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Award Number:
W81XWH-09-1-0137

TITLE:
Protein Phosphatase 2A signaling in human prostate cancer

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REPORT DATE: June 2011

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

x Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
06-JUN-2011	Annual	May 07, 2010- May 06, 2011

4. TITLE AND SUBTITLE

Protein Phosphatase 2A signaling in human prostate cancer

5a. CONTRACT NUMBER
W81XWH-09-1-0137

5b. GRANT NUMBER
PC080496

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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Ajay Singh, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of South
Alabama
Mobile, AL 36688

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US Army Medical Research and
Materiel Command, Fort
Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

To determine the role of PP2A, a serine/threonine phosphatase, in human prostate cancer (PCa) progression, we have conducted a series of experiments. Specifically, we have investigated the effect of PP2A activity modulation on androgen-independent (AI) growth of prostate cancer cells and defined underlying mechanisms. Our data show that the downregulation of *PP2A* activity by pharmacological inhibition or siRNA-mediated *PPP2CA* silencing sustains the growth of AD PCa (LNCaP) 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. These findings are further supported by the effect of ceramide, a potent PP2A activator, on AI PCa (C4-2) prostate cancer cells. Ceramide suppresses AI growth of C4-2 cells, which could be rescued by pre-treatment with PP2A inhibitor. Altogether, these initial findings identify a novel PP2A-mediated signaling mechanism that support AI growth of prostate cancer cells.

15. SUBJECT TERMS
PPP2CA, protein phosphatase 2A, androgen-independent prostate cancer

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	25	**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U			

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INTRODUCTION

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 pathological conditions, including malignant transformation. The **overall objective of this research** is to investigate the role of protein phosphatase 2A (PP2A) signaling in human prostate cancer (PC). Supporting preliminary evidence include our demonstration of downregulated expression of *PPP2CA* (PP2Acα, catalytic subunit of PP2A) in LNCaP-C81 (androgen independent) cells as compared to LNCaP-C33 (androgen-dependent) cells (Singh *et al*., 2008). A similar observation was also made in clinical samples by immunohistochemical analysis (Singh *et al*., 2008). Furthermore, data mining of 'Oncomine cancer profiling database' (www.oncomine.org) also indicated a progressive loss (Normal>Primary>Metastatic) of PP2Ac in prostate cancer. Another study also reported the downregulated expression of β-isoform of PP2A catalytic subunit ($PP2A_{C\beta}$) in PCa (Prowatke *et al*., 2007). $PP2A_{C\alpha}$ and $PP2A_{C\beta}$ share 97% identity and are ubiquitously expressed; however, $PP2A_{C\alpha}$ is about 10 times more abundant than $PP2A_{C\beta}$ (Khew-Goodall and Hemmings, 1988). $PP2A_{C\alpha/\beta}$ 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 (Janssens and Goris, 2001). All these studies strongly suggested a role of PP2A in prostate cancer and led us to hypothesize that *dysregulation of PP2A plays an important role in the progression of prostate cancer*.

To test our hypothesis, we have proposed three specific aims:

1) Investigate the biological role of PP2Ac in growth and malignant properties of the prostate cancer cells.
2) Delineate the molecular pathways that are responsive for the changes in PP2A signaling and establish their association with observed phenotype.
3) Determine the expression and/or activation profiles of PP2Ac, Erk and Akt in human prostate cancer.

We expect that the proposed investigations will provide experimental evidence for a role of PP2A signaling in PC progression and may aid in designing of novel therapeutic approach(es) against PC to improve the patient's survival.

BODY:

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

We currently are working with three prostate cancer cell lines: LNCaP (androgen-dependent; AR positive; high PP2Acα expression), C4-2 (androgen-independent; AR positive; low PP2Acα expression), and PC3 (androgen-independent, AR negative; low PP2Acα expression). After confirming the efficacy of *PPP2CA* expression plasmid (pCMV6-PPP2CA) in transient assays, we stably transfected the C4-2 and PC-3 cells to generate *PPP2CA*- overexpressing C4-2 (C4-2-*PPP2CA*) and PC-3 (PC-3-PPP2CA) sub-lines (from pooled *PPP2CA*-overexpressing clones) along with their control transfectants (C4-2-Neo and PC-3-Neo). These cells have been characterized for $PP2A_{C\alpha}$ expression and PP2A activity (**Figure 1**). $PP2A_{C\alpha}$ expression is upregulated in both C4-2-PPP2CA (≥ 3.8 fold) and PC-3-PPP2CA (≥ 4.2 fold) cells as compared

to their respective controls. Similarly, activity of PP2A is also increased in C4-2-PPP2CA (≥ 2.9 fold) and PC-3-PPP2CA (≥ 3.3 fold) cells as compared to their respective controls.

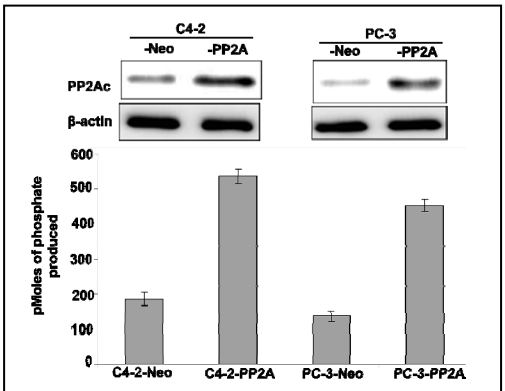

Fig. 1: Ectopic overexpression of *PPP2CA* and resulting enhanced PP2A activity in androgen-independent C4-2 (AR positive) and PC3 (AR negative cell lines.

We are, however, still working to generate stable PPP2CA-knockdown clones through gene silencing in LNCaP cells.

Task 2: To examine the effect of PP2A 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 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.

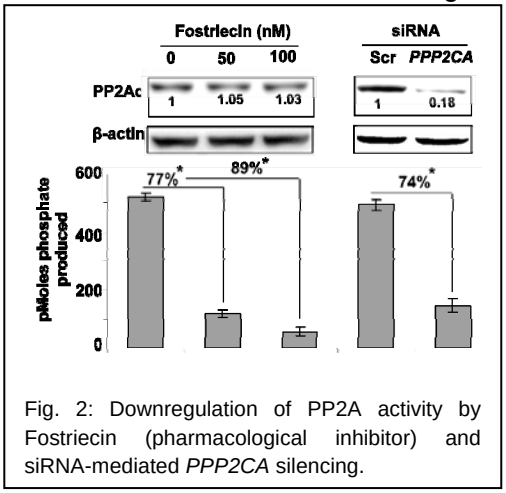

Fig. 2: Downregulation of PP2A activity by Fostriecin (pharmacological inhibitor) and siRNA-mediated *PPP2CA* silencing.

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

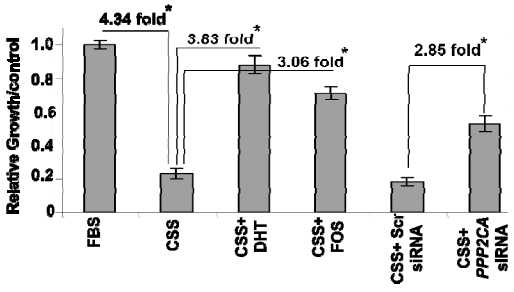

Fig. 3: Effect of PP2A inhibition on androgen-independent growth of LNCaP cells. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydro-testerone, Fos: fostriecin, Scr: scrambled sequence.

and flow cytometry (**Figure 4).** In accordance with previously published reports (Eto *et al*., 2003; Kazi *et al*., 2002), 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**). 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-deprdemonstrate that PP2A inhibition supports the growth of LNCaP cells under androgen-depleted condition by preventing cell cycle arrest and apoptosis.

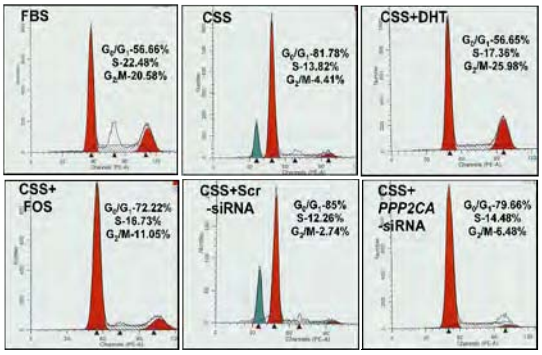

Fig. 4: Effect of PP2A inhibition on relieving steroid-deprivation-induced cell cycle arrest. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydrotesterone, Fos: fostriecin, Scr: scrambled sequence.

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 (Law and Rossie, 1995; Ruvolo *et al*., 1999) 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.

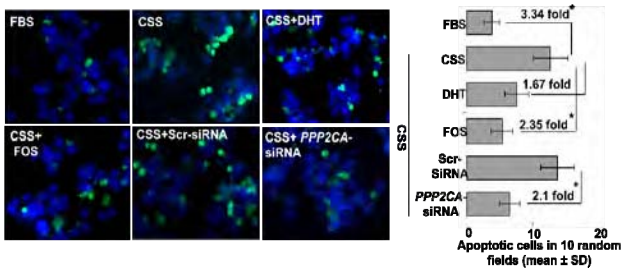

Fig. 5: PP2A inhibition suppresses steroid-deprivation-induced apoptosis. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydro-testerone, Fos: fostriecin, Scr: scrambled sequence.

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 (Janssens and Goris, 2001). 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 (Carson *et al*., 1999; Grethe and Porn-Ares, 2006; Murillo *et al*., 2001). 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 upon PP2A inhibition. Our immunoblot data with total and phospho-form-specific antibodies (**Figure 7**) 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.

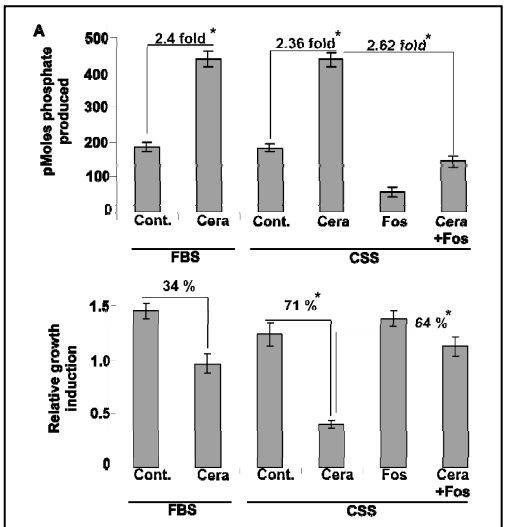

Fig. 6: A. Ceramide induces PP2A activity in C4-2 cells, which could be effectively suppressed by fostricin. B. Ceramide-induced growth suppression of C4-2 cells and rescue effect of fostriecin.

Androgen receptor (AR) plays important roles in both androgen-dependent and –independent growth of prostate cancer cells (Feldman and Feldman, 2001). It has been established that AR can maintain its transcriptional activity even under androgen-deprived condition through ligand-independent activation (Murillo *et al*., 2001). Notably, it has been shown earlier that both Akt and ERK can induce phosphorylation of AR at serine residues leading to its activation (Murillo *et al*., 2001; Shigemura *et al*., 2009). Therefore, we examined the effect of PP2A inhibition on the phosphorylation of AR in LNCaP cells under steroid-depleted condition (**Figure 8A**). 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 immunoblotting data also demonstrated an induced expression of AR and its target gene, PSA/KLK3 upon treatment with DHT or PP2A inhibition (**Figure 8A**). 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 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 8B**). Our data show a limited induction of AR activity in LNCaP cells treated with fostriecin (1.57 fold)

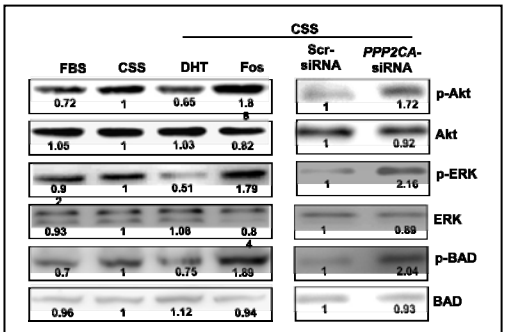

Fig. 7: Effect of PP2A inhibition on Akt and ERK signaling pathways and subsequent inactivating phosphorylation of BAD. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydro-testerone, Fos: fostriecin, Scr: scrambled sequence.

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.

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).

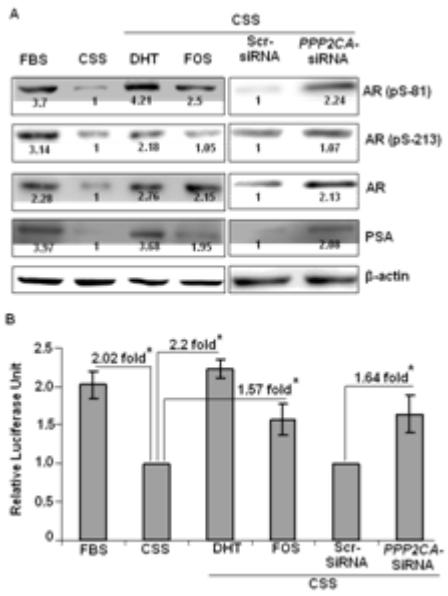

Fig. 8: A. PP2A inhibition in LNCaP cells leads to enhanced AR phosphorylation (at Serine-81) and overexpression of AR and PSA. B. AR activity is partially sustained in PP2A inhibited LNCaP cells. FBS: fetal bovine serum (steroid)-containing media, CSS: charcoal-stripped serum (steroid-deprived) containing media, DHT: dihydro-testerone, Fos: fostriecin, Scr: scrambled sequence.

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.

KEY RESEARCH ACCOMPLISHMENTS:

- We have established C4-2 and PC3 sublines exhibiting stable ectopic *PPP2CA* overexpression and enhanced PP2A activity.
- We have provided experimental evidence (*in vitro*) for a role of PP2A downregulation in androgen-independent growth 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.

REPORTABLE OUTCOMES

We submitted two abstracts and presented our findings in US Army/PCRP-sponsored IMPaCT-2011 (Innovative Minds in Prostate Cancer Today, March 2011) and AACR (American Association for Cancer Research, April 2011) meetings.

Our research findings have also been accepted for publication in "Molecular Cancer Therapeutics" journal:

CONCLUSION

Our research findings provide *in vitro* evidence and mechanistic insight into the role of PP2A in androgen-independent growth of prostate cancer cells. Subsequent *in vivo* studies and correlation of experimental findings in clinical specimen will further validate the functional and clinical significance of PP2A in prostate cancer progression and support its translational potential. Altogether, our data identify a novel mechanism underlying the androgen-independent progression of prostate cancer.

REFERENCES

Carson JP, Kulik GWeber MJ. (1999). Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3'-kinase and Akt/protein kinase B. *Cancer Res* **59:** 1449-1453.

Eto M, Bennouna J, Hunter OC, Hershberger PA, Kanto T, Johnson CS et al. (2003). C16 ceramide accumulates following androgen ablation in LNCaP prostate cancer cells. *Prostate* **57:** 66-79.

Feldman BJ and Feldman D. (2001). The development of androgen-independent prostate cancer. *Nat Rev Cancer* **1:** 34-45.

Grethe S and Porn-Ares MI. (2006). p38 MAPK regulates phosphorylation of Bad via PP2A-dependent suppression of the MEK1/2-ERK1/2 survival pathway in TNF-alpha induced endothelial apoptosis. *Cell Signal* **18:** 531-540.

Janssens V and Goris J. (2001). Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* **353:** 417-439.

Kazi A, Smith DM, Zhong QDou QP. (2002). Inhibition of bcl-x(l) phosphorylation by tea polyphenols or epigallocatechin-3-gallate is associated with prostate cancer cell apoptosis. *Mol Pharmacol* **62:** 765-771.

Khew-Goodall Y and Hemmings BA. (1988). Tissue-specific expression of mRNAs encoding alpha- and beta-catalytic subunits of protein phosphatase 2A. *FEBS Lett* **238:** 265-268.

Law B and Rossie S. (1995). The dimeric and catalytic subunit forms of protein phosphatase 2A from rat brain are stimulated by C2-ceramide. *J Biol Chem* **270:** 12808-12813.

Murillo H, Huang H, Schmidt LJ, Smith DITindall DJ. (2001). Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. *Endocrinology* **142:** 4795-4805.

Prowatke I, Devens F, Benner A, Grone EF, Mertens D, Grone HJ et al. (2007). Expression analysis of imbalanced genes in prostate carcinoma using tissue microarrays. *Br J Cancer* **96:** 82-88.

Ruvolo PP, Deng X, Ito T, Carr BKMay WS. (1999). Ceramide induces Bcl2 dephosphorylation via a mechanism involving mitochondrial PP2A. *J Biol Chem* **274:** 20296-20300.

Shigemura K, Isotani S, Wang R, Fujisawa M, Gotoh A, Marshall FF et al. (2009). Soluble factors derived from stroma activated androgen receptor phosphorylation in human prostate LNCaP cells: roles of ERK/MAP kinase. *Prostate* **69:** 949-955.

Singh AP, Bafna S, Chaudhary K, Venkatraman G, Smith L, Eudy JD et al. (2008). Genome-wide expression profiling reveals transcriptomic variation and perturbed gene networks in androgen-dependent and androgen-independent prostate cancer cells. *Cancer Lett* **259:** 28-38.

APPENDICES

Singh AP, Bhardwaj A, Singh S, and Srivastava SK. (**2011**) 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. “Innovative Minds in Prostate Cancer today (IMPaCT) meeting”, Orlando, Florida, March 9-12

Bhardwaj A, Singh S, Srivastava SK, Honkanen RE, and **Singh AP (2011)** Inhibition of protein phosphatase 2A supports androgen-independent growth of prostate cancer cells. “American Association for Cancer Research (AACR) 102nd Annual Meeting”, Orlando, Florida, April 2-6.

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

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

Ajay Pratap Singh, Arun Bhardwaj, Seema Singh, and Sanjeev Srivastava

Background and objectives: Clinical progression of prostate cancer is characterized by a transition from an androgen-dependent to an androgen-independent phenotype. Once the prostate cancer has recurred in androgen-independent form, it progresses to a highly aggressive disease with frequent metastasis and poses an increased risk of morbidity and death. Therefore, significant research has been carried out to identify novel targets in androgen-independent prostate cancer and understand the disease mechanisms. In an earlier study, we identified *PPP2CA*, which encodes for alpha-isoform of the protein phosphatase 2A (PP2A) catalytic subunit (PP2Ac), as one of the downregulated genes in androgen-independent prostate cancer cells. PP2A is a serine/threonine phosphatase and a potent tumor suppressor; however, its role in prostate cancer has not yet been determined. Our objective under this project is to demonstrate functional significance of downregulated *PPP2CA* expression in prostate cancer. We hypothesize that downregulated *PPP2CA* expression causes loss of PP2A activity, which, in turn, impacts multiple cell signaling pathways associated with prostate cancer proliferation, survival, aggressiveness and metastatic behavior.

Methods: To investigate the role of PP2A in prostate cancer, we inhibited its activity by utilizing specific pharmacological inhibitor and/or by silencing the expression of *PPP2CA* in androgen-dependent (AD) LNCaP cells. Cell growth was assessed by MTT assay. Apoptosis was determined by staining of the apoptotic cells by a fluorescently-labeled pan-caspase inhibitor. Expression and activation of Akt, ERK and androgen receptor (AR) was examined by immunoblotting with their normal and phospho-form specific antibodies. AR transcriptional activity was determined by promoter-reporter (luciferase) assay and by analyzing its target gene (KLK3) expression.

Results to date: An elevated expression of *PPP2CA* was observed in androgen-dependent LNCaP cells as compared to its androgen-independent derivative cell line, C4-2, that correlated with increased serine/threonine phosphatase activity. Downregulation of PPP2CA by siRNA-mediated silencing or treatment with fostriecin (a poent inhibitor of PP2A) sustained the growth of androgen-dependent LNCaP prostate cancer cells under androgen-deprived condition by potentiating survival. Immunoblot analysis revealed enhanced phosphorylation of ERK and Akt upon PP2A downregulation and an increased expression of androgen-receptor (AR) and its target gene, *KLK3*, encoding for prostate-specific antigen(PSA)/kallikrein-3. Enhanced transcriptional activity of AR-responsive promoter was confirmed by luciferase reporter assay.

Conclusions: Downregulation of PP2A permits prostate cancer cell growth under androgen-deprivation by promoting survival signaling plausibly through activation of Akt and Erk, and partially sustained androgen receptor signaling.

Impact: Elucidation of mechanism(s) underlying androgen-independent progression of prostate cancer will aid in the development of novel therapeutic strategies and/or better treatment planning.

INHIBITION OF PROTEIN PHOSPHATASE 2A SUPPORTS ANDROGEN-INDEPENDENT GROWTH OF PROSTATE CANCER CELLS

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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 (PP2A) catalytic subunit, 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 restoration of PP2A activity may represent an alternative therapeutic approach for the treatment of advanced androgen-independent prostate cancer.

Molecular Cancer Therapeutics

Highlights

April 2011 • Volume 10 • Number 5

Selected Articles from This Issue

hTERT Promotes Imatinib Resistance in CML

Deville *et al.*_____Page 711

Despite satisfactory remission rates, resistance to imatinib is an important issue for therapy of chronic myeloid leukemia. Deville and colleagues showed that the emergence of resistance occurred faster in cells overexpressing the catalytic subunit of the telomerase, showing that this enzyme represents an additional factor in the development of imatinib resistance. Furthermore, strategies targeting either telomerase expression or activity restored imatinib sensitivity in the resistant cells. Therefore, combining antitelomerase strategies to imatinib treatment represents an attractive approach to prevent the emergence of imatinib-resistant clones and increase the probability to eradicate the disease.

PP2A: A Novel Therapeutic Target in Prostate Cancer

Bhardwaj *et al.*_____Page 720

PP2A is a major serine/threonine phosphatase and a potent tumor suppressor; however, its role in prostate cancer has remained underexplored. Bhardwaj and colleagues have now shown that PP2A activity is inversely associated with androgen-independent growth of prostate cancer cells. Their data reveal a novel mechanism, whereby loss of PP2A-mediated checkpoints leads to the activation of Akt and ERK and partially sustains androgen-receptor signaling under steroid-deprived condition. Their findings offer potential therapeutic implications for targeting PP2A in castration-resistant prostate cancer.

Chemical Modulation of the Mitotic Checkpoint

Riffell *et al.*_____Page 839

Exposure of cells to microtubule-targeting cancer drugs such as paclitaxel causes mitotic arrest by activation of the mitotic checkpoint. Some cells can escape mitotic arrest by entering interphase without dividing, a process termed mitotic slippage. Riffell and colleagues examine mechanisms underlying mitotic slippage using two chemicals found to induce slippage. SU6656 and geraldol induced mitotic slippage through caspase-3-dependent degradation of the checkpoint kinase BubR1, thus permitting proteasome-dependent degradation of cyclin B1 and escape from drug-induced mitotic arrest. The identification of this pathway linking apoptosis with mitotic control may have implications for cancer therapy.

Monitoring Drug Efficacy in Hepatocellular Carcinoma

van Zijl *et al.*_____Page 850

The epithelial to mesenchymal transition (EMT) of malignant hepatocytes is a crucial event in hepatocellular carcinoma (HCC) progression and recurrence. In this study, van Zijl and colleagues established a novel and unique cellular EMT model of human HCC to identify molecular mechanisms and to assess therapeutic drug efficacy during liver carcinoma progression. Most remarkably, they found that the combined treatment with doxorubicin and sorafenib caused increased susceptibility of HCC cell types before and after EMT, resulting in enhanced drug efficacy. This model of EMT that reliably reflects human HCC progression is an invaluable tool in preclinical studies for the identification of molecular mechanisms underlying HCC progression, the pharmacological determination of dose-effect relationships and thus the efficacy of single and combined treatments with novel and currently used anti-cancer drugs, and the (re)-evaluation of drug target specificity and pleiotropic effects.

Published OnlineFirst March 10, 2011; DOI:10.1158/1535-7163.MCT-10-1096

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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doi: 10.1158/1535-7163.MCT-10-1096

Published OnlineFirst March 10, 2011; DOI:10.1158/1535-7163.MCT-10-1096

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.

Published OnlineFirst March 10, 2011; DOI:10.1158/1535-7163.MCT-10-1096

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

Published OnlineFirst March 10, 2011; DOI:10.1158/1535-7163.MCT-10-1096

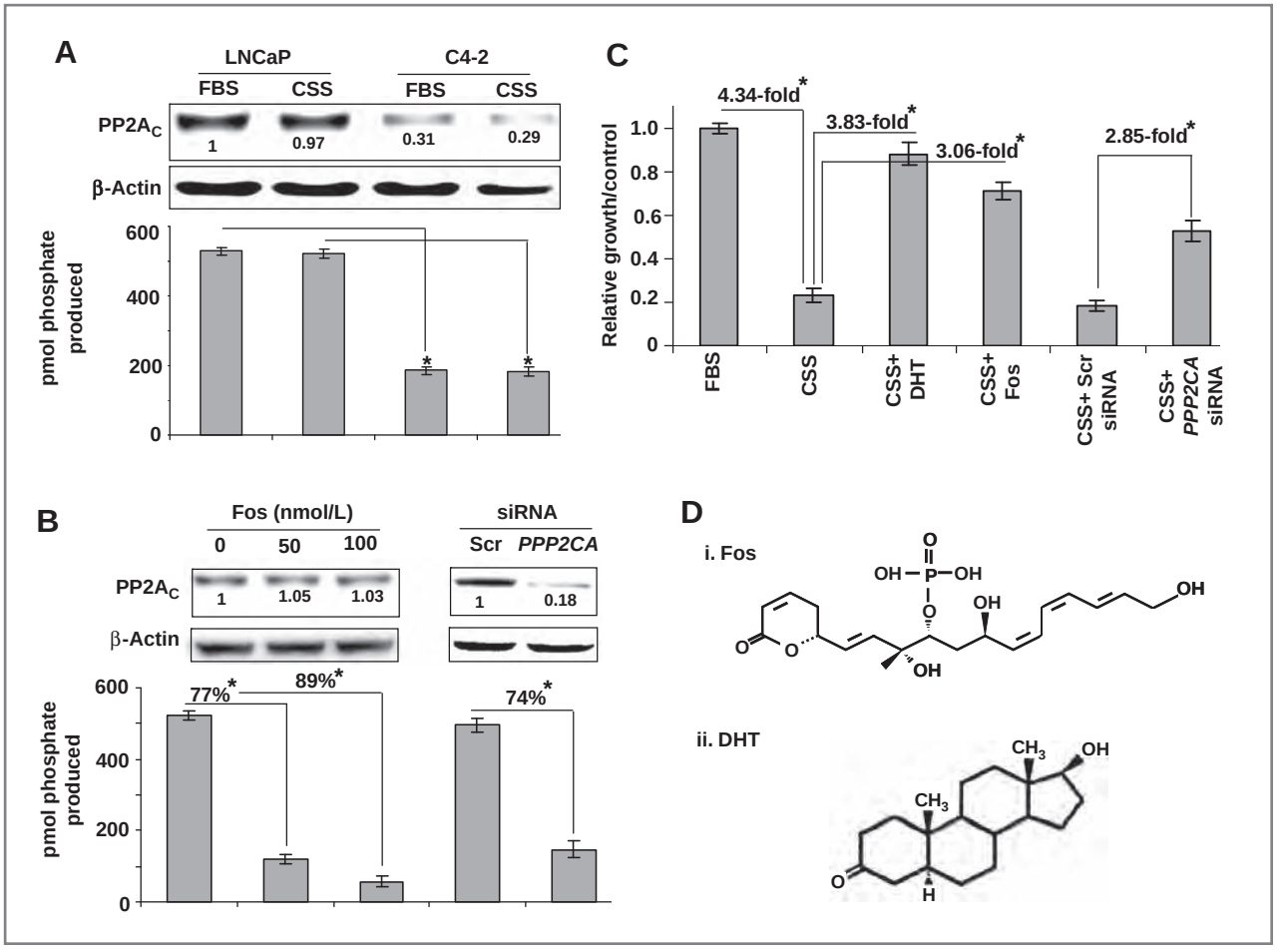

Figure 1. PP2A activity is downregulated in AI prostate cancer cells and its inhibition sustains the growth of AD prostate cancer cells under steroid-depleted condition. A, total protein from LNCaP (androgen dependent) and C4-2 (androgen independent) prostate cancer cells was resolved and immunoblotted 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

Published OnlineFirst March 10, 2011; DOI:10.1158/1535-7163.MCT-10-1096

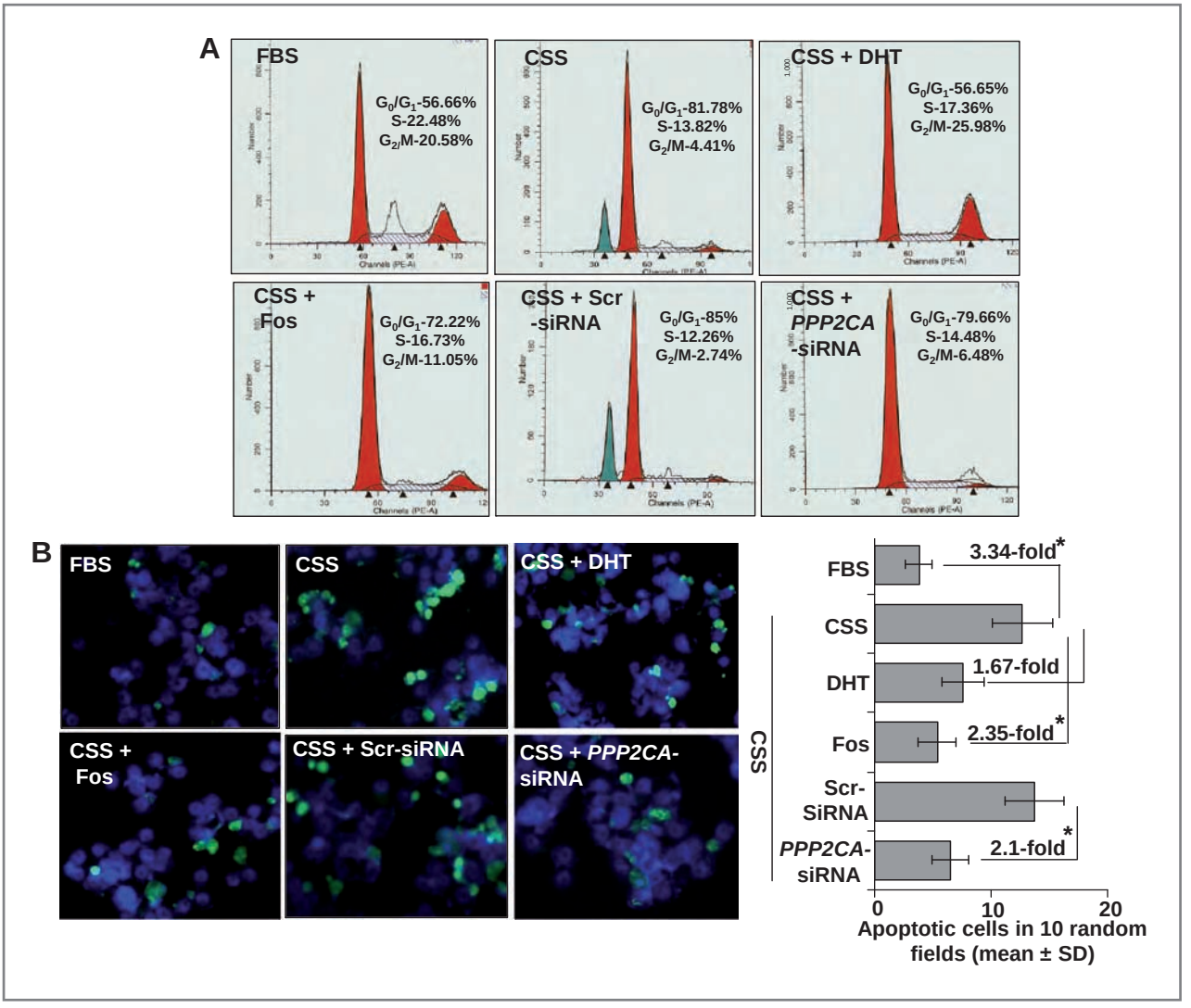

Figure 2. PP2A inhibition relieves hormone deprivation–induced G_0-G_1 arrest and suppresses apoptosis. A, LNCaP cells were synchronized by serum starvation and treated with DHT (1 nmol/L) or fostriecin (Fos; 100 nmol/L) for 24 hours in steroid-reduced (CSS) media. After treatments, distribution of cells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

Published OnlineFirst March 10, 2011; DOI:10.1158/1535-7163.MCT-10-1096

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

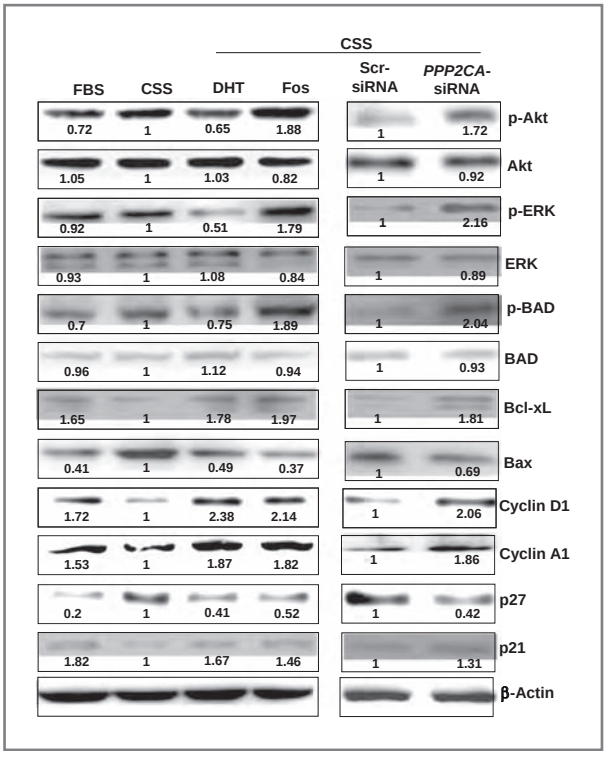

Figure 3. Inhibition of PP2A alters the expression and/or activation of survival and cell-cycle–associated proteins. LNCaP cells under steroid-reduced condition were treated with DHT (1.0 nmol/L) or fostriecin (Fos; 100 nmol/L) or silenced for *PPP2CA* expression. Following treatment, immunoblot analyses 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

Published OnlineFirst March 10, 2011; DOI:10.1158/1535-7163.MCT-10-1096

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.

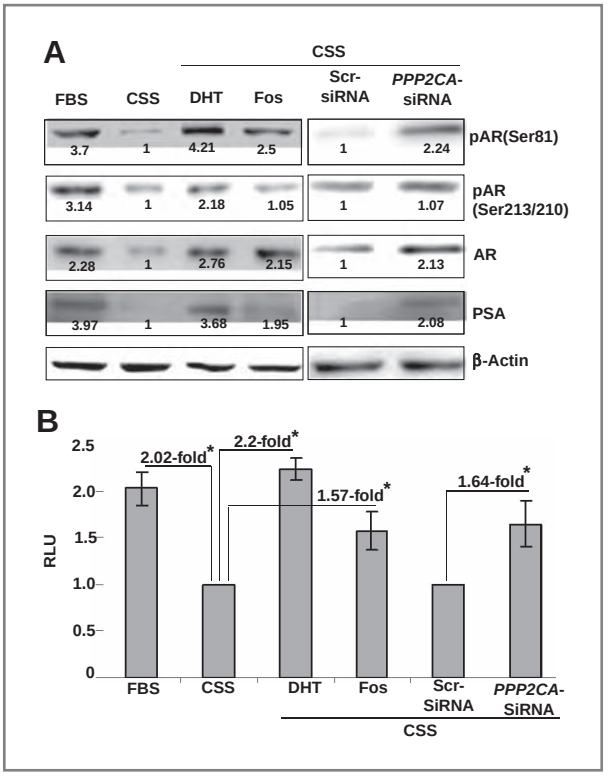

Figure 4. Inhibition of PP2A leads to induction of AR expression and its ligand-independent activation. A, LNCaP cells under steroid-reduced condition were treated with DHT (1.0 nmol/L) or fostriecin (100 nmol/L) or silenced for *PPP2CA* expression. Following treatment, immunoblot analyses were carried 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

Published OnlineFirst March 10, 2011; DOI:10.1158/1535-7163.MCT-10-1096

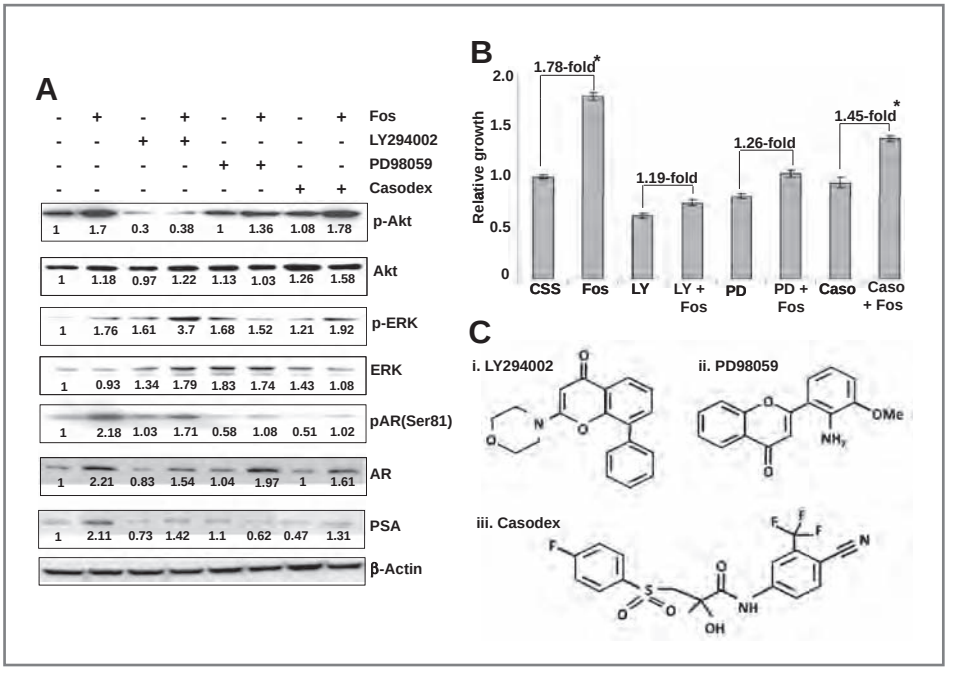

Figure 5. Pharmacologic repression of Akt, ERK, and AR signaling pathways suppresses androgen-independent growth of PP2A-inhibited cells. A, LNCaP cells were pretreated for an hour with LY294002 (20 μmol/L), PD98059 (25 μmol/L), and Casodex (5 μmol/L) followed by treatment with fostriecin (Fos; 100 nmol/L) 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,

Published OnlineFirst March 10, 2011; DOI:10.1158/1535-7163.MCT-10-1096

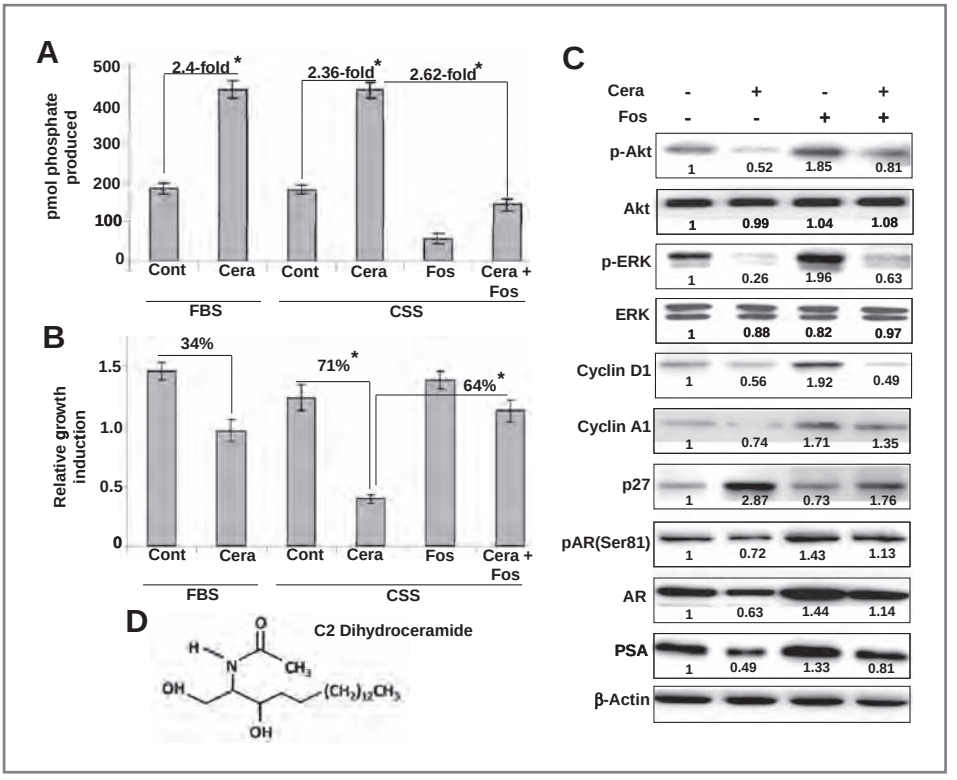

Figure 6. Ceramide (Cera) activates PP2A and suppresses the growth of androgen-independent prostate cancer C4-2 cells. A, C4-2 prostate cancer cells under steroid-supplemented (FBS) or -reduced (CSS) conditions were treated with ceramide (20 μmol/L; with or without pretreatment with fostriecin, Fos). 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

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.

Acknowledgments

The authors thank Steve McClellan (USAMCI Flow Cytometry Core Facility) for technical support and Dr. Joel Andrews (USAMCI) for help with fluorescence microscopy.

Grant Support

The study was supported by Department of Defense/U.S. Army (W81XWH-09-1-0137), NIH/National Cancer Institute (CA137513), and USAMCI.

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Received December 7, 2010; revised February 7, 2011; accepted February 23, 2011; published OnlineFirst March 10, 2011.

References

1. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer 2001;1:34–45.

2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225–49.

Published OnlineFirst March 10, 2011; DOI:10.1158/1535-7163.MCT-10-1096

3. Culig Z, Hobisch A, Cronauer MV, Cato AC, Hittmair A, Radmayr C, et al. Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. Mol Endocrinol 1993;7:1541–50.
4. Gregory CW, He B, Johnson RT, Ford OH, Mohler JL, French FS, et al. A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. Cancer Res 2001;61:4315–9.
5. Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, et al. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res 1997;57:314–9.
6. Craft N, Shostak Y, Carey M, Sawyers CL. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat Med 1999;5:280–5.
7. Kim O, Jiang T, Xie Y, Guo Z, Chen H, Qiu Y. Synergism of cytoplasmic kinases in IL6-induced ligand-independent activation of androgen receptor in prostate cancer cells. Oncogene 2004;23:1838–44.
8. Cooperberg MR, Park S, Carroll PR. Prostate cancer 2004: insights from national disease registries. Oncology 2004;18:1239–47.
9. Joly F, Tannock IF. Chemotherapy for patients with hormone-refractory prostate cancer. Ann Oncol 2004;15:1582–4.
10. Tannock IF, de WR, Berry WR, Horti J, Pluzanska A, Chi KN, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 2004;351:1502–12.
11. Singh AP, Bafna S, Chaudhary K, Venkatraman G, Smith L, Eudy JD, et al. Genome-wide expression profiling reveals transcriptomic variation and perturbed gene networks in androgen-dependent and androgen-independent prostate cancer cells. Cancer Lett 2008;259:28–38.
12. Prowatke I, Devens F, Benner A, Grone EF, Mertens D, Grone HJ, et al. Expression analysis of imbalanced genes in prostate carcinoma using tissue microarrays. Br J Cancer 2007;96:82–8.
13. Khew-Goodall Y, Hemmings BA. Tissue-specific expression of mRNAs encoding alpha- and beta-catalytic subunits of protein phosphatase 2A. FEBS Lett 1988;238:265–8.
14. Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J 2001;353:417–39.
15. Grethe S, Porn-Ares MI. p38 MAPK regulates phosphorylation of Bad via PP2A-dependent suppression of the MEK1/2-ERK1/2 survival pathway in TNF-alpha induced endothelial apoptosis. Cell Signal 2006;18:531–40.
16. Sontag E, Sontag JM, Garcia A. Protein phosphatase 2A is a critical regulator of protein kinase C zeta signaling targeted by SV40 small t to promote cell growth and NF-kappaB activation. EMBO J 1997;16:5662–71.
17. Van Kanegan MJ, Adams DG, Wadzinski BE, Strack S. Distinct protein phosphatase 2A heterotrimers modulate growth factor signaling to extracellular signal-regulated kinases and Akt. J Biol Chem 2005;280:36029–36.
18. Gotz J, Probst A, Ehler E, Hemmings B, Kues W. Delayed embryonic lethality in mice lacking protein phosphatase 2A catalytic subunit Calpha. Proc Natl Acad Sci U S A 1998;95:12370–5.
19. Silverstein AM, Barrow CA, Davis AJ, Mumby MC. Actions of PP2A on the MAP kinase pathway and apoptosis are mediated by distinct regulatory subunits. Proc Natl Acad Sci U S A 2002;99:4221–6.
20. Strack S, Cribbs JT, Gomez L. Critical role for protein phosphatase 2A heterotrimers in mammalian cell survival. J Biol Chem 2004;279:47732–9.
21. Chowdhury D, Keogh MC, Ishii H, Peterson CL, Buratowski S, Lieberman J. gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. Mol Cell 2005;20:801–9.
22. Miyabayashi T, Teo JL, Yamamoto M, McMillan M, Nguyen C, Kahn M. Wnt/beta-catenin/CBP signaling maintains long-term murine embryonic stem cell pluripotency. Proc Natl Acad Sci U S A 2007;104:5668–73.
23. Singh S, Srivastava SK, Bhardwaj A, Owen LB, Singh AP. CXCL12-CXCR4 signalling axis confers gemcitabine resistance to pancreatic cancer cells: a novel target for therapy. Br J Cancer 2010;103:1671–9.
24. Eto M, Bennouna J, Hunter OC, Hershberger PA, Kanto T, Johnson CS, et al. C16 ceramide accumulates following androgen ablation in LNCaP prostate cancer cells. Prostate 2003;57:66–79.
25. Kazi A, Smith DM, Zhong Q, Dou QP. Inhibition of bcl-x(l) phosphorylation by tea polyphenols or epigallocatechin-3-gallate is associated with prostate cancer cell apoptosis. Mol Pharmacol 2002;62:765–71.
26. Knudsen KE, Arden KC, Cavenee WK. Multiple G1 regulatory elements control the androgen-dependent proliferation of prostatic carcinoma cells. J Biol Chem 1998;273:20213–22.
27. Carson JP, Kulik G, Weber MJ. Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3′-kinase and Akt/protein kinase B. Cancer Res 1999;59:1449–53.
28. Murillo H, Huang H, Schmidt LJ, Smith DI, Tindall DJ. Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. Endocrinology 2001;142:4795–805.
29. Rokhlin OW, Taghiyev AF, Guseva NV, Glover RA, Syrbu SI, Cohen MB. TRAIL-DISC formation is androgen-dependent in the human prostatic carcinoma cell line LNCaP. Cancer Biol Ther 2002;1:631–7.
30. Baron S, Manin M, Beaudoin C, Leotoing L, Communal Y, Veyssiere G, et al. Androgen receptor mediates non-genomic activation of phosphatidylinositol 3-OH kinase in androgen-sensitive epithelial cells. J Biol Chem 2004;279:14579–86.
31. Shigemura K, Isotani S, Wang R, Fujisawa M, Gotoh A, Marshall FF, et al. Soluble factors derived from stroma activated androgen receptor phosphorylation in human prostate LNCaP cells: roles of ERK/MAP kinase. Prostate 2009;69:949–55.
32. Law B, Rossie S. The dimeric and catalytic subunit forms of protein phosphatase 2A from rat brain are stimulated by C2-ceramide. J Biol Chem 1995;270:12808–13.
33. Ruvolo PP, Deng X, Ito T, Carr BK, May WS. Ceramide induces Bcl2 dephosphorylation via a mechanism involving mitochondrial PP2A. J Biol Chem 1999;274:20296–300.
34. Corcoran NM, Hovens CM, Michael M, Rosenthal MA, Costello AJ. Open-label, phase I dose-escalation study of sodium selenate, a novel activator of PP2A, in patients with castration-resistant prostate cancer. Br J Cancer 2010;103:462–8.
35. Agus DB, Cordon-Cardo C, Fox W, Drobnjak M, Koff A, Golde DW, et al. Prostate cancer cell cycle regulators: response to androgen withdrawal and development of androgen independence. J Natl Cancer Inst 1999;91:1869–76.
36. McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, et al. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 1992;52:6940–4.
37. Schmitz M, Grignard G, Margue C, Dippel W, Capesius C, Mossong J, et al. Complete loss of PTEN expression as a possible early prognostic marker for prostate cancer metastasis. Int J Cancer 2007;120:1284–92.
38. Graff JR, Konicek BW, McNulty AM, Wang Z, Houck K, Allen S, et al. Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. J Biol Chem 2000;275:24500–5.
39. Malik SN, Brattain M, Ghosh PM, Troyer DA, Prihoda T, Bedolla R, et al. Immunohistochemical demonstration of phospho-Akt in high Gleason grade prostate cancer. Clin Cancer Res 2002;8:1168–71.
40. Kim SW, Kim HJ, Chun YJ, Kim MY. Ceramide produces apoptosis through induction of p27(kip1) by protein phosphatase 2A-dependent Akt dephosphorylation in PC-3 prostate cancer cells. J Toxicol Environ Health A 2010;73:1465–76.
41. Li B, Sun A, Youn H, Hong Y, Terranova PF, Thrasher JB, et al. Conditional Akt activation promotes androgen-independent progression of prostate cancer. Carcinogenesis 2007;28:572–83.
42. Gao H, Ouyang X, Banach-Petrosky WA, Gerald WL, Shen MM, Abate-Shen C. Combinatorial activities of Akt and B-Raf/Erk signaling in a mouse model of androgen-independent prostate cancer. Proc Natl Acad Sci U S A 2006;103:14477–82.

Published OnlineFirst March 10, 2011; DOI:10.1158/1535-7163.MCT-10-1096

43. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, et al. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res 1994;54:5474–8.
44. Yeh S, Lin HK, Kang HY, Thin TH, Lin MF, Chang C. From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. Proc Natl Acad Sci U S A 1999;96:5458–63.
45. Lin HK, Yeh S, Kang HY, Chang C. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proc Natl Acad Sci U S A 2001;98:7200–5.
46. Wen Y, Hu MC, Makino K, Spohn B, Bartholomeusz G, Yan DH, et al. HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. Cancer Res 2000; 60:6841–5.
47. Xin L, Teitell MA, Lawson DA, Kwon A, Mellinghoff IK, Witte ON. Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor. Proc Natl Acad Sci U S A 2006;103:7789–94.
48. Chen S, Kesler CT, Paschal BM, Balk SP. Androgen receptor phosphorylation and activity are regulated by an association with protein phosphatase 1. J Biol Chem 2009;284:25576–84.