

ORIGINAL ARTICLE

Characterization of the androgen receptor in a benign prostate tissue-derived human prostate epithelial cell line: RC-165N/human telomerase reverse transcriptase

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The majority of prostate epithelial cell lines stably expressing wild-type (wt) or mutant (mt) androgen receptor (AR) are derived from metastatic prostate cancers. Therefore, the wt AR-expressing RC-165N/human telomerase reverse transcriptase (hTERT) cell line derived from the benign prostate tissue of an African-American patient provides a unique opportunity to assess the functional status of AR in a cellular context not studied before. Although androgen-induced expression of known androgen responsive genes such as *PMEPA1*, and *NDRG1* was observed in RC-165N/hTERT, this cell line expresses prostate-specific antigen (PSA) at significantly lower levels. Chromatin immunoprecipitation assay revealed androgen-dependent binding of AR to androgen response elements of *PSA*, *PMEPA1* and *NDRG1* genes. Similarities, as well as differences were noted in the expression of androgen responsive genes between RC-165N/hTERT and LNCaP cells. Comprehensive evaluations of AR functions in RC-165N/hTERT cells suggest that whereas some features of known AR functions are maintained in this benign prostatic tissue-derived cell line, other AR functions are not retained. Objective evaluations of similar cell lines will lead to the understanding of AR functions in prostate growth and differentiation.

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Introduction

Carcinoma of the prostate (CaP) is the most common non-cutaneous malignancy and the third leading cause of cancer-related deaths in American males.¹ However, a large part of the molecular mechanisms of prostate tumorigenesis remains to be defined. Cell culture models effectively representing *in vivo* characteristics of CaP can greatly facilitate research focusing on prostate cancer biology. Although, several metastatic tissue-derived CaP cell models are being extensively used such as LNCaP, LAPC4, PC-3 and DU-145, there is a continued need for the establishment of *in vitro* models derived from benign and primary prostate cancer tissues. We and others have recently reported establishment of new cell lines from benign and primary prostate cancer tissues.^{2–6}

Prostate cancer is a major health concern in older American males with higher incidence in African-American males, with almost twice the morbidity as Caucasian American males.⁷ Therefore, the establishment of *in vitro* cell models isolated from African-American patients may contribute to better understanding of the biology of prostate growth as well as prostate cancer of African-American men. Among previously established prostate cell lines, only a few were established from African-American patients: MDA PCa 2a and 2b,⁸ E006AA⁴ and the RC-165N/human telomerase reverse transcriptase (hTERT).² The MDA cell lines were derived from advanced androgen-independent prostate cancer of a metastatic bone tumor.⁸ Another recently established cell line, the E006AA was derived from a pathologically confined tumor of an African-American patient, and this cell line may represent the characteristics of localized human prostate cancer.⁴ We have recently reported the establishment of a cell line (RC-165N/hTERT) from a histologically defined benign tissue of African-American patient using overexpression of hTERT.^{2,3} Assessment of expressed markers in RC-165N/hTERT indicated luminal characteristics.³ This cell line constitutively expressed wild-type (wt)-androgen receptor (AR) protein and responded to both androgen hormone stimuli and

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AR antagonist (flutamide). As, stable AR expression represents unique feature of this benign tissue-derived immortalized prostatic epithelial cells, here we report the evaluation of AR downstream functions in RC-165N/hTERT cells.

In this report, we examined the AR binding to androgen responsive elements within the promoter regions of androgen-regulated genes. Quantitative expression of the representative androgen responsive genes was also evaluated by quantitative polymerase chain reaction (QPCR) and supersensitive enzyme-linked immunosorbent assay (ELISA) assays. Furthermore, we examined the features of AR-signaling pathways using temporal gene expression profiles in response to DHT.

Materials and methods

Cell culture and androgen treatment

Epithelial cells (RC-165N) were derived from benign prostate tissue of an African-American patient who underwent radical prostatectomy. Tissue sections adjacent to tissues used for cell culture showed histological features of benign non-malignant prostate glands. Primary cultures were established according to Walter Reed Army Medical Center (WRAMC) and Uniformed Services University of the Health Sciences Internal Review Board (USU IRB) protocols by the explant-outgrowth method as described in elsewhere.² For serial passages, routine trypsinization was used. Keratinocyte serum-free medium (Cat. 10724-011, LifeTechnologies, Gaithersburg, MD, USA) supplemented with bovine pituitary extract (BPE), recombinant epidermal growth factor (rEGF), 1% (v/v) penicillin-streptomycin-neomycin (PSN) antibiotic mixtures and 1% (v/v) amphotericin B (LifeTechnologies, Gaithersburg, MD, USA) were used for growing and maintaining the cells and designated as KGM medium.² For androgen treatment experiment, BPE and rEGF were replaced by 0.1% bovine serum albumin solution (Sigma, St Louis, MO, USA). Both DHT (0, 1.0, 10 and 100 nM) (Sigma, St Louis, MO, USA) and R1881 (10 nM) (Perkin Elmer, Boston, MA, USA) were sequentially diluted by absolute ethanol to desired concentration, and the final concentration of ethanol in the cell culture was adjusted to 0.1% (v/v) in control and hormone-treated cultures. After treating the cells with androgen at approximately 50% confluence, the cells were allowed to grow in an incubator at 37°C with 5% humidity and 5% CO₂ for 48 h. Cells were then processed for chromatin immunoprecipitation (ChIP), real-time reverse transcription (RT)-PCR and GeneChip experiments. The cell culture medium treated with DHT or R1881 for 120 h (5 days) were used for prostate-specific antigen (PSA) protein detection with the Super-ELISA system, which employed the multi-photon detection methods in ELISA setup.^{9,10} LNCaP was maintained in Rosewell Park Memorial Institute medium (Invitrogen, Carlsbad, CA, USA).

Immunoblot analysis

Total proteins of the RC-165N/hTERT cells without androgen treatment were extracted with a commercial

lysis buffer (T-PER, Pierce, IL, USA) including protease inhibitor mixtures (Complete Mini, Roche, IN, USA). To detect AR protein with immunoblot, 30 µg of total proteins were separated in a 4–12% gradient gel (Invitrogen, Carlsbad, CA, USA). Proteins were transferred to a polyvinylidene difluoride membrane (Invitrogen), then membranes were blocked in 10% non-fat dry milk (Bio-Rad, Hercules, CA, USA) in 1 × phosphate-buffered saline (PBS) including 0.1% Tween-20 for 1 h, and incubated with anti-AR primary antibodies (SC-7305, Santa Cruz Biotech, CA, USA) at 150 × dilution for 1.5 h at room temperature. The blots were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, NJ, USA) at a dilution of 1:5000 for 1 h at room temperature. After washing, the blots were developed with the Enhanced Chemiluminescence kit according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA).

ChIP in prostate cell cultures

ChIP procedures^{11–13} were modified to adopt the protocols to prostate cell cultures. RC-165 N/hTERT cells (0.5×10^6) were washed twice with ice-cold PBS and were harvested in ice-cold 1 × PBS, pH 7.4 containing 1% formaldehyde and Complete Mini protease inhibitors (Roche) and collected by centrifugation. Cells were incubated at 37°C for 15 min to complete the chemical crosslinking reaction. The reaction was stopped by washing the cells three times with 0.5 ml of PBS at 4°C. The cells were lysed by 0.5 ml RT lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulphate (SDS) and Complete Mini protease inhibitor), which was followed by incubation on ice for 10 min. To disintegrate the cells by sonication we used VisSonic 100 sonicator (VITRIS). We applied 10-s bursts at energy level four on ice followed by 30 s cooling on ice for a total of four times. The suspension was diluted 10-fold with dilution buffer (16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl, 0.01% SDS, 1.1% Triton-X 100, 1.2 mM EDTA and Complete Mini protease inhibitors). The suspension was precleared with 40 µl of 50% protein A/G-coupled sepharose bead slurry (PIERCE, Rockford, IL, USA) by rotating for 1 h at 4°C before the experiment. One microgram aliquot of an equal volume mixture of anti-AR antibodies H-280 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), PG-21 (Upstate, Lake Placid, NY, USA) and AR441 (Alexis Biochemicals, Lausen, Switzerland) were incubated with the chromatin suspension 12 h at 4°C. The immunoprecipitated chromatin was recovered by A/G-coupled sepharose beads. The beads were washed and the DNA was released by digesting the samples with Proteinase K (Roche, Indianapolis, IN, USA) for 12 h at 24°C. The crosslinks were reverted by incubating the samples at 65°C for 6 h and the proteins were removed by phenol/chloroform extraction. The DNA was recovered by ethanol precipitation and dissolved in 100 µl of Tris-EDTA buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA). AR-binding sites within the promoter regions of *PMEPA1*, *NDRG1* and *PSA (KLK3)* genes were predicted *in silico* by the Genomatix Software (www.genomatix.de). PCR primers were designed by the Primer3 program of the Whitehead Institute.¹⁴ To amplify the predicted *PMEPA1*

AR binding sites at the distal -2134 position the 5'-CCCTGGCACATCTAGGGTTA-3' and 5'-TGGACTG CCAGCACTCATAG-3' primer pair was used. To assess *PMEPA1* androgen response element (ARE) at -230, primers 5'-CAGGGAGGGGAGGTCTCTTA-3' and 5'-TCAAAGGGGTATGAGCAGG-3' were used. For the amplification of ARE (-984) in the *NDRG1* promoter we designed the primers 5'-GCCACCTGGGTAGCTTTGTA-3' and 5'-AGAGGAGCCGCCAAATTA-3' primers. The 5'-ACAGACCTACTCTGGAGGAAC-3' and 5'-AA GACAGCAACACCTTTTT-3' primers were used for amplifying the PSA/AREIII (-3999) element. PCR was carried out in 43 cycles for ChIP products and in 38 cycles for input DNA. Each cycle included 10 s denaturation at 94°C, 15 s annealing at 50°C and 60 s extension at 72°C. The PCR products were separated on 2.5% agarose gel by electrophoresis along with a 100 kb molecular weight ladder.

Reverse transcription and real-time PCR

Total RNA from cell cultures was isolated by the RNAzol B (Tel-Tes Inc., Friendswood, TX, USA) method. Two micrograms of total RNA were used in the reverse transcription reaction, by using random hexamer primer and Omniscript reverse transcriptase (Qiagen, Germantown, MD, USA) in 20 µl reaction volume, following the supplier's recommendations. PCR primers and probes for *PSA*, *PSMA* and *NDRG1* were designed by Primer Express software (Applied Biosystems, Foster City, CA, USA). Each 30 µl volume of the PCR reaction included 2 µl 2 × diluted cDNA (equal 100 ng RNA), 300 nM sense primer, 300 nM antisense primer, 225 nM 6'-FAM-labeled fluoresce target probes, 1.5 µl 20 × glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control (primers plus VIC-labeled probe) and 15 µl Universal Master Mixture (Applied Biosystems, Foster City, CA, USA). The PCR reactions were run in ABI PRISM 7700 sequence detection system. PCR amplification included one stage at 50°C 2 min, one step at 95°C for 10 min and 50 cycles at 95°C for 15 s and 60°C for 30 s two-step PCR, triplicate RT-PCR reactions and one control reaction without RT (No-RT) were performed for each gene. Amplification of an endogenous control was performed in same well to standardize the amount of the expression target RNA. The CT data in real-time PCR is used to determine ΔCT , $\Delta\Delta CT$ and the relative amount of the target messenger RNA (mRNA). The formula for expression of targets mRNA: $2^{-\Delta\Delta CT}$. The ΔCT is obtained by subtracting the average GAPDH CT from the average target gene CT, $\Delta\Delta CT$ is obtained by subtracting the ΔCT from the target gene in treated DHT to untreated control samples.

Primers and probes used for quantitative RT-PCR amplification of PSA were as follows: Sense: CCCACTG CATCAGGAACAAA. Antisense: GAGCGGGTGTGGG AAGCT. Probe: 6FAM-ACACAGGCCAGGTATTTCAGG TCAGCC-TAMRA. *PSMA* probes were as follows. Sense: GGCGCTGGTGCTGGC. Antisense: CAATTCATCCAA AAATGCTTTTCAT. Probe: 6FAM-TCGGCTTCCTCTTCG GGTGGTTTATAAAATCC-TAMRA. Probes to detect *NDRG1* expression were as follows. Sense: GCCAGCA CATTGTGAATGACA. Antisense: GCAGGGTGACTG TGTGGGT. Probe: 6FAM-AATGCCTACAACAGCCGG CGCGA-TAMRA.

GeneChip analysis

Total RNA was used to hybridize to high-density oligonucleotide human genome array HG U133A GeneChip (Affymetrix, Santa Clara, CA, USA) that contains 22283 probe sets which represented about 18000 annotated genes and the rest represented expressed sequence tags and hypothetical genes. Biotinylation of complementary RNA (cRNA), purification of the

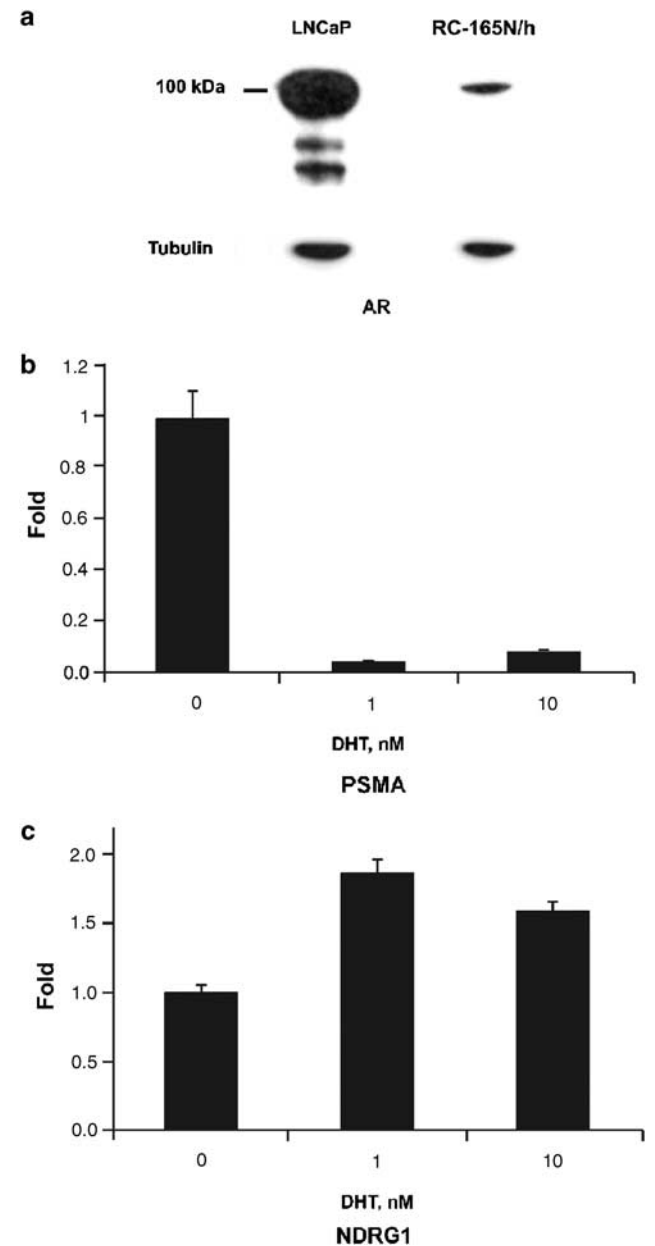


Figure 1 Detection of AR protein in RC-165N/hTERT cells by immunoblot. (a) Twenty micrograms of LNCaP and RC-165N/hTERT lysates were loaded on SDS/PAGE gels for immunoblot. (b, c) Effect of androgen (DHT) on the expression of *PSMA*, and *NDRG1* mRNA in the RC-165N/hTERT cells. Cells were treated by DHT as described in Materials and methods. Quantitative RT-PCR was performed on total RNA isolated from the cells. Relative abundance of mRNAs was calculated with respect to the standard calibration curve and by the comparative C_T method $2^{-\Delta\Delta CT}$. The expression levels of individual genes were normalized to the expression of *GAPDH*. Each experiment was carried out triplicate and was repeated twice.

biotinylated cRNA, fragmentation of the probes, hybridization, staining and scanning of the hybridized GeneChips were carried out as described earlier.¹⁵ For Image Analysis and data extraction Affymetrix GeneChip Microarray Analysis Software, version 3.1, Affymetrix Micro DB, Data Mining Tool version 2.0 (Affymetrix, Santa Clara, CA, USA), Microsoft Excel 2000 (Microsoft, Seattle, WA, USA) and Statistica version 4.1 (Stat Soft Inc., Tulsa, OK, USA) were used. For further details and advanced bioinformatic analysis we used the GeneSpring software (Silicon Genetics, CA, USA) as we previously described.¹⁶ Pathway analysis was performed by the Ingenuity Pathway Analysis Software (Ingenuity, Mountain View, CA, USA).

Results

RC-165N/hTERT cells express AR protein and AR regulates the expression of downstream target genes
AR protein was detected in RC-165N/hTERT cells by immunoblot assay. The results of the assay were

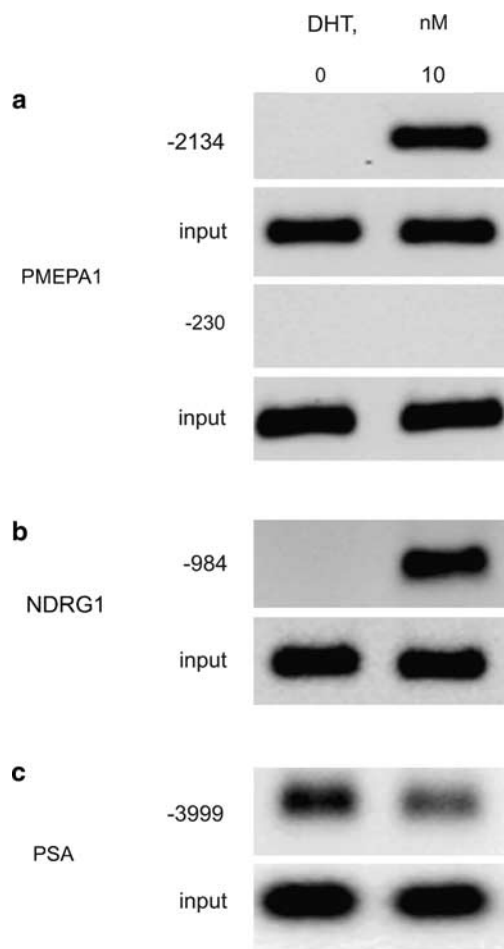


Figure 2 ChIP assay reveals AR binding to *PMEPA1*, *NDRG1* and *PSA(KLK3)* promoter upstream sequences. Specific sets of primer pairs were selected in order to amplify the regions of predicted ARE sequences within the *PMEPA1* (a), *PSA(KLK3)* (b) and *NDRG1* (c) genes. To achieve linear amplifications ranges the ChIP and input products were amplified with 43 and 38 cycles, respectively.

consistent with our previous report on AR mRNA expression in RC-165N/hTERT cells where we reported the presence of several prostate-epithelial markers in this cell line^{2,3} (Figure 1). To assess the integrity of the AR pathway, gene expression levels of AR-regulated genes were screened using QPCR. *PSMA* and *NDRG1*¹⁶ were selected, and then examined as representatives for the integrity of AR-pathway. Consistent with previous reports, quantitative assessment of DHT-responsive *PSMA* gene expression revealed a dose-dependent downregulation in response to DHT.¹⁷ Although *NDRG1* gene expression was previously reported to reach saturation levels at 10^{-9} – 10^{-8} M¹⁸ and our data on *NDRG1* showed modest decrease (~0.2-fold decrease) at 10^{-8} M compared to 10^{-9} M DHT, its expression in the RC-165N/hTERT cells was generally upregulated by increasing DHT, which was similar to the previous observations.^{16,18}

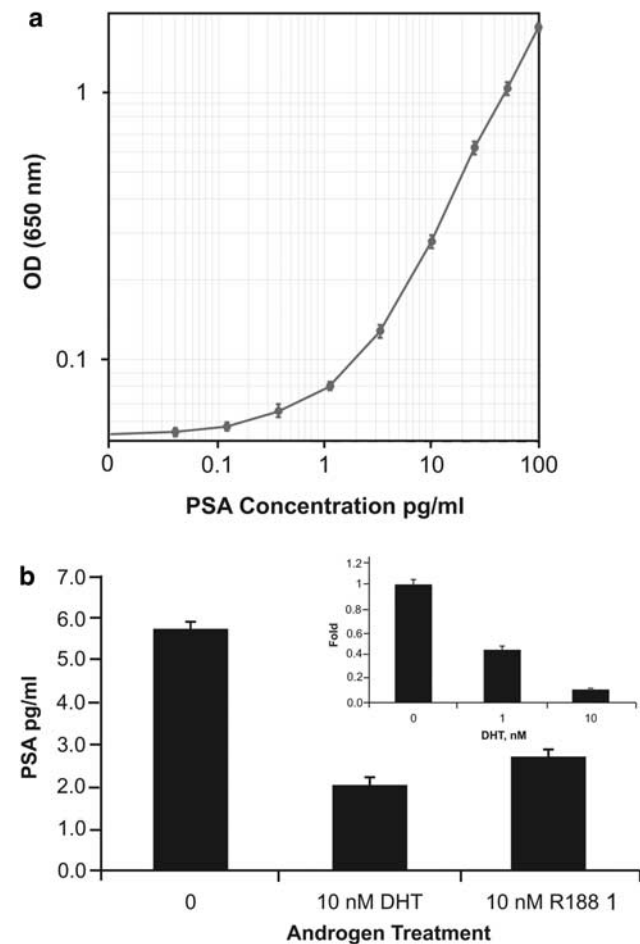


Figure 3 Expression of *PSA (KLK3)* mRNA and secretion of *PSA* protein in response to androgen. (a) Cells were treated with DHT as described in the Materials and methods. Linear detection of purified *PSA* protein concentration standards at picogram levels (1–100 pg/ml) was achieved ($R^2 > 0.998$). (b) Secreted *PSA* concentrations at various DHT-induction levels were assessed by matching the detected signals to the standard curve in the region of antigen concentrations between 0 and 100 pg/ml. The standard curve was compiled by triplicate experiments and independently repeated twice. Inset graph represents the expression fold changes of *PSA* mRNA measured by QPCR from cell lysates. Each experiment was carried out in triplicate and independently repeated twice.

Table 1 Androgen-upregulated genes in RC165N/hTERT cells after 24 h treatments with 100, 10 and 1 nM of DHT (normalized to 0 nM DHT treatment)

Genbank	RC165-treated with DHT			Gene name	Map	Reported in cancer
	100.0 nM	10.0 nM	1.0 nM			
NM_024512	47.54	49.92	3.92	LRRC2	2	Tumor Suppressor
NM_001175	15.33	7.34	5.35	ARHGDIB	12p12.3	Cancer metastasis
NM_000900	15.23	5.06	7.39	MGP	12p13.1-p12.3	Prostate
NM_001704	14.50	5.81	6.41	BAI3, KIAA0550	6q12	Malignant gliomas
BC005224	14.29	5.17	2.54	SCC, T4-A	18q21.3	Prostate, malignant squamous cells, liver, uterine cervix, head and neck, lung and esophagus
AF021834	14.15	4.89	10.81	TFPI	2q31-q32.1	Prostate
M31159	9.44	3.79	2.76	IGFBP3	7p13-p12	Prostate
NM_003155	8.85	2.49	1.83	STC	8p21-p11.2	Prostate and breast
BG251266	8.19	7.64	8.57	FOSL1	11q13	Pancreatic ductal epithelium
NM_007231	7.30	9.26	6.81	ATB (0+)	Xq23-q24	Colorectal
U19557	7.15	2.89	2.75	PI11, SCCA2	18q21.3	Squamous cell carcinomas
AI300520	6.61	1.01	1.60	STC1	8p21-p11.2	Primary breast tumors
NM_001146	5.75	2.87	2.11	AGPT, ANG	8q22.3-q23	Prostate
AK000970	5.69	3.42	3.77	TEB4	5	Discriminate the invasive front and the tumor center
NM_000956	5.68	2.86	4.00	EP2	14q22	Prostate
U94592	4.85	5.94	3.46	UCP2	11q13	Colon
AI189753	4.26	2.89	1.58	TM4SF1	3q21-q25	Colorectal
NM_006096	3.99	1.30	1.54	NDRG1	8q24	Prostate
AI346835	3.94	1.68	1.64	TM4SF1	3q21-q25	Colorectal
AA573523	3.93	0.80	1.37	EPB41L1	20q11.2-q12	Breast, lung and brain
BF973178	3.82	3.34	2.53	GTSE1	22	N/A
NM_000598	3.75	2.98	2.39	IGFBP3	7p13-p12	N/A
NM_005585	3.70	3.16	2.22	MADH7, SMAD6	15q21-q22	Breast and ovarian tumorigenesis
NM_002450	3.47	1.57	1.26	MT1	16q13	Prostate, cervical, bladder and ovary
NM_004878	3.44	3.23	3.53	PGES, PIG12	9q34.3	Gastric and in gastric cancer cell lines
AF348514	3.37	2.02	1.39	PTMA	2q35-q36	Ovarian cancer cells
NM_002276	3.35	2.18	3.99	KRT19	17q21	Thyroid ovary, gastric, liver and lung
AW025529	3.34	3.51	3.31	CALML4	15	Lung cancer
R78668	3.32	2.31	1.96	CDKN1C	11p15.5	Putative tumor suppressor, bladder, urothelial, colorectal and esophagus
AI989477	3.28	1.18	1.07	SOX4	6p22.3	Bladder, breast
NM_001150	3.25	1.78	2.54	CD13, GP150	15q25-q26	Prostate, lung
BF672975	3.24	1.99	1.81	LPL	8p22	Prostate, ovary
NM_003069	3.21	1.70	2.07	SNF2L	Xq25	Breast
AI335208	3.12	3.00	1.11	ITGB5	3q21.2	Gastrointestinal tract
X15132	3.12	2.20	2.33	MNSOD	6q25.3	Prostate, gastric, colorectal, breast and skin
NM_001630	3.09	2.56	2.51	ANXA8	10q11.2	Acute promyelocytic leukemia (APL)
M90657	3.07	1.51	1.43	TM4SF1, L6	3q21-q25	Lung, breast, colon, and ovary and colorectal cancer
BF686442	3.06	2.08	1.55	PTMA	2q35-q36	Ovarian cancer cells
NM_020037	3.05	2.81	1.96	MRP3, ABC31	17q22	Breast, pancreatic cancer
BC001188	3.05	1.45	1.51	TFR, CD71	3q26.2-qter	Prostate, potential molecular imaging marker
NM_012242	3.03	1.98	1.74	SK, DKK-1	10q11.2	Tumor Suppressor, proapoptotic gene

Abbreviations: DHT, dihydrotestosterone; hTERT, human telomerase reverse transcriptase.

Wt AR in RC-165N/hTERT cells binds to specific androgen responsive elements

To examine AR binding to androgen responsive elements of target gene promoter regions, ChIP assay was performed assessing AREs of PSA (*KLK3*), *PMEPA1* and *NDRG1* gene upstream sequences in DHT treated or untreated cells. A distal ARE element (-2134) of the *PMEPA1* gene promoter showed AR binding in DHT-inducible manner whereas AR binding was not detected at the proximal region (-230) (Figure 2a). Furthermore, ChIP assay revealed DHT-dependent binding of AR to the ARE (-984) of *NDRG1* promoter (Figure 2b). Interestingly, AR-binding was detected in non-stimulated cells at the AREIII of PSA (*KLK3*) gene enhancer that was somewhat decreased by the addition of DHT (Figure 2c).

Expression of PSA

Using conventional Northern blot and Immunoblot methods, the detection of PSA protein expression from RC-165N/hTERT cells was previously not feasible.² However, with the Super-ELISA system that can detect proteins at sub-nanogram levels^{9,10} secreted PSA protein was detectable (Figure 3). The Super-ELISA system can reproducibly detect PSA protein between 1 and 100 pg/ml (Figure 3a) with remarkable linearity ($R^2 > 0.998$). The Super-ELISA system reproducibly detected PSA protein (2.0-5.7 pg/ml) from the cell culture medium in the presence or absence of 10 nM DHT or 10 nM R1881 (Figure 3b). Ten nanomolar of DHT appeared to decrease PSA protein levels. This observation was consistent with changes in PSA mRNA levels assessed by QRT-PCR (Figure 3b, inset). Previous reports

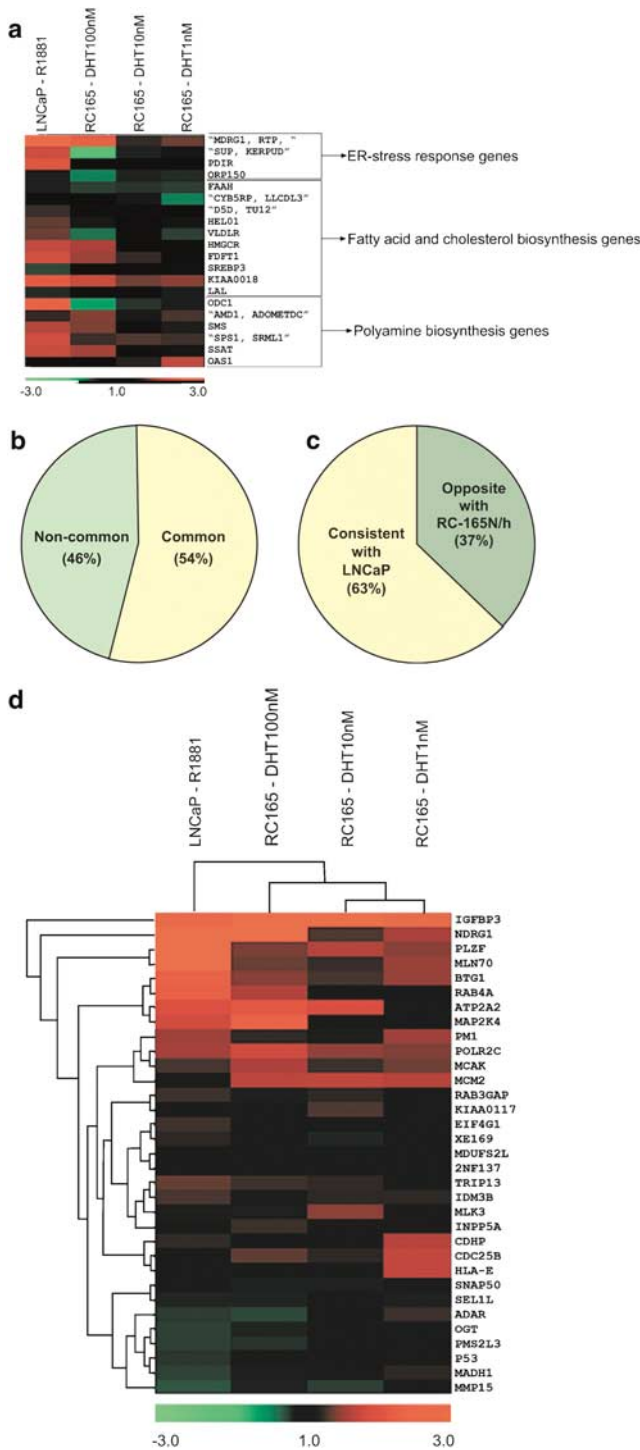


Figure 4 Analysis of gene expression response of RC-165N/hTERT cells to DHT treatment reveals changes in the expression of thirteen genes with prostate cancer association. (a) These genes represent three major pathways: endoplasmic reticulum stress response, fatty acid and cholesterol biosynthesis and polyamine biosynthesis pathways, respectively. (b) Comparison of expressed genes in LNCaP cells to the expression of genes in RC165 N/hTERT cells in response to androgens. Expression changes were detected in 54% of the examined genes as shown in the left pie chart. (c) The up- or downregulation of the genes were consistent between LNCaP and RC165 N/hTERT cells in 63% of the observed gene expression changes as shown in the right pie-chart. (d) Heat map demonstrates consistent gene expression responses between androgen-treated LNCaP and RC165 N/hTERT cells.

from androgen responsive CaP cell lines of distinct origin indicated that PSA expression was increased in response to DHT.¹⁹ Intriguingly, in RC-165N/hTERT cells secreted PSA protein levels were decreased, expression of PSA mRNA was decreased, and binding of AR to the AREIII enhancer of PSA gene was also decreased (Figure 2c) in response to 10 nM DHT.

Androgen-regulated transcriptome in RC-165N/hTERT cells

To assess the expression of androgen-induced genes in RC-165N/hTERT cells, androgen-regulated transcriptome (ART) was analyzed. In this experiment cells were treated for 48 h with 1, 10 and 100 nM DHT and the expression profiles were normalized to 0 nM DHT-treated cells. Using the data computed from Affymetrix Gene-Chips, at an arbitrary cutoff of threefolds increases or < -3 folds decreases over the untreated control level of gene expression was applied to evaluate ART in RC-165N/hTERT cells. In RC-165N/hTERT cells, 99 genes were induced by DHT including known androgen-inducible genes such as, *NDRG1* and *IGFBP3*. Forty-one genes out of the 99 upregulated genes were previously reported in CaP and other cancers (Table 1). Thirteen of the 41 cancer-related genes were involved in prostate cancer. Out of the 13 genes, *MGP* was highly expressed in metastatic prostate tumor-derived cell lines.²⁰ Similarly, *TFPI*,²¹ *IGFBP3*²²⁻²⁴ *MNSoD*^{25,26} and *MT1*²⁷ were shown to be associated with prostate cancer. The association of DHT-induced genes identified in RC-165N/hTERT cells with CaP support the potential of this cell culture model in evaluating the AR-signaling pathways involved in prostate epithelial cell growth and differentiation. Moreover, pathway analysis of the 13 identified prostate tumor associated genes indicated that some of these genes represent the endoplasmic reticulum stress response, fatty acid and cholesterol biosynthesis and polyamine biosynthesis pathways that is consistent with our previous studies in elucidating of androgen-regulated pathways in LNCaP cells¹⁶ (Figure 4a). We found that 140 genes were repressed by DHT from ~9696 expressed transcripts in RC-165N/hTERT. The repression of 20 genes was consistent between the 1, 10 and 100 nM DHT treatment groups (Table 2).

LNCaP cell line has been the prototypical model for evaluating ART in the context of prostate cell biology. Therefore, we compared published data on LNCaP-ART¹⁶ to the RC165 N/hTERT -ART measurements (Figure 4b). Fifty-two (54%) of the 96 previously described androgen-induced genes did show common expression changes in androgen-treated LNCaP and RC165 N/hTERT cells. Those 52 genes were categorized into two sub-classes. Thirty-three (63%) of the 52 genes showed consistent expression patterns in LNCaP and RC165 N/hTERT in response to androgens despite the different types of cell culture conditions or androgens (R1881 or DHT, respectively) were used in these experiments. Overall 33 (34.38%) genes out of the total 96 genes showed similar expression pattern in both the androgen-treated LNCaP cells and DHT-treated RC165 N/hTERT cells. This data supports that a significant component of AR signaling is intact in RC165 N/hTERT cells.

Table 2 Genes downregulated in 100, 10 and 1 nM DHT-treated RC165N/hTERT cells (normalized to 0 nM DHT treatment)

Genbank	RC165-treated with DHT			Gene name	Map	Reported in cancer
	100.0 nM	10.0 nM	1.0 nM			
U75667	-9.35	-3.01	-2.41	ARG2	14q24.1-q24.3	Arginase, type II
BE535746	-2.50	-2.06	-2.09	FLJ13110	2	Chromosome 2 open reading frame 23
AL109928	-2.90	-2.19	-2.15	DJ551D2.5	20	Protein phosphatase 1, regulatory subunit 3D
BF439316	-4.15	-2.72	-2.35	TMEFF1	9q31	Transmembrane protein with EGF-like and two Follistatin-like domains 1
NM_000600	-4.14	-3.04	-2.48	BSF2, IFNB2	7p21	Interleukin 6 (interferon, beta 2)
NM_005025	-4.80	-4.18	-2.43	PI12	3q26.31	Serine (or cysteine) proteinase inhibitor, clade I (neuroserpin), member 1
NM_004362	-5.72	-2.61	-3.28	CLGN	4q28.3-q31.1	Calmegin
NM_002852	-11.15	-2.42	-2.60	PTX3	3q25	Pentraxin-related gene, rapidly induced by IL-1 beta
NM_002090	-3.14	-12.97	-3.44	MIP2B, SCYB3	4q21	Chemokine (C-X-C motif) ligand 3
U96845	-2.19	-5.69	-2.02	NKG2F, NKG2-F	12p13.2-p12.3	Killer cell lectin-like receptor subfamily C, member 4
BE786164	-2.82	-3.16	-2.16	Hs cDNA DKFZp586C1723	2	Activating transcription factor 2
Z98884	-3.19	-9.58	-2.25	PER3	1p36.23	Calmodulin binding transcription activator 1
AB011131	-4.28	-2.05	-2.33	ACZ, KIAA0559	7q11.23-q21.3	Piccolo (presynaptic cytomatrix protein)
AL080170	-2.87	-4.49	-4.29	DKFZP434C091	1q44	Tripartite motif-containing 58
AL161999	-2.32	-8.92	-2.88	PIR121	5q34	Cytoplasmic FMR1 interacting protein 2
AF288391	-5.24	-2.37	-2.01	NIBAN	1q25	Chromosome 1 open-reading frame 24
NM_016098	-4.43	-2.02	-2.44	LOC51660	6q27	Brain protein 44-like
NM_024592	-4.91	-3.67	-2.46	FLJ13352	4q12	Steroid 5 alpha-reductase 2-like
NM_013381	-10.21	-2.30	-2.27	TRHDE	12q15-q21	Thyrotropin-releasing hormone degrading enzyme
NM_030953	-2.07	-2.01	-3.56	DKFZP761E2110	5q34	Tigger transposable element derived 6

Abbreviations: DHT, dihydrotestosterone; hTERT, human telomerase reverse transcriptase.

Discussion

We previously reported² that the RC-165N/hTERT cells express wt-AR, that showed modest biphasic growth profile in the presence of increasing concentration of androgen (DHT), and cell growth was inhibited by flutamide. This cell line did express cytokeratin 8 and AR, pointing to luminal cell characteristics.² Furthermore, we found no evidence of p63 expression in this cell line arguing against basal cell origin.³ As the RC-165N/hTERT is the only available cell line derived from benign prostate tissue of an African-American CaP patient that stably expresses wt-AR, we characterized the biochemical functions of AR. Stable expression of AR is a requirement for a prostate epithelial cell model owing to its critical roles in prostate cell growth, differentiation and tumorigenesis.²⁸⁻³⁰

Ligand-activated AR can regulate the transcription of a series of genes promoting growth and differentiation of male urogenital structures and the expression of prostate-specific markers such as PSA^{31,32}, NKX3.1³³⁻³⁶ and prostate-specific membrane antigen (PSMA).³² The integrity of AR-signaling pathways in cell culture models is often assessed by addressing the question of whether or not AR-regulated genes are expressed and modulated in response to androgens.^{37,38} We found significant expression and regulation of three examined genes, PSMA (Figure 1b), *NDRG1* (Figure 1c) and *PMEPA1* (Figure 2a) by androgen treatment, which were also well characterized by others and us^{16-18,39}. In comparison to LNCaP cells, the level of secreted PSA protein was substantially lower in RC-165N/hTERT cells (>1:400 ratio). In RC-165N/hTERT cells, the downregulation of

PSA at 10 nM DHT is a distinct response that is in contrast to androgen-sensitive CaP cells and may reflect inhibition of *KLK3* gene transcription at and above 10 nM DHT.³¹ This intriguing observation supported by ChIP as well as PSA (*KLK3*) QPCR assay that may in fact reflect the intrinsic nature of wt AR functions in RC165N/hTERT cellular context.

As expected, analysis of the androgen inducible *NDRG1* gene expression by QPCR and by microarray revealed consistent upregulation of the gene in response to DHT. Androgen-induced binding of AR to the upstream ARE of *NDRG1* promoter supported these findings.¹² Although, we found the distal ARE occupied within the *PMEPA1* promoter upstream sequences, the lack of AR binding at the proximal ARE is in contrast to our previous findings in LNCaP cells where binding of AR to both sites were required for the transcription activation of *PMEPA1* gene.¹²

We challenged RC165 N/hTERT cells with androgen hormone to evaluate the integrity of androgen signaling functions. The experiment revealed the activation of androgen-inducible genes as well as the expression of genes signifying cancer progression. However, the most important finding of the experiment was that pathways previously identified in cancer progression such as, endoplasmic reticulum stress response^{16,39}, fatty acid and cholesterol biosynthesis,⁴⁰ and polyamine biosynthesis pathways⁴¹ were activated in DHT-treated RC165 N/hTERT.

To reveal the similarities and differences between RC165 N/hTERT and LNCaP cells we compared androgen-induced gene expression changes between these two cell models. LNCaP cells are malignant cells harboring

AR mutation. In contrast, RC165 N/hTERT cells are derived from a benign prostate gland followed by immortalization with telomerase harboring wt AR. The effects of mutation in AR have been well studied on *in vivo* malignant cell growth⁴² and *in vitro* cell proliferation.⁴³ These studies suggested that the presence of mutation in AR could be responsible for diversified AR-signaling pathways, resulting in independent regulation of molecular events. The observed differences of androgen response transcriptome between LNCaP and RC165 N/hTERT highlight the contributions of both distinct nature of AR and the cellular context of the RC165N/hTERT cells.

In this study, we characterized the RC-165N/hTERT cell line for AR and its functions. RC-165N/hTERT is a unique cell line derived from an African-American CaP patient's benign gland tissue. Wt AR in this cell line can bind to AR binding elements of target gene promoters in response to DHT. RC-165N/hTERT cells can express and secrete the prostate-specific antigen, however at very low levels. PSA expression is intriguingly downregulated with increasing androgen treatment in this cell line that may reflect the functions of a wt AR in a benign-derived cell line. GeneChip analysis demonstrated the expression of androgen-inducible genes in RC-165N/hTERT including known androgen-regulated and cancer progression-associated genes and the subsequent pathway analysis supported these findings. Therefore, it is important to report the nature of androgen signaling in a benign prostate epithelium-derived cell line which might be illustrative for future studies of androgen-regulated genome in this type of cellular context. As AR is often lost in cell lines derived from primary prostate cancers and benign prostate, retention of AR expression and partial AR responsiveness of RC-165N/hTERT cells is suggestive of adaptive nature of AR signaling in a given cellular context. The unexpected DHT response of PSA regulation in RC-165N/hTERT cells may be revealing some aspect of AR functions during prostate epithelium growth and/or differentiation. In conclusion, RC-165N/hTERT as benign prostate-derived epithelial cell culture model with wt AR expression and functional AR in many respects has potential utility as a new experimental model in prostate biology.

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