Role of protein S in castration-resistant prostate cancer-like cells

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Abstract

Understanding how castration-resistant prostate cancer (CRPC) cells survive the androgen-deprivation condition is crucial for treatment of this advanced prostate cancer (PCa). Here, we reported for the first time the up-regulation of protein S (PROS), an anticoagulant plasma glycoprotein with multiple biological functions, in androgen-insensitive PCa cells and in experimentally induced castration-resistant PCa cells. Overexpression of exogenous PROS in LNCaP cells reduced androgen deprivation-induced apoptosis and enhanced anchorage-dependent clonogenic ability under androgen deprivation condition. Reciprocally, *PROS1* knockdown inhibited cell invasiveness and migration, caused the growth inhibition of castration-resistant tumor xenograft under androgen-depleted conditions, and potentiated Taxol (a widely prescribed anti-neoplastic agent)-mediated cell death in PC3 cells. Furthermore, PROS overexpression significantly stimulated AKT activation but failed to evoke oxidative stress in LNCaP cells under normal condition, suggesting that the malignance-promoting effects of the above-mentioned pathway may occur in the order of oxidative stress/PROS/AKT. The potential mechanism may be due to control of oxidative stress-elicited activation of PI3K-AKT-mTOR pathway. Taken together, our gain-of-function, loss-of-function analyses suggest that PROS may facilitate cell proliferation and promote castration resistance in human castration-resistant PCa-like cells via its apoptosis-regulating property. Future study emphasizing on delineating how PROS regulate cellular processes controlling transformation during the development of castration resistance should open new doors for the development of novel therapeutic targets for CRPC.

Key Words

- castration-resistant prostate cancer
- prostate cancer
- protein S
- apoptosis
- AKT
- oxidative stress

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Introduction

Prostate cancer (PCa) is the most common malignancy affecting men in the western world, with >233,000 new cases estimated for 2014 (Adams & Ferrington 2014). In some Asian countries, PCa has become one of the leading male cancers (Zhang *et al.* 2011). Although newly diagnosed

localized disease, which accounts for approximately 12% of PCa patients, may be cured with interventional therapies, about 30% of patients finally develop recurrent disease and may progress to castration-resistant prostate cancer (CRPC) (Karzai *et al.* 2015). Several chemical

agents such as docetaxel, although previously reported to improve overall survival in men with CRPC, fail to prolong the prognosis of CRPC patients by >6 months (Suzman & Antonarakis 2014). These facts suggest that progression to the stage of castration resistance remains the major obstacle to the effective control of advanced PCa, thus emphasizing the need for understanding and identification of the molecular mechanisms mediating the CRPC development.

Protein S (PROS) is a vitamin K-dependent protein encoded by the *PROS1* gene in humans and by *Pros1* in mice (Dahlback 2007). Protein S circulates in plasma and serves as an anticoagulant by working as a nonenzymatic cofactor for activated protein C in the breakdown of coagulation factors (F) Va and FVIIIa (Fernandez et al. 2009). Ablation of *Pros1* in mice results in the severe impairment of the hemostatic function, which has been shown to be associated with the disruption of TAM receptor (Tyro3, Axl, and Mer) pathways (Burstyn-Cohen et al. 2009). Independent of its anticoagulant activity, protein S exerts direct cellular effects such as cell survival, adhesion, and migration. For example, PROS blocks the extrinsic apoptotic cascade in tissue plasminogen activator/*N*-methyl *D*-aspartate-treated neurons (Guo et al. 2011). Very recently, Saraon and colleagues demonstrated that PROS is elevated in high grade and CRPC using proteomic profiling analysis, indicative of a potential involvement of PROS in the pathogenesis (Saraon et al. 2012). However, the function details of PROS in CRPC remain to be established.

The aims of this study are therefore (i) to determine the expression levels of PROS in different PCa cells, (ii) to assess any alterations in PROS expression resulting from CRPC development, (iii) to evaluate the therapeutic effects of PROS inhibition/overexpression on CRPC progress, and (iv) to identify the associated molecular events most affected so as to provide some insights into the mechanisms by which PROS may possibly become activated in high grade and aggressive PCa.

Materials and methods

Cell culture and treatment

Human prostate cancer cell lines were purchased from ATCC. Normal human prostate epithelial cells (PEC) were purchased from Clonetics (Nanjing, China). All of the cells were recently authenticated in August 2013 by the short tandem repeat analysis method using Promega PowerPlex1.2 analysis system (Genewiz Inc, Suzhou, China). Upon receipt, cells with lower passage number were

frozen in multiple aliquots in liquid nitrogen. Cells used for the current study had less than 30 passages. RWPE-1 cells were cultured in keratinocyte serum-free medium (K-SFM, Life Technologies) supplemented with 50 mg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor (EGF; Life Technologies), 100 U/mL penicillin, and 100 U/mL streptomycin (Zhang et al. 2014b). The rest cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 U/mL penicillin, and 100 U/mL streptomycin. To generate castration-resistant LNCaP sublines, LNCaP cells were incubated in media containing 10% charcoal-stripped FBS (FBS-CS) in the presence of 10 μ M bicalutamide (Selleck, Shanghai, China). After a 4-month incubation, the surviving cells were selected and designated as LNCaP-R (Ishikura et al. 2010). To test whether PROS expression was androgen receptor (AR) signaling-dependent, hormone-depleted LNCaP cells were treated with R1881 (1 nM; Sigma-Aldrich) for 12 h, followed by other biochemical analysis. To investigate whether PROS function directly or indirectly, LNCaP cells were cultured in RPMI-1640 medium supplemented either with FBS or with FBS-CS, in the presence or absence of human recombinant PROS (100 nM; ABM, Richmond, BC, Canada), for 3 days before being subjected to apoptosis ELISA as described below. To answer whether moderate oxidative stress was involved in the up-regulation of PROS, PC3 cells were treated with H₂O₂ or diamide for various concentrations, in the presence or absence of 20 μ mol/L diphenyleneiodonium chloride, for 5 min. To investigate the specific involvement of PI3K-AKT-mTOR signaling, cells were preincubated with two inhibitors (25 μ mol/L LY294002 for 1 h or 100 nmol/L rapamycin for 2 h) before other assays. To induce the chemotherapeutic damage, cells were treated with taxol (paclitaxel, from *Taxus brevifolia*; Sigma-Aldrich, 30 nM for 48 h) or the antiandrogen bicalutamide (10 μ M for 48 h), respectively.

Cell proliferation

Androgen-depleted cells were prepared by preincubation in media containing 10% FBS-CS for 3 days and the cells were then plated onto a 96-well plate at 1×10^4 cells/well (LNCaP) or 5×10^3 cells/well (LNCaP-R) in media containing 10% FBS-CS and incubated overnight at 37°C in 5% CO₂, followed by incubation with different concentrations of synthetic androgen R1881 (Sigma). After a further 9 days of incubation, viable cell numbers were determined using a hemocytometer (Beckman Coulter, Fullerton, CA, USA).

Prostate-specific antigen (PSA) and AR quantification

After androgen depletion for 3 days, the cells were plated onto a 24-well plate at 2×10^5 cells/well in media containing 10% FBS-CS and cultured overnight, followed by R1881 incubation. After 2 days of incubation, the cells were lysed with ice-chilled cell lysis buffer (Cell Signaling) supplemented with complete proteinase-inhibitor cocktail tablets (Roche Diagnostics). The final PSA content in the supernatant was measured using PSA ELISA kit (Abcam). To further confirm the effect of hormone ablation condition during the genesis of LNCaP-R, we monitored the expression level of PSA, as described above, at different time points following the establishment of LNCaP-R. As for the latter, intracellular AR levels were quantified using AR ELISA assay (Active Motif, Shanghai, China) at different time points following the establishment of LNCaP-R.

Generation of stable cell lines

pCMV6-XL5-*PROS1* plasmid was obtained from OriGene (Rockville, MD, USA). LNCaP cells stably overexpressing exogenous *PROS1* were established according to our previous work (Li et al. 2008a).

Assessment of apoptosis

LNCaP cells transfected with pCMV6-XL5-*PROS1* or vector were cultured in media containing either 10% fetal bovine serum (FBS) or 10% FBS-CS for 3 days. The extent of apoptosis was quantitatively measured using apoptosis ELISA kit (Roche Diagnostics), according to the manufacturer's instruction. In this spectrophotometric assay, histone-complexed DNA fragments (mono- and oligonucleosomes, namely low-molecular-weight DNA (LMW DNA)) are released from the cytoplasm of apoptotic cells after lysis and the LMW DNA can be separated from nuclear high-molecular-weight DNA (HMW DNA) by centrifugation. The supernatant that contains LMW DNA can be analyzed by ELISA method. The final spectrophotometric assay was developed using peroxidase substrate and the absorbance was measured in triplicate with a microplate reader at 405 nm (Bio-Rad 680).

Colony formation assay

Anchorage-dependent clonogenic ability was assessed as described previously (Yu et al. 2013). Approximately

400 cells (transformed with the pCMV6-XL5-*PROS1* or vector)/10 cm plastic dish (Falcon; Becton Dickinson, Lincoln Park, NJ, USA) were cultured in media containing either 10% fetal bovine serum (FBS) or 10% FBS-CS at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The plates were left undisturbed for 14 days until colonies were large enough to be clearly discerned. Colonies were stained for 5 min with a solution containing 0.5% crystal violet and 25% methanol, followed by three rinses with tap water to remove excess dye. The colonies, defined as groups of >50 cells, were finally scored using Colony Counter software (Syngene, Frederick, MD, USA).

In vitro siRNA treatment

We designed and synthesized chemically two siRNA sequences targeting *PROS1* (GenBank accession number: NM_000313.3) (Ruibo Co, Shanghai, China). The oligo sequences used were as follows: si-1 (sense: 5'-AUUCAUUUAUGUCAAAUUCACdTdT-3', antisense: 3'-dTdT GAAUUUGACAUAAAUGAAUGC-5'); si-2 (sense: 5'-UAAUCUAUGUGAAAUGAGCAdTdT-3', antisense: 3'-dTdT CUCAAUUUCACAUAGAUUAUA-5'). The off-target effects of siRNA treatment are very frequent if not universal, and the standard in the field is to simultaneously use two different RNAi's (Huppi et al. 2005). Therefore, after confirmation of the knock down efficiency of these two siRNAs by immunoblotting assay, we mixed these two siRNAs together to achieve the ablation of *PROS* expression in PCa cells. *PROS1*-siRNAs or a non-silencing scrambled control siRNA (Altogen Biosystems, Las Vegas, NV, USA) were transfected into PC3 cells using Lipofectamine 2000. After 48 h of transfection, cells were collected and subjected to other experiments.

Invasion assay

Invasion assay was performed using a BD BioCoat Matrigel Invasion Chamber (BD Biosciences, MA, USA) with an 8 mm PET membrane, uniformly coated with BD Matrigel Matrix (Yan et al. 2013). Serum-starved cancer cells were transferred to the top chambers (1×10^4 /well), while the bottom chambers containing 0.5 mL of conditioned medium from NIH 3T3 fibroblasts. After 24 h of incubation, cells migrating to the lower surface were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min and then counted under a fluorescence microscope (Axio Imager M1; Zeiss), and the relative number to the vector control was calculated.

Wound healing assay

The indicated cells were plated and grown to confluence in six-well plates. Streaks were created in the monolayers with P200 pipette tips. The progression of migration was observed and photographed at 24 h after wounding.

In vivo tumor growth assay

Male nude mice aged 8 weeks were purchased from Animal Facility of our university and maintained on a 12 h light:12 h darkness cycle (lights on at 8:00 h) with constant temperature 20–26°C and humidity (35–75%). Food and water were available *ad libitum*. LNCaP-R cells were infected with lentiviruses encoding shRNAs specific for the control vector (sh-Ctrl) or *PROS1* (sh-*PROS1*). At 48 h post-infection, cells were injected into left (sh-Ctrl) and right (sh-*PROS1*) flanks of castrated male nude mice. The tumor size was measured by caliper and expressed as cubic millimeters using the formula $0.5236 \times r_1^2 \times r_2$ (where $r_1 < r_2$) (Long et al. 2000). All procedures involving animals were approved by the local ethics committee. All surgery was performed under sodium pentobarbital anesthesia (0.04–0.05 mg/g body weight, i.p.), and all efforts were made to minimize suffering.

Measurement of intracellular oxidation

Non-treated LNCaP cells and LNCaP cells transfected with pCMV6-XL5-*PROS1* or vector were cultured in media containing 10% FBS for 3 days, followed by the measurement of intracellular oxidation using OxiSelect Intracellular ROS Assay Kit, according to the manufacturer's instruction.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-PCR was performed as described previously (Yu et al. 2013). Amplification of 18S served as the internal control. Primer sequences used were *PROS1*, 5'-TCTCAGAGGCAAACCTTTTGT-3' and 5'-GGCTTCTTCTTATTGCACAG-3' (gene access no. NM_000313.3); *AR*, 5'-GTGTTGTATGCCTTTAAATC-3' and 5'-GATAAGTAACGTTTGTGTG-3' (gene access no. M23263.1); *18S*, 5'-CTCGCCGCGCTCTACCTACCTA-3' and 5'-ATGAGCCATTCGACAGTTTCACTGTA-3' (gene access no. M10098.1). PCR products were quantified by SYBR green intercalation using the MiniOpticon system (Bio-Rad Laboratories). The relative abundance of each target transcript was quantified using the comparative $\Delta\Delta C_t$ method.

Immunoblotting

Total or nuclear protein samples were prepared according to our previous work (Li et al. 2008b). Immunoblotting assays were performed as described in our previous work (Zhang et al. 2014a). Protein was separated on SDS/PAGE and transferred to nitrocellulose membrane (Millipore). Membranes were then incubated with primary antibodies as listed in Table 1, in blocking solution overnight at 4°C. Positive signals were finally detected by using an ECL kit (Amersham Biosciences). Densitometric analysis of immunoblots was performed using Image J (<http://rsbweb.nih.gov/ij/>).

Statistical analysis

Results are presented as mean $\pm$ S.E.M. from at least three independent experiments and were analyzed for statistically significant differences using Student's *t*-test or

Table 1 Sources of antibodies and the working dilutions that were used for immunoblotting experiments in this study.

Antibody	Vendor	Catalog no.	Dilutions
Rabbit anti-PROS	Sigma-Aldrich	HPA023974	1:800
Goat anti-TUBULIN	Santa Cruz Biotechnology	sc-9935	1:2000
Rabbit anti-AR	Santa Cruz Biotechnology	sc-816	1:2000
Mouse anti-histone H1	Santa Cruz Biotechnology	sc-8030	1:1000
Rabbit anti-cleaved caspase 3	Santa Cruz Biotechnology	sc-23461-R	1:1000
Rabbit anti-BCL-XL	Santa Cruz Biotechnology	sc-7195	1:1000
Rabbit anti-pAKT	Cell Signaling Technology	4060	1:1500
Rabbit anti-AKT	Cell Signaling Technology	4085	1:2000
Rabbit anti-pS6K	Cell Signaling Technology	9205	1:1200
Rabbit anti-S6K	Cell Signaling Technology	9202	1:2000
Goat anti-Rabbit IgG-HRP secondary antibody	Abcam	ab97200	1:10,000
Bovine anti-goat IgG-HRP secondary antibody	Santa Cruz Biotechnology	sc-2350	1:5000
Bovine anti-mouse IgG-HRP secondary antibody	Santa Cruz Biotechnology	sc-2371	1:5000

one-way ANOVA. Data returning a value of $P < 0.05$ were considered statistically significant.

Results

Up-regulation of PROS in CRPC cells

We firstly established the expression profiles of PROS in human PCa cells. Genetic backgrounds of different PCa cell lines used in this study were indicated in Fig. 1A. Our RT-qPCR analysis revealed that *PROS1* mRNA expression was weak in PEC and RWPE-1 cells, moderate in 22Rv1 and LNCaP cells, and high in PC3 and DU145 cells (Fig. 1B). We then validated this observation at the translational level. As shown in Fig. 1C and 1D, PC3 and DU145 cells exhibited the highest expression of PROS compared with the moderate expression in 22Rv1 and LNCaP cells. No positive signals were detected in PEC and RWPE-1 cells. These data indicate that PROS is irrelevantly correlated with androgen receptor expression in PCa cells.

PROS induction during the pathogenesis of castration resistance

We then generated castration-resistant sublines (LNCaP-R) according to a previously validated protocol (Ishikura et al. 2010). LNCaP-R exhibited faster growth and higher PSA production in the androgen-depleted medium compared with LNCaP (4.4- and 13.2-fold, respectively, Fig. 2A and B). Although LNCaP-R grew

in an androgen-independent manner, R1881 treatment further stimulated its cell growth and PSA production (Fig. 2A and B), with a relatively lower increase in the latter (Fig. 2B). Interestingly, along the pathogenesis of castration resistance, PROS expression was gradually up-regulated, with the highest levels observed at 3 and 4 months after establishment of LNCaP-R (Fig. 2C). The up-regulation of PROS level at the late phase of the genesis of LNCaP-R was well correlated with the high PSA production (Fig. 2D) and high AR expression (Fig. 2E), which confirmed the effect of hormone ablation condition during the establishment of castration-resistant LNCaP-R. To further answer whether PROS expression was modulated directly by androgen signaling, we treated AR-positive LNCaP cells with R1881 (1 nM). Supplement with this synthetic androgen, methyltrienolone significantly inhibited AR mRNA expression and stimulated AR protein expression as reported previously (Kumar & Thakur 2004), but failed to notably evoke the PROS expression (Fig. 2F and G). These data, together with the above-mentioned irrelevant correlation between PROS and AR expression (Fig. 1), suggest that PROS function may be independent of AR in PCa cells.

Ectopic overexpression of PROS1 protects LNCaP cells in the androgen-depleted condition

To further assess whether enhanced PROS expression is causative of or a result of castration resistance in PCa,

Figure 1

Up-regulation of PROS in androgen-insensitive prostate cancer (PCa) cells. (A) Genetic backgrounds of prostate cells used in the study. (B) Relative expression levels of *PROS1* mRNA in different PCa cells were evaluated using RT-qPCR as described in the 'Materials and methods' section. Parallel amplification of *18S* mRNA served as internal control. Quantitative values are the mean $\pm$ S.E.M. of at least three independent determinations. Different superscript letters denote groups that are statistically different ($P < 0.05$; Student's *t*-test). (C) Immunoblotting analysis of PROS protein levels in different PCa cells. TUBULIN served as a loading control. (D) Densitometric scanning of immunoblots was performed in which the level of a target protein was normalized against the protein level in 22Rv1 cells, which was arbitrarily set at 1. Different superscript letters denote groups that are statistically different ($P < 0.05$; Student's *t*-test). UN, undetectable.

Figure 2

PROS induction by castration-resistant development in LNCaP cells. (A) The castration-resistant LNCaP sublines (LNCaP-R) were developed as described in the 'Materials and methods' section. Cells were preincubated in media containing 10% FBS-CS for 3 days and were then maintained in media containing 10% FBS-CS, followed by incubation with different concentrations of synthetic androgen R1881 for 9 days. Viable cell numbers were finally determined and relative cell growth was calculated. (B) Intracellular PSA levels of LNCaP and LNCaP-R cells were measured in the presence of R1881. (C) Expression level of PROS protein at different time points after establishment of LNCaP-R was determined by immunoblotting. TUBULIN served as a loading control. (D) Intracellular PSA levels were determined by ELISA at different time points after induction of LNCaP-R cells. (E) Intracellular AR levels were determined by ELISA at different time points after induction of LNCaP-R cells. Hormone-depleted LNCaP cells were treated with R1881 (1 nM) for 12 h, followed by qPCR (F) and immunoblotting (IB) analysis (G) of AR and *PROS1*/PROS expression (* $P < 0.05$, ** $P < 0.01$; Student's *t*-test). *18S* and TUBULIN served as loading controls. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0126>.

we overexpressed pCMV6-XL5-*PROS1* in LNCaP cells (Fig. 3B). The androgen-depleted condition (FBS-CS culture)-induced apoptosis was significantly attenuated in the cells overexpressing *PROS1* when compared with that in control cells (Fig. 3A). This decreased apoptotic rate was further confirmed by the down-regulated expression of cleaved caspase 3 fragment and the elevated expression of anti-apoptotic Bcl-XL in the pCMV6-XL5-*PROS1*-transfected cells (Fig. 3B). Consistently, *PROS1* overexpression significantly increased the cell viability in the presence of FBS-CS (Fig. 3C). Moreover, *PROS1* overexpression promoted colony formation efficiency in the presence or absence of androgen-depleted condition (Fig. 3D). To further study whether PROS function directly or indirectly in the castration condition, we

added purified PROS protein into the culture media of LNCaP cells. Supplement with the recombinant PROS protein efficiently reduced the apoptosis and promotes the cell viability in the presence of FBS-CS, with a dose-dependent effect being detected in the former (Fig. 3D). Thus, PROS may function directly upon androgen depletion condition.

Endogenous PROS promotes cancer progression in PCa cells

We next explored the role of PROS in the androgen-insensitive PC3 cells, which represent the more castration-resistant phenotype, and in the LNCaP-R cells. To select the effective *PROS1*-siRNA, two different

Figure 3

PROS1 overexpression protects LNCaP cells from androgen deprivation condition. (A) LNCaP cells transfected with pCMV6-XL5-*PROS1* or vector were cultured in media containing either 10% FBS or 10% FBS-CS for 3 days. The extent of apoptosis was quantitatively measured using apoptosis ELISA. * $P < 0.05$ indicates that values were statistically significantly different from those for vector control in FBS-CS condition. (B) Expression levels of apoptosis-related molecules in the above-mentioned cells were evaluated using immunoblotting. TUBULIN served as a loading control. (C) After 3-day culture of the above cells, total cell numbers were determined using a hemocytometer. * $P < 0.05$ indicates that values were statistically significantly different from those for vector control in FBS-CS condition. (D) Anchorage-dependent clonogenic ability of the above cells was assessed using colony formation assay as described in the 'Materials and methods' section. * $P < 0.05$ or ** $P < 0.01$ indicates that values were statistically significantly different from those for vector control under different culture conditions. LNCaP cells were cultured in RPMI-1640 medium supplemented either with FBS or with FBS-CS, in the presence or absence of human recombinant PROS (100 nM) for 3 days, followed by apoptosis ELISA (E) and cell number count analysis (F) as described above. * $P < 0.05$ indicates that values were statistically significantly different.

siRNAs against *PROS1* were tested, with a non-silencing scrambled siRNA as a negative control. Immunoblotting analysis demonstrated that si-2 was more effective in suppressing the PROS expression (Fig. 4A), and these two siRNAs were therefore selected together for further studies to avoid off-target effects. Interestingly, selective knockdown of *PROS1* reduced the invasive property of PC3 cells, as revealed by the Transwell–Matrigel assay (Fig. 4B). Furthermore, regardless of cell type, ablation of *PROS1* slowed down the healing speed of wounded cells compared with those observed in cells transfected with the control siRNA at the end of 24 h after streaks creation (Fig. 4C and D), suggesting that PROS

knockdown could affect cell proliferation under both normal and castration conditions. From a therapeutic standpoint, PROS inhibition significantly enhanced the Taxol sensitivity in PC3 cells. This therapeutic effect was not observed in cells exposed to bicalutamide (Fig. 4E). To provide the *in vivo* evidence that the overexpressed PROS in hormone refractory PCa cells may be required for tumor cell growth under androgen-depleted conditions, PROS expression was knocked down by specific shRNA in LNCaP-R cells. Subsequent xenograft experiment showed that ablation of PROS expression resulted in a significant tumor growth inhibition in castrated immunodeficient mice (Fig. 4F).

Figure 4

Silencing of *PROS1* expression enhances the androgen deprivation-induced damage in PC3 cells. (A) Verification of *PROS1* knock down effect by siRNA treatment was performed using immunoblotting analyses. TUBULIN was used as an internal control. (B) Matrigel invasion assay was performed as described in the 'Materials and methods' section. Migrated tumor cells were highlighted by 4',6-diamidino-2-phenylindole (DAPI) staining. * $P < 0.05$ indicates that values were statistically significantly different from those for scrambled control. Effects of *PROS1* knock down on PC3 cell (C) and LNCaP-R cell (D) migration was evaluated using wound healing assay as described in the 'Materials and methods' section. * $P < 0.05$ or ** $P < 0.01$ indicates that values were statistically significantly different from those for scrambled control at post-treatment 24 h. (E) Cells were treated with Taxol (30 nM for 48 h) or the antiandrogen bicalutamide (10 μM for 48 h) respectively. Subsequently, total cell numbers were determined using a hemocytometer. * $P < 0.05$ indicates that values were statistically significantly different from those for DMSO control. (F) LNCaP-R cells were infected with lentiviruses encoding shRNAs specific for the control vector (sh-Ctrl) or *PROS1* (sh-*PROS1*). At 48 h post-infection, cells were injected into left (sh-Ctrl) and right (sh-*PROS1*) flanks of castrated male nude mice. Growth of tumors was examined as described in the Experimental Procedures method. Results represent mean tumor sizes $\pm$ S.E.M. ($n = 5$ mice per group). * $P < 0.05$ indicates that values were statistically significantly different from those for sh-Ctrl. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0126>.

PROS induction by oxidative stress augments the PI3K-AKT-mTOR signaling in PC3 cells

To understand how PROS functions in CRPC, we treated PC3 cells with hydrogen peroxide (H_2O_2), as castration induces oxidative stress *in vivo*, and treatment with H_2O_2 can partially mimic cellular events after castration (Zhang et al. 2014b). Unexpectedly, H_2O_2 exposure substantially induced PROS expression in PC3 cells. This stimulatory effect was significantly suppressed in the presence of oxidative stress inhibitor diphenyleneiodonium chloride (Fig. 5A). We then asked whether oxidative stress other than H_2O_2 can cause PROS up-regulation. Diamide oxidizes cellular thiols,

especially protein-integrated cysteines, provoked a rapid decrease in cellular glutathione, and hence caused oxidative stress (Ho et al. 2013). Diamide treatment also promoted PROS expression in the absence of diphenyleneiodonium (Fig. 5B). Considering that the PI3K-AKT-mTOR pathway has an established role in CRPC (Bitting & Armstrong 2013), we asked whether PROS is required for its activation. As shown in Fig. 5C, *PROS1* knockdown caused a reduction of the levels of phospho-AKT (S473) and phospho-S6 upon oxidative stress (Fig. 5C), suggesting that PROS acts upstream of the PI3K-AKT-mTOR pathway. To further confirm that oxidative stress-induced activation of AKT is PROS

Figure 5

Oxidative stress activates the PI3K-AKT-mTOR pathway in a PI3K-dependent manner in PC3 cells. (A) PC3 cells were treated with or without 1 mmol/L of H₂O₂ in the presence or absence of 20 mmol/L of diphenyleneiodonium chloride (an inhibitor of NADPH oxidase), for 5 min and harvested for anti-PROS immunoblotting. (B) PC3 cells were treated with diamide for various concentrations as indicated for 5 min and harvested for anti-PROS immunoblotting. (C) PC3 cells were depleted of PROS by siRNA treatment. Cells were then treated with 1 mmol/L of H₂O₂ for 5 min and harvested for immunoblotting analysis. (D) PC3 cells were pretreated with 25 μmol/L LY294002 for 1 h. Cells were then treated with 1 mmol/L of H₂O₂ for 5 min and harvested for immunoblotting analysis. (E) PC3 cells were pretreated with 100 nmol/L rapamycin for 2 h. Cells were then treated with 1 mmol/L H₂O₂ for 5 min and harvested for immunoblotting analysis. (F) LNCaP cells transfected with pCMV6-XL5-PROS1 or vector and non-treated naïve LNCaP cells were cultured in media containing 10% FBS for 3 days and harvested for measurement of intracellular oxidation using OxiSelect Intracellular ROS Assay Kit. No significant difference was detected between different experimental groups. (G) LNCaP cells transfected with pCMV6-XL5-PROS1 or vector and non-treated naïve LNCaP cells were cultured in media containing 10% FBS for 3 days, followed by immunoblotting analysis of AKT activity. TUBULIN served as a loading control.

dependent, we also inhibited the PI3K-AKT-mTOR pathway using various inhibitors and found that these inhibitors did not affect oxidative stress-induced PROS elevation (Fig. 5D and E), supporting that the PI3K-AKT-mTOR pathway acts downstream of PROS. We were then curious whether PROS overexpression could directly influence the oxidative stress status or AKT activation in PCa cells. LNCaP cells stably transfected with pCMV6-XL5-PROS1 or vector, along with the naïve control cells,

were cultured in media containing 10% FBS for 3 days and were then subjected to the intracellular oxidation assay. The results revealed that ROS accumulation under normal condition was unchanged in the absence or presence of PROS overexpression (Fig. 5F). In contrast, the expression level of phospho-AKT (S473) was significantly stimulated in the LNCaP cells transfected with pCMV6-XL5-PROS1, when compared with that in naïve cells and cells transfected with vector (Fig. 5G).

Figure 6

Inhibition of AKT pathway potentiates androgen deprivation-induced cell death in LNCaP cells stably overexpressing exogenous *PROS1*. (A) LNCaP cells transfected with pCMV6-XL5-*PROS1* or vector were cultured in media containing either 10% FBS or 10% FBS-CS, in the presence or absence of the pretreatment with 25 μ M LY294002, for 3 days and harvested for immunoblotting analysis. (B) Effect of LY294002 pretreatment on *PROS1* overexpression-induced cell growth in different culture conditions was evaluated using a hemocytometer. * $P < 0.05$ indicates that values were statistically significantly different from those for vector control in FBS-CS condition. (C) A working model depicts that *PROS* is responsible for the direct control of oxidative stress-elicited activation of PI3K-AKT-mTOR pathway in castration-resistant PCa-like cells.

Inhibition of AKT pathway promotes apoptosis in LNCaP cells stably overexpressing *PROS1*

Lastly, we tested whether inhibition of AKT activity could reverse the above phenotype in LNCaP cells stably overexpressing *PROS1*. When the activation of AKT signaling was blocked by LY294002, *PROS1* overexpression-induced cell growth was abolished both in FBS and in androgen-deprived FBS-CS conditions (Fig. 6A and B). These results indicate that deregulation of AKT pathway may be terminally responsible for the pathogenesis of castration resistance caused by *PROS* overexpression in LNCaP cells.

Discussion

The progression to androgen independence after androgen deprivation therapy represents one of the greatest challenges to PCa treatment. So far, many advances have been made, but the molecular

mechanisms underlying this complicated process remain largely unknown (Wyatt & Gleave 2015). Our study herein begins to provide novel insight into this issue by showing the clear-cut up-regulation of *PROS*, an anticoagulant plasma glycoprotein with multiple biological functions, in castration-resistant PCa cells. In line with the previous human tissue data (Saraon et al. 2012), *PROS* level was higher in androgen-insensitive PC3 and DU145 cells than that in androgen-sensitive LNCaP, PEC, and RWPE-1 cells (Fig. 1C), suggesting a close association between *PROS* and malignant status. *PROS* expression was even more robust in experimentally induced castration-resistant LNCaP cells (Fig. 2E), further validating the potential involvement of *PROS* in the pathogenesis of CRPC. Of note, *PROS* expression appears to be not closely correlated with androgen receptor expression (Fig. 1B, C, and D). We reason that additional genetic modifications during PCa progression might be responsible for this non-perfect correlation.

Accumulated evidence points to a frequent participation of tyrosine kinases in CRPC. For example, protein tyrosine kinases have been implicated in supporting AR activation under castrate conditions (Kung 2011). CRPC develops mechanisms that reactivate the AR axis via oncogenic pathways, in which tyrosine kinases have a crucial role (Egan et al. 2014). The Lyn tyrosine kinase, a member of the src family, regulates AR stability and transcriptional activity, thus accelerating CRPC progression (Zardan et al. 2014). Reciprocally, evidence from both experimental and clinical studies suggest that pharmaceutical inhibition using small-molecule receptor tyrosine kinase inhibitors (such as lapatinib and sunitinib) represents a therapeutic relevant strategy for CRPC treatment (Whang et al. 2013, Basch et al. 2014, Nishikawa et al. 2015, Shiota et al. 2015). Earlier *in vitro* studies have displayed that PROS is the ligand for tyrosine-protein kinase receptor TYRO3 (Deng et al. 2012). Recent studies have demonstrated that PROS is also a biologically relevant ligand for tyrosine-protein kinase MER and TYRO3 receptors among different systems (Recarte-Pelz et al. 2013). In this context, deregulation of tyrosine kinases or their receptors may at least in part explain the elevated expression of PROS in CRPC, although the mechanisms whereby PROS expression is up-regulated in human castration-resistant PCa-like cells remain to be further defined.

PROS acts as an anti-apoptotic factor in distinct cell types. Specifically, it can inhibit N-methyl-D-aspartate (NMDA) excitotoxicity in neuron by activating the PI3K/Akt pathway that blocks the key steps in the extrinsic apoptotic cascade (Guo et al. 2011). Because androgen deprivation treatment induces androgen-sensitive prostate epithelial cells to undergo apoptotic cell death, it is therefore a logical hypothesis that PROS may directly participate in the androgen deprivation-induced cell death in PCa. Our results showed that overexpression of *PROS1* promoted cell proliferation and protected androgen-sensitive LNCaP cells from apoptotic cell death induced by androgen deprivation (Fig. 3). Consistently, *PROS1* knockdown in PC3 cells inhibited the ability to resist apoptosis and thereafter resulted in decreased migration and invasiveness (Fig. 4A, B, C and D). Consistently in our xenograft experiment, ablation of PROS expression resulted in a significant tumor growth inhibition in castrated immunodeficient mice (Fig. 4E). These data collectively support our emerging view that PROS probably serves as a key apoptotic modulator during the pathogenesis of CRPC.

Compelling evidence has established that the PI3K-AKT-mTOR pathway plays a critical role in PCa cell survival. Deregulated AKT activity has been frequently reported in CRPC progression (Kaarbo et al. 2010, Chang et al. 2014, Fisher et al. 2015). Although prostate-specific knockout of PTEN (phosphatase and tensin homolog) leads to invasive PCa and ultimately to metastatic cancer in mice, loss-of-function PTEN mutations are detected in less than 5% of primary PCa, suggesting that additional mechanisms might be responsible for activation of the PI3K-AKT-mTOR pathway in PCa (Ellis et al. 2013, Zhang et al. 2014b). Our findings extend these understanding by identifying PROS as a potent upstream regulator of AKT signaling in CRPC. Two lines of observations may support this assumption: (i) PROS expression in different PCa cell lines was irrelevant to PTEN status (Fig. 1); (ii) pharmacological inhibition of AKT activity promotes apoptosis in LNCaP cells stably overexpressing *PROS1* both in androgen-supported and androgen-deprived conditions (Fig. 6B). However, oxidative stress is inherent in PCa cells and is increased in CRPC (Zhang et al. 2014b). In particular, castration, the major approach for the treatment of late-stage prostate cancer, significantly increases oxidative stress of PCa (Shiota et al. 2011). However, how castration-induced oxidative stress contributes to CRPC is still elusive. Herein, we showed that oxidative stress led to PROS up-regulation in PC3 cells (Fig. 5A), and the elevated PROS expression turned out to be a critical molecule that links oxidative stress and activation of the PI3K-AKT-mTOR pathway (Fig. 5C), two hallmarks of CRPC. Of note, 1 mmol/L H₂O₂ treatment might not be physiologically relevance. Additional experiments without H₂O₂ treatment further confirmed that oxidative stress-elicited PROS expression is a general phenomenon (Fig. 5B). These results together argued that the potential mechanism underlying PROS action may be due to control of oxidative stress-elicited activation of PI3K-AKT-mTOR pathway. To be noted, because AKT signaling is a main pathway for cell survival and this pathway is so frequently deregulated in human PCa, our current study could not rule out the possibility that abolishing the *PROS1* overexpression-induced cell growth is due to the general effect of inactivation of AKT signaling. Employment of a prostate-specific PROS transgenic mouse along with a prostate-specific Akt knockout mouse will be helpful to understand the exact role of AKT signaling in PROS action, which merits further investigation.

In summary, we showed here for the first time that PROS level is significantly elevated in androgen-insensitive PCa cells and that PROS promotes castration resistance in

human castration-resistant PCa-like cells via its apoptosis-regulating property. A major pathway in this response is the direct control of oxidative stress-elicited activation of PI3K-AKT-mTOR pathway, which in turn governs the ability of PROS to reduce androgen deprivation-induced apoptosis in PCa cells (Fig. 6C).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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