

# Androgen Receptor Mediates the Reduced Tumor Growth, Enhanced Androgen Responsiveness, and Selected Target Gene Transactivation in a Human Prostate Cancer Cell Line<sup>1</sup>

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## ABSTRACT

The growth and development of the prostate gland are regulated by the androgen and the androgen receptor (AR). Despite our molecular understanding of the roles of the AR regulating; a downstream target gene transcription, the direct or indirect (stromally mediated) actions of the androgen in controlling prostate cell growth and differentiation are still unclear. In this report, an invasive; and metastatic human prostate tumor cell line, androgen-repressed human prostate cancer cell line (ARCaP), either transduced with wild-type human AR (*hAR*) or a control neomycin-resistant plasmid DNA, was used to evaluate the direct role of AR in regulating prostate tumor cell growth and gene transcription. Results showed that: (a) introduction of wild-type *hAR* to ARCaP cells restored positive androgen regulation of prostate tumor cell growth *in vitro* through an enhanced cell-cycle progression from G<sub>0</sub>/G<sub>1</sub> to S and G<sub>2</sub>-M phases; (b) *hAR* was shown to transactivate glucocorticoid-responsive element but not prostate-specific antigen promoter-directed reporter gene expression; and (c) *hAR*-transduced ARCaP cells exhibited reduced growth, invasion, and migratory behavior *in vitro* and tumor growth *in vivo*. These results suggest that the introduction of *hAR* into the invasive human prostate cancer ARCaP cell line restored its androgen-regulated cell growth, decreased the rate of tumor growth, and selectively activated AR target gene expression. These cellular functions in response to androgen are commonly associated with increased differentiation of prostate epithelial cells.

## INTRODUCTION

Steroid hormones have long been thought important in the etiology of cancers from hormonally responsive tissues like prostate (1–3). The biological activities of steroid hormones are mediated by their specific receptors, namely androgen, estrogen, progesterone, glucocorticoid, and mineralocorticoid receptors, which are members of the nuclear receptor superfamily (4–6). All of the members of this family have a similar structural organization with a highly variable NH<sub>2</sub>-terminal transactivation domain, followed by a well-conserved DNA-binding domain consisting of two zinc finger DNA-binding motifs, a hinge region consisting of nuclear localization signals, and a moderately conserved COOH-terminal ligand-binding domain. Steroid receptors regulate the transcriptional activity of target genes by binding to the cognate DNA sequence of hormone response elements, followed by the initiation, assembly, and stabilization of the preinitiation complex in the region of the target gene promoter (7–13).

Despite clear evidence that the regulation of gene expression by steroid hormones is mediated by the interaction of steroid hormone

receptors and their respective cofactors on the *cis*-DNA element of target gene promoters, only very limited and somewhat controversial information is available on how cell growth is regulated by specific molecular interactions between steroid hormones and cellular target genes. One of the difficulties in elucidating the direct action of steroid hormone receptors controlling target cell growth is that steroid hormone receptors may act on target cells indirectly, with the action mediated by stromal cells (14–18). Steroid hormone action on target tumor epithelial cells may be mediated by a wide spectrum of “cross-talk” between soluble growth factors, insoluble matrix proteins, and steroid hormone receptors in the stromal and/or epithelial cells (19–22). Another difficulty is the lack of appropriate tumor cell models that provide a suitable framework to decipher how steroid hormones may regulate target gene expression and cell growth *in vitro*. Steroid hormones and their receptors may affect the growth of hormone-sensitive tumors by a number of mechanisms including the direct regulation of epithelial cells alone, stromal-epithelial communication, tumor angiogenesis, and host immune reactivity around tumor epithelium (14–25). Most data reported in the literature citing steroid hormone regulation of target tissue growth were obtained by *in vivo* evaluation.

The goal of this study is to evaluate the possible direct role of AR<sup>3</sup> regulating both prostate tumor epithelial cell growth and gene expression. The *hAR* gene encodes 919 amino acids with a M<sub>r</sub> 110,000 protein (26–29) that is a ligand-activated transcription factor (30–32) and recognizes the ARE as a homodimer (33–37). AR is essential in regulating the development and maintenance of male reproductive systems by controlling prostate cell proliferation, apoptosis, differentiation, and senescence (38–43). Liganded AR is also pivotal to the onset of PCa and its subsequent progression to androgen independence (44–48). Using animal models bearing human prostate tumor xenograft, Thalmann *et al.* (49, 50) and others (51, 52) have shown that on androgen withdrawal, PCa can progress from an androgen-dependent to an AI state and eventually become refractory to hormonal therapy. Our laboratory developed a novel PCa cell model, ARCaP. The ARCaP cell line was derived from the ascites fluid of a PCa patient with advanced metastatic disease and characterized to express the low level of AR and PSA (53). ARCaP cell growth *in vitro* and tumor growth *in vivo* were also repressed by androgen (53).

In this paper, we seek to define how AR may regulate PCa growth and target gene transactivation. By introducing *hAR* to ARCaP cells, we observed that AR plays a critical role in promoting target gene transactivation, mediating androgen-induced cell growth, and inhibiting tumor cell migration, invasion *in vitro*, and tumor growth *in vivo*.

<sup>3</sup> The abbreviations used are: AR, androgen receptor; AI, androgen-independent; ARCaP, androgen-repressed human prostate cancer cell line; PSA, prostate-specific antigen; *hAR*, human AR; *hAR* wt, wild-type human AR; ARE, androgen-responsive element; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammoniummethyl sulfate; GRE, glucocorticoid response element; PCa, prostate cancer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EtOH, ethanol; RT-PCR, reverse transcription PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCC, dextran-coated charcoal; FBS, fetal bovine serum; *his*, histidine; 4-OH-FL, 4-hydroxy flutamide.

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## MATERIALS AND METHODS

### Cell Cultures, Chemicals, and Extracellular Matrices

PCa cell lines LNCaP, PC-3, and an ARCaP subline, AI8, were routinely cultured in T medium (Ref. 54; Life Technologies, Inc., Rockville, MD) containing 5% FBS in a 37°C incubator supplemented with 5% CO<sub>2</sub>. Green monkey kidney CV-1 cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured in the same manner as described above. *Neo* or *hAR*-expressing ARCaP cell clones were routinely cultured in 5% FBS/T medium containing 0.7 mg/ml Geneticin (G418; Life Technologies, Inc.). RPMI 1640 phenol red-free medium (Life Technologies, Inc.) supplemented with 5% DCC-stripped serum was used in transfection and growth assays. R1881, a synthetic analogue of androgen, was purchased from New England Nuclear (Boston, MA). The antiandrogen 4-OH-FL was obtained as a gift from Schering-Plough Institute (Kenilworth, NJ). Bicalutamide (casodex, a synthetic antiandrogen) was kindly provided by Zeneca, Inc. (Wilmington, DE). Type 1 laminin purified from Englebreth-Holm-Swarm mouse tumor was obtained as a gift from Dr. Roy Ogle (University of Virginia, Charlottesville, VA). Matrigel matrix was obtained from BD Biosciences (Bedford, MA). Propidium iodide, MTT, protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin A), and crystal violet were purchased from Sigma Chemical Co. (St. Louis, MO). DOTAP was obtained from Roche (Indianapolis, IN).

### Transfections

**Stable Transfections.** Stable AR-expressing ARCaP cell clones were generated by transfecting the full-length *hAR* cDNA into AI8, which preserved the characteristics of parental ARCaP cells, as described by Cleutjens *et al.* (55) with modifications. Briefly,  $3 \times 10^5$  cells/well in six-well plates were transfected with 3  $\mu$ g of wild-type *hAR* cDNA expression vector, designated pcDNA-*hAR*, or pcDNA-*neo* control vector (Invitrogen, Carlsbad, CA). The AR gene was expressed under the control of cytomegalovirus promoter. The pcDNA-*hAR* construct was kindly provided by Dr. Sue-Hwa Lin (University of Texas, M.D. Anderson Cancer Center, Houston, TX). Gene transfection was carried out in RPMI 1640 phenol red-free medium via a DOTAP liposome-mediated procedure according to the manufacturer's protocol. After gene transfection (6 h), the cell culture medium was replaced with fresh medium and cultured overnight. Subsequently, cells were trypsinized and plated in 100-mm culture plates in 5% FBS/T medium. After overnight incubation, the medium was replaced with "*neo*" selection medium (5% FBS/T medium/0.7 mg/ml G418). *Neo*-resistant control and *hAR*-transfected single cell clones (36) were selected in 3 weeks and amplified. All of the selected clones were routinely maintained under 0.7 mg/ml G418 and tested for positive *neo* or AR expression.

**Transient Transfections.** The AR-positive or *neo* control clones were subjected to transient transfection of the reporter gene constructs via the DOTAP liposomes according to the manufacturer's protocol (Roche). In brief,  $3 \times 10^5$  cells/well in six-well plates were transfected either with 2.5  $\mu$ g of GRE4-TATA-Luc or p61PSA-Luc reporter plasmid DNA as described previously (56). GRE4-TATA-Luc and PSA-Luc reporter plasmids were kindly provided by Dr. C. Kao (University of Indiana, Indianapolis, IN) and Fan Yeung (University of Virginia, Charlottesville, VA), respectively. The AR-mediated p61PSA-Luc (2.5  $\mu$ g) reporter activity was also assessed in LNCaP cells. To assess *hAR* transcriptional activity, CV-1 cells were cotransfected with 0.5  $\mu$ g of pcDNA-*hAR* and 2.5  $\mu$ g of GRE4-TATA-Luc plasmids. pCMV- $\beta$ -galactosidase plasmid DNA (0.5  $\mu$ g) was included in all of the transfection assays to determine the transfection efficiency. After 6-h incubation with DNA-DOTAP mixture, the culture medium was completely removed. The cells were incubated in culture medium in the absence (EtOH vehicle) or presence of  $10^{-8}$  M R1881 for at least 24–36 h. Specificity of AR-mediated reporter gene transactivation under R1881 induction was confirmed by the addition of antiandrogen  $10^{-5}$  M casodex. Transfection experiments were repeated at least three times in duplicates using different batches of purified plasmid DNA.

### Luciferase Assay

Luciferase reporter gene activities were measured according to the Luciferase Assay System (Promega, Madison, WI). Briefly, cells were washed two

times with PBS and lysed in 250  $\mu$ l of Luciferase Lysis Buffer (25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, and 1% Triton X-100; Promega) at room temperature for 15 min with constant rocking. Cell lysate was vortexed briefly and centrifuged for 3 min at 4°C. Supernatant (20  $\mu$ l) was mixed with 100  $\mu$ l of luciferase substrate. Reporter gene activities were measured using Monolight 2010 Luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Either  $\beta$ -galactosidase activity as assessed according to manufacturer instruction (Promega) or total protein as determined by Bradford assay (Pierce, Rockford, IL) was used to normalize the relative luciferase activity. No difference was observed using either of these methodologies. The background relative luciferase activity of the vector backbones (pGL3/TATA for GRE4-TATA-Luc or pGL3 basic for p61PSA-Luc) was subtracted from all of the assays. The fold induction was calculated based on the ratios of R1881: EtOH.

### RNA Isolation

Total RNA was isolated from LNCaP, *Neo* control, or AR-expressing ARCaP cell clones or frozen (–80°C) primary tumor tissues (formed by *Neo*1 or C-18 clone) using RNazol B reagent (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's protocol. Briefly, 80% confluent monolayer cells in 100-mm culture dishes were lysed in 2.5 ml of RNazol B. Homogenized tissue specimens were prepared with RNazol B (3 ml/100 mg tissue) using a polytron homogenizer. The total RNA from cell lysate and homogenized tissues was extracted by addition of 0.2 ml of chloroform/2 ml of homogenates. The RNA was precipitated by adding one volume of isopropanol to one volume of aqueous phase, washed two times with 75% EtOH, and dissolved in RNase-free water.

### RT-PCR

Oligo-pdT<sub>12–18</sub> primer (Amersham-Pharmacia Biotech, Piscataway, NJ) and reverse transcriptase enzyme (Life Technologies, Inc.) were used to synthesize single-strand cDNA using 2  $\mu$ g of total RNA as the template in 20- $\mu$ l reaction volume according to the standard method (57). A portion (1/20) of the cDNA was used to amplify the fragment of AR either from ligand or the NH<sub>2</sub>-terminal domain in the presence of 1.25 units of Taq DNA polymerase. Primer sets used to run PCR reactions were P1 = 5' CAGAAGCTGACAGTGTCACA (forward), P2 = 5' CAAGGCACACTGCAG (reverse), P3 = 5' TAGGACTCAC-TATAGGGAG (forward), and P4 = 5' CTGGAAAGCTCCTCGGTA (reverse). Thirty cycles of 1 min at 94°C (pre-denaturation), 30 s at 94°C (denaturation), 1 min at 55°C (annealing temperature), and 45 s at 72°C (extension) were performed, and 5–10  $\mu$ l of each PCR product was resolved on a 1.2% agarose gel containing 0.5  $\mu$ g/ml ethidium bromide.

### Western Blot

Monolayer cells were washed twice with ice cold PBS and lysed in lysis buffer [1  $\times$  PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors (10  $\mu$ g/ml leupeptin, 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride)]. The protein concentration of clear lysate was determined by bicinchoninic acid protein assay using BSA as the standard (Pierce). Total cell lysate (7.5  $\mu$ g) was resolved on a 7.5% SDS-PAGE and transferred to nitrocellulose membrane (NitroPure; Osmonics, Westborough, MA). The membrane was blocked 1 h at room temperature with TBST blocking buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20, and 5% nonfat milk]. The membrane was incubated for 1 h at room temperature with primary anti-AR polyclonal antibody PG21 at 2  $\mu$ g/ml in TBST blocking buffer. The secondary antibody used was directed against rabbit IgG conjugated to horseradish peroxidase (Amersham-Pharmacia) at 1:2000 dilutions in TBST blocking buffer and incubated for 1 h at room temperature. The signal was detected by enhanced chemiluminescence on Hyperfilm (Amersham-pharmacia Biotech).

### Cell Growth

Cells/well ( $2 \times 10^3$ ) were seeded in 96-well plate. After overnight incubation, the medium was replaced by 5% DCC/RPMI 1640 phenol-red free medium and incubated for an additional 24 h. Cells were incubated in the presence or absence of increasing concentrations of a synthetic androgen

agonist R1881, ranging from  $10^{-10}$  M to  $10^{-6}$  M, for 6 days. The medium was changed at day 3. For growth inhibition studies, cells were incubated in the presence or absence of  $10^{-7}$  M of the antiandrogen 4-OH-FL along with  $10^{-8}$  M R1881. Cell growth was determined by crystal violet assay as described previously (53). Briefly, cells were fixed by 1% glutaraldehyde for 15 min and stained with 0.5% crystal violet solution for 15 min at room temperature. Plates were washed with tap water several times, incubated in distilled water for 10 min, and air-dried. The dye from fixed cells was eluted by Sorenson's solution for 30 min at room temperature with constant shaking. ELISA reader was used to read aliquots of eluant at 590 nm. Experiments were performed in quadruplicates and repeated at least three times.

### Flow Cytometric Analysis

Cells synchronized by serum starvation were grown in phenol red-free RPMI 1640 containing 5% DCC in the absence or presence of  $10^{-8}$  M R1881 for 48 h. Cell cycle analysis was conducted according to Mendonca *et al.* (58) with modifications. Briefly,  $2.5 \times 10^5$  cells were harvested, fixed with 70% EtOH in PBS, and incubated on ice for 30 min. Subsequently, cells were pelleted, washed with PBS, and resuspended in 0.4 ml of PBS containing propidium iodide (25  $\mu$ g/ml) and DNase-free RNase (90  $\mu$ g/ml). Cells ( $5 \times 10^4$ ) were counted for cell cycle analysis. Experiments were performed in duplicate and repeated three times.

### Substrate Attachment, Invasion, and Migration

Cell adhesion assays were performed as described previously (59) with modifications. The 96-well cell culture plates were precoated with 100  $\mu$ l of serum-free T medium containing type 1 laminin (50  $\mu$ g/ml) at 4°C overnight. The precoated wells were blocked with heat inactivated 0.1% BSA/T medium. Subconfluent cells were lifted up from culture plates and suspended as single cells using a brief treatment in 10 mM EDTA and 20 mM HEPES buffer (pH 7.4) in T medium. After neutralizing the EDTA with  $\text{CaCl}_2$  and  $\text{MgSO}_4$ , cell pellets were washed once and suspended in T medium containing 0.1% BSA. Cells ( $\times 10^3$ ) were placed on laminin-coated wells and allowed to adhere for various times at 37°C in a 5%  $\text{CO}_2$  incubator. Attached cells were fixed in 3.7% paraformaldehyde. Percentage attachment is expressed by counting the spread cells with enumerated from random triplicate fields containing  $\geq 100$  cells.

Invasion and migration assays were performed using Boyden chambers, BIOCOAT, purchased from Becton Dickinson Labware (Bedford, MA) with 6.4-mm insert size and 8- $\mu$ m pores. Matrigel Matrix (100  $\mu$ g/cm<sup>2</sup> surface area) in T medium (1:5 dilution) was placed in 35  $\mu$ l on the inner layer of the invasion chamber and incubated at 37°C for 30 min before the addition of cells. The chambers with or without Matrigel Matrix placed into the wells of 24-well culture plates were used for invasion and migration assays, respectively. Cells ( $5 \times 10^4$ ) were added in 500  $\mu$ l to the inner side of chambers; 1 ml of T medium/0.1% BSA was added to the lower chamber. The chambers were incubated for 20 h at 37°C in a 5%  $\text{CO}_2$  atmosphere in the presence and absence of  $10^{-8}$  M R1881. Cell invasion and migration were quantified by standard MTT assay as described by Lu *et al.* (41). Briefly, 2.5 mg/ml MTT solution was added into the inner and outer chamber and incubated at 37°C for 4 h. The medium were collected separately from both sides of the chambers, and the residual MTT crystals were precipitated. The emergent cells on the underside of the membrane were "scrubbed" with filter paper and combined with the crystal collected from the outer side of the chamber. The MTT crystals from both sides were dissolved separately in 500  $\mu$ l DMSO. The color intensity, correlated linearly with cell number, was measured at 590 nm against the blank, consisting of 0.1% BSA/T medium with MTT solution and 500  $\mu$ l of DMSO. The percentage invasion is expressed as the number of cells that invade through the occluded Matrigel-coated membrane (Invasion Chamber) divided by the total number of cells. Percentage migration is expressed as the number of cells that migrate through the uncoated membrane divided by the total number of cells. Both assays were performed at least in triplicates.

### Animal Study and Histochemistry

Tumorigenicity and the rate of ARCaP tumor growth in athymic mice (CD-1 *nu/nu*; Charles River Breeding Laboratories, Wilmington, MA) inoculated with ARCaP cell clones transfected either with *neo* or *hAR* plasmid

cDNA constructs were evaluated. Intact young male mice were injected orthotopically at the dorsolateral lobe of the prostate gland with ARCaP cells ( $2 \times 10^6$  cells/25  $\mu$ l; Refs. 49, 53). All University of Virginia policies concerning the humane care and use of laboratory animals were strictly adhered. Mice were sacrificed and evaluated 16 weeks after tumor cell inoculation. Tumor volumes in prostate gland were assessed by caliper according to procedures described previously (53). Routine tumor histopathology was performed. ARCaP-induced tumors were fixed in 3.7% formaldehyde and paraffinized. Other sections were "snap" frozen with liquid nitrogen and stored at  $-80^\circ\text{C}$  until RT-PCR studies.

To detect *hAR* expression in the primary tumor tissue, an immunohistochemistry assay was performed as described (60) for which paraffinized prostate tumor specimens were subjected to antigen retrieval (microwaved in 10 mM citric acid (pH 4.0) for 8 min), incubated with 3%  $\text{H}_2\text{O}_2$ , blocked with Superblock (SeyTek Laboratories, Logon, UT), and reacted with an anti-AR polyclonal antibody, PG21, at 4  $\mu$ g/ml. The AR signals were amplified by biotinylated-peroxidase-conjugated streptavidin system (Bio-Genex Laboratories, San Ramen, CA) with diaminobenzidine as a substrate. Native and transfected *hAR* gene expression was also evaluated by RT-PCR in tumor tissues as described above.

### Statistical Analysis

Where appropriate, Student's *t* test was performed to analyze for differences between various treatments. Statistical significance was determined at  $P \leq 0.05$ .

## RESULTS

### Establishment of *hAR*-expressing ARCaP Cell Transfectants.

ARCaP cells were shown to express low levels of AR and PSA (53). To determine whether the low level of PSA production might be related to the low level of AR protein expression, we generated both *hAR* and *neo*-vector transfected ARCaP cell clones. Fig. 1A shows the designated pcDNA-*hAR* expression vector containing the structure of a *his*-tagged *hAR* wt cDNA. We selected two *neo*-vector transfected control (Neo1 and Neo2) and 5 *hAR*-expressing (C-18, C-20, C-22, C-23, and C-24) clones for the comparative study. As shown in Fig. 1B, the levels of AR expression in *hAR*-transfected ARCaP cell clones were comparable with the AR level detected in the positive-control LNCaP cells. We devised a RT-PCR assay using the primer sets indicated in Fig. 1A to distinguish the endogenous from the transfected *hAR* in ARCaP cell clones (see "Materials and Methods"). Fig. 1C (*top panel*) demonstrates that when P1 and P2 primers that recognize *hAR* ligand-binding domain were used, we detected AR in LNCaP and transfected *hAR* in ARCaP cell clones (330-bp band). The RT-PCR with P1 and P2 primers detected the very low level of endogenous AR in Neo1 and Neo2 clones (Fig. 1C, *top panel*, Lanes 2 and 3). When P3 and P4, which specifically recognize the *his*-tagged  $\text{NH}_2$ -terminal region of *hAR*, were used as primers, we detected a 190-bp *hAR* fragment only in *hAR*-transfected ARCaP cell clones. This amplified DNA sequence was absent in control clones and the LNCaP-positive control specimens (Fig. 1C, *middle panel*).

**Transfected *hAR* Differentially Regulates Target Gene Expression in ARCaP Cells.** We evaluated the functionality of transfected *hAR* on gene transactivation in ARCaP cell clones. Two reporter genes, GRE4-TATA-Luc, which contains four copies of GRE (165 bp) linked to TATA-Luc reporter, and a 5.8-kb full-length PSA promoter-directed luciferase expression vector were transfected into both control *neo*- and *hAR*-expressing ARCaP cell clones. GRE was characterized as a high affinity-binding site for AR as well as glucocorticoid receptor (61). The reporter gene expression was evaluated in the presence and absence of  $10^{-8}$  M R1881 or  $10^{-5}$  M casodex. Fig. 2A (*left panel*) shows that transfected *hAR* can enhance luciferase reporter activity under the control of a GRE *cis*-element by 5- to



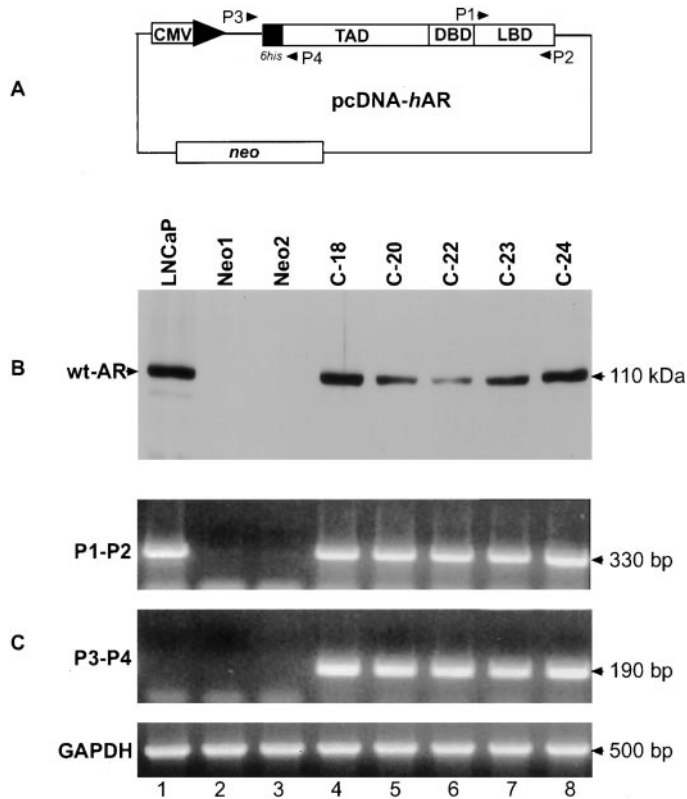


Fig. 1. Establishment of stable AR-expressing ARCaP cells clones by stable transfections. **A**, schematic representation of pcDNA-*hAR* plasmid used in stable and transient transfections assays. The “6his” represents six *his* tags. P1, P2, P3, and P4 represent the primers used to amplify the fragments of AR. *TAD*, transactivation domain; *DBD*, DNA-binding domain; *LBD*, ligand-binding domain of AR; *neo*, neomycin gene served as selection marker. **B**, Western blot analysis of AR protein in ARCaP cell clones. Total cell lysate (7.5  $\mu$ g) from LNCaP cells (as positive control), *neo* vector-transfected control clones (Neo1 and Neo2), and various *hAR* cDNA-transfected ARCaP cell clones (C-18, C-20, C-22, C-23, and C-24) were subjected to Western blot analysis using AR polyclonal antibody. The expected  $M_r$  110,000 and clearly detectable wild type-AR (*wt-AR*) protein are marked. **C**, transcription of *hAR* in ARCaP cell clones as determined by RT-PCR. P1-P2 primer set amplified the ligand-binding portion of AR (*top panel*). P3-P4 primer set amplified the NH<sub>2</sub>-terminal fragment, including His-tag, of AR (*middle panel*). *GAPDH* gene was used as an internal control in PCR reactions (*bottom panel*). The expected sizes of the PCR product were indicated on the left. Lane 1, LNCaP positive cell control; Lanes 2 and 3, *neo*-control clones; and Lanes 4–8: *hAR* expressing clones 18, 20, 22, 23, and 24, respectively.

8-fold in an AR and ligand-dependent manner, and such enhancement can be blocked by casodex. Similarly, CV-1 cells cotransfected with AR and GRE4-TATA-Luc also responded markedly R1881 induction (Fig. 2A, right panel). These results suggest that *hAR* is functional in activating ARE-responsive target gene in ARCaP cell clones.

In contrast to GRE4-TATA-Luc, p61PSA-Luc failed to respond to R1881 when transiently transfected into *hAR*-expressing ARCaP cell clones (Fig. 2B, right panel); p61PSA-Luc, as expected, is highly responsive to androgen induction (Fig. 2B, left panel; Refs. 36, 62). These results, taken together, demonstrate that selective activation of AR target genes may occur in ARCaP cell clones. Similar observations were also documented in PC-3 cells expressing stably transfected *hAR* (data not shown), suggesting that a differential loss of regulation of AR target gene transactivation in prostate tumor epithelial cells may occur on AI progression.

**Transfected *hAR* Restores Androgen Regulation of ARCaP Cell Growth *in Vitro*.** Because *hAR* is transcriptionally active in ARCaP cell clones, we tested whether transfected *hAR* can promote ARCaP cell growth *in vitro* in a ligand-dependent manner. Fig. 3A shows that in comparison with control clones the synthetic androgen

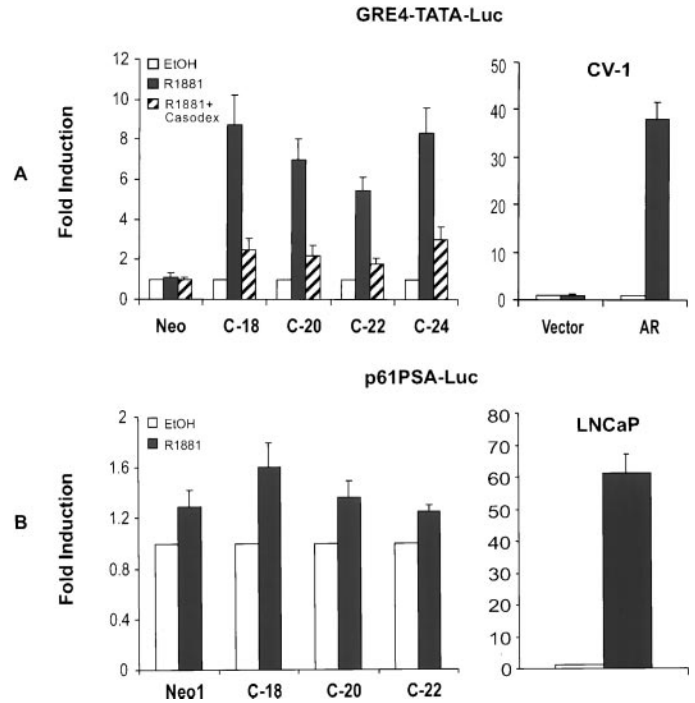


Fig. 2. The transcriptional activity of *hAR* in ARCaP cell line as determined by transient transfections. **A**, GRE4-TATA-Luc activity in ARCaP cell clones (*right panel*) or in CV-1 control cell line (*left panel*). **B**, p61PSA-Luc activity in ARCaP cell clones (*right panel*) or in positive control LNCaP cell line (*left panel*). Data presented as the mean of values from three independent experiments; bars,  $\pm$  SD. EtOH control-induced gene activation was arbitrarily set to 1 so the fold induction of R1881-induced or casodex-blocked reporter gene activity is relative to EtOH control.

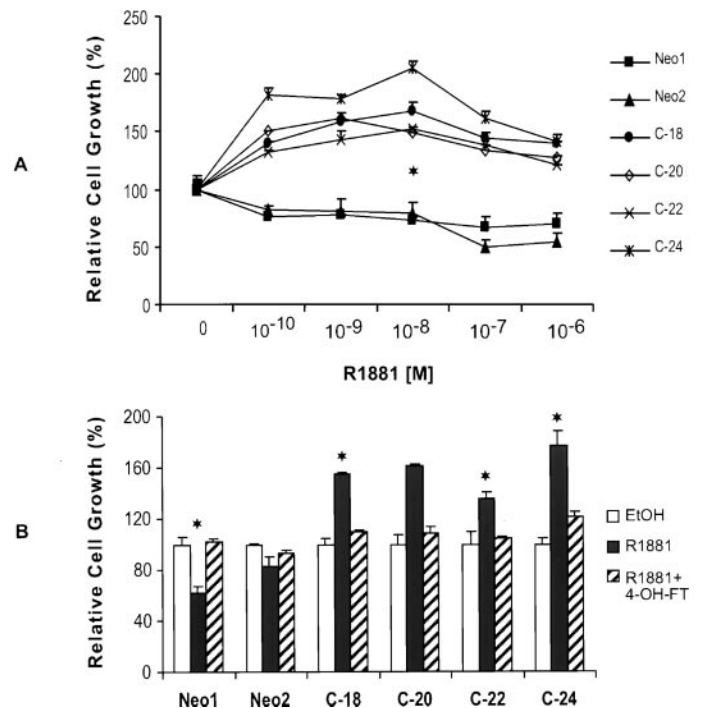


Fig. 3. Effects of level of *hAR* expression on androgen-regulated PCa cell growth as determined by crystal violet assay. **A**, dose-dependent effects of R1881 on the growth of control or *hAR*-transfected ARCaP cell clones. **B**, androgen-regulated growth inhibition of *hAR*-expressing ARCaP cells clones by 4-OH-FL *in vitro*. EtOH control-induced growth arbitrarily set to 100% so the percentage of R1881-induced growth or 4-OH-FL-blocked (*B*) growth is relative to EtOH control. Data represent the mean of four values repeated at least four times; bars,  $\pm$  SD.  $P \leq 0.007$  was used to compare the mean value of growth (EtOH control versus  $10^{-8}$  M R1881 induction).

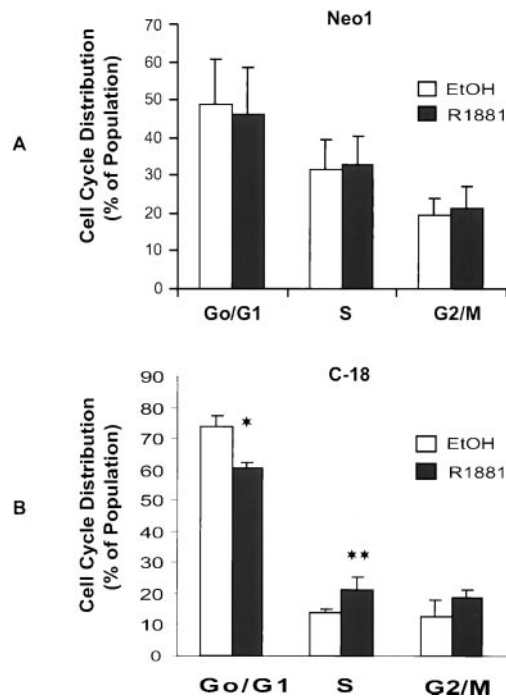


Fig. 4. *hAR*-mediated cell growth in response to androgen action as determined by fluorescence-activated cell sorter analysis of cell cycle. Neo1 (*neo* control) and *hAR*-transfected ARCaP cell (C-18) clones were incubated in the presence or absence of  $10^{-8}$  M R1881. Cell cycle analyses were performed after 48 h. Data represent the mean of triplicate values repeated at least three times; bars,  $\pm$  SD. \*,  $P \leq 0.03$  and \*\*,  $P \leq 0.02$  were shown to compare the mean value of cell cycle progression (EtOH control versus R1881 induction).

agonist R1881 stimulated the growth of *hAR*-transfected ARCaP cell clones by 50 to 100% in a biphasic manner, with maximal growth stimulation observed at  $10^{-8}$  M R1881 and a decreased growth stimulation observed at  $10^{-6}$  M R1881. Fig. 3B shows that the growth stimulatory effect of androgen in the *hAR*-expressing ARCaP cell clones and growth-inhibitory effect in the control *neo*-transfected ARCaP cell clones are mediated through *hAR*, because these observed effects can be antagonized by the coadministration of the synthetic androgen against R1881 and its antagonist, 4-OH-FL. These observations suggest that AR expression is crucial in mediating androgen action on PCa cell growth.

**Transfected *hAR* Mediates Androgen-induced Cell Cycle Progression in ARCaP Cells.** To test if transfected *hAR* impairs basal cell growth and restores positive androgen-regulated growth through altered cell cycle progression, we assayed the cell cycle progressions of Neo1 and C-18 ARCaP cell clones treated either with  $10^{-8}$  M R1881 or vehicle (EtOH control) for 48 h before fluorescence-activated cell sorter analysis. Fig. 4, A and B, show that compared with Neo1 ARCaP clone, *hAR*-expressing C-18 ARCaP cell clone had a lower basal growth fraction (S phase,  $13.7 \pm 1.2\%$  as opposed to  $31.5 \pm 7.8\%$ ). On R1881 stimulation, there was statistically significant increase in the fraction of cell proliferation in C-18 clone (S phase increased from  $13.7 \pm 1.2\%$  to  $20.9 \pm 3.2\%$ , whereas G<sub>2</sub>-M increased from  $12.8 \pm 3.8\%$  to  $18.8 \pm 1.8\%$ ) at the expense of a decreased pool of cells in G<sub>0</sub>/G<sub>1</sub> (decreased from  $73.4 \pm 2.9\%$  to  $60.2 \pm 1.3\%$ ). There appear to be minimal changes in the cell cycle progression of the control Neo1 clone when treated with R1881 (S phase,  $31.5 \pm 7.8\%$  as opposed to  $32.5 \pm 7.9\%$ ). Similar results were also obtained with Neo2 control and C-20, C-22, and C-24 *hAR*-expressing ARCaP cell clones (data not shown).

**Transfected *hAR* Decreases ARCaP Cell Adhesion, Invasion, and Migration *in Vitro*.** One of the characteristics of cancer cells is loss of contact inhibition associated with increased cell invasion and

migration (63). As demonstrated previously, ARCaP cells are highly invasive and metastatic *in vivo* (53). The goal of this experiment was to determine whether transfected *hAR* affects such cell behaviors (*e.g.*, cell adhesion, invasion, and migration) *in vitro* in chemically defined growth conditions. In comparison with control clones (Fig. 5A), *hAR*-expressing ARCaP cell clones exhibited decreased cell adhesion (*i.e.*, expressed as cell spreading) to laminin matrix. Likewise, the basal rate of invasion but not migration by *hAR*-transfected cell clones was slower than that of control clones (Fig. 5B). On addition of  $10^{-8}$  M R1881, the invasion and migration of ARCaP cells *in vitro* on Matrigel was additionally attenuated (Fig. 5C). Therefore, AR may mediate decreased cell adhesion, invasion, and migration onto extracellular matrices surrounding ARCaP cells in a ligand-dependent manner. Parallel results were obtained when Neo2 control and C-20 and C-22 *hAR*-expressing clones were used for this study (data not included).

**Transfected *hAR* Reduces ARCaP Tumor Growth.** We compared the rate of tumor growth in intact male athymic mice inoculated orthotopically with *hAR* expressing C-18 or Neo1 control clone. Local tumor growth was evaluated by tumor volume 16 weeks after the initial tumor cell inoculation and confirmed by histomorphology (Fig. 6A, bottom panel). Orthotopic ARCaP tumors were induced in 60% of animals inoculated either with Neo1 or C-18 cell clone. Mean tumor volumes harvested from the primary site of growth were significantly lower in the C-18 inoculated animals than that of the Neo1 control clone inoculated animals (Fig. 6A, top panel, and 6B). The immunohistochemical study confirmed *hAR* expression in the

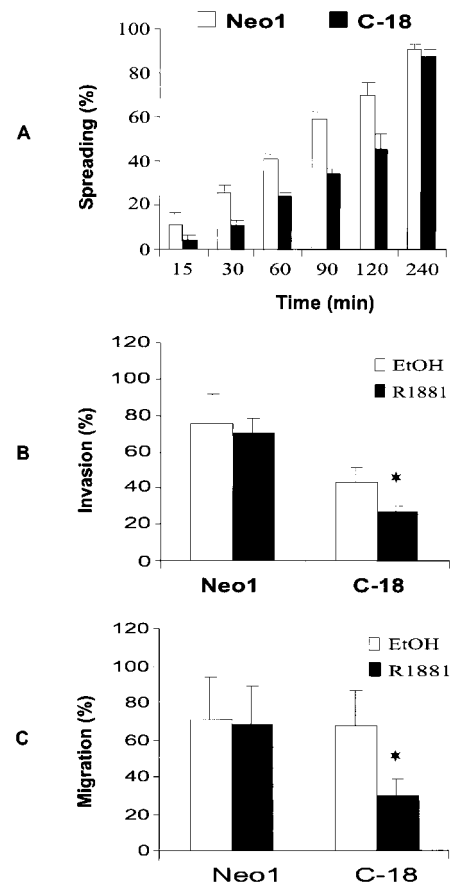


Fig. 5. *hAR* expression reduces the cell attachment, migration, and invasion ability of ARCaP cells *in vitro*. A, cell spreading on laminin matrix. B, migratory response. C, invasiveness of *neo*- or *hAR*-expressing ARCaP cell clones in the presence or absence of  $10^{-8}$  M R1881. Data represent the mean of triplicate values; bars,  $\pm$  SD. \*  $P \leq 0.02$  was used to compare the mean value of EtOH control versus R1881 induction.

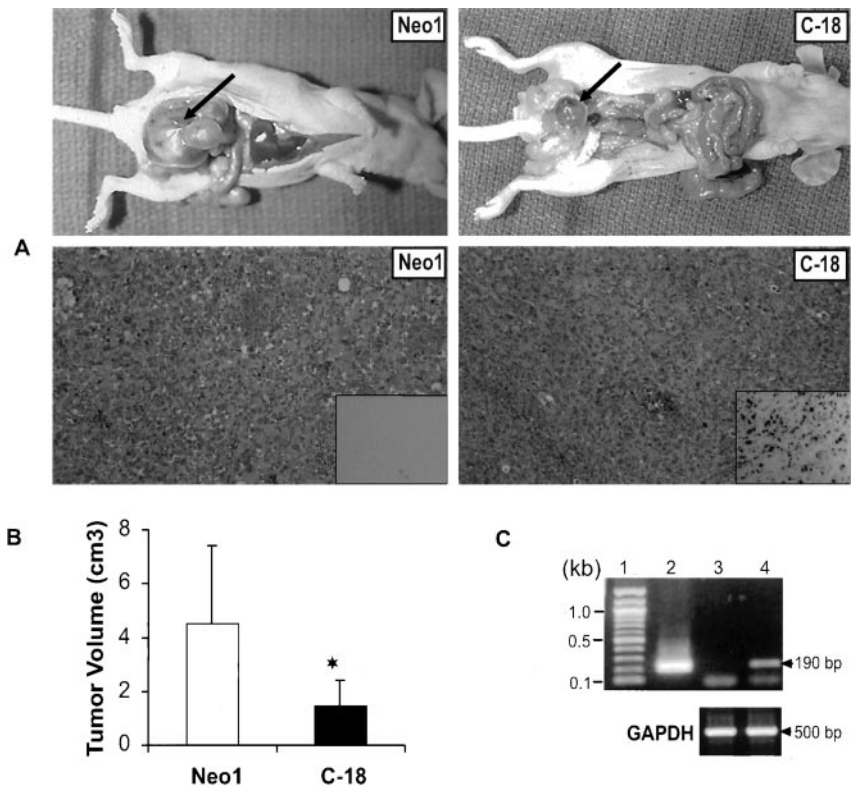


Fig. 6. *hAR* expression reduces the tumorigenicity of orthotopically injected-ARCaP cells in intact male (CD-1 *nu/nu*) athymic mice. *A*, representative gross morphology of the primary tumors as pointed with arrows (*top panels*) and histopathology (*bottom panel*). Insets represent the AR immunostaining in Neo1 and C-18 transfected tumor tissues. *B*, a graph of primary tumor volume. *C*, RT-PCR analysis of his-tag AR expression in primary prostate tumor tissue of C-18 versus Neo1 control clone. Data represent the mean tumor volumes of 60% tumor uptakes; bars,  $\pm$  SD. \*,  $P \leq 0.03$  was used to compare the mean tumor volumes of Neo1 versus C-18 clone. Lane 1, 100-bp size marker; Lane 2, pcDNA-*hAR* expression vector used as a positive control (Fig. 1A); Lane 3, Neo1; and Lane 4, C-18. GAPDH was used as a loading control in PCR reactions.

C-18 primary tumor; however, no *hAR* signals were detected in Neo1 cell inoculated tumor specimens (Fig. 6A, *bottom panel insets*). We also analyzed total RNA from the snap frozen tumor tissues by RT-PCR using the P3-P4 primer set (Fig. 1A). RT-PCR showed that C-18 clone expressed *hAR* (190 bp) during tumor growth *in vivo* (Fig. 6C). These results are consistent with the immunohistochemical staining of AR data. Because of the exceedingly fast growth rate of the Neo1 control clone, we did not observe any distant metastasis of ARCaP tumor cells beyond the lymph nodes.

## DISCUSSION

AR, a member of the steroid hormone superfamily, is a master switch that controls prostate cell gene expression, growth, and differentiation (4–6, 34–43). Despite much effort in delineating the molecular mechanisms of AR regulation of downstream target gene expression in androgen-responsive target cells, little progress has been made in elucidating the molecular mechanisms underlying androgen regulation of normal prostate growth and the loss of androgen regulation of PCa growth after prolonged hormonal therapy. Currently, several attractive hypotheses may help to explain the mechanisms by which AR regulates PCa cell growth and differentiation: (a) AR prevents cell death through the expression of genes that encode antiapoptotic factors (52); (b) AR stimulates cell cycle progression by stimulating the expression of certain cyclin-dependent kinases and decreasing the expression of cyclin-dependent kinase inhibitors (41); (c) AR may be responsible for downstream cell signaling by “cross-talk” with other putative soluble growth factors (19, 21, 48) and extracellular matrix-mediated signaling pathways (14–17, 22); and (d) AR may promote prostate cell terminal differentiation by directly enhancing cell death through DNA fragmentation (40) or indirectly by secretion of soluble factors that inhibit cell growth and promote cell differentiation (39) in a highly spatio-temporal regulated manner in cells of different lineages and genetic backgrounds (64, 65).

The objective of the present study was to define the possible roles of *hAR* in transactivating target genes, mediating androgen-induced growth and behavioral changes of ARCaP cells *in vitro*, and affecting the tumor growth *in vivo*. Our results clearly indicate that by introducing *hAR* into ARCaP cells, the following effects were observed. First, *hAR* transactivated target genes with stimulation observed for GRE4-TATA-Luc but not for p61PSA-Luc reporter (Fig. 2). Second, although *hAR* slowed down the growth of ARCaP cells under androgen stimulation, it restored the ability of ARCaP to respond positively to androgen-induced cell growth *in vitro* (Fig. 3). The primary action of AR appears to enhance the larger fraction of cell population to progress toward the S phase of the cell cycle (Fig. 4). In comparison with parental or control *neo*-transfected ARCaP cells, unliganded AR decreased the ability of cells to adhere and invade. Cell invasion and migration additionally decreased in the presence of an androgen agonist R1881 (Fig. 5). AR attenuated ARCaP tumor growth both *in vitro* (data not shown) and *in vivo* (Fig. 6). The ability of AR to selectively transactivate gene transcription and alter prostate cell growth, differentiation, and behavioral changes seems to be highly dependent on the genetic background of the cell (64, 65). As illustrated in Fig. 7, our laboratory demonstrated that during acquisition of androgen-independence by metastatic LNCaP sublines C4–2 and C4–2B no obvious additional structural or functional changes of AR were noted (49, 50, 64, 65). These cell behavioral changes could be influenced by the functional AR, whereby the addition of androgen decreased cell growth, enhanced androgen regulation of cell proliferation, and attenuated cell migration and invasion (Figs. 3, 5, 6, and 7). Thus, a normal prostate cell that can undergo genetic/epigenetic changes to become Pre-PCa, a transformed preneoplastic cell displaying a cancer phenotype. There are two potential pathways that could lead to PCa cells to acquire increased malignant potential: one pathway is represented by the LNCaP cell and its sublines (C4–2 and C4–2B; Ref. 64), where AI PCa progression can be achieved through



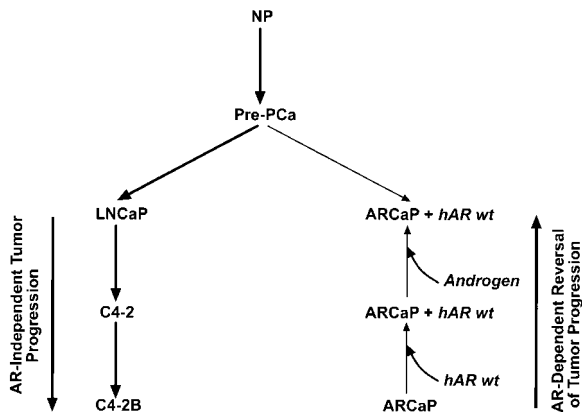


Fig. 7. A proposed model of the role of AR in the progression of PCa. In the LNCaP PCa progression model, no obvious structural or quantitative alterations of AR were detected despite the progression of tumor cells to acquire increased AI and metastatic potential (Refs. 49, 50, 64, 65). In the ARCaP model, the introduction of *hAR wt* exhibited decreased tumor growth, enhanced androgen responsiveness, and reduced the malignant potential of the cell line herein. NP, normal prostate cell; Pre-PCa, Pre-PCa cell.

no obvious additional structural, functional, or level of AR abnormalities. The other pathway is represented by the ARCaP model, where AI progression is inversely related to the level of AR expression in ARCaP cells, suggesting that AR is intimately involved in promoting differentiated function of PCa cells. Several previous reports also support this proposal. For example, p69, a large T-antigen transformed human prostatic epithelial cell line transduced stably with AR exhibited reduced cell growth and tumorigenicity in athymic mice (66). In PC-3 cells transduced with AR, androgen treatment activated the ARE-responsive promoter reporter gene; however, it also depressed cell growth and enhanced apoptosis (40). Eder *et al.* (67) recently observed that LNCaP cells transduced with an antisense AR cDNA showed reduced cell growth both *in vivo* and *in vitro*. These differences in biological responses of PCa cell lines to the introduction of the AR gene or the antagonism of AR function by antisense AR could be attributed to differences in the genetic background of the target cells. In this context, it is interesting to note that all of the androgen-unresponsive or repressed PCa cell lines lacking endogenous AR responded to the introduction of *hAR* by decreased basal cell proliferation *in vitro* and tumor growth *in vivo*, whereas in an androgen-responsive and AR-positive LNCaP cell line, removal rather than introduction of AR resulted in decreased tumor cell growth *in vitro*. If one considers modulation of AR activity a potential therapeutic target for the treatment of androgen-unresponsive and AR-negative human prostate tumors, it would be highly likely that introducing rather than removing AR in PCa cells could lead to decreased tumor cell growth and invasion, prolonged cell cycle progression, and increased tumor cell apoptosis.

On the basis of the proposed cell model, it could be predicted that despite the introduction of functional AR into PCa cells, there is a high likelihood that cells containing *hAR* will not express and secrete PSA. It is possible that failure to transactivate PSA promoter in many human PCa cell lines could be attributable to the presence of suboptimal levels of critical AR-interactive and cell-specific AR-coactivators or corepressors, which bind AR and modulate AR function in target cells. A number of candidate transcription factors for PSA promoter were recently described, such as ESE-1 (68), ESE-2 (69), ESE-3 (70), PDEF (71), and p45 (62), which regulate PSA gene expression by binding to its cognate consensus DNA sequences suggesting that other factors along with the AR were also involved in the regulation of PSA gene expression.

In conclusion, we used a highly invasive and metastatic human PCa

cell line, ARCaP, to evaluate *hAR* expression and function in ARCaP cell background. The *hAR* expression in ARCaP cells restored androgen responsiveness; increased ligand-dependent target gene transactivation; decreased cell attachment, invasion, and migration *in vitro*; and decreased tumor growth *in vivo* in athymic mice. These results taken together suggest that the introduction of AR into poorly differentiated, highly invasive AR-negative or low AR-containing PCa cells may enhance in an improvement of overall status of tumor differentiation. Finally, we propose that the induction of PCa differentiation can be achieved either through introducing AR into AR-negative (1) or low AR-expressing PCa cell line herein, or blocking AR function in AR-expressing prostate tumor cells (66).

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