

Tissue Prostate-Specific Antigen Facilitates Refractory Prostate Tumor Progression via Enhancing ARA70-Regulated Androgen Receptor Transactivation

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Abstract

Despite being well recognized as the best biomarker for prostate cancer, pathophysiologic roles of prostate-specific antigen (PSA) remain unclear. We report here that tissue PSA may be involved in the hormone-refractory prostate cancer progression. Histologic analyses show that the increased tissue PSA levels are correlated with lower cell apoptosis index and higher cell proliferation rate in hormone-refractory tumor specimens. By stably transfecting PSA cDNA into various prostate cancer cell lines, we found that PSA could promote the growth of androgen receptor (AR)-positive CWR22rv1 and high-passage LNCaP (hormone-refractory prostate cancer cells) but not that of AR-negative PC-3 and DU145 cells. Surprisingly, the protease activity of PSA is not crucial for PSA to stimulate growth and promote AR transactivation. We further showed that increased PSA could enhance ARA70-induced AR transactivation via modulating the p53 pathway that results in the decreased apoptosis and increased cell proliferation in prostate cancer cells. Knockdown of PSA in LNCaP and CWR22rv1 cells causes cell apoptosis and cell growth arrest at the G₁ phase. *In vitro* colony formation assay and *in vivo* xenografted tumor results showed the suppression of prostate cancer growth via targeting PSA expression. Collectively, our findings suggest that, in addition to being a biomarker, PSA may also become a new potential therapeutic target for prostate cancer. PSA small interfering RNA or smaller molecules that can degrade PSA protein may be developed as alternative approaches to treat the prostate cancer. [Cancer Res 2008;68(17):7110–9]

Introduction

Prostate-specific antigen (PSA), a member of the kallikrein gene family, is a serine protease with chymotrypsin-like activity that is expressed mainly in the prostate (1). Extensive evidence indicates that PSA and other tissue kallikrein members are involved in hormone-related tumorigenesis (2), including ovarian cancer (3), breast cancer (4), prostate cancer, lung adenocarcinoma (5),

pancreatic ductal adenocarcinomas (6), and lymphoblastic leukemia (7). PSA has been known as the best biomarker for monitoring prostate cancer progression (8). In prostate tissue, PSA expression levels in prostate tissue are correlated with clinical stages and histologic grades of prostate cancer (9). In localized prostate cancer, locally advanced (T3) tumors produced higher tissue PSA than organ confined (T2) tumors, whereas metastatic tumors have relatively low PSA expression (9). Early data suggested that PSA might promote prostate cancer growth and metastasis via its protease activity to digest insulin-like growth factor-binding protein-3 (IGFBP-3; ref. 10) or hydrolyze several extracellular matrixes (11).

The androgen receptor (AR) is the primary regulator of PSA expression through androgen response elements (ARE) located in the PSA promoter (12). PC-3 cells with stably transfected AR, but not parental PC-3 cells lacking AR protein, could express PSA (13). Therefore, during the hormone treatment sensitive state, surgical or medical castration can significantly reduce PSA expression in prostate cancer tissue. But in the hormone-refractory cancer, although AR still exists (14), the abnormal PSA elevation may have little linkage with AR status. It has been reported that, in addition to androgens, PSA expression may be induced by glucocorticoids (15), progestin (16), and Ets transcription factors (17). However, the significance of androgen/AR-independent PSA expression remains unclear.

Here, we find that PSA may promote hormone-refractory prostate cancer growth via enhancing ARA70-induced AR transactivation without involving its protease activity. Under the treatment of hydroxyflutamide (HF) or $\Delta 5$ -androstenediol (Adiol), the increased tissue PSA may activate ARA70/AR transcription function, which may then result in tumor cell survival in the hormone-refractory tissue. Therefore, targeting tissue PSA by PSA small interfering RNA (siRNA) or smaller molecules may be developed as alternative approaches to suppress prostate cancer growth.

Materials and Methods

Cell cultures, transient DNA transfection, and promoter reporter assay. All cell lines were obtained from the American Type Culture Collection. The COS-1 and PC-3 cells were maintained at 37°C in DMEM (Life Technologies) supplemented with 10% charcoal-deprived serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin under 5% CO₂. High-passage LNCaP cells were grown in RPMI 1640 (Life Technologies) with 10% charcoal-deprived serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin under 5% CO₂. 5 α -Dihydrotestosterone (DHT) and $\Delta 5$ -androstenediol (Adiol) were obtained from Sigma and HF was from Schering. For details of transfection and promoter reporter assay, please see our publications (18, 19). In brief, COS-1, PC-3, DU145, LNCaP, and CWR22rv1 cells, grown in appropriate medium at 1×10^5 to 4×10^5

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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cells in 24-well plates, were transfected with indicated plasmids using SuperFect (Qiagen).

Plasmids. pSG5-AR, pSG5-ARA70N (NH₂ terminus), pSG5-ARA70f (full length), pGEX-GST-ARA70, MMTV-Luc, pVP16-ARA70, and pGL-TK were constructed as described previously (18, 19). Forward primer 5'-GCGG-ATCCGGGGAGCCCAAGCTTACC-3' and reverse primer 5'-CGTCTA-GAGGGTGCTCAGGGGTTGGC-3' were used to PCR amplify full-length PSA cDNA and cloned into *Bam*HI- and *Xba*I-digested pCDNA3 vector. The mutant pCDNA-PSA (S213A) plasmid was generated by mutating the 213th amino acid residue from serine to alanine using QuikChange XL Site-Directed Mutagenesis kit (Stratagene). The siRNA target sites for PSA and ARA70 [GTGGATCAAGGACACCATC (753-771) and GAGGAGACACTTCAA-CAGC (384-402)], respectively, were selected by OligoEngine siRNA designing software. The negative controls for each of them were generated as scramble siRNA. The positive controls were applied following "knockin after knockdown" strategy by generating pCDNA3-PSAkb and pSG5-ARA70kb plasmid, respectively. pCDNA3-PSAkb was generated from parental pCDNA3-PSA plasmid by mutating PSA siRNA target sequence to GTGGATCAAAAACACCATC (753-771), whereas pSG5-ARA70kb was generated from parental pSG5-ARA70 plasmid by mutating ARA70 siRNA target sequence to GAGGAGACACCCCAACAGC (384-402). All the constructs were verified by DNA sequencing.

Immunohistochemical staining of clinical cancer tissues. Hormone-refractory prostate cancer specimens, defined by failure following HF treatment and acquired by transurethral resection of the prostate management of urethral obstruction, were chosen for the study by evaluating the clinical records. They included 20 no hormone treatment, 4 hormone-refractory, and 15 HF treatment sensitive prostate cancer specimens. All samples were collected from the Department of Pathology of the Tianjin Institute of Urological Surgery and the Sir Run Run Shaw Hospital of Zhejiang University Medical School.

Samples were fixed in formalin and embedded in paraffin. The AR, ARA70, and PSA protein expression levels were determined by immunohistochemical method in four pairs of samples. The rabbit anti-PSA polyclonal antibody (DAKO), the mouse anti-ARA70 antibody, and the rabbit anti-AR (N20) antibody (Santa Cruz Biotechnology) were used in immunohistochemical staining. The bound primary antibody was recognized by the biotinylated secondary antibody (Vector) and visualized by Vectastain avidin-biotin complex peroxidase system and peroxidase substrate 3,3'-diaminobenzidine kit (Vector). The positive staining signals were semiquantitated by Image J software.

Establishment of stably transfected cell lines. CWR22rv1, high-passage LNCaP, PC-3, and DU145 cells were cultured to the mid- or late-logarithmic phase of growth. After trypsinization, the cells were resuspended and washed twice in 2.5% fetal bovine serum (FBS) medium without antibiotics. We then transferred 400 μ L of the cell suspension (10^7 cells) into the electroporation cuvettes (VWR), set the voltages of the electroporator (Bio-Rad Gene Pulser II) to 300V and hinge capacity to 950 μ F, added 20 μ g of total plasmid DNA to each cuvette, and incubated for 5 min at room temperature. After pulse charge, the cells were incubated on ice for 5 min and transferred to a 35-mm culture dish. After culturing in complete medium for 72 h, the transfected cells were cultured in the appropriate selection medium and medium was changed every 3 d for 2 to 3 wk until colonies of resistant cells formed.

For pCDNA3-PSA-transfected cells, we used 1,000 μ g/mL neomycin for selection, whereas for p^{Superior}-siPSA- or p^{Superior}-siARA70-transfected cells we used 5 μ g/mL puromycin.

Cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and cell cycle flow cytometry. Cells of stable transfected LNCaP, CWR22rv1, PC-3, and DU145 sublines were seeded in 24-well plates at a density of 5,000 per well in medium containing 10% charcoal-deprived FBS with or without 1 nmol/L DHT. At the indicated time point, medium was removed and serum-free medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/mL; Sigma) was then added into each well. Four hours after incubation at 37°C, cellular formazan product was dissolved with acidic isopropanol, and the absorbance at 595 nm was measured by spectropho-

tometry (Beckman Du640B). We used six replicate wells for each sample at each time point. α -Antichymotrypsin (MP Biomedicals, Inc.) at a concentration of 1,000 ng/mL was used as a PSA proteinase inhibitor to treat the LNCaP cell sublines. For the cell cycle flow cytometry assay, we digested the cells by trypsin-EDTA, harvested as many as 1×10^6 cells, and fixed them in 70% ethanol at 4°C. After 12 h, cells were centrifuged ($1,000 \times g$, 7 min, 4°C), resuspended in PBS containing 0.05 mg/mL RNase A (Sigma), and then incubated at room temperature for 30 min. After washing and staining with 10 mg/mL propidium iodide, cells were filtered through a 60- μ m mesh, and 10,000 cells were analyzed by flow cytometry (FACSCalibur, BD Company) with ModFit software (Verity Software House, Inc.).

Cell death analysis (7-aminoactinomycin D staining) and caspase-3 activation. 7-Amino-actinomycin D (7-AAD; Sigma) was dissolved in acetone, diluted in PBS at a concentration of 200 μ g/mL, and kept at 20°C without light until use. 7-AAD solution (100 μ L) was added to 2.5×10^6 cells in 1 mL PBS and incubated for 20 min at 4°C in the dark. Cells were then washed and total DNA was stained with 7-AAD (20 μ L per sample) followed by flow cytometric analysis using FACSort (Becton Dickinson). To assess apoptosis, 5×10^5 cells were resuspended in PBS and analyzed by flow cytometry. The total DNA content (7-AAD) was determined using CellQuest (Becton Dickinson) and FCS Express software (De Novo Software). We challenged LN-vehicle and LN-PSA cells with 1 nmol/L 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 1 nmol/L DHT for 24 h to induce apoptosis and detected cell apoptosis by 7-AAD flow cytometry. Simultaneously, we measured caspase-3 activity using Western blot by detecting its activated subunits, 17 and 12 kDa (Rabbit Anti-Active Caspase-3, BD Biosciences).

RNA extraction, reverse transcription, and real-time quantitative PCR. Total RNA (5 μ g) was extracted using Trizol and reverse transcribed into 20 μ L cDNA immediately by the SuperScript III kit (Invitrogen) with oligo(dT) primer. Real-time quantitative PCR was performed on iCycler IQ multicolor real-time PCR detection system with 1.5 μ L cDNA amplified by SYBR Green PCR Master Mix. We designed primers by Beacon Designer 2 software as follows: *NKX3.1*, 5'-ATGGTTCAGAACAGACGCCTAT-3' (forward) and 5'-TGCCACGCAGTACAGGTAT-3' (reverse); *PSP94*, 5'-TCCTGG-GCAGCGTTGTGA-3' (forward) and 5'-TTGGGTGTTTCTTCCTTTGAG-3' (reverse); *PSMA*, 5'-AAGGAAGGGTGGAGACCTAG-3' (forward) and 5'-ACT-GAACTCTGGGAAGGAC-3' (reverse); β -actin, 5'-TGTGCCATCTACGAGG-GGTATGC-3' (forward) and 5'-GGTACATGGTGGTCCGCCAGACA-3' (reverse). The β -actin expression was used as internal control. We calculated Δ threshold (C_T) values by subtracting the control C_T value from the corresponding β -actin C_T from each time point. We confirmed the absence of nonspecific amplification products by agarose gel electrophoresis.

Tumorigenesis in nude mice. Ten athymic male nude mice were castrated at 12 wk old. One week following castration, we injected 5×10^7 high-passage LNCaP and LN-siPSA cells into the left and right dorsal part of these nude mice, respectively. Twelve weeks later, the nude mice developed obvious xenograft tumors. We harvested the tumors, measured the size, and determined the PSA protein level.

Glutathione S-transferase pull-down assay. We transformed the pGEX-GST-ARA70 plasmid into BL21-CodonPlus Competent cells (Stratagene) to express glutathione S-transferase (GST) fusion ARA70 protein and purified the fusion protein with glutathione beads. We incubated the radiolabeled PSA protein for 2 h with the GST-ARA70 fusion protein attached to glutathione beads. The protein complex beads were washed four times, resuspended in SDS-PAGE loading buffer, and resolved on 12% SDS-PAGE gel followed by autoradiography.

Coimmunoprecipitation. We incubated 500 μ g protein from LNCaP cell lysates with 2 μ g anti-ARA70 monoclonal antibody or normal mouse IgG for 4 h at 4°C with agitation. To each sample we added protein A/G plus agarose (50 μ L), incubated for 1 h, and washed thrice with radioimmunoprecipitation assay (RIPA) buffer. We resolved the complex on a 10% SDS-polyacrylamide gel, transferred to the membrane, and blotted with anti-ARA70, anti-AR, or anti-PSA monoclonal antibody, respectively. Immuno-responsive bands were developed by an alkaline phosphatase detection kit (Bio-Rad).

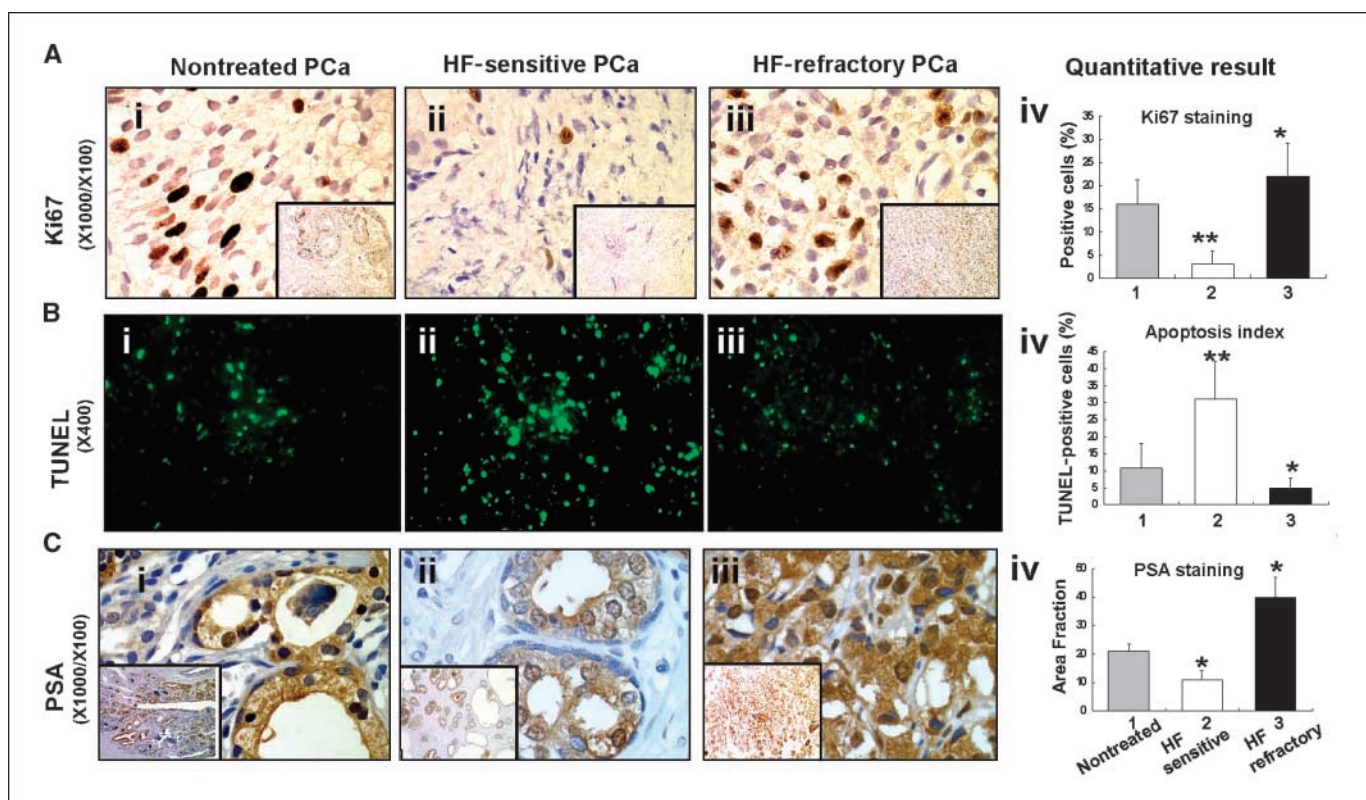


Figure 1. The growth of human prostate tumors is correlated with their tissue PSA levels. Histologic analyses of the cell proliferation marker Ki67, cell apoptosis (TUNEL), and the tissue expressions of PSA in non-hormone-treated and HF-treated sensitive and refractory prostate tumor specimens. *A*, immunohistochemical staining for Ki67 shows lower levels of cell proliferation in HF-sensitive (*ii*) and higher levels of cell proliferation in HF-refractory prostate cancer (PCa) specimens (*iii*) compared with non-hormone-treated specimens (*i*), as shown by counting the percentage of positive staining cells with results from the average of a total of 30 fields on each specimen (*iv*). *B*, TUNEL assay shows that the cell apoptosis signals in HF-sensitive tumors (*ii*) are higher than those in non-hormone-treated tumors (*i*) and refractory tumors (*iii*). *C*, immunohistochemical staining shows that tissue PSA levels are higher in hormone-refractory tumors (*iii*) and lower in HF-sensitive tumors (*ii*) compared with non-hormone-treated tumors (*i*). The increased tissue PSA levels in the hormone-refractory tumors are correlated with higher cell proliferation and lower apoptosis index in those samples. The Ki67, TUNEL, and PSA stainings are quantitated by Image J software (*iv*, $P < 0.01$).

Western blot analysis of AR, PSA, ARA70, p53, and others. Cell were lysed in RIPA buffer, separated on SDS-10% PAGE gel, and then transferred to a polyvinylidene difluoride membrane. After blocking by 5% nonfat milk and 5% FBS in PBST buffer, we immunoblotted the membrane with the primary antibody followed by incubation with alkaline phosphatase-conjugated second antibody (Santa Cruz Biotechnology). Our laboratory generated the monoclonal anti-ARA70 antibody. The rabbit polyclonal anti-AR (N20) and mouse monoclonal anti-PSA antibodies, rabbit anti-cyclin-dependent kinase (cdk) 2, rabbit anti-cyclin D1, rabbit anti-p21, rabbit anti-proliferating cell nuclear antigen (PCNA), rabbit anti-RFC1, rabbit anti-bax, mouse anti-tubulin, and goat anti- β -actin were from Santa Cruz Biotechnology. Anti-p53 monoclonal and anti-bcl2 monoclonal antibodies were from DAKO. Rabbit anti-phospho-p53 (Ser³⁹²) antibody was from Cell Signaling Technology, Inc. We detected PSA protein amounts in equal amounts of total proteins from cell lysates and equal volumes of concentrated cultured medium.

Colony formation assay. We determined the cell survival of LN-PSA and LN-siPSA in a clonogenic assay. Briefly, we plated cells (200 per well) in six-well plates and cultured them in normal medium for 2 wk. Then, we fixed and stained the cells with 0.25% crystal violet in 80% methanol for 30 min, washed them with water, and counted the number of colonies that contained >50 cells. We determined the plating efficiency as the fraction of cells that were attached to the plate and grew into colonies larger than 1 mm in diameter.

Results

Accelerated growth of the hormone-refractory prostate tumor correlates with the increased tissue PSA expression.

By histologic analysis of Ki67 expression in the refractory prostate tumor specimens that were treated with HF, we found that the cell proliferation signals were higher in the hormone-refractory tumors than those in the hormone-sensitive tumors (Fig. 1*A*). In addition, the cell apoptosis index of the hormone-refractory tumors was lower than those of the hormone-sensitive tumors using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Fig. 1*B*). Tissue PSA levels are significantly higher in the hormone-refractory tumors than those in hormone-sensitive tumors (Fig. 1*C*). Higher PSA levels (Fig. 1*C*, *iv*) in the hormone-refractory tissues are coincident with higher proliferation rates and lower apoptosis index in these tissues ($P < 0.05$; Fig. 1*A* and *B*, *iv*).

PSA increases cell growth in AR-positive LNCaP and CWR22rv1 cells but not in AR-negative PC-3 and DU145 cells.

To test the PSA effects on the growth of prostate cancer cells, we stably transfected PSA cDNA into high-passage number LNCaP (named LN-PSA), CWR22rv1 (named CWR-PSA), PC-3 (named PC3-PSA), and DU145 cells (named Du-PSA). Western blot was applied to examine the expression of ARA70 and PSA (Supplementary Fig. S2*A* and *B*) in parental cells and overexpression of PSA in LNCaP, CWR22rv1, PC-3, and DU145 cells. The growth rates of high-passage number ($n > 70$) of LNCaP and CWR22rv1 cells are not sensitive to the androgen. Therefore, those two cell lines could represent AR-positive hormone-refractory prostate cells. The cell viability assays by MTT showed that addition of PSA

resulted in the increased number of living cells in AR-positive LNCaP and CWR22rv1 (Fig. 2A and B, left) cell lines but not in AR-negative PC-3 and DU145 (Fig. 2C and D, left) cell lines with 1 nmol/L DHT (human prostate DHT concentration after androgen deprivation therapy). The data of cell number differences between controlled groups were further interpreted into the different patterns of cell cycle distribution (Fig. 2A-D, middle) and cell death by flow cytometry analyses (Fig. 2A-D, right). Overexpression of PSA in LNCaP and CWR22rv1 cells (LN-PSA and CWR-PSA) resulted in the decreased G_0 - G_1 phase from 69.1% to 54.7% and 49.2% to 40.4% and increased S phase from 18.8% to 29.4% and 37.5% to 44.2% (Fig. 2A and B, middle), respectively. However, ectopic PSA expression in PC-3 and DU145 cells (PC3-PSA and Du-PSA in Fig. 2C and D, middle) had little influence on the cell cycle. Meanwhile, increased PSA expression in LN-PSA and CWR-PSA (Fig. 2A and B, right) cells resulted in the decreased cell death, whereas overexpression of PSA in PC3-PSA and Du-PSA (Fig. 2C and D, right) showed little change in the cell death. These results suggest the PSA effects on the cell cycle are evident by significant reduction in G_0 - G_1 phase cell population. In contrast, the G_0 - G_1 phase population of Du-vector versus Du-PSA is 53.5%

versus 51.7%, which indicates little difference. The total amount of the cells reentering the cell cycle, by counting percentage of cells into both S and G_2 -M phases, is also similar between Du-vector and Du-PSA cells. Together with results from MTT assay, the overall cell number in proliferative (S and G_2 -M) and quiescent (G_0 - G_1) phases of the cell cycle showed no significant difference between Du-vector and Du-PSA cells, suggesting that adding PSA had little influence on the cell proliferation of DU145 cells. A similar conclusion was also reported previously by Denmeade and colleagues (11), showing that PSA had little effect on the DU145 cell growth. The different PSA effects between different prostate cancer cell lines, which express AR differently, indicate that the growth stimulation activity of PSA could be an AR-dependent event.

Knockdown of endogenous PSA via siRNA results in the suppression of cell growth. To further confirm the PSA effects on the growth of AR-positive prostate cancer cells, we stably transfected PSA siRNA into high-passage LNCaP (named LN-siPSA; Fig. 3A) and CWR22rv1 cells (named CWR-siPSA; Fig. 3C). Knockdown of endogenous PSA in LN-siPSA and CWR-siPSA cells resulted in retarded cell growth in MTT assays (Fig. 3A and C), consistent with increase in G_0 - G_1 phase from 61.6% to 79.5% and

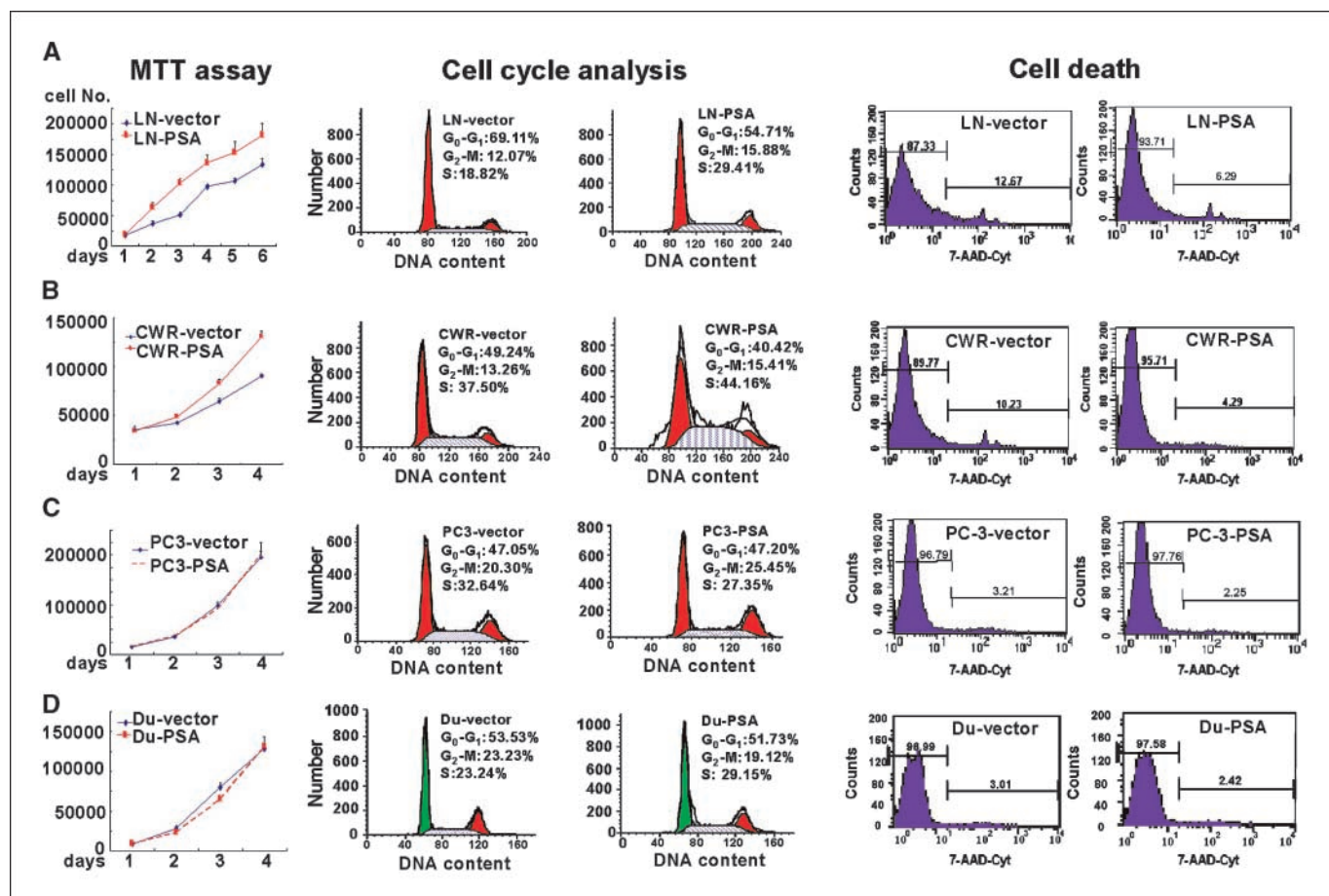


Figure 2. PSA accelerated cell growth in AR-positive but not in AR-negative prostate cancer cells. Two AR-positive hormone-refractory models, high-passage LNCaP and CWR22rv1 cells, and two AR-negative hormone-refractory models, PC-3 and DU145 cells, were stably transfected with pCDNA3-PSA. Cell viability was determined by MTT assay, which is interpreted into cell cycle and cell death analysis. **A**, stable overexpression of PSA promotes cell growth of high-passage LNCaP cells (LN-PSA) with 1 nmol/L DHT treatment using MTT assay. **Middle**, flow cytometry analysis of cell cycle by detecting propidium iodide staining reveals that the higher expression of PSA in LN-PSA cells resulted in the decreased G_0 - G_1 phase from 69.1% to 54.7% and increased S phase from 18.8% to 29.4%. Flow cytometry analysis of cell apoptosis by detecting 7-AAD staining shows fewer apoptotic cells in LN-PSA cells than control LN-vector cells. **B**, PSA has similar effects on AR-positive CWR22rv1 cell as on high-passage LNCaP cells. **C**, overexpression of PSA in PC-3 cells (PC3-PSA) does not show the change of cell growth and apoptosis compared with parental PC-3 cells transfected with pCDNA3 vector. **D**, the same conclusion was drawn in PSA-overexpressed DU145 cells (Du-PSA).

47.8% to 61.7% (Fig. 3B and D, top) and increased cell death (Fig. 3B and D, bottom, respectively).

Our results revealed that the higher expression of PSA in human prostate refractory tumors may facilitate tumor cell survival from HF treatment by resistance to cell death and accelerating cell cycle. In contrast, we found that PSA did not significantly alter the growth of AR-negative PC-3 and DU145 cell lines. Interestingly, we found that PSA, unlike other general secretory proteins (20–24), could be found in the cytosol outside of the Golgi apparatus (Supplementary Fig. S1C and D). We performed chromatin immunoprecipitation assay to analyze whether the PSA-ARA70-AR complex can bind onto the promoter of the AR target gene, and

the results suggested that PSA-ARA70-AR complex might not form on the chromosome DNA. In contrast, using yeast two-hybrid screen, we found that PSA inside the cell might function as an associated protein of ARA70 (data not shown). Using the coimmunoprecipitation assay, we also proved that PSA-ARA70-AR form a complex (Supplementary Fig. S1A and B). Confocal microscope further clearly showed the existence of this AR-ARA70-PSA complex within the same cells (Supplementary Fig. S1C). Together, these results suggested that ARA70 might be required to coordinate for the formation of this complex.

To investigate whether PSA, as an ARA70-associated protein, could go through AR signals to increase cell growth, we applied AR

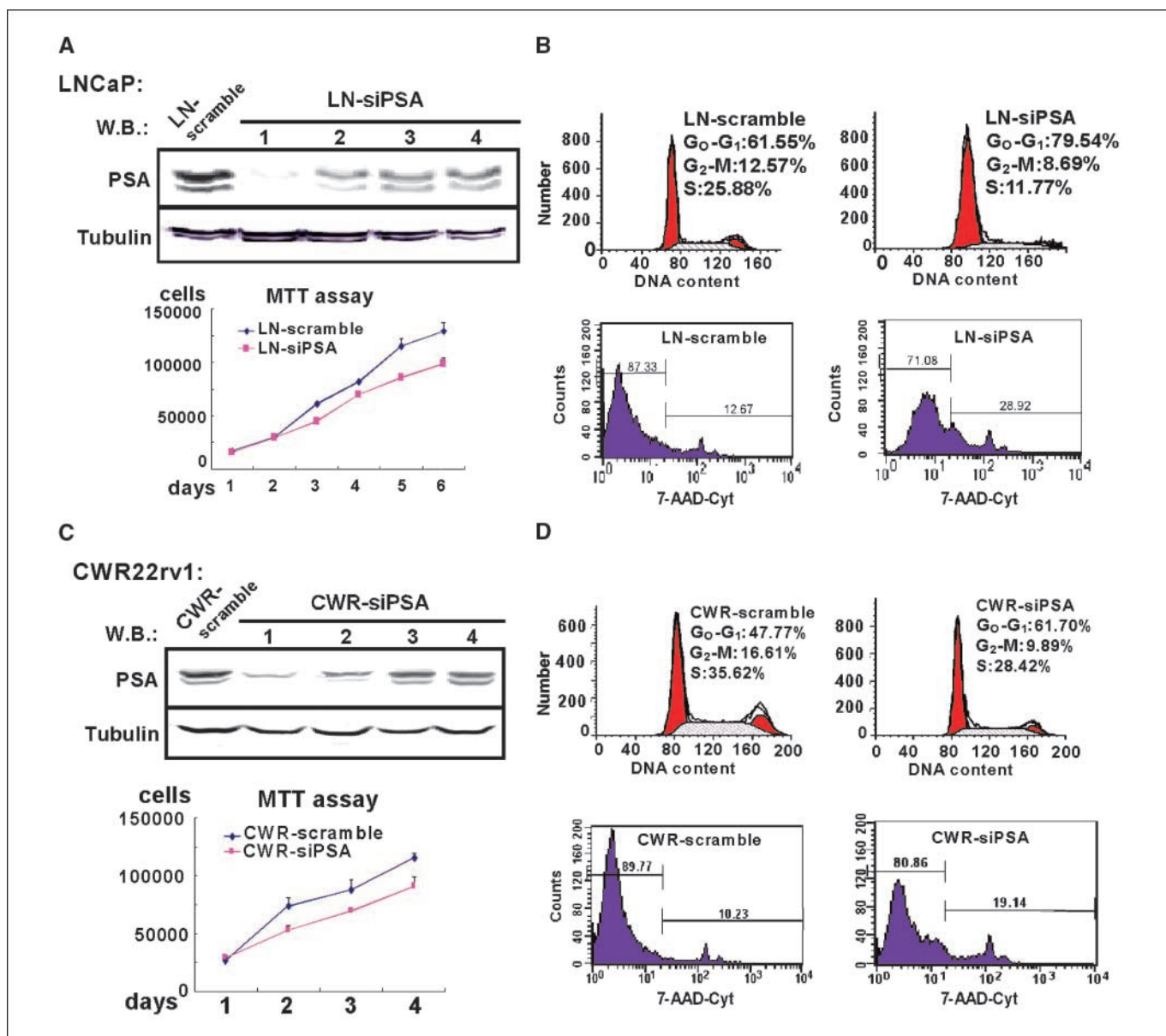


Figure 3. Knockdown of PSA reduces cell growth in AR-positive LNCaP and CWR22rv1 cells. *A*, PSA expression has been knocked down in high-passage LNCaP cells as shown by Western blotting of LN-siPSA clones 1, 2, 3, and 4 using siRNA strategy (top) and cell viability was determined using MTT assay. *B*, knockdown of endogenous PSA via PSA siRNA reduces growth rates of high-passage LNCaP cells in the presence of 1 nmol/L DHT. Our results showed that the LN-siPSA cells have the increased G_0 - G_1 phase from 61.6% to 79.5% and decreased S phase from 25.9% to 11.8% (top) and induction of apoptosis (bottom). *C*, knockdown of endogenous PSA via PSA siRNA in CWR22rv1 cells (CWR-siPSA clones 1, 2, 3, and 4). Top, knockdown of PSA level was shown by Western blot; bottom, CWR-siPSA cells have reduced growth rates using MTT assay. *D*, CWR-siPSA cells have the increased G_0 - G_1 phase and decreased S phase (top) and induction of apoptosis (bottom).

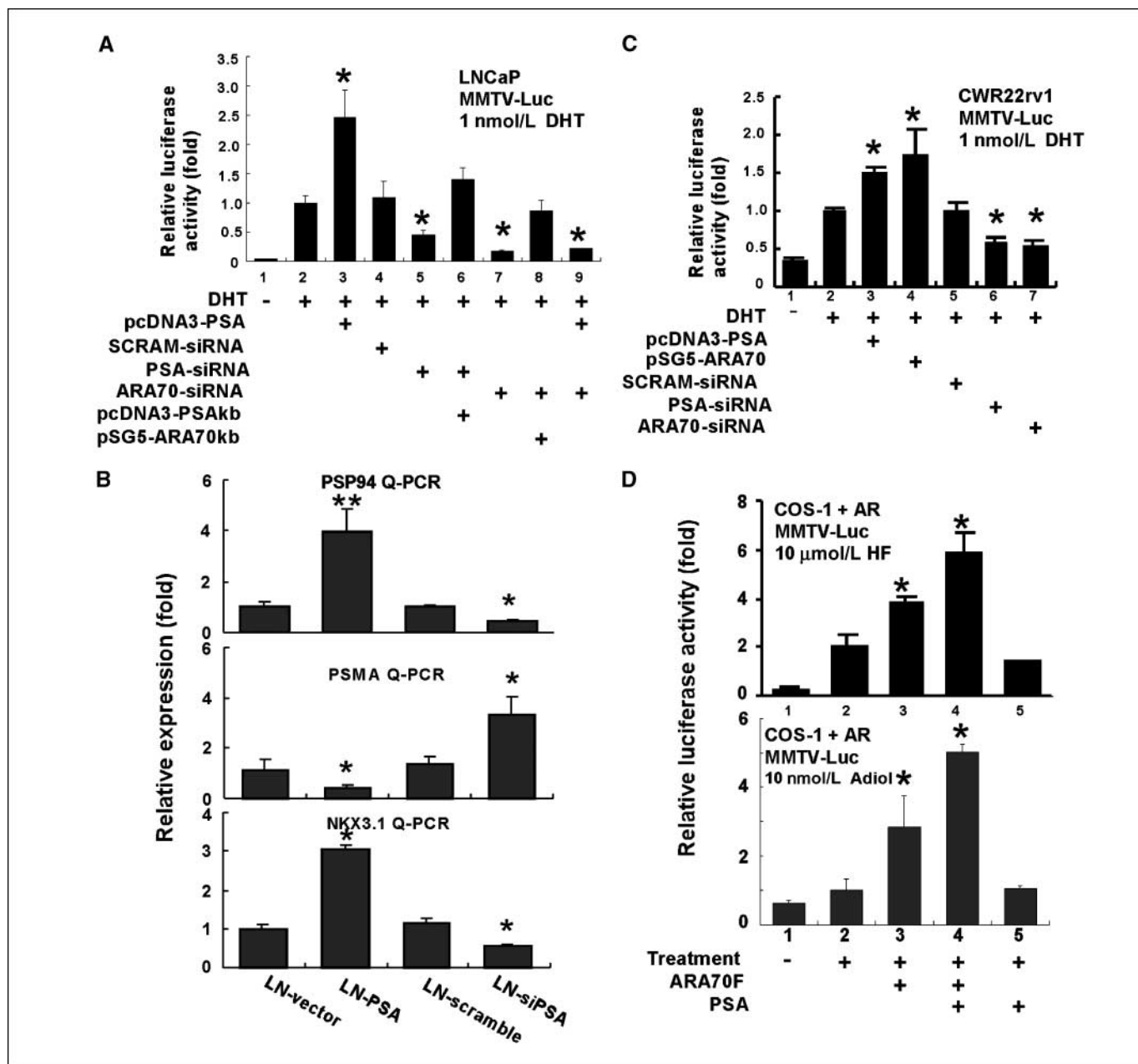


Figure 4. PSA cooperates with ARA70 to enhance AR transactivation. **A**, PSA enhances AR transactivation and PSA siRNA inhibits AR transactivation in stably transfected LNCaP cells. MMTV-luciferase activity was measured when PSA was overexpressed or knocked down in LNCaP cells. Using the LNCaP stable cell lines, AR transactivation was effectively suppressed by PSA siRNA and ARA70 siRNA and further enhanced by overexpression of PSA. **B**, PSA regulates the expression of AR target genes, *PSP94*, *PSMA*, and *NKX3.1*, in LNCaP cells in real-time PCR assay. *, $P < 0.05$, LN-PSA versus LN-scramble; **, $P < 0.01$, LN-PSA versus LN-vector. **C**, overexpression of PSA enhances AR transactivation and knockdown of PSA or ARA70 inhibits AR transactivation in CWR22rv1 cells. Using the CWR22rv1 stable cell lines, AR transactivation on MMTV-ARE luciferase reporter (MMTV-Luc) was suppressed by PSA siRNA and ARA70 siRNA and could be enhanced by overexpression of PSA. **D**, PSA and ARA70 collaboratively enhance HF- or Adiol-mediated AR transactivation. COS-1 cells were transfected with MMTV-Luc and pSG5-AR in the presence or absence of pSG5-ARA70F or pCDNA3-PSA. The cotransfection of ARA70 and PSA further triggers the 10 $\mu\text{mol/L}$ HF-induced or 10 nmol/L Adiol-induced AR transactivation.

functional study by MMTV-ARE luciferase assay. As shown in Fig. 4, addition of PSA could increase the AR transactivation in LNCaP (Fig. 4A) and CWR22rv1 cells (Fig. 4C), whereas suppression of endogenous PSA expression in LNCaP and CWR22rv1 cells reduced AR transactivation in the presence of 1 nmol/L DHT (Fig. 4A and C), normalized by both positive (Fig. 4A, lane 6) and negative control (Fig. 4A, lane 4). Furthermore, the knockdown of ARA70 by siRNA could diminish the PSA-enhanced AR activity (Fig. 4A, lane 9

versus lane 3), suggesting that ARA70 is important for the PSA-enhanced AR transactivation.

To reduce the potential artificial effects due to transient transfection assays, we stably transfected either PSA cDNA or PSA siRNA into high-passage LNCaP and CWR22rv1 cells. We then examined the PSA effects on AR transactivation in multiple sublines and results showed that PSA could further enhance AR transactivation in both LNCaP and CWR22rv1 cells (data not

shown). Using the stably transfected cell lines, we found that PSA could also induce endogenous AR-positive regulated target genes, such as *PSP94* (25) and *NKX3.1* (26), as well as suppress endogenous AR-negative regulated target genes, such as *PSMA* (Fig. 4B; refs. 27, 28). In contrast, addition of PSA siRNA into LNCaP cells results in opposite effects on AR target gene expressions (Fig. 4B). Western blot also shows that PSA and ARA70 are indeed silenced by PSA siRNA and ARA70 siRNA (Fig. 3; Supplementary Fig. S2D).

As early studies suggested that the higher expression level of ARA70 could enhance the antiandrogen HF-induced and Adiol-induced AR transactivation (29, 30), we tested whether PSA can cooperate with ARA70 to enhance HF- or Adiol-induced AR transactivation. As expected, we found that PSA could enhance the ARA70-induced AR transactivation in the presence of 10 $\mu\text{mol/L}$ HF or 10 nmol/L Adiol (Fig. 4D). These data revealed that increased tissue PSA in the hormone-refractory state (Fig. 1C, *iv*) could help tumor cell survival after treatment of HF by activating AR transcription.

To further strengthen the above results showing that PSA might need to go through the interaction with certain selective AR coregulators, such as ARA70, to induce AR transactivation, we tested whether reduced endogenous ARA70 (via siRNA) might interrupt the PSA-induced AR transactivation. As shown in both LNCaP and CWR22rv1 cells, knockdown of ARA70 by stably transfected ARA70 siRNA results in the reduction of the PSA-induced AR transactivation [Fig. 4A (*lane 9* versus *lane 3* in LNCaP)

and C (*lane 7* versus *lane 2* in CWR22rv1)], suggesting that PSA might go through interaction with ARA70 to enhance its coactivity that results in the induction of AR transactivation. These data showed that the existence of ARA70 is critical for PSA-enhanced AR transactivation.

Together, using several cell lines with either transient transfection or stable transfection of PSA, PSA siRNA, or ARA70 siRNA to assay AR transactivation or AR endogenous target gene expression, we found that PSA could promote cell growth possibly through the ARA70-induced AR transactivation.

Protease activity is not crucial for PSA to stimulate growth and promote AR transactivation. We applied two different approaches to test whether the increased cell growth via increased PSA is protease activity dependent. We first added the PSA protease inhibitor α_1 -antichymotrypsin (1,000 ng/mL) to the LN-PSA cells and parental control LNCaP cells and results showed that α_1 -antichymotrypsin has limited influence on the PSA-induced cell growth in LN-PSA cells (Fig. 5A). We then mutated the essential protease domain (213 serine to 213 alanine) that inactivates the protease activity of PSA (31) and stably transfected this mutated mPSA-S213A cDNA into LNCaP cells (LN-mPSA) and showed that PSA expression levels are comparable between LN-PSA and LN-mPSA stable cells (Fig. 5B, *top*). The results again showed that protease activity-null PSA still stimulates the growth of AR-positive prostate cancer cells (Fig. 5B). Interestingly, we also found that higher expression of wild-type (wt) PSA and mPSA could

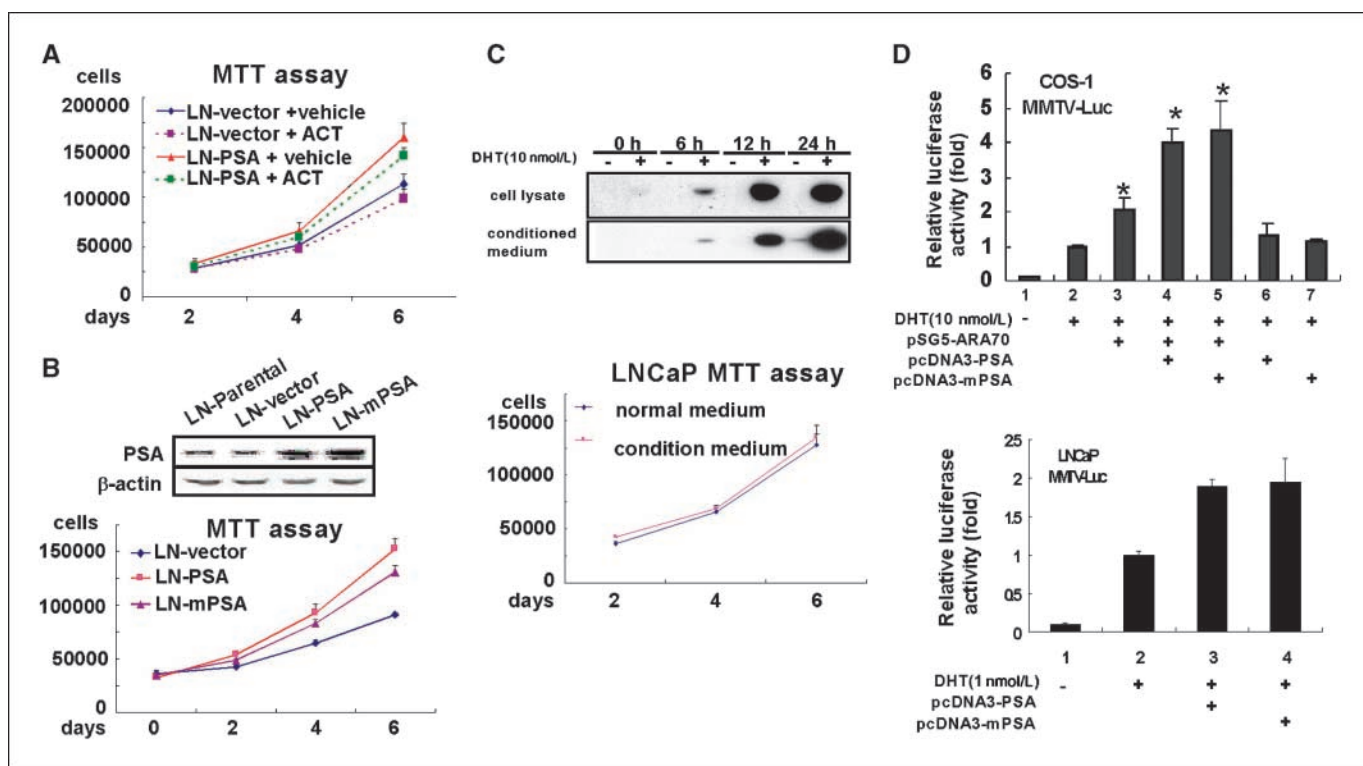


Figure 5. PSA protease activity is not crucial for its effects on cell growth and AR transactivation in prostate cancer cells. **A**, PSA-overexpressed high-passage LNCaP cells (LN-PSA) and control LN-vector cells were treated with the PSA proteinase inhibitor α_1 -antichymotrypsin (ACT; 1,000 ng/mL) for the indicated time courses. The growth of enzyme inhibitor-treated cells was not significantly changed compared with the vehicle ($1 \times$ PBS)-treated cells. **B**, both wt PSA and enzyme activity-null mutant PSA function as growth stimulators in high-passage LNCaP cells. wt and mutant PSA were stably introduced into LNCaP cells, LN-PSA and LN-mPSA, respectively. Comparable wt and mutant PSA expression levels were examined using Western blotting. The cell growth rates were determined by MTT assay. **C**, the secreted PSA does not further enhance the growth of prostate cancer cells. We have detected that the increase of secreted PSA follows the increase of cellular PSA after LNCaP cells were treated with 10 nmol/L DHT for 6, 12, and 24 h. The 24-h culture medium from LN-PSA cells was collected and used as the conditioned medium to grow LNCaP cells. The proliferation rate was compared with that of cells grown under normal medium. Cells were then collected and proliferating rates were examined using MTT assay. **D**, MMTV-Are luciferase assays show that both wt PSA and enzyme activity-null PSA can cooperate with ARA70 to enhance AR transactivation in COS-1 cells (*top*) and high-passage LNCaP cells (*bottom*).

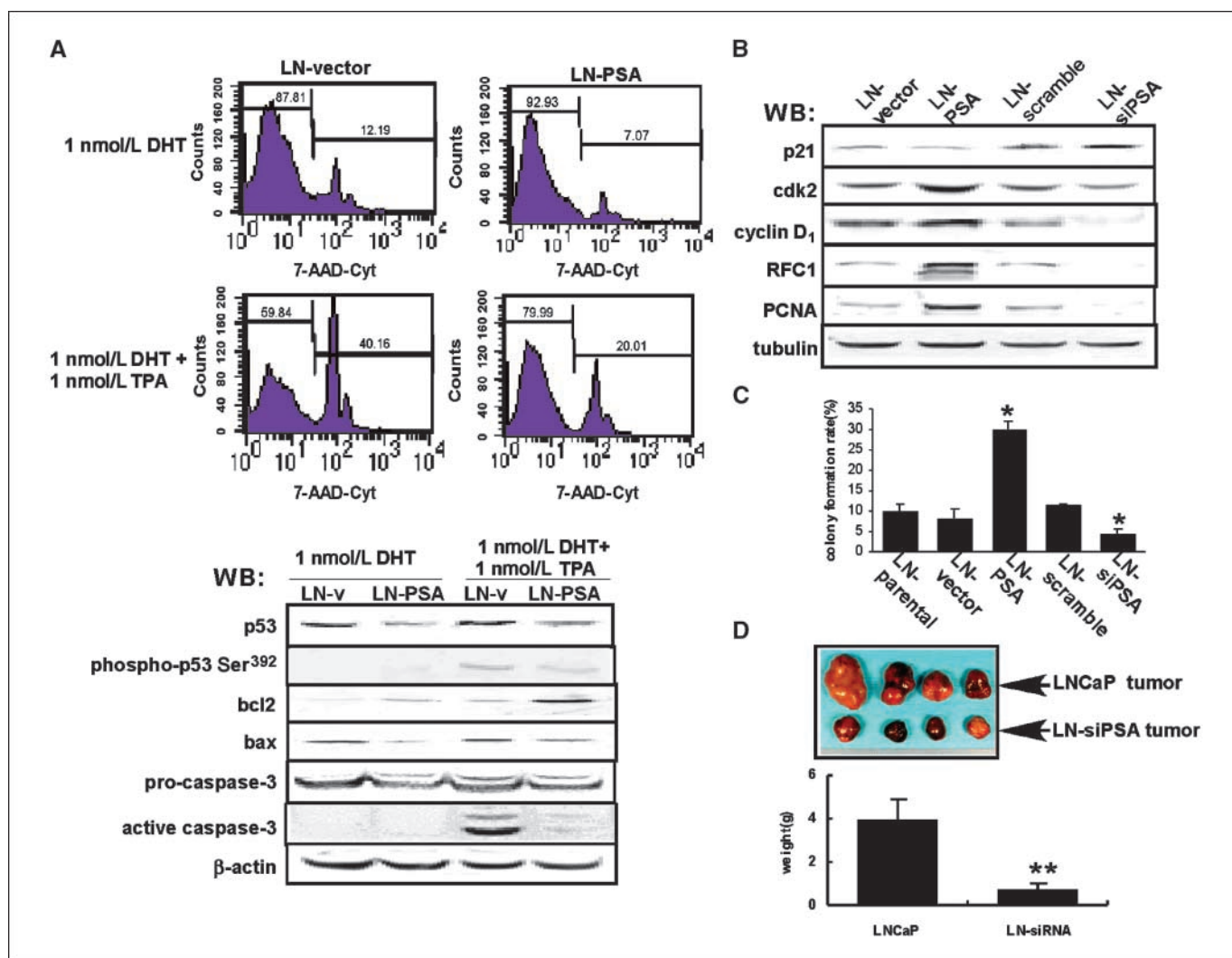


Figure 6. PSA-ARA70-AR may modulate prostate cancer cell death and proliferation via regulating p53 and cdk2/cyclin D1 expression. *A*, the differences of the apoptosis signal between LN-PSA and LN-vector are further magnified by treatment with 1 nmol/L TPA. Following 24-h challenge with 1 nmol/L TPA, the expression of apoptosis-associated proteins bcl2, bax, and total and active forms of p53 (phospho-p53 Ser³⁹²) in LN-PSA and LN-vector cells were analyzed by Western blotting. Two caspase-3 activated subunits, 17 and 12 kDa, were also measured. Overexpression of PSA in LN-PSA cells significantly reduces apoptosis induced by 1 nmol/L TPA via decreasing total p53 expression. *Bottom*, the consequence of reduced total p53 expression may result in decreased phospho-p53 levels, elevated bcl2/bax ratio, and diminished active caspase-3 in LN-PSA cells. *B*, PSA expression status affects the expression levels of cell cycle proteins (p21, cdk2, and cyclin D1) and cell proliferation markers (PCNA and RFC1) in AR-positive prostate cancer cells. Western blot analyses show that overexpression of PSA in high-passage LNCaP cells results in higher expression of p21, cdk2, cyclin D1, ORC1, and RFC1, whereas knockdown of PSA has opposite effects on the markers. *C*, colony formation assays of high-passage LNCaP cells showed that PSA affects the colony-forming capacity and cell viability of prostate cancer cells. Colonies with cell numbers higher than 50 were counted. *D*, PSA expression level is positively correlated with the tumorigenicity of prostate cancer xenografts. The high-passage LNCaP cells expressed higher levels of PSA and generated larger-size xenograft tumors than LN-siPSA cells. Equal numbers of high-passage LNCaP and LN-siPSA cells were mixed with Matrigel and then inoculated into the left and right flanks of precastated athymic nude mice, respectively. Tumors were harvested and weighed 12 wk after the xenograft implantation. Four representative tumors from LNCaP and LN-siPSA were shown. The average tumor weights were quantitated ($n = 10$ for each group).

increase the LNCaP cell growth (Fig. 5*B*, *bottom*). Although mPSA is slightly less effective than wt PSA to enhance the prostate cancer cell growth, the difference in cell number between LN-PSA and LN-mPSA groups is not as dramatic as that between LN-mPSA and LN-vector, suggesting that PSA protease activity may have few, yet not significant, effects on LNCaP growth. Furthermore, we detected both cytosol PSA and secreted (into medium) PSA 24 h after adding 10 nmol/L DHT to the LNCaP cells with the passage number less than 50, which still respond to the DHT stimulation (Fig. 5*C*, *top*). However, adding these media with secreted PSA into LN-PSA cells results in little influence on the cell growth (Fig. 5*C*), suggesting that the PSA-induced cell growth effect might be elicited by the PSA existing inside the LN-PSA cells (named Tissue-PSA).

Consistent with cell viability data, ectopic expression of the mutated PSA (mPSA-S213A) also enhances the ARA70-induced AR transactivation in COS-1 cells (Fig. 5*D*, *top*) and LNCaP cells (Fig. 5*D*, *bottom*). These results clearly showed that PSA, without its protease activity, could enhance ARA70-induced AR transactivation and stimulate cell growth.

Mechanisms by which PSA-enhanced ARA70-induced AR transactivation results in the increased prostate cancer cell growth. Early studies suggested that androgen/AR might induce cell growth via modulation of p53-mediated cell growth arrest and apoptosis (32–34). Other studies also showed that AR could modulate p53 expression (35) via several key factors, such as MDM2, HoxA5, and Egr-I. We therefore hypothesized that

PSA-enhanced ARA70-induced AR transactivation might result in the increased cell growth via modulation of p53-mediated cell growth arrest and apoptosis. We first challenged the LN-vector control cells and LN-PSA cells with 1 nmol/L TPA and 1 nmol/L DHT, a condition that was reported previously to accelerate cell apoptosis (36). Western blot analysis was also used to examine the apoptosis-related markers and showed lower expression of p53 and bax and higher expression of bcl2 in LN-PSA cells compared with control LN-vector cells (Fig. 6A). Furthermore, lower expression of p53 was consistent with lower phosphorylation of p53 at Ser³⁹² and lower activated form of caspase-3 (Fig. 6A) in LN-PSA cells treated by TPA compared with LN-vector cells.

Under environmental changes, such as DNA damage or oxidative stress, p53 can be activated/stabilized to modulate a series of genes that facilitate cell cycle arrest and apoptosis (33). Functioning as a key downstream target of p53, the p21 might mediate G₁ arrest via inhibition of cdk2 (33, 37). We found that LN-PSA cells with decreased G₁ phase expressed a lower p21 and higher cdk2, cyclin D1, PCNA, and RFC1, whereas LN-siPSA cells with G₁ arrest expressed a higher p21 and lower expression of cdk2, cyclin D1, PCNA, and RFC1 compared with parental LNCaP cells (Fig. 6B). Together, these results show that PSA might go through the AR-p53 pathway to promote cell growth via the G₁-S cell cycle checkpoint.

PSA as a potential new therapeutic target to control prostate cancer growth. All the above data indicate that PSA can induce cell growth via ARA70/AR→p53→cell apoptosis and G₁ arrest, which suggests that PSA might become a new therapeutic target to treat the prostate cancer. To test this hypothesis, we applied different approaches to see if reducing endogenous PSA expression can result in the suppression of prostate cancer growth. Using the MTT cell viability assay, we first found that stably transfecting PSA siRNA into high-passage LNCaP cells (LN-siRNA) and CWR22rv1 cells (CWR-siRNA) results in suppression of cell growth in the presence of 1 nmol/L DHT (Fig. 3). Using colony formation assays, we also found more colonies in LN-PSA cells, and less colonies in LN-siPSA cells, compared with control LNCaP cells (Fig. 6C). Finally, *in vivo* tumor growth assays using xenografted LNCaP (into the left flank) and LN-siPSA (into the right flank) cells in castrated nude mice also showed smaller tumors in LN-siPSA xenografts compared with LNCaP xenografts. Four representative tumors from LNCaP and LN-siPSA were shown and quantitative tumor weight results are attached (Fig. 6D, *n* = 10 for each group). Together, cell line MTT assays, colony formation assays, and *in vivo* xenograft tumor growth assays all show that targeting PSA via PSA siRNA to reduce endogenous PSA expression is a potential new therapeutic approach to suppress prostate cancer cell growth.

Discussion

Pathophysiologic roles of PSA in prostate cancer. Early studies suggested that PSA might modulate growth of PSA-producing cells and their surrounding cells (38, 39) via its serine protease activity. PSA might promote the growth and invasion of prostate cancer via degradation of IGFBP-3, fibronectin, and laminin (2, 10, 40). Interestingly, Fortier and colleagues (41) presented evidence that PSA protein itself, without its protease activity, could also function as an endothelial cell-specific inhibitor of angiogenesis. Their findings, however, were countered by later findings from Denmeade and colleagues (11) showing that the antiangiogenic effects of PSA are not significant enough in PSA-

producing cells to appreciably affect tumor growth *in vivo*. Therefore, the significance of pathophysiologic roles of PSA in prostate cancer, from the above studies, might depend predominantly, if not completely, on the protease activity from PSA. The development of inhibitors to block the protease activity of PSA might then have potential therapeutic advantages to battle the prostate cancer. Our findings here showing PSA, without involving its protease activity, can promote prostate cancer cell growth. Therefore, PSA may have two functions, one in invasion and one in proliferation, and that its protease activity could be important for one and not the other. These findings suggest that targeting PSA itself, instead of just blocking its protease activity, might be needed to stop the PSA-induced prostate cancer progression.

Clinical linkage: Tissue-PSA increased in prostate cancer patients treated with androgen ablation therapy. An early study showed that 31 of 63 (49%) of the patients died of prostate cancer with their Tissue-PSA values increased (from 0.054 to 0.204 μg Tissue-PSA/μg DNA) during androgen ablation treatment that includes either surgical or chemical castration. The average of their pretreatment Tissue-PSA values was significantly lower compared with other groups of patients (0.063 versus 0.381 μg Tissue-PSA/μg DNA) who were alive at the end of the observation period or died of causes other than prostate cancer (42). This agrees with our immunohistochemical staining (Fig. 1), showing that the tissue PSA level was also higher in the hormone-refractory samples than the hormone-sensitive control group. The rationale for those patients that had increased Tissue-PSA during treatment, yet failed to respond to androgen ablation therapy and died of prostate cancer, could be that the Tissue-PSA synthesis in these patients has become androgen insensitive (42) and other inducers, such as antiandrogens or Adiol, can then stimulate Tissue-PSA synthesis (29, 30).

New signaling pathways from PSA→HF/Adiol-ARA70/AR→p53→cell apoptosis and cell growth arrest. One possible explanation for the above clinical observations and our experiments is that with expression of AR and ARA70 in those patients at the hormone-refractory stage (14, 43, 44), antiandrogen HF or Adiol could induce AR transactivation (Fig. 4D), which results in the increased Tissue-PSA. The increased Tissue-PSA could then go through positive feedback regulation to further enhance ARA70-induced AR transactivation that results in the suppression of p53 expression via modulation of MDM2/HoxA5/Egr-1 signaling pathways (35). The consequence of AR suppression of p53 might result in the cell survival via the decrease of cell apoptosis via bax/bcl2/caspase-3 signaling pathways as well as in the decrease of cell G₁ arrest via modulation of p21/cdk2/cyclin D1 signaling pathways (Fig. 6). Previous reports showed that increase in expression of the p53 and p21/WAF1 proteins is the early event during standard androgen withdrawal therapy (45). Besides, p53 was identified as a critical molecule in response to androgen deprivation in prostate from mouse model and LNCaP cells (35). *In vitro* studies on both LNCaP and LAPC4 cells indicated that AR promotes cell growth by abrogation of p53-mediated apoptosis (34), and mutant p53 can facilitate the androgen-independent growth of LNCaP cells (46). On the other hand, inhibition of p53 function diminishes AR-mediated signaling in prostate cancer cell lines (47). In addition, a functional role for the wt p53 gene in suppressing prostatic tumorigenesis was well documented (48, 49), and it was also documented that amplification of AR gene was associated with p53 mutation in hormone-refractory prostate cancer (50). Taken together, the AR-regulated p53 signaling pathway was significant for tumor progression, and PSA may play an important role in this pathway.

In summary, results from these studies show that tissue PSA, without involving its protease activity, can promote ARA70-AR-mediated cell growth and facilitate refractory tumor development. This observation suggests that PSA might be treated as a new potential therapeutic target to battle the prostate cancer. Small molecules that can degrade PSA protein or siRNAs that can knock down PSA expression might be able to be developed in the future to inhibit the AR-mediated prostate cancer growth with fewer side effects from the AR-influenced physiologic functions in other tissues.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

- Diamandis EP. Prostate-specific antigen: its usefulness in clinical medicine. *Trends Endocrinol Metab* 1988;9:310-6.
- Borgono CA, Michael IP, Diamandis EP. Human tissue kallikreins: physiologic roles and applications in cancer. *Mol Cancer Res* 2004;2:257-80.
- Obiezu CV, Sorilas A, Katsaros D, et al. Higher human kallikrein gene 4 (KLK4) expression indicates poor prognosis of ovarian cancer patients. *Clin Cancer Res* 2001;7:2380-6.
- Anisowicz A, Sotiropoulou G, Stenman G, Mok SC, Sager R. A novel protease homolog differentially expressed in breast and ovarian cancer. *Mol Med* 1996;2:624-36.
- Bhattacharjee A, Richards WG, Staunton J, et al. Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc Natl Acad Sci U S A* 2001;98:13700-5.
- Iacobuzio-Donahue CA, Ashfaq R, Maitra A, et al. Highly expressed genes in pancreatic ductal adenocarcinomas: a comprehensive characterization and comparison of the transcription profiles obtained from three major technologies. *Cancer Res* 2003;63:8614-22.
- Roman-Gomez J, Jimenez-Velasco A, Aguirre X, et al. The normal epithelial cell-specific 1 (NES1) gene, a candidate tumor suppressor gene on chromosome 19q13.3-4, is down-regulated by hypermethylation in acute lymphoblastic leukemia. *Leukemia* 2004;18:362-5.
- Bok RA, Small EJ. Bloodborne biomolecular markers in prostate cancer development and progression. *Nat Rev Cancer* 2002;2:918-26.
- Grande M, Carlstrom K, Rozell BL, Eneroth P, Stege R, Pousette A. Tissue concentrations of tissue polypeptide antigen (TPA) and prostatic specific antigen (PSA) in 42 patients with prostatic carcinoma. *Prostate* 2000;45:299-303.
- Cohen P, Graves HC, Peehl DM, Kamarei M, Giudice LC, Rosenfeld RG. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. *J Clin Endocrinol Metab* 1992;75:1046-53.
- Denmeade SR, Litvinov I, Sokoll LJ, Lilja H, Isaacs JT. Prostate-specific antigen (PSA) protein does not affect growth of prostate cancer cells *in vitro* or prostate cancer xenografts *in vivo*. *Prostate* 2003;56:45-53.
- Cluetjens KBJM, van der Korput HAGM, van Eekelen CCEM, van Rooij HCJ, Faber PW, Trapman J. An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. *Mol Endocrinol* 1997;11:148-61.
- Kollara A, Diamandis EP, Brown TJ. Secretion of endogenous kallikreins 2 and 3 by androgen receptor-antagonized PC3 prostate cancer cells. *J Steroid Biochem Mol Biol* 2003;84:493-502.
- Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. *Nat Med* 2004;10:33-9.
- Cluetjens CBJM, Steketee K, van Eekelen CCEM, van der Korput JAGM, Brinkmann AO, Trapman J. Both androgen receptor and glucocorticoid receptor are able to induce prostate-specific antigen expression, but differ in their growth stimulating properties of LNCaP cells. *Endocrinology* 1997;138:5293-300.
- Yu H, Diamandis EP, Zarghami N, Grass L. Induction of prostate-specific antigen production by steroids and tamoxifen in breast cancer cell lines. *Breast Cancer Res Treat* 1994;32:291-300.
- de Winter JAR, Janssen PJA, Sleddens HMEB, et al. Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer. *Am J Pathol* 1994;144:735-46.
- Hu YC, Yeh S, Yeh SD, et al. Functional domain and motif analyses of androgen receptor coregulator ARA70 and its differential expression in prostate cancer. *J Biol Chem* 2004;279:33438-46.
- Yeh S, Chang C. Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. *Proc Natl Acad Sci U S A* 1996;93:5517-21.
- Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell JE. *Molecular cell biology*. 4th edition. New York: W.H. Freeman and Company; 2000. p. 691-6.
- Novick P, Field C, Schekman R. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* 1980;21:205-15.
- Novick P, Ferro S, Schekman R. Order of events in the yeast secretory pathway. *Cell* 1981;25:461-9.
- Kaiser CA, Schekman R. Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell* 1990;61:723-33.
- Schekman R. SEC mutants and the secretory apparatus. *Nat Med* 2002;8:1055-8.
- Matusik RJ, Kreis C, McNicol P, et al. Regulation of prostatic genes: role of androgens and zinc in gene expression. *Biochem Cell Biol* 1986;64:601-7.
- Prescott JL, Blok L, Tindall DJ. Isolation and androgen regulation of the human homeobox cDNA, NKX3.1. *Prostate* 1998;35:71-80.
- Israeli RS, Powell CT, Fair WR, Heston DW. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res* 1993;53:227-30.
- Wright GL, Jr., Grob BM, Haley C, et al. Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy. *Urology* 1996;48:326-34.
- Yeh S, Miyamoto H, Chang C. Hydroxyflutamide may not always be a pure antiandrogen. *Lancet* 1997;349:852-3.
- Miyamoto H, Yeh S, Lardy H, Messing E, Chang C. D⁵-Androstenediol is a natural hormone with androgenic activity in human prostate cancer cells. *Proc Natl Acad Sci U S A* 1998;95:11083-8.
- Nelson PS, Gan L, Ferguson C, et al. Molecular cloning and characterization of prostate, an androgen-regulated serine protease with prostate-restricted expression. *Proc Natl Acad Sci U S A* 1999;96:3114-9.
- Ikezoe T, Yang Y, Saito T, Koeffler HP, Taguchi H. Proteasome inhibitor PS-341 down-regulates prostate-specific antigen (PSA) and induces growth arrest and apoptosis of androgen-dependent human prostate cancer LNCaP cells. *Cancer Sci* 2004;3:271-5.
- Hastak K, Agarwal MK, Mukhtar H, Agarwal ML. Ablation of either p21 or Bax prevents p53-dependent apoptosis induced by green tea polyphenol epigallocatechin-3-gallate. *FASEB J* 2005;19:789-91.
- Sun C, Shi Y, Xu LL, et al. Androgen receptor mutation (T877A) promotes prostate cancer cell growth and cell survival. *Oncogene* 2006;25:3905-13.
- Nantermet PV, Xu J, Yu Y, et al. Identification of genetic pathways activated by the androgen receptor during the induction of proliferation in the ventral prostate gland. *J Biol Chem* 2004;279:1310-22.
- Altuwajri S, Lin HK, Chuang KH, et al. Interruption of nuclear factor κ B signaling by the androgen receptor facilitates 12-O-tetradecanoylphorbolacetate-induced apoptosis in androgen-sensitive prostate cancer LNCaP cells. *Cancer Res* 2003;63:7106-12.
- Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. *Oncogene* 2005;24:2899-908.
- Webber MM, Waghray A, Bello D. Prostate-specific antigen, a serine protease, facilitates human prostate cancer cell invasion. *Clin Cancer Res* 1995;1:1089-94.
- Sutkowski DM, Goode RL, Banial J, et al. Growth regulation of prostatic stromal cells by prostate-specific antigen. *J Natl Cancer Inst* 1999;91:1663-9.
- Veveris-Lowe TL, Lawrence MG, Collard RL, et al. Kallikrein 4 (hK4) and prostate-specific antigen (PSA) are associated with the loss of E-cadherin and an epithelial-mesenchymal transition (EMT)-like effect in prostate cancer cells. *Endocr Relat Cancer* 2005;12:631-43.
- Fortier AH, Nelson BJ, Grella DK, Holaday JW. Antiangiogenic activity of prostate-specific antigen. *J Natl Cancer Inst* 1999;91:1635-40.
- Stege R, Grande M, Carlstrom K, Tribukait B, Pousette A. Prognostic significance of tissue prostate-specific antigen in endocrine treated prostate carcinomas. *Clin Cancer Res* 2000;6:160-5.
- Rahman MM, Miyamoto H, Takatera H, Yeh S, Altuwajri S, Chang C. Reducing the agonist activity of antiandrogens by a dominant-negative androgen receptor coregulator ARA70 in prostate cancer cells. *J Biol Chem* 2003;278:19619-26.
- Gregory CW, Hamil KG, Kim D, et al. Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. *Cancer Res* 1998;58:5718-24.
- Agus DB, Cordon-Cardo C, Fox W, et al. Prostate cancer cell cycle regulator: response to androgen withdrawal and development of androgen independence. *J Natl Cancer Inst* 1999;93:1867-76.
- Vinall RL, Tepper CG, Shi X-B, Xue LA, Gandour-Edwards R, de Vere White RW. The R273H p53 mutation can facilitate the androgen-independent growth of LNCaP by a mechanism that involves H2 relaxin and its cognate receptor LGR7. *Oncogene* 2006;25:2082-93.
- Cronauer MV, Schulz WA, Burchardt T, Ackermann R, Burchardt M. Inhibition of p53 function diminishes androgen receptor-mediated signaling in prostate cancer cell lines. *Oncogene* 2004;23:3541-9.
- Isaacs WB, Carter BS, Ewing CM. Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. *Cancer Res* 1991;51:4716-20.
- Srivastava S, Katayose D, Tong YA, et al. Recombinant adenovirus vector expressing wild-type p53 is a potent inhibitor of prostate cancer cell proliferation. *Urology* 1995;46:843-8.
- Koivisto PA, Rantala I. Amplification of the androgen receptor gene is associated with P53 mutation in hormone-refractory recurrent prostate cancer. *J Pathol* 1999;187:237-41.