorylation of Akt and ERK in *PPP2CA*-overexpressing C4-2 and PC-3 cells as compared to their respective controls or vice versa in *PPP2CA* silenced LNCaP cells (Figure 18).

Androgen receptor (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 (15). Notably, it has been shown earlier that both Akt and ERK can induce phosphorylation of AR at serine residues leading to its activation (15;16). Therefore, we examined the effect of PP2A inhibition on the phosphorylation of AR in LNCaP cells under steroid-depleted condition (Figure 19A). We observed that the inhibition of PP2A either by fostriecin or siRNA led to an increased phosphorylation of AR at serine-81 residue, while no change was detected at the serine-213. In contrast, stimulation with DHT induced phosphorylation at both the serines (81 and 213). Our immunoblot data also demonstrated an induced expression of AR and its target gene, PSA/KLK3 upon treatment with DHT or PP2A inhibition (Figure 19A). 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 h post-transfection, treated with either DHT or fostriecin under steroid-depleted condition for next 24 h. In parallel, cells were also co-transfected with scrambled or *PPP2CA*-specific siRNAs for 48 h. Transcriptional activity of AR is presented as the relative

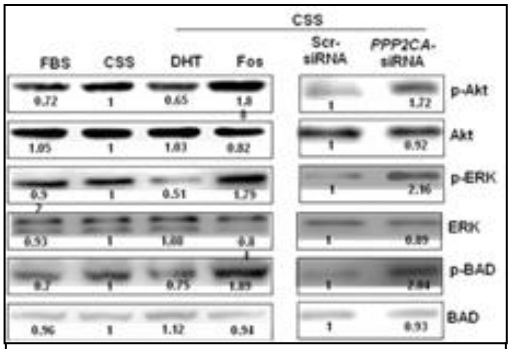

Fig. 17: Effect of PP2A inhibition on Akt and ERK signaling pathways and subsequent inactivating phosphorylation of BAD. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydro-testerone, Fos: fostriecin, Scr: scrambled sequence.

luciferase units (RLUs), which is the ratio between firefly (for AR activity) and renilla (transfection efficiency control) luciferase activity (Figure 19B). 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 to 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 (Figure 19B).

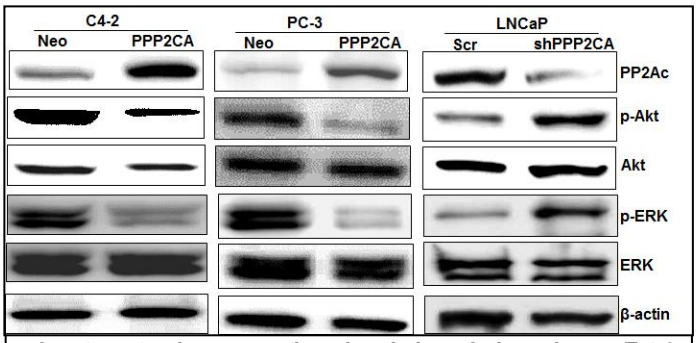

Fig. 18: PP2A decreases phosphorylation of Akt and ERK. Total protein was isolated and effect of PP2A modulation on Akt and ERK activation was examined by immunoblot assay. β-actin was used as internal control.

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 androgen-independent growth of LNCaP cells. To examine this, we used pharmacological inhibitors of Akt (LY294002) and ERK (PD98059) and anti-androgen (Casodex) to obstruct their activation prior to PP2A inhibition under steroid-deprived condition (data not shown, see appendix). Evaluation of LNCaP cell growth upon repression of Akt, ERK and AR prior to PP2A inhibition suggested a major role of Akt and ERK signaling pathways in supporting the androgen-independent growth of LNCaP cells. 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 –independent manners (data not shown, see appendix). Our signaling data also demonstrated that ceramide treatment decreased the phosphorylation of Akt and ERK, which could be reversed by pre-treatment with fostriecin (data not shown, see appendix). It was also observed that the expression of cyclins (D1 and A1), AR,

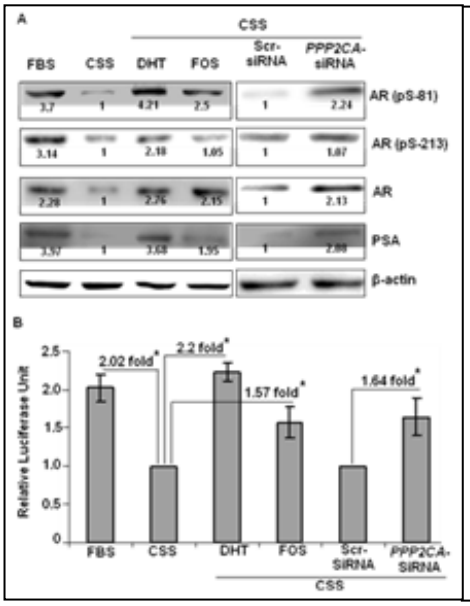

Fig. 19: Effect of PPP2CA on androgen receptor signaling. A. PP2A inhibition in LNCaP cells leads to enhanced AR phosphorylation (at Serine-81) and overexpression of AR and PSA. B. AR activity is partially sustained in PP2A inhibited LNCaP cells. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydro-testerone, Fos: fostriecin, Scr: scrambled sequence.

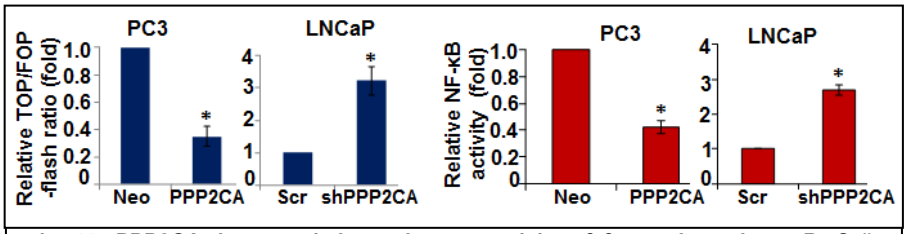

Fig. 20: PPP2CA downregulation enhances activity of β-catenin and NF-κB. Cells were grown in six well plate and transiently cotransfected with the luciferase promoter-reporter constructs (TOPflash or FOPflash, or pGL4.32[luc2P/NF-kB-RE/Hygro]) and pRL-TK construct (transfection efficiency control) for 24h. Thereafter, total protein was collected in passive lysis buffer and firefly and Renilla luciferase activities were measured using a dual-luciferase assay system. Data presented as fold change in normalized luciferase activity. Bars represent mean ± S.D. (n=3); *, $p < 0.05$.

pS81-AR and PSA was downregulated, whereas, the expression of p27 was upregulated upon treatment of C4-2 cells with ceramide (see appendix). Downregulation of PP2A with fostriecin abrogated ceramide-induced effect on cyclin A1, D1, p27, AR and PSA (see appendix).

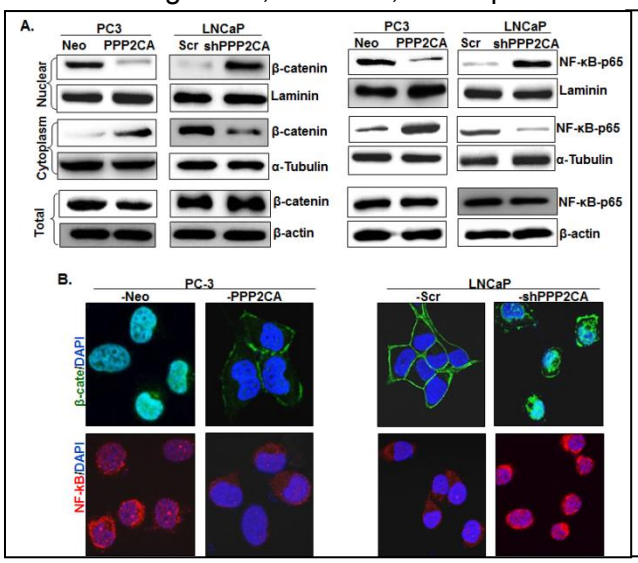

Fig. 21: PPP2CA downregulation enhances nuclear accumulation of of β-catenin and NF-κB. (A) Immunoblot analysis of β-catenin and NF-κB in different cellular compartment. β-actin, α-tubulin and laminin were used as loading controls for total, cytoplasmic and nuclear protein, respectively. **(C)** Immuno-fluorescence analysis of β-catenin and NF-κB in PPP2CA-overexpressing (PC3-PPP2CA and LNCaP-Scr) and PPP2CA-silenced (PC3-Neo and LNCaP-shPPP2CA) prostate cancer cells. Data show that PPP2CA-silenced cells exhibit higher levels of both β-catenin and NF-κB in nucleus as compared to PPP2CA-overexpressing cells.

To explore mechanistic basis of altered expression of EMT markers, we focused on β-catenin and NF-κB, which have earlier been shown to be aberrantly activated in PCa and implicated in transcriptional regulation of EMT markers (17-19). Our data from luciferase-based promoter reporter assays show increased transcriptional activities of both TCF/LEF/β-catenin and NF-κB responsive promoters (>60 %) in PCa cells having low PPP2CA expression (PC3-Neo and LNCaP-shPPP2CA) as compared to high PPP2CA expressing cells (PC3-PPP2CA and LNCaP-Scr) (Figure 20). In accordance to these findings, our immunoblot analysis revealed enhanced nuclear accumulation of β-catenin as well as NF-κB that correlated with their decreased cytoplasmic level in PPP2CA-silenced (PC3-Neo and LNCaP-shPPP2CA) cells in contrast to their respective PPP2CA-overexpressing sub lines (PC3-PPP2CA and LNCaP-Scr) (Figure 21A). To further confirm these findings and to

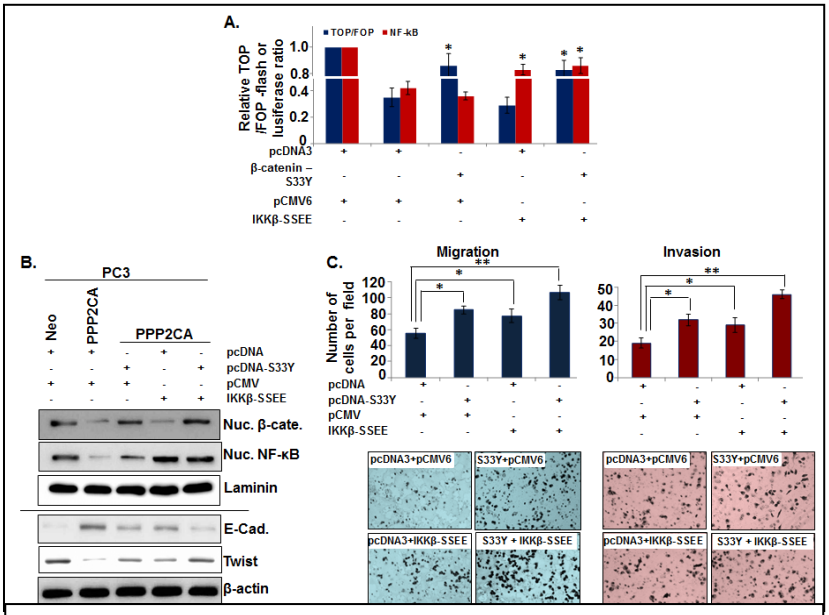

Fig. 22: Suppression of β-catenin and NF-κB is responsible for *PPP2CA*-mediated reversal of EMT and reduced aggressiveness. (A) Cells were grown in six well plate and transiently transfected with constitutively active β-catenin (pcDNA-S33Y) and IKKβ (pCMV-IKKβ-SSEE) mutants (to activate β-catenin and NF-κB, respectively). 24 h post-transfection, cells were again transfected with TOPflash-/ FOPflash- and NF-κB -luciferase promoter-reporter constructs to examine LEF/ TCF and NF-κB activity as described previously. Bars represent mean ± S.D. (n=3); *, $p < 0.05$. **(B)** Cells were transfected with constitutive active β-catenin and IKKβ mutants or their respective control plasmids. Thereafter, nuclear (Nuc) and total protein lysates were prepared after 24 h and 48 h of transfection and expression level of β-catenin and NF-κB (after 24 h in nuclear lysate) and E-cadherin and Twist (after 48 h in total lysate) were examined by immunoblot analysis. Laminin (for nuclear fraction) and β-actin (for total protein) were used as loading controls. **(C)** Cells were transiently transfected with constitutive active β-catenin and IKKβ mutants. After 48 h of transfection cells were trypsinized, counted and equal number of cells were seeded in a transwell chambers and number of migrated/invaded cells were examined as described previously. Bars represent mean + S.D. (n=3); *, $p < 0.05$

visualize the precise sub cellular localization of β-catenin and NF-κB, we performed immunofluorescence assay. Similar to immunoblot data, our immunofluorescence data also revealed the association of PPP2CA downregulation with increased β-catenin and NF-κB localization in PCa cells (Figure 21B). Next, to confirm the role of β-catenin and NF-κB in PP2A downregulation-induced EMT. PC-3-PPP2CA cells (with decreased β-catenin and NF-κB activity) were transfected with constitutive active β-catenin and IKKβ mutants (to activate β-catenin and NF-κB, respectively). Thereafter, effect on transcriptional activity of TCF/LEF/β-catenin and NF-κB responsive promoters, EMT markers and aggressive behavior of PCa cells was examined. Our data show that transfection of active β-catenin and IKKβ mutants specifically block the inhibitory effect of PPP2CA on β-catenin and NF-κB, respectively, which was depicted by increased transcriptional activities of their respective responsive promoters (Figure 22A) and enhanced their nuclear accumulation in PC3-PPP2CA cells (Figure 22B, upper panel). Furthermore, we observed that activation of either β-catenin or NF-κB alone, in part, led to regain of mesenchymal markers (Figure 22B, lower panel) and increased migration and invasive potential (Figure 22C), whereas their combined activation have more potent effect. Together, these findings indicate that both the β-catenin and NF-κB are cooperatively involved in PPP2CA-mediated inhibition of malignant properties in PCa cells.

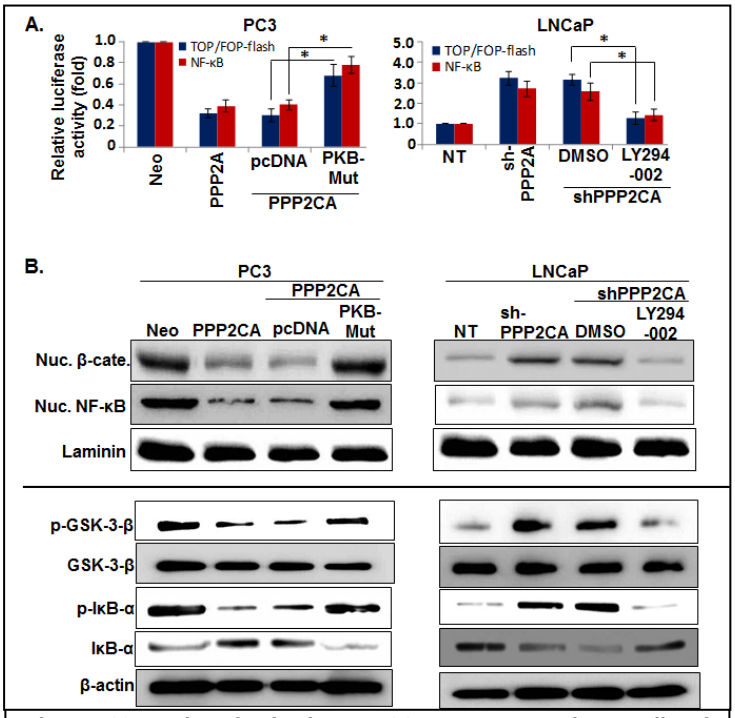

Figure 23: Role of Akt in *PPP2CA* overexpression–mediated suppression of β-catenin and NF-κB. (A) PC3-PPP2CA cells were transiently transfected with constitutively active PKB mutant (to activate Akt) or control plasmid for 24 h, and LNCaP-shPPP2CA cells were treated with PI3K/Akt inhibitor (LY294002; to inhibit Akt) for 1 h. After respective transfection or treatment, cells were co-transfected with TOPflash/ FOPflash/ NF-κB luciferase promoter-reporter constructs along with control plasmids for 24 h and luciferase activities were measured as described previously. Bars represents mean ± SD (n=3), * $p < 0.05$. **(B)** PC3-PPP2CA cells were transiently transfected with constitutively active PKB mutant or control plasmid for 24 h, and LNCaP-shPPP2CA cells were treated with PI3K/Akt inhibitor for 12 h. Thereafter, nuclear and total proteins were prepared and effect on localization of β-catenin and NF-κB (in nuclear protein; upper panel) and on p-Gsk3-β/Gsk3-β and p-IκB-α/IκB-α (in total protein; lower panel) was examined by immunoblot analysis. Laminin (for nuclear protein) and β-actin (for total protein) were used as loading controls.

As we observed that PPP2CA inhibits malignant properties of PCa via inactivation of β-catenin or NF-κB. In addition negative regulation of Akt, an upstream regulator of both β-catenin and NF-κB pathway; by PPP2CA in PCa cells was also observed. Next, we investigated whether PPP2CA-mediated Akt inhibition has any role in the suppression of β-catenin and NF-κB. For this, we activated Akt in PC3-PPP2CA cells (exhibiting decreased activated Akt) and inhibited its activation in LNCaP-shPPP2CA cells (having enhanced activated Akt). Thereafter, transcriptional activity of TCF/LEF/β-catenin and NF-κB was examined. Our data show that inhibitory effect of PPP2CA overexpression on transcriptional activity of TCF/LEF/β-catenin and NF-κB was diminished after re-activation of Akt in PC3-PPP2CA cells (Figure 23A). Whereas, vice versa was observed in LNCaP-shPPP2CA cells upon Akt inhibition (Figure 23A). In accordance to this, our immunoblot data show an enhanced nuclear localization of both β-catenin and NF-κB in PC3-PPP2CA cells upon Akt activation and opposite was observed upon

Akt inhibition in LNCaP-shPPP2CA cells (Figure 23B; upper panel). Altogether, our findings clearly suggest that Akt is the key intermediate signaling molecule which is involved in the PPP2CA-mediated decreased activation of β-catenin and NF-κB in PCa cells. To delineate the mechanism(s) involved in the Akt-mediated regulation of β-catenin and NF-κB in PCa cells, we analyzed effect on their biological inhibitors i.e. Gsk3-β and IκB-α, respectively. We observed that PPP2CA expression is inversely associated with phosphorylated/inactive Gsk3-β in PCa cells (Figure 23B; lower panel). Furthermore, we also observed that PPP2CA overexpression led to a drastic increase in IκB-α level, which was associated with a concomitant decrease in its phosphorylation, thus indicating the stabilization of IκB-α after PPP2CA overexpression (Figure 23B; lower panel). Moreover, our data show that effects of PPP2CA alteration on Gsk3-β and IκB-α were reversed upon activation (by PKB-mutant; in PC3-PPP2CA) and inhibition (by LY294002; in LNCaP-shPPP2CA) of Akt (Figure 23B; lower panel). Together, our data clearly suggest the role of PP2A/Akt axis regulates nuclear accumulation of β-catenin and NF-κB through Gsk3-β and IκB-α, respectively.

Task 4: To examine the expression, localization and/or activation profiles of PP2Acα, Akt, AR, and ERK in human prostate cancer.

To determine the clinical significance of our experimental findings, we proposed to examine the expression and localization of PP2Acα and its targets such as Akt in clinical specimens of normal, primary and metastatic PCa by immune-histochemical (IHC) analysis. We performed the IHC analysis on paraffin embedded tissue micro-arrays containing tissue sections of normal/ benign (n=34) and prostate tumor (n=109) using PP2ACα and p-Akt specific antibodies. Our analyses show an overall downregulation of PP2Acα in prostate cancer tissues as compared to the normal/benign prostate tissues (Figure 24). Importantly, we found that expression of its target i.e. p-Akt is high in tumor tissues specimens whereas, in case of normal/benign prostate tissues weak staining of p-Akt is observed (Figure 24). Together, our data highlight the clinical importance of PP2ACα in prostate cancer and suggest that combination of low PP2Acα and high p-Akt can be used to differentiate between normal/benign and prostate tumors cases.

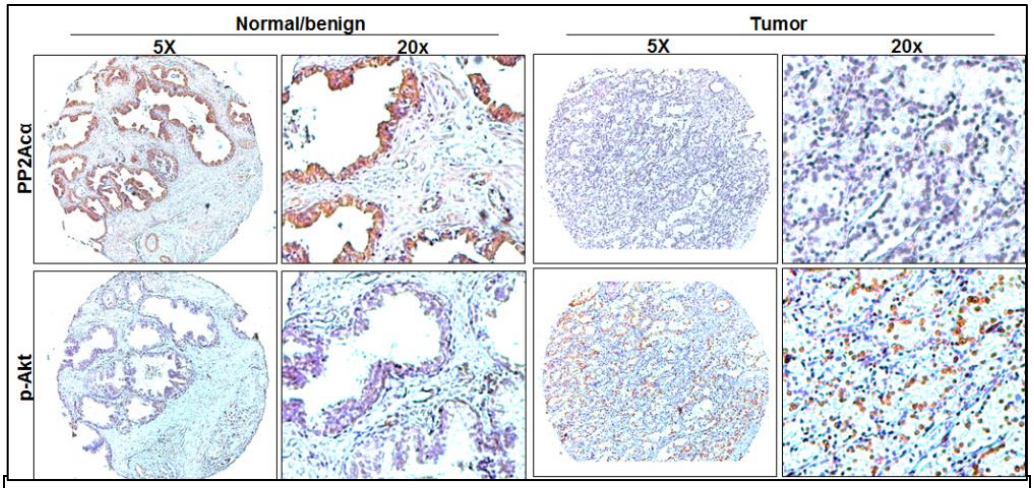

Fig. 24: Immunohistochemical analysis of PP2ACα and p-Akt expression on prostate cancer tissues. Immunohistochemical assay was performed on paraffin embedded tissue microarrays containing tissue sections of normal/benign (n=34) and prostate tumor (n=109) using PP2ACα and p-Akt specific antibodies. A decreased expression of PP2ACα is observed in prostate cancer tissues as compared to normal/benign tissues. In contrast, expression of p-Akt is high in case of tumor tissues as compare to normal/benign prostate tissues.

KEY RESEARCH ACCOMPLISHMENTS:

- We have established C4-2 and PC3 sublines exhibiting stable *PPP2CA* overexpression and enhanced PP2A activity and LNCaP sublines exhibiting stable *PPP2CA* downregulation and decreased PP2A activity.
- We have obtained experimental evidence (*in vitro*) for the role of PP2A downregulation in growth, androgen depletion-resistance and aggressive behavior of prostate cancer cells.
- We have developed mechanistic insight into the PP2A-mediated growth effects in prostate cancer cells. Our data indicate that PP2A downregulation facilitates androgen-independent growth of prostate cancer cells in both androgen receptor (AR)- dependent and –independent manners in AR expressing (LNCaP and C4-2) cells.
- We have delineated the mechanism involved in the PPP2CA downregulation-mediated epithelial-mesenchymal transition (EMT), migration and invasion of PCa cells. Our data show that PPP2CA downregulation favors EMT, migration and invasion of PCa cells through Akt-dependent activation of β-catenin and NF-κB pathway.
- We have also developed *in vivo* experimental support for a suppressor role of PP2A in prostate cancer progression using orthotopic mouse model.
- We have provided the evidence that expression of PP2A is also developed *in vivo* experimental support for a suppressor role of PP2A in prostate cancer progression using orthotopic mouse model.
- We show that downregulation of PP2Acα correlated with increased expression of p-Akt in tumor tissues.

REPORTABLE OUTCOMES (during this funding period)

2011:

- We presented a poster entitled “Downregulation of Protein phosphatase 2A enables androgen-dependent prostate cancer cells to grow under steroid-depleted condition: role of ERK, Akt and androgen-receptor signaling pathways” by Singh AP, Bhardwaj A, Singh S, and Srivastava SK. in “Innovative Minds in Prostate Cancer today (IMPaCT) meeting”, Orlando, Florida, March 9^{th}-12^{th} (2011).

- We presented a poster entitled “Inhibition of protein phosphatase 2A supports androgen-independent growth of prostate cancer cells” by Bhardwaj A, Singh S, Srivastava SK, Honkanen RE, and Singh AP in “American Association for Cancer Research (AACR) 102nd Annual Meeting”, Orlando, Florida, April 2^{nd}-6^{th} (2011).

- We published a manuscript entitled “Modulation of protein phosphatase 2A (PP2A) activity alters androgen-independent growth of prostate cancer cells: therapeutic implications” by Bhardwaj A, Singh S, Srivastava SK, Honkanen RE, Reed E, and Singh AP, in Mol Cancer Ther 10(5):720-731, (2011). **(featured in highlights of the issue, p709).**

2012:

- We presented a poster entitled “Protein phosphatase 2A (PP2A) downregulation is associated with aggressive and castration-resistant phenotypes in prostate cancer” by Bhardwaj A, Srivastava SK, Singh S, Arora A, Honkanen RE, Grizzle WE, Reed E and

Singh AP, in 103rd Annual Meeting of American Association for Cancer Research (AACR), held at Chicago, Illinois, March 31st-April 4th (2012).

- We presented a poster entitled "PP2A downregulation induces epithelial to mesenchymal transition, and promotes prostate cancer progression and metastasis" by Bhardwaj A, Singh S, Srivastava SK, Arora A, Hyde SJ, Grizzle WE, and Singh AP, in 2012 SBUR (Society For Basic Urologic Research) Fall Symposium held at Miami, FL November 15 – 18, 2012. **(Post-Doctoral Fellow received "Travel Award" to attend the Fall Symposium of SBUR).**

2013:

- We presented a poster entitled "Downregulation of protein phosphatase 2A promotes prostate cancer progression and metastasis" by Bhardwaj A, Singh S, Srivastava SK, Arora A, Hyde SJ, Honkanen RE, Grizzle WE, and Singh AP, in 104th Annual Meeting of American Association for Cancer Research (AACR), held at Washington, DC, April 6th – April 10th (2013).

2014:

- We published a manuscript entitled "Restoration of PPP2CA expression reverses epithelial to mesenchymal transition and suppresses prostate tumor growth and metastasis in an orthotopic mouse model" by Bhardwaj, A., Singh, S., Srivastava, S.K., Arora, S., Hyde, J.H., Andrews, J., Honkanen, R.E., Grizzle, W.E., & Singh, A.P, in Brit. J. Cancer. 110(8):2000-10, (2014).

MANUSCRIPTS UNDER PREPARATION:

- We are writing a manuscript for publication on "the correlative expression of PPP2CA and its downstream mediators in prostate cancer".

- We are preparing a review manuscript on role of PP2A in prostate cancer with tentative title being "Protein phosphatase 2A and prostate cancer connection: mechanistic insight and functional significance" for publication.

CONCLUSION:

Downregulation of PP2A is associated with progression and metastasis of human prostate cancer. Thus, restoration of PP2A activity could serve as an effective preventive and /or therapeutic approach against this deadly disease.

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APPENDICES:

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Presentation Abstract

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Abstract Number: 249

Presentation Title: Inhibition of protein phosphatase 2A supports androgen-independent growth of prostate cancer cells

Presentation Time: Sunday, Apr 03, 2011, 1:00 PM - 5:00 PM

Location: Exhibit Hall A4-C, Poster Section 10

Poster Section: 10

Poster Board Number: 20

Author Block: Arun Bhardwaj[1], Seema Singh[1], Sanjeev K. Srivastava[1], Richard E. Honkanen[2], Eddie Reed[1], Ajay P. Singh[1]. [1]University of South Alabama Mitchell Cancer Institute, Mobile, AL; [2]Biochemistry and Molecular Biology, College of Medicine, University of South Alabama, Mobile, AL

Abstract Body: Clinical progression of prostate cancer (PCa) is characterized by a transition from androgen-dependent (AD) to androgen-independent (AI) stage. Once the PCa has recurred in AI form, it progresses to a highly aggressive disease and poses an increased risk of morbidity and death. Therefore, understanding the mechanisms involved in AI progression of PCa is a significant area of research. Earlier, we identified *PPP2CA*, which encodes for alpha-isoform of the protein phosphatase 2A catalytic subunit (PP2Acα), as one of the downregulated genes in AI PCa cells. PP2A is a ser/thr phosphatase and a potent tumor suppressor involved in broad cellular functions; however, its role in PCa has not yet been determined. Here, we have investigated the effect of PP2A downregulation on the growth of AD PCa (LNCaP) cells under steroid-deprived condition. Furthermore, we have examined the effect of PP2A inhibition on the signaling pathways and delineated their role in AI growth of LNCaP cells. Our data show that the downregulation of *PP2A* activity by pharmacological inhibition or siRNA-mediated *PPP2CA* silencing sustains the growth of AD PCa cells under androgen-deprived condition by relieving the androgen-deprivation-induced cell cycle arrest and preventing apoptosis. Immunoblot analysis revealed enhanced phosphorylation of Akt, ERK, BAD, increased expression of cyclins (cyclin A1 and cyclin D1) and decreased expression of cyclin inhibitor (p27) upon PP2A downregulation. Furthermore, our data show that PP2A inhibition partially maintains AR signaling through its increased expression and ligand-independent phosphorylation, which is also supported by AR transcriptional activity assay and its target gene, *KLK3*, expression. Pharmacological inhibition of Akt, ERK and AR confirmed a role of these signaling pathways in facilitating the AI growth of LNCaP cells. Altogether, our findings suggest that modulation of PP2A activity may represent an alternative therapeutic approach for the treatment of advanced androgen-independent prostate cancer.

American Association for Cancer Research
615 Chestnut St.
17th Floor
Philadelphia, PA
19106

Presentation Abstract

Abstract Number: 3991

Presentation Title: **Protein phosphatase 2A (PP2A) downregulation is associated with androgen-independent and aggressive phenotypes in prostate cancer**

Presentation Time: Tuesday, Apr 03, 2012, 1:00 PM - 5:00 PM

Location: McCormick Place West (Hall F), Poster Section 3

Poster Section: 3

Poster Board Number: 5

Author Block: Arun Bhardwaj[1], Sanjeev K. Srivastava[1], Seema Singh[1], Sumit Arora[1], Richard E. Honkanen[2], William E. Grizzle[3], Eddie Reed[1], Ajay P. Singh[1]. [1]Department of Oncologic Sciences, Mitchell Cancer Institute, University of South Alabama, Mobile, AL; [2]Department of Biochemistry and Molecular Biology, University of South Alabama, Mobile, AL; [3]Department of Pathology, University of Alabama at Birmingham, Birmingham, AL

Abstract Body: First line of therapy for advanced prostate cancer (PCa) is androgen-deprivation through surgical or chemical castration; however, in majority of cases, tumors relapse in an androgen-independent (AI) form. Once the PCa has recurred in AI form, it progresses to a highly aggressive disease with frequent metastasis and poses an increased risk of morbidity and death. Previously, we demonstrated that *PPP2CA*, which encodes for alpha-isoform of the protein phosphatase 2A (PP2A) catalytic subunit, is downregulated in prostate cancer. Furthermore, we showed that PP2A activity is inversely associated with AI growth of PCa cells through a novel mechanism, whereby loss of PP2A-mediated checkpoints leads to the activation of Akt and ERK and partially sustains androgen receptor (AR) signaling under steroid-deprived condition. Since AI phenotype of PCa is associated with enhanced metastatic potential, we have investigated, in this study, a role of *PPP2CA* in the aggressive behavior of the PCa cells. For this, we overexpressed *PPP2CA* in AI (C4-2 and PC-3) PCa cells, while silenced its expression in AD (LNCaP) PCa cells. We observed that overexpression of *PPP2CA* in C4-2 and PC-3 cells not only decreased their AI growth and clonogenic ability, but also led to reduced motility and invasion and enhanced cell-cell interaction. Conversely, we observed increased cell motility and invasion and decreased cell-cell interaction upon *PPP2CA* silencing in LNCaP cells. Immunoblot analyses demonstrated gain of epithelial and loss of mesenchymal markers in *PPP2CA*-overexpressing PCa cells or *vice versa* indicating a role of PP2A in opposing epithelial to mesenchymal transition (EMT). Altogether, these studies provide evidence for a functional role of *PPP2CA* *in* aggressive behavior of AI PCa cells.

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Presentation Abstract

Abstract Number: 3861

Presentation Title: Downregulation of protein phosphatase 2A promotes prostate cancer progression and metastasis

Presentation Time: Tuesday, Apr 09, 2013, 1:00 PM - 5:00 PM

Location: Hall A-E, Poster Section 17

Poster Board Number: 02

Author Block: Arun Bhardwaj[1], Seema Singh[1], Sanjeev K. Srivastava[1], Sumit Arora[1], Stephen J. Hyde[1], Richard E. Honkanen[2], William E. Grizzle[3], Ajay P. Singh[1]. [1]Mitchell Cancer Institute, University of South Alabama, Mobile, AL; [2]Department of Biochemistry and Molecular Biology, University of South Alabama, Mobile, AL; [3]Department of Pathology, University of Alabama at Birmingham, Birmingham, AL

Abstract Body: Clinical progression of prostate cancer (PCa) is characterized by a transition from castration-sensitive (CS) to castration-resistant (CR) phenotype. The resulting CR tumors are highly aggressive and metastatic, and thus pose increased risk of morbidity and death to PCa patients. Therefore, identification of novel gene targets associated with CR growth and metastatic behavior remain a priority area in PCa research. Previously, we identified that *PPP2CA*, which encodes for the α−isoform of catalytic subunit of PP2A (a serine/threonine phosphatase), is downregulated in CRPCa. In additional findings, we reported that PP2A downregulation sustained the growth of PCa cells under steroid−deprived conditions. In the present study, we examined the role of PP2A in malignant behavior of PCa cells using *in vitro* and *in vivo* functional assays. Furthermore, we also delineated the underlying molecular mechanisms. Our data demonstrated that downregulation of *PPP2CA* (in CS LNCaP cells) promoted, whereas its overexpression (in CR C4-2 and PC3 cells) decreased the migration and invasion of human PCa cells. Similarly, we observed a loss of homotypic interactions in *PPP2CA*-silenced LNCaP cells, while it increased in *PPP2CA*-overexpressing C4-2 and PC3 cells. These changes were associated with epithelial to mesenchymal transition (EMT) or vice versa in *PPP2CA*-silenced or overexpressing PCa cells, respectively. When examined *in vivo* in an orthotopic mouse model, *PPP2CA*-overexpressing PC3 cells exhibited dramatic decrease in tumorigenesis due to diminished proliferation and enhanced apoptosis as compared to the control cells. Moreover, significant reduction in metastatic incidence was also observed. Mechanistic studies revealed that *PPP2CA* downregulation increases nuclear accumulation of β-catenin and NF-κB and subsequent activation of transcriptional activity of the responsive gene promoters. We also observed cooperative involvement of both β-catenin and NF-κB in the *PPP2CA* downregulation-induced EMT and invasiveness of PCa cells. Lastly, our data demonstrated a role of PP2A/Akt axis in enhanced nuclear accumulation of β-catenin and NF-κB through phosphorylation-induced inactivation of Gsk3-β and IkB-α, respectively. Altogether, our data suggest that loss of *PPP2CA* is associated with PCa progression and metastasis, and restoration of PP2A activity could serve as an effective preventive and/or therapeutic approach against the advanced disease.

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Therapeutic Discovery

Molecular Cancer Therapeutics

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

Arun Bhardwaj[1], Seema Singh[1], Sanjeev K. Srivastava[1], Richard E. Honkanen[1,2], Eddie Reed[1], and Ajay P. Singh[1,2]

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. *Mol Cancer Ther; 10(5); 720–31. ©2011 AACR.*

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

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expression profiling and identified many differentially expressed genes (11). *PPP2CA*, which encodes the catalytic subunit (α-isoform) of the protein phosphatase 2A ($PP2A_{C\alpha}$), was one of the genes of interest that exhibited a downregulated expression in androgen-independent prostate cancer cells. The level of $PP2A_{C\alpha}$ 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 $PP2A_{C\alpha}$ 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 ($PP2A_{C\beta}$) in prostate cancer has also been reported (12). $PP2A_{C\alpha}$ and $PP2A_{C\beta}$ share 97% identity and are ubiquitously expressed; however, $PP2A_{C\alpha}$ is about 10 times more abundant than $PP2A_{C\beta}$ (13). $PP2A_{C\alpha/\beta}$ 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 immunoprecipitation 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 $PP2A_C$, 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), antiandrogen 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% CO_2 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.

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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: $PP2A_C$, Akt, pAkt, ERK1/2, pERK1/2, BAD, pBAD, AR, pAR (Ser81), Bcl-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, $PP2A_{C\alpha}$ was immunoprecipitated with anti-$PP2A_{C\alpha}$ monoclonal antibody and Protein A Agarose beads. $PP2A_{C\alpha}$-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 5×10^3 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 $= [(A/B) - 1] \times 100$, 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 Canto™ II (Becton Dickinson) and percentage of cell population in various phases of cell cycle was calculated using ModFit LT software (Verity Software House).

Apoptosis 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-VAD-FMK solution in PBS for 2 hours at 37°C. CaspACE FITC-VAD-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% confluence 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 $P \leq 0.05$ 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 $PP2A_{C\alpha}$ in androgen-independent prostate cancer cells as compared with the androgen-dependent prostate cancer cells (11). Here, we examined the expression and activity of $PP2A_{C\alpha}$ 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 $PP2A_{C\alpha}$ is significantly downregulated in C4-2 (androgen independent) cells as

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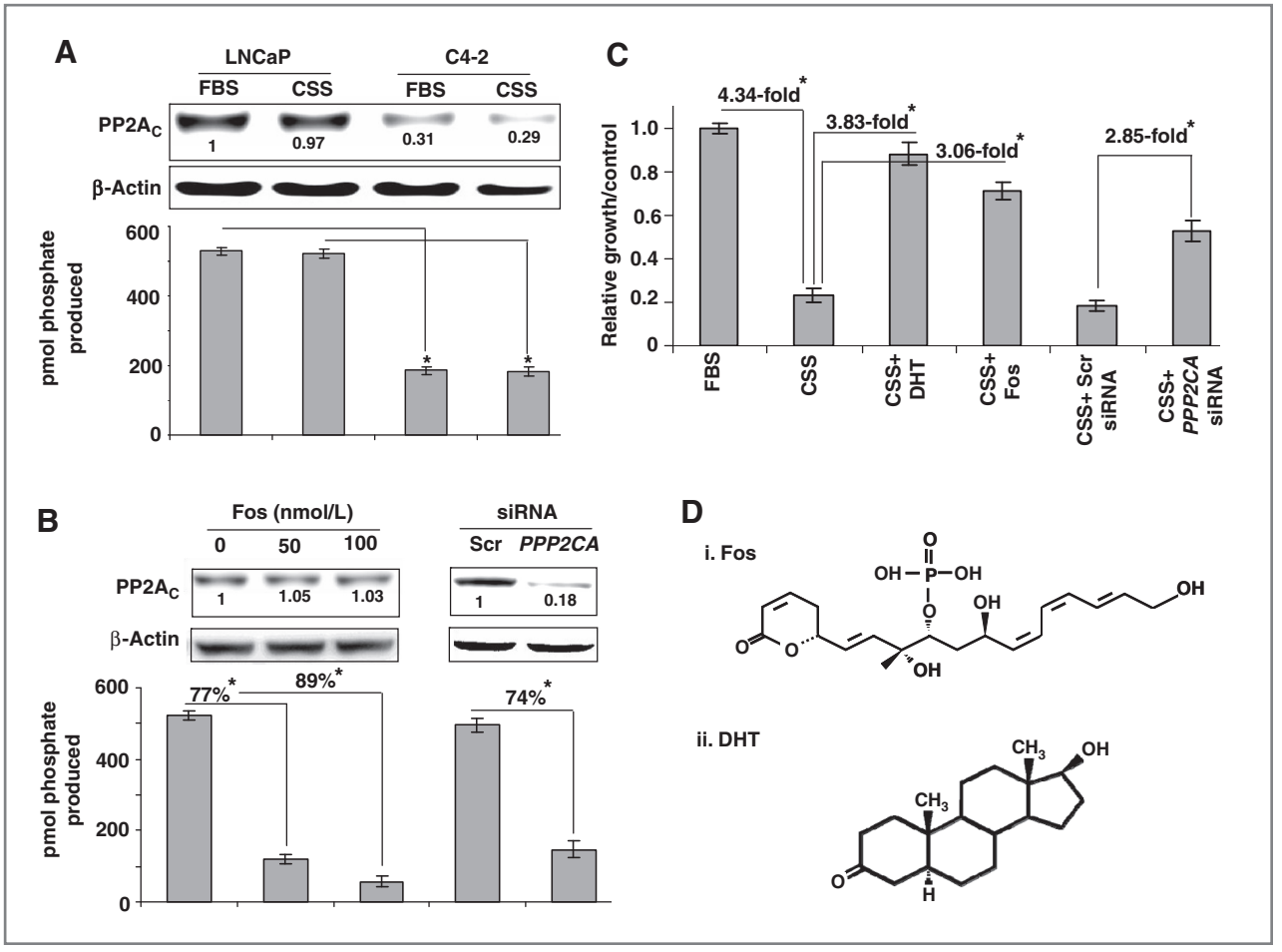

Figure 1. PP2A activity is downregulated in AI prostate cancer cells and its inhibition sustains the growth of AD prostate cancer cells under steroid-depleted condition. A, total protein from LNCaP (androgen dependent) and C4-2 (androgen independent) prostate cancer cells was resolved and immunoblotted for $PP2A_C$ and β-actin (internal control). PP2A activity was determined by malachite green–based phosphatase assay. $PP2A_C$ 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 $PP2A_C$ was decreased in LNCaP cells after treatment with Fos (~77.27% and 89.32% at 50 and 100 nmol/L, respectively) and knockdown of $PP2A_C$ 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).

compared with LNCaP (androgen dependent) cells, and there is no significant change in the expression or activity of $PP2A_{C\alpha}$ 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) or transfection with *PPP2CA*-specific siRNA (~74%) that resulted in over 80% reduction in gene expression (Fig. 1B). In the next set of experiments, we analyzed the effect of PP2A inhibition on the growth of LNCaP cells under steroid-depleted condition. LNCaP cells were treated with fostriecin (100 nmol/L) or DHT (1 nmol/L) under steroid-reduced condition. Alternatively, following transfection with scrambled or *PPP2CA*-specific siRNAs for 24 hours, LNCaP cells were placed in steroid-reduced growth media. Growth of the LNCaP cells was analyzed by MTT assay after 96 hours of treatments (Fig. 1C). We observed that LNCaP cells under steroid-depleted condition had about 4.3-fold decreased cell growth as compared with the cells grown in regular media. The treatment with either DHT or fostriecin had a rescue effect exhibiting about 3.83- and 3.06-fold growth induction, respectively. Similarly, siRNA-mediated silencing of *PPP2CA* also resulted in increased growth (~2.85-fold) as compared with the scrambled siRNA–transfected control cells under steroid-depleted condition (Fig. 1C). These findings

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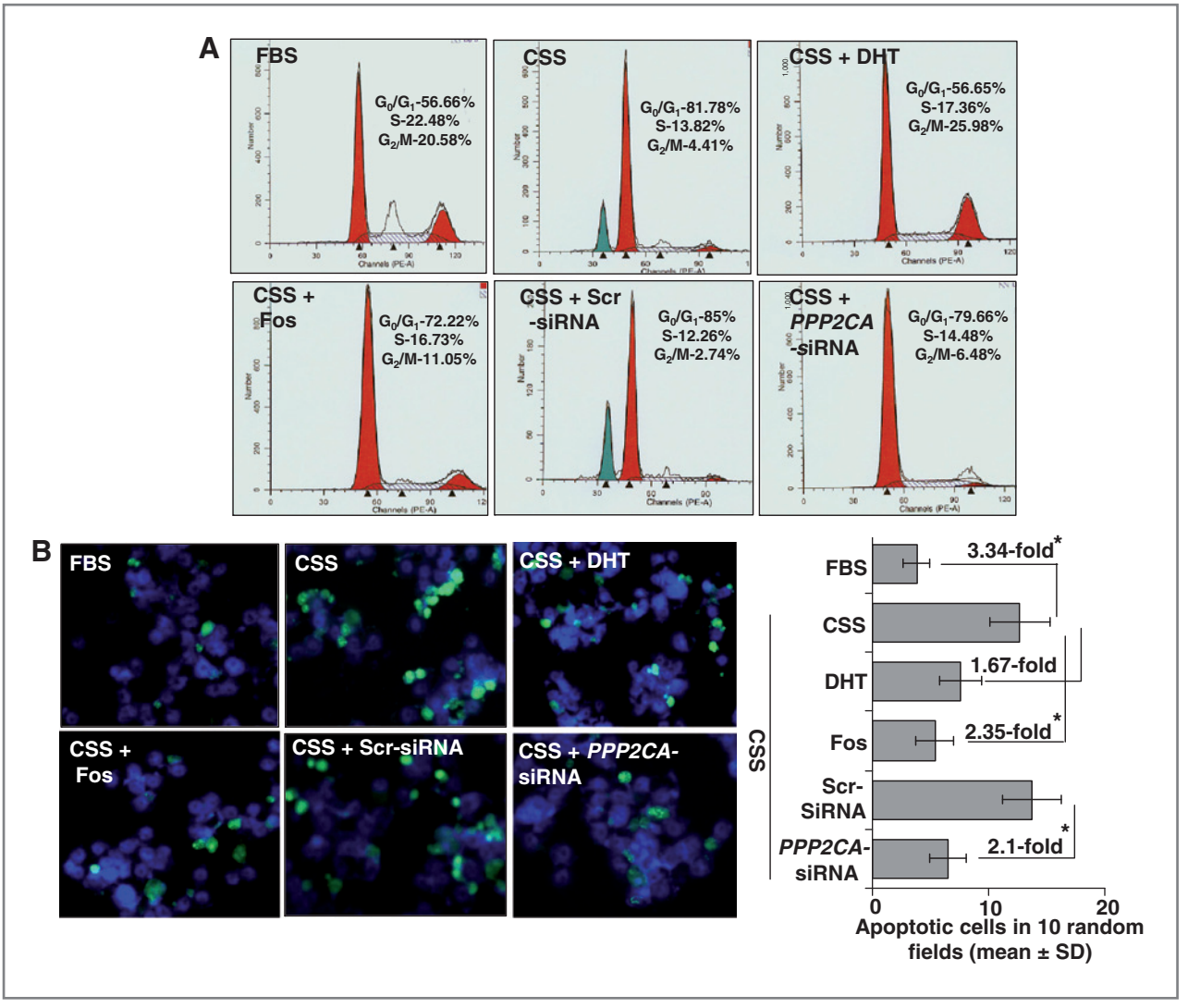

Figure 2. PP2A inhibition relieves hormone deprivation–induced G_0-G_1 arrest and suppresses apoptosis. A, LNCaP cells were synchronized by serum starvation and treated with DHT (1 nmol/L) or fostriecin (Fos; 100 nmol/L) for 24 hours in steroid-reduced (CSS) media. After treatments, distribution of cells 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 G_0-G_1 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 florescent) 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 ($P < 0.05$).

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 treatments 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 G_0-G_1 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

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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 G_2-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 G_2-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 cells in 10 random fields of view under a fluorescence microscope (Fig. 2B). Our data showed that steroid depletion led to enhanced apoptosis of LNCaP cells (3.34-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. Similarly, 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

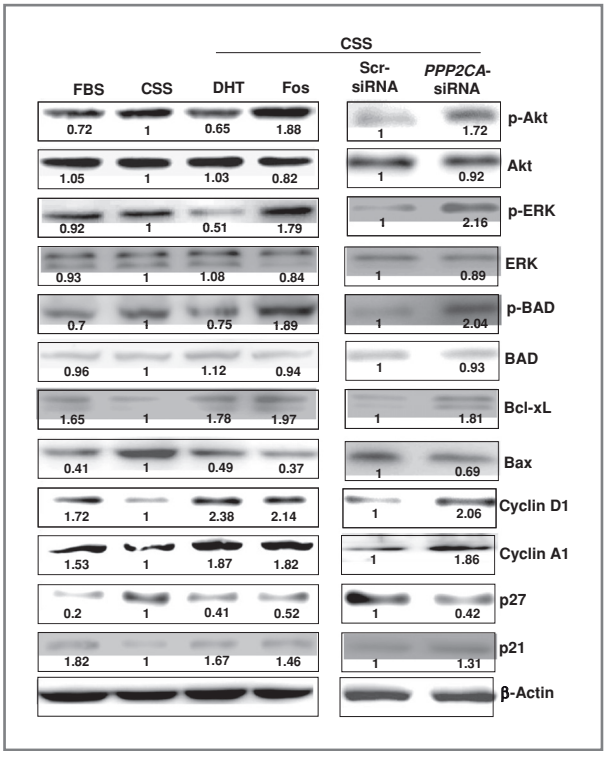

Figure 3. Inhibition of PP2A alters the expression and/or activation of survival and cell-cycle–associated proteins. LNCaP cells under steroid-reduced condition were treated with DHT (1.0 nmol/L) or fostriecin (Fos; 100 nmol/L) or silenced for *PPP2CA* expression. Following treatment, immunoblot analyses were carried out for p-Akt/Akt, p-ERK/ERK, p-BAD/BAD, Bcl-xL, Bax, cyclin A1, cyclin D1, p27, p21, and β-actin (used as internal control). Phosphorylation of Akt, ERK, and BAD was increased on treatment with fostriecin or *PPP2CA*-specific siRNAs. Moreover, expression of antiapoptotic Bcl-xL protein, cyclin A1, and cyclin D1 was increased, whereas expression of proapoptotic Bax protein and cyclin inhibitor p27 was decreased on PP2A downregulation. Interestingly, treatment with DHT exhibited contrasting effects on Akt, ERK, and BAD, whereas the expression of cyclin inhibitor p21 was increased in both DHT-treated and PP2A-inhibited cells. Numbers below the bands represent the fold ratio of densitometric quantification relative to corresponding control. Scr, scrambled.

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 G_0-G_1 phase, we examined the expression of cyclins (D1 and A1) and their inhibitors (p27 and p21), which are involved during G_1-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

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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.

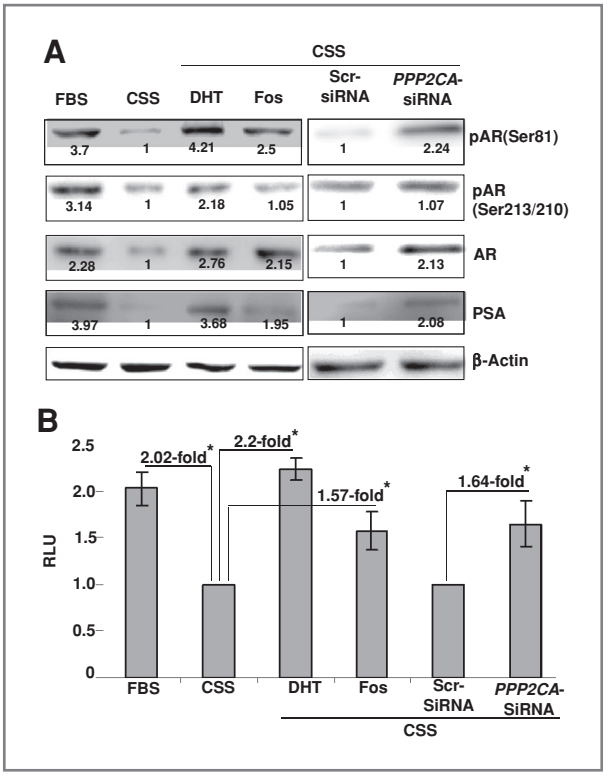

Figure 4. Inhibition of PP2A leads to induction of AR expression and its ligand-independent activation. A, LNCaP cells under steroid-reduced condition were treated with DHT (1.0 nmol/L) or fostriecin (100 nmol/L) or silenced for *PPP2CA* expression. Following treatment, immunoblot analyses were carried out for AR, phospho-AR (Ser81 and Ser213/210) and PSA. β-Actin was used as an internal control. Treatment with DHT or PP2A inhibition led to upregulation of AR and PSA and enhanced pSer81-AR phosphorylation. Phosphorylation at the Ser213/210 was only observed in DHT-treated cells. Numbers below the bands represent the fold ratio of densitometric quantification relative to corresponding control. B, LNCaP cells were transfected with a mixture of control *Renilla* reporter and androgen receptor element-luciferase reporter plasmids. After 24 hours of the transfection, cells were treated with either DHT (1 nmol/L) or fostriecin (Fos; 100 nmol/L) in steroid-reduced medium for next 24 hours. In parallel experiments, cells were cotransfected with scrambled or *PPP2CA*-specific siRNAs along with *Renilla* or androgen receptor element reporter plasmids for 48 hours. Luciferase activities were estimated using a Dual-Luciferase Assay System Kit. RLUs (the ratio of firefly/*Renilla* luciferase) were calculated as a measure of AR transcriptional activity. Bars represent the means ± SD ($n = 3$); *, statistically significant ($P < 0.05$). A partial activation of AR is reported in PP2A-inhibited LNCaP cells as compared with DHT-treated cells.

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

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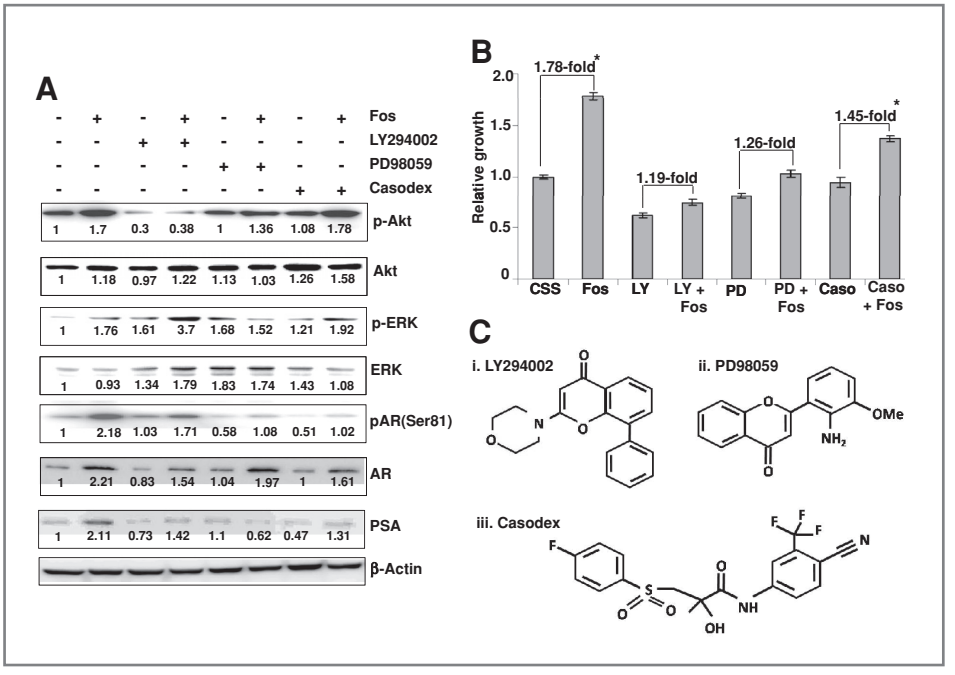

Figure 5. Pharmacologic repression of Akt, ERK, and AR signaling pathways suppresses androgen-independent growth of PP2A-inhibited cells. A, LNCaP cells were pretreated for an hour with LY294002 (20 μmol/L), PD98059 (25 μmol/L), and Casodex (5 μmol/L) followed by treatment with fostriecin (Fos; 100 nmol/L) for 24 hours. Total protein was isolated and effect on the activation of Akt, ERK, and AR was examined by immunoblotting with their total and phospho-form–specific antibodies. Data indicate that the induction of AR expression on PP2A inhibition occurs through Akt pathway, whereas its ligand-independent phosphorylation involves ERK activation. Numbers below the bands represent the fold ratio of densitometric quantification relative to corresponding control. B, in parallel experiments, effect of Akt, ERK, and AR inhibition was observed on the growth of LNCaP cells under steroid-reduced condition following PP2A downregulation. Cell growth was assessed after 48 hours of treatment by MTT assay. Bars represent the means ± SD ($n = 3$); *, statistically significant ($P < 0.05$). Our data indicate that androgen-independent growth of LNCaP cells on PP2A inhibition is facilitated through a concerted action of Akt, ERK, and AR signaling pathways. C, chemical structures of LY294002, PI3K inhibitor (i), PD98059, ERK inhibitor (ii), and Casodex, anti-androgens (iii).

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,

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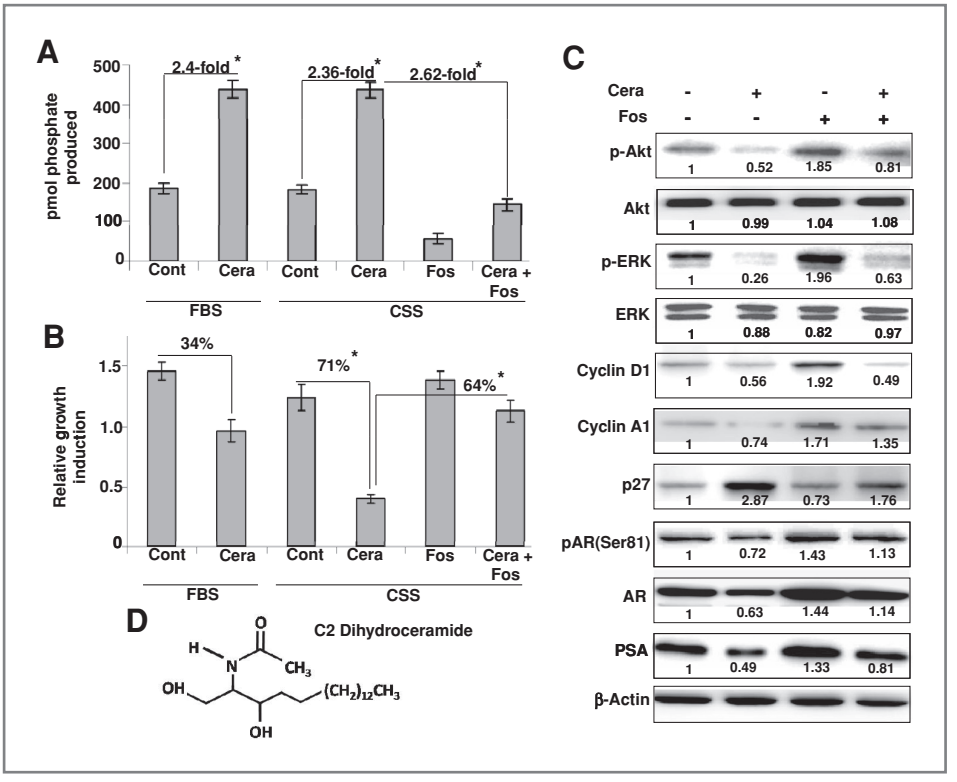

Figure 6. Ceramide (Cera) activates PP2A and suppresses the growth of androgen-independent prostate cancer C4-2 cells. A, C4-2 prostate cancer cells under steroid-supplemented (FBS) or -reduced (CSS) conditions were treated with ceramide (20 μmol/L; with or without pretreatment with fostriecin, Fos). PP2A activity was assessed after 24 hours as previously described. Treatment with ceramide led to the activation of PP2A, which could be inhibited by pretreatment with fostriecin. B, in parallel experiments, the effect of ceramide treatment was monitored on the growth of C4-2 cells under steroid-supplemented (FBS) or -reduced (CSS) conditions after 96 hours using MTT assay. Ceramide led to the suppression of growth of C4-2 cells under both steroid-supplemented and -reduced conditions; however, the effect was more prominent under steroid-reduced condition. Pretreatment with fostriecin attenuated ceramide-induced growth suppression. Bars represent the means ± SD ($n = 3$); *, statistically significant ($P < 0.05$). C, to examine the signaling changes, immunoblot analyses were carried out for p-Akt/Akt, p-ERK/ERK, cyclin A1, cyclin D1, p27, phospho-AR (Ser81), AR, PSA, and β-actin (used as internal control). Ceramide treatment led to the dephosphorylation of endogenously activated Akt and ERK, decreased the expression of cyclins, and induced the expression of p27. Moreover, reduced expression of AR and PSA and decreased AR phosphorylation (Ser81) was also observed in ceramide-treated cells. Pretreatment of C4-2 cells with fostriecin abrogated the ceramide-induced changes in signaling/effector proteins. Numbers below the bands represent the fold ratio of densitometric quantification relative to corresponding control. D, chemical structure of C2 dihydroceramide, a potent activator of PP2A. Cont, control.

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 overexpress 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

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suppress MAP/ERK kinase (MEK)/ERK pathway (15, 17), and both Akt and ERK have been shown to potentiate the proliferation and survival of cancer cells (38, 41). In fact, it has been reported that forced activation of either Akt or ERK signaling in an androgen-responsive prostate cancer cell line could induce hormone-independent growth in culture (42). Furthermore, it was observed that these pathways act synergistically *in vivo* to promote tumorigenicity and androgen independence.

As majority of AI prostate tumors retain AR expression and overexpress androgen-regulated genes (*PSA* etc.), a pathogenic role of aberrant AR signaling is also considered central to the androgen-independent progression of prostate cancer (1, 6, 7). One of the important mechanisms proposed to explain the androgen-independent growth of prostate cancer implicates an important role of ligand-independent activation of AR signaling. It has been shown that certain growth factors (insulin like growth factor I, keratinocyte growth factor, and epidermal growth factor) can activate the AR in the absence of androgen in prostate cancer cells (43). In other studies, overexpression of ErbB2/HER2 has been shown to activate the expression of AR-dependent genes (6, 44). It is shown that such ligand-independent activation of AR signaling may involve MAPK pathway (44). However, the role of PI3K/Akt pathway in AR-mediated PC cell growth has been controversial and largely unclear. In some cases, Akt has been shown to suppress AR activity (45), whereas in other reports, it is also shown to potentiate AR action (46, 47). In this study, we report that PP2A downregulation leads to partially sustained AR signaling. Our data indicate that AR signaling is maintained through induced expression of AR and its ligand-independent activation. These observations are in corroboration with recently published report, where PP2A inhibition was shown to cooperate with DHT to induce AR expression and phosphorylation (48). In addition, our studies utilizing pharmacologic inhibitors against Akt and MEK/ERK indicate that induction of AR expression on PP2A inhibition is mediated through the activation of Akt, whereas its ligand-independent phosphorylation (on serine-81) is caused by ERK activation. An earlier study also reported that AR phosphorylation at Ser-81 is mediated through ERK pathway (31). In other studies, AR phosphorylation on serine-213 by Akt has also been reported; however, we did not observe such phosphorylation despite activation of Akt in response to PP2A inhibition. Nonetheless, our data on AR transcriptional activity and PSA expression confirmed the partial activation of AR on downregulation of PP2A under steroid-depleted condition, and thus holds mechanistic significance. Our data also highlighted the importance of these signaling pathways in sustaining androgen-independent growth of LNCaP cells on PP2A inhibition. Whereas we noted almost complete abrogation of androgen-independent growth in Akt- and ERK-inhibited cells, a minimal, but significant effect of AR inhibition was also observed. These findings are 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 the 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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Restoration of *PPP2CA* expression reverses epithelial-to-mesenchymal transition and suppresses prostate tumour growth and metastasis in an orthotopic mouse model

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Background: Emergence of castration-resistance in prostate cancer (PCa) is invariably associated with aggressive and metastatic disease. Previously, we reported promotion of castration-resistance upon downregulation of *PPP2CA* (encoding catalytic subunit of protein phosphatase 2A (PP2A), α-isoform); however, its role in PCa growth and metastasis remained undetermined.

Methods: *PPP2CA* was overexpressed/silenced in PCa cells by stable transfection. Gene expression was examined by reverse transcription polymerase chain reaction, immunoblot and immunofluorescence analyses, and transcriptional activity measured by luciferase-based promoter-reporter assay. Effect on PCa phenotype was studied *in vitro* and in orthotopic mouse model, and immunohistochemical/histological analyses performed to assess proliferation/apoptosis and confirm metastatic lesions.

Results: An inverse association of *PPP2CA* expression was observed with epithelial-to-mesenchymal transition (EMT) and aggressive PCa phenotype. *PPP2CA* restoration resulted in decreased nuclear accumulation and transcriptional activity of β-catenin/NF-κB, and restitution of their activity abrogated *PPP2CA*-induced EMT reversal and suppression of PCa invasiveness. Akt mediated *PPP2CA* loss-induced nuclear accumulation of β-catenin/NF-κB through inactivation of Gsk3-β and IκB-α, respectively. Animal studies revealed a suppressive effect of *PPP2CA* expression on PCa growth and metastasis.

Conclusions: Our findings suggest that *PPP2CA* downregulation serves as a molecular link between gain of castration-resistance and aggressive PCa phenotype, and its restoration could be an effective preventive/therapeutic approach against the advanced disease.

Prostate cancer (PCa) is the most commonly diagnosed non-cutaneous malignancy and second leading cause of cancer-related death in American men. The American Cancer Society estimates that in 2014, there will be 233 000 new diagnoses of PCa, and nearly 29 480 patients will die of this disease (Siegel *et al*, 2014). Owing to widespread screening, most patients are now diagnosed with localised prostate tumours; however, a significant proportion still continues to present with locally advanced or metastatic disease (Cooperberg *et al*, 2005). At the present time, there is no effective cure for the advanced disease and androgen deprivation therapy (ADT) remains the principal treatment option (Feldman and Feldman, 2001). Unfortunately, most PCa patients treated with ADT eventually acquire castration-resistant phenotype, a form, which is highly aggressive and unresponsive to other therapies (Joly and Tannock, 2004; Sridhar *et al*, 2013). Therefore, characterisation of molecular targets facilitating PCa progression

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and metastasis, and deciphering their mechanisms of action is critically important to develop effective treatment approaches.

Protein phosphatase 2A (PP2A) is the most abundant serine/threonine phosphatase in mammals and has important roles in several biological processes (Mumby, 2007; Perrotti and Neviani, 2013). It consists of a common heteromeric core enzyme, which is composed of a catalytic subunit and a constant regulatory subunit that associates with a variety of regulatory subunits (Janssens and Goris, 2001). Previously, we identified that *PPP2CA*, which encodes the alpha isoform of the catalytic subunit (PP2Acα), is one of the downregulated genes in castration-resistant PCa cells (Singh *et al*, 2008). More importantly, an inverse correlation of *PPP2CA* expression with increasing Gleason grades and tumour stage was also observed (Singh *et al*, 2008). In other recent studies, we have observed that *PPP2CA* downregulation confers androgen depletion resistance to PCa cells (Bhardwaj *et al*, 2011). The data revealed that the loss of PP2A-mediated checkpoints led to activation of Akt and ERK, and partially sustained androgen receptor signalling under steroid-deprived condition (Bhardwaj *et al*, 2011). Interestingly, studies from other groups have reported loss and functional significance of additional PP2A subunits (PP2A-Cβ, PP2A-Bγ and PP2A-Aα) as well; suggesting that dysregulation of PP2A may be a frequent occurrence in PCa pathogenesis (Prowatke *et al*, 2007; Bluemn *et al*, 2013; Pandey *et al*, 2013).

As emergence of castration-resistance in PCa is invariably associated with highly aggressive and metastatic disease (Jennbacken *et al*, 2006; Srivastava *et al*, 2012), it appears that there may be a common molecular thread for these distinct phenotypes. If such an association is characterised at the molecular level, it can have significant impact on the management of PCa. Therefore, this study was aimed at determining the role of *PPP2CA* in PCa progression and metastasis. We developed 'paired' PCa cell lines, in which *PPP2CA* expression is either stably 'restored' or 'silenced' through genetic engineering approaches. Using these cell lines, we demonstrate that *PPP2CA* downregulation promotes migration and invasion of PCa cells. Furthermore, our data show that *PPP2CA* expression is associated with loss of mesenchymal and gain of epithelial characteristics. Mechanistically, we find important roles of Akt-driven β-catenin and NF-κB activation in *PPP2CA* downregulation-induced epithelial-to-mesenchymal transition (EMT) and invasiveness of PCa cells. Finally, our data demonstrate that restoration of *PPP2CA* expression attenuates PCa growth and metastasis in an orthotopic mouse model. Thus, our findings suggest that *PPP2CA* downregulation may be a common link between castration-resistance and aggressive tumour phenotypes, and its targeting may be useful in stalling PCa progression as well as in the treatment of the advanced disease.

MATERIALS AND METHODS

Cell lines, antibodies and plasmids. All cell lines (LNCaP, C4-2 and PC3) were procured, maintained and validated intermittently as described earlier (Srivastava *et al*, 2012). Anti-PP2Acα (rabbit polyclonal) and anti-cytokeratin 18 (mouse monoclonal) antibodies were purchased from Abcam (Cambridge, MA, USA). Antibodies against ERK1/2, NF-κB/p65, Slug (rabbit monoclonal), pERK1/2, IκB-α (mouse monoclonal) and p-IκB-α (rabbit polyclonal) were from Cell Signaling Technology (Beverly, MA, USA). Antibodies against Akt, p-Akt and vimentin (rabbit monoclonal) were from Epitomics (Burlingame, CA, USA). Antibodies against β-catenin, E-cadherin and N-cadherin (all mouse monoclonal) were from BD transduction laboratories (Bedford, MA, USA). Anti-β-actin (mouse monoclonal) antibody was from Sigma-Aldrich (St Louis, MO, USA). Anti-Twist (rabbit polyclonal), anti-α-tubulin, anti-laminin (mouse monoclonal), and all HRP-, FITC- and TRITC-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). *PPP2CA*-short hairpin RNA expression plasmid (*PPP2CA*-shRNA-pGFP-V-RS), non-targeted control plasmid (NT-shRNA-pGFP-V-RS), *PPP2CA* overexpression (*PPP2CA*-pCMV6) and empty vector (pCMV6) constructs were purchased from Origene (Rockville, MD, USA). pGL4.32 (luc2P/NF-B-RE/Hygro) and pRL-TK plasmids were from Promega (Madison, WI, USA). TOPflash or FOPflash reporter plasmids were kindly provided by Dr R Samant, UAB, Birmingham, AL, USA. pcDNA3-β-catenin S33Y (plasmid number 19286), pCMV-IKKβ S177E S181E (plasmid number 11105) and pcDNA3-HA PKB T308D S473D (plasmid number 14751) were from E Fearon, A Rao and J Woodgett Laboratories, respectively, and procured through Addgene (Cambridge, MA, USA).

Transfections and treatments. For overexpression (in PC3 and C4-2) and knockdown (in LNCaP) of *PPP2CA*, cells were transfected with *PPP2CA*-overexpressing or shRNA plasmids, respectively, along with their control plasmids (empty vector for overexpression and non-targeted control vector for shRNA) using FuGENE (Roche, Mannheim, Germany) as a transfection reagent following the manufacturer's instructions. Stable pooled population of transfected cells were obtained using selective antibiotic containing media (G418, 200 $\mu g\,ml^{-1}$ or puromycin 2 $\mu g\,ml^{-1}$). Following selection, cells were expanded and examined for stable *PPP2CA* overexpression or silencing. Cells were transiently transfected with constitutively active mutant plasmids of IKKβ, β-catenin and PKB, or with respective empty vector plasmids using FuGENE as per the manufacturer's instructions. To dissect the role of the Akt signalling pathway, cells were treated with 20 mM LY294002 (PI3K inhibitor; Cell Signaling Technology) for various time intervals as described in respective figure legends.

RNA isolation and reverse transcription polymerase chain reaction (RT–PCR). Total RNA was isolated from the cultured cells using RNeasy Kit (Qiagen, Gaithersburg, MD, USA). Subsequently, complementary DNA (cDNA) was synthesised using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative real-time PCR was performed in 96-well plates using SYBR Green Master Mix (Applied Biosystems) on an iCycler system (Bio-Rad, Hercules, CA, USA). Specific sequences of PCR primers used in this study are listed in Supplementary Table S1. The thermal conditions for real-time PCR assays were as follows: cycle 1: 95 °C for 10 min, cycle 2 (× 40): 95 °C for 10 s and 58 °C for 45 s. Threshold cycle (C_T) values for each were separately normalised against C_T values for GAPDH, and a relative fold change in expression with respect to a reference sample was calculated by the $2^{-\Delta\Delta Ct}$ method.

PP2A activity assay. Protein phosphatase 2A activity was determined using PP2A immunoprecipitation phosphatase assay kit as described earlier by us (Bhardwaj *et al*, 2011).

Nuclear and cytoplasmic fractionation. The preparation of cytoplasmic and nuclear extracts was performed using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) as described previously by us (Arora *et al*, 2011).

Immunoblot analysis. Immunoblotting was performed as described earlier (Bhardwaj *et al*, 2011) using specific antibodies against various proteins. β-Actin, α-tubulin and laminin were used as loading controls for total, cytoplasmic and nuclear proteins, respectively. All the primary antibodies were used at 1 : 1000 dilution except antibodies against Twist, α-tubulin and laminin, which were used at 1 : 200 dilution. All the secondary antibodies were used at 1 : 2500 dilution.

Immunofluorescence assay. Actin staining was performed as described previously by us (Srivastava *et al*, 2012). For β-catenin and NF-κB/p65 staining, cells were fixed in ice-cold methanol, washed, blocked and incubated with respective antibodies diluted in antibody diluent (1 : 50) for 90 min at room temperature followed by washing. Cells were then incubated with FITC- or TRITC-conjugated goat anti-rabbit secondary antibodies (1 : 500) for 60 min. Thereafter, cells were washed, mounted with antifade Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA) and observed under Nikon Eclipse TE2000-U fluorescent microscope (Nikon Instruments Inc., Melville, NY, USA).

TCF/LEF/β-catenin and NF-κB transcriptional activity assays. To examine the effect of *PPP2CA* modulation on transcriptional activity of LEF/TCF and NF-κB, cells were transiently co-transfected with the luciferase promoter–reporter constructs (TOPflash or FOPflash, or pGL4.32 (luc2P/NF-κB-RE/Hygro)) and pRL-TK (control reporter plasmid containing a *Renilla reniformis* luciferase gene downstream of the TK promoter). After 24 h of transfection, total protein was harvested in reporter lysis buffer (Promega). Firefly and Renilla luciferase activities were measured using a dual-luciferase assay kit (Promega) according to the manufacturer's instructions.

Motility and invasion assays. Effects of the modulation of *PPP2CA* expression on the migration and invasion ability of PCa cells were examined using non-coated or Matrigel-coated transwell chamber by following the previously described procedure (Srivastava *et al*, 2012).

Orthotopic xenograft mouse tumour model. All animal experiments were performed in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines. Immunodeficient male mice (4- to 6-week old; Harlan Laboratories, Prattville, AL, USA) were anaesthetised with intraperitoneal injection of ketamine ($100\,mg\,kg^{-1}$) and xylazine ($15\,mg\,kg^{-1}$). After cleaning their abdomen, a small midline incision was made to expose the prostate gland, and cells (1×10^6 suspended in 50 μl of HBSS medium) were injected into the dorsal prostatic lobe. The abdominal wound was closed in two layers and animals were monitored every alternate day. At the end point (30 days post-implantation), mice were killed by CO_2 asphyxiation and autopsied. Prostate tumours were resected, weighed and measured for their dimensions using Vernier Calipers. Tumour volume was calculated by the following formula: $(A \times B^2)/2$, where A is the larger and B is the smaller of the two dimensions. To examine the metastases, distinct organs (liver, lung, bone and lumbar lymph nodes) were dissected and fixed in Bouin's solution. Numbers of visible metastatic nodules were counted (in case of liver and lung) and total mass of collected lumbar lymph nodes was recorded.

IHC and histological analyses. Immunohistochemical (IHC) analysis was performed on deparaffinised and rehydrated tissue sections from formalin-fixed, paraffin-embedded blocks of orthotopically developed prostate tumours. In brief, 5-μm thick tumour sections were deparaffinised using EZ-Dewax (Biogenex, Fremont, CA, USA) and incubate in methanol containing 3.0% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Thereafter, antigen retrieval was achieved by using decloaking Chamber (Biocare Medical, Concord, CA, USA) according to the manufacturer's protocol. Later sections were blocked for 10 min with Background Sniper (Biocare Medical) and incubated with the following primary antibodies for 60 min at room temperature. Subsequently, sections were incubated at room temperature with recommended polymer and probe (Biocare Medical) according to the manufacturer's protocol. Immunoreactivity was visualised by using DAB Chromogen followed by haematoxylin counterstain. Negative control tissues were also incubated in all reagents with no primary antibody. Apoptotic cells in tumour samples were identified by using DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. Presence of a reddish brown precipitate indicated a positive reaction. The number of proliferative and apoptotic cells were counted in ten random view fields (× 200 magnification) in a double-blinded manner and expressed as average number of cells per field view. Histological examination followed by haematoxylin and eosin (H&E) staining was performed on the Bouin's solution-fixed, paraffin-embedded tissue sections of the livers, lungs, lymph nodes and bones to examine the presence of metastatic tumour nodules. In case of bone, specimens were decalcified in 10% EDTA in PBS for 14 days at 4 °C before paraffin embedding.

Statistical analysis. All the experiments were performed at least three times, independently and all data are expressed as 'mean ± s.d.' Wherever appropriate, the data were also subjected to unpaired two-tailed Student's *t*-test. $P < 0.01$ was considered statistically significant.

RESULTS

***PPP2CA* expression is inversely associated with migratory and invasive potential of PCa cells.** To investigate the role of *PPP2CA* in the aggressive malignant behaviour of PCa cells, we overexpressed it in C4-2 and PC3 (low *PPP2CA* expressing) and silenced in LNCaP (high *PPP2CA* expressing) cells by stable transfection. Expression and activity of its encoded protein (PP2Acα) were assessed in stable transfectants by immunoblot and malachite green-based assays, respectively. The data demonstrate significant overexpression and enhanced activity of PP2Acα in C4-2-PPP2CA and PC3-PPP2CA cells as compared with their respective controls (C4-2-Neo and PC3-Neo; Figures 1A and B). Similarly, *PPP2CA*-knockdown LNCaP cells (LNCaP-shPPP2CA) exhibit reduced expression and activity of PP2Acα in comparison with its non-targeted scrambled sequence expressing control (LNCaP-NT) cells (Figures 1A and B). Furthermore, consistent with our prior observation in transient assays (Bhardwaj *et al*, 2011), *PPP2CA*-overexpressing C4-2 and PC3 cells exhibit decreased phosphorylation of Akt and ERK (PP2A substrates), while it is increased in *PPP2CA*-silenced LNCaP cells (Supplementary Figure S1).

We next examined the effect of *PPP2CA* overexpression or silencing on the migration and invasive potential of PCa cells. Our data show that *PPP2CA*-overexpressing C4-2 and PC3 cells have significantly ($P < 0.01$) reduced migratory (2.3- and 2.2-fold, respectively) and invasive (2.7- and 2.8-fold, respectively) potential as compared with their respective controls (Figure 1C). Similarly, we also observe a significant gain ($P < 0.01$) in migratory (2.4-fold) and invasive (3.0-fold) behaviour of *PPP2CA*-knockdown LNCaP cells (Figure 1C). Together, these data indicate that *PPP2CA* suppresses aggressive behaviour of the PCa cells.

Loss of *PPP2CA* facilitates EMT transition in PCa cells. Several lines of evidence indicate that increased malignant potential of cancer cells is associated with their transition from epithelial-to-mesenchymal phenotype, a process referred as EMT (Kang and Massague, 2004; Nauseef and Henry, 2011). Therefore, we investigated whether altered expression of *PPP2CA* had an impact on EMT process. For this, we first examined actin organisation in *PPP2CA*-overexpressing or *PPP2CA*-knockdown PCa cells considering the fact that actin-dependent membrane protrusions act as critical determinants of EMT (Shankar *et al*, 2010). Staining of filamentous-actin with FITC-conjugated phalloidin revealed the presence of many filopodial structures in low *PPP2CA*-expressing (C4-2-Neo, PC3-Neo and LNCaP-shPPP2CA) cells, while they were absent or less obvious in the high

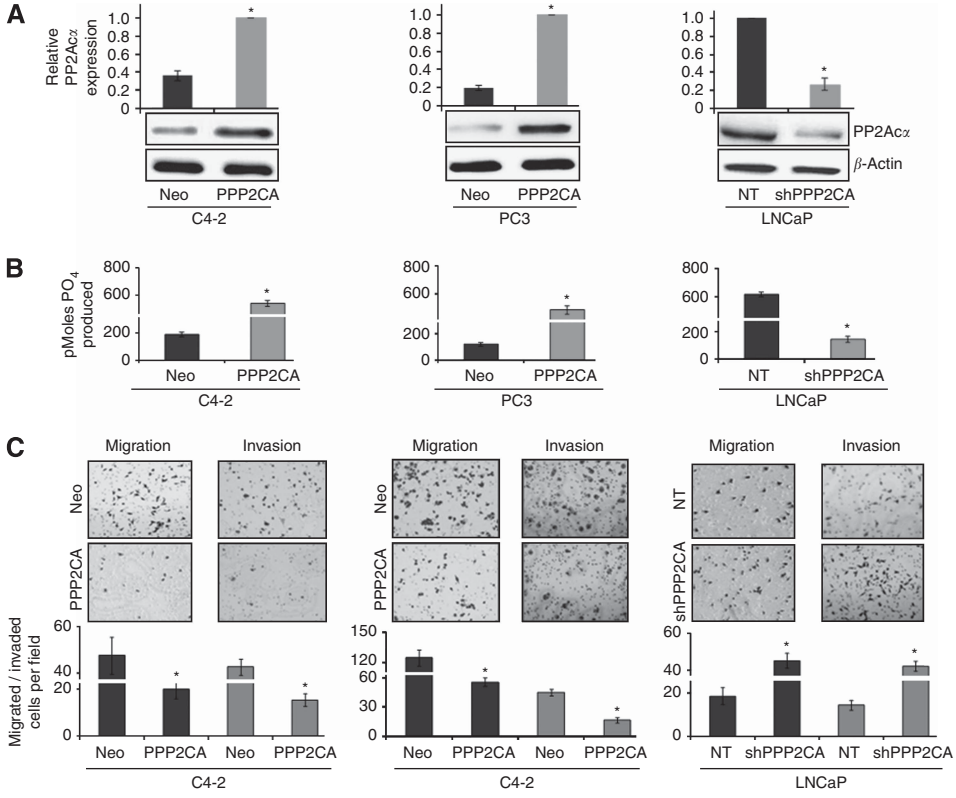


Figure 1. Overexpression of *PPP2CA* suppresses malignant behaviour of PCa cells. Expression (**A**) and activity (**B**) of PP2Acα in stable pooled populations of *PPP2CA*-overexpressing (C4-2- and PC3-PPP2CA) and *PPP2CA*-silenced (LNCaP-shPPP2CA) PCa cells, along with their respective empty vector (-Neo) and non-targeting scrambled-shRNA (-NT) transfected control cells were examined by immunoblot analysis and malachite green-based assay, respectively. β-Actin was used as a loading control in immunoblot analysis. (**C**) Cells were seeded in a transwell chamber (8 μM pore size, non-coated or Matrigel-coated) and allowed to migrate or invade overnight under chemotactic drive. After overnight incubation, the cells that did not migrate or invade through the membranes were removed and migrated/invaded cells were stained using a commercial kit. Images were taken in 10 random fields (magnification × 100) and cell number counted. The data are presented as mean ± s.d., ($n=3$).*$P<0.01$.

PPP2CA-expressing (C4-2-PPP2CA, PC3-PPP2CA and LNCaP-NT) cells (Figure 2A). To further confirm the role of *PPP2CA* on EMT, we examined the expression of markers associated with epithelial (E-cadherin and cytokeratin-18) and mesenchymal (N-cadherin, vimentin, Twist and Slug) phenotypes by qRT–PCR and immunoblot assays. Our data show that suppression of *PPP2CA* in LNCaP cells causes increased expression of mesenchymal markers, whereas those associated with epithelial phenotype are suppressed both at transcriptional (Figure 2B) and protein levels (Figure 2C). On the other hand, *PPP2CA*-overexpressing C4-2 and PC3 cells exhibit greater expression of epithelial and reduced expression of mesenchymal markers as compared with their respective controls (Figures 2B and C). Together, these findings suggest that loss of *PPP2CA* expression facilitates EMT in PCa cells.

Activation of β-catenin and NF-κB is involved in *PPP2CA* downregulation-induced EMT and aggressive behaviour of PCa cells. To explore the mechanistic basis of *PPP2CA* loss-induced EMT, we focused on β-catenin and NF-κB, which have earlier been shown to be aberrantly activated in PCa (Ross *et al*, 2004; Jaggi *et al*, 2005; Lessard *et al*, 2006), and implicated in transcriptional regulation of EMT markers (Min *et al*, 2008; Li *et al*, 2012, 2013). Our data from luciferase-based promoter–reporter assays show decreased transcriptional activity (>2.4-fold) of both LEF/TCF/β-catenin and NF-κB in *PPP2CA*-overexpressing PC3 cells as compared with that in control cells (Figure 3A). Similarly, we observe gain of transcriptional activity (>2.8-fold) of both LEF/TCF/β-catenin and NF-κB in *PPP2CA*-silenced LNCaP cells, when compared with non-targeted siRNA-expressing cells (LNCaP-NT) (Figure 3A). In accordance with these findings, our immunoblot analysis reveal decreased nuclear accumulation of β-catenin and NF-κB that correlate with their increased cytoplasmic levels in high *PPP2CA*-expressing (PC3-PPP2CA and LNCaP-NT) cells as compared with their low *PPP2CA*-expressing sublines (PC3-Neo and LNCaP-shPPP2CA) (Figure 3B). These findings are further confirmed in immunofluorescence assay. The data show that β-catenin is predominantly localised in the membrane region in *PPP2CA*-overexpressing LNCaP (endogenous) and PC3-PPP2CA (exogenous) cells, whereas its nuclear staining is prominent in *PPP2CA*-silenced LNCaP or low endogenous *PPP2CA*-expressing PC3 cells. Similarly, we observe reduced nuclear localisation of NF-κB in *PPP2CA*-overexpressing LNCaP-NT and PC3-PPP2CA cells as compared with low *PPP2CA*-expressing (PC3-Neo and LNCaP-shPPP2CA) cells (Figure 3C).

We next investigated the involvement of β-catenin and NF-κB in *PPP2CA*-induced EMT reversal and suppression of migration and invasion. For this, we transfected *PPP2CA*-overexpressing PC3 cells with constitutively active mutants of β-catenin (β-catenin-S33Y) and/or IKKβ (IKKβ-SSEE) along with their respective control vectors (pcDNA3 and pCMV6). Our data show that transfection of active β-catenin and IKKβ mutants efficiently restores the β-catenin and NF-κB transcriptional activity, respectively, in PC3-PPP2CA cells, while it remains suppressed in control-transfected cells as observed in promoter–reporter assay (Figure 3D). This is accompanied by enhanced nuclear

accumulation of β-catenin and NF-κB in active mutant-transfected PC3-PPP2CA cells (Figure 3E, upper panel). Interestingly, we also observe that activation of either β-catenin or NF-κB led to partial loss of epithelial (E-cadherin) and regain of mesenchymal (Twist) markers, while their simultaneous activation reverse *PPP2CA*-induced EMT completely (Figure 3E, lower panel). In addition, migration and invasive potential of PC3-PPP2CA cells is also restored upon forced activation of β-catenin and NF-κB (Figure 3F). Together, these findings indicate that both β-catenin and NF-κB cooperatively mediate *PPP2CA* loss-induced EMT and invasiveness of PCa cells.

Akt mediates *PPP2CA* silencing-induced activation of β-catenin and NF-κB. Having observed important roles of β-catenin and NF-κB in mediating the effect of *PPP2CA* downregulation, we next investigated the mechanism(s) underlying their suppression in *PPP2CA*-overexpressing PCa cells. As Akt is a target of PP2A and its downstream signalling has been shown to promote EMT and aggressive tumour phenotype (Grille *et al*, 2003; Bhardwaj *et al*, 2011; Yoo *et al*, 2011), we examined if its activation is involved in mediating enhanced transcriptional activity of β-catenin and NF-κB upon *PPP2CA* silencing. For this, *PPP2CA*-silenced LNCaP were treated with pharmacological inhibitors of Akt (LY294002) and their effect on transcriptional activity of β-catenin and NF-κB was examined. The data reveal that Akt inhibition leads to abrogation of *PPP2CA* silencing-induced stimulation of transcriptional activity of β-catenin and NF-κB (Figure 4A, left panel). We later confirmed this finding by transfecting the PC3-PPP2CA cells

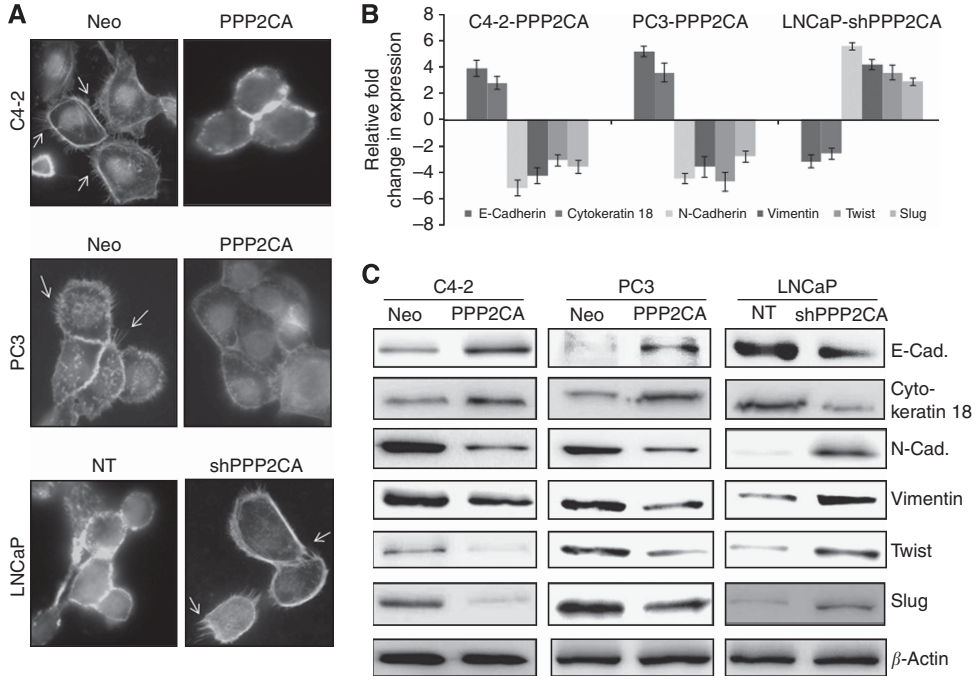
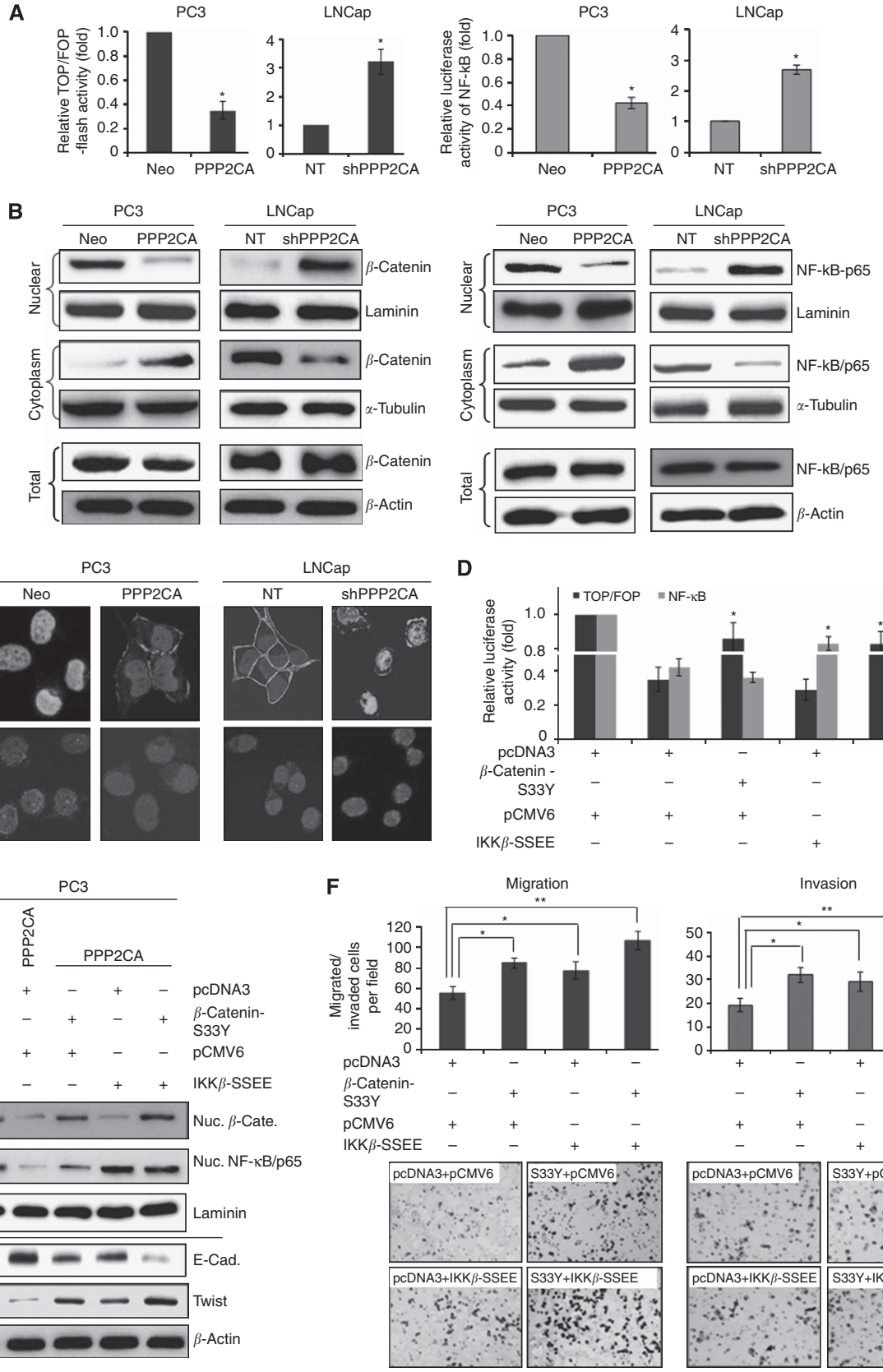


Figure 2. *PPP2CA* overexpression causes reversal of EMT in PCa cells. (**A**) *PPP2CA*-overexpressing or *PPP2CA*-silenced PCa cells were grown on fluorodish, fixed and stained with Alexa Fluor 488-conjugated phalloidin. Images were taken under confocal microscope. Data show that low *PPP2CA*-expressing (C4-2-Neo and PC3-Neo and LNCaP-shPPP2CA) cells exhibited several filopodia- and lamellipodia-like projections (arrows) as compared with *PPP2CA*-overexpressing (PC3-PPP2CA and C4-2-PPP2CA and LNCaP-NT) cells. Expression of various epithelial (E-cadherin and cytokeratin-18) and mesenchymal (N-cadherin, vimentin, Slug and Twist) markers at (**B**) transcript and (**C**) protein levels was examined by qRT–PCR and immunoblot assays, respectively. Data are presented as fold change in expression upon *PPP2CA* overexpression and silencing. Bars represent mean ± s.d., $n=3$.

Figure 3. Suppression of β-catenin and NF-κB is responsible for *PPP2CA*-mediated reversal of EMT and reduced aggressiveness. (**A**) Transcriptional activities of β-catenin/LEF/TCF and NF-κB in *PPP2CA*-overexpressing or *PPP2CA*-silenced PCa cells were measured as described in Materials and Methods section. Data are presented as fold change in normalised luciferase activity. (**B**) Expression level of β-catenin and NF-κB in different cellular fractions was examined by immunoblot analysis. Laminin, α-tubulin and β-actin were used as loading controls for nuclear, cytoplasmic and total protein, respectively. (**C**) Cells grown on glass bottom were fixed, incubated with β-catenin or NF-κB antibodies for 90 min at room temperature and subsequently stained using FITC-conjugated (for β-catenin) or TRITC-conjugated (for NF-κB) goat anti-rabbit secondary antibodies for 60 min. Thereafter, cells were washed, mounted and observed under confocal fluorescent microscope. (**D**) PC3-PPP2CA cells were grown in six-well plate and transiently transfected with constitutively active β-catenin (β-catenin-S33Y) and IKKβ (IKKβ-SSEE) mutants (to activate β-catenin and NF-κB, respectively) along with controls vectors. At 24 h post-transfection, cells were again transfected with TOPflash/FOPflash and NF-κB luciferase promoter–reporter constructs to examine LEF/TCF and NF-κB activity as described previously. (**E**) PC3-PPP2CA cells were transfected with constitutively active β-catenin and IKKβ mutants or their respective control plasmids. Thereafter, nuclear (Nuc) and total protein lysates were prepared after 24 and 48 h of transfection and expression level of β-catenin and NF-κB (after 24 h in nuclear lysate) and E-cadherin and Twist (after 48 h in total lysate) were examined by immunoblot analysis. Laminin (for nuclear fraction) and β-actin (for total protein) were used as loading controls. (**F**) Cells were transiently transfected with constitutive active β-catenin and IKKβ mutants, trypsinised after 48 h, and re-seeded at equal density in transwell chambers (uncoated or Matrigel-coated). Numbers of migrated/invaded cells were counted in random fields following fixation and staining. Bars represent mean ± s.d. ($n=3$); *$P<0.05$ and **$P<0.01$.

with constitutively active protein kinase B/Akt mutant or its vector only control. The data show that inhibitory effect of *PPP2CA* overexpression on transcriptional activity of TCF/LEF/β-catenin and NF-κB is diminished after re-activation of Akt in PC3-PPP2CA cells (Figure 4A, right panel). Accordingly, we observe an enhanced nuclear localisation of both β-catenin and NF-κB in PC3-PPP2CA cells upon Akt activation, whereas the opposite is observed upon Akt inhibition in LNCaP-shPPP2CA cells (Figure 4B, upper panel). In additional assays, we find an inverse association of *PPP2CA* expression with Gsk3-β and IκB-α



phosphorylation in PCa cells (Figure 4B, lower panel). Although Gsk3-β phosphorylation leads to its inactivation, phosphorylation of IκB-α is associated with its destabilisation as observed in our data (Figure 4B, lower panel). Together, these findings suggest a role of PP2A/Akt axis in regulation of β-catenin and NF-κB in PCa cells.

Restored *PPP2CA* expression suppresses prostate tumour growth and metastasis. To examine the effect of *PPP2CA* restoration on tumourigenicity and metastatic potential of PCa cells, control (PC3-Neo) and *PPP2CA*-overexpressing PC3 cells (PC3-PPP2CA) were injected into the dorsal prostatic lobe of immunodeficient male mice (4 to 6-week old). Tumour growth was monitored by palpation and mice were killed 30 days post-injection. We observed 100% tumour incidence in both the control and PC3-PPP2CA injected mice; however, tumours in the latter group were significantly smaller. Average volume and weight of tumours in PC3-PPP2CA group were 317.1 mm^3 (range from 171.5 to 490.8 mm^3) and 0.31 g (range from 0.13 to 0.46 g), respectively, as compared with 1803.98 mm^3 (range from 1369.9 to 2254.0 mm^3) and 1.56 g (range from 1.1 to 2.11 g) in PC3-Neo group (Figures 5A and B). Immunohistochemical analyses on paraffin-embedded tumour sections revealed significant decrease (>40%; $P<0.01$) in Ki67-positive cells, whereas a greater percentage of TUNEL-positive cells (>50%; $P<0.01$) was observed in tumours generated from PC3-PPP2CA cells as compared with those developed from PC3-Neo cells (Figures 5C and D).

In parallel, we also examined the effect of *PPP2CA* restoration on PCa metastasis. Tissues from suspected sites of metastasis (liver, lungs and lumber lymph nodes) were collected from tumour-bearing mice and fixed in Bouin's solution, and visible metastatic nodules were quantified. Data demonstrate high metastases in case of PC3-Neo mice as evident from the presence of multiple metastatic nodules in lungs and livers (Figure 6A). Furthermore, all of the tumour-bearing mice from PC3-Neo group carried enlarged lumber lymph nodes and exhibited significant differences in average weight (Figure 6A). To further confirm the presence of tumour cells in the suspected lesions, we sectioned the tissue following paraffin embedding and stained with H&E. Microscopic examination revealed the presence of tumour cell nests in stained tissue sections from PC3-Neo group as depicted in representative photomicrographs (Figure 6B). Furthermore, bone (femur) specimens from the mice were also collected, decalcified and examined for metastasis. No visible metastatic characteristics were observed in any of the bone from PC3-Neo and PC3-PPP2CA group (data not shown). Interestingly, when we examined H&E-stained tissue sections, we found presence of micro-metastatic colonies in PC3-Neo group, whereas no metastatic colonies were observed in bone specimens collected from PC3-PPP2CA group (Figure 6B, lower panel). Altogether, our data provide strong evidence for the role of *PPP2CA* downregulation in the progression and metastasis of PCa cells.

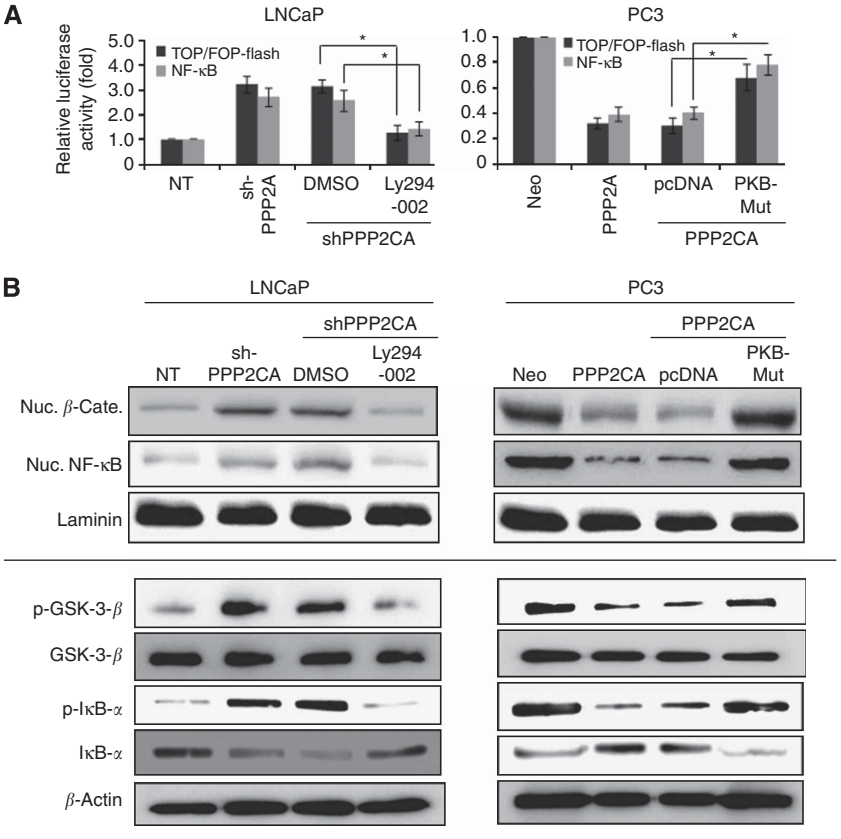


Figure 4. Role of Akt in *PPP2CA* overexpression-mediated suppression of β-catenin and NF-κB. (**A**) PC3-PPP2CA cells were transiently transfected with constitutively active PKB mutant or control plasmid for 24 h, and LNCaP-shPPP2CA cells were pre-treated (for 1 h) with PI3K/Akt inhibitor (LY294002). Subsequently, cells were co-transfected with TOPflash/FOPflash/NF-κB luciferase promoter–reporter constructs along with control plasmids for 24 h and luciferase activities were measured. Bars represents mean ± s.d. ($n=3$), *$P<0.01$. (**B**) Following transfection with active PKB mutant or PI3K inhibitor treatment, nuclear and total proteins were prepared and effect on localisation of β-catenin and NF-κB (in nuclear protein; upper panel) and on p-Gsk3-β/Gsk3-β and p-IκB-α/IκB-α (in total protein; lower panel) was examined by immunoblot analysis. Laminin (for nuclear protein) and β-actin (for total protein) were used as loading controls.

DISCUSSION

Several recent observations, including ours, have suggested an important role of PP2A in PCa progression and castration-resistance (Bhardwaj *et al*, 2011; Bluemn *et al*, 2013; Pandey *et al*, 2013). In the same line, this study revealed that loss of *PPP2CA*, a gene encoding the catalytic subunit of PP2A, promotes metastatic progression of PCa cells (Figure 7). Our data from 'gain' and 'loss' of function studies, provide compelling evidence for a role of PPP2CA in malignant behaviour of prostate tumour cells by modulating EMT process. Mechanistic studies reveal the involvement of Akt-mediated activation of β-catenin and NF-κB in *PPP2CA* loss-induced EMT and potentiation of PCa cell migration and invasion. Finally, our data from *in vivo* studies establish a direct inverse association of *PPP2CA* expression with prostate tumour growth and metastasis.

Metastasis is a complex and multistep phenomena, which initiates and progresses as a result of several molecular alterations (Valastyan and Weinberg, 2011). The regulatory networks that control such molecular alterations in PCa cells are, however, poorly understood. Our investigations reveal a significant role of *PPP2CA* in reducing aggressive phenotypes, that is, invasion and migration, of PCa cells. This is highly significant considering the fact that the castration-resistant PCa cells are also highly aggressive and more metastatic than their castration-sensitive counterparts (Jennbacken *et al*, 2006; Srivastava *et al*, 2012). Epithelial-to-mesenchymal transition is an important event that aids in invasion and subsequent metastatic dissemination of tumour cells to secondary sites (Nauseef and Henry, 2011). During EMT, tumour cells acquire the expression of mesenchymal markers such as vimentin, N-cadherin, Twist and Slug, whereas epithelial markers, such as E-cadherin that promote cell–cell contact, are usually lost (Kang and Massague, 2004; Li *et al*, 2012; Srivastava *et al*, 2012). These molecular changes facilitate detachment of the tumour cell from the primary site and migration and invasion across the extracellular matrix (Valastyan and Weinberg, 2011). In accordance with this, we observed an increased expression of epithelial and decreased expression of mesenchymal markers in *PPP2CA*-overexpressing cells. A similar role of PP2A in the regulation of malignant behaviour and EMT has been reported in other cancer types as well (Ito *et al*, 2000; Xu and Deng, 2006). Our *in vivo* data show that the overexpression of *PPP2CA* decreases the metastatic potential of PC3 cells to the various organs including liver, lung, lymph nodes and bone. Metastasis of PC3 cells to liver, lung and lymph nodes in orthotopic mouse model of PCa is widely reported (Rembrink *et al*, 1997; Wu *et al*, 2010). However, contradictory reports are available on the bone metastasis of PC3 cells upon intra-prostate implantation (Rembrink *et al*, 1997; Yang *et al*, 1999). Our findings are in consistence with the previous study published by Yang *et al* (1999), in which they observed skeletal metastases (skull, rib, pelvis, femur and tibia) of intra-prostate implanted PC3 cells.

Prostatic epithelial cells undergo EMT in response to the abnormal activation of several signalling pathways (Nauseef and Henry, 2011). Aberrant activation of Wnt/β-catenin and NF-κB has been linked with the high-grade PCa (Ross *et al*, 2004; Jaggi *et al*, 2005; Lessard *et al*, 2006). Moreover, multiple reports document the involvement of Wnt/β-catenin as well as NF-κB in the regulation of EMT and aggressive phenotypes of various tumour cells (Ganesan *et al*, 2008; Pantuck *et al*, 2010; Wu *et al*, 2012; Li *et al*, 2012, 2013). In these contexts, we investigated the involvement of β-catenin and NF-κB in the *PPP2CA*-mediated regulation of EMT and aggressive phenotype of PCa cells. The data demonstrate that activation status of both β-catenin and NF-κB is inversely correlated with the expression of *PPP2CA*. In general, activation of the Wnt pathway is associated with the nuclear accumulation of β-catenin, which then enhanced the transcriptional activity of LEF/TCF-responsive promoter through complex formation (Moon *et al*, 2004; Kypta and Waxman, 2012). A complex set of factors including GSK-3β are involved in this signalling process. GSK-3β acts as an inhibitor of β-catenin by inducing its phosphorylation-mediated proteolytic degradation (Moon *et al*, 2004; Kypta and Waxman, 2012). Similarly,

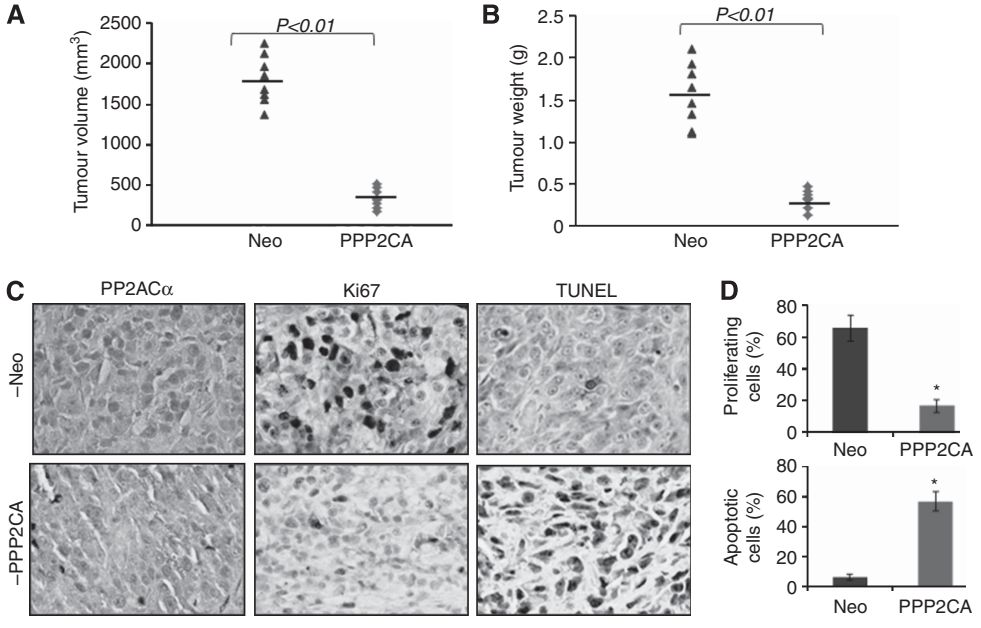


Figure 5. *PPP2CA* overexpression decreases tumourigenicity of PCa cells in orthotopic mice model. PC3 (control and *PPP2CA*-overexpressing) cells were injected into the prostate of mice ($n=8$ per group) and killed after 30 days. (**A**) Volume and (**B**) weight of the tumours from the each animal of the control and *PPP2CA*-overexpressing group were calculated. Dark line indicates the median value of the respective group. (**C**) Immunohistological analysis of paraffin-embedded prostate tumours for PP2Acα expression (left panel), Ki67 expression (a proliferative marker; middle panel) and TUNNEL assay (right panel). (**D**). Number of proliferating (upper panel) and apoptotic (lower panel) cells were counted in 10 random view fields (magnification $\times 100$) in a double-blinded manner and expressed as mean ± s.d., $n=3$, per field view. *$P<0.01$.

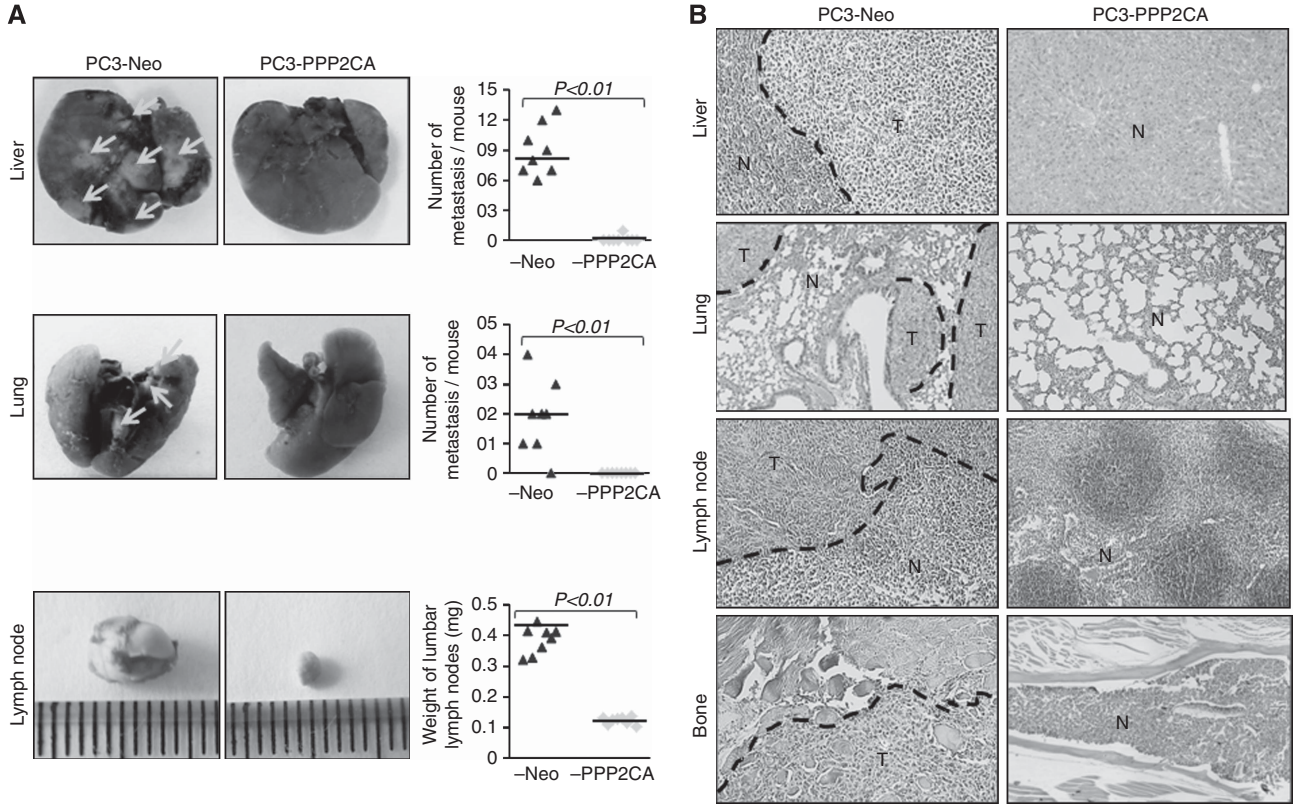


Figure 6. *PPP2CA* restoration decreases incidence of metastasis. (**A**) Liver (upper), lungs (middle) and lumber lymph nodes (lower) from mice were excised out and fixed in Bouin's solution and number of metastatic colonies were counted (in case of liver and lungs) and total mass of all the lumber lymph nodes was taken. Data presented as number of metastatic modules per mouse in case of liver and lungs and, total weight of the lumber lymph nodes per mouse. Dark line indicates the median value of the group. (**B**) Haematoxylin and eosin staining of liver, lung, lymph node and bone sections from control and *PPP2CA*-overexpressing group. Mice group injected with PC3-Neo cells show metastatic deposits of cancer cells in liver, lung, lymph node and bone, whereas *PPP2CA*-overexpressing group had no gross evidence of deposits of cancer cells. Abbreviations: N = normal; T = tumour.

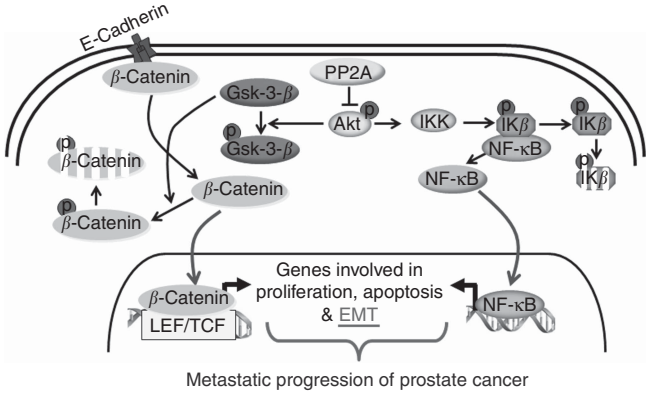


Figure 7. Diagrammatic representation of molecular pathways targeted by PP2A to suppress metastatic progression of PCa. Restoration of PP2A activity inhibits Akt phosphorylation, which then suppresses β-catenin and NF-κB through activation of GSK3β and inhibition of IKK, respectively. Inactivation of β-catenin and NF-κB leads to reversal of EMT and attenuation of tumour cell aggressiveness.

inactivation of GSK-3β leads to stabilisation of β-catenin, which subsequently enters the nucleus and promotes the expression of EMT-associated genes (Moon *et al*, 2004). On the other hand, activation of IKKβ leads to phosphorylation of Ikβ-α, a protein that keeps NK-κB sequestered in the cytoplasm (Gilmore, 2006). Phosphorylation of Ikβ-α induces its degradation and release of NF-κB, which then translocates into the nucleus to induce gene expression (Gilmore, 2006). Our data using active β-catenin and IKKβ mutants provide convincing evidence to suggest the participation of both β-catenin and NF-κB in *PPP2CA*-mediated regulation of EMT and malignant properties in PCa cells.

Accumulating evidence suggests that PP2A can regulate multiple signalling pathways involved in cancer pathogenesis (Janssens and Goris, 2001; Perrotti and Neviani, 2013). In an earlier study, we also demonstrated that downmodulation of PP2A led to the activation of Akt and ERK, which sustained the growth of prostate tumour cells in androgen-depleted media (Bhardwaj *et al*, 2011). These signalling nodes were also involved in partial activation of androgen receptor signalling in a ligand-independent manner (Bhardwaj *et al*, 2011). In this study, our data reveal a role of PP2A in negative regulation of β-catenin and NF-κB through Akt inactivation. Akt-mediated regulation of NF-κB through IKK has been shown by others as well (Dan *et al*, 2008). Furthermore, a recent report suggested that carnosic acid induced growth inhibition in PCa cells involved PP2A-mediated suppression of Akt/IKK/NF-κB pro-survival signalling (Kar *et al*, 2012). In additional studies, PP2A has been shown to regulate the activity of β-catenin. Seeling *et al* (1999) reported that the regulatory subunit of PP2A (PP2A-B) interfered with the formation of APC/Axin/Gsk3β complex, an inhibitory complex of β-catenin, and thus regulated the stabilisation and nuclear translocation of β-catenin (Seeling *et al*, 1999). In addition, based on the study performed in *Drosophila*, it was proposed that PP2A55α subunit of PP2A directly interacts with β-catenin and subsequently promotes its phosphorylation and degradation (Zhang *et al*, 2009). Findings

from our work suggest that PP2A-mediated β-catenin suppression in PCa cells is mediated through GSK-3β, which remains activated because of suppressive effect of PP2A on Akt. Similarly, Akt-mediated activation of NF-κB through IKK is also lost, when *PPP2CA* expression is restored in PCa cells. These findings are suggestive of a context-dependent impact of PP2A on signalling networks, which may underlie its functional diversity in biological processes.

In summary, our findings have provided compelling support for a tumour-suppressive role of PP2A in PCa and suggest that it likely serves as a mechanistic link between castration-resistance and aggressive tumour phenotypes. In light of other recent observations, it appears that dysregulation of PP2A through altered expression of its catalytic or regulatory subunits may be a common phenomenon in advanced PCa. On these bases, it can be suggested that PP2A signalling is a key regulator of PCa progression and metastasis and its targeting may be useful in preventing the disease progression and/or treatment of the disease that has already advanced.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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