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Development of an Androgen-Deprivation Induced and Androgen Suppressed Human Prostate Cancer Cell Line

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BACKGROUND. Emergence of recurrent cells during androgen-deprivation therapy and intermittent androgen suppression therapy suggest that a subset of prostate cancer cells survive and proliferate in the androgen deprivation condition. Some of the recurrent cells that emerge during the androgen-deprivation therapy and intermittent androgen suppression therapy could be suppressed by replacement of androgen. In an attempt to recapitulate the clinical phenomenon, we developed an androgen-deprivation induced and androgen suppressed human prostate cancer cell line.
METHODS. LNCS, an androgen-deprivation induced and androgen suppressed human prostate cancer cell line, was generated from an androgen-sensitive LNCaP cells cultured in PRMI-1640 media containing charcoal-stripped FBS for a prolong period for more than a year. The responsiveness to androgen in vitro and in vivo was examined. The characteristics of this subline including activation of signaling pathways were investigated.
RESULTS. LNCS, an androgen-deprivation induced and androgen suppressed human prostate cancer cell line, was developed. LNCS cells express considerably lower levels of androgen receptor than LNCaP cells and grow vigorously in androgen deprived condition in vitro, and develop tumors in castrated male mice in vivo. Addition of androgen inhibits cell growth in vitro and tumor growth in vivo. LNCS cells are more resistant to etoposide, a typical apoptotic inducing agent. Although AR signaling is less active in LNCS cells, Stat3 is constitutively activated. Down regulation of Stat3 activation inhibits LNCS cell growth in vitro.
CONCLUSIONS. We have developed an androgen-deprivation induced and androgen suppressed human prostate cancer cell line. This cell line is a valuable tool to study molecular mechanisms of the progression of androgen refractory prostate cancer and intermittent androgen suppression therapy. *Prostate 67: 1293–1300, 2007.* © 2007 Wiley-Liss, Inc.

KEY WORDS: **androgen; hormone therapy; Stat3**

INTRODUCTION

Prostate cancer remains the second leading cause of cancer death among American men with an increasing incidence [1]. Initially, the growth of prostate cancer is dependent on androgen and can be effectively treated by androgen-deprivation therapy either by bilateral orchiectomy or anti-androgens such as gonadotropin-releasing hormone agonists. However, androgen-deprivation therapy only causes a temporary regression of prostate cancer, as all tumors will eventually progress

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to refractory to hormonal therapy after 1–3 years of treatment [2–4]. Understanding the mechanisms by which hormonally sensitive tumor cells lose hormone dependency or otherwise acquire the ability to grow in hormone deprivation conditions has therefore been an important objective of finding of the effective treatments of prostate cancer. Recent advances identified several possible mechanisms involved in the progression of hormone refractory prostate cancer cells. Prostate cancer cells can survive in low levels of androgens by several mechanisms including androgen receptor mutation or amplification [5,6]; or increasing 5α-reductase activity to convert testosterone to dihydrotestosterone [7]. Several different growth factors such as EGF and cytokines also stimulate proliferation by ligand-independent mechanism. Androgen receptor (AR) can be sensitized by growth factors, cytokines, and co-regulators [8–12]. In addition, clonal expansion of cells with neuroendocrine (NE) differentiation may also involve in hormone refractory prostate cancer progression [13].

One of the critical questions for treatment of advanced prostate cancer remains how to overcome or delay the development of hormonal resistance to androgen-deprivation therapy. Clinical and experimental studies showed that some of the patients with hormone refractory prostate cancer respond to secondary hormonal manipulation, such as intermittent androgen suppression in which androgen-deprivation is followed by a period of androgen recovery, and then successive rounds of androgen-deprivation and replacement [14–19]. Clinical trials demonstrate that patients with biochemical recurrence after irradiation for localized prostate cancer are responsive to intermittent androgen suppression therapy with relatively fewer adverse events [15,20]. The rationale is to allow prostate cancer cells to recover responsiveness to androgen during the periods of androgen replacement and thereby prolong overall responsiveness of the prostate cancer to antiandrogen and/or androgen-deprivation in the patient. Clinical and experimental studies suggest that androgen deprivation therapy may trigger some set of tumor population to proliferate in the androgen deprivation condition. Some of the recurrent cells that emerge during the androgen-deprivation therapy and intermittent androgen suppression therapy could be suppressed by replacement of androgen [16,21,22]. Development of a cell culture model that represents such a phenotype (i.e., androgen-deprivation induced and androgen suppressed cells) would be valuable for understanding both the progression of androgen refractory prostate cancer and intermittent androgen suppression therapy.

In this study, we developed an androgen-deprivation induced and androgen suppressed human prostate cancer cell line, named as LNCS, from an androgen-sensitive LNCaP human prostate cancer cells by prolonged culture of LNCaP cells in charcoal-stripped serum condition. These cells express considerably lower levels of AR than LNCaP cells and grow vigorously in charcoal-stripped FBS condition in vitro, and develop tumors in castrated male mice. Addition of androgen inhibits cell growth in vitro and tumor growth in vivo. Although AR signaling is inactive in LNCS cells, Stat3 is constitutively activated. Inhibition of Stat3 activation in LNCS cells suppressed cell growth in androgen deprivation condition.

METHODS

Cell Culture

LNCaP cells were grown in RPMI 1640 media containing 10% FBS and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% carbon dioxide. LNCS cells were developed by culturing LNCaP cells in RPMI 1640 media containing 10% charcoal-stripped FBS continuously for a prolonged time. Initially after two passages in media containing 10% charcoal-stripped FBS, most of the LNCaP cells died and very few cells survived. Even though cell density was very low, cells still grew slowly and were passaged when they reached to 40–50% of confluence. At the passage number of 21, almost after 1 year of culturing in the RPMI-1640 media containing 10% charcoal-stripped FBS, cells suddenly began to grow well. We named them as LNCS cells since they grow well in medium containing charcoal-stripped FBS. LNCS cells were maintained in RPMI-1640 media containing 10% charcoal-stripped FBS.

Morphological Analysis

LNCaP cells and LNCS cells were imaged by photomicoscopy using phase-contrast optics (Leica, Rijswijk, The Netherlands).

In Vitro Cell Growth assay

LNCaP cells and LNCS cells were cultured in RPMI1640 media containing 10% charcoal stripped FBS in the presence of different concentrations of R1881. Cell number was determined by Coulter Counter. For growth comparison, LNCaP cells and LNCS cells were plated in 12-well culture plate (1×10^4 cell/well). LNCaP cells were grown in RPMI1640 media containing 10% FBS, and LNCS cells were grown in RPMI1640 media containing 10% charcoal-stripped FBS. Cells were counted each day using Coulter Counter.

In Vivo Growth Study

LNCaP and LNCS cells (2×10^6) were subcutaneously injected into either castrated (1 week prior to injection) or intact male nude mice. LNCaP cells were injected into intact mice as controls. Tumor volume was measured twice a week. When tumor volume reached to about 300 mm^3, the castrated male mice injected with LNCS cells were divided into two groups. Testosterone (100 μg/ day, suspended as 500 μg/ml in vegetable oil) was given into one group intraperitoneally and the other group was injected with vehicle (vegetable oil) as controls.

Western Blot analysis

Whole cell extracts were obtained using high salt buffer (10 mM, HEPES-KOH (pH7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 mM NaV, 20 mM NaF, 20% glycerol, and 1 μg/ml protease inhibitor) with freezing-thawing procedure as described previously [23]. Whole cell extracts were resolved in 8 or 10 % SDS–PAGE. Proteins were then transferred to nitrocellulose membrane. After blocking overnight at 4°C in 5% milk in PBS-0.1% Tween-20, membranes were incubated for 1 hr at room temperature with antibodies against AR, Stat3 (Santa Cruz, CA), and phosphorylated Stat3 (Cell Signaling, CA) in 1% milk in PBS-Tween-20. Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buchinghamshire, England).

Measurement of PSA

LNCaP and LNCS cells were grown in 24 well plates in 5% CS-FBS in the absence and presence of androgen. After 24 hr of incubation, PSA in supernatants were measured using ELISA kit according to the manufacturer's instruction and as described previously [24]. PSA levels were normalized based on cell number.

Small Interfering RNA (siRNA) Preparation and Transfection

The Stat3 siRNA and scramble siRNA was described previously [25]. One day prior to transfection, 3×10^5 cells were seeded in 6-well plate. Cells at 40–60% confluence were transfected with the scramble and Stat3 siRNAs, respectively. Serum-free media (100 μl) containing 3 μg siRNA were mixed with 100 μl serum-free media containing 9 μl Lipofectamine reagents and held at room temperature. The medium in a 6-well plate was replaced by serum-free medium (800 μl/well), the siRNA-Lipofectamine mixture prepared above was added onto each well in the 6-well plate within 20–45 min after the mixture was prepared. The number of cells were counted using Coulter Counter 72 hr post-transfection. Stat3 expression was analyzed by Western blots.

Statistical Analysis

Student's t test (two-tailed) was used to determine the significance between control and the treatment groups, and $P < 0.05$ was considered significant.

RESULTS

Development of Androgen-Deprivation Induced and Androgen Suppressed LNCS Cell Line

LNCS cell line was established by long-term culture of androgen-sensitive human LNCaP (passage number less than 20) prostate cancer cells in RPMI-1640 media containing charcoal-stripped FBS. Initially, most of the cells died and a few cells survived during the first two passages of LNCaP cells cultured in RPMI-1640 media containing 10% charcoal-stripped FBS. The cells that survived displayed a neuroendocrine-like morphology, similar to the reports by several other laboratories [26,27]. Even though cell density was very low, cells still grew slowly and were passaged when they reached 40–50% of confluence. At the passage number of 21, almost after 1 year of culturing in the RPMI-1640 media containing 10% charcoal-stripped FBS, cells suddenly began to grow well. We named them as LNCS cells since they grow well in medium containing charcoal-stripped FBS. LNCS cells were maintained in RPMI-1640 media containing 10% charcoal-stripped FBS (Fig. 1).

Androgen responsiveness of LNCS cells was compared with parental LNCaP cells in vitro under phenol red-free conditions containing 10% charcoal-stripped FBS in the presence of different concentrations of R1881. As expected, LNCaP cells respond to R1881 in a biphasic manner (Fig. 2A). In contrast, R1881

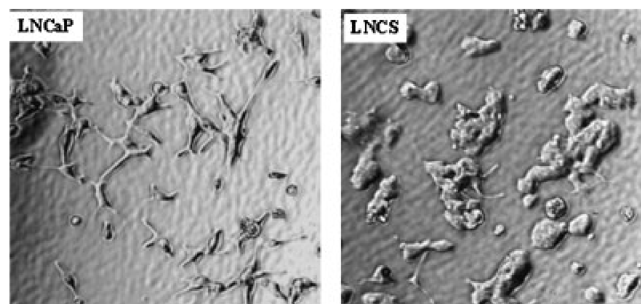

Fig. 1. Cell morphology. LNCaP and LNCS cells were grown in 100 mm culture dish in RPMI 1640 medium containing 10% FBS and 10% charcoal stripped-FBS, respectively. Cell image was visualized using photomicroscopy.

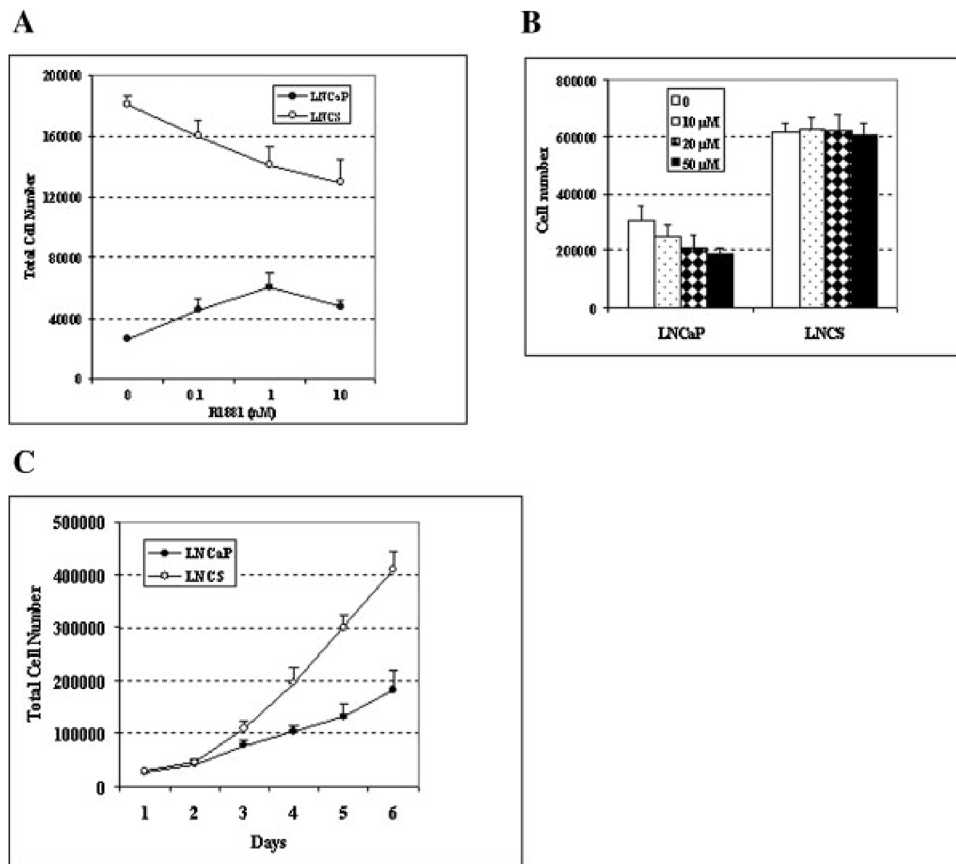

Fig. 2. Growth comparison between LNCaP and LNCS cells. **A**: The effects of androgen on LNCaP and LNCS cell growth. LNCaP and LNCS cells were cultured in RPMI1640 media containing 10% charcoal stripped FBS in the presence of different concentrations of R1881. Cell number was determined by Coulter Counter. Each point represents at least four independent experiments. **B**: The effects of bicalutamide on LNCaP and LNCS cell growth in vitro. LNCaP and LNCS cells were plated in 12-well culture plate (1×10^4 cell/well). LNCaP cells were grown in RPMI1640 media containing 10% FBS, and LNCS cells were grown in RPMI1640 media containing 10% charcoal-stripped FBS in the presence of different concentrations of bicalutamide as indicated. Cells were counted after 3 days using Coulter Counter. Each point represents at least four independent experiments. **C**: LNCS cells grow faster than LNCaP cells. LNCaP and LNCS cells were plated in 12-well culture plate (1×10^4 cell/well). LNCaP cells were grown in RPMI1640 media containing 10% FBS, and LNCS cells were grown in RPMI1640 media containing 10% charcoal-stripped FBS. Cells were counted at each day using Coulter Counter. Each point represents at least four independent experiments.

suppressed the growth of LNCS cells (Fig. 2A). Addition of antiandrogen, such as bicalutamide, was not able to reverse androgenic suppression of growth of LNCS cells (Fig. 2B). We next compared the growth of LNCS cells and LNCaP cells. LNCS cells grow at much accelerated rate in RPMI-1640 media containing 10% charcoal-stripped FBS compared to LNCaP cells grown in RPMI-1640 media containing 10% FBS (Fig. 2C).

In order to investigate whether LNCS cells grow in the absence of androgen in vivo as well, we performed animal study. One group of male mice was castrated prior to injection of cells. LNCS cells were injected into either castrated or intact mice. The tumor growth was monitored twice a week. As shown in Figure 3, LNCS cells developed tumors within 10 days of injection and the incidence of tumor development was higher than 90%. The tumors grew more vigorously in castrated mice group than the intact one (Fig. 3). More interestingly, when testosterone was given to the castrated mice, the tumor growth was decreased dramatically (Fig. 3). These results suggest that LNCS grow tumors subcutaneously, and androgen-deprivation accelerates the tumor growth, which can be suppressed by the addition of exogenous androgen.

AR Signaling is Suppressed in LNCS Cells

Parental LNCaP cells express mutant but functional AR protein. We next examined AR expression in LNCS cells. Surprisingly, the levels of AR protein expression were not detectable in LNCS cells by Western blot analysis (Fig. 4A). Similarly, when we analyzed tumor samples obtained from LNCS cells, the level of AR protein was not detectable by Western blot analysis

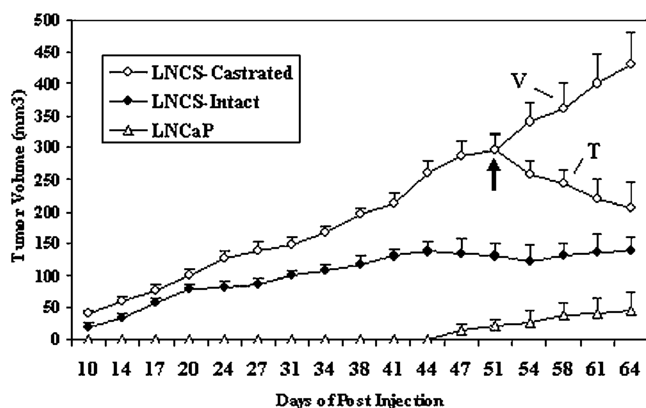

Fig. 3. Progression of LNCaP and LNCS tumor xenografts in athymic nude mice. LNCS cells (2×10^6) were subcutaneously injected into either 16 castrated (1 week prior to injection) or 10 intact male nude mice, respectively. The same number of LNCaP cells was also injected into 10 intact mice as controls. The tumor volume was measured and monitored twice a week. When tumor volume reached about to 300 mm^3, the castrate mice that have been injected with LNCS cells were divided into two groups with 8 mice for each group. One group was given testosterone (100 μg/day) intraperitoneally (T) for 14 days, and the other group was injected with vehicle as controls (V). The arrow indicates starting point of testosterone injection. Points, mean of tumor volumes; bars, SE.

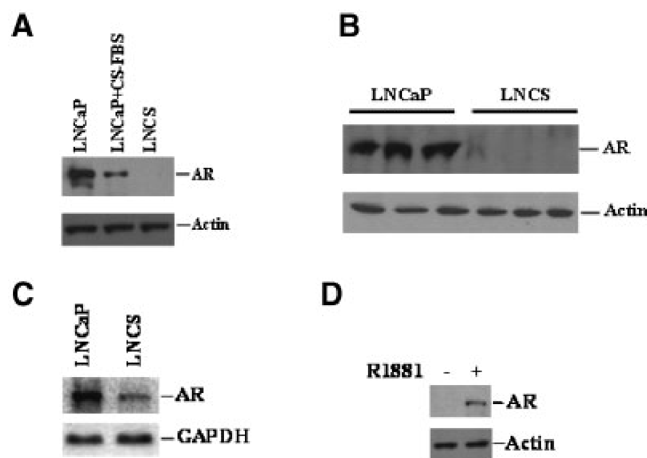

Fig. 4. AR expression is decreased in LNCS cells. **A**: AR protein expression. LNCaP cells were grown in 100 mm culture dish in RPMI 1640 containing either 10% FBS or 10% charcoal-stripped FBS for 3 days. LNCS cells were grown in RPMI 1640 containing 10% charcoal-stripped FBS. Whole cell extracts were isolated and subjected to Western blot analysis. **B**: AR protein expression in tumor tissues. Total protein extracts were isolated from tumors of mice injected with LNCaP cells or LNCS cells and subjected to Western blot analysis. Actin is the loading control. **C**: AR mRNA expression in LNCaP and LNCS cells by Northern blot analysis. **D**: AR protein expression in LNCS cells treated with R1881. LNCS cells were cultured in RPMI1640 containing either 10% charcoal-stripped FBS or 10% charcoal-stripped FBS plus 10 nM R1881 for 3 days. Whole cell extracts were isolated and subjected to Western blot analysis.

(Fig. 4B). However, Northern blot analysis indicated that AR mRNA is expressed in LNCS cells, albeit in a much lower levels compared to LNCaP cells (Fig. 4C). We next tested whether addition of androgen in LNCS cell culture recovers AR protein expression. LNCS cells were treated with 10 nM R1881 for 3 days and whole cell extracts were isolated and subjected to Western blot analysis. The levels of AR protein were detectable in LNCS cells after R1881 treatment, but at much lower levels compared to LNCaP cells (Fig. 4D).

The levels of PSA, a typical androgen-responsive gene, were compared between LNCaP and LNCS cells. As expected, the expression of PSA was severely repressed compared to LNCaP cells (Fig. 5A). This repression was also shown in serum sample obtained from LNCS tumor bearing mice (Fig. 5B). While significant amount of PSA secretion was detected in serum of LNCaP tumor bearing mice, no detectable PSA protein was observed in serum of the LNCS tumor bearing mice (Fig. 5B).

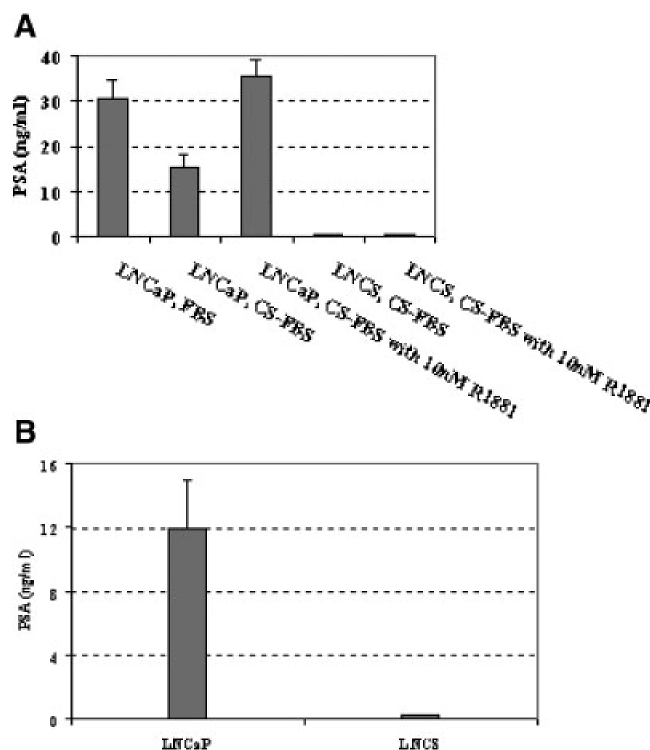

Fig. 5. PSA secretion in LNCaP and LNCS cells. **A**: PSA secretion in culture supernatant of LNCaP and LNCS cells. LNCaP cells were grown in 100 mm culture dish in RPMI 1640 containing either 10% FBS or 10% charcoal-stripped FBS for 3 days in the presence and absence of 10 nM R1881. LNCS cells were grown in RPMI 1640 containing 10% charcoal-stripped FBS for 3 days in the presence and absence of 10 nM R1881. Culture supernatants were obtained and the levels of PSA protein secretion were determined by PSA ELISA analysis. **B**: Secretion of PSA in serum of LNCaP and LNCS tumor bearing mice. Sera were taken from LNCaP or LNCS derived tumor bearing mice. PSA amount was analyzed by PSA ELISA.

Stat3 is Constitutively Activated in LNCS Cells

Constitutive activation of Stat3 is linked to tumor growth and androgen independent progression [10,28]. We next examined whether alteration of Stat3 is associated with the growth of LNCS cells. The levels of phosphorylated Stat3 are not detectable in LNCaP cells grown in RPMI-1640 media containing 10% charcoal-stripped FBS for 3 days. However, phosphorylated Stat3 is highly expressed in LNCS cells grown in RPMI-1640 media containing 10% charcoal-stripped FBS (Fig. 6A). To determine whether Stat3 activation is involved in LNCS growth in RPMI-1640 media containing 10% charcoal-stripped FBS, we used Stat3 siRNA to knockdown Stat3 expression (Fig. 6B). Down-regulation of Stat3 by Stat3 siRNA significantly inhibited LNCS cell growth in RPMI-1640 media containing 10% charcoal-stripped FBS compared to the scramble siRNA control, while have little effect on LNCaP cell growth (Fig. 6C). These results suggest that Stat3 activation plays a critical role in mediating LNCS cell growth in androgen deprived condition.

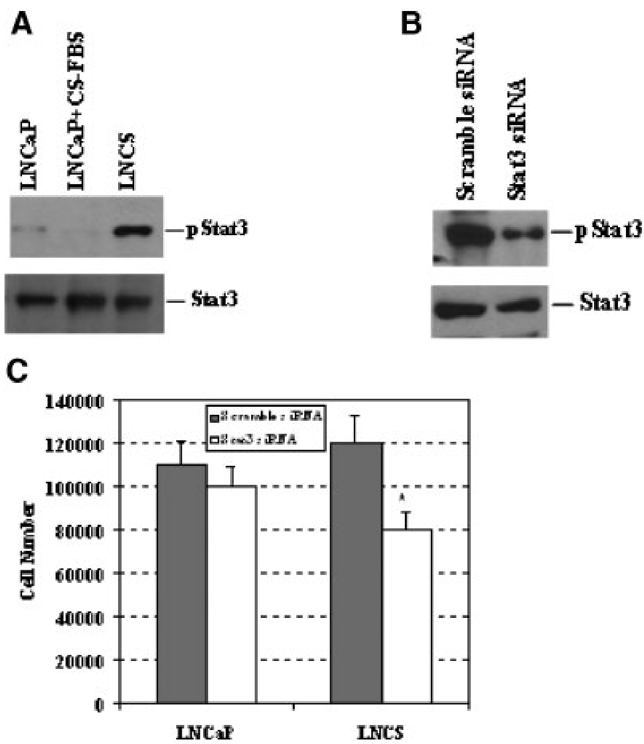

Fig. 6. The effects of Stat3 activation on the growth of LNCS cells. **A**: Stat3 is constitutively activated in LNCS cells. LNCaP cells were grown in 100 mm culture dish in RPMI 1640 containing either 10% FBS or 10% charcoal-stripped FBS for 3 days before harvest. LNCS cells were grown in RPMI 1640 containing 10% charcoal-stripped FBS. Whole cell extracts were isolated and subjected to Western blot analysis using antibodies against either phosphorylated Stat3 or total Stat3. **B**: The levels of phosphorylated Stat3 was decreased by Stat3 siRNA. LNCS cells were transfected with either Stat3 siRNA or scramble siRNA as a control. The whole cell extracts were isolated 3 days following transfection and subjected to Western blot analysis using antibodies against either phosphorylated Stat3 or total Stat3. **C**: Inhibition of Stat3 activation suppresses LNCS cell growth in vitro. LNCS cells and LNCaP cells were transfected with either Stat3 siRNA or scramble siRNA as a control. Total cell number was determined by Coulter Counter 3 days following the transfection. Each column represents three independent experiments. * indicates statistical significance.

LNCS Cells are More Resistant to Apoptosis Inducing Agent

Since constitutive activation of Stat3 is associated with cell survival and anti-apoptosis, we determined whether LNCS cells are become more resistant to apoptotic inducing agents such as etoposide compared to LNCaP cells. LNCaP and LNCS cells were treated with various concentrations of etoposide. As shown in Figure 7, LNCS cells are more resistant to etoposide compared to LNCaP cells. These results suggest that LNCS cells are resistant to anti-apoptotic treatment.

DISCUSSION

Androgen-deprivation therapy only causes a temporary regression of prostate cancer, as all tumors will eventually progress to refractory to hormonal therapy after 1–3 years of treatment. Clinical and experimental studies suggest that androgen-deprivation therapy

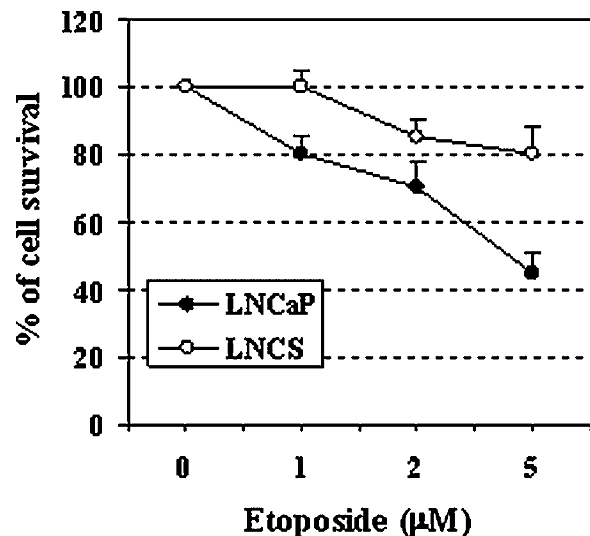

Fig. 7. The effects of etoposide on cell growth. LNCaP cells and LNCS cells (1×10^5) were plated in 12-well culture plate. Twenty four hours later, cells were treated with various concentrations of etoposide as indicated. After 3 days of incubation, the number of cells were counted using Coulter Counter. Cell survival was expressed as percentage of cell number in the treatment group compared to the untreated control group.

and intermittent androgen suppression therapy may trigger some set of tumor population to proliferate in the androgen-deprivation condition. Some of the recurrent cells that emerge during the androgen-deprivation therapy and intermittent androgen suppression therapy could be suppressed by replacement of androgen [16,21,22]. Development of a cell culture model that represents such a phenotype (i.e., androgen-deprivation induced and androgen suppressed cells) would be valuable for understanding both the progression of androgen refractory prostate cancer and intermittent androgen suppression therapy. In this study, we developed an androgen-deprivation induced and androgen suppressed human prostate cancer cell line, LNCS, from androgen-sensitive LNCaP human prostate cancer cells by a prolonged culture of LNCaP cells in charcoal-stripped serum condition. LNCS cells grow vigorously in androgen deprived condition both in vitro and in vivo. Exogenous addition of androgen suppressed the growth of LNCS cells. In contrast to parental LNCaP cells, LNCS cells expressed substantially lower levels of AR protein, but Stat3 is constitutively activated.

Human LNCaP are androgen sensitive prostate cancer cells that have been widely used for studying the mechanism of prostate cancer progression. Many sublines of LNCaP cells have been developed including androgen less sensitive C4-2 and androgen-independent LNCaP 104-R2 [29,30]. Unlike these sublines that express increased levels of AR and PSA, LNCS cells express decreased levels of AR and PSA protein. Liao et al. have acquired an androgen repressive cell line from LNCaP cells cultured in media containing charcoal-stripped FBS for a prolonged period of time [30,31]. Unlike LNCS cells, these cells express increased levels of AR and PSA. Similar findings of the expression of lower levels of AR and PSA were reported in ARCaP human prostate cancer cells derived from the ascites fluid of a patient with advanced metastatic prostate cancer [21]. Interestingly, the hormone responsiveness of LNCS is similar to ARCaP cells in that cells grow well in androgen deprivation condition both in vitro and in vivo, addition of exogenous androgen suppress their grow [21].

The incidence of tumor development of LNCS cells in castrated male mice was higher than 90 % and developed tumors within 10 days after injection. The tumors grow more vigorously in castrated male mice than the intact one. More interestingly, when testosterone was given to the castrated male mice, the tumor growth was decreased dramatically. These results suggest that LNCS cells grow well in androgen-deprivation condition; addition of exogenous androgen suppresses the tumor growth. Upon analysis of tumor protein extracts, no AR protein was detectable in LNCS tumor bearing mice. Accordingly, no PSA secretion was observed in serum of LNCS tumor bearing mice.

It is interesting to note that Stat3 is constitutively activated in LNCS cells and inhibition of Stat3 activation suppresses LNCS cell growth in androgen deprived media. Stat3 is a key signal transduction protein that mediates signaling by numerous cytokines, peptide growth factors, and oncoproteins [32]. Upon stimulation, Stat3 can be activated by tyrosine or serine phosphorylation or acetylation [32–34]. Accumulating evidence demonstrates that Stat3 activation plays important roles in cell differentiation, proliferation, development, apoptosis, and inflammation [35]. Elevated activity of Stat3 has been found frequently in a wide variety of human tumors, including hematologic malignancies, head and neck, breast, and prostate cancer [35]. Constitutively activated Stat3 is linked to tumor growth and androgen-independent progression of prostate cancer cells. Previous studies showed that overexpression of constitutively activated Stat3 promoted LNCaP cells growth in castrated male mice. Taken together, our results suggest that constitutive activation of Stat3 in LNCS cells is one of the factors that support the cell survival under the androgen-deprivation condition.

In summary, we developed an androgen-deprivation induced and androgen suppressed human prostate cancer cell line, LNCS, from androgen sensitive LNCaP human prostate cancer cells by prolonged culture in media containing charcoal-stripped FBS. LNCS cells express a lower level of AR compared to LNCaP cells and grow vigorously in androgen-deprivation condition in vitro and develop tumors in castrated male mice. The growth of LNCS cells can be suppressed by androgen in vitro and in vivo. This cell line should be a useful tool for studying the molecular mechanisms of both the progression of androgen refractory prostate cancer and intermittent androgen suppression therapy.

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