

Prostate Specific Antigen Expression Is Down-Regulated by Selenium through Disruption of Androgen Receptor Signaling

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Abstract

A previous controlled intervention trial showed that selenium supplementation was effective in reducing the incidence of prostate cancer. Physiological concentrations of selenium have also been reported to inhibit the growth of human prostate cancer cells *in vitro*. The present study describes the observation that selenium was able to significantly down-regulate the expression of prostate-specific antigen (PSA) transcript and protein within hours in the androgen-responsive LNCaP cells. Decreases in androgen receptor (AR) transcript and protein followed a similar dose and time response pattern upon exposure to selenium. The reduction of AR and PSA expression by selenium occurred well before any significant change in cell number. With the use of a luciferase reporter construct linked to either the PSA promoter or the androgen responsive element, it was found that selenium inhibited the *trans*-activating activity of AR in cells transfected with the wild-type AR expression vector. Selenium also suppressed the binding of AR to the androgen responsive element site, as evidenced by electrophoretic mobility shift assay of the AR-androgen responsive element complex. In view of the fact that PSA is a well-accepted prognostic indicator of prostate cancer, an important implication of this study is that a selenium intervention strategy aimed at toning down the amplitude of androgen signaling could be helpful in controlling morbidity of this disease.

Introduction

Prostate cancer is the second most common cancer as well as the second most common cause of cancer death in men in the United States. Every year, there are ~190,000 new cases and 30,000 deaths from prostate cancer (1). Age is a major risk factor; the incidence is 1 in 53 for men in their 50s but 1 in 7 for men from 60 to 80 years of age. A chemopreventive modality that can suppress or delay the clinical symptoms of prostate cancer would be well suited for preserving the quality of life in high-risk elderly men. In a previous randomized, placebo-controlled cancer prevention trial in which prostate cancer was evaluated as a secondary end point (974 of the 1312 subjects in the cohort were men), supplementation with a nutritional dose of selenium was found to reduce prostate cancer incidence by 50% (2, 3). Recent studies by Dong *et al.* (4) showed that selenium inhibited human prostate cancer cell growth, blocked cell cycle progression at multiple transition points, and induced programmed cell death. Prostate specific antigen (PSA) is a gene known to be under the control of the androgen receptor (AR) and is a well-accepted marker for the diagnosis and prognosis of prostate cancer. In view of the clinical observation of the effectiveness of selenium in prostate cancer

prevention, it is reasonable to believe that selenium might be able to reduce the expression of PSA. If confirmed, this attribute obviously has the advantage of forecasting the responsiveness to selenium intervention. In this report, we describe a series of experiments that were designed to test the hypothesis that selenium is capable of down-regulating PSA through a mechanism of attenuating the functional intensity of the AR signal transduction pathway.

As discussed previously (4), cultured prostate cells respond poorly to selenomethionine (a commonly used selenium reagent) and only when it is present at supraphysiological levels in the medium. A plausible explanation is that prostate cells have a low capacity in metabolizing selenomethionine to methylselenol (CH_3SeH), which is believed to be the active species for selenium chemoprevention (5). This process normally takes place in the liver and kidney. For this reason, methylseleninic acid ($\text{CH}_3\text{SeO}_2\text{H}$, abbreviated to MSA) was developed by Ip *et al.* (6) specifically for *in vitro* experiments. Once taken up by cells, MSA is readily reduced by glutathione and NADPH to methylselenol (which is rather unstable in itself) via a non-enzymatic reaction. The cellular and molecular responses of prostate cells to physiological concentrations of MSA have been documented in a number of publications (4, 7–10). Thus, we believe we have the right tool to study the effect of selenium on AR signaling.

Materials and Methods

Selenium Reagent, Prostate Cell Culture, and Cell Growth Analysis.

MSA was synthesized as described previously (6). The LNCaP human prostate cancer cells were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml of penicillin, and 100 $\mu\text{g}/\text{ml}$ of streptomycin (11). In some experiments, cells were cultured in an androgen-defined condition by using charcoal-stripped FBS in the presence of 10 nM R1881 (a potent synthetic androgen). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed 24, 48, or 72 h after MSA treatment as described previously (11). For the quantitative determination of AR and PSA transcripts and proteins, cells were exposed to MSA for much shorter periods of time, usually 24 h or less. Total RNA and protein were isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA).

Real-Time Reverse Transcription-PCR. First-strand cDNA was synthesized from 100 ng of total RNA by SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's protocol. The PCR primers and TaqMan probes for β -actin, AR, and PSA were Assays-on-Demand products from Applied Biosystems. Two μl of first-strand cDNA were mixed with 25 μl of 2 \times Taqman Universal PCR Master Mix (Applied Biosystems) and 2.5 μl of 20 \times primers/probe mixture in a 50- μl final volume. Temperature cycling and real-time fluorescence measurement were performed using an ABI prism 7700 Sequence Detection System (Applied Biosystems). The PCR conditions were as follows: an initial incubation at 50°C for 2 min, then a denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

The relative quantitation of gene expression was performed using the comparative C_T ($\Delta\Delta C_T$) method (12). Briefly, the threshold cycle number (C_T) was obtained as the first cycle at which a statistically significant increase in fluorescence signal was detected. Data normalization was carried out by subtracting the C_T value of β -actin from that of the target gene. The $\Delta\Delta C_T$ was calculated as the difference of the normalized C_T values (ΔC_T) of the treatment

Received 9/4/03; revised 10/17/03; accepted 11/03/03.

Grant support: Department of Defense Postdoctoral Fellowship Award and an AACR-Cancer Research Foundation of America Fellowship in Prevention Research Award (to Y. D.), Grant CA90271 (to A. C. G.) and Grant CA91990 (to C. I.) from the National Cancer Institute, and also in part by Cancer Center Support Grant P30 CA16056 (to R. P. C. I.) from the National Cancer Institute.

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and control samples: $\Delta\Delta C_T = \Delta C_{T \text{ treatment}} - \Delta C_{T \text{ control}}$. Finally, $\Delta\Delta C_T$ was converted to fold of change by the following formula: Fold of change = $2^{-\Delta\Delta C_T}$.

Western Blot Analysis. Details of the procedure were described previously (4). Immunoreactive bands were quantitated by volume densitometry and normalized against either glyceraldehyde-3-phosphate dehydrogenase or α -actin. The following monoclonal antibodies were used (source): anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Temecula, CA), anti- α -actin (Sigma Chemical Co., St. Louis, MO), anti-AR (BD Transduction Laboratory, San Jose, CA), and anti-PSA (Santa Cruz Biotechnology, Santa Cruz, CA).

Transfection and Luciferase Assay. An aliquot of 3×10^5 cells was placed in a 6-well plate and then transfected with a total amount of 5 μ g of DNA using Superfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. Two different constructs were evaluated: the PSA promoter-luciferase reporter plasmid (13) and the androgen responsive element (ARE)-luciferase reporter plasmid (14). The total amount of plasmid DNA was normalized to 5 μ g/well by the addition of empty plasmid. The DNA/liposome mixture was removed 3 h later, and cells were treated with different concentrations of MSA in the presence of 10 nM R1881. Cell extracts were obtained after 24 h, and luciferase activity was assayed using the Luciferase Assay System (Promega, Madison, WI). Protein concentration in cell extracts was determined by the Coomassie Plus protein assay kit (Pierce, Rockford, IL). Luciferase activities were normalized by the protein concentration of the sample. All transfection experiments were performed in triplicate wells and repeated at least four times.

Nuclear Lysate Preparation. Nuclear protein extract was prepared as described previously (15). Cells were harvested, washed with PBS twice, and resuspended in a hypotonic buffer [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.1% NP40] and incubated on ice for 10 min. Nuclei were precipitated with $3000 \times g$ centrifugation at 4°C for 10 min. After washing once with the hypotonic buffer, the nuclei were lysed in a lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Triton X-100] and incubated on ice for 30 min. The nuclear lysate was precleared by $20,000 \times g$ centrifugation at 4°C for 15 min. Protein concentration was determined by the Coomassie Plus protein assay kit.

Electrophoretic Mobility Shift Assay (EMSA). A quantity of 20 μ g of nuclear protein extract was incubated in a 20- μ l solution containing 10 mM HEPES (pH 7.9), 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 μ g/ml poly(deoxyinosinic-deoxycytidylic acid), and the radiolabeled double-stranded AR consensus binding motif 5'-CTAGAAGTCTGGTACAGGGT-GTTCTTTTGGCA-3' (Santa Cruz Biotechnology). The protein-DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel containing 2.5% glycerol in 0.25 \times Tris-borate EDTA at room temperature, and the results were autoradiographed. Quantitation of AR DNA-binding activity in the "protein-DNA" bandshift was measured using the Molecular Imager FX System (Bio-Rad, Hercules, CA). For the supershift experiment, 20 μ g of cell extract protein were incubated with the monoclonal AR antibody (Santa Cruz Biotechnology) for 1 h at 4°C before incubation with the radiolabeled probe.

Results

MSA Inhibits LNCaP Cell Growth in a Dose- and Time-dependent Manner. Table 1 shows the results of the effect of MSA treatment on cell growth. The data are expressed as percentages of the untreated control. A concentration of 2.5 μ M MSA produced essentially no change, even after 3 days of treatment. Increasing the concentration of MSA to 5 μ M inhibited cell growth by about 25%,

Table 1 Effect of MSA on the accumulation of LNCaP cells^a

| Treatment | Treatment duration (h) ^b | | |
|----------------|-------------------------------------|-----------------------------|-----------------------------|
| | 24 | 48 | 72 |
| MSA (μ M) | | | |
| 2.5 | 102.5 \pm 4.0 | 106.6 \pm 6.2 | 102.5 \pm 1.9 |
| 5 | 93.7 \pm 3.1 | 96.4 \pm 2.9 | 72.6 \pm 1.9 ^c |
| 10 | 77.1 \pm 8.4 ^c | 61.1 \pm 1.7 ^c | 55.4 \pm 3.7 ^c |

^a As a percentage of untreated control.

^b Results are expressed as mean \pm SE ($n = 4$ independent experiments).

^c Significantly different compared with the corresponding control value ($P < 0.05$).

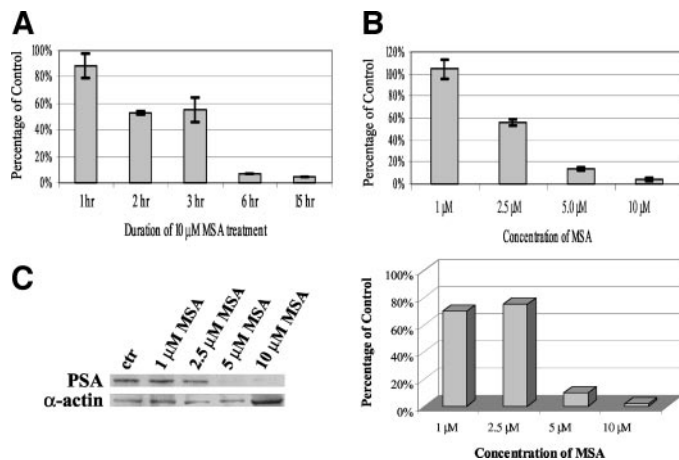


Fig. 1. Effect of MSA on PSA expression. *A* and *B*, changes in PSA mRNA, as determined by quantitative RT-PCR, as a function of time of treatment with MSA or as a function of MSA concentration. With the exception of the 1-h and the 1 μ M MSA data points, the remaining data points are significantly different ($P < 0.01$) from the control, which is set as 100%. Bars, SE. *C*, Western blot data of changes in PSA protein level as a function of different concentrations of MSA (left side) and normalized quantitative changes compared with the control value of 100% (right side).

but the effect was not observed until the 72-h time point. The same magnitude of growth inhibition was observed at 24 h with 10 μ M MSA, and by 72 h, there were 50% fewer cells compared with the untreated culture. The experiment therefore established the dose and time response to MSA with respect to growth inhibition. This information is important because the down-regulation of PSA and AR by selenium occurs well before the onset of growth inhibition (see below).

MSA Suppresses PSA mRNA and Protein Expression in LNCaP Cells. The modulation of PSA mRNA by MSA was assessed quantitatively by real-time RT-PCR. Cells were treated with 10 μ M MSA for various lengths of time; the PSA results are shown in Fig. 1A. A marked decrease in PSA mRNA was detected as early as 2 h after exposure to MSA; the mRNA level dropped to <10% of the control value by 6 and 15 h. As shown in Fig. 1B, the depression of PSA mRNA was dependent on the concentration of MSA in the range between 2.5 and 10 μ M; the assay was performed after exposure to MSA for 15 h. As little as 2.5 μ M MSA reduced PSA mRNA level by 40%. This level of MSA had no effect on cell growth. Even with 10 μ M MSA, the near complete elimination of PSA mRNA expression occurred before there was any detectable change in growth. The decrease in PSA protein level by MSA at 15 h, as determined by Western blot analysis, is shown in Fig. 1C, left panel. The right panel shows the normalized quantitative changes compared with the control value of 100%. Small decreases in PSA protein were evident with 1 or 2.5 μ M MSA. At 5 or 10 μ M MSA, the level of PSA protein became very low or hardly detectable. The experiments described in Fig. 1 were done with cells cultured in 10% FBS. In addition, we carried out another set of experiments with cells cultured in charcoal-stripped FBS containing 10 nM R1881 (a potent synthetic androgen). The down-regulation of PSA mRNA by MSA, as a function of dose and time, was qualitatively similar to that observed with the FBS culture (data not shown).

MSA Suppresses AR mRNA and Protein Expression in LNCaP Cells. The expression of PSA is known to be regulated by AR, which is a ligand-activated transcription factor. Our next step was to investigate the expression of AR mRNA in response to MSA by real-time RT-PCR. Fig. 2A shows the time course of response to 10 μ M MSA. Within the first hour, there was a 50% decrease in AR mRNA. The transcript level continued to drop down to 20% or below with longer

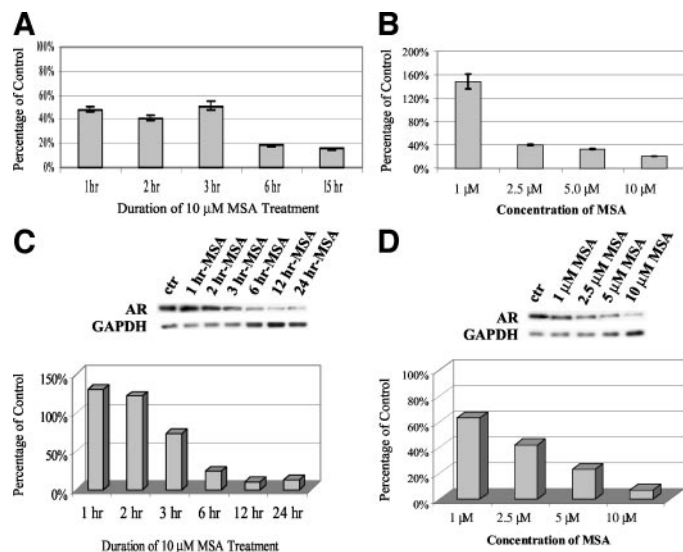


Fig. 2. Effect of MSA on AR expression. *A* and *B*, change in AR mRNA, as determined by quantitative RT-PCR, as a function of time of treatment with MSA or as a function of MSA concentration. All of the data are significantly different ($P < 0.01$) from the control, which is set as 100%. Bars, SE. *C*, Western blot data of changes in AR protein level as a function of time of treatment with 10 μM MSA. *D*, Western blot data of changes in AR protein level as a function of different concentrations of MSA. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

treatment with MSA. The dose response to MSA is shown in Fig. 2*B*; these assays were done at the 15-h time point. Interestingly, 1 μM MSA actually increased slightly the level of AR mRNA. However, raising the concentration of MSA to 2.5 μM or above caused a very significant depression of the AR transcript to 40% or less of the control value. We next examined AR protein expression in response to 10 μM MSA. As shown in Fig. 2*C*, MSA down-regulated AR protein level progressively as a function of time over a period of 24 h. Initially, the reduction in protein appeared to lag behind the reduction in transcript by at least 2–3 h. The delay in response might be reflective of the time needed for protein turnover. Fig. 2*D* shows the effect of different concentrations of MSA on expression of the AR protein. MSA produced a graded suppression of the AR protein in a dose-dependent manner. In general, the changes in protein level were consistent with the real-time RT-PCR results with the exception of the 1 μM MSA data point.

MSA Inhibits AR *trans*-Activating Activity. LNCaP cells have a mutant but functional AR. In an attempt to determine the ability of MSA to interfere with AR *trans*-activating activity, we transiently transfected LNCaP cells with an expression vector for the wild-type AR and the PSA promoter-luciferase reporter plasmid. This region of the PSA regulatory element contains the promoter and enhancer and has been demonstrated to be responsive to androgen stimulation (13). As shown in Fig. 3*A*, MSA inhibited the luciferase reporter in a dose-dependent manner. Thus, the PSA promoter activity was decreased by 50, 67, 93, or 96% in the presence of 1, 2, 5, or 10 μM MSA, respectively.

Activated AR exerts its function by binding to the ARE site. Because the PSA promoter contains many regulatory elements in addition to the ARE, one could argue that the decrease in PSA promoter activity might not necessarily be attributable to a change in AR *trans*-activating activity. To address this issue, we transiently transfected LNCaP cells with an expression vector for the wild-type AR and the ARE-luciferase reporter plasmid. This construct contains three repeats of the ARE region ligated in tandem to the luciferase reporter (14). As shown in Fig. 3*B*, the ARE-luciferase activity was inhibited by 50, 60, or 75% in the presence of 1, 2, or 5 μM MSA,

respectively. The result obtained with 10 μM MSA was similar to that with 5 μM MSA.

MSA Decreases Binding of AR to ARE. To determine whether MSA might reduce the DNA binding activity of the AR protein to the ARE, we performed EMSA using radiolabeled oligonucleotides of the ARE with nuclear extract from LNCaP cells treated for 30 min with various concentrations of MSA. As shown in Fig. 4, *A* and *B*, a decrease in AR-ARE complex formation was evident with MSA treatment compared with the untreated control. We can rule out the reduced availability of the AR protein as a contributing factor, because there was no change in AR protein after only 30 min of

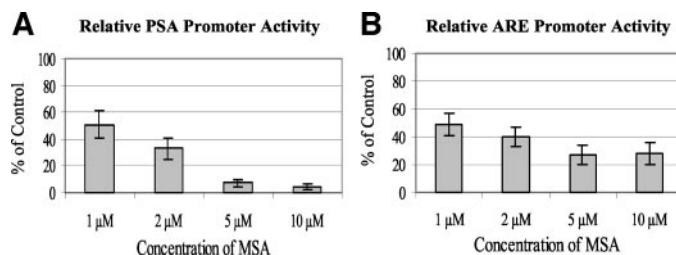


Fig. 3. Effect of MSA on PSA promoter activity (*A*) and ARE promoter activity (*B*). The cells were cultured in charcoal-stripped FBS containing 10 nM R1881. The results are expressed as percentages of untreated control. All of the data points are significantly different ($P < 0.01$) from the control value. Bars, SE.

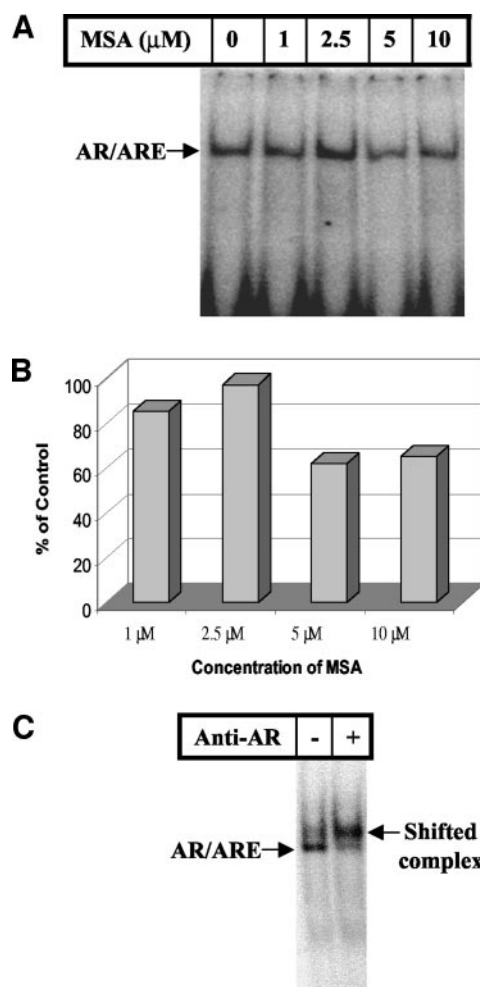


Fig. 4. EMSA results of AR binding to ARE as a function of different concentrations of MSA. *B*, quantitative determination of the EMSA results. *C*, supershift of the AR/ARE complex with antibody against AR.

treatment with MSA (see Fig. 2C). The specificity of the AR-ARE complex was demonstrated by the supershift assay using an antibody against AR (Fig. 4C).

Discussion

This report is the first to show that a selenium metabolite is able to down-regulate the expression of PSA in human prostate cancer cells via a mechanism involving disruption of the androgen signal transduction pathway. On the basis of the information from this study, selenium decreases AR transcript and protein and inhibits AR *trans*-activating activity. Selenium can also diminish the binding of AR to the ARE site. However, we cannot at this time distinguish whether this is attributable to a block in nuclear translocation of the activated AR or a physical interference of AR association with the ARE through modulation of other co-regulators. These various possibilities will be investigated systematically in the future. It is noteworthy that the reduction in AR and PSA expression occurs at least 20 h before any significant decrease in cell number. This kind of bellwether change at the molecular level might be one of the causes underlying the sensitivity of prostate cells to selenium treatment.

In a recent paper, Bhamre *et al.* (16) reported that although supra-physiological levels of selenomethionine inhibited LNCaP cell growth, selenomethionine did not specifically affect the production of PSA when the results were normalized to the decreased number of viable cells. As explained in the "Introduction," selenomethionine is not a suitable selenium reagent for cell biology studies *in vitro*, because it is poorly metabolized by cultured epithelial cells to the active monomethylated intermediate. Not surprisingly, many cellular and molecular events that are normally sensitive to modulation by physiological levels of MSA (4, 7–11) respond very sluggishly to selenomethionine, and only when it is present at excessively high levels in the medium. Thus, the discrepancy between our study and that of Bhamre *et al.* (16) can be reconciled by the differences in biochemical reactivity between MSA and selenomethionine.

The clonal expansion of prostate cancer at the early stage is mostly dependent on androgen stimulation. A selenium intervention strategy aimed at dampening the amplitude of androgen signaling could be helpful for controlling prostate cancer in high-risk men. PSA is a well-accepted diagnostic and prognostic biomarker of prostate cancer progression. The down-regulation of PSA by selenium therefore has significant clinical implication. In patients treated with selenium, the monitoring of PSA in the circulation could potentially be evaluated as a barometer to gauge the efficacy of intervention. The benefit might also be extended to the prevention of relapses after endocrine therapy. Recurrent prostate cancer is generally hormone refractory, although the expression of AR is maintained regardless of the clinical stage of the disease (17, 18). The fact that PSA continues to be produced by the pathologically advanced cancer suggests that the AR signal transduction pathway is still intact. Several hypotheses have been proposed to explain this phenomenon. Mutations of the AR may enable cells to be sensitized by very low levels of androgens, or perhaps even by non-androgen steroids (19). Alternatively, the receptor may become promiscuous and can be activated by non-steroidal growth factors and cytokines (20). Prostate cancer may also adapt to androgen deprivation by increasing the expression of AR through gene amplification (21, 22). We have developed a LNCaP subline that is not responsive to androgen but is capable of producing a copious amount of PSA. We are planning to use this cell model to further investigate the role of

selenium in AR function when the presence of androgen is no longer required.

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