Poly r(C) Binding Protein-1 is Central to Maintenance of Cancer Stem Cells in Prostate Cancer Cells

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Key Words
Poly r(C) binding protein-1 • PCBP1 • Prostate cancer • Stemness • Metastasis • Cancer stem cells

Abstract
Aims: To investigate global proteomic changes induced in CD44+CD24- stem cells isolated from the prostate cancer cell lines, LNCaP and DU145, post prolonged TGF-β treatment in order to understand underlying mechanisms that promote stemness in prostate cancer cells. Methods: CD44+CD133+α2β1Integrin+CD24- population was isolated from mock or TGF-β treated (7 days) prostate cancer cell line, LNCaP through fluorescent activated cell sorting. Cell lysates were obtained from the ±TGF-β cell population and proteomics profiling (MS/MS) was performed by mass spectrometry. Relative enrichment or depletion in the CD44+CD24- population post-TGF-β treatment was determined relative to mock-treated CD44+CD24- cells post normalization to GAPDH expression levels. Results obtained from MS/MS were validated using immunoblotting. Functional validation of one putative regulator was performed using gain-of-function strategy to investigate its role in rendering stemness in LNCaP and DU145 cells in vitro and in promoting tumorigenicity in vivo. Results: TGF-β treatment caused significant enrichment of CD44+CD24- population in LNCaP cells (22.35 ± 0.94% in mock treated vs 95.23 ± 2.34% in TGF-β treated cells; P < 0.01), which were also positive for CD133 and α2β1Integrin. Mass spectrometry analysis of the enriched cell population revealed that sixty-three proteins were either up- or down-regulated greater than five folds, out of which the poly r(C) binding protein (PCBP)-1 was the most down-regulated (9.31 ± 0.05 folds). Ectopic overexpression of PCBP1 in LNCaP and DU145 cells not only attenuated enrichment of CD44+CD133+CD24- population in these cells following TGF-β treatment, but also significantly decreased tumorigenicity of the stem cell subset, as assessed by in vitro soft agar colony formation and in vivo xenograft assays. Conclusion: Our proteomic profiling and subsequent validation indicate that PCBP1 is central to CSCs enrichment and functionality in prostate cancer.

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Introduction

Prostate cancer (PCa) is the second leading cause of cancer mortality and is the one that has shown the maximum increase of incidence in the last decade (World Health Organization, 2012). Despite the intensive and extensive studies on its cause, early detection, and therapy, there are still many unanswered questions about the molecular mechanisms underlying the uncontrolled expansion of PCa cells, as well as tumor recurrence and metastasis, the latter being attributed to be responsible for 90% of the deaths associated with PCa. Emerging evidence suggest that a minor population of cells in prostate tumors with stem cell properties called cancer stem cells (CSCs) driving metastatic progression, recurrence and resistance to different treatment regimens [1-4].

CSC populations may be phenotypically purified based on the expression of cell surface markers like CD44, CD34, CD133, and epithelial cell adhesion molecule (EpCAM) [5, 6]. CD44 has been widely used for enriching the stem cell populations in cancers of the prostate [7, 8]. Alongside, several PCa cell populations with enhanced tumor-initiating capacities that fit the definitions of CSCs have been identified [8-11]. The CD44+ PCa cells have been shown to express higher levels of ‘stemness’ genes including Oct-3/4, Bmi-1, β- catenin, and Smoothened [8]. Clonal analysis have indicated that a small subset of the CD44+ cells can undergo asymmetric cell division, with only one of the daughter cells retaining CD44 expression [8].

It has been elucidated by various research groups that TGF-β secreted from the tumor microenvironment and bone marrow derived mesenchymal cells both contribute to stemness of prostate cancer cells [12, 13]. However, not much, if any, is known about the underlying mechanisms that contribute to the maintenance of PCa stem cell population in the presence of secreted TGF-β. Hence, the objective of the current study was to investigate global proteomic changes induced in CD44+CD133+CD24- stem cells isolated from the prostate cancer cell lines, DU145 and LNCaP, post prolonged TGF-β treatment in order to understand underlying mechanisms that promote stemness in prostate cancer cells.

Materials and Methods

Cell lines and reagents

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Shanghai Jiaotong University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. LNCaP and DU145 cell lines were purchased from ATCC (Manassas, VA). LNCaP cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% HEPES, 1% sodium-pyruvate and 10 ml/L of 100 × antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO). DU145 cells were grown in DMEM supplemented with 10% fetal bovine serum and 10 ml/L of 100 × antibiotic-antimycotic solution. Cells were maintained in a humidified atmosphere (5% CO₂) at 37°C. Where indicated, LNCaP and DU145 cells were treated with TGF-β1 (R&D Systems, Minneapolis, MN) at a final concentration of 5 ng/ml for up to 7 days. The anti–PCBP1 and anti-GAPDH antibodies were purchased from Abcam (Cambridge, MA). Western blot reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Fluorescence-activated cell sorting

Anti-CD24-APC and anti-CD44-PE antibodies were purchased from BD Biosciences. Anti-CD133-Biotin and anti-Alpha-2 Beta-1 (α2β1) Integrin-FITC antibody were purchased from Miltenyi Biotech and Abcam, respectively. Cell labeling with fluorescent-conjugated antibodies was performed according to the manufacturer’s recommendations. Sorting of antibody-labeled cells was carried out on a FACSArray cell sorter (BD Biosciences). Sorted CD24 CD44+ cells from LNCaP or DU145 cells were cultured in MEM containing 10% fetal bovine serum and insulin (10 μg/ml) at 37°C with 5% CO₂. Sorted CD24 CD44+ and CD24-CD44- cells were subsequently tested for expression of CD133 and α2β1 Integrin.
Quantitative Real-Time RT-PCR (qRT-PCR)

qRT-PCR was performed using an Applied Biosystems Prism 7300 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems, Inc., Foster City, CA, USA). Primers were designed to be specific for CD44 variants:

CD44s: forward primer 5'-TCC AAC ACC TCC CAG TAT GAC A-3' and reverse primer 5'-GGC AGG TCT GTG ACT GAT GTA CA-3';

CD44v3: forward primer 5'-GCA GGC TGG GAG CCA AAT-3' and reverse primer 5'-GAG GTG TCT GTC TTC TTC ATT CTC ATC ATT-3';

CD44v6: forward primer 5'-GGA ACA GTG GTT TGG CAA CAG-3' and reverse primer 5'-TTG GGT GTT TGG GCA TAT CC-3';

CD44v10: forward primer 5'-CAG GTG GAA GAA GAG ACC CAA A-3' and reverse primer 5'-GGA TGA AGG TCC TGC TTT CCT T-3'.

CD133 forward and reverse primers used were 5'-GGA CCC ATT GGC ATT CTC-3' and 5'-CAG GAC ACA GCA TAG AAT C-3'; CD24 forward and reverse primers were 5'-TGC TCC TAC CCA CGC AGA TT-3' and 5'-GGC CAA CCC AGA GTT GGA A-3'.

Amplification data were analyzed by 2-ΔCT method with an Applied Biosystems Prism Sequence Detection Software (Version 2.1). The cycle threshold (CT) value corresponding to the PCR cycle number at which fluorescence emission in real time reached a threshold above the baseline emission was determined for each gene of interest and normalized to a cycle threshold for a housekeeping gene (TBP) determined in parallel. All qRT-PCR reactions were performed in triplicate and three independent sets of samples were used in each experiment. Data is represented as means ± standard deviation (SD).

Mass spectrometry and data analysis

Sorted CD44+CD24- cells from mock or TGF-β treated (for 7 days) LNCaP cells in triplicate were lysed using NET buffer [50 mmol/L Tris-HCl, (pH 7.4), 150 mmol/L NaCl, 0.1% NP40, 1 mmol/L EDTA, 0.25% gelatin, 0.02% sodium azide, 1 mmol/L phenylmethylsulfonyl fluoride, and 1% aprotinin]. The lysates were centrifuged at 15,000 × g for 30 minutes at 4°C. Collected supernatant was dialyzed against PBS, proteins were reduced with 5 mM Tris 2-carboxyethyl phosphine (TCEP) and alkylated with 10 mM iodoacetamide. Samples were digested with trypsin (Promega) in a 1:50 ratio for 12 h at room temperature. Peptides were desalted on Ultra MicroTIP Columns (The Nest Group, Southborough, MA, USA) and dried in a SpeedVac concentrator. Dried peptides were resolubilized in 20 μL HPLC grade water containing 0.1% formic acid. Sample analysis was performed on a linear ion trap LTQ mass spectrometer (Thermo Electron, San Jose, CA) equipped with a nanoelectrospray ion source (Thermo Electron) coupled to an Agilent 1100 micro HPLC system. Peptides were loaded with a cooled Agilent autosampler on a 2 cm long pre-column filled with C18 resin (Magic C18 AQ 5 μm; Michrom Bioresources, Auburn, CA, USA). A linear gradient of 80 minutes from 5% to 40% acetonitrile in H2O with 0.1% formic acid was used to separate peptides on a 10 cm long fused silica emitter packed with C18 resin spraying directly into the mass spectrometer at a flow rate of 0.5 μl/min. The MS instrument was operated in positive ion mode. The data-dependent acquisition mode was set to acquire one MS scan followed by three collision induced dissociation MS/MS scans. The MS full scans were recorded over a mass range of 400-1600 m/z. Dynamic exclusion was enabled, the repeat count was set to 2 and the exclusion duration to 30s. Further MS conditions were set as following: spray voltage 1.95 kV, transfer capillary temperature 230 °C, normalized collision energy 35%, activation q 0.25 and activation time 30 ms.

The acquired raw files were converted to mzXML files using ReAdW with default settings and searched against the mouse IPI database version 3.26 with the Sequest search algorithm. The Sequest search parameters contained the static modification of cysteine +57.02 Da, at least one tryptic terminus and one missed cleavage was allowed. The data were further processed using the the Trans-Proteomic Pipeline TPP including PeptideProphet and ProteinProphet to estimate the false discovery rate in the datasets. A protein probability of 0.5 was set as a cutoff corresponding to a false discovery rate of approximately 5%. The protein list was annotated for secreted proteins using the algorithm SignalP and further manually curated for secreted proteins using UniProt database and literature search. Functional annotation was assigned using the PANTHER Classification system.

Relative enrichment or depletion in the CD44+CD24- population post-TGF-β treatment was determined relative to mock-treated CD44+CD24- cells and data was normalized to GAPDH expression levels (relative
change in GAPDH expression was 1.2 fold increase post TGF-β treatment). Fold-changes (increase or decrease) above 5 was considered significant.

**Preparation of cell extracts**

Untreated or TGF-β treated LNCaP and DU145 cells were washed twice in PBS (pH 7.4), scraped into 15-mL conical tubes, and centrifuged at 1,000 × g at 4°C for 5 minutes. Cell extracts were prepared by lysis in NET buffer [50 mmol/L Tris-HCl, (pH 7.4), 150 mmol/L NaCl, 0.1% NP40, 1 mmol/L EDTA, 0.25% gelatin, 0.02% sodium azide, 1 mmol/L phenylmethylsulfonyl fluoride, and 1% aprotinin]. The lysates were centrifuged at 15,000 × g for 30 minutes at 4°C. The protein concentration of the supernatants was determined according to the method of Bradford using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

**Western blot analysis**

Ten micrograms of total LNCaP or DU145 cell protein extracts were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in PBS/Tween 20 (0.05%), followed by incubation with an anti-PCBP1 antibody (1:2000 dilution in 10% milk/PBS-T). Post-exposure blots were stripped and probed with anti-GAPDH antibody (1:5000 dilution). The detection was done using horseradish peroxidase–labeled secondary antibodies and enhanced chemiluminescence detection reagent.

**Overexpression of PCBP1**

Human PCBP1 plasmid in pCMV6-AC-GFP backbone and the empty vector were obtained from Origene. CD24-CD44+ population isolated from LNCaP or DU145 cells (4 × 10^5) were transfected with the PCBP1 construct or the empty vector using Lipofectamine LTX (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were split 1:10 and subsequently selected with G418 (250 μg/ml) (Teknova, Hollister, CA) for approximately 2 weeks.

**Soft-agar assay**

Parental or PCBP1 overexpressing CD44+CD24- subset (5 × 10^3 cells per 35-mm well) isolated from LNCaP or DU145 cells were resuspended in complete medium containing 0.35% agarose. Cells were grown on tissue culture dishes containing a 2-mL layer of solidified 0.7% agar in a complete medium. After 14 days, number of colonies was quantified from two randomly taken micrographs per well (original magnification, ×20). For visualization, foci were methanol-fixed and stained with 0.005% crystal violet.

**In vivo tumorigenesis assays**

4 clones each of pCMV6-AC-GFP (vector control) and pCMV6-AC-PCBP1-GFP (PCBP1 overexpressing) expressing CD44+CD24- population sorted from LNCaP or DU145 cells were resuspended in 50 μl RPMI1640 or DMEM medium, respectively, and mixed with 50 μl Matrigel (Becton Dickinson) at a 1:1 ratio and held on ice. The entire 100 μl sample (5 × 10^4 cells) was injected into each flank of 6-8 weeks old NOD/SCID mice anesthetized with isoflurane (n = 5 for each construct). Animals were accessed at the end of 4 weeks for the formation of tumors.

**Statistical analysis**

Statistical analysis was performed using SPSS 19.0 software. The results were expressed as mean ± SD. The data were treated by Student’s t test to determine statistical significance. P < 0.05 was considered statistically significant.

**Results**

CD44+CD24- population in LNCaP and DU145 cells are enriched following TGF-β treatment

Since it has been previously suggested that TGF-β can contribute to stemness of prostate cancer cells [12, 13], we sought to determine the effect of TGF-β on the abundance of CD44+CD24- population in the LNCaP cell line. Fluorescent activated cell sorting (FACS)
revealed that CD44+CD24- were significantly enriched in LNCaP cells treated with TGF-β for 7 days (22.35 ± 0.94% in mock treated vs 95.23 ± 2.34% in TGF-β treated cells; P < 0.01) (Fig. 1A, B). The enrichment in CD44+CD24- population was accompanied by a significant depletion of CD44+CD24+ population during the same time span (76.93 ± 2.31% in mock treated vs 3.78 ± 0.59% in TGF-β treated cells; P < 0.01) (Fig. 1A, B). To confirm that this observation is not limited to one cell type we assessed the enrichment of CD44+CD24- population in another cell line, DU145 (Fig. 1C-E). In addition, we also assessed the expression levels of other prostate cancer stem cell makers, CD133 (Fig. 1C, D) and α2β1Integrin (Fig. 1E). The CD24-CD44- cell population obtained after 7 days of TGF-β treatment were enriched for both CD133 and α2β1Integrin in both LNCaP and DU145 cell lines (Fig. 1C-E). No significant CD133 expression was observed in the CD24+CD44- cell population in untreated LNCaP or DU145 cells (Fig. 1C, D; blue dots).

**PCBP1 is significantly downregulated in enriched CD44+CD24- population**

In order to determine the underlying mechanism driving enrichment of CD44+CD24- population in LNCaP cells following TGF-β treatment we performed proteomic profiling of CD44+CD24- cells isolated from mock or 7 days TGF-β treated LNCaP cells. Following mass spectrometry, data analysis and normalization to GAPDH expression levels, 63 proteins showed either a 5 fold up- or down-regulation (Fig. 2A). Of these, 34 proteins were upregulated and 29 proteins were downregulated in the TGF-β-enriched CD44+CD24- cells. The protein that was maximally downregulated was poly r(C) binding protein-1 (PCBP1) or heterogeneous nuclear ribonucleoprotein (hnRNP)-E1 (9.31 ± 0.05 folds) (Fig. 2A). We subsequently validated the steady state expression level of PCBP1 protein in CD44+CD24- cells isolated from LNCaP (Fig. 2B) and DU145 (Fig. 2C) cells for the indicated time points. PCBP1
protein expression was significantly downregulated after 5 days of TGF-β treatment (Fig. 2B, C), further validating our proteomics data (Fig. 2A). Of note, the steady state expression level of PCBP1 was higher in DU145 cells compared to LNCaP cells. Cumulatively, our data suggest that PCBP1 is robustly downregulated when the CD44+CD133+α2β1IntegrinCD24- population is progressively enriched concomitant with prolonged TGF-β treatment. Since LNCaP cells are largely considered CD44- [14], we evaluated expression of different CD44 isoforms in the CD44+CD24- cell population sorted from LNCaP and DU145 cells (Fig. 2D). DU145 cells showed robust transcription of CD44s, CD44v3, CD44v6, and CD44v10 in comparison to only CD44s expression in the LNCaP cells.

**Overexpression of PCBP1 in either LNCaP or DU145 cells can attenuate enrichment of stem cell population following TGF-β treatment**

Our next objective was to determine if downregulation of PCBP1 is functionally required for observed enrichment of stem cell population in LNCaP and DU145 cells following TGF-β treatment. For the same, we transfected a pCMV-AC-PCBP1-GFP plasmid into LNCaP and DU145 cells and generated both stable pools and clones for subsequent downstream experiments. Successful overexpression following stable selection was tested by fluorescence microscopy for GFP; both empty vector and PCBP1 overexpressing cells showed robust GFP expression in comparison to the mock transfected cells (Fig. 3A). Successful transfection was also confirmed via western blot (data not shown). FACS analysis revealed that CD44+CD24-
were significantly enriched in LNCaP cells transfected with the empty vector (LNCaP/GFP cells) post treatment with TGF-β for 7 days (98.09 ± 4.29%) (Fig. 3B), similar to what was observed initially (Fig. 1A, B). However, overexpression of PCBP1 (LNCaP/PCBP1) significantly attenuated the enrichment of CD44+CD24- population during the same time span, even after TGF-β treatment for 7 days (5.11 ± 1.99%) (P < 0.001) (Fig. 3B). We also tested CD133 expression post overexpression of PCBP1. Whereas CD133+CD24- cells were significantly enriched in DU145 cells transfected with the empty vector (DU145/GFP cells) post treatment with TGF-β for 7 days (98.37 ± 1.38%), overexpression of PCBP1 (DU145/GFP-PCBP1) significantly attenuated the enrichment of CD133+CD24+ population during the same time span, even after TGF-β treatment for 7 days (0.2 ± 0.09%) (P < 0.001) (Fig. 3C). Similar changes in CD133 expression was observed in LNCaP/PCBP1 cells (data not shown). However, loss of CD44 expression seemed to be more drastic than corresponding loss of CD133 expression (Fig. 3B, C and data not shown). Cumulatively, our data suggested that downregulation of PCBP1 protein expression is necessary for observed enrichment of CD44+CD133+CD24- stem cells in LNCaP and DU145 cell lines following TGF-β treatment.

Quantitative real-time PCR (qRT-PCR) revealed that PCBP1 overexpression was correlated to significant (P<0.05) upregulation of CD24 and significant (P<0.05) downregulation of CD133 mRNA levels (Fig. 3D). No significant variation was observed in
CD44 mRNA expression levels. Our results are indicative of PCBP1 potentiating signaling mechanisms that transcriptionally regulate CD24 and CD133 expression, which subsequently through an unknown mechanism downregulates CD44 expression.

**Downregulation of PCBP1 protein expression is required for potent pro-tumorigenic activity observed in enriched CD44+CD24− population**

In order to further determine the role of PCBP1 in regulating the stemness of LNCaP and DU145 cells, we performed soft agar colony formation assay using the mock transfected and PCBP1 overexpressing LNCaP and DU145 CD44+CD133+CD24− (stem) cells. As expected, TGF-β treated parental LNCaP or DU145 stem cells formed soft agar colonies in a vast majority of the cases (96 ± 12% in LNCaP and 96.3 ± 3.4% in DU145); in comparison, the PCBP1 overexpressing cells showed a significant decrease in colony formation (18.3 ± 1.83% in LNCaP and 19.2 ± 2.3% in DU145; P < 0.01 in each case) (Fig. 4A). We furthermore tested the ability of the aforementioned stem cells to form tumors. Since stable pools can give false positives we used four different clones, each from empty vector and PCBP1 overexpressing LNCaP stem cells (Fig. 4B). Each clone was injected into flanks of five 6-8 weeks old NOD/SCID mice and tumor formation was compared four weeks after injection. After 4 weeks, distinctive tumors were observed in a vast majority of mice injected with LNCaP enriched stem cells carrying the empty vector (81.39 ± 8.96%). However, in mice injected with PCBP1 overexpressing LNCaP enriched stem cells, tumors formed in significantly less percentage of mice (5.93 ± 4.23%; P<0.001, compared to empty vector control group) (Fig. 4B). Not much heterogeneity was observed among the different clones tested in this assay. Cumulatively, our results showed that downregulation of PCBP1 is important for increase of stemness of LNCaP cells post TGF-β treatment and is also perhaps central to tumorigenicity of these cells as revealed by lack of potent tumorigenic activity post-overexpression of PCBP1 in the LNCaP cells.
Discussion

It has been widely proposed that both tumorigenesis and metastatic progression is contingent on transformation of a subpopulation of normal stem or progenitor cells, characterized by low expression of CD24 and high expression of CD44 [15-17]. The inherent properties of stem/progenitor cells may impart their transformed counterparts with the ability to evade traditional antitumor therapies and to establish metastasis [15-17]. Previous studies of CSCs and therapy resistance in other tumor systems have shown that CSCs appear to resist chemo- and radio-therapy, whereas therapy resistant cancer cells possess enhanced CSCs properties. Hence, it is pertinent to discover druggable mechanistic targets that drive the enrichment and maintenance of CSCs in prostate tumors.

Our proteomic profiling and subsequent validation indicate that PCBP1 is central to CSCs enrichment and functionality in prostate cancer. To the best of our knowledge this is the first report of any direct regulator of CSC enrichment in prostate cancer. LNCaP cells were originally classified as CD44 negative through global studies involving multiple prostate cancer cell lines and allocating cut-off values to protein expression and calling negative if expression of a protein did not meet the cut-off. But this really did not indicate complete absence of the secreted product. It must be noted that such experiments were conducted in whole cell lysates prepared from the entire population of LNCaP cells and not any subset population as in the current case.

It has been recently shown that PCBP1 regulates the stability of the pro-oncogenic and pro-stemness p63 transcript in pancreatic, ovarian and breast cancer cell line [18]. However, in the aforementioned context PCBP1 downregulation caused p63 degradation, which would suggest that PCBP1 is a positive regulator of stemness, in apparent contradiction to our current findings. However, two separate studies corroborate our findings. In one of those, a transcript-selective translational regulatory pathway was described in which a ribonucleoprotein (mRNP) complex, consisting of Poly(C) binding protein (PCBP) 1, silences translation of Dab2 and ILEI mRNAs, which are involved in mediating EMT [19]. It was shown that TGFβ activates a kinase cascade terminating in the phosphorylation of PCBP1 by isoform-specific stimulation of protein kinase Bα/Akt2, inducing the release of the mRNP complex from the 3'-UTR element, in turn resulting in the reversal of translational silencing and increased expression of transcripts that mediates EMT [19-21]. In the second study, PCBP1 was shown to downregulate production of the pro-metastatic PRL-3 phosphatase [22]. Thus it might be possible that downregulation of PCBP1 dictates stemness and mesenchymal cell formation in a context dependent fashion, as observed by us in the current study and the others [19, 22].

It would be interesting to identify what potentially causes downregulation of PCBP1 following TGF-β treatment in these cells. Our preliminary results suggest that it is not at the level of mRNA transcript and is perhaps being mediated by ubiquitin-mediated degradation of PCBP1 following TGF-β treatment (data not shown). Another interesting objective would be to determine if a similar mechanism is operative in the sustained enrichment of CSCs in other tumor types.

Our current study had some potential limitations including the study being conducted in cell line rather than primary tumor cells and that the study was based on proteomic profiling in only one androgen sensitive prostate tumor cell line. Obviously it is difficult to do such a scale of experiments in primary tumor samples. However, further validation in a large number of prostate cancer cell lines is warranted and which is currently being conducted.

Disclosure Statement

None.
Acknowledgements

This work was granted by Program of Shanghai City Committee of Science and technology (No. 134119a9800) and National Science Foundation of China (81172450 and 81202008).

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