Androgen Receptor Remains Critical for Cell-Cycle Progression in Androgen-Independent CWR22 Prostate Cancer Cells

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The majority of prostate cancers (PCa) that relapse after androgen deprivation therapy (androgen-independent PCa) continue to express androgen receptor (AR). To study the functional importance of AR in these tumors, we derived androgen-independent CWR22 PCa xenografts in castrated mice and generated a cell line from one of these xenografts (CWR22R3). Similarly to androgen-independent PCa in patients, the relapsed xenografts and cell line expressed AR and were resistant to treatment with bicalutamide. However, expression of the AR-regulated PSA gene in the CWR22R3 cell line was markedly decreased compared to the relapsed xenografts in vivo. Transfections with androgen-regulated reporter genes further indicated that the cells lacked androgen-independent AR transcriptional activity and were not hypersensitive to low androgen concentrations despite constitutive activation of the Erk/MAP kinases. Nonetheless, AR remained essential for androgen-independent growth because retroviral shRNA-mediated AR down-regulation resulted in marked long-term growth suppression. This was associated with increased levels of p27kip1 and hypophosphorylation of retinoblastoma protein but not with decreases in D-type cyclin levels or MAP kinase activation. These results reveal a potentially critical function of AR in androgen-independent PCa that is distinct from its previously described transcriptional or nontranscriptional functions. (Am J Pathol 2006, 169:682–696; DOI: 10.2353/ajpath.2006.051047)

Prostate cancer (PCa) most frequently presents as an androgen-dependent disease, and suppression of androgen receptor (AR) activity by surgical or medical castration (androgen-deprivation therapy) results in tumor regression and alleviation of cancer-related symptoms in most patients. However, patients with initially favorable responses to androgen-deprivation therapy eventually relapse with tumors that are termed hormone refractory or androgen-independent PCa. Importantly, the high-level expression of the AR and renewed expression of androgen-regulated genes such as prostate-specific antigen (PSA) suggest that the AR regains its activity and plays an important role at this stage of the disease. Some patients who relapse after androgen-deprivation therapy may still respond to secondary hormonal treatments with AR antagonists such as bicalutamide or with agents such as ketoconazole that suppress residual androgen production from the adrenal glands, suggesting that the tumors are responding to the low levels of androgen that remain after surgical or medical castration. Indeed, studies using androgen-dependent PCa cells have shown that long-term growth under reduced androgen conditions can select for cells in which the AR can be activated by very low levels of androgen, and down-regulation of AR expression in these cells can suppress their growth.

Mechanisms that may mediate PCa adaptation to decreased androgen levels include AR amplification, AR mutations, increased expression of transcriptional co-activator proteins, and activation of other signal transduction pathways. Previous studies showed that AR mRNA was highly expressed, the AR gene was frequently amplified in androgen-independent PCa, and increased expression of AR was a con-
sistent finding in multiple xenograft models of androgen-independent PCa. The overall incidence of AR mutations in androgen-independent PCa is low, but the mutations that do occur enhance AR responsiveness to nonandrogen steroid hormones and to AR antagonists such as flutamide and bicalutamide.\textsuperscript{12,14–17} Moreover, AR mutations that convert flutamide from an AR antagonist to an agonist occur primarily in patients treated with combined flutamide and androgen-deprivation therapy, indicating a selective pressure to maintain AR transcriptional activity in androgen-independent PCa.\textsuperscript{16} Increased expression of AR transcriptional co-activator proteins, such as steroid receptor co-activator (SRC)-1 and -2, can enhance AR activity and has been reported in androgen-independent PCa.\textsuperscript{18} Alternatively, activation of extracellular signal-regulated kinase/mitogen-activated protein kinase (Erk/MAP kinases) and the phosphatidylinositol 3-kinase (PI3 kinase) signal transduction pathway (the latter through the loss of the phosphatase and tensin homologue deleted on chromosome 10, PTEN) occur frequently in advanced androgen-independent PCa and may enhance the activity of AR co-activator proteins.\textsuperscript{19–21}

Although the current data indicate that multiple mechanisms may enhance AR transcriptional activity at low androgen levels in androgen-independent PCa, the responses to secondary hormonal therapies designed to further suppress AR activity are generally partial and most tumors progress despite postcastration androgen levels and treatment with even high doses of AR antagonists such as bicalutamide.\textsuperscript{4,22} These progressive androgen-independent tumors continue to express AR and AR-regulated genes such as PSA. It is not clear whether AR still plays a critical role in this stage of disease and thus remains a valid target for more effective therapy. To assess the role of AR in bicalutamide-resistant, progressive androgen-independent PCa, we examined the CWR22 PCa xenograft model.\textsuperscript{23,24} Androgen-dependent CWR22 xenografts responded initially to castration and then relapsed with androgen-independent tumors that were resistant to high-dose bicalutamide treatment and maintained AR and PSA expression at levels comparable to the androgen-dependent CWR22 xenografts. A cell line (CWR22R3) from a relapsed and bicalutamide-resistant xenograft was established and propagated long term in steroid hormone-depleted medium. Similarly to its parental androgen-independent xenograft in vivo, the cell line continued to express AR and was resistant to bicalutamide. However, in the absence of added ligand, the expression of the AR-regulated PSA gene was markedly lower than the xenografts. Transient transfections with androgen-responsive element (ARE)-regulated reporter genes further indicated that the AR in the CWR22R3 cell line lacked androgen-independent transcriptional activity and was not hypersensitive to androgen at low levels. Nonetheless, AR down-regulation resulted in a marked and was not hypersensitive to androgen at low levels. The CWR22 PCa xenograft model is very generously provided by Dr. Tom Pretlow (Case Western Reserve University, Cleveland, OH).\textsuperscript{25,26} The cells from CWR22 xenografts (~10⁶) in 50% Matrigel (Becton Dickinson, Bedford, MA) were implanted into the flanks of male Ncr nude mice (Taconic, Germantown, NY), which were supplemented with 12.5 mg of sustained-releasing testosterone pellets (Innovative Research of America, Sarasota, FL). When the tumors were established and reached the size of 1 cm in largest dimension, the host mice were castrated and the testosterone pellets removed. Tumors that relapsed in castrated mice were subsequently treated with 1 mg of bicalutamide (Astra-Zeneca, Wilmington, DE) in dimethyl sulfoxide as a 0.1-ml intraperitoneal injection three times per week. Mice were bled periodically through the retro-orbital sinus to assess serum PSA. Tumor excision biopsies obtained before castration, at the initiation of bicalutamide treatment, or at the time of sacrificing, were either frozen at −80°C or fixed in 10% buffered formalin.

At the time of sacrificing, tumors were aseptically excised under anesthesia and minced into pieces of ~1 mm³ in phosphate-buffered saline (PBS). The tissues were then spun down and treated with trypsin-ethylene diamine tetraacetic acid (EDTA) (0.05% trypsin/0.53 mmol/L EDTA; Life Technologies, Inc., Rockville, MD) at 37°C for 10 minutes with frequent agitation. The cell suspensions were again centrifuged and plated on 10-cm tissue culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) or 10 to 20% charcoal/dextran-striped FBS (steroid hormone-depleted) (CD3-FBS; Hyclone, Logan, UT) and antibiotics. The tumor cells derived from the initial xenografts grew more rapidly in the CDS medium; thus all androgen-independent PCa that is independent of its conventional transcriptional activity and distinct from previously reported nontranscriptional functions.

Materials and Methods

Plasmids and Antibodies

Expression vectors for AR, SRC-1, SRC-2, SRC-3, ARE\textsubscript{2} luciferase reporter, PSA-luciferase reporter, and pGL3-promoter and Renilla luciferase control vector (pGL3, pRL-CMV; Promega, Madison, WI) have been described previously.\textsuperscript{25,26} Antibodies were from the following sources: Ber-EP4 (DAKO, Carpinteria, CA), anti-PSA (Biodesign, Saco, ME), anti-tubulin (Sigma, St. Louis, MO), and anti-AR (Upstate Biotechnology, Lake Placid, NY, and Santa Cruz Biotechnology, Santa Cruz, CA). Additional antibodies were from Cell Signaling Technology (Beverly, MA) including, anti-phospho-Erk1 and 2 (T202/Y204), anti-phospho-Akt (S473), anti-Erk1 and 2, anti-Akt, anti-phospho-pRb (S780), anti-p27\textsuperscript{kip1}, and anti-PARP. Secondary anti-mouse and anti-rabbit antibodies were from Promega.

CWR22 Xenografts and Cell Lines

CWR22 xenografts were very generously provided by Dr. Tom Pretlow (Case Western Reserve University, Cleveland, OH).\textsuperscript{23,24} The cells from CWR22 xenografts (~10⁶) in 50% Matrigel (Becton Dickinson, Bedford, MA) were implanted into the flanks of male Ncr nude mice (Taconic, Germantown, NY), which were supplemented with 12.5 mg of sustained-releasing testosterone pellets (Innovative Research of America, Sarasota, FL). When the tumors were established and reached the size of 1 cm in largest dimension, the host mice were castrated and the testosterone pellets removed. Tumors that relapsed in castrated mice were subsequently treated with 1 mg of bicalutamide (Astra-Zeneca, Wilmington, DE) in dimethyl sulfoxide as a 0.1-ml intraperitoneal injection three times per week. Mice were bled periodically through the retro-orbital sinus to assess serum PSA. Tumor excision biopsies obtained before castration, at the initiation of bicalutamide treatment, or at the time of sacrificing, were either frozen at −80°C or fixed in 10% buffered formalin.

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of the subsequent cultures were performed with medium containing 10 to 20% CDS-FBS. The cell were trypsinized and passaged approximately every 5 to 10 days.

Differential trypsinization was performed to separate epithelial tumor cells from stromal cells. Cells were treated with trypsin/EDTA for 1 to 5 minutes and followed microscopically for the detachment of epithelial cells, which were collected and replated. Alternatively, completely trypsinized cells were placed into medium, and stromal cells were depleted by allowing them to adhere to tissue culture plates for 10 to 60 minutes, at which time the less adherent epithelial tumor cells were recovered and plated in a separate dish. Additional enrichment of epithelial cells was performed with immunomagnetic beads. Cells were trypsinized and incubated with magnetic protein A beads conjugated with a monoclonal antibody against human epithelial antigen (Ber-EP4) (Dynabeads Protein A; Dynal, Oslo, Norway). After three rounds of washing, the cells attached to the magnetic beads were replated in Dulbecco’s modified Eagle’s medium with 20% CDS-FBS medium. After several passages the cultures were weaned to medium with 10% CDS-FBS.

**Cell Growth and PSA Measurements**

For cell growth measurement, cells were plated in 96-well culture dishes and treated as indicated. The viable cells at the end of the treatment were measured either using the CellTiter 96 AQueous One Solution cell proliferation assay (Promega), as suggested by the manufacturer; or the cells were directly counted using a hemocytometer or a Coulter Z1 counter (Coulter, Miami, FL). PSA in serum from xenografted animals was measured by enzyme-linked immunosorbent assay using a clinical kit (Hybridtech, San Diego, CA).

**Immunoblotting**

Tumor xenografts were excised and minced in PBS into 1-mm³ pieces, homogenized with a glass Dounce homogenizer, and sonicated in RIPA lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/L EDTA, and 1 mmol/L EGTA) containing protease and phosphatase inhibitors. Cells were directly lysed with RIPA buffer containing protease and phosphatase inhibitors. Protein quantity was determined by Brad- ford assay (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and then transferred to 0.45-μm nitrocellulose membranes by electroblotting. The membranes were blocked with 5% nonfat powdered milk in PBS and then probed with primary antibodies at a 1:1000 dilution in Tris-buffered saline containing 0.2% Tween 20 (TBST) with 5% milk. The membranes were then washed extensively with TBST and probed with horseradish peroxidase-conjugated secondary antibodies at 1:2000 dilutions in TBST with 5% milk. After further washing in TBST, the membranes were developed with the ECL Western blotting detection system (Pierce Biotech, Rockford, IL). The Imagej program (Wayne Rasband, National Institutes of Health, Bethesda, MD) was used to quantify the interested protein band density on Western blots according to the author’s instruction.

**Immunohistochemistry**

Five-μm sections from paraffin-embedded tissue blocks were deparaffinized, rehydrated, and subjected to antigen retrieval by autoclaving for 20 minutes in 50 mmol/L citrate buffer (pH 6.0). After cooling to room temperature, the tissue sections were blocked using 10% goat serum and avidin blocking solution (Vector Laboratories, Burlingame, CA). Primary antibodies were then added and incubated overnight at 4°C. The anti-phospho-Erk antibody was used at 1:100 whereas the anti-AR antibody was used at 1:50. After four washes in PBS, the antibodies were detected using biotinylated goat anti-rabbit antibody at 1:400 followed by streptavidin-horseradish peroxidase at 1:400 (Vector Laboratories). Slides were developed with 3,3’-diaminobenzidine and counterstained with hematoxylin.

**Immunofluorescence**

The cells were grown on a cover slide and fixed with 5% paraformaldehyde at room temperature for 10 minutes. The cells were washed in PBS and permeabilized in PBS with 1% Triton X-100 (PBST). The cells were then incubated with an anti-AR antibody (1:30 in PBST) at room temperature for 45 minutes. The cells were subsequently washed three times in PBST and incubated with an Alexa Fluor 488-conjugated anti-rabbit antibody (1:400 in PBST; Invitrogen, Carlsbad, CA) for an additional 45 minutes at room temperature. The cells were then washed and cover slides mounted and examined with a Nikon fluorescent microscope (Nikon Corporation, Tokyo, Japan).

**Fluorescence-Activated Cell Sorting**

CWR22R3 or LNCaP cells were trypsinized and fixed and permeabilized with BD Cytofix/Cytoperm Plus/Fixation/Permeabilization kit according to the manufacturer’s suggestion. The cells were incubated with anti-Ber-EP4 or anti-AR antibodies at 1:50 on ice for 30 minutes. After washing, the cells were labeled with fluorescein isothiocyanate-anti-mouse antibody (1:100) for 30 minutes on ice. After washing, the cells were analyzed with flow cytometry.

**Transfection**

One day before transfection, cells were plated into a 24- or 48-well plate at a density of 70 to 80%. The cells were transfected with mixtures of DNA and/or siRNA and Lipofectamine 2000 (Invitrogen) for 24 hours, according to the manufacturer’s recommendations. Cells were then...
switched to fresh medium containing various treatment reagents for another 24 hours, and then lysed with passive lysis buffer and analyzed for luciferase activity using the dual-luciferase measurement system (Promega). The anti-AR siRNA sequence was AR siRNA (5’-AAAGGUUCUCUGCUAGACGAC-3’). Its control Mut siRNA (5’- AAAGUGULCCUCUGUACGAC-3’) differed from AR siRNA in position switches among three neighboring nucleotide pairs (bold letters). The siRNA sequences were compared to sequences in GenBank and revealed to have no greater than 76% homology to any genes other than human AR.

**Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

Total RNA was isolated from CWR22 xenografts or cultured cells using the RNeasy protect mini kit (Qiagen, Valencia, CA). The amount of total RNA from each sample was determined by spectrophotometer, and 100 ng of total RNA from each sample were used to determine the specific RNA level by TaqMan real-time RT-PCR using ABI Prism 7000 (Applied Biosystems, ABI, Foster City, CA). The primer sequences for human PSA are: 5’-TCTCCATGACGTACAGGCC-3’ (forward), 5’-GGAGGGAAGTACACTGTCG-3’ (reverse) and 5’-TTCGATCCAGGTTGATCTGAAATTGAC-3’ (probe). The manufacturer quality-controlled and -validated 18S human rRNA and OAS1 primers and probes were purchased from ABI. The amounts of the target mRNA in each sample were normalized to its 18S rRNA and presented as the relative fold difference of a reference sample (relative quantification method recommended by ABI).

**Retrovirus-Mediated shRNA**

The following oligomers were used: 5’-GATCCCCAGGGTCTCTGTGACACGACTCAAGAGTCTAGTGCTAGAGAACAGCTTTTTT-3’ (pSuper-1981 sense), 5’-AGCTAAGAAGGTTTCTCTGAGAC-GACTCTCTGAAAGTCGTCTAGCAAGAACACTGTTGG-3’ (pSuper-2001 anti-sense), 5’-GATCCCCGCAAATTACATTAGGGGCTTCAAGAGAGCCCCCTAAGAATTGTCCTGCGG-3’ (pSuper-1981 anti-sense), 5’-GATCCCCAGGTGCTCCTGTCGAGCAGTGTCCTTGTTAGG-3’ (pSuper-1981 sense), 5’-AGCTAAGAAGGTTTCTCTGAGAC-GACTCTCTGAAAGTCGTCTAGCAAGAACACTGTTGG-3’ (pSuper-2001 sense), 5’-AGCTAAAAAGGTTTCTCTGAGAC-GACTCTCTGAAAGTCGTCTAGCAAGAACACTGTTGG-3’ (pSuper-1981 mut sense), 5’-AGCTAAAAAGGTTTCTCTGAGAC-GACTCTCTGAAAGTCGTCTAGCAAGAACACTGTTGG-3’ (pSuper-1981mut sense). The corresponding pairs of primers (pSuper-1981, or pSuper-1981mut sense) were annealed and ligated into BglII-HindIII linearized pSuper-retro puro vector (Oligogeneing, Seattle, WA). The correct inserts were verified by DNA sequencing. To generate retroviruses, the pSuper vectors were co-transfected with PCG-gagpol and PCG-VSV-G into amphotropic Phoenix cells (Orbigen, San Diego, CA), and the media were collected during days 2 to 3 after transfection. CWR22R3 cells (2 × 10⁶) were plated into a 10-cm² culture dish the day before infection, and were then infected with the retrovirus by incubating with the Phoenix cell culture medium mixed with 4 µg/ml of polybrene (Calbiochem, La Jolla, CA). During days 3 to 5 after infection, the infected cells were selected with 2 µg/ml puromycin, and the surviving infected cells were further studied.

**Cell-Cycle Analysis**

At 7 days after retroviral infection, cells were rinsed in PBS, trypsinized, washed three times with PBS, and fixed with 95% ethanol at 4°C for 15 minutes. The cells were then treated with propidium iodide and RNase A at room temperature for 30 minutes and subsequently analyzed by flow cytometry. The results were analyzed using CellQuest-Pro software.

**Results**

**Androgen-Independent and Bicalutamide-Resistant CWR22 Xenografts**

To develop progressive androgen-independent PCA models, we started with CWR22, which is a human androgen-dependent PCA xenograft that undergoes regression after castration and can relapse after a relatively long interval of many months as androgen-independent tumors that continue to express AR and AR-regulated genes such as PSA. Androgen-independent CWR22 PCA xenografts were established subcutaneously in the flanks of male NCr nude mice supplemented with sustained-release testosterone pellets. When tumors reached ~1 cm in diameter, the testosterone pellets were removed and the mice were castrated. Consistent with previous results, the CWR22 xenografts regressed in response to castration, and two of the four mice followed long term relapsed ~7 months after castration with increasing tumor size and serum PSA levels.

To determine whether the relapsed CWR22 xenografts (CWR22R3 and CWR22R4) would respond to further direct AR blockade, the tumor-bearing mice were treated with high doses of the AR antagonist bicalutamide, which is effective against the H874Y mutant AR expressed by CWR22. The bicalutamide dose used (1 mg intraperitoneally three times per week) was ~50-fold greater than the dose used in patients (50 mg/day orally) and was confirmed to be effective during initial pilot experiments based on rapid involution of seminal vesicles in mice with intact testes (data not shown). There was no regression in either mouse throughout a 4-week treatment period based on tumor size or tumor-derived serum PSA, indicating that the relapsed tumors were intrinsically resistant to bicalutamide.

Immunoblotting of protein extracts from the relapsed and bicalutamide-treated CWR22 tumors demonstrated that they expressed AR and PSA, with AR levels being somewhat reduced relative to the parental androgen-
dependent CWR22 xenografts (Figure 1, A and B). DNA sequencing of the entire AR mRNA coding region from the relapsed tumors showed the codon 874 mutation identical to that in the parental CWR22 xenograft30 but did not reveal any additional mutations (data not shown). Overall, these results demonstrated that the relapsed CWR22 tumors, with respect to expression of AR and PSA and resistance to AR antagonists, were similar to androgen-independent PCa that occur in patients after initial responses to androgen deprivation therapies.
Establishment of CWR22R3 Cell Line

To further assess AR function in the relapsed and bicalutamide-resistant tumors, we established cell lines from the two relapsed and bicalutamide-resistant CWR22R3 xenografts. The cells were cultured in steroid hormone-depleted medium (Dulbecco’s modified Eagle’s medium with 10% charcoal-dextran-stripped FBS, CDS medium). During the initial passages, there was an outgrowth of both tumor and stromal cells. Differential trypsinization, as well as immunomagnetic beads conjugated with a monoclonal antibody against an epithelial antigen (Ber-EP4), were used during some passages to positively select for tumor cells. The tumor cells initially grew clustered in small aggregates surrounded by stromal cells and continued to grow in clusters even after the depletion of stromal cells. One of the cell lines, CWR22R3, was established as a long-term (>2 years) line free of stromal cell contamination because flow-cytometry analysis demonstrated a similar profile to LNCaP cells in their expression of Ber-EP4 epithelial marker and AR (Figure 1C). This line was the focus of further detailed studies.

After more than 2 years of culture in CDS medium, the CWR22R3 cell line continued to express AR at high levels comparable to the AR levels in the LNCaP PCa cell line (Figure 1D). AR DNA sequencing again revealed the codon 874 mutation and no other mutations in the CWR22R3 cell line (data not shown). The amount of AR in both cell lines was increased after dihydrotestosterone (DHT; 10 nmol/L) treatment for 24 hours, consistent with the stabilization of AR protein by ligand.7 The DHT-mediated increase in AR protein level was also demonstrated in CWR22R3 cells by immunofluorescence, with AR being predominantly nuclear in either the absence or presence of DHT (Figure 1E). No PSA protein was detected in CWR22R3 cells, even after DHT stimulation, whereas PSA protein was readily detected in LNCaP cells and was markedly increased by DHT (Figure 1D). Assessment of PSA mRNA levels by quantitative real-time RT-PCR confirmed that expression was markedly decreased in the CWR22R3 cell line relative to the in vivo xenografts (Figure 1F).

PSA mRNA expression in the CWR22R3 cells could be stimulated by DHT, but the level of stimulation (40% increase) was small relative to the DHT-stimulated increase in the LNCaP cell line. Consistent with the lack of response to bicalutamide in the in vivo xenografts, growth of the CWR22R3 cell line was not repressed by bicalutamide, whereas growth could be modestly stimulated by DHT (Figure 1G).

Activation of Erk/MAP Kinase and PI3 Kinase in CWR22R3 Cells

The progression of PCs in patients to more aggressive, androgen-independent disease has been associated with activation of the Erk1 and Erk2 MAP kinases as well as with PTEN loss and the subsequent activation of the PI3 kinase/Akt pathway.31,32 Therefore, the CWR22R3 cells were next assessed for activation of these kinases. Significantly, CWR22R3 cells grown in medium with 10% FBS or steroid hormone-depleted CDS medium had high levels of activated Erk1 and 2, as assessed by immunoblotting with a phospho-specific Erk1/2 antibody (Figure 2A). This activation was not diminished by serum starvation for 24 hours and was only modestly enhanced by serum stimulation of the starved cells (Figure 2A). As shown previously, Erk phosphorylation was not detectable in LNCaP cells grown in FBS or CDS medium. In contrast to Erk activation, Akt was phosphorylated at very low levels in the CWR22R3 cells in comparison to the PTEN-deficient LNCaP cells (Figure 2B). Consistent with this result, PTEN expression was readily detectable by immunoblotting in the CWR22R3 cells, indicating that progression to androgen independence was not associated with PTEN loss or PI3 kinase activation in these cells (data not shown).

Immunohistochemistry was next used to determine whether MAP kinase activation occurred in vivo during the progression of the CWR22 xenograft to androgen independence. Anti-phospho-Erk1/2 immunostaining of biopsies from the CWR22 xenograft, which ultimately gave rise to the CWR22R3 cell line, immediately before castration showed only rare weak staining of scattered tumor cells and occasional positive staining of endothelial cells (less than 2% of tumor cells) (Figure 3A). In contrast, there were increased numbers of cells with strong nuclear phospho-Erk1/2 staining in the relapsed and bicalutamide-resistant CWR22R3 xenografts (Figure 3, B and C, respectively). The staining pattern is patchy and within...
positively stained area, increased percentage of tumor cells is positive for p-Erk (20 to 30% in postcastration xenograft and 50 to 60% in postcastration and bicalutamide-resistant xenograft). The arrangement of positive cells varied from scattered to large aggregates. AR expression in above described xenografts by immunostaining demonstrated a more even distributed pattern and the vast majority of cells (80 to 90%) in all tumors were AR-positive (Figure 3, D–F). In summary, these results indicated that Erk1/2 activation in the CWR22R3 xeno-

Figure 2. Erk activation in the CWR22R3 cell line. CWR22R3 or LNCaP cells were grown in FBS (FBS+) or CDS medium (CDS+) for 24 hours. Additional CWR22R3 cells were grown in serum-free medium (FBS−, CDS−, stim−) for 24 hours, with or without subsequent serum stimulation with CDS medium for 20 minutes (FBS−, CDS−, stim+, or FBS−, CDS−, stim+). The cell lysates were blotted with anti-phospho-Erk (p-p44 Erk1/p-p42 Erk2) and anti-total Erk (total Erk) antibodies (A, top), or anti-phospho-Akt (p-Akt) and anti-total Akt (total Akt) antibodies (B, top). The bottom panels showed the quantification of Erk phosphorylation (A, p-p42/p42) or Akt phosphorylation (B, p-Akt/Akt) by ImageJ analysis of the respective top panels.

Figure 3. Erk activation during the relapse of CWR22R3 xenograft. Immunohistochemical staining with phospho-Erk-specific antibodies (A–C) or anti-AR antibodies (D–E) on CWR22R3 xenograft biopsy samples prepared before castration (A, D), after castration but before the initiation of bicalutamide treatment (B, E), or at the end of bicalutamide treatment (C, F). Original magnifications, ×200.
graft occurred in vivo during the progression to androgen independence, and Erk1/2 activation was maintained in the CWR22R3 cell line.

CWR22R3 Cells Are Not Hypersensitive to Low Levels of Androgen

Previous studies have shown that long-term culture of PCa cells in androgen-depleted medium can select for cells that are hypersensitive to androgen, being stimulated by low concentrations (picomolar) of DHT and repressed by nanomolar concentrations that stimulate their parental cells.6,7 As the relapsed CWR22 xenografts were similarly adapted to low castrated androgen levels and the cell line was grown long term in androgen-depleted medium, the CWR22R3 cells were further studied for their response to low androgen concentrations. Cells cultured in CDS medium were supplemented with DHT, and cell growth was assessed by MTS assays on days 2 and 4. As shown in Figure 4A, cell growth was not stimulated by picomolar concentrations of DHT and was only stimulated by 1 to 10 nmol/L DHT. Comparable results were obtained by direct cell counting (Figure 4B). Examination of PSA mRNA levels in CWR22R3 cells by quantitative real-time RT-PCR, throughout a range of DHT concentrations from $10^{-14}$ to $10^{-18}$ mol/L, similarly showed that PSA expression was not hypersensitive to low levels of DHT and was stimulated by DHT at nanomolar range (Figure 4C). This DHT dose response is similar to that in LNCaP cells, an androgen-dependent PCa cell line, although the magnitude of the response is much greater in LNCaP (Figure 4D). Taken together, these data indicate that despite long-term propagation in androgen-depleted medium, CWR22R3 cells remain modestly responsive to androgen, but are not hypersensitive to low androgen levels.

Lack of Androgen-Independent AR Transcriptional Activity in CWR22R3 Cells

The low level of PSA expression in the CWR22R3 cells cultured in hormone-depleted medium and the lack of stimulation by low concentrations of DHT suggested that AR was not transcriptionally active in the absence of added androgen. However, it was also possible that the AR remained transcriptionally active and that the lack of PSA expression was because of endogenous PSA gene methylation or other mechanisms unrelated to AR activity. Therefore, transfection studies with AR-regulated reporter genes were next performed to more directly assess ligand-dependent or -independent AR transcriptional activity. CWR22R3 cells were transfected with a luciferase reporter regulated by four tandem consensus androgen-responsive elements (ARE4-luciferase), and the luciferase activity was determined in the absence or presence of DHT. There was a low level of reporter activity in the absence of androgen (comparable to the levels with the parental pGL3-promoter reporter, lacking the AREs), which was stimulated approximately fivefold by DHT (Figure 5A). Significantly, bicalutamide suppressed the DHT-stimulated AR transcriptional activity, confirming that the drug still functioned as an AR antagonist in these cells. However, bicalutamide had no effect on the basal androgen-independent luciferase activity.

RNA interference was next used to further address whether basal androgen-independent and bicalutamide-resistant reporter activity was mediated by the AR. Cotransfection of CWR22R3 cells with the ARE4-luciferase reporter and siRNA directed against AR, but not a mutant control, caused a marked decrease in DHT-stimulated transcriptional activity (Figure 5B). In contrast, the androgen-independent activity was not specifically suppressed by the
This experiment was also performed using a luciferase reporter controlled by the PSA gene regulatory region. The PSA-luciferase reporter was stimulated approximately sixfold by DHT, and this DHT-stimulated activity could be completely suppressed by the AR siRNA, but not the mutant siRNA (Figure 5C). However, as observed with the ARE4-luciferase reporter, the basal androgen-independent luciferase activity was not specifically suppressed by the AR siRNA. These results indicated that AR lacked transcriptional activity in CDS medium without added androgen.

**Androgen-Independent or Hypersensitive AR Transcriptional Activity Is Not Restored by p160 Steroid Receptor Co-Activators**

Previous studies have shown that increased expression of p160 steroid receptor co-activator proteins (SRC-1, -2, and -3) can enhance AR responses to low levels of androgen and may enhance AR activity in androgen-independent PCa.

Moreover, Erk MAP kinase activation can further enhance the interactions between steroid hormone receptors and p160 co-activator proteins, which in the case of AR may be mediated by phosphorylation of SRC-2 (TIF-2). Therefore, we assessed whether increased expression of SRC-1, -2, or -3 in CWR22R3 cells, which maintain a high level of constitutive Erk MAP kinase activation, might amplify any weak androgen-independent AR transcriptional activity that was not detectable using reporter genes alone. As shown in Figure 6A, co-transfection of SRC proteins (in particular SRC-3) could enhance DHT-dependent reporter gene activity. However, these co-activators did not stimulate androgen-independent AR transcriptional activity.

We next addressed whether increased co-activator expression could enhance AR responses to low levels of androgen stimulation. CWR22R3 cells co-transfected with SRC-2 and an ARE4-luciferase reporter were assessed for luciferase activity throughout a broad range of DHT concentrations. Consistent with the above data, SRC-2 began to enhance AR activity at 10 nmol/L DHT (Figure 6B). However, SRC-2 did not render the AR hypersensitive to lower levels of DHT. Co-transfections with SRC-3 or SRC-1 similarly failed to enhance AR responses to low levels of DHT (Figure 6C, and data not shown). Together, these results further supported the conclusion that AR in the CWR22R3 cells lacked androgen-independent transcriptional activity on ARE-regulated genes.

**AR Down-Regulation Causes a Growth Retardation and G0/G1 Cell-Cycle Arrest of CWR22R3 Cells**

CWR22R3 cells maintain a high level of AR expression during long-term growth under androgen-depleted conditions, despite lacking detectable AR transcriptional activity. This suggested that AR might continue to play a critical role in these cells. The importance of AR in CWR22R3 cells was further examined by stable AR down-regulation through retrovirus-mediated expression of AR-specific short hairpin RNA (shRNA). CWR22R3 cells were infected with retroviruses encoding AR-specific shRNA (pSuper-1981 or pSuper-1774) or control shRNA (pSuper-1981mut), and infected cells were selected briefly with puromycin. AR down-regulation was demonstrated by anti-AR immunoblotting of the infected cells, which showed that AR expression in cells infected with pSuper-1981 or pSuper-1774 was reduced to 29% and 37%, respectively, of the level in the control infected cells (Figure 7A).

AR down-regulation allowed us to further address whether the AR in the CWR22R3 cells had any androgen-

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**Figure 5.** AR transcriptional activity on AR-responsive reporters. CWR22R3 cells grown in CDS medium were transfected with ARE-less pGL3 promoter (pGL3) or with AR-responsive element-driven firefly luciferase reporter, ARE4-Luc or PSA-Luc, as well as a pRL-CMV Renilla luciferase reporter. Twenty-four hours after transfection, the culture media were replaced with CDS medium with or without 10 nmol/L DHT for an additional 24 hours. The cells were lysed at the end of the treatment and AR transcriptional activity is presented as relative light units (firefly luciferase over Renilla luciferase activity, RLU). **A** The posttransfected cells were co-treated with various concentrations of bicalutamide (Bical) during the last 24 hours. **B** and **C** ARE4-Luc (B) and PSA-Luc (C) were co-transfected with AR-specific (AR siRNA) or control (mut siRNA) siRNAs and cultured with or without DHT during the last 24 hours.
independent transcriptional activity on the endogenous PSA gene. RNA was isolated from the cells infected with pSuper-1981 and control pSuper-1981mut at 7 to 8 days after infection and was assessed for PSA expression by real-time RT-PCR. Significantly, there was no decrease in PSA expression in the AR down-regulated cells, further indicating that basal PSA expression was not mediated by androgen-independent AR transcriptional activity (Figure 7B). Indeed, PSA expression in the AR-silenced cells was higher than in the control-treated cells in two independent infections. The increase was modest in absolute terms but appears more substantial as fold of change due to the low basal level (Figure 1F). In any case, this increase could reflect decreased co-repressor recruitment (NCoR or SMRT) by the unliganded AR, or an indirect effect on other transcriptional factors mediating the low basal PSA expression.

Strikingly, the growth of the AR shRNA-expressing CWR22R3 cells was markedly decreased relative to the mutant control infected cells (Figure 7, C and D). Morphologically, the infected cells expressing AR shRNA showed reduced cell density and an increase in dendritic projections, without an increase in cellular debris or apoptotic bodies (Figure 7D). To assess the long-term effects of AR down-regulation on cell growth in CDS medium, equal numbers of puromycin-selected infected cells were plated and followed for growth. The growth suppression lasted more than 5 weeks (Figure 7E), and the cells that continued to grow after pSuper-1981 infection had residual AR expression as demonstrated by AR immunofluorescence staining (data not shown). Similar results have been obtained in three separate infections. This growth retarding effect appears to be directly related to AR down-regulation because it was not seen with the control shRNA virus-infected cells. To exclude the potential effects of nonspecific interferon induction by shRNA, we assessed induction of 2′,5′-oligo(a-deoxyadenylate synthetase (OSA1), a classical interferon target gene. Previous studies have shown that an interferon response induces >50-fold induction of OSA1, while most siRNAs induce changes that are insignificant (less than three-fold).33,34 As shown in Figure 7F, none of the virus-mediated AR shRNA induced significant changes in OSA1 mRNA levels compared with the control shRNA, indicating that the effect of AR shRNA was not mediated by interferon.

To examine further whether the growth retardation in the pSuper-1981-infected cells was because of an increase in apoptosis, the extent of PARP cleavage was determined. As shown in Figure 7G, there was no detectable PARP cleavage in the cells infected with pSuper-1981 or control pSuper-1981mut. As a positive control, PARP cleavage products were readily detected in LNCaP cells treated with the PI3 kinase inhibitor LY294002. Comparison of cell apoptosis in pSuper-1981- versus control-infected cells by terminal dUTP nick-end labeling assays also did not reveal any significant differences (data not shown). Thus, the reduced cell growth after AR down-regulation was not because of increased apoptosis.

We next examined DNA content to determine whether AR down-regulation affected a particular stage in the cell cycle. The fraction of cells in S and G2/M phases was markedly decreased in pSuper-198-infected cells, with a corresponding increase of cells in G0/G1 phase (Figure 8A). This cell-cycle analysis also confirmed the absence of sub-G0/G1 apoptotic cells. Consistent with the cells being arrested in G0/G1, there was an increase in the expression of p27kip1 and a marked decrease in pRb phosphorylation in the pSuper-198-infected cells versus the pSuper-1981mut control-infected cells (Figure 8, B and C, respectively).

Previous studies in androgen-dependent LNCaP cells, CWR22 xenografts, and in rodent prostate have shown that androgen withdrawal can similarly cause a G0/G1 cell-cycle arrest, with increased p27kip1 expression and recruitment (NCoR or SMRT) by the unliganded AR, or an indirect effect on other transcriptional factors mediating the low basal PSA expression.
Figure 7. Growth retardation of CWR22R3 cells because of viral-mediated AR down-regulation. A: AR down-regulation by shRNA. CWR22R3 cells were infected with AR-specific (pSuper-1981 or pSuper-1774) or control (pSuper-1981mut) shRNA-generating retroviruses for 2 days. The infected cells were then selected in CDS medium with 2 μg/ml of puromycin for 3 days. The cells were allowed to recover in puromycin-free medium for an additional 2 days before lysis. The cell extracts were blotted for AR and α-tubulin (tubulin) (left). The right panel quantified the AR levels, relative to the respective α-tubulin level in each sample by analyzing the left panel blot using the ImageJ program. B: PSA gene expression after AR down-regulation. In two independent experiments (Exp.1 and 2), CWR22R3 cells were infected with pSuper-1981mut (mut) or pSuper-1981(1981), and the infected cells were selected with puromycin as in A. Total RNA was isolated at days 7 (Exp. 1) or 8 (Exp. 2) after infection. Quantitative real-time RT-PCR analysis of PSA mRNA levels was presented as the relative fold of change (relative level) compared to the PSA mRNA level in mut infected cells. C: Cells counted at 9 days after infection with AR-specific (1981 or 1774) or control (mut) shRNA-generating retroviruses and after puromycin selection. D: Phase contrast images of CWR22R3 cells at 9 days after infection with AR-specific (pSuper-1981 or pSuper-1774) or control (pSuper-1981mut) shRNA-generating retroviruses and after puromycin selection. E: OAS1 induction in CWR22R3 cells at 11 days after infection infected with AR-specific (1981 or 1774) or control (mut) shRNA-generating retroviruses and after puromycin selection. The induction was measured by quantitative real-time RT-PCR and presented as relative fold of change (relative level) compared to that in mut-infected cells. F: Cell extracts from CWR22R3 cells at 3 days after infection without puromycin selection were blotted for PARP and α-tubulin (tubulin). An asterisk indicates the cleaved PARP products. Original magnifications, ×200.
decreased pRb phosphorylation. Significantly, cell-cycle arrest after androgen withdrawal in LNCaP cells is also associated with decreased expression of D-type cyclins. In contrast, the levels of cyclin D1, D2, and D3 were not decreased in the AR shRNA-expressing CWR22R3 cells versus those in control cells, indicating that cell-cycle progression was arrested by a distinct mechanism that was independent of D-type cyclin expression levels (Figure 8D).

Taken together, these results demonstrated that AR continued to have a critical function in regulating the growth of CWR22R3 cells in steroid hormone-depleted medium and indicated that this function was distinct from its conventional transcriptional activity on ARE-regulated genes. Previous studies have identified nontranscriptional functions for the agonist liganded AR and other steroid hormone receptors, leading to the activation of MAP kinases and PI3 kinase. Therefore, we finally examined whether the Erk1 and Erk2 constitutive activation in the CWR22R3 cells was affected in the AR shRNA-expressing cells versus control. As shown in Figure 8E, there was no detectable decrease in Erk1 or Erk2 phosphorylation in cells expressing the AR shRNA. Similarly, we did not detect any change in PI3 kinase/Akt activity (data not shown).

**Discussion**

The CWR22 xenograft was used as a model to determine whether the AR, through transcriptional or nontranscriptional functions, continues to play a critical role in ad-
Advanced androgen-independent PCa. Relapsed CWR22 tumors in castrated mice expressed AR and PSA at levels that were comparable to the androgen-dependent CWR22 xenografts and were resistant to high doses of bicalutamide. The CWR22R3 cell line established from a relapsed CWR22 xenograft after long-term culture in steroid hormone-depleted medium continued to express AR and was resistant to bicalutamide, but PSA expression was markedly reduced relative to the in vivo xenograft. Consistent with this low level of PSA expression, transient transfection experiments with AR-regulated reporter genes indicated that the AR in CWR22R3 cells lacked androgen-independent transcriptional activity and was not hypersensitive to low levels of androgen. Nonetheless, stable shRNA-mediated down-regulation of AR expression resulted in marked suppression of cell growth and an accumulation of cells in G0/G1. These results identify a critical function for AR in regulating cell-cycle progression in androgen-independent PCa, and demonstrate that this AR function is not mediated through its conventional transcriptional activity.

Similar to other steroid hormone receptors, the AR has been reported to have rapid nontranscriptional signaling functions in response to its ligands, with several studies indicating that AR can directly interact with c-Src and PI3 kinase. The biological significance of nontranscriptional AR functions has been most clearly demonstrated in oocytes, in which the hormone-stimulated AR initiates oocyte maturation via such a nontranscriptional mechanism. However, these reported AR nontranscriptional functions (similarly to transcriptional activities) are ligand stimulated, and their physiological importance in normal prostate epithelium or in PCa has not been established. Significantly, in CWR22R3 cells, AR remains functional in the absence of ligand stimulation, and we did not observe changes in Erk1, Erk2 MAP kinase or PI3 kinase/Akt activities in response to AR down-regulation, further indicating that AR was functioning through distinct mechanisms.

Androgen binding induces a conformational change in the AR ligand-binding domain that stimulates homodimerization and generates a binding site for transcriptional co-activator proteins through LXXLL motifs. Nonetheless, multiple proteins can also bind to the AR via sites that are not dependent on androgen-mediated conformational changes in the ligand-binding domain, and such interactions could possibly mediate androgen-independent effects of AR on cell-cycle progression. These candidate AR-interacting proteins include cyclin D1 and pRb, which have been reported to interact with the AR N-terminus and suppress (cyclin D1) or enhance (pRb) androgen-stimulated AR transcriptional activity. The molecular mechanism of AR-mediated growth maintenance in CWR22R3 cells, including the roles of AR-interacting proteins are currently under investigation. Finally, it should be emphasized that although our studies did not detect residual AR transcriptional activity in the absence of ligand on conventional androgen-regulated genes that contain multiple ARES, it cannot be completely excluded that AR may nonetheless remain critical for the expression of some genes through alternative mechanisms that are not dependent on ARE binding, such as through interactions with other transcription factors.

Previous studies have shown that the growth of LNCap or LAPC4 PCa cells that have been adapted to grow under androgen-depleted conditions could also be suppressed when AR was down-regulated by a transfected AR-specific siRNA, ribozyme, or by anti-AR antibody injection. These findings are consistent with our results in CWR22R3 cells. However, in these reported studies, the androgen-independent PCa cells expressed substantial levels of PSA, suggesting that AR remained transcriptionally active. Therefore, they did not address whether growth suppression was due to abrogation of AR transcriptional activity mediated by residual androgens (with one study using cells grown in medium that was not depleted of steroid hormones) or by other mechanisms. Moreover, these previous studies did not assess effects of AR down-regulation on cell cycle or cell-cycle regulatory proteins, in particular on D-type cyclin expression (which was not repressed by AR shRNA in the CWR22R3 cells). In addition, AR down-regulation in LNCap-ABL cells results in significant apoptotic cell death, which is not observed in CWR22R3 cells. Therefore, although these previous studies further support the hypothesis that AR remains as a critical regulator of cell growth and survival in androgen-independent PCa, it remains to be determined whether AR has similar or distinct functions in the LNCap and LAPC4 PCa cells versus in the CWR22R3 cells.

Although the AR in the CWR22R3 cell line had no detectable transcriptional activity in the absence of androgens, the CWR22R3 and CWR22R4 xenografts in vivo in castrated mice expressed PSA at levels that were comparable to the androgen-dependent CWR22 xenograft. The high-level expression of PSA and other androgen-regulated genes in the relapsed CWR22 xenograft model, and in patients with androgen-independent PCa, strongly indicates that the AR retains transcriptional activity during the progression to androgen independence. Although relapsed PCa after castration and anti-androgen treatment are termed androgen-independent PCa, in reality these tumors may still be responsive to androgen. The AR transcriptional activity may be mediated by low levels of residual androgens in vivo, with multiple mechanisms including MAP kinase activation enhancing androgen responses. Significantly, the relapsed CWR22 xenografts were also resistant to bicalutamide, which may reflect the low AR affinity of this antagonist and is consistent with the intrinsic resistance to bicalutamide in most patients who relapse after androgen deprivation therapy. The loss of AR transcriptional activity in the CWR22R3 cell line is consistent with the more controlled and stringent depletion of androgens in vitro in the medium with charcoal-dextran-stripped serum. In addition, this loss of transcriptional activity may also be due to the loss of additional factors because the AR activity in the CWR22R3 cells was not hypersensitive to low levels of DHT and was not restored by adding back AR cofactors, SRC1 to SRC3.

In summary, this study demonstrates that the AR remains critical for androgen-independent growth of the CWR22R3 cell line. Moreover, the lack of detectable AR
transcriptional activity in the absence of androgens or in response to low levels of added androgens indicates that AR function in the CWR22R3 cells is mediated by a mechanism that is distinct from its conventional transcriptional function. Further characterization of this in vitro critical androgen-independent AR activity, whether a nontranscriptional or a novel transcriptional function, is necessary to determine the extent to which it contributes to in vivo tumor growth and whether it may provide a new therapeutic target for drug development.

Acknowledgments

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