



Published in final edited form as:

*Prostate*. 2011 November ; 71(15): 1595–1607. doi:10.1002/pros.21375.

## Enzymatically Active Prostate-Specific Antigen Promotes Growth of Human Prostate Cancers

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### Abstract

**Background**—Prostate specific antigen (PSA) is the best-known member of the kallikrein-related peptidase family, with an established role as a prostatic disease biomarker. Although it is produced at high levels by all stages of prostate cancer, it is uncertain if PSA plays a role in prostate cancer initiation and progression. We decided to investigate the impact of PSA and its enzymatic activity on tumor cell growth rates.

**Methods**—A gene-specific shRNA lentiviral construct reduced endogenous PSA expression in the LNCaP human prostate cancer cell line. Resulting changes in growth rates in vitro and in vivo were determined. Using a mass spectroscopy-based approach, alterations to the LNCaP proteome due to reduced PSA were measured. Finally, to evaluate the importance of PSA's proteolytic activity, the PSA-null Du145 human prostate cancer cell line was engineered to express either enzymatically inactive pro-PSA (WT) or a furin-activated variant (FR) with high enzymatic activity. The resulting clones were evaluated for PSA-induced changes in growth rates in vivo and in vitro.

**Results**—Lowered PSA levels dramatically reduced LNCaP growth rates. Expressing active PSA (FR), but not the inactive WT variant, conferred a growth advantage on Du145 cells. Proteomics analysis revealed global changes to the LNCaP proteome as a result of reduced PSA expression.

**Conclusions**—These studies demonstrate the importance of PSA to prostate cancer cell growth. We also show that the enzymatic activity of PSA confers an enhanced growth rate to human prostate cancer cells, suggesting a causal role in prostate cancer progression.

### Keywords

PSA; kallikrein; prostate; enzyme

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### Conflict of interest

The authors declare no conflict of interest.

## Introduction

Prostate Specific Antigen (PSA) is the most widely utilized cancer biomarker. PSA is used extensively to screen for prostate cancer, to detect recurrence following local therapies, and to follow response to systemic therapies for metastatic disease [1–3]. A number of epidemiologic studies have demonstrated that PSA levels and the rate of PSA increase over time are associated with increased risk for developing prostate cancer [4,5]. Using data from the Baltimore Longitudinal Study of Aging, Carter et al. demonstrated that rates of change in serum PSA levels over time (i.e. PSA velocity) could be used to identify men with life-threatening prostate cancer many years before diagnosis [4]. In this study, PSA velocity measured 10–15 years before a diagnosis of prostate cancer was associated with cancer-specific survival 25 years later [4]. PSA has also been demonstrated to be an independent risk factor for progression of prostate cancer outside of the prostate, for development of extension of prostate cancer to pelvic lymph nodes, for the development of distant metastases and for mortality [6–9].

One interpretation of these studies is that PSA is merely a marker for underlying pathology. However, an alternative hypothesis is that the chronic production and leakage of PSA from the prostatic ducts across the luminal and basal epithelial cells into the stromal compartment, and ultimately into the circulation is itself responsible, in part, for either the development of prostate cancer and/or its progression from localized to metastatic stage [10]. A variety of clinical and laboratory observations support this hypothesis. Unlike other tissue differentiation markers, PSA continues to be expressed at high levels by prostate cancer, even as these cells lose the morphologic characteristics of the normal prostate gland and become less differentiated [11]. PSA is a serine protease secreted as a zymogen, with a prodomain that must be removed by a second protease to produce enzymatically active, mature protein. Remarkably high levels of enzymatically active PSA are present in the extracellular fluid surrounding early and advanced human prostate cancers, within sites of bony and soft tissue metastases [12]. PSA expression is under the transcriptional regulation of the androgen receptor (AR), both in the normal and cancerous prostate. However, castration-resistant prostate cancer cells continue to produce PSA at high levels possibly due to an AR that remains active despite low levels of its normal ligand, dihydrotestosterone [13]. The high level of PSA produced at all stages of the disease retains its enzymatic activity, suggesting that the biochemical mechanisms required to convert the zymogen to mature enzyme are also retained. These observations point to PSA playing some role in the lethal progression of prostate cancer.

As a member of the kallikrein-related peptidase (KLK) family of serine proteases, PSA is present mainly in its mature form at mg/mL concentrations in the prostatic ducts and seminal fluid. It cleaves its known physiologic substrates, principally the gel-forming proteins semenogelin I and II, produced by the seminal vesicles [14,15]. Increased leakage of PSA into the circulation occurs, presumably, when the normal prostate glandular architecture is disrupted by inflammation or neoplasia. It is on this basis that PSA is useful as a biomarker for prostate cancer. However, previous studies have suggested that PSA may play a role in prostate cancer pathobiology through a variety of mechanisms that include alterations in prostate cancer cell invasive capabilities, gene expression, and morphology [16–19]. Additional studies, using purified proteins, have documented that PSA can directly cleave or release from binding proteins cytokines present in the stromal compartment that are involved in growth stimulation and inflammation. For example, PSA can cleave insulin-like growth factor binding proteins (IGFBP) resulting in local release of IGF-1 [20], and can specifically activate the small latent form of transforming growth factor beta-2 (TGF $\beta$ 2) [21]. The enzyme can also hydrolyze parathyroid hormone related protein (PTHrP), which may convert it from an osteoclastic to an osteoblastic growth factor, capable of playing a

role in the osteoblastic bone metastasis phenotype typically observed in men with advanced prostate cancer [22,23]. While these studies document that PSA can activate or modify various growth factors in vitro, there is no data demonstrating that PSA-mediated proteolysis of any of these factors occurs as part of the development and/or progression of human prostate cancers or preclinical models.

The interpretation of PSA's function has been complicated by the observation by us and others that the majority of PSA produced by the available human prostate cancer cell lines in vitro has little enzymatic activity. Similar results have been reported by a number of groups that have transfected the gene into a variety of mammalian cell lines. In the cases where it has been assayed, very little enzymatically active PSA is produced due to a combination of incomplete processing of the zymogen and internal cleavage of the PSA protein by unidentified proteases [24–28]. Thus, while it has been documented that the increased expression of the PSA protein itself, independent of its activity, may alter specific and/or global gene expression, activate signaling pathways, inhibit angiogenesis, and effect the overall growth of prostate cancer cells, it is difficult to draw firm conclusions from any of these published studies because the enzymatic activity of the PSA produced by the model systems was either not assayed or adequately documented [29–32].

In this study, we sought to use cellular systems to investigate the effect of PSA on prostate cancer cell growth and tumorigenicity. We stably reduced endogenous PSA expression levels in the LNCaP human prostate cancer cell line and investigated the resulting phenotypic differences, while using mass spectrometry (MS)-based proteomics analysis of conditioned media to identify candidate mediators. To investigate the contribution of enzymatically active PSA, we used the PSA-null Du145 human prostate cancer cell line to compare the effect of expressing wild-type pro-PSA, that is known not to be processed to active enzyme in this line, to a PSA variant that is activated in vitro and in vivo because it contains a modified pro-peptide activation sequence that is recognized as a substrate for ubiquitously expressed furin proteases. Using these approaches, we demonstrate that PSA is a mediator of prostate cancer cell growth in vitro and in vivo, and produces alterations in gene expression in eliciting its effect. PSA must be enzymatically active to have these oncogenic effects and can do so independent of the androgen receptor.

## Materials and Methods

### Generation of PSA-targeted shRNA-expressing LNCaP cells

shRNA lentivirus expression vectors containing sequences specific to regions of the PSA cDNA with minimal homology to other kallikreins (i.e. KLK2) or a control were developed by the MISSION™ TRC-Hs 1.0 library based on their algorithm (Sigma-Aldridge, St. Louis, MO). The vectors were packaged in lentiviral particles isolated from HEK293T cells and used to transduce LNCaP cells (ATCC, Manassas, VA) following the vendor's recommendations. In brief, low-passage (below passage 30) LNCaP cells were transferred to 96-well plates at 5000 cells per well in 200µL of media. 48 hours later, the media was removed and replaced by 110µL media with 8µg/mL hexadimethrine bromide (Sigma-Aldridge, St. Louis, MO). 10µL of viral particles was added to each of 12 wells per construct before an overnight incubation. The media was then replaced with 200µL of fresh media and incubated overnight. Puromycin at 1µg/mL was added to the cells until resistant populations emerged for subsequent characterization.

## PSA Measurements

Levels of free and total PSA in media and mouse plasma were determined by the Clinical Chemistry laboratory at Johns Hopkins using the Hybritech assays on the Beckman Access Immunoassay System (Beckman Coulter, Inc., Brea, CA).

## Cell line and viability assay

The human prostate cancer cell lines LNCaP and Du-145 were obtained from ATCC (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% serum, 1% pen/strep, and 2 mM L-glutamine (Invitrogen, Carlsbad, California) as previously described [33]. For in vitro proliferation assays, cells were seeded into 96-well plates at 5,000 (LNCaP) or 1,000 (DU145) cells per well. At each time point cell viability was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT Dye Solution, Promega, Madison, WI) according to the manufacturer's instructions. Samples were measured at 570 nm using a spectrophotometer (Spectramax Plus, Molecular Devices, Sunnyvale, CA).

## Generation of LNCaP variant-conditioned media

LNCaP populations stably expressing either control (shC) or PSA-specific (shPSA) shRNA constructs were placed on 150 mm poly-D-lysine-coated dishes (Fisher) to 70% confluence in 20 mL of standard tissue culture media. A dish containing no cells was also incubated with media and treated the same as the cell-containing dishes. After a 48 hour incubation period, media was removed from the dishes, which were then gently washed 3 times with phenol-red-free HBSS. The plates were then incubated overnight in 20 mL of serum-free, phenol-red-free RPMI, supplemented with the synthetic androgen methyltrienolone (Perkin Elmer) at 10 nM. This was followed by another 3 washes with HBSS and a 24 hour incubation in 20 mL of the androgen-supplemented serum-free, phenol-red-free RPMI. The conditioned media was gently removed from plates and placed into 50 mL conical tubes containing Complete Protease Inhibitor Cocktail with EDTA (Roche) and PMSF (Sigma) in methanol to final concentrations of 1X and 1mM respectively. Intact cells were removed by centrifugation at  $500 \times g$  for 5 mins at 4°C. The supernatant was transferred to fresh tubes before cell debris was removed by centrifugation  $8000 \times g$  for 10 mins at 4°C. Particulate matter was removed by syringe-driven filtration of the supernatant through a 0.2  $\mu\text{m}$  pore filter disc. The conditioned media was concentrated by placement into an Amicon Ultra 3kDa filter followed by centrifugation at  $4000 \times g$  for 30 mins at 4°C. The concentrated conditioned media was washed in the filter by the addition of 15 mL saline followed by another round of centrifugation in the previous conditions. The protein concentration of the retentate was determined with the BCA assay (Pierce) and normalized by subtracting value of the cell-free conditioned media reading. The protein quality of the conditioned media was determined by electrophoretic separation of 10  $\mu\text{g}$  of protein on a 12% gel and coomassie dye staining for visualization. For the proteomics analysis, 50  $\mu\text{g}$  of concentrated conditioned media was precipitated in a 10  $\times$  volume of prechilled acetone and stored at  $-70^\circ\text{C}$ , along with the unused concentrated conditioned media.

## Proteomics analysis

The 50  $\mu\text{g}$  of conditioned media was digested with 0.5  $\mu\text{g}$  of trypsin prepared in 1 mM HCl, incubated overnight at 37°C. Resultant peptides were isolated, and lyophilized to dryness before mass spectrometric analysis. Once pelleted, all samples were reconstituted in 12  $\mu\text{L}$  of water with 0.1% trifluoroacetic acid (TFA) (Pierce, Rockford, IL). For HPLC-ESI-MS/MS analysis, 10  $\mu\text{L}$  of the tryptic peptide mixtures were placed into an Agilent 1200 autosampler (Agilent, Santa Clara, CA). Each 10  $\mu\text{L}$  sample was loaded by the autosampler onto a fused silica PicoFrit (New Objective, Woburn, MA) capillary column, 75  $\mu\text{m}$  inner

diameter (i.d.)  $\times$  150 mm long column packed in-house with 5  $\mu$ m, 300 Å BioBasic C18 (Thermo Electron, Bremen, Germany) stationary phase. Peptides were separated on-line via reversed phase nano high-performance liquid chromatography using the Eksigent Nano 2D high-performance liquid chromatography (HPLC) pumping system (Eksigent, Dublin, CA). The Eksigent Nano 2D HPLC system was controlled by XCalibur software, Version 2.0 (Thermo Electron, San Jose, CA). Nano-c18 HPLC separations were performed over a linear gradient for 200 minutes. The outlet flow of the nano-HPLC interfaced directly with the inlet of an LTQ-Orbitrap-XL (Thermo Electron, San Jose, CA) allowing for introduction of the analyte into the mass spectrometer. The LTQ-OrbitrapXL mass spectrometer was operated in data dependent mode. MS precursor scan spectra ( $m/z$  300–2000) were acquired in the Orbitrap with mass resolution of one part in 60,000; the five most intense ions from each MS scan were automatically targeted for fragmentation (MS/MS) in the ion trap. Using helium as the collision gas, collision induced dissociation (CID) mediated peptide fragmentation in the linear ion trap. The LTQ-OrbitrapXL was controlled using XCalibur software. For each experiment, the source voltage was set at 2.4 V, capillary voltage at 48 V, and the capillary temperature was set at 200°C. Sheath and auxiliary gases were not necessary because nanoflow parameters were being used. The tube lens voltage was kept at 105 V, and the ion gauge pressure was  $1.5 \times 10^{-5}$  Torr. The ion trap AGC was set at 30,000. Normalized collision energy was established at 35% for MS/MS. The default charge state was set at two. The isolation window for the ion gauge was fixed at two Daltons. After an initial MS/MS fragmentation event, ions were excluded from additional fragmentation rounds for 45 seconds using dynamic exclusion. The ion selection threshold, the minimum signal required to trigger tandem mass spectrometry, was set to 1000. The activation Q was set at 0.25. Peptides were identified and confirmed via automated database searching using the MASCOT search algorithm (Matrix Science, Boston, MA). Our experimental data was searched against the mammalian RefSeq 35 FASTA database with a mass tolerance of 10 ppm established for MS species and 1 Da for MS/MS. Trypsin was selected as the proteolytic enzyme. Automatic charge state assignment was applied for all MS data. Oxidized methionine (M) and deamidation of asparagine (N) and glutamine (Q) were chosen as variable modifications. Search results were filtered using standard reporting criteria. All protein species identified using the MASCOT search engine with a p value greater than or equal to 0.05 were the exported as .XML files and introduced into the proteomics platform Protein Center (Thermo Fisher Scientific, Palo Alto). The identified proteome for each sample was automatically curated within the program for protein taxonomy, signal peptides, transmembrane regions, alternative splicing, biological function, subcellular location, and cellular roles as defined by Gene Ontology. Any ambiguities resulting from peptides matching different members of protein families were automatically resolved as well. An initial merging of data was performed within ProteinCenter such that entries with at least 70% sequence homology and grouped homologous sequences were collapsed and reported as a single protein entry. Secondly, only proteins identified as Homo Sapien in taxonomy were included in subsequent data comparisons and analysis.

### Generation of PSA expression constructs

The PSA wild type cDNA was cloned into the pcDNA3.1 vector using the BamHI and XbaI restriction sites. The DNA sequence representing the PSA pro domain (APLILSR) was subjected to site-directed mutagenesis to convert it to a sequence derived from the bacterial proaerolysin gene (KVRRAR) recognized and cleaved by the ubiquitous furin family of peptide convertases. With use of the Gene Editor (Promega, Madison, WI) product and directions we subcloned the wild-type PSA cDNA into the pGEM-11Zf(+) vector (Promega, Madison, WI). Mutagenic oligonucleotides for the PSA pro domain facilitated new strand synthesis. The incorporation of the mutation into the PSA prodomain was confirmed by sequencing, and the gene was subcloned back into the pcDNA3.1 vector. Both the wild type



(WT) and mutant (FR, furin-recognized) PSA variant constructs were used to transfect the DU145 human prostate cancer cell line which was then subjected to selective pressure in 500 $\mu$ g/mL of zeocin (Invitrogen, Carlsbad, CA). Clonal outgrowths were tested for their production of PSA before subsequent characterization.

### **In vitro PSA enzymatic PSA activity assays**

Du145 -derived populations expressing the PSA variants were transferred to 60 cm tissue culture dishes. Following a day of incubation in media with 10% FBS, the cells were washed 3 times in HBSS before serum-free media was added. Following 96 hours of incubation, samples of the conditioned media was concentrated using filter units with a 10kD molecular weight cut off (Millipore, Billerica, MA). Concentrated samples were subjected to analyses of protein concentration and PSA levels. Between 200ng and 1 $\mu$ g of PSA was isolated from the conditioned media by immunoprecipitation and mixed with a 40 $\mu$ M solution of a PSA-specific peptide conjugated to a fluorogenic substrate (His-Ser-Ser-Lys-Leu-Gln-aminomethylcoumarin) [31]. Change in Relative Fluorescence Units (RFU) generated as a result of PSA-mediated substrate cleavage was recorded at 3 minute intervals.

### **Tumor xenograft studies**

Mouse care and treatment was approved by and performed in accordance with the guidelines of the animal care and use committee of the Johns Hopkins University School of Medicine. Cells maintained under standard conditions were detached by treatment with 0.25% trypsin-EDTA solution and washed in Hank's balanced salt solution (HBSS). They were then suspended in a 60% mixture of Matrigel Matrix (BD Biosciences, San Jose, CA) in HBSS at a concentration  $5.0 \times 10^6$  (LNCaP) or  $1 \times 10^6$  (Du145) cells per 200  $\mu$ L of solution. The cells were then injected into the subcutis overlying the rear flanks of 6-week-old male nude mice ( $n = 10$ ). Weekly tumor measurements were made with calipers. Tumor volume (in  $\text{cm}^3$ ) was calculated by the formula  $0.5236 \times L \times W \times H$ . The mice were euthanized by  $\text{CO}_2$  overdose shortly after the last measurement. Blood was collected by cardiac puncture and used to determine plasma PSA levels. The tumors were surgically removed, weighed, fixed in a 10% formalin solution in phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA), and embedded in paraffin.

### **Hematoxylin and eosin tissue staining**

Hematoxylin and eosin (H&E) staining was performed using standard protocols as previously described [25]. Slides were then evaluated under brightfield illumination to assess tissue morphology.

### **Immunohistochemistry**

PSA staining was performed as previously described [25]. Slides were then overlaid with a coverslip and then evaluated under brightfield illumination to assess PSA distribution.

## **Results**

### **Generation of PSA-targeted shRNA LNCaP human prostate cancer cells**

To first evaluate the importance of endogenously produced PSA on the growth of prostate cancer cells in vitro and in vivo, we assessed the effect of decreased PSA expression on growth of the well-characterized LNCaP human prostate cancer model. LNCaP is an androgen responsive cell line that generates serum PSA levels in vivo that approximate those produced by human prostate cancers [12]. In a previous study, we documented that, in vitro, only 10–15% of the PSA isolated from serum-free LNCaP-conditioned media is

enzymatically active [25]. However, this amount of mature PSA is sufficient to activate PSA-activated prodrugs and protoxins under development in our laboratory [34,35].

Using a lentivirus-mediated shRNA approach to stably reduce PSA expression in the LNCaP line, we generated cell populations expressing one of five distinct shRNA constructs targeting PSA (Fig. 1A). Niu et al. used RNAi in a plasmid-based approach to generate one clone with 20% reduced PSA expression compared to wild type LNCaP [32]. The populations derived from our lentivirus strategy were first assayed for PSA levels in the media (Fig. 1B). Of the three PSA-targeted shRNA constructs that produced cell populations, two had modest decreases in PSA levels compared to controls. However, one construct, shPSA1, produced a 95% reduction in PSA production over 10 separate evaluated cell populations (Fig. 1B).

### Loss of endogenous PSA slows LNCaP cell growth in vitro and in vivo

Three of these distinct shPSA1 populations expressing the lowest levels of PSA were selected for further in vitro studies. While these cells exhibited no obvious differences in morphology under brightfield microscopy (data not shown), they had significantly reduced growth rates, with average doubling times increasing from 2 days for the control LNCaP cells expressing the non-targeted shRNA construct to 11 days for the low-PSA populations. All three low-PSA populations grew significantly more slowly than these three control populations (Fig. 2A).

Next, the in vivo growth of LNCaP shPSA1 and control shRNA populations was compared (Fig. 2B). Mice were inoculated subcutaneously with cells suspended in Matrigel and evaluated at weekly intervals for the appearance of tumor and changes in tumor volume. In the control group, 10 of 18 animals (55%) had measurable tumors at the end of 8 weeks. Serum PSA levels were detectable (i.e.  $\geq 0.1$  ng/mL) in 12/18 of these animals, with serum PSA levels ranging from 0.16 to 608 ng/mL with a median of 79.7 ng/mL and a mean of 146.7 ng/mL. In the shPSA1 populations 7/20 (35%) had measurable tumors with PSA levels detectable in 4/20 animals ranging from 0.28 to 1.38 ng/mL with a mean of 0.76 ng/mL. The average weight of detectable tumor from the control animals was  $1.05 \pm 0.35$  grams (Fig. 2C). In contrast, the average tumor weight for the shPSA1 was  $0.11 \pm 0.044$  grams (Fig. 2C). While there was a 10-fold difference in tumor weight between the two groups, there was a 45-fold difference in PSA levels when normalized for tumor weight (Fig. 2D). Immunohistochemical analysis revealed high levels of PSA surrounding control LNCaP cells compared to barely detectable PSA staining above background in shPSA1-expressing xenografts (Fig. 2E). In contrast to the earlier report by Niu et al., no nuclear staining for PSA was observed in any tissue (Fig. 2E).

Compared to the low level of enzymatically active PSA produced in vitro, we determined that the majority of detectable PSA produced by LNCaP cells in vivo can be found in the serum complexed to alpha-1-anti-chymotrypsin (ACT) serum protease inhibitor, consistent with the production of enzymatically active protein. The PSA-ACT complex is the major form of PSA found in the serum of men with prostate cancer, and is also the major form in the serum of the mice bearing LNCaP tumors (Fig. 2F). While there was a pronounced difference in total PSA production between the shRNA control and shPSA1 xenografts, the percentage of alpha-1-antichymotrypsin-bound enzyme in the serum was somewhat comparable (55.4% for shPSA1 vs 66.9% for controls) (Fig. 2F). These results indicate that in both sets of xenografts the majority of the PSA released into the blood is enzymatically active. Also, since the total amount of PSA produced by the shPSA1 tumor-bearing mice is markedly decreased, the amount of active enzyme made by those tumors is decreased to a similar extent.

The combined results from these studies demonstrate that the amount of total and enzymatically active PSA produced by the LNCaP shPSA1 populations is significantly decreased both in vitro and in vivo compared to control. This decrease in PSA production is associated with a dramatic decrease in growth between these different populations of otherwise isogenic cells both in tissue culture and when growing as subcutaneously implanted xenografts.

### PSA-dependent proteomic differences

Previously, the effect of PSA on cellular global gene expression profiles was conducted by expressing the gene in PSA-null prostate cancer cells [16]. The shPSA1 LNCaP cell populations appeared to be a useful model for proteomics analysis since the loss of endogenous protein production would result in a more relevant proteomics profile, especially given the profound change in the growth phenotype. Therefore, serum-free media lacking serum protease inhibitors, supplemented with androgen to maintain PSA production, was conditioned by the LNCaP variants for 48 hours followed by protein quality control, concentration, MS analysis, and data interpretation. Of the 6497 proteins identified as reproducibly present in conditioned media across populations and experimental replication, 84.38% were designated with intracellular origins (5474/6487, Fig. 3A). While 27.9% of the proteins (1810/6487) were found common to both populations, we chose to focus on those detected exclusively in either group. Our strategy did not quantify the levels of protein production but did allow for a ranking of the protein abundance within each group (Fig. 3B). The 25 most highly expressed proteins exclusive to either group (i.e. control vs shPSA1) are presented, ranked by normalized abundance, and annotated with their documented role in cancer or specifically prostate cancer (Fig. 3B).

### Generation of a high activity PSA variant

The data from the LNCaP shRNA populations establish that PSA production is an important factor in the growth and progression of established prostate cancer. However, both the amount of total and enzymatically active PSA is decreased in the shPSA1 LNCaP populations. Therefore, to determine the role of PSA's enzymatic activity in growth promotion, the generation of isogenic cells producing active vs. inactive PSA protein was required.

Previously, we demonstrated that the transfection of prostate cancer cells with the wild type PSA gene produces clones that generate high levels of PSA with little enzymatic activity both in vivo and in vitro [24], in part due to the lack of proper processing of the zymogen. Pro-PSA can be converted to its enzymatically active form by the human kallikrein-related peptidases 2, 4 and 15 (KLK2, 4, 15), with KLK2 as the most likely physiologic PSA processing enzyme [26–28,36,37]. PSA-expressing clones derived from human (PC-3, DU145) and rat (AT2, AT6) prostate cancer cell lines all lack endogenous AR and expression of its transcriptional targets, PSA and KLK2. Therefore, the lack of PSA activity in cells transfected with full length Pre-Pro-PSA appears to be due to a lack of concomitant expression of the appropriate PSA processing protease.

To overcome this limitation, we mutagenized the pro-domain of the wild type PSA gene (WT), replacing it with an amino acid sequence recognized and cleaved by the ubiquitously expressed furin convertase family of proteases (FR) (Fig. 4A). This approach was based on earlier studies where we documented that the furin-activated bacterial toxin, proaerolysin, was readily activated by human prostate cancer cell lines [35]. Both WT and FR constructs were stably expressed in PSA-null Du145 human prostate cancer cell line, with the resulting secreted protein tested for activity against a previously validated fluorogenic peptide substrate selective for PSA (Fig. 4B). The DU145 WT clones had a low level of PSA



activity compared to the activity of an equivalent amount of active PSA isolated from human seminal plasma. In contrast, compared to the WT protein, the FR clones displayed almost 10-fold more PSA activity (Fig. 4B).

### PSA-mediated growth induction of prostate cancer cells is dependent on enzymatic activity

To evaluate the effects of PSA variant expression on growth in vitro, the Du145-derived clones were subjected to the MTT-based proliferation assay. Growth over a 5 day period revealed that each of the individual FR clones grew significantly faster than either the WT or VC clones (Fig. 5A). Subsequently we conducted analyses of animals bearing the WT, FR, or VC Du145 clones growing subcutaneously in nude mice (Fig. 5B). By the end of 5 weeks, tumors derived from the FR clones were significantly larger than either the WT or the VC-derived tumors (Fig. 5B). This difference was also seen in the weight of the tumors (Fig. 5C). In addition, the overall “take rate” was also increased in the mice inoculated with the FR clones. In this FR group, measurable tumors were observed in 80% (12/15) animals compared to 40% (6/15) of the VC-derived tumors and 46% (7/17) of the WT clones (data not shown). Serum PSA levels from these groups of animals, normalized to tumor weight, demonstrated that the PSA-expressing clones generated approximately equal amounts of PSA in the circulation, (Fig. 5D). The percent of enzymatically active PSA bound to alpha-1-antichymotrypsin showed that ~12% of the PSA in circulation from the WT clones was in the complexed form and therefore secreted initially in an enzymatically active form (Fig. 5F). In contrast, ~90% of the PSA in the circulation from the FR clones was enzymatically active.

### Discussion

In this study we used two separate approaches to challenge our hypothesis that enzymatically active PSA plays a role in the growth of human prostate cancer cells. Reducing endogenous PSA production by over 90% resulted in a marked decrease in the growth rate of androgen sensitive LNCaP human prostate cancer cells in vitro. Decreased PSA production in LNCaP cell populations also resulted in both a decreased rate of tumor establishment and tumor growth when inoculated subcutaneously into nude mice.

Genomics and proteomics-based approaches had previously been used to assess changes in expression caused by PSA. Bindukumar et al. added isolated PSA to the PC-3M human prostate cancer cell line, briefly reporting a growth-inhibitory phenotype in addition to changes to the cell lysate proteome [38]. Also, in an attempt to investigate any potential role PSA may play in osteoblastic effect frequently observed in prostate cancer bone metastases, Nadiminty et al. reported changes to the genomic expression profile of SaOS-2 osteosarcoma cells transfected with the gene [31]. Our choice of conditioned media analysis is based on the secreted nature of PSA, leading us to argue that the first point of influence on cancer cell behavior is occurring in the extracellular space. Proteomics analyses of LNCaP-conditioned media are rare. Of these, the report by Sardana et al. is the most notable attempt [39]. In a study of comparison between cell lines, this group analyzed serum-free media conditioned for 48 hours by PC-3, CWR22Rv1, or LNCaP cells. While their group identified 1242 proteins in LNCaP-conditioned media, in comparison to the 6497 identified here, Sardana et al. had a comparable rate of intracellular proteins identified (88% vs 90%). Almost half of the proteins we identified were uniquely found in the shPSA1 cell populations. In addition, while more than half (28/50) of the most abundantly expressed proteins in both control and shPSA1 cells have no known cancer association, a number of known tumor suppressors were more prevalent at the top of the shPSA1 list. Proteins of interest high on the shControl LNCaP abundance list include a member of the olfactory receptor family (OR52E8) [40]. Another member of this family, prostate-specific G-protein-

coupled receptor (PSGR), is overexpressed in prostate cancer progression [41] but, antithetically, causes an elevation of intracellular calcium levels, slowing cell growth[42]. High on the shPSA LNCaP list is the ZIP1 zinc transporter, which is downregulated in prostate cancer and causes apoptotic cell death when overexpressed in the RWPE2 human prostate epithelial cell line [43]. While validating the expression and contribution of this list of proteins is beyond the scope of this report, this strategy has been useful in identifying candidate mediators of the PSA effect on LNCaP cells that can be evaluated in future studies.

Since this PSA-targeted shRNA approach reduces the total level of PSA (i.e. both active and inactive) it does not address whether it is PSA's enzymatic activity or the PSA protein itself that is important in prostate cancer cell growth. Therefore, to dissect the contribution of active vs. inactive PSA, a complementary approach was developed, using a PSA-null Du145 human prostate cancer background engineered to express either active or inactive PSA. Having previously documented that cells transfected with wild type PSA produced primarily enzymatically inactive protein in vitro, we modified the pro-domain of PSA by introducing a cleavage site recognized as a substrate by furin to generate cells that produced primarily enzymatically active PSA [44]. In these studies, we confirmed our earlier observations demonstrating that the production of high levels of total PSA, of which only a small percentage is enzymatically active, by the AR-negative Du145 cell line did not produce a significant enhancement in growth either in vitro or in vivo. In contrast, transfection of the FR construct into DU145 cells resulted in clones that generated high levels of total PSA, which consisted of predominantly enzymatically active enzyme. These clones had significantly higher growth rates both in vitro and in vivo compared to WT and parental controls. These findings further support our hypothesis that it is the enzymatic activity of PSA, not the PSA protein itself that is involved in growth promotion. In contrast to the findings of Niu et al [32], this effect appears to be independent of AR function as it was observed in an AR negative prostate cancer cell line.

Our findings also highlight the importance of documentation of PSA activity in prostate cancer model systems designed to assess the role of PSA in prostate cancer growth and progression. In addition to this study, multiple earlier studies have confirmed that cells transfected with wild type PSA do not generate significant amounts of active PSA either in vitro or in vivo [24–28]. In addition, in both human serum and fetal calf serum containing media, active PSA binds rapidly to the abundant serum protease inhibitor alpha-2-macroglobulin (A2M) and, more slowly, to alpha-1-antichymotrypsin (ACT) [1,45]. Therefore, in our studies, cells were grown in serum free media prior to assaying for PSA activity in order to eliminate complex formation with serum protease inhibitors.

These studies with prostate cancer models provide compelling evidence supporting our hypothesis that active PSA is important in the growth of established prostate cancers. Previously, we tried to address whether PSA was also involved in the initiation of prostate cancer using transgenic mouse models [37]. Using the prostate tissue-specific (ARR)<sub>2</sub> PB promoter, we achieved prostate-targeted expression of the WT-PSA protein. However, analysis of the enzymatic activity showed that, as was the case with cell lines, only a low level of PSA activity was observed in the prostates of these transgenic animals. No pathology was observed in mice up to 2 years of age compared to age matched controls in this study. Unfortunately, we were unsuccessful in generating mice retaining the FR-PSA construct, and circumvented this limitation by developing prostate-targeted KLK2 transgenic mice. While the subsequent PSA/KLK2 double transgenic offspring generated PSA that had increased enzymatic activity, these animals also did not develop any malignant lesions out to 10 months of age [37].

In conclusion, in this report we have confirmed that PSA plays a role in the growth of established human prostate cancers and that this growth promotion is dependent on the protein's enzymatic activity. The next frontier in delineating the role of PSA in prostatic disease must involve the discovery of the key proteolytic targets of the enzyme. PSA is a serine protease, which, although classified as "chymotrypsin-like" due to its ability to cleave after tyrosine and phenylalanine residues, also has the relatively unique ability to cleave after glutamine and methionine [25]. PSA has been demonstrated to cleave growth factors, growth factor binding proteins and extracellular matrix proteins. The model systems described in this report can be used to evaluate whether cleavage of these known protein substrates by PSA are important in the progression of prostate cancer. The current list of some dozen proteins is limited by the candidate gene approach used in their discovery. Systems that use non-biased, proteomics-based methods are available and can be adapted to discover additional PSA substrates in more appropriate biological contexts.

Finally, exposing the growth-promoting characteristics of PSA as we have done in this report moves the active protease from its thoroughly characterized role as biomarker, to that of a target of therapy. Previously, we utilized the tissue and tumor-specific production of high levels of PSA in conjunction with its unique amino acid sequence cleavage preference to mediate the activation of prostate-targeted prodrug and protoxin therapies [34,35]. The data reported here also suggest that inhibition of PSA enzymatic activity may also produce a therapeutic benefit. Beta-lactam based inhibitors of PSA have been previously described and other alternative small molecule platforms have been also been evaluated as PSA inhibitors [46,47], while our group has generated a peptide-based inhibitory approach that is under evaluation in our laboratory as a therapeutic modality using these PSA-producing model systems [48].

## Acknowledgments

We thank Marc Rosen and Rebecca Ricklis for their excellent technical assistance.

This project was supported by funding from the NCI Prostate SPOR (P50CA58236, SRD and JTI), the U.S. Department of Defense Prostate Cancer Research Program (PC040107, SAW), and the One-in Six Foundation, Akron OH (SRD).

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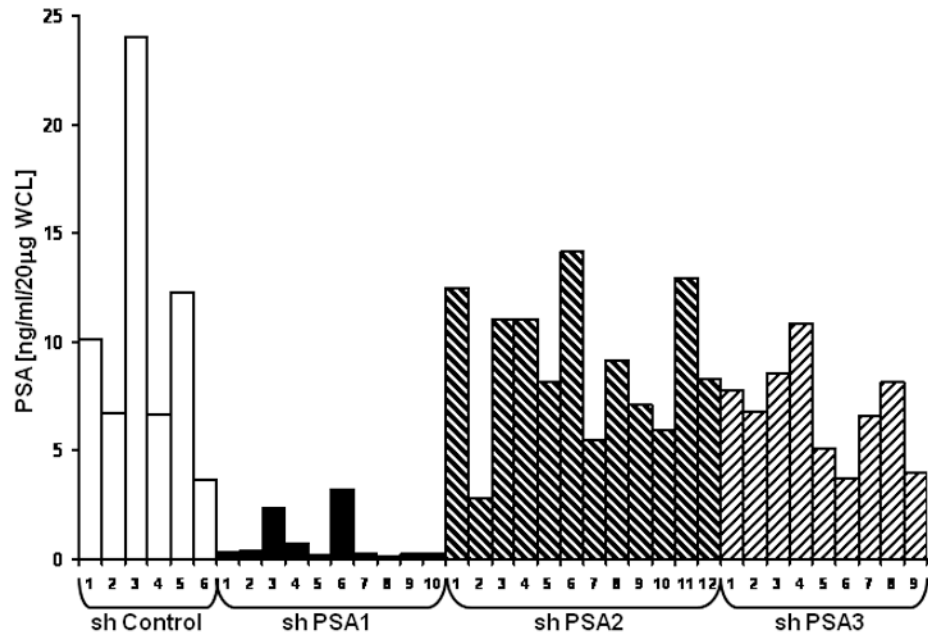


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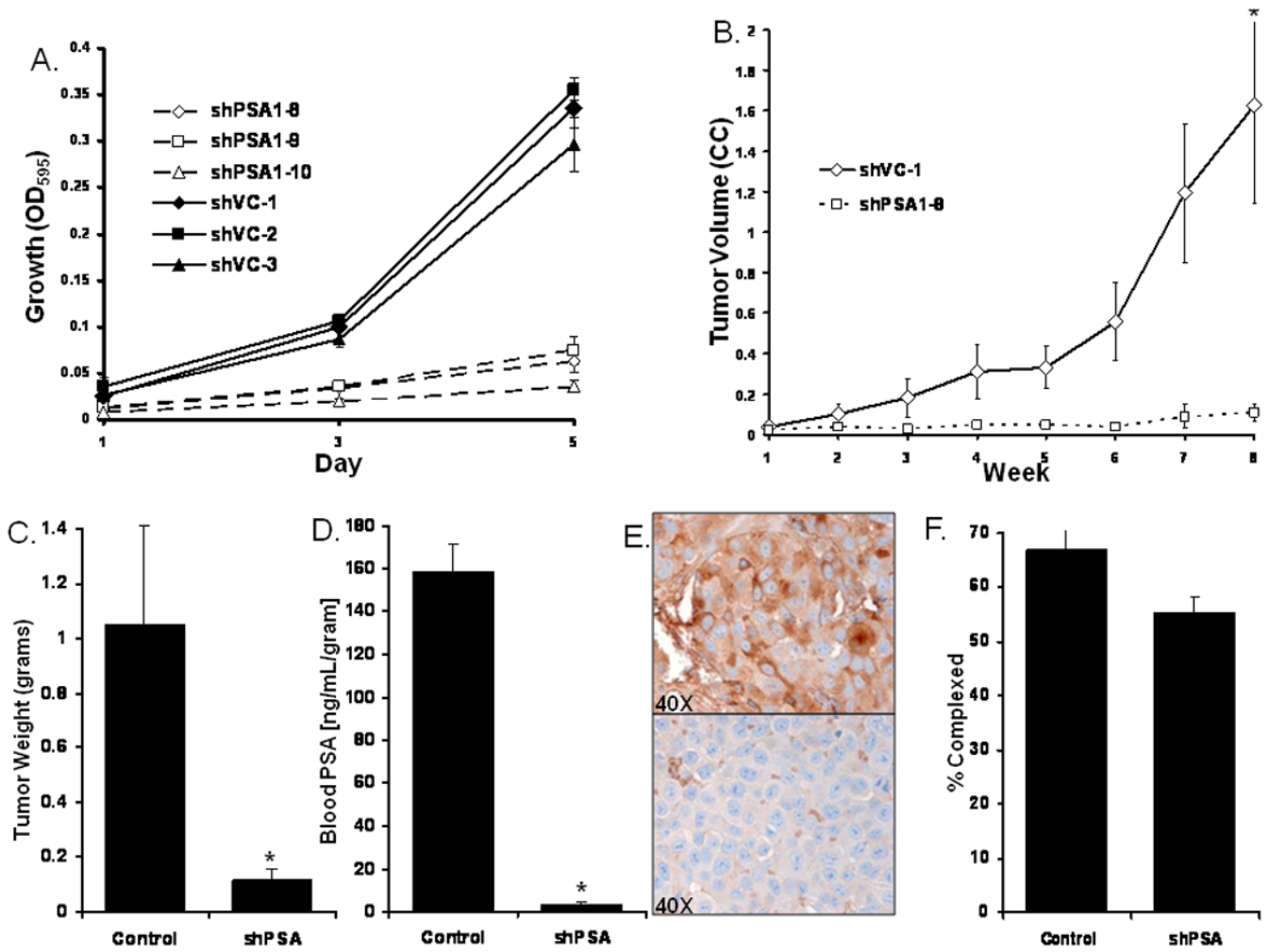
A

Construct Name	shRNA Sequence	Colonies?	PSA knockdown?
sh PSA1	1020GCCTGGAGACATATCACTCAA <sup>1041</sup>	Yes	Yes
sh PSA2	253GGCACAGCCTGTTTCATCCTGAA <sup>276</sup>	Yes	No
sh PSA3	550GGACCTCCATGTTATTTCCAAT <sup>572</sup>	Yes	No
sh PSA4	332GAGCCTCCTGAAGAATCGATTCT <sup>356</sup>	No	N/A
sh PSA5	541TGTGTGGACCTCCATGTTATT <sup>561</sup>	No	N/A

B.

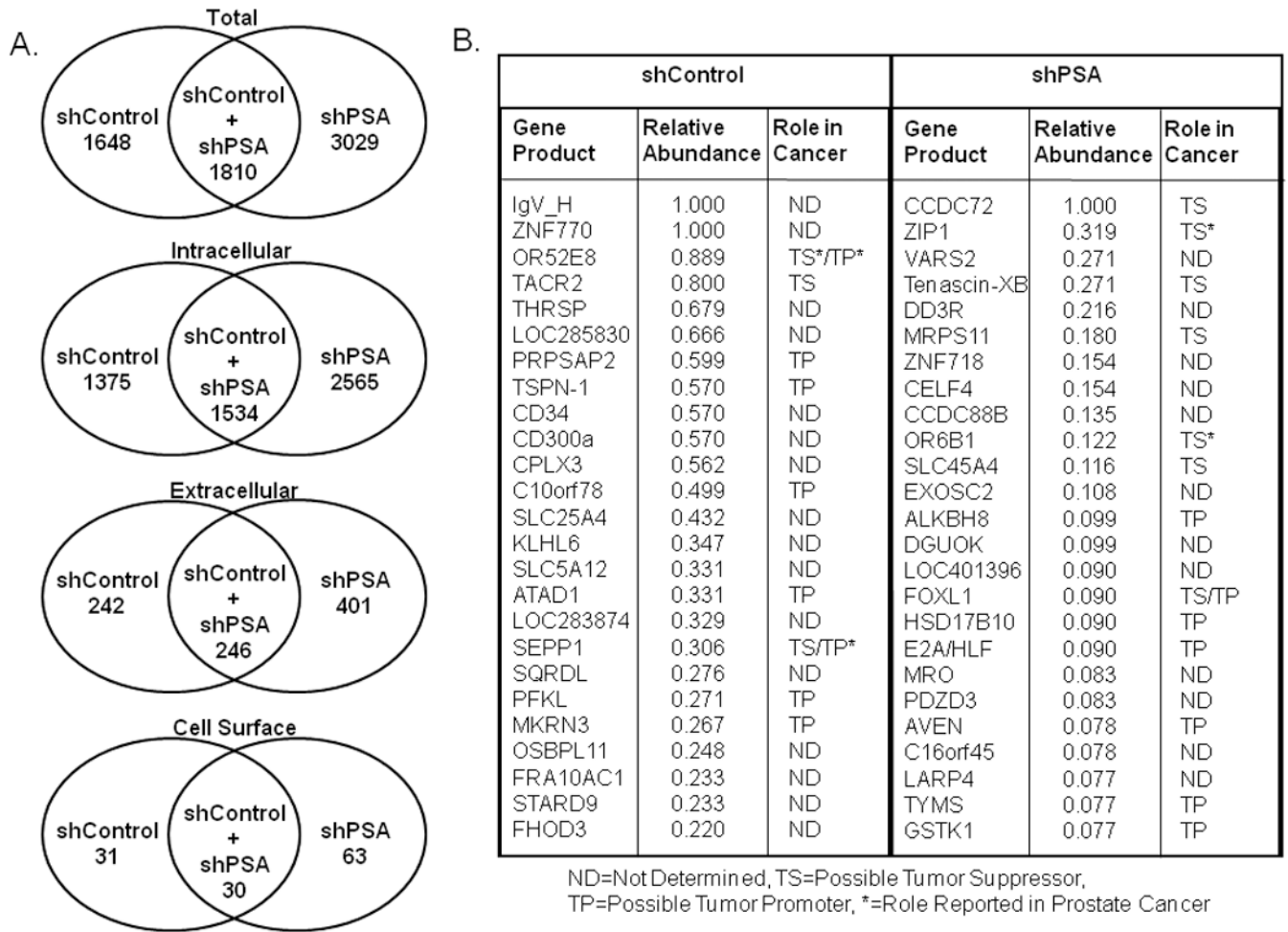
**Figure 1.**

Targeted disruption of PSA expression by shRNA. **A**, Sequences used for targeting. Five sequences based on the PSA cDNA were used to develop lentiviral shRNA expression vectors. **B**, Puromycin-resistant colonies derived from LNCaP cells transduced with the shRNA lentiviral constructs were initially characterized for their PSA protein production rates. Total protein levels were determined from whole cell lysates (WCL) and PSA concentrations in the conditioned media were subsequently measured using the Hybritech PSA assay.

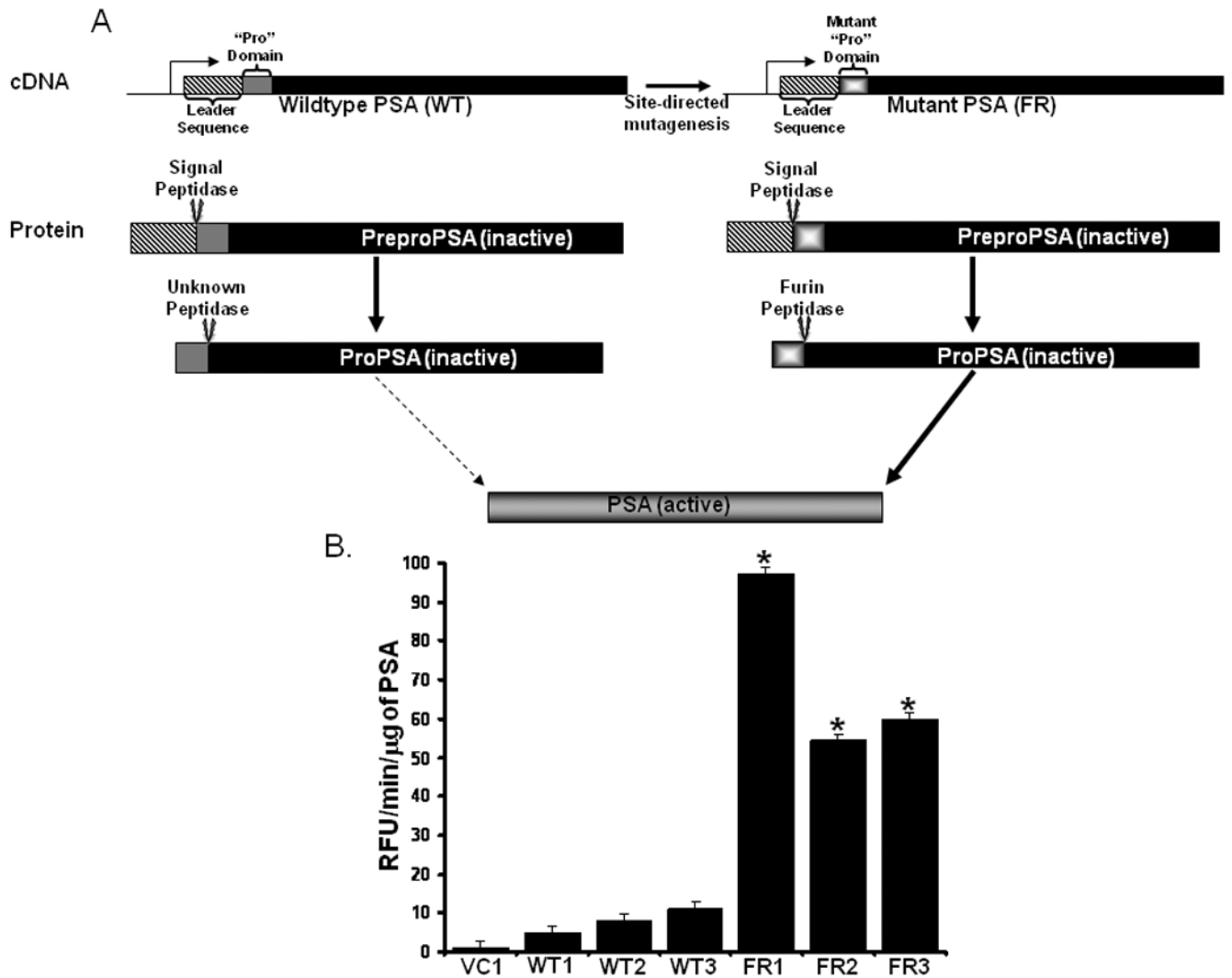


**Figure 2.**

Effect of reduced PSA production on LNCaP cell growth rates. **A**, In vitro growth. LNCaP-derived populations expressing PSA-targeted shRNA (**dashed lines, open symbols**), or a control construct (**solid lines, closed symbols**), were subjected to the MTT assay. Means and 95% confidence intervals shown for experiments performed in replicates of eight. **B**, In vivo growth. Populations of LNCaP cells expressing the PSA-targeted shRNA construct [shPSA1-8 (n=10) and shPSA-9 (n=10)] (**squares**) or vector control constructs [shVC-2 (n=9) and shVC-4 (n=9)] (**diamonds**) were injected into the flanks of nude mice. Tumor volume measurements were taken weekly. Combined average tumor volumes from both populations (shPSA1-8 and shVC-1) with 95% confidence intervals are shown. \* describes p<0.05. **C**, Tumor weights. Tumors were harvested and weighed at the end of the study. Means and 95% confidence intervals are shown. \* describes p<0.05. **D**, Plasma PSA. Tumor-bearing mice were sacrificed and whole blood was harvested by cardiac puncture. Plasma was subjected to PSA analysis and the results were normalized to tumor weight. Combined Means and 95% confidence intervals are shown. \* describes p<0.05. **E**, PSA staining of xenografts. Upper image is an example of tissue from a tumor expressing the control construct. Lower image represents tissue from the shPSA1-8 tumors. **F**, Active PSA levels in plasma. In addition to total PSA level measurements, plasma from tumor-bearing mice was subjected to free PSA analysis. ACT-complexed PSA levels were then calculated (=total PSA – free PSA). 95% confidence intervals are shown.

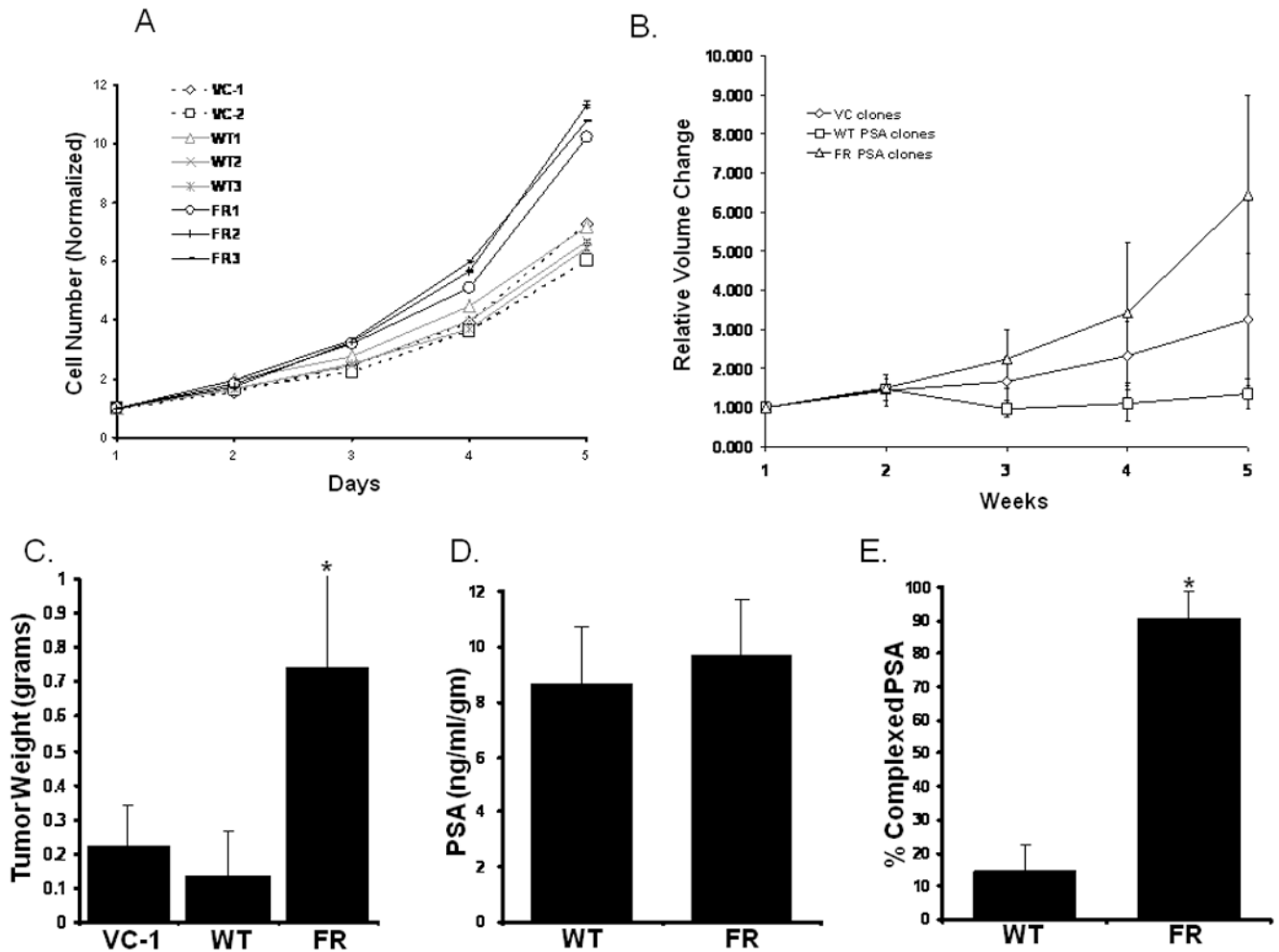
**Figure 3.**

Summarized proteomic analysis of LNCaP variant conditioned media. LNCaP shPSA and shControl populations were used to condition serum-free media for 48 hours, the protein from which was subjected to proteomics analysis. **A**, Overall distribution of LNCaP conditioned media proteome. **B**, Table of proteins with highest abundance in LNCaP-variant conditioned media. The Protein Center proteomics platform used the exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount based on the number of sequenced peptides per protein [49]. These findings were normalized to the most abundant protein in each condition. The top 25 proteins reproducibly expressed into the conditioned media of either variant are presented, along with an annotation of any reported roles in cancer or prostate cancer in particular. The table describes proteins found in all 3 populations of either variant, and in 2 of 3 experiments.



**Figure 4.** Design and enzymatic activity of PSA variant expression constructs. **A**, Illustration of wild-type (WT) and furin (FR) expression constructs and protein processing in PSA-null background cells. While the leader sequence of the newly synthesized proteins are removed, only the prodomain of the FR construct is removed at significant enough levels to generate active protein. **B**, Du145 clones expressing either the WT or the FR constructs were incubated in serum-free media for 5 days. This conditioned media was filter-concentrated (CCM) and then assayed for its PSA content by immune assay. Equal amounts of PSA were isolated from the CCM by immunoprecipitation and incubated with a PSA-selective fluorogenic peptide substrate. The mean change in fluorescence over time (RFU/min)/μg of PSA is presented for indicated clones with 95% confidence intervals. Experiments are performed in triplicate. \* describes  $p < 0.05$ .





**Figure 5.** Characteristics of stable PSA variant expression in Du145-derived cells. **A**, In vitro growth. Du145-derived clones expressing the empty vector (P, dashed lines), or wild-type (WT, grey lines) or active mutant (FR, black solid lines) PSA variants were subjected to the MTT assay. Means and 95% confidence intervals shown for experiments performed in replicates of eight. **B**, In vivo growth. Du145-derived clones expressing the empty vector (VC, solid lines), or wild-type (WT, dashed lines) or active mutant (FR, wide dashed lines) were injected into the flanks of nude mice. Tumor volume measurements were taken weekly. Means and 95% confidence intervals are shown. **C**, Tumor weights. Tumors were harvested and weighed at the end of the study. Tumor-bearing mice were sacrificed and whole blood was harvested by cardiac puncture. **D**, Plasma was subjected to PSA analysis with results normalized to tumor weights. **E**, Blood Free PSA readings were used to determine complexed protein levels (complexed = total - free). Only active enzyme can form complexes with antichymotrypsin. Means and 95% confidence intervals are shown. \* describes  $p < 0.05$ .