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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 (PP2AC α), is downregulated in CR PCa. The level of PP2AC α 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- κ B 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.					
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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 (PP2A α), is downregulated in CR PCa (2). The level of PP2A α 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 β) in PCa (3). PP2A α and PP2A β share 97% identity and are ubiquitously expressed; however, PP2A α is about 10 times more abundant than PP2A β (4). PP2A α/β is a well conserved subunit of PP2A serine/threonine phosphatases, and the *in vivo* activity of PP2A is provided by related complexes that exist either as 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.

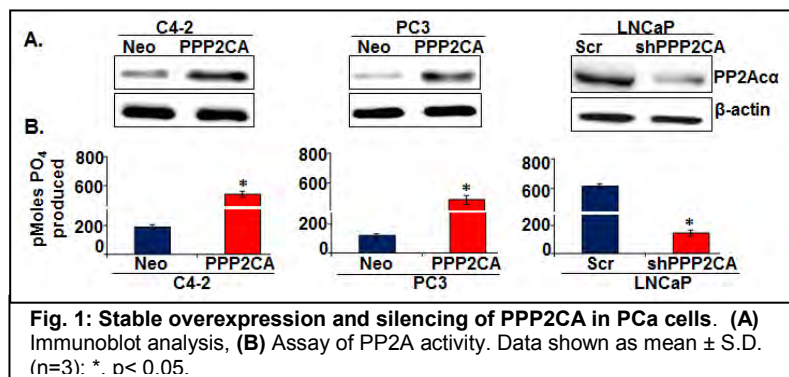
Aim 2: Define the molecular pathways that are responsive for the changes in PP2A signaling and establish their association with observed phenotype.

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 PP2A α .

We are working with three prostate cancer cell lines: LNCaP (castration- sensitive; AR positive; high PP2A α expression), C4-2 (castration-resistant; AR positive; low PP2A α expression), and PC3 (castration-resistant, AR negative; low PP2A α 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-

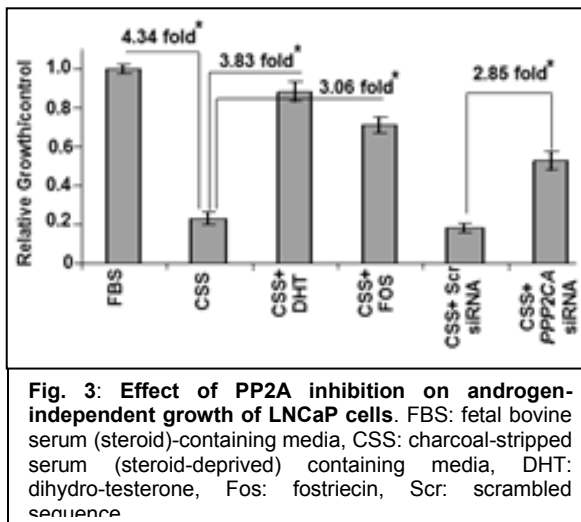
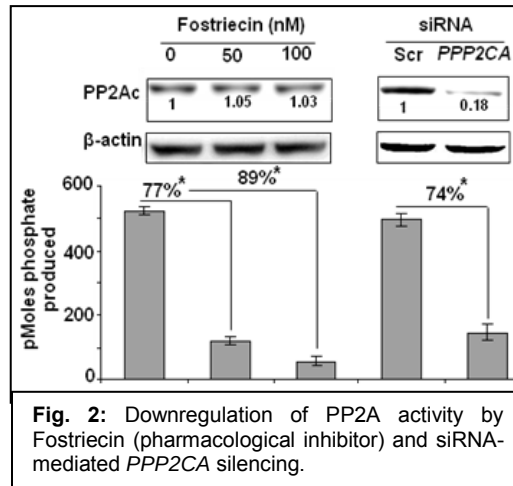


knockdown clones, respectively) along with their respective control transfectants. Later, these cells were characterized for the PP2A α expression and activity by immunoblot and malachite green based assay, respectively. Data demonstrate that expression and activity of PP2A α both are upregulated in 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.

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 fostriecin 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.

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). In accordance with previously published reports (6;7), our data showed arrest of LNCaP cells in G₀/G₁ phase of cell cycle under steroid-reduced condition, an effect that was abrogated upon treatment with DHT (1 nM) (Figure 4). 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 G₂/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 G₂/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

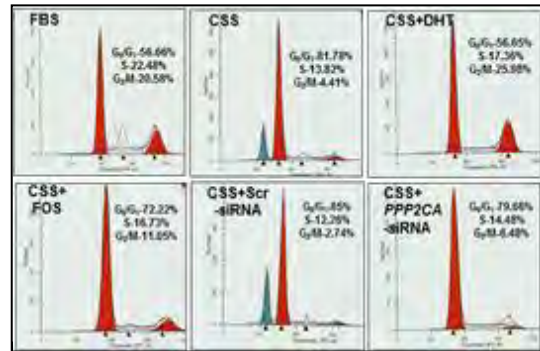


Fig. 4: Effect of PP2A inhibition on relieving steroid-deprivation-induced cell cycle arrest. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydrotestosterone, Fos: fostriecin, Scr: scrambled sequence.

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-deprivation. Our data thus demonstrate that PP2A inhibition supports the growth of LNCaP cells under androgen-depleted condition by preventing cell cycle arrest and apoptosis.

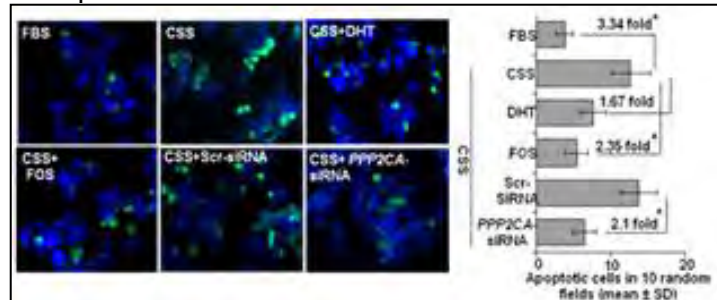


Fig. 5: PP2A inhibition suppresses steroid-deprivation-induced apoptosis. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydro-testosterone, Fos: fostriecin, Scr: scrambled sequence.

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 ($\sim 34\%$) in regular media, whereas in steroid-deprived media, ceramide treatment showed even more potent effect ($\sim 71\%$ 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.

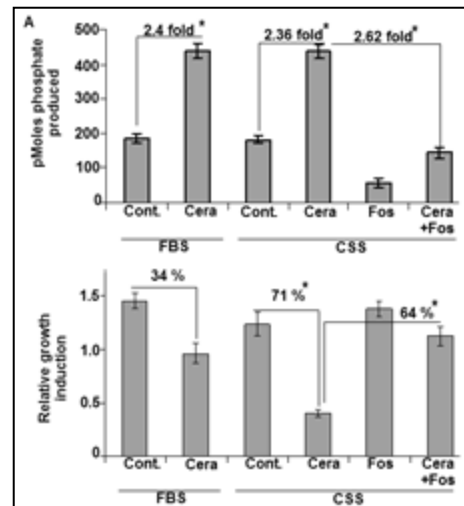


Fig. 6: A. Ceramide induces PP2A activity in C4-2 cells, which could be effectively suppressed by fostriecin. B. Ceramide-induced growth suppression of C4-2 cells and rescue effect of fostriecin.

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 (1×10^4) 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.

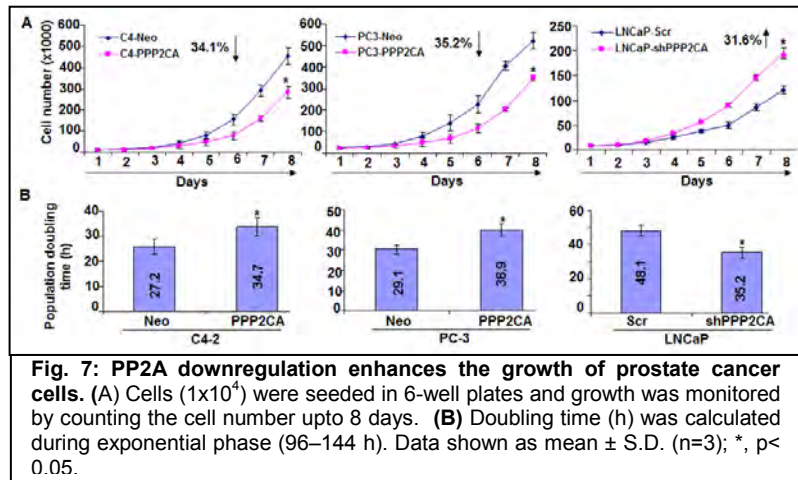


Fig. 7: PP2A downregulation enhances the growth of prostate cancer cells. (A) Cells (1×10^4) were seeded in 6-well plates and growth was monitored by counting the cell number upto 8 days. (B) Doubling time (h) was calculated during exponential phase (96–144 h). Data shown as mean $\pm$ S.D. (n=3); *, $p < 0.05$.

Above, we showed that downregulation of PP2A (by transient silencing or pharmacological 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 (1×10^3 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

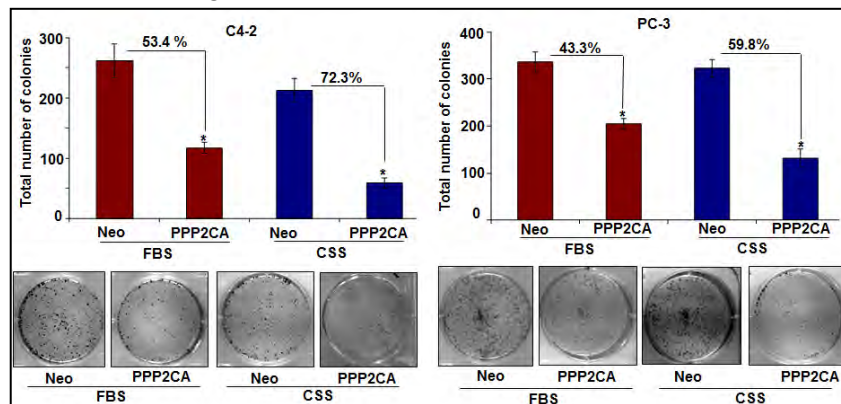


Fig. 8. Upregulation of PP2A suppresses androgen-depletion resistance of prostate cancer cells. Cells were seeded at low density (1×10^3 cells/well) in steroid-supplemented (FBS) and -reduced (CSS) media. After 2 weeks, colonies were stained with crystal violet, and visualized and photographed using imaging system. Bars represent mean $\pm$ S.D; n=3; *, $p < 0.05$.

PC-3-PPP2CA, respectively) under steroid-deprived condition (Figure 8). Thus, our data provide 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

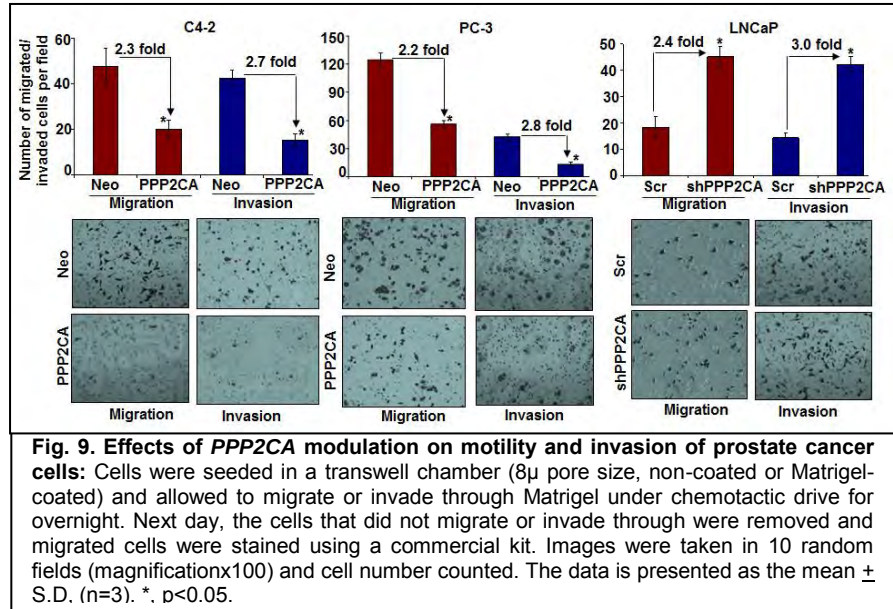


Fig. 9. Effects of *PPP2CA* modulation on motility and invasion of prostate cancer cells: Cells were seeded in a transwell chamber (8μ pore size, non-coated or Matrigel-coated) and allowed to migrate or invade through Matrigel under chemotactic drive for overnight. Next day, the cells that did not migrate 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.

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.

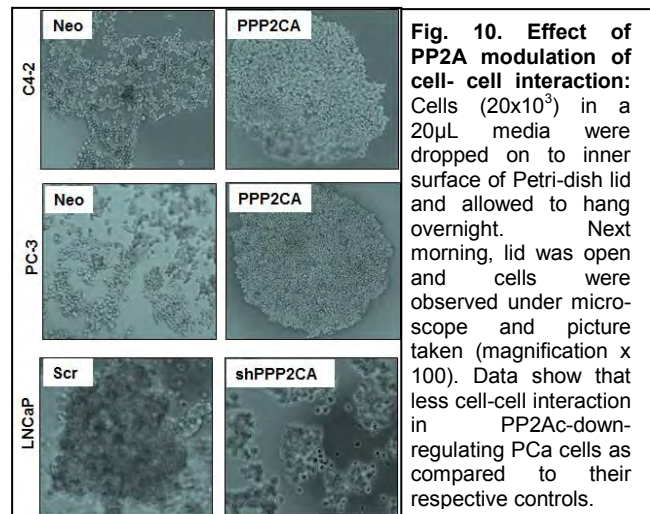


Fig. 10. Effect of PP2A modulation of cell-cell interaction: Cells (20×10^3) in a 20μL media were dropped on to inner surface of Petri-dish lid and allowed to hang overnight. Next morning, lid was open and cells were observed under microscope and picture taken (magnification x 100). Data show that less 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-knockdown (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).

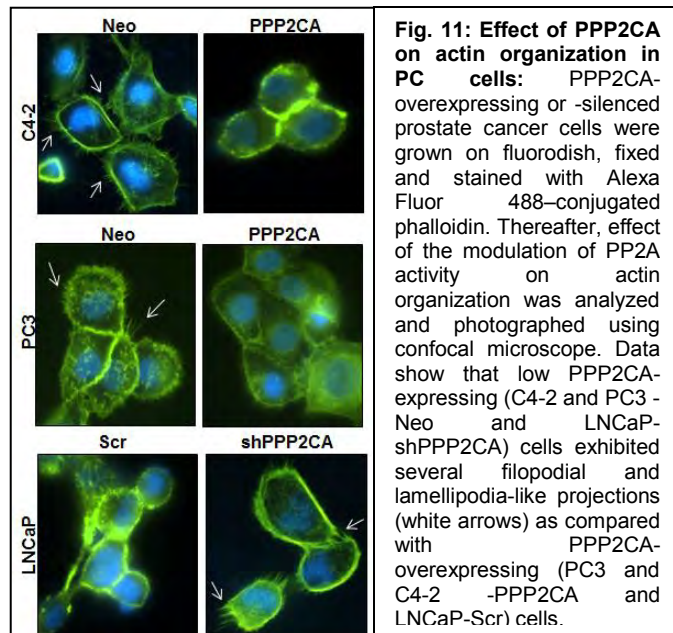


Fig. 11: Effect of PPP2CA on actin organization in PC cells: PPP2CA-overexpressing or -silenced prostate cancer cells were grown on fluorodish, fixed and stained with Alexa Fluor 488-conjugated phalloidin. Thereafter, effect of the modulation of PP2A activity on actin organization was analyzed and photographed 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 from PC3-PPP2CA group are significantly smaller as compared to mice group injected with the control (PC3-Neo) cells (Figure 13 A). 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, we performed immunohistochemistry (IHC) analysis on paraffin-embedded tissue sections to examine PP2A expression and proliferative and apoptotic markers. Our IHC data show intense PP2Ac staining in tissue section of PC3-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

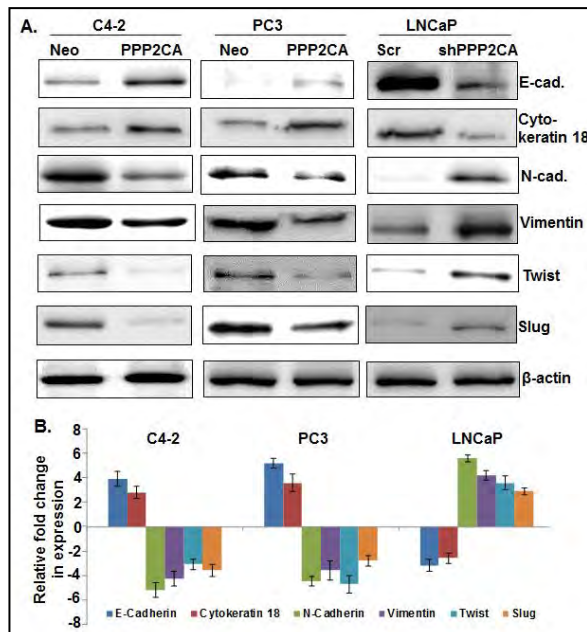
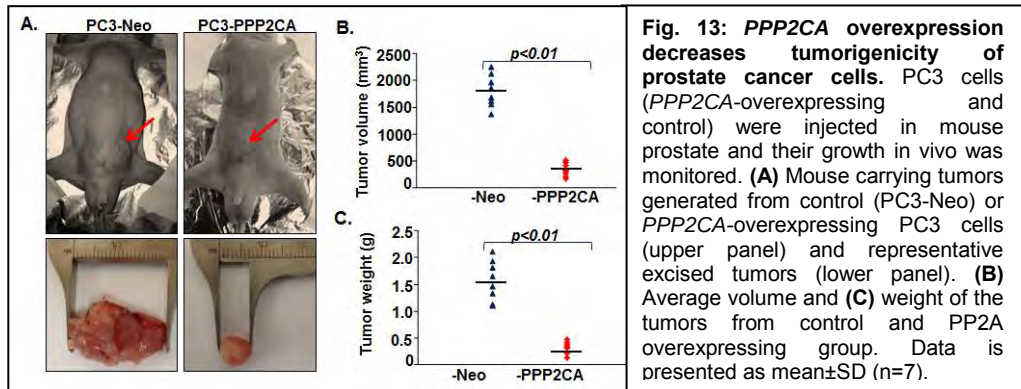
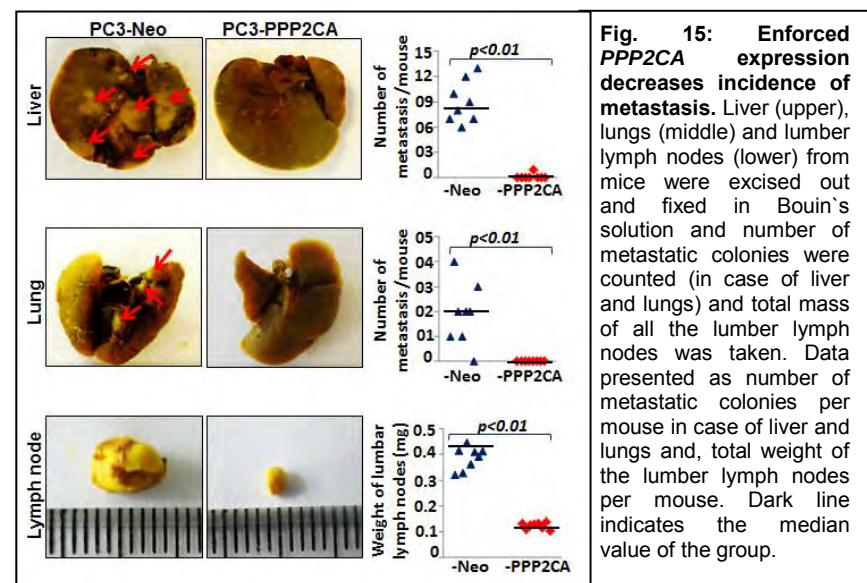
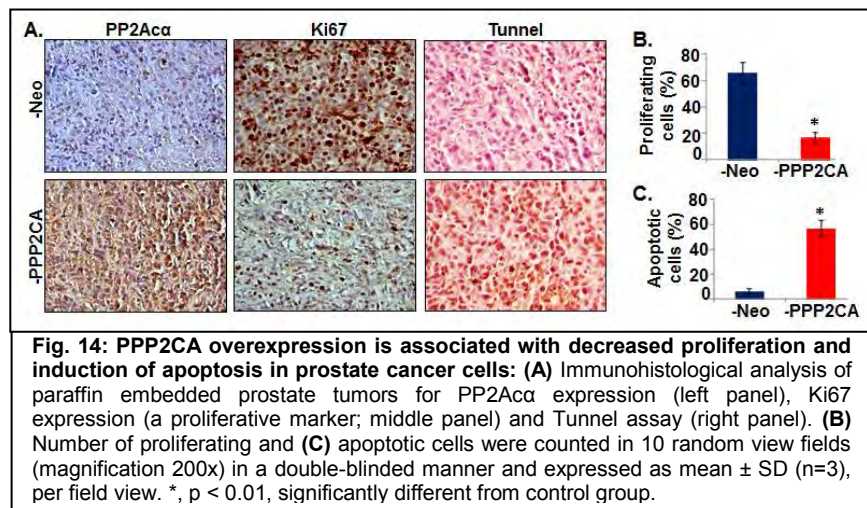


Fig. 12: Loss of PPP2CA favors epithelial to mesenchymal transition in prostate cancer cells. Expression of various epithelial (E-cadherin and cytokeratin-18) and mesenchymal (N-cadherin, Vimentin, Slug and Twist) markers at (A) protein and (B) transcript level was examined by immunoblot and real-time 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 $\pm$ 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.

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).



To analyze the effects of PPP2CA downregulation on prostate cancer metastasis, distinct organs (liver, lungs, lumbar 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). Altogether, our data provide strong evidence of role of PP2A downregulation in the progression and metastasis of prostate cancer.



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

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 phosphoform-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

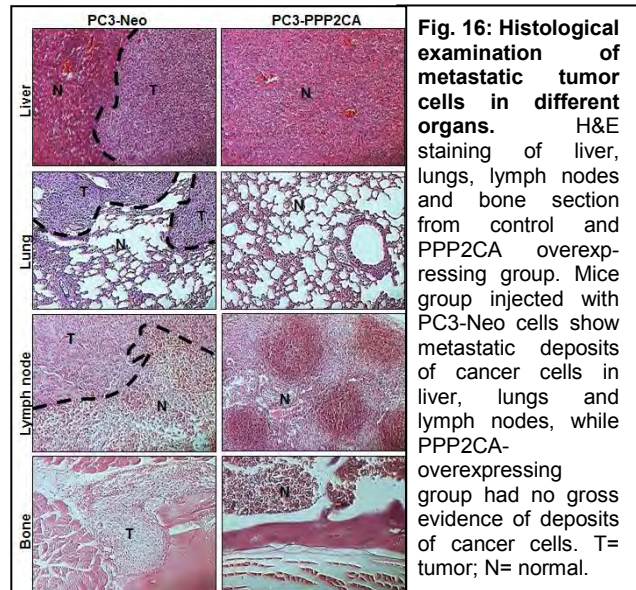


Fig. 16: Histological examination of metastatic tumor cells in different organs. H&E staining of liver, lungs, lymph nodes and bone section from control and PPP2CA overexpressing group. Mice group injected with PC3-Neo cells show metastatic deposits of cancer cells in liver, lungs and lymph nodes, while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

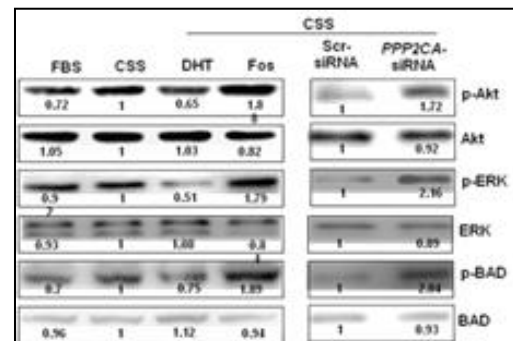


Fig. 17: Effect of PP2A inhibition on Akt and ERK signaling pathways and subsequent inactivating phosphorylation of BAD. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydro-testosterone, Fos: fostriecin, Scr: scrambled sequence.

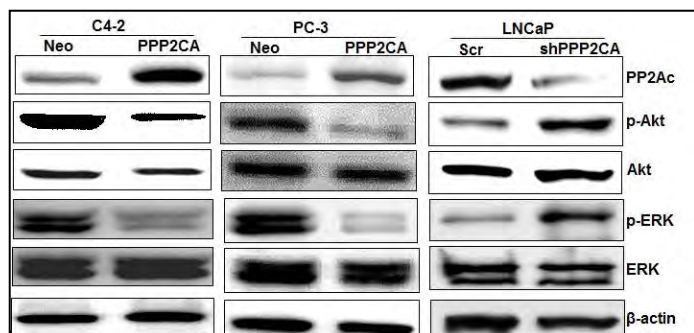
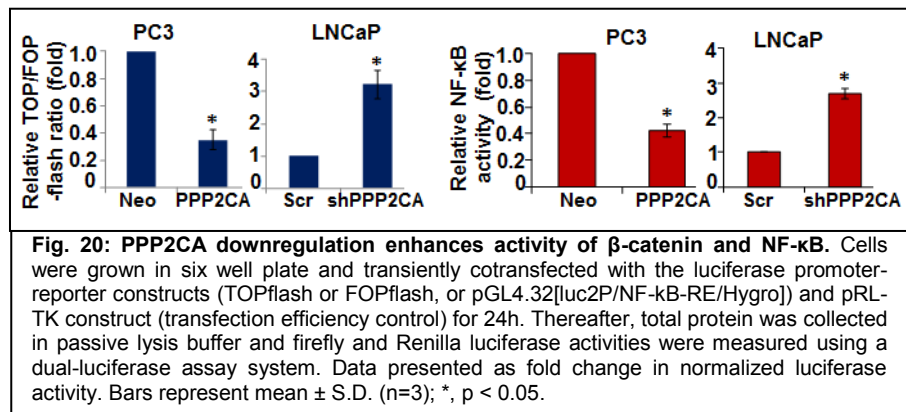
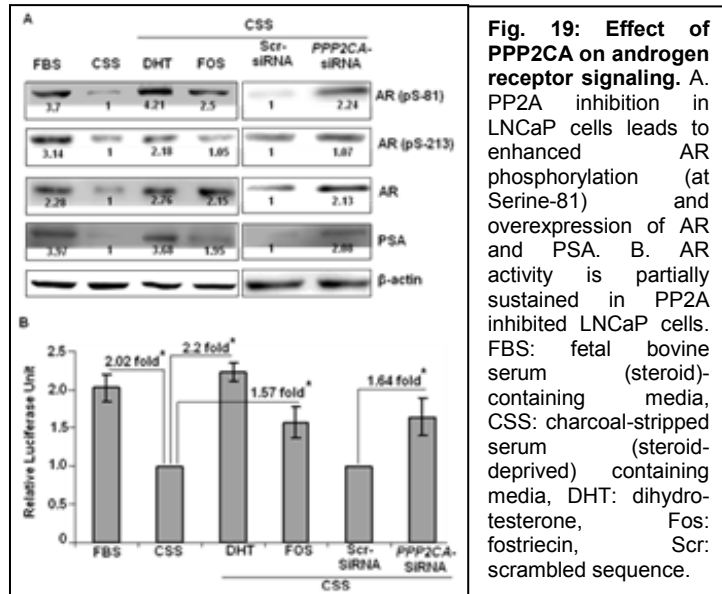


Fig. 18: PP2A decreases phosphorylation of Akt and ERK. Total protein was isolated and effect of PP2A modulation on Akt and ERK activation was examined by immunoblot assay. β-actin was used as internal control.

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 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).

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

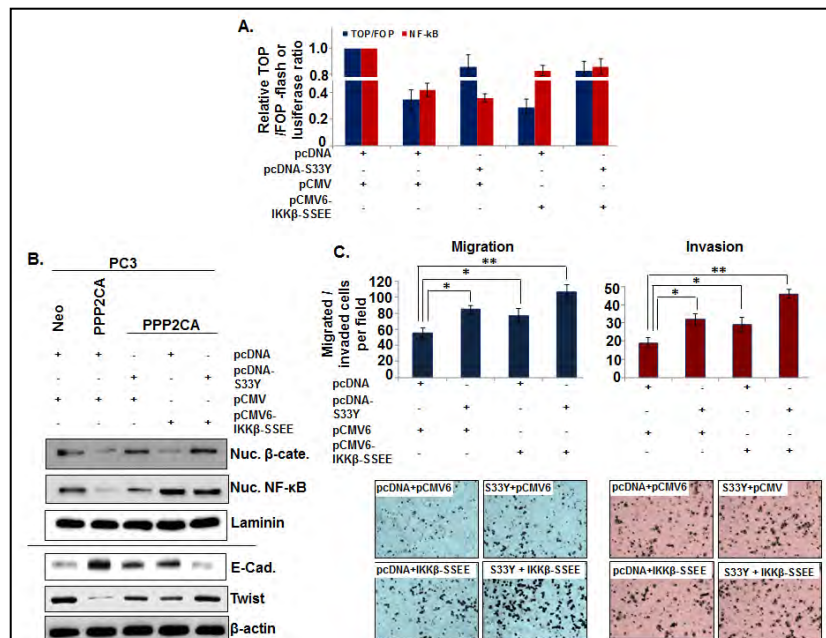
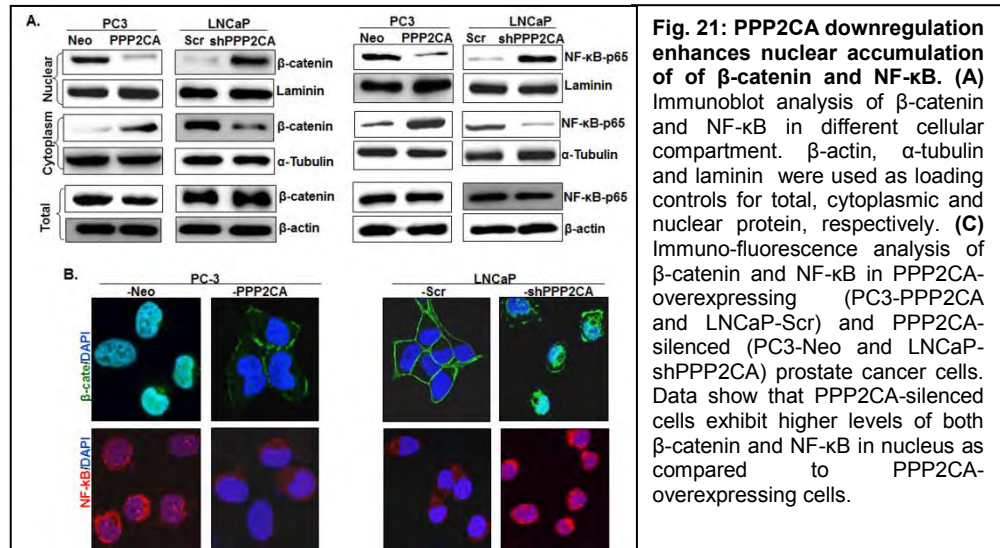
growth of LNCaP cells under androgen-deprived condition. These findings suggest that the inhibition of PP2A leads to the activation of Akt and ERK, which supports androgen-independent growth of LNCaP cells in AR-dependent (through partial activation) and – independent manners (data not shown, see appendix). 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, 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).



To explore mechanistic basis of altered expression of EMT markers, we focused on β -catenin and NF- κ B, which have earlier been shown to be aberrantly activated in PCa and implicated in transcriptional regulation of EMT markers (17-19). Our data from

promoter reporter assays show increased transcriptional activities of both TCF/LEF/ β -catenin and NF- κ B 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- κ B 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 visualize the precise sub cellular localization of β -catenin and NF- κ B, we performed immunofluorescence assay. Similar to immunoblot data, our immunofluorescence data also

revealed the association of PPP2CA downregulation with increased β -catenin and NF- κ B



localization in PCa cells (Figure 21B). Next, to confirm the role of β -catenin and NF- κ B in PPP2CA downregulation-induced EMT. PC-3-PPP2CA cells (with decreased β -catenin and NF- κ B activity) were transfected with constitutive active β -catenin and IKK β mutants (to activate β -catenin and NF- κ B, respectively). Thereafter, effect on transcriptional activity of TCF/LEF/ β -catenin and NF- κ B responsive promoters, EMT markers and aggressive behavior of PCa cells was examined. Our data show that transfection of active β -catenin and IKK β mutants specifically block the inhibitory effect of PPP2CA on β -catenin and NF- κ B, 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- κ B 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- κ B are cooperatively involved in PPP2CA-mediated inhibition of malignant properties in PCa cells.

As we observed that PPP2CA inhibits malignant properties of PCa via inactivation of β -catenin or NF- κ B. In addition negative regulation of Akt, an upstream regulator of both β -catenin and NF- κ B 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- κ B. 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- κ B was examined. Our data show that inhibitory effect of PPP2CA overexpression on transcriptional activity of TCF/LEF/ β -catenin and NF- κ B 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- κ B 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- κ B in PCa cells. To delineate the mechanism(s) involved in the Akt-mediated regulation

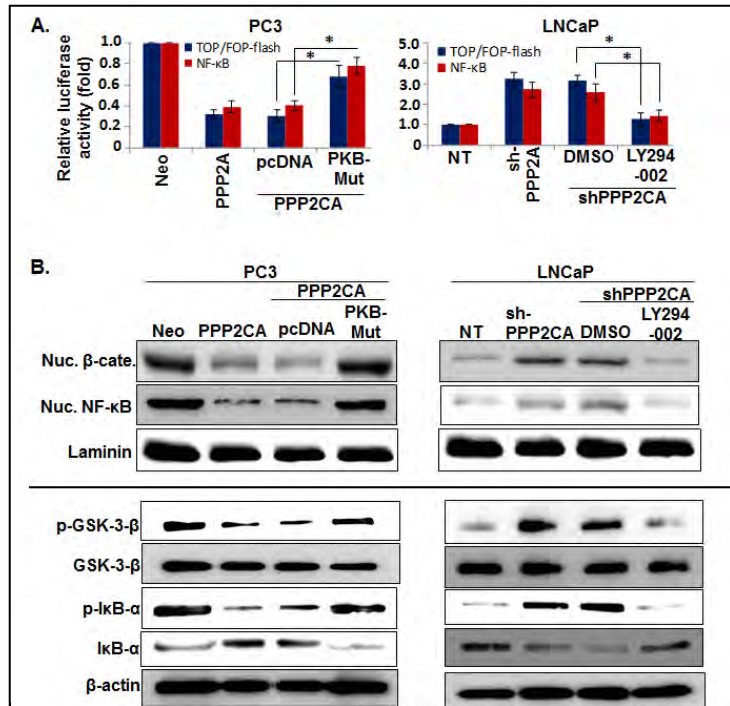
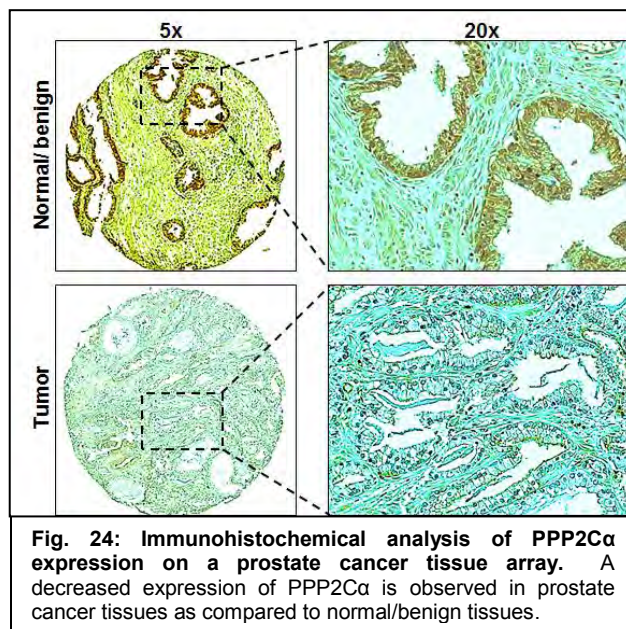


Figure 23: Role of Akt in PPP2CA overexpression-mediated suppression of β -catenin and NF- κ B. (A) PC3-PPP2CA cells were transiently transfected with constitutively active PKB mutant (to activate Akt) or control plasmid for 24 h, and LNCaP-shPPP2CA cells were treated with PI3K/Akt inhibitor (LY294002; to inhibit Akt) for 1 h. After respective transfection or treatment, cells were co-transfected with TOPflash/ FOPflash/ NF- κ B luciferase promoter-reporter constructs along with control plasmids for 24 h and luciferase activities were measured as described previously. Bars represents mean $\pm$ 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- κ B (in nuclear protein; upper panel) and on p-Gsk3- β /Gsk3- β and p-I κ B- α /I κ B- 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.

of β -catenin and NF- κ B in PCa cells, we analyzed effect on their biological inhibitors i.e. Gsk3- β and I κ B- 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 I κ B- α level, which was associated with a concomitant decrease in its phosphorylation, thus indicating the stabilization of I κ B- α after PPP2CA overexpression (Figure 23B; lower panel). Moreover, our data show that effects of PPP2CA alteration on Gsk3- β and I κ B- 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- κ B through Gsk3- β and I κ B- α , respectively.

Task 4: To examine the expression, localization and/or activation profiles of PP2Ac, AR, Akt and ERK in human prostate cancer.

To determine the clinical significance of our experimental findings, we proposed to examine the expression and localization of PP2Ac and its targets such as Akt, Erk1/2 and AR in clinical specimens of normal, primary and metastatic PCa by immunohistochemical (IHC) analysis. We have standardized IHC protocol for PP2Ac α and examined its expression in clinical samples using PCa tissue microarray [containing cancerous tissues (n=32) along with corresponding normal (n=32) tissues]. Our analysis showed an overall downregulation of PP2Ac α in cancer tissues as compared to the normal/benign tumor tissues (Figure 24).



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- κ B pathway.
- We have also developed *in vivo* experimental support for a suppressor role of PP2A in prostate cancer progression using orthotopic mouse model.

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 9th-12th (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 2nd-6th (2011).
- We published a manuscript “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). **(Manuscript near submission).**

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 the advanced disease.

ONGOING WORK:

- 1) Finalizing the manuscript for submission.
- 2) Conducting in situ expression analysis of pAkt, Akt, p-ERK, ERK, p-AR and AR in prostate cancer tissues through immunohistochemical (IHC) assay.
- 3) Pathological examination of the stained sections for assessment of staining intensity and incidence (extent of tissue stained).
- 4) Statistical analysis of the data obtained from IHC study for the comparison of expression of different proteins in paired tissue samples (benign and malignant tissue) and their correlation with different TNM stages (I-IV) and Gleason grades of prostate cancer.
- 5) Writing another manuscript for publication of IHC data.

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Mol Cancer Ther. 2011 May;10(5):720-31. doi: 10.1158/1535-7163.MCT-10-1096. Epub 2011 Mar 10.

Modulation of protein phosphatase 2A activity alters androgen-independent growth of prostate cancer cells: therapeutic implications.

[Bhardwaj A](#), [Singh S](#), [Srivastava SK](#), [Honkanen RE](#), [Reed E](#), [Singh AP](#).

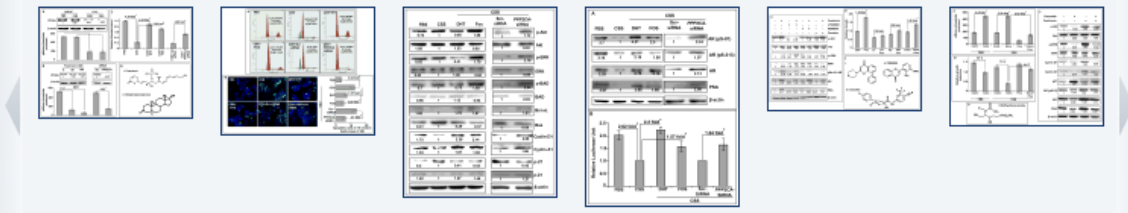
Department of Oncologic Sciences Mitchell Cancer Institute, University of South Alabama 1660 Springhill Avenue, Mobile, AL 36604, USA.

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.

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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 Bhardwaj¹, Seema Singh¹, Sanjeev K. Srivastava¹, Richard E. Honkanen², Eddie Reed¹, Ajay P. Singh¹, ¹University of South Alabama Mitchell Cancer Institute, Mobile, AL; ²Biochemistry 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 (PP2A α), 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.

Poster Presentations - Cytoplasmic Oncogenes and Tumor Suppressors

Abstract 3991: Protein phosphatase 2A (PP2A) downregulation is associated with androgen-independent and aggressive phenotypes in prostate cancer

Arun Bhardwaj¹, Sanjeev K. Srivastava¹, Seema Singh¹, Sumit Arora¹, Richard E. Honkanen², William E. Grizzle³, Eddie Reed¹, and Ajay P. Singh¹

¹Department of Oncologic Sciences, Mitchell Cancer Institute, University of South Alabama, Mobile, AL

²Department of Biochemistry and Molecular Biology, University of South Alabama, Mobile, AL

³Department of Pathology, University of Alabama at Birmingham, Birmingham, AL

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.

Citation Format: {Authors}. {Abstract title} [abstract]. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2012;72(8 Suppl):Abstract nr 3991. doi:1538-7445.AM2012-3991

doi: 10.1158/1538-7445.AM2012-3991
Cancer Res April 15, 2012; 72(8 Supplement): 3991

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Abstract Number: 3581

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-C, Poster Section 17

Poster Board Number: 2

Author Block: Arun Bhardwaj¹, Seema Singh¹, Sanjeev K. Srivastava¹, Sumit Arora¹, Stephen J. Hyde¹, Richard E. Honkanen², William E. Grizzle³, Ajay P. Singh¹, ¹Mitchell Cancer Institute, University of South Alabama, Mobile, AL; ²Department of Biochemistry and Molecular Biology, University of South Alabama, Mobile, AL; ³Department 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 CRPCs. 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 IκB-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.