Androgen-independent prostate cancer cells acquire the complete steroidogenic potential of synthesizing testosterone from cholesterol.

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Androgen-independent prostate cancer cells acquire the complete steroidogenic potential of synthesizing testosterone from cholesterol

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Abstract

The proliferation and differentiation of normal prostate epithelial cells depends upon the action of androgens produced by the testis. Prostate cancers retain the ability to respond to androgens in the initial stages of cancer development, but progressively become independent of exogenous androgens in advanced stages of the disease while maintaining the expression of functional androgen receptor (AR). In the present study, we have determined the potential of prostate cancer cells to synthesize androgens from cholesterol which may be involved in intracrine regulation of AR in advanced stages of the disease. Established androgen-independent prostate cancer cell lines, PC3 and DU145 cells, expressed mRNA and proteins for scavenger receptor type B1 (SRB1), steroidogenic acute regulatory (StAR) protein, cytochrome P450 cholesterol side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD) and other enzymes involved in androgen biosynthesis. Expression of all these proteins and enzymes was significantly higher in the androgen-independent derivative of LNCaP prostate cancer cells (C81) than in the androgen-dependent cell line (C33). In serum-free cultures, the androgen-independent C81 cells secreted ~5 fold higher testosterone than C33 cells as determined in the conditioned media by immunoassays. These cells could also directly convert radioactive cholesterol into testosterone which was identified by thin layer chromatography. These results for the first time show that prostate cancer cells in advanced stages of the disease could synthesize androgens from cholesterol and hence are not dependent upon testicular and/or adrenal androgens.

Keywords
prostate cancer; androgen-independence; androgen receptor; androgen biosynthesis; steroidogenesis; intracrine regulation

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**Introduction**

Prostate cancer is the most frequently diagnosed malignancy in men and the second leading cause of male cancer related deaths in the United States (Jemal, et. al., 2005). The majority of prostate cancers are dependent upon androgens at initial diagnosis, and endocrine therapy is directed toward removal of androgen source (castration) or inhibition of androgen synthesis in the testicular Leydig cells (Griffiths, et. al., 1997). However, there is a progressive loss of androgen dependency (castration-resistance) during advanced stages of prostate cancer and the tumor cells do not respond to hormonal treatments (Feldman and Feldman, 2001) indicating that these cells acquire the potential to survive and proliferate in the absence of exogenous testosterone (Chen, et. al., 2004).

Studies using archival patient samples have shown increased expression of androgen receptor (AR) in advanced stages of prostate cancer and/or that AR becomes supersensitive to androgens and antiandrogens (Chen, et. al., 2004,Gelmann, 2002,Mohler, et. al., 2004); a two to five fold increase in AR mRNA is the only gene expression change consistently associated with hormone refractory disease (Chen, et. al., 2004). Immunohistochemical localization studies show that AR is present in primary site and metastatic prostate cancers regardless of stage and grade, as well as in hormone refractory cancers (Mostaghel, et. al., 2007). These findings are supported by **in vitro** data showing that inhibition of AR expression in hormone refractory prostate cancer cells results in inhibition of growth and survival, indicating an essential role of AR in advanced stages of prostate cancer (Chen, et. al., 2004). These **in vivo** and **in vitro** studies suggest that some unknown factor(s) produced in prostate cancer cells is able to activate and maintain the function of AR (Gelmann, 2002). It has been recently shown that the levels of testosterone, DHT and androgen precursors in the prostate tissue of patients with recurrent prostate cancers following medical or surgical castration, are present at levels sufficient for AR activation (Mohler, et. al., 2004,Titus, et. al., 2005). The possibility that the prostate cancer cells may acquire steroidogenic potential and may produce androgens which bind to AR and stimulate gene expression has also been explored (Ellem, et. al., 2004,Holzbeierlein, et. al., 2004). Several studies have indicated that other steroidal ligands such as estradiol may be able to activate AR (Kaplan, 1969). The AR in these cells may be mutated causing hypersensitivity of the receptor and the promiscuous usage of other steroid ligands such as cortisol, estrogen, and progesterone (Gelmann, 2002). Expression of aromatase which converts androgens to estrogens, is also altered in prostate cancer cells (Ellem and Risbridger, 2006). Therefore, it has been postulated that prostate cancer cells are capable of converting steroid precursors into androgens and estrogens which serve as ligands for their respective receptors (El-Alfy, et. al., 1999,Nakamura, et. al., 2005). The central hypothesis in the previous studies have focused on either the role of low (castration) levels of serum androgens or androgens synthesized from adrenal precursors as potential regulators of AR in hormone refractory advanced prostate cancers. However, the possibility that prostate cancer cells acquire complete steroidogenic potential to synthesize androgens from cholesterol has not been explored, although upregulation of genes involved in cholesterol biosynthesis from acetate in androgen independent prostate cancers has been reported (Holzbeierlein, et. al., 2004,Stanbrough, et. al., 2006). In this study we demonstrate for the first time the expression of cellular proteins involved in key steps in steroidalogenesis in the prostate cancer model cell lines and show that prostate cancer cells are capable of producing testosterone directly from cholesterol.
Materials and Methods

Cell lines and cell culture

Human prostate cancer cell lines DU145 and PC3 were purchased from American Type Cell Culture Collection (Rockville, MD). Clonetics® normal human prostate epithelial cells (PrEC) were purchased from Lonza (Allendale, NJ). For these experiments, LNCaP prostate cancer cell progression model (Igawa, et. al., 2002) includes cells that have passage numbers less than 33 are designated as clone 33 (C33) and the passage numbers over 80 as clone 81 (C81). These cell lines recapitulate the phenomenon of clinical tumor progression and C81 cells express PSA in the absence of exogenous androgens (Igawa, et. al., 2002). DU145 and PC3 cells were cultured in Eagle’s minimum essential medium with Earle’s salts and amino acid supplements (Millena, et. al., 2004). LNCaP -C33, and –C81 cells were routinely maintained in RPMI 1640 (Igawa, et. al., 2002) containing 10% fetal bovine serum (HyClone, South Logan, Utah). PrEC cells were cultured in Prostate Epithelial Cell Basal Medium according to the supplier’s instructions. RNA and total proteins from H295R adrenal carcinoma and JEG3 choriocarcinoma cells were provided by Dr. Marion Sewer (Georgia Institute of Technology, Atlanta) and were used as positive controls for steroidogenic proteins and enzymes.

RNA isolation, cDNA synthesis and RT-PCR

For RT-PCR analysis, cells were cultured in 6-well plates overnight in the presence of 10% FBS. After 24h, cells were washed with 1× PBS, and cultured in serum-free media for 48 h. Total RNA was isolated from the cells using TRIzol (Invitrogen, Carlsbad, CA) and quantified by optical density reading at 260 nm as described earlier (Millena, et. al., 2004). PCR reactions were performed using MasterCycler gradient (Eppendorf AG, Hamburg, Germany) using the procedures described previously (Millena, et. al., 2004). For PCR, 2 µl of cDNA were added to separate PCR reaction mixtures (total 10 µl) containing 0.1 mM dNTPs, (Promega, Madison, WI), 0.5 U Taq DNA polymerase (PGC Scientifics, Frederick, MD), 1× standard PCR buffer with 1.5 mM MgCl₂ and 25 pmol of gene-specific primers. Cycling conditions for the MasterCycler were 94°C for 2 min (initial denaturation), 94°C for 1 min, 60°C or 62°C for 1 min, 72°C for 2 min for 30–35 cycles, and 72°C for 5 min (final extension). L19 (a ribosomal protein) primer pair was used as an internal control. The oligonucleotide pair for L19 was synthesized according to previously published report (Millena, et. al., 2004). All other gene-specific primers were designed using Beacon Designer 5.0 (Premier Biosoft International, Palo Alto, CA) software program. Sequences of all primers used in RT-PCR experiments are shown in Table 1. The PCR products were visualized on 1.5% agarose gels stained with ethidium bromide. Relative amounts of PCR products were estimated by digital densitometry (GE-Amersham, Piscataway, NJ). Density values for each gene-specific PCR band was normalized with L19 bands. The PCR products were sequenced to confirm the identities of the specific mRNAs. Three independent experiments were performed and the data analyzed using student t-test and ANOVA. Representative RT-PCR results are shown in the figures.

Western blot analyses

The antibodies against StAR, P450scc and Cyp 17 were obtained from Dr. Buck Hales (University of Illinois, Chicago). Cells were cultured in 10 cm² dishes to 70% confluency, washed twice with PBS and lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA) and the protein concentrations were determined as described previously (Millena, et. al., 2004). Cell lysates were mixed with Laemmli’s buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol and 10% glycerol) and individual samples (30 µg protein) were subjected to SDS-PAGE in 10% gels and transferred to PVDF membranes (Millipore, Bedford, MA). Western blot analyses were performed by the procedures described previously (Millena, et. al., 2004). The membranes were incubated with appropriate dilutions of specific primary antibodies (over night at 4°C) and with secondary antibodies (anti-rabbit or anti-mouse...
immunoglobulin coupled to horseradish peroxidase) at appropriate dilutions for 1h at room temperature. Western blots for β-actin were carried out in parallel as loading controls. The relative intensities of specific protein bands were determined by densitometric analysis of images using ImageQuant software (GE-Amersham).

**Radioimmunassay of testosterone**

LNCaP-C33, -C81, DU145 and PC3 cells were plated in 6-well plates and cultured in serum free medium for 48 hours, after which conditioned media were collected and analyzed for total androgens using a radioimmunoassay (Millena, et. al., 2004). The antiserum employed in this immunoassay exhibits 77 and 26% cross-reactivity to 5α-dihydrotestosterone and 5α - androstanediol, respectively and hence is ideal for determination of total androgens (Ismail, et. al., 1972).

**Thin layer chromatography (TLC)**

LNCaP-C33, -C81, DU145, and PC3 cells were plated in six well plates (10^6 cells/well), treated with 3H-Cholesterol (1 µCi, 34 µM) in 1 ml of serum-free medium and incubated for 48 hours. The cells and media were extracted twice with chloroform/acetone (1:1, v/v) (Schmidt, et. al., 2005). The combined extracts were dried at 76°C, subjected to TLC on silica gel G plates and developed with chloroform/diethylether (1:1, v/v) (Schmidt, et. al., 2005). Purified unlabeled cholesterol and testosterone standards were either added to the cell extracts or ran in parallel on the TLC plates. For identification of spots the silica gel plates were stained using phosphomolybdic acid then dried with a heat gun (Schmidt, et. al., 2005). Blue-black spots were visualized identifying the locations of the testosterone and cholesterol standards. Spots were excised, added to liquid scintillation fluid and radioactivity was determined using a scintillation counter (Schmidt, et. al., 2005).

**Results and Discussion**

The results described in this study show for the first time that androgen-independent human prostate cancer cells are able to acquire complete steroidogenic potential and are capable of synthesizing testosterone from cholesterol, indicating an intracrine regulation of AR in advanced stages of prostate cancer. Several studies have shown the expression of key steroidogenic enzymes in prostate cancer cells indicating that these cells are able to synthesize androgens from adrenal precursors (El-Alfy, et. al., 1999,Nakamura, et. al., 2005,Stanbrough, et. al., 2006). The presence of functional AR in advanced stages of the disease and the presence of testosterone and DHT, sufficient to activate the AR, in cancer tissues under androgen ablation therapy, also support this notion (Gelmann, 2002,Mohler, et. al., 2004,Titus, et. al., 2005). The purpose of our studies was to determine whether prostate cancer cells in advanced stages of the disease can synthesize testosterone from cholesterol hence making them completely independent of serum testosterone and/or adrenal steroid precursors.

Figure 1 shows the key components of steroidogenic machinery required for cholesterol uptake (SRB-1), cholesterol transport into inner mitochondrial membrane (StAR protein) and enzymes required to convert cholesterol into pregnenolone (P450scc) and subsequently into testosterone (Stocco, 2001). As a first step we determined the expression of SRB-1, StAR, P450scc, 3βHSD, CYP17 and 17βHSD1 in normal prostate epithelial cells (PrEC) and two established androgen-independent prostate cancer cell lines (DU145 and PC3), along with human adrenal carcinoma (H295R) and choriocarcinoma (JEG3) cell lines (Figure 2). RT-PCR analyses revealed very low or undetectable expression of STAR, P450scc and CYP17 in PrEC cells. However, expression of all components of steroidogenesis was detected in both DU145 and PC3 cells. The expression of StAR was detected at a low level in these cells compared to H295R cells. The placental cells (JEG3) are known not to express StAR protein and to use other
members of this protein family to transport cholesterol into mitochondria (Watari, et. al., 1997). All cell lines including PrEC cells expressed comparable levels of 17β-HSD1 which was confirmed by sequencing the PCR-product. Previous studies have identified different isoforms of 17β-HSD in several tissues have shown that while 17β-HSD1 and 17β-HSD5 are present in prostate cancer cells, 17β-HSD3 is primarily expressed in the testis (Labrie, et. al., 1997). Previous studies have shown the presence of 17β-HSD1 in the prostate, and in LNCap cells using RNAse protection assays (Labrie, et. al., 1997). However, another study failed to detect 17β-HSD1 in prostate cancer cell lines (LNCap, DU145, PC3) using northern blotting (Castagnetta, et. al., 1997). The reasons for this discrepancy could be due to the differential sensitivity of methods employed by the two studies.

To determine whether the expression of steroidogenic machinery correlates with independence from exogenous testosterone, we employed cell lines derived from LNCaP cells, which represent this phenomenon in vitro. C33 and C81 cells are both AR positive, however C33 cells are androgen dependent and C81 cells do not require exogenous androgens for survival and proliferation (Igawa, et. al., 2002). These cell lines recapitulate the phenomenon of clinical tumor progression and have been validated and used as models of androgen-independent cells in subsequent studies from different laboratories (Cai, et. al., 2007;Cao, et. al., 2006;Denmeade, et. al., 2003;Unni, et. al., 2004). PSA secretion by C81 cells in the absence of androgens is much higher than that secreted by C33 cells which is a biomarker for androgen-independence and is seen in other in vitro models of androgen independent prostate cancers (Chen, et. al., 2007;Hara, et. al., 2003). Data presented in Figure 3A confirmed the presence of AR mRNA and protein in C33 and C81 cells (Igawa, et. al., 2002) and showed no expression of AR in PC3 and DU145 cells. The presence of functional AR makes C33 and C81 cells a better model for these studies. We determined the expression of all steroidogenic enzymes and proteins in C33 and C81 cells by RT-PCR as described above. The results of these determinations are presented in Figure 3B. All key components of steroidogenic machinery are expressed in androgen-independent C81 cells. Both C33 and C81 cells expressed SRB1, P450scc, 3β-HSD and 17β-HSD1 at comparable levels; however StAR expression was low in C33 cells compared to C81 cells and no expression of CYP17 was detected in C33 cells. The steady state mRNA levels for StAR and P450scc in C81 cells were 4.7-fold and 1.3-fold higher than those in C33 cells, respectively. Similar differences in the expression of StAR mRNA were observed using real time PCR analysis (data not shown). These results were confirmed using Western blot analysis as shown in Figure 3C. StAR protein was not detected in PrEC cells and is present at low levels in C33 cells. C81 cells contained higher amount of StAR protein when compared with C33 cells. The protein levels of StAR and P450scc in C81 cells were 2.6-fold and 1.7-fold higher than those in C33 cells. We also detected presence of StAR protein in PC3 and DU145 cells. Our results also showed that CYP17 is not expressed either at mRNA or at protein level in PrEC and C33 cells; however, significant expression of CYP17 mRNA and protein were present in C81 cells. Since StAR is critical for cholesterol transport into mitochondria and CYP17 is required for androgen synthesis from progesterone, C33 cells may have very low or limited capacity to synthesize androgen from cholesterol (Stocco, 2001).

These data suggest that androgen independence in prostate cancer cell lines correlates with the acquisition of proteins required for androgen biosynthesis from cholesterol. To determine whether these cells synthesize androgens in vitro, the cells were cultured for 48 hr in serum-free media and androgen levels were determined in conditioned media using RIA. As shown in Figure 4A, detectable levels of androgens were present in the conditioned media from both C33 and C81 cells cultured in the absence of serum. Nevertheless, C81 cells secreted approximately five-fold higher androgens than C33 cells. Levels of androgens in the conditioned media from PC3 and DU145 cells and also in unrelated control cells or in the culture medium without the cells were undetectable (HeLa, COS; data not shown). These data show that androgen independence is indeed associated with androgen secretion by C81 cells.
in the absence of exogenous steroid precursors. The reason for undetectable levels of testosterone in conditioned media from PC3 and DU145 cells could be due to a) low production of testosterone by these cells as a consequence of low expression of StAR, or b) due to its conversion to estradiol by aromatase. The second possibility is supported by high levels of aromatase in these cell lines (Dillard et al, unpublished data; Ellem and Risbridger, 2006); androgens may be irrelevant in these cell lines since they do not express AR.

To confirm that the C33 and C81 cells are capable of using cholesterol uptake and its conversion into androgens, we determined the conversion of labeled exogenous cholesterol to testosterone in these cells. The cells were cultured in the presence of 1α, 2α [N]-3H-cholesterol for 48 hr in serum-free medium. The conditioned media and cell extracts were then analyzed for the presence of labeled testosterone by TLC. Figure 4B shows the separation of testosterone and cholesterol by TLC (upper panel). The spots representing cholesterol and testosterone were scraped and counted in the scintillation counter. The results showed conversion of radiolabeled cholesterol into testosterone by both cell lines. The radioactivity observed in C33 cells was 3613 ± 1004 cpm and 191±45 cpm (Mean±SEM; n=3) for cholesterol and testosterone spots, respectively. The corresponding radioactive in C81 cells was 3268±1309 and 225±45, for cholesterol and testosterone, respectively. The differences in the levels of radioactive testosterone were not statistically significant and were different than those observed for RIA. The reason for this discrepancy were probably due to the fact that RIA determines the levels of all androgens while only testosterone spots were analyzed in the TLC. Any conversion of testosterone into DHT or other metabolites may account for these differences. In addition, while only conditioned media were analyzed in the RIA, both cell extracts and media were combined for TLC analysis. Nevertheless, it should also be noted that DHT is more active form of androgens than T in prostate cells. Additional studies involving separation of all androgenic steroids and analysis will be required to clarify this point. However, the fact that we did find radioactive testosterone in these cells and since radioactive testosterone could only be synthesized from radioactive cholesterol, this experiment conclusively demonstrated that these cells are capable of making androgens directly from exogenous cholesterol. The specificity of this conversion was also supported by the fact that we did not detect any radioactive testosterone in DU145 and PC3 cells (data not shown) confirming our RIA results.

Mohler and collaborators (Mohler, et. al., 2004,Titus, et. al., 2005) reported the presence of significant amounts of testosterone and DHT in the recurrent prostate cancer tissues and suggested that prostate cancer cells may synthesize androgens from adrenal precursors or cholesterol (El-Alfy, et. al., 1999,Nakamura, et. al., 2005,Stanbrough, et. al., 2006). Our studies support the notion that this testosterone can be synthesized directly from cholesterol. This possibility is also supported by the fact that genes regulating cholesterol biosynthesis are upregulated in androgen ablation resistant prostate cancer cells (Holzbeierlein, et. al., 2004) suggesting that prostate cancer cells may also be able to increase biosynthesis of intracellular cholesterol.

In conclusion, our results clearly show for the first time that advanced androgen independent prostate cancer cells acquire complete steroidogenic ability to synthesize androgens and underline the fact that castration and inhibition of testosterone production in the testes may not achieve androgen deficiency in prostate cancer cells in advanced stages of the disease. Our results also explain the essential role of AR in survival and proliferation of androgen-independent prostate cancers under androgen-ablation therapy and suggest that inhibitors of steroid biosynthesis in prostate cancer cells may be required to completely abolish the androgens in these tumors for its therapy.


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**References**


Fig 1.
Diagram illustrating pathways involved in biosynthesis and metabolism of testosterone.
Fig 2.
RT-PCR analysis of mRNAs for steroidogenic enzymes and other proteins required for androgen biosynthesis in normal prostate epithelial cells (PrEC; 2A) and androgen independent prostate cancer cell lines (PC3 and DU 145; 2B). Established steroidogenic cell lines (H295R and JEG3) served as positive controls.
Expression of AR and steroidogenic machinery in prostate cancer cells

A. RT-PCR (upper panel) and Western blot (lower panel) analysis of AR mRNA and protein in C33 (androgen dependent), C81 (androgen independent), and AR negative DU145, and PC3 prostate cancer cell lines. Ribosomal L19 and β-actin were used as controls in RT-PCR and Western blots, respectively.

B. RT-PCR analysis of steroidogenic enzymes and other proteins required for androgen biosynthesis in C33 and C81 prostate carcinoma cells.

C. Western blotting for StAR, P450scc (upper panel), and CYP17 (lower panel) protein in C33, C81, DU145 and PC3, PrEC, and H295R cells.
Fig 4.
Androgen production in androgen dependent (C33) and androgen independent (C81) prostate carcinoma cells
A. The cells were cultured in serum-free medium for 48 hr and the conditioned media were collected and analyzed for androgen content by radioimmunoassay. Each bar represents Mean ± SD from triplicate determinations.
B. Thin layer chromatography (TLC) separation of testosterone and cholesterol in C33 and C81 prostate cancer cells. The cells were cultured in serum-free medium containing $^{3}$H-cholesterol (1µci/ml) for 48 hr and the cells and conditioned media were collected. The cells and media were combined and extracted and the extracts were subjected to TLC. Purified unlabeled cholesterol and testosterone standards were added to the cell extracts for identification of spots using phosphomolybdic acid. Blue-black spots identifying the locations of the testosterone and cholesterol standards (4B) were excised, added to liquid scintillation fluid and radioactivity was determined using a scintillation counter. An equivalent spot from an unrelated area of the TLC plate was used as background.
Table 1

Primer Sequences for RT-PCR

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